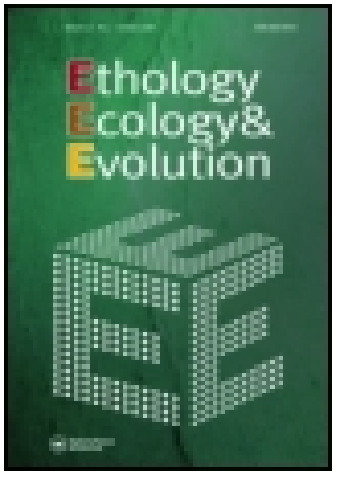

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Reactions of leopard geckos (Eublepharis macularius) to defensive secretion of Graphosoma lineatum (Heteroptera Pentatomidae): an experimental approach

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Reactions of leopard geckos (*Eublepharis macularius*) to defensive secretion of *Graphosoma lineatum* (Heteroptera Pentatomidae): an experimental approach

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Chemical protection of Heteroptera is mostly based on repellent secretion, which might signal the unpalatability of the bug to its potential predators or be directly toxic to predators. The aversive reactions of leopard geckos (*Eublepharis macularius*) were tested towards the major compounds of defensive secretion of *Graphosoma lineatum*: (1) a mixture of three aldehydes: (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (2) a mixture of three aldehydes and tridecane; (3) oxoaldehyde: (E)-4-oxohex-2-enal; (4) extracted metathoracic scent-glands secretion of *Graphosoma lineatum* adults and (5) hexane as a non-polar solvent. Additionally, (6) 2-isobutyl-3-methoxypyrazine was used to exclude the effect of neophobia. All chemicals were applied on a palatable food (*Tenebrio molitor* larvae). The aversive reactions of leopard geckos towards the mealworms were evaluated by observing the approach latencies, attack latencies and approach–attack intervals. Leopard geckos exhibited aversive reactions to the mixture of three aldehydes and also to this mixture and tridecane. Oxoaldehyde did not have any aversive effect. The whole metathoracic scent-glands secretion clearly had an aversive effect on geckos. Furthemore, when a living specimen of *Graphosoma lineatum* was offered to the 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.

KEY WORDS: aposematism, aversive reaction, repellent secretion, *Graphosoma lineatum*, *Eublepharis macularius*.

INTRODUCTION

Chemical signals can act as an important defence mechanism (Gohli & Högstedt 2009). The chemical signal could stimulate aversive reactions to visual signals, accelerate aversive learning and improve memorisation of the conspicuous prey (Marples &

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Roper 1996; Lindström et al. 2006; Skelhorn & Rowe 2006a, 2006b; Gohli & Högstedt 2009). The compounds of the chemical defence could be unpalatable, malodourous or directly toxic (Aldrich 1988); they may also cause nausea or vomiting (Staples et al. 2002; Ruxton et al. 2004). The compounds affect the predator before, during and/or after the attack (Skelhorn & Rowe 2005a, 2005b, 2006a, 2006b, 2006c, 2006d). The effectiveness of the chemical defence depends on the speed at which predators are able to associate warning signals with noxious toxins (Brower 1984; Skelhorn & Rowe 2010). Thus, visually oriented predators, such as birds or lizards, easily learn to avoid toxic insects (Benes 1969; Guilford 1990; Krall et al. 1999; Kelly & Marples 2004; Bonacci et al. 2008; Shanbhag et al. 2010).

The chemical defence among insect species shows great variability. True bugs (Heteroptera) obtain the chemical components by sequestration from host plants (Aliabadi et al. 2002) or de novo synthesis (Aldrich 1988). The following short-chained aldehydes belong to the most common compounds of Heteropteran defensive secretion: (E)-2-hexenal, (E)-2-decenal, (E)-2-octenal, 4-oxo-(E)-2-octenal and (E)-4-oxohex-2-enal; as well as other compounds such as n-tridecane (Aldrich 1988; Farine et al. 1992; Krall et al. 1999; Aliabadi et al. 2002; Šanda et al. 2012). The short-chained aldehydes are highly volatile and odorous, and they could act as irritants or be directly toxic (Eisner 1970; Hamilton et al. 1985). Irritants, such as n-tridecane (Gunawardena & Herath 1991), are effective against arthropod predators (Aldrich 1988), while toxins, such as α,β-unsaturated oxoaldehydes (Šanda et al. 2012), could protect bugs mostly against birds and other vertebrates, e.g. lizards (Aldrich 1988).

In lizards, the senses that mediate food chemical discrimination are vomerolfaction, olfaction and gustation (Schwenk 1985, 1993; Bonacci et al. 2008). In geckos, two major senses are involved in detecting the prey – vomerolfaction and olfaction (Schwenk 1993; Rehorek et al. 2000). According to Cowles and Phelan's hypothesis (Cowles & Phelan 1958), olfaction and vomerolfaction are functionally linked. Specifically, Cowles & Phelan (1958) state that the initial detection of chemical volatiles by the olfactory system triggers tongue-flicking, thus activating the vomeronasal system. Vomeronasal organs play a 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 the dual olfactory system (Schwenk 1993). Gustation is poorly developed in geckos (Schwenk 1985). There is no evidence of taste buds in leopard geckos (Schwenk 1985; Jamniczky et al. 2009). Therefore, the tongue-flicking may be directly linked to vomerolfaction (Schwenk 1993).

Since geckos have a dual olfactory system (Halpern 1980, 1987; Schwenk 1993; Dial & Schwenk 1996), the chemical defence of the striated shieldbug (*Graphosoma lineatum*), which is mainly composed of volatiles, could be aimed at this type of predator (a lizard with well-developed nasal senses – olfaction and vomerolfaction – or a combination of these two senses). Therefore, such a lizard predator – leopard gecko (*Eublepharis macularius*) – was chosen for this model study, and a striated shieldbug (*Graphosoma lineatum*) served as a model example of chemically defended prey. The repellent secretion of *G. lineatum* is well known and, according to the recent detailed analysis by Šanda et al. (2012), the following aldehydes belong to the most common compounds of the *G. lineatum* repellent secretion: (E)-2-hexenal, (E)-2-decenal, (E)-2-octenal, tridecane, (E)-4-oxohex-2-enal. The present study is focused on these compounds from the adult metathoracic scent-glands secretion.

(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 & 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 pers. comm.). The aldehyde mixture enriched with tridecane was tested to evaluate the hypothesis that tridecane serves as a catalyst for the aldehydes (Gunawardena & Herath 1991). In contrast, oxoaldehyde was included among the 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 geckos towards highly odorous compounds.

The present study had the following objectives: (1) to assess the aversive effect of particular chemical compounds of *Graphosoma* metathoracic scent-glands secretion, (2) to compare the aversive effect of selected chemical compounds with the whole metathoracic scent-glands secretion of *G. lineatum* and (3) to evaluate how the presence of a living specimen of *G. lineatum* influences reactions of leopard geckos to the mixture of aldehydes.

METHODS

Eublepharis macularius

Leopard geckos (*Eublepharis macularius*) were captured originally in the wild (Pakistan) as fully grown adults, and they have been kept under the defined laboratory conditions for 10 years. All tested geckos were adults, of both sexes. Geckos were kept in glass terraria of size 30 × 40 × 20 cm, temperature 27 °C, 50% humidity, 12 hr period light/dark cycle (06:00–18:00). The terraria were supplied with a drinking dish, a calcium dish and a box for laying eggs. Geckos were housed in the groups of three – one male, 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. Between 2010 and 2012, 77 leopard geckos were employed in experiments to examine the reaction of the geckos to various chemical compounds. The experiments were executed between September and the first week in December, which is after breeding season and before hibernation in autumn. One week before the experiments, geckos were removed from their breeding groups, and they were housed individually in terraria of sizes 20 × 40 × 20 cm to allow habituation to the laboratory environment. During the 1-week acclimation period, the geckos were kept at a temperature of 27 °C and 50% humidity, without feeding but offering water ad libitum. In captivity, geckos are standardly fed once a week. Therefore, feed deprivation for 1 week did not have any negative influence on their behaviour. The light conditions were set according to the 12 hr (06:00–18:00) period. Every gecko was weighed before the experiment. Sex was checked according to Seufer et al. (2005). Each gecko was put back into the breeding group the day after the experiments.

Graphosoma lineatum

Striated shieldbugs were picked up at several locations in Prague and kept in a thermostat-controlled environment at long-day photoperiod (16 hr light:8 hr dark) with the temperature oscillating between 24 °C (day) and 20 °C (night). They were supplied with water and with green tops, leaves and seeds of their host plants: carrot, *Daucus carota*; cow parsley, *Anthriscus sylvestris*; and garden angelica, *Angelica archangelica*.

Palatable prey

Mealworms (larvae of *Tenebrio molitor*, length ca 20 mm) were used for the experiments as a palatable prey, because geckos were normally fed with them. Therefore, it was possible to exclude the effect of neophobia towards experimental prey (see Methods – *Eublepharis macularius*). Tested chemicals were applied on the surface of the middle part of the dorsal side of a mealworm to simulate the situation in the wild when *G. lineatum* ejects the secretion on the surface of its body (Skelhorn & Rowe 2009). Adding chemicals on the surface of the middle part of the dorsal side of mealworms did not change their behaviour in any way.

Chemicals

The tested chemicals represent the major components of adult metathoracic scent-glands (MTG) secretion of striated shieldbug *G. lineatum* (Stránský et al. 1998; Šanda et al. 2012). The chemicals and mixtures tested were: (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 the other chemicals. (6) pyrazine (PYR): 2-isobutyl-3-methoxypyrazine was used as a positive control in order to exclude the effect of neophobia towards new malodours. This pyrazine is highly odorous (it occurs, for example, in wine) and represents another type of repellent signal.

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 & 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 pers. comm.). 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. Untreated mealworms (UM) without any chemicals added were used as controls.

Experimental equipment

Experiments were carried out in terraria of size 20 × 40 × 20 cm (length × depth × 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.

Testing procedure

The leopard geckos were split into eight experimental groups, which were balanced according to the sex of the geckos. Each gecko was tested only once. Geckos in each testing group were tested against one of the particular chemical compound and/or untreated mealworm (UM). The following compounds were tested in individual groups: the mixture of three aldehydes (3A), the

same mixture of aldehydes and tridecane (TA), oxoaldehyde (OXO), *Graphosoma* secretion (GS), hexane (HX), Living *Graphosoma* (LG/3A) followed by the chemical 3A, and pyrazine (PYR). The control group (UM) consisted of seven animals, whereas the remaining groups consisted of 10 animals. In each group, three males were present.

The testing sequence was composed of 10 mealworms presented sequentially in 5-min 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. Geckos from the control group (UM) were offered 10 untreated mealworms. Geckos from the hexane group (HX) were offered 10 hexane-treated mealworms.

For the testing of geckos' reactions to the living specimen of *G. lineatum* (LG/3A), the alternation of the untreated mealworm and the bug was used until the gecko had rejected the bug 3 times without any handling (manipulation by touching and/or taking it into the mouth). The bug was offered a maximum of 5 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. After this regime, the geckos were tested with the standard sequence corresponding to 3A to test the hypothesis that the presence of the living specimen of *G. lineatum* could increase the potency of the mixture of three aldehydes (3A), which could serve as a sufficient signal to avoid prey.

Behaviour was compared in different parts of the experimental sequence: (1) 'pre-chemical' trials in the beginning (mealworm no. 1), (2) 'chemical' trials with tested chemicals (mealworms no. 2–6), and (3) 'post-chemical' trials following the experience with chemicals (mealworms no. 7–10) to differentiate between immediate and persisting effect of the tested chemicals. In each trial, the gecko was allowed for 5 min to attack and potentially consume the mealworm; otherwise the trial was stopped. The trial was stopped earlier if the gecko consumed the prey. In each trial, the following behavioural characteristics were evaluated: (1) approach latencies – representing the time when the gecko started to come purposefully towards the prey; (2) attack latencies – representing the time when the gecko 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.

Statistical analyses

The data were analysed using the statistical program R 3.0.1. The original recorded values (i.e. observed time of reactions) are captured in Figs 1–3. Since in no case did the data show a normal distribution (Shapiro–Wilk normality test), numerical analyses based on ranks were applied.

Analysis of covariance (ANCOVA) was used to estimate the underlying model and to evaluate the impact of the chemicals. Simple analysis of variance (ANOVA), concerning only the effect of a chemical, would not have been sufficient because there were also other characteristics in the data, which could influence the time of reaction (like sex, age, etc.). ANCOVA enables us to compare the groups when controlling for other covariates. 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 characteristics) were used as the dependent variable instead of the real time values, and we 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.

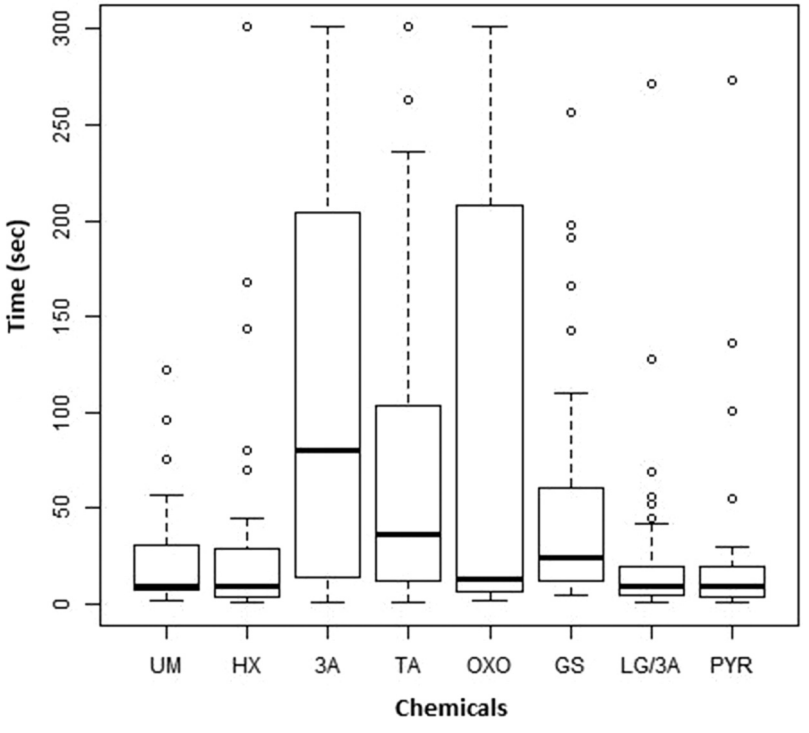

Fig. 1. — Approach latencies in trials following the experience with the chemicals – post-chemical trials (original values). Approach latencies are presented on the y-axis. The figures reflect the original recorded values (the time when the gecko started to come purposefully towards the prey). Band inside the box = median; box = lower and upper quartiles; whiskers = nonoutlier range; circles = outlier data. 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 *Graphosoma lineatum* followed by the mixture of three aldehydes; PYR – pyrazine.

A type II ANOVA table was used to evaluate the impact of the particular covariates. This type of ANOVA table is used to evaluate 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 interval) were estimated with a coefficient of the rank-based regression model (Estimate) – the higher its value, the slower the reaction of the animal and thus the stronger the aversion towards the particular chemical (Table 3).

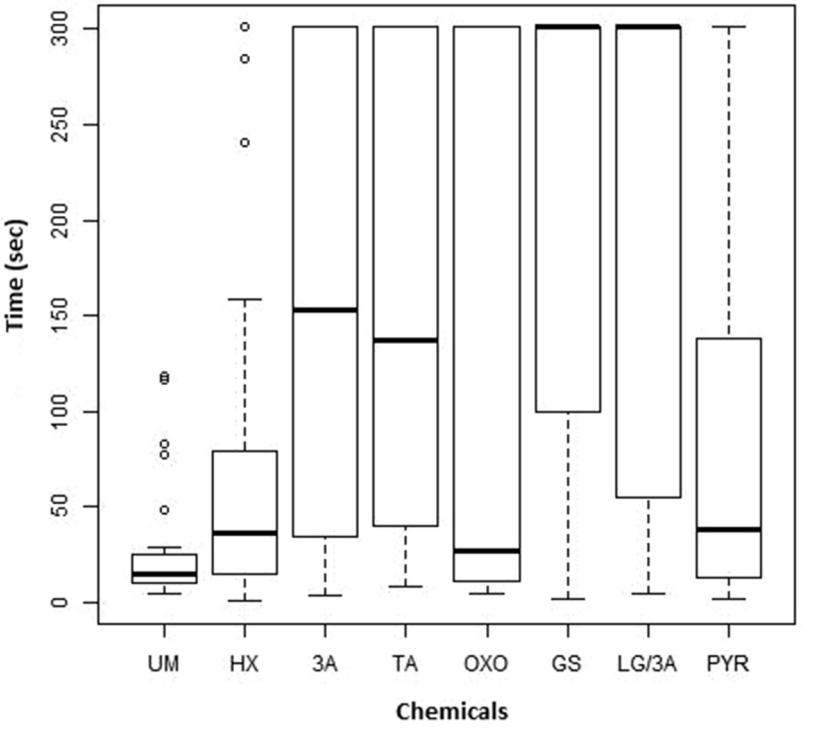

Fig. 2. — Attack latencies in trials with tested chemicals – chemical trials (original values). Attack latencies are presented on the y-axis. The figures reflect the original recorded values (the time when the gecko started to handle the prey). Band inside the box = median; box = lower and upper quartiles; whiskers = nonoutlier range; circles = outlier data. 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 *Graphosoma lineatum* followed by the mixture of three aldehydes; PYR – pyrazine.

Ethical note

Keeping of leopard geckos and experiments were carried out under permission no. 24773/2008-10001 and CZ 00059 issued by the Central Commission for Animal Welfare of the Czech Republic (UKOZ).

RESULTS

The design of the experiment included two additonal controls to the untreated mealworm control group (UM). Hexane (HX) was used as a non-polar solvent for the other chemicals of MTG secretion of *G. lineatum*, and pyrazine (2-isobutyl-3-methoxypyrazine) was used as a positive control to exclude the effect of neophobia to new malodours (PYR). For all behavioural characteristics (approach latencies, attack latencies and approach–attack intervals) and in all parts of the experimental sequence (pre-chemical trials, chemical trials and post-chemical trials), the reactions of leopard

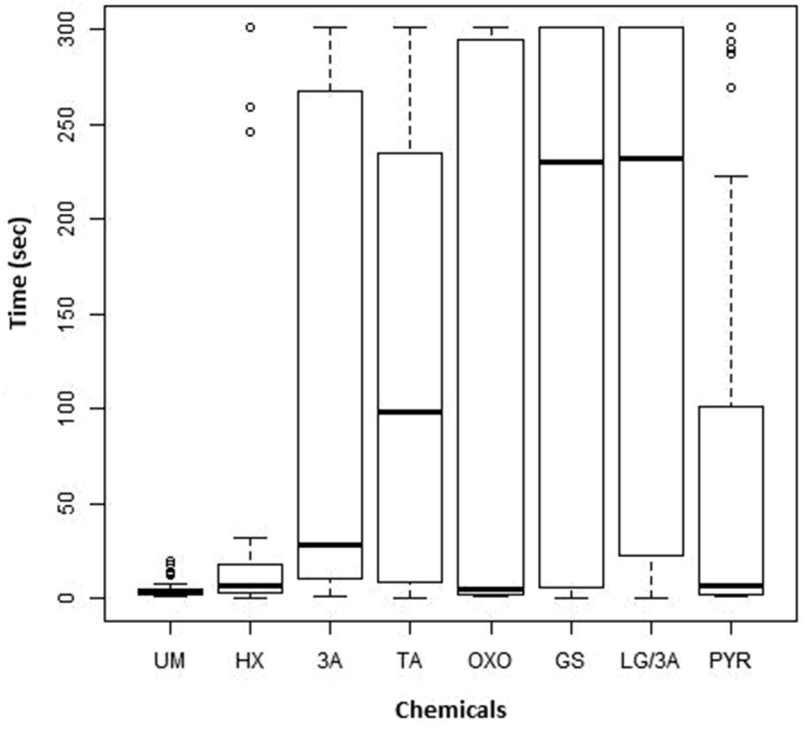

Fig. 3. — Approach–attack intervals in trials with tested chemicals – chemical trials (original values). Approach–attack intervals are presented on the y-axis. The figures reflect the original recorded values (the degree of hesitation between approaching the prey and attacking the prey). Band inside the box = median; box = lower and upper quartiles; whiskers = nonoutlier range; circles = outlier data. 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 *Graphosoma lineatum* followed by the mixture of three aldehydes; PYR – pyrazine.

geckos from the hexane and pyrazine groups did not significantly differ from those of the UM control group. The corresponding *P* values are shown in Table 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 (approach latencies, attack latencies and approach–attack intervals), 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 2A–C). Therefore, all geckos started the experiment with the same motivation.

The following sections describe the detailed results for individual behavioural characteristics and for all tested groups (3A-TA-OXO-GS-LG/3A). The corresponding results are summarised in Tables 2 and 3.

Table 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. HX and PYR groups consisted of 10 animals; the UM group consisted of seven animals. In each group, three males were present. 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

Finally, Table 4 summarises the impact of particular covariates (ANOVA type II) on individual behavioural characteristics.

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 the part of the experimental sequence ($P < 0.05$; $F = 1.971$; df1 = 14; df2 = 734). Statistical values are summarised in Table 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 ($P < 0.001$). However, approach latencies of leopard geckos tested with the rest of the chemicals did not significantly differ from geckos' reactions in the hexane group (see Table 2A).

In trials following the experience with the chemicals (post-chemical trials), leopard geckos that had previous experience with the mixture of aldehydes, with aldehyde mixture and tridecane and with *Graphosoma* secretion hesitated significantly longer

Table 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. Each tested group consisted of 10 animals, three of them were males. 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 *Graphosoma 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 3.

The aversive effect of the tested chemical compounds on the individual behavioural characteristics of leopard geckos. The UM group consisted of seven animals; all other tested groups consisted of 10 animals. In each group, three males were present. 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 *Graphosoma 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

than geckos from the hexane group before approaching the mealworms, even when they were no longer treated with the chemicals ($P < 0.01$; $P < 0.05$; $P < 0.05$; Fig. 1). 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 2A.

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 summarised in Table 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 (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

Table 4.

The impact of particular covariates on individual behavioural characteristics evaluated by using Type II analysis of variance (ANOVA) table (see Statistical analyses section in Methods).

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

significantly differ from geckos' reactions in the hexane group ($P = 1.000$). For details refer to Table 2B and Fig. 2.

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 (see Table 2B).

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 summarised in Table 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 (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 $P < 0.01$). 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 to Table 2C and Fig. 3.

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 (see Table 2C).

Manipulation of living specimens of G. lineatum

During the testing of geckos' reactions to the living specimen of *G. lineatum* (see Testing procedure), six geckos out of 10 manipulated the bug twice (out of a maximum of five offered bugs), two geckos only once and the remaining two geckos did not manipulate any of the three offered bugs, suggesting 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 five offered bugs was a sufficient number to gain the experience to avoid the bugs.

Aversive effects

Both the original observed data (Figs 1–3) and the results of the numerical analyses based on ranks (Tables 2 and 3) demonstrated (1) a highly significant aversive effect of the mixture of three aldehydes (3A), and an even more pronounced aversive effect of the same aldehyde mixture enriched with tridecane (TA); (2) persistence of the aversive effects indicated by a significant prolongation of approach latencies in post-chemical trials (3A-TA-GS); (3) non-significant aversive effects of hexane (HX), pyrazine (PYR) and oxoaldehyde (OXO). (4) By far the most pronounced aversive effects were demonstrated for *Graphosoma* secretion (GS) and for the aldehyde mixture presented after the geckos had been offered a living specimen of *Graphosoma lineatum* (LG/3A).

DISCUSSION

The experiments demonstrated that the major chemical compounds of MTG secretion of *G. lineatum* are aversive for leopard geckos. Together with green lizards (Gregorovičová & Černíková 2015), these are probably the first studies on the effects of individual compounds of the defensive secretion of true bugs against lizard predators. In some studies, such as Krall et al. (1999), an analysis of the particular true bug species (*Cosmopepla bimaculata*) was performed, but the individual chemical compounds were never tested against lizard predators; instead, only the predators' reactions towards the living bugs were evaluated.

Hexane, as a non-polar solvent for the other tested chemicals, did not have an aversive effect for leopard geckos in any scored behaviour. Therefore, the hexane group was used as the control group for the other chemical groups.

Since the geckos were captured as fully grown adults with unknown ages and life histories, they could not be treated as naïve animals (L. Kratochvíl pers. comm.). Nevertheless, a positive control – pyrazine – was chosen to test the hypothesis that leopard geckos could be neophobic towards new malodours. 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, we could use 2-isobutyl-3-methoxypyrazine as the positive control. The selected pyrazine did not cause any aversive reactions of leopard geckos in any scored behaviour. Moreover, there were no significant differences among all control groups (UM, HX, PYR) in any behavioural characteristics, nor in any part of the experimental sequence. Therefore, it was possible to exclude the effect of neophobia towards new malodours. Hence, leopard geckos could be exposed directly to *G. lineatum* and its secretion.

The mixture of three aldehydes had an aversive effect for leopard geckos, but geckos reacted differently in separately scored behaviours. The mixture of three aldehydes had the strongest aversive effect for approach latencies only in the trials following the experience with tested chemicals (post-chemical trials). The mixture of three aldehydes may play a role as a chemical signal of unpalatability of the prey, based on the previously obtained association between the visual image of the prey and the noxious 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 & Roper 2004) and it can elicit generalisation (Sexton 1960, 1964; McLain 1984). In attack latencies and approach–attack intervals, the mixture of three aldehydes had a significant aversive effect in the trials with tested chemicals, but there was no significant aversive effect in the trials following the experience with tested chemicals. Therefore, it appears that the mixture of three aldehydes might have an aversive effect on attacking and eating the prey only if it is present on the mealworm (chemical trials). The attack appears to depend strongly on the presence of the aldehydes on the prey when the predator overcomes the hesitation caused by the previous negative experience with the chemically treated prey. The same situation was observed for the remainder of the chemicals (except for oxoaldehyde, hexane and pyrazine) and MTG secretion.

The mixture of three aldehydes and tridecane had a strong aversive effect on leopard geckos, supporting the idea that combined aldehydes and n-tridecane are effective repellents (Gunawardena & Herath 1991). The results agree with the hypothesis that chemicals, which could have a synergic effect, increase the potency of joint toxic loads compared to the effect of each chemical tested alone (Skelhorn & Rowe 2005b). In attack latencies and approach–attack intervals, the geckos hesitated more with the mixture of three aldehydes and tridecane than with the mixture of three aldehydes. Geckos may react aversively towards tridecane due to the dual olfactory mechanism (Schwenk 1993). Since geckos have extremely well-developed olfaction and vomerolfaction, which are functionally linked (Cowles & Phelan 1958), compared to other lizards (Halpern 1980; Schwenk 1985, 1993), it seems that tridecane may play a role as a catalyst to the aldehyde mixture.

Oxoaldehyde may not have any aversive effect on leopard geckos because it is odourless. It seems that oxoaldehyde might be mediated by gustation, which is poorly developed in leopard geckos (Schwenk 1985; Jamniczky et al. 2009).

The *Graphosoma* secretion exhibited a particularly strong aversive effect. Leopard geckos hesitated most in approach/attack latencies in the corresponding 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. The repellent secretion of aposematic Heteroptera can have two functions – signalling the unpalatability and secondly, being toxic for the predators (Aldrich 1988; Gohli & Högstedt 2009). Since the whole MTG secretion of *G. lineatum* contains more than 100 chemical compounds (Šanda et al. 2012), this double function of the secretion cannot be excluded.

The presence of a 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. The significant aversive effect was similar to that of the whole MTG secretion, when geckos attacked the prey (attack latencies). Furthermore, when evaluating approach–attack intervals, geckos hesitated even more with the mixture of three aldehydes in trials with tested chemicals, when the living specimen of *G. lineatum* was previously presented. The presence of the living striated shieldbug did not increase the aversive effect of the mixture of three aldehydes on approach latencies. Therefore, it seems that the mixture of three aldehydes could play a role as a signal to the predator with prior experience with the striated shieldbug in the decision whether to attack the prey.

Leopard geckos were chosen as a model lizard predator to test their aversive reactions towards the major compounds of the repellent secretion of *G. lineatum*. However, both species might encounter each other in the nature because, according to P. Štys (pers. comm.), bugs of genus *Graphosoma* sleep on the ground. Therefore, leopard geckos, as nocturnal active foragers, may have the opportunity to encounter them.

The results agree with the hypothesis that repellency is dependent mostly on the aldehydes (Eisner 1970; Hamilton et al. 1985; Gunawardena & Herath 1991; present study). It was observed very often that geckos rejected the mealworms treated with the particular chemical after manipulating the mealworm, and also that they left the mealworm untouched after approaching it. Geckos cleaned their heads towards the substrate after attacking a mealworm treated with the particular chemical compound or MTG secretion of *Graphosoma*. Geckos manipulated the living specimen of *G. linetaum* very carefully; they killed only two bugs and also showed a defensive posture towards the shieldbug. The results indicate that predator rejects chemically defended prey relatively unharmed (Boyden 1976; Wiklund & Järvi 1982; Skelhorn & Rowe 2006a).

Geckos also exhibited the aversive behaviour from a distance such as closing the eyes in the presence of mealworm with the particular chemical (not in the presence of oxoaldehyde, hexane and pyrazine) and with the whole MTG secretion. Therefore, it seems that some applied chemicals and the whole MTG secretion have a strong odorous function as a signal from a distance as well as the potential to elicit pain when inhaled (eye, respiratory system). This may be attributed to 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 previously described behaviour, geckos showed also a 'grinning' behaviour (A. Exnerová pers. comm.), which typically consists of shaking themselves when searching/approaching or attacking the prey with a particular chemical. The geckos did not exhibit any avoidance behaviour when approaching/attacking the prey treated with hexane, oxoaldehyde and pyrazine.

The rejection of chemically defended prey in geckos is probably based on olfaction/vomerolfaction (Halpern 1987; Schwenk 1993). Therefore, olfactory aposematism may play the major role (Eisner & Grant 1981). Geckos are highly sensitive to airborne volatiles, more than the other lizard species (Schwenk 1993). Since MTG secretion is highly odorous and volatile (e.g. aldehydes; Durak & Kalender 2009; Šanda et al. 2012), it seems that geckos can avoid such a prey based on odorous signal alone.

In all reactions heavier animals were slower in response, which may be related to the relatively lower nutritional impact of the prey and the existing fat deposits in heavier animals. Therefore, heavier animals were not forced to hunt (Trnik et al. 2011). Sex was a significant variable only in approach latencies, when males were faster

than females, which could be caused by female caution towards new prey/situation – greater risk-sensitivity (Martín & López 1999).

Chemical defence is widespread across the animal kingdom, but our understanding of its principles is still not sufficient. Therefore, more comparative studies on which chemical compounds are responsible for aversive reactions in different types of predators will have to be performed in order to deepen our knowledge.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

REFERENCES

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

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

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. 28:1749–1761.

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

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.

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

Brower LP. 1984. Chemical defense in butterflies. In: Vane-Wright RI, Ackery PR, editors. The biology of butterflies. New York: Academic Press; p. 109–134.

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.

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

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.

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. 332:34–42.

Eisner T. 1970. Chemical defense against predation in arthropods. In: Sondheimer E, Simeone SB, editors. Chemical ecology. New York: Academic Press; p. 157–217.
Eisner T, Grant RP. 1981. Toxicity, odor aversion and "olfactory aposematism". Science. 213:476.
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. 18:1673–1682.
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: e5779.
Gregorovičová M, Černíková A. 2015. Reactions of green lizards (*Lacerta viridis*) to major repellent compounds secreted by *Graphosoma lineatum* (Heteroptera: Pentatomidae). Zoology. doi:10.1016/j.zool.2015.02.001.
Guilford T. 1990. The evolution of aposematism. In: Evans DL, Schmidt JO, editors. Insect defenses: adaptive mechanisms and strategies of prey and predators. Albany: State University of New York Press; p. 23–61.
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. 17:2449–2458.
Halpern M. 1980. Chemical ecology of terrestrial vertebrates. In: Gilles R, editor. Animals and environmental fitness. New York: Pergamon Press; p. 263–282.
Halpern M. 1987. The organization and function of the vomeronasal system. Annu Rev Neurosci. 10:325–362.
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. 11:1399–1409.
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. 36:397–406.
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.
Krall BS, Bartlet RJ, Lewis CJ, Whitman DW. 1999. Chemical defense in the stink bug *Cosmopepla bimaculata*. J Chem Ecol. 25:2477–2494.
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.
Marples NM, Roper TJ. 1996. Effects of novel colour and smell on the response of naive chicks towards food and water. Anim Behav. 51:1417–1424.
Marples NM, Roper TJ. 2004. Warning colours and warning smells: how birds learn to avoid aposematic insects. In: Van Emdem HF, Rothschild M, editors. Insect and bird interactions. Andover: Intercept; p. 185–192.
Martín J, López P. 1999. When to come out from a refuge: risk-sensitive and state-dependent decisions in an alpine lizard. Behav Ecol. 10:487–492.
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. 43:143–148.
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. 25:173–179.
Ruxton GD, Sherratt TN, Speed MP. 2004. Avoiding attack: the evolutionary ecology of crypsis, warning signals, and mimicry. New York: Oxford University Press.
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.

Schwenk K. 1985. Occurence, distribution and functional significance of taste buds in lizards. Copeia. 1985:91–101.
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. 10:7–12.
Seufer H, Kaverkin Y, Kirschner A, editors. 2005. The eyelash geckos: care, breeding and natural history. Karlsruhe: Kirschner & Seufer Verlag.
Sexton OJ. 1960. Experimental studies of artificial Batesian mimics. Behaviour. 15:244–252.
Sexton OJ. 1964. Differential predation by the lizard, *Anolis carolinensis*, upon unicoloured and polycoloured insects after an interval of no contact. Anim Behav. 12:101–110.
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.
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. 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. 277:1729–1734.
Staples JK, Krall BS, Bartlet RJ, Whitman DW. 2002. Chemical defense in the plant bug *Lopidea robiniae* (Uhler). J Chem Ecol. 28:601–615.
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 Chromatogr. 21:475–476.
Trnik M, Albrechtová J, Kratochvíl L. 2011. Persistent effect of incubation temperature on stress-induced behavior in the Yucatan Banded gecko (*Coleonyx elegans*). J Comp Psychol. 125:22–30.
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. 36:998–1002.