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Spiders as senders and receivers of antipredatory warning signals

Pavouci jako zdroje a příjemci antipredačních varovních signálů

Doctoral thesis

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Contents

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Abstract

The introductory part of this thesis sums up the state of knowledge on aposematism and mimicry, the effect of aposematic and mimetic signals on spider predators, and cases when spiders do not receive but send such signals. Attachments of the thesis include four original manuscripts. In the first study, we presented jumping spiders (*Evarcha arcuata*, Salticidae) with different colour forms (red-and-black, yellow-and-black, white-and-black) of the firebug (*Pyrrhocoris apterus*, Pyrrhocoridae). Our goal was to compare reactions of the spiders to various intensity of aposematic signalization, expecting red-and-black coloration to have the strongest effect. Aversive learning of all colour forms was equally effective, but generalization of the learned avoidance to other colour forms was more effective after switch from less (white-and-black, yellow-and-black) to more (red-and-black) conspicuously coloured prey. When tested the next day, avoidance of the white-and-black prey got mostly forgotten. In the second study, we assessed little studied sensitivity of spiders to smells of unpalatable prey. After jumping spiders learned to avoid firebugs, most of them avoided the firebug smell, showing their sensitivity not only to optical, but also to chemical part of signalization of the unpalatable prey. In the third study, we approached spiders not only as receivers, but as senders of aposematic signalization. In two species of red-and-black ladybird spiders (*Eresus kollari*, *E. moravicus*) that are considered Batesian mimics (warningly signalling, but palatable prey), we attempted to specify their model – i.e., a species protected against predators and mimicked by the spiders. The key factors were relative abundances of species similar to ladybird spiders in their localities and degree of similarity between these species and ladybird spiders based on objective criteria (colour, coloration pattern, body shape, body size). The results have shown that the most likely model of the ladybird spiders is the seven-spotted ladybird (*Coccinella septempunctata*, Coccinellidae). In the fourth study, we used jumping spiders as model predators for analysis of mimetic relationship between larvae of two true bug species, the firebug and the Mediterranean red bug (*Scantius aegyptius*, Pyrrhocoridae). The composition of the red bug's secretion notably differed from that of the firebug and was slightly less effective against spiders. Nonetheless, avoidance learning was highly effective and the aversion was well memorable in both species. The generalization was only slightly asymmetric, favouring the red bug. Mimetic relationship between the species was therefore rather Müllerian, although under specific conditions, the firebug may be put into disadvantage due to weaker defence of the red bug and slightly asymmetric generalization. Altogether, the results reveal high complexity of spider reactions towards mimetic and aposematic signals and provide support for their further use in studies of these phenomena.

Abstrakt

Úvodní část této práce shrnuje stav poznání aposematismu a mimese, vlivu mimetických a aposematických signálů na pavoučí predátory a případů, kdy pavouci naopak tyto signály vysílají. Součástí disertační práce jsou i čtyři původní vědecké práce. V první studii jsme skákavkám černým (*Evarcha arcuata*, Salticidae) předkládali larvy různě zbarvených forem (červenočerná, žlutočerná, bíločerná) ruměnice pospolné (*Pyrrhocoris apterus*, Pyrrhocoridae). Cílem bylo srovnat reakce pavouků vůči různé intenzitě aposematické signalizace, přičemž předpokladem bylo, že červenočerné zbarvení bude mít nejvýraznější efekt. Averzivní učení probíhalo u všech barevných forem stejně rychle, ale generalizace naučené averze na další barevné formy byla účinnější při přechodu z méně (bíločerná, žlutočerná) na více (červenočerná) nápadně zbarvenou kořist. U bíločerné barevné formy byla averze do druhého dne do značné míry zapomenuta. Ve druhé studii jsme zkoumali málo zkoumanou citlivost pavouků na pachy nepoživatelné kořisti. Poté, co se skákavky černé naučily vyhýbat ruměnicím, vyhýbaly se jejich pachu, čímž byla prokázala jejich citlivost nejen na optickou, ale i na pachovou složku signalizace aposematické kořisti. Ve třetí studii jsme zkoumali pavouci nikoliv jako příjemce, ale jako zdroje varovné signalizace. U dvou červenočerných druhů stepníků (*Eresus kollari*, *E. moravicus*), považovaných za batesovské mimetiky (varovně zbarvenou, ale nechráněnou kořist), jsme se pokusili určit model stepníků – tedy druh chráněný před predátory, který tito pavouci v přírodě napodobují. Klíčovými faktory přitom byli relativní abundance stepníkům podobných druhů na jejich lokalitách a míra podobnosti těchto druhů stepníkům na základě objektivních kritérií (barva, barevný vzor, tvar těla, velikost). Výsledky ukázaly, že nejpravděpodobnějším modelem stepníků slunéčko sedmitečné (*Coccinella septempunctata*, Coccinellidae). Ve čtvrté studii jsme použili skákavky černé jako modelové predátory pro analýzu mimetického vztahu larev dvou druhů ploštic, ruměnice pospolné a *Scantius aegyptius* (Pyrrhocoridae). Sekrece *S. aegyptius* měla výrazně odlišné složení od sekrece ruměnic a proti pavoukům byla trochu méně účinná. Přesto u nich averzivní učení probíhalo rychle u obou druhů a averze byla stejně dobře zapamatovatelná. Při generalizaci mezi oběma druhy byl *S. aegyptius* mírně zvýhodněn. Mimetický vztah mezi oběma druhy je tedy spíše müllerovský, prospěšný pro obě strany, i když slabší obrana a mírná asymetrie generalizace může za určitých podmínek ruměnici znevýhodňovat. Dohromady výsledky odhalují vysokou komplexitu reakcí pavouků na aposematické a mimetické signály a poskytují podporu dalšímu využití pavouků jako modelových predátorů při studiu těchto jevů.

1 Introduction

Aposematism and mimicry are popular cases of predator-prey interaction, being well-known even to general public. However, there are large gaps in knowledge on these topics. One of them is the role of spiders in communication by means of aposematic and mimetic signals.

Most hypotheses concerning aposematism and mimicry are tested on avian predators (Ruxton et al. 2019). Birds are important predators in nature (reviewed in Whelan et al. 2008) and their complex cognitive abilities allow testing various aposematic signals, especially visual ones. However, use of other experimental predators than birds, especially those as phylogenetically distant and neurologically different as spiders, may provide a key additional information about the functioning of aposematic and mimetic signals, especially in two aspects. Firstly, theories regarding antipredatory defence should be tested on multiple predatory taxa before being considered a general principle. Secondly, many non-avian taxa are key predators in nature, and their reactions to prey signals may reflect the actual effect of the signals on prey fitness.

As the following pages will hopefully prove, some spiders, despite having synganglions ("brains") of a size of a pinhead (Land 2004), respond to aposematic and mimetic signals with complexity close to that observed in birds. Aside from this ability, spiders play an unenviable role of prey combining warning signals with almost no direct protection from their predators. This combination is rare in arthropods (as many insects are at least evasive or distasteful) and makes some spider taxa suitable for use in experiments concerning the effect of mimetic signals on predators.

The main aims of this thesis are:

1) Assessment of the effect of warning colouration on behaviour of jumping spiders (*Evarcha arcuata*) and comparison of the results with those previously obtained from bird predators.

2) Experimental study of assumed mimetic relationship between two pyrrhocorid aposematic species, *Pyrrhocoris apterus* and *Scantius aegyptius*, using jumping spiders as predators.

3) Testing the little known role of olfactory chemoreception in perception of aposematism by jumping spiders.

4) Identification of a natural model of ladybird spiders (*Eresus* spp.), warningly coloured but undefended group that probably mimics some similar, aposematic species.

2 Aposematism

2.1 Definition of aposematism

The effect of warning signalization on predators was first thoroughly analysed by Wallace (1867), who even formulated some rules of its functioning that have stayed valid until nowadays (see below). Aposematism has been one of the most popular subjects of study of evolutionary ecology ever since, but the definitions of and general approaches to the phenomenon have changed since the first use of the term (Poulton 1890). According to a relatively recent, broadly applicable conception, aposematism can be defined as an association between (1) a signal sent to a predator by a prey and (2) unprofitability of the prey to the predator (Mappes et al., 2005). The term "unprofitability" may not only include a distasteful, noxious, or dangerous prey, but also a prey with low nutritional value (such as ants, McNab 1984) or a prey which is difficult to catch or handle (such as agile butterflies or beetles, Balgooyen 1997, Ruxton et al. 2004a; see Marples et al. 2018 for general discussion).

2.2 Types of aposematic signalization

Traditionally, aposematic signals are considered to be primarily visual. The reason for their dominance is that once present, they require virtually no energy to maintain (Ruxton et al. 2018). Typical means of visual aposematic signalization is warning coloration, often combining black colour with either middle- or long-wave (yellow, orange, red) colours, or with white (Cott 1940, Svádová et al. 2009). Some patterns, especially striping, may serve dual function: when observed closely, the pattern is conspicuous, but from longer distance, it often has disruptive effect, making the signaller cryptic (reviewed in Ruxton et al. 2018).

Acoustic and vibratory signals, unlike visual ones, are energy-consuming, so signallers produce them only in proximity of a potential predator. They may not only serve as aposematic signals, but may even be unpleasant to predators by themselves, making them less willing to capture and handle the prey (Mitchell Masters 1979). The most widespread means of production of acoustic/vibratory aposematic signals in arthropods is stridulation, a sound or vibrations produced by rubbing two body parts against each other. Warning stridulation occurs in numerous arthropod taxa, especially insects such as Hymenoptera, Coleoptera, Heteroptera, and Lepidoptera (e.g., Mitchell Masters 1979, Schilman et al. 2001, Bura et al. 2009). Other means of acoustic/vibratory aposematic signalling include buzzing, common in Hymenoptera

(Brower & Brower 1965, Kirchner & Röschard 1999), tymbalation (i.e., sound produced by vibrations of tymbal, a specialized cuticular organ), used as an aposematic signal by arctiid moths (reviewed in Conner 2014), and air flow modification, used only by a few taxa such as hissing cockroaches (*Gromphadorhina* spp., Blattodea: Blaberidae) or hawk moths (Lepidoptera: Sphingidae) (Bura 2012 and references therein). On the contrary, hissing and other means of air flow modification are typical warning signals in terrestrial vertebrate taxa (Silaeva 1996).

Chemical aposematism, airborne (olfactory) and/or contact, is common in many taxa, especially in insects such as true bugs (Aldrich 1988) and beetles (e.g., Pettersson 2012). The main advantage of aposematic allomones (i.e. chemicals used for interspecific communication, advantageous for the signaller) is that they may serve as both in signalization and in defence itself (Ruxton et al. 2018 and references therein). Nature provides countless examples of antipredatory allomones that are also distasteful, irritating, repellent, noxious, toxic, or at least unpleasant to potential predators. Aside from having defensive or aposematic effects, some chemicals have other antipredatory functions. For example, the main function of pyrazines, commonly excreted by unpalatable ladybirds, is to trigger hidden aversions of other aposematic signals (such as warning colouration) in potential predators (Rowe & Guilford 1996).

Behavioural aposematism, although being considered a separate category, typically enhances a signal already present, often using the same sensory mode. For example, warningly coloured animals do not hide from predators, but display their signal so it can be seen from long distance. This gives predators more time to evaluate the prey and lowers the probability of recognition error in experienced predators (Guilford 1986). Many displays of behavioural aposematism are relatively simple and do not require special morphological prerequisities, but are relatively effective against predators and therefore widely used. Such signals include, among others, slow movement of prey (Wallace 1889, Hatle et Faragher 1998, Hatle et al. 2002), striking lack of cryptic behaviour (Wallace 1889, Pröhl & Ostrowski 2011), or aggregatory behaviour (Cott 1940, Gamberale & Tullberg 1996a, Hatle et al. 2002). However, behavioural signalling can eventually become so sophisticated that it can involve originally semantically neutral activities of the signaller. For example, typical ant-like behaviour, such as fast erratic locomotion combined with antennal and abdominal movement (McIver & Stonedahl 1993, Cushing 2012, Rubio et al. 2013), can act as a strong aposematic signal, as ants are usually avoided by predators, especially arthropod ones (Simmons 1957, Seifert 2007, Cushing 2012).

2.3 Effects of aposematic signalling

Aposematic signals are almost exclusively conspicuous. This enables potential predators to distinguish such prey from a cryptic, usually unprotected one (Ruxton et al. 2004b). Moreover, intense conspicuous stimuli elicit corresponding reactions in predators, and avoidance of conspicuous aposematic stimuli have often been fixated in predators during predator-prey co-evolution. Most notably, predators may possess innate, unlearned avoidance of signals such as red, orange, or yellow coloration (e.g., Schuler & Hesse 1985, Lindström et al. 1999, Pegram & Rutowski 2014). Subsequently, it is often difficult to distinguish whether the predator's reactions during experiments is based on the signal's conspicuousness, or on a hidden innate bias against a particular colour.

Bird predators learn to avoid widespread warning signals (especially red colour) faster than atypical or cryptic ones (e.g., Gittleman et al. 1980, Sillén-Tullberg 1985, reviewed in Skelhorn et al. 2016, but see, e.g., Ham et al. 2006), and detailed experiments have shown that atypical, but conspicuous signals have the same effect (Roper & Wistow 1986, Roper & Redston 1987). Furthermore, if predators learned to avoid a prey, the avoidance persists longer if the prey signal is conspicuous (e.g., Exnerová et al. 2008, reviewed in Ruxton et al. 2004, but see Ham et al. 2006, Svádová et al. 2009). Another advantage of conspicuous signals is that they provide advantage during generalization, which is more effective when the novel aposematic signal is stronger than the one already avoided (Gamberale-Stille & Tullberg 1999, Pegram & Rutowski 2014). The avoidance of a highly conspicuous novel prey may be even stronger than that of a previously avoided one (Gamberale & Tullberg 1996b, Svádová et al. 2009). One of possible explanations is that predators learn to discriminate between unprofitable and profitable prey on a basis of a particular trait (e.g., colour wavelength) and consequently avoid a novel prey to a greater extent if it is located farther from the profitable prey in the trait dimension (e.g., when its colour has longer wavelength) ("peak-shift", reviewed in Ten Cate & Rowe 2007).

It should be noted that nearly all information about the effect of aposematic signalization has been obtained from studies of bird predators. However, occasional experiments with non-avian predators, especially mantises (Bowdish & Bultman 1993, Prudic et al. 2006) and spiders (Taylor et al. 2014, Raška et al. 2017, Vickers & Taylor 2018) provide some support to broad generalization of theories that were originally tested on birds only.

Despite advantages of conspicuous aposematic coloration mentioned above, avoidance learning in predators represents a critical phase to aposematic prey. Before predators learn to associate the signal with prey unprofitability, signallers suffer from high mortality due to their conspicuousness. This threshold of temporarily decreased fitness is overcome by several mechanisms. One of the most important ones is kin selection: an aposematic individual may get killed by a naive predator, but if the predator learns to avoid the aposematic signal after this experience, the survival rate of relatives (i.e. bearers of the mutation for aposematic signalization) of the killed individual is increased, and the mutation can persist in population (Fisher 1930).

However, mechanisms of individual selection can help an aposematic prey to survive an encounter with a naive predator as well, still providing the predator with sufficient information about the prey unprofitability. For example, defensive chemicals of true bugs (Hemiptera: Heteroptera) are often excreted onto the body surface and may even be sprayed towards an approaching predator (Carayon 1971); these mechanisms decrease the chance that the prey would get injured or killed during the encounter with the predator (e.g., Exnerová et al. 2003, Raška et al. 2017).

Special attention has been paid to seemingly paradoxical initial stage of the evolution of aposematism. As only few novel aposematic mutants occur in this phase, they should theoretically be killed off (due to their easier detectability) before predators learn to avoid the signal. Numerous hypotheses have been formulated to explain the very possibility of establishment of an aposematic prey population, suggesting critical effects of phenomena such dietary conservatism and neophobia, innate biases in predators, or phenological, geographical, or individual variability in responses of potential predators (Lindström 1999, Mappes et al. 2005, Ruxton et al. 2018).

3 Mimicry

3.1 Definition and basic categories of mimicry

The term “mimicry” was introduced to biology at the beginning of the 19th century (Kirby & Spence 1817), referring to what is now classified as masquerade, an atypical, problematic subcategory of mimicry (Ruxton et al. 2018). It took almost half a century later since mimicry was used in a modern context to describe, among others, a case of antipredatory protection when a harmless species, a mimic, imitates a protected one, a model, to deceive potential predators (Bates 1862, Ruxton et al. 2018). This model-mimic relationship, later termed as Batesian mimicry, is parasitic – the mimic disrupts the ability of potential predators to associate the model’s signal with its protection, and therefore the mimic’s fitness is increased at the expense of the model (Mappes & Alatalo 1997 and references therein).

To benefit from mimicry, a Batesian mimic has to fulfil two critical conditions: it has to be less abundant than its model, and it has to co-occur with its model both spatially and temporarily (Wallace 1867; Pfennig et al. 2001). As usually, there are exceptions from these rules. For example, a model and a mimic do not have to co-occur when their predators (such as birds) are highly mobile (Ruxton et al. 2004b), and mimics in fact tend to occur later during season than their models – this is adaptive both for the model (its signal is not disrupted by Batesian mimics) and the mimic (by the time it occurs, the predators have already learned to avoid the signalization) (Waldbauer et al. 1988 and references therein).

The theory of mimicry was later complemented by category of Müllerian mimicry, involving a model and a mimic which are both protected against potential predators (Müller 1878). Unlike Batesian mimicry, the relationship between a model and its Müllerian mimic is mutualistic. Since predators typically have to learn to avoid a defended prey and the process is associated with prey mortality, Müllerian mimicry enables the model and the mimic to share the cost of this process (Müller 1879, Ruxton et al. 2018). Since Müllerian mimicry are advantageous to both involved sides, its evolution differs from that of Batesian mimicry. Most importantly, it is convergent rather than advergent (Turner 1987) and it is less dynamic, rather evolving by gradual steps (Fisher 1930, Balogh & Leimar 2005).

3.2 Mimicry under natural conditions

The two categories of antipredatory mimicry, Batesian and Müllerian, are in fact not as clearly defined as it may seem from the textbook examples of wasps, bees, and hoverflies. Some mimics that are harder to categorize, such as sweat bees (Hymenoptera: Halictidae) with only mildly painful stings (Schmidt 2017). Mimics therefore rather form a Batesian-Müllerian continuum of mimics with various effect on their model, from purely mutualistic to purely parasitic. The mimics that are protected against predators, but still decrease their models' fitness due to relatively low efficacy of their defence, are considered quasi-Batesian, rather parasitic (Speed 1993, 1999).

Müllerian and quasi-Batesian mimics could be distinguished based on their effect on the model's fitness. Quasi-Batesian mimics are less harmful to their models that classical Batesian mimics, but are still parasitic, increasing mortality of their models (Speed 1999, reviewed in Ruxton et al. 2018). Müllerian mimicry, on the other hand, allows the costs of avoidance learning to be shared between the model and the mimic (Müller 1878). Unfortunately, the whole phenomenon is difficult to assess experimentally and the experimental setup (e.g., relative abundances of the model and the mimic, presence of an alternative prey, predator feeding motivation) significantly affect the results (Speed et al. 2000, Rowland et al. 2007, 2010, Ihalainen et al. 2012).

Mimetic relationships also do not necessarily include only one model and one mimic, as involvement of more than two species in a Müllerian mimetic relationship further decreases the cost of avoidance learning. In nature, this often results into coevolution of broad mimicry rings including numerous species from various taxa (Ruxton et al. 2018). Among the best-known ones is the wasp-like mimicry ring, with hymenopteran models and/or Müllerian mimics, hoverfly Batesian mimics, some moth species positioned along the whole Batesian-Müllerian axis, and others (Chittka & Osorio 2007). In Europe, red-and-black mimicry ring includes broad spectrum of arthropods, such as true bugs (Heteroptera) (Hotová Svádová et al. 2010) and ladybird beetles (Coleoptera: Coccinellidae) (Arenas et al. 2015), but probably also taxa such as leaf beetles (e.g., *Cryptocephalus* spp. and *Crioceris* spp., Coleoptera: Chrysomelidae), leafhoppers (*Cercopis* spp., Cicadomorpha: Cercopidae), and even some spiders (*Eresus* spp. (Araneae: Eresidae), *Philaeus chrysops* (Poda 1761) (Araneae: Salticidae)).

3.3 3 Imperfect mimicry

Just like aposematism, mimicry has some topics that are difficult to explain and therefore fascinate researchers for decades. One of them is the case of imperfect mimicry. One would assume that mimic would be strongly selected for evolution of perfect mimicry, but many mimics, such as some hoverflies (e.g., *Melanostoma* spp.) or spiders (e.g., *Eresus* spp.), seem to get away with what can hardly confuse a careful observer. Some reasons for this apparent paradox may relate to the signaller itself (e.g., developmental constraints, trade-offs between different life aspects such as defensive and foraging behaviour), some may relate to their potential models (as one mimic can evolve intermediate signal based on multiple models, "jack-of-all-trades"), and some can be explained by characteristics of predators which, for example, may perceive signals only vaguely and may therefore not act as selective agents for mimicry perfection (Sherratt 2002, Kikuchi & Pfennig 2013). Perfect mimicry may also be a handicap during an encounter with a predator specialized on preying on the model (especially myrmecophagous specialists that may represent a threat to myrmecomorphic mimics) (Pekár et al. 2011).

4 Cognition of spiders

4.1 Spider senses

Spider sensory modes often have strikingly different priorities than in human or bird observers. In this chapter, spider senses are discussed, with emphasis on sight which, according to the current paradigm, is a dominant mediator of aposematic signalization (Ruxton et al. 2018).

Image resulution strongly depends on absolute eye diameter value (Land & Barth 1992). Spiders and other arthropods therefore inevitably have worse initial conditions for acute vision than vertebrates. Nonetheless, principal eyes may be modified to compensate this handicap. For example, jumping spiders have tubular rather than round eyes, which enables them to see objects in their focal point as if their eyes were much larger (Land 1969a), and the eyes are equipped with six eye-moving muscles, the same number as in humans, to adjust the focal point (Land 1969b; Fig. 1).

The significance of visual cues in spiders largely depends on thir hunting strategy. Generally, diurnal cursorial spiders have larger eyes than web-building or nocturnal ones, although contrarily, some nocturnal spiders (e.g., Lycosidae, Deinopidae) have the eyes enlarged to obtain more visual information. Spiders of some families (e.g., Gnaphosidae) have their eyes reduced so they do not provide detailed information, but complete eye reduction is common only in troglomorphic species (Culver & Pipan 2010).

According to the information about the number of photoreceptor cell types detected in main (anterior median) eyes, it seems that spiders‘ colour vision is highly variable. It ranges from monochromatic, such as in Sparassidae (Nørgaard 2008), through probably the most common dichromatic system, present in Lycosidae, Salticidae pt., Ctenidae, and Thomisidae (DeVoe 1972, Blest et al. 1981; Walla et al. 1996, Defrize et al. 2011, respectively), up to trichromatic, such as in Salticidae pt. and Araneidae (DeVoe 1975, Yamashita & Tateda 1978, respectively), and even tetrachromatic vision (Salticidae pt.; Land 1969a, Yamashita & Tateda 1976). Colour vision has usually been studied on a single species or genus per family, so it is unknown whether high variability observed in salticids is present in other spiders as well, or whether it is unique to this family of visually oriented spiders.

Although colour vision largely depends on the number and characteristics of photoreceptor cell types, it can be further modified morphologically, physiologically, or neurologically. Thus, supposedly dichromatic *Cupiennius salei* (Keyserling 1877) (Ctenidae) turned out to be colour-blind in physiological tests (Orlando & Schmid 2011). On the other hand, *Habronattus pyrrithrix* (Chamberlin 1924), a dichromatic salticid, shifts wavelength of light reaching one layer of its photoreceptor cells, making its eyes functionally trichromatic and sensitive even to long-wavelength light (Zurek et al. 2015). True long-wavelength photoreceptors are present in tetrachromatic salticids (Land 1969a, Yamashita & Tateda 1976), and both physiological (Peaslee & Wilson 1989) and behavioural (Chang 2012) tests performed on other species suggest that such broad sensitivity spectrum is in fact probably common in jumping spiders.

Mechanoreception, including perception of vibrations and sound, is the most important sense in most spiders (Foelix 2011). Mechanic information is mediated by several receptor types with partially overlapping function: tactile hairs, densely distributed on spider body surface; trichobothriae, hairs connected to innervated pits; and slit sensilla, which are often merged into unique lyriform organs (Foelix 2011). Tactile hair, despite having relatively complex structure, especially compared to their insect counterparts (Foelix & Chu-Wang 1973a), provide rather simple information acout contact stimuli (Foelix 2011). Trichobothriae, on the other hand, mediate non-contact and more complex information about air currents and low-frequency sounds (Barth 2004, Foelix 2011). They are important especially during foraging behavior of cursorial spiders and may be so sensitive that they nearly reach the maximum sensitivity level possible for this type of sensory organs (Barth 2004). Slit sensilla, especially those formed into lyriform organs, are principal vibroreceptors and auditory organs especially in web-building spiders (Foelix 2011). Among other functions, slit sensilla inform spiders about presence, position, and qualities of the prey (Uhl & Elias 2011).

Analogically to mechanoreceptors, spider chemoreceptors can be classified into two categories, contact and non-contact. Contact chemoreception is only partially analogous to the sense of taste in human and other terrestrial vertebrates, as spiders have chemoreceptive organs ("taste hairs") not only in their mouthparts, but also on pedipalps and legs, especially in distal parts (Foelix & Chu-Wang 1973b). Contact chemoreception is used in many aspects of spider behaviour, especially mating (reviewed in Gaskett 2007, Uhl & Elias 2011) and, to a lesser extent, foraging (Foelix 2011).

Non-contact chemoreception (olfactory chemoreception, "smell") is largely unknown in spiders. Even olfactory chemoreceptors have not been unambiguously specified yet, although tarsal organs, located on legs, are the most likely candidates for this function (Foelix & Chu-Wang 1973b, Foelix 2011). With an exception of specialized predators (e.g., Allan et al. 1996, Jackson et al. 2002, Cross & Jackson 2010), olfactory chemoreception is probably of lesser importance in foraging behaviour of spiders. However, increasing amount of data (e.g., Hostettler & Nentwig 2006, Raška et al. 2018, reviewed in Uhl 2013) suggests that relevance of olfactory stimuli in predation may be underrated in spiders.

4.2 Perception of aposematism and mimicry

Current knowledge on perception of aposematic and mimetic signals by spiders can hardly be considered comprehensive, especially compared to the state of knowledge on birds. Still, the data are sufficient to illustrate a striking contrast in perception of prey signals by the two taxa.

Although the ability to perceive visual aposematic signals has been observed in a few spider families, such as Salticidae (see the next chapter) and Thomisidae (Morris & Reader 2016), visual signals typically play a secondary role in spider cognition (Foelix 2011). It would therefore seem that spiders are probably generally not very sensitive to aposematic, usually visual (Ruxton et al. 2018), warning signals.

However, there may be an alternative interpretation of the available data. It is likely that our understanding of aposematism as a visually-dominated phenomenon is biased, as both bird predators and human observers are highly dependent on visual information (Zeigler & Bischof 1993, Van Essen et al. 1992, respectively). However, occasional studies of non-visual aposematic signalization show that alternative means of aposematic signalling, especially warning vibrations, are produced by numerous taxa, such as true bugs (Hemiptera: Heteroptera; reviewed in Gogala 2006), Coleoptera of many families (reviewed in Wessel 2006), moths (Lepidoptera: Erebidae; e.g., Dunning 1968, Hristov & Conner 2005) and Hymenoptera, such as mutillid wasps (Mitchell Masters 1979) or ants (Santos et al. 2005). Due to small size of these animals, their signals usually have either high frequency and limited range, or are transfered via substrate rather than via air (Michelsen et al. 1982). In such cases, they are not well perceptible to vertebrate predators, but represent strong stimuli in an *umwelt* of an arthropod predator. So, while typical receiver of a visual aposematic signal is a vertebrate predator, vibratory signals are more likely to be aimed at arthropods, including spiders.

Different perception of acoustic and vibratory signals by spiders (substrate-borne and plain air flow, reviewed in Foelix 2011) than in humans (airborne) is probably the reason of insufficient knowledge on the use of vibratory cues in spiders and other arthropod predators. It is no surprise, however, that vibratory signals allow spiders, among others, to perceive unprofitability of a potential prey. For example, wolf spiders (*Lycosa ceratiola* (Gertsch & Wallace 1935), *Geolycosa ornatipes* (Bryant 1935)) attacked an unprofitable prey (mutillid wasps and ground beetles (Coleoptera: Carabidae)) more readily when stridulatory organs of the prey were disabled (Mitchell Masters 1979). The same effect occurred when an artificial vibrating prey was used (Mitchell Masters 1979), which suggests presence of a broad spectrum of aversions towards vibrating prey. This hypothesis has been supported by experiments with jumping spiders and neutral stimuli, one of which was associated with vibratory signal (Long et al. 2015). Spider sensitivity to vibratory aposematic signals can be quite broad and non-specific. For example, orb-web spiders (*Araneus diadematus* Clerck 1757) learned to associate presentation of a quinine-coated prey with a tone of a tuning fork (Bays 1962).

Unlike visual and vibratory aposematic signals, olfactory (non-contact) traits seem to have a similar, usually secondary effect on both birds and spiders. Although spiders of many families have shown aversion towards strong odours, such as peppermint or lavender oil (Peckham & Peckham 1887), knowledge on the relevance of olfactory information to spiders in nature is only fragmental, and few studies of the effect of olfactory aposematic signals on spiders (Raška et al. 2018, Vickers & Taylor 2018) provide only a fragmental insight into the topic. For example, jumping spiders that had learnt to avoid unpalatable firebugs (*Pyrrhocoris apterus* L. 1758, Heteroptera: Pyrrhocoridae) also avoided the distinct smell of crushed firebugs (Raška et al. 2018). However, more subtle smell of dangerous, previously encountered paper wasp prey, *Polistes dominula* Christ 1791, was not recognized by crab spider *Synema globosum* (Morris & Reader 2016). Olfactory signals may have an indirect effect on spider predators: a novel odour released into the experimental arena primed avoidance of conspicuous red prey (Vickers & Taylor 2018).

The perception of contact defensive chemicals, on the other hand, is widespread in spiders. The advantage of such substances is that they may serve both as a signal and as a defence (Holen 2013). Since spiders are able perceive chemicals not only by mouthparts, but also by legs and palps (Foelix 2011), contact chemoreception is especially important to them compared to vertebrate predators, as they can assess the qualities of their prey before attacking it. The most striking examples of spiders‘ ability to perceive contact defensive chemicals include cutting out the distasteful prey from webs by *Nephila* spp. (Masters 1990 and references therein) and wiping the mouthparts against substrate after encountering unlapatable seed bugs (Heteroptera: Lygaeidae) by jumping spiders (Hill 2006). Spiders are able to detect both prey (e.g., Johnson et al. 2011 and references therein, reviewed in Uhl 2013) and predator (e.g., Eiben & Persons 2007, reviewed in Uhl & Elias 2011) indirectly via cues on a substrate, but so far no study has focused on whether they are able to perceive defensive chemicals this way.

Hypotheses concerning effects of behavioural aposematism on spiders have not been thoroughly tested on spiders yet. It is known that fast movement of a prey can have a stimulating effect on predatory behaviour of spiders (e.g., Persons & Uetz 1997, Bednarski et al. 2012), and more frequent encounters with prey may enhance avoidance learning when a prey is aposematic. Although the effect of behavioural aposematism has not been studied directly, its potential significance in spider predatory behaviour can be deduced from spiders‘ reactions towards ant mimics. When encountering a non-visually oriented spider predator (*Lampona murina* Koch 1873, Lamponidae), the myrmecomorphic jumping spider *Myrmarachne luctuosa* (Koch 1873) starts moving its front legs as if they were ant antennae; this behaviour eventually increases its chance to survive the encounter (Pekár et al. 2017, Pekár, pers. comm.) Ant-like behaviour may even help juvenile *M. luctuosa* to protect themselves against cannibalistic adults (Huang et al. 2011). Aggregations of *Myrmarachne melanotarsa* Wesolowska & Salm 2002, atypical of spiders and effective against other spider predators, might have not only increased the intensity of visual mimicry, but also mimicked social behaviour of ants (Nelson & Jackson, 2009). It should be noted that observations of behavioural aposematism are in general often not validated in experiments with natural predators (e.g., Mochida 2009, Pröhl & Ostrowski 2011) and are seldom paid sufficient attention to.

4.3 Unique case of jumping spiders

Percetion of aposematic signalization by jumping spiders, with their predominant visual orientation, acute vision, and complex cognitive abilities (reviewed in Richman & Jackson 1992, Jackson & Pollard 1996) may be considered more similar to that of vertebrate predators such as lizards or birds (or humans, for that matter) than to perception by other spider taxa or insect predators. Not only are jumping spiders able to perceive long-wavelength warning colours such as red, orange, or yellow, but are also able to associate them with the signaller’s defence and have potential for responsiveness to various changes of experimental conditions. For these reasons, jumping spiders represent an excellent alternative model predators for studies of effects of aposematism and associated antipredatory defence. During the 20th century, however, studies of these subjects using these predators were scarce and superficial (e.g., Eisner 1980, Eisner et al. 1981, McIver & Lattin 1990), and only in the last two decades have jumping spiders become widely used as model predators.

During pioneering experiments with differently coloured heated plates, jumping spiders have shown their ability to associate a colour with negative experience (Nakamura & Yamashita 2000). Following experiments with live prey have provided the same results, along with data concerning effects of environment changes, presentation of an alternative prey, duration of intervals between prey presentation etc. (Skow & Jakob 2005, Hill 2006). In following years, jumping spiders exhibited many behavioural patterns previously observed in the most thoroughly studied predators, birds. First, when offered differently-coloured prey , jumping spiders avoided red and yellow individuals, showing an innate bias against these typical aposematic colours (Taylor et al. 2014, but see Raška et al. 2017). If spiders had to learn to avoid protected, warningly coloured prey, they subsequently generalized the association between the signal and the protection to a similar prey, be it artificially coloured crickets (Taylor et al. 2015), a second true bug species involved in a mimetic relationship with the first one (Raška et al., to be submitted), or differently coloured individuals of the same species (Raška et al. 2017). The last case was especially intriguing. Not only did the spiders generalized between similar prey, but due to peak-shift in the response, the aversion to the presented prey even increased after switch from a yellow or white colour form to a novel red one, but not vice versa (Raška et al. 2017). This response, surprisingly complex in predators with such simple neural system (Steinhoff et al. 2017), was very similar to reactions of bird predators in previous experiments (Svádová et al. 2009), suggesting presence of convergent evolutionary processes on a wide taxonomical scale not only in prey species (such as in formation of mimetic complexes), but correspondingly also in their potential predators.

5 Spiders as senders of warning signals

5.1 Warning signals of spiders

Spiders do not only perceive aposematic signalization as predators, but can also send warning signals to their potential predators, especially birds and other vertebrates. However, aposematism *sensu stricto*, i.e. association between a signal and the signaller‘s unprofitability (Mappes et al. 2005), is quite rare in spiders. Most truly aposematic species belong to only a few families, and even in those few ones, they usually represent a small fraction of all species (Pekár 2014a).

The most conspicuous truly aposematic spiders are araneids armed with hard cuticle appendages which make them harder or even impossible to swallow or digest. This defence is typical of *Gasteracantha* spp. but can be found in other spiders belonging to two phylogenetically distant (Kallal et al. 2018) araneid subfamilies, Micratheninae (*Micrathena* spp.) and Gasteracanthinae (e.g., *Macracantha arcuata* (F. 1793), *Thelacantha brevispina* (Doleschall 1857)).

Colouration of these spiders is peculiar: not only do they exhibit unique colouration patterns that to not match those of potential Müllerian mimics, but some species exhibit high colouration polymorphism (Gawryszewski & Motta 2012, Truong 2012). The former phenomenon could be explained by specific signalization associated with the spiders‘ relatively uncommon means of defence, as the thorns themselves are often coloured in contrasting colours. The explanation of the latter phenomenon is still unclear. Experiments testing effect of different colouration on predation success have provided ambiguous results (Hauber et al. 2002, Gawryszewski & Motta 2012, Kemp et al. 2013, Rao et al. 2015, White & Kemp 2016) and its effect on thermoregulation (Rao & Mendoza-Cuenca 2016) is probably only secondary. The same problem arises in another polymorphic (e.g., Sen et al. 2010) conspicuous spider group, *Nephila* spp. Their large, resilient webs allow these spiders to catch and subdue even birds (Brooks 2012, Walther 2016) and their conspicuous colouration may warn flying predators about this risk. However, this hypothesis has not been supported yet, and just as in other conspicuous araneids, the colouration may serve other functions, such as prey attraction (Tso et al. 2002).

Unambiguously aposematic colouration can be found in venomous representatives of the *Latrodectus* genus (Theridiidae), yet even in these spiders, the colouration may be highly polymorphic (e.g., *L. mactans* (F. 1775), Levi 1959). The reason for the polymorphism is even more enigmatic than in aposematic Araneidae. Since *Latrodectus* spp. mostly prey on nocturnal arthropods (Hódar & Sánchez-Piñero 2002), the hypothesis tested in araneids (prey attraction effect) is unlikely to function in these spiders. This phenomenon, which may be associated with multiple potential models of these spiders (Kikuchi & Pfennig 2013) or with disruption of search image of the spiders' potential predators, has not been directly assessed by any study so far.

Other cluster of aposematic spider species, *Phoneutria* spp. and *Cuppienius* spp. (both Ctenidae), uses its aposematic colouration as a part of behavioural warning signalling. These spiders raise their front legs when threatening potential attackers (Peigneur et al. 2018). Such behaviour is common in wide spectrum of spider taxa, such as theraphosids (West et al. 2008) or salticids (Clark & Uetz 1990), but some ctenid spiders enhance its effect by displaying red (e.g., *C. coccineus* Pickard-Cambridge 1901), yellow (e.g., *P. boliviensis* (Pickard-Cambridge 1897)), or black-and-white (e.g., *P. nigriventer* (Keyserling 1891)) lower sides of their legs. This signalling, supported by colourful chelicerae in some species, is so characteristic that *Phoneutria* spp. are commonly known as "armed spiders" (Peigneur et al. 2018).

Although its function has not been sufficiently supported by experimental studies yet, it is very likely that aposematic colouration of some mygalomorph spiders, usually with red and white elements (e.g., *Brachypelma* spp.), less often blue-dominated (e.g., *Poecilotheria metallica* Pocock 1899), serves aposematic function. Moreover, when threatened, *Brachypelma* spp. spiders stridulate, producing sound clearly audible even to vertebrates (Pérez-Miles & Perafán 2017).

Despite seemingly high number of taxa discussed above, only a small proportion of spiders is truly aposematic (Pekár 2014a). This fact may be surprising, considering that nearly all spiders have functional venom glands (Foelix 2011). Even spiders with strong chelicerae, however, usually use them against predators only as a last resort, and rather rely on other means of active defence, such as escape (Cloudsley-Thompson 1995, Arbuckle 2015), or passive defences such as anachoresis, crypsis, or Batesian mimicry (Pekár 2014a).

5.2 Mimicry in spiders, case study: *Eresus* spp.

Batesian mimicry is more or less important part of defence in about 30 % of all spider species (Pekár 2014a). Great majority of spider mimics imitate ants (Pekár 2014a), but accurate ant mimicry is common only in two families, Salticidae and Corinnidae (Pekár 2014b). Aside from ants, spiders imitate taxa such as other Hymenoptera, or beetles (Pocock 1909, Pekár 2014a).

The latter is probably the case of ladybird spider males (*Eresus* spp.). These spiders are among the most widely known examples of warningly coloured arachnids. However, these spiders are probably not truly aposematic, as they seem to possess no effective defence that could be associated with their warning coloration. Although they are endowed with relatively strong chelicerae, they are unable to penetrate specialized organs used during predation, such as mantid forelegs (Raška et al., unpublished) or, analogically, a bird beak. Combining this drawback with soft cuticle and relatively low body flexilibity, ladybird spider males seem to represent a harmless and profitable prey to their potential predators. However, further empirical support is necessary for this hypothesis. For example, the risk of a spider bite to a non-protected body part may not be negligible in young, inexperienced birds.

If ladybird spiders‘ warning colouration does not signal their own defence, they are most likely Batesian mimics of some other, protected species. Considering how popular with arachnologists ladybird spiders are, it is surprising that the identification of the spiders‘ model was long neglected. Although, as the ladybird spiders‘ name suggests, the models may be ladybirds, especially the seven-spotted ladybird (*Coccinella septempunctata* L. 1758) (Cloudsley-Thompson 1995), other taxa, such as some true bugs, may in fact play this role (Askins 2002). Identification of the ladybird spiders‘ model is complicated by the fact that four *Eresus* species occuring in central Europe differ in phenology. None of them occur in early summer, a period when naive predators are abundant and Batesian mimics would be put under high predatory pressure (Waldbauer et al. 1988 and references therein). However, while adult males of *E. kollari* Rossi 1846, the most widely distributed species, actively search for females in late summer and early autumn, males of the other three species occuring in central Europe emerge in spring, with slightly shifted activity periods (*E. hermani* Kovács et al. 2015 is active in early spring, *E. moravicus* Řezáč 2008 in mid-spring, and *E. sandaliatus* (Martini & Goeze 1778) in mid- and late spring) (Řezáč et al. 2008, Kovács et al. 2015, Kůrka et al. 2015). According to hypotheses concerning phenology, a Batesian mimic should occur either at the same time as its model (Wallace 1867), or preferably a little later (Waldbauer 1988). Thus, it is not impossible for spring- and autumn-occuring ladybird spiders, although looking almost the same, to form mimetic relationships with completely different species.

The hypothesis suggesting a close mimetic relationship between ladybird spiders and ladybird beetles has been recently supported by phenological and morphometrical data. During the emergence periods of *E. moravicus* (spring) and *E. kollari* (early autumn), *C. septempunctata* was abundant at all studied localities of the spiders in Southern Moravia (Czech Republic). Analysis of similarity, based on close-to-objective criteria (colouration pattern, colouration reflectance, body shape and size), showed that among species more abundant than *Eresus* spp. (and therefore suitable for a role of a model), it was again *C. septempunctata* that was the most similar to the spiders (Raška & Pekár 2018).

These results show that *C. septempunctata* is most likely the key model species of the two spider species despite their different phenology. Although field data were obtained in a relatively small area and only from *E. moravicus* and *E. kollari*, the information can be broadly generalized not only to all four central European *Eresus* species (as *E. hermani* and *E. sandaliatus* have similar phenology as *E. moravicus*), but also to the whole distribution areas of these spider species. Since *C. septempunctata* is a common species in whole western Palaearctic region, it is probably sympatric with the spiders in their whole distribution area (e.g., Řezáč et al. 2008, Mihajlov 2013, Nikitsky & Ukrainsky 2016, Pasqual et al. 2019).

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STUDY 1

How variation in prey aposematic signals affects avoidance learning, generalization and memory of a salticid spider

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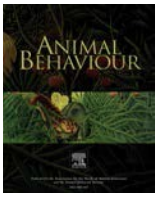

How variation in prey aposematic signals affects avoidance learning, generalization and memory of a salticid spider

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Most studies of aposematism focus on the effect of warning signals on vertebrate predators, especially birds. In our experiments, we used jumping spiders, *Evarcha arcuata* (Araneae: Salticidae) as predators, and larvae of three colour forms (red, white, yellow) of an unpalatable firebug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) as prey. The experiments were divided into four successive steps, focusing on different aspects of predator–prey interaction. (1) When presented with a firebug for the first time, the spiders captured the white, least conspicuous colour form more often than the other two. No differences in the attack latencies were observed between the colour forms. (2) In the avoidance-learning test, the spiders were offered in succession five firebugs of one of the three colour forms. The attack and capture rate decreased in all colour forms, more notably in the red, most conspicuous form. (3) After five presentations of the same prey, the spiders were presented with a different firebug colour form. The results of the generalization process were asymmetric: spiders' attack rate increased when the red prey was followed by the yellow or white one, but decreased when the red form was presented after the other colour forms. (4) Spiders attacked the same prey more often the next day, but the attacks were seldom fatal. Similarly to the initial reaction, spiders captured the white firebugs more often. Our results show that for *E. arcuata*, the red coloration can represent an effective aposematic signal. Red prey coloration decreased the attack rate during the avoidance-learning process and favoured the prey in generalization between different colour forms. Yellow coloration was moderately effective against *E. arcuata*, whereas white coloration was the least effective because of low innate bias against this signal.

Aposematism can be defined as an association between a prey's unprofitability and a relevant signal sent by the prey to a potential predator (Mappes, Marples, & Endler, 2005). This may involve optical signals (colour, pattern, contrast, shape, etc.) as well as other modes of signalling, such as warning sounds, tastes or smells (reviewed in Komárek, 2003).

Aposematic signals usually make the prey conspicuous, which enhances the avoidance-learning process of predators (Aronsson & Gamberale-Stille, 2009; Roper & Wistow, 1986). The attacked prey may often be killed even though it is unpalatable or noxious, but the negative association made by a predator potentially protects its relatives from further attacks. Aposematism can thus be maintained by means of kin selection (Fisher, 1930). However, aposematic prey individuals may frequently survive the attack unharmed, especially when predators sample the 'suspicious' prey (e.g. Gamberale-Stille & Guilford, 2004; Guilford, 1994; Järvi, Sillen-Tullberg, & Wiklund, 1981; Skelhorn & Rowe, 2006). The fitness of the aposematic prey can therefore be increased directly by individual selection (Järvi et al., 1981; Wiklund & Järvi, 1982).

An aposematic signal can affect the behaviour of its receiver at several levels. Even naïve predators can be affected by a signal due to the presence of an innate bias. For example, when presented with a painted novel palatable food (*Tenebrio molitor* larvae), domestic chicks, *Gallus gallus domesticus*, preferred olive and green prey items over the conspicuous (yellow, black and yellow) ones (e.g. Hauglund, Hagen, & Lampe, 2006; Schuler & Hesse, 1985).

The most prominent aspect of aposematism is that the association between signal and unprofitability can be learned by predators in a process of avoidance learning. The more salient the signal is, the easier it is for the predator to associate it with the sender's characteristics (Gamberale-Stille, Balogh, Tullberg, & Leimar, 2012; Rescorla & Wagner, 1972). This hypothesis has been supported by numerous experimental studies (reviewed in Ruxton, Sherratt, &

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Speed, 2004). Combining two or more warning signals can further increase the predator's response, as seen in experiments using optical signalling combined with acoustic or chemical cues (e.g. Marples & Roper, 1996; Rowe & Guilford, 1996).

When a predator learns to avoid a particular aposematic prey, it can generalize this experience towards a similar prey. Under some circumstances, the generalization may be asymmetric due to sensory or learning biases (ten Cate & Rowe, 2007). For example, when a predator has learned to discriminate between palatable and unpalatable prey, it may show stronger avoidance of a novel prey that differs more from the palatable one than the prey encountered during the avoidance-learning process. This phenomenon has been termed peak shift as the theoretical peak of the aversive response is shifted along the discriminative-stimulus dimension away from the original aversive stimulus (Ghirlanda & Enquist, 2003; Spence, 1937). This way predators may generalize their aversive response more effectively towards a novel prey that is more conspicuously coloured (Aronsson & Gamberale-Stille, 2008; Gamberale-Stille & Tullberg, 1999), larger (Gamberale & Tullberg, 1996a), or presented in an aggregation (Gamberale & Tullberg, 1996b).

Stronger aposematic signals may also help the learned avoidance to be remembered for longer (e.g. Guilford & Dawkins, 1991; Speed, 2000). In some experiments, conspicuous colour (Exnerová et al., 2008; but see Ham, Ihalainen, Lindström, & Mappes, 2006) or contrast against background (Alatalo & Mappes, 1996; Roper & Redston, 1987) indeed increased the maintenance of learned association in bird predators.

Most experimental data on the function of aposematic signals have been obtained from bird predators (Exnerová et al., 2008), and knowledge of how aposematism functions is especially limited for arthropod predators. However, most aposematic species are insects, and therefore are small enough to be preyed upon by other insects and spiders. Arthropod predators and parasitoids are in fact the main biotic cause of mortality of arthropod species (Symondson, Sunderland, & Greenstone, 2002) and may be crucial agents for the evolution of aposematic signals. The only two arthropod predatory taxa in which the reactions towards aposematic prey have been studied in detail are mantids (Mantodea; Berenbaum & Miliczky, 1984; Bowdish & Bultman, 1993; Prudic, Skemp, & Papaj, 2007) and jumping spiders (Araneae: Salticidae). Because of complex cognitive abilities (reviewed in Richman & Jackson, 1992), acute vision (Land, 1969; Yamashita, 1985) and tetrachromatic colour perception ranging from UV to red (Nakamura & Yamashita, 2000; Peaslee & Wilson, 1989), the jumping spiders are an excellent model for studies of the effect of aposematism.

Recently, several phenomena, first described in birds, have also been observed in jumping spiders. Jumping spiders have learned to avoid unpalatable prey (Hill, 2006; Skow & Jakob, 2006), generalized information about prey palatability to a similar prey (Taylor, Amin, Maier, Byrne, & Morehouse, 2016), and even avoided red and yellow prey without any prior experience, suggesting presence of an innate bias (Taylor, Maier, Byrne, Amin, & Morehouse, 2014). Maintenance of the learned avoidance, however, is problematic in jumping spiders: after five trials of avoidance learning, the attack rate towards unpalatable large milkweed bugs, *Oncopeltus fasciatus* (Heteroptera: Lygaeidae) on the next day increased to about 80% of that observed in naïve spiders on the previous day (Hill, 2006).

In this study, we used *Evarcha arcuata*, a common European jumping spider, as a model predator, and the third-instar larvae of (1) red-and-black, (2) yellow-and-black and (3) white-and-black colour forms of the European firebug, *Pyrrhocoris apterus*, as prey. Red-and-black, yellow-and-black and white-and-black colour combinations represent typical aposematic signals (Cott, 1940). However, the aposematic function of black-and-white coloration is context dependent (e.g. Lyytinen, Alatalo, Lindström, & Mappes, 1999; Stimson & Berman, 1990); moreover, black-and-white coloration seems to have a smaller effect on predators than the red-and-black and yellow-and-black ones (Exnerová et al., 2006; Svádová et al., 2009). We wanted to test whether the antipredator defence of the third-instar larvae of *P. apterus* is effective against jumping spiders, and how the spider's predatory behaviour is affected by the prey's colour. Specifically, we tested whether prey colour affects the degree of unlearned wariness, the process of avoidance learning and signal memorability, and how the spiders generalize between the different colour forms.

METHODS

Predators

Evarcha arcuata is a jumping spider (Araneae: Salticidae) with a Palaearctic distribution (Platnick, 2016), living in grass habitats (Buchar & Kůrka, 2001). Its body length is about 5 mm in males and 6 mm in females. It is a cursorial generalist predator feeding on other spiders and various insects, including true bugs from the family Miridae (Dobroruka, 1997; Nentwig, 1986). Jumping spiders are visually oriented and experimental studies have revealed that the species *Hasarius adansoni* could discriminate between red and yellow, red and grey, and yellow and grey colour stimuli (Nakamura & Yamashita, 2000). Although these experiments have not been performed with *E. arcuata*, we assumed that its colour perception would be similar.

Altogether we tested 200 spiders (68 males, 67 females and 65 juveniles of older instars, at least 3 mm long); each spider was tested only once. All spiders were collected in the meadows in Dalejské údolí, Prague (50°02′34.0″N, 14°21′26.1″E) between April and August 2010–2014. They were kept at 27 °C and under a natural light cycle (between 12:12 and 16:8 h light:dark) in transparent cylindrical plastic containers (70 mm tall, 30–33 mm in diameter) for at least 2 weeks before the experiments. After this period, the spiders can be considered quasinaïve, having forgotten all the potential prey preferences based on previous experience (Hill, 2006; Taylor et al., 2016). The spiders had access to water ad libitum and were fed twice a week with three to four micropterous fruit flies (*Drosophila melanogaster*); the number of fruit flies depended on the spiders' age and sex (adult females were fed more than males and juveniles). Each spider had been fed at least three times in captivity; the last feeding took place 1 week before the experiment to increase and standardize the foraging motivation of the spiders. We did not use females that laid eggs in captivity prior to the experiments.

Prey

We used the third-instar larvae of *P. apterus* (Heteroptera: Pyrrhocoridae) as prey. *Pyrrhocoris apterus* is a common, nearly pan-Palearctic true bug (Kerzhner, 2001; Moulet, 1995) feeding mainly on seeds of lime trees (*Tilia cordata*, *Tilia platyphylos*), other Malvaceae sensu stricto and black locust, *Robinia pseudacacia* (Fabaceae) (Kristenová, Exnerová, & Štys, 2011). The firebugs are mostly brachypterous and live on the ground under their host plants or upon them. The adults are about 7–12 mm long (Puchkov, 1974); the third-instar larvae are about 2–3 mm long. This size makes the third-instar larvae suitable prey for *E. arcuata*, as its highest capture rate is with prey that are 50–75% of its own size (Nentwig & Wissel, 1986). The red, wild-type firebug colour form has warning coloration (red-and-black); these colours are produced by high concentrations of red erythropterin (Bel, Porcar, Socha, Němec, & Ferre, 1997; Merlini & Nasini, 1966; Socha, 1993) and black melanin (Henke, 1924), respectively; other pteridines, such as xanthopterin and violapterin, can occur in this colour form as well (Krajíček et al., 2014; Socha, 1993). White (rather white-and-black) colour mutants differ from the natural colour form by the absence of erythropterin and low overall amount of pteridine pigments; yellow (rather yellow-and-black) colour mutants have a lower concentration of erythropterin and higher concentration of other pigments, especially xanthopterin (Bel et al., 1997; Socha, 1993). All three colour forms have melanized head, legs, wing lobes, antennae and vicinity of the dorsoabdominal scent gland openings, and partly melanized pronotum. In characters other than colour, the white and yellow mutants do not differ from the wild-type red form (Svádlová et al., 2009). White and yellow mutants occasionally occur in nature but are very rare and these phenotypes do not persist in populations of *P. apterus* (Exnerová et al., 2006). Note that while the red and the yellow larvae were clearly conspicuous on the white filter paper used in the experimental arena (see below), the white larvae were partly cryptic.

Both larvae and adult firebugs are chemically protected. The defensive secretion of adults consists mostly of aldehydes, and the dominant chemicals in the larval secretion are oxoaldehydes and aldehydes (Farine, Bonnard, Brossut, & Le Quere, 1992). These chemicals are known to act as repellents and contact poisons to ants (Remold, 1963) and are toxic to mantids (Prudic, Noge, & Becerra, 2008). Also, adults of *P. apterus* are generally unpalatable for bird predators (e.g. Exnerová et al., 2003, 2006; Hotová Svádlová, Kopečková, Exnerová, & Štys, 2010; Wiklund & Järvi, 1982). The defensive secretion in larvae of all three colour forms of *P. apterus* contains mostly aldehydes and ketoaldehydes. The dominant compound is always 4-oxo-*trans*-2-octenal, with *trans*-2-octenal, *trans*-2-decenal and 4-oxo-*trans*-2-decenal occurring in lesser amounts (Farine, n.d.). The chemical secretion of all three colour forms is equally effective against bird predators (Exnerová et al., 2006; Svádlová et al., 2009).

The red, naturally coloured wild-type form of *P. apterus* was collected in Dalejské údolí (50^{0}02^{0}34.0^{0}N, 14^{0}21^{0}18.0^{0}E), Prague, in a different habitat from *E. arcuata*; we used F1eF3 laboratory-reared generations in the experiments. The white and yellow colour mutants were obtained from laboratory cultures of the Institute of Entomology, Academy of Sciences of the Czech Republic, České Budějovice. Bugs of each colour form were reared separately in 500e750 ml plastic containers at 23e27 ^{0}C and 14:10 h light:dark cycle. The bugs were fed on crushed seeds of small-leaved lime, *T. cordata*, and provided with water ad libitum.

Micropterous fruit flies, *D. melanogaster*, which we used as food prior to the experiments and as a palatable control prey during the experiments, were reared on nutritive substrate at 20e25 ^{0}C.

Experimental Design

The experiments consisted of three parts: avoidance-learning test, generalization test and memory test. Each of these parts consisted of a series of consecutive 10 min trials with 50 min intertrial intervals (see Skow & Jakob, 2006). In the first trial of the avoidance-learning test we tested the initial reaction of spiders to the prey presented. During each prey presentation, the spiders received a single prey individual. Recorded reactions of spiders included attack (spider bit or attempted to bite the prey) and contact (spider touched the prey with chemoreceptive organs on the front legs). If the spider captured the prey (i.e. held it by chelicerae for more than 2 min, killing and feeding on it), the trial was ended and the prey was removed to prevent the spider's satiation. Latency between the presentation of the prey and the first attack in each trial and prey activity level (percentage of time spent moving, see Taylor et al., 2014, 2016) were also recorded.

Before the experiments, the spiders were divided into four groups according to the prey presented in the avoidance-learning test: red-and-black ('red'), yellow-and-black ('yellow') and white-and-black ('white') firebugs (always third-instar larvae) and adult fruit flies as a control palatable prey. After the avoidance-learning test, the groups were further divided into subgroups according to the prey presented in the generalization and memory tests (see Table 1). The spiders were divided semirandomly, and each group and subgroup contained an equal proportion of male, female and juvenile spiders. The sample size in each subgroup was 25 spiders. The experiments were performed in petri dishes (90 mm diameter, 14e17 mm height) with white filter paper covering their bottom. A white environment was used in several recent studies (Hill, 2006; Skow & Jakob, 2006; Taylor et al., 2014, 2016) and we used it to prevent the effect of chromatic contrast.

The experiments were carried out under natural daylight, or daylight with an additional fluorescent tube (Philips Master PL-S 11W/840/2P) if necessary. The light source had no significant effect on the spiders' reactions (Wald test: attack rate: C^2_1 ¼ 2.45, *P* ¼ 0.118; capture rate: C^2_1 ¼ 0.004, *P* ¼ 0.949). We allowed the spiders to acclimate in the experimental arena for 1 h before the experiments, which always started between 0900 and 1000 hours CEST.

The experiments were filmed using digital cameras (Canon HG 20, Canon Legria FS 22) and the spiders' behaviour was recorded in Observer XT 8.0 (Noldus Information Technology, Wageningen, Netherlands).

Initial Reaction

The initial reactions of spiders towards firebugs of different colour forms were assessed in the first trial of the avoidance-learning test, when the spiders encountered the firebugs for the first time. If the spider did not attack or contact the prey within 10 min, the experiment was terminated, and the data were not used in the analyses. In this case, the whole experiment was repeated with a new spider, so the resulting number of spiders in each experimental group/subgroup remained the same (see Table 1).

Nearly all spiders (198 of 200) that passed the abovementioned criterion attacked (not only contacted) the prey, and therefore it was not possible to analyse differences in the attack rate during trial 1. The analysed variables of the initial reaction included latency of the first attack and the capture rate, which informed us about a possible existence of innate wariness associated with the prey's colour, or about different prey detectability. Two spiders that only contacted the prey during trial 1 were excluded from the analysis of latency of the first attack.

Avoidance-Learning Test

The avoidance-learning test consisted of a sequence of five consecutive 10 min trials, in which the spider was presented repeatedly with the same prey type according to its experimental group (Table 1), with 50 min intertrial intervals (see Skow & Jakob, 2006). We analysed a change in the attack rate towards the prey and the capture rate of the prey in each group to find out whether the spiders learned to avoid the particular prey. Moreover, we analysed differences in these changes between the groups to compare the effectiveness of avoidance learning.

Generalization Test

The generalization test followed 50 min after the avoidance-learning test. This interval was the same as the intertrial intervals of the avoidance-learning test, because we wanted to separate the effect of generalization from the possible effect of prey

Table 1
List of experimental groups and subgroups based on prey types

Group	Avoidance-learning test (day 1, trial 1e5)	Subgroup	Generalization test (day 1, trial 6e8)	Memory test (day 2, trial 9)
Group 1 (*N*¼75)	Red firebug	1a (*N*¼25)	Red firebug (control)[a]	Red firebug[b]
		1b (*N*¼25)	White firebug[b]	e
		1c (*N*¼25)	Yellow firebug[b]	e
Group 2 (*N*¼50)	White firebug	2a (*N*¼25)	White firebug (control)[a]	White firebug[b]
		2b (*N*¼25)	Red firebug[b]	e
Group 3 (*N*¼50)	Yellow firebug	3a (*N*¼25)	Yellow firebug (control)[a]	Yellow firebug[b]
		3b (*N*¼25)	Red firebug[b]	e
Group 4 (*N*¼25)	Fruit fly	4a (*N*¼25)	Fruit fly	Fruit fly[b]

Prey types: third-instar larvae of red, white and yellow colour forms of the firebug *Pyrrhocoris apterus* and adult fruit fly *Drosophila melanogaster*. *N* is the number of spiders used in the analyses. Subgroups that were offered the same firebug colour form during all three parts of the experiment were used as controls for the generalization test.

[a] Control subgroup to the generalization test.

[b] Followed by a control trial with a fruit fly.

memorability. Previous experimental data have shown that learned avoidance may fade in a matter of hours in jumping spiders (Hill, 2006).

The test consisted of three 10 min trials (trials 6e8) with 50 min intertrial intervals. The spiders were divided into subgroups and their prey was either altered or remained the same as in the avoidance-learning test (see Table 1). Only the spiders in which the prey was changed were used in the generalization test.

The reactions of spiders to a novel prey during the first trial of the generalization test were compared to their reactions to the prey previously encountered during the avoidance-learning test. Also, possible asymmetry in generalization was analysed in opposite prey combinations (e.g. the change from the red to the white firebug was opposite to the change from the white to the red firebug).

Memory Test

The memory test took place on day 2, and was carried out only with the spiders that were presented with the same prey during trials 1e5 (five trials of the avoidance-learning test) and trials 6e8 (control groups in three trials of the generalization test; see Table 1). After the last trial of the first day, the spiders were provided with a few drops of water and kept in the experimental arenas overnight. Next day, they received the same prey as on the previous day (trial 9); the trial took place approximately 24 h after the first trial of the first day. The change in the attack and capture rate between the trials of the first day and the first trial of the second day was used for comparing memorability of different prey types.

Control Trial

To test the possibility that the decreased frequency of attacks by spiders during the sequence of trials was due to satiation (see Skow & Jakob, 2006), running out of venom or other reasons not specific to the firebugs, we provided the spiders with control palatable prey (fruit fly, *D. melanogaster*). The control fruit fly was offered immediately after the end of the experiments (after the generalization test if the prey was altered, or after the memory test if the prey remained the same for the whole experimental test; see Table 1). If the spider did not capture the prey during the first 5 min of the control trial, we added another fruit fly for the next 5 min to exclude the possibility that this result was due to prey inactivity. If the spider did not attack the prey during the whole 10 min control trial, we did not use the data in the analyses and the whole experiment was later repeated with another spider, so the number of spiders in each group/subgroup remained the same. Ten spiders in total failed to attack the control prey and their data were not used in the analyses.

Data Analyses

The data were analysed in R 2.12.1 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and Statistica 8 (Statsoft, Tulsa, OK, U.S.A.). All the tests were two tailed, with the level of significance a ¼ 0.05. We refer to $0.05 < a < 0.1$ as a ‘nonsignificant trend‘.

In the analyses, dependent variables included the attack rate (the rate of spiders attacking the prey, binomial distribution), the capture rate (the rate of spiders capturing the prey, binomial distribution), the attack latency (the latency between the presentation of the prey and the first attack, normalized distribution) or the prey activity level (percentage of time spent moving, normalized distribution). The explanatory variables of models included, if applicable, trial number (factor), day of the trial (factor), prey type (factor), spider sex/life stage (factor; levels: male, female, juvenile) or spider life stage only (factor; levels: adult, juvenile), depending on an explanatory value for a particular model and/or prey activity level (covariate), and all possible two-level interactions between the variables. All generalized estimating equations (GEE) included individual spider in the model. Models were further reduced according to the Akaike informational criterion (linear models, generalized linear models) or quasi-informational criterion (generalized estimation equation) (Pekár & Brabec, 2016). We used spider sex and life stage as explanatory variables because these aspects have been shown to affect predatory behaviour by means of different predator/prey size ratios (highest in females, lowest in juveniles), nutritional demand (higher in females, lower in males (Givens, 1978; Taylor et al., 2014, 2016)), or level of exploratory and potentially risky behaviour (high in males, low in females; Jackson, 1979). We used the prey activity level as a covariate because jumping spiders are motion-oriented and higher prey activity elicits more intense predatory reaction (e.g. Bednarski, Taylor, & Jakob, 2012; Freed, 1984; Tarsitano & Jackson, 1992, 1994).

We performed analyses of the effect of light source (factor, levels: natural light, natural light with an additional fluorescent light tube) on the attack and capture rates. The models included these explanatory variables: trial, day, prey type, life stage, light and prey activity level, and all possible two-level interactions. The model was further reduced according to the quasi-informational criterion.

We performed an overall analysis of spiders’ attack rate and capture rate during all experimental trials to assess the effect of sex/life stage, life stage and the prey activity level, using GEE. Subsequently, we compared the activity level of true bug colour forms and control *D. melanogaster* prey, as it might explain potential differences in the spiders' approach towards different prey. The prey activity level was logit transformed to fit a normal distribution and analysed using a linear model (LM) with the prey type as an explanatory variable.

We compared the rate of spiders succeeding (i.e. attacking or contacting the prey) during trial 1 to estimate the level of spiders' innate bias towards different prey. A low rate of success would suggest the presence of an innate bias, but this test was not fully representative, as it could reflect some other aspect (e.g. low feeding motivation of a particular spider). We conducted the analysis using a generalized linear model (GLM) and the prey type as a fixed factor. To compare the initial reactions of the spiders to different prey types, we also analysed the attack latencies and the capture rates in the first trial of the avoidance-learning sequence. Shorter latencies would reflect either an innate bias towards a certain prey type or better prey detectability. A lower capture rate during the initial reaction would suggest more careful approach towards a certain prey type, which might be a possible effect of innate bias against the prey. The first-attack latencies were log-transformed to fit a normal distribution and analysed with an LM; the capture rates were compared with a GLM.

For the analyses of avoidance learning, generalization and memory tests, we always used two dependent variables: the attack rate and the capture rate. They were analysed with a GEE model.

To assess the learning process during the avoidance-learning test, we analysed the reactions (attack rate, capture rate) of spiders towards presented prey during the whole avoidance-learning test to compare the level of protection of the firebug colour forms against spiders during the learning process. Owing to the bias caused by the design of trial 1 (exclusion of spiders not attacking or contacting the prey, see above), only trials 2e5 were used in comparison of the attack rates. Moreover, we used the learning curves (i.e. the effect of trial number on spiders' reactions) to compare the learning process between groups presented with different prey (i.e. interaction between the trial and prey type variables): the more rapidly a curve descended, the more effective the learning process was.

When we analysed the process of generalization, we compared the change in reactions of spiders when presented with a novel prey during the first trial of the generalization test (trial 6) to its reactions towards the previous prey during the avoidance-learning test (trials 1e5). The null hypothesis was that we would observe the same trend as in later trials of the avoidance-learning test (between trials 3e4 and 4e5), i.e. slow decrease in reactions towards firebugs. Then we tested the symmetry in generalization: we compared the change in reactions towards familiar (trials 1e5) and novel (trial 6) prey in opposite subgroups, e.g. during the switch from red to yellow colour forms and during the switch from yellow to red colour forms (i.e. interaction between subgroup and prey novelty (levels: familiar prey, novel prey)). If the change differed between the subgroups (i.e. the attack rate increased during a switch, but decreased during the opposite one), the generalization process was asymmetric, suggesting presence of a peak shift. We did not use data from trials 7e8 for the analyses of generalization, as they provided no additional information to results of previous analyses.

The spiders used in the memory test were those that were used as controls in the generalization test (trials 6e8), being presented with the same prey as in the avoidance-learning test (trials 1e5). Next day, we presented them with the same prey again and using day as a new explanatory variable, we compared the reaction during the first presentation of the prey on day 2 (trial 9) to those observed during previous trials (1e8). The null hypothesis was that the trend would be similar to that observed in late trials of the previous day (trials 6e8), i.e. slow decrease in reactions towards firebugs. After analysing the changes within each experimental subgroup, we compared them with each other (i.e. analysed interactions between day and prey type) to assess relative memorability of each firebug colour form. If reactions towards one firebug colour form increased more than towards other colour forms between days 1 and 2, the colour signal was easier to forget.

Ethical Note

No ethics approval was necessary for experiments with the tested species. We carefully handled the experimental animals and provided them with sufficient food and water. For 1 week after the experiments, we provided the spiders with *D. melanogaster* and water, and then released them back into the locality of their origin.

RESULTS

Twenty-four spiders (10.7%) did not attack or contact the prey during trial 1. This proportion was significantly lower in the experiments with the yellow form of the third-instar larvae of *P. apterus* (2%) than with the red form (16.7%; chi-square test: C_1^2 ¼ 8.804, *P* ¼ 0.003), and there was a similar, but nonsignificant trend when we compared the yellow and white forms (10.7%; chi-square test: C_1^2 ¼ 3.729, *P* ¼ 0.053). The difference in proportion of spiders that failed to attack or contact the red and white forms was not significant (chi-square test: C_1^2 ¼ 1.028, *P* ¼ 0.311). There was no significant difference in the number of spiders failing to attack or contact the prey between any of the firebug colour forms and the control fruit flies (7.4%; chi-square test: red: C_1^2 ¼ 1.626, *P* ¼ 0.202; white: C_1^2 ¼ 0.238, *P* ¼ 0.625; yellow: C_1^2 ¼ 1.329, *P* ¼ 0.249).

The results of the overall analysis of all experimental trials showed that higher prey activity level (i.e. percentage of time spent moving) significantly increased the chance that the prey would be attacked (Wald test: C_1^2 ¼ 32.537, *P* < 0.001) and captured (Wald test: C_1^2 ¼ 9.897, *P* ¼ 0.002). The initial model revealed no significant effect of spider sex or life stage on attack rate (Wald test: C_2^2 ¼ 2.714, *P* ¼ 0.257) or capture rate (Wald test: C_2^2 ¼ 3.046, *P* ¼ 0.218), and subsequent analyses of effects of life stage revealed that although the attack rates of adults (67.8%) and juveniles (72.8%) did not differ significantly (Wald test: C_1^2 ¼ 2.6, *P* ¼ 0.107), a nonsignificant trend could be observed in success of the attacks, as juveniles captured the prey less often (20.8%, compared to 24.8% in adults; Wald test: C_1^2 ¼ 2.873, *P* ¼ 0.09). We found no significant differences between the reactions of male and female adult spiders (attack rate: males: 67.5%; females: 68.1%; Wald test: C_1^2 < 0.114, *P* ¼ 0.736; capture rate: males: 24.6%; females: 25.1%; Wald test: C_1^2 ¼ 0.277, *P* ¼ 0.599).

Analysis of the overall prey activity level revealed a significant difference in activity between the firebug colour forms. Yellow firebugs moved around significantly less (median 49%) than the other two colour forms (red: median 69.3%; ANOVA: $F_{1,1048}$ ¼ 57.345, *P* < 0.001; white: median 68.2%; ANOVA: $F_{1,848}$ ¼ 33.228, *P* < 0.001). The difference between the red and white forms was not significant (ANOVA: $F_{1,1048}$ ¼ 1.328, *P* ¼ 0.25). The activity level of the control *D. melanogaster* prey (median 79.7%) was significantly higher than that of any firebug colour form (ANOVA: red: $F_{1,848}$ ¼ 15.347, *P* < 0.001; white: $F_{1,648}$ ¼ 20.44, *P* < 0.001; yellow: $F_{1,648}$ ¼ 97.103, *P* < 0.001).

Initial Reaction

We found a nonsignificantly different trend in first-attack latencies towards red (median 24.9 s) and yellow (median 35.6 s) colour forms (ANCOVA: $F_{1,119}$ ¼ 2.829, *P* ¼ 0.095). First-attack latencies towards the white form (median 32.8 s) did not differ significantly from those towards the other two forms (ANCOVA: red and white: $F_{1,122}$ ¼ 0.455, *P* ¼ 0.27; white and yellow: $F_{1,95}$ ¼ 0.186, *P* ¼ 0.668).

The spiders were more likely to capture white firebugs (52%) during the initial presentation, compared to the red (29.33%; chi-square test: C^2_1 ¼ 6.5, P ¼ 0.011) or yellow (28%; chi-square test: C^2_1 ¼ 6.07, P ¼ 0.014) ones. The difference between the capture rate of the red and the yellow firebugs was not significant (chi-square test: C^2_1 ¼ 0.026, P ¼ 0.872). All three firebug colour forms were captured significantly less often than control fruit flies (88%; chi-square test: red: C^2_1 ¼ 28.083, $P < 0.001$; white: C^2_1 ¼ 10.432; P ¼ 0.001; yellow: C^2_1 ¼ 26.211, $P < 0.001$; Fig. 1, trial 1).

Avoidance Learning

During the five trials of the avoidance-learning test, the firebugs of all three colour forms were attacked at a lower frequency than the control fruit flies (Wald test: red: C^2_1 ¼ 26.787, $P < 0.001$; white: C^2_1 ¼ 17.529, $P < 0.001$; yellow: C_1 ¼ 16.503, $P < 0.001$). The attack rate towards the red firebugs was lower than that towards the white (Wald test: C^2_1 ¼ 8.267, P ¼ 0.004) and the yellow (Wald test: C^2_1 ¼ 9.45, P ¼ 0.002) ones. There was no difference in the attack rate towards the white and the yellow firebugs (Wald test: C^2_1 ¼ 0.085, P ¼ 0.77; Fig. 1).

Spiders learned to avoid the firebugs, and their attack rate decreased significantly during the avoidance-learning test when tested with any of the colour forms (Wald test: red: C^2_1 ¼ 69.166, $P < 0.001$; white: C^2_1 ¼ 47.595, $P < 0.001$; yellow: C^2_1 ¼ 22.59, $P < 0.001$). There were no significant differences in the decrease in the attack rate (i.e. interactions between prey type and trial) towards different colour forms (Wald test: red and white: C^2_1 ¼ 0.551, P ¼ 0.458; red and yellow: C^2_1 ¼ 0.384, P ¼ 0.535; white and yellow: C^2_1 ¼ 1.527, P ¼ 0.217; Fig. 1).

The attack rate towards the control fruit flies remained high (Wald test: C^2_1 ¼ 1.785, P ¼ 0.182) and differed from that for all three firebug colour forms (Wald test: red: C^2_1 ¼ 26.536, $P < 0.001$; white: C^2_1 ¼ 17.404, $P < 0.001$; yellow: C^2_1 ¼ 16.493, $P < 0.001$; Fig. 1).

The spiders captured firebugs of all the colour forms less frequently than the control fruit flies (Wald test: red: C^2_1 ¼ 82.166, $P < 0.001$; white: C^2_1 ¼ 55.747, $P < 0.001$; yellow: C^2_1 ¼ 69.473, $P < 0.001$). The white firebugs were more likely to be captured than the yellow ones, but this trend was nonsignificant (Wald test: C^2_1 ¼ 3.765, P ¼ 0.052). Other differences were not significant (Wald test: red and white: C^2_1 ¼ 0.606, P ¼ 0.436; red and yellow: C^2_1 ¼ 1.741, P ¼ 0.187).

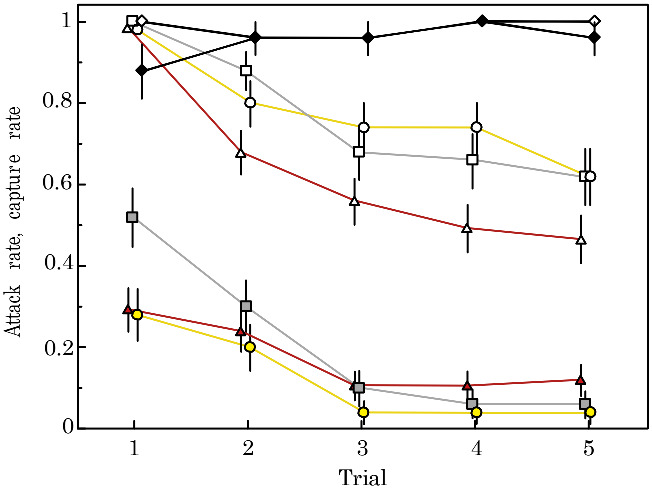

Figure 1. Spider attack rate towards the prey ± SE (open symbols) and capture rate of the prey ± SE (filled symbols) during the five trials of the learning test: triangles ¼ red firebugs (N ¼ 75), squares ¼ white firebugs (N ¼ 50), circles ¼ yellow firebugs (N ¼ 50), diamonds ¼ fruit flies (N ¼ 25).

During the five trials of the avoidance-learning test, the proportion of spiders capturing the firebugs decreased significantly (Wald test: red: C^2_1 ¼ 12.072, $P < 0.001$; white: C^2_1 ¼ 29.294, $P < 0.001$; yellow: C^2_1 ¼ 12.661, $P < 0.001$). The decrease in the capture rate was more gradual for the red form than for the other colour forms: the difference was significant when compared to the white form (Wald test: C^2_1 ¼ 7.301, P ¼ 0.007) and the trend was nonsignificantly different when compared to the yellow form (Wald test: C^2_1 ¼ 2.891, P ¼ 0.089). The difference in the decrease in the capture rate between the white and the yellow firebugs was not significant (Wald test: C^2_1 ¼ 0.341, P ¼ 0.559; Fig. 1).

In spiders tested with the fruit flies, the capture rate remained high during the avoidance-learning sequence (Wald test: C^2_1 ¼ 1.515, P ¼ 0.218). This result differed significantly from the decreasing tendency found in spiders tested with any of the firebug colour forms (Wald test: red: C^2_1 ¼ 5.333, P ¼ 0.021; white: C^2_1 ¼ 10.978, $P < 0.001$; yellow: C^2_1 ¼ 9.562, $P < 0.001$; Fig. 1).

Generalization

After the avoidance-learning test, we changed the colour form of firebugs in some experimental subgroups and compared the reactions of the spiders encountering the novel prey with their reactions towards previous prey during the avoidance-learning test. When we replaced the white colour form with the red one, the attack rate continued to decrease, not differing from the tendency observed during the avoidance-learning test (Wald test: C^2_1 ¼ 0.006, P ¼ 0.938). When we switched from the red colour form to the white one, however, the proportion of attacking spiders increased, and therefore differed significantly from the decreasing tendency observed during the avoidance-learning test (Wald test: C^2_1 ¼ 9.894, P ¼ 0.002). A similar response was observed when the spiders were presented with the yellow and the red firebugs: when we switched from the yellow to the red colour form, the attack rate kept decreasing (Wald test: C^2_1 ¼ 0.856, P ¼ 0.355), but when the prey colour was switched from red to yellow, the attack rate increased (Wald test: C^2_1 ¼ 10.503, P ¼ 0.001).

The spiders' ability to generalize different colour forms was asymmetrical both in red-white (Wald test: C^2_1 ¼ 7.334, P ¼ 0.007) and red-yellow combinations (Wald test: C^2_1 ¼ 14.458, $P < 0.001$; Figs. 2 and 3). In both cases, it was caused by a decrease in the attack rate when a red firebug was presented as a novel prey and by an increase in the attack rate when white and yellow colour forms were presented as a novel prey after the red colour form.

The rate of spiders capturing the prey increased significantly between the learning test and the first trial of the generalization test in most colour combinations, and this response differed significantly from the decreasing tendency observed during the avoidance-learning test (Wald test: red to white: C^2_1 ¼ 6.061, P ¼ 0.014; white to red: C^2_1 ¼ 6.5, P ¼ 0.011; yellow to red: C^2_1 ¼ 5.446, P ¼ 0.02). The only combination with no significant change in the capture rate was red to yellow (Wald test: C^2_1 ¼ 0.047, P ¼ 0.828).

Comparison of the capture rates between opposite subgroups (red-white and white-red, red-yellow and yellow-red) showed no significant asymmetry in generalization between red and white (Wald test: C^2_1 ¼ 0.558, P ¼ 0.455) and red and yellow (Wald test: C^2_1 ¼ 0.514, P ¼ 0.473) colour forms (Figs. 2 and 3).

Memory

The proportion of attacking spiders increased significantly between day 1 and day 2 towards all firebug colour forms (Wald test:

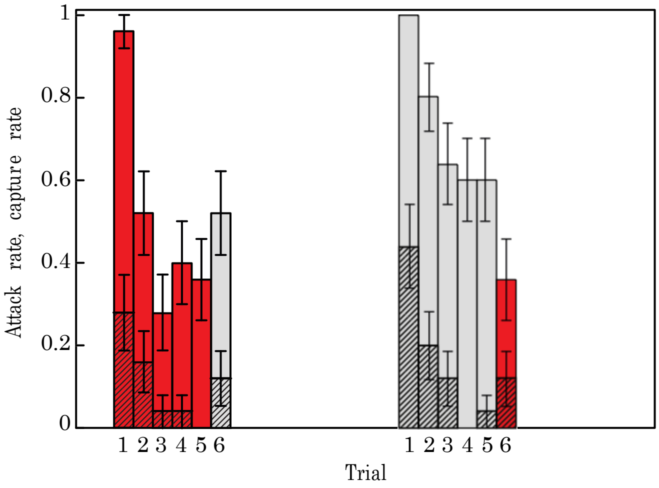

Figure 2. Spider attack rate (open bars) and capture rate of the prey (hatched bars) of the subgroup of spiders presented with red (red bars) versus white (grey bars) firebug colour forms. Either red or white prey were provided in trials 1e5 (avoidance-learning test) and then the prey were switched to the other colour in trial 6 (the first trial of the generalization test). The sample size in each subgroup was 25 spiders.

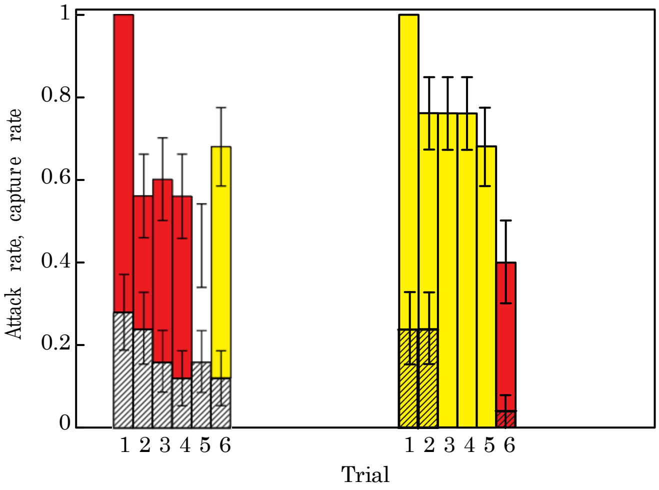

Figure 3. Spider attack rate (open bars) and capture rate of the prey (hatched bars) of the subgroup of spiders presented with red (red bars) versus yellow (yellow bars) firebug colour forms. Either red or yellow prey were provided in trials 1e5 (avoidance-learning test) and then the prey were switched to the other colour in trial 6 (the first trial of the generalization test). The sample size in each subgroup was 25 spiders.

red: C^2_1 ¼ 19.331, $P < 0.001$; white: C^2_1 ¼ 12.801, $P < 0.001$; yellow: C^2_1 ¼ 10.491, P ¼ 0.001). There were no differences (i.e. interactions between prey type and day) in the increase in the attack rate between spiders tested with different firebug colour forms (Wald test: red and white: C^2_1 ¼ 0.483, P ¼ 0.487; red and yellow: C^2_1 ¼ 0.974, P ¼ 0.324; white and yellow: C^2_1 ¼ 0.024, P ¼ 0.878; Fig. 4).

The capture rate increased significantly between day 1 and day 2 only when the white firebugs were presented as a prey (Wald test: C^2_1 ¼ 19.001, $P < 0.001$). In the other two colour forms, there was either a nonsignificant trend in the increase in the capture rate, as with red firebugs (Wald test: C^2_1 ¼ 3.67, P ¼ 0.055), or no significant change, as with yellow firebugs (Wald test: C^2_1 ¼ 2.34, P ¼ 0.126). The increase was most noticeable in the white colour form, compared to the red one (Wald test: C^2_1 ¼ 10.692, P ¼ 0.001) and (with a nonsignificant trend) to the yellow one (Wald test: C^2_1 ¼ 2.754, P ¼ 0.097). The increase in the rate of spiders capturing the red and the yellow firebugs did not differ significantly (Wald test: C^2_1 ¼ 0.66, P ¼ 0.416; Fig. 4).

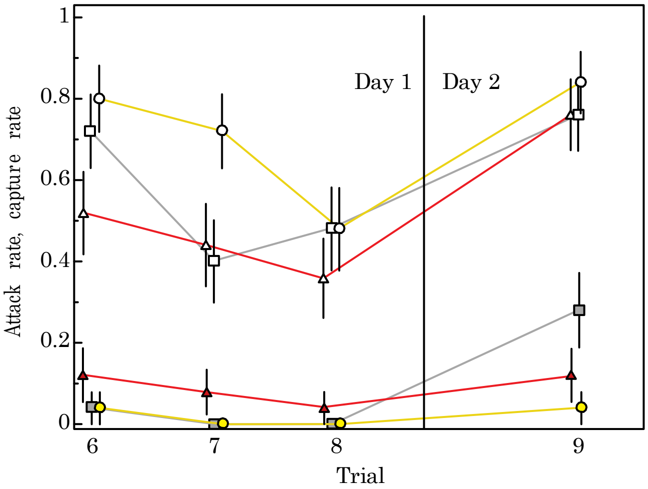

Figure 4. Spider attack rate ± SE (open symbols) and capture rate ± SE (filled symbols) during trials 6e8 on day 1 and trial 9 on day 2: triangles ¼ red firebugs ($N = 25$), squares ¼ white firebugs (N ¼ 25), circles ¼ yellow firebugs (N ¼ 25).

DISCUSSION

Our results show that the colour of an unpalatable prey affects the reactions of jumping spiders. According to the current theory of aposematism (reviewed in Ruxton et al., 2004), aposematic signals trigger unlearned avoidance, decrease the attack rate and prey mortality during avoidance learning, are advantageous during generalization from a weaker signal, and increase memorability. These effects were generally most prominent when the red colour form of *P. apterus* was presented to the spiders. Yellow coloration had an intermediate effect on the spiders, and white coloration was the least effective signal among the three presented types.

The spiders‘ reactions were also strongly affected by the intensity of prey movement: the more the prey moved around, the more likely it was to be attacked and captured. This effect has been observed in previous studies with jumping spiders and live prey (e.g. Bednarski et al., 2012; Freed, 1984; Tarsitano & Jackson, 1992, 1994). Some studies, however, have used immobilized prey instead (Hill, 1979; Jackson & Tarsitano, 1993; see Jackson & Pollard, 1996 for review). For example, the rate of jumping spiders attacking motionless fruit flies was only 6e24% (Jackson & Tarsitano, 1993); in our study, when live fruit flies were used as a control prey, almost all spiders attacked the prey. The use of living prey is therefore suggested for future studies on predatory behaviour of jumping spiders, as it more accurately reflects the perception of the prey by spiders.

The activity level differed between the firebug colour forms, as the yellow form moved significantly less than the other ones. Because the more the prey moved, the more likely it was to be attacked and captured, we would expect the yellow form to be partially protected by its lower activity. However, the reactions towards the yellow form usually did not differ significantly from more than one colour form, and the reactions towards it could therefore be considered intermediate. In a single case the reactions did differ from both other colour forms (percentage of spiders attacking or contacting the prey during trial 1), but this result was opposite to the above-stated prediction.

The effect of sex and/or life stage of spiders on their predatory reactions was not significant. The results might be affected by generally bigger adult females having more prey before the experiments; nevertheless, the results do not support the traditional point of view that male jumping spiders are less suitable for foraging experiments (Jackson & Pollard, 1996) which has even resulted in exclusion of males from experiments (e.g. Hill, 2006; Jackson, Pollard, Li, & Fijn, 2002; Nentwig, 1986; Nentwig & Wissel, 1986). During an experiment by Taylor et al. (2014), the wild-caught females, some of which were mated, were more responsive to the presented prey than males or juveniles; this effect was not observed in the laboratory-reared virgin females. No females used in our experiments laid eggs in captivity, so this effect could not be verified.

Initial Reaction

Previous studies using different predator species revealed two contradictory effects of prey coloration on the predator's initial reaction. Potential innate biases should reduce predatory reactions towards an aposematic prey (e.g. Hauglund et al., 2006; Schuler & Hesse, 1985). On the other hand, aposematic coloration is usually conspicuous, and predators are therefore able to detect (and possibly attack) aposematic prey sooner than the cryptic one (Fabricant & Herberstein, 2015; Roper & Wistow, 1986).

The majority of spiders in our experiments attacked or contacted the prey during the first encounter. This suggests that *E. arcuata* probably does not possess any strong innate bias against the firebugs of any of the three colour forms. Furthermore, yellow firebugs were attacked or contacted more often than the red and white ones when presented to the spiders for the first time. This suggests an innate preference for yellow prey. This result would be contradictory to those found in previous experiments with jumping spiders (Taylor et al., 2014) as well as birds (Hauglund et al., 2006), in which predators showed innate bias against yellow prey. It is therefore possible that the reaction could be affected by the similarity between the yellow firebugs and some yellowish brown palatable prey preferred by *E. arcuata* (such as aphids, psyllids and true bugs, *Plagiognathus* sp., Miridae; Nentwig, 1986). Another possibility is that the spiders generalized the yellow form with the palatable *D. melanogaster* they were fed prior to the experiments.

The analyses of first-attack latencies showed no significant differences in reactions towards the three firebug colour forms, and therefore did not support the prediction of shorter latencies towards the red, presumably the most conspicuous form, and longer latencies towards the white, the least conspicuous form. An alternative hypothesis, which suggests an opposite result due to the presence of an innate bias, was also not supported by these results.

The white form suffered from higher mortality than the other colour forms during the first encounter with spiders (Fig. 1, trial 1). The difference found in the capture rate but not in the tendency to attack can be explained via the ‘go-slow’ signalling theory (Guilford, 1994). According to this theory, the aposematic prey may not only be more likely to be avoided by predators but also handled more carefully if they decide to attack it. Although this theory was designed to explain the phenomenon of automimicry, it can also explain the predator's behaviour during encounters with a novel prey. Our results therefore suggest the presence of an innate bias, but on a different level than hypothesized. All colour forms may be attacked at a high rate, but the conspicuous red and yellow ones may be handled more carefully and therefore suffer from lower mortality than the less conspicuous white form.

The different approach towards the white colour form than to the other two may be caused by innate bias (e.g. Hauglund et al., 2006; Roper, 1990; Schuler & Hesse, 1985) against certain prey colours, especially red and yellow (Hauglund et al., 2006; Roper, 1990; Schuler & Hesse, 1985; Taylor et al., 2014), or against their higher contrast against the background (Roper & Wistow, 1986). However, some other mechanisms with no effect of aposematism or of any bias may affect the predator's reaction during the first encounter. For instance, a white colour with minor black elements may make the white form appear smaller. Jumping spiders generally approach small prey with less caution (Jackson & Pollard, 1996, and references therein), which may be why they rejected the white unpalatable bugs less than the red or yellow ones after attacking them.

Avoidance Learning

During the five avoidance learning trials, the spiders attacked the red colour form less frequently than the other two, presumably less conspicuous ones (Fig. 1). These results agree with the theory that conspicuous coloration affects avoidance learning. This has repeatedly been supported by experimental studies with vertebrate predators (e.g. Gittleman & Harvey, 1980; Hauglund et al., 2006; Roper & Wistow, 1986; Sillén-Tullberg, 1985), but our results suggest the occurrence of the same phenomenon in an arthropod predator.

Although the attack rate was lower towards the red form than towards the other two colour forms, the decrease in the attack rate was not significant. The likely reason for this discrepancy is that spiders learned to avoid the red form faster than the other two colour forms. The attack rate towards red bugs decreased rapidly, but the decrease slowed down and the learning curve became parallel to those of the other colour forms (Fig. 1). Still, this rapid decrease alone was enough to create a significant difference in the overall attack rate. Red and yellow are generally considered to be aposematic colours (Cott, 1940), but some studies of aposematism successfully used other colours, such as green and purple (e.g. Gittleman & Harvey, 1980; Roper, 1994), or contrast against the background (Prudic et al., 2007; Roper & Wistow, 1986). Also, each predator species may have different sensitivity to different aposematic signals. An extreme model predator is a colour-blind mantis, which learned to avoid unpalatable prey faster when it contrasted with the background; the discrimination cue was luminance contrast (Prudic et al., 2007). Further research would be needed to investigate the effect of colour and contrast on the response of *E. arcuata*. As only a uniform background (white filter paper) was used in this study, our data do not provide any pertinent information.

The decrease in the capture rate seemed to be steeper when the white form was presented than when the red form was presented (Fig. 1). This was probably because spiders captured white bugs more often during the first presentation of the prey, and therefore had more intense initial experience with them. Nonsignificant trends involving differences in the capture rate were probably caused by low absolute numbers of spiders capturing the firebugs.

It is intriguing that while the capture rate of *P. apterus* decreased rapidly during learning, the attack rate was higher than 50% in all the colour forms even in trial 5. In other words, although many spiders attacked the bugs even after several encounters, their attacks were seldom fatal to the prey. This suggests that there are two distinct steps on which the spiders assess the palatability of their prey, each with different dynamics.

Previous studies suggest that if there is a possibility of encountering Batesian mimics (including automimics), predators tend to ‘go slow’ (Guilford, 1994) and sample seemingly dangerous prey before consuming it (e.g. Gamberale-Stille & Guilford, 2004; Järvi et al., 1981; Skelhorn & Rowe, 2006). This strategy is more likely to occur when it is not very costly to the predator (Guilford, 1994), which is probably the case during an encounter with relatively harmless *P. apterus*.

Generalization

Our results show that the generalization was asymmetrical between the red form and the other two colour forms. Comparison of the overall attack rates showed that generalization worked better towards the red, presumably more conspicuous form; this effect was consistent in both color combinations, red/white and red/yellow (Figs. 2 and 3). These results agree with theoretical predictions (Leimar, Enquist, & Sillen-Tullberg, 1986) as well as with experimental observations. In experiments on generalization between unpalatable prey, bird predators were more successful when they generalized towards more conspicuous prey: either larger (Gamberale & Tullberg, 1996a, 1996b), with more intense hue (Gamberale-Stille & Tullberg, 1999), or with more conspicuous colour (Svádová et al., 2009; but see Ham et al., 2006).

When we presented the red colour form as a novel prey, the attack rate towards it even decreased, compared to the reactions to the previously presented colour forms. These results indicate the presence of a peak shift towards the red coloration during generalization (ten Cate & Rowe, 2007), possibly based on its higher contrast against the background, longer wavelengths or lower resemblance to fruit flies (i.e. previously encountered positive stimulus). Peak shift is a widespread phenomenon observed both in vertebrates and in arthropods (e.g. Lynn, Cnaani, & Papaj, 2005), and our results suggest that this response towards aposematic stimuli may be similar across the various predator taxa.

An alternative explanation is the presence of a hidden colour bias, triggered by a previous negative experience with chemically defended prey. Hidden biases against novel or conspicuous prey colours are well documentad in avian predators (e.g. Kelly & Marples, 2004; Marples & Roper, 1996; Rowe & Guilford, 1996), and they may be elicited by chemical stimuli, both olfactory (Jetz, Rowe, & Guilford, 2001; Lindström, Rowe, & Guilford, 2001) and gustatory ones (Rowe & Skelhorn, 2005; Skelhorn, Griksaitis, & Rowe, 2008). For example, in Rowe and Skelhorn (2005) study, after experience with distasteful naturally coloured (brownish grey) seeds, domestic chicks were simultaneously presented with green and either yellow- or red-coloured distasteful seeds. Although all these colour forms were novel to them, chicks ate more green seeds than conspicuously coloured ones. Moreover, these results were more striking in green/red colour choice.

The ability of jumping spiders to generalize between differently coloured prey has not been tested before. However, they can generalize the avoidance of red, chemically protected *O. fasciatus* towards a novel red-coloured prey (crickets; Taylor et al., 2016). However, this generalization ability was dependent on several factors: for example, wild-caught spiders were not able to generalize between the prey types, and the spiders required the same environment for the training and the generalization task (Taylor et al., 2016).

Memory

Better memorability may be one of the effects of aposematic signals (Endler & Mappes, 2004; Guilford & Dawkins, 1991; Ruxton et al., 2004; Speed, 2000), but our results do not show clear support for this effect in jumping spiders. The attack rate increased significantly between days 1 and 2, reaching about 80% towards all colour forms (Fig. 4). This rate was similar to that observed by Hill (2006) in experiments testing the retention of aversive experience with aposematic prey in jumping spiders between 2 consecutive days. The white, less conspicuous colour form suffered from higher mortality than the other two colour forms. This may suggest either that the white coloration is less memorable, or that all spiders forgot the information to the same degree, and their approach towards the prey on day 2 was analogous to the initial reaction. The capture rate increased between days 1 and 2 only when the white colour form was presented (Fig. 4). In other words, spiders often attacked the red and the yellow firebugs on day 2, but rarely killed them. This behaviour would be in accordance with predictions of the go-slow theory which suggests that when predators sample ‘suspicious’ prey, they tend to attack it carefully, and often leave it unharmed (Guilford, 1994). The red and the yellow colour forms (but not the white one) may therefore be protected by means of individual selection.

Prey memorability may be enhanced by particular colours and patterns (Exnerová et al., 2008; Svádová et al., 2009; but see Ham et al., 2006) or contrast against the background (Alatalo & Mappes, 1996; Lindström, Alatalo, & Mappes, 1999; Roper, 1994; Roper & Redston, 1987). In an experiment with great tits, *Parus major*, the yellow colour form of *P. apterus* was less memorable than the red and brown ones when presented on a beige background (Svádová et al., 2009). Because we used white filter paper as a substrate during the experiments, higher mortality of the white colour form could have been caused by its lower contrast against the background, when compared to the other two colour forms.

Evolutionary Implications

As jumping spiders are unusual among arthropods in possessing tetrachromatic vision and complex cognitive abilities (see above), our results cannot be generalized to other predatory arthropod taxa, or even to other spider families. Still, they extend our concept of aposematism by checking its tenets on predators rarely used as models.

The great majority of hypotheses related to aposematism have been tested with bird predators, especially two species: the domestic chicken and great tit. The number of neurons in the central neural system of a great tit is over 2 000 000 (Olkowicz et al., 2016), which is about 2000 times more than the number found in the wandering spider, *Cupiennius salei* (Ctenidae; Babu & Barth, 1984). The spider umwelt is therefore considerably different from that of a bird, and probably much less complex. Although the cognitive abilities of jumping spiders and birds differ, their reactions towards aposematic signals are surprisingly similar. For example, the high effectiveness of the red warning coloration observed in our study (as well as in the study by Taylor et al., 2014) corresponds to its effectiveness in analogous studies carried out with birds (Rowe & Skelhorn, 2005; Svádová et al., 2009). This consistency in reactions of phylogenetically distant predators is advantageous for the prey as the variability in the reactions of predators is usually considered to be a limiting factor for the evolution and function of aposematic signals.

Fixation of a novel, originally rare aposematic signal in a population seems hardly possible as its bearers do not profit from the predators‘ learned avoidance (Mappes et al., 2005). Several hypotheses have been proposed to explain this apparent paradox: predator neophobia or innate bias, existence of a peak shift in generalization of aposematic signals, or similarity to an already known unprofitable prey (reviewed in Lindström, 1999; Mappes et al., 2005). Our results suggest that thanks to biased generalization, the novel red coloration may be advantageous for the prey even when the predator has previously encountered only other colour forms. The red colour form can then withstand the initial predatory pressure and persist in a population of yellow and white bugs. Red coloration is one of the most common aposematic signals, especially in true bugs (Hotová Svádová, Kopečková, Exnerová,

& Štys, 2010), and our findings suggest an explanation for the widespread occurrence of this warning signal.

Acknowledgments

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STUDY 2

Perception of olfactory aposematic signals by jumping spiders

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Perception of olfactory aposematic signals by jumping spiders

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Abstract

Studies of aposematism are mostly focused on visual aspects of warning signalization and based on experiments with avian predators. This study presents results of experiments with an arthropod predator, a jumping spider *Evarcha arcuata* (Araneae: Salticidae) and olfactory (i.e., noncontact chemical) aposematic signals. Spiders were presented with chemically protected firebug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). After acquiring the negative experience with the bugs, the spiders were offered a choice between the firebug olfactory signal and a scentless control. Spiders actively avoided the firebug signal, showing that they are able to recognize an unprofitable prey by means of olfactory chemoreception. The results show that olfactory signals alone may function as effective means of aposematic signalization to spiders.

KEYWORDS
aposematism, araneae, chemical defence, heteroptera, olfaction, salticidae

1 | INTRODUCTION

Olfaction, that is, noncontact chemoreception, is probably the least studied among five traditionally recognized senses in spiders (Uhl & Elias, 2011). The current hypothesis suggests that olfactory chemoreception is carried out mainly by tarsal organs shaped as small pits on the dorsal side of each tarsus (Ehn & Tichy, 1994). The sensitivity of these organs to airborne chemicals is assumed to be low (Foelix, 2011). Along with the tarsal organs, the contact chemoreceptive setae, situated mainly on distal parts of palps and legs, probably perceive some olfactory cues, especially pheromones (Foelix, 2011, Ganske & Uhl, 2018, and references therein).

The most common ecologically relevant use of olfactory cues is the pheromone-mediated intraspecific communication, especially sexual attraction, observed in species of many families (reviewed in Gaskett, 2007). Use of airborne prey signals during foraging seems to be relatively uncommon and has been mostly observed in context of search for prey by specialized predators (e.g., Allan, Elgar, & Capon, 1996; Jackson & Cross, 2015, and references therein). However, several studies have shown that even generalistic spiders are able to perceive and use olfactory cues from potential prey (Hostettler & Nentwig, 2006; Riechert, 1985).

If spiders respond to attractive prey olfactory cues, is it possible that they may also react to repellent ones? This hypothesis has not yet been supported. Recent study of reactions of the crab spider *Synema globosum* (F., 1775; Thomisidae) did not reveal any ability of the spiders to discriminate between the odour of protected wasps and bees and unprotected hoverflies (Morris & Reader, 2016). However, these species signal their unprofitability by means of visual or acoustic signals rather than by olfactory ones (Morris & Reader, 2016). A prey with more effective warning chemicals will be more suitable for testing the ability of spiders to perceive repellent olfactory signals.

We therefore carried out experiments with a chemically protected prey, third-instar larvae of the firebug *Pyrrhocoris apterus* (L. 1758), and a generalist, primarily visually oriented (Uhl & Elias, 2011) jumping spider predator, *Evarcha arcuata* (Clerck, 1757; Salticidae). The secretion of firebugs is primarily effective in contact chemical defence, but owing to its volatile compounds, it may also function as an airborne aposematic signal, especially to predators that had a previous experience with contacting the chemically defended bug. The aim of this study was to test whether *E. arcuata* is able to perceive and avoid olfactory signals of previously encountered firebugs.

2 | METHODS

2.1 | Spiders

Evarcha arcuata (Araneae: Salticidae) is a medium-sized (5–6 mm) spider with Palaearctic distribution (World Spider Catalog, 2018). It is a cursorial generalist predator feeding on small spiders and insects (Dobroruka, 1997; Nentwig, 1986).

Altogether, we tested 19 spiders (eight adult males, eight adult females, one subadult male and two juvenile males); each spider was tested only once. All spiders were collected in meadows in Dalejské údolí, Prague (50°2′37.343" N, 14°21′31.157" E) in late July 2015 and were used in the experiments in August 2015. The spiders were kept in captivity according to methods described in Raška, Štys, and Exnerová (2017).

2.2 | Prey

As a chemically defended prey, we used third-instar larvae of the firebug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Their body length of 2–3 mm (Puchkov, 1974) makes them a potential prey of these spiders (Nentwig & Wissel, 1986). *P. apterus* is an aposematic species, combining warning signalization (especially its red and black coloration) with unprofitability. The defensive secretion of firebug larvae mostly consists of ketoaldehydes and aldehydes (Farine, Bonnard, Brossut, & Quere, 1992), chemicals common in true bug defensive secretions (Blum, 1981). Secretions of third-instar larvae of *P. apterus* are effective defence against *E. arcuata*: The spiders attacked the bugs initially, but the prey mortality was low and the spiders gradually learned to avoid the bugs (Raška et al., 2017).

The firebugs used in the experiments were collected under linden trees in Botanic Garden of Charles University in Prague (50°04′20.5" N, 14°25′25.2" E) 1–5 days prior to the experiments. They were kept in 500-ml plastic containers at 23–27°C and 14:10 light:dark cycle, fed on crushed seeds of the small-leaved lime (*Tilia cordata* Miller, 1768) and provided with water ad libitum.

2.3 | Experimental design

2.3.1 | Pretraining

To ensure the spiders would have sufficient negative experience with the firebug secretion, they were offered third-instar larvae of *P. apterus* in a sequence of trials until they learned to avoid them. Each trial took 10 min; the intertrial interval was 20 min. Spiders which did not attack the firebugs during trial 1 or at least did not touch them with legs, on which the contact chemoreceptors are situated (Foelix & Chu-Wang, 1973), were excluded from the experiments. Remaining spiders were tested immediately after they refused to attack or touch the firebugs in two consecutive trials. If a spider kept attacking the firebugs after 10 trials, the pretraining was terminated and the spider was excluded from the test.

2.3.2 | Test

The test was based on the design by Cross and Jackson (2009, Experiment 2). It was performed in a cardboard arena eliminating unwanted visual stimuli, and equipped with a fluorescent tube (Osram L 18 W/965 Biolux) as the only light source, located above and in front of the olfactometer.

The glass Y-olfactometer (see Figure 1 and Cross & Jackson, 2009, 2010, for further details and the olfactometer terminology) consisted of a test arm and two "experimental" arms. The spider contained in a holding chamber was put into the test arm of the olfactometer. The test arm was 320 mm long, but the opening of the holding chamber was situated closer to the branching of the olfactometer at the beginning of the experiment, so the functional length of the test arm was the same as of the other two arms. Two parallel experimental chambers were connected to one arm of the olfactometer each. They were spheric rather than square (compare with Cross & Jackson, 2009, 2010) to enable more even air flow through the chamber. The volume of air pushed through each chamber was set to 500 ml/min and was regulated by a single flow

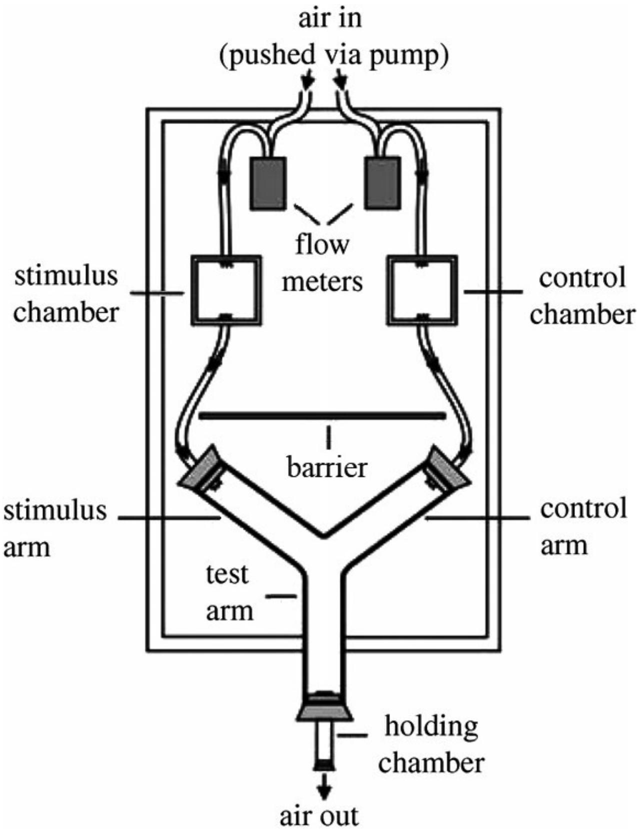

FIGURE 1 Olfactometer (not drawn to scale). Arrows indicate the direction of airflow (modified after Cross & Jackson, 2010). Stimulus and control chambers: 285 ml in volume. Stimulus, control and test arms: 90 mm long (functional length in the test arm), 22 mm diameter. Holding chamber: 10 mm long, 7 mm internal diameter. See the Methods section for further specifications and modifications of the original design

meter Omega FL 3803ST. A cardboard barrier between the chambers and the olfactometer ensured that the spiders could not see inside.

Chemicals of five freshly crushed third-instar firebug larvae on gauze placed in one experimental chamber were used as a stimulus and provided in one experimental chamber, while the control (clean gauze) was placed in the other. Left/right orientation of the set-up was changed after each trial, and the spiders were ordered randomly for the experiment.

A single 10-min trial was carried out with each spider during the test. The criterion for a preference was set to spending at least 30 s either in the arm with a stimulus or with a control (see e.g., Cárdenas, Jiroš, & Pekár, 2012; Cross & Jackson, 2009, 2010). The spider was free to move to the other arm of the olfactometer afterwards, but only the first choice was recorded. After each test, we cleaned the olfactometer consecutively with a common detergent, water and 96% ethanol, and dried it up in 170°C.

2.4 | Statistical analysis

The data were analysed in R 2.12.1 (R Foundation for Statistical Computing, 2010). The effect of spiders' preference of either the stimulus or the control was analysed by exact binomial test. The effect of the spiders' sex on their responsiveness was analysed by generalized linear model with binomial distribution (levels: 0—the spider was either not used in the test or did not choose any experimental arm of the olfactometer; 1—the spider was used in the test and chose one of the experimental arms). The statistical tests were two-tailed, with the level of significance $\alpha = 0.05$.

3 | RESULTS

Of 19 individuals, three spiders failed to attack the presented firebug during trial 1 of the pretraining, and two spiders failed to learn to avoid the firebugs during 10 trials of the session. Fourteen spiders that successfully learned to avoid the firebugs proceeded to the test.

When put into a Y-olfactometer with the stimulus in one arm and a control (clean gauze) in the other, two spiders did not choose any of them. Among the 12 spiders that entered one of the experimental arms, significantly more spiders preferred the control cue ($n = 10$) over the stimulus ($n = 2$; two-tailed binomial test, $p = 0.039$).

Only three of eight adult females successfully performed in both parts of the experiment (i.e., learned to avoid the firebugs and chose one of the experimental arms of the olfactometer), but all eight adult males succeeded in both tasks. The spiders' sex therefore had a significant effect on their responsiveness towards the stimuli during the experiment (chi-square test, $\chi^2_{(1)} = 9.29$, $p = 0.002$). The sample of juvenile spiders ($N = 3$) was too small to be comparable with those of adult spiders.

4 | DISCUSSION

Our study provides the evidence that jumping spiders are able to perceive olfactory warning signals from their potential prey. Jumping spiders do not seem to be exceptional among spiders when it comes to chemoreception (Gaskett, 2007), so it is quite likely that this ability is present also in other spider families. We set up a rather conservative experimental design by combination of prey (larvae of firebug *Pyrrhocoris apterus*) with known and effective chemical defences (Farine et al., 1992; Raška et al., 2017) and a jumping spider (*Evarcha arcuata*) able to learn to avoid firebug larvae after several encounters (Raška et al., 2017). Moreover, we used crushed bugs as a stimulus source during the experiments. Aside from the need of a stable source of semiochemicals during the experiments, we wanted to use a stimulus that would be at least as strong as (or even stronger than) the one that could be encountered by the spiders in nature. Sufficient intensity and consistency of stimuli are essential for an experimental design (Jakob & Long, 2016), and low intensity of prey signals might be the reason why spiders did not respond properly in previous experiments (Morris & Reader, 2016).

Higher responsiveness of adult male spiders to stimuli during the experiments as compared to females was surprising, as the females are usually considered more responsive in behavioural experiments and this assumption even resulted in exclusion of male spiders from some studies (e.g., Cross & Jackson, 2009; Li, Jackson, & Cutler, 1996; Nentwig, 1986). However, low activity of female jumping spiders has been observed in some previous experiments (Hoefler & Jakob, 2006; Taylor, Maier, Byrne, Amin, & Morehouse, 2014). Under natural conditions, male spiders are more likely than females and juveniles to leave the nest and explore. This has been explained by the males' need to search for females despite the higher exposure to predators and other risks associated with such behaviour (Jackson, 1979). Exploratory behaviour seems to be sexually dimorphic in jumping spiders, and the effect of sex should not be neglected in future studies.

Studies on aposematism focus mainly on visual signalling (Mappes, Marples, & Endler, 2005; Ruxton, Sherratt, & Speed, 2004). Visual cues are the most important source of information for birds (McFadden, 1993), the predators most frequently used in studies of aposematism (Ruxton et al., 2004). This bias is even stronger in jumping spiders, a primarily visually oriented group, and the role of olfaction in most aspects of their behaviour is unknown. Our study shows that olfactory chemoreception itself may be sufficient for their perception of aposematic signals. Ecological significance of such information is probably not critical in predatory behaviour of grass-dwelling *E. arcuata*. However, in spiders with different predatory strategies, especially in the night-active or web-building ones, the situation may be different. Aside from the need of involvement of more spider families, future studies should focus especially on the sensitivity of spiders to individual chemicals, the role of chemicals acquired from plants in defence of *P. apterus* and the correlation between jumping spider sex and/or stage and their performance in behavioural experiments.

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STUDY 3

Do ladybird spiders really mimic ladybird beetles?

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Do ladybird spiders really mimic ladybird beetles?

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Adult male ladybird spiders (*Eresus* spp., Araneae: Eresidae) possess conspicuous red and black coloration. As these spiders are palatable to many predators, they are likely to be Batesian mimics of some other, defended species. We therefore aimed to identify potential models of two ladybird spider species, the spring-breeding *Eresus moravicus* and the autumn-breeding *Eresus kollari*, by assessing the co-occurence and similarity of the spiders and their putative models. The abundance of potential models of *Eresus* spp. was monitored at nine sites where the spiders are found. Phenotypic similarity between the spiders and their potential models was assessed by comparing their colour, coloration pattern, and body size and shape. The ladybirds *Coccinella septempunctata* and *Hippodamia variegata* co-occurred with *Eresus* spp. at most sites during the whole season and were among the most abundant species found; the leafhopper *Cercopis sanguinolenta* was common only in spring and the stink bug *Graphosoma lineatum* only in late summer and early autumn. Analyses of similarity showed that coccinellids, including *Coccinella septempunctata*, were relatively similar to *Eresus* spp. We conclude that the seven-spot ladybird beetle *Coccinella septempunctata* is probably the key model of the ladybird spiders. Other taxa may serve as secondary models, further enhancing the spiders' defence against predators.

ADDITIONAL KEYWORDS: aposematism – Araneae – Batesian mimicry – Coccinellidae – *Eresus*.

INTRODUCTION

Prey that are distasteful, dangerous or otherwise unprofitable to their potential predators often advertise this fact by using warning (aposematic) signals that their predators can associate with unprofitability (Mappes *et al.*, 2005). The signals are often visual (e.g. colour, colour pattern, movement), but can also be manifested mechanically (vibrations, sounds) or chemically (characteristic odour or taste) (Ruxton *et al.*, 2004). The negative association between the signal and the sender's unprofitability is sometimes exploited by palatable prey that imitate signals of the defended prey. Adaptive resemblance between signals of a palatable mimic and a defended model is called Batesian mimicry (Bates, 1862; Mappes & Alatalo, 1997, and references therein).

Ladybird spiders (*Eresus* spp., Araneae: Eresidae) are believed to mimic chemically defended ladybird beetles, especially the seven-spotted ladybird (*Coccinella septempunctata* L. 1758, Coleoptera: Coccinellidae) (Cloudsley-Thompson, 1995). Their resemblance to ladybird spiders is sex- and stage-limited, however, in that it is only the adult males that are characterized by a bright red abdomen with four to six black spots (Fig. 1). Females and juveniles, in comparison, are darkish with no prominent colour pattern. The restriction of the mimicry to adult males is likely to reflect their behaviour of actively searching for females, while juveniles and adult females hide in their burrows (Řezáč *et al.*, 2008).When searching, male spiders are virtually defenceless and highly profitable prey (Cloudsley-Thompson, 1995). In fact, due to their high nutritional value, spiders are a common prey of many arthropod and vertebrate predators, such as birds, lizards, wasps and other spiders (e.g. Polis *et al.*, 1989; Gunnarsson, 2007; reviewed by Foelix, 2011).

Although it has been suggested that ladybird spiders mimic ladybird beetles (Cloudsley-Thompson, 1995), this hypothesis has never been verified. It is possible that the resemblance to ladybird beetles is

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Figure 1. Adult males of *Eresus moravicus* (A) and *E. kollari* (B).

perceived only by human observers, and is ecologically irrelevant (eye-of-the-beholder hypothesis, see Kikuchi & Pfennig, 2013). Ladybird spiders might therefore mimic a different model that has the widespread red–black aposematic coloration instead, for example some true bug species (Askins, 2002).

The key prerequisite for a mimetic relationship is co-occurrence of the model and the mimic. Models should be more abundant than their Batesian mimics, so the association between the warning signal and the model's unprofitability, which leads to their avoidance by predators, would not be disrupted by presence of the mimic ('relative abundance hypothesis': Wallace, 1867; Lindström *et al.*, 1997). Mimics should also tend to occur later in the season than their models, so that naive predators (especially fledgling birds) can learn to associate the warning signal with prey unprofitability ('phenological shift hypothesis': Waldbauer & Sheldon, 1971). This shift in phenology is advantageous for both the model (its aposematic signal is not disrupted by the presence of mimics) and the mimic (because predators have already learned to avoid the aposematic signal by the time the mimic emerges). In the temperate zone, this leads to a low abundance of Batesian mimics in summer and high abundance in late spring and early autumn (e.g. Waldbauer & Sheldon, 1971; Waldbauer, 1988; reviewed by Evans, 1990; but see Howarth & Edmunds, 2000). Indeed, males of central European ladybird spiders search for females for a short period either in spring or in late summer and early autumn. The former is the case for *Eresus moravicus* Řezáč 2008, which is most active in May, while the latter is the case for *Eresus kollari* (Rossi 1846), which is most active during September (NCA CR, 2018).

The effect of a mimetic signal on predators also depends on the degree of similarity between a model and its mimic. It is often difficult to specify this similarity, as the perception of similarity depends on the cognitive abilities of the predators involved. For instance, most arthropods lack red opsin and are therefore insensitive to the corresponding part of the light spectrum (reviewed by Osorio & Vorobyev, 2005), and some of them, such as mantises, may even be colour-blind (Barry *et al.*, 2015, and references therein). However, physiological constraints are not the only factors affecting the perception of prey signals – further cognitive processes can dramatically alter the way the perceived information is stored and applied. To filter out the specifics of observer perception, several studies have used objective methods to quantify similarity unaffected by observer perception (Cuthill & Bennett, 1993; Dittrich *et al.*, 1993; Pekár & Jarab, 2011). The results were similar, but not identical, to those obtained with pigeon and human observers (Dittrich *et al.*, 1993).

Here we aimed to identify the potential model of adult males of *Eresus* spp. and to test the relative abundance and phenological shift hypotheses. We focused on two central European ladybird spider species (Fig. 1), the spring breeder *E. moravicus* and the autumn breeder *E. kollari* (together referred to hereafter as *Eresus* spp.). First, we performed a field study on the occurrence and abundance of *Eresus* spiders and their potential models. We then measured the phenotypic resemblance of *Eresus* spiders to potential models by using a series of objective methods that focused on size, shape, colour pattern and colour reflectance.

MATERIAL AND METHODS

We studied the association of *Eresus* spp. with their potential models at nine sites in southern Moravia where the spiders occur: Stránská skála, Brno (49°11′25″N, 16°40′30″E), Svatý kopeček, Mikulov (48°48′22″N, 16°38′47″E), and Pouzdřanská step, Pouzdřany (48°56′33″N, 16°38′34″E), where *E. kollari* occurs; Čebínský kopec, Čebín (49°18′12″N, 16°29′04″E), Ve Stráních, Dolní Kounice (49°04′12″N, 16°28′23″E), and Květnice, Tišnov (49°21′10″N 16°25′13″E), where *E. moravicus* occurs; and Váté Písky, Bzenec (48°55′52″N, 17°16′45″E), Mohelenská hadcová step, Mohelno (49°06′18″N, 16°11′24″E), and Děvín, Pavlov (48°52′01″N 16°39′08″E), where both species occur.

Sampling

To obtain data on the seasonal occurrence of potential models of *Eresus* spp., for assessment of the ecological significance of the models to the spider, we conducted surveys at each study site once a month: in late August and September 2016, and in late April, May, June and July 2017. Central European arthropods are mostly univoltine and occur for longer than a few weeks, so this sampling rate was sufficient to record phenological shifts of the study species.

To study the relative abundances of mimics and models at the study sites, we used individual sampling and net sampling methods. We sampled all potentially aposematic species with a dorsal red/black colour pattern and an average body length of between 4 and 20 mm, i.e. very roughly of a similar size to *Eresus* spp. A single collector (J.R.) collected the specimens on sunny days, in an area of *c.* 0.4 ± 0.1 ha, during a 2-h period between 09.00 and 15.00 h CEST, i.e. when the spiders are active on the soil surface. Within this period, we conducted six sampling sessions during each survey – four individual samplings (10 min each) of arthropods occurring on the ground and vegetation up to a height of 120 cm, and two net samplings (net 350 mm in diameter, 3 × 50 sweeps per sampling, about 10 min each) of arthropods occurring on vegetation up to a height of 80 cm.

Phenotypic Similarity

To obtain data for analyses of the visual similarity of putative models of *Eresus* spp., we photographed 3–5 specimens of each species. If both larvae and adults of a single species were aposematic, or if a species had two distinct colour forms, we took pictures of both stages/forms and they were treated as separate models in the analyses. If species were indistinguishable by eye, such as imagoes of *Zygaena purpuralis* (Brünnich 1763) and *Z. minos* (Denis & Schiffermüller, 1775) or aposematic lygaeid larvae, they were treated as a single model. Additionally, we included a common wolf spider, *Pardosa* sp. (Araneae: Lycosidae), as a non-mimetic control.

Individuals were killed with ethyl acetate and images were taken less than 1 h later. If the colouration of a specimen was altered by ethyl acetate [e.g. in Coccinellidae, *Corizus hyoscyami* (L. 1758)], we killed it by freezing at −30 °C for around 10 min. We mounted freshly killed specimens on a glass plate using sticky tape in order to hold their natural body posture.

We photographed the specimens using an Olympus SC50 camera installed on an Olympus X12 stereomicroscope, which was lit from the anterior and posterior sides with fluorescent bulbs (13-W daylight Repti Glo 2.0 UVB) with a light spectrum similar to natural light. We took images by means of Stream Motion 1.9.4 image analysis software (Olympus Soft Imaging Solutions, 2014), which produces composite fully focused images.

We then analysed the images by means of custom-made image analysis software (Ježek, 2015) to obtain data on body shape and size and dorsal colour pattern. When assessing body shape and size, we straightened the image of each specimen according to the axis of the body, measured the length of the body axis, placed 40 evenly distributed points along the body axis relative to the body length of each specimen, and measured the distance from each of these points to the body edge (without legs, which are not visible from above in some models). We then estimated the local binary pattern (LBP, i.e. differences between nearby pixels after conversion to greyscale) (Ojala *et al.*, 2002) to measure the colour pattern separately for head and thorax/cephalothorax and abdomen.

After the specimen was photographed, we measured its reflectance by means of a USB4000 spectrometer with a PX-2 pulsed xenon light source (Ocean Optics, Largo, FL, USA) emitting light from 220 to 750 nm. The read probe was at a 45° angle to the specimen and 9 mm from the specimen. We used STAN-SSH (Pixelteq, Largo, FL, USA) as a high-reflectance standard and the background of specimens (black polypropylene adhesive tape) as a low-reflectance standard. We took three measurements of each specimen from the anterior, posterior and lateral sides and recorded average values.

Data analySeS

Overall, we found three individuals of *E. kollari* (two in August, one in September) and four individuals of *E. moravicus* (two in April, two in May); the overall abundance threshold (i.e. when the abundance of a potential model could be considered as equal to or greater than that of the mimic) for the use of a potential model in principal component analysis from the package vegan (Oksanen *et al.*, 2013) was therefore set to four. According to this criterion, 24 models (along with both *Eresus* spp. and a non-mimetic control *Pardosa* sp.) were used in the analyses (Supporting Information, Fig. S1).

We compared model occurrence (binaries) and abundances (counts) between April and May (for localities of *E. moravicus*) and between August and September (for localities of *E. kollari*) by means of a generalized linear model (GLM). The linear predictor included mimetic model, site, month and all interactions between these variables. We assumed a binomial distribution (GLM-b) for occurrence data and a negative binomial distribution (GLM-nb) for counts because the data showed strong overdispersion when fitted with a Poisson distribution (Pekár & Brabec, 2016). We consequently reduced the linear predictor of models the using Akaike's information criterion (AIC).

We analysed similar morphometric variables among 127 individuals (3–5 individuals for each model) separately by means of multivariate methods in order to reduce multidimensional variation to two dimensions. Specifically, we carried out three separate principal components analyses (PCAs) of the following variables: (1) body shape and length (40 measures of shape and one measure of length); (2) local binary pattern (20 measures of the head and thorax/cephalothorax and abdomen); and (3) colour reflectance (90 measures from 300 to 750 nm). The former two variables were based on analyses of images, while the third was based on measurements obtained by a spectrophotometer. Body shape and length and local binary pattern were column-scaled. PCA was used as an indirect ordination method because the gradient of the first two axes was low (Lepš & Šmilauer, 2003). We then took PCA scores for each individual along the first two axes from each separate PCA and used them in the final PCA. Therefore, six variables in total (row-scaled) were subjected to the final PCA.

When producing figures depicting the results of PCAs (see Fig. 5), we used median values for the model in order to avoid a large overlap of points if all data points were used. We performed all analyses in R 3.3.1 (R Core Team, 2017).

RESULTS

Occurrence

We found 36 potential models of *Eresus* spp. at sites of their occurrence (Table S1). The models most commonly belonged to the beetle families Chrysomelidae ($N = 9$) and Coccinellidae ($N = 8$). Of the 36 potential models, 24 occurred either a month before or during the main period of emergence of adult spider males.

During April, before the peak of emergence of *E. moravicus* males, *Coccinella septempunctata* was the most frequent model and was found at five of the six study sites where this spider occurred; during May, when the spiders usually emerge, *Coccinella septempunctata* occurred at all six sites. Other potential models found frequently at the sites of *E. moravicus* in spring were *Hippodamia variegata* Goeze 1777 and *Cercopis sanguinolenta* (Scopoli 1763); the latter model species occurred only in May. Other models were found only at one or occasionally at two sites (Fig. 2A). Model species composition at the studied sites was significantly different in April and May (GLM-b, $\chi^2_1 = 119.82$, $P = 0.001$) – in May, almost twice as many models per site were found than in April.

In late summer and early autumn, *Coccinella septempunctata* was found at all six studied localities

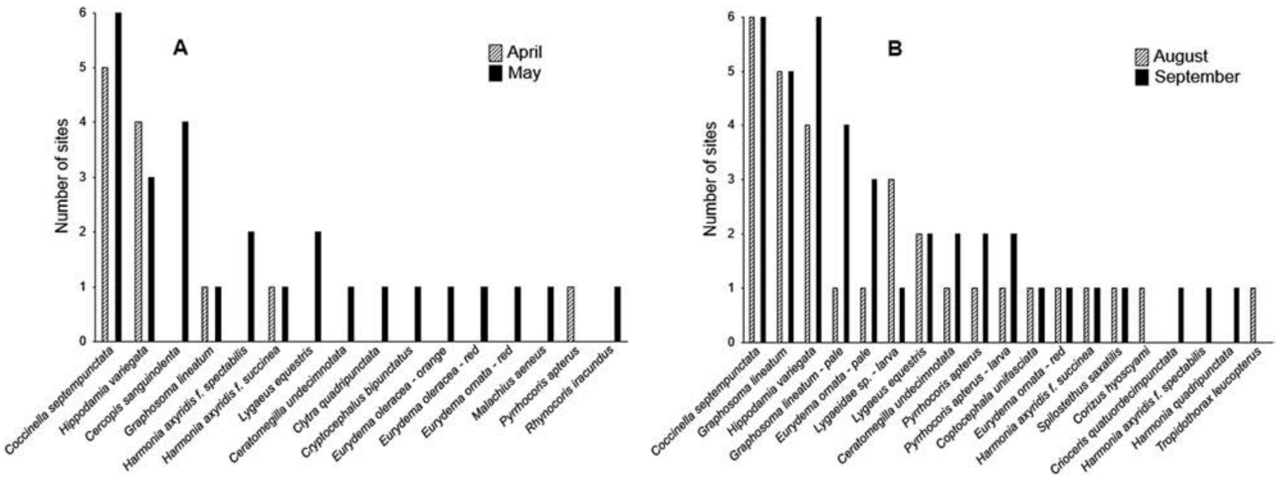

Figure 2. Number of studied sites (out of six) where potential models co-occurred with *Eresus moravicus* in April and May (A) and with *Eresus kollari* in August and September (B).

of *E. kollari* both in August (before the main period of emergence of *E. kollari* males) and in September (when the spider males usually emerge). Only the red colour form of *Graphosoma lineatum* (L. 1758) and *Hippodamia variegata* occurred at most studied sites, and except for *Ceratomegilla undecimnotata* and adults and larvae of *Lygaeus equestris* (L. 1758) and *Pyrrhocoris apterus* (L. 1758), no other potential model was found at more than one site either in August or in September (Fig. 2B). Slightly more potential models per site were found in September than in August, but the difference in species composition was not significant (GLM-b, $\chi^2_1 = 180.99$, $P = 0.095$).

Abundance

We collected 6346 individuals of potential models of *Eresus* spp. at the study sites. Most of the collected specimens belonged to seven families of three orders: (1) Hemiptera: Cicadomorpha: Cercopidae, Heteroptera: Pentatomidae, Lygaeidae, Pyrrhocoridae; (2) Coleoptera: Coccinellidae, Chrysomelidae; and (3) Lepidoptera: Zygaenidae.

At localities of *E. moravicus*, the most abundant model during April, i.e. before the peak of emergence of adult spider males, was *Pyrrhocoris apterus* (7.5 individuals per survey), followed by *Hippodamia variegata* (5.5) and *Coccinella septempunctata* (3.2) (Fig. 3A). During May, when *E. moravicus* males are generally active on the surface, the most abundant model was *Coccinella septempunctata* (3.2 individuals per survey), followed by *Cercopis sanguinolenta* (1.2) and *Cryptocephalus bipunctatus* (L. 1758) (1.2) (Fig. 3B). The abundances of potential models differed significantly between April and May (GLM-nb, $\chi^2_1 = 445.56$, $P < 0.001$), with a higher mean number of individuals of each model in April.

In August, at localities of *E. kollari* before the main period of emergence of adult males, the most abundant model was the red colour form of *G. lineatum* (28.7 individuals per survey), followed by *Hippodamia variegata* (6.2) and *Coccinella septempunctata* (5.5) (Fig. 4A). In September, when *E. kollari* males are most active, by far the most abundant model was *Hippodamia variegata* (808.2 individuals per survey), followed by *Ceratomegilla undecimnotata* Schneider 1792 (12.2) and the red form of *G. lineatum* (6.0) (Fig. 4B). The abundances of potential models of *Eresus* spp. differed significantly between August and September (GLM-nb, $\chi^2_1 = 3632$, $P < 0.001$), and the mean number of individuals of each model was higher in September than in August.

Phenotypic Similarity

The gradient along PC1 axis in the PCA of body shape and size (Fig. 5A) can be largely attributed to body size (Table S2). The position of *Eresus* spp. on the PC2 axis suggests that the spiders are not very similar in shape to other taxa, such as beetles, moths or true bugs. According to the PCA of the local binary pattern (Fig. 5B), *Eresus* spp. are not very similar to *Coccinella septempunctata* or other coccinellids. Instead, they shared a low LBP value (i.e. rather low inner pattern contrast) with *Zygaena* spp., *Trichodes apiarius* (L. 1758) and *Cercopis sanguinolenta*. Finally, the PCA of reflectance (Fig. 5C) suggests that *Eresus* spp. are similar to models that reflect a high proportion of the red part of the colour spectrum, such as *Trichodes apiarius* (Coleoptera: Cleridae), *Crioceris* spp. (Coleoptera: Chrysomelidae) and *Coccinella septempunctata* (Fig. S2).

The final PCA combined the first two axes of the three previous analyses. In two-dimensional ordination space, potential models of *Eresus* spp. formed the

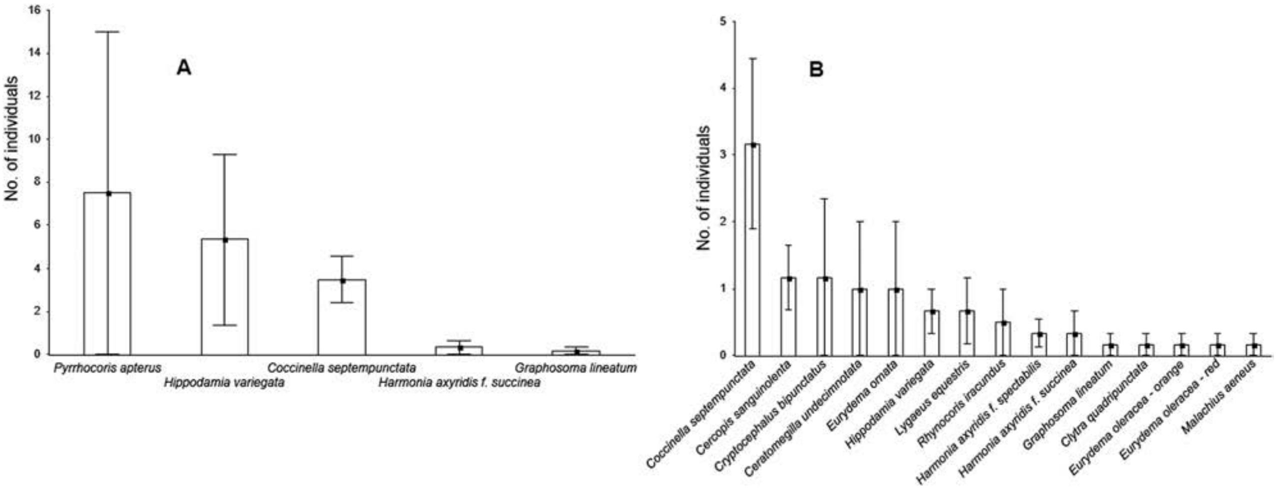

Figure 3. Abundance of potential models found per site where *Eresus moravicus* occurred in April (A) and in May (B). Bars = mean values, whiskers = ±SE.

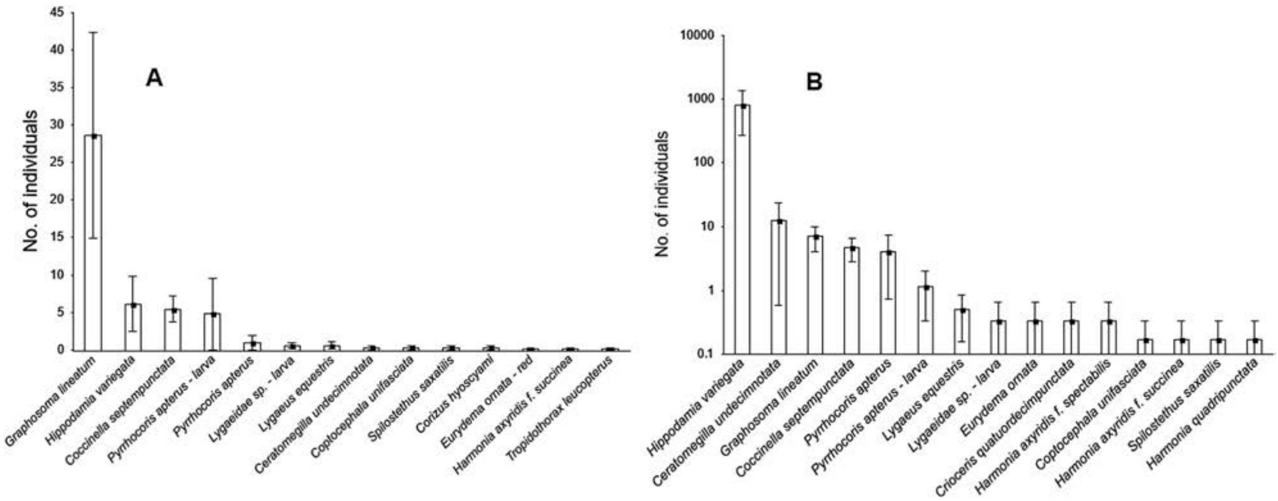

Figure 4. Abundance of potential models found per site where *Eresus kollari* occurred in August (A) and in September (B). Bars = mean values, whiskers = ±SE. Note that in B the ordinate is on a log scale.

following clusters: (1) medium-sized insects with red coloration and black spots, (2) small beetles and true bugs, and (3) a rather inconsistent group of mostly large insects with an atypical body shape (such as *Zygaena* spp. moths) or colour pattern (e.g. stripes in *G. lineatum*). *Eresus* spp. (along with *T. apiarius*) did not belong to any of these clusters and were situated far from all of them. However, the nearest was cluster 1, and the models closest to both *Eresus* species were *Coccinella septempunctata*, *Cercopis sanguinolenta* and *Harmonia axyridis* Pallas 1773 f. *succinea* (Fig. 5D).

DISCUSSION

Ladybird spiders of the genus *Eresus* frequently co-occurred at our study sites with several potential model species, e.g. *Coccinella septempunctata*, *Hippodamia variegata* or the red form of *G. lineatum*. The closest in appearance to the spiders were large coccinellid beetles, namely *Coccinella septempunctata*, *Harmonia axyridis* f. *succinea* and *Ceratomegilla undecimnotata*. Overall, the spiders were not very similar to any of their potential models and phenotypically they were not intermediate in appearance between multiple potential models.

Only 12 potential models were found at more than one site during the period of ladybird spider emergence, and only eight of these species were found at more than half of the studied sites. These findings indicate that the number of potential models that are ecologically relevant for *Eresus* spp. is low. Co-occurrence on a regular basis is a key assumption for a stable mimetic relationship. If this requirement is not fulfilled, the mimic's phenotype should be selected either to shift away to either another model, or become cryptic ('mimetic breakdown': Kikuchi & Pfennig, 2013). Stable mimetic relationships involve models that are 'reliable' in terms of consistent occurrence at sites suitable for the mimic – *Coccinella septempunctata*, *Hippodamia variegata*, *Cercopis sanguinolenta* or *G. lineatum* in this case. The effect of the model's abundance is similar: the more abundant the model is compared to the mimic, the more stable and effective the whole mimetic relationship should be (e.g. Huheey, 1964, 1980; Lindström *et al.*, 1997). Therefore, it would be adaptive for ladybird spiders to rely on one or several of the most abundant potential models. If we accept this conclusion, the above-mentioned species would be even more likely candidates as mimics of *Eresus* spp.

The co-occurrence and relative abundance of *Eresus* spp. and their potential models were assessed only at sites in southern Moravia. However, several *Eresus* species with a ladybird-like colour pattern are known to occur outside this region (*E. kollari* having the widest distribution) (Řezáč *et al.*, 2008). Collectively, *Eresus* spp. with the mimetic phenotype range from the Iberian Peninsula through almost the whole of Europe to Siberia (Řezáč *et al.*, 2008; Mihajlov, 2013). What then do the mimetic relationships look like in different parts of the spiders' distribution area? The answer might, in fact, be quite simple: the ladybird spiders occur within the distribution areas of the four most common potential model species: *Coccinella septempunctata* (Nikitsky & Ukrainsky, 2016; Pasqual *et al.*, 2017), *Cercopis sanguinolenta* (Soulier-Perkins, 2017), *G. lineatum* (Rider, 2006) and *Hippodamia variegata* (Agarwala & Ghosh, 1988; Pasqual *et al.*, 2017). Thus, the mimetic relationships observed in

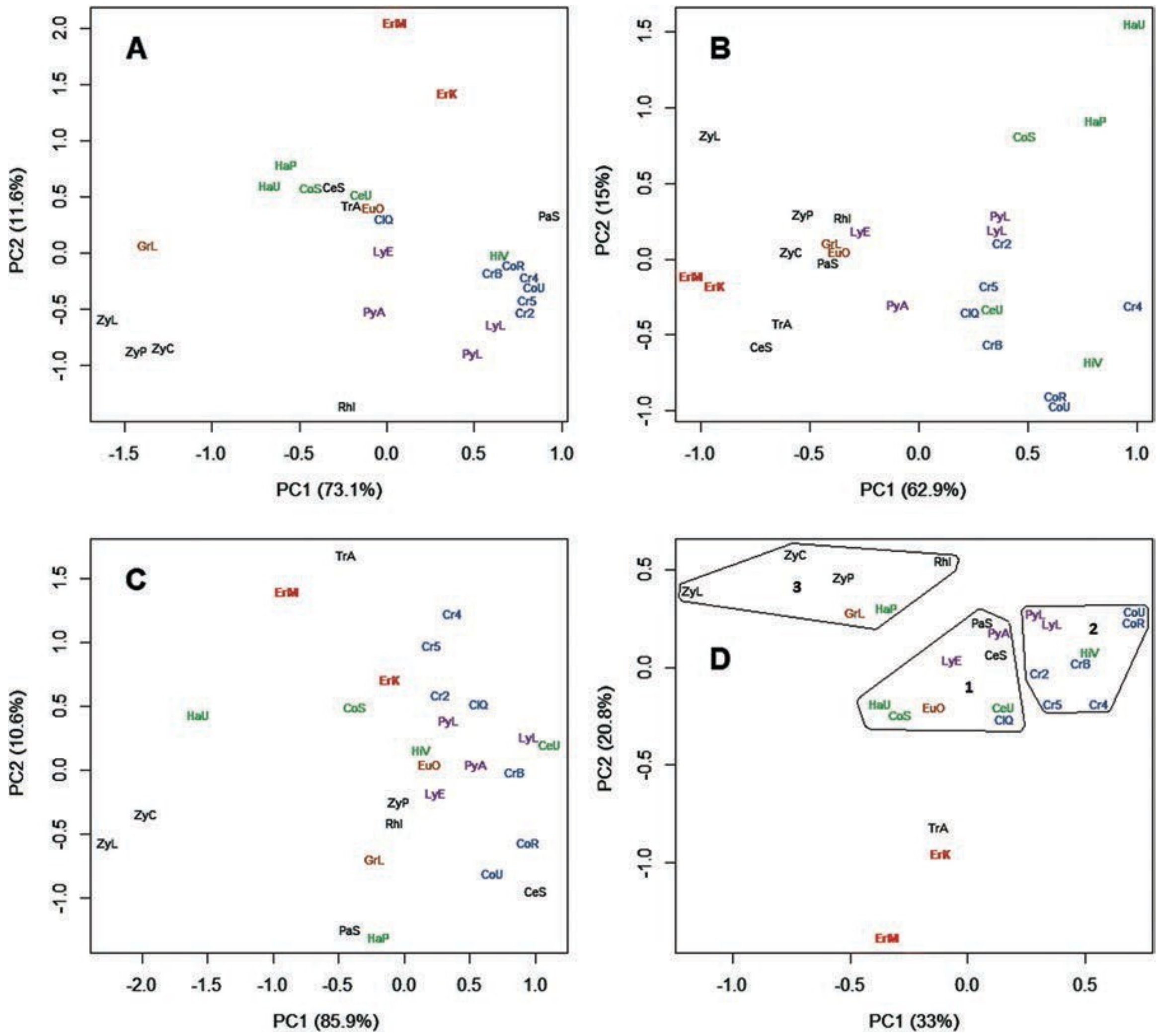

Figure 5. Ordination diagrams of PCAs of body shape and length (A), of the local binary pattern (B), of the reflectance (C), and combined analysis using PCA1 and PCA2 values of analyses A–C for each individual as input variables, with highlighted model clusters (D). Only two axes, PC1 and PC2, are shown, with the amount of variability explained by each axis. The analysis was performed using several individuals for each model/mimic; however, each type is represented by a single point, the model-type median. Colour indicates ladybird spiders and the most important model groups: red – Araneae: Eresidae, brown – Heteroptera: Pentatomidae, purple – Heteroptera: Lygaeidae and Pyrrhocoridae, blue: Coleoptera: Chrysomelidae, green – Coleoptera: Coccinellidae. Abbreviations for model species: CeS – *Cercopis sanguinolenta*, CeU – *Ceratomegilla undecimnotata*, ClQ – *Clytra quadripunctata*, CoR – *Coptocephala rubicunda*, CoS – *Coccinella septempunctata*, CoU – *Coptocephala unifasciata*, Cr2 – *Crioceris duodecimpunctata*, Cr4 – *Crioceris quatuordecimpunctata*, Cr5 – *Crioceris quinquepunctata*, CrB – *Cryptocephalus bipunctatus*, ErK – *Eresus kollari*, ErM – *Eresus moravicus*, EuR – *Eurydema ornata*, red form, GrR – *Graphosoma lineatum*, red form, HaP – *Harmonia axyridis*, f. *spectabilis*, HaU – *Harmonia axyridis*, f. *succinea*, HiV – *Hippodamia variegata*, Ly5 – Lygaeidae sp., 4th–5th instar, LyE – *Lygaeus equestris*, PaS – *Pardosa* sp., Py5 – *Pyrrhocoris apterus*, 4th–5th instar, PyA – *Pyrrhocoris apterus*, RhI – *Rhynocoris iracundus*, TrA – *Trichodes apiaries*, ZyC – *Zygaena carniolica*, ZyL – *Zygaena loti*, ZyP – *Zygaena purpuralis* complex.

Moravia may be common for all ladybird-like spiders of the genus *Eresus*.

The results of the analysis of body shape suggest that ladybird spiders are not very similar to potential model taxa. This result is not very surprising, given that spiders and insects have different body plans. Legs were not taken into account in the analysis, but we can assume that the eight thick, long legs of ladybird spiders would make them even more distant from insect models. Body shape, therefore, seems to be a key limitation in achieving perfect mimicry of insect models in spiders. In *Eresus* spp., the black coloration of the cephalothorax may be cryptic on a dark surface, making the spiders look smaller and more compact in terms of body shape, and therefore more similar to, for example, coccinellids. However, the occurrence of *Eresus* spp. did not appear to depend on the colour of the surface, so this effect is probably of minor ecological relevance for the spiders.

Surprisingly, the local binary pattern of *Eresus* spp. was quite dissimilar to that of *Coccinella septempunctata* and other coccinellids (Table S3). According to the analysis, the dorsal pattern of the spiders was most similar to those of zygaenid moths, possibly because the colour patterns in both the spiders and the moths consist of large monochromatic areas.

Interestingly, ladybird spiders (especially *E. moravicus*) reflected more light in the red part of the spectrum and, therefore, may produce a stronger aposematic signal (Gamberale-Stille & Tullberg, 1999) compared to most of their potential models. Such results may indicate the presence of perceptual exploitation of the sensory bias of the observer (Kikuchi & Pfennig, 2013) – in this case, the negative association of red colour with unprofitability. Perceptual exploitation is considered to be too costly to become widespread among mimics and has been suggested for epigamic signals only (Kikuchi & Pfennig, 2013, and references therein). *Eresus* spp., therefore, represent an intriguing case of mimics that exaggerate their models' signal to achieve better protection against potential predators. The pigments responsible for red coloration in *Eresus* spp. have not yet been determined (Holl, 1987), but it is possible that due to different colour production mechanisms (e.g. cuticular pigments in insect models vs. hair pigments in spiders), the spiders evolved a coloration that is unavailable to insects.

When multiple traits were considered collectively, *Eresus* spp. were clearly separated from almost all potential models, which suggests that their mimicry is quite poor. The persistence of imperfect mimicry in these spiders may have several explanations. First, numerous traits (number of legs, absence of antennae, compact cephalothorax) have made it almost impossible for spiders to become perfect mimics of insects during evolution. Also, perfecting a particular mode of defence is usually constrained by another, antagonistic selection pressure (e.g. the need for foraging behaviour in cryptic species) (Pekár, 2014). In ladybird spiders, short legs and a compact body, common adaptations of beetle-mimicking spiders (Pocock, 1909), could negatively affect the mobility of male spiders searching for females.

Predators probably do not select for evolution of multi-trait perfect mimicry. Rather, they may focus on a single dominant trait that produces overall similarity (e.g. Chittka & Osorio, 2007; Gamberale-Stille *et al.*, 2018). Experiments with birds have shown that birds do indeed discriminate prey based on colour but, surprisingly, not the colour pattern (Aronsson & Gamberale-Stille, 2008) or shape (Kišelová, 2014). Another argument against selection for perfect mimicry is that only moderately accurate overall resemblance may be a sufficiently effective mimetic signal. Experiments comparing pigeons' perceptions of similarity between wasps and their hoverfly mimics revealed that a 50% objective resemblance between the compared species was enough for them to be treated as perfect mimics by the birds (Dittrich *et al.*, 1993). Further analyses have shown that human perception of mimetic similarity is, in fact, more similar to the classification performed by pigeons than to the results of objective methods (Penney *et al.*, 2012). We weighted all variables in the analysis of phenotypic similarity equally to keep our analyses rather conservative and observer-independent; ultimately, only presentation of the mimics and models to their potential predators, especially birds, will determine the actual importance of each potential mimetic trait.

Our study indicates that ladybird spiders of the genus *Eresus* co-occur at the same sites and at the same time as several members of the hypothetical European red/black insect mimicry complex, which includes many species of heteropterans (Hotová Svádová *et al.*, 2010), coccinellid beetles (Brakefield, 1985) and other taxa, but are relatively dissimilar to its members. We conclude that although several species are likely to be involved in mimetic relationships with *Eresus* spp., the seven-spot ladybird *Coccinella septempunctata* is probably the key mimetic model of the ladybird spiders.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Potential models of *Eresus* spp. that were sufficiently abundant at the studied sites. Cicadomorpha: Cercopidae: A – *Cercopis sanguinolenta*; Heteroptera: Reduviidae: B – *Rhynocoris iracundus*; Heteroptera: Pentatomidae: C – red form of *Eurydema oleracea*, D – red form of *Graphosoma lineatum*; Heteroptera: Lygaeidae: E – *Lygaeus equestris*, F – lygaeid larva, aposematic morphotype; Heteroptera: Pyrrhocoridae: G – *Pyrrhocoris apterus*, H – *P. apterus* larva; Coleoptera: Cleridae: I – *Trichodes apiarius*; Coleoptera: Coccinellidae: J – *Ceratomegilla undecimnotata*, K – *Coccinella septempunctata*, L – *Harmonia axyridis*, forma *spectabilis*, M – *H. axyridis*, f. *succinea*, N – *Hippodamia variegata*; Coleoptera: Chrysomelidae: O – *Clytra quadripunctata*, P – *Coptocephala rubicunda*, Q – *C. unifasciata*, R – *Crioceris duodecimpunctata*, S – *C. quatuordecimpunctata*, T – *C. quinquepunctata*, U – *Cryptocephalus bipunctatus*; Lepidoptera: Zygaenidae: V – *Zygaena carniolica*, W – *Z. loti*, X – *Z. purpuralis* group

Figure S2. Median reflectance spectrum of *Eresus kollari*, *E. moravicus*, selected potential models and the control non-mimetic spider (*Pardosa* sp.), compared to the white standard.

Table S1. List of studied ladybird spider species, potential models of the spiders found at their localities, and the control non-mimetic spider (*Pardosa* sp.), along with their taxonomic classification.

Table S2. Median body length and median maximum body width (based on 40 measurements taken evenly across body axis) of *Eresus* spp., their potential models and the control non-mimetic spider (*Pardosa* sp.). Species are arranged according to body length.

Table S3. Sums of column-scaled median local binary pattern values (10 values, each with range 0–1) for the dorsal side of the front part of the body (cephalothorax in spiders, head + thorax in insects), back body (abdomen), and sum of these values for *Eresus* spp., their potential models and the control non-mimetic spider (*Pardosa* sp.). Species are arranged according to the sum of values.

SHARED DATA

The data from this study can be downloaded from a website of Masaryk University (http://www.sci.muni.cz/zoolecol/inverteb/?page_id=18).

STUDY 4

Mimicry in larvae of pyrrhocorid true bugs as seen by spider predators

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Alice Exnerová

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Mimicry in larvae of pyrrhocorid true bugs as seen by spider predators

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Abstract

Adults and larvae of a true bug *Scantius aegyptius* (Heteroptera: Pyrrhocoridae) closely resemble a sympatric pyrrhocorid species, *Pyrrhocoris apterus*, and most likely form a mimetic relationship with this species. *S. aegyptius* adults, although producing secretion atypical of true bugs, are to some extent chemically protected against predators. In this study, we analyzed composition and function of larval secretion in *S. aegyptius*, and attempted to determine mimetic relationship between larvae of *S. aegyptius* and *P. apterus*. The main component of larval secretion in *S. aegyptius* is 2-heptanol, a chemical not known to function in antipredatory defence, followed by (*E*)-2-octenal, a common true bug defensive chemical. When larvae of both species were presented to jumping spiders (*Evarcha arcuata*), *S. aegyptius* was slightly less protected than *P. apterus*, but the spiders behaved towards the two species in a similar way: they quickly learned to avoid the bugs, but usually attacked them again on the second day. The spiders also generalized their learned avoidance from one true bug species to the other (with only slight asymmetry favouring *S. aegyptius*), suggesting that the bugs‘ mimetic relationship is most likely Müllerian, advantageous to both species.

Keywords

Aposematism, Heteroptera, mimicry, Salticidae, spider

Introduction

Antipredatory mimicry refers to a situation, when one species (mimic) is protected against predators by possessing a signal (or a set of signals) that resembles a signal of another species (model), which is avoided by predators. The key aspect of mimicry is an antipredatory defence of the mimic itself. If the mimic is undefended (Batesian), the relationship is parasitic –

protecting the mimic but harmful to the model (Bates 1862, Huheey 1988). An honest, Müllerian mimic, on the other hand, is unprofitable for predators by itself, and its presence is advantageous to the model. Most predators have to encounter (and frequently kill) one or more defended prey individuals before they learn to avoid prey of a particular appearance, and Müllerian mimics share this "cost of predation" with their models (Müller 1879). It is often impossible to identify a model and a mimic in Müllerian mimicry, as the signals involved might have evolved to resemble each other by convergence rather than advergence (Turner 1987, Balogh & Leimar 2005).

In his model, Müller (1879) implicitly expected defended mimics to have equally effective defence as their models, which is virtually never the case in nature. The relative efficacy of the mimic's defence compared to that of the model, positions the mimic onto a continuous Batesian-Müllerian axis. Simply said, mimics that are beneficial their model are located in the Müllerian part of the axis and mimics that harm their model are located in the Batesian part of the axis (Wallace 1882, Huheey 1976, Speed 1993). Even some defended mimics may harm their model if their protection is considerably lower than that of their model (quasi-Batesian mimicry, Speed 1993). However, distinction between Müllerian and quasi-Batesian mimics is problematic, reflecting high complexity of the topic. The results depend on the classification criterion (e.g., absolute vs. relative mortality, Rowland et al. 2007, 2010) and factors such as relative abundances of models and mimics, model-mimic similarity, or prey community complexity (Speed et al. 2000, Rowland et al. 2007, 2010, Ihalainen et al. 2012). Moreover, most studies are either theoretical (Speed 1993, Balogh et al. 2008), or are limited to artificial prey and bird predators (Speed et al. 2000, Rowland et al. 2007, 2010, Ihalainen et al. 2012). Use of natural prey or broader spectrum of predatory taxa may provide more ecologically relevant information about the phenomenon.

Some well-defended species share their warning signals with more than one mimic, which leads to formation of mimetic complexes (Poulton 1909, Brower 1958). These complexes may include numerous species from various parts of the Batesian-Müllerian spectrum. One of the most distinct mimetic complexes in West Palearctic is composed of red-and-black insects and spiders. The complex includes species from numerous true bug families (e.g. Lygaeidae, Pentatomidae, Pyrrhocoridae, Rhopalidae) and several beetle families (especially Coccinellidae and Chrysomelidae), along with taxa such as ladybird spiders (*Eresus* spp.) and zygaenid moths (Hotová Svádová et al. 2010, Raška & Pekár 2018). Two red-and-black species of pyrrhocorid true bugs exhibit particularly close similarity in their colour patterns: the firebug, *Pyrrhocoris apterus* (L. 1758) (referred to as “Pyrrhocoris“ further in the text) and the Mediterranean red bug, *Scantius aegyptius* (L. 1758) (“Scantius“ further in the text) (Fig. 1). Despite being members of the same family, the two species belong to different genera, each of which includes several other species, none of them exhibiting the contrasting red-and-black colour pattern. Even though the phylogeny of the group is still unknown, it is therefore possible that the red-and-black colouration evolved in each of the studied species independently. Distribution areas of these species overlap (Kerzhner 2001) and they can even be found in mixed aggregations on their host plants (Exnerová & Štys, pers. obs). The resemblance between the two species is therefore very likely to enhance one or both species' antipredatory protection under natural conditions.

True bugs (Heteroptera) possess two primary systems of exocrine scent glands often used in antipredatory defence: dorsoabdominal glands in larvae and metathoracic glands in adults (Staddon 1979, Aldrich 1988). In Pyrrhocoris, adults produce aldehyde-rich secretion (Krajicek et al. 2016), which is effective as an antipredatory defence against birds (Exnerová et al. 2003), ants (Remold 1963), and mantises (Raška et al., unpubl. data). Secretion of Pyrrhocoris larvae contains mainly ketoaldehydes (Farine et al. 1992) and acts as an effective antipredatory defence against birds (Prokopová et al. 2010) and spiders (Raška et al. 2017, 2018). These defensive effects are not surprising, as both aldehydes and ketoaldehydes belong to the most widespread defensive chemicals in true bugs (Aldrich 1988). In Scantius, the function of scent-gland secretions is less clear. Adult secretion is dominated by atypical alcohols, such as 2-cyclohexen-1-ol and cyclopentanemethanol (Krajicek et al. 2016), and larval secretion, which in true bugs usually differs from secretion of the adults (Aldrich 1988), has not been analysed so far. Chemical defence of Scantius adults against birds appears to be less effective than that of Pyrrhocoris (Exnerová et al. 2005).

Predators are supposed to learn to avoid highly unprofitable prey faster and remember their experience for longer, compared to the prey with only moderately effective defences (theoretical and experimental studies reviewed in Skelhorn et al. 2016, Ruxton et al. 2018). As Scantius seems to be less well defended than Pyrrhocoris, it may gain protection from its resemblance to this better defended and more abundant species. However, mimicry between the two species have not been studied so far, and it is unknown whether mimetic relationships between Scantius and Pyrrhocoris are rather Batesian or Müllerian. Moreover, the comparative data on effectiveness of antipredatory defence in the two species are based solely on experiments with avian predators (Exnerová et al. 2005), and it is not clear, whether other predators, especially arthropods, would react in the same way.

In our study, we assessed the effectiveness of antipredatory defences in larvae of Pyrrhocoris and Scantius, and the mimetic relationships between the two species. First, we filled a gap in knowledge of the defensive secretions of the two species by analysing the composition of the larval dorsoabdominal scent-gland secretion of Scantius by means of GC-MS analysis. Then we compared antipredatory effects of the defensive secretions of 3rd-instar larvae of Pyrrhocoris and Scantius in experiments with jumping spiders, *Evarcha arcuata* (Clerck 1757). Jumping spiders (Salticidae) possess complex cognitive abilities (reviewed in Richman & Jackson 1992), including acute vision with spectral sensitivity ranging from UV to dark red (Peaslee & Wilson 1989), and they are responsive to nuances of aposematic signals (e.g. Taylor et al. 2014, Raška et al. 2017). We compared reactions of spiders to the bugs during their first encounter and rate of subsequent avoidance learning. Once the spiders gained experience with one of the two prey species, we presented one group of the spiders with the other prey to test for the degree and symmetry of their mimetic relationships. The other group was presented with the same prey species for two consecutive days to assess memorability of the learned avoidance.

Methods

Predators

Evarcha arcuata is a jumping spider (Araneae: Salticidae) common in grass habitats of the Palearctic region (Buchar & Kůrka 2001, World Spider Catalog 2019). It is a medium-sized (5-6 mm) cursorial generalist predator, which is able to feed on various arthropods including true bugs from the family Miridae (Nentwig 1986, Dobroruka 1997).

We collected the spiders in meadows in Dalejské údolí, Prague (50°02‘35“N, 14°21‘28“E) between April and August 2010-2014. We kept the spiders in the laboratory for two weeks. After such period, the spiders can be considered quasi-naive, having forgotten all prey preferences based on previous experience (Hill 2006). We kept the spiders in small plastic containers and fed them with micropterous fruit flies, housing and feeding conditions were identical to those described in Raška et al. (2017).

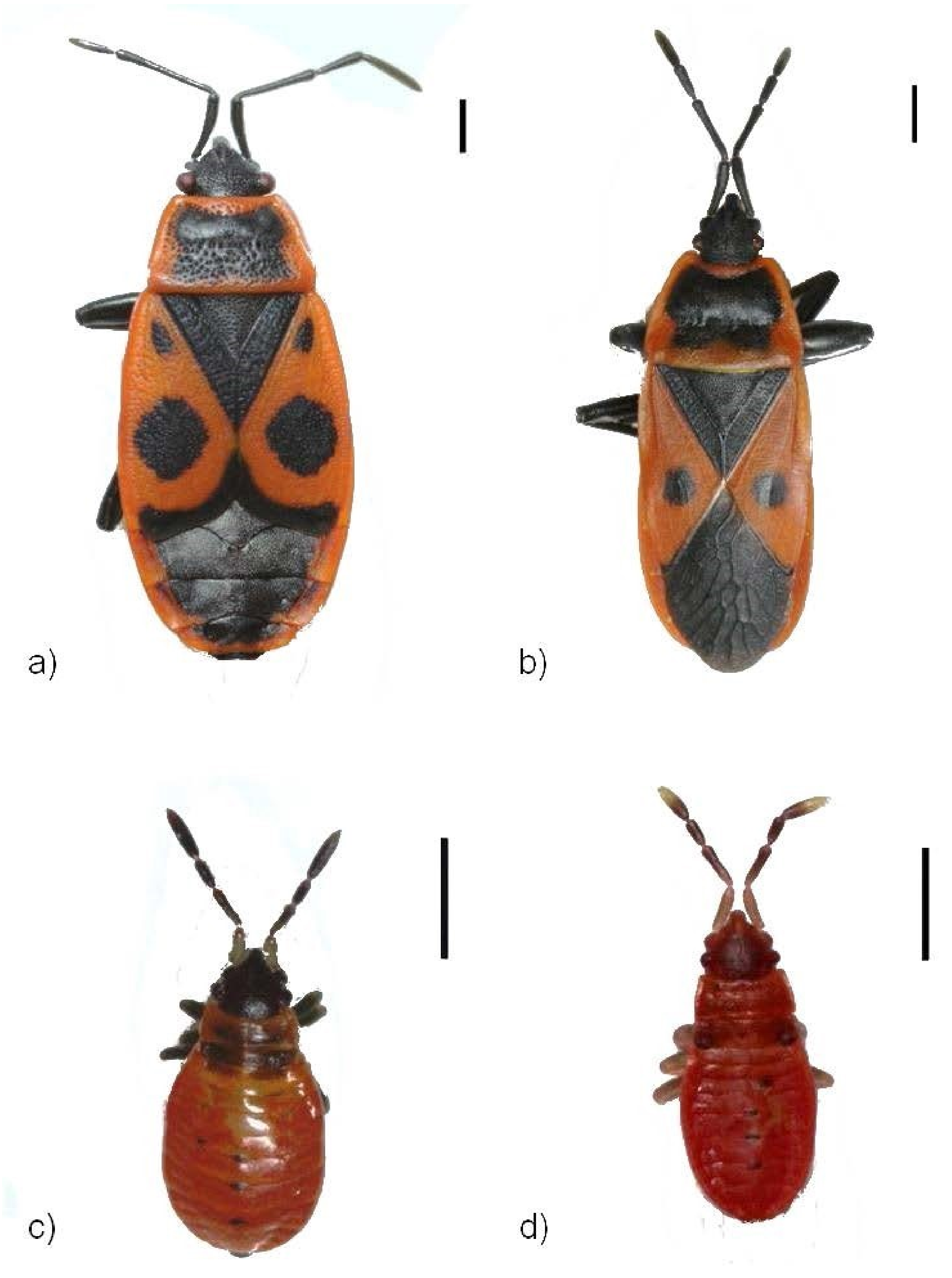

Fig. 1: Adults of the firebug *Pyrrhocoris apterus* (a) and the red bug *Scantius aegyptius* (b), and 3rd instar larvae of both species – the firebug (c) and the red bug (d). Scale = 1 mm.

Prey

We used 3rd-instar larvae of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae; referred to as "Pyrrhocoris" further in the text) and *Scantius aegyptius* (Heteroptera: Pyrrhocoridae; referred to as "Scantius" further in the text) as a prey. Both species have Palaearctic distribution. Pyrrhocoris is nearly pan-Palaearctic, whereas Scantius is rather subtropical, with its natural distribution area ranging from western Mediterranean to India (Puchkov 1974, Kerzhner 2001). Both Pyrrhocoris and Scantius have also been recently introduced to North America (Hodgson 2008, Bryant 2009, Rojas & Jackson 2018). Pyrrhocoris is slightly larger than Scantius (Puchkov 1974), its 3rd instar larvae are 2-3 mm long compared to 2-2.5 mm in Scantius. Larvae of both species are therefore of adequate size to be an acceptable prey to *E. arcuata*, which prefers prey size about 50-75% of its own body length (Nentwig & Wissel, 1986).

Both species are defended against predators by a combination of aposematic coloration and defensive secretion. In both species, the warning red-and-black coloration is produced mainly by high concentration of red erythropterin and black melanin in the bugs' cuticle (Krajíček et al. 2014). The composition of defensive secretions of the two species, on the other hand, seems to be completely different. While the secretion of Pyrrhocoris is mostly composed chemicals typical of true bug defensive secretion (aldehydes and alkanes in adults, ketoaldehydes and aldehydes in larvae, Farine et al. 1992, Krajicek et al. 2016), the composition of secretion of Scantius adults includes unique chemicals such as cyclic alcohols (Krajicek et al. 2016).

We collected Pyrrhocoris under lime trees (*Tilia* sp.) in Dalejské údolí, Prague (50°02'34"N, 14°21'18"E), and used F1–F3 laboratory-reared generations in the experiments. We reared the bugs in 500–750 ml plastic containers at 23–27 °C and 14:10 h light:dark cycle. We fed the bugs on crushed seeds of small-leaved lime (*Tilia cordata* Mill. 1768) and provided with water ad libitum.

Scantius was collected on several sites: Kos island, Greece; Brač island, Croatia; Athens, Greece; and Palermo, Italy. We used F1-F3 laboratory-reared generations in the experiments. We reared the bugs in 250-500 ml plastic containers at 27°C and 14:10 light:dark cycle, fed them on seeds of the common hollyhock (*Alcea rosea* (L. 1758)), and provided with water *ad libitum*.

We reared micropterous fruit flies (*Drosophila melanogaster* Meigen 1830), the prey the spiders were fed on before the experiments and presented with as a control prey, on nutritive substrate in 20-25°C.

Analysis of scent-gland secretion (GC-MS)

We performed the analysis of secretion of dorsoabdominal grands of Scantius larvae using a GCMS-QP2010 Plus instrument (Shimadzu, Japan), equipped with 20 m × 0.15 mm i.d., 0.15 µm film thickness Rtx-200 column (trifluoropropylmethyl polysiloxane stationary phase, Restek, USA). We used helium (99.999 %, Linde, Czech Republic) as the carrier gas at a constant linear flow rate of 35 cm s^{-1}, and employed splitless-mode injection with SPME liner at 250 °C (split valve closed for 1 min). We maintained the oven temperature at 35 °C for 3 min, ramping it at 5 °C min^{-1} to 130 °C, then ramping it at 20 °C min^{-1} to 300 °C and then maintaining it for 5 min (total run time, 35.50 min). We operated the mass spectrometer in the scan mode (m/z 35–500). The ion source and interface temperatures were 200 and 250 °C, respectively.

We collected and evaluated the data using the GCMS software (Shimadzu, Japan), Origin 8 (Origin Lab corporation, Northampton, MA, USA), Microsoft Excel 2003 (Microsoft Corporation, Redmond, DC, USA) and Minitab 16 (Minitab Inc., State College, PA, USA) programs. We identified the secretion components by comparing the obtained spectra with those in the NIST 2008 Mass Spectra Library. We analyzed series of *n*-alkanes (C8—C20) and reference compounds under the same experimental conditions as those used for the samples to either establish the retention indices or confirm the identity of the analytes. We measured retention times of reference compounds by injection of their diluted solutions in pentane. We selected the dilution for each standard individually in such a way that its retention time was constant and reproducible and did not change with higher dilution. We performed the identity confirmation of the separated compounds by comparing the experimental retention times, retention indices and mass spectra of the compounds with those of concurrently analyzed reference standards.

We adopted the procedure of secretion sampling from Krajicek at al. 2016 to allow better comparison of secretions of Scantius larvae (this article) and adults (Krajicek et al. 2016). We placed three larvae in a syringe with a barrel volume of 12.5 mL (Eppendorf, Hamburg, Germany), and placed the syringe in an incubated shaker for tempering the specimens at 40 °C for 1 min (bioSan, Riga, Latvia). After that, we slightly compressed the larvae with the plunger of a syringe until they discharged the secretion which appeared as a thin liquid film appeared on their abdominal dorsum. Then we inserted an SPME (solid phase microextraction) fiber based on 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA) into the tip of the barrel to sample volatile compounds. Before each analysis of defensive secretion, we performed a control analysis of SPME sampler itself and SPME sampling of the syringe itself (blank).

Experimental design

Altogether we tested 125 spiders (44 males, 41 females, and 40 juveniles of older instars, at least 3 mm in length). We divided the spiders semirandomly into three groups (five subgroups) so that each group would include about the same number of males, females, and juveniles. We performed the experiments simultaneously with our previous study (Raška et al. 2017) and data of two subgroups have already been published in the study (see Table 1).

The experiments consisted of a series of consecutive 10-minute trials separated by 50-minute inter-trial intervals. During each prey presentation, we provided the spiders with a single prey individual. We recorded whether spiders attacked (i.e. bit or attempted to bite) or at least contacted (i.e. touched with chemoreceptive organs on front legs) the prey, and we recorded latency between the presentation of the prey and the first attack in each trial. If a spider captured the prey (i.e. held it by chelicerae for more than two minutes and was feeding on it), we removed the prey to prevent the spider's satiation.

We performed the experiments in Petri dishes (90 mm diameter, 14–17 mm height) with white filter paper covering their bottom. We carried out the experiments under daylight, supplemented with fluorescent tube (Philips Master PL-S 11W/840/2P) if necessary. We filmed the experiments using digital cameras (Canon HG 20, Canon Legria FS 22) and recorded the spiders' behaviour using Observer XT 8.0 (Noldus Information Technology, 2008).

Avoidance-learning test

We allowed the spiders to acclimate in the experimental arena for 1 hour before the experiments, which always started between 9 and 10 AM CEST. The avoidance-learning test consisted of 5 trials, in which we compared changes of the attack rates and the capture rates during repeated presentation of the same prey species. If a spider did not attack or contact the prey within the 10-minute limit of trial 1, the experiment was terminated, the spider was not used in subsequent trials, and its data were not used in analyses unless stated otherwise. In such case, the whole experiment was repeated with a new spider, so the resulting number of spiders in each experimental group/subgroup would remain the same. The numbers of excluded spiders were also analysed to test whether the spiders were initially equally attracted to the two prey species, and whether their behaviour was not affected by previous experience, unlearned avoidance or other unforeseen factors.

After the avoidance-learning test, two subgroups of spiders were presented with a different prey and used in the generalization test (trials 1–8) and three subgroups of spiders were presented with the same prey and used in the memory test (trials 1–9, with trial 9 being performed on a second day) (Table 1).

Generalization test

The generalization test consisted of three 10-minute trials (trials 6–8) and followed 50 minutes after the avoidance-learning test. We presented the spiders with the other true bug species than they were presented in the avoidance-learning test (see Table 1). The reactions of the spiders towards a novel prey during trial 6 were compared (1) to their responses to previously presented prey during trial 5 of the avoidance-learning test to test how the spiders' reactions change directly after the prey switch (2) to reactions of spiders presented with the same prey during trial 1 to find out whether the spiders' experience with a similar prey decreases their predatory response to the presented true bug species, compared to naive spiders.

Memory test

The memory test was divided into two parts. The first part (trials 6–8) was performed to prolong the avoidance-learning process. Previous experiments have shown that after 8 presentations of unpalatable true bugs, almost all jumping spiders learn to avoid the prey (Skow and Jakob 2005). After trial 8, the spiders were provided with a few drops of water and kept in the experimental arenas overnight.

The second part of the memory test took part the next day (trial 9), approximately 24 hours after trial 1. We presented the spiders with the same prey as on the previous day to find out whether and to what degree they have remembered the learned avoidance acquired on day 1.

Control trial

To account for the possibility that the spiders' attack frequency decreased due to satiation or other reasons not specific to the true bugs, we provided the spiders with a control palatable prey (micropterous fruit fly *D. melanogaster*). We offered a single fruit fly to each spider immediately after the end of the experiments (after trial 8 of the generalization test or after trial 9 of the memory test, see Table 1). If a spider did not capture the prey during the first 5 minutes of the control trial, we added an additional fruit fly for the next five minutes to reduce the possibility that the spider did not respond to the prey due to its inactivity. If a spider did not attack during the whole 10-minute control trial, we excluded its data from the analyses and the whole experiment was later repeated with another spider, so the number of spiders in each subgroup would remain the same.

Data analyses

Dependent variables included attack rate (rate of spiders attacking the prey), capture rate (rate of spiders capturing the prey), attack latency (latency between the presentation of the prey and the first attack). All models initially included all possible second-level interactions of explanatory variables and were simplified according to Akaike information criterion (AIC) in linear models and generalized linear models with binomial distribution (GLM(b)) and Quasi-information criterion (QIC) in general estimation equation models with binomial distribution (GEE(b)).

We used GEE(b) models to assess which variables could potentially affect dependent variables and as such should be used in partial analyses. The model initially included a single dependent variable (attack rate or capture rate) and illumination (levels: natural only, with an additional light source), sex and life stage (levels: male, female, juvenile), trial (1–9), day (1–2), and prey species (levels: fruit fly, Pyrrhocoris, Scantius) as explanatory variables. According to the overall model (and contrary to the previously published results, Raška et al. 2017), the additional light source significantly increased both the attack rate (GEE(b), $\chi^2 = 5.047$, $p = 0.025$) and the capture rate (GEE(b), $\chi^2 = 4.03$, $p = 0.045$). We therefore used illumination and its second-level interactions as random factors in initial versions of all models. Sex and life stage of the spiders, on the other hand, had no significant effect in the overall model (GEE(b), attack rate: $\chi^2 = 0.457$, $p = 0.796$; capture rate: $\chi^2 = 0.107$, $p = 0.948$) and could be excluded according to QIC. We therefore decided not to use the sex/life stage variable in further analyses.

To rule out a possibility that some factor (e.g. unlearned avoidance) could influence the initial part of the experiment, we compared the rate of spiders failing to respond to the prey (i.e. to attack or contact it) during trial 1 by means of GLM(b) models with illumination as a random factor and the prey species as a fixed factor. To obtain more detailed information, we compared attack latencies and capture rates in spiders attacking the presented prey during the first trial of the avoidance-learning test. The first-attack latencies were log-transformed to fit normal distribution and analysed by means of linear models, capture rates were compared by means of GLM(b) models. We used illumination as a random factor and the prey species as a fixed factor in both analyses.

We assessed changes in prey attack rate and prey capture rate during the avoidance-learning test (trials 1–5) by means of GEE(b) models with illumination as a random factor and the trial number as a fixed factor. When comparing the trend between two prey species, we added the prey species and its interaction with the trial number as a fixed factor.

To find out whether the spiders were able to generalize between the two true bug species, we first compared the attack rate and the capture rate between the last trial of the avoidance-learning test (trial 5) and the first trial of the generalization test, after we switched the prey species (trial 6). We used GEE(b) models with illumination as a random factor and trial number as a fixed factor for each prey switch (from Pyrrhocoris to Scantius, from Scantius to Pyrrhocoris) separately and then added the prey switch as a fixed factor to account for potential asymmetry of the generalization process (i.e. interaction between the trial number and the prey switch).

An additional method to approach the generalization process was to compare reactions (attack rates, capture rates) towards the novel prey in truly naive spiders (trial 1) and in spiders that had an experience with a similar prey (trial 6, i.e. the first trial of the generalization test). The two groups were not fully comparable, as we took into account even naive spiders that did not attack or at least contact the prey in trial 1 and were not used in subsequent trials. This fact, however, made our analyses more conservative, as the use of foraging-motivated spiders only in the generalization test could have an effect opposite to potential generalization between unprofitable prey species. Still, the rate of attacking spiders was clearly higher in trial 1 (ca 90 %) than in trial 6 (ca 60-70 %) and capture rate values were affected by this difference. We therefore decided to assess the capture rates in attacking spiders only to obtain more comparable results. We compared the attack rates and the attack susses rates by means of a GLM(b) models. We used illumination as a random factor and the trial number as a fixed factor in both analyses. To test for difference between trends in the two true bug species, we added the prey species as a fixed factor and focused on the interaction between the trial number and the prey species.

We tested whether the attack rate and capture rate changed between the prolonged avoidance-learning test (trials 1–8) and the memory test (trial 9, performed on day 2) to assess memorability of the avoidance the spiders have learned during day 1. For that, we used GEE(b) models with illumination and trial number as random factors, and day as a fixed factor. We compared the changes in two prey species by adding the prey species its interaction with day as fixed factors. We did not use data from trials 10–11 due to their low informative value.

We analysed the data in R 3.5.2 (R Foundation for Statistical Computing, 2018). All the tests were two-tailed, with the level of significance $\alpha = 0.05$. We refer to $0.05 < \alpha < 0.1$ as the "marginal significance". All GEE models were analysed using package geepack (Halekoh et al. 2006), QIC was calculated using package MuMIn (Bartoń 2009).

Table 1: List of experimental groups and subgroups based on prey species: 3rd instar larvae of Pyrrhocoris (*P. apterus*), 3rd instar larvae of Scantius (*S. aegyptius*), and adult fruit fly (*Drosophila melanogaster*), with numbers of spiders in each (sub)group.

Group	Learning (day1, trial1-5)	Subgroup	Generalization (day 1, trial 6-8)	Memorability (day 2, trial 9-11)
Group 1 (N = 50)	Pyrrhocoris	1a (N = 25)*	Pyrrhocoris	Pyrrhocoris **
		1b (N = 25)	Scantius**	-
Group 2 (N = 50)	Scantius	2a (N = 25)	Scantius	Scantius **
		2b (N = 25)	Pyrrhocoris**	-
Group 3 (N = 25)	fruit fly	3a (N = 25)*	fruit fly	fruit fly**

*Previously published results (Raška et al. 2017), recalculated and compared with data not published before.

** Followed by a control trial with the fruit fly prey.

Results

Composition of scent-gland secretions

Analyses of larval secretion were carried out for the same number of individuals (n = 3) as in the analyses of male and female secretions (Krajicek et al. 2016). Identifications of the individual peaks of dominant secretion components obtained for males, females and larvae, the corresponding retention times, their standard deviations and relative abundance (only peaks with response (A) greater than 20,000 a.u. and similarity higher than 85 % were selected for the identification) are summarized in Table 2. Both adult (Krajicek et al. 2016) and larval secretions mostly composed of alcohols, alkanes, aldehydes, and esters. Compounds dominant in the secretion of the adults were cyclopentanemethanol, 2-cyclohexen-1-ol, 1-dodecanol, and tetradecane (Krajicek et al. 2016). The larval secretion, on the other hand, was dominated by 2-heptanol and (*E*)-2-octenal. Some compounds, such as tetradecane, 4-*tert*-butylcyclohexyl acetate, 1-dodecanol, and limonene, were present in both adult (Krajicek et al. 2016) and larval secretions.

Initial reaction

In trial 1, only fifteen spiders (9.09 %) did not attack or at least contact the prey. The proportion did not differ significantly between the prey species, including the control fruit flies (GLM(b), Pyrrhocoris vs. Scantius $\chi^2_{(1)} = 0.068$, $p = 0.794$; Pyrrhocoris vs. fruit fly $\chi^2_{(1)} = 0.191$, $p = 0.662$; Scantius vs. fruit fly $\chi^2_{(1)} = 0.411$, $p = 0.521$).

Compared to Pyrrhocoris, Scantius suffered from higher mortality during its first presentation in trial 1, although the difference in capture rates was only marginally significant (GLM(b), $\chi^2_{(1,97)} = 2.753$, $p = 0.097$) (Fig. 2, Trial 1). Still, both true bugs were captured less often than control fruit flies (GLM(b), Pyrrhocoris: $\chi^2_{(1,73)} = 86.939$, $p < 0.001$; Scantius: $\chi^2_{(1,73)} = 83.688$, $p = 0.022$).

In trial 1, spiders attacked Pyrrhocoris sooner (median 19.8 s) than Scantius (median 40 s) (ANOVA, $F_{1,98} = 5.881$, $p = 0.017$). Also, unlike Pyrrhocoris (ANOVA, $F_{1,72} = 0.004$, $p = 0.953$), Scantius was attacked later than control fruit flies (median 21.4 s) (ANOVA, $F_{1,73} = 4.994$, $p = 0.028$) when first presented to the spiders.

Table 2. The dominant chemicals identified in defensive secretion of Scantius larvae (n = 3), compared with secretions of males (n = 3) and females (n = 3) (Krajicek et al. 2016); t_{ret} is the retention time of the relevant substance, SD standard deviation, relative abundances (% areas of the relevant peaks) as a result of chromatogram internal normalization. The methods used for the identification: A – retention time of the relevant substance was compared with the reference compound; B – the mass spectrum of the relevant substance was compared with NIST 2008 mass spectra library; S – similarity of the compound spectrum with the spectrum in the NIST 2008 database. Only peaks with response (A) greater than 20,000 a.u. and similarity (match factor in NIST 08 database) higher than 85 % were selected for the identification and following normalization.

Compound	t_{ret} (min) ± SD	Relative abundance (%) and spectra similarity (S)						Identification
		Males	S	Females	S	Larvae	S	
2-heptanol	6.39 ± 0.04	-	-	-	-	20.81	97	A, B
cyclopentanemethanol	6.42 ± 0.05	11.42	96	12.96	94	-	-	A, B
2-cyclohexen-1-ol	6.88 ± 0.05	12.07	85	18.85	88	-	-	A, B
cyclopentanone	6.27 ± 0.05	3.77	97	0.34	85	-	-	A, B
limonene	7.65 ± 0.07	3.93	93	3.67	91	2.83	87	A, B
(*E*)-2-octen-1-ol	11.16 ± 0.05	-	-	-	-	4.90	95	A, B
dodecane	11.86 ± 0.07	10.84	97	2.92	97	1.21	97	A, B
1-dodecene	11.97 ± 0.03	2.89	93	1.13	95	0.47	94	A, B
(*E*)-2-octenal	14.06 ± 0.04	-	-	-	-	14.06	95	A, B
tetradecane	16.91 ± 0.02	7.62	97	9.01	95	3.71	95	A, B
4-*tert*-butylcyclohexyl acetate [a]	18.64 ± 0.02	4.37	91	3.90	92	2.93	89	A, B
(*E*)-2-decenal	19.26 ± 0.03	-	-	-	-	2.81	90	A, B
4-*tert*-butylcyclohexyl acetate [a]	19.65 ± 0.06	0.18	81	0.58	82	0.35	85	A, B
pentadecane	19.52 ± 0.06	2.08	92	2.54	89	1.21	84	A, B
1-dodecanol	21.10 ± 0.02	9.21	97	10.01	96	5.28	94	A, B
hexadecane	21.41 ± 0.07	1.95	94	2.42	92	1.21	85	A, B

[a] *E* or *Z* isomer

Avoidance-learning test

Spiders have learned to avoid both true bug species during five trials, as evidenced by a rapid decrease of attack rates (GEE(b), Pyrrhocoris: $\chi^2_{(1)} = 38.799$, $p < 0.001$; Scantius: $\chi^2_{(1)} = 38.163$, $p < 0.001$) and capture rates (GEE(b), Pyrrhocoris $\chi^2_{(1)} = 12.975$, $p < 0.001$; Scantius $\chi^2_{(1)} = 21.93$, $p < 0.001$) over time. The efficiency of the avoidance-learning process did not differ significantly between the two true bug species (GEE(b), attack rate: $\chi^2_{(1)} = 0.04$, $p = 0.841$, capture rate: $\chi^2_{(1)} = 0.362$, $p = 0.548$) (Fig. 2). The spiders also attacked (GEE(b), $\chi^2_{(1)} = 1.811$, $p = 0.178$) and captured (GEE(b), $\chi^2_{(1)} = 1.9$, $p = 0.168$) both true bug species with similar frequency during the whole avoidance-learning test.

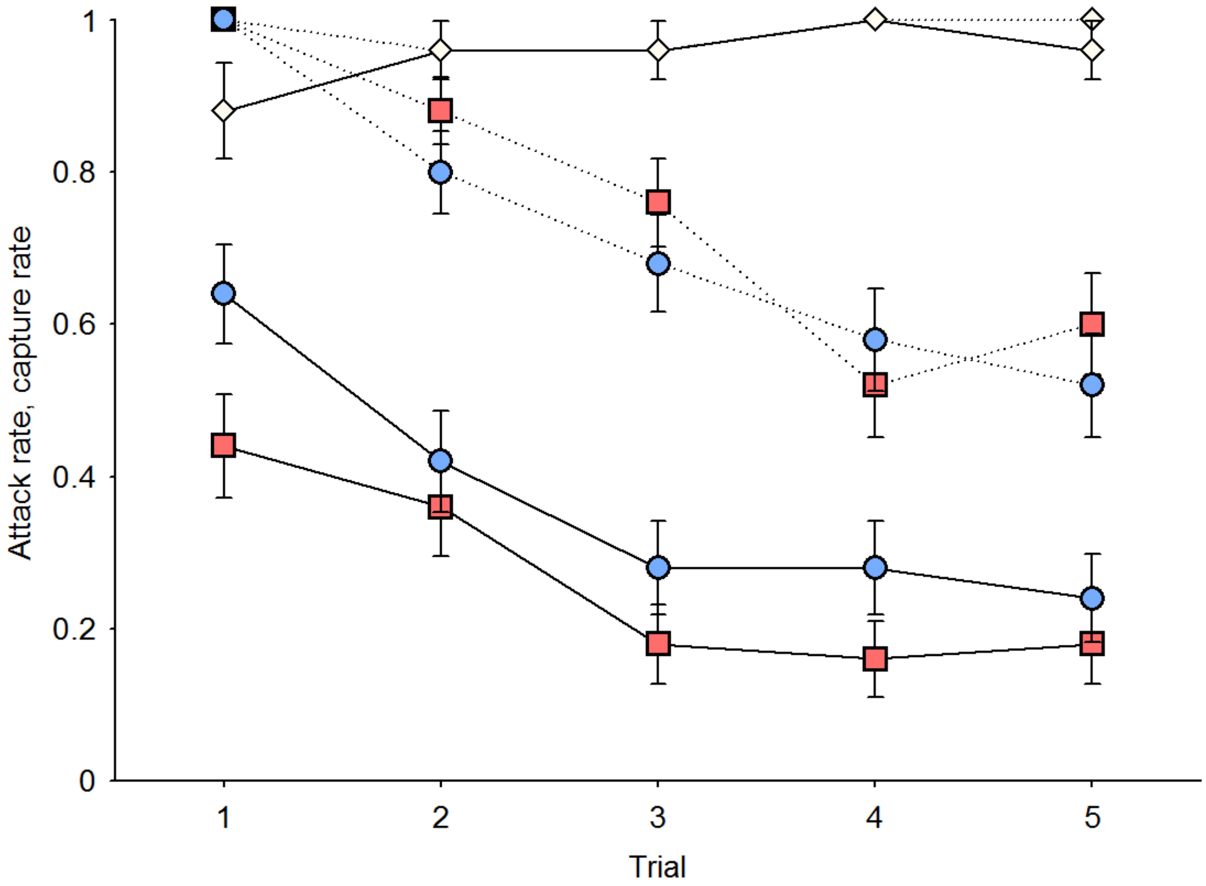

Fig. 2. Attack rates ± SE (dotted lines) and capture rates ± SE (full lines) during five trials of the avoidance-learning test. Pink squares = Pyrrhocoris (N = 50), blue circles = Scantius (N = 50), white diamonds = control fruit flies (N = 25).

On the contrary, when we presented the spiders with the control fruit flies, the attack rate (GEE(b), $\chi^2_{(1)} = 1.78$, $p = 0.182$) and the capture rate (GEE(b), $\chi^2_{(1)} = 1.515$, $p = 0.218$) did not change significantly over the trials, differing from trends observed in Pyrrhocoris (GEE(b), attack rate: $\chi^2_{(1)} = 18.638$, $p < 0.001$; capture rate: $\chi^2_{(1)} = 18.638$, $p < 0.001$) and Scantius (GEE(b), attack rate: $\chi^2_{(1)} = 17.746$, $p < 0.001$; capture rate: $\chi^2_{(1)} = 5.118$, $p = 0.024$) (Fig. 2).

Generalization test

When we switched the presented true bug species after the last trial of the avoidance-learning test (between trials 5 and 6), the attack rate increased significantly following the switch from Scantius to Pyrrhocoris (GEE(b), $\chi^2_{(1)} = 3.884$, $p = 0.049$), but not following the switch from Pyrrhocoris to Scantius (GEE(b), $\chi^2_{(1)} = 0.334$, $p = 0.563$). However, the difference between the change of attack rate during switch from Scantius to Pyrrhocoris and from Pyrrhocoris to Scantius was not significant (GEE(b), $\chi^2_{(1)} = 2.291$, $p = 0.130$). The capture rates did not change significantly after we switched the prey species (GEE(b), from Scantius to Pyrrhocoris: $\chi^2_{(1)} = 0.671$, $p = 0.563$, from Pyrrhocoris to Scantius: $\chi^2_{(1)} = 0.671$, $p = 0.563$) and the trend was the same in both prey combinations (GEE(b), $\chi^2_{(1)} = 0$, $p = 1$) (Fig. 3).

The attack rate towards both true bug species was lower when the spiders had an experience with a similar species (trial 6), compared to their naive counterparts in trial 1 (GLM(b), Pyrrhocoris: $\chi^2_{(1)} = 8.721$, $p = 0.003$, Scantius: $\chi^2_{(1)} = 4.225$, $p = 0.04$). The trend did not depent significantly on which prey was presented first and which second (GLM(b), $\chi^2_{(1)} = 0.362$, $p = 0.547$). Also, if a spider attacked Scantius in trial 6, after a five-trial experience with Pyrrhocoris, it was less likely to capture the prey (GLM(b), $\chi^2_{(1)} = 87.416$, $p = 0.039$). We did not observe this effect when Pyrrhocoris was presented after Scantius (GLM(b), $\chi^2_{(1)} = 88.783$, $p = 0.783$). However, the effect of the prey order (Scantius-Pyrrhocoris vs. Pyrrhocoris-Scantius) was not significant in direct comparison (GLM(b), $\chi^2_{(1,128)} = 176.2$, $p = 0.225$).

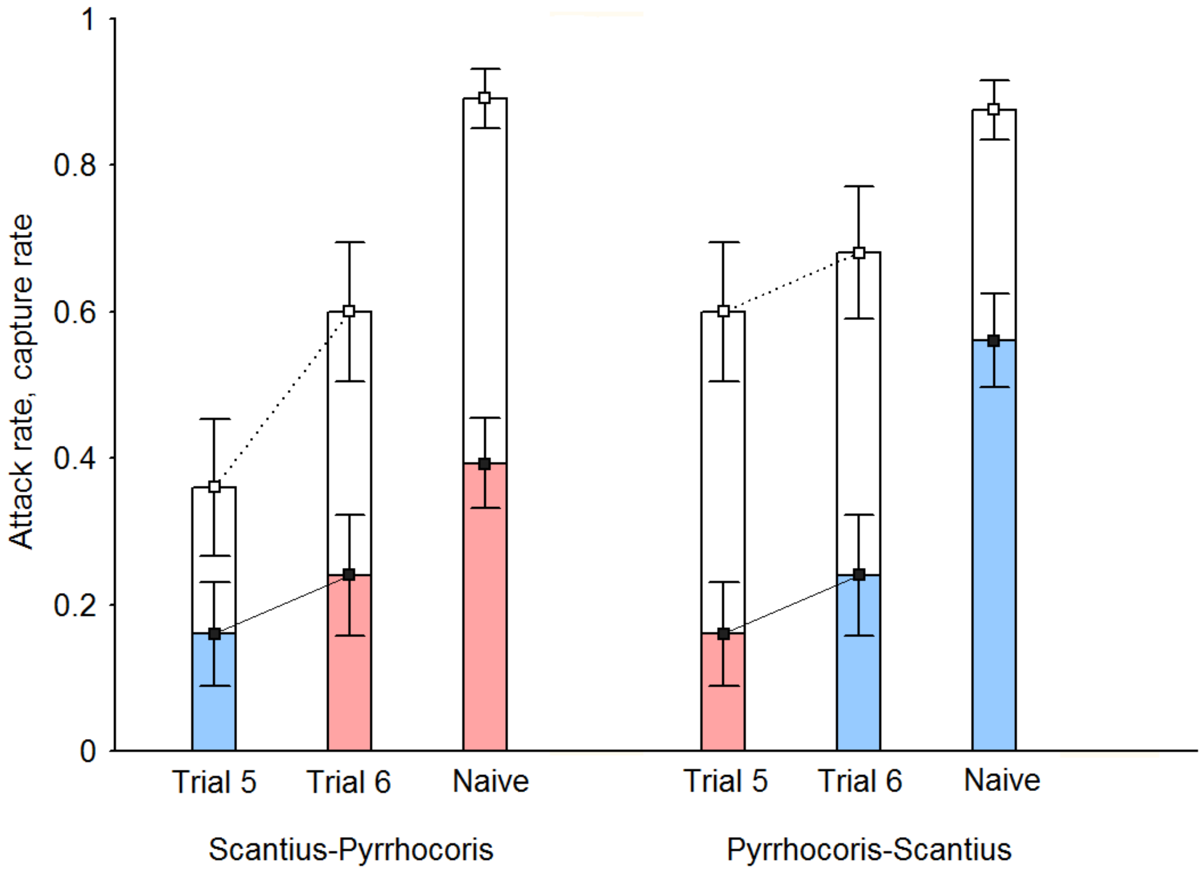

Fig. 3. Attack rates ± SE (open bars connected with dotted lines) and capture rates ± SE (coloured bars connected with full lines) before and after switch from a familiar (trial 5) to a novel prey species (trial 6) (N = 2 × 25) and in comparison with naive spiders (trial 1). Pink = Pyrrhocoris (N = 56), blue = Scantius (N = 57).

Memory test

The attack rate towards both true bug species increased between the last trial of the prolonged avoidance-learning test (trial 8) and the memory test on the second day (trial 9) (GEE(b), Pyrrhocoris: $\chi^2_{(1)} = 7.843$, $p = 0.005$, Scantius: $\chi^2_{(1)} = 4.926$, $p = 0.026$), but the capture rate did not (GEE(b), Pyrrhocoris: $\chi^2_{(1)} = 1.589$, $p = 0.208$, Scantius: $\chi^2_{(1)} = 2.098$, $p = 0.148$) (Fig. 4). The capture rate was also lower in the memory test than in trial 1, when the spiders were naive (although the difference was only marginally significant in Scantius) (Pyrrhocoris: $\chi^2_{(1)} = 0.509$, $p = 0.019$, Scantius: $\chi^2_{(1)} = 3.742$, $p = 0.053$).

Overall trends did not differ significantly between reactions towards Pyrrhocoris and Scantius in the last trial of the prolonged avoidance-learning test and in the memory test (GEE(b), attack rates: $\chi^2_{(1)} = 0.525$, $p = 0.489$; capture rates: $\chi^2_{(1)} = 1.124$, $p = 0.289$; capture rates, trial 1 vs. memory test: $\chi^2_{(1)} = 0.033$, $p = 0.855$).

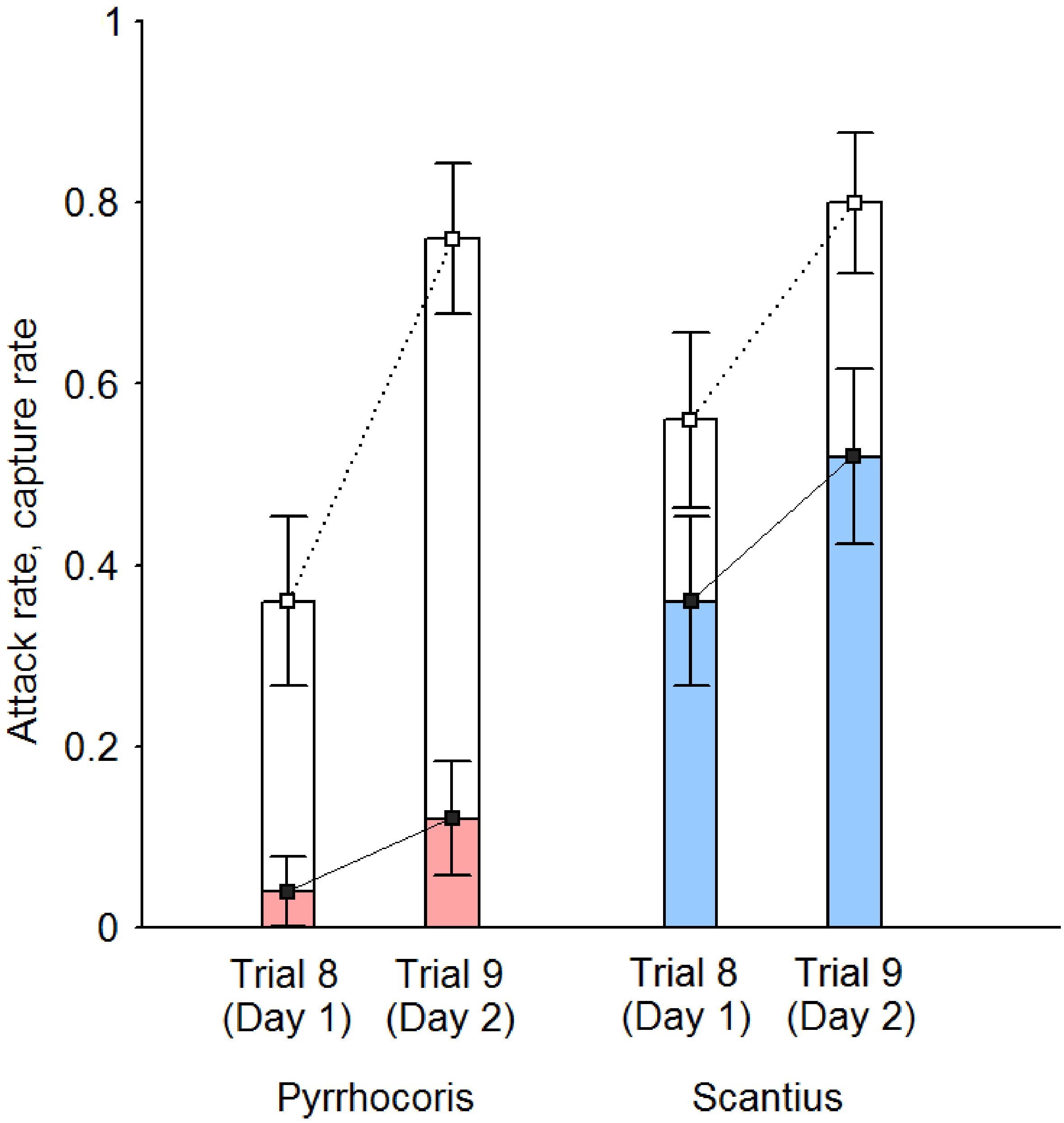

Fig. 4. Attack rates ± SE (open bars connected with dotted lines) and capture rates ± SE (coloured bars connected with full lines) during presentation of the same prey in the last trial of the prolonged avoidance-learning test (trial 8) and in the memory test on the second day (trial 9). Pink = Pyrrhocoris (N = 25), blue = Scantius (N = 25).

Discussion

Secretions of both Pyrrhocoris and Scantius larvae contained several chemicals effective in true bug defence (Aldrich 1988, Farine 1992), but these chemicals represented only a minor fraction of Scantius secretion. Correspondingly, Scantius larvae suffered from slightly higher mortality during spider attacks due to their less effective chemical defence. Nonetheless, spiders effectively learned to avoid both true bug species despite their unequal defence, and retained the learned avoidance until the next day at similar rates. During generalization experiments, spiders were able to generalize learned avoidance to a newly presented true bug species, but the process was asymmetric, providing higher advantage to Scantius larvae.

Secretions

Defensive secretions of Pyrrhocoris and Scantius larvae were markedly different, considering how closely both species are related. On the other hand, interspecific differences in larvae were less striking than differences in adults, probably due to lack of epigamic pheromonal function in the secretion.

Dominant components of larval secretion in Pyrrhocoris are (4)-oxo-(*E*)-2-octenal, (4)-oxo-(*E*)-2-decenal, (*E*)-2-decenal, and (*E*)-2-octenal, altogether representing more than 90 % of the secretion (Farine et al. 1992). These chemicals, along with other unsaturated aldehydes and ketoaldehydes (usually C_6, C_8, or C_{10}), are among the most common components of true bug defensive secretions (Blum 1981, Aldrich 1988), and have been proven to have repellent and/or toxic effects on insects (Remold 1963, Gunawardena & Herath 1991, Prudic et al. 2008). Similar chemicals (especially (*E*)-2-hexenal) have been found in Pyrrhocoris adults (Farine 1992), suggesting defensive function for secretions of both stages.

Although the secretion of Scantius larvae also contains some typical true bug defensive chemicals (e.g., (*E*)-2-hexenal), the main component of the secretion is 2-heptanol. This chemical is relatively common alarm pheromone of bees and ants (e.g., Blum 1981, Schorkopf et al. 2009). Ant alarm pheromones are often synthetized by true bugs, supposedly being effective in defence against these predators (Aldrich 1988). This may be the case of 2-heptanol in Scantius larval secretion. Ants may represent a serious threat to Scantius larvae but not adults, which may be the reason why this chemical is absent in adult secretion. They are replaced with cyclic compounds (Krajicek et al. 2016), which probably serve pheromonal function, but their unique complex structures are still surprising, considering that true bug pheromones are almost always rather simple molecules (Aldrich 1988).

Initial reaction

Great majority of spiders (88–93 %) attacked or at least contacted presented prey species in trial 1. We can therefore conclude that (1) all prey species were sufficiently attractive to the spiders, and (2) the spiders did not show initial avoidance of any of the prey species, either innate or learned in the wild prior to the experiments. The former conclusion supports the hypothesis that 3rd instar larvae of European pyrrhocorids are of an adequate size (50–75 % of the spiders' body length, Nentwig & Wissel 1986) to represent an acceptable prey to *Evarcha arcuata*. The latter conclusion supports the results of previous studies (e.g. Hill 2006) that after a two-week period in captivity, the spiders could be treated as quasi-naive.

We expected that, similarly to results of experiments with bird predators (Exnerová et al. 2005), defence of Pyrrhocoris would be more effective than that of Scantius. Our results suggest that Pyrrhocoris may be the better defended species also against spiders, since they successfully captured Scantius more frequently than Pyrrhocoris in their first encounter with the bugs. Nevertheless, this difference was only marginally significant, and other aspects of behaviour of the spiders were similar towards both species.

Surprisingly, spiders attacked Pyrrhocoris sooner than Scantius during their first encounter. One hypothesis that could explain this difference is that Pyrrhocoris simply moved around more and attracted the spiders' attention. The three pairs of salticid secondary eyes serve mainly as movement detectors (Richman and Jackson 1992), so moving prey can be detected and eventually attacked sooner than a static one. However, post-hoc analysis of prey activity revealed that in fact, it was Scantius which moved around slightly more (69 % of the time on average, compared to 59 % in Pyrrhocoris; ANOVA, $F_{1,98} = 96.402$, $p = 0.059$). It is therefore possible that the spiders' reactions could be affected by some other aspect of Pyrrhocoris's behaviour, for example speed of movement. Fast-moving aposematic prey is frequently attacked sooner than slow-moving one (Hatle & Faragher 1998, Hatle et al. 2002). Since we did not record prey movement speed during our experiments, this hypothesis could be to be tested in further experiments.

Avoidance learning

The attack rate towards both Pyrrhocoris and Scantius dropped by ca 45 % and the capture rate by ca 60 % between trials 1 and 5. These results, contrasting with constantly high attack and capture rates towards the control prey, support the hypothesis that the both true bug species are protected against predation by jumping spiders. In similar experiments with jumping spiders (*Phidippus princeps* (Peckham & Peckham 1883)) and larvae of a milkweed bug *Oncopeltus fasciatus* (Dallas 1852; Lygaeidae: Lygaeinae) the attack rate dropped between trials

1 and 2 even further, becoming only 5 % (Hill 2006), which suggests considerably more effective protection of this species compared to those used in our experiments.

Similar rates at which the spiders learned to avoid both Pyrrhocoris and Scantius, does not fit the experimentally supported (Skelhorn & Rowe 2006, Zvereva et al. 2018) prediction that, of the two species, Pyrhocoris possesses more effective antipredatory defence, since the stronger defence is supposed to induce more rapid avoidance learning (reviewed in Skelhorn et al. 2016, Ruxton et al. 2018). Differences reported in the previous study (Exnerová et al. 2005) may therefore be specific for adult bugs and/or for the defence against avian predators. Defensive secretions of larvae of both species, notwithstanding their differences, may be both sufficiently effective against arthropod (spider) predators. Similar avoidance rates for prey with different chemical defences have been observed also in avian predators (e.g. Skelhorn & Rowe 2005, Chouteau et al. 2019).

Generalization

Our data show that shared colour of the prey species may be sufficient for generalization between them by jumping spiders even if their other characters, such as body shape, pattern, and chemical defence, are similar only partially. Pyrrhocoris has a more rounded body shape than Scantius, is more melanized (Fig. 1) and possesses different defensive chemicals, but after an experience with one of the species, spiders attacked the other one with considerably lower frequency.

Generalization between same-coloured insects is not surprising in jumping spiders, as they have already shown this ability in experiments with red-coloured milkweed bugs and red-painted crickets (Taylor et al. 2015). Colour is critical for recognition of similarity between different prey species, and particularly for birds it is so important that they often do not generalize between closely similar aposematic prey if it differs in warning colour (Aronsson & Gamberale-Stille 2008, 2012, Rönkä et al. 2018, but see Ham et al. 2006, Svádová et al. 2009). Jumping spiders, however, were also able to generalize between different colour morphs of Pyrrhocoris (Raška et al. 2017). Therefore, unlike in birds, shared colour seems to be an effective but not necessary part of mimetic signalization to jumping spiders.

Compared to encounters with naive spiders, both true bug species were attacked substantially less when the spiders had the experience with the other species, but some aspects of the generalization process suggest slight asymmetry of the relationship. The attack rate and the proportion of spiders attacking successfully were generally higher following the switch from Scantius to Pyrrhocoris than vice versa, so it was Scantius that profited more from the species‘ similarity. The most probable reason for this result is more effective defence of Pyrrhocoris, which may subsequently provide better protection to the similar, less protected species.

Memorability

Spiders’ attack rates towards true bugs increased significantly between days 1 and 2. The increases were similar in both presented prey species, although the stronger defence of Pyrrhocoris should theoretically be better memorable than the weak one of Scantius (Skelhorn & Rowe 2006, Ruxton et al. 2018). Increases in attack rates are consistent with results of previous experiments with jumping spiders as predators and true bugs as prey (Hill 2006, Raška et al. 2017). However, the capture rate did not increase significantly between the two days, and was lower on day 2 compared to first trial of day 1, when the spiders were naive. The increase in the attack rate but not the capture rate may indicate that the spiders attacked the prey with caution on day 2, and as a result they killed the bugs with relatively low frequency. Careful handling of a potentially dangerous prey has been proposed as an optimal predator strategy especially in cases when prey individuals within a population vary in presence or intensity of their antipredatory defence (automimicry and go-slow signalling, Guilford 1994), but may also be advantageous in situations when the predator becomes uncertain about the previously learned warning signal and resumes sampling the prey to get new information about its palatability (e.g., Sherratt 2011).

Mimetic relationship

Composition of secretions of Pyrrhocoris and Scantius larvae notably differed, and typical true bug defensive compounds were more abundant in secretion of the former species. Correspondingly, the effectiveness of the secretions in antipredatory defence was slightly higher in Pyrrhocoris. Nevertheless, the decrease of attack rates towards the two true bug species showed that the both species are well protected against spider predators. This information alone, however, still does not say anything about their mimetic relationship. When we compared reactions between naive spiders and spiders experienced with the other bug species, the experienced spiders attacked novel prey significantly less often than the naive ones. It seems that jumping spiders perceive both true bug species as reasonably accurate mimics (the attack rate increased only slightly after the prey change), but certainly not perfect ones (in which case the attack rate would further decrease). The key aspect of similarity between the two species is their shared colour, which is a fundamental trait for recognition of mimicry by birds (Aronsson & Gamberale-Stille 2008, Kazemi et al. 2014) that has also been shown to allow generalization between otherwise different prey in jumping spiders (Taylor et al. 2015).

These results indicate that both true bug species gained some profit from their mutual resemblance. Even though, the mimetic relationship between Pyrrhocoris and Scantius was slightly asymmetric, and Scantius gained more protection from from its similarity to Pyrrhocoris. This asymmetry could be ecologically relevant especially in situations of scarcity of an alternative prey, when Scantius would become acceptable to some predators and the co-occurrence of the two species could be therefore harmful to Pyrrhocoris (e.g. Kokko et al. 2003, Sherratt et al. 2004 and references therein). Under other circumstances, the mimetic relationship between Pyrrhocoris and Scantius appears to be most likely Müllerian, advantageous to both species.

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