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Role of individual chemical compounds of repellent secretion of *Graphosoma lineatum* towards different predator species

Význam jednotlivých složek repelentní sekrece *Graphosoma lineatum* vůči
různým druhům predátorů

Ph.D. THESIS

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BEWARE OF PREDATORS!

They are waiting for you.

Declaration:

I hereby declare that I elaborated the final thesis individually and I cited all used information sources and literature. Neither this thesis nor its substantial part has been presented elsewhere in order to obtain another or same academic title. My involvement in the research presented in this thesis is represented by author's order in included publications and manuscripts.

Prague, 31 July 2015 Signature:

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CONTENTS

ABBREVIATIONS

AOB – accessory olfactory bulb

MOB – main olfactory bulb

MTG – metathoracic scent glands

OR – olfactory receptor

VNO – vomeronasal organ

UM – untreated mealworm

HX – hexane

PYR – pyrazine

3A – mixture of three aldehydes

TA – mixture of three aldehydes and tridecane

OXO – oxoaldehyde

GS – *Graphosoma lineatum* secretion

LG/3A – living specimen of *Graphosoma linetaum* offered before the trials with the mixture of three aldehydes

LP/3A – living specimen of *Pyrrhocoris apterus* offered before the trials with the mixture of three aldehydes

ABSTRACT

The chemical defence of Heteroptera is based on the repellent secretion that is very complex and consists of dozens chemical compounds. Heteroptera have good ability to produce/store large amounts of chemical components. The repellent secretion of *Graphosoma lineatum* is composed of many chemicals, such as short-chained aldehydes, which may signal the unpalatability of the bug to its potential predators or be directly toxic for them.

The thesis is aimed at the major components of defensive secretion of *Graphosoma lineatum* – aldehydes – as well as the whole metathoracic scent-glands secretion of *Graphosoma lineatum*. The aversive reactions of four selected predators were evaluated: (1) leopard gecko (*Eublepharis macularius*); (2) green lizard (*Lacerta viridis*); (3) great tit (*Parus major*) and (4) blue tit (*Cyanistes caeruleus*).

The following major compounds of the repellent secretion were tested: (1) the mixture of three aldehydes: (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) the mixture of three aldehydes and tridecane; (3) oxoaldehyde: (E)-4-oxohex-2-enal; (4) extracted metathoracic scent-glands secretion of *Graphosoma lineatum* adults; (5) hexane as a non-polar solvent and (6) pyrazine: 2-isobutyl-3-methoxypyrazine in experiments with leopard geckos as a positive control for excluding the effect of neophobia. All chemicals were applied on a palatable food (*Tenebrio molitor* larvae).

The aversive reactions of predators were evaluated by observing following behavioural characteristics: (1) approach latencies, (2) attack latencies, (3) approach-attack intervals and (4) attack-eating intervals towards the mealworms.

Leopard geckos exhibited aversive reactions to the mixture of three aldehydes and also to this mixture and tridecane. The mixture enriched by tridecane had even stronger aversive effect. On the other hand, oxoaldehyde did not have any aversive effect. The whole metathoracic scent-glands secretion had clearly an aversive effect on leopard geckos. Furthermore, when living specimen of *Graphosoma lineatum* was offered to leopard geckos before the trials with the mixture of three aldehydes, the impact of this mixture was enhanced thus acting as a potential signal of unpalatability.

Green lizards exhibited an aversive reaction to the mixture of three aldehydes. Tridecane reduced the aversive effect of the aldehydes mixture. Oxoaldehyde had the weakest but still significantly aversive effect on green lizards. The whole metathoracic scent-glands secretion had clearly an aversive effect for green lizards. Moreover, when living specimen of *Graphosoma lineatum/ Pyrrhocoris apterus* was presented to green lizards before the trials with the mixture of three aldehydes, the effect of this mixture was enhanced hence acting as a potential signal of unpalatability.

The results of great tits and blue tits showed that both bird species had aversive reactions to the mixture of three aldehydes. On the other hand, the mixture of three aldehydes and tridecane did not have any aversive effect in case of great tits. Oxoaldehyde had strong aversive effect for great tits, whereas for blue tits this effect was delayed. The whole metathoracic scent-glands secretion of *Graphosoma lineatum* had clearly an aversive effect for both bird species. Great tits hesitated most to oxoaldehyde, while blue tits hesitated most to the whole metathoracic scent-glands secretion of *Graphosoma lineatum*.

In conclusion, aldehydes show a promise as deterrents for different types of chosen predators. The mixture of three aldehydes plays role as a strong signal of unpalatability of *Graphosoma lineatum*.

Key words: aldehyde, aversive reaction, leopard gecko, green lizard, great tit, blue tit, repellent secretion

ABSTRAKT

Skupina ploštice (Heteroptera) má vynikající schopnost produkovat/uchovávat velké množství chemických látek, jež tvoří základ jejich komplexní repelentní sekrece. Mezi nejlépe prostudované repelentní sekrece patří sekrece *Graphosoma lineatum* skládající se z mnoha složek, které mohou působit jako iritanty nebo přímo jako toxiny. Mezi hlavní chemické složky sekrece patří aldehydy s krátkými řetězci.

Disertační práce se zaměřuje na hlavní chemické složky této repelentní sekrece – aldehydy – stejně jako na extrahovanou sekreci metathorakálních pachových žláz *Graphosoma lineatum*. Celkem byly testovány aversivní reakce čtyř vybraných druhů predátorů: (1) gekončík noční (*Eublepharis macularius*); (2) ještěrka zelená (*Lacerta viridis*); (3) sýkora koňadra (*Parus major*) a (4) sýkora modřinka (*Cyanistes caeruleus*).

Vybrané druhy predátorů byly konfrontovány s majoritními složkami obranné sekrece *Graphosoma lineatum*: (1) směs tří aldehydů: (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) směs těchto tří aldehydů obohacená o tridekan; (3) oxoaldehyd: (E)-4-oxohex-2-enal; (4) extrahovaná sekrece metathorakálních pachových žláz dospělé *Graphosoma lineatum*; (5) hexan, jakožto nepolární rozpouštědlo a (6) pyrazín: 2-isobutyl-3-methoxypyrazín u experimentů s gekončíky nočními jako pozitivní kontrola k vyloučení efektu neofobie. Všechny chemikálie byly aplikovány na poživatelnou kořist (*Tenebrio molitor* larva).

Aversivní reakce jednotlivých druhů predátorů byly vyhodnocovány na základě sledování následujících charakteristik chování: (1) latence přiblížení se ke kořisti, (2) latence zaútočení na kořist, (3) interval mezi přiblížením a vlastním útokem na kořist a (4) interval mezi útokem a vlastní konzumací kořisti.

U gekončíků nočních výsledky ukazují, že gekončík reagoval aversivně vůči směsi tří aldehydů. Stejná směs obohacená o tridekan vykazovala dokonce silnější aversivní reakci. Oxoaldehyd nevyvolal žádný aversivní efekt. Celková sekrece metathorakálních pachových žláz měla jasný aversivní účinek. Přítomnost živé ploštice *Graphosoma lineatum* před vlastní testovanou sekvencí směsi tří aldehydů zesílila aversivní reakce na tuto směs. Tato směs může tedy fungovat jako potenciální signál nepoživatelnosti této kořisti.

V případě ještěrek zelených byla aversivní reakce na směs tří aldehydů silnější, než reakce na tuto směs obohacenou o tridekan. Směs s tridekanem měla však silnější aversivní efekt, než tomu bylo u oxoaldehydu. Aversivní reakce na oxoaldehyd byla sice nejslabší, ale stále signifikantní. Celková sekrece metathorakálních pachových žláz měla jasný aversivní účinek. Navíc, pokud byla přítomna ploštice *Graphosoma lineatum*/*Pyrrhocoris apterus* před vlastní sekvencí směsi tří aldehydů, byl tento efekt zesílený a díky tomu může být tato směs potenciálním signálem nepoživatelnosti dané kořisti.

Výsledky experimentů u obou ptačích druhů, sýkory koňadry a sýkory modřinky, ukazují, že oba druhy reagovaly aversivně na směs tří aldehydů. U sýkor koňader byla navíc testována tato směs tří aldehydů obohacená o tridekan a přítomnost tridekanu nevyvolala žádnou aversivní reakci. Oxoaldehyd měl silný aversivní efekt u sýkor koňader, zatímco u sýkor modřinek byla aversivní reakce opožděná. Celková sekrece metathorakálních pachových žláz měla jasný aversivní účinek pro oba ptačí druhy. Koňadry váhaly nejvíce na přítomnost oxoaldehydu, zatímco modřinky váhaly nejvíce na celkovou sekreci *Graphosoma lineatum*.

Závěrem lze říci, že všechny vybrané druhy predátorů reagovaly aversivně vůči aldehydům. Navíc směs tří aldehydů fungovala jako signál nepoživatelnosti ploštice *Graphosoma lineatum*.

Klíčová slova: aldehydy, aversivní reakce, gekončík noční, ještěrka zelená, sýkora koňadra, sýkora modřinka, repelentní sekrece

1. INTRODUCTION

The chemical signalization is an important part of the aposematic signal and it is widespread across not only animal kingdom (Cott 1940). Many insect species protect themselves using unpalatable, malodorous or directly toxic compounds, which can be found in e.g. butterflies, ladybird beetles, leaf beetles or bugs, (Fink and Brower 1981; Rowell-Rahier et al. 1995; Aldrich et al. 1997; Nishida 2002; Camarano et al. 2006; Pareja et al. 2007; Moraes et al. 2008; Speed et al. 2012).

The Heteroptera represent a group rich in taxa with well-developed chemical defensive secretion towards predators. The defensive secretion of true bugs is very complex and it contains numerous chemical compounds (Aldrich 1988). The antipredatory function is supposed to be mediated by the compounds, which are abundant in the secretion – such as aldehydes or tridecane (Aldrich 1988; Farine et al. 1992; Šanda et al. 2012).

These chemical compounds, which are present across the species of Heteroptera (Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996, 1997; Pareja et al. 2007, Šanda et al. 2012), could serve as a signal of unpalatability or as a direct defence for different types of predators. Although the chemical analysis is well-known in many heteropteran species (Hamilton et al. 1985; Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996, 1997; Krall et al. 1999; Aliabadi et al. 2002; Prudic et al. 2008; Fávaro et al. 2011; Šanda et al. 2012), it is still unknown, which components are responsible for the aversive reactions of different types of predator species.

Many studies have been focused on testing chemicals towards predators, but these chemicals were mostly artificial as bitrex (Skelhorn and Rowe 2005b; Skelhorn and Rowe 2006e) or natural products such as methyl anthranilate, amygdalin, vanilin or chinin (Marples and Roper 1997; Roper and Marples 1997; Rowe and Skelhorn 2004; Skelhorn and Rowe 2006c). Some studies used pyrazines as potential deterrent chemicals (Marples and Roper 1996; Rowe and Guilford 1999; Lindström et al. 2001; Kelly and Marples 2004), which could be responsible for unpalatability of the prey in many aposematic heteropteran species (Aldrich 1995; Aldrich et al. 1997).

Yet, only few studies evaluated predators' reactions towards aposematic insect (Krall et al. 1999; Exnerová et al. 2006; Bonacci et al. 2008; Svádová et al. 2009) and/or effects of the defensive secretion on vertebrate predators, such as Benfield (1972) or Härlin (2005) using the defensive secretion of beetles of family Gyrinidae.

The roles of individual components of the chemical defence secretion of heteropteran aposematic insect in relation to vertebrate predators have not been rigorously tested at all. Therefore, the present study of the aversive reactions of selected potential predators towards the defensive repellent secretion of the striated shieldbug *G. lineatum* provides such data probably for the first time.

This study is focused on the major compounds of the repellent secretion – aldehydes of *G. lineatum* (Šanda et al. 2012). These compounds are highly volatile and odorous and can function as a potential signal of unpalatability as well as a secondary chemical defence itself (Eisner 1970; Hamilton et al. 1985). However, little is known how the major compounds of the repellent secretion of the striated shieldbug precisely work.

Following predators were chosen: two species of lizards – *Eublepharis macularius* and *Lacerta viridis*, with different life history (nocturnal and diurnal life style), but with well-developed chemical discrimination and with same foraging mode – active foragers (Schwenk 1993; Cooper 1996; Cooper 2007), and two bird species, which are potential predators of Heteroptera in the wild (Exnerová 2003a) – *Parus major* and *Cyanistes caeruleus*.

The objective of the present study was to test the selected major compounds of the metathoracic scent-glands secretion of *G. lineatum* adults, which could have potential repellent and antipredatory function towards those types of selected predators.

1.1. Aposematism

Aposematism is a successful antipredatory strategy, when the prey uses warning signals to inform predators about its unpalatability – bright and conspicuous colours associated with some sort of other defence, such as stings, toxicity or aggressive behaviour (Cott 1940; Härlin and Härlin 2003; Niskanen and Mappes 2005). The theory of warning signals dates back to Wallace (Mappes et al. 2005).

The warning signals (colour, odour or behaviour) can influence predator‘s foraging behaviour and show that the prey is unprofitable (Cott 1940; Ruxton et al. 2004; Mappes et al. 2005). The signals can be divided into visual – such as colour (Sexton 1960; Aronsson and Gamberale-Stille 2008), pattern (Smith 1980) or contrast against background (Gamberale-Stille 2001); acoustic (Rowe and Guilford 1999; Rowe

2002) or chemical (Rowe and Guilford 1996; Skelhorn and Rowe 2006 a,c; Siddall and Marples 2008). They may also act together as a multimodal signal, which is more effectively detected by the predators (Rowe and Guilford 1999; Rowe and Halpin, 2013).

These signals work best when they are easily detectable and also memorable, which could ease avoidance learning (Ruxton et al. 2004).

Aposematism is often viewed as a distinctive strategy to crypsis, which means being inconspicuous and palatable. But according to Mappes et al. (2005), aposematism and crypsis should be interpreted as part of a continuum of strategies from very protected highly conspicuous to weakly protected less conspicuous forms (Mappes et al. 2005). Thus crypsis and aposematism are not mutually exclusive (Niskanen and Mappes 2005; Tullberg et al. 2005).

According to Edmunds (1987) ex Härlin and Härlin (2003), an animal, which is considered as an aposematic, should be sufficiently noxious, conspicuously coloured or it should have some other type of signals, and some predators avoid it because of these signals. Such conspicuous signals afford better protection for the individual. There is also an important detail that mimicry could not work if the predator was not able to think and not able to learn such signals (Speed 1993; Lindström et al. 2006; Skelhorn and Rowe 2006a).

In principle, there are three major modes of mimicry. The first one is called Batesian mimicry, where species are edible and copy the warning signals of defended aposematic species (so called model) (Huheey 1961; Speed 1999). Batesian mimics should be expected as polymorphic in their mimicry (Turner 1987 ex Speed 1999). The other aspect of mimesis is remembered by Müllerian theory, which stress a common benefit for all included species from sharing the same warning signal (Speed 1999). In other words, when two or more aposematic species share the same, most often, visual resemblance, predators have to learn to avoid only one colour pattern (or other type of signal) (Lindström et al. 2006). This prediction infers that there is a fixed number of prey killed during the education of naive predators and also if two or more defended species have the same warning signal then the fixed cost of predator education can be shared among mimetic species (Mallet and Joron 1999; Speed 1999). And it is also true that Müllerian mimics are the least protected when they are rare (MacDougall and Dawkins 1998), and therefore aposematic species very often aggregate (Aldrich and Blum 1978; Gamberale and Tullberg 1996).

The another aspect of the organisation of mimetic complex is remembered with a concept of quasi-Batesian mimicry. This theory suggests that, because of differences in unpalatability, the less toxic mimics act as a parasite on the more defended prey and therefore they decrease its fitness (Mallet and Joron 1999; Speed 1999; Lindström et al. 2006).

Traditionally, discussions of aposematism focused on the visual displays of the prey predict that visual signal could play the best role in learning such a prey. Chemical aposematism could cause concurrent selection when chemical signal elicits chemosensory avoidance responses in signal receivers, and therefore, it could play a significant function in avoidance and learning the aposematic prey (Eisner and Grant 1981; Weldon 2013; Weldon and Burghardt 2015).

The following chapters will be focused on the particular types of warning signals, which influence learning of aposematic prey – visual, chemical, acoustic and multimodal signals.

1.1.1. Visual signals

Predators discriminate aposematic prey based on the different type of signals. The most studied are visual signals. These signals are the most important for the animals that are visually oriented (such as birds or lizards) and therefore, they can easily avoid toxic insects (Sexton 1960; Benes 1969; Guilford 1990; Krall et al. 1999; Kelly and Marples 2004; Bonacci et al. 2008; Shanbhag et al. 2010).

The visual signalization contains more components such as colour, pattern, shape, size, symmetry, contrast against background, inner contrast among different coloured parts of the body, moving etc. (Cott 1940; Roper 1994; Mastrota and Mench 1995; Forsman and Marilaita 1999; Gamberale-Stille 2001; Hatle et al. 2002; Gamberale-Stille and Guilford 2003; Exnerová et al. 2006; Prudic et al. 2006; Ruxton and Sherratt 2009; Svádová et al. 2009).

Colour is the best studied example of visual signal in birds (Mastrota and Mench 1995; Gamberale-Stille and Guilford 2003; Aronsson and Gamberale-Stille 2008) as well as in lizards (Boyden 1976; Terrick et al. 1995; Schall 2000; Clark et al. 2014). Conspicuous colours are warning signals and these signals might be easily detected and learned by potential predators (Guilford and Dawkins 1993). The aim of the conspicuous colour is always to deter an attack of the predator (Nilsson and Forsman

2003). The predators avoid such conspicuous colour (Sexton 1960, 1964; Exnerová et al. 2006).

The most common aposematic colour is red. This colour is often combined with the black pattern, but only the pattern is not sufficient to function as a warning signal (Exnerová et al. 2006; Hotová-Svádová et al. 2010). It was shown that red colour causes innate aversive reaction (Matsrocha and Mench 1995). According to Svádová et al. (2009), red colour is a very effective signal compared to other types of aposematic colours such as yellow, orange or white (Cott 1940). The colour in combination with contrast against background also enables easier discrimination of the prey (Gamberale-Stille and Guilford 2003).

The aposematic animals show their unpalatability not only by colour but also by their pattern. The most common combinations of the aposematic colour and pattern are bright colours (such as red, orange, yellow or white) with the black colour (Cott 1940). This black colour makes a pattern, which could also function as an inner contrast among differently coloured parts of the body (Svádová et al. 2009). The potential predators learn probably only the parts of conspicuous patterns of the prey (Gamberale- Stille and Guilford 2003; Exnerová et al. 2006). Therefore, for detection of the aposematic prey the colour is more important than the pattern itself (Svádová et al. 2009).

Symmetric pattern probably facilitates aversive learning and discrimination for predators (Forsman and Marilaita 1999; Forsman and Herrstrom 2004). According to Kirkpatrick and Rosenthal (1994), there is a hypothesis that disrupted bilateral symmetry could reduce the effect of visual warning signals of the prey such as colour or pattern. Similarly, the asymmetry of the pattern probably decreases the influence of the aposematic signal of the prey.

Shape is an important part of signalization of unpalatability of the prey (Poulton 1890 ex Rotheray 1986). Except the shape, animals very often use horns, spines, thorns or seths (as other type of defence compared to the repellent secretion or direct toxins) also with combination of aposematic coloration (Kaupinnen and Mappes 2003; Inbar and Lev-Yadun 2005). Unfortunately, for the recognition of known aposematic prey the shape is not sufficient (Exnerová et al. 2006; Svádová et al. 2009), because the specific colour overshadows the shape and other types of aposematic components.

The theory of the contrast against the background presumes that predators easily learn to avoid aposematic prey, which has strong contrast against its background (Gamberale-Stille 2001). This aversive reaction is therefore the strongest and more permanent. Additionally to the chromatic contrast, characteristic luminance is also included as a key aposematic signal (Ruxton et al. 2004; Prudic et al. 2006). According to Prudic et al. (2006), if the prey increases the luminance, it increases the risk of its detection, but this makes easier aversive reaction and memorability to predator. The luminance is detected also by non-colour oriented predators (Prudic et al. 2006).

Size as an aposematic signal can be considered either as the size of the individual or the size of the aggregation. There is a possibility that the size of the individual could increase the efficiency of the other visual warning signals (Roper 1994). Generally, the bigger size of the body of the individual is better for the aposematic prey rather than for the cryptic one (Nilsson and Forsman 2003).

There is no doubt about the size of the aggregation, which increases intensity of warning signals (Forsman and Merilaita 1999; Ruxton and Sherratt 2009). In case of the aggregation, many aposematic species live in big aggregations, e.g *Pyrrhocoris apterus* (Socha 1993). The aggregation therefore strengthens discrimination learning and aversive reactions, thus increasing function of visual warning signalization of the prey (Mappes and Alatalo 1997).

The manner of moving is a less common way of how the prey could inform the predator about its unpalatability, but there is a prediction that the movement could possibly play a role in the whole defence strategy (Yamawaki 2003). More common type of the movement of the aposematic prey is the ‘sluggish’ movement” (Hatle et al. 2002). The advantages of this type of the movement are not clear but it is very common for the aposematic prey (Hatle and Faragher 1998). There are three main hypotheses about slow movement of the aposematic prey.

Firstly, the aposematic prey does not have any reason to move quickly away from the predator, because it is unpalatable (Chai and Srygley 1990). Secondly, conspicuous prey provides the predator with sufficient time to evaluate its disadvantageousness (Hatle and Faragher 1998). Finally, an attack of the predator could be elicited only by specific intensity of the movement and therefore, when the prey moves slowly, predator does not launch the attack (Hatle and Faragher 1998).

1.1.2. Chemical signals

The aposematic prey signals its unpalatability also by chemical compounds. These compounds are perceived by taste, smell or chemesthesis. The chemical signals can strengthen the visual signals and often constitute multimodal signals.

Some insect species can produce/sequester and store chemical compounds. Most of the compounds have bitter taste (Nishida 2002). Taste signal could increase aversion to visual signals and therefore, accelerate aversive learning or memorization and thus increase defence of the prey. The probability that the predator avoids unpalatable prey after initial attack increases with ascending content of defensive compounds, thus predator can drop such a prey relatively unharmed (Wiklund and Järvi 1982). Also two different defensive chemicals can accelerate learning of the predator and improve its memory. This type of chemical defence is present very often among Müller's mimetics (McLain 1984; Skelhorn and Rowe 2005b), which have different chemical defence and therefore, could be better protected than aposematic preys, which have same chemical defence.

Secretion is a very effective defence mechanism if it is ejected on the surface of the body of the aposematic insect (Skelhorn and Rowe 2009). Thanks to this, the predator accelerates its learning and the ejection of secretion decreases the risk of predation compared to the situation when insect stores its secretion inside of the body (Skelhorn and Rowe 2006c). Furthermore, predators can release the prey relatively unharmed based on only manipulation and taste (Schlee 1986).

Taste signals can have function in recognition of the aposematic prey individually or in the interaction with the visual signals (multimodal signals). Predators can distinguish to avoid the prey using the taste based on the individual level of chemical defence (Holen 2013).

Studies on odorous signals have been mostly focused on the function of pyrazines, which are often linked with conspicuous colour. According to Rotschild et al. (1984), there is a hypothesis that pyrazines function as a trigger for the other signal compounds. On the other hand, the smell of pyrazine did not cause aversive reaction, but caused the aversion to red colour (Marples and Roper 1996; Kelly and Marples 2004). Except for pyrazines, many studies were focused on the chemicals such as amygdalin and vanilin (Roper and Marples 1997). In such studies the results showed that aversive reaction was caused only by amygdalin and not by vanilin. Moreover, amygdalin overshadowed the effect of colour.

Chemesthetic signals represent different possibility how to perceive the warning signals in other manner than using taste or smell senses. Chemesthesis has ability to sense chemical components and elicit irritation such as burning, warming, coldness or stabbing thanks to chemoreceptive fibres of trigeminal nerve. These receptors are inside of the nasal and oral cavity and they are also present in the eye and they may cause nausea or sneezing (Lin et al. 2008).

The most studied chemesthetic irritants are capsaicin in mammals or methyl anthranilate, which is used as defensive compound by some species of ants (Clark 1998). At last it needs to be said that mammalian and avian morphological organization of peripheral trigeminal nerve is similar. The difference is mostly in function in response to these chemical irritants (Mason et al. 1991). Unfortunately, in case of reptiles, little is known about the chemesthetic signals and their function.

1.1.3. Acoustic signals

Acoustic signals can be found in different species of insects. This is another way how to deter predator and show him its unpalatability (Rowe and Guilford 1999; Rowe 2002). One part of such studies is focused on the effectiveness of acoustic signalization towards predators, which orient acoustically or by perceiving vibrations (Chapman 1998; Ratclife et al. 2008). The second part of the studies is based on the effectiveness of acoustic signal towards predators, which are oriented visually (Rowe 2002; Hauglund et al. 2006).

The most complete picture provide experiments with bats as acoustically oriented predators and tiger moth (Arctiidae). Some species of tiger moths are chemically defended by pyrolizidine alkaloids, which in combination with the acoustic signal act as a multimodal signal (Chapman 1998). According to Ratclife et al. (2008), tiger moths, which are chemically defended and produce ‘clacking’ sound, did not need to decrease their flight activity.

Fullard et al. (1994) suggested three possible hypotheses. Firstly, ‘clacking’ sound can have function in tiger moths as an aposematic signal, which informs bat about its noxiousness. Secondly, the ‘clacking’ sound can disturb echolocation of the bats (jamming), and thirdly, these sounds can deter or rouse (startle) the bat.

Similar strategy of using sound to deter the predator is hissing, e.g. in *Bombus terrestris* (Kirchner and Röschard 1999). These authors suggested that this hissing sound serves as a warning signal towards small mammals, such as mouse.

Another type of acoustic signal is stridulation (Masters 1979), which is common in Mutillidae, Hydrophilidae and Carabidae. The experiments, where a spider from the family Lycosidae was chosen as selected predator, proved that stridulation is the sufficient signal, which deters spiders from attacking such a prey.

The sound functions as an aposematic signal (Fullard et al. 1994) and the presence of the sound (e.g. vibration) can accelerate discrimination learning (VanderSal and Hebets 2007). However, acoustic signals as well as chemical signals have been less studied than visual signals and the results are yet not sufficient.

1.1.4. Multimodal signals

Warning signals can arise as multimodal if they relate to more than one sensory modality, e.g. visual and acoustic signalling, or visual and chemical signalling (Guilford and Dawkins 1993; Rowe and Guilford 1996; Hauglund et al. 2006). The prey can use not only combination of two modalities, but it can use three or more (Rowe and Halpin 2013). In principle, the modality means mode, with which signalling specimen sends a signal and this signal is accepted by the same or different sense of receiver (Smith and Evans 2013).

There are also multicomponent signals, which means that these signals are multiple and they are identified only by one sense. For example, the predator can observe colour, contrast, pattern and also shape of the prey (Rowe 1999).

According to Partan and Marler (2005), multimodal signals are divided into redundant and non-redundant. Redundant signal, if it is presented individually, has the same function as more redundant signals displayed together. Non-redundant components have different effects and if they are joined together to multimodal signal, then they have new significance. Such information could inform the predator in a more complex way than unimodal signal (Partan and Marler 2005). Multimodal signalization is very useful in the environment, which is often changing and these changes favour another modality (Grafe et al. 2012). Disadvantages of multimodal signalling could be higher energy costs, coordination and reception of the signals (Partan and Marler 2005).

Multimodal signals in the aposematic prey very often combine visual signals with signals from other senses such as noxious odour, toxic compounds or unpleasant sounds (Rowe and Guilford 1999). Multiple signals are more robust in providing the information about the aposematic prey to receiving predator and they are also identified faster than one signal. Therefore, they increase the chances of surviving the potential attack (Rowe 1999).

Multimodal warning signals can function as the defence against different types of predators (Vallin et al. 2006) or individual modalities interact and increase potency towards one particular predator (Avery and Nelms 1990; Rowland et al. 2013).

The most frequent combination of multimodal signals are visual – acoustic (Wiklund et al. 2008) and visual – chemical signalling: visual – olfaction (Woolfson and Rothschild 1990) and visual – taste (Skelhorn et al. 2008).

According to experiments of Rowe and Halpin (2013), it follows that visual – acoustic signalling dominates the defence of the orders Diptera and Hymenoptera, whereas for the order Orthoptera, Hemiptera (Heteroptera), Coleoptera and Lepidoptera the defence is mostly based on visual – chemical multimodal signalling.

The multimodal signal is better than individual components of the signal for several reasons. Firstly, multimodal signal acts faster (Rowe 1999), secondly, naive predators avoid the aposematic prey more likely, if this prey produces another warning signal (Jetz et al. 2001). Also the predator can learn faster and better, if there is a multimodal signal instead of only unimodal signalling (Siddall and Marples 2008). Finally, the information obtained by multimodal signalling persists in predator's memory longer (Roper and Marples 1997).

1.2. Chemical defence

Across nature – defence is a common strategy of living creatures not only in the animal kingdom but also among the plants and fungi. Defence is a basic strategy of surviving predation or inter – intraspecific agresivity among species. The defence itself can have many forms - behavioural, mechanical (Gross, 1993) or chemical (Brower 1984; Aldrich 1988, 1995).

Chemical defence can function as a signal and/or as the defence itself (Gohli and Högstedt 2009). The chemical signal could increase aversive reactions to visual signals, accelerate aversive learning and improve memorization of the conspicuous prey (Marples and Roper 1996; Lindström et al. 2006; Skelhorn and Rowe 2006 a, b; Gohli and Högstedt 2009). On the other hand, the chemical signal could serve also within members of the same species for communication (e.g. causing dispersion, Evans and Schmidt 1990). However, how exactly chemical signalization works, is still unknown.

The components of the chemical defence could be unpalatable, malodourous or directly toxic (Aldrich 1988) and they affect the predator before, during and/or after the attack (Skelhorn and Rowe 2005 a, b, 2006 a, b, c, d). They can also cause nausea or vomiting (Staples et al. 2002, Ruxton et al. 2004). The effectiveness of the chemical defence is based on the rapid learning of associating warning signals with noxious toxins (Brower 1984; Skelhorn and Rowe 2010). Therefore, visually hunting predators, such as birds or lizards can easily avoid noxious insects (Benes 1969; Guilford 1990; Krall et al. 1999; Kelly and Marples 2004; Bonacci et al. 2008; Shanbhag et al. 2010).

The chemical defence shows great variability among insect species, e.g. butterflies, ladybird beetles, leaf beetles or bugs (Fink and Brower 1981; Rowell-Rahier et al. 1995; Aldrich et al. 1997; Nishida 2002; Camarano et al. 2006; Pareja et al. 2007; Moraes et al. 2008; Speed et al. 2012). Moreover, it differs also due to various chemical compounds that are used (as a signal and/or defence) - cardenolides, pyrrolizidine alkaloids, phenolic glycosides, aldehydes, (Waterhouse and Gilby 1964; Scudder and Meredith 1982; Aldrich 1988, 1995; Farine et al. 1992; Klitzke and Trigo 2000; Aliabadi et al. 2002; Ghostin et al. 2007; Trigo 2011).

1.2.1. Metathoracic scent-glands

In Heteroptera there are two types of scent-glands mainly involved in the chemical defence: the dorsoabdominal glands, which are primarily developed in larvae (but often persisting to adulthood), and metathoracic glands, which are exclusive for adults (Staddon 1979). The present chapter will be focused on the metathoracic scent-glands (MTG) with particular attention of MTG of chosen true bug model species: the striated shieldbug *G. lineatum*.

Generally, MTG are placed in a ventral position in the hind part of the metathorax (Staddon 1979) and the gland itself does not usually extend over the edge of the metathorax (Hepburn and Yonke 1971). According to Carayon (1971), two basic types of MTG are classified: omphalien type with one opening to metasternum and diastomien type with two openings in both metacoxal cavities (Fig. 1.1). The omphalien type is probably primitive and the diastomien type is derived. However, MTG are often reduced or secondary divided, so the situation is not completely clear (Staddon 1979).

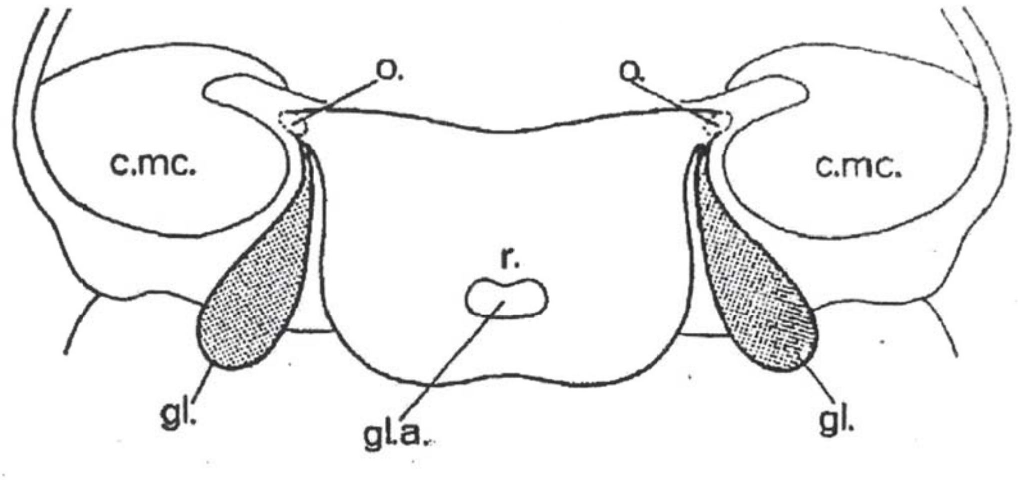

Fig. 1.1: Diastomien MTG with undivided median reservoir. c.mc. – metacoxal comb; gl. – lateral glandular reservoir; gl.a. – accessory gland; r. – median reservoir; o. – orifice (after Carayon 1971).

According to Staddon (1979), typical diastomien MTG apparatus is composed of paired lateral reservoirs with primary glands (branching secretory tubules), unpaired median reservoir with accessory gland and paired excretory tubules. However, the morphology of MTG varies among Heteropteran families. MTG are associated with a cuticle called evaporatorium (Carayon 1971). This cuticle is often situated on metapleura, however it can also cover lateral and ventral parts of thorax (Carayon 1971). The fine structure of evaporatorium is mushroom-shaped sculpture and this sculpture holds the fluid. These fine structures are taxon – specific (Hepburn and Yonke 1971, Durak and Kalender 2009).

In family Pentatomidae, evaporatorium is bigger than in the other families of Heteroptera and it also extends to the mesothorax. The accessory gland is long and wavy (Nagnan et al. 1994) – the reason could be to increase the surface for the effective synthesis of secondary MTG products.

In pentatomid secretion two phases of the liquid were found – clear fluid (containing nonpolar molecules – alkanes etc.) and yellow/orange fluid, which contains polar molecules – such as aldehydes (Waterhouse and Gilby 1964; Games and Staddon 1973; Staddon 1979).

The morphology and structure of MTG of *G. lineatum* (Fig. 1.2) were studied by Durak and Kalender (2009). *G. lineatum* MTG belong to the diastomien type with scent glands always open to the outside with two ostioles. MTG have well-developed reservoir and paired glands, which are located in the lateral of this reservoir. The reservoir is bag-shaped and it is connected to the lateral glands by a canal in the apical surface (Durak and Kalender 2009). MTG are opened thanks to paired ostioles and they have globular shapes in *G. lineatum*.

The mushroom-like structure was observed on the evaporatorium surface and these structures are connected to each other by ridges (Durak and Kalender 2009). According to Durak and Kalender (2009), the reservoir of MTG contains two types of cells called Type I (columnar epithelial cells surrounded by a thin basal lamina and cuticular intima layer at the apical surface) and Type II, which are secretory cells found in a certain area of reservoir walls.

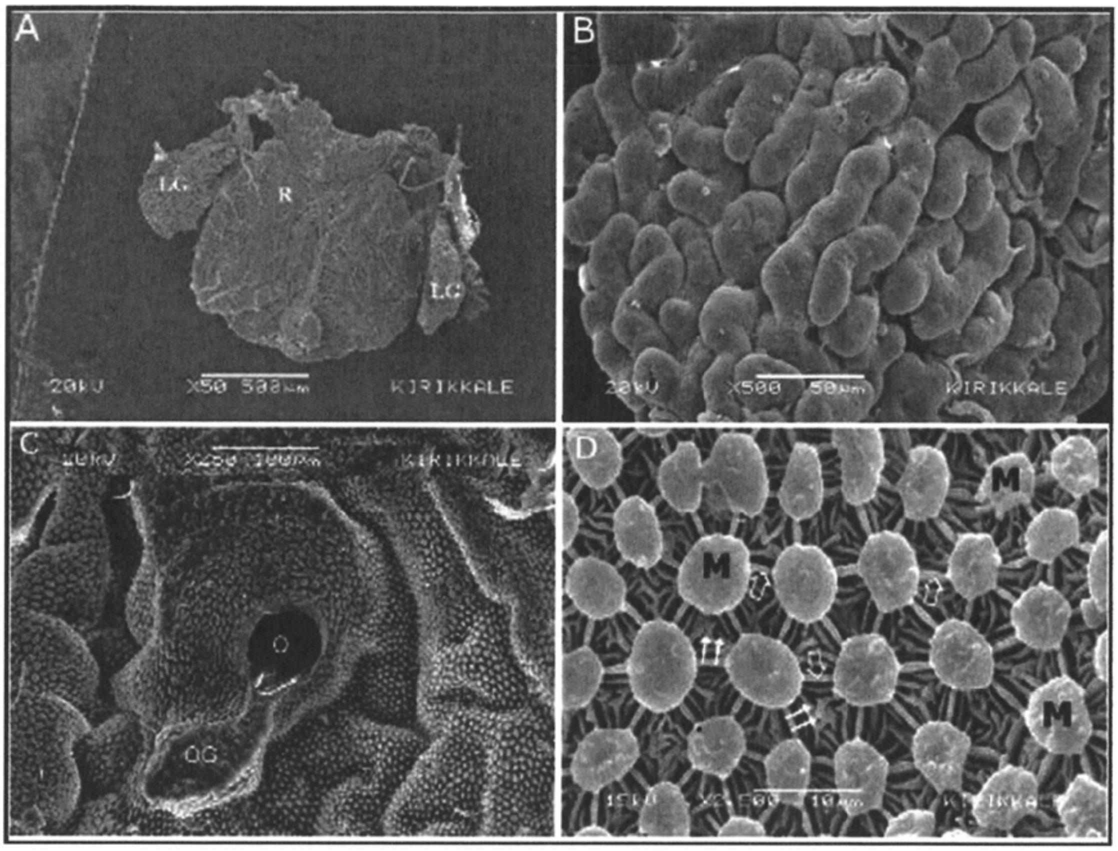

Fig. 1.2: (A) Reservoir and lateral glands of MTG; (B) Lateral glands of MTG; (C) Ostiole and evaporative area of MTG of *Graphosoma lineatum*; (D) Structure of evaporative area of MTG of *Graphosoma lineatum*. R: reservoir; LG: lateral gland; O: ostiole; OG: ostiolar groove; M: mushroom-like structure; ⇨ : ridge; ⇉ : trabecule (after Durak and Kalender 2009).

1.2.2. Sequestration

The most common definition of sequestration is that phytophagous insect species store toxins directly from the host-plants as a basis of the chemical defence against predators (Duffey 1980; Opitz and Müller 2009). Duffey (1980) also described a phenomenon called potentiator sequestration, which describes a situation in which sequestered chemical serves as an initiator or a precursor of biosynthetic reactions.

The sequestration is highly adaptive and has a considerable variability (Blum 1996; Opitz and Müller 2009). Except for its defensive mechanism, the sequestration often has a function in intraspecific communication (Pasteels 2007). Besides sequestration, *de novo* synthesis of chemical defensive compounds could be also found in Heteroptera (Aldrich 1988). According to Pasteels et al. (1990), *de novo* synthesis seems to be the primitive state and the sequestration is derived in chrysomelid beetles. This situation could be similar in Heteroptera, in which almost all defensive compounds are sequestered (Aldrich 1988; Aldrich et al. 1997; Pareja et al. 2007; Moraes et al. 2008). However, there is another opinion that the sequestration is the primitive state and *de novo* synthesis is the derived one (Browers 1992). Nevertheless, *de novo* synthesis is costly (Nishida 2002).

Disadvantages of the sequestration are dependent on the seasonal variation of quality, quantity and concentration of chemicals in the host-plants. Therefore, true bugs could not be identically defended during the whole season against predators (Browers 1992; Aliabadi et al. 2002; Pasteels 2007). On the other hand, true bugs store the chemical compounds in haemolymph or in the special organs for such situations (Aldrich 1988; Aldrich et al. 1997).

In Heteroptera, the sequestration mostly occurs in families Miridae, Lygaeidae, Rhopalidae and Pentatomidae (Aldrich 1988). Most common defensive chemicals are cyanolipids (Aldrich et al. 1990), glycosides (Aliabadi et al. 2002), cardiac glycosides (Evans et al. 1986) and pyrrolizidine alkaloids (McLain 1984). Sequestered chemicals are often taxonomically specific (Aldrich 1988).

1.2.3. Common chemical compounds in Heteroptera

The defensive secretion of Heteroptera is complex and varies among families and also from species to species (Aldrich 1988; Aldrich et al. 1995; Aldrich et al. 1997). Furthermore, nearly all Heteroptera have scent glands; however, in some families MTG are reduced (Aldrich 1988). Among common compounds of the repellent secretion of

Heteroptera belong alkanes, aldehydes, esters, alcohols, ketons, acids and other components such as isoprenoids or pyrazines (Hamilton et al. 1985; Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996, 1997; Krall et al. 1999; Aliabadi et al. 2002; Prudic et al. 2008; Fávaro et al. 2011; Šanda et al. 2012).

The following section will focus on families of Heteroptera (in some families the chemical compounds of their secretion have not been described) and the most common chemical compounds in their MTG secretion – according to Aldrich (1988):

1) **Coreidae** – MTG secretion is a mixture of saturated or α,β – unsaturated aldehydes and alcohols. The secretion also contains acetate or butyrate esters of these alcohols.
2) **Alydidae** – MTG secretion contains rancid butyric and hexanoic acids.
3) **Rhopalidae** – MTG secretion is extremely reduced, but contains aliphatic carbonyls ((E)-2-hexenal, (E)-2-octenal and 4-oxo-(E)-2-octenal).
4) **Pyrrhocoridae** – MTG secretion is also reduced but according to Farine et al. (1992), it contains aldehydes, saturated hydrocarbons, alcohols, ketones, lactones terpenes, one phenol and one ester.
5) **Berytidae** – MTG secretion has not been chemically investigated.
6) **Lygaeidae** – MTG secretion contains (E)-2-hexenal, (E)-2-octenal and tridecane and it also contains cardiac glycosides from the host-plant. Females in danger release almost purely aldehydes.
7) **Pentatomidae** – MTG secretion is highly developed and contains hydrocarbons, aldehydes, alkanes, alkenals, ketones, esters and alcohols.
8) **Scutelleridae** – MTG secretion is composed mostly of (E)-2-decenal, 4-oxo-(E)-2-hexenal and tridecane.
9) **Plataspidae** – MTG secretion has pentatomid pattern, but little is known about particular components.
10) **Cydnidae** – Similar to Plataspidae, MTG secretion has probably pentatomid pattern.
11) **Acanthosomatidae** – Little is known about the chemical compounds of MTG secretion.
12) **Reduviidae and Phymatidae** – MTG are small, but produce 3-methyl-2-hexanone.

13) **Cimicidae** – MTG are reduced, but produce mostly C_6 and C_8 alk-2-enals, but also 2-butanone and acetaldehyde.

14) **Anthocoridae** – Little is known about the semiochemistry of these predators. MTG are present.

15) **Miridae** – MTG secretion is based also on sequestered chemicals from host-plants (similarly to Pentatomidae). The major compounds are butyl and hexyl butyrates.

16) **Tingidae** – MTG secretion has not been analysed chemically.

17) **Pleidae** – MTG secretion contains mostly hydrogen peroxide and carbonyl compounds.

18) **Notonectidae** – MTG is missing in one subfamily.

19) **Naucoridae** – MTG produce phenolics.

20) **Corixidae** – MTG secretion contains mostly 4-oxo-alk-2-enals.

21) **Gelastocoridae** – MTG secretion consists of 4-oxo-2-hexenal and 2-octenal.

22) **Belostomatidae** – MTG secretion contains (E)-2-hexenyl acetate in the species *Lethocercus*.

23) **Amphibicorisae** – Little is known about the chemical investigation of MTG secretion.

24) **Enicocephalidae** – MTG are present only in males and little is known about the chemical compounds themselves.

1.2.4. Repellent secretion of *Graphosoma lineatum*

The striated shieldbug is a widely used model for chemical analysis of the MTG secretion (Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012). The chemical defence of *G. lineatum* is composed of a highly volatile liquid, which contains irritants as well as toxins (Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012) and it comes from MTG in adults (Aldrich 1988). Thanks to this volatile composition , the repellent secretion could operate over greater distance and therefore, there is a bigger chance that potential predator, bird or lizard, could discriminate and avoid such a prey due to olfactory aposematism (Eisner and Grant 1981).

While irritants, such as *n*-tridecane (Gunawardena and Herath 1991), are effective against arthropod predators, such as mantids, spiders or ants (Aldrich 1988); toxins, such as α,β - unsaturated oxoaldehydes (Šanda et al. 2012), can protect the bugs mostly against birds and other vertebrates (Aldrich 1988).

According to Stránský et al. (1998), the main components are series of *n*-alkanes (C_{10}-C_{13}) dominated by *n*-tridecane (C_{13}). The presence of *n*-alkanes was described also by Durak and Kalender (2009) as well as the presence of aldehydes. However, according to Šanda et al. (2012), the repellent secretion of both sexes is based mostly on aldehydes, (E)-2-decenal and (E)-4-oxohex-2-enal, respectively. Furthermore, there are no differences between sexes of *G. lineatum* in the chemical character and relative percentage of the volatile secretion components (Šanda et al. 2012). The results of this study support the hypothesis that the secretion primarily deters predators.

The following aldehydes belong to the most common compounds of the striated shieldbug repellent secretion: (E)-2-hexenal, (E)-2-decenal, (E)-2-octenal, tridecane, (E)-4-oxohex-2-enal (Šanda et al. 2012). The present study is therefore focused on these compounds from adult MTG secretion.

Moreover, *G. lineatum* can avoid the attack by spraying this secretion from its MTG towards the predator (M. Šanda, personal communication) and hits very often the eye of the predator (M. Gregorovičová, personal observation).

According to personal communication with Ludvík Streinz and Bohumír Koutek from the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, (E)-2-hexenal, (E)-2-decenal and (E)-2-octenal were tested together as a mixture, because of their common occurrence in the repellent secretion of true bugs (Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996; Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012). Therefore, this mixture could function as a potential olfactory signal – typical nasty smell of the striated shieldbug (L. Streinz, personal communication).

1.3. Chemical discrimination

Discrimination is the learning process, during which animal distinguishes different reaction/situation and/or distinct stimuli. The chemical discrimination enables animals to learn and avoid potential chemically defended prey, detect predators or find a partner (Cooper 1997, 2007; Mason and Clark 2000; Aragón et al. 2001; Pough et al. 2005), whereas the chemoreception is the ability to perceive chemical substances using chemoreceptors such as taste buds, nasal epithelium with cilia etc. (Luu et al. 2004).

According to Eisthen (1997), distinct subsystems for the chemical discrimination are widely present in vertebrates (aquatic or terrestrial) and the additional vomeronasal organs (VNOs) arose in tetrapods.

Vertebrates discriminate mostly based on the following senses: (1) gustation; (2) olfaction; (3) vomerolfaction and (4) chemesthesis. These senses have a key position in life-history of the animal, such as the identification of intra-inter relationship, mate recognition, exploration, social behaviours or prey detection (Burghardt 1970; Cooper 1991; Halpern 1992; Aragón et al. 2001). The ability how to discriminate the prey also influences foraging mode of the species (Huey and Pianka 1981) – sit-and-wait predator (or ambush predator) and active forager.

The chemical discrimination is always linked to good chemoreception, which enables to perceive stimuli of the chemical substances thanks to chemoreceptors. Chemoreceptors are able to detect the presence and amounts of different types of molecules, pheromones, predator's odours etc. (Halpern 1992; Cooper 1997; Luu et al. 2004). Chemoreception is well-developed in Squamata, which use mostly nasal senses – the olfaction and vomerolfaction, especially in more derived taxa in the group Autarchoglossa (Schwenk 1985; Schwenk 1995; Vitt et al. 2003). On the other hand, some of these derived taxa use, except the nasal senses, also gustation as was observed in e.g. Lacertidae (Schwenk 1985).

Nevertheless, gustation is mainly used by lizards (e.g. Iguania), which are mostly ambush predators and also their nasal senses are not so well-developed (Schwenk 1985; Schwenk 1995; Vitt et al. 2003). The types of tongue indicate the level of gustation development (Schwenk 1995; Cooper 1997). The taste buds disappear according to the higher phylogenetic level of lizard taxa (Schwenk 1985; Cooper 1997). This hypothesis is based on previous morphological studies (Estes 1988; Gauthier et al. 2012). This phylogenetic analysis also very well corresponds with the foraging mode.

In case of birds, there is still speculation about their actual ability to use olfaction such as in case of Paridae (Bang and Cob 1968), but many groups of birds including Passeriformes have apparently an acute sense of smell and they also rely on olfaction (Steiger et al. 2009). However, it does not mean that all birds have similar olfactory abilities (Steiger et al. 2009).

Birds do not have vomerolfaction at all (Wenzel 1987). Thus their main senses are olfaction, gustation and chemesthesis (Schlee 1986; Mason and Clark 2000).

Chemoreception is able to discriminate thousands of different odorants by the vertebrates olfactory system, Fig. 1.3, (Luu et al. 2004). The detection of odorants is mediated by olfactory receptors (ORs). ORs are G-protein coupled receptors that form large protein superfamily in vertebrate genome and they are expressed in sensory neurons within olfactory epithelium (Steiger et al. 2009). The sensitivity of neurons, which are responsible for sensitisation to different odours, is determined by which from the thousands of odour receptor proteins are expressed on their surface (Araneda et al. 2000).

Each of these receptors may harbour multiple binding sites – therefore, they possess an enormous diversity of chemical structure (Araneda et al. 2000). It is also true that many odours are recognized by more than one receptor and reversely most receptors could recognize multiple odours (Mori et al. 1999; Araneda et al. 2004). So, the chemical discrimination using chemoreception gives basically unlimited possibilities.

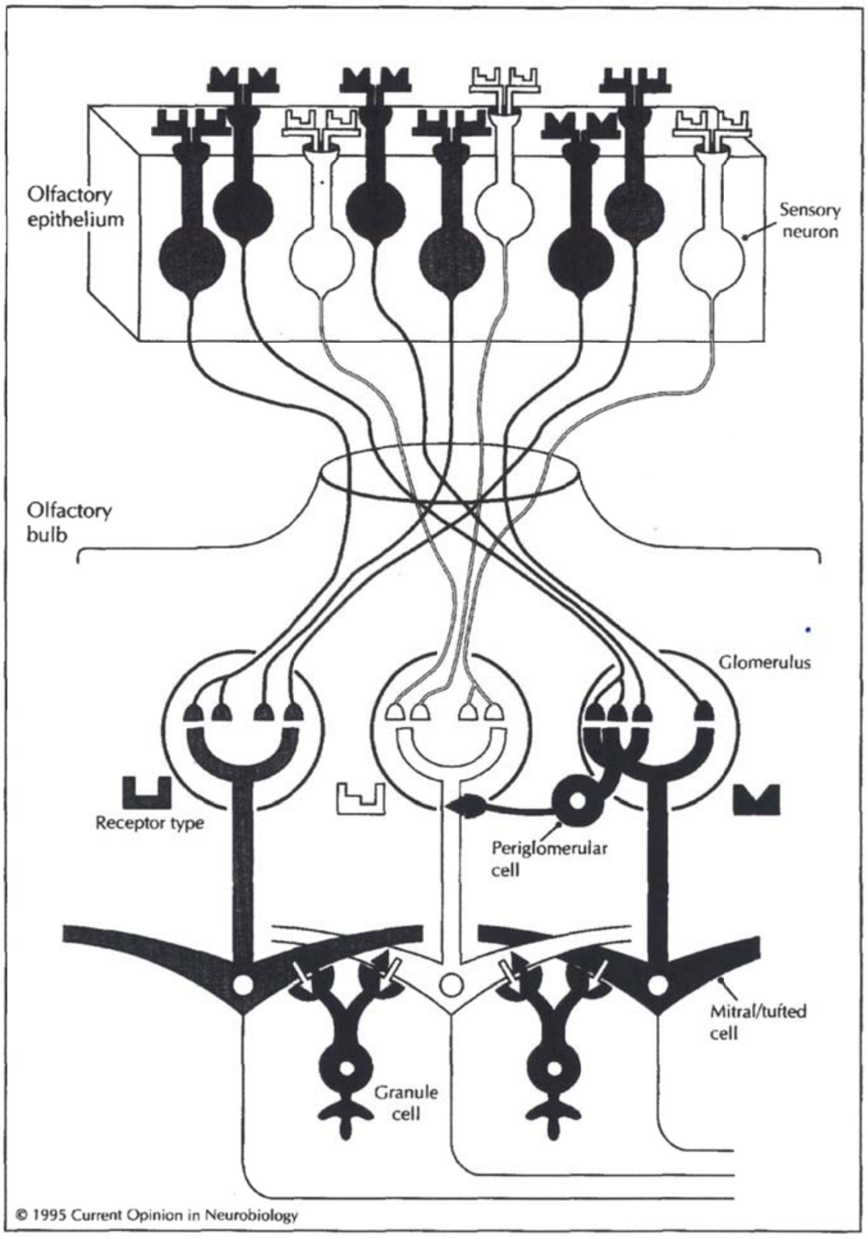

Fig. 1.3: Schematic diagram depicting the convergence onto glomerulus of axon originating from olfactory sensory neurons expressing the same type of odour receptors (after Mori 1995).

1.3.1. Nasal senses in lizards

Nasal chemical senses in lizards are represented by olfaction and vomerolfaction (Moulton and Biedler 1967; Burghardt 1970; Halpern 1992; Dial and Schwenk 1996; Cooper 1997; Cooper 2007). According to Cowles and Phelan hypothesis (1958), both olfaction and vomerolfaction are functionally linked. This hypothesis says that initial detection of chemical volatiles by olfactory system triggers tongue-flicking thus activating the vomeronasal system. So VNOs play role as a proximate chemoreceptor. Additionally, acording to Schwenk (1995) olfaction reacts mainly to airborne volatiles (such as volatiles of repellent secretion), whereas VNOs analyse the nonvolatile components of the chemical source by tongue-flicking towards the source (such as aposematic insect). So, it seems, that repellent secretion of *G. lineatum* could be aimed at this type of predators with well developed nasal senses – vomerolfaction as well as olfaction.

Olfaction is projected into main olfactory organ, which is covered by epithelium with three types of cells: a) supporting cells; b) bipolar neurons and c) basal undifferentiated cells (Ferri et al. 1982 ex Halpern 1992). The morphology of cilia may differ among lizard species (Wang and Halpern 1983 ex Halpern 1992). Epithelium is the product of Bowman's glands, because these glands are supposed to function as a source for the mucus (Halpern 1992). Bipolar neurons in basal lamina pass into cranial vault and terminate in glomerular layer of the Main Olfactoric Bulb (MOB), which projects to telencephalon structures (Halpern 1992). The nervus olfactorius enters the main olfactory chamber, which is located in the most posterodorsal part of the chamber, just medial to the lateral nasal gland and nasal concha (Dakrory 2011). Projections go predominantly to the nucleus sphericus (Martínez-Marcos et al. 1999).

In Squamata reptiles, the vomeronasal chemosensory system is anatomically and functionally distinct from the main olfactory system (Halpern 1987, 1992; Cooper 1997; Halpern and Martínez-Marcos 2003). Vomeronasal organs (VNOs) are paired chemosensors that lost their connection to the main olfactory system. VNOs communicate exclusively with the oral cavity through two openings, vomeronasal fenestrae (Halpern 1987, 1992; Halpern and Martínez-Marcos 2003; Filoramo and Schwenk 2009). The vomeronasal organs are located at the base of the nasal cavity, above the palate (Filoramo and Schwenk 2009). The vomeronasal organs are innervated by two nerves distinct from the olfactory nerve: the terminal nerve and vomeronasal nerve. Additionally, they are structurally isolated from the main olfactory system, so the vomeronasal nerves project to separate part of the olfactory bulb (AOB) until at least amygdaloid nuclei (Halpern 1992; Halpern and Martínez-Marcos 2003; Filoramo and Schwenk 2009).

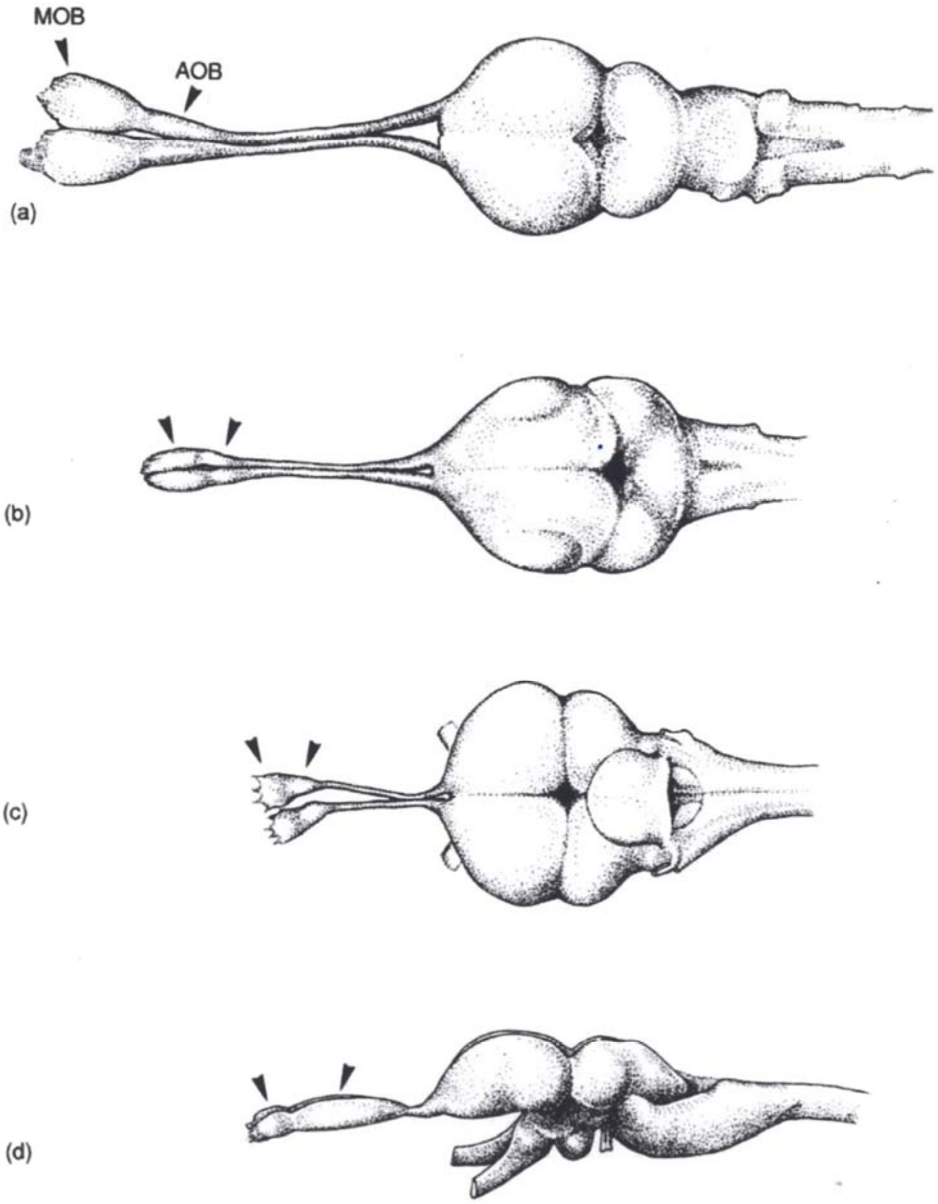

Fig. 1.4: Squamate brains showing relative size of MOB (main olfactoric bulb) and AOB (accessory olfactoric bulb). A) *Gecko gecko*; B) *Anolis garmani*; C) *Dipsosaurus dorsalis*; D) *Varanus flavescence*. A)-C) dorsal view; D) lateral view; bold darts show olfactory bulbs (after Schwenk 1993).

When comparing the brains of selected lizards, there are considerably differences among MOB and AOB (Fig. 1.4). In *Gecko* MOB and AOB are well-developed; in *Anolis* MOB and AOB are also developed, but these structures are much smaller than in geckos. In case of *Dipsosaurus* both olfactory bulbs are atrophied. At last, in *Varanus* the MOB is significantly smaller than the AOB, which is abnormally well-developed showing that in Varanidae the VNOs function as the main nasal sense.

Thanks to all these adaptations, lizards have great ability to detect prey odours and discriminate them from nonprey (e.g. predator) odours (Cooper 1990; Halpern 1992) based on MOB, AOB, or both olfactory systems working together.

1.3.2. Nasal sense in birds

Although birds discriminate mostly visual (Gamberale-Stille 2001; Hatle et al. 2002; Gamberale-Stille and Guilford 2003; Exnerová et al. 2006; Ruxton and Sherratt 2009; Svádová et al. 2009), their ability of chemical discrimination has been taken to account as well because of highly volatile repellent secretion of *G. lineatum* (Šanda et al. 2012), which is highly odorous.

The nasal region of birds follows the reptilian plan: two elongated nasal chambers, separated by the septum, leading from the external nares to choana in the buccal cavity. Each chamber is divided into three distinct chambers. The second or main chamber contains the conchae, which are homologous to structures present in reptiles (Portmann 1961 ex Stager 1967). The avian olfactory system has the same features as in other vertebrates (e.g. innervation by olfactory nerve). Thus, there is no doubt that olfaction is a functional sensory modality for most birds (Wenzel 1987).

However, this functional modality is well-developed only in a few families, such as Procellariiformes. On the other hand, Passeriformes have relatively poorly developed olfactory capacities (Bang and Cobb 1968; Mason and Clark 2000).

The olfactory receptors are located in the olfactory epithelium in the caudal conchae, where each receptor cell is surrounded by a cluster of supporting cells (Mason and Clark 2000). To gain access to chemoreceptors, odour molecules have to diffuse through a mucous membrane, because the cilia of the sensory cells don’t have transport function (Mason and Clark 2000). Olfactory threshold and relative size of the olfactory bulb is species-dependent (Clark et al. 1993).

Some birds use scent to locate prey, such as kiwis, but birds mostly use olfaction for orientation and navigation (Mason and Clark 2000). The well-developed nasal bulbs present in colonial species suggest the possibility that they use olfaction for social functions (Pough et al. 2005).

1.3.3. Gustation

Taste is the sensation produced when a substance in the oral cavity and pharyngeal epithelia interacts with the taste receptor cells (gustatory cells) located on taste buds (Kardong 2012). Taste, along with the smell (olfaction) and trigeminal nerve stimulation (e.g. pain and temperature), determine flavours of food or other substances (Green et al. 2005). In amphibians, reptiles and birds, the taste buds are located in the mouth, but also on the tongue, such as in case of lizards (Schwenk 1985) or birds (Mason and Clark 2000). Mammalian taste buds tend to be distributed mostly on the tongue (Kardong 2012).

The nerves transmitting the taste – are the chorda tympani, glossopharyngeal and greater superficial petrosal nerves (Green et al. 2005). The sense of taste is mostly conserved across vertebrates (Roura et al. 2013). The number of taste buds varies among animal species and the differences among the taste systems are linked to their adaptation to dietary regimes (Jiang et al. 2012). Following chapters are focused on gustation in lizards and birds.

1.3.3.1. Gustation in lizards

Basically, there are not many studies focused on lizard taste buds occurrence (Schwenk 1985, 1995). The taste buds consist of at least three types of cells in reptiles: type I, II and III. The type I cells are characterized by the presence of dense secretory granules containing polysaccharides. The type II cells contain numerous tubular, vesicular and lamellated structures. The type III cells contain dense cored vesicles and they are responsible for afferent synaptic contact (Uchida 1980).

The taste buds are present on the tongues as well as oral epithelium of maxillae and mandibles in lizards. In snakes, it was observed that the taste buds covered squamous epithelium located along the dental arch (Uchida 1980). In lizards, the taste buds are remarkably abundant and they are not restricted only to oral and pharyngeal epithelia. Therefore, they may reach the greatest densities on the tongue of some species (Schwenk 1985).

According to Schwenk (1985), the taste buds are present principally in the regions of stratified squamous epithelium moderate in thickness. The large amount of the taste buds is suggested as a primitive state in phylogenesis (Iguania), because the loss of the taste buds, e.g. in *Varanus*, and reduction or loss of lingual taste buds in teiids, eublepharids and snakes could be interpreted as a derived state (Schwenk 1985; Jamniczky et al. 2009). This interpretation of primitive/derived state follows phylogenetic system (Estes et al. 1988; Gauthier et al. 2012). The number of the taste buds is also influenced by the shape of the tongue, Fig. 1.5 (Schwenk 1993).

The occurrence and distribution of the taste buds in selected taxa follow (Schwenk 1985):

1) **Iguania** – taste buds are abundant, mostly concentrated on the tongue tip.
2) **Agamidae** – taste buds are scattered and more or less evenly distributed.
3) **Chamaeleonidae** – taste buds are also scattered in the glandular portion of the tongue tip and foretongue.
4) **Gekkonidae** – in *Gecko gecko* the taste buds are numerous (Nonoyama 1936 ex Schwenk 1985) and according to Schmidt et al. (2010), there is a presence of taste buds close to VNOs in four tested gecko species.
5) **Eublepharidae** – in species *Eublepharis macularius* no taste buds were found (Jamniczky et al. 2009).
6) **Pygopodidae** – in *Liasis burtonis* taste buds are numerous.
7) **Xantusiidae** – taste buds are scattered and widely distributed (most frequent on dorsolateral margins of the tongue tip and foretongue).
8) **Scincidae** – taste buds are numerous and abundant on the tongue, especially on the tongue tip.
9) **Lacertidae** – taste buds are also numerous on the foretongue and become scattered in the hindtongue. In case of *Lacerta viridis* taste buds are even present on the long tines of the forked tongue tip.
10) **Teiidae** – taste buds are rare.
11) **Cordylidae** – different abundance among species.
12) **Anguidae** – taste buds are rare or absent on the tongue tips, they are most common on the sides of the foretongue.
13) **Varanidae** – no taste buds were found in *Varanus indicus*, which is similar to the snakes, where they are present rarely along the dental arch (Uchida 1980).

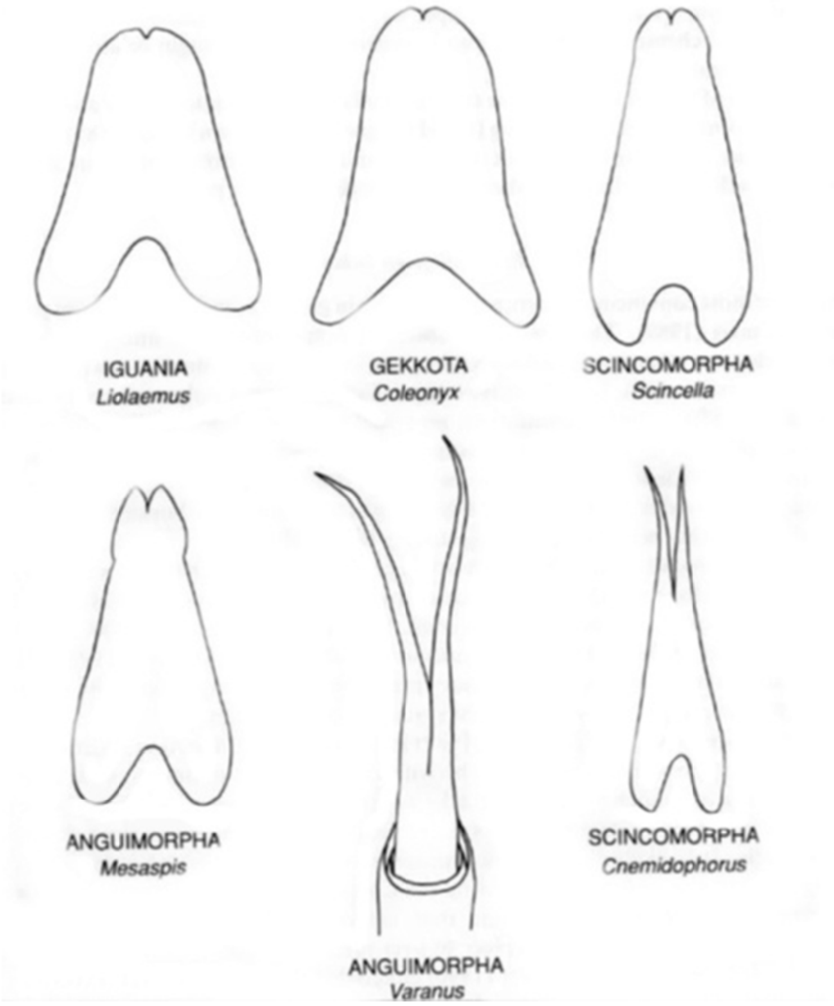

Fig. 1.5: Tongue shapes in squamata lizards (after Schwenk 1993)**. The more forked tongue the less taste buds.**

1.3.3.2. Gustation in birds

In comparison to other vertebrates, birds have only very few taste buds. These are mostly distributed throughout the oral mucosa, but the most frequent occurrence is associated with salivary gland openings (Berkhoudt 1985 ex Mason and Clark 2000). There are also age-dependent changes in the number of the taste buds, e.g. the adult chicken has twice as high number of the taste buds than a day-old chick (Mason and Clark 2000). However, within adults the numbers of the taste buds decline with age (Duncan 1960 ex Mason and Clark 2000).

In birds, there are several types of taste buds. First type has spindle/pear shape and it is located in foveas. This type occurs in e.g. Passeriformes, Galliformes and Columbidae. The second type is slim and elongated and it occurs in families e.g.

Anatidae, Phoenicopteridae and in some Charadrii. The last type is rounded and exists in e.g. Psittacidae (Veselovský 2001).

Saliva is crucial for the transport of taste stimuli to the receptors (Belman and Kare 1961 ex Mason and Clark 2000). Most experiments have been focused not on the occurrence or distribution of the taste buds, but on the behavioural reactions to the five types of taste – sweet, bitter, umami, salty, sour (Mason and Clark 2000; Roura et al. 2013). Within the class Aves, the taste buds distribution and taste sensitivity vary and reflect different feeding regimes and also feeding strategies. This is valid also for the number of the taste buds (Roura et al. 2013) – see Table 1.1.

Table 1.1: Number of the taste buds in selected bird species (according to Roura et al. 2013).

Species	Scientific name	Number of taste buds
Blue tit	*Cyanistes caeruleus*	24
Chicken	*Gallus gallus*	312
Duck	*Anatidae* spp.	375
Parrot	*Psittacidae* spp.	350
Pigeon	*Columba livia domestica*	56
Quail	*Coturnix japonica*	62
Sparrow	*Zonotrichia albicolis*	0
European starling	*Sturnus vulgaris*	200
Turkey	*Meleagris gallopavo*	0
Zebra finch	*Taenopygia guttata*	0

1.3.4. Chemesthesis

Chemesthesis or common chemical sense is the ability to perceive chemically induced pain caused by the chemical components, which trigger irritating and painful sensation. This sensation is mediated by the trigeminal nerve fibers, located on the inside of the nasal and oral cavity and in the eye (Lin et al. 2008).

Trigeminal nerve has chemoreceptive fibres with chemoreceptors (Green et al. 1990). Unfortunately, little is known how chemesthesis precisely works in birds and reptiles. Most studies have been focused on humans (Green et al. 2005) with irritant chemicals such as vanilin, capsaicin or menthol (Clark 1998). In the experiments with birds, the most tested chemical was methyl antranilate/methyl-N-methyl antranilate (Mason et al. 1989; Clark and Shah 1991; Conner et al. 2007).

These irritants raise sensation of burning, bitter taste (capsaicin) or sensation of coolness or picking (menthol). These components can activate trigeminal nerve and run defensive reflexes such as nausea or sneezing (Staples et al. 2002). Furthermore, there are experiments, which proved that some irritants could cause innate aversive reaction in birds (Mason et al. 1989).

How irritants work in reptiles is an open question. Therefore, it is neccessary to perform the experiments with reptilian taxa. In addition, there are many possibilities how chemesthesis could work, because different species react differently towards the same stimuli. So, it is still not clear whether chemesthesis works independently or in combination with other senses such as olfaction or taste (Green et al. 2005).

1.3.5. Chemical discrimination and Foraging mode in lizards

Foraging mode is the phenomenon, which has been studied for more than three decades (Pianka 1966; Huey and Pianka 1981; Cooper 1995; Perry and Pianka 1997; Cooper et al. 2005) and it is very closely connected with the chemical discrimination and tongue-flicking (Cooper 1995, 1997, 2007). In ecological context, there are two principle strategies – active foragers and sit-and-wait or ambush predators (Huey and Pianka 1981; Perry and Pianka 1997). Active foragers (such as Eublepharidae, Lacertidae or Varanidae) are defined as hunters, which are very agile and in order to find a prey they use mostly the chemical discrimination by vomeronasal olfaction, olfaction itself and they use tongue-flicking (Huey and Pianka 1981; Vitt et al. 2003; Cooper 2007). On the other hand, sit-and-wait foragers (such as Iguania or Gekkonidae) wait for a prey and discriminate this prey by vision and also gustation (Huey and Pianka 1981; Schwenk 1985; Vitt et al. 2003; Cooper 2007). Moreover, in some studies a theory was postulated that active foragers and ambush predators represent two extremes of one continuum of various foraging modes (Perry 1999; Butler 2005).

The chemical discrimination increases with the degree of active foraging (Cooper 1995, 1997, 1999, 2007; Vitt et al. 2003; Cooper et al. 2005). It means that lizards, which are phylogenetically more derived (such as Teiidae or Anguidae), use more and more olfaction and vomerolfaction rather than gustation, which is the primitive state in the basal groups such as Iguania (Schwenk 1985; Cooper 1995; Vitt et al. 2003; Gauthier et al. 2012). And finally, lizards placed in crown groups, such as Varanidae or snakes, are vomerolfaction specialists (Schwenk 1985; Estes et al. 1988; Cooper 1995; Vitt et al. 2003; Gauthier et al. 2012).

There are many factors, such as morphology or behaviour, which influence foraging mode via changes in the chemical discrimination (Fig. 1.6).

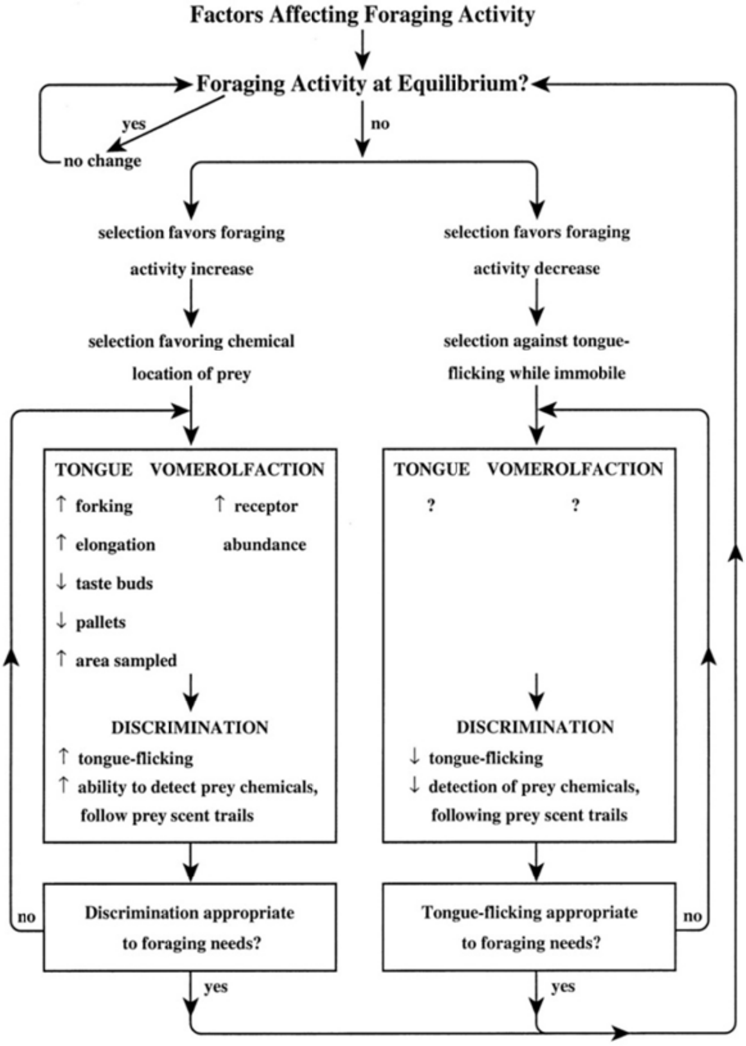

Fig. 1.6: Hypothesized effects of natural selection on relationships among prey chemical discrimination, foraging mode and lingual-vomeronasal system (after Cooper 1997).

The most likely interpretation of Fig. 1.6 is that there is a shift in foraging mode thanks to the changes in chemosensory behaviour and morphology of the predators (Cooper 1997).

The phylogenetic background of the adaptations related to the chemical discrimination and foraging mode in squamate reptiles represents an exciting yet still only poorly comprehended aspect of the topic. There are two major phylogenetic hypotheses of Squamata: the one based on morphology, directly linked to the characters promoting particular modes of foraging strategy (Estes et al. 1988; Gauthier et al. 2012), and the other one is based on molecular data (Vidal and Hedges 2005, 2009; Pyron et al. 2013). The latter reveals numerous discrepancies in states of particular characters and in contrast to Gauthier et al. (2012) suggests rather a mosaic evolution of the adaptations in question.

Nevertheless, the present thesis is not focused on the phylogeny – rather it is aimed at the ecological consequenses of the aversive reactions of different types of predators. The studied lizard predators represent quite distant clades of Squamata (Gekkota vs. Lacertilia), yet both could be described as active foragers in the true sense of the word (Cooper 1990, 1995, 1997, 2007). Both selected lizard predators use the chemical discrimination but in different ways according to the development of their chemoreceptors.

1.4. Model organisms

For the present study six model organisms were selected. Four model organisms were chosen as predators: two species of lizards – leopard gecko (*Eublepharis macularius*) and green lizard (*Lacerta viridis*) and two species of birds – great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*). Two true bug species were chosen as a prey: striated shieldbug (*Graphosoma lineatum*) and firebug (*Pyrrhocoris apterus*).

The two lizard species represent opposing types of the predator – diurnal and nocturnal. Both are active foragers (Cooper 1995) and insectivorous predators (Angelici et al. 1997; Seufer et al. 2005) and they are more dependent on the chemical senses than sit-and-wait predators (Huey and Pianka 1981). However, the prey discrimination in these two types of predators is slightly different.

In leopard gecko, two major senses can be used for the prey detection – olfaction and vomerolfaction (Schwenk 1993, Rehorek 2000). According to Cowles and Phelan hypothesis (Cowles and Phelan 1958), both olfaction and vomerolfaction are functionally linked. Specifically, Cowles and Phelan (1958) state that initial detection of chemical volatiles by olfactory system triggers tongue-flicking, thus activating the vomeronasal system. Vomeronasal organs play role as proximate chemoreceptors. Additionally, according to Schwenk (1995) olfaction reacts mainly to airborne volatiles (such as volatiles of the repellent secretion), whereas vomeronasal organs analyse the nonvolatile components of the chemical source by tongue-flicking towards the source (e.g. aposematic insect) – this could be named dual olfactory system (Schwenk 1993).

In geckos, the olfactory chamber is well developed as well as the olfactory bulbs in brain (Pratt 1948). On the other hand, gustation is poorly developed (Schwenk 1985). Even more, there is no evidence of the taste buds in leopard geckos (Schwenk 1985, Jamniczky et al. 2009).

In green lizard, the senses, which can mediate the chemical discrimination of the prey, are vomerolfaction, olfaction and gustation (Schwenk 1985, 1993; Bonacci et al. 2008). The olfaction is similarly well-developed as in case of geckos (Gabe and Saint Girons 1976; Cooper 1996). As for the gustation, which is well developed sense in the family Lacertidae (Schwenk 1985), the taste buds are numerous, especially on the ventrolateral surfaces of the foretongue and become scattered in the glandular portion of the hindtongue (Schwenk 1985). In green lizard, the taste buds are even present on the long tines of the forked tongue tip (Schwenk 1985).

The other sense, which could be responsible for food discrimination, is vomerolfaction that is also well developed in green lizards (Cooper 1991, 1996). Both strategies could be usefull in avoiding chemically defended prey such as striated shieldbug *G. lineatum*. In fact, the chemical defence of Heteroptera, which is mainly composed of volatiles (Šanda et al. 2012), could be aimed at this type of predator – a lizard with well-developed olfactory senses and gustation or combination of these senses.

In case of selected bird species, the finding that both species avoid red-and-black Heteroptera was taken into account (Hotová Svádová et al. 2010). The question is, which chemical compound could be responsible for the aversive reactions of particular species. Both species are insectivorous (del Hoyo et al. 2007) and their diet contains true bugs (Exnerová et al. 2003a; del Hoyo et al. 2007). Although the nasal sense in birds is not well developed (Mason and Clark 2000), the chemical defence of *G. lineatum* still could play a role as signal in olfactory aposematism (Eisner and Grant 1981) as well as in gustation (Schlee 1986) or chemesthesis (Mason and Clark 2000; Conner 2007) thanks to its volatile composition (Šanda et al. 2012). Exnerová et al. (2003b) demonstrated that even closely related species of Paridae reacted differently in avoiding similar aposematic prey.

Two true bug species were chosen as a prey. Firstly, the striated shieldbug (*Graphosoma lineatum*), on which defence secretion this study is based, and secondly the firebug (*Pyrrhocoris apterus*), which was used as another chemically defended prey of red-and-black Central European aposematic Heteroptera (Hotová Svádová et al. 2010). The focus was on the adults of both species because of their MTG defensive secretion.

The striated shieldbug was chosen because the compounds of its repellent secretion are well known (Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012), and because of its widespread distribution across the Western Palaearct (Aukema and Rieger 2006). Distribution of *G. lineatum* in the Czech Republic overlaps with three of our four selected predators – green lizards (Arnold 2002; Aukema and Rieger 2006), great tits (Aukema and Rieger 2006, del Hoyo et al. 2007) and blue tits (Aukema and Rieger 2006; del Hoyo et al. 2007). In case of leopard geckos, the distribution of both species might potentially overlap as well (Linnavuori 2008; Rastegar-Pouyani et al. 2008).

As for the firebug, it was chosen as a second model organism in order to compare it with the striated shieldbug, because of the similar chemical compounds of its adult repellent secretion (Farine et al. 1992; Šanda et al. 2012) and its widespread distribution in Palaearct and North Africa (Moulet 1995).

1.4.1. Leopard gecko (*Eublepharis macularius*, Blyth, 1854)

Leopard gecko is a member of family Eublepharidae, which inhabits the eastern and southern Afghanistan, Pakistan and north-western India (Fig. 1.7) (Seufer et al. 2005; Sindaco and Jeremčenko 2008).

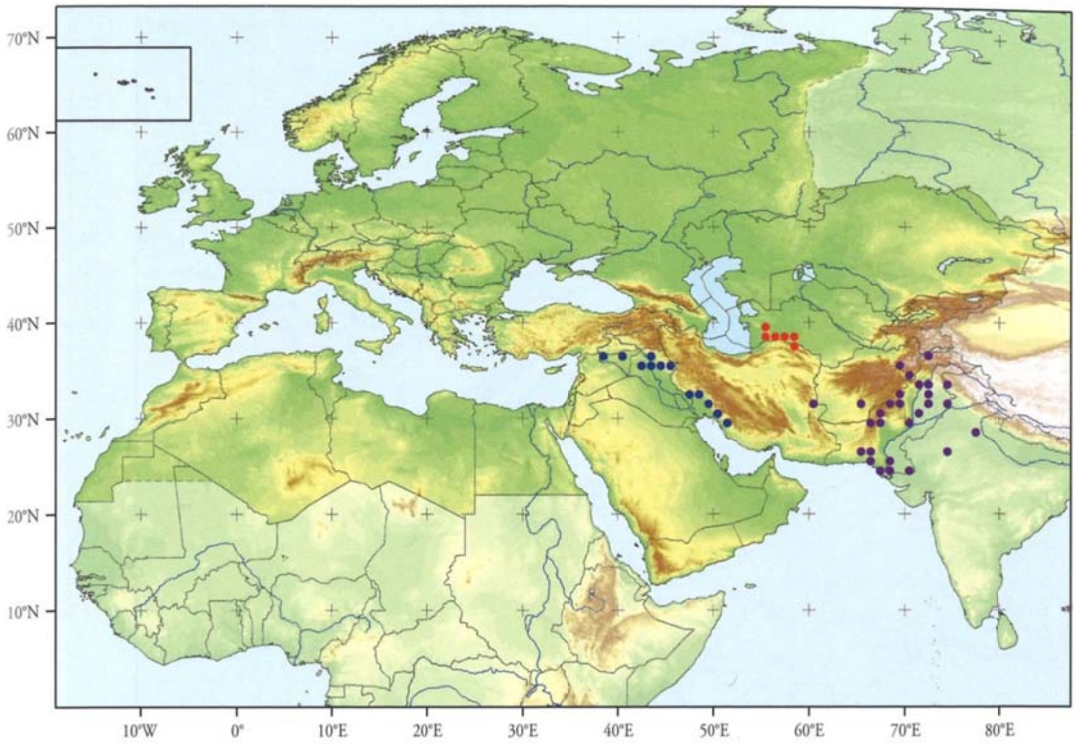

Fig. 1.7: Distribution of Leopard gecko (*Eublepharis macularius*, violet colour) (Sindaco and Jeremčenko 2008).

It is a large and fairly slender gecko (Fig. 1.8) and it varies in colouration and pattern with sex-linked dimorphism in size (Seufer et al. 2005). Male leopard geckos have the length of 11 – 16 cm from snout to vent, whereas females grow to 10 – 13 cm (Minton 1966).

The tail of both sexes is always shorter than snout-vent length. The males can be easily distinguished from the females by their clearly visible hemipenes pockets and they also have preanal pores, which are arranged at an angle (Seufer et al. 2005). Juveniles have different colour pattern than the adults (Seufer et al. 2005).

Fig. 1.8: Adult specimen of Leopard gecko (*Eublepharis macularius*) (from www.algrains.net).

This species inhabits areas of steppe vegetation from the plains (sea level) up to the mountains (Himalayas) (Khan 1999; Seufer et al. 2005). The leopard gecko is ground-dwelling and nocturnal. It spends the day hiding under the rocks, large stones or in crevices (Seufer et al. 2005). The period of gecko's activity is strictly limited to dusk, night and dawn (Seufer et al. 2005) and it is obviously linked to the low temperature of about 26.5 °C, which the animal prefers (Kratochvíl and Frynta 2002).

The habitat, which gecko prefers, frequently consists of hart and stony soil, on which bushes (*Zygophyllum* sp) grow and it may be also found in dry or bushy forests (Seufer et al. 2005). Areas that contain only sand are avoided (Minton 1966; Seufer et al. 2005). Optimal habitats could be shared by large colonies of the geckos (Minton 1966).

Within its range the leopard gecko hunts for a variety of insects, spiders, scorpions and small vertebrates such as small reptiles (*Agamura sp.*) (Minton 1966; Schifter 1988 ex Seufer et al. 2005).

According to Minton (1966), mating starts in March and April and the eggs are deposited between April and August. The females have typically invariant clutches of two eggs (Werner 1972; Kratochvíl and Frynta 2006) and they can lay several clutches within the season depending on their nutritional conditions (Seufer et al. 2005). Juveniles hatch about one and half month after starting incubation at 28 °C (Seufer et al. 2005). Winter dormancy should be at least for 6 – 10 weeks (Seufer et al. 2005).

1.4.2. Green lizard (*Lacerta viridis*; Laurenti, 1768)

Green lizard is a large member of family Lacertidae and represents probably a complex of species (Godinho et al. 2005; Böhme et al. 2006). It inhabits Europe to Turkey (Fig. 1.9) (Arnold 1987; Arnold 2002; Sindaco and Jeremčenko 2008), including Central and South-East Europe (Arnold 2002; IUCN redlist.org/details/61530/0). In the Czech Republic, it is critically endangered species and it inhabits lowlands at several localities of the central and western Bohemia and southern Moravia (Mikátová et al. 2001).

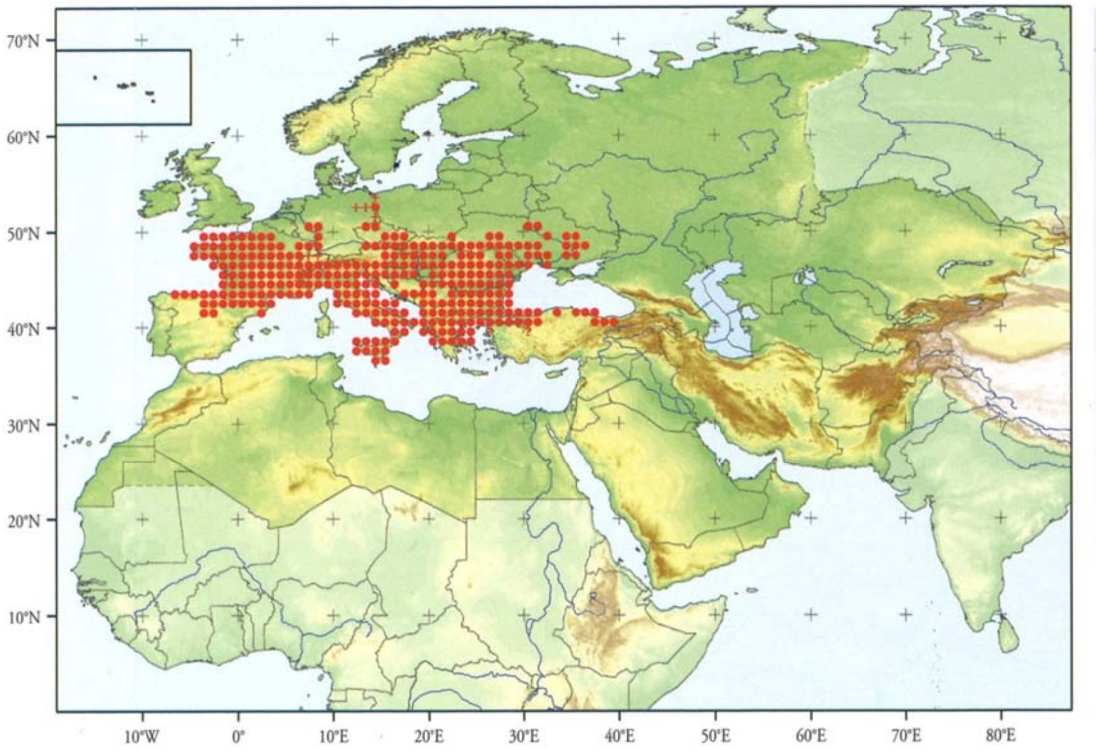

Fig. 1.9: Distribution of Green lizard (s.s.) (*Lacerta viridis*) (Sindaco and Jeremčenko 2008).

Green lizard is fairly large – up to about 13 cm from snout to vent (Arnold 2002) and the total length between 35 – 40 cm (Václav et al. 2007). This species has distinctive sexual dimorphism (Fig. 1.10), where males are usually almost green with fine black stippling above and a darker light-spotted head (Arnold 2002).

In breeding seasons males have blue-coloured throat. Females are very variable – from uniform green to brown or with blotches. The belly in both sexes is yellow. Younger animals are often beige, uniform with a few light spots, sometimes with narrow light lines (Arnold 2002).

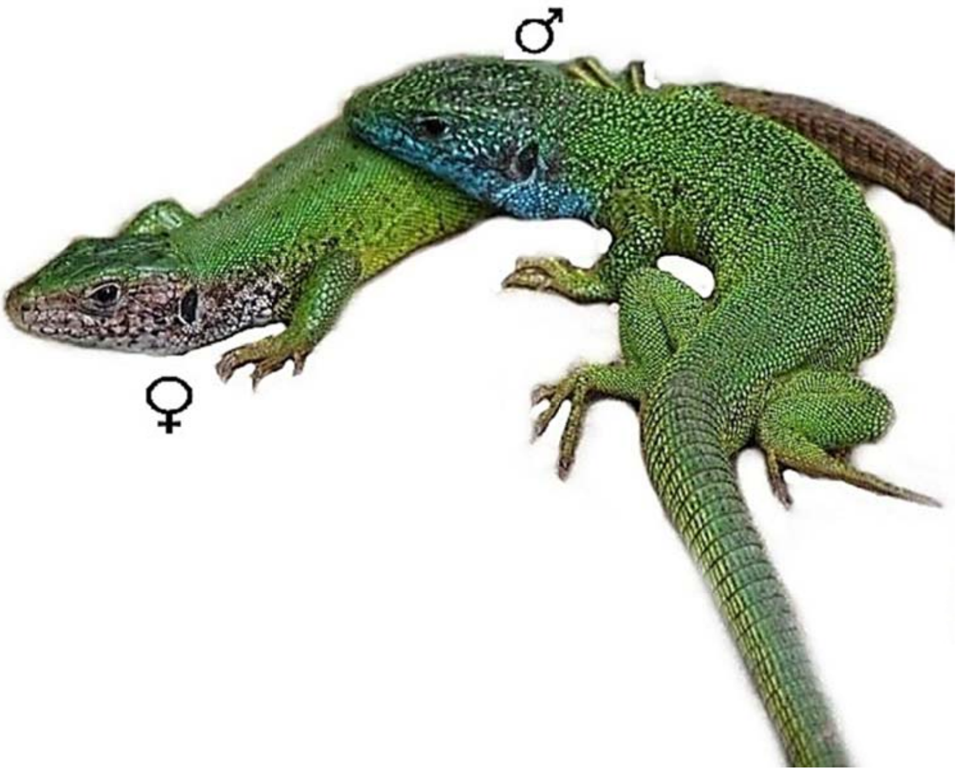

Fig. 1.10: Adult male and female of Green lizard (*Lacerta viridis*) (adjusted after www.aquapage.cz).

Green lizard prefers bushy forest-steppe habitats with good exposure to sun, which involves open woods, hedgerows and field edges or walls (Mikátová et al. 2001; Arnold 2002). The lizards form home ranges, which are occupied mostly by one pair of adult animals; nevertheless, some older and bigger males have in their home range up to three females (Mikátová et al. 2001).

Lizards feed mostly on invertebrates, such as spiders or orthopterans as well as small vertebrates and fruits (Arnold 1987; Arnold 2002). But they also consume Heteroptera, especially younger green lizards (Angelici et al. 1997). They can hunt even on vegetation (Arnold 2002).

Green lizards become sexually mature in the age of three and their breeding season begins after hibernation in April and lasts until June (Václav et al. 2007). Female usually lay from 6 to 23 eggs in one clutch. Younglings hatch after 7 – 15 weeks after laying the eggs (Arnold 2002). Green lizards hibernate from September to spring and they become active when the temperature rises to about 15 °C (Mikátová et al. 2001).

1.4.3. Great tit (*Parus major*; Linnaeus, 1758)

Great tit is a larger member of family Paridae. Its widespread distribution (Fig. 1.11) across the Palaearct (North Siberia, Mongolia) is well documented as well as its distribution in the south part of Asia (del Hoyo et al. 2007). In the Czech Republic it is common bird species, which lives from lowlands to mountains (Šťastný et al. 2006).

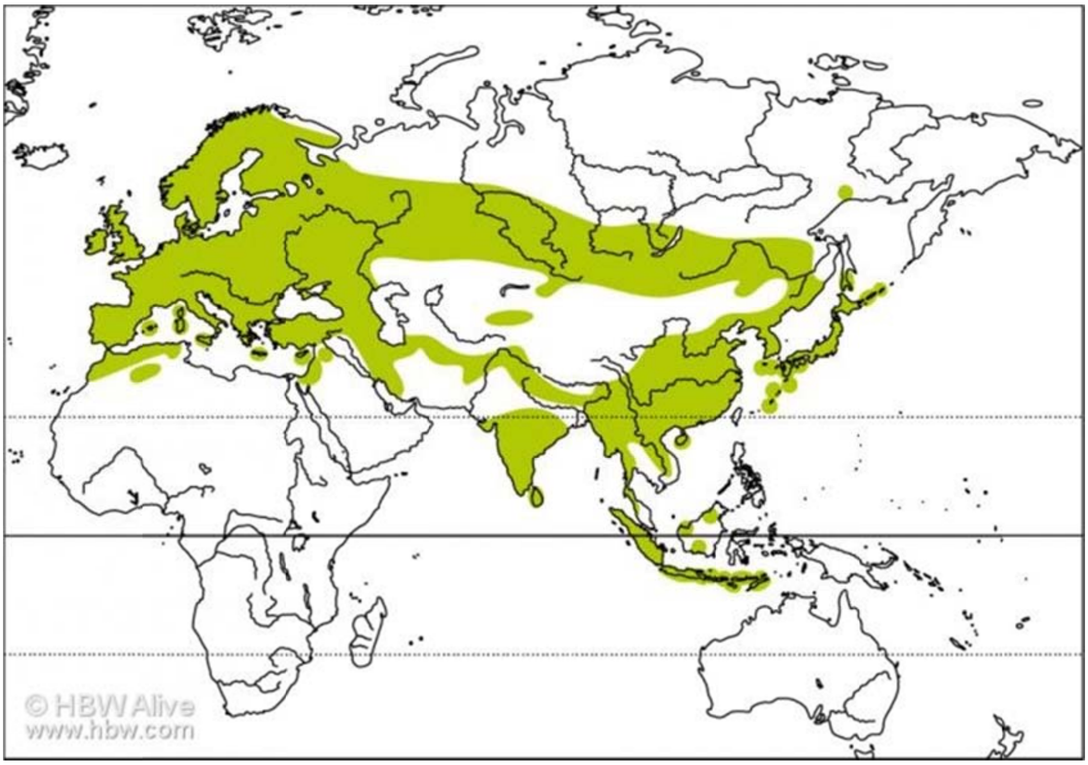

Fig. 1.11: Area of distribution of Great tit (*Parus major*) (from www.hbw.com).

It is a large, black-headed tit with large white face patch, pale or yellow underparts divided by black ventral line (del Hoyo et al. 2007; Svensson 2009). This line could be used as a clue in gender determination, where in males it is broad (Fig. 1.12) and in female (Fig. 1.13) it is disappearing in yellow underparts (del Hoyo et al. 2007; Svensson 2009).

Fig. 1.12: Adult male of Great tit (*Parus major*) (from www.hbw.com).

Fig. 1.13: Adult female of Great tit (*Parus major*) (from www.hbw.com).

The size of the bird is between 12.5 – 14 cm in the body length and 11.9 – 22 g of the body weight (Cramp and Perrins 1993; Harrap and Quinn 1996; del Hoyo et al. 2007).

Great tit occurs mostly in open deciduous and mixed forest and can be found also in urban and suburban areas (Cramp and Perrins 1993; del Hoyo et al. 2007). In Europe great tit prefers oaks (*Quercus*) (del Hoyo et al. 2007).

It feeds mostly on small invertebrates and larvae in summer. During other seasons, it consumes also seeds and various other items (del Hoyo et al. 2007). The composition of invertebrate part of the diet includes different species of insect and spiders (Cramp and Perrins 1993; del Hoyo et al. 2007). Great tit also feeds on Heteroptera mostly from family Miridae and Pentatomidae (Cramp and Perrins 1993; Exnerová et al. 2003a).

The breeding season starts from late March in Western Palaearct (del Hoyo et al. 2007). The clutch size is generally between 5 – 12 eggs. The eggs are incubated by female, which is fed on the nest by male. The incubation period is between 12 – 15 days and the chicks are fed by both parents during the next 16 – 22 days (del Hoyo et al. 2007). The breeding success varies and is dependent on wide range of factors such as the age of adults, rate of predation, starvation or changes in the temperature (del Hoyo et al. 2007).

1.4.4. Blue tit (*Cyanistes caeruleus*; Linnaeus, 1758)

Blue tit is a lesser member of family Paridae. The distribution range (Fig. 1.14) covers Western Palaearct (del Hoyo et al. 2007). Blue tit is a common small bird species in the Czech Republic and it inhabits lowland to mountain habitats (Šťastný et al. 2006).

Blue tit (Fig. 1.15) is a small billed compact tit. It has the size of 11 – 12 cm in body length and 7. 5 – 14.7 g of the body weight (Cramp and Perrins 1993; Harrap and Quinn 1996; del Hoyo et al. 2007). In this species, it is hard to identify well the sex of the bird. Nevertheless, the males have more intensely blue-coloured head and they also have well-marked neckband (Hromádko et al. 1993; Svensson 2009).

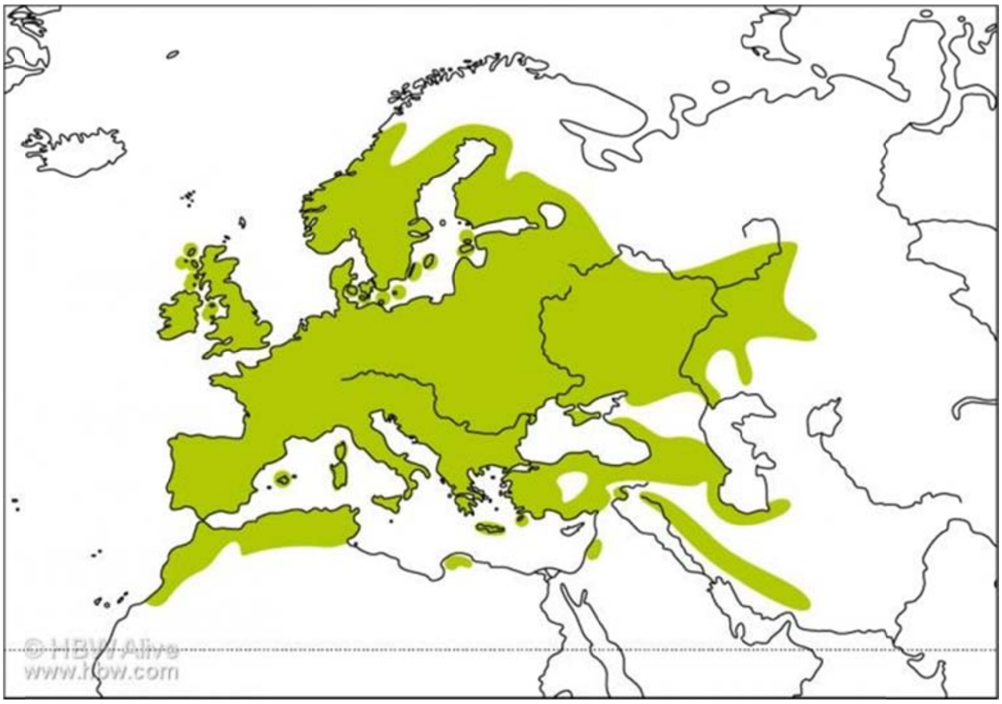

Fig. 1.14: Area of distribution of Blue tit (*Cyanistes caeruleus*) (from www.hbw.com).

Fig. 1.15: Adult Blue tit (*Cyanistes caeruleus*) (from www.hbw.com).

In Europe, blue tit inhabits mostly lowlands and submonate deciduous woodlands, principally containing oak (*Quercus*) and birch (*Betula*) (del Hoyo et al. 2007). It is also common in parks and gardens including suburban areas and city centres (Cramp and Perrins 1993; del Hoyo et al. 2007).

Food preferences include small invertebrates and also fruits and seeds (del Hoyo et al. 2007). Blue tit hunts mostly on insects, such as grasshoppers or moths (family Torticidae), including Heteroptera (del Hoyo et al. 2007). Fruit and seed are eaten mainly in non-breeding season (del Hoyo et al. 2007).

In blue tits there are usually two broods from April to June. The clutch size varies geographically, by altitude, and also with the size of nest-cavity and quality of the surrounding habitat (del Hoyo et al. 2007). The common size of clutch is between 7 – 13 eggs, which are incubated by the female for about 12 – 16 days. The chicks are fed by both parents for about 16 – 23 days (del Hoyo et al. 2007). The breeding success is dependent mostly on weather and predation.

1.4.5. Striated shieldbug (*Graphosoma lineatum*; Linnaeus, 1758)

G. lineatum (Heteroptera: Pentatomidae) is a common European oligophagous true bug species (Fig. 1.16), which is widely distributed through Western Palaearct (Aukema and Rieger 2006) with common distribution in the Czech Republic (Wagner 1965 ex Tullberg et al. 2008). It feeds mainly on the host-plants of family Apiaceae such as *Angelica silvestris, Daucus carota, Anthriscus silvestris* etc. (Stránský et al. 1998; Wachmann et al. 2008). The adults are fairly large bug species (Fig. 1.17) having the size of 10 – 12 mm. Adult colouration is red-and-black. The abdominal pattern is formed by six black symetrical stripes, ventral part is covered by black spots (Tietz and Zrzavý 1996).

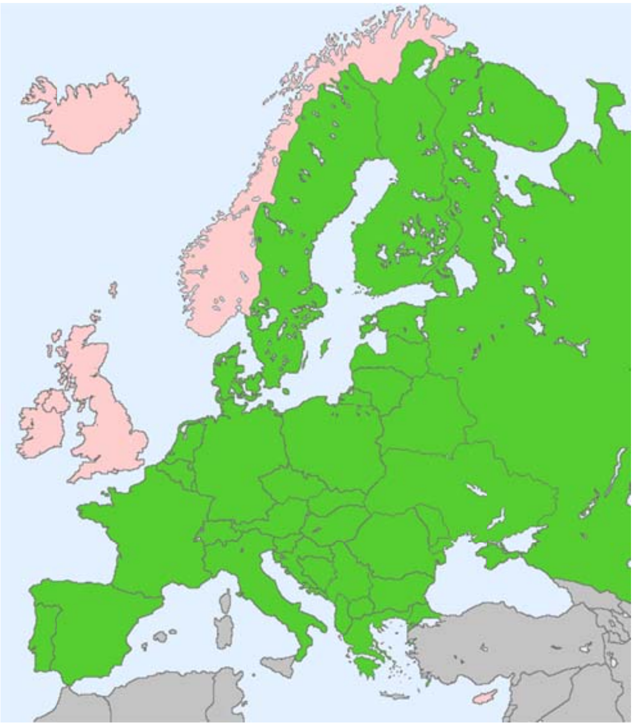

Fig. 1.16: Distribution of *Graphosoma lineatum* in Europe (from www.faunaeur.org). Green: present; pink: absent; grey: data not available.

Fig. 1.17: Adult specimen of *Graphosoma lineatum* (from www.alexhydephotography.com).

The life cycle (Fig. 1.18) of *G. lineatum* involves five larval instars with cryptic colouration and imago with aposematic colouration (Johansen et al. 2010). In the northern parts of its distribution two colour polymorphisms can be observed – at the end of the summer pale adults arise and therefore, they are cryptic for the bird predators especially on the dried vegetation. After hibernation in the spring they have typical red-and-black colouration (Johansen et al. 2010).

Although in Central Europe this phenomenon was also observed, the most common and widely distributed colouration is the typical aposematic red-and-black pattern (Wagner 1965 ex Tullberg et al. 2008).

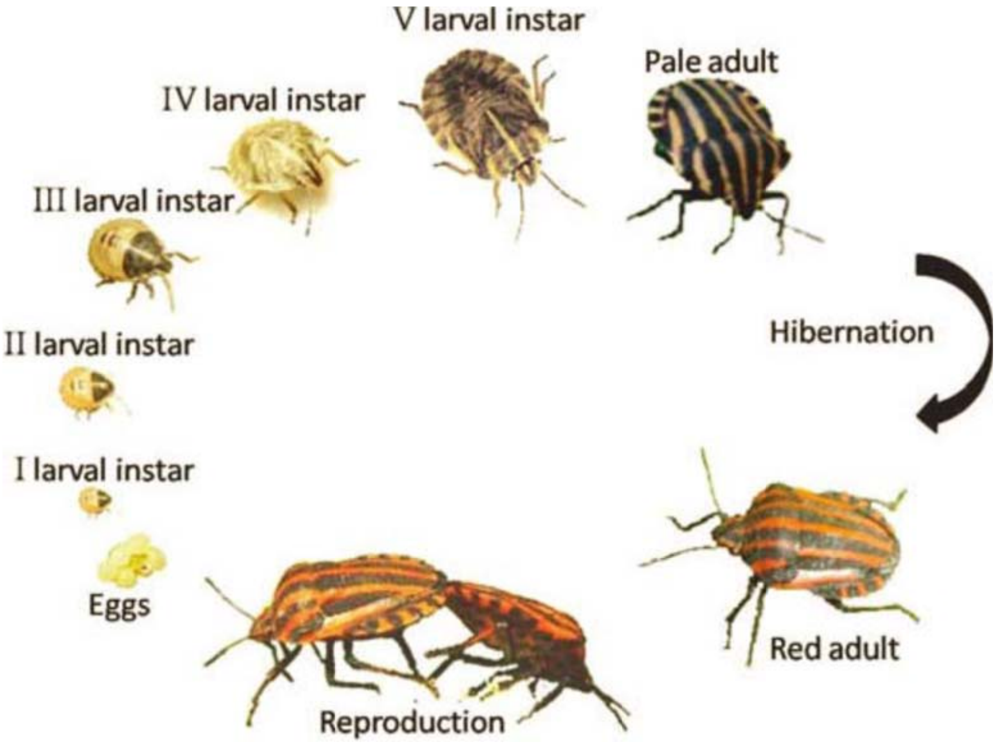

Fig. 1.18: Life cycle of *Graphosoma lineatum* with two colour form from Sweden (from Johansen et al. 2010).

G. lineatum produces and sequestrates lots of chemical compounds for deterring the predators, mostly birds such as great tit or blue tit (Veselý et al. 2006; Exnerová et al. 2008; Hotová Svádová et al. 2010). The question we were posing is how the defensive secretion works towards another type of heteropteran predator – green lizard (Castilla et al. 1991; Díaz and Carrascal 1993; Angelici et al. 1997) or potential predator – leopard gecko (Linnavuori 2008; Rastegar-Pouyani et al. 2008).

1.4.6. Firebug (*Pyrrhocoris apterus*; Linnaeus, 1758)

P. apterus is a common Palaearctic true bug species from family Pyrrhocoridae. Its distribution extends from the entire Europe (except for Norway and Finland) (Fig. 1.19) to Asia (East China) and North Africa – Algeria, Morocco and Tunis (Moulet 1995). In the Czech Republic it is very common true bug species (Socha 1993).

Adults (Fig. 1.20) have the size of 7 – 12 mm and they are more slender than the striated shieldbug, which is more robust. The life cycle involves five larval instars and imago (Socha 1993). *P. apterus* feeds mostly on the seeds of families Tiliaceae and Malvaceae (Socha 1993). During one season firebugs can have one or two generations (Košťál and Šimek 2000) and they winter in forest litter near to *Tilia cordata* and *Aesculus hippocastanum*.

Firebugs join together to form aggregations (Borden 1984 ex Farine et al. 1992), which could potent aposematic signal and therefore, could ensure better protection against predators (Mappes and Alatalo 1997). They have typical red-and-black colour pattern (Bohlin et al. 2012) and they are part of mimetic complex of Central European red-and-black Heteroptera (Hotová Svádová et al. 2010).

The chemical defence of *P. apterus* is composed mostly of short-chained aldehydes (Farine et al. 1992) as well as in case of striated shieldbug. The most common compounds are: (E)-2-hexenal, (E)-2-octenal and tridecane in adults (Farine at al. 1992). However, the present study is focused on the chemical defence of adult *G. lineatum* and firebug *P. apterus* was used only in one set of the experiments with green lizard in order to have the comparison to the striated shieldbug.

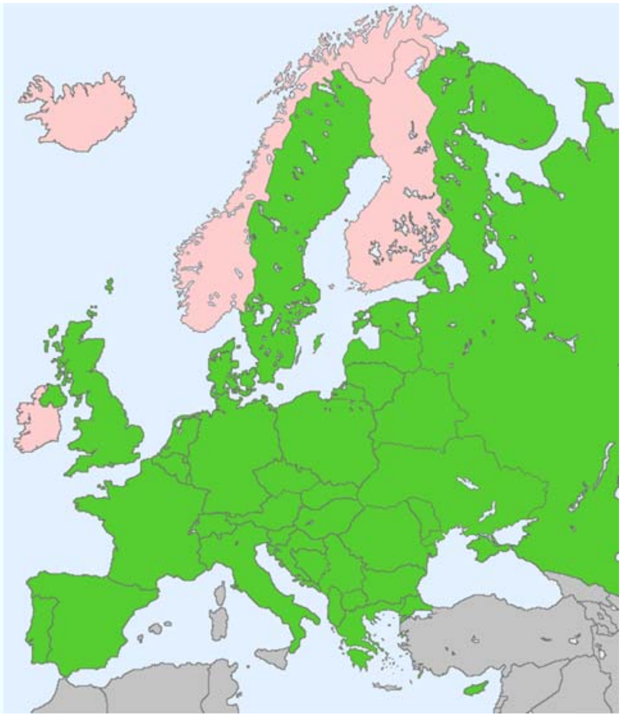

Fig. 1.19: Distribution of *Pyrrhocoris apterus* in Europe (from www.faunaeur.org). Green: present; pink: absent; grey: data not available.

Fig. 1.20: Adult specimen of *Pyrrhocoris apterus* (from www.flickr.com)**.**

2. AIMS OF THE THESIS

The aim of the present project was to reveal the effects of particular chemical compounds of MTG secretion of *G. lineatum* towards different types of potential predators and to test the hypothesis that the repellent potency of this secretion is dependent mostly on the aldehydes. The specific aims of the thesis were:

(1) To assess the aversive effect of particular chemical components of MTG secretion and the whole MTG secretion of *G. lineatum* towards four different types of predators – leopard geckos (*Eublepharis macularius*), green lizards (*Lacerta viridis*), great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*).

(2) To compare the aversive effect of selected chemical compounds and the whole MTG secretion of *G. lineatum* for all selected types of predators.

(3) To evaluate whether the mixture of three aldehydes could function as a potential signal of unpalatability for all tested predators.

(4) To verify the hypothesis that tridecane may function as catalyst for the mixture of three aldehydes thus potentiating aversive reactions of tested predators (leopard geckos, green lizards and great tits).

(5) To evaluate the hypothesis that oxoaldehyde has function as a direct toxin for all tested predators.

(6) To investigate how the presence of living specimen of *G. lineatum* influences aversive reactions of leopard geckos to the mixture of three aldehydes.

(7) To investigate how the presence of living specimen of *G. lineatum* and *P. apterus* influences aversive reactions of green lizards to the mixture of three aldehydes.

3. MATERIALS AND METHODS

3.1. Leopard geckos

In total 77 leopard geckos (*Eublepharis macularius*) were tested during the years 2010 – 2012. The experiments were carried out in the period after breeding season and before hibernation: in autumn from September to the first week in December.

Geckos were originally from the wild (Pakistan). They were captured as fully grown adults and they have been kept under the defined laboratory conditions for 10 years. All of them were adults of both sexes. They were kept in glass terraria of size 30x40x20 cm, temperature 27 °C, 50 % humidity, twelve hour period of light/dark cycle (6:00 am – 6:00 pm).

The terraria were supplied with a drinking dish, calcium dish and a box for laying eggs. Geckos were housed in the groups of three – one male and two females – and fed once a week with various type of prey (adult crickets, mealworms, locusts, cockroaches or pinky mice) fortified with vitamin powder for reptiles.

One week before the experiments, geckos were removed from their breeding groups and were housed individually in terraria of sizes 20x40x20 cm for allowing habituation to the laboratory environment. At this time, they were kept at temperature 27 °C, 50 % of humidity, without feeding but offering water ad libitum.

The light conditions were set according to the twelve hour light/dark cycle (6:00 am – 6:00 pm). Every gecko was weighed before the experiment. The sex check followed Seufer et al. (2005). Each gecko was then put back to the breeding group the day after the experiment.

3.2. Green lizards

In total 84 green lizards (*Lacerta viridis*) were tested during the years 2010 – 2012. Lizards were captured in Podyjí National Park (48° 48’ 59.20’’N – 15° 58’ 37.80’’E of Greenwich) in South Moravia after the breeding season and before hibernation: from July to early August.

Lizards were housed individually in glass terraria of size 20x40x20 cm, temperature 29 °C, 45 % humidity, twelve hour period of light/dark cycle (6:00 am – 6:00 pm). The terraria were supplied with a drinking dish and a small hiding place. Lizards were fed immediately after housing by adult crickets fortified with vitamin powder for reptiles but they were fed only once before the experiments.

Lizards were allowed to habituate to the laboratory environment for one week before the experiments with offering water ad libitum. Each lizard was weighed before the experiment. Two categories of age were recognized – adults and subadults. Three categories of sex were recorded – juvenile, female and male. Sex and age was checked according to Arnold (2002). Each lizard was released back to the wild (the exact location of the capture) the next week after the experiment.

3.3. Great tits and Blue tits

In total 196 great tits (*Parus major*) and 91 blue tits (*Cyanistes caeruleus*) were tested. Birds were captured using the mist nets in Prague from September 2009 to March 2011. Captured birds were housed individually in plastic cages of size 50x40x40 cm with a wire-mesh front wall. Cages were equipped with wooden perches, water bowls and feeders.

Birds were allowed to habituate to the laboratory conditions for 2 – 7 days before the experiments. The light conditions were set according to the outdoor photoperiod and the temperature was between 18 – 22 °C. The birds were provided with mealworms, sunflower seeds and water ad libitum.

Before the experiments, the birds were placed into the experimental cage and were allowed to habituate for half an hour to the new conditions and to learn to search for mealworms in the feeding tray. Then the birds were deprived of food for two hours before the start of the experiments. Sex and age determination followed Svensson (2009). Two age categories were recognized: yearlings and adults. Each bird was ringed and released at the locality of the capture the day after the experiment.

3.4. *Graphosoma lineatum*

Striated shieldbug (*Graphosoma lineatum,* Heteroptera: Pentatomidae) was selected as a primary model true bug species. Shieldbugs were picked up at several locations in Prague and kept in a thermostat-controlled environment at long-day photoperiod (16L:8D) and the temperature oscillating between 24 °C (day) and 20 °C (night).

They were supplied with tops, leaves and seeds of their host plants: carrot (*Daucus carota*), cow parsley (*Anthriscus sylvestris*) and garden angelica (*Angelica archangelica*) and water.

3.5. *Pyrrhocoris apterus*

The firebug (*Pyrrhocoris apterus*) was chosen as a second model organism only for the tests with green lizards as another living specimen of red-and-black aposematic Heteroptera (Hotová Svádová et al. 2010).

Firebugs were collected at several localities in Prague and kept in captivity under natural conditions similarly to the striated shieldbug. The firebugs were fed on host plants and seeds of Malvaceae, Tiliaceae, Bombacaceae and Sterculiaceae with supplement of water.

3.6. Larvae of *Tenebrio molitor*

Mealworms (larvae of *Tenebrio molitor,* length approx. 20 mm) were used for the experiments as a palatable prey. Tested chemicals were applied on the middle part of the dorsal side of the body of a mealworm.

The chemical compounds were applied on the surface of the mealworm to simulate the situation in the wild (Skelhorn and Rowe 2009) when *G. lineatum* ejects the secretion on the surface of its body. Adding chemicals on the surface of the middle part of the dorsal side of mealworms did not change their behaviour in any way. Untreated mealworms were used as a control prey.

3.7. Chemicals

Tested chemicals represent major components of adult MTG secretion of striated shieldbug *G. lineatum* (Stránský et al. 1998; Šanda et al. 2012). Following chemicals and mixtures were tested: (1) the mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal at a volume ratio 10:1:10; (2) the mixture of three aldehydes and tridecane (TA), ratio 10:1:10:10; (3) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (4) extracted MTG secretion of *G. lineatum* adults (GS); (5) hexane (HX) – it was used as a non-polar solvent for all the other chemicals and (6) pyrazine (PYR): 2-isobutyl-3-methoxypyrazine as a positive control to exclude the effect of neophobia in the experiments with leopard geckos.

Aldehydes, tridecane, pyrazine and hexane were purchased commercially (Sigma-Aldrich), mixed and stored in glass vials under argon in the freezer (at – 20 °C) before the experiment. Oxoaldehyde ((E)-4-oxohex-2-enal) was synthesised at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, and stored similarly to the other chemicals.

The mixtures of three aldehydes, tridecane and oxoaldehyde were used as their 2 % solution in hexane; pyrazine was dissolved in the small amount of glycerol and then diluted in distilled water to form its 0.003 % solution, which was sufficient to elicit potential aversive reactions in chicks (Marples and Roper, 1996). Therefore, this concentration was chosen for geckos as well due to their better nasal/vomeronasal sensitivity.

All chemicals were applied using a Hamilton syringe on the middle part of the dorsal side of the mealworms in the amount of 2 μl, an amount of secretion that is usually discharged by the striated shieldbug (M. Šanda, personal communication). Metathoracic scent-glands secretion (GS) was obtained by simulated attacks to the striated shieldbugs. When the shieldbug had released the secretion, it was applied directly on the dorsal side of the mealworm.

3.8. Experimental equipment

Leopard geckos

The experiments were carried out in terraria of size 20x40x20 cm (length x depth x height). Prey was offered by direct insertion to the terrarium. The experiments were performed during the active time period for geckos – during the night.

The behaviour of geckos was recorded with a SONY HDR-XR550VE video camera equipped with night vision mode, and simultaneously behavioural elements were recorded using Observer XT 8.0.

Green lizards

The experiments were performed in terraria of size 20x40x20 cm. Prey was offered by direct insertion to the terrarium. The experiments were carried out during the active time period for lizards – during the day.

The behaviour of lizards was recorded with a SONY HDR-XR550VE video camera, and simultaneously behavioural elements were captured using Observer XT 8.0.

Great tits and Blue tits

The experiments were carried out in wooden cages (70x70x70 cm) with wire-mesh walls and ceiling and the one-way mirror front wall through which the birds were observed. The cages were equipped with perch, water bowl, and a circular feeding tray with cups (6 cm in diameter), in which the tested prey was offered in transparent glass Petri dishes. Each cage was illuminated with two daylight simulating bulbs (Biolux Combi 18 W, Osram). The behaviour of birds was recorded with several types of CANON video cameras, and simultaneously behavioural elements were recorded using Observer XT 8.0.

3.9. Testing procedure

The same basic testing sequence was used for all types of the predators with the exception of blue tits, where the testing sequence was shortened (see below). Each animal was tested only once.

The testing sequence consisted of ten mealworms presented successively in five-minute trials. For the experimental groups tested with the chemicals (3A-TA-OXO-GS-PYR), the sequences started with a hexane-treated mealworm followed by five mealworms treated with the particular chemical corresponding to the experimental group, and ended with a sequence of four hexane-treated mealworms. Animals from the control group (UM) were offered ten untreated mealworms; animals from the hexane group (HX) were offered ten hexane-treated mealworms. This way, it was possible to compare the reactions of animals towards the tested chemicals with potentially repellent function with their reactions to hexane and to untreated mealworms.

The behaviour in different parts of the experimental sequence was also compared: (1) “pre-chemical“ trials in the beginning, (2) “chemical“ trials with tested chemicals, and (3) “post-chemical“ trials following the experience with chemicals to differentiate between immediate and persisting effect of the tested chemicals. In each trial, the animal was allowed for five minutes to attack and potentially consume the mealworm, otherwise the trial was stopped. The trial was stopped earlier, if the animal consumed the prey.

For lizard’s predator species following behavioural characteristics were recorded: (1) Approach latencies – representing the time when the animal started to come purposefully towards the prey; (2) Attack latencies – representing the time when the animal started to handle the prey (after approaching it); and (3) Approach-attack intervals – representing the degree of hesitation between approaching the prey and attacking the prey. The whole time interval is evaluated during which the tested chemical could influence the predator’s behaviour.

For bird's predators following behavioural characteristics were recorded: (1) Attack latencies – representing the time when the bird started to handle the prey (touching, pecking or seizing); and (2) Attack-eating intervals – representing the interval between the first attack and the moment the bird started eating the prey.

Approach latencies were recorded only for lizard's predators since it was possible to evaluate the purposeful approach towards the prey. In case of bird's predators the attack latencies were sufficient since they immediately followed the approach behavior, i.e. both latencies followed the same pattern. Moreover, the attack latencies were more convincing.

Specific details of the testing procedures for each type of predator follow.

Leopard geckos

The leopard geckos were split into eight experimental groups, which were balanced as for the sex of the geckos. In each group, 3 males were present. Geckos were tested with following chemicals: a mixture of aldehydes (3A), the same mixture of aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), hexane (HX), untreated mealworm (UM), Living *Graphosoma* (LG/3A) followed by chemical 3A and pyrazine (PYR).

For the testing of gecko's reactions to the living specimen of *G. lineatum* (LG/3A), the alternation of the untreated mealworm and the bug was used until the gecko rejected the bug three times without any handling (manipulation by touching and/or taking it into the mouth). The bug was offered maximally five times. Three bugs were offered in case the gecko did not manipulate any offered bug. If the gecko manipulated a bug only once, it was offered four bugs. Five bugs were offered only in case the gecko manipulated bug twice (successively). The alternation of the striated shieldbug with mealworms was used to reinforce the geckos towards aposematic prey. This sequence was followed by the standard sequence of ten mealworms treated by the mixture of three aldehydes.

Green lizards

The lizards were split into eight experimental groups, which were equalized as for the sex and age of the green lizards. Lizards were tested with following chemicals: 3A, TA, OXO, GS, HX, UM, Living *Graphosoma* (LG/3A) followed by chemical 3A and Living *Pyrrhocoris* (LP/3A) followed by chemical 3A.

For the testing of lizard's reactions to the living specimen of *G. lineatum* (LG/3A) and *P. apterus* (LP/3A) the same procedure as in case of leopard geckos was followed for both bug species.

Great tits

The great tits were divided into six experimental groups, which were balanced as for the age and sex of the birds. Great tits were tested with following chemicals: 3A, TA, OXO, GS, HX and UM.

Blue tits

The blue tits were divided into five groups, which were balanced as for the age and sex of the birds. Blue tits were tested with following chemicals: 3A, OXO, GS, HX and UM.

Due to the smaller size and faster satiation of the blue tits, the testing sequence had to be shortened to six trials. For the experimental groups tested with chemicals (3A-OXO-GS), the sequence started with a hexane-treated mealworm followed by three mealworms treated with the particular chemical corresponding to the experimental group, and ending with two hexane-treated mealworms.

Similarly to the great tits, birds from the control group (UM) were offered only untreated mealworms; birds from the hexane group (HX) were offered only hexane-treated mealworms.

3.10. Statistical analyses

The data were analysed in the statistical program R 3.0.1. The data were first judged using standard summary statistics (extremes, quartiles, median, mean, standard deviation). The Shapiro-Wilk normality test showed that the data were highly non-normally distributed, which is mainly caused by zero response of some of the animals. Such type of non-normality can be hardly resolved using any transformation type. Therefore, it was decided to apply the robust methods of analyses based on ranks.

The main aim was to compare the particular chemicals, when controlling for age sex and weight. Analysis of covariance (ANCOVA) was used to estimate the underlying model and to evaluate the impact of the chemicals. One of the assumptions of classical ANCOVA is normal distribution of the data. Since this assumption was violated, the original method had to be adjusted being inspired by Kruskal-Wallis anova. The ranks of recorded data (latencies of chosen behavioural elements) were used as the dependent variable instead of the real time values, and it was evaluated how these ranks depend on the other covariates: chemicals, part of the experimental sequence (pre-chemical trials, chemical trials and post-chemical trials), age, sex and weight (age and weight enter the model as numerical variables, the other covariates as categorical variables). An interaction between the time period and the chemical was also assumed.

Type II ANOVA table was used to evaluate the impact of the particular covariates. This type of ANOVA table is evaluating the impact of each covariate controlling for the other covariates (their main effect), but not for interactions. Since all types of interactions were not anticipated in the model, this type of ANOVA table is the most plausible for the situation. The optimal (final) model was determined by backward stepwise selection and Akaike's information criterion (AIC) was used for the selection.

The differences among chemicals within each of the three experimental sequences were assessed by Multiple Comparison of Means (Tukey Contrasts) when controlling for the other covariates with significant impact on the dependent variable. This means that for the evaluation of the differences, the optimal model was used. A new "interaction variable" (chemical vs part of the experimental sequence) was used for this purpose. In all tests, significance was assumed at $\alpha = 0.05$ significance level.

The aversive effects of the particular chemical on the recorded behavioural characteristics (approach latency, attack latency, approach-attack and/or attack-eating interval) were estimated with a coefficient of the rank-based regression model (Estimate) – the higher its value the slower reaction of the animal and thus stronger aversion towards the particular chemical.

Note, that the figures reflect the original recorded values (i.e. observed time of reactions), whereas the numerical results come from the ranks of these times.

3.11. Ethical note

The experiments were carried out under the permission no. 24773/2008-10001 and CZ 00059 issued by the Central Commission for Animal Welfare of the Czech Republic (UKOZ). Green lizards were catched under the permission obtained from Podyjí National Park in headquarter Znojmo (SZ NPP 0108/2010/8, NPP 0967/2010).

Bird capturing and experiments were carried out under the permissions 29532/2006-30, CZU150/99 and CZ 00059 issued by Central Commission for Animal Welfare of the Czech Republic (UKOZ), and MHMP-154521/04/OOP-V-25/R-

40/09/Pra issued by Prague City Hall. Catching and ringing birds were performed under the licenses from Czech Ringing Centre in Prague (Nos 876, 1110).

4. RESULTS

Individual chemical compounds selected for the experiments were chosen based on their common occurrence in the repellent secretion of true bugs with the specific focus on the composition of *G. lineatum* secretion (Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012). (E)-2-hexenal, (E)-2-decenal and (E)-2-octenal were tested together as a mixture, which is commonly found in the repellent secretion of true bugs (Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996; Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012). This aldehyde mixture could function as a potential olfactory signal – typical noxious smell of the striated shieldbug (L. Streinz, personal communication). The aldehyde mixture enriched with tridecane was tested to evaluate the hypothesis that tridecane serves as catalyst for the aldehydes (Gunawardena and Herath 1991). In contrast, oxoaldehyde was included among tested chemical compounds because it could function as a direct toxin (Aldrich 1988). Finally, 2-isobutyl-3-methoxypyrazine, which is not included in the *G. lineatum* secretion, was used to exclude the effect of neophobia of leopard geckos towards highly odorous compounds.

The whole MTG secretion of *G. lineatum* was included in the experiment because it may function as a signal of unpalatability as well as a secondary chemical defence. The presence of living specimens of *G. lineatum* or *P. apterus* before the trials with mealworms was used to test the hypothesis that the previous experience with the aposematic red-and-black bugs may increase the repellent potency of the mixture of three aldehydes (Gregorovičová and Černíková 2015a).

4.1. Reactions of Leopard geckos

First predator chosen for the present study is leopard gecko (*Eublepharis macularius*). Since it is dual olfactory specialist (Halpern 1987; Schwenk 1993; Dial and Schwenk 1996), it is an ideal model organism for testing the chemical defence of aposematic Heteroptera, which is mainly composed of volatiles (Šanda et al. 2012). Such chemical defence could be aimed at this type of predator (Gregorovičová and Černíková 2015b). Following chemicals and mixtures were tested: (1) the mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) the mixture of three aldehydes and tridecane (TA); (3) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (4) extracted MTG secretion of *G. lineatum* adults (GS) and (5) hexane (HX) as a non-polar solvent for the other chemicals; (6) pyrazine (PYR): 2-isobutyl-3-methoxypyrazine, was used as a positive control to exclude the effect of neophobia towards new malodours.

It was also tested how the presence of living specimen of *G. lineatum* influences repellent potency of chosen particular chemical – the mixture of three aldehydes (LG/3A).

The following behavioural characteristics were evaluated – approach latencies, attack latencies and approach-attack intervals.

For all behavioural characteristics and in all parts of the experimental sequence (pre-chemical trials, chemical trials and post-chemical trials), the reactions of leopard geckos from the hexane (HX) and pyrazine (PYR) groups did not significantly differ from untreated mealworm (UM) control group. Corresponding p values are in Table 4.1. These results proved that the effect of neophobia could be excluded as well as the effect of hexane as a non-polar solvent for the other chemicals of MTG secretion of *G. lineatum*. Therefore, the reactions of leopard geckos in the other groups (3A-TA-OXO-GS-LG/3A) were compared with those of the hexane group.

For all behavioural characteristics, the reactions of leopard geckos for all tested groups (3A-TA-OXO-GS-LG/3A) in the first control (pre-chemical) trial did not significantly differ compared to the hexane group (Table 4.2A-C). Therefore, all geckos started the experiment with the same motivation.

Following sections describe detailed results for individual behavioural characteristics and for all tested groups (3A-TA-OXO-GS-LG/3A). The corresponding results are summarized in Tables 4.2 and 4.3.

Finally, Table 4.4 summarizes the impact of particular covariates (ANOVA type II) on individual behavioural characteristics.

4.1.1. Approach latencies

Approach latencies were affected by tested chemicals ($p < 0.001$; $F = 13.539$; df1 = 7; df2 = 734), sex of the leopard geckos ($p < 0.01$; $F = 7.371$; df1 = 1; df2 = 734) and their weight ($p < 0.001$; $F = 37.064$; df1 = 1; df2 = 734). Heavier animals usually hesitated longer than lighter animals before approaching the mealworms. Females mostly hesitated longer than males before approaching the mealworms. There was also a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.05$; $F = 1.971$; df1 = 14; df2 = 734). Statistical values are summarized in Table 4.4A.

In chemical trials, leopard geckos tested with *Graphosoma* secretion hesitated significantly longer before approaching the chemical-treated mealworms compared to the geckos from the hexane group (Tukey Contrasts: $p < 0.001$). However, approach latencies of leopard geckos tested with the rest of the chemicals did not significantly differ from geckos' reactions in the hexane group (Table 4.2A, Fig. 4.1).

Although the rest of the chemicals did not cause significant difference compared to the hexane group, an influence on the approach latencies in chemical trials could be observed. The aversive effects of the particular chemical were estimated with a coefficient of the rank-based regression model – the higher its value the slower reaction of the animal and thus stronger aversion towards the particular chemical. When the effects of tested chemicals on the approach latencies were compared, following sequence was obtained (Table 4.3A): *Graphosoma* secretion, the mixture of three aldehydes, Living *Graphosoma*/mixture of aldehydes, the mixture of three aldehydes and tridecane, oxoaldehyde, hexane and untreated mealworm. The approach latencies in the untreated mealworm group were thus the shortest.

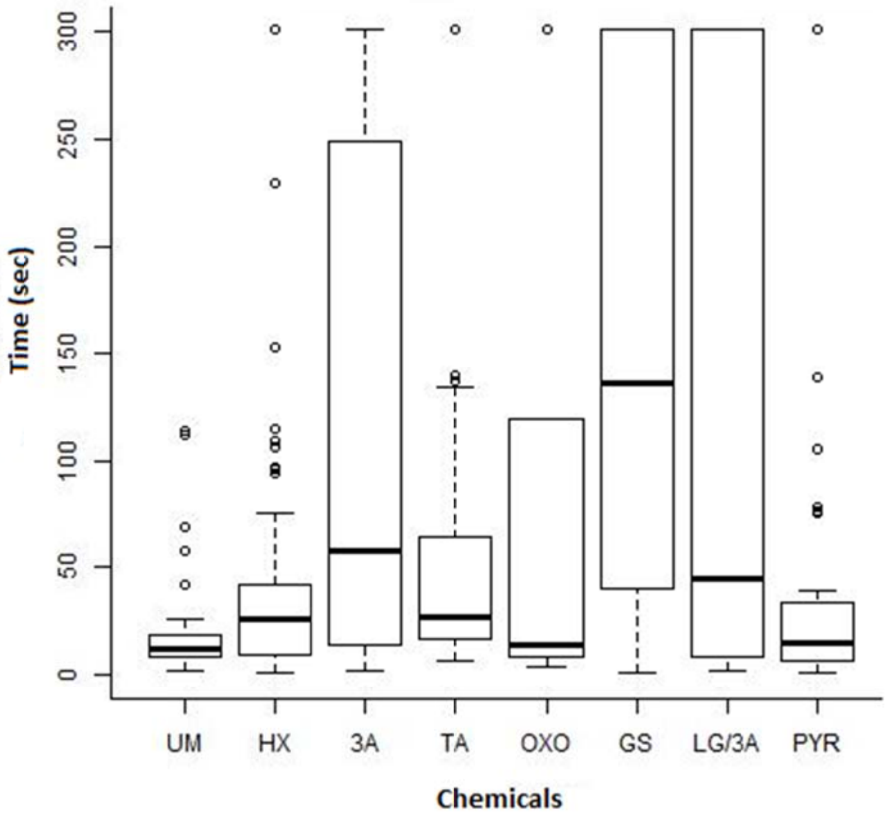

Fig. 4.1: Approach latencies in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), pyrazine (PYR).

Approach latencies are presented on y-axis. The figures reflect the original recorded values (the time when the gecko started to come purposefully towards the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

In trials following the experience with the chemicals (post-chemical trials), leopard geckos that had previous experience with the mixture of aldehydes, with this mixture and tridecane and with *Graphosoma* secretion hesitated significantly longer than geckos from the hexane group before approaching the mealworms, even when they were no longer treated with the chemicals (Tukey Contrasts: $p < 0.01$; $p < 0.05$; $p < 0.05$ respectively, Table 4.2A, Fig. 4.2). Approach latencies of the group previously treated with oxoaldehyde ($p = 0.824$) and Living *Graphosoma*/mixture of aldehydes ($p = 1.000$)

did not significantly differ from the hexane group. All statistical values are in Table 4.2A.

In post-chemical trials, the approach latencies could be again ordered based on the effect of the tested chemicals. The approach latencies were the longest in the group previously treated with the mixture of three aldehydes (Table 4.3A). The effect of other chemicals on approach latencies was following: the mixture of aldehydes and tridecane, *Graphosoma* secretion, oxoaldehyde, untreated mealworm, Living *Graphosoma* /mixture of aldehydes and hexane. The approach latencies in the hexane group were the shortest.

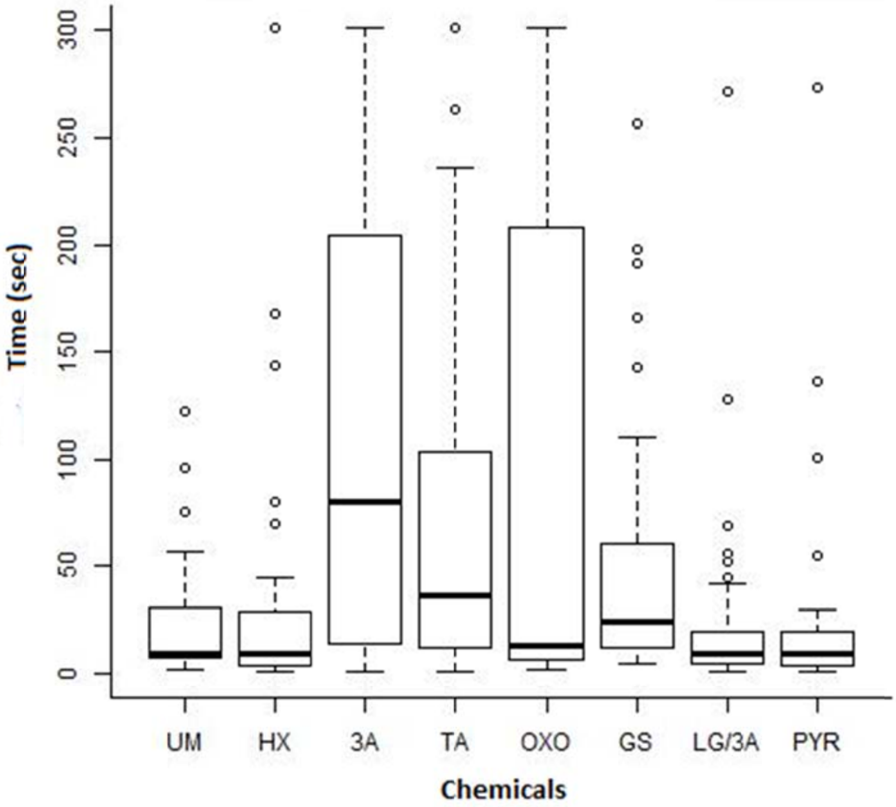

Fig. 4.2: Approach latencies in trials following the experience with chemicals – post-chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), pyrazine (PYR).

Approach latencies are presented on y-axis. The figures reflect the original recorded values (the time when the gecko started to come purposefully towards the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.1.2. Attack latencies

Attack latencies were affected by the tested chemicals ($p < 0.001$; $F = 14.384$; df1 = 7; df2 = 734) and the weight of leopard geckos ($p < 0.001$; $F = 18.041$; df1 = 1; df2 = 734), but not by their sex ($p = 0.903$; $F = 0.015$; df1 = 1; df2 = 734). Heavier animals usually hesitated longer than lighter animals before attacking the mealworms. There was also a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.001$; $F = 3.381$; df1 = 14; df2 = 734). Statistical values are summarized in Table 4.4B.

In chemical trials, leopard geckos tested with *Graphosoma* secretion and Living *Graphosoma*/mixture of aldehydes hesitated significantly longer before attacking the chemical-treated mealworms compared to the geckos from the hexane group (Tukey Contrasts: both $p < 0.001$). Attack latencies were also significantly longer in the group treated with the mixture of aldehydes and tridecane ($p < 0.01$) and the mixture of three aldehydes ($p < 0.05$). Attack latencies of leopard geckos tested with oxoaldehyde did not significantly differ from geckos' reactions in the hexane group ($p = 1.000$). For details refer Table 4.2B and Fig. 4.3.

The attack latencies in chemical trials were the longest in the group treated with *Graphosoma* secretion. The effect (Table 4.3B) of other chemicals on attack latencies was following: Living *Graphosoma*/mixture of aldehydes, the mixture of aldehydes and tridecane, the mixture of three aldehydes, oxoaldehyde, hexane and untreated mealworm. The attack latencies in the untreated mealworm group were the shortest.

In trials following the experience with the chemicals (post-chemical trials), the attack latencies of leopard geckos did not significantly differ among the groups of tested animals (Tukey Contrasts) – see Table 4.2B.

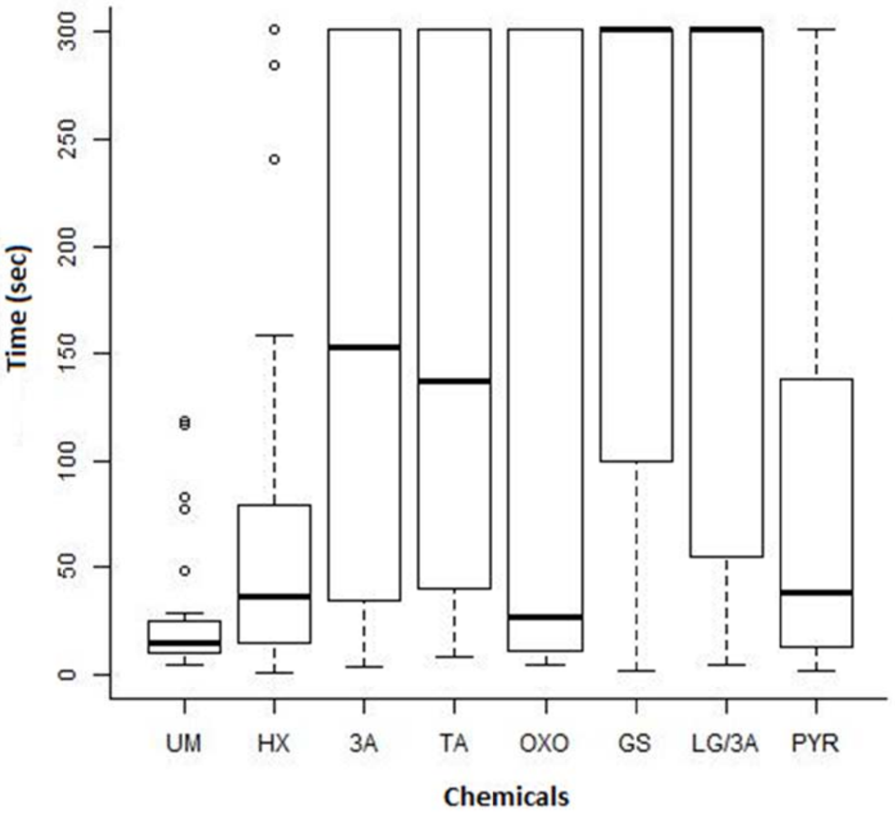

Fig. 4.3: Attack latencies in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), pyrazine (PYR).

Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the gecko started to handle the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.1.3. Approach-attack intervals

Approach-attack intervals were affected by the tested chemicals ($p < 0.001$; F = 12.768; df1 = 7; df2 = 734) and the weight of leopard geckos ($p < 0.001$; F = 10.925; df1 = 1; df2 = 734), but not by their sex (p = 0.348; F = 0.883; df1 = 1; df2 = 734). Heavier animals were slower when evaluating approach-attack intervals. There was also a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.001$; F = 3.563; df1 = 14; df2 = 734). Statistical values are summarized in Table 4.4C.

In chemical trials, when evaluating the approach-attack intervals, leopard geckos tested with *Graphosoma* secretion and Living *Graphosoma*/mixture of aldehydes hesitated significantly longer compared to the geckos from the hexane group (Tukey Contrasts: both $p < 0.001$). Approach-attack intervals were also significantly longer in the group treated with the mixture of three aldehydes and the same mixture and tridecane (both $p < 0.001$). Approach-attack intervals of leopard geckos tested with oxoaldehyde did not significantly differ from geckos' reactions in the hexane group (p = 0.998). For details refer Table 4.2C and Fig. 4.4.

The approach-attack intervals in chemical trials were the longest in the group treated with Living *Graphosoma*/mixture of aldehydes. The effect of other chemicals on approach-attack intervals was following: *Graphosoma* secretion, the mixture of aldehydes and tridecane, the mixture of three aldehydes, oxoaldehyde, hexane and untreated mealworm. The approach-attack intervals in the untreated mealworm group were the shortest (Table 4.3C).

In trials following the experience with the chemicals (post-chemical trials), the approach-attack intervals did not significantly differ among the groups of tested geckos (Tukey Contrasts) – see Table 4.2C.

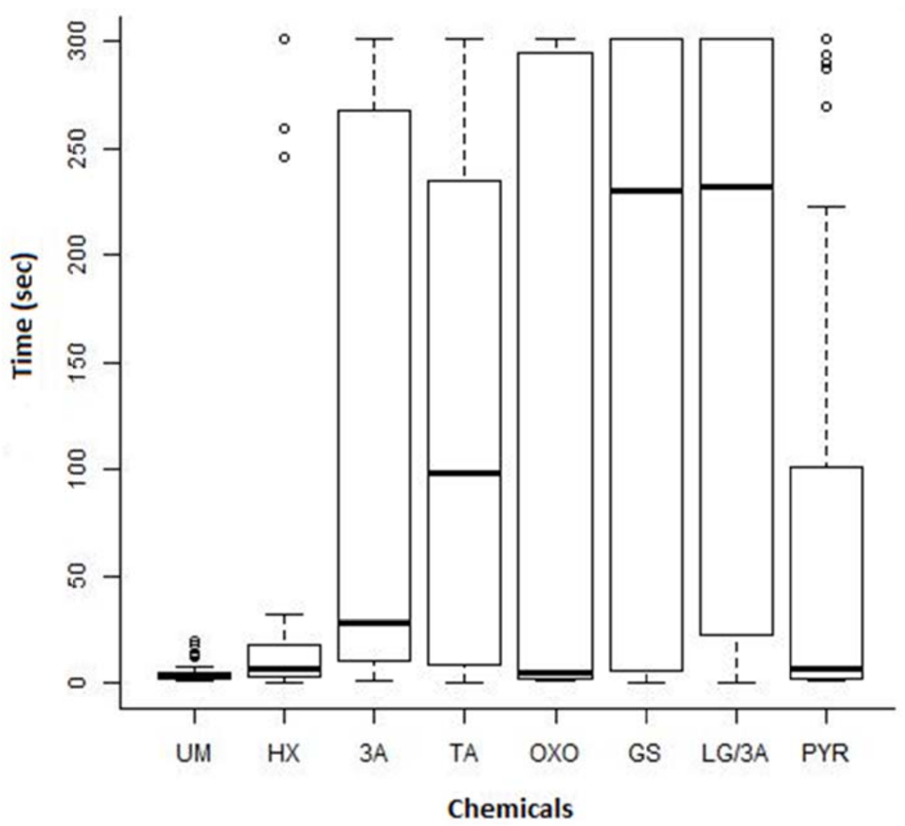

Fig. 4.4: Approach-attack intervals in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), pyrazine (PYR).

Approach-attack intervals are presented on y-axis. The figures reflect the original recorded values (the degree of hesitation between approaching the prey and attacking the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.1.4. Manipulation with *Graphosoma lineatum*

During the testing of gecko's reactions to the living specimen of *G. lineatum* following characteristics in the bug handling were observed. Out of 10 tested animals, 6 geckos manipulated the bug twice (out of a maximum of 5 offered bugs), 2 geckos only once and remaining 2 geckos did not manipulate any of three offered bugs. It means that leopard geckos manipulated the bug maximally twice. As a result of the manipulation, only two bug specimens were killed, the remaining bugs were released unharmed. The results indicated that 5 offered bugs was sufficient number to gain the experience to avoid the bugs.

Table 4.1: The reactions of leopard geckos towards mealworms treated with hexane (HX) and mealworms treated with pyrazine (PYR) compared with the reactions of leopard geckos towards untreated mealworms (UM)
All behavioural characteristics were evaluated. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with untreated mealworm).

Control	HX			PYR		
	p value	Est.	SE	p value	Est.	SE
A – Approach latencies						
Pre-chemical trial	1.000	52.55	99.40	1.000	77.71	99.25
Chemical trials	1.000	– 47.99	44.74	1.000	– 51.35	44.41
Post-chemical trials	1.000	50.36	49.94	1.000	49.11	49.65
B – Attack latencies						
Pre-chemical trial	1.000	– 5.52	97.32	1.000	41.52	97.22
Chemical trials	0.948	– 85.47	43.73	0.199	– 136.01	43.49
Post-chemical trials	1.000	– 2.96	48.84	1.000	16.47	48.62
C – Approach-attack intervals						
Pre-chemical trial	1.000	– 49.15	97.78	1.000	– 23.78	97.67
Chemical trials	0.957	– 84.53	43.93	0.135	– 143.00	43.69
Post-chemical trials	1.000	– 56.50	49.06	1.000	– 12.69	48.85

Table 4.2: The reactions of leopard geckos in the tested groups (3A-TA-OXO-GS-LG/3A) compared to the hexane group (HX)
All behavioural characteristics were evaluated. Abbreviations: 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion; LG/3A – living specimen of *G. lineatum* followed by the mixture of three aldehydes. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with hexane).

Chemicals	**3A**			**TA**			**OXO**			**GS**			**LG/3A**		
	p value	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**
A – Approach latencies															
Pre-chemical trials	0.660	– 229.15	92.53	1.000	39.23	90.10	1.000	– 57.37	90.06	1.000	75.21	90.23	1.000	– 88.28	90.09
Chemical trials	0.302	– 121.88	41.38	0.798	92.17	40.35	1.000	28.01	40.28	**< 0.001**	230.48	40.66	0.754	– 94.83	40.36
Post-chemical trials	**< 0.01**	– 193.94	46.27	**< 0.05**	177.28	45.10	0.824	100.88	45.03	**< 0.05**	171.65	45.38	1.000	– 2.23	45.10
B – Attack latencies															
Pre-chemical trials	0.836	– 201.37	90.64	1.000	– 16.50	88.24	1.000	– 119.08	88.22	1.000	0.56	88.34	1.000	64.32	88.24
Chemical trials	**< 0.05**	– 153.42	40.54	**< 0.01**	171.58	39.50	1.000	45.98	39.46	**< 0.001**	238.91	39.72	**< 0.001**	– 219.55	39.51
Post-chemical trials	0.064	– 159.90	45.32	0.228	35.39	44.16	0.992	73.17	44.11	0.973	81.51	44.35	1.000	– 14.30	44.16
C – Approach-attack intervals															
Pre-chemical trials	1.000	– 102.63	91.06	1.000	– 74.49	88.65	0.951	– 172.47	88.63	1.000	– 23.12	88.75	1.000	– 88.42	88.65
Chemical trials	**< 0.01**	– 172.28	40.73	**< 0.01**	173.47	39.69	0.998	59.93	39.65	**< 0.001**	208.30	39.90	**< 0.001**	– 240.25	39.69
Post-chemical trials	0.526	– 120.30	45.53	0.973	81.68	44.36	1.000	23.70	44.32	1.000	– 53.68	44.55	1.000	– 26.29	44.36

Table 4.3: The aversive effect of the tested chemical compounds on the individual behavioural characteristics of leopard geckos

Abbreviations: UM – untreated mealworm; HX – hexane; PYR – pyrazine; 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion; LG/3A – living specimen of *G. lineatum* followed by the mixture of three aldehydes. Estimate: effect on behavioural characteristics estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

Chemicals	UM	HX	PYR	3A	TA	OXO	GS	LG/3A
	Estimate (regression coefficient)							
A – Approach latencies								
Chemical trials	– 263.7	– 215.8	– 212.4	– 93.9	– 123.6	– 187.7	14.7	– 120.9
Post-chemical trials	– 260.0	– 310.4	– 309.2	– 116.5	– 133.1	– 209.5	– 138.8	– 308.2
B – Attack latencies								
Chemical trials	– 301.9	– 216.8	– 165.8	– 63.4	– 45.1	– 170.8	22.5	2.9
C – Approach-attack intervals								
Chemical trials	– 219.0	– 131.8	– 77.0	40.3	40.4	– 72.2	73.7	107.2

Table 4.4: The impact of particular covariates on individual behavioural characteristics of leopard geckos evaluated by using Type II ANOVA table

Covariate	p value	F value	df1	df2
A – Approach latencies				
Chemical	< 0.001	13.539	7	734
Weight	< 0.001	37.064	1	734
Sex	< 0.01	7.371	1	734
Chemical: part	< 0.05	1.971	14	734
B – Attack latencies				
Chemical	< 0.001	14.384	7	734
Weight	< 0.001	18.041	1	734
Sex	0.903	0.015	1	734
Chemical: part	< 0.001	3.381	14	734
C – Approach-attack intervals				
Chemical	< 0.001	12.768	7	734
Weight	< 0.001	10.925	1	734
Sex	0.348	0.883	1	734
Chemical: part	< 0.001	3.563	14	734

4.1.5. Summary

(1) Hexane did not have aversive effect on leopard geckos. Therefore, hexane could be used as a non-polar solvent for the other chemical compounds.
(2) Pyrazine did not have aversive effect on leopard geckos as well as hexane. Therefore, the effect of neophobia could be excluded.
(3) The mixture of three aldehydes had an aversive effect and it could also play a role as a signal of unpalatability.
(4) The mixture of three aldehydes and tridecane had even more pronounced aversive effect than the mixture of three aldehydes. Tridecane probably increases the impact of the mixture of aldehydes to leopard geckos.
(5) Oxoladehyde alone did not have any aversive effect for leopard geckos.
(6) Whole MTG secretion had a strong aversive effect for leopard geckos.
(7) Presence of living specimen of *G. lineatum* increased the effect of the mixture of three aldehydes as a signal of unpalatability.

4.2. Reactions of Green lizards

Green lizard was chosen as a second type of lizard predator in order to compare the results with leopard geckos. Moreover, members of family Lacertidae are known as predators of Heteroptera (Castilla et al. 1991; Díaz and Carrascal 1993; Angelici et al. 1997). Following chemicals were tested: (1) the mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) the mixture of three aldehydes and tridecane (TA); (3) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (4) extracted MTG secretion of *G. lineatum* adults (GS) and (5) hexane (HX) as a non-polar solvent for the other chemicals.

Additional experiments were performed using the living specimen of two true bug species *G. lineatum* and *P. apterus* in order to compare, which bug species can more potentiate the chosen chemical – the mixture of three aldehydes (LG/3A, LP/3A).

Both true bug species are found in green lizard's habitat (M. Gregorovičová, personal observation). Since green lizard has well developed chemical discrimination, principally vomerolfaction (Cooper 1991, 1996) and gustation (Schwenk 1985), the heteropteran repellent secretion could be targeted at this type of predator (Gregorovičová and Černíková 2015a).

Similarly to leopard geckos, the following behavioural characteristics were evaluated – approach latencies, attack latencies and approach-attack intervals.

For all behavioural characteristics in the chemical trials, the reactions of green lizards from the hexane (HX) group significantly differed from untreated mealworm (UM) control group. Corresponding p values are in Table 4.5. Therefore, the reactions of green lizards in the other groups (3A-TA-OXO-GS-LG/3A-LP/3A) were compared with those of the untreated mealworm control group.

For all behavioural characteristics, the reactions of green lizards for all tested groups (HX-3A-TA-OXO-GS-LG/3A-LP/3A) in the first control (pre-chemical) trial did not significantly differ compared to the untreated mealworm control group (Tukey Contrasts, Table 4.5A-C). Therefore, all lizards started the experiment with the same motivation.

Following sections describe detailed results for individual behavioural characteristics and for all tested groups (HX-3A-TA-OXO-GS-LG/3A-LP/3A). The corresponding results are summarized in Tables 4.5 and 4.6.

Finally, Table 4.7 summarizes the impact of particular covariates (ANOVA type II) on individual behavioural characteristics.

4.2.1. Approach latencies

Approach latencies were influenced only by the tested chemicals ($p < 0.001$; F = 13.438; df1 = 7; df2 = 812), but not by the weight of the animals (p = 0.453; F = 0.565; df1 = 1; df2 = 812), their sex (p = 0.095; F = 2.359; df1 = 2; df2 = 812) nor their age (p = 0.555; F = 0.348; df1 = 1; df2 = 812). An interaction between the effect of chemicals and part of the experimental sequence was also not significant (p = 0.067; F = 1.626; df1 = 14; df2 = 812). Statistical values are summarized in Table 4.7A.

In chemical trials, all tested chemicals had significant effect on the approach latencies of green lizards compared to the untreated mealworm group. Hexane had the weakest aversive effect (Tukey Contrasts: $p < 0.01$) in comparison with the other tested chemicals (Tukey Contrasts: all $p < 0.001$, Table 4.5A, Fig. 4.5).

The aversive effect of the tested chemicals on the approach latencies of the green lizards was evaluated similarly to leopard geckos – based on a coefficient of the rank-based regression model – the higher its value the slower reaction of the animal and thus stronger aversion towards the particular chemical. Following sequence was obtained (Table 4.6A): *Graphosoma* secretion, Living *Graphosoma*/mixture of aldehydes, Living *Pyrrhocoris*/mixture of aldehydes, the mixture of three aldehydes, the mixture of aldehydes and tridecane, oxoaldehyde, hexane and untreated mealworm. The approach latencies in the untreated mealworm group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), green lizards that had previous experience with *Graphosoma* secretion and Living *Pyrrhocoris/*mixture of aldehydes hesitated significantly longer than lizards from the untreated mealworm group before approaching the mealworms, even when they were no longer treated with the chemicals (Tukey Contrasts: both $p < 0.01$). Approach latencies of the groups previously treated with the other chemicals did not significantly differ from the untreated mealworm group (Tukey Contrasts). All statistical values are in Table 4.5A.

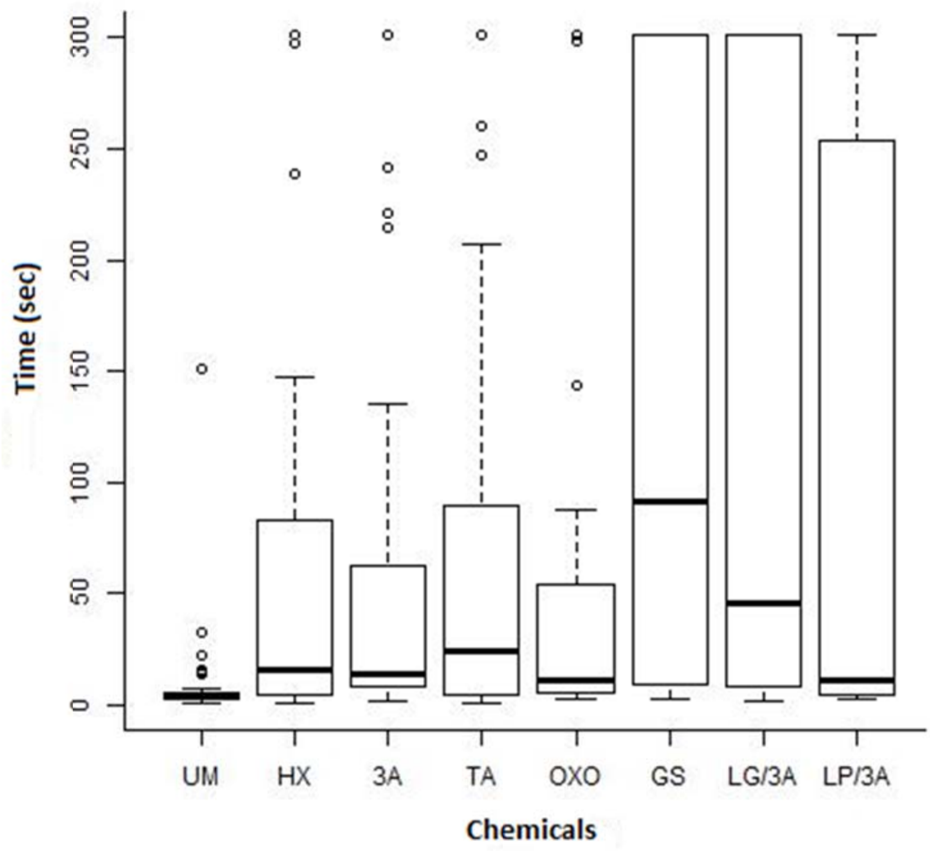

Fig. 4.5: Approach latencies in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), living specimen of *P. apterus* followed by the mixture of three aldehydes (LP/3A).

Approach latencies are presented on y-axis. The figures reflect the original recorded values (the time when the lizard started to come purposefully towards the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.2.2. Attack latencies

Attack latencies were influenced only by the tested chemicals ($p < 0.001$; F = 14.806; df1 = 7; df2 = 812), but not by the weight of the animals (p = 0.373; F = 0.793; df1 = 1; df2 = 812), their sex (p = 0.162; F = 1.825; df1 = 2; df2 = 812) nor their age (p = 0.541; F = 0.374; df1 = 1; df2 = 812). There was a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.05$; F = 2.047; df1 = 14; df2 = 812). Statistical values are summarized in Table 4.7B.

In chemical trials, all tested chemicals had significant effect on the attack latencies of green lizards compared to the untreated mealworm group (Tukey Contrasts: all $p < 0.001$, Table 4.5B, Fig. 4.6). The attack latencies were the longest in the group treated with the Living *Graphosoma*/mixture of aldehydes. The effect of the other chemicals on attack latencies was following (Table 4.6B): *Graphosoma* secretion, Living *Pyrrhocoris*/mixture of aldehydes, the mixture of three aldehydes, the mixture of aldehydes and tridecane, oxoaldehyde, hexane and untreated mealworm. The attack latencies in the untreated mealworm group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), green lizards that had previous experience with *Graphosoma* secretion and Living *Pyrrhocoris/*mixture of aldehydes hesitated significantly longer than lizards from the untreated mealworm group before attacking the mealworms, even when they were no longer treated with the chemicals (Tukey Contrasts: $p < 0.05$; $p < 0.01$ respectively). Attack latencies of the groups previously treated with the other chemicals did not significantly differ from the untreated mealworm group (Tukey Contrasts). All statistical values are in Table 4.5B.

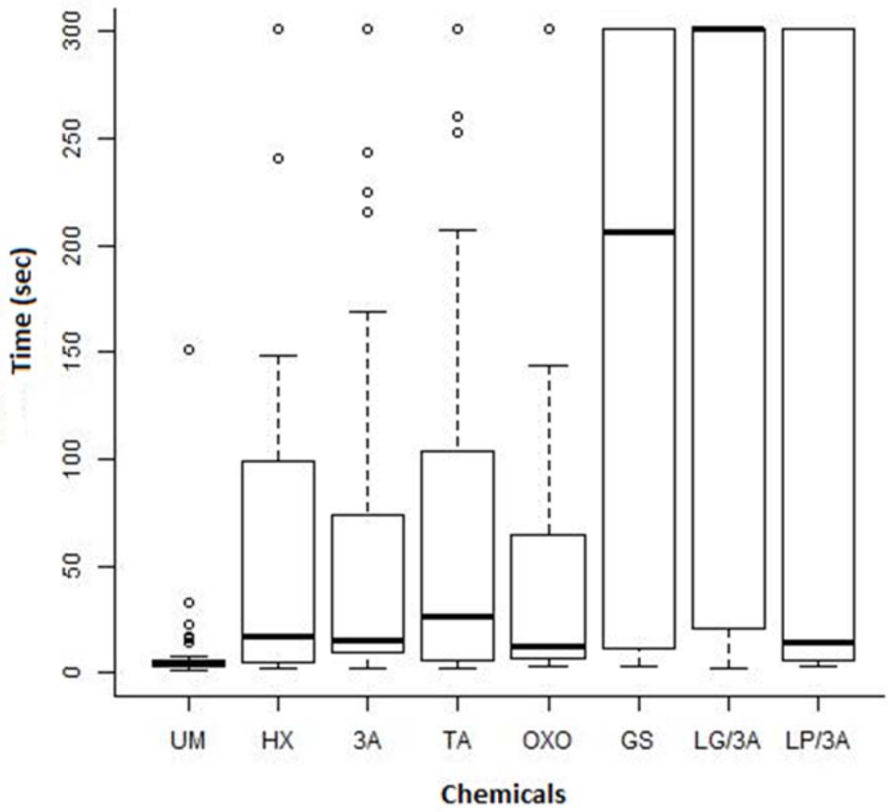

Fig. 4.6: Attack latencies in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), living specimen of *P. apterus* followed by the mixture of three aldehydes (LP/3A).

Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the lizard started to handle the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.2.3. Approach-attack intervals

Approach-attack intervals were influenced by the tested chemicals ($p < 0.001$; F = 14.138; df1 = 7; df2 = 812) and the weight of green lizards ($p < 0.01$; F = 7.360; df1 = 1; df2 = 812), but not by their sex (p = 0.200; F = 1.614; df1 = 2; df2 = 812) nor their age (p = 0.435; F = 0.609; df1 = 1; df2 = 812). Heavier animals were faster when assessing approach-attack intervals. Additionally, there was a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.001$; F = 2.693; df1 = 14; df2 = 812). Statistical values are summarized in Table 4.7C.

In chemical trials, all tested chemicals had significant effect on the approach-attack intervals of green lizards compared to the untreated mealworm group (Tukey Contrasts: all $p < 0.001$, Table 4.5C, Fig. 4.7). The approach-attack intervals were the longest in the group treated with Living *Graphosoma*/mixture of aldehydes. The effect of other chemicals on approach-attack intervals was following (Table 4.6C): *Graphosoma* secretion, Living *Pyrrhocoris*/mixture of aldehydes, the mixture of aldehydes and tridecane, the mixture of three aldehydes, oxoaldehyde, hexane and untreated mealworm. The approach-attack intervals in the untreated mealworm group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), when evaluating the approach-attack intervals, green lizards that had previous experience with *Graphosoma* secretion hesitated significantly longer than lizards from the untreated mealworm group, even when the mealworms were no longer treated with the chemicals (Tukey Contrasts: $p < 0.05$). Approach-attack intervals of the groups previously treated with the other chemicals did not significantly differ from the untreated mealworm group (Tukey Contrasts). All statistical values are in Table 4.5C.

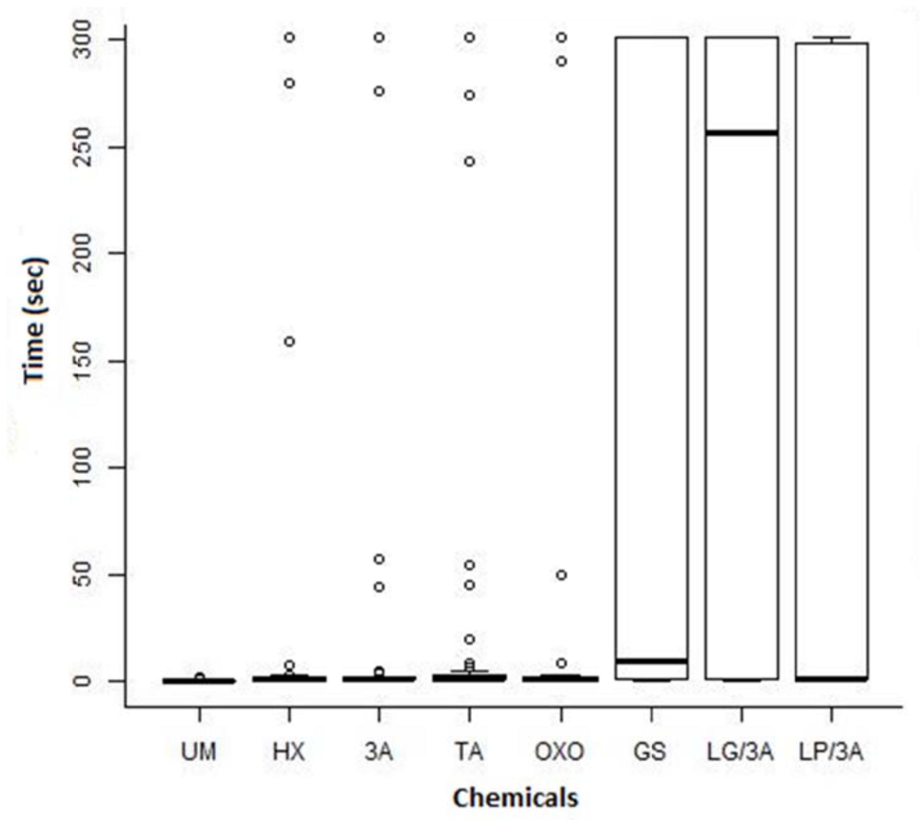

Fig. 4.7: Approach-attack intervals in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), living specimen of *G. lineatum* followed by the mixture of three aldehydes (LG/3A), living specimen of *P. apterus* followed by the mixture of three aldehydes (LP/3A).

Approach-attack intervals are presented on y-axis. The figures reflect the original recorded values (the degree of hesitation between approaching the prey and attacking the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.2.4. Manipulation with *Graphosoma lineatum*

During the testing of lizard's reactions to the living specimen of *G. lineatum* following characteristics in the bug handling were observed. Out of 8 tested animals, 3 lizards manipulated the bug twice (out of a maximum of 5 offered bugs), 3 lizards only once and remaining 2 lizards did not manipulate any of three offered bugs. It means that green lizards manipulated the bug maximally twice. All bugs were released unharmed, no one was killed.

4.2.5. Manipulation with *Pyrrhocoris apterus*

Lizard's reactions to the living specimen of *P. apterus* were different from the observation made with *G. lineatum*. Following characteristics in the bug handling were observed. Out of 7 tested animals, only 1 manipulated and killed the firebug. Remaining 6 animals did not manipulate any of three offered firebugs. All bugs were thus untouched, except for one.

In conclusion, the results indicated that 5 offered bugs was sufficient number to gain the experience to avoid the bugs.

Table 4.5: The reactions of green lizards in the tested groups (HX-3A-TA-OXO-GS-LG/3A-LP/3A) compared to the untreated mealworm control group (UM)

All behavioural characteristics were evaluated. Abbreviations: HX – hexane; 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion; LG/3A – living specimen of *G. lineatum* followed by the mixture of three aldehydes; LP/3A – living specimen of *P. apterus* followed by the mixture of three aldehydes. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with untreated mealworm).

Chemicals	**HX**			**3A**			**TA**			**OXO**			**GS**		
	p value	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**
A – Approach latencies															
Pre-chemical trials	1.000	– 102.65	99.82	0.991	–167.97	99.82	0.987	– 172.19	99.82	1.000	– 93.69	99.82	0.634	– 250.03	59.82
Chemical trials	**< 0.01**	– 202.69	44.64	**< 0.001**	– 253.81	44.64	**< 0.001**	– 230.64	44.64	**< 0.001**	– 226.19	44.64	**< 0.001**	– 372.38	44.64
Post-chemical trials	0.713	– 119.95	49.91	0.766	– 116.32	49.91	1.000	– 53.57	49.91	0.486	– 134.24	49.91	**< 0.01**	– 211.74	49.91
B – Attack latencies															
Pre-chemical trials	1.000	– 109.25	98.56	0.990	– 167.17	98.56	0.985	– 172.58	98.56	1.000	– 93.17	98.56	0.664	– 243.29	98.56
Chemical trials	**< 0.001**	– 208.41	44.08	**< 0.001**	– 252.10	44.08	**< 0.001**	– 244.82	44.08	**< 0.001**	– 229.53	44.08	**< 0.001**	– 392.67	44.08
Post-chemical trials	0.821	– 110.65	49.28	0.769	– 114.64	49.28	0.999	– 71.72	49.28	0.588	– 126.33	49.28	**< 0.05**	– 199.39	49.28
C – Approach-attack intervals															
Pre-chemical trials	0.691	– 238.06	97.82	0.444	– 268.50	97.92	0.950	– 191.01	97.89	1.000	– 81.10	97.92	0.939	– 194.84	98.09
Chemical trials	**< 0.001**	– 217.80	43.76	**< 0.001**	– 245.75	43.99	**< 0.001**	– 267.13	43.92	**< 0.001**	– 210.10	43.98	**< 0.001**	– 393.62	44.35
Post-chemical trials	0.495	– 131.18	48.92	0.771	– 114.06	49.13	0.466	– 133.31	49.06	0.562	– 127.51	49.12	**< 0.05**	– 186.50	49.45

Chemicals	LG/3A			LP/3A		
	p value	Est.	SE	p value	Est.	SE
A – Approach latencies						
Pre-chemical trials	1.000	– 140.78	110.00	0.991	– 191.28	114.08
Chemical trials	**< 0.001**	– 357.74	49.19	**< 0.001**	– 263.50	51.02
Post-chemical trials	0.985	– 96.34	55.00	**< 0.01**	– 257.76	57.04
B – Attack latencies						
Pre-chemical trials	1.000	– 137.25	108.61	0.992	– 186.71	112.64
Chemical trials	**< 0.001**	– 416.90	48.60	**< 0.001**	– 283.30	50.37
Post-chemical trials	0.980	– 96.86	54.30	**< 0.01**	– 237.52	56.32
C – Approach-attack intervals						
Pre-chemical trials	1.000	– 54.61	107.79	1.000	– 149.78	111.79
Chemical trials	**< 0.001**	– 406.36	48.22	**< 0.001**	– 289.80	50.00
Post-chemical trials	0.994	– 87.53	53.91	0.061	– 197.65	55.90

Table 4.5: The reactions of green lizards in the tested groups (HX-3A-TA-OXO-GS-LG/3A-LP/3A) compared to the untreated mealworm control group (UM) – continuation

Table 4.6: The aversive effect of the tested chemical compounds on the individual behavioural characteristics of green lizards

Abbreviations: UM – untreated mealworm; HX – hexane; 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion; LG/3A – living specimen of *G. lineatum* followed by the mixture of three aldehydes; LP/3A – living specimen of *P. apterus* followed by the mixture of three aldehydes. Estimate: effect on behavioural characteristics estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

Chemicals	UM	HX	3A	TA	OXO	GS	LG/3A	LP/3A
	Estimate (regression coefficient)							
A – Approach latencies								
Chemical trials	– 256.2	– 59.1	3.2	– 22.2	– 25.9	127.1	92.6	5.4
B – Attack latencies								
Chemical trials	– 248.4	– 45.1	11.2	– 0.9	– 12.8	157.9	162.0	33.2
C – Approach-attack intervals								
Chemical trials	– 245.1	– 22.1	17.8	31.1	– 16.2	171.3	174.1	45.2

Table 4.7: The impact of particular covariates on individual behavioural characteristics of green lizards evaluated by using Type II ANOVA table

Covariate	**p value**	**F value**	**df1**	**df2**
A – Approach latencies				
Chemical	< 0.001	13.438	7	812
Weight	0.453	0.565	1	812
Sex	0.095	2.359	2	812
Age	0.555	0.348	1	812
Chemical: part	0.067	1.626	14	812
B – Attack latencies				
Chemical	< 0.001	14.806	7	812
Weight	0.373	0.793	1	812
Sex	0.162	1.825	2	812
Age	0.541	0.374	1	812
Chemical: part	< 0.05	2.047	14	812
C – Approach-attack intervals				
Chemical	< 0.001	14.138	7	812
Weight	< 0.01	7.360	1	812
Sex	0.200	1.614	2	812
Age	0.435	0.609	1	812
Chemical: part	< 0.001	2.693	14	812

4.2.6. Summary

(1) Hexane had the weakest aversive effect on green lizards. Lizards hesitated with mealworms treated with hexane in chemical trials. In the following trials (post-chemical trials) they were already habituated to hexane.

(2) The aversive effect of the mixture of aldehydes was moderately stronger than the same mixture enhanced by tridecane, which is in contradiction with hypothesis of tridecane acting as a catalyst.

(3) The mixture of aldehydes enhanced by tridecane had a stronger aversive effect than oxoaldehyde.

(4) Oxoaldehyde had the weakest aversive effect on green lizards from the tested chemicals, which may be attributed to its odourless nature.

(5) Whole MTG secretion had a strong aversive effect for green lizards.

(6) The presence of living *G. lineatum*/ *P. apterus* increased the effect of the mixture of three aldehydes. The strongest effect was observed in the presence of living specimen *G. lineatum*, rather than *P. apterus*.

4.3. Reactions of Great tits

Great tits were chosen as a bird predator because they also feed on Heteroptera, mostly from families Miridae and Pentatomidae (Cramp and Perrins 1993; Exnerová et al. 2003a). Therefore, they are an ideal model for testing major compounds of defensive secretion of *G. lineatum* against such type of insectivorous bird.

Following chemicals and mixtures were tested: (1) the mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) the mixture of three aldehydes and tridecane (TA); (3) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (4) extracted MTG secretion of *G. lineatum* adults (GS) and (5) hexane (HX) as a non-polar solvent for the other chemicals.

The following behavioural characteristics were evaluated: attack latencies and attack-eating intervals.

For all behavioural characteristics and in all parts of the experimental sequence (pre-chemical trials, chemical trials and post-chemical trials), the reactions of great tits from the hexane (HX) group did not significantly differ from untreated mealworm (UM) control group. Corresponding p values are in Table 4.8. These results proved that the effect of hexane as a non-polar solvent for the other chemicals of MTG secretion of *G. lineatum* could be excluded. Therefore, the reactions of great tits in the other groups (3A-TA-OXO-GS) were compared with those of the hexane group.

For all behavioural characteristics, the reactions of great tits for all tested groups (3A-TA-OXO-GS) in the first control (pre-chemical) trial did not significantly differ compared to the hexane group (Table 4.9A-B). Therefore, all great tits started the experiment with the same motivation.

Following sections describe detailed results for individual behavioural characteristics and for all tested groups (3A-TA-OXO-GS). The corresponding results are summarized in Tables 4.9 and 4.10.

Finally, Table 4.11 summarizes the impact of particular covariates (ANOVA type II) on individual behavioural characteristics.

4.3.1. Attack latencies

Attack latencies were influenced by the tested chemicals ($p < 0.001$; $F = 41.777$; df1 = 5; df2 = 1940) and sex of the great tits ($p < 0.001$; $F = 14.630$; df1 = 1; df2 = 1940), but not by their age ($p = 0.857$; $F = 0.032$; df1 = 1; df2 = 1940). Females were faster than males. There was also a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.001$; $F = 3.129$; df1 = 10; df2 = 1940). Statistical values are summarized in Table 4.11A.

In chemical trials, the attack latencies were significantly longer in the group treated with *Graphosoma* secretion and oxoaldehyde (Tukey Contrasts: both $p < 0.001$) compared to the birds from the hexane group. The mixture of three aldehydes had also significant effect on attack latencies (Tukey Contrasts: $p < 0.05$), but its strength was weaker compared to previous chemicals. Attack latencies of birds tested with the mixture of aldehydes and tridecane did not significantly differ from birds‘ reactions in the hexane group (Tukey Contrasts: $p = 0.884$). For details refer Table 4.9A and Fig. 4.8.

The aversive effect of the tested chemicals on the attack latencies of the great tits was evaluated similarly to lizard predators – based on a coefficient of the rank-based regression model – the higher its value the slower reaction of the animal and thus stronger aversion towards the particular chemical. The great tits hesitated most before attacking the prey treated with oxoaldehyde (Table 4.10A). The effect of the other chemicals on attack latencies was following: *Graphosoma* secretion, the mixture of three aldehydes, untreated mealworm, the mixture of aldehydes and tridecane, and hexane. The attack latencies in the hexane group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), great tits that had previous experience with *Graphosoma* secretion, oxoaldehyde and with the mixture of three aldehydes hesitated significantly longer before attacking the prey, even when the mealworms were no longer treated with the chemical (Tukey Contrasts: all $p < 0.001$). Attack latencies in the group previously treated with the mixture of aldehydes and tridecane did not significantly differ from the hexane group (Tukey Contrasts: $p = 0.839$). For details refer Table 4.9A and Fig. 4.9.

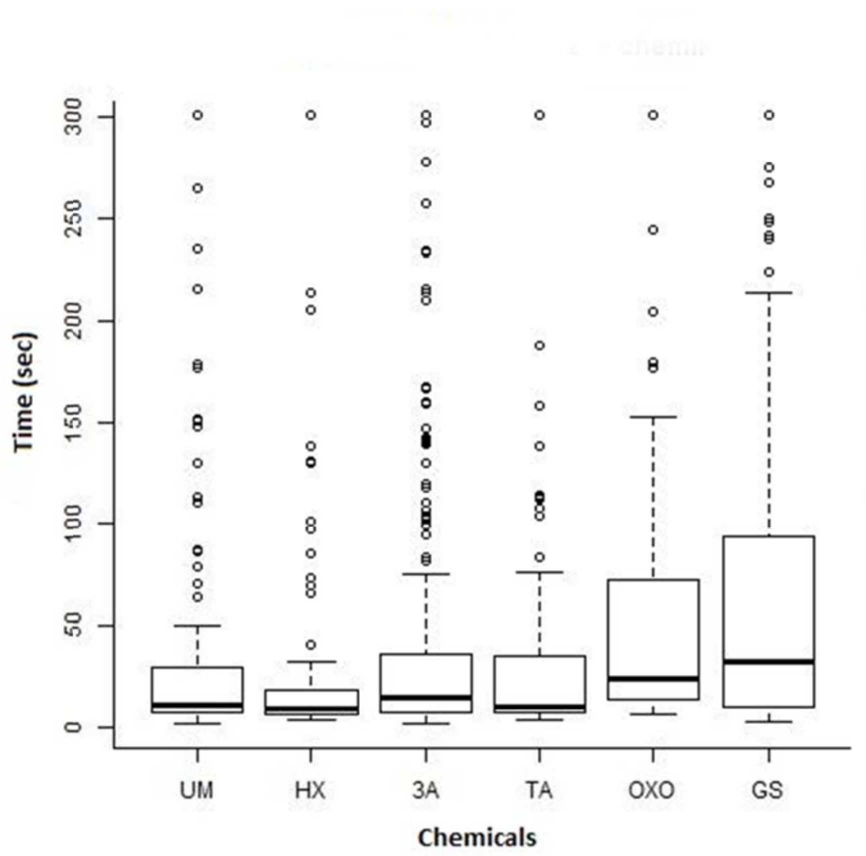

Fig. 4.8: Attack latencies in trials with tested chemicals – chemical trials (original values)
Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO) and *Graphosoma* secretion (GS).
Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the bird started to handle the prey (touching, pecking or seizing)).
(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

The great tits hesitated most before attacking the prey in the group previously treated with oxoaldehyde (Table 4.10A). Although the mealworms were no longer treated with the chemicals, the persistent effect of the other chemicals on attack latencies could be assessed: *Graphosoma* secretion, the mixture of three aldehydes, untreated mealworm, the mixture of aldehydes and tridecane, and hexane. The attack latencies in the hexane group were again the shortest.

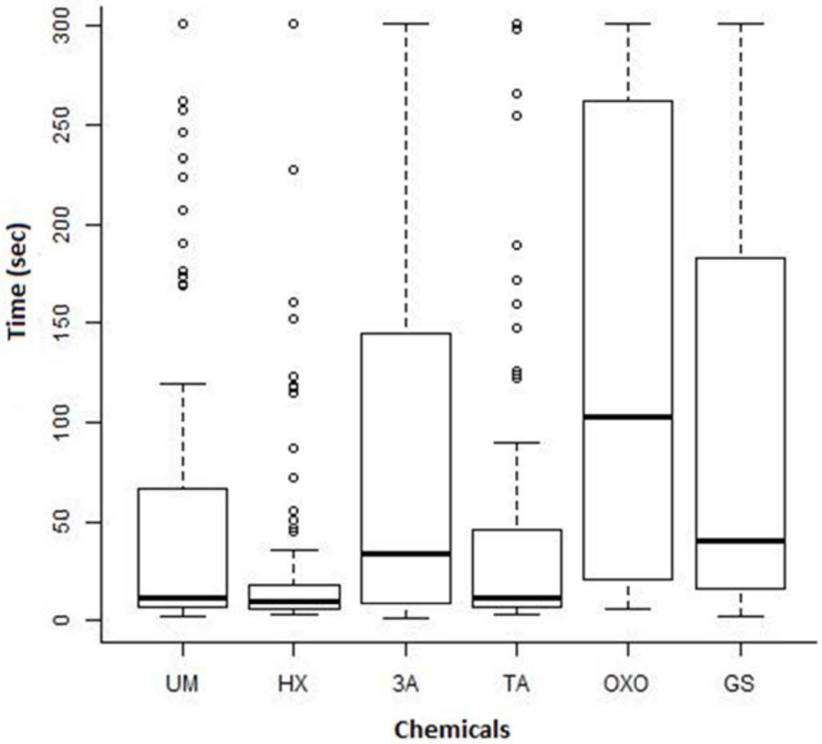

Fig. 4.9: Attack latencies in trials following the experience with chemicals – post-chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS)

Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the bird started to handle the prey (touching, pecking or seizing)).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.3.2. Attack-eating intervals

Attack-eating intervals were affected by the tested chemicals ($p < 0.001$; F = 82.401; df1 = 5; df2 = 1940) and the age of great tits ($p < 0.001$; F = 11.061; df1 = 1; df2 = 1940), but not by their sex (p = 0.827; F = 0.048; df1 = 1; df2 = 1940). Younger birds were slower than older birds. There was also a significant interaction between the effect of chemicals and part of the experimental sequence ($p < 0.001$; F = 3.138; df1 = 10; df2 = 1940). Statistical values are summarized in Table 4.11B.

In chemical trials, when evaluating attack-eating intervals, great tits tested with the mixture of three aldehydes, oxoaldehyde and *Graphosoma* secretion hesitated significantly longer compared to the birds from the hexane group (Tukey Contrasts: all $p < 0.001$). The mixture of aldehydes and tridecane had also significant effect on attack-eating intervals ($p < 0.01$), but it was weaker compared to previous chemicals. For details refer Table 4.9B and Fig. 4.10.

When assessing attack-eating intervals, the great tits hesitated most to the prey treated with oxoaldehyde in chemical trials (Table 4.10B). The effect of the other chemicals on attack-eating intervals was following: *Graphosoma* secretion, the mixture of three aldehydes, the mixture of aldehydes and tridecane, untreated mealworm and hexane. The attack-eating intervals in the hexane group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), when assessing attack-eating intervals, the great tits that had previous experience with *Graphosoma* secretion, oxoaldehyde and with the mixture of three aldehydes hesitated significantly longer than birds from the hexane group, even when the mealworms were no longer treated with the chemicals (Tukey Contrasts: all $p < 0.001$). Attack-eating intervals in the group previously treated with the mixture of aldehydes and tridecane did not significantly differ from the hexane group (p = 0.955). For details refer Table 4.9B and Fig. 4.11.

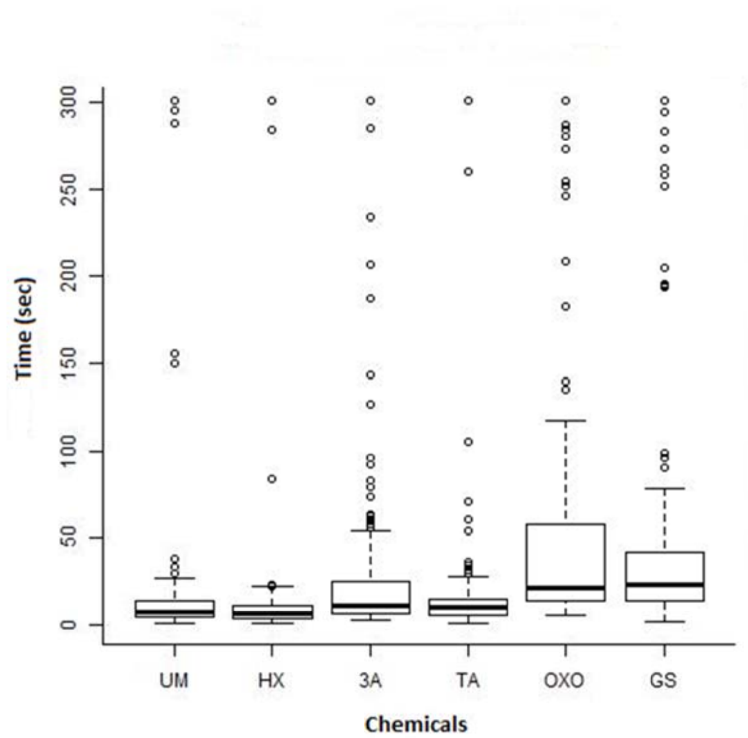

Fig. 4.10: Attack-eating intervals in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS).

Attack-eating intervals are presented on y-axis. The figures reflect the original recorded values (the interval between the first attack and the moment the bird started eating the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

The attack-eating intervals were the longest in the group previously treated with *Graphosoma* secretion (Table 4.10B). The persistent effect of the other chemicals on attack-eating intervals was following: oxoaldehyde, the mixture of three aldehydes, untreated mealworm, the mixture of aldehydes and tridecane, and hexane. The attack-eating intervals in the hexane group were again the shortest.

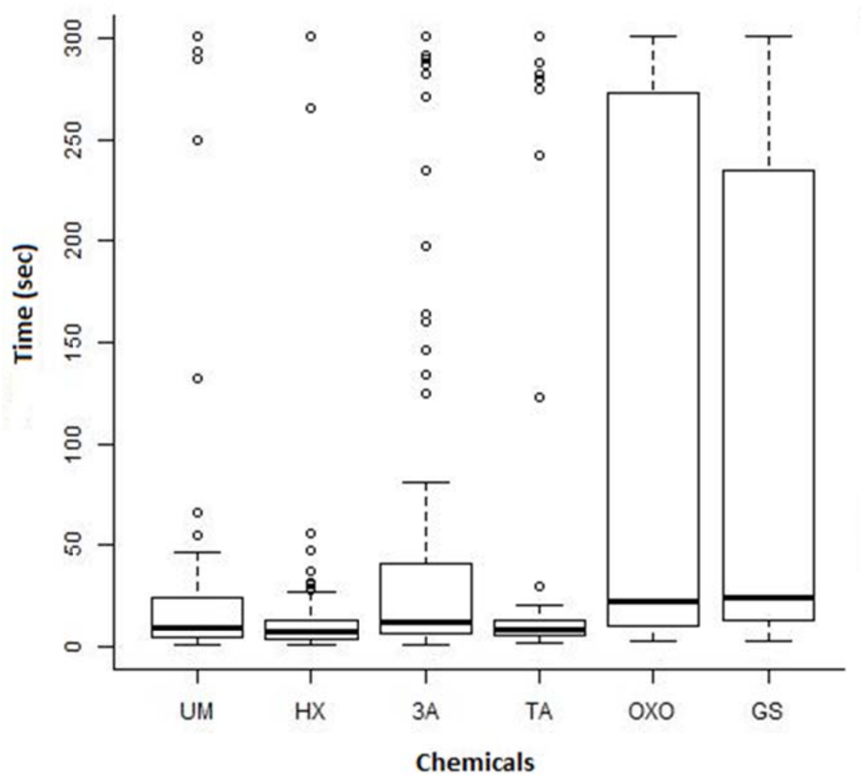

Fig. 4.11: Attack-eating intervals in trials following the experience with the chemical – post-chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), the mixture of three aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS).

Attack-eating intervals are presented on y-axis. The figures reflect the original recorded values (the interval between the first attack and the moment the bird started eating the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

Table 4.8: The reactions of great tits towards mealworms treated with hexane (HX) compared with the reactions of great tits towards untreated mealworms (UM)

All behavioural characteristics were evaluated. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with untreated mealworm).

Control		**HX**	
	p value	**Est.**	**SE**
A – Attack latencies			
Pre-chemical trial	0.998	190.43	149.75
Chemical trials	0.869	131.62	67.11
Post-chemical trials	0.384	198.33	74.99
B – Attack-eating intervals			
Pre-chemical trial	1.000	70.48	143.20
Chemical trials	0.944	113.07	64.17
Post-chemical trials	0.376	190.67	71.71

Table 4.9: The reactions of great tits in the tested groups (3A-TA-OXO-GS) compared to the hexane group (HX)

All behavioural characteristics were evaluated. Abbreviations: 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with hexane).

Chemicals	**3A**			**TA**			**OXO**			**GS**		
	p value	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**
A – Attack latencies												
Pre-chemical trials	1.000	– 13.85	129.29	1.000	– 6.02	149.77	1.000	94.28	149.69	0.991	192.69	132.06
Chemical trials	**< 0.05**	– 216.85	57.98	0.884	129.41	67.15	**< 0.001**	446.70	66.98	**< 0.001**	426.62	59.18
Post-chemical trials	**< 0.001**	– 441.85	64.88	0.839	151.38	75.03	**< 0.001**	714.71	74.78	**< 0.001**	580.33	66.13
B – Attack-eating intervals												
Pre-chemical trials	1.000	– 19.98	123.63	1.000	0.90	143.22	1.000	119.64	143.15	0.976	202.93	126.29
Chemical trials	**< 0.001**	– 361.60	55.45	**< 0.01**	256.26	64.22	**< 0.001**	731.11	64.05	**< 0.001**	710.21	56.60
Post-chemical trials	**< 0.001**	– 346.10	61.95	0.955	123.47	71.75	**< 0.001**	620.51	71.61	**< 0.001**	653.59	63.24

Table 4.10: The aversive effect of the tested chemical compounds on the individual behavioural characteristics of great tits

Abbreviations: UM – untreated mealworm; HX – hexane; 3A – the mixture of three aldehydes; TA – the mixture of three aldehydes and tridecane; OXO – oxoaldehyde; GS – *Graphosoma* secretion; Estimate: effect on behavioural characteristics estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

Chemicals	UM	HX	3A	TA	OXO	GS
		Estimate (regression coefficient)				
A – Attack latencies						
Chemical trials	92.2	– 39.4	177.4	90.0	407.2	387.2
Post-chemical trials	149.3	– 49.1	392.8	102.3	665.6	531.3
B – Attack-eating intervals						
Chemical trials	31.2	– 81.9	279.7	174.4	649.2	628.3
Post-chemical trials	181.1	– 9.5	336.5	113.9	611.0	644.0

Table 4.11: The impact of particular covariates on individual behavioural characteristics of great tits evaluated by using Type II ANOVA table

Covariate	p value	F value	df1	df2
A – Attack latencies				
Chemical	< 0.001	41.777	5	1940
Sex	< 0.001	14.630	1	1940
Age	0.857	0.032	1	1940
Chemical: part	< 0.001	3.129	10	1940
B – Attack-eating intervals				
Chemical	< 0.001	82.401	5	1940
Sex	0.827	0.048	1	1940
Age	< 0.001	11.061	1	1940
Chemical: part	< 0.001	3.138	10	1940

4.3.3. Summary

(1) Great tits did not show any aversive reaction to hexane.

(2) The mixture of three aldehydes had aversive effect on great tits when they attacked the prey and when evaluating attack-eating intervals. Therefore, it could play a role as a signal of unpalatability.

(3) The mixture of three aldehydes and tridecane did not have any aversive effect on great tits when they attacked the prey. Weak aversive effect was observed when evaluating attack-eating intervals in the chemical trials. Tridecane probably decreases the impact of the mixture of three aldehydes on great tits.

(4) Oxoaldehyde had a strong aversive effect for great tits.

(5) Whole MTG secretion had clearly an aversive effect for great tits.

4.4. Reactions of Blue tits

Blue tit was chosen as a second bird predator because of its predation on Heteroptera (Exnerová 2003a, b; del Hoyo 2007) and its close relation to the great tit. Since great tit is bigger than blue tit, it is obvious that the ability of predator to avoid or eat chemically defended true bug could be linked with body weight and its foraging strategy (Exnerová et al. 2003b; Hotová Svádová et al. 2010).

Following chemicals were tested: (1) the mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (3) extracted MTG secretion of *G. lineatum* adults (GS) and (4) hexane (HX) as a non-polar solvent for the other chemicals.

There was one difference between the experiments with blue tits and great tits – due to the obtained results that the mixture of three aldehydes and tridecane (TA) does not have any aversive effect on the great tits (see the chapter 4.3.), this mixture (TA) was eliminated from the group of tested chemicals.

Similarly to the great tits, the following behavioural characteristics were evaluated: attack latencies and attack-eating intervals.

In case of attack latencies in the post-chemical trials and attack-eating intervals in the chemical trials, the reactions of blue tits from the hexane (HX) group significantly differed from untreated mealworm (UM) control group. Corresponding p values are in Table 4.12. Therefore, the reactions of blue tits in the other groups (3A-OXO-GS) were compared with those of the untreated mealworm control group.

For all behavioural characteristics, the reactions of blue tits for all tested groups (HX-3A-OXO-GS) in the first control (pre-chemical) trial did not significantly differ compared to the untreated mealworm control group (Tukey Contrasts, Table 4.12A-B). Therefore, all birds started the experiment with the same motivation.

Following sections describe detailed results for individual behavioural characteristics and for all tested groups (HX-3A-OXO-GS). The corresponding results are summarized in Tables 4.12 and 4.13.

Finally, Table 4.14 summarizes the impact of particular covariates (ANOVA type II) on individual behavioural characteristics.

4.4.1. Attack latencies

Attack latencies were affected by the chemicals ($p < 0.001$; $F = 25.128$; $df1 = 4$; $df2 = 529$), but not by the sex of the birds ($p = 0.390$; $F = 0.739$; $df1 = 1$; $df2 = 529$) nor their age ($p = 0.536$; $F = 0.384$; $df1 = 1$; $df2 = 529$). An interaction between the effect of chemicals and part of the experimental sequence was also not significant ($p = 0.113$; $F = 1.630$; $df1 = 8$; $df2 = 529$). Statistical values are summarized in Table 4.14A.

In chemical trials, the attack latencies were significantly longer only in the group treated with *Graphosoma* secretion (Tukey Contrasts: $p < 0.001$) compared to the birds in the untreated mealworm group. The attack latencies of birds tested with the mixture of three aldehydes, oxoladehyde and hexane did not significantly differ from birds' reactions in the untreated mealworm group (Tukey Contrasts: $p = 0.122$; $p = 0.873$; $p = 0.994$ respectively). For details refer Table 4.12A and Fig. 4.12.

The blue tits hesitated most before attacking the prey treated with *Graphosoma* secretion (Table 4.13A). The effect of the other chemicals on attack latencies was following: the mixture of three aldehydes, oxoaldehyde, hexane and untreated mealworm. The attack latencies in the untreated mealworm group were thus the shortest.

In trials following the experience with the chemical (post-chemical trials), blue tits that had previous experience with *Graphosoma* secretion and with the mixture of three aldehydes hesitated significantly longer before attacking the prey, even when the mealworms were no longer treated with the chemical (Tukey Contrasts: $p < 0.001$; $p < 0.01$ respectively). The weaker aversive effect was observed in the group previously treated with oxoaldehyde as well as with hexane (Tukey Contrasts: both $p < 0.05$). For details refer Table 4.12A and Fig. 4.13.

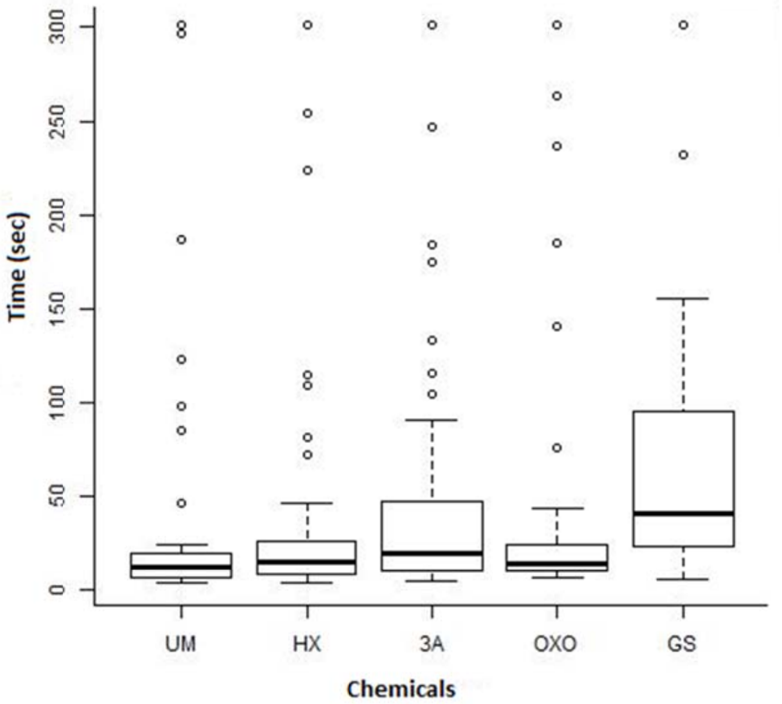

Fig. 4.12: Attack latencies in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), oxoaldehyde (OXO) and *Graphosoma* secretion (GS).

Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the bird started to handle the prey (touching, pecking or seizing)).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

The blue tits hesitated most before attacking the prey in the group previously treated with *Graphosoma* secretion (Table 4.13A). Although the mealworms were no longer treated with the chemicals, the persistent effect of the other chemicals on attack latencies could be evaluated: the mixture of three aldehydes, hexane, oxoaldehyde and untreated mealworm. The attack latencies in the untreated mealworm group were again the shortest.

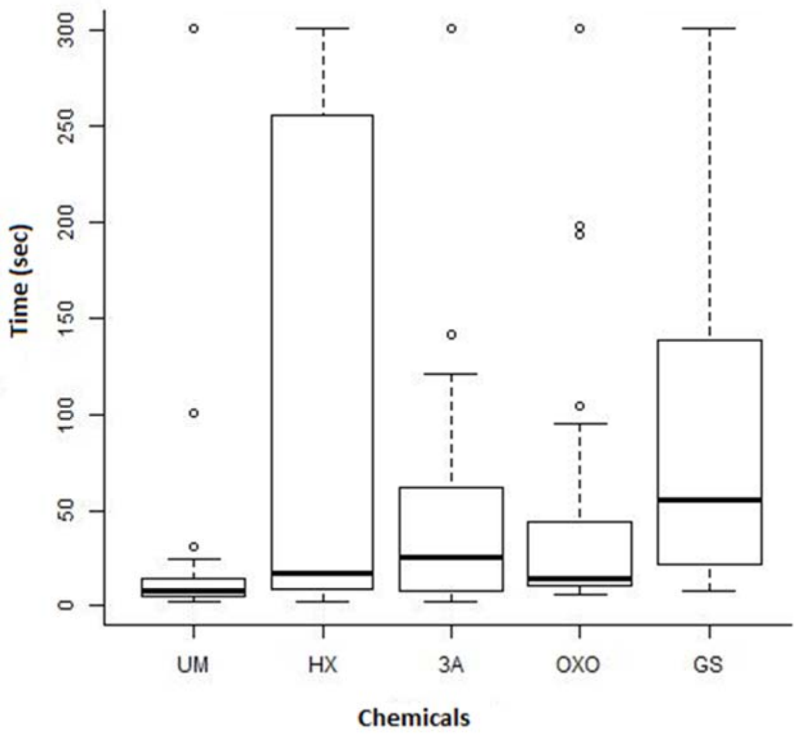

Fig. 4.13: Attack latencies in trials following the experience with chemicals – post-chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), oxoaldehyde (OXO), *Graphosoma* secretion (GS)

Attack latencies are presented on y-axis. The figures reflect the original recorded values (the time when the bird started to handle the prey (touching, pecking or seizing)).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

4.4.2. Attack-eating intervals

Attack-eating intervals were influenced only by the tested chemicals ($p < 0.001$; $F = 16.697$; $df1 = 4$; $df2 = 529$), not but the sex of blue tits ($p = 0.095$; $F = 2.802$; $df1 = 1$; $df2 = 529$) nor their age ($p = 0.249$; $F = 1.330$; $df1 = 1$; $df2 = 529$). An interaction between the effect of chemicals and part of the experimental sequence was not significant ($p = 0.359$; $F = 1.103$; $df1 = 8$; $df2 = 529$). Statistical values are summarized in Table 4.14B.

In chemical trials, when evaluating attack-eating intervals, blue tits tested with *Graphosoma* secretion, oxoaldehyde and hexane hesitated significantly longer compared to the untreated mealworm group (Tukey Contrasts: $p < 0.001$; $p < 0.01$; $p < 0.01$ respectively). On the other hand, the mixture of three aldehydes did not have any significant aversive effect on attack-eating intervals (Tukey Contrasts: $p=1.000$). For details refer Table 4.12B and Fig. 4.14.

When evaluating attack-eating intervals, the blue tits hesitated most before attacking the prey treated with *Graphosoma* secretion (Table 4.13B). The effect of the other chemicals on attack-eating intervals was following: hexane, oxoaldehyde, the mixture of three aldehydes and untreated mealworm. The attack-eating intervals in the untreated mealworm group were thus the shortest.

In trials following the experience with the chemicals (post-chemical trials), the attack-eating intervals did not significantly differ among the groups of tested birds (Tukey Contrasts).

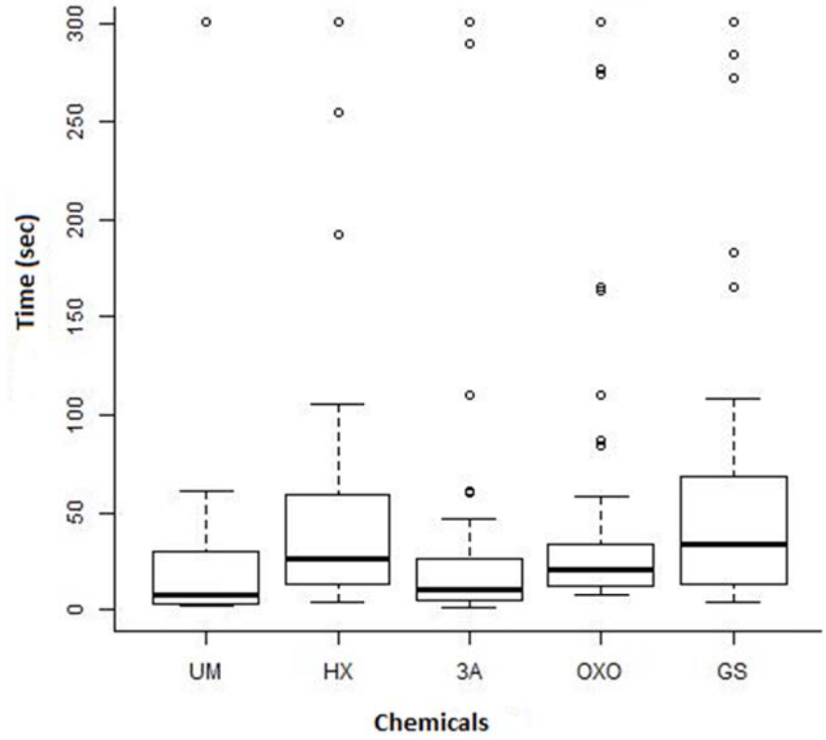

Fig. 4.14: Attack-eating intervals in trials with tested chemicals – chemical trials (original values)

Abbreviations: untreated mealworm (UM), hexane (HX), the mixture of three aldehydes (3A), oxoaldehyde (OXO) and *Graphosoma* secretion (GS).

Attack-eating intervals are presented on y-axis. The figures reflect the original recorded values (the interval between the first attack and the moment the bird started eating the prey).

(band inside the box = median; box = lower and upper quartile; whiskers = nonoutlier range; circles = outlier data)

Table 4.12: The reactions of blue tits in the tested groups (HX-3A-OXO-GS) compared to the untreated mealworm control group (UM)

All behavioural characteristics were evaluated. Abbreviations: HX – hexane; 3A – the mixture of three aldehydes; OXO – oxoaldehyde; GS – *Graphosoma* secretion. Est.: estimate of difference between pairs of the chemicals obtained by a rank-based regression model (selected chemical compared with untreated mealworm).

Chemicals	**HX**			**3A**			**OXO**			**GS**		
	p value	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**	**p value**	**Est.**	**SE**
A – Attack latencies												
Pre-chemical trials	1.000	− 42.84	52.74	0.999	− 54.63	48.86	0.990	66.95	49.48	0.445	− 121.99	49.31
Chemical trials	0.994	− 38.96	30.49	0.122	− 86.93	28.30	0.873	− 52.94	28.79	**< 0.001**	− 176.93	28.48
Post-chemical trials	**< 0.05**	− 130.67	37.32	**< 0.01**	− 135.76	34.60	**< 0.05**	− 119.80	35.13	**< 0.001**	− 245.56	34.87
B – Attack-eating intervals												
Pre-chemical trials	0.878	− 98.44	53.82	1.000	− 13.00	49.87	1.000	23.69	50.50	0.840	− 95.87	50.32
Chemical trials	**< 0.01**	− 137.35	31.12	1.000	− 20.65	28.88	**< 0.01**	− 118.26	29.39	**< 0.001**	− 139.19	29.07
Post-chemical trials	0.106	− 119.14	38.09	1.000	− 13.63	35.32	0.383	−91.85	35.85	0.064	− 117.59	35.59

Table 4.13: The aversive effect of the tested chemical compounds on the individual behavioural characteristics of blue tits

Abbreviations: UM – untreated mealworm; HX – hexane; 3A – the mixture of three aldehydes; OXO – oxoaldehyde; GS – *Graphosoma* secretion; Estimate: effect on behavioural characteristics estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

Chemicals	**UM**	**HX**	**3A**	**OXO**	**GS**
	Estimate (regression coefficient)				
A – Attack latencies					
Chemical trials	– 72.2	– 33.2	14.8	– 19.2	104.8
Post-chemical trials	– 122.1	8.5	13.6	– 2.3	123.4
B – Attack-eating intervals					
Chemical trials	7.3	144.7	28.0	125.6	146.5

Table 4.14: The impact of particular covariates on individual behavioural characteristics of blue tits evaluated by using Type II ANOVA table

Covariate	**p value**	**F value**	**df1**	**df2**
A – Attack latencies				
Chemical	< 0.001	25.128	4	529
Sex	0.390	0.739	1	529
Age	0.536	0.384	1	529
Chemical: part	0.113	1.630	8	529
B – Attack-eating intervals				
Chemical	< 0.001	16.697	4	529
Sex	0.095	2.802	1	529
Age	0.249	1.330	1	529
Chemical: part	0.359	1.103	8	529

4.4.3. Summary

(1) For blue tits hexane had an aversive after-effect when attacking the prey. On the other hand, when evaluating attack-eating intervals blue tits were able to overcome the toxin burden of hexane in the post-chemical trials.

(2) The mixture of three aldehydes had clearly an aversive effect on blue tits in the post-chemical trials when evaluating attack latencies.

(3) For blue tits oxoaldehyde had delayed effect when attacking the prey (post-chemical trials), whereas it had aversive effect in the chemical trials when evaluating attack-eating intervals. Therefore, oxoaldehyde could function as a direct toxin.

(4) Whole MTG secretion had clearly an aversive effect for blue tits.

(5) Blue tits hesitated most to the whole MTG secretion of *G. lineatum*.

5. DISCUSSION

The thesis presents probably for the first time the reactions of selected predators towards individual compounds of the defensive secretion of striated shiledbug *G. lineatum*. Many studies provided results related to the chemical composition of the repellent secretion of aposematic insect (Hamilton et al. 1985; Aldrich 1988; Farine et al. 1992; Aldrich et al. 1996, 1997; Krall et al. 1999; Aliabadi et al. 2002; Prudic et al. 2008; Fávaro et al. 2011; Šanda et al. 2012), but there are only few studies (Benfield 1972; Härlin 2005) related to predators' reactions towards individual compounds of the defensive secretion. More comparative studies will have to be performed to explain true roles of particular chemical compounds contributing to the studied complex of chemical signalling and the mechanisms promotiong aversive reactions in different types of predators.

The results surveyed in this thesis provide reliable information concerning wild-caught green lizards, great tits and blue tits with unknown histories. As for the leopard geckos, they were originally from the wild (Pakistan), but they have been kept under the lab conditions for 10 years. Since their life histories before the capture is not known (captured as fully grown adults with unknown age), they were approached as potentionally non-naive animals (L. Kratochvíl, personal communication) and hence the experiments with pyrazine were added to exclude the effect of neophobia of leopard geckos towards new malodours.

The experiments showed that major chemical compounds of MTG secretion of *G. lineatum* as well as the whole *G. lineatum* secretion are aversive for selected predators. MTG secretion of *G. lineatum* is a highly volatile liquid, which contains irritants and toxins (Stránský et al. 1998; Durak and Kalender 2009; Šanda et al. 2012). Irritants, such as tridecane, are effective against invertebrate predators (e.g. spiders, mantids or ants) whereas toxins, such as aldehydes, have function to deter vertebrate predators, e.g. insectivorous birds, lizards or small mammals (Aldrich 1988; Gunawardena and Herath 1991).

The present study revealed the behavioural aspects of aversive reaction towards repellent secretion of *G. lineatum* and its individual components and two true bug species. The differences among the selected predators could be alternatively explained also by differences in structural setting of their olfactory/vomerolfactory and oral cavity epithelium – presence of the "right" receptors. Detailed studies examining that alternative (Breer 2003; Araneda et al. 2004; Luu et al. 2004;) led to the conclusion that one single olfactory receptor (OR) can recognize multiple odorants, but on the other hand a single odorant could be recognized by multiple receptors (Araneda et al. 2000; Breer 2003). Short-chained and also unsaturated aldehydes show great potency as agonists and they may induce large activation of ORs (Araneda et al. 2000; Araneda et al. 2004). However, most of these studies were tested on mammalian olfactory epithelium and olfactory bulbs (see Araneda et al. 2000; Araneda et al. 2004); no data are available on taxa studied in this project.

It is poorly understood, how ORs influence olfactory bulbs and brain in birds, whose olfaction is often linked with navigation (Wallraff 2004; DeBose and Nevitt 2008). Information on reptiles is then more or less lacking. Since little is known about ORs in the predators selected for this study, this topic is here not explicitly included in discussion of the present results. Yet, it should be taken into account and undoubtedly would be worth of a detailed study.

5.1. Comparison of chosen lizard predators

The two lizard predators under study, leopard gecko and green lizard, represent quite distant clades of squamate reptiles, and, consequently, considerable differences between them can be expected. Yet, in addition to certain differences in their aversive reactions toward particular tested chemicals discussed below, a broad measure of similarities between them was found. The common features in their reactions can thus be tentatively ascribed to general characteristics of squamate reptiles in these respects.

Hexane did not have aversive effect for leopard geckos in any scored behaviour. Therefore, it was confirmed that hexane can be used as a solvent for the other chemical compounds. On the other hand, hexane had a slight aversive effect for green lizards in the chemical trials in scored behaviour (actually the weakest aversive effect from all tested chemicals). In the post-chemical trials, green lizards were already habituated to it and no aversive effect was recorded. It seems that green lizards as well as blue tits (when evaluating attack-eating intervals) can overcome the amount of toxin in the prey (Fink and Brower 1981; Skelhorn and Rowe 2006a,b,c,d).

Although the methoxypyrazines were found in some heteropteran species such as *Oncopeltus fasciatus* or *Murgantia histrionica* (Aldrich et al. 1996, 1997), no methoxypyrazines were found in the repellent secretion of *G. lineatum* (Šanda et al. 2012). Therefore, 2-isobutyl-3-methoxypyrazine was chosen as a positive control. This pyrazine did not have any aversive effect in experiments with leopard geckos in any scored behaviour. Moreover, there were no significant differences among all control groups (untreated mealworm, hexane and pyrazine). Therefore, it was possible to exclude the effect of neophobia of leopard geckos towards new malodours.

The mixture of three aldehydes had aversive effect for leopard geckos, but geckos reacted differently in separately scored behaviours. As for the approach latencies, the mixture of three aldehydes had the second strongest aversive effect in the chemical trials and the strongest aversive effect in the post-chemical trials. It seems that in the decision whether to approach the prey, the mixture of three aldehydes could play a role as a chemical signal of unpalatability of the prey, based upon the previously obtained association between the visual image of the prey and the nasty odour of the aldehydes.

Therefore, it seems that the chemical signal of aldehydes can act as a cue for learned avoidance in experienced predators (Marples and Roper 2004) and it can elicit generalization (Sexton 1964; McLain 1984) – leopard geckos hesitated significantly before approaching the prey even when the mealworms were no longer treated with the mixture of three aldehydes (post-chemical trials). On the other hand, in attack latencies and approach-attack intervals the mixture of three aldehydes had significant aversive effect in the chemical trials, but there was no significant aversive effect in the post-chemical trials. So, it seems that the mixture of three aldehydes might have the aversive effect on attacking and eating the prey only if it is present on the mealworm (chemical trials). In other words, when the predator overcomes the hesitation caused by the previous negative experience with the chemically treated prey, the attack itself depends strongly on the presence of the aldehydes on the prey. Similar situation was observed for green lizard predators in the chemical trials, where in all scored behaviours the effect of the mixture of three aldehydes could serve as a signal of unpalatability.

The mixture of three aldehydes and tridecane had a strong aversive effect on leopard geckos. For green lizards there was observed aversive effect but much weaker (however stronger than for oxoaldehyde – see below), than in the mixture of the aldehydes itself. In case of leopard geckos the results are in accordance with the hypothesis that aldehydes and n-tridecane are effective repellents when combined (Gunawardena and Herath 1991), but for other chosen predators the results disagree with this hypothesis – especially in the experiments with the bird predators (Gregorovičová et al. in preparation).

The results agree with the hypothesis that chemicals, which could have synergic effect, increase the potency of joint toxic loads compared to the effect of each chemical tested alone in case of leopard geckos but not in case of green lizards, where this combination decreases the potency (Skelhorn and Rowe 2005b) similarly to the birds. In attack latencies and approach-attack intervals, the geckos hesitated more to the mixture of three aldehydes and tridecane than to the mixture of three aldehydes. The reason why geckos and lizards reacted aversively towards tridecane could be explained by the olfaction or vomerolfaction mechanism (Halpern 1987; Schwenk 1993). Since leopard geckos reacted to the mixture of three aldehydes and tridecane more strongly, it seems that it could be mediated by dual olfactory mechanism (Schwenk 1993). So tridecane could play a role as effective repellent towards gecko predators (Gregorovičová and Černíková 2015b).

Oxoaldehyde did not have any aversive effect on leopard geckos. This could be caused by the fact that oxoaldehyde does not have a typical odour for human and probably even for birds. It seems that oxoaldehyde might be mediated by gustation, which is poorly developed in leopard geckos (Schwenk 1985; Jamniczky et al. 2009). Nevertheless, in case of green lizards oxoaldehyde had mild aversive effect on them (chemical trials). But this effect was weaker than aversive effect of the mixture of three aldehydes and tridecane. Therefore, oxoaldehyde might be mediated by gustation, which is well developed in green lizards (Schwenk 1985; Cooper 1991). Similar but much stronger aversive effect was observed in case of bird predators (both chemical and post-chemical trials – great tits). They have also well-developed gustation (Mason and Clark 2000) and reject potentially noxious prey based on the taste (Schlee 1986).

The *Graphosoma* secretion also had a greatly pronounced aversive effect. Leopard geckos hesitated most in approach and attack latencies in the chemical trials. These results indicate that the whole MTG secretion of *G. lineatum* may function as a signal as well as a secondary chemical defence. For green lizards the strongest aversive effect was observed when approaching the prey in the chemical trials. When evaluating attack latencies and approach-attack intervals the *Graphosoma* secretion had the second strongest aversive effect on green lizards. Moreover, the *Graphosoma* secretion had also the significant aversive effect for green lizards when evaluating all three scored behavioural elements in the post-chemical trials when the mealworms were no longer treated with the secretion. It seems that the *Graphosoma* secretion could play a role as a chemical signal of unpalatability of the prey, based upon the previously obtained association between the visual image of the prey and the nasty odour/taste of the *Graphosoma* secretion. Therefore, it seems that the chemical signal of *Graphosoma* secretion can act as a cue for learned avoidance in experienced predators (Marples and Roper 2004) and it can elicit generalization (Sexton 1964; McLain 1984) similarly to the mixture of three aldehydes.

For leopard geckos the presence of living specimen of *G. lineatum* before the trials with mealworms increased the repellent potency of the mixture of three aldehydes when attacking the prey (attack latencies) and when evaluating approach-attack intervals. Significant aversive effect was similar to the whole MTG secretion, when geckos attacked the prey (attack latencies). Furthermore, when evaluating approach-attack intervals, geckos hesitated even more to the mixture of three aldehydes in trials with tested chemicals, when the living specimen of *G. lineatum* was previously presented. On the other hand, the presence of the living striated shieldbug did not increase the aversive effect of the mixture of three aldehydes on the approach latencies at all. Therefore, it seems that the mixture of three aldehydes could function as a signal to the predator with prior experience with the striated shieldbug in the decision whether to attack the prey. It could be explained by associative learning of predators (Sexton 1964; Sexton et al. 1966; Shanbhag et al. 2010).

For green lizards the situation was a little bit more complicated, because they faced two true bug species – *Graphosoma lineatum* and *Pyrrhocoris apterus*.

The presence of living specimen of *G. lineatum* or *P. apterus* before the trials with mealworms increased the repellent potency of the mixture of three aldehydes in all scored behaviours in the chemical trials. Green lizards hesitated most to the mixture of aldehydes when attacking the prey (attack latencies) and when evaluating approach-attack intervals, in case the living shieldbug *G. lineatum* was previously presented. Therefore, the presence of shieldbug had stronger effect on green lizards than the presence of firebug, which is in agreement with hypothesis that shieldbug has more effective defence by spraying repellent secretion towards predator (M. Šanda, personal communication). Thanks to this type of defence, it seems that aldehydes have function as an odorous signal of unpalatable prey for green lizards.

On the contrary, when evaluating approach and attack latencies in the post-chemical trials, when the mealworms were no longer treated with the mixture of three aldehydes, the presence of living specimen of *P. apterus* before the trials with mealworms significantly increased the aversive effect of the mixture of three aldehydes and also elicited generalization (Sexton 1964; McLain 1984). This result could be attributed to the possible role of the mixture of three aldehydes as a potential chemical signal and its role in the prey generalization (Sexton 1964; Gregorovičová and Černíková 2015a,b) for the predator with prior experience with the firebug in the decision whether to approach and/or attack the prey. Additionally, this result could be also explained by previous negative experience of the predator from the wild with firebugs that could be demonstrated by the minimal bug manipulation in our experiments.

The obtained results agree with the hypothesis, that repellency is dependent mostly on the aldehydes (Eisner 1970; Hamilton et al. 1985; Gunawardena and Herath 1991). Geckos and lizards faced the predator's dilemma – to starve or to eat a potentially toxic prey (Glendinning 2007). It was observed very often that geckos rejected the mealworms previously treated with the particular chemical based on the manipulation with the mealworm, and also that they left the mealworm without any manipulation after approaching it. The same reactions were observed also for green lizards. Geckos cleaned their heads towards the substrate after attacking mealworm treated with the particular chemical compound or MTG secretion of *Graphosoma*. This behaviour was not observed for the green lizards. Geckos manipulated the living specimen of *G. linetaum* very carefully; they killed only two bugs and also showed defensive posture towards the shieldbug. Green lizards also manipulated *G. lineatum* very carefully – they even did not kill any of the offered bugs. In one case, lizard showed menace by opening mouth towards the shieldbug. As for *P. apterus*, there was no manipulation with the firebug at all except for one animal (younger one), which may be caused by possible previous negative experience with the firebug in the wild. Similar observation was made on the bird predators (Exnerová et al. 2007).

The firebug is not protected by spraying the secretion towards the predator as the striated shieldbug is (P. Štys, personal communication). This seems to have an influence on learning such a species of aposematic prey (and has a relation to the strength of aversive effect in the present study). It means that predator can easily learn based on the repellent secretion and therefore, it can learn to avoid such a prey without manipulation with the striated shieldbug (A. Exnerová, personal communication). Despite the fact that firebug is not able to protect itself by direct spraying the repellent secretion, it was observed that bird species can manipulate the firebug very carefully with little mortality of the firebug (Exnerová et al. 2006, 2007). Similar effect was observed in case of green lizards (the firebug was not tested against leopard geckos).

All this leads to one conclusion, that predator rejects chemically defended prey relatively unharmed – similarly to the bird predators (Boyden 1976; Wiklund and Järvi 1982; Skelhorn and Rowe 2006a).

It was also observed the aversive behaviour from a distance such as closing the eyes in the presence of the mealworm with the particular chemical (not in the presence of oxoaldehyde, hexane and pyrazine in case of leopard geckos) and with the whole MTG secretion. Therefore, it seems that some applied chemicals (such as the mixture of three aldehydes) and the whole MTG secretion have strong odorous function as a signal from distance as well as the potential to elicit pain when inhaled (eye, respiratory system). This could be triggered by short-chained aldehydes (e.g. trans-2-hexenal and trans-2-octenal) that show promise as trigeminal stimulants (Conner et al. 2007). Apart from the above described behaviour, a "grinning" behaviour was also observed in geckos (not in lizards) – similarly to birds (A. Exnerová, personal communication), which typically consists of shaking themselves when searching/approaching or attacking the prey with the particular chemical. Such behaviour was not observed when approaching/attacking the prey treated with hexane (geckos, lizards), oxoaldehyde (geckos) and pyrazine (geckos).

The rejection of chemically defended prey in geckos is probably based on olfaction/vomerolfaction (Halpern 1987; Schwenk 1993). Therefore, the major role may play olfactory aposematism (Eisner and Grant 1981; Weldon 2013). Geckos are highly sensitive to airborne volatiles, more than the other lizard species (Schwenk 1993). They have a well-developed olfactory chamber, and also the vomeronasal system and the olfactory bulbs are very large (Pratt 1948). Whereas olfaction is involved particularly in the detection of food and potential predators and responds primarily to volatiles, the vomeronasal system is focused on novel stimuli and reproductive behaviour and it is sensitive also to nonvolatiles (Schwenk 1993).

For green lizards rejection of repellent secretion is probably based on (1) gustation (Schwenk 1985; Bonacci et al. 2008) and (2) olfaction/vomerolfaction (Cooper 1991, 1996). The previous experiments showed that prey chemical discrimination is mediated by vomerolfaction rather than olfaction in lizards (see Cooper 1997). Since MTG secretion is highly odorous and volatile (e.g. aldehydes)

(Durak and Kalender 2009; Šanda et al. 2012), it seems that geckos as well as lizards can avoid such a prey based on odorous signal alone.

In leopard geckos the influence of the sex and weight was observed and there were differences between scored behaviour reactions. In all scored behaviour reactions heavier animals were slower. This may be caused by relatively lower nutritional impact of the prey and the existing fat deposits in heavier animals. Therefore, heavier animals were not forced to hunt that much (Trnik et al. 2011). Sex had a significant effect only on approach latencies, when males were faster than females. This could be caused by female caution towards new prey/situation – greater risk-sensitivity (Martín and López 1999), neophobia or dietary conservatism (Marples and Kelly 1999).

In case of green lizards there was neither impact of the sex nor the age at all. The weight influenced only approach-attack intervals, when heavier animals were faster and thus they could risk eating potentially dangerous prey. This could be explained by differences among tested individuals – inter-specific differences (Castilla et al. 2008).

5.2. Comparison of chosen bird predators

Two bird predators were studied: great tits and blue tits. The following paragraphs will again discuss the effect of the tested chemicals on aversive reactions of selected predators.

Hexane did not have any aversive effect for great tits, but it was specifically aversive for blue tits – hexane had an aversive after-effect (in the post-chemical trials) when blue tits attacked the prey, whereas when evaluating attack-eating intervals blue tits were able to habituate to it. Similar effect was observed also in case of green lizards (Gregorovičová and Černíková 2015a). It seems that this difference may be connected with predator's size and with ability to overcome toxin burdens. It seems that in case of hexane, blue tits mediated their response via post-ingestive feedback (Glendinning 2007). It is known that birds can overcome the amount of toxin in the prey (Fink and Brower 1981; Skelhorn and Rowe 2006a,b,c,d) and they may also learn to accept nasty taste as long as there is no toxic effect (Marples 2004). Similar situation could be possible also for lizards. Hexane could be an example of such a situation in blue tits and it may cause an after-effect as toxin burden when blue tits attacked the prey.

The mixture of three aldehydes had aversive effect for both species; however there is a difference between behavioural reactions (attack latencies and attack-eating intervals) to this compound. Whereas in case of great tits the mixture of three aldehydes had aversive effect in both behavioural reactions (attack latencies and attack-eating intervals), and therefore, the mixture could function as a chemical signal of unpalatability of the prey; in case of blue tits the same mixture caused aversive reaction only when birds attacked the prey in the post-chemical trials when the mealworms were no longer treated with the chemical. When evaluating attack-eating intervals when blue tits must decide to eat or not to eat, it seems that birds can overcome the mixture of three aldehydes in accordance with nutritive benefits of eating such a prey (Skelhorn and Rowe 2007).

The mixture of three aldehydes and tridecane did not have any effect on great tits when attacking the prey. It caused only a weak aversive reaction when evaluating attack-eating intervals. This result is in contradiction to the hypothesis that aldehyde and n-tridecane are effective repellents when combined (Gunawardena and Herath 1991). On the other hand, the results agree with the hypothesis that chemicals, which could have synergic effect, decrease the potency of the joint toxic loads compared to the effect of each chemical tested alone (Skelhorn and Rowe 2005b). This finding is in contradiction to other chosen predator – leopard gecko (Gregorovičová and Černíková 2015b). It seems that tridecane may have decreased the impact of the mixture of three aldehydes on its function as a signal. Therefore, tridecane was not tested against blue tits due to prediction, that there will be no effect of tridecane on blue tits, because of same type of chemical discrimination (Schlee 1986; Mason and Clark 2000).

Reactions of great tits support the hypothesis that tridecane is more effective towards invertebrate predators than vertebrate ones. This is partially true for birds (Gregorovičová et al. in preparation), but not for other vertebrate predators – leopard geckos and green lizards (Gregorovičová and Černíková 2015a,b).

Oxoaldehyde does not have typical odour for humans and probably not even for birds and it seems that it may function as a direct toxin. In great tits it had clear aversive effect in both scored behavioural reactions. However, in blue tits the situation was again slightly different. Whereas in attack latencies oxoaldehyde, similarly to the mixture of aldehydes, did not have aversive effect in the chemical trials, it had aversive effect in the post-chemical trials. This could be caused by after-effect of toxin burdens (Skelhorn and Rowe 2006a,b,c,d). When evaluating attack-eating intervals, oxoaldehyde caused aversive reaction in the chemical trials. This could mean that the decision of rejections could be made based on bird's intake according to their physiological state (Skelhorn and Rowe 2007) and/or whether toxin has delayed effect (Lett 1980; Franchina et al. 1997).

The *Graphosoma* secretion had strong aversive effect for both species. Blue tits avoided most the *Graphosoma* secretion in both scored behaviours – attack latencies (in both the chemical and post-chemical trials) and attack-eating intervals (the chemical trials). On the other hand, great tits hesitated most to oxoladehyde in the chemical trials in both scored behaviours as well as in the post-chemical trials when attacking the prey (attack latencies) – the *Graphosoma* secretion caused the second strongest aversive reaction.

When evaluating attack-eating intervals great tits hesitated most to oxoaldehyde in the chemical trials, whereas in the post-chemical trials they avoided the *Graphosoma* secretion most. The results clearly show differences between closely related bird species. Great tits avoided most oxoaldehyde, which could function as a direct toxin (Šanda et al. 2012), while blue tits hesitated most to *Graphosoma* secretion, which may function as a signal as well as a secondary chemical defence (Gregorovičová and Černíková 2015a,b; Gregorovičová et al. in preparation).

In agreement with the literature the results showed that repellent protection is dependent mostly on the aldehydes (Eisner 1970; Hamilton et al. 1985; Gunawardena and Herath 1991). Birds in the experiments were put under the predator's dilemma – to starve or to eat a potentially toxic prey (Glendinning, 2007) – similarly to the lizard predators. Therefore, it was observed that birds ate very often only parts of the prey. In that case they ate only inner parts of mealworm and dropped empty cuticle, where the particular chemical compound or MTG secretion of *Graphosoma* had been applied. This indicates that birds are able to detect the toxin not only at different concentrations but also the place of toxin storage/secretion (Fink and Brower 1981; Skelhorn and Rowe 2005a,b, 2006a,b,c,d, 2007, 2009, 2010; Skelhorn and Ruxton 2007; Holen 2013).

It is very profitable to secret repellent chemicals on the surface of the body as bugs do in the wild. It may accelerate learning and reduce the risk of predation compared to the storage of toxins inside the body of the prey (Skelhorn and Rowe 2009). For that reason, chemical compounds were applied on the surface of the mealworm to simulate the situation in the wild.

The rejection of chemically defended prey is probably based on (1) taste (Schlee 1986; Skelhorn and Rowe 2006a,b,c,d), but role may play also (2) olfactory aposematism (Eisner and Grant 1981; Weldon 2013) and (3) chemesthesis (Conner et al. 2007). In olfactory aposematism predators can learn an avoidance response based on odour. Since volatile compounds of MTG secretion are highly odorous (e.g. aldehydes), it seems that birds can avoid such a prey based on an odorous signal. These signals can be directly noxious or relatively innocuous and therefore, they represent warning signals for other toxic (non-volatile) compounds (Brower 1984). In our study it seems that aldehydes may have function as noxious volatile compounds as well as warning signals for oxoaldehyde for bird predators, because it is odourless for human and probably also for birds.

Birds have very different behavioural strategies when dealing with prey that have internal/external chemical defences (Skelhorn and Rowe 2006a). These "handling techniques" avoid or minimize contact with the secretion (Schlee 1986). In our experiments birds clearly used such handling techniques. They ate only inner parts, tore mealworms into pieces and wiped their beaks on perches during/after eating the mealworms. Birds can selectively reject visually identical prey based on their chemical investment (Skelhorn and Rowe 2006d). It was also observed aversive behaviour from distance such as blinking in the presence of the mealworm treated with the mixture of aldehydes and the whole MTG secretion. This indicates that such chemicals/whole secretion have strong odorous function as signals from a distance as well as they may elicit pain when inhaled (eye, respiratory system). This could be reliable signal related to the level of defence and it also indicates that chemical secretion could work as a signal and a secondary defence component (Gohli and Högstedt 2009). The third mechanism, which could be responsible for aversive reactions of birds, can be chemesthesis.

The mixture of aldehydes as well as the whole MTG secretion are burning substances for humans and they may work towards birds in a similar way. Aldehydes as well as MTG secretion could work as chemesthetic signal, causing pain in the eyes or in the respiratory system, because short-chained aldehydes (including trans-2-hexenal and trans-2-octenal) show promise as trigeminal stimulants (Conner et al. 2007). Except for this behaviour, a "grinning" behaviour was observed very often in birds (A. Exnerová, personal communication), which typically consists of ruffling their feathers and shaking themselves when searching the mealworm with the particular chemical compound from a distance and also before attacking such a prey. This behaviour indicates that bird can rouse recall associated with chemically defended prey previously eaten.

Since the tested bird species differed in body size, it could be concluded that differences between species may be caused by the level of toxin burden of particular bird species (Skelhorn and Rowe 2007). Therefore, it seems that hexane can cause aversive effect for the lesser of the tested bird species – blue tits. Cautious reactions to the mealworms in case of blue tits could be in accordance with greater innate neophobia (Exnerová et al. 2007) and with food conservatism (Marples et al. 1998; Marples et al. 2005; Marples and Kelly 1999; Kelly and Marples 2004). However, according to Beranová (personal communication), blue tit adults are less neophobic and more food-competitive compared to great tits, which may explain the differences in scored behaviours of both species. Although blue tit adults are less neophobic they tend to hesitate more in the beginning of the scored behavioural elements (Dana Adamová, personal communication).

Impact of the age and sex was observed only for great tits, but there were differences between scored behavioural reactions. In attack latencies the females were faster than the males and the age was not significant. This difference may be caused by nutritional impact of the prey for females during the winter (experiments were carried out during winter season). Situation for attack-eating intervals was quite opposite.

Whereas sex was not significant, younger birds were slower than older birds. This could be caused by innate caution, which is overcome by life experience (Exnerová et al. 2007; Skelhorn and Rowe 2006d). For blue tits, the age and sex were not significant in any scored behavioural reactions.

6. CONCLUSIONS

The comparative study of aversive effects of individual chemical compounds of repellent secretion of *Graphosoma lineatum* towards four different predator species revealed a broad measure of similarities among them particularly in response to a complete MTG secretion. It also confirmed the essential role of aldehydes in the repellent effect, yet in the response towards particular aldehydes the tested taxa significantly differ. It suggests that the chemical complexity of the repellent secretion might result from subsequent selection by predators of different groups. The outputs of particular experiments can be summarized as follows:

1. Hexane (used as a non-polar solvent for the other chemicals)

Hexane did not have any aversive effect on leopard geckos and great tits. In case of green lizards hexane had slightly aversive effect in the chemical trials, whereas in the post-chemical trials (when hexane was still present on the mealworms) green lizards were already habituated to it. The same behaviour was observed in case of blue tits when evaluating attack-eating intervals. On the other hand, hexane had an aversive after-effect on blue tits when birds attacked the prey in the post-chemical trials.

2. Pyrazine (positive control in the experiments with leopard geckos)

Pyrazine was used as a positive control in the experiments with leopard geckos in order to exclude the effect of neophobia towards new malodours. The results showed that pyrazine did not have any aversive effect on leopard geckos in any scored behaviour. Therefore, neophobia could be excluded for leopard geckos.

3. The mixture of three aldehydes

The mixture of three aldehydes had an aversive effect for all chosen predator species although the predators reacted differently. In case of lizard predators, this mixture caused aversive reaction when present on the mealworms (chemical trials). On the other hand, great tits were able to generalize the prey previously treated with the mixture of three aldehydes. In case of blue tits, this mixture caused an aversive after-effect (attack latencies). It seems that the mixture of three aldehydes could play a role as a signal of unpalatability and it could elicit generalization.

4. The mixture of three aldehydes and tridecane

The mixture of three aldehydes and tridecane had a strong aversive effect for leopard geckos. In this case tridecane probably increases the impact of the mixture of aldehydes to leopard geckos. In case of green lizards, tridecane decreased the potency of the mixture of three aldehydes, but still there was a stronger effect of this mixture and tridecane compared to oxoaldehyde. Similar effect was observed also for great tits where the mixture of three aldehydes and tridecane had only a weak effect in one of the two scored behaviours (attack-eating intervals).

5. Oxoaldehyde

Oxoaldehyde did not have any aversive effect for leopard geckos, whereas in case of green lizards there was observed a weak aversive effect. As for the bird predators, oxoaldehyde had a strong aversive effect for great tits, whereas for blue tits this effect was delayed. Oxoaldehyde could function as a direct toxin for great tits. For blue tits it had a strong after-effect. The reason why the predators reacted so differently towards oxoaldehyde could be explained by different levels of the gustation among the predators.

6. *Graphosoma* secretion

For all four chosen predators MTG secretion of *G. lineatum* had clearly an aversive effect and may function as a signal as well as a secondary chemical defence.

7. Presence of living specimen of *G. lineatum* before chemical sequence test

The presence of living specimen of *G. lineatum* increased the effect of the mixture of three aldehydes as a signal of unpalatability in leopard geckos as well as in green lizards.

8. Presence of living specimen of *P. apterus* before chemical sequence test

The presence of living specimen of *P. apterus* also increased the effect of the mixture of three aldehydes and also elicited generalization in green lizards. However, the strongest effect was observed in the presence of *G. lineatum,* rather than *P. apterus*.

7.REFERENCES

* secondary citation

Aldrich JR, Blum MS (1978) Aposematic aggregation of a bug (Hemiptera: Coreidae): The defensive display and formation of aggregations. Biotropica 10(1): 58 – 61

Aldrich JR (1988) Chemical ecology of the Heteroptera. Ann Rev Entomol 33: 211 – 238

Aldrich JR, Carroll SP, Lusby WR, Thompson MJ, Kochansky JP, Waters RM (1990) Sapindaceae, cyanolipids and bugs. J Chem Ecol 16(1): 199 – 210

Aldrich JR (1995) Chemical communication in true bugs and exploitation by parasitoids and commensals.In: Cardé RT, Bell WJ, eds. Chemical Ecology of Insects II. New York: Chapman & Hall, pp: 318 – 363

Aldrich JR, Avery JW, Lee CJ, Graf JC, Harrison DJ, Bin F (1996) Semiochemistry of Cabbage Bugs (Heteroptera: Pentatomidae: *Euryderma* and *Murgantia*). J Entomol Sci 31(2): 172 – 182

Aldrich JR, Leal WS, Nishida R, Khrimian AP, Lee CJ, Sakuratani Y (1997) Semiochemistry of aposematic seed bugs. Entomol Exp Appl 84: 127 – 135

Aliabadi A, Renwick JAA, Whitman DW (2002) Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. J Chem Ecol 28(9): 1749 – 1761

Angelici FM, Luiselli L, Rugiero L (1997) Food habits of the green lizard, *Lacerta bilineata*, in central Italy and a reliability test of faecal pellet analysis. Ital J Zool 64: 267 – 272

Aragón P, López P, Martín J (2001) Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav Ecol Sociobiol 50: 128 – 133

Araneda RC, Kini AD, Firestein S (2000) The molecular receptive range of an odorant receptor. Nat Neurosci 3: 1248 – 1255

Araneda RC, Peterli Z, Zhang X, Chesler A, Firestein S (2004) A pharmacological profile of the aldehyde receptor repertoire in rat olfactory epithelium. J Physiol 555(3): 743 – 756

Arnold EN (1987) Resource partitioning among lacertid lizards in southern Europe. J Zool 1(4): 739 – 782

Arnold EN (2002) Reptiles and Amphibians of Europe. Princeton University Press, Princeton and Oxford

Aronsson M, Gamberale-Stille G (2008) Domestic chicks primarily attend to colour, not pattern, when learning an aposematic coloration. Anim Behav 75: 417 – 423

Aukema B, Rieger CH (eds.) (2006) Catalogue of Heteroptera of the Palaearctic region. Wageningen: Ponsen & Looijen

Avery ML, Nelms CO (1990) Food avoidance by red-winged blackbirds conditioned with pyrazine odor. Auk 107(3): 544 – 549

Bang BG, Cob S (1968) The size of the olfactory bulb in 108 species of birds. Auk 85(1): 55 – 61

*Belman AL, Kare MR (1961) Character of salivary flow in the chicken. Poult Sci 40: 1377

Benes ES (1969) Behavioral evidence for color discrimination by the whiptail lizard, *Cnemidophorus tigris*. Copeia 4: 707 – 722

Benfield EF (1972) A Defensive Secretion of *Dineutes discolor* (Coleoptera: Gyrinidae). Ann Entomol Soc Am 65(6): 1324 – 1327

*Berkhoudt H (1985) Structure and function of avian taste receptors. In Form and function in birds III. Levy AS, McLelland (eds), Academic Press New York, pp: 463 – 496

Blum MS (1996) Semiochemical parsimony in the Arthropoda. Ann Rev Entomol 41: 353 – 374

Bohlin T, Gamberale-Stille G, Merilaita S, Exnerová A, Štys P, Tullberg B (2012) The detectability of the colour pattern in the aposematic firebug, *Pyrrhocoris apterus*: an image-based experiment with human 'predators'. Biol J Linnean Soc 105: 806 – 816

Böhme MU, Fritz U, Kotenko T, Džukić G, Ljubisavljevi K, Tzankov N, Berendonk T (2006) Phylogeography and cryptic variation within the *Lacerta viridis* complex (Lacertidae, Reptilia). Zool Scr doi:10.1111/j.1463-6409.2006.00262.x

Bonacci T, Aloise G, Brandmayr P, Brandmayr TZ, Capula M (2008) Testing the predatory behaviour of *Podarcis sicula* (Reptilia: Lacertidae) towards aposematic and non-aposematic preys. Amphibia-Reptilia 29: 449 – 453

*Borden JH (1984) Aggregation pheromons. In Kerkut GA, Gilbert LI (eds) Comprehensive Insect Physiology vol 9. Pergamon Press, Oxford, pp: 257 – 285

Boyden TC (1976) Butterfly palatability and mimicry: experiments with ameiva lizards. Evolution 30(1): 73 – 81

Breer H (2003) Olfactory receptors: molecular basis for recognition and discrimination of odors. Anal Bioanal Chem 377: 427 – 433

Brower LP (1984) Chemical defense in butterflies. IN: Vane-Wright RI, Ackery PR, EDS. The biology of butterflies. New York: Academic Press, pp: 109 – 134

Browers MD (1992) The ecolution of unpalatability and the cost of chemical defence in insects. Isman M, Roitberg BD (eds.) Evolutionary perspective in insect chemical ecology. Chapman and Hall London: 216 – 244

Burghardt GM (1970) Chemical perception in reptiles. Johnston JW, Moulton DG, Turk A (eds.) Advances in Chemoreception. I. Communication by chemical signals. Appleton – Century – Crofts, New York, pp: 241 – 308

Butler MA (2005) Foraging mode of the chameleon, *Bradypodion pumilum*: a challenge to the sit-and-wait versus active forager paradigm? Biol J Linnean Soc 84(4): 797 – 808

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

Castilla AM, Bauwens D, Llorente GA (1991) Diet composition of the lizard *Lacerta lepida* in Central Spain. J Herpetol 25(1): 30 – 36

Castilla AM, Herrel A, Gosá A (2008) Mainland versus island differences in behaviour of *Podarcis* lizards confronted with dangerous prey: the scorpion *Buthus occitanus*. J Nat Hist 42(35-36): 2331 – 2342

Carayon J (1971) Notes et documents sur l'appareil odorant métathoracique des Hémipteres. Ann Soc Entomol Fr 7(4): 737 – 770

Chai P, Srygley RB (1990) Predation and the flight, morphology and temperature of neotropical rain-forest butterflies. Am Nat 135(6): 758 – 765

Chapman RF (1998) The Insects: structure and function, 4th edition. Cambridge University Press

Clark L (1998) A review of bird repellents. Proceedings of the 20th Vertebral Pest Conference 18: 300 – 337

Clark L, Shah P (1991) Nonlethal bird repellents: in search of a general model relating repellency and chemical structure. J Wild Man 55: 538 – 545

Clark L, Avilova kv, Bean NJ (1993) Odor thresholds in passerines. Comp Biochem Physiol A 104: 305 – 312

Clark BF, Amiel JJ, Shine R, Noble DW, Whiting MJ (2014) Colour discrimination and associative learning in hatchling lizards incubated at ’hot’ and ‘cold’ temperatures. Behav Ecol Sociobiol 68(2): 239 – 247

Conner WE, Alley KM, Barry JR, Harper AE (2007) Has vertebrate chemesthesis been a selective agent in the evolution of arthropod chemical defenses? Biol Bull 213: 267 – 273

Cooper WE, Jr (1990) Prey odor detection by Teiid and Lacertid lizards and the relationship of prey odor detection to foraging mode in lizard families. Copeia 1: 237 – 242

Cooper WE, Jr. (1991) Responses to prey chemicals by a lacertid lizard, *Podarcis muralis*: prey chemical discrimination and poststrike elevation in tongue-flick rate. J Chem Ecol 17(5): 849 – 863

Cooper Jr, W. E. (1995). Foraging mode, prey chemical discrimination, and phylogeny in lizards. Anim Behav 50(4): 973 – 985

Cooper WE, Jr. (1996) Preliminary reconstructions of nasal chemosensory evolution in Squamata. Amphibia-Reptilia 17(4): 395 – 415

Cooper WE (1997) Correlated evolution of prey chemical discrimination with foraging, lingual morphology and vomeronasal chemoreceptor abundance in lizards. Behav Ecol Sociobiol 41: 257 – 265

Cooper WE (1999) Supplementation of phylogenetically correct data by two-species comparison: support for correlated evolution of foraging mode and prey chemical discrimination in lizards extended by first intrageneric evidence. Oikos 87(1): 97 – 104

Cooper WE (2007) Lizard chemical senses, chemosensory behaviour and foraging mode. Reilly SM, McBrayer LB, Miles DB (eds.) Lizard Ecology. Cambridge University Press, pp: 237 – 270

Cooper WE, Jr., Vitt LJ, Caldwell JP, Fox SF (2005) Relationships among foraging variables, phylogeny and foraging modes, with new data for nine north american lizard species. Herpetologica 61(3): 250 – 259

Cott HB (1940) Adaptive coloration in animals. London: Methuen & Co. Ltd

Cowles RB, Phelan RL (1958) Olfaction in rattlesnakes. Copeia 2: 77 – 83

Cramp S, Perrins C (1993) Handbook of the birds of Europe, the Middle East and North Africa. The birds of the western Palearctic, Vol. VII flycatchers to shrikes. Oxford University Press, New York

Dakrory AI (2011) Innervation of the olfactory apparatus of *Varanus niloticus* (Squamata – Lacertilia – Varanidae). J AM Sci 7(9): 118 – 125

DeBose JL, Nevitt GA (2008) The use of odors at different spatial scales: Comparing birds with fish. J Chem Ecol 34: 867 – 881

del Hoyo J, Elliott EA, Sargatal J, Christie DA (eds) (2007) Handbook of the Birds of the World. 12 Picathartes to Tits and Chickadees. Lynx Edicions, Barcelona

Dial BE, Schwenk K (1996) Olfaction and predator detection in *Coleonyx brevis* (Squamata: Eublepharidae), with comments on the functional significance of buccal pulsing in geckos. J Exp Zool 276: 415 – 424

Díaz JA, Carrascal LM (1993) Variation in the effect of profitability on prey size selection by the lacertid lizard *Psammodromus algirus*. Oecologia 94: 23 – 29

Duffey SS (1980) Sequestration of plant natural products by insects. Ann Rev Entomol 25: 447 – 477

*Duncan CJ (1960) The sense of taste in birds. Ann Appl Biol 48: 409 – 414

Durak D, Kalender Y (2009) Fine structure and chemical analysis of the metathoracic scent gland secretion in *Graphosoma lineatum* (Linnaeus, 1758) (Heteroptera, Pentatomidae). C R Biol doi: 10.1016/j.crvi.2008.10.004

*Edmunds M (1987) Color in opisthobranchs. Am Malacol Bull 5: 185 – 196

Eisner T (1970) Chemical defense against predation in arthropods. In: Sondheimer E, Simeone SB (eds). Chemical Ecology. New York: Academic Press

Eisner T, Grant RP (1981) Toxicity, odor aversion and “olfactory aposematism“. Science 213: 476

Eisthen HL (1997) Evolution of vertebrate olfactory systems. Brain Behav Ecol 50: 222 – 233

Estes R, de Queiroz K, Gauthier J (1988) Phylogenetic relationships within Squamata. Phylogenetic relationships of the lizard families 119: 281

Evans DL, Castoriades N, Badruddine H (1986) Cardenolides in the defense of *Caenocoris nerii* (Hemiptera). Oikos 46(3): 325 – 329

Evans DL, Schmidt JO, (eds) (1990) Insect defenses: adaptive mechanisms and strategies of prey and predators. Albany: State University of New York Press

Exnerová A, Štys P, Krištín A, Volf O, Pudil M (2003a) Birds as predators of true bugs (Heteroptera) in different habitats. Biologia 58: 253 – 264

Exnerová A, Landová E, Štys P, Fuchs R, Prokopová M, Cehláriková P (2003b) Reactions of passerine birds to aposematic and non-aposematic firebugs (Pyrrhocoris apterus; Heteroptera). Biol J Linnean Soc 78: 517 – 525

Exnerová A, Štys P, Barcalová S, Landová E, Prokopová M, Fuchs R, Socha R (2006) Importance of colour in the reaction of Passerine predators to aposematic prey: Experiments with mutants of *Pyrrhocoris apterus* (Heteroptera). Biol J Linnean Soc 88: 143 – 153

Exnerová A, Štys P, Fucíková E, Veselá S, Svádová K, Prokopová M, Jarošík V, Fuchs R, Landová E (2007) Avoidance of aposematic prey in European Tits (Paridae): Learned or Innate? Behav Ecol 18: 148 – 156

Exnerová A, Svádová K, Fousová P, Fučíková E, Ježová D, Niederlová A, Kopečková M, Štys P (2008) European birds and aposematic Heteroptera: review of comparative experiments. B Insectol 61(1): 163 – 165

Farine JP, Bonnard O, Brossut R, Le Quere JL (1992) Chemistry of defensive secretions in nymphs and adults of fire bug, *Pyrrhocoris apterus* L. (Heteroptera, Pyrrhocoridae). J Chem Ecol 18(10): 1673 – 1682

Fávaro CF, Rodrigues MACM, Aldrich JR, Zarbin PHG (2011) Identification of semiochemicals in adults and nymphs of the stink bug *Pallantia macunaima* Grazia (Hemiptera: Pentatomidae). J Braz Chem Soc 22(1): 58 – 64

*Feri D, Liguori GE, Lbate M (1982) La mucosa del *cavum nasi proprium* della lucertola campestre (*Podarcis sicula campestris* de betta). Atti Soc Pelorit Sc Fis Mat Natur 28: 75 – 81

Filoramo NI, Schwenk K (2009) The mechanism of chemical delivery to the vomeronasal organs in Squamate reptiles: A comparative morphological approach. J Exp Zool 311A: 20 – 34

Fink LS, Brower LP (1981). Birds can overcome the cardenolide defence of monarch butterflies in Mexico. Nature 291(7): 67 – 69

Forsman A, Merilaita S (1999) Fearful symmetry: pattern size and asymmetry affects aposematic signal efficacy. Evol Ecol 13: 131 – 140

Forsman A, Herrstrom J (2004) Asymmetry in size, shape, and color impairs the protective value of conspicuous color patterns. Behav Ecol 15: 141 – 147

Franchina JJ, Moon C, Peters S (1997) Effects of toxin magnitude on taste aversion and taste-potentiated aversion to visual cues in chicks (Gallus domesticus). Physiol Behav 62: 605 – 609

Fullard JH, Simmons JA, Sailant PA (1994) Jamming bat echolocation: the dogbane tigermoth *Cycnia tenera* times its click to terminal attack calls of the big brown bat *Eptesicus fuscus*. J Exp Biol 194: 285 – 298

Gabe M, Saint Girons H (1976) Contribution a la morphologie compareé des fosses nasales et de leur annexes chez les Lépidosauriens. Mém Mus Nat Hist Nat A98: 1 – 87

Gamberale G, Tullberg BS (1996) Evidence for a more effective signal in aggregated aposematic prey. Anim Behav 52: 597 – 601

Gamberale-Stille G (2001) Benefit by contrast: an experiment with live aposematic prey. Behav Ecol 12: 768 – 772

Gamberale-Stille G, Guilford T (2003) Contrast versus colour in aposematic signals. Anim Behav 65: 1021 – 1026

Games DE, Staddon BW (1973) Chemical expression of a sexual dimorphism in tubular scent glands of milkweed bug *Oncopeltus fasciatus* (Heteroptera: Lygaeidae). Experientia 29(5): 532 – 533

Gauthier JA, Kearney M, Anderson Maisano J, Rieppel O, Behlke ADB (2012) Assembling the Squamate tree of life: perspective from phenotype and fossil record. Bull Peabody Mus Nat Hist 53(1): 3 – 308

Ghostin J, Habib-Jiwan JL, Rozenberg R, Daloze D, Pasteels JM, Braekman JC (2007) Triterpene saponin hemi-biosynthesis of a leaf beetle's (*Platyphora kollari*) defensive secretion. Naturwissenschaften 94: 601 – 605

Glendinning JI (2007) How do predators cope with chemically defended foods? Biol Bull 213(3): 252 – 266

Godinho R, Crespo EG, Ferrand N, Harris DJ (2005) Phylogeny and evolution of the green lizards, *Lacerta* spp. (Squamata: Lacertidae) based on mitochondrial and nuclear DNA sequences. Amphibia-Reptilia 26: 271 – 285

Gohli J, Högstedt G (2009) Explaining the evolution of warning coloration: secreted secondary defence chemicals may facilitate the evolution of visual aposematic signal. Plos One 4(6): e5779

Grafe TU, Preininger D, Sztatecsny M, Kasah R, Dehling JM, Proksch S, Hödl W (2012) Multimodal communication in a noisy enviroment: A case study of the Bornean rock frog *Staurois parvus.* PLoS One 7(5): e37965

Green BG, Mason JR, Kare MR (1990) Chemical Senses, Vol. 2. Irritation. Marcel Dekker, New York

Green BG, Alvarez-Reeves M, George P, Akirav C (2005) Chemesthesis and taste: Evidence of independent processing of sensation intensity. Physiol Behav 86: 526 – 537

Gregorovičová, M., Černíková A. (2015a): Reactions of green lizards (*Lacerta viridis*) to major repellent compounds secreted by *Graphosoma lineatum* (Heteroptera: Pentatomidae). Zoology 118: 176 – 182 doi: 10.1016/j.zool.2015.02.001

Gregorovičová, M., Černíková A. (2015b): Reactions of leopard geckos (*Eublepharis macularius*) to defensive secretion of *Graphosoma lineatum* (Heteroptera Pentatomidae): an experimental approach. Ethology Ecology & Evolution doi: 10.1080/03949370.2015.1059895

Gross P (1993) Insect behavioral and morphological defenses against parasitoids. Annu Rev Entomol 38: 251 – 273

Guilford T (1990) The evolution of aposematism. In: Evans DL, Schmidt JO (eds) Insect defenses: Adaptive mechanisms and strategies of prey and predators. Albany: State University of New York Press, pp: 23 – 61

Guilford T, Dawkins MS (1993) Receiver psychology and the design of animal signals. Trends Neurosci 16: 430 – 436

Gunawardena NE, Herath HMWKB (1991) Significance of medium chain n-alkanes as accompanying compounds in hemipteran defensive secretions: An investigation based on the defensive secretion of *Coridius janus*. J Chem Ecol 17(12): 2449 – 2458

Halpern M (1987) The organization and function of the vomeronasal system. Annu Rev Neurosci 10: 325 – 362

Halpern M (1992) Nasal chemical senses in reptiles: structure and function. Gans C, Crews D (eds) In Hormones, Brain and Behavior. Biology of Reptilia, New York

Halpern M, Martínez-Marcos A (2003) Structure and function of the vomeronasal system: an update. Prog Neurobiol 70: 245 – 318

Hamilton JGC, Gough AJE, Staddon BW, Games DE (1985) Multichemical defense of plant bug *Hotea gambiae* (Westwood) (Heteroptera: Scutelleridae): (E)-2-Hexenol from abdominal gland in adults. J Chem Ecol 11(10): 1399 – 1409

Härlin C (2005) To have and have not: volatile secretions make a difference in gyrinid beetle predator defence. Anim Behav 69(3): 579 – 585

Härlin C, Härlin M (2003) Towards a historization of aposematism. Evol Ecol 17: 197 – 212

Harrap S, Quinn D (1996) Tits, nuthatches and treecreepers. Christopher Helm Publishers, London

Hatle JD, Faragher SG (1998) Slow movement increases the survivorship of a chemically defended grasshopper in predátory encounters. Oecologia 115: 260 – 267

Hatle JD, Salazar BA, Whitman DW (2002) Survival advantage of sluggish individuals in aggregations of aposematic prey, during encounters with ambush predators. Evol Ecol 16: 415 – 431

Hauglund K, Hagen SB, Lampe HM (2006) Responses of domestic chicks (*Gallus gallus domesticus*) to multimodal aposematic signals. Behav Ecol 17: 392 – 398

Hepburn HR, Yonke TR (1971) The metathoracic scent glands of coreoid Heteroptera. J Kans Entomol Soc 44(2): 187 – 210

Holen ØH. 2013. Disentangling taste and toxicity in aposematic prey. Proceedings of the Royal Society B 280: 20122588

Hotová Svádová K, Exnerová A, Kopečková M, Štys P (2010) Predator dependent mimetic complexes: Do passerine birds avoid Central European red-and-black Heteroptera? Europ J Entomol 107: 349 – 355

Hromádko M, Horáček J, Chytil J, Pithart K, Škopek J (1993) Příručka k určování našich pěvců. Část 2. pěnicovití - ťuhýkovití. Invence Litomyšl a Kroužkovací stanice NM Praha

Huey RB, Pianka E (1981) Ecological consequences of foraging mode. Ecology 62: 991 – 999

Huheey JE (1961) Studies in warning coloration and mimicry. III. Evolution of Müllerian Mimicry. Evolution 15(4): 567 – 568

Inbar M, Lev-Yadun S (2005) Conspicuous and aposematic spines in the animal kingdom. Naturwissenschaften 92: 170 – 172

Jamniczky HA, Russell AP, Johnson MK, Montuelle SJ, Bels VL (2009). Morphology and histology of the tongue and oral chamber of *Eublepharis macularius* (Squamata: Gekkonidae), with special references to the foretongue and its role in fluid uptake and transport. Evol Biol doi: 10.1007/s11692-009-9072-9

Jetz W, Rowe C, Guilford T (2001) Non-warning odors trigger innate color aversions – as long as they are novel. Behav Ecol 12(2): 134 – 139

Jiang P, Josue J, Li X, Glaser D, Li W, Brand JG, Margoslee RF, Reed DR, Beauchamp GK (2012) Reply to Zhao and Zhang: loss of taste receptor function in mammals is directly related to feeding specialization. Proc Natl Acad Sci 109: E1465

Johansen AI, Exnerová A, Hotová Svádová K, Štys P, Gamberale-Stille G, Tullberg BS (2010) Adaptive change in protective coloration in adult striated shieldbugs *Graphosoma lineatum* (Heteroptera: Pentatomidae): test of detectability of two colour forms by avian predators. Ecol Entomol 35: 602 – 610

Kardong K (2012) Vertebrates: comparative anatomy, function, evolution. Sixth edition. McGraw-Hill, New York

Kauppinen J, Mappes J (2003) Why are wasps so intimidating: field experiments on hunting dragonflies (Odonata: *Aeshna grandis*). Anim Behav 66: 505 – 511

Kelly DJ, Marples NM (2004). The effects of novel odour and colour cues on food acceptance by the zebra finch, *Taeniopygia guttata*. Anim Behav 68: 1049 – 1054

Khan MS (1999) Herpetology of habitat types of Pakistan. Pakistan J Zool 31(3): 275 – 289

Kirchner WH, Röschard J (1999) Hissing in bumblebees: an interspecific defence signal. Insectes Soc 46: 239 – 243

Kirkpatrick M, Rosenthal GG (1994) Symmetry without fear. Nature 372: 134 – 135

Klitzke CF, Trigo JR (2000) New records of pyrrolizidine alkaloid-feeding insects Hemiptera and Coleoptera on *Senecio brasiliensis*. Biochem Syst Ecol 28: 313 – 318

Košťál V, Šimek P (2000) Overwintering strategy in *Pyrrhocoris apterus* (Heteroptera): the relations between life-cycle, chill tolerance and physiological adjustments. J Insect Physiol 46: 1321 – 1329

Krall BS, Bartlet RJ, Lewis CJ, Whitman DW (1999) Chemical defense in the stink bug *Cosmopepla bimaculata*. J Chem Ecol 25(11): 2477 – 2494

Kratochvíl L, Frynta D (2002) Body size, male combat and the evolution of sexual dimorphism in eublepharid geckos (Squamata: Eublepharidae). Biol J Linnean Soc 76: 303 – 314

Kratochvíl L, Frynta D (2006) Body-size effect on egg size in eublepharid geckos (Squamata: Eublepharidae), lizards with invariant clutch size: negative allometry for egg size in ectotherms is not universal. Biol J Linnean Soc 88: 527 – 532

Lett BT. 1980. Taste potentiates color-sickness associations in pigeons and quail. Anim Learn Behav 8: 193 – 198

Lin W, Ogura T, Margolskee RF, Finger TE, Restrepo D (2008) TRPM5-expressing solitary chemosenrory cells respond to odour irritants. J Neurophysiol 99: 1055 – 1056

Lindström L, Rowe C, Guilford T (2001) Pyrazine odour make visually conspicuous prey aversive. Proc Roy Soc Lond B 268: 159 – 162

Lindström L, Lyytinen A, Mappes J, Ojala K (2006) Relative importance of taste and visual appearance for predator education in Müllerian mimicry. Anim Behav 72: 323 – 333

Linnavuori RE (2008) Studies on Acanthosomatidae, Scutelleridae and Pentatomidae (Heteroptera) of Gilan and the adjacent provinces in northern Iran. AEMNP 48(1): 1 – 21

Luu P, Acher F, Bertrand HO, Fan J, NGai J (2004) Molecular determinants of ligand selectivity in a vertebrate odorant receptor. J Neurosci 24(45): 10128 – 10137

MacDougall A, Dawkins MS (1998) Predator discrimination erreor and the benefits of Müllerian mimicry. Anim Behav 55: 1281 – 1288

Mallet J, Joron M (1999) The evolution of diversity in warning colour and mimicry. Ann Rev Ecol Syst 30: 201 – 233

Mappes J, Alatalo RV (1997) Effect of novelty and gregariousness in survival of aposematic prey. Behav Ecol 8: 174 – 177

Mappes J, Marples N, Endler JA (2005) The complex business of survival by aposematism. Trends Ecol Evol 20(11): 598 – 603

Marples NM, Roper TJ (1996) Effects of novel colour and smell on the response of naive chicks towards food and water. Anim Behav 51(6): 1417 – 1424

Marples NM, Roper TJ (1997) Response of domestic chicks to methyl anthranilate odour. Anim Behav 53: 1263 – 1270

Marples NM, Roper TJ, Harper DG (1998) Responses of wild birds to novel prey: Evidence of dietary conservatism. Oikos 83(1): 161 – 165

Marples NM, Kelly DJ (1999) Neophobia and dietary conservatism: two distinct processes? Evolutionary Ecology 13: 641 – 653

Marples NM (2004) The chemical defences of an aposematic complex: A case study of two-spot and seven-spot ladybirds. In: Van Emdem HF, Rothschild M, eds. Insect and Bird Interaction. Intercept

Marples NM, Roper TJ (2004) Warning colours and warning smells: How birds learn to avoid aposematic insects. In: Van Emdem HF, Rothschild M (eds) Insect and Bird Interactions. Intercept

Marples NM, Kelly DJ, Thomas RJ (2005) Perspective: The evolution of warning coloration is not paradoxical. Evolution 59(5): 933 – 940

Martín J, López P (1999) When to come out from a refuge: risk-sensitive and state-dependent decisions in an alpine lizard. Behav Ecol 10(5): 487 – 492

Martínez-Marcos A, Lanuza E, Halpern M (1999) Organization of teh ophidian amygdala: Chemosensory pathways to the hypotalamus. J Comp Neurol 412: 51 – 68

Mason JR, Adams MA, Clark L (1989) Anthranilate repellency to starlings: chemical correlates and sensory perception. J Wild Man 53(1): 55 – 64

Mason JR, Bean NJ, Shah PS, Clark L (1991) Taxon-specific differences in responsiveness to capsaicin and several analogues: correlates between chemical structure and behavioral aversivness. J Chem Ecol 17: 2539 – 2551

Mason RJ, Clark L (2000) The chemical senses in birds. In: Whittow GC, ed. Sturkie's Avian Physiology, fifth edition. Online access via Elsevier

Masters WM (1979) Insect disturbance stridulation: its defensive role. Behav Ecol Sociobiol 5: 187 – 200

Mastrota FN, Mench JA (1995) Colour avoidance in northern bobwhites: effects of age, sex and previous experience. Anim Behav 50: 519 – 526

McLain DK (1984) Müllerian mimicry between a plant bug (Miridae) and a seed bug (Lygaeidae) and the relationship between host plant choice and unapalatability. Oikos 43(2): 143 – 148

Mikátová B, Vlašín M, Zavadil V (eds) (2001) Atlas of the distribution of reptiles in the Czech Republic. Agentura ochrany přírody a krajiny České republiky Brno

Minton SA (1966) A contribution to the herpetology of West Pakistan. Bull AM Mus Nat Hist 134(2): 27 – 184

Moraes MCB, Pareja M, Laumann A, Borges M (2008) The chemical volatiles (semiochemicals) produced by neotropical stink bugs (Hemiptera: Pentatomidae). Neotrop Entomol 37(5): 489 – 505

Mori K (1995) Relation of chemical structure to specifity of respons in olfactory glomeruli. Curr Opin Neurobiol 5: 467 – 474

Mori K, Nagao H, Yoshihara Y (1999) The olfactory bulbs: coding and processing of odor molecule information. Science 286: 711 – 715

Moulet (1995) Hémiptéres Coreoidae, Pyrrhocoridae et Stenocephalidae euro-mediterranéens. Faune de France, France et régions limitrophes 81. Féd Franc Soc Sc Nat Paris: 1 – 336

Moulton DG, Beidler LM (1967) Structure and function in the peripheral olfactory systém. Physiol Rev 47: 1 – 52

Nagnan P, Cassier P, Andre M, Llosa JF, Guillaumin D (1994) Fine structure and physicochemical analysis of the metathoracic scent glands of *Lincus malevolus* (Rolston) and *Lincus spurcus* (Rolston) (Heteroptera: Pentatomidae). Int J Insect Morphol Embryol 23(4): 355 – 370

Nilsson M, Forsman A (2003) Evolution of conspicuous colouration, body size and gregariousness: a comparative analysis of lepidoptera larvae. Evol Ecol 17: 51 – 66

Nishida R (2002) Sequestration of defensive substances from plants by lepidoptera. Annu Rev Entomol 47: 57 – 92

Niskanen M, Mappes J (2005) Significance of the dorsal zig-zag pattern of *Vipera latastei graditana* against avian predators. J Anim Ecol 74: 1091 – 1101

*Nonoyama J (1936) The distribution of the taste buds on the tongue of some Reptilia. J Sci Hiroshima Univ Serv B 5: 57 – 66

Opitz SEW, Müller C (2009) Plant chemistry and insect sequestration. Chemoecology 19: 117 – 154

Pareja M, Borges M, Laumann RA, Moraes MCB (2007) Inter- and intraspecific variation in defensive compounds produced by five neotropical stink bug species (Heteroptera: Pentatomidae). J Ins Physiol 53: 639 – 648

Partan SR, Marler P (2005) Issues in the clasification of multimodal communication signals. Am Nat 166(2): 209 – 215

Pasteels JM (2007) Chemical defence, offence and alliance in ants-aphids-ladybirds relationships. Popul Ecol 49: 5 – 14

Pasteels JM, Duffey S, Rowell-Rahier M (1990) Toxins in chrysomelid beetles: possible evolutionary sequence from de novo synthesis to derivation from food plant chemicals. J Chem Ecol 16(1): 211 – 222

Pianka ER (1966) Convexity, desert lizards and spatial heterogeneity. Ecology: 1055 – 1059

Perry G (1999) The evolution of search modes: ecological versus phylogenetic perspectives. Am Nat 153(1): 98 – 109

Perry G, Pianka ER (1997) Animal foraging: past, present and future. Trends Ecol Evol 12: 306 – 364

*Portmann A (1961) Olfaction. In Marshall AJ (ed). Biology and comparative physiology of birds. Academic Press New York 2, pp: 42 – 48

Pough HF, Janis CM, Heiser JB (2005) Vertebrate life. Seventh edition. Pearson Education, Inc New Jersey

*Poulton EB (1890) The colours of animals: Their meaning and use especially considered in the case of insect. London: Kegan Paul, Trench, Trubner and Co Ltd

Pratt CWMcE (1948) The morphology of the ethmoidal region of Sphenodon and lizards. Proc Zool Soc 118: 171 – 201

Prudic KL, Skemp AK, Papaj DR (2006) Aposematic coloration, luminance contrast and the benefit of conspicuousness. Behav Ecol 18: 41 – 46

Prudic KL, Noge K, Becerra JX (2008) Adults and nymphs do not smell the same: The different defensive compounds of the gian mesquite bug (*Thasus neocalifornicus*: Coreidae). J Chem Ecol 34: 734 – 741

Pyron RA, Burbrink FT, Wiens JJ (2013) A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. BMC evolutionary biology 13(1): 93 – 146

Rastegar-Pouyani N, Gholi Kami H, Rajabzadeh M, Shafiei S, Anderson SC (2008) Annotated checklist of Amphibians and Reptiles of Iran. IJAB 4(1): 7 – 30

Ratcliffe JM, Soutar AR, Muma KE, Guignion c, Fullard JH (2008) Anti-bat flight activity in sound producing versus silent moths. Can J Zool 86: 582 – 587

Rehorek SJ, Firth BT, Hutchinson MN (2000) The structure of the nasal chemosensory system in squamate reptiles. 1. The olfactory organ, with special reference to olfaction in geckos. J Biosci 25(2): 173 – 179

Roper TJ (1994) Conspicuousness of prey retards reversal of learned avoidance. Oikos 69: 115 – 118

Roper TJ, Marples NM (1997) Odour and colour as cues for taste avoidance learning in domestic chicks. Anim Behav 53(6): 1241 – 1250

Rotheray GE (1986) Colour, shape and defence in aphidophagous syrphid larvae (Diptera). Zool J Linn Soc 88(23): 201 – 216

Rothschild M, Moore BP, Brown WV (1984) Pyrazines as warning odour components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera *Zygaena* and *Amata* (Lepidoptera). Biol J Linn Soc 23: 375 – 380

Roura E, Baldwin MW, Klasing KC (2013) The avian taste system. Potential implications in poultry nutrition. Anim Feed Sci Tech 180: 1 – 9

Rowe C (1999) Receiver psychology and the evolution of multicomponent signals. Anim Behav 58(5): 921 – 931

Rowe C (2002) Sound improves visual discrimination learning in avian predators. Proc R Soc B 269(1498): 1353 – 1357

Rowe C, Guilford T (1996) Hidden colour aversions in domestic chicks triggered by pyrazine odours of insect warning displays. Nature 383: 520 – 522

Rowe C, Guilford T (1999) The evolution of multimodal warning displays. Evol Ecol 13(7): 655 – 671

Rowe C, Halpin C (2013) Why are warning displays multimodal? Behav Ecol Sociobiol 67: 1425 – 1439

Rowe C, Skelhorn J (2004) Colour biases are a question of taste. Anim Behav 69: 587 – 594

Rowell-Rahier M, Pasteels JM, Alonso-Mejia A, Brower LP (1995) Relative unpalatability of leaf beetles with either biosynthesized or sequestered chemical defence. Anim Behav 49: 709 – 714

Rowland HM, Ruxton GD, Skelhorn J (2013) Bitter taste enhances predatory biases against aggregations of prey with warning coloration. Behav Ecol doi:10.1093/beheco/art013

Ruxton GD, Sherratt TN, Speed MP (2004) Avoiding Attack: The evolutionary ecology of crypsis, warning signals, and mimicry. New York: Oxford University Press

Ruxton GD, Sherratt TN (2009) Aggregation, defence and warning signals: the evolutionary relationship. Proc R Soc B 273: 2417 – 2424 doi:10.1098/rspb.2006.3570

Scudder GGE, Meredith J (1982). Morphological basis of cardiac glycoside sequestration by *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). Zoomorphology 99: 87 – 101

*Schifter H (1988) Bemerkenswerte Lebensdauer eines *Eublepharis macularius* (Blyth, 1854) (Sauria: Gekkonidae). In Gefangenschaft, Salamandra, Bonn 24 (4): 310 – 311

Seufer H, Kaverkin Y, Kirschner A (eds) (2005) The eyelash geckos: Care, breeding and natural history. Karlsruhe: Kirschner & Seufer Verlag

Sexton OJ (1960) Experimental studies of artificial Batesian mimics. Behaviour 15(3-4): 244 – 252

Sexton OJ (1964) Differential predation by the lizard, *Anolis carolinensis*, upon unicoloured and polycoloured insects after an interval of no contact. Anim Behav 12(1): 101 – 110

Sexton OJ, Hoger C, Ortleb E (1966) *Anolis carolinensis*: effects of feeding on reaction to aposematic prey. Science 153: 1140

Shanbhag BA, Ammanna VHF, Saidapur SK (2010) Associative learning in hatchlings of the lizard *Calotes versicolor*: taste and colour discrimination. Amphibia-Reptilia 31: 475 – 481

Schall JJ (2000) Learning in free-ranging populations of the whiptail lizard *Cnemidophorus murinus*. Herpetologica 56(1): 38 – 45

Schlee MA (1986) Avian predation on Heteroptera: Experiments on the European Blackbird *Turdus m. merula* L. Ethology 73(1): 1 – 18

Schmidt M, Nowack C, Wöhrmann-Repenning A (2010) On the presence of taste buds close to vomeronala organs in Gekkonidae. Amphibia-Reptilia 31: 355 – 361

Schwenk K (1985) Occurence, distribution and functional significance of taste buds in lizards. Copeia: 91 – 101

Schwenk K (1993) Are gecko olfactory specialist? J Zool 229: 289 – 302

Schwenk K (1995) Of tongues and noses: chemoreception in lizards and snakes. Trends Ecol Evol 10(1): 7 – 12

Siddall EC, Marples NM (2008) Better to be bimodal: the interaction of color and odor on learning and memory. Behav Ecol doi: 10.1093/beheco/arm155

Sindaco R, Jeremčenko VK (2008) The Reptiles of the Western Palearctic. 1. Annotated checklist and distributional atlas of the turtles, crocodiles, amphisbaenians and lizards of Europe, North Africa, Middle East and Central Asia. Edizioni Belvedere

Skelhorn J, Rowe C (2005a) Frequency-dependent taste-rejection by avian predation may select for defence chemical polymorphisms in aposematic prey. Biol Lett 1: 500 – 503

Skelhorn J, Rowe C (2005b) Tasting the difference: do multiple defence chemicals interact in Müllerian mimicry? Proc R Soc B 272: 339 – 345

Skelhorn J, Rowe C (2006a) Predator avoidance learning of prey with secreted or stored defences and the evolution of insect defences. Anim Behav 72: 827 – 834

Skelhorn J, Rowe C (2006b) Prey palatability influences predator learning and memory. Anim Behav 71: 1111 – 1118

Skelhorn J, Rowe C (2006c) Avian predators taste-reject aposematic prey on the basis of their chemical defence. Biol Lett 2: 348 – 350

Skelhorn J, Rowe C (2006d) Taste-rejection by predators and the evolution of unpalatability in prey. Behav Ecol Sociobiol 60: 550 – 555

Skelhorn J, Rowe C (2006e) Do the multiple defense chemicals of visually distinct species enhance predator learning? Behav Ecol doi:10.1093/beheco/arl028.

Skelhorn J, Rowe C (2007). Predator's toxin burdens influence their strategic decisions to eat toxic prey. Current Biology 17: 1479 – 1483

Skelhorn J, Griksaitis D, Rowe C (2008) Colour biases are more than a question of taste. Anim Behav 75(3): 827 – 835

Skelhorn J, Rowe C (2009) Distastefulness as an antipredator defence strategy. Anim Behav 78: 761 – 766

Skelhorn J, Rowe C (2010) Birds learn to use distastefulness as a signal of toxicity. Proc R Soc B 277, doi: 10.1095/rspb.2009.2092

Skelhorn J, Ruxton GD (2007) Ecological factors influencing the evolution of insects‘ chemical defenses. Behav Ecol doi: 10.1093/beheco/arm115

Smith SM (1980) Responses of naive temperate birds to warning coloration. Am Midl Nat 103: 346 – 352

Smith CL, Evans CS (2013) A new heuristic for capturing the complexity of multimodal signals. Behav Ecol Sociobiol 67: 1389 – 1398

Socha R (1993) *Pyrrhocoris apterus* (Heteroptera) – an experimental model species: A review. Eur J Entomol 90: 241 – 286

Speed MP (1993) Muellerian mimicry and the psychology of predation. Anim Behav 45: 571 – 580

Speed MP (1999) Batesian, quasi-Batesian or Müllerian mimicry? Theory and data in mimicry research. Evol Ecol 13: 755 – 776

Speed MP, Ruxton GD, Mappes J, Sherratt TN (2012) Why are defensive toxins so variable? An evolutionary perspective. Biol Rev doi: 10.1111/j.1469-185X.2012.00228.x

Staddon BW (1979) The scent glands of Heteroptera. Adv Insect Physiol 14: 351 – 418

Stager KE (1967) Avian olfaction. Am Zool 7(3): 415 – 420

Staples JK, Krall BS, Bartlet RJ, Whitman DW (2002) Chemical defense in the plant bug *Lopidea robiniae* (Uhler). J Chem Ecol 28(3): 601 – 615

Steiger SS, Kuryshev VY, Stensmyr MC, Kempenaers B, Mueller JC (2009) A comparison of reptilian and avian olfactory receptor gene repertoires: Species-specific expansion of group ɣ genes in birds. BMC Genomics 10:446 doi:10.1186/1471-2164-10-446

Stránský K, Valterová I, Ubik K, Čejka J, Křeček J (1998) Volatiles from stink bug, *Graphosoma lineatum* (L.), and from green shield bug, *Palomena prasina* (L.) (Heteroptera: Pentatomidae). J High Resolut Chrom 21(8): 475 – 476

Svádová K, Exnerová A, Štys P, Landová E, Valenta J, Fučíková A, Socha R (2009) Role of different colours of aposematic insects in learning, memory and generalization of naïve bird predators. Anim Behav 77: 327 – 336

Svensson L (2009) Identification guide to European passerines. Fourth Edition. Norwich: Page Bross.

Šanda M, Žáček P, Streinz L, Dračínský M, Koutek B (2012) Profiling and characterization of volatile secretions from the European stink bug *Graphosoma lineatum* (Heteroptera: Pentatomidae) by two dimensional gas chromatography/time-og-flight mass spektrometry. J Chromatogr B 881-882: 69 – 75

Šťastný K, Bejček V, Hudec K (2006) Atlas hnízdního rozšíření v ČR 2001-2003. Aventinum, Praha

Terrick TD, Mumme RL, Burghardt GM (1995) Aposematic coloration enhances chemosensory recognition of noxious prey in the garter snake *Thamnophis radix*. Anim Behav 49: 857 – 866

Tietz D, Zrzavý J (1996) Dorsoventral pattern formation: morphogenesis of longitudinal coloration in *Graphosoma lineatum* (Heteroptera: Pentatomidae). Eur J Entomol 93 15 – 22

Trigo JR (2011) Effects of pyrrolizidine alkaloids through different trophic levels. Phytochem Rev 10: 83 – 98

Trnik M, Albrechtová J, Kratochvíl L (2011) Persistent Effect of Incubation Temperature on Stress-Induced Behavior in the Yucatan Banded gecko (*Coleony elegans*). J Comp Psychol 125(1): 22 – 30

Tullberg BS, Merilaita S, Wiklund C (2005) Aposematism and crypsis combined as a result of distance dependence: functional versatility of the colour pattern in the swallowtail butterfly larva. Proc R Soc Lond B Biol Sci 272: 1315 – 1321

Tullberg BS, Gamberale-Stille G, Bohlin T, Merilaita S (2008) Seasonal ontogenetic colour plasticity in the adult striated shieldbug *Graphosoma lineatum* (Heteroptera) and its effect on detectability. Behav Ecol Sociobiol 62: 1389 – 1396

*Turner JRG (1987) The evolutionary dynamics of batesian and muellerian mimicry: similarities and differences. Ecol Entomol 12: 81 – 95

Uchida T (1980) Ultrastructural and histochemical studies on the taste buds in some reptiles. Arch Histol Jap 43(5): 459 – 478

Václav R, Prokop P, Fekiač V (2007) Expression of breeding coloration in European Green Lizards (Lacerta viridis): variation with morphology and tick infestation. Can J Zool 85: 1199 – 1206

Vallin A, Jakobson S, Lind J, Wiklund C (2006) Crypsis versus intimidation – anti-predation defence in three closely related butterflies. Behav Ecol Sociobiol 59(3): 455 – 459

VanderSal ND, Hebets EA (2007) Cross-modal effects on learning: a seismic stimulus improves color discrimination learning in a jumping spider. J Exp Biol 210: 3689 – 3695

Veselovský Z (2001) Obecná ornitologie. Akademie věd České Republiky, Praha

Veselý P, Veselá S, Fuchs R, Zrzavý J (2006) Are gregarious red-black shieldbugs, *Graphosoma lineatum* (Hemiptera: Pentatomidae), really aposematic? An experimental approach. Evol Ecol Res 8: 881 – 890

Vidal N, Hedges SB (2005) The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes. C R Biol 328(10): 1000 – 1008

Vidal N, Hedges SB (2009) The molecular evolutionary tree of lizards, snakes, and amphisbaenians. C R Biol 332(2): 129 – 139

Vitt LJ, Pianka ER, Cooper WE, Jr., Schwenk K (2003) History and the global ecology of Squamate reptiles. Am Nat 162(1): 44 – 60

*Wagner E (1965) Zur Systematic der Gattung *Graphosoma* Lap. (Hem., Het. Pentatomidae). Entomol Ber 16:110 – 116

Wachmann E, Melber A, Deckert J (2008) Wanzen, 4: Pentatomomorpha II. Die Tierwelt Deutschlands, 81. Goecke & Evers, Keltern

Wallraff HG (2004) Avian olfactory navigation: its empirical foundation and conceptual state. Anim Behav 67: 189 – 204

*Wang RT, Halpern M (1983) Surface modifications of the olfactory cilia in some reptiles. Anat Rec 205: 209A – 210A

Waterhouse DF, Gilby AR (1964) The adult scent glands and scent of nine bugs of the superfamily Coreoidea. J Ins Physiol 10: 977 – 987

Weldon PJ (2013) Chemical aposematism. Chemoecology 23: 201 – 202

Weldon PJ, Burghardt GM (2015) Evolving détente: the origin of warning signals via concurrent reciprocal selection. Biol J Linnean Soc doi: 10.1111/bij.12565

Wenzel BM (1987) The olfactory and related systems in Birds. Ann NY Acad Sci 519: 137 – 149

Werner YL (1972) Observations on eggs of eublepharid lizards, with comments on the evolution of the Gekkonoidea. Zool Med Leiden 47: 211 – 224

Wiklund C, Järvi T (1982) Survival of distasteful insects after being attacked by naive birds: A reappraisal of the theory of aposematic coloration evolving through individual selection. Evolution 36(5): 998 – 1002

Wiklund C, Vallin A, Friberg M, Jakobsson S (2008) Rodent predation on hibernating peacock and small tortoiseshell butterflies. Behav Ecol Sociobiol 62(3): 379 – 389

Woolfson A, Rothschild M (1990) Speculating about pyrazines. Proc. R. Soc. Lond. B 242(1304): 113 – 119

Yamawaki Y (2003) Responses to worm-like-wriggling models by the praying mantis: effects of amount of motion on prey recognition. J Ethol 21: 123 – 129

Internet sources:

1) www.iucnredlist.org
2) www.algrains.net
3) www.aquapage.cz
4) www.flickr.com
5) www.hbw.com
6) www.alexhydephotography.com
7) www.faunaeur.org

8.APPENDIX – ORIGINAL RESEARCH PAPERS