Cracking the chemical code: European common lizards (*Zootoca vivipara*) respond to an hexane soluble predator kairomone

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Abstract

In many animals, chemosensation acts as a first line of defence against snake predators. However, in spite of their obvious importance, the chemical nature of cues used by prey to detect snakes remains to be discovered. Here, we analyse which neutral lipids, extracted with n-hexane, are present in the skin of the European adder (*Vipera berus*) using Gas Chromatography – Mass Spectrometry. The analyses revealed that the washes held a complex cocktail of chemical compounds, with a total of 165 different molecules, mostly steroids (82% of the total ion current) and alkanes (13%), and smaller amounts of carboxylic acids, wax esters, ketones, amides and alcohols. Using bio-assays in which we confronted individuals of a prey species (the European common lizard, Z*ootoca vivipara*) with these washes, we were able to confirm that the kairomones can be extracted using n-hexane. In fact, lizards did not respond to chemical cues still present in adder skin after washing, indicating that the kairomones are indeed strongly n-hexane soluble. Consequently, we have set a next step in deciphering the chemical nature of the predator-prey interaction between the European adder and the European common lizard. We hope our results facilitate further investigation into the chemical ecology of snakes and their prey.

Keywords: non-polar lipids; anti-predator behaviour; Viperidae; Lacertidae; chemodetection; GC-MS

Introduction

In many animals, chemosensory recognition of predators functions as an important first line defence system (e.g. rotifers: Gilbert 1999; insects: Chivers et al. 1996; fish: Wisenden 2000; amphibians: Troyer and Turner 2015; reptiles: Thoen et al. 1986; mammals: Jedrzejewsky et al. 1993). Chemical cues are especially germane in situations where the visual and/or auditory information channels are obstructed, e.g. in the dark, or in densely vegetated habitats. Also, in contrast to visual and auditory cues, chemical cues tend to linger in the environment and may, therefore, signal to the prey that it is treading on dangerous grounds, even if the predator has temporarily left the area, or is lying in ambush (Kats and Dill 1998).

Despite their obvious importance, the exact nature of predator kairomones (i.e. predator-derived chemical cues detected by the prey) remains largely unknown. Even in aquatic systems, where their ecological role has received considerable attention, the chemical characterization of kairomones is lagging behind (Ferrari et al. 2010). Research on terrestrial model systems has almost exclusively targeted chemicals that are used by two rodent species (mice and rats) to detect feline or canine predators (Vernet-Maury 1980; Hendriks et al. 1995; Fendt 2006; Ferrero et al. 2011). These studies have identified a number of candidate-kairomones typically present in the waste of carnivores. Examples include 2,3,5-trimethyl-3-thiazoline (TMT), a characteristic compound of the faeces and urine of red foxes (*Vulpes vulpes,* Vernet-Maury 1980; Fendt et al. 2005), 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (felinine), found in the urine of several cat species (Hendriks et al. 1995; Voznessenskaya and Malanina 2015), and 2-phenylethylamine (PEA), which is found at characteristically high concentrations in the excreta of a wide range of mammalian carnivores (Ferrero et al. 2011).

Virtually nothing is known on the kairomones of non-mammalian terrestrial predators, such as snakes. A large body of literature testifies to how a diverse array of prey animals can detect the odours of snakes (primates: Sündermann et al. 2008; rodents: e.g. Weldon et al. 1987 and Pillay et al.

2003; frogs: e.g. Supekar and Gramapurohit 2018; salamanders: e.g. Murray and Jenkins 1999; lizards: e.g. Thoen et al. 1986 and Ortega et al. 2018; snakes: e.g. Cooper et al. 2000), but which individual or combination of compounds reveals a snake's presence, remains unexplored. While at least some prey species recognise odours emanating from snake faeces (Pillay et al. 2003), most studies seem to suggest that compounds found in the skin of snakes could also be used as kairomones. Snakes tend to have a much lower defecation rate (vipers evacuate once every 18-77 days; Lillywhite et al. 2002) compared to mammals and, consequently, faeces may not be a reliable information source regarding a snake's whereabouts. Therefore, although studies on mammalian kairomones have targeted molecules in the urine or faeces of the predator (Apfelbach et al. 2015), we here chose to focus on body odour.

We investigated the neutral-lipid fraction of the European adder's skin chemicals (*Vipera berus*) and its possible use as kairomones by their prey. The odours of this snake species elicit a clear fear response in a prey animal, the European common lizard *Zootoca vivipara* (Thoen et al. 1986; Van Damme et al. 1990). We washed samples of freshly-shed skin of several individual wild adder specimens in n-hexane and ran Gas Chromatography-Mass Spectrometry (GC-MS) analyses on the lipophilic fraction of compounds in the residues. Then, to test whether the washing procedure had effectively removed the kairomones used by the prey species, we presented samples of the washes and of washed skin to common lizards and noted their chemosensory and antipredatory behaviour.

Material and Methods

Snake skin samples

We obtained the skin samples of thirteen individuals (ten males, two females and one of undetermined sex) of the European adder from a population in the north of Antwerp (nature reserve Marum, Brecht, Belgium; permit reference number: ANB/BL-FF/V16-00002 and ANB/BL/FF-V17-00018). All but one of these samples were taken directly from animals that were moulting when

caught in the field. In this case, the sex, snout-vent length (SVL) and body mass of the snake was noted (see Bauwens et al. 2018 for methodology). We could not collect this data for one sample because it was obtained from a freshly shed skin in the field. All skins were handled with rubber gloves and transported to the lab in Antwerp on ice, in separate and marked ziplock bags. There, each skin sample was weighed and stored in a freezer at -20 °C until the start of the chemical extraction procedure.

Chemical extraction

Chemical extractions were performed within one month after collecting the skins in the field, following procedures outlined in Baedke et al. (2019). N-hexane was chosen instead of other solvents (e.g. methanol or dichloromethane) to enable the assessment of the kairomonal role of neutral adder-skin lipids (see Ball 2004 and references therein). All lab utensils were rinsed with n-hexane (Merck, Emplura grade) before use. Each skin sample was left to soak overnight in n-hexane (Merck, Suprasolv grade) in a glass container which we stored in a fridge. A volume of 50 mL was used for small pieces of tail skin and 70 mL for complete skins. The containers were wrapped in tinfoil and in parafilm for health reasons. The next day, the solvent was filtered through glass wool and collected in a second glass container. Any residues of lipophilic compounds that remained in the original glass container were washed out with 20 mL of n-hexane (Merck, Suprasolv), filtered through the glass wool and added to the rest of the solvent. The resulting volume was left to evaporate at room temperature under a fume hood to a volume of approximately 200 µL, which was then pipetted into a 250 µL glass vial with Teflon cap. These samples were kept at – 20 °C until analyses with GC-MS (see next section) were carried out. The extraction steps were repeated without using an adder skin sample to control for contaminants. This control sample was also analysed through GC-MS.

For one of the complete skins we divided the solvent in two equal volumes of 45 mL after filtration. Both volumes were processed as described above. Whereas one of the volumes ended up being used

in GC-MS analysis as was the case for the other samples, the second volume was used to prepare twenty skin extract swabs for presentation in focal observations to *Z. vivipara* lizards (see further).

Gas Chromatography – Mass Spectrometry of snake skin

Extract samples were analysed using a gas chromatograph (Agilent 7890A, Santa Clara, CA, USA) equipped with an Agilent HP5-MS column (5% diphenyl, 95% dimethylsiloxane, 30 m length × 0.25 mm ID, 0.25 µm film thickness), coupled to a mass spectrometer (Agilent 5975C with triple axis detector). Sample injections (2 µl of the n-hexane extract) were performed in splitless mode using helium as the carrier gas at a constant 30 cm/s flow, with injector and detector temperatures at 250 °C and 280 °C, respectively. The oven temperature programme was as follows: 45 °C isothermal for 10 min, then increased to 280 °C at a rate of 5 °C/min, and then isothermal (280 °C) for 15 min. The mass spectrometer was operated at an ionization voltage of 70 eV and with scanning between m/z 30-500 at 3.9 scans/s. Impurities identified in the solvent and/or the control vial samples are not reported.

We tentatively identified chemicals by comparison of mass spectra in the NIST/EPA/NIH 2002 library, and later confirmed them, when possible, with authentic standards (from Sigma-Aldrich Chemical Co., St. Louis, MO, USA; Table 1). From the chromatograms, we calculated, using the Xcalibur software, the percentage of the total ion current (TIC) to determine the relative amount of each compound (García-Roa et al. 2018).

Bio-assays

To test whether the snake-skin hexane washes contained kairomones, we offered cotton swabs dipped in the extracted liquid to European common lizards. Thirty-one adult male lizards of this species were caught from the same nature reserve (Marum) as the adders and transported to the lab in individual cloth bags (permit reference numbers: ANB/BL/FF-V17-00007 and ANB/BL/FF-V17-00018). There, lizards were housed individually in terrariums of 100 × 50 × 50 cm (length × width × height), which had the bottom covered with sand, stones and moss to mimic the lizards' natural

environment. The walls of the terraria were lined with paper in order to prevent the lizards from interacting and exchanging behavioural cues, thereby reducing impact during focal observations. A 60 Watt incandescent lamp above one end of the terrarium was switched on 12 hours per day, offering the lizards the opportunity to regulate their body temperature. The bulb was switched off for half an hour at noon, to prevent overheating. Water was available ad libitum and the lizards were fed vitamin E dusted crickets (*Acheta domesticus*) twice a week and wax moth larvae (*Galleria mellonella*) once a week. Water was sprayed inside the terrariums daily to guarantee adequate humidity. After the experiment all of the animals were released in good condition at their capture location.

The bio-assays followed procedures outlined by Cooper et al. (2000). A swab containing the experimental or control substance was mounted on a 60 cm wooden peg. The experimenter carefully approached the lizard's home terrarium and manoeuvred the swab just in front of the animal's snout. Once the swab was in place, the lizard's behaviour was scored for one minute using JWatcher v1.0 software (Blumstein & Daniel 2007). Whenever the lizard averted its body or ran away, the swab was carefully repositioned anterior to the lizard's snout and behavioural scoring continued. We counted the number of tongue flicks that were directed towards the swab (Directed tongue flicks), and those that were performed when the head was tilted away from the swab (Undirected tongue flicks). Directed tongue flicks touched the swab in at least three out of four cases. We also noted the number of Foot Shakes, Tail vibrations, Startles, Bites and the number of times that the lizard averted its snout away from the swab at an angle of more than 90 degrees (hereafter called Head turns). Tail vibrations and Foot shakes were too rare to be analysed separately so we grouped them in a new variable, Flutters, which is simply the sum of Foot shakes and Tail vibrations. Both Foot shakes and Tail vibrations are considered as signs of stress or antipredatory responses in lizards (Mori 1990; Ruxton et al. 2004; Telemeco et al. 2011; Font et al. 2012; see Verbeek 1972 and Thoen et al. 1986 for detailed descriptions). Handling and housing of lizards was in accordance with prevailing local and

European regulations and all experiments were approved by the ethical committee of the University of Antwerp (2015-34).

Experiment A

Experiment A was designed to test whether the snake kairomones invoking anxiety in lizards included some of the lipophilic compounds that readily dissolve in n-hexane. To that end, we compared the lizards' responses to (1) cotton swabs dipped in clean hexane (hexane control) and (2) swaps dipped in the solution obtained by washing skin with n-hexane (experimental treatment; see above). A total of twenty male adult lizards were tested. Half of them were confronted with clean n-hexane swabs first and skin extract next, for the other half the order was reversed. Lizards were tested between 9 am and 4 pm with at least one full day between both trials. This experiment was performed in March 2017, within a week after the lizards were caught.

Experiment B

Experiment B was designed to test whether washing with n-hexane effectively removed all the compounds from adder skin that may be used as kairomones by lizards. Here, we compared the lizards' responses to (1) sterile swabs (odourless control), (2) swabs dipped in clean hexane (hexane control) and (3) swabs rubbed over a snake's shed skin that had previously been washed with n-hexane (experimental treatment). A total of eleven male adult lizards were tested. The order in which the control and experimental swabs were offered was randomized per individual. Lizards in experiment B were caught and tested in July 2017; observations were conducted between 9 am and 4 pm and with at least one full day between subsequent trials.

Statistics

We used nonlinear regression to describe the relationship between adder skin sample mass and the number of compounds retrieved with GC-MS. In particular, we fitted a three parameter exponential

rise to maximum ($y=y_0+a(1-e^{-bx})$) and used the equation to predict how much skin was needed to obtain 80, 90 or 100% of all compounds.

To test whether lizards in experiments A and B reacted differently to control and experimental treatments, we ran generalized linear mixed-effect models (GLMM; lme4 package, Bates et al. 2014 in R version 3.3.0, R Core Team 2016). Since all behavioural variables scored were counts, we used a Poisson fit or a negative binomial fit (depending on which distribution fitted the data best) and a log link function. In each model, Individual was included as a random effect to account for the repeated measures design. The data was checked for overdispersion, heteroscedasticity and any deviations from linearity. When overdispersion was detected, an observation-level random effect was added to the model (Harrison 2014). We compared models with and without the treatment variable as a fixed effect and selected the best model based on the Akaike Information Criterion (AIC). To test for differences between specific pairs of treatments, post-hoc multiple comparisons were carried out with a Bonferroni correction using the lsmeans package in R (Lenth 2016). Data from experiments A and B was analysed separately because these experiments were performed on different individuals.

Results

GC-MS analyses

Gas chromatography-mass spectrometry revealed a total of 165 distinct compounds in the n-hexane washes of adder skin (Table 1). The washing procedure mobilised 22 different steroids that together made up more than 82% of the total TIC. Cholesterol, representing 67% of the TIC, was by far the most ubiquitous compound in the washes. The washes also contained a diverse cocktail of alkanes, 25 of which had a linear structure (C_{11} to C_{36}) and 44 were branched. The alkane group as a whole accounted for 13% of the TIC. We also detected smaller amounts of carboxylic acids (N=12 different compounds, from C_9 up to C_{20}), wax esters (N=8), ketones (N=7), squalene, amides (N=2), alcohols

(N=9, from C_8 up to C_{28}), ethyl and methyl esters of carboxylic acids (N=16, from C_{14} to C_{24}), aromatic compounds with benzene rings (N=3), aldehydes (N=10, from C_9 up to C_{20}), tocopherols (N=2) and the furanone 4,8,12,16-Tetramethylheptadecan-4-olide. In three samples we found high concentrations of carboxylic acids, one sample contained up to twelve of these compounds (14.45 % of its TIC).

The number of compounds detected per sample rose rapidly between 0.01 and 0.20 g of skin material and then levelled off. Fitting an exponential-rise-to-a-maximum function ($y=y_0+a(1-e^{-bx})$) through the raw data resulted in a fair fit ($r^2=0.52$). From this equation, it follows that 80%, 90% and 100% of compounds can be retrieved from skin samples weighing 0.060 g, 0.10 g and 0.20 g, respectively.

Bio-assays

In experiment A, swabs dipped in n-hexane washes of adder skin elicited far more Directed tongue flicks ($Z = 3.31$, $P < 0.001$), Startles ($Z = 3.18$, $P = 0.0015$) and Flutters ($Z = 3.89$, $P < 0.001$) than swabs dipped in pure n-hexane (Fig. 1, Table 2). In contrast, no significant effect of Treatment was evident in the number of Undirected tongue flicks, the number of swab Bites, or the number of Head turns (Table 2).

In experiment B, Treatment had no effect on the incidence of any of the behaviours recorded. Flutters were observed on only two occasions, so no analyses were performed on this variable. The overall GLMM model suggested a marginally significant effect of Treatment on the number of Head turns (Table 2), but post-hoc testing failed to find significant differences among pairs of treatments (sterile versus clean hexane: $Z = 2.061$, $P = 0.079$; depleted shed versus clean hexane: $Z = 1.903$, $P = 0.11$).

Discussion

Our chemical analyses revealed the presence of a wide array of lipophilic compounds in adder skins. Probably, several of these chemicals are involved in the primary functions of the animal's skin. For instance, cholesterol is a major component of vertebrate tissue. It has been found in abundance in the epidermis of many squamates (Weldon et al. 2008), including several snake species (Ahern and Downing 1974; Mason et al. 1987; Jacob et al. 1993; Ball 2000). Experimental research has revealed that cholesterol plays an important role in maintaining a barrier to water permeation in these snakes (Burken et al. 1985a) and, thereby, it protects them against dehydration. Several other molecules found in the adder's skin have also been implicated to play a role in the maintenance of the water balance: linoleic acid (Elias et al. 1980), long-chained alkanes (Lillywhite & Maderson, 1988) and wax esters (Koch et al. 2007; Nickerl et al. 2014) may have such properties. Other molecules such as lauric acid (Nakatsuji et al. 2009; Fischer et al. 2014), the methyl ester of palmitic acid and two amides (Medeiros dos Reis et al. 2019), on the other hand, may function in the deterrence of harmful microorganisms. Furthermore, it has been suggested that some of the carboxylic acids promote wound-healing (Oh et al. 2015) and/or are known anti-inflammatory agents (Lin et al. 2018). Chemicals with antioxidant properties, such as tocopherols (Mardones and Rigotti 2004) and phenols (Lin et al. 2018), protect membrane lipids against free radicals. Wax esters (Pappas 2009) and amides (Getachew et al 2016) probably protect the skin against fouling; fatty alcohols tend to have emollient properties (Fillet and Adrio 2016). Notably, the two amides (oleamide and erucamide) that we found in a subset of the adder skin washes, are used in the packaging industry as slip agents on films that guarantee an easy opening (Poisson et al. 2010). For snakes, a high slippability seems a desirable trait during locomotion, so it would be interesting to test whether these amides serve similar purposes in adders.

In addition to these protective purposes, the skin is increasingly considered to play a role in communication. Also in European adders, there are strong behavioural indications that sex and reproductive status can be deduced from compounds in, or secreted by, an individual's dorsal skin

(Andrén 1982). We indeed found molecules in adder sheds with potential pheromonal properties. The long-chained methyl ketones 2-heptacosanone and 2-nonacosanone are part of a multi-compound sex pheromone in Canadian red-sided garter snakes (*Thamnophis sirtalis parietalis*), attracting males to potential female partners (Mason et al. 1989; Mason et al. 1990). Together with the remaining saturated methyl ketones detected in our study, these could have a similar pheromonal function in adders. Furthermore, in garter snakes, squalene is a key molecule in the male sex recognition system (Mason et al. 1989) and in the Iberian worm lizard (*Blanus cinereus*) it has been shown to provoke agonistic behaviour in males (López and Martín 2009). Alas, in our male-biased dataset of *V. berus*, we were unable to statistically test differences in squalene concentrations between sexes. For other reptiles, male agonistic behaviour has also been found in response to carboxylic acids (gopher tortoise, *Gopherus polyphemus*: Rose 1970), certain alcohols (Bosc's fringe-toed lizard, *Acanthodactylus boskianus*: Khannoon et al. 2011), and cholesterol (*A. boskianus*: Khannoon et al. 2011; and Iberian rock lizard, *Iberolacerta cyreni*: Martín and López 2007). Whether all of these molecules serve similar purposes in the European adder requires further investigation.

Furthermore, many of the compounds detected in the skin washes have a distinctive smell and could, therefore, have a (secondary) role in communication. The strong, sour odour of carboxylic acids, the sweet smell of wax and carboxylic acid esters and fatty alcohols, and the floral scent of aldehydes are all detectable by us, chemically deprived humans. Therefore, it seems likely that chemosensory specialist reptiles would use these volatiles as a source of information. Particularly, male adders have been suspected of emitting airborne cues during spring molting, indicating their readiness to mate and provoking aggressive behaviour in competing males (Andrén 1982). These low-weight molecules may be present in our subset of adder-derived chemicals. Alternatively, because n-hexane is highly non-polar, it extracted solely neutral lipids such as steroids, hydrocarbons, carboxylic acids and waxy esters (Ball 2004). The chemical cocktail exuded from these snakes should consequently be even richer than described in this study and pheromones may be present, as well, in the non-hexane extractable fraction of an adder's skin.

It should be noted that, although we corrected for contaminants resulting from the extraction procedure, there could still have been compounds present on the skins which are not a product of an adder's physiology. We expect these to be minor compounds. Nevertheless, if an environmental chemical would excite a certain benefit onto the snake, its presence on the skin may not be a mere coincidence. For instance, lup-20(29)-en-3-one found in our samples is known to stimulate melanin biosynthesis in murine cells which could protect against UVB light induced skin cancers (Villareal et al. 2013). This chemical is known to be present in leaf extracts of *Erica multiflora*, which is a heath plant closely related to *Erica tetralix* and *Calluna vulgaris* which grow at our sampling site. Perhaps adders purposefully rub their bodies onto these plants for protection against disease. Increasing evidence is found of self-medication in animals (de Roode and Hunter 2019; Domínguez-Martín et al. 2020). However, in reptiles, the presence of such behaviour has not been scientifically assessed.

Our bio-assays indicated that at least one of the 165 adder skin-derived compounds is used by common lizards in assessing predation risk by this snake. During focal observations we observed lizards exhibiting increased tongue flicking directed towards swabs that had been dipped in n-hexane extract of adder sheds. They also displayed more Startles, Foot shakes and Tail vibrations – behaviours associated with stressful situations. However, we observed a complete lack of such behaviours towards swabs taken from depleted adder sheds. This indicates that n-hexane washes out all kairomones from the adder's skin and, consequently, lizards are unable to assess potential danger when confronted with such depleted cues.

Which molecule(s) in the adders' sundry blend serve as kairomones and consequently give away the predator's imminence to *Z. vivipara*? Common lizards can distinguish between odours of saurophagous and harmless snakes (Thoen et al. 1986). Therefore, skin chemicals that carry out primary functions in a wide array of snake species do not seem to be likely candidate-kairomones. It seems more probable that lizards eavesdrop on adder-specific molecules, perhaps pheromones. As previously discussed, current knowledge on the chemical nature of pheromones remains practically

nonexistent. Therefore, any thoughts or suggestions on candidate compounds remain purely guesswork.

However, we want to draw attention to a particular group of molecules. Hydrocarbons, and more specifically alkanes, make up the most diverse chemical group in adder sheds. Although many remain unidentified after our GC-MS analyses (especially when molecules are branched), single compounds are often consistently found over the various samples. This type of molecule has been observed before in squamate skins and secretions. However, alkane diversity is seldom so pronounced (Jacob et al. 1993; Weldon et al. 2008; Schulze et al. 2017; Baeckens et al. 2018). Compounds that have not before been detected in animals, such as 4,5-diethyl-octane, 5-methyl-nonane or potentially currently unidentified molecules, may be ideal candidates for adder-specific pheromones and, therefore, also good indicators of adder presence towards lizards. Or, lizards may rather get their information from a unique combination of hydrocarbons and/or their relative proportions in the total odour blend (Apps 2013; Wen et al. 2017). Alkanes have been proposed before as kairomone candidates warning pit vipers of the genera *Agkistrodon, Crotalus* and *Sistrurus* (Crotalinae) for ophiophagous king snakes (*Lampropeltis getula*) (Gutzke et al. 1993). Furthermore, no clear anti-predatory behaviour is observed in desert iguanas (*Dipsosaurus dorsalis*) when these were confronted with solely polar lipids and lipids of intermediate polarity of kingsnake sheds (Bealor and O'Neil Krekorian 2006). Perhaps here as well, predator-recognition works through alkanes which would not have been collected in a sufficient amount by the chloroform and methanol solvents used by the researchers (Ball 2000; Cequier-Sánchez et al. 2008). Consequently, alkanes are promising subjects for future research. Evidently, other adder-unique compounds described in Table 1 are not to be neglected, either. The next step in the current research will be to fractionate the n-hexane extract from adder skins and test the potency of different fractions to elicit anti-predatory defences in lizards (Baedke et al. 2019).

Considerably more is known on kairomones in mammalian interactions. Individual molecules, such as 2-phenylethylamine found in the urine of lions, servals and tigers (Ferrero et al. 2011), pyrazine analogues from wolf urine (Osada et al. 2013) and 2,5-dihydro-2,4,5-trimethylthiazoline from fox faeces (Vernet-Maury 1980) suffice to evoke avoidance behaviour of mammalian prey species (rats and mice). None of these molecules were found in our analyses. Note, however, that the identified mammalian kairomones are predominantly isolated from excrements whereas our analyses focussed on skin chemicals. Therefore, it could still be possible that these kairomones do occur in adder faeces and are, in fact, interpreted by mammalian prey species. However, snake-skin derived kairomones have been shown to evoke responses in mammals, as well. To date, their isolation and identification remains unsuccessful (Papes et al. 2010). Therefore, in future research, lizards and mammals may still prove to interpret the same non-polar, snake-skin derived kairomones. Whether these are single compounds, as for excrement-derived mammalian kairomones, still needs to be explored.

To conclude, in the current study, we have succeeded in confirming a source (i.e. the skin) of adder kairomone and have found that this semiochemical comprises of at least one n-hexane extractable and therefore neutral lipid. In doing so, we have set the next step in deciphering the chemical nature of the prey-predator interaction between the European common lizard and the European adder. Additionally, through means of our chemical analyses, we hope to facilitate further investigation into the European adder's chemical ecology.

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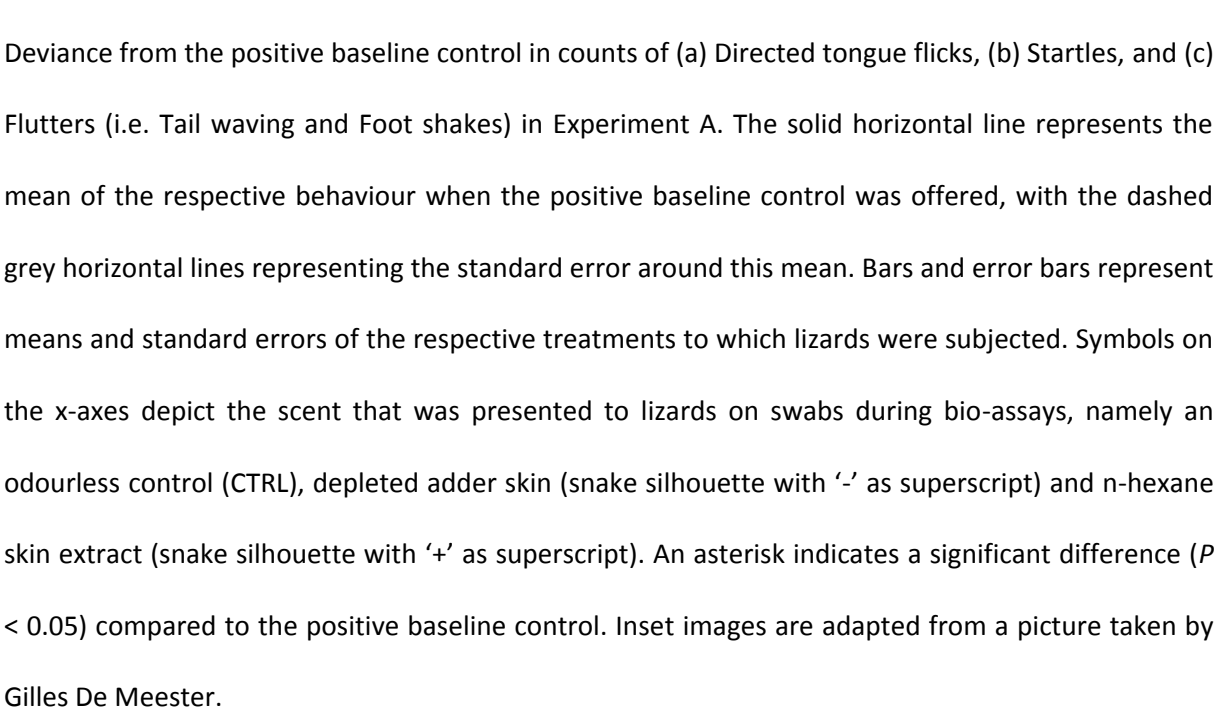
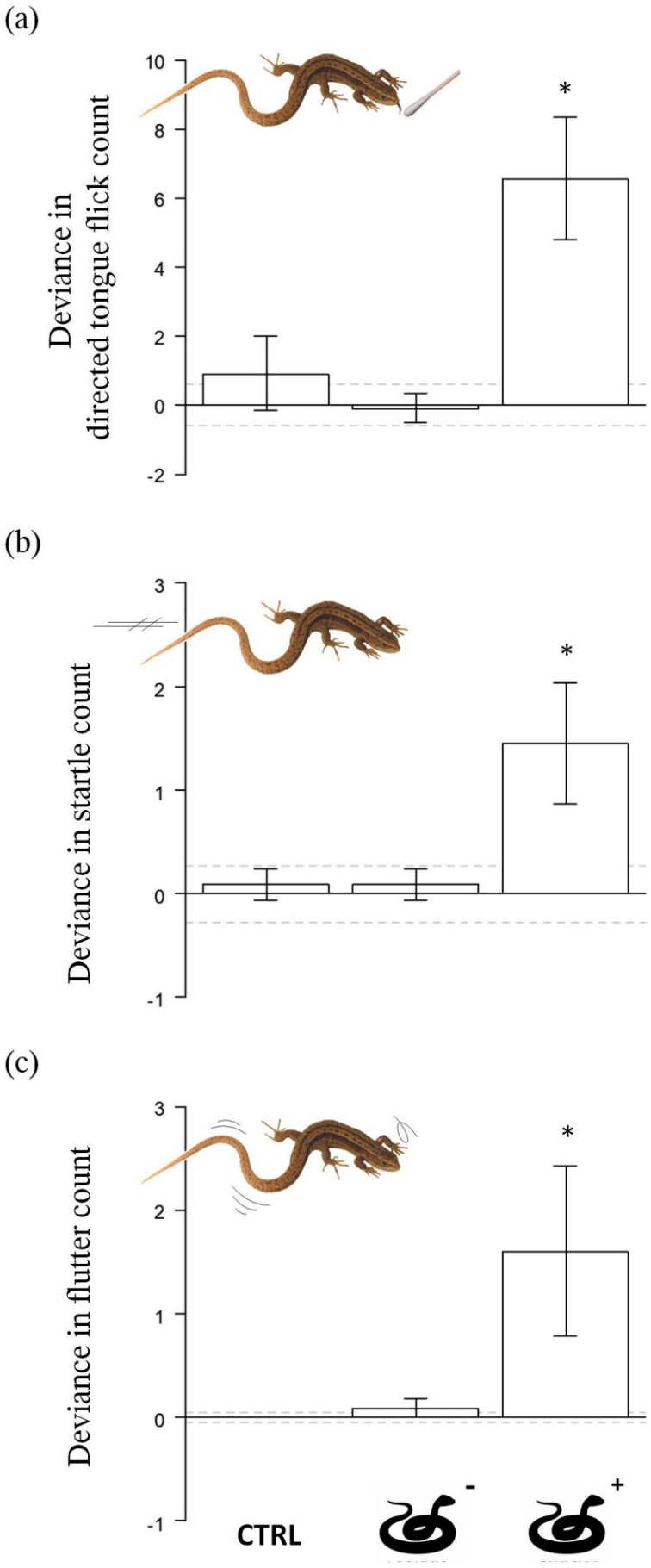
Figures

Fig. 1



Deviance from the positive baseline control in counts of (a) Directed tongue flicks, (b) Startles, and (c) Flutters (i.e. Tail waving and Foot shakes) in Experiment A. The solid horizontal line represents the mean of the respective behaviour when the positive baseline control was offered, with the dashed grey horizontal lines representing the standard error around this mean. Bars and error bars represent means and standard errors of the respective treatments to which lizards were subjected. Symbols on the x-axes depict the scent that was presented to lizards on swabs during bio-assays, namely an odourless control (CTRL), depleted adder skin (snake silhouette with '-' as superscript) and n-hexane skin extract (snake silhouette with '+' as superscript). An asterisk indicates a significant difference ($P < 0.05$) compared to the positive baseline control. Inset images are adapted from a picture taken by Gilles De Meester.


(a)
Deviance in directed tongue flick count
10
8
6
4
2
0
-2
*
(b)
Deviance in startle count
3
2
1
0
-1
*
(c)
Deviance in flutter count
3
2
1
0
-1
*
CTRL
-
+

Tables

Table 1.

Relative proportion of lipophilic compounds (%; mean ± SE) in skin samples of European adders with their retention times (RT). An asterisk after the compound name indicates that the identification was confirmed with standards. The other compounds were tentatively identified based on mass spectra and retention times. A '+' sign indicates a compound detected in very low proportion (< 0.01 %). Also indicated is the number of individual skin samples in which the compound was detected in this study (between brackets: in males and females) and whether the compound has been listed as possible semiochemical in the literature, in arthropods (Ar), amphibians (Am), lizards (Li), snakes (Sn) and mammals (Ma). Studies that have described the compound in specific genera of snakes are indicated in the subscript to this table.

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
CARBOXYLIC ACIDS									
18.9	Nonanoic acid (pelargonic acid)*	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12]
21.5	Decanoic acid (caproic acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Naja*[11], *Pantherophis*[12]
24.8	Dodecanoic acid (lauric acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12]
30.4	Tetradecanoic acid (myristic acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12], *Drymarchon*[13]
32.3	Pentadecanoic acid (pentadecylic acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Echis*[7], *Loxocemus*[8], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12], *Drymarchon*[13]
33.9	9-Hexadecenoic acid (palmitoleic acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Pantherophis*[12], *Drymarchon*[13]
34.3	Hexadecanoic acid (palmitic acid)	0.14 ± 0.11	2 (0 ♀, 1 ♂)	✓	✓	✓	✓	✓	*Rena*[1], *Acrantophis*[3,4], *Thamnophis*[6], *Echis*[7],

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
									Vipera[7], *Loxocemus*[8], *Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Pantherophis*[12,14], *Naja*[11], *Ptyas*[11], *Drymarchon*[13], *Hydrophis*[15]
36.3	Heptadecanoic acid (margaric acid) *	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Acrantophis*[3], *Vipera*[7], *Loxocemus*[8], *Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12], *Drymarchon*[13]
37.6	(Z,Z)-9,12-Octadecadienoic acid (linoleic acid)*	0.52 ± 0.45	3 (1 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Thamnophis*[6], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12,14]
37.7	(Z)-9-Octadecenoic acid (oleic acid) *	0.68 ± 0.44	3 (1 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Acrantophis*[3,4], *Echis*[7], *Loxocemus*[8], *Python*[10], *Elaphe*[11], *Naja*[11], *Deinagkistrodon*[11], *Ptyas*[11], *Pantherophis*[12,14], *Drymarchon*[13], *Hydrophis*[15]
38.1	Octadecanoic acid (stearic acid) *	0.07 ± 0.05	2 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Acrantophis*[3,4], *Echis*[7], *Vipera*[7], *Loxocemus*[8], *Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12,14], *Drymarchon*[13], *Hydrophis*[15]
40.7	5,8,11,14-Eicosatetraenoic acid (arachidonic acid) *	0.02 ± 0.02	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Rena*[1], *Loxocemus*[8], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12,14]
ESTERS OF CARBOXYLIC ACIDS									
29.5	Tetradecanoic acid, methyl ester *	+	1 (0 ♀, 1 ♂)	✓		✓		✓	
33.6	Hexadecanoic acid, methyl ester *	0.03 ± 0.02	4 (1 ♀, 2 ♂)	✓	✓	✓		✓	
34.9	Hexadecanoic acid, ethyl ester *	+	4 (1 ♀, 2 ♂)	✓	✓	✓		✓	
35.4	Heptadecanoic acid, methyl ester *	+	1 (0 ♀, 1 ♂)	✓				✓	
35.8	7,10,13-Eicosatrienoic acid, methyl ester	+	1 (0 ♀, 1 ♂)						
36.8	9,12-Octadecadienoic acid, methyl ester *	+	2 (1 ♀, 1 ♂)	✓	✓	✓		✓	
36.9	9-Octadecenoic acid, methyl ester *	0.03 ± 0.01	5 (1 ♀, 3 ♂)	✓				✓	
37.0	10-Octadecenoic acid, methyl ester	+	3 (1 ♀, 2 ♂)						
37.4	Octadecanoic acid, methyl ester *	0.01 ± 0.01	3 (1 ♀, 1 ♂)	✓	✓	✓		✓	
38.0	9,12-Octadecadienoic acid, ethyl ester *	0.01 ± 0.01	3 (1 ♀, 1 ♂)	✓		✓		✓	
38.1	9-Octadecenoic acid, ethyl ester *	0.05 ± 0.03	5 (1 ♀, 3 ♂)	✓	✓	✓		✓	

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
38.6	Octadecanoic acid, ethyl ester *	0.01 ± 0.01	3 (1 ♀, 1 ♂)	✓	✓	✓		✓	
39.7	5,8,11,14-Eicosatetraenoic acid, methyl ester	+	2 (1 ♀, 1 ♂)	✓		✓			
44.0	Docosanoic acid, methyl ester	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
46.1	6,9,12,15-Docosatetraenoic acid, methyl ester	0.03 ± 0.09	7 (1 ♀, 5 ♂)						
47.1	Tetracosanoic acid, methyl ester	+	1 (0 ♀, 1 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Ptyas*[11]
ALCOHOLS									
12.8	3,7-Dimethyl-octanol	0.01 ± 0.01	7 (1 ♀, 5 ♂)				✓		*Naja*[11]
16.1	Undecanol *	+	1 (0 ♀, 0 ♂)	✓		✓		✓	
16.5	Decenol	+	1 (0 ♀, 0 ♂)						
21.6	Dodecanol *	+	1 (0 ♀, 0 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11], *Pantherophis*[12]
27.0	Dodecenol	+	2 (0 ♀, 1 ♂)						
38.9	Octadecanol *	0.04 ± 0.03	2 (0 ♀, 2 ♂)	✓		✓	✓	✓	*Python*[10], *Elaphe*[11,14], *Naja*[11], *Ptyas*[11], *Pantherophis*[12]
42.2	Eicosanol *	0.02 ± 0.02	2 (0 ♀, 2 ♂)	✓		✓	✓	✓	*Python*[10], *Pantherophis*[12]
50.1	Hexacosanol *	+	3 (1 ♀, 1 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11]
52.2	Octacosanol *	0.32 ± 0.22	2 (1 ♀, 0 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11]
ALKANES									
12.1	Undecane *	0.05 ± 0.01	10 (2 ♀, 7 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
12.3	2,4,6-Trimethyl-decane	+	6 (1 ♀, 4 ♂)						
13.4	4,5-Diethyl-octane	0.11 ± 0.04	9 (1 ♀, 7 ♂)						
13.6	Unknown branched alkane	+	5 (1 ♀, 3 ♂)						
14.0	5-Methyl-nonane	0.16 ± 0.06	7 (1 ♀, 5 ♂)						
14.4	5,6-Dimethyl-decane	0.05 ± 0.02	6 (1 ♀, 4 ♂)					✓	
14.5	2,3-Dimethyl-heptane	0.05 ± 0.02	6 (1 ♀, 4 ♂)	✓				✓	

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
14.9	4-Ethyl-decane	+	3 (0 ♀, 2 ♂)	✓				✓	
15.1	5-Methyl-undecane	0.26 ± 0.22	9 (1 ♀, 7 ♂)	✓					
16.4	Dodecane *	+	3 (0 ♀, 2 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Ptyas*[11]
18.6	Tridecane *	0.01 ± 0.01	6 (1 ♀, 4 ♂)	✓		✓	✓	✓	*Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
19.9	3,7-Dimethyl-undecane	0.01 ± 0.01	6 (0 ♀, 5 ♂)	✓				✓	
21.8	Tetradecane *	+	4 (0 ♀, 3 ♂)	✓		✓	✓	✓	*Python*[10], *Naja*[11], *Ptyas*[11]
24.2	Pentadecane *	0.01 ± 0.01	6 (1 ♀, 4 ♂)	✓	✓	✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Ptyas*[11]
25.3	Hexadecane *	0.02 ± 0.01	8 (1 ♀, 6 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
28.9	Heptadecane *	0.01 ± 0.01	9 (1 ♀, 7 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Ptyas*[11]
29.2	Octadecane *	0.02 ± 0.01	7 (1 ♀, 6 ♂)	✓	✓	✓	✓	✓	*Python*[10]
30.1	Unknown branched alkane	0.02 ± 0.01	9 (1 ♀, 7 ♂)						
33.1	Nonadecane *	+	4 (0 ♀, 3 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
33.6	Unknown branched alkane	0.03 ± 0.01	7 (2 ♀, 5 ♂)						
35.0	Eicosane *	0.02 ± 0.01	9 (1 ♀, 7 ♂)	✓		✓	✓	✓	*Python*[10]
36.9	Unknown branched alkane	+	1 (0 ♀, 1 ♂)						
38.4	Unknown branched alkane	0.03 ± 0.01	7 (1 ♀, 6 ♂)						
38.7	Docosane *	0.06 ± 0.03	4 (0 ♀, 4 ♂)	✓		✓	✓	✓	*Python*[10]
40.4	Tricosane *	0.17 ± 0.08	9 (2 ♀, 6 ♂)	✓		✓	✓	✓	*Python*[10]
42.0	Tetracosane *	0.05 ± 0.04	2 (0 ♀, 2 ♂)	✓		✓	✓		*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Ptyas*[11]
43.0	Unknown branched alkane	0.01 ± 0.01	5 (1 ♀, 3 ♂)						
43.2	Unknown branched alkane	+	2 (0 ♀, 2 ♂)						
43.7	Pentacosane *	0.48 ± 0.25	12 (2 ♀, 9 ♂)	✓		✓	✓	✓	*Python*[10]
44.1	Unknown branched alkane	0.01 ± 0.01	4 (1 ♀, 2 ♂)						
44.6	Unknown branched alkane	0.02 ± 0.01	6 (1 ♀, 4 ♂)						
44.7	Unknown branched alkane	0.45 ± 0.43	8 (1 ♀, 7 ♂)						
44.9	Unknown branched alkane	+	2 (1 ♀, 1 ♂)						
45.1	Hexacosane *	0.60 ± 0.31	11 (2 ♀, 8 ♂)	✓		✓	✓	✓	*Python*[10]

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
45.2	Unknown branched alkane	+	4 (1 ♀, 2 ♂)						
46.0	Unknown branched alkane	0.03 ± 0.01	5 (0 ♀, 5 ♂)						
46.1	Heptacosane *	0.04 ± 0.04	2 (0 ♀, 2 ♂)	✓	✓	✓	✓	✓	*Python*[10]
46.2	Unknown branched alkane	0.01 ± 0.01	5 (1 ♀, 4 ♂)						
46.6	Unknown branched alkane	0.79 ± 0.40	12 (2 ♀, 9 ♂)						
47.1	Unknown branched alkane	0.01 ± 0.01	4 (2 ♀, 1 ♂)						
47.5	Unknown branched alkane	0.03 ± 0.01	6 (1 ♀, 4 ♂)						
47.6	Unknown branched alkane	0.03 ± 0.01	8 (1 ♀, 6 ♂)						
48.0	Octacosane *	0.98 ± 0.53	12 (2 ♀, 9 ♂)	✓		✓	✓	✓	*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
48.8	Unknown branched alkane	0.59 ± 0.54	9 (2 ♀, 7 ♂)						
48.9	Unknown branched alkane	0.04 ± 0.02	8 (2 ♀, 5 ♂)						
49.4	Unknown branched alkane	1.23 ± 0.69	12 (2 ♀, 9 ♂)						
49.8	Unknown branched alkane	0.02 ± 0.01	7 (2 ♀, 4 ♂)						
50.2	Unknown branched alkane	0.02 ± 0.01	4 (1 ♀, 3 ♂)						
50.4	Unknown branched alkane	0.04 ± 0.01	9 (2 ♀, 6 ♂)						
50.6	Unknown branched alkane	0.60 ± 0.56	6 (1 ♀, 5 ♂)						
50.7	Nonacosane *	1.18 ± 0.69	12 (2 ♀, 10 ♂)	✓		✓	✓	✓	*Python*[10], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
50.8	Unknown branched alkane	0.03 ± 0.02	6 (2 ♀, 4 ♂)						
51.2	Unknown branched alkane	0.02 ± 0.01	6 (1 ♀, 5 ♂)						
51.5	Unknown branched alkane	0.06 ± 0.02	8 (0 ♀, 8 ♂)						
51.7	Unknown branched alkane	0.03 ± 0.01	4 (1 ♀, 3 ♂)						
52.0	Unknown branched alkane	1.12 ± 0.63	11 (2 ♀, 9 ♂)						
53.2	Triacontane *	0.90 ± 0.51	11 (2 ♀, 9 ♂)	✓		✓	✓		*Python*[10]
53.5	Unknown branched alkane	0.10 ± 0.03	8 (1 ♀, 7 ♂)						
54.2	Unknown branched alkane	0.02 ± 0.01	2 (0 ♀, 2 ♂)						
54.6	Hentriacontane *	0.73 ± 0.41	7 (1 ♀, 6 ♂)	✓		✓	✓	✓	*Python*[10]
54.7	Unknown branched alkane	0.08 ± 0.05	6 (2 ♀, 4 ♂)						
55.5	Unknown branched alkane	0.04 ± 0.03	3 (0 ♀, 3 ♂)						

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
55.8	Unknown branched alkane	0.02 ± 0.02	3 (0 ♀, 3 ♂)						
56.2	Dotriacontane *	0.43 ± 0.27	7 (0 ♀, 7 ♂)	✓		✓			
58.0	Tritriacontane *	0.47 ± 0.23	8 (1 ♀, 7 ♂)	✓		✓			
59.2	Unknown branched alkane	0.04 ± 0.02	5 (1 ♀, 3 ♂)						
60.2	Tetratriacontane *	0.18 ± 0.13	4 (0 ♀, 4 ♂)	✓		✓		✓	
62.8	Pentatriacontane *	0.11 ± 0.08	4 (0 ♀, 4 ♂)	✓		✓	✓		*Elaphe*[11], *Ptyas*[11]
65.9	Hexatriacontane *	0.06 ± 0.04	4 (0 ♀, 4 ♂)	✓				✓	
ALDEHYDES									
13.5	Nonanal *	0.02 ± 0.01	7 (2 ♀, 4 ♂)	✓		✓		✓	
16.8	Decanal *	+	1 (0 ♀, 1 ♂)	✓		✓		✓	
19.6	Undecanal *	+	1 (0 ♀, 1 ♂)	✓				✓	
22.1	Dodecanal *	+	2 (0 ♀, 1 ♂)	✓		✓		✓	
27.0	Tetradecanal *	+	3 (0 ♀, 3 ♂)	✓		✓	✓	✓	*Pantherophis*[12]
31.4	Pentadecanal *	+	2 (0 ♀, 1 ♂)	✓		✓		✓	
35.4	Hexadecanal *	+	3 (1 ♀, 1 ♂)	✓		✓		✓	
37.3	Octadecanal *	+	2 (1 ♀, 0 ♂)	✓		✓		✓	
39.1	Octadecenal	0.04 ± 0.03	3 (1 ♀, 1 ♂)						
40.9	Eicosanal *	0.02 ± 0.01	6 (1 ♀, 4 ♂)	✓		✓			
AROMATICS									
19.4	4-Butyl-4-cyanophenyl ester-benzoic acid	0.04 ± 0.01	9 (2 ♀, 6 ♂)						
24.7	Butylated hydroxytoluene *	0.01 ± 0.01	7 (1 ♀, 5 ♂)	✓				✓	
45.6	3,4-Dihydro-6,7-dimethoxy-1-phenyl-isoquinoline	0.02 ± 0.01	2 (0 ♀, 2 ♂)						
KETONES									
32.0	6,10,14-Trimethyl-2-pentadecanone	+	3 (0 ♀, 2 ♂)	✓		✓		✓	

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
40.5	2-Nonadecanone *	0.01 ± 0.01	3 (0 ♀, 2 ♂)	✓		✓		✓	
43.8	Docosa-2,21-dione	+	1 (0 ♀, 1 ♂)						
46.8	2-Pentacosanone *	0.05 ± 0.02	6 (2 ♀, 3 ♂)	✓		✓	✓		*Drymarchon*[13]
49.6	2-Heptacosanone *	0.14 ± 0.06	9 (1 ♀, 7 ♂)	✓			✓		*Thamnophis*[9], *Drymarchon*[13]
52.3	2-Nonacosanone *	0.13 ± 0.07	6 (2 ♀, 4 ♂)	✓			✓		*Thamnophis*[9], *Drymarchon*[13]
55.0	2-Heneicosanone	0.13 ± 0.05	6 (1 ♀, 4 ♂)	✓		✓	✓	✓	*Drymarchon*[13]
AMIDES									
41.4	9-Octadecenamide (oleamide) *	0.07 ± 0.04	5 (0 ♀, 4 ♂)	✓		✓		✓	
47.7	13-Docosenamide (erucamide) *	0.35 ± 0.16	11 (2 ♀, 8 ♂)			✓			
TERPENES & TERPENOIDS									
11.2	Limonene *	0.11 ± 0.03	12 (2 ♀, 9 ♂)	✓	✓			✓	
31.1	Limonen-6-ol, pivalate	0.04 ± 0.01	10 (2 ♀, 7 ♂)	✓					
48.5	Squalene *	0.42 ± 0.10	12 (2 ♀, 9 ♂)	✓	✓	✓	✓	✓	*Acrantophis*[3], *Thamnophis*[5], *Python*[10]
STEROIDS									
48.6	Cholesta-2,4-diene *	0.08 ± 0.02	11 (2 ♀, 8 ♂)			✓		✓	
49.0	Cholesta-3,5-diene *	0.06 ± 0.02	10 (2 ♀, 7 ♂)	✓		✓	✓	✓	*Python*[10]
49.2	Cholesta-4,6-dien-3-ol *	0.13 ± 0.02	12 (2 ♀, 9 ♂)	✓		✓		✓	
49.5	Cholesta-3,5-diene (unknown derivative)?	0.24 ± 0.03	12 (2 ♀, 9 ♂)						
49.9	Unknown steroid (m/z: 119,325,351)	0.03 ± 0.02	8 (2 ♀, 5 ♂)						
51.0	3-Methoxy-cholest-5-ene *	0.06 ± 0.02	10 (2 ♀, 8 ♂)			✓			
51.7	3-Methoxy-cholest-5-ene (unknown derivative)?	0.08 ± 0.03	6 (1 ♀, 5 ♂)						

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
52.5	Cholesterol *	65.19 ± 4.36	13 (2 ♀, 10 ♂)	✓	✓	✓	✓	✓	*Boa*[2,18], *Coluber*[2], *Lampropeltis*[2,18], *Pituophis*[2], *Thamnophis*[2,6], *Tropidoclonion*[2], *Heterodon*[2], *Naja*[2,11], *Morelia*[2], *Liasis*[2], *Morelia*[2], *Malayopython*[2], *Pantherophis*[2,12,14,17], *Crotalus*[2,18], *Bitis*[2], *Agkistrodon*[2,18], *Acrantophis*[3,4], *Echis*[7] , *Vipera*[7], *Gloydius*[7], *Python*[10], *Deinagkistrodon*[11], *Ptyas*[11], *Elaphe*[11], *Hydrophis*[15], *Crotalus*[16], *Drymarchon*[18], *Pituophis*[18], *Nerodia*[18], *Calloselasma*[18]
52.6	Cholestan-3-ol *	8.79 ± 1.14	13 (2 ♀, 10 ♂)			✓		✓	
53.0	Cholestan-3-one *	0.88 ± 0.13	12 (2 ♀, 10 ♂)	✓		✓		✓	
53.2	Cholestan-3-one (unknown derivative)?	0.03 ± 0.02	5 (1 ♀, 4 ♂)						
53.3	Ergosta-7,22-dien-3-ol	0.09 ± 0.05	7 (1 ♀, 5 ♂)			✓			
53.6	Stigmastan-3-en-6-ol	0.83 ± 0.23	12 (2 ♀, 9 ♂)						
53.9	Campesterol *	0.24 ± 0.23	5 (1 ♀, 3 ♂)	✓	✓	✓	✓	✓	*Patherophis*[12]
54.1	Cholest-4-en-3-one *	3.31 ± 0.48	13 (2 ♀, 10 ♂)	✓		✓		✓	
54.6	Cholesta-4,6-dien-3-one *	0.32 ± 0.12	6 (1 ♀, 4 ♂)			✓		✓	
55.3	β-Sitosterol *	0.33 ± 0.23	8 (1 ♀, 6 ♂)	✓	✓	✓	✓		*Python*[10], *Deinagkistrodon*[11], *Elaphe*[11], *Naja*[11], *Ptyas*[11]
55.4	Olean-12-en-28-ol	0.11 ± 0.05	5 (1 ♀, 4 ♂)						
55.8	Stigmastanol *	0.17 ± 0.14	4 (1 ♀, 2 ♂)			✓	✓		*Python*[10]
56.1	Lup-20(29)-en-3-one	0.78 ± 0.40	8 (1 ♀, 6 ♂)						
56.6	Cholestane-3,6-dione	0.13 ± 0.07	6 (1 ♀, 4 ♂)			✓			
57.4	Stigmast-4-en-3-one *	0.26 ± 0.23	5 (1 ♀, 3 ♂)			✓			
TOCOPHEROLS									
51.5	γ-Tocopherol *	+	1 (0 ♀, 1 ♂)	✓		✓			
52.0	D-α-Tocopherol *	0.01 ± 0.01	1 (0 ♀, 1 ♂)	✓		✓		✓	
WAXY ESTERS									

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
42.5	Octadecyl-9-octadecenoate *	0.01 ± 0.01	5 (2 ♀, 3 ♂)	✓		✓			
43.7	Unknown wax ester of hexadecanoic acid	+	1 (0 ♀, 1 ♂)						
50.2	Unknown wax ester of 9-octadecenoic acid	+	3 (0 ♀, 3 ♂)						
57.2	Unknown wax ester of 9-octadecenoic acid	0.03 ± 0.01	3 (0 ♀, 3 ♂)						
61.2	Nonyl-docosanoate	0.10 ± 0.05	8 (1 ♀, 7 ♂)			✓			
61.9	Unknown wax ester	0.12 ± 0.07	5 (1 ♀, 4 ♂)						
63.7	Octadecyl-eicosanoate	0.54 ± 0.41	7 (1 ♀, 6 ♂)			✓			
64.4	Unknown wax ester of hexadecanoic acid	0.09 ± 0.04	6 (1 ♀, 4 ♂)						
OTHERS									
41.3	4,8,12,16-Tetramethylheptadecan-4-olide	+	3 (1 ♀, 1 ♂)	✓		✓		✓	
57.5	Unknown compound (m/z: 167 185)	0.45 ± 0.20	8 (2 ♀, 6 ♂)						

[1] Blum et al. 1971; [2] Burken et al. 1985b; [3] Simpson et al. 1993; [4] Simpson et al. 1988; [5] Mason et al. 1989; [7] Razakov & Sadykov 1986; [8] Schulze et al. 2017; [9] Mason et al. 1990; [10] Jacob et al. 1993; [11] Chunfu et al. 2019; [12] Ball 2000; [13] Ahern & Downing 1974; [14] Ball 2004; [15] Weldon et al. 1991; [16] Weldon et al. 1990; [17] Roberts & Lillywhite 1980; [18] Schell & Weldon 1985

Table 2.

Mixed effect models describing the causes of variance within behavioural variables extracted from focal observations. The data is considered for the two experiments separately. The random effect (1|IND) accounts for repeated measurements on the same individual. (1|ObsID) is an observation-level random effect and accounts for overdispersion (Harrison 2014). The increment of AIC (Akaike information value) indicates the difference between two models which differ only in the inclusion of Treatment. An asterisk indicates a significantly ($P < 0.05$) better fitting model

Best model	ΔAIC	Chi-square	Degrees of freedom	*P*-value
Experiment A: skin extract				
Undirected tongue flick = 1 + (1|IND) + (1|ObsID)	- 1.96	0.041	3	0.839
Directed tongue flick = Treatment + (1|IND)	**- 8.82**	**10.828**	**4**	**0.001 ***
Bite = 1 + (1|IND) + (1|ObsID)	- 0.99	1.002	3	0.317
Startle = Treatment + (1|IND)	**- 8.90**	**10.899**	**3**	**0.001 ***
Head turn = 1 + (1|IND)	- 1.87	0.133	2	0.715
Flutter = Treatment + (1|IND)	**- 32.46**	**34.458**	**3**	**< 0.001 ***
Experiment B: skin residue				
Undirected tongue flick = 1 + (1|IND) + (1|ObsID)	- 2.65	1.353	3	0.508
Directed tongue flick = 1 + (1|IND)	- 2.45	1.552	3	0.460
Bite = 1 + (1|IND)	- 3.55	0.455	2	0.797
Startle = 1 + (1|IND)	- 3.81	0.188	2	0.910
Head turn = Treatment + (1|IND)	- 1.83	5.833	4	0.054