School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

Aposematism and toxicity of Coccinellidae

Ph.D. Thesis

Muhammad Aslam, MSc

Supervisor: doc. RNDr. OLDŘICH NEDVĚD, CSc.
Department of Zoology, Faculty of Science, University of South Bohemia,
Institute of Entomology, Biology Centre, Czech Academy of Sciences

České Budějovice 2020

This thesis should be cited as:
Aslam, M. (2020). Aposematism and toxicity of Coccinellidae. Ph.D. Thesis Series, No. 5. in English, University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 137 pp.

Annotation

This thesis highlights the significance of conspicuous colouration and toxicity in ladybirds. We have shown the responses of three bird species to the larvae and pupae of *H. axyridis* to examine whether their colouration has warning sense, showing the deterrent effect of their odour and taste or toxic effect of their chemical protection. The importance of larval colour pattern has been emphasized by presenting models of aposematic ladybird larvae to wild and naïve birds. We have measured the deleterious effect of set of concentrations of extracts from several species of ladybirds by laboratory experiments with captive colonies of ants such as *Lasius niger*. Likewise, laboratory toxicological experiments with extracts from several species of ladybirds have shown specific differences for *Daphnia,* a standard organism used in toxicological assessment. The rate of disappearance of fresh cadavers of various small edible and poisonous invertebrates exposed on soil surface in semi-natural habitat has established how much are ladybirds protected chemically and mechanically without the contribution of their behaviour. Carotenoids are important pigments of ladybirds providing bright red colouration. We have measured the rate of deposition of carotenoids with age in elytra of two ladybird species in ethanol extracts, as well effects of sex and temperature.

Declaration

I hereby declare that my Ph.D. thesis is my work alone and that I have used only those sources and literature detailed in the list of references. Further, I declare that, in accordance with Article 47b of Act No. 111/1998 Coll. in the valid wording, I agree to the publication of my Ph.D. thesis in electronic form in a publically accessible part of the STAG database operated by the University of South Bohemia in České Budějovice on its web page, with the preservation of my rights of authorship to the submitted text of this thesis. Further, I agree to the publication, via the same electronic portal, in accordance with the detailed regulations of Act 111/1998 Coll., of the reviews of the supervisor and opponents of the thesis as well as the record of proceedings and result of the defence of the thesis. I also agree to the comparison of the text of my thesis with the Theses.cz database operated by the National Registry of Theses and the Plagiarism Tracing System.

České Budějovice --

Date: Muhammad Aslam

This thesis originated from a partnership of Faculty of Science, University of South Bohemia and Institute of Entomology, Biology Centre of the ASCR, supporting doctoral studies in the Zoology study programme.

Přírodovědecká
fakulta
Faculty
of Science

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

BIOLOGY
CENTRE
ASCR

Financial support

The study was supported by grants 152/2016/P and 038/2019/P provided by the Grant Agency of University of South Bohemia.

Acknowledgments

I praise Al-mighty ALLAH for enabling me to complete this study.

I would like to express my special thanks of gratitude to my supervisor doc. RNDr. Oldřich Nedvěd, CSc. for his expert, sincere and valuable guidance and encouragement throughout my study.

I am also thankful to RNDr. Petr Veselý, Ph.D. of Department of Zoology, Faculty of Science, University of South Bohemia and Mgr. Kateřina Sam, Ph.D. of Institute of Entomology, Biology Centre, CAS, for their help and cooperation.

Also, I would like to extend my gratitude to Dr John J Sloggett, Lecturer Zoology and Ecology, Maastricht Science Programme,

Maastricht university, The Netherlands for his kind supervision and assistance during my study stay in The Netherlands.

The desire of my father, the priceless support, prayers and love of my mother and my loving sister and the smiles of Muhammad Ammar and Muhammad Talha encouraged me during my stay abroad.

List of articles and author's contribution

This thesis is based on the following articles (listed chronologically) and one summary:

1. **Aslam, M.**, Veselý, P., & Nedvěd, O. (2019). Response of passerine birds and chicks to larvae and pupae of ladybirds. *Ecological Entomology*. 44 (6): 792-799 (IF= 2.073) https://doi.org/10.1111/een.12756
   Muhammad Aslam carried out experimental work and contributed to manuscript writing.

2. **Aslam, M.**, Nedvěd, O. & Sam. K. (2020). Attacks by predators on artificial cryptic and aposematic insect larvae. *Entomologia Experimentalis et Applicata*. (In press) (IF= 1.623)
   Muhammad Aslam carried out experimental work and contributed to manuscript writing.

3. Nedvěd O., **Aslam M.**, Abdolahi R., Sakaki S., Soares A.O. (2019). Age and temperature effects on accumulation of carotenoids in ladybirds. *IOBC WPRS Bulletin*, 145: 33-36. (Summary)
   Muhammad Aslam carried out experimental work related to Harmonia axyridis.

4. **Aslam, M.**, & Nedvěd, O. (a). Response of the ant *Lasius niger* (Hymenoptera: Formicidae) to extracts from ladybirds (Coleoptera: Coccinellidae) (manuscript)

Muhammad Aslam carried out experimental work and contributed to manuscript writing.

5. **Aslam, M.**, & Nedvěd, O. (b). Scavenging rate of palatable and toxic arthropod carrions during day and night (manuscript)
   Muhammad Aslam carried out experimental work and contributed to manuscript writing.

6. **Aslam, M.**, & Nedvěd, O. (c). Toxicity of extracts from ladybirds (Coleoptera: Coccinellidae) for water flea *Daphnia magna* (Cladocera) (manuscript)
   Muhammad Aslam carried out experimental work and contributed to manuscript writing.

TABLE OF CONTENTS

INTRODUCTION

1. Aposematism

Many animals discourage predators through advertisement (warning signals) about their toxicity, unpalatability or otherwise unsuitability as prey items. This is known as aposematism and is usually beneficial to both predators and prey as predators evade the costs of utilizing inappropriate food (including wastage of energy to sickness or even death) and the prey eludes from the predator's attack. Most animals possess bright colours to warn predators of their defences, nonetheless, it is uncertain whether the levels of defence are reliably designated by the strength of warning colours. Two constituents of aposematism include: a primary defence like characteristic colour or smell which indicates the unsuitability of the prey and thus helps to avoid attack; and a secondary defence that includes chemical toxicity and is exercised by the prey when attacked by the predator. For example, the bright colouration (red, orange or yellow) of the wings of ladybirds along with black spots is an advertisement to the predators that they are toxic (Arenas et al., 2015).

Wallace (1889) originally put forward the concept of warning signals for the brightly coloured caterpillars which were apparently incongruous with the theory of natural selection at that time (Winters, 2018). Wallace mentioned the expression of poisonous features as "warning colouration" and the early studies of brightly coloured aposematic signals were mostly limited to the visual perspective only. Now, conspicuous colouration, sounds, odours or other perceivable characteristics have been included among the aposematic signals (Rowe & Guilford, 1999). "Aposematism" is a Greek word used for the first time by Edward Poulton (1890) to depict the Wallace's perception of warning colouration.

1.1. Evolution of aposematism

From the evolutionary perspective, aposematism seems to be paradoxical because aposematic displays make the bearers noticeable to the predators, as a result of which they may be eliminated by killing and the trait can be lost before the predator's learning to avoid it (Briolat et al., 2019). Although the advertisement of unpalatability is clearly beneficial to predators and many species are characterized by aposematic displays, the origin of aposematic traits is less straight. Darwin (1874) speculated that sexual selection is responsible for the origin of bright plumage and other conspicuous traits and these are used primarily by the males to attract females.

The efficacy of aposematic displays increases with their density/frequency. Multiple theories have been suggested to explain the survival of first individuals with mutations that strengthen conspicuousness to assist predator learning and consequently accomplish a selective advantage (Skelhorn et al., 2016). Amongst these, some are concerned with the stochastic situations like the absence of predators temporarily, survival of the mutants by chance or arbitrary shifts in prey population dynamics (Winters, 2018) while others hypothesize non-mutation based origin of aposematism (Ruxton et al., 2004). Aposematic signals might evolve due to their role in other activities like mating (i.e. sexual selection), searching for food, or temperature regulation with the continuation of evolutionary process including multiple selective pressures. Likewise, unperturbed selection on crypsis may contribute to the evolution of aposematic signals in case of dense population of the prey and high possibility of finding by the predators. An abrupt environmental change that initiates a previously cryptic signal to modify into a conspicuous one may also be the cause of aposematism.

The above mentioned hypotheses are, however, not mutually exclusive and the evolution of aposematism occurred through different routes in different species with the passage of time. Advances in the study of evolutionary processes such as phylogenetic analyses and reconstructions may be helpful in understanding the origin of aposematic organisms (Härlin, C, & Härlin, M. 2003).

1.2. Characteristics of aposematic display

Generally, an effective warning display is one that facilitates long-term learning and avoidance by the predator. In spite of the diversity of aposematic displays, most of them also have several common features, which are associated with their functions. Usually, the aposematic displays exhibit conspicuousness including elements like bright colouration or powerful odours. Several reasons have been listed to explain the suitability of conspicuous colours to an aposematic display (Stevens & Ruxton, 2011).

Due to conspicuousness, detection and species identity becomes easy for the predator at a distance. Conspicuous aposematic displays may add to the predator vigilance and increase predator learning, remembering and prey identification precision as compared to other types of signals.

Apart from general conspicuousness, aposematic displays are mostly characterized by other related features (Stevens & Ruxton, 2011). Aposematic warning signals mostly comprise similar colours, usually red, yellow, and black. The reason for this may be that the usage of similar colours enables the aposematic species to minimize species-level learning costs when the avoidance of these aposematic colours is learnt by the predators either individually or through evolutionary time. Many aposematic species share same phenotype to get benefit in Müllerian mimicry. Nevertheless, the common use of red, yellow and black is important with respect to the environment. In comparison to other colours, these colours have a strong contrast with green foliage background, persist distinct in shadow and lighting, show distinctiveness in both colouration as well as brightness and exhibit distance dependent camouflage (Stevens & Ruxton, 2011).

Regardless of the above-mentioned general features, aposematic displays show variations both within species and among closely associated species. Environmental variation or predator community, with respect to seasons or space, are important to maintain this diversity which may be helpful for different morphs to flourish in different times, areas or contexts. There might be involvement of further selective pressures with phenotypes showing an association between aposematism and other phenomena such as sexual selection.

1.3. Signal honesty

Honest signalling has been described in association with handicap hypothesis, which suggests that displays are honest because high costs are related to their production (Penn & Számadó, 2019). However, aposematic displays are not essentially handicaps and there is no consistent link between the mechanisms of aposematic display production and secondary defences (Guilford & Dawkins, 1993). Qualitatively, prey unpalatability is often honestly revealed by the conspicuous aposematic displays as prey lacking secondary defences, such as toxicity, is incapable to disburse for the survival costs of being more prominent to predators (Summers et al., 2015). In absence of secondary defence, conspicuous aposematic displays will result in high rates of predation and reduced fitness. Batesian mimicry is the major exception whereby an undefended species mimics an aposematic species for the sake of getting fitness advantages of the aposematic one without experiencing the costs of defence.

Exhibition of quantitative honest displays by some aposematic species indicates that the strength of aposematic display positively correlates to the degree of predator’s unpalatability (Summers et al., 2015). Qualitative honesty seems to be more common than this, though it needs further research. Quantitatively honest aposematic displays may be predominantly liable when there is morphological or physiological relation of the signal to the mode of defence (Winters, 2018).

2. Aposematism in Coccinellidae

Ladybirds (Coleoptera: Coccinellidae) are famous for their bright colouration and for their important role in the biological control of pests of valuable crops like aphids and scale insects. Nevertheless, they possess unpleasant and toxic defensive chemicals which along with bright colouration make ladybirds truly aposematic (Ceryngier et al., 2012). Especially the best known tribe Coccinellini is characterized by colourful adults with contrasting patterns of red-and-black or yellow-and-black on their elytra serving as aposematic (warning) coloration (Escalona et al., 2017). The body surface of larvae and pupae also consists of aposematically coloured dark and bright areas (Holloway et al., 1991). For some optically oriented vertebrate predators like the great tit (*Parus major*), any spotted pattern and general body shape (oval and convex) may also serve as signal of prey unprofitability besides warning colouration (Dolenská et al., 2009).

A wide range of variation in colouration is common not only among the members of Coccinellidae family but also within some peculiar species exhibiting several colour forms (e.g. *Adalia bipunctata* and *Adalia decempunctata*: Honěk et al., 2005; *Harmonia axyridis*: Kholin, 1990). There is a distinct variation in the number and size of the spots. Plain colouration is characteristic for smaller species such as black Scymnini, unspotted reddish *Coccidula rufa* and brownish *Cynegetis impunctata* (Majerus, 1994).

2.1. Pigments in ladybirds

Carotenoid pigments are found in many insects (Goodwin, 1971), for example, beetles, including orange-red colour of the ladybird beetles. Generally, *de novo* synthesis of carotenoids has never been reported in animals including insects, consequently, those found in animals are of dietary origin (Goodwin, 1952). Metabolic reactions can be responsible for their partial modification (Liaaen-Jensen, 1990; Matsuno, 1989). The carotenoids identified in *Coccinella septempunctata* are probably of microbial, not plant, origin indicating the contribution of symbiotic microorganisms (Britton, et al., 1977).

3. Toxicity of Coccinellidae

Ladybirds are chemically defended against predators by the possession of unpleasant and toxic chemical compounds present in their haemolymph, the most common of which are alkaloids, polyazamacrolides, and polyamines (Laurent et al., 2005). In addition to these compounds, haemolymph of ladybirds also contains chemicals known as pyrazines, which provide olfactory long-distance anti-predatory protection (Guilford et al., 1987; Rothschild & Moore, 1987). Olfactory orienting predators like ants (Sloggett et al., 1998), spiders (Camarano et al., 2006), lacewings (Lucas, 2005), and other ladybirds (Agarwala & Dixon, 1992) avoid to attack ladybirds due to their chemical signals. Some ladybird species are found very toxic to birds (Marples, 1993; Marples et al., 1989). The ladybird haemolymph contains the following major toxic elements:

3.1. Alkaloids

Many ladybirds produce alkaloids which are basic, nitrogen-containing natural compounds and have bitter taste and toxic properties (Daloze et al., 1995). Nearly 50 different types of alkaloids have been studied in ladybirds (Laurent et al., 2005) which are manufactured in their haemolymph including acyclic amines, pyrrolidines, piperidines, 9-azabicyclononanes (homotropanes), 2-methylperhydro-9b-azaphenalenes and azamacrolides. On disturbance, ladybirds emit droplets of their haemolymph at their tibio-femoral joints, which is known as 'reflex blood' and this emission is called 'reflex bleeding'. This mechanism is an efficient defence against predators due to the presence of distasteful and toxic alkaloids (Daloze et al., 1995). Reflex bleeding is common in adults of most ladybird species while in some species, larvae and/or pupae also reflex bleed through the pores of the dorsal surface of the body (Holloway et al., 1991).

The correlation of the existence of alkaloids with the aposematic colours and not with being phytophagous or carnivorous has been established from a survey of 30 different ladybird species (Braekman et al., 1998). Several studies have shown the deterrence activity of the bitter-tasting alkaloids of ladybirds towards ants (Pasteels et al., 1973; Aslam & Nedvěd, unpublished) and jumping spiders (Eisner et al., 1986). Although the toxic effects of ladybirds to nestlings of birds are relatively variable, *Coccinella septempunctata* has been found to be highly toxic to nestling blue tits (Marples et al., 1989).

3.2. Pyrazines

The idea of "olfactory aposematism" proposed by Eisner & Grant (1980) reveal an association of odours and toxicity and resulting avoidance to attack toxic or unpalatable organisms based on their smell. Chemically, 2-methoxy-3-alkylpyrazines found in the haemolymph of ladybirds are aromatic heterocyclic nitrogen containing compounds. When these insects reflex bleed, pyrazines produce the typical odour which, together with aposematic colouration, warns the predators of the unpalatability of ladybirds (Moore et al., 1990).

3.3. Polyamines

Polyamines are organic compounds comprising more than two amino groups. Mixture of macrocyclic polyamines constitutes the major portion of the pupal defensive secretion of phytophagous ladybird *Epilachna borealis*. These compounds include very large-ring lactonic structures derivative of 2-hydroxyethylamino alkanoic acids. A high structural diversity is generated by the combinatorial assembly of the small building blocks with an additional intramolecular reorganisation of the macrocycles (Schröder et al., 1998).

4. Relationship between conspicuousness and toxicity

The signalling role of carotenoids and melanin-based colours in invertebrates is not well investigated. *Harmonia axyridis* may be a model organism for such type of studies due to the great variation of red and black patches on the elytra and the chemical defence with an alkaloid, harmonine. It has been revealed that the visual signal strength is positively correlated with the level of toxicity in the harlequin ladybird *H. axyridis* (Bezzerides et al., 2007). Similar results have been found in the conspicuously coloured poison frog family (Dendrobatidae) (Summers & Clough, 2001). *Harmonia axyridis* females were particularly found having more red (or less black) elytra with high alkaloid content as compared to males, and non-melanics have better chemical defence (Bezzerides et al., 2007). In a supportive argument for this, use of antioxidant molecules for both pigmentation and protection against accumulated toxins has been proposed; hence, conspicuousness and toxicity may be described to be positively correlated due to the presence of these molecules (Blount et al., 2009).

5. Studies of avoidance of ladybirds by birds

Warning coloration of ladybirds is often associated with advertisement of their unpalatability, toxicity, or nutritional unprofitability to potential predators (Blount et al., 2009), particularly birds, which are the most common visual predators (Exnerová et al., 2006). Besides warning colours, any spotted pattern and general appearance of ladybird (oval and convex body shape) may appear to be signals of prey unprofitability for some optically oriented vertebrate predators like the great tit (*Parus major*), as shown by Dolenská et al. (2009).

Although ladybirds are often distasteful and toxic (Daloze et al., 1995), there is substantial variability in predator response. Among vertebrates, predation of ladybirds by birds has been analysed in detail by Mizer (1970), who detected ladybirds belonging to 23 species in the stomach of birds. Ladybird larvae, pupae, and adults make up a major proportion of the nestling diet of tree sparrows (*Passer montanus*) (Wieloch, 1975;

Kříštín, 1986). Experiments with adult tree sparrows (*Passer montanus*) showed that these are able to cope with ingesting chemicals produced by ladybirds and may consume ladybirds during shortage of preferred insect (Veselý et al., 2016). This is in contrast to strong avoidance shown by great tits to several ladybird species (Dolenská et al., 2009) including *H. axyridis* (Průchová et al., 2014). High levels of avoidance can also be expected in domestic chicks (*Gallus gallus domesticus*), which are commonly used in tests of aposematic signals, showing aversion to certain colour patterns (e.g. Schuler & Hesse, 1985; Gamberale & Tullberg, 2001). The study carried out by Marples et al. (1994) using taxonomically related naïve Japanese quail (*Coturnix japonica*) showed that the colour pattern of ladybirds was used as one of the cues for avoiding attack.

The harlequin ladybird (*Harmonia axyridis*) is an optimal model for studying ladybird warning signals. It is a large and highly toxic ladybird (Nedvěd et al., 2010) found in many colour forms (induced genetically – Gautier et al., 2018 – or environmentally – Michie et al., 2010), which are similar in size and shininess (Tan, 1946). Their patterns are formed by combinations of orange to red (carotenoids) and black (melanin; Ando et al., 2018). Larvae and pupae of this species may also be aposematically coloured, combining black and orange (Nedvěd, 2014) which, like adult beetles (Dolenska et al., 2009) are avoided by birds such as *Parus major* (Aslam et al., 2019).

6. Studies of avoidance of ladybirds by ants

The release of reflex blood by adult ladybirds in reaction to ant attacks differs among species and conditions (Pasteels et al., 1973; Sloggett, 1998). According to Majerus (1994), adult ladybirds may use reflex bleeding as a last defence against ants after the failure of other defensive strategies. Chemical extracts from *Epilachna paenulata* applied on a mealworm (*Tenebrio molitor*) deterred ants *Linepithema humile* from feeding on it (Camarano et al., 2006). The synthetic alkaloid adaline originally found in *Adalia bipunctata* was an effective feeding deterrent against fire ants (*Solenopsis invicta*) (Hill & Renbaum, 1982). Less aposematic ladybirds (*Aphidecta obliterata*, *Rhizobius litura* and *Subcoccinella vigintiquatuorpunctata*) were thought to lack alkaloids. Pasteels et al. (1973) presented these three species of ladybirds to ants and *Aphidecta obliterata*, despite a bitter taste was accepted as food while some *R. litura* and *S. vigintiquatuorpunctata* were refused.

Larvae reflex bleed against ant attack (Sloggett, 1998) because they have more chances to be attacked and injured by ants than adult ladybirds (Majerus, 1994). Ladybird eggs and pupae also exhibit chemical defences. Some predators, including ants, are deterred by these defensive chemicals in ladybird eggs (Hemptinne et al., 2005). Reflex bleeding of pupae of some ladybirds (e.g. Chilocorini) and alkaloids found in pupal hairs in *Epilachna varivestis* have also been demonstrated to deter ants (Attygale et al., 1993).

AIMS AND OBJECTIVES OF THESIS

Most ladybirds show an excellent example of aposematism due to their conspicuous coloration. In this PhD thesis, the significance of colouration and toxicity in ladybirds has been highlighted and we studied these aspects of ladybirds with respect to:

I. Protection against vertebrate predators

1. Information about the anti-predatory protection of developmental stages (larvae and pupae) of ladybirds (Coleoptera: Coccinellidae) against vertebrates is very limited, despite their visual look and content of defensive compounds express the definition of an aposematic prey. The responses of three bird species to the larvae and pupae of *H. axyridis* have been observed in laboratory to examine whether their colouration has warning sense, showing the deterrent effect of their odour and taste and possible toxic effect of their chemical protection. Tree sparrows, great tits, and domestic chicks were used as predators, to see if there is a variability in response of predators that differ in their diet, foraging tactics, individual experience, and attitude to ladybird adults.
2. To test the importance of aposematic larval colour pattern of *H. axyridis*, we presented the models of larvae to wild and naïve birds. Specifically, we addressed the following questions: (1) Do visually oriented predators (i.e. birds) attack conspicuously coloured artificial larvae (black) more than cryptic larvae (green) or does the black colouration serve as semi-aposematic signal? (2) Are purely black larvae considered to be more edible than aposematic (black and orange) larvae? (3) Do invertebrate predators also distinguish between the colour modifications? (4) How does the

reaction of naïve and experienced birds to aposematic larvae differ? We predicted that the black colour itself can be sufficient to decrease predators attack (in contrast to attacks to green colour). However, we also predicted (5) that the complete orange and black pattern will prevent predators from attacks significantly more than black colour only. If the ability of the birds to react to the aposematic coloration is not innate, but birds need some earlier negative experience with the signal, we expected that naïve birds will not be deterred by black colour or orange and black pattern and will attack all larvae with similar intensity.

II. Properties of warning colouration

3. Carotenoids are important pigments of ladybirds providing bright red colouration. We measured the rate of deposition of carotenoids in elytra of two ladybird species during aging, as well as the effects of sex and temperature. Carotenoids in ethanol extracts were measured on spectrophotometer.

III. Protection against invertebrate predators

4. We compared the response of the ant *Lasius niger* to increasing concentrations of extracts from several species of ladybirds, to determine the variation in deterrence by different compounds. We expected more deterrence in aposematic and large-sized ladybirds. We also wanted to see whether aphidophagous ladybirds differed from mycophagous ladybirds in their defence against *L. niger*.
5. The rate of disappearance of fresh cadavers of various small edible and poisonous invertebrates exposed on soil surface in semi-natural habitat should show how much are ladybirds protected chemically and mechanically without the contribution of their

behaviour. We hypothesized i) quicker removal of palatable items; ii) quicker removal of native ladybirds than the invasive *Harmonia axyridis*.

IV. Toxicity to invertebrates

6. We tested the toxicity of whole body extract from several species of ladybirds on the water flea *Daphnia magna* that cannot avoid toxins in water solution. We expected that i) young ladybirds would be less toxic to *D. magna* than older ones; ii) more aposematic (red and black) species would be generally more toxic than less aposematic and cryptic ones.; iii) colour morphs of a polymorphic ladybird species would not differ in effects of their defensive chemicals.

CHAPTER – I

Article 1

Article 2

ARTICLE 1

Aslam, M., Veselý, P., & Nedvěd, O. (2019). Response of passerine birds and chicks to larvae and pupae of ladybirds. *Ecological Entomology*. 44 (6): 792-799. https://doi.org/10.1111/een.12756

Ecological Entomology

Ecological Entomology (2019), **44**, 792–799 DOI: 10.1111/een.12756

Response of passerine birds and chicks to larvae and pupae of ladybirds

MUHAMMAD ASLAM,[1,2] PETR VESELÝ[1] and OLDŘICH NEDVĚD[1,2] [1]Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic and [2]Institute of Entomology, Biology Centre, České Budějovice, Czech Republic

Abstract. 1. Few, if any, experimental tests have demonstrated the anti-predator protection of the developmental stages of ladybirds (Coleoptera: Coccinellidae) against vertebrates, despite the fact that both the visual appearance of ladybirds and the content of defensive compounds fulfil the definition of an aposematic prey.

2. In this study, avian predators of three species were confronted with fourth-instar larvae and pupae of the harlequin ladybird (*Harmonia axyridis*) – a large, conspicuous, and toxic ladybird species.

3. The selected predators differed in their individual experience and attitude to ladybirds. Wild-caught great tits (*Parus major*) strongly avoided attacking ladybirds in general, whereas wild-caught tree sparrows (*Passer montanus*) were willing to include ladybirds in their diet. Domestic chicks (*Gallus gallus domesticus*) have never been confronted with ladybirds but usually show avoidance of aposematic signals. In this study, great tits and domestic chicks avoided both developmental stages, but in the case of chicks the avoidance had to be learned over the course of repeated encounters. Sparrow avoidance was significantly lower, with more than one-third of the prey being attacked and eaten.

4. The protection of both developmental stages of ladybirds is similar to adults, despite substantially different visual appearance.

Key words. Alkaloids, aposematism, avoidance learning, pyrazines, warning coloration.

Introduction

Most ladybirds (Coleoptera: Coccinellidae) use alkaloids and pyrazines for chemical protection against predators (Majerus, 1994; Pasteels, 2007; Petersson, 2012). In addition, adults are often brightly coloured with contrasting red-and-black or yellow-and-black patterns on their elytra (mainly the tribes Coccinellini and Chilocorini; Escalona *et al.*, 2017), which fulfils the definition of aposematic (warning) coloration (Moore *et al.*, 1990; Joron, 2003). Warning coloration of ladybirds is often associated with advertisement of their unpalatability, toxicity, or nutritional unprofitability to potential predators (Blount *et al.*, 2009).

It has been demonstrated that due to strong toxicity in some species of ladybirds (Marples *et al.*, 1989; Marples, 1993), it is essential for at least some bird predators to recognise ladybirds accurately. Besides warning colours, any spotted pattern and general appearance of ladybirds (oval and convex body shape) may appear to be signals of prey unprofitability for some optically oriented vertebrate predators such as the great tit (*Parus major*), as shown by Dolenská *et al.* (2009).

Larvae and pupae of ladybirds may also be aposematically coloured with dark and bright areas on their surface (Holloway *et al.*, 1991; Nedvěd, 2015) and also possess toxins (Hautier *et al.*, 2011; Deyrup *et al.*, 2014). Spiny projections or waxy covers of larvae (sometimes shown by pupae as well) are conspicuous morphological anti-predator adaptations (Pope, 1979; Richards, 1980; Majerus *et al.*, 2007).

The harlequin ladybird (*Harmonia axyridis* Pallas) is an optimal model for studying warning signalling by ladybirds. It is a large and highly toxic ladybird (Nedvěd *et al.*, 2010) found in many colour forms [induced genetically (Gautier *et al.*, 2018) or environmentally (Michie *et al.*, 2010)], which are equal in size and shininess (Tan, 1946) and their patterns are formed by combinations of orange to red (carotenoids) and black

Correspondence: Muhammad Aslam, Faculty of Science, University of South Bohemia, Branišovská 1760, 37005 České Budějovice, Czech Republic. E-mail: maslam@icp.edu.pk

(melanin; Ando *et al.*, 2018). Larvae and pupae of this species may also be aposematically coloured, combining black and orange (Nedvěd, 2014), but little is known about their toxicity or predator responses to them. Artificial larvae resembling *H. axyridis* were attacked much less often than green or black dummies (Aslam *et al.*, 2019).

Although ladybirds are often distasteful and toxic (Daloze *et al.*, 1995), there is substantial variability in predator response. Among vertebrates, predation of ladybirds by birds has been analysed in detail by Mizer (1970), who detected ladybirds belonging to 23 species in the stomachs of birds. Ladybird larvae, pupae, and adults make up a major proportion of the nestling diet of tree sparrows (*Passer montanus*) (Wieloch, 1975; Krištín, 1986; Krištín & Lebedeva, 1995). Veselý *et al.* (2016) showed experimentally that adult tree sparrows (*Passer montanus*) are able to cope with ingesting chemicals produced by ladybirds and may consume ladybirds during shortages in preferred insect availability. This is in contrast to the strong avoidance shown by great tits to several ladybird species (Dolenská *et al.*, 2009), including *H. axyridis* (Průchová *et al.*, 2014). We can also expect high levels of avoidance in domestic chicks (*Gallus gallus domesticus*), which are commonly used in tests of aposematic signals, showing aversion to certain colour patterns (e.g. Schuler & Hesse, 1985; Gamberale & Tullberg, 2001). Nevertheless, we are not aware of any study testing their responses to ladybirds experimentally. The study carried out by Marples *et al.* (1994) using taxonomically related naïve Japanese quail (*Coturnix japonica*) showed that the colour pattern of ladybirds was used as one of the cues for avoiding attack.

In this study, we observed the responses of three bird species to the larvae and pupae of *H. axyridis* to investigate whether their coloration has warning meaning, advertising the deterrent effect of their smell and taste or the toxic effect of their chemical protection. We used tree sparrows, great tits, and domestic chicks as predators, to see if there is variability in the response of predators that differ in their diet, foraging tactics, individual experience, and attitude to ladybird adults.

Materials and methods

Predators

We used three species of predators. Wild ranging great tits (hereafter called '*Parus*') and tree sparrows (hereafter called '*Passer*'), both native and common in the surroundings of České Budějovice (Czechia, 49°00′N, 14°27′E), were caught by a licensed ringer (PV) using mist nets during the non-breeding season (September–April, in 2017 and 2018). After tagging with a metal ring, each bird was subsequently kept in a commercially sold birdcage (40 × 40 × 20 cm) in a room at lowered room temperature (*c.* 15 °C) and natural photoperiod (LD 12:12 h during winter). There was more than one cage in the room so that birds could see each other in order to reduce stress from separation. Diet for the birds included mealworms, sunflower seeds, and commercial feed for insectivorous birds. Water was supplied *ad libitum*. After the experiments, birds were released at the place of the capture. Each bird spent 2–4 days in the laboratory.

Domestic chicks (hereafter called '*Gallus*'), 1 or 2 days old, were obtained from factory farms, transported to the birdcages in a room at room temperature and natural photoperiod, and supplied with mealworms, commercially sold fodder for chickens, and water. After the experiments, chicks were returned to the farms. Chicks were always kept in pairs to avoid stress from separation.

Prey

The prey used in our experiments were larvae and pupae of the ladybird *H. axyridis* forma *novemdecimsignata* [described by Faldermann (1835) but usually referred to in the literature as forma *succinea*, which was described by Hope (1843) and included only the phenotype without black spots]. Larvae reach 12 mm in length and their coloration is predominantly black with some of the abdominal scoli orange and with small white lateral spots (Fig. 1a). The head capsule and legs of the larvae are hard, but otherwise the body is soft. Laboratory-reared fourth-instar larvae were used. They were fed with aphids *Acyrthosiphon pisum* reared on *Vicia faba*, kept at an average temperature of 20 °C and in LD 18:6 h conditions.

Pupae of *H. axyridis* are orange with black spots (Fig. 1b), although there is great variation in the extent of melanised spots depending on ambient temperature during late fourth-instar larval development (Nedvěd, 2014). The pupae are not fully immobile. If attacked, the predator can be deterred by rapid up-and-down flicking movements of the anterior end of the pupa. Against small insect predators, these movements represent an effective defence (called a 'gin trap'). Pupae were stored at 17 °C prior to the experiment to prevent rapid development to adult.

Experimental equipment

The experimental cage was made of wooden cubic frames (70 cm × 70 cm × 70 cm) with the front wall made from a one-way mirror, while the other walls were covered with fine wire mesh (2 mm × 2 mm). The cage was equipped with a perch, a bowl of water, and a rotating circular feeding tray containing small cups, where only the cup nearest to the perch contained prey during each trial. The bottom of each cup was white to provide sufficient contrast for the birds to distinguish the prey. The distance between the tray and the perch was *c.* 25 cm. Standard illumination was obtained by a light source (Lumilux Combi 18 W; Osram Bruntál, Czech Republic) that simulates a full daylight spectrum, including UV radiation.

Trials

A total of 120 birds were used in the experiments, including 40 great tits (*Parus*), 40 tree sparrows (*Passer*), and 40 chicks (*Gallus*). From each species 20 birds were offered larvae and the other 20 birds were offered pupae. After being moved from breeding cages to the experimental cage, each bird was trained to eat a mealworm immediately after offering. After this training, the bird was deprived of food for 2 h to be motivated

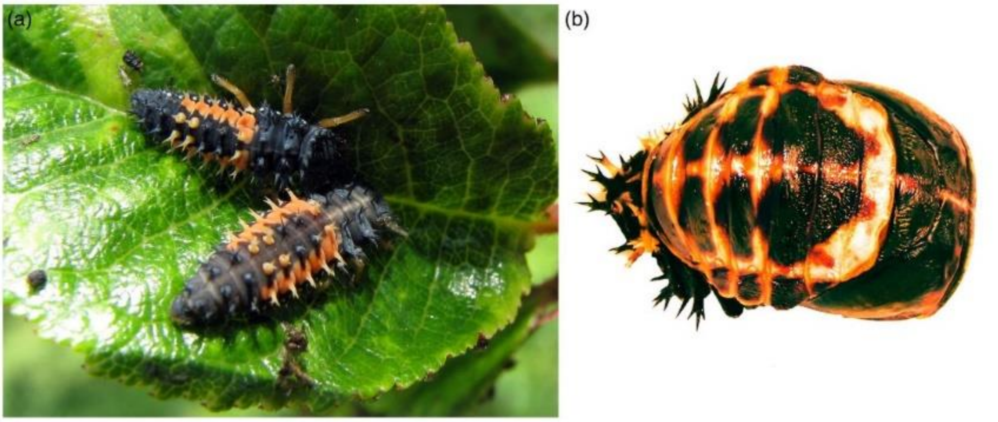


Fig. 1. (a, b) Larvae (fourth larval instar) (a) and pupa (b) of *Harmonia axyridis*. Photograph by Oldřich Nedvěd. [Colour figure can be viewed at wileyonlinelibrary.com].

to forage but not stressed by starvation (as highlighted by Exnerová *et al.*, 2003).

Every bird was used in a single experiment comprising five trials. Each trial consisted of offering a mealworm to examine the readiness of the bird to forage, followed immediately by the presentation of the experimental prey (one larva or one pupa). Altogether, each bird was offered five mealworms and five experimental prey individuals in an alternating sequence. This repeated sequence of presentation was employed to decrease the potential effects of neophobia in the birds (Marples & Kelly, 1999). Complete swallowing of the mealworm (control prey) was followed by presentation of larva or pupa. Every presentation of a larva or pupa lasted 5 min. All the successive trials were carried out with minimal delay (only an event of seconds required to rotate the feeding tray). All experiments were conducted during the daytime, between 10.00 and 16.00 hours. The OBSERVER 3.0 ethological software package (Noldus Information Technology, Wageningen, the Netherlands) was used to record the birds' behaviour.

Statistical analyses

In our analyses we focused on three behavioural responses of tested birds:

1. The occurrence of attacking the larva or pupa within the prey trial (five for each individual bird) was counted as '1' when the tested bird attacked and '0' when it did not. This response variable had a binomial distribution of variability. We analysed the effect of the predictors (see later) with a generalised linear mixed effect model (GLMM), where the bird's identity was included as a random factor, as each bird was offered five prey individuals. A likelihood ratio test following a χ^2 test with binomial distribution was used to assess the effects of the predictor variables. Fisher's least significant difference (LSD) test (z-value) with the Tukey adjustment for repeated comparisons was used as a *post hoc* test.
2. Each occurrence of prey consumption was scored as '1' when at least part of the offered prey was eaten. This response variable thus had a binomial distribution of variability. We also analysed these predictors with a GLMM, where bird identity was again included as a random factor. We used the same likelihood ratio test following the χ^2 test with binomial distribution to assess the effect of the predictor variables, as well as a Fisher LSD test (z-value) with the Tukey adjustment for repeated comparisons as a *post hoc* test.
3. We also tested the latency to the first attack by each tested bird by recording the difference (in s) between the start of the first trial and the first attack (touching or taking the prey). The first attacked prey might be any of the five offered prey, and therefore the latency was additive. When the first prey was not attacked, we scored 300 s until any prey was attacked. If no prey of the five offered was attacked, we scored 1500 s (five 5-min-long trials). The residual variability of this response variable followed a gamma distribution and thus the effect of predictors was assessed using the generalised linear model (GLM). A likelihood ratio test following the gamma distribution with the χ^2 test was used to assess the effect of the predictor variables, and a Fisher's LSD test (z-value) with the Tukey adjustment for repeated comparisons was used as the *post hoc* test.

In the first two models (occurrence of attacking and eating the prey) we included the following predictor variables: interaction of the bird species (*Gallus*, *Parus*, *Passer*) and prey type (larva, pupa) and interaction of the bird species and ordinal number of the trial (one to five for each bird).

Table 1. Effects of predictors in particular models.

Response variable	Interaction of predictors	d.f.	AIC	BIC	χ^2	P
Occurrence of attack	Null model		502.41	511.18		
	Bird × prey	**7**	**486.94**	**517.62**	**25.474**	**< 0.001**
	Bird × trial	**19**	**465.14**	**548.43**	**45.797**	**≪ 0.001**
Occurrence of eating	Null model		511.38	529.15		
	Bird × prey	**7**	**493.11**	**534.16**	**18.254**	**0.003**
	Bird × trial	19	490.36	536.97	7.256	0.159
Latency to the attack	Null model		159.24	211.78		
	Bird	**2**	**114.73**	**254.17**	**13.602**	**≪ 0.001**
	Prey	1	111.77	257.35	0.931	0.337
	Bird × prey	5	109.58	259.48	1.111	0.333

AIC, Akaike criterion; BIC, Bayesian information criterion; Bird, tested bird species (*Gallus*, *Parus*, *Passer*); prey, prey type (larva or pupa); trial, order number of trial within the series of five.
Significant effects are highlighted in bold.

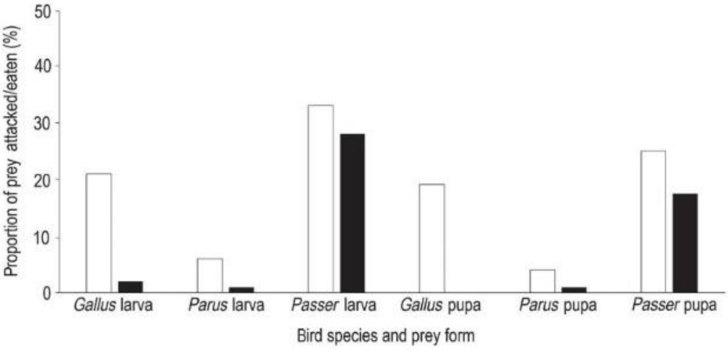


Fig. 2. Proportion of larvae and pupae of *Harmonia axyridis* attacked (white columns) and eaten (black columns) by particular bird species. *Gallus*, young domestic fowl (*Gallus gallus domesticus*); *Parus*, great tit (*Parus major*); *Passer*, tree sparrow (*Passer montanus*).

Results

Occurrence of attack

The probability of attack to offered prey was significantly affected by the interaction of bird species and prey type. We decided to analyse reactions to larvae and pupae separately. Furthermore, the attack probability was affected by the interaction of the factors bird species and trial number. We therefore decided to look at the learning process within individual predator species (Table 1). Other factor interactions had no significant effect on the data variability (Table 1).

Passer ($z = 3.152$, d.f. = 99, $P = 0.016$; Fig. 2) and *Gallus* ($z = 2.935$, d.f. = 99, $P = 0.039$; Fig. 2) attacked larvae significantly more often than did *Parus*. There was no difference in the attack rate of larvae between *Gallus* and *Passer* ($z = 1.458$, d.f. = 99, $P = 0.287$; Fig. 2). *Passer* ($z = 3.268$, d.f. = 99, $P = 0.010$; Fig. 2) as well as *Gallus* ($z = 2.959$, d.f. = 99, $P = 0.036$; Fig. 2) attacked pupae significantly more often than *Parus*. *Passer* attacked pupae at equal rates to *Gallus* ($z = 1.615$, d.f. = 99, $P = 0.582$; Fig. 2). There were no differences in attack rates towards larvae or pupae by any of the tested bird species (z-values < 1.587, d.f. = 99, P-values > 0.721; Fig. 2).

There was a significant effect of trial order number (within the sequence of five trials) in the case of *Gallus*, where the attack rate to the first offered prey (larvae and pupae together) was significantly higher than the attack rate to the second ($z = 2.931$, d.f. = 99, $P = 0.039$; Fig. 3), third ($z = 2.724$, d.f. = 99, $P = 0.045$; Fig. 3), fourth ($z = 3.154$, d.f. = 99, $P = 0.015$; Fig. 3) and fifth ($z = 3.154$, d.f. = 99, $P = 0.015$; Fig. 3) prey. There was no difference among trials of *Parus* (z-values < 2.105, P-values > 0.251; Fig. 3) or *Passer* (z-values < 1.325, d.f. = 99, P-values > 0.843; Fig. 3).

Occurrence of eating

The probability of eating the offered prey was significantly affected by the interaction of bird species and prey type (Table 1) – therefore we have to analyse predator response to larvae and pupae separately. Other factor interactions had no significant effect on the data variability (Table 1).

Passer ate larvae significantly more often than *Parus* ($z = 4.231$, d.f. = 99, $P = 0.001$; Fig. 2) and *Gallus* ($z = 3.935$,

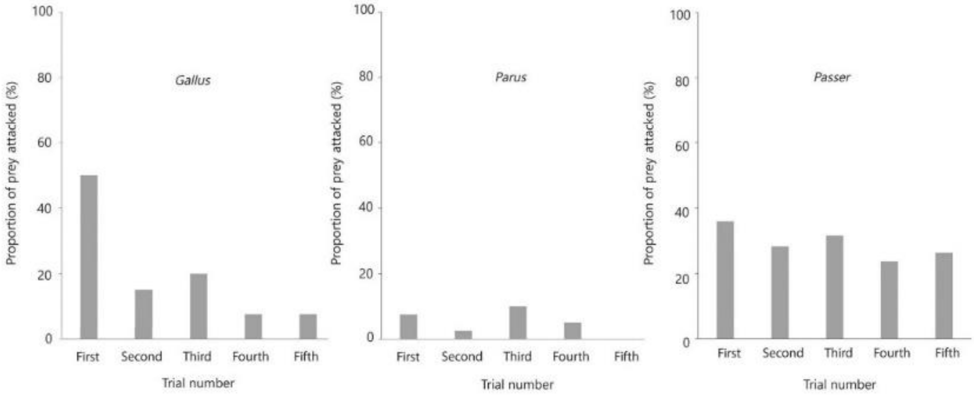


Fig. 3. Proportion of larvae and pupae (together) of *Harmonia axyridis* attacked by particular bird species in the course of five successive trials. *Gallus*, young domestic fowl (*Gallus gallus domesticus*); *Parus*, great tit (*Parus major*); *Passer*, tree sparrow (*Passer montanus*).

d.f. = 99, $P = 0.003$; Fig. 2). There was no difference in the eating rate of larvae between *Gallus* and *Parus* ($z = 1.031$, d.f. = 99, $P = 0.489$; Fig. 2). *Passer* ate pupae significantly more often than *Parus* ($z = 3.615$, d.f. = 99, $P = 0.005$; Fig. 2) as well as *Gallus* ($z = 4.268$, d.f. = 99, $P = 0.001$; Fig. 2). *Parus* and *Gallus* ate pupae equally scarcely ($z = 0.658$, d.f. = 99, $P = 0.985$). There were no differences between the consumption rate of larvae or pupae in any of tested bird species (z-values < 1.124, d.f. = 99, P-values > 0.726; Fig. 2).

Latency to the first attack

The latency until the first attack by particular birds was affected by bird species. There was no effect of prey type and the interaction of bird species and prey type (Table 1). *Gallus* attacked the prey significantly quicker than *Parus* ($z = 2.875$, d.f. = 19, $P = 0.013$). The latency to the first attack of *Passer* was somewhere in between, not differing from *Parus* ($z = 1.700$, d.f. = 19, $P = 0.210$) or *Gallus* ($z = 1.175$, d.f. = 19, $P = 0.471$). This effect was equal for pupae and larvae (Fig. 4).

Discussion

Our experiments reveal that the immature developmental stages (larvae and pupae) – and not just adults – of ladybird beetles (Coccinellidae) are protected against predation by potential bird predators. The low number of our experimental birds attacking either larvae or pupae of the ladybird generally suggests that there is visual aversion based on the appearance of the insects. Although larvae and pupae differ in their appearance, they were both avoided at similar rates by individuals of each predator species. We hypothesise that their coloration (orange and black) may be responsible for this aversion, separately for each life stage and its specific pattern. This partly contrasts with the findings of Dolenská *et al.* (2009), who showed that the body shape of adult ladybirds plays an important role in their protection. Larvae, pupae, and adults of the same species of ladybird have different body shape as well as different patterns, consisting of black, red, yellow and white patches (e.g. colour appendix in Nedvěd, 2015). Therefore, visually oriented predators could not generalise between these types of unpalatable prey, even though all the developmental stages occur together in the same habitat. An exception is *Psyllobora vigintiduopunctata*, which has a lemon yellow background with small black spots in all three developmental stages (Nedvěd, 2015: appendix). Such intraspecific Müllerian mimicry warrants further study.

The aversion of birds to black and orange coloration of their prey can be both partially innate and learned. Only one-half of naïve chicks of *Gallus* attacked larvae or pupae of ladybirds during 5 min in the first trial, although they ate mealworms without hesitation. The proportion of *Gallus* attacking immature ladybirds then decreased and remained at < 10% in the fourth and fifth trials. No such learning was found in *Parus* and *Passer* during the feeding trials, which corresponds to the expected experience with diverse aposematically coloured insects in these wild-caught birds.

The chemical defence of immature ladybirds was also supported by our findings. It was most pronounced in *Gallus*, where the number of ladybirds eaten was very low despite a moderate attack rate. Chemical defence most probably resulted in the avoidance learning by *Gallus*. Also, *Parus* consumed much lower numbers of attacked ladybirds, suggesting an effect of chemical protection. The chemical-based avoidance in *Parus* has already been shown in studies with other ladybird species (Dolenská *et al.*, 2009) and other unpalatable aposematic insects (Svadova *et al.*, 2013).

The only predatory species that readily ate immature ladybirds was *Passer*; not surprisingly, because they have been shown to feed on adult ladybirds both in the wild (Krištín, 1986) and in the laboratory (Veselý *et al.*, 2016). Although the chemical defence of *H. axyridis* against birds is considered to be particularly strong (Průchová *et al.*, 2014), this species is still acceptable to

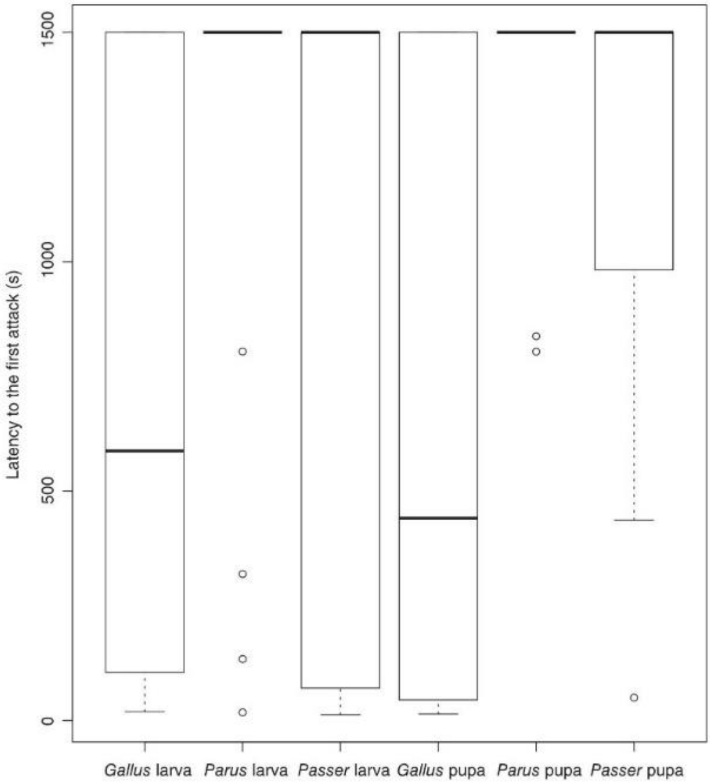


Fig. 4. Latency (time) to the first attack by particular birds towards larvae and pupae of *Harmonia axyridis*. *Gallus*, young domestic fowl (*Gallus gallus domesticus*); *Parus*, great tit (*Parus major*); *Passer*, tree sparrow (*Passer montanus*).

Passer without any apparent physiological problems. However, the attack and feeding rates of *Passer* on adult ladybirds in winter (the same season as this study) were higher (Veselý *et al.*, 2016) than on immatures in this study. Our previous study suggests that tree sparrows in winter include ladybirds in their diet due to the shortage of food availability. We hypothesise that there was not a strong shortage of food in the seasons of this study compared with the winter of the previous one, so that *Passer* did not switch to less profitable prey to a strong degree. Alternatively, the presence of larvae and pupae of ladybirds may be unusual for *Passer* during winter, as they are normally present from May to August in the wild (Kula & Nedvěd, 2011). This might result in some hesitancy to attack them, due to unfamiliarity, whereas they may be more willing to attack adult ladybirds that overwinter on tree branches (Holecová *et al.*, 2018).

Conclusions

We used feeding trial experiments to show that larvae and pupae of ladybirds (Coccinellidae) are protected against predation by potential bird predators. There is some variability depending on the particular bird species, but most individuals avoided attacking larvae and pupae of *H. axyridis*. We hypothesise that coloration may protect the ladybird larvae and pupae from being attacked, in addition to chemical protection, which resulted in avoidance learning in domestic chicks.

Acknowledgements

We thank Joel Brown for English edits. We thank the Selgen company in Stupice, Czech Republic, for providing seeds of *Vicia faba* for free. Experiments carried out in the course of this research comply with the current laws of the Czech Republic and follow all animal care and ethics guidelines of the University of South Bohemia. The authors are licensed for animal experimentation (Ministry of Agriculture, CZ 02766) and for conducting experiments with animals (Ministry of the Environment, 833/630/15). The Faculty of Science has accreditation for breeding birds (Ministry of Agriculture, 8760/2013-MZE-17214). A licensed bird ringer (PV, license no. 1004) performed bird captures. The research was funded by the University of South Bohemia (GA JU 152/2016/P). The authors declare they have no conflicts of interest.

Author contributions

MA designed the experiments, conducted all experiments and helped to write the manuscript; PV designed the experiments, performed the data analyses and helped to write the manuscript; and ON designed the experiments and helped to write the manuscript.

References

Ando, T., Matsuda, T., Goto, K., Hara, K., Ito, A., Hirata, J. *et al.* (2018) Repeated inversions within a pannier intron drive diversification of intraspecific colour patterns of ladybird beetles. *Nature Communications*, **9**, 3843.

Aslam, M., Nedvěd, O. & Sam, K. (2019) Attacks by predators on artificial cryptic and aposematic insect larvae. *Entomologia Experimentalis et Applicata*, submitted.

Blount, J.D., Speed, M.P., Ruxton, G.D. & Stephens, P.A. (2009) Warning displays may function as honest signals of toxicity. *Proceedings of Royal Society of London (B)*, **276**, 871–877.

Daloze, D., Braekman, J.-C. & Pasteels, J.M. (1995) Ladybird defence alkaloids: Structural, chemotaxonomic and biosynthetic aspects (Col: Coccinellidae). *Chemoecology*, **5**(6), 173–183.

Deyrup, S.T., Eckman, L.E., Lucadamo, E.E., McCarthy, P.H., Knapp, J.C. & Smedley, S.R. (2014) Antipredator activity and endogenous biosynthesis of defensive secretion in larval and pupal *Delphastus catalinae* (Horn) (Coleoptera: Coccinellidae). *Chemoecology*, **24**, 145–157.

Dolenská, M., Nedvěd, O., Veselý, P., Tesařová, M. & Fuchs, R. (2009) What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? *Biological Journal of the Linnean Society*, **98**, 234–242.

Escalona, H.E., Zwick, A., Li, H.-S., Li, J., Wang, X., Pang, H. *et al.* (2017) Molecular phylogeny reveals food plasticity in the evolution of true ladybird beetles (Coleoptera: Coccinellidae: Coccinellini). *BMC Evolutionary Biology*, **17**, 151.

Exnerová, A., Landová, E., Štys, P., Fuchs, R., Prokopová, M. & Cehláriková, P. (2003) Reactions of passerine birds to aposematic and nonaposematic firebugs (*Pyrrhocoris apterus*; Heteroptera). *Biological Journal of the Linnean Society*, **78**, 517–525.

Faldermann, F. (1835) Coleopterorum abb ill. Bungio in China boreale, Mongolia et montibus Altaicus collectorum nec non ab ill. Turczaninoffio et Stschukino e provincia Irkutzk missorum illustrationes. *Mémoires de l'Académie des Sciences de Saint Pétersburg*, **2**, 337–464.

Gamberale, G. & Tullberg, B.S. (2001) Fruit or aposematic insect? Context-dependent colour preferences in domestic chicks. *Proceedings of Royal Society of London*, **268**, 2525–2529.

Gautier, M., Yamaguchi, J., Foucaud, J., Loiseau, A., Ausset, A., Facon, B. *et al.* (2018) The genomic basis of color pattern polymorphism in the harlequin ladybird. *Current Biology*, **28**, 3296–3302.e7.

Hautier, L., Martin, G., Gomez, G., Callier, P., de Biseau, J.C. & Gregoire, J.C. (2011) Alkaloids provide evidence of intraguild predation on native coccinellids by *Harmonia axyridis* in the field. *Biological Invasions*, **13**, 1805–1814.

Holecová, M., Zach, P., Hollá, K., Šebestová, M., Klesniaková, M., Šestáková, A. *et al.* (2018) Overwintering of ladybirds (Coleoptera: Coccinellidae) on Scots pine in Central Europe. *European Journal of Entomology*, **115**, 658–667.

Holloway, G.J., De Jong, P.W., Brakefield, P.M. & De Vos, H. (1991) Chemical defence in ladybird beetles (Coccinellidae). I. Distribution of coccinelline and individual variation in defence in 7-spot ladybird (*Coccinella septempunctata*). *Chemoecology*, **2**, 7–14.

Hope, F.W. (1843) Descriptions of the coleopterous insects sent to England by Dr. Cantor from Chusan and Canton, with observation on the entomology of China. *The Annals and Magazine of Natural History*, **11**, 62–66.

Joron, M. (2003) Aposematic colouration. *Encyclopedia of Insects* (ed. by R. T. Carde and V. H. Resh), pp. 39–45. Academic Press, New York, New York.

Krištín, A. (1986) Heteroptera. Coccinea. Coccinellidae and Syrphidae in the food of *Passer montanus* L. and *Pica pica* L. *Biologia (Bratislava)*, **41**, 143–150.

Krištín, A., Lebedeva, N. & Pinowski, J. (1995) The diet of nestling tree sparrows (*Passer montanus*). Preliminary report. *International Studies on Sparrows*, **20-21**, 3–19.

Kula, E. & Nedvěd, O. (2011) *Chilocorus renipustulatus* (Coleoptera: Coccinellidae) dominates predatory ladybird assemblages on *Sorbus aucuparia* (Rosales: Rosaceae). *European Journal of Entomology*, **108**, 603–608.

Majerus, M.E.N. (1994) *Ladybird*, p. 367. Harper Collins, London.

Majerus, M.E.N., Sloggett, J.J., Godeau, J.-F. & Hemptinne, J.-L. (2007) Interactions between ants and aphidophagous and coccidophagous ladybirds. *Population Ecology*, **49**, 15–27.

Marples, N. (1993) Toxicity assays of ladybirds using natural predators. *Chemoecology*, **4**, 33–38.

Marples, N.M. & Kelly, D.J. (1999) Neophobia and dietary conservatism: two distinct processes? *Evolutionary Ecology*, **13**, 641–653.

Marples, N.M., Brakefield, P.M. & Cowie, R.J. (1989) Differences between the 7-spot and 2-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. *Ecological Entomology*, **14**, 79–84.

Marples, N.M., Van Veelen, W. & Brakefield, P.M. (1994) The relative importance of color, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour*, **48**, 967–974.

Michie, L.J., Mallard, F., Majerus, M.E.N. & Jiggins, F.M. (2010) Melanic through nature or nurture: genetic polymorphism and phenotypic plasticity in *Harmonia axyridis*. *Journal of Evolutionary Biology*, **23**, 1699–1707.

Mizer, A.V. (1970) On eating of beetles from Coccinellidae family by birds. *Vestnik Zoologie*, **6**, 21–24.

Moore, B.L., Brown, W.V. & Rothschild, M. (1990) Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology*, **1**, 43–51.

Nedvěd, O. (2014) Slunéčko východní (*Harmonia axyridis*) – pomocník v biologické ochraně nebo ohrožení biodiverzity? The harlequin ladybird (*Harmonia axyridis*) - helper in biological control or threat to biodiversity? Jihočeská univerzita, České Budějovice, pp. **65**. (In Czech)

Nedvěd, O. (2015) *Brouci čeledi slunéčkovití (Coccinellidae) střední Evropy. Ladybird beetles (Coccinellidae) of Central Europe*, p. 304+LXXIX. Academia, Praha.

Nedvěd, O., Kalushkov, P., Fois, X., Ungerová, D. & Rozsypalová, A. (2010) *Harmonia axyridis*: six-legged alligator or lethal fugu? *IOBC/WPRS Bulletin*, **58**, 65–68.

Pasteels, J.M. (2007) Chemical defence, offence and alliance in ants-aphids-ladybirds relationships. *Population Ecology*, **49**, 5–14.

Petersson, J. (2012) Coccinellids and semiochemicals. *Ecology and Behaviour of the Ladybird Beetles (Coccinellidae)* (ed. by I. Hodĕk,

H. van Emden and A. Honěk), pp. 444–464. Wiley-Blackwell, London.

Pope, R.D. (1979) Wax production by coccinellid larvae (Coleoptera). *Systematic Entomology*, **4**, 171–196.

Průchová, A., Nedvěd, O., Veselý, P., Ernestová, B. & Fuchs, R. (2014) Visual warning signals of the ladybird *Harmonia axyridis*: the avian predators' point of view. *Entomologia Experimentalis et Applicata*, **151**, 128–134.

Richards, A.M. (1980) Defensive adaptations and behaviour in *Scymnodes lividigaster* (Coleoptera: Coccinellidae). *Journal of Zoology*, **192**, 157–168.

Schuler, W. & Hesse, E. (1985) On the function of warning coloration: a black and yellow pattern inhibits prey-attack by naive domestic chicks. *Behavioral Ecology and Sociobiology*, **16**, 249–255.

Svadova, K.H., Exnerova, A., Kopeckova, M. & Stys, P. (2013) How do predators learn to recognize a mimetic complex: experiments with naive great tits and aposematic Heteroptera. *Ethology*, **119**, 814–830.

Tan, C.C. (1946) Mosaic dominance in the inheritance of color patterns in the lady-bird beetle, *Harmonia axyridis*. *Genetics*, **31**, 195–210.

Veselý, P., Ernestová, B., Nedvěd, O. & Fuchs, R. (2016) Do predator energy demands or previous exposure influence protection by aposematic coloration of prey? *Current Zoology*, **63**, 259–267.

Wieloch, M. (1975) Food of nestling house sparrows, *Passer domesticus* L. and tree sparrows, *Passer montanus* l. in agrocenoses. *Polish Ecological Studies*, **1**, 227–242.

Accepted 13 May 2019

Associate Editor: Shannon Murphy

ARTICLE 2

Aslam, M., Nedvěd, O. & Sam. K. (2020). Attacks by predators on artificial cryptic and aposematic insect larvae.
Entomologia Experimentalis et Applicata. (In press)
DOI: 10.1111/eea.12877

Attacks by predators on artificial cryptic and aposematic insect larvae

Muhammad Aslam [1,2], Oldřich Nedvěd[1,2], Kateřina Sam[1,2]

[1] Faculty of Science, University of South Bohemia, Branisovska 1760, Ceske
Budejovice, Czech Republic;

[2] Biology Centre of Czech Academy of Sciences, Institute of Entomology, Ceske
Budejovice, Czech Republic

Abstract

Colour and colour patterns seem to be especially important visual warning signals for predators, who might have innate or learned ability to avoid aposematic prey. To test the importance of larval colour pattern of aposematic *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) – an invasive alien species in Europe, we presented the models of aposematic larvae to wild and naïve birds. We studied the attacks to aposematic larvae of various patterns and colours in nature and in outdoor aviary. The larvae were cryptic (green), aposematic (resembling those of the ladybird *H. axyridis* larvae), and semi-aposematic (i.e. black but missing the typical orange patches of *H. axyridis* larvae).

We detected attacks on 71 larvae out of 450 (i.e. 2.56% daily predation). Twenty-nine attacks were made by birds, 37 attacks by arthropods and 5 by gastropods. Wild birds attacked green and black larvae significantly more often than aposematic larvae. Colour did not have an effect on attacks by arthropods.

The experiment with naïve birds was conducted in an outdoor aviary, where naïve great tits (*Parus major*) were offered the same artificial larvae as in the first experiment. In total, 57 of 90 exposed larvae were attacked

by birds (i.e. 28% daily predation), and green larvae were attacked significantly more than the aposematic larvae (but not more than black larvae).

Our results imply that aposematic larvae of *H. axyridis* are more than 12 times less likely to be predated by birds than green larvae in nature. Aposematic pattern thus represented more effective signal than semi-aposematic signal. The ability to reject aposematic prey seemed to be innate in our birds.

CHAPTER – II

Article 3

ARTICLE 3

Nedvěd O., **Aslam M.**, Abdolahi R., Sakaki S., Soares A.O. (2019). Age and temperature effects on accumulation of carotenoids in ladybirds. *IOBC WPRS Bulletin*, 145: 33-36

Summary

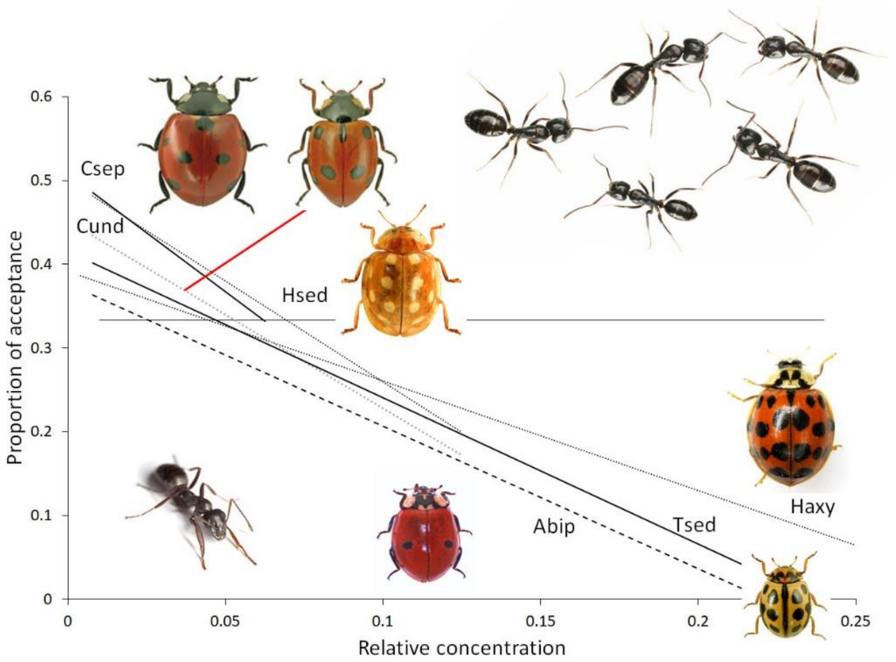
Nedvěd O., Aslam M., Abdolahi R., Sakaki S., Soares A.O. (2019). Age and temperature effects on accumulation of carotenoids in ladybirds. *IOBC WPRS Bulletin*, 145: 33-36.

Description: Carotenoids are important pigments of ladybirds providing bright red colouration. We measured the rate of deposition of carotenoids with age in elytra of two ladybird species in ethanol extracts, as well effects of sex and temperature. In *Coccinella undecimpunctata*, content of carotenoids continued to increase after 60 days of adult age at both 20 and 25 °C. Males had slightly higher pigment concentration than females. Carotenoid content in adult *Harmonia axyridis* (forma succinea) increased up to four months of the adult stage. No difference was found between individuals with contrasting extent of melanic pattern or between sexes. In a later experiment, the carotenoid content increased up to three months of the adult stage similarly for three levels of melanisation. There was no decrease of carotenoids in individuals with large melanic patterns. This means that carotenoids are not deposited under black spots. After four months, there was little decrease in carotenoid content. Slower rate of carotenoid deposition during adult stage after 1 week and 1 month was observed at higher temperatures (25, 30 °C) than at lower ones (15, 20 °C).

CHAPTER – III

Article 4

Article 5



ARTICLE 4

Aslam, M., & Nedvěd, O. (a). Response of the ant *Lasius niger* (Hymenoptera: Formicidae) to extracts from ladybirds (Coleoptera: Coccinellidae) (*Manuscript under review in Insectes Sociaux*)

insects

Article

Response of the ant *Lasius niger* (Hymenoptera: Formicidae) to extracts from ladybirds (Coleoptera: Coccinellidae)

Muhammad Aslam [1,2] * and Oldřich Nedvěd [1,2]

[1] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic; maslam@icp.edu.pk
[2] Czech Academy of Sciences, Biology Centre, Institute of Entomology, České Budějovice, Czech Republic; nedved@prf.jcu.cz
* Correspondence: maslam@icp.edu.pk;

Received: date; Accepted: date; Published: date

Abstract: Alkaloids are important in the defence strategy of ladybirds against arthropod and vertebrate predators. We observed the response of the ant *Lasius niger* to extracts from several ladybird species mixed at diverse concentrations with honey solution. The ladybird species tested included *Adalia bipunctata*, *Ceratomegilla undecimnotata*, *Coccinella septempunctata*, *Harmonia axyridis*, *Halyzia sedecimguttata*, and *Tytthaspis sedecimpunctata*, with *Alphitobius diaperinus* (commonly known as lesser mealworm) as a control. We estimated the degree of deterrence to ants as concentration necessary to deter half of the ants and concentration to deter all of them. Increase of deterrence with concentration of extract was statistically significant among all ladybird species. However, the differences were too small and variable to have biological meaning.

Keywords: toxicity; repellence; avoidance; predation; aphids; reflex bleeding; pyrazines

1. Introduction

1.1. Ants – ladybirds association

Myrmecophilous aphids and ladybirds live in association with ants and exhibit resultant behavioural and structural adaptations. This association is beneficial to ants as they get aphid-provided honeydew with carbohydrates, proteins, minerals and vitamins [1,2]. Aphids get advantages including better hygiene through elimination of caste skins, dead aphids, and honeydew [3]; stimulating development rate, adult body size, fecundity and reproductive rate [4]; and protection against enemies [5]. Ant-ladybird interactions include competing with aphidophagous or coccidophagous ladybirds for the utilization of resources. Some ladybirds (*Ortalia pallens*) feed on ants [6]. Ladybirds may also be preyed upon by ants [7,8].

1.2. Coccinellid defences against ants

The defensive potential of ladybirds is important in their tolerance to ants. Ladybirds employ different mechanisms when confronted by aggressive ant behaviour [9,10]. Such defence mechanisms include behavioural, physical or chemical strategies. Some of these are exhibited at certain periods of the life cycle, while others are employed at both immature and adult stages [8].

1.3. Behavioural defences

Attack by ants is mostly prevented through specific ladybird defensive behaviour. Most larvae escape by running away or dropping to the soil. Adults, beyond these strategies, have the ability to fly [11]. In case of danger, some adult ladybirds belonging to the tribe Chilocorini "clamp down"

ARTICLE 5

Aslam, M., & Nedvěd, O. (b). Scavenging rate of palatable and toxic arthropod carrions during day and night.
(*Manuscript under review in Journal of Zoology*)

Scavenging rate of palatable and toxic arthropod carrions during day and night

Muhammad Aslam[1,2], Oldřich Nedvěd[1,2]

[1] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

[2] Institute of Entomology, Biology Centre, České Budějovice, Czech Republic

Corresponding author: Muhammad Aslam, e-mail: maslam@icp.edu.pk

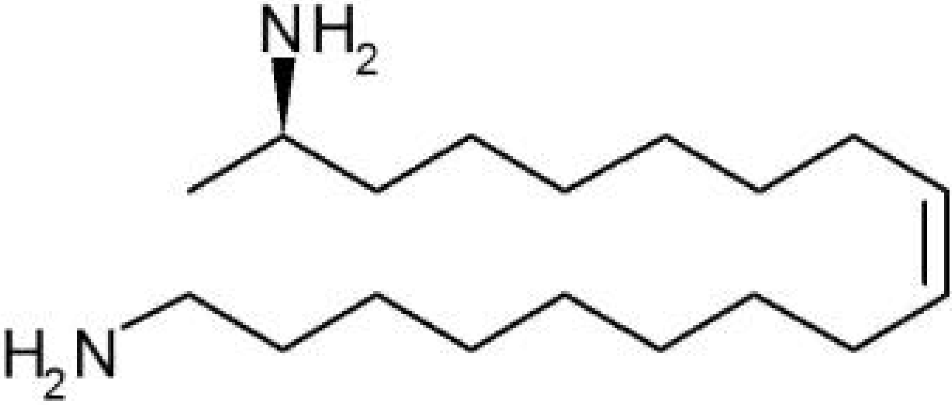
Abstract

Rate of disappearance of fresh carcasses of various small palatable and unpalatable invertebrates exposed on soil surface in a semi-natural habitat showed how much they are protected chemically and mechanically without the contribution of their behaviour. Highly accepted species included woodlice *Porcellionides pruinosus*, young crickets *Acheta domesticus,* larvae of mealworm *Tenebrio molitor* and earwigs *Forficula auricularia* with median survival time up to 2 h and mean survival time up to 4 h. Moderately accepted was the lesser mealworm *Alphitobius diaperinus* with survival times 8 and <12 h. Least accepted species with survival times mostly >24 and >17 h were firebugs *Pyrrhocoris apterus* and all ladybirds: *Coccinella septempunctata, Ceratomegilla undecimnotata*, *Harmonia axyridis* (adults and larvae), and *Tytthaspis sedecimpunctata*. Scavenging at night was slightly quicker than at day.

Keywords: alkaloid, methoxypyrazine, benzoquinon, predator, scavenger, defence, survival, cadaver.

CHAPTER – IV

Article 6



ARTICLE 6

Aslam, M., & Nedvěd, O. (c). Toxicity of extracts from ladybirds (Coleoptera: Coccinellidae) for water flea *Daphnia magna* (Cladocera). (*Manuscript under review in Entomological Science*)

Toxicity of extracts from ladybirds (Coleoptera: Coccinellidae) for water flea *Daphnia magna* (Cladocera)

Muhammad Aslam [1,2] *** and Oldřich Nedvěd** [1,2]
[1] Faculty of Science, University of South Bohemia, Czech Republic
[2] Czech Academy of Sciences, Biology Centre, Institute of Entomology, České Budějovice, Czech Republic
*Correspondence: maslam@icp.edu.pk

Abstract

Ladybird toxicity was studied using bioassays on water flea *Daphnia magna* Straus (Cladocera) with set of diverse concentrations of whole body water extracts of diverse ladybird species. We examined *Harmonia axyridis* Pallas of diverse age (2 weeks, 4 weeks and three months old adults) and of two different morphs (f. *novemdecimsignata* and f. *spectabilis*), *Adalia bipunctata* Linnaeus (1 month old adults), *Calvia quatuordecimguttata* Linnaeus (overwintering adults), *Coccinella septempunctata* Linnaeus (overwintering adults), and *Cynegetis impunctata* Linnaeus (4th instar larvae). No effect of small difference in age of *H. axyridis* (2 weeks vs. 4 weeks) was observed but the toxicity of much older adults (3 months) was 3.8 times higher. Difference between melanic and non-melanic morphs of *H. axyridis* was negligible. *H. axyridis* was more toxic than all the other species examined: aposematic *A. bipunctata* > cryptic *C. impunctata* > aposematic *C. septempunctata* > slightly aposematic *C. quatuordecimguttata*.

Keywords: coccinellid, ladybug, reflex bleeding, alkaloid, methoxypyrazine, defensive chemical, succinea, melanism, defence, predation.

SUMMARY OF RESULTS

Chapter I. We proved that the protection of both tested developmental stages of ladybirds, larvae and pupae, was similar to adult ladybirds despite substantially different visual appearance. Wild-caught great tits (*Parus major*) in general strongly avoid to attack ladybirds (Dolenská et al., 2009).), whereas wild-caught tree sparrows (*Passer montanus*) are often willing to include ladybirds in their diet (Krištín, 1986). Domestic chicks (*Gallus gallus domesticus*) have never been confronted with ladybirds but usually show avoidance to aposematic signals (Gamberale & Tullberg, 2001). We showed in the first study in laboratory **(Aslam et al., 2019)**, that great tits and domestic chicks avoided both developmental stages of *Harmonia axyridis,* an invasive alien species in Europe. In the case of chicks, the avoidance had to be learnt over the course of repeated encounters. Sparrow avoidance was significantly lower, with more than one third of the prey being attacked and eaten.

In the second study **(Aslam et al., 2020, in press)** we exposed plasticine models of aposematic larvae of *H. axyridis* to wild and naïve great tits *Parus major* to test the importance of larval colour pattern. Aposematic artificial larvae resembling larvae of *H. axyridis* (black with orange stripes) exposed in nature on shrubs were more than 12 times less likely to be attacked by birds than green artificial larvae. Purely black artificial larvae (considered partially aposematic) were 9 times less often attacked. Aposematic pattern thus represented more effective signal than semi-aposematic signal. Comparison of the nature data to cage experiment with naïve birds showed the ability to reject aposematic prey similarly. The avoidance behaviour seemed to be innate in our birds.

According to Průchová et al. (2014), red and black colouration of the ladybird *H. axyridis* is the main part of the visual warning signal for the great tit (*Parus major*). In contrast to our study, Prokopová et al. (2010) showed that larval red-black coloration of the red fire bug (*Pyrrhocoris apterus*) did not represent a full-value warning signal for the great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*), while a similar conspicuous red-black colouration of the adults reliably protected them. Similarly, most of the red and black coloured adults and larvae of the bug *Lygaeus equestris* were mostly avoided by the great tit (*Parus major*) (Sillén-Tullberg et al., 1982) exhibiting the importance of aposematic red and black colour pattern in the avoidance of avian predators.

Chicks (*Gallus gallus domesticus*) learned to avoid the aposematic caterpillars of the butterfly *Methona themisto* while naïve chicks did not avoid eating the mealworms painted like the caterpillars indicating that they do not innately avoid this specific colour pattern (Massuda & Trigo, 2009). This is in accordance to our findings of domestic chicks, which learnt to avoid the aposematic ladybird larvae (Aslam et al., 2019).

Chapter II. The rate of deposition of carotenoids, an important part of the aposematic signalling by ladybirds to vertebrate predators, increased with age in elytra of two ladybird species **(Nedvěd et al., 2019)**. It was revealed that in *Coccinella undecimpunctata*, content of carotenoids continued to increase after 60 days of adult age at both 20 and 25 °C. Males had slightly higher pigment concentration than females. Carotenoid content in adult *Harmonia axyridis* (forma *succinea*) increased up to four months of the adult stage. No difference was found between individuals with contrasting extent of melanic pattern or between sexes. In a later experiment, the carotenoid content increased up to three months of the adult stage similarly for three levels of melanisation. There was no decrease of carotenoids in individuals with large melanic patterns. In the same time, it is known (Ando et al., 2018) that carotenoids are not deposited under black spots. This means that carotenoids are deposited in higher concentration in smaller red are\ of more melanic individuals. Slower rate of carotenoid deposition during adult stage after 1 week and 1 month was observed at higher temperatures (25, 30 °C) than at lower ones (15, 20 °C).

Conspicuousness and toxicity are significantly correlated which indicates that conspicuousness serves as an honest signal when signifying level of defence and provides evidence for aposematism in opisthobranchs (Cortesi & Cheney, 2010). Similarly, a positive correlation has been shown between signal strength and level of toxicity in the conspicuous and highly toxic dendrobatid frog family (Summers & Clough, 2001) and in *H. axyridis* (Bezzerides et al., 2007). It is also apparent from these results that elytra color patterns have the potential to reveal information about chemical defensiveness to mates or predators (Bezzerides et al., 2007).

An important aspect of communication is the honesty of animal signals (Stuart-Fox, 2005). Generally, aposematic signals of ladybirds are honest, both among and within species, for both the elytra coloration and perhaps more significantly their conspicuousness against natural backgrounds (Arenas et al., 2015).

Chapter III. We quantified the avoidance of ladybirds by invertebrate predators and scavengers and compared it to other arthropods. The response of the ant *Lasius niger* to extracts from several ladybird species mixed at decreasing concentrations with honey solution was quantified as concentration necessary to deter half of the ants and concentration necessary to deter all of them **(Aslam & Nedvěd, submitted a).** The ladybird species tested included *Adalia bipunctata*, *Ceratomegilla undecimnotata*, *Coccinella septempunctata*, *H. axyridis*, *Halyzia sedecimguttata*, and *Tytthaspis sedecimpunctata*. Although the degree of deterrence to ants was statistically significant among ladybird species, the differences between species were too small and changing with concentration to have biological meaning. Extract from the lesser mealworm *Alphitobius diaperinus* used as a control did not deter ants even at high concentrations.

Despite great differences (0.4x–50x) between deterrent concentrations of extracts of eight species of ladybirds that we found in a preliminary study (Nedvěd et al., 2010), we found less than 3fold (15x–40x) differences in the partially overlapping set of species. The reaction of ants to the extracts was not correlated with the body size of ladybirds, contrary to the findings by Marples (1993) that *L. niger* killed *A. bipunctata* and *A. decempunctata* but not *C. septempunctata*. The extract from large and small ladybirds were both deterring for ants.

Contrary to the preliminary study, the reaction to two less aposematically coloured mycophagous species *T. sedecimpunctata* and *H. sedecimguttata* did not differ from brightly coloured aphidophagous species, where mycophagous *H. sedecimguttata* and *Psyllobora vigintiduopunctata* were moderately deterrent (Nedvěd et al., 2010). Deterrence by the successful invasive species *H. axyridis* did not differ from native and less common species.

The rate of disappearance of fresh carcasses of various small palatable and unpalatable invertebrates exposed on soil surface in a semi-natural habitat showed how much they are protected chemically and mechanically without the contribution of their behaviour **(Aslam & Nedvěd, submitted**

b). Highly accepted species included woodlice *Porcellionides pruinosus*, young crickets *Acheta domesticus*, larvae of mealworm *Tenebrio molitor* and earwigs *Forficula auricularia* with median survival time up to 2 h and mean survival time up to 4 h. Moderately accepted was the lesser mealworm *A. diaperinus* with survival times 8 and <12 h. Least accepted species with survival times mostly >24 and >17 h were firebugs and all ladybirds. Scavenging at night was slightly quicker than at day. Because less than 50% of the exposed individuals of ladybirds were removed which makes calculation of survival times difficult, we propose to simple measure the proportion disappeared during 24 hours. Aposematic species did not differ much in this characteristic: *Pyrrhocoris apterus* (0.33): *C. septempunctata* (0.30), *C. undecimnotata* (0.37), *H. axyridis* adults (0.29) and larvae (0.30), and *T. sedecimpunctata* (0.13).

Thus, all the studied aposematic insects seemed to be well protected against invertebrate predators such as ants, but there were little interspecific differences in the avoidance reaction. We did not find any role of body size, food specialization and colouration on the level of avoidance of the ladybirds.

In **chapter IV (Aslam & Nedvěd, submitted c)**, ladybird toxicity was studied using bioassays on water flea *Daphnia magna* Straus (Cladocera) with set of diverse concentrations of whole body water extracts of diverse ladybird species. We examined *H. axyridis* of diverse ages (2 weeks, 4 weeks and three months old adults) and of two different morphs (f. *novemdecimsignata* and f. *spectabilis*), *A. bipunctata* (1 month old adults), *Calvia quatuordecimguttata* (overwintering adults), *C. septempunctata* (overwintering adults), and *Cynegetis impunctata* (4th instar larvae). No effect of little difference in age of *H. axyridis* (2 weeks vs. 4 weeks) was observed but the toxicity of much older adults (3 months) was 3.8 times higher. Difference between melanic and non-melanic morphs of *H. axyridis* was negligible. *Harmonia axyridis* was more toxic than all the other species examined: aposematic *A. bipunctata* > cryptic *C. impunctata* > aposematic *C. septempunctata* > slightly aposematic *C. quatuordecimguttata*.

Toxicity of the defensive chemical compounds of ladybirds *C. septempunctata* for nestling blue tit (*P. caeruleus*) was proved experimentally (Marples et al., 1989) – the birds suffered from liver damage. The ladybird homogenate proved to be fatal for the guinea pig (*Cavia porcellus*) when introduced into its body (Frazer & Rothschild, 1960). Other studies on vertebrates cannot show ladybird toxicity because the beetles are simply refused by the predators and not ingested (Dolenská et al., 2009; Průchová et al., 2014).

Ladybirds are known for their cannibalism including eggs, so that they do not hesitate to eat eggs of other species. However, eggs of some ladybirds are known to be toxic to intraguild ladybird predators (Hemptinne et al., 2000). Thus, it is possible to measure the level of toxicity of diverse ladybird species for the others. The eggs of *Calvia quatuordecimguttata* (Linnaeus) (Coleoptera: Coccinellidae) appear to be extremely well protected from attack by the invasive ladybird *H. axyridis* due to the compounds present on egg surface (Ware et al., 2007). After consuming a few eggs of *A. bipunctata*, larvae of *C. septempunctata* developed more slowly and died more often than *A. bipunctata* fed with *C. septempunctata* (Agarwala & Dixon 1992; Hemptinne et al., 2000).

CONCLUSIONS

We used feeding experiments to show that larvae and pupae of ladybirds (Coccinellidae) are protected against predation by potential bird predators, similarly to adult beetles. There was some variability depending on the particular bird species, but most individuals avoided to attack larvae and pupae of *H. axyridis*. Similarly, the avoidance of artificial plasticine models of *H. axyridis* larvae by experienced wild birds during breeding season, and in aviary by naïve birds, showed high protection of aposematic larvae of ladybirds against vertebrate predators comparable to the adults.

Colouration itself protects the ladybird larvae and pupae from being attacked, in addition to real chemical protection. Rate of carotenoid deposition in elytra of laboratory reared adults and those of wild caught ladybirds allows estimation of their age. We suggest that adults of different age can cause different avoidance behaviour of birds.

Bitter-tasting alkaloids of ladybirds, together with foul-smelling methoxypyrazines, due to their deterrent features, play a major role in the defence strategy of ladybird beetles against ants and other invertebrate predators. Small differences in the effective concentrations between extracts from several ladybird species were observed, but these differences may have little biological significance.

Invertebrate predators/scavengers distinguished strongly between palatable dead prey (soft and non-toxic cricket and woodlouse, moderately hard mealworm and earwig), less palatable prey (hard and foul-smelling lesser mealworm), and toxic prey (firebug, ladybirds), but there were little differences between individual ladybird species. Nocturnal invertebrate predators/scavengers distinguished between the prey properties similarly to the diurnal ones, and were slightly more active.

Coccinellid toxicity was assessed using *D. magna* as a test organism due to its significance in ecotoxicological procedures and its sensitivity to low quantities of chemicals in water. We observed higher toxicity in old adults of *H. axyridis* compared to young ones. No difference between melanic and non-melanic morphs of *H. axyridis* was found. All other species examined were less toxic than *H. axyridis* which may contribute to the invasiveness of this species.

In the real toxicity for arthropods, there were bigger differences than in the repellency of extracts and carcasses of ladybirds. It was hypothesized that conspicuousness and toxicity should be correlated, however, our study revealed that the repellency and toxicity of cryptic and less aposematic ladybird species were not systematically lower than those of conspicuous species. It can mean that the toxicity is more targeted against arthropod predators that have limited vision abilities or even forage during night, while the colouration is targeted against visually oriented vertebrate predators (birds).

REFERENCES

Agarwala, B.K. & Dixon, A.F.G. (1992). Laboratory study of cannibalism and interspecific predation in ladybirds. *Ecological Entomology*, 17: 303–309.

Ando, T., Matsuda, T., Goto, K., Hara, K., Ito, A., Hirata, J., Yatomi, J., Kajitani, R., Okuno, M. & Yamaguchi, K. (2018). Repeated inversions within a pannier intron drive diversification of intraspecific colour patterns of ladybird beetles. *Nature Communications*, 9: 3843.

Arenas, L. M., Walter, D., & Stevens, M. (2015). Signal honesty and predation risk among a closely related group of aposematic species. *Scientific reports*, 5, 11021.

Aslam, M., Veselý, P., & Nedvěd, O. (2019). Response of passerine birds and chicks to larvae and pupae of ladybirds. *Ecological Entomology*, 44(6):792-799.

Aslam, M., Nedvěd, O. & Sam, K. (2020). Attacks by predators on artificial cryptic and aposematic insect larvae. *Entomologia Experimentalis et Applicata*, (in press).

Attygalle, A.B., McCormick, K.D., Blankespoor, C.L., Eisner, T. & Meinwald, J. (1993). Azamacrolides: a family of alkaloids from the pupal defensive secretion of a ladybird beetle (Epilachna varivestis). *Proc. Natl. Acad. Sci*. USA. 90: 5204–5208.

Bezzerides, A.L., McGraw, K.J., Parker, R.S. & Husseini, J. (2007). Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behav. Ecol. Sociobiol*. 61: 1401–1408.

Blount, J. D., Speed, M. P., Ruxton, G. D. & Stephens, P. A. (2009). Warning displays may function as honest signals of toxicity. *Proc. R. Soc. Lond*. (B). 276:871-877.

Braekman, J. C., Daloze, D. & Pasteels, J. M. (1998). Alkaloids: Biochemistry, Ecology and Medicinal Applications. Ed: Roberts and Wink. Plenum Press, New York.

Brakefield, P.M. (1985). Polymorphic Müllerian mimicry and interactions with thermal melanism in ladybirds and a soldier beetle: a hypothesis. *Biol. J. Linn. Soc*. 26: 243–267.

Briolat, E. S., Burdfield-Steel, E. R., Paul, S. C., Rönkä, K. H., Seymoure, B. M., Stankowich, T., & Stuckert, A. M. (2019). Diversity in warning coloration: selective paradox or the norm? *Biological Reviews*, 94(2), 388-414.

Britton, G., Lockley, W. J., Harriman, G. A., & Goodwin, T. W. (1977). Pigmentation of the ladybird beetle *Coccinella septempunctata* by carotenoids not of plant origin. *Nature*, 266(5597), 49.

Camarano, S., Gonzalez, A. & Rossini, C. (2006). Chemical defence of the ladybird beetle *Epilachna paenulata*. *Chemoecology*, 16: 179–184.

Ceryngier, P., Roy, E. H., & Poland, R. L. (2012). Natural enemies of ladybird beetles. In: Ecology and Behaviour of the Ladybird Beetles (Coccinellidae). Blackwell Publishing Ltd.

Cortesi, F., & Cheney, K. L. (2010). Conspicuousness is correlated with toxicity in marine opisthobranchs. *Journal of Evolutionary Biology*, 23(7): 1509-1518.

Daloze, D., Braekman, J. C., & Pasteels, J. M. (1995). Ladybird defence alkaloids: structural, chemotaxonomic and biosynthetic aspects (Col.: Coccinellidae). *Chemoecology,* 5(3-4): 173-183.

Darwin, C. (1874). The descent of man and selection in relation to sex. John Murray, London.

Dolenska, M., Nedved, O., Vesely, P., Tesarova, M. & Fuchs, R. (2009). What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: colour, pattern or general look? *Biological Journal of the Linnean Society,* 98: 234–242.

Eisner, T. & Grant, R. P. (1980). Toxicity, odor aversion, and ‘‘olfactory aposematism’’. *Science,* 213:476.

Eisner, T., Goetz, M., Aneshansley, D., Ferstandig-Arnold, G., & Meinwald, J. (1986). Defensive alkaloid in blood of Mexican bean beetle (Epilachna varivestis). *Experientia,* 42(2): 204-207.

Escalona, H. E., Zwick, A., Li, H.-S., Li, J., Wang, X., Pang, H., Hartley, D., Jermiin, L. S., Nedvěd, O., Misof, B., Niehuis, O., Ślipiński, A., Tomaszewska, W. (2017). Molecular phylogeny reveals food plasticity in the evolution of true ladybird beetles (Coleoptera: Coccinellidae: Coccinellini). *BMC Evolutionary Biology,* 17: 151.

Exnerová, A., Štys, P., Fučíková, E., Veselá, S., Svádová, K., Prokopová, M., and Landová, E. (2006). Avoidance of aposematic prey in European tits (Paridae): Learned or innate? *Behavioral Ecology,* 18(1): 148-156.

Frazer, J. F. D., & Rothschild, M. (1960). Defence mechanisms in warningly coloured moths and other insects. In Proceedings 11th International Congress of Entomology, Wien, Verhandlungen B (Vol. 3, pp. 249-256).

Gamberale, G. & Tullberg, B. S. (2001). Fruit or aposematic insect? Context-dependent colour preferences in domestic chicks. *Proceedings of Royal Society of London*, 268: 2525 – 2529.

Gautier, M., Yamaguchi, J., Foucaud, J., Loiseau, A., Ausset, A., Facon, B., Gschloessl, B., Lagnel, J., Loire, E., Parrinello, H., Severac, D., Lopez-Roques, C., Donnadieu, C., Manno, M., Berges, H., Gharbi, K., Lawson-Handley, L., Zang, L.S., Vogel, H., Estoup, A. & Prud'homme, B. (2018). The genomic basis of color pattern polymorphism in the harlequin ladybird. *Current Biology*, 28: 3296+.

Goodwin, T. W. (1952). The Comparative Biochemistry of the Carotenoids. Chapman and Hall, London.

Goodwin, T. W. (1971). In Chemical Zoology VI, Arthropoda B (eds Florkin, M. and Scheer, B. T.), 290–306 (Academic, New York).

Guilford, T. I. M., Nicol, C., Rothschild, M., & Moore, B. P. (1987). The biological roles of pyrazines: evidence for a warning odour function. *Biological Journal of the Linnean Society*, 31(2): 113-128.

Guilford, T., & Dawkins, M. S. (1993). Are warning colors handicaps? *Evolution*, 47: 400–416.

Härlin, C., & Härlin, M. (2003). Towards a historization of aposematism. *Evolutionary Ecology*, 17: 197–212.

Hemptinne, J. L., Lognay, G., Gauthier, C., & Dixon, A. F. G. (2000). Role of surface chemical signals in egg cannibalism and intraguild predation in ladybirds (Coleoptera: Coccinellidae). Chemoecology, 10(3): 123-128.

Hemptinne, J-L., Magro, A. & Majerus, M. E. N. (2005). Les Coccinelles. Delachaux et Niestle, Paris.

Hill, R.K. & Renbaum, L.A. (1982). Asymetric syntheses of the ladybug alkaloid adaline and 1-methyl-9-azabicyclo [3.3.1] nonan-3-one. *Tetrahedron*, 38: 1959-1963.

Holloway, G. J., de Jong, P. W., Brakefield, P. M., & de Vos, H. (1991). Chemical defence in ladybird beetles (Coccinellidae). I. Distribution of coccinelline and individual variation in defence in 7-spot ladybirds (*Coccinella septempunctata*). *Chemoecology*, 2(1): 7-14.

Honěk, A., Martínková, Z. & Pekar, S. (2005). Temporal stability of morph frequency in central European populations of *Adalia bipunctata* and *A. decempunctata* (Coleoptera: Coccinellidae). *European Journal of Entomology*, 102: 437–442.

Kholin, S.K. (1990). Stability of the genetic-polymorphism in color of *Harmonia axyridis* Pall. (Coccinellidae, Coleoptera) in maritime province, USSR. *Genetika*, 26: 2207–2214.

Krištín, A. (1986). Heteroptera. Coccinea. Coccinellidae and Syrphidae in the food of *Passer montanus* L. and *Pica pica* L. *Biologia* (Bratislava), 41: 143-150.

Laurent, P., Braekman, J.C. & Daloze S. (2005). Insect chemical defence. Chemistry of Pheromones and other Semiochemicals II: 167–229.

Liaaen-Jensen, S. (1990). Marine carotenoids-selected topics. New J. Chem., 14: 747-759.

Lucas, E. (2005). Intraguild predation among aphidophagous predators. *European Journal of Entomology*, 102: 351–363.

Majerus, M.E.N. (1994). Ladybird. Harper Collins. London. 367 pp.

Marples, N.M., Brakefield, P.M. & Cowie, R.J. (1989). Differences between the 7-spot and 2-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. *Ecological Entomology*, 14: 79–84.

Marples, N. (1993). Toxicity assays of ladybirds using natural predators. *Chemoecology,* 4: 33–38.

Marples, N. M., Van Veelen, W. & Brakefield, P.M. (1994) The relative importance of color, taste and smell in the protection of an aposematic insect *Coccinella septempunctata*. *Animal Behaviour*, 48: 967–974.

Massuda, K. F., & Trigo, J. R. (2009). Chemical defence of the warningly coloured caterpillars of *Methona themisto* (Lepidoptera: Nymphalidae: Ithomiinae). *European Journal of Entomology,* 106(2): 253-259.

Matsuno, T. (1989). Marine carotenoids. Marine biogenic lipids, fats, and oils. 1: 251-388.

Michie, L.J., Mallard, F., Majerus, M.E.N. & Jiggins, F.M. (2010). Melanic through nature or nurture: genetic polymorphism and phenotypic plasticity in Harmonia axyridis. *Journal of Evolutionary Biology*, 23: 1699–1707.

Mizer, A. V. (1970). On eating of beetles from Coccinellidae family by birds. *Vestnik Zoologie*, 6: 21-24.

Moore, B. L., Brown, W. V. & Rothschild, M. (1990). Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology,* 1: 43-51.

Nedvěd, O., Kalushkov, P., Fois, X., Ungerová, D. & Rozsypalová, A. (2010). *Harmonia axyridis*: six-legged alligator or lethal fugu? *IOBC/WPRS Bulletin,* 58: 65–68.

Nedvěd, O. (2014). Slunéčko východní (*Harmonia axyridis*) – pomocník v biologické ochraně nebo ohrožení biodiverzity? The harlequin ladybird (*Harmonia axyridis*) - helper in biological control or threat to biodiversity? *Jihočeská univerzita, České Budějovice,* pp. 65. (In Czech)

Nedvěd, O., Muhammad, A., Abdolahi, R., Sakaki, S., & Soares, A. O. (2019). Age and temperature effects on accumulation of carotenoids in ladybirds. *IOBC-WPRS Bull*, 145: 33-36.

Pasteels, J. M., Deroe, C., Tursch, B., Braekman, J. C., Daloze, D., & Hootele, C. (1973). Distribution et activites des alcaloides defensifs des Coccinellidae. *J. Insect. Physiol*. 19: 1771-1784.

Penn, D. J. & Számadó, S. (2019). The Handicap Principle: how an erroneous hypothesis became a scientific principle. *Biological Reviews*.

Poulton, E. B. (1890). The colours of animals: Their meaning and use, especially considered in the case of insects. New York: D. Appleton and Company.

Prokopova, M., Veselý, P., Fuchs, R., & Zrzavý, J. (2010). The role of size and colour pattern in protection of developmental stages of the red firebug (*Pyrrhocoris apterus*) against avian predators. *Biological journal of the Linnean Society*, 100(4): 890-898.

Průchová, A., Nedvěd, O., Veselý, P., Ernestová, B. & Fuchs, R. (2014). Visual warning signals of the ladybird *Harmonia axyridis*: the avian predators' point of view. *Entomologia Experimentalis et Applicata*, 151 (2): 128-134.

Rothschild, M. & Moore, B. (1987). Pyrazines as alerting signals in toxic plants and insects. In: Labeyrie V, Fabres G, Lachaise D, eds. Insects-Plants. Dordrecht: Dr W. Junk, 97–101.

Rowe, C. & Guilford, T. (1999). The evolution of multimodal warning displays. *Evolutionary Ecology*, 13: 655-671.

Ruxton, G. D., Sherratt, T. N., & Speed, M. P. (2004). Avoiding attack: The evolutionary ecology of crypsis, warning signals and mimicry. New York: Oxford University Press.

Schröder, F. C., Farmer, J. J., Attygalle, A. B., Smedley, S. R., Eisner, T., & Meinwald, J. (1998). Combinatorial chemistry in insects: a library of defensive macrocyclic polyamines. *Science*, 281(5375): 428-431.

Schuler, W. & Hesse, E. (1985) On the function of warning coloration: a black and yellow pattern inhibits prey-attack by naive domestic chicks. *Behavioral Ecology and Sociobiology*, Vol, 16. pp. 249-255.

Sillén-Tullberg, B., Wiklund, C., & Järvi, T. (1982). Aposematic coloration in adults and larvae of *Lygaeus equestris* and its bearing on Müllerian mimicry: an experimental study on predation on living bugs by the great tit *Parus major*. *Oikos*: 131-136.

Skelhorn, J., Halpin, C. G., & Rowe, C. (2016). Learning about aposematic prey. *Behavioral Ecology*, 27: 955–964.

Sloggett, J. J. (1998). Interactions between coccinellids (Coleoptera) and ants (Hymenoptera: Formicidae), and the evolution of myrmecophily in Coccinella magnifica Redtenbacher. Unpublished PhD thesis, University of Cambridge.

Sloggett, J.J., Wood, R.A., & Majerus, M.E.N. (1998). Adaptations of *Coccinella magnifica* Redtenbacher, a myrmecophilous coccinellid, to aggression by wood ants (*Formica rufa* group). I. Adult behavioral adaptation, its ecological context and evolution. *Journal of Insect Behavior*, 11: 889–904.

Stevens, M., & Ruxton, G. D. (2011). Linking the evolution and form of warning coloration in nature. *Proceedings of the Royal Society B: Biological Sciences*, 279(1728): 417-426.

Stuart-Fox, D. (2005). Deception and the origin of honest signals. *Tr. Ecol. Evol*. 20: 521–523.

Summers, K. & Clough, M.E. (2001). The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). *Proc. Nat. Acad. Sci*. USA. 98: 6227–6232.

Summers, K., Speed, M. P., Blount, J. D., & Stuckert, A. M. M. (2015). Are aposematic signals honest? A review. *Journal of Evolutionary Biology*, 28: 1583–1599.

Tan, C. C. (1946). Mosaic dominance in the inheritance of color patterns in the lady-bird beetle, *Harmonia axyridis*. *Genetics*, 31: 195–210.

Veselý, P., Ernestová, B., Nedvěd, O., & Fuchs, R. (2016). Do predator energy demands or previous exposure influence protection by aposematic coloration of prey? *Current Zoology*, 63(3): 259-267.

Wallace, A. R. (1889). Darwinism: An exposition of the theory of natural selection with some of its applications. London: Macmillan and Co.

Ware, R. L., Ramon-Portugal, F., Magro, A., Ducamp, C., Hemptinne, J. L., & Majerus, M. E. (2007). Chemical protection of *Calvia quatuordecimguttata* eggs against intraguild predation by the invasive ladybird *Harmonia axyridis*. *In*: From Biological Control to Invasion: the Ladybird *Harmonia axyridis* as a Model Species (pp. 189-200). Springer, Dordrecht.

Wieloch, M. (1975). Food of nestling house sparrows, *Passer domesticus* L. and tree sparrows, *Passer montanus* L.in agrocenoses. *Polish Ecological Studies*, 1: 227-242.

Winters, S. (2018). Aposematism. In: Vonk J., Shackelford T. (eds) Encyclopedia of Animal Cognition and Behavior. Springer, Cham.

e-mail: maslam@icp.edu.pk

Aposematism and toxicity of Coccinellidae
Ph.D. Thesis Series, 2020, No. 5

Printed in the Czech Republic by Typodesign
Edition of 20 copies

University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz