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Reactions of green lizards (*Lacerta viridis*) to major repellent compounds secreted by *Graphosoma lineatum* (Heteroptera: Pentatomidae)

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ABSTRACT

The chemical defence of Heteroptera is primarily based on repellent secretions which signal the potential toxicity of the bug to its predators. We tested the aversive reactions of green lizards (*Lacerta viridis*) towards the major compounds of the defensive secretion of *Graphosoma lineatum*, specifically: (i) a mixture of three aldehydes: (E)-hex-2-enal, (E)-oct-2-enal, (E)-dec-2-enal; (ii) a mixture of these three aldehydes and tridecane; (iii) oxoaldehyde: (E)-4-oxohex-2-enal; (iv) secretion extracted from metathoracic scent glands of *G. lineatum* adults and (v) hexane as a non-polar solvent. All chemicals were presented on a palatable food (*Tenebrio molitor* larvae). The aversive reactions of the green lizards towards the mealworms were evaluated by observing the approach latencies, attack latencies and approach–attack intervals. The green lizards exhibited a strong aversive reaction to the mixture of three aldehydes. Tridecane reduced the aversive reaction to the aldehyde mixture. Oxoaldehyde caused the weakest, but still significant, aversive reaction. The secretion from whole metathoracic scent glands also clearly had an aversive effect on the green lizards. Moreover, when a living specimen of *G. lineatum* or *Pyrrhocoris apterus* (another aposematic red-and-black prey) was presented to the green lizards before the trials with the aldehyde mixture, the aversive effect of the mixture was enhanced. In conclusion, the mixture of three aldehydes had the strong aversive effect and could signal the potential toxicity of *G. lineatum* to the green lizards.

1. Introduction

Chemical defence is widespread across the animal kingdom (Ruxton et al., 2004) and is very often linked with aposematism (Skelhorn and Rowe, 2006c). Chemical defence can function as a signal and/or as the defence itself (Gohli and Högstedt, 2009). The chemical signal may enhance aversive reactions to visual signals, accelerate aversive learning and improve memorisation of the conspicuous prey (Marples and Roper, 1996; Lindström et al., 2006; Skelhorn and Rowe, 2006a,b). Various compounds that are either unpalatable, malodourous or directly toxic are used in chemical defence (Aldrich, 1988). Some compounds are also known to cause nausea or vomiting (Staples et al., 2002; Ruxton et al., 2004). The potency of the chemical defence depends on the speed at which predators are able to associate the warning signals with noxious toxins (Brower, 1984; Skelhorn and Rowe, 2010).

True bugs (Heteroptera) have the ability to produce and/or store large amounts of chemical compounds (Aldrich, 1988). The bugs obtain the chemical components by sequestration from host plants (Aliabadi et al., 2002) or de novo synthesis (Aldrich, 1988). The most common compounds of heteropteran defensive secretions are: (E)-2-hexenal, (E)-2-decenal, (E)-2-octenal, 4-oxo-(E)-2-octenal, (E)-4-oxohex-2-enal, and *n*-tridecane (Aldrich, 1988; Aldrich et al., 1997; Krall et al., 1999; Durak and Kalender, 2009; Šanda et al., 2012). These short-chained aldehydes are highly volatile, malodorous (Šanda et al., 2012), and may function as irritants or direct toxins (Eisner, 1970; Hamilton et al., 1985). Whereas toxins such as α,β-unsaturated oxoaldehydes (Šanda et al., 2012) are effective primarily against birds and other vertebrates (Aldrich, 1988), other compounds, such as *n*-tridecane (Gunawardena and Herath, 1991), are effective against arthropod predators (Aldrich, 1988).

Lizards sensu lato (order Squamata) are also important insectivorous predators (Mitchell, 1979; Arnold, 1987) with specific

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predation of aposematic prey (Boyden, 1976). The lizards are able to discriminate aposematic prey based on e.g. visual and/or olfactory signals (Sexton, 1960; Burghardt et al., 1973). Furthermore, they are able to learn warning signals and to avoid aposematic prey based on previous experience (Sexton, 1964). Chemical discrimination is well developed in lizards (Burghardt et al., 1973; Cooper, 1995; Terrick et al., 1995; Besson et al., 2009). The senses that mediate chemical discrimination of food are olfaction, vomerolfaction and gustation (Schwenk, 1985, 1993; Bonacci et al., 2008). In green lizards, all three senses are involved in detecting the prey (Schwenk, 1993; Bonacci et al., 2008). In Lacertidae, olfaction (Gabe and Saint Girons, 1976; Cooper, 1996) and gustation (Schwenk, 1985; Bonacci et al., 2008) are well developed. In green lizards (*Lacerta viridis*), the taste buds are well developed on the ventrolateral surfaces of the foretongue and on the long tines of the forked tongue tip (Schwenk, 1985). Another sense that could be responsible for food discrimination is vomerolfaction (Cooper, 1991, 1996). Rapid tongue-flicking might help detect specific prey chemical patterns, which suggests the possibility of a chemical search image of the prey (Cooper, 1991).

Since green lizards have well developed olfactory, vomerolfactory and gustatory senses, the chemical defence of aposematic Heteroptera might function against this type of predator. Moreover, it is known that the members of the family Lacertidae are natural predators of heteropteran species (Castilla et al., 1991; Díaz and Carrascal, 1993; Angelici et al., 1997). Especially younger green lizards are known to hunt Heteroptera (Angelici et al., 1997). Therefore, *L. viridis* was chosen as a model predator. It inhabits the same type of habitat (Arnold, 2002; Sindaco and Jeremčenko, 2008) as the striated shieldbug (*Graphosoma lineatum)*, a chemically defended prey which has a widespread distribution in Central Europe (Wagner, 1965; Aukema and Rieger, 2006). All three model species (*L. viridis*, *G. lineatum* and *Pyrrhocoris apterus*) used in this study were observed in the same habitat–bushy forest-steppe with good exposure to sunlight (Arnold, 2002; Rabitsch, 2005). The repellent secretion of *G. lineatum* was described in detail by Šanda et al. (2012) and according to that study the major compounds of the secretion were selected for our experiments.

The objectives of the present study were (i) to evaluate the aversive effect of particular chemical compounds of the *Graphosoma* metathoracic scent gland secretion, (ii) to compare the aversive effect of selected chemical compounds with that of the whole metathoracic scent gland secretion of *G. lineatum*, and (iii) to investigate how the presence of living specimens of *G. lineatum* and *P. apterus* influences the reactions of green lizards to the mixture of three selected aldehydes.

2. Materials and methods

2.1. Green lizards

Between 2010 and 2012, 84 green lizards (*L. viridis*) 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). The lizards were housed individually in glass terraria (20 cm × 40 cm × 20 cm) at a temperature of 29 °C, 45% humidity, and a 12 h period of light/dark cycle (6:00 am–6:00 pm). The terraria were supplied with a drinking dish and a small hiding place. Immediately after housing, the lizards were fed with adult crickets fortified with vitamin powder for reptiles, but they were fed only once before the experiments. The lizards were allowed to habituate to the laboratory environment for 1 week before the experiments, without further feeding, but with water ad libitum. Each lizard was weighed before the experiment. Two categories were determined: adults and subadults. Sex and age were checked according to Arnold (2002). Each lizard was released back to the wild at the exact location of the capture the week after the experiments.

Capturing of the lizards and all experiments were carried out under permits no. 24773/2008-10001 and CZ 00059 issued by the Central Commission for Animal Welfare of the Czech Republic (UKOZ). Permits to catch green lizards were obtained from Podyjí National Park (SZ NPP 0108/2010/8, NPP 0967/2010).

2.2. Graphosoma lineatum

The striated shieldbug (*G. lineatum*, Heteroptera: Pentatomidae) was chosen as a model true bug species. Shieldbugs were gathered from several localities in Prague and kept in a thermostat-controlled environment, with the temperature oscillating between 24 °C (day) and 20 °C (night), at a long-day photoperiod (16L:8D), thus simulating natural conditions. 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 ad libitum.

2.3. Pyrrhocoris apterus

The firebug (*Pyrrhocoris apterus*, Heteroptera: Pyrrhocoridae) was chosen as a second model organism to test the lizards' reactions to another living specimen of aposematic red-and-black Heteroptera (Hotová Svádová et al., 2010), which lives in the same habitat as the captured green lizards (Podyjí National Park). The firebugs were kept in captivity under simulated natural conditions, similarly to the striated shieldbug. The firebugs were fed on host plants and seeds of Malvaceae, Tiliaceae, Bombacaceae and Sterculiaceae, supplemented with water ad libitum.

2.4. Chemicals

The tested chemicals represent major components of the metathoracic scent gland (MTG) secretion of adult *G. lineatum* (Stránský et al., 1998; Šanda et al., 2012). The following chemicals and mixtures were tested: (i) a mixture of three aldehydes (3A): (E)-hex-2-enal, (E)-oct-2-enal, and (E)-dec-2-enal at a volume ratio 10:1:10; (ii) a mixture of these three aldehydes and tridecane (TA), ratio 10:1:10:10; (iii) oxoaldehyde (OXO): (E)-4-oxohex-2-enal; (iv) extracted MTG secretion of *G. lineatum* adults (GS); (v) hexane (HX) (as a control since it was used as a non-polar solvent for the other chemicals).

Aldehydes, tridecane and hexane were purchased commercially (Sigma–Aldrich, St. Louis, MO, USA), 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.

The mixtures of aldehydes, tridecane and oxoaldehyde were prepared as 2% solutions in hexane, and aliquots of 2 μl were applied (using a Hamilton syringe) onto the middle part of the dorsal side of the mealworms (larvae of *Tenebrio molitor*, length approx. 20 mm) that were offered to the lizards as prey. The volume of 2 μl corresponds to the amount that is typically released by the shieldbugs as a reaction to simulated attacks (M. Šanda, personal communication). Metathoracic scent gland secretion (GS) was obtained by simulated attacks on the shieldbugs. When the shieldbug had ejected the secretion, it was applied directly onto the dorsal side of the mealworm to simulate the situation in the wild where *G. lineatum* ejects the secretion onto the surface of its body (Skelhorn and Rowe, 2006a). Untreated mealworms (UM) without any chemicals added were used as controls.

2.5. Experimental procedures

Experiments were performed in the same terraria where the animals were housed (20 cm × 40 cm × 20 cm). Prey was offered by direct insertion into the terrarium. Experiments were carried out during the active time period for lizards, i.e. during the day. The behaviour of the lizards was recorded with a video camera (Sony HDR-XR550VE; Sony Corp. Tokyo, Japan), and simultaneously behavioural elements were captured using Observer XT 8.0 (Noldus Information Technology, Wageningen, The Netherlands).

The lizards were split into eight experimental groups, which were equalised as to their sex and age. The groups were offered the following prey: mealworms with 3A, TA, OXO, GS, HX, living *Graphosoma* (LG) followed by mealworm with 3A, living *Pyrrhocoris* (LP) followed by mealworm with 3A, and untreated mealworm (UM). Each lizard was tested only once. The testing sequence consisted of 10 mealworms. The mealworms were offered sequentially in 5-min trials. For the experimental groups tested with chemicals (3A, TA, OXO, GS groups), the sequences started with a hexane-treated mealworm followed by 5 mealworms treated with the particular chemical corresponding to the experimental group, and ended with a sequence of 4 hexane-treated mealworms. Lizards in the control group (UM) were offered 10 untreated mealworms; lizards in the hexane group (HX) were offered 10 hexane-treated mealworms.

To test the lizards' reactions to living specimens of *G. lineatum* (LG) and *P. apterus* (LP), an untreated mealworm and the bug were offered alternately until the lizard rejected the bug three times without any handling (manipulation by touching and/or taking it into the mouth). The bug was offered a maximum of five times. This sequence was followed by the standard testing sequence with 3A.

Behaviour was compared in different parts of the experimental sequence: (i) "pre-chemical" trials at the beginning (mealworm no. 1), (ii) "chemical" trials with the tested chemicals (mealworms no. 2–6), and (iii) "post-chemical" trials following the experience with chemicals (mealworms no. 7–10) to distinguish between immediate and persistent effects of the tested chemicals. In each trial, the lizard was given 5 min to attack and potentially consume the mealworm, otherwise the trial was terminated. The trial was stopped earlier if the lizard consumed the prey within the 5-min interval. In each trial, (i) the latency of approaching the prey, (ii) the latency of attacking the prey (capturing), and (iii) the interval between the time of first approach and the moment the lizard started attacking the prey (approach–attack interval) were evaluated.

2.6. Statistical analyses

The data were analysed using the statistical program R 3.0.1 (R Core Team, 2014). Since the data were not normally distributed (Shapiro–Wilk normality test), robust methods of analysis based on ranks were applied.

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 (inspired by Kruskal–Wallis ANOVA). Instead of the real time values, the ranks of the recorded data (latencies of chosen behavioural elements) were used as the dependent variable, and it was evaluated in which way these ranks depended on the other covariates: chemicals, part of the experimental sequence (pre-chemical, chemical and post-chemical trials), age, sex and weight (with age and weight entering 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, and Tukey contrasts were used to compare chemicals within parts of the experimental sequence. Type II ANOVA, which assesses the impact of each covariate, controlling for the other covariates (their main effect) but not for interactions, was chosen since not all types of interactions were anticipated in the model. The comparison of particular chemicals was carried out in the final model which included only the covariates with a significant impact on the dependent variable. A new "interaction variable" (chemical vs. part of the experimental sequence) was used for this purpose. In all tests, significance was assumed at $p < 0.05$.

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

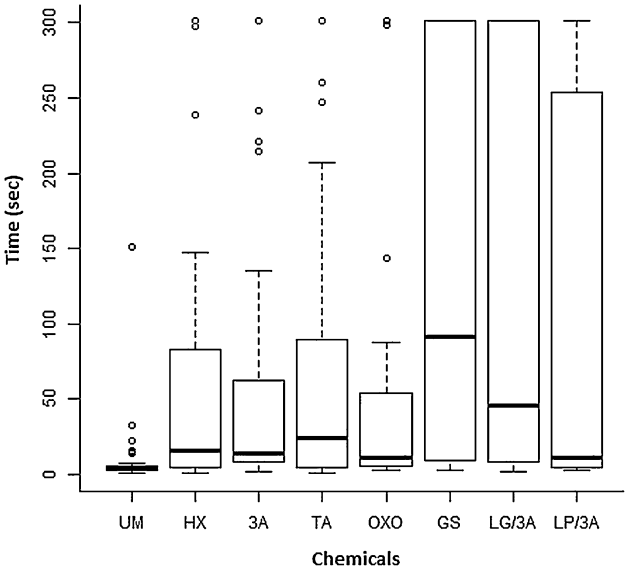

Fig. 1. Approach latencies in trials with tested chemicals–chemical trials. Approach latencies are presented on the *y*-axis. The figure reflects the original recorded values (the reaction time when approaching a prey). Band inside the box = median; box = lower and upper quartile; whiskers = non-outlier range; circles = outlier data. Abbreviations: UM, untreated mealworm; HX, hexane; 3A, mixture of three aldehydes; TA, 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; LP/3A, living specimen of *Pyrrhocoris apterus* followed by the mixture of three aldehydes.

3. Results

3.1. Approach latencies

Approach latencies were influenced only by the tested chemicals ($p < 0.001$), but not by the weight of the animals ($p = 0.4526$), their sex ($p = 0.0952$) nor their age ($p = 0.5552$). The interaction between the chemicals' effect and part of the experimental sequence was also not significant ($p = 0.0669$).

In the first control (pre-chemical) trial, approach latencies did not significantly differ among the groups of tested lizards. Hence all lizards started the experiment with the same motivation.

In the chemical trials, green lizards from the hexane group exhibited significantly longer approach latencies compared with the control group tested with untreated mealworms ($p < 0.01$). Therefore, the reactions of the other groups were compared with those of the UM group. All tested chemicals significantly extended the approach latencies of green lizards compared to the UM group (all $p < 0.001$; Fig. 1).

Regression based on ranks was used to evaluate the effect of the particular chemical on the approach latency. The approach latencies in the UM group were the shortest, whereas the *Graphosoma*

Table 1
The aversive effect of the tested chemicals on the approach latencies (the reaction time when approaching a prey) of green lizards in chemical trials (see text for details). Chemicals are sorted by estimate values.

Chemical	UM	HX	OXO	TA	3A	LP/3A	LG/3A	GS
Estimate	−256.170	−59.063	−25.901	−22.172	3.158	5.374	92.649	127.100

UM, untreated mealworm; HX, hexane; 3A, mixture of three aldehydes; TA, 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; LP/3A, living specimen of *Pyrrhocoris apterus* followed by the mixture of three aldehydes. Estimate: effect on approach latencies estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

secretion had the strongest aversive effect (Table 1). Presenting living specimens before the aldehyde mixture also had a strong aversive effect, followed by the mixture of aldehydes (3A) and the same mixture with tridecane (TA) and oxoaldehyde (OXO). Of all the tested chemicals, hexane had the weakest effect.

In the trials following the experience with the chemicals (post-chemical trials), the approach latencies of the green lizards were also compared to those of the UM group. Green lizards that had previous experience with *Graphosoma* secretion (GS) and living *P. apterus* followed by the aldehyde mixture (LP/3A) hesitated significantly longer than lizards from the UM group before approaching the mealworms, even when they were no longer treated with the tested chemical (both $p < 0.01$). Approach latencies of the groups previously confronted with the other chemicals did not significantly differ from those of the UM group.

3.2. Attack latencies

Attack latencies were influenced only by the tested chemicals ($p < 0.001$), but not by the weight of the animals ($p = 0.3734$), their sex ($p = 0.1619$) nor their age ($p = 0.5409$). There was a significant interaction between the chemicals' effect and part of the experimental sequence ($p < 0.05$).

In the first control (pre-chemical) trial, attack latencies did not significantly differ among the groups of tested lizards. Hence all lizards started the experiment with the same motivation.

In the chemical trials, green lizards from the hexane group exhibited significantly longer attack latencies compared with the control group tested with untreated mealworms ($p < 0.001$). Therefore, the reactions of the other groups were compared with those of the UM group. All tested chemicals significantly extended the attack latencies of green lizards compared to those of the UM group (all $p < 0.001$, Fig. 2).

Regression based on ranks was used to evaluate the aversive effect of the particular chemical on the attack latencies. The attack latencies in the UM group were the shortest, whereas offering a living specimen of *G. lineatum* before the aldehyde mixture had the strongest aversive effect (Table 2). The *Graphosoma* secretion and the living specimen of *P. apterus* before the aldehyde mixture also had a strong aversive effect, followed by the mixture of three aldehydes (3A), the same mixture with tridecane (TA) and oxoaldehyde (OXO). Of all the tested chemicals, hexane had the weakest effect.

In the trials following the experience with the chemicals (post-chemical trials), the attack latencies of green lizards were also compared to those of the UM group. Green lizards that had previous experience with *Graphosoma* secretion (GS) and living *P. apterus* followed by the aldehyde mixture (LP/3A) hesitated significantly longer than lizards from the UM group before attacking the mealworms, even when they were no longer treated with the tested chemical ($p < 0.05$ and $p < 0.01$, respectively). Attack latencies of the groups previously confronted with the other chemicals did not significantly differ from those of the UM group.

3.3. Approach–attack intervals

Approach–attack intervals were influenced by the tested chemicals ($p < 0.001$) and the weight of the green lizards ($p < 0.01$), but not

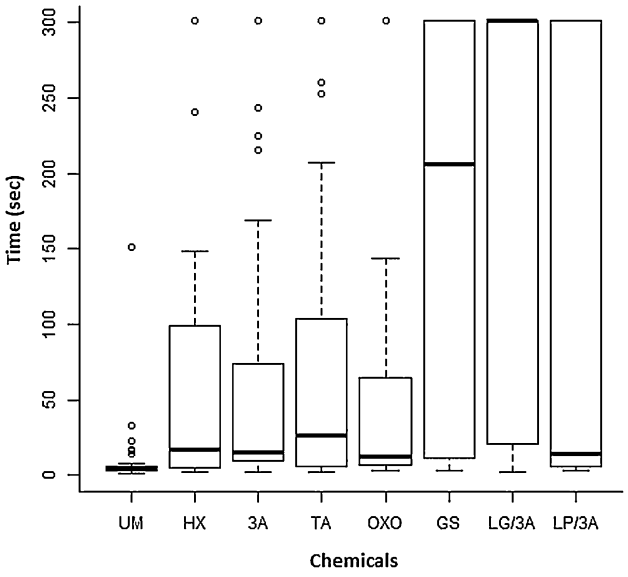

Fig. 2. Attack latencies in trials with tested chemicals–chemical trials. Attack latencies are presented on the *y*-axis. The figure reflects the original recorded values (the reaction time when attacking a prey). Band inside the box = median; box = lower and upper quartile; whiskers = non-outlier range; circles = outlier data. Abbreviations as in Fig. 1.

by their sex ($p = 0.1997$) or their age ($p = 0.4353$). Heavier animals had shorter approach–attack intervals. Additionally, there was a significant interaction between the chemicals' effect and part of the experimental sequence ($p < 0.001$).

In the first control (pre-chemical) trial, approach–attack intervals did not significantly differ among the groups of tested green lizards. Hence all lizards started the experiment with the same motivation.

In the chemical trials, green lizards from the hexane group exhibited significantly longer approach–attack intervals compared with the control group tested with untreated mealworms ($p < 0.001$). Therefore, the reactions of the other groups were compared with those of the UM group. All tested chemicals significantly extended the approach–attack intervals of the green lizards compared to those of the UM group (all $p < 0.001$, Fig. 3).

Regression based on ranks was used to evaluate the aversive effect of the particular chemical on the approach–attack interval. The approach–attack intervals in the UM group were the shortest, whereas the living specimen of *G. lineatum* before the aldehyde mixture had the strongest aversive effect (Table 3). The *Graphosoma* secretion and the living specimen of *P. apterus* before the aldehyde mixture (LP/3A) also had a strong aversive effect, followed by the aldehyde mixture with tridecane (TA) and oxoaldehyde (OXO). Of all the tested chemicals, hexane had the weakest effect.

In the trials following the experience with the chemicals (post-chemical trials), the lizards' approach–attack intervals were also compared to those of the UM group. When evaluating the approach–attack intervals, green lizards that had previous experience with *Graphosoma* secretion (GS) hesitated significantly longer than lizards from the UM group, even when the

Table 2
The aversive effect of the tested chemicals on the attack latencies (the reaction time when attacking a prey) of green lizards in chemical trials (see text for details). Chemicals are sorted by estimate values.

Chemical	UM	HX	OXO	TA	3A	LP/3A	GS	LG/3A
Estimate	−248.396	−45.085	−12.839	−0.945	11.208	33.205	157.877	161.994

UM, untreated mealworm; HX, hexane; 3A, mixture of three aldehydes; TA, 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 attack latencies estimated by a rank-based regression model (the lower the number the faster the reaction to the chemical).

Table 3
The aversive effect of the tested chemicals on the approach–attack intervals (time interval between the first approach and the first attack of a prey) of green lizards in chemical trials (see text for details). Chemicals are sorted by estimate values.

Chemical	UM	HX	OXO	3A	TA	LP/3A	GS	LG/3A
Estimate	−245.052	−22.062	−16.242	17.825	31.084	45.162	171.337	174.050

UM, untreated mealworm; HX, hexane; 3A, mixture of three aldehydes; TA, 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 approach-attack intervals estimated by a rank-based regression model (adjusted weight) (the lower the number the faster the reaction to the chemical).

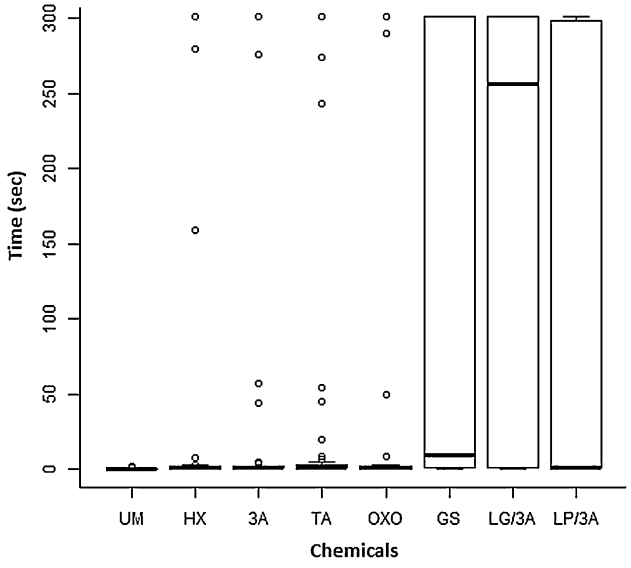

Fig. 3. Approach–attack intervals in trials with tested chemicals–chemical trials. Approach–attack intervals are presented on the *y*-axis. The figure reflects the original recorded values (time interval between the first approach and the first attack of a prey). Band inside the box = median; box = lower and upper quartile; whiskers = non-outlier range; circles = outlier data. Abbreviations as in Fig. 1.

mealworms were no longer treated with the tested chemical ($p < 0.05$). Approach–attack intervals of the groups previously confronted with the other chemicals did not significantly differ from those of the UM group.

3.4. Manipulation of living specimens of *G. lineatum* and *P. apterus*

During the testing of the lizards' reactions to the living specimen of *G. lineatum* (see Section 2.5), 3 lizards out of 8 manipulated the bug twice (out of a maximum of 5 offered bugs), 3 lizards only once and the remaining 2 lizards did not manipulate any of the three offered bugs. This means that the green lizards manipulated this bug twice at a maximum. All bugs were released unharmed, none were killed.

The lizards' reactions to the living specimens of *P. apterus* (see Section 2.5.) were different from those observed for *G. lineatum*. Out of 7 tested animals, only 1 manipulated and killed a firebug. The remaining 6 animals did not manipulate any of the three offered firebugs.

4. Discussion

In the present study we investigated the aversive effect of various chemical compounds of the repellent secretion of *G. lineatum* on green lizards. The major chemical compounds of the MTG secretion of *G. lineatum* appear to have aversive effects on green lizards.

Hexane was used as a non-polar solvent for the tested chemicals. Its influence was expected to be minimal, which was supported by the post-chemical trials, when mealworms were still treated with hexane but no aversive effects were recorded. Green lizards hesitated when offered mealworms treated with hexane in the chemical trials in all scored behaviours. However, hexane had the weakest aversive effect (the shortest latencies following the UM control group – see Tables 1–3) on green lizards.

The mixture of three aldehydes (3A) had an aversive effect on green lizards in all scored behaviours in the chemical trials. The mixture of three aldehydes and tridecane (TA) also had an aversive effect, but it was weaker than that of the aldehyde mixture itself (except for the approach–attack intervals). This contradicts the hypothesis that tridecane acts as a catalyst for the aldehyde mixture (Gunawardena and Herath, 1991) and supports the hypothesis that chemicals which might have a synergistic effect decrease the potency of the joint toxic loads (Skelhorn and Rowe, 2005). The parallel results of two other studies suggest that tridecane might play a role as an effective repellent against another lizard predator (leopard geckos; Gregorovičová and Černíková, unpublished data), but that it has no effect on bird predators (Gregorovičová et al., unpublished data). These results indicate that the effect of tridecane might be mediated by olfaction and/or vomerolfaction, which is well developed in lizards (Halpern, 1987; Schwenk, 1993). Since birds have poorly developed olfaction (Mason and Clark, 2000), there was no aversive effect of tridecane.

The weaker aversive effect of oxoaldehyde as compared to the mixture of three aldehydes and tridecane might be related to the presumably odourless nature of oxoaldehyde. In this case, the aversive effect could be mediated by gustation, which is also well developed in green lizards (Schwenk, 1985; Cooper, 1991). The absence of any aversive effect of oxoaldehyde on leopard geckos (Gregorovičová and Černíková, unpublished data) may be related to their poorly developed sense of gustation (Schwenk, 1985; Jamniczky et al., 2009). On the other hand, oxoaldehyde has a strong aversive effect on birds (Gregorovičová et al., unpublished data), presumably due to their relatively well developed gustatory sense (Mason and Clark, 2000).

The *Graphosoma* secretion had a strong aversive effect on green lizards, because they hesitated most 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 (Tables 2 and 3), indicating that the MTG secretion may have an impact as a signal as well as a secondary chemical defence. Moreover, the *Graphosoma* secretion also had a significant aversive effect 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 a previously developed association between the visual image of the prey and the nasty odour/taste of the secretion. Therefore, it seems that the chemical signal of the *Graphosoma* secretion can act as a cue for learned avoidance in experienced predators (Marples and Roper, 2004) and can elicit generalisation (Sexton, 1964; McLain, 1984).

The presence of living specimens of *G. lineatum* or *P. apterus* before the trials with mealworms increased the repellent effect of the mixture of three aldehydes in all scored behaviours in the chemical trials. Attack latencies (Table 2) and approach–attack intervals (Table 3) were highest when a living specimen of *G. lineatum* had previously been presented. Thus, the presence of *G. lineatum* had a stronger effect on green lizards than the presence of *P. apterus*, which is in agreement with the observation that *G. lineatum* has a more effective defence by spraying the repellent secretion towards the predator (M. Gregorovičová, personal observation). Thus it seems that aldehydes function as an odorous signal of unpalatability for green lizards.

The presence of a living specimen of *P. apterus* before the chemical trials with mealworms also significantly increased the aversive effect of the aldehyde mixture. This might be attributed to the possible role of the aldehyde mixture as a chemical signal for a predator with prior experience with *P. apterus* in the decision whether to approach and/or attack the prey. The chemical defence of *P. apterus* also consists mostly of short-chained aldehydes (Farine et al., 1992), as in the case of *G. lineatum*. The predominant compounds in adults are (E)-2-hexenal, (E)-2-octenal and tridecane (Farine et al., 1992) – similarly to the secretion of *G. lineatum* (Šanda et al., 2012). Therefore, the aldehydes found in the defensive secretion of *P. apterus* may also convey a signal of unpalatability. Moreover, the aversive effect of *P. apterus* may also be attributed to previous negative experience of the predator with firebugs in the wild. The co-existence of the three investigated species (*L. viridis*, *G. lineatum* and *P. apterus*) in the same habitat (M. Gregorovičová, personal observation) supports the idea that previous experience leads to avoidance behaviour. The present results support the hypothesis that repellency in these two bugs is mostly dependent on the aldehydes (Eisner, 1970; Hamilton et al., 1985; Gunawardena and Herath, 1991).

The green lizards faced a predator's dilemma – to starve or to consume a potentially toxic prey (Glendinning, 2007). In the present study, the lizards rejected mealworms previously treated with the particular chemical. They manipulated *G. lineatum* very carefully and did not even kill the bug. In one case, the lizard showed menace by opening the mouth towards *G. lineatum*. There was no manipulation of *P. apterus* except by one young lizard, which may be due to the older lizards' previous negative experience with firebugs in the wild. Similar observations were made for bird predators (Exnerová et al., 2007). Taken together, the present results confirm that predators reject chemically defended prey relatively unharmed (Boyden, 1976; Wiklund and Järvi, 1982; Skelhorn and Rowe, 2006a).

Based on additional observations, the aldehydes and the MTG secretion may have caused pain to the eyes and the respiratory system of the lizards, because they cleaned their heads after attacking mealworms treated with aldehydes or MTG secretion of *G. lineatum* or after manipulating living specimens of *G. lineatum*. This may be due to the effect of these short-chained aldehydes as trigeminal stimulants (Conner et al., 2007). The avoidance behaviours (closing eyes, cleaning heads) were not observed in the hexane group.

The results showed no impact of sex or age. Weight had an impact only on the approach–attack intervals when heavier animals were faster. This might be explained by the fact that heavier animals can risk eating potentially dangerous prey because it may not present such a burden for them. A similar behaviour was observed in the case of *Podarcis* lizards when consuming dangerous prey (Castilla et al., 2008).

Chemical defence is commonly used in nature. However, its principles are still poorly understood. More comparative studies will have to be performed to better understand the specific chemical compounds that may be responsible for aversive reactions in different types of predators.

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References

Aldrich, J.R., 1988. Chemical ecology of the Heteroptera. Annu. Rev. Entomol. 33, 211–238.
Aldrich, J.R., Leal, W.S., Nishida, R., Khrimian, A.P., Lee, C.J., Sakuratani, Y., 1997. Semiochemistry of aposematic seed bugs. Entomol. Exp. Appl. 84, 127–135.
Aliabadi, A., Renwick, J.A.A., Whitman, D.W., 2002. Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. J. Chem. Ecol. 28, 1749–1761.
Angelici, F.M., 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.
Arnold, E.N., 1987. Resource partitioning among lacertid lizards in southern Europe. J. Zool. 1, 739–782.
Arnold, E.N., 2002. Reptiles and Amphibians of Europe. Princeton University Press, Princeton/Oxford.
Aukema, B., Rieger, C.H. (Eds.), 2006. Catalogue of Heteroptera of the Palaearctic Region. Ponsen & Looijen, Wageningen.
Besson, A.A., Thierry, A., Boros, E., Allen, K., Bradley, S., Norrie, C., Cree, A., 2009. Evidence of food chemical discrimination in tuatara (O. Rhynchocephalia): comparison with a gekkotan lizard (O. Squamata). J. Herpetol. 43, 124–131.
Bonacci, T., Aloise, G., Brandmayr, P., Brandmayr, T.Z., Capula, M., 2008. Testing the predatory behaviour of *Podarcis sicula* (Reptilia: Lacertidae) towards aposematic and non-aposematic preys. Amphib. Reptil. 29, 449–453.
Boyden, T.C., 1976. Butterfly palatability and mimicry: experiments with *Ameiva* lizards. Evolution 30, 73–81.
Brower, L.P., 1984. Chemical defense in butterflies. In: Vane-Wright, R.I., Ackery, P.R. (Eds.), The Biology of Butterflies. Academic Press, New York, pp. 109–134.
Burghardt, G.M., Wilcoxon, H.C., Czaplicki, J.A., 1973. Conditioning in garter snakes: aversion to palatable prey induced by delayed illness. Anim. Learn. Behav. 1, 317–320.
Castilla, A.M., Bauwens, D., Llorente, G.A., 1991. Diet composition of the lizard *Lacerta lepida* in Central Spain. J. Herpetol. 25, 30–36.
Castilla, A.M., 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, 2331–2342.
Conner, W.E., Alley, K.M., Barry, J.R., Harper, A.E., 2007. Has vertebrate chemesthesis been a selective agent in the evolution of arthropod chemical defenses? Biol. Bull. 213, 267–273.
Cooper Jr., W.E., 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, 849–863.
Cooper Jr., W.E., 1995. Prey chemical discrimination and foraging mode in gekkonid lizards. Herpetol. Monogr. 9, 120–129.
Cooper Jr., W.E., 1996. Preliminary reconstructions of nasal chemosensory evolution in Squamata. Amphib. Reptil. 17, 395–415.
Díaz, J.A., Carrascal, L.M., 1993. Variation in the effect of profitability on prey size selection by the lacertid lizard *Psammodromus algirus*. Oecologia 94, 23–29.
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, S.B. (Eds.), Chemical Ecology. Academic Press, New York, pp. 157–217.
Exnerová, A., Štys, P., Fučíková, 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.
Farine, J.P., Bonnard, O., Brossut, R., Le Quere, J.L., 1992. Chemistry of defensive secretions in nymphs and adults of fire bug, *Pyrrhocoris apterus* L. (Heteroptera Pyrrhocoridae). J. Chem. Ecol. 18, 1673–1682.
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.
Glendinning, J.I., 2007. How do predators cope with chemically defended foods? Biol. Bull. 213, 252–266.
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.
Gunawardena, N.E., Herath, H.M.W.K.B., 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., 1987. The organization and function of the vomeronasal system. Annu. Rev. Neurosci. 10, 325–362.
Hamilton, J.G.C., Gough, A.J.E., Staddon, B.W., Games, D.E., 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.
Hotová Svádová, K., Exnerová, A., Kopečková, M., Štys, P., 2010. Predator dependent mimetic complexes: do passerine birds avoid Central European red-and-black Heteroptera? Eur. J. Entomol. 107, 349–355.
Jamniczky, H.A., Russell, A.P., Johnson, M.K., Montuelle, S.J., Bels, V.L., 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.
Krall, B.S., Bartlet, R.J., Lewis, C.J., Whitman, D.W., 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, N.M., Roper, T.J., 1996. Effects of novel colour and smell on the response of naive chicks towards food and water. Anim. Behav. 51, 1417–1424.
Marples, N.M., Roper, T.J., 2004. Warning colours and warning smells: how birds learn to avoid aposematic insects. In: Van Emdem, H.F., Rothschild, M. (Eds.), Insect and Bird Interactions. Intercept, Hampshire, pp. 185–192.
Mason, J.R., Clark, L., 2000. The chemical senses in birds. In: Whittow, G.C. (Ed.), Sturkie's Avian Physiology. , 5th ed. Academic Press, San Diego, pp. 39–56.
McLain, D.K., 1984. Müllerian mimicry between a plant bug (Miridae) and a seed bug (Lygaeidae) and the relationship between host plant choice and unpalatability. Oikos 43, 143–148.
Mitchell, J.C., 1979. Ecology of southeastern Arizona whiptail lizards (*Cnemidophorus*: Teiidae): population densities, resource partitioning, and niche overlap. Can. J. Zool. 57, 1487–1499.
R Core Team, 2014. R – A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org
Rabitsch, W., 2005. Die Wanzenfauna (Insecta Heteroptera) im Nationalpark Thayatal (Niederösterreich). Beitr. Entomofaun. 6, 87–106.
Ruxton, G.D., Sherratt, T.N., Speed, M.P., 2004. Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals, and Mimicry. Oxford University Press, New York.
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-of-flight mass spectrometry. J. Chromatogr. B 881/882, 69–75.
Schwenk, K., 1985. Occurrence, distribution and functional significance of taste buds in lizards. Copeia 1985, 91–101.
Schwenk, K., 1993. Are geckos olfactory specialists? J. Zool. 229, 289–302.
Sexton, O.J., 1960. Experimental studies of artificial Batesian mimics. Behaviour 15, 244–252.
Sexton, O.J., 1964. Differential predation by the lizard, *Anolis carolinensis*, upon unicoloured and polycoloured insects after an interval of no contact. Anim. Behav. 12, 101–110.
Sindaco, R., Jeremčenko, V.K., 2008. The reptiles of the Western Palearctic. 1. In: Annotated Checklist and Distributional Atlas of the Turtles, Crocodiles, Amphisbaenians and Lizards of Europe, North Africa, Middle East and Central Asia, Edizioni Belvedere, Latina, Italy.
Skelhorn, J., Rowe, C., 2005. 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., 2010. Birds learn to use distastefulness as a signal of toxicity. Proc. R. Soc. B. 277, 1729–1734.
Staples, J.K., Krall, B.S., Bartlet, R.J., Whitman, D.W., 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.
Terrick, T.D., Mumme, R.L., Burghardt, G.M., 1995. Aposematic coloration enhances chemosensory recognition of noxious prey in the garter snake *Thamnophis radix*. Anim. Behav. 49, 857–866.
Wagner, E., 1965. Zur Systematik der Gattung *Graphosoma* Lap. (Hem., Het. Pentatomidae). Entomol. Ber. 16, 110–116.
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.