Chemosensory predator detection in lacertid lizards

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Podarcis and *Zootoca* lizard drawings, and *Herpestes* mongoose drawing are made by Wendy Vrijdag.

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Summary

For many animals, the ability to detect and recognise predators is crucial for their survival. Accordingly, species have evolved multiple sensory systems warning them of imminent dangers. One of these, the sense of smell, is probably the oldest and most widespread system, but likely also the least understood. Technological constraints have long time hampered the study of chemical cues. As a result, we know little about how chemosensory predator detection works, how it helps animals to survive, and which factors and mechanisms power or constrain its evolution.

Lacertid lizards (Lacertidae, Squamata, Reptilia) are excellent study models to change this. These organisms use chemical cues in many contexts, including predator detection and recognition. When confronted with danger-indicating kairomones, they will immediately raise the frequency with which they flick their tongue and exhibit a number of highly stereotyped behaviours, elements that can be conveniently used to quantify and interpret their interest in particular chemical cues. In addition, lacertid species are abundant in a wide variety of habitats that differ in the composition and density of their predator communities. This offers opportunities for identifying possible (evolutionary) drivers of variation in chemosensory abilities.

This thesis contributes in advancing our knowledge on lizard chemosensory predator detection in two complementary ways. First, I have conducted behavioural experiments, confronting two species of lacertid lizards with chemical cues from a divergent set of terrestrial predators, in order to test the effects of predator type (mammal or snake), origin (native, invasive or allopatric) and insularity (mainland or island prey populations). Second, I have explored and tested a number of new techniques that may revolutionise chemical ecology in the years to come.

Studies on chemosensory predator recognition in lacertids hitherto have focused on their ability to detect and recognise chemicals left behind by snakes. Intriguingly, no one had considered mammalian predator cues. Instigated by the alarming spread of the invasive mongoose (*Herpestes auropunctatus*, a notorious predator of reptiles) in the Balkan region, I set out to test whether lacertid lizards were able to detect and recognise its odour. Surprisingly, individuals of the Asian grass lizard (*Takydromus sexlineatus*), a lacertid species that inhabits the native range of the mongoose, exhibited no signs of stress or overt anti-predatory

behaviour when experimentally confronted with mongoose chemicals; while chemicals of a sympatric snake predator (the Oriental whip snake, *Ahaetulla prasina*) elicited the behaviours typical for lacertids in dangerous situations. Even more puzzling, Dalmatian wall lizards (*Podarcis melisellensis*) from a mainland habitat in Croatia did mount the typical anti-predatory response when brought into contact with mongoose scent. However, wall lizards of the same species, but living on islands, failed to recognise mongoose chemicals, or ignored them. This was irrespective of whether mongooses had been introduced to the island or not. In fact, island lizards proved to have remarkably low chemosensory responsiveness in general: they also showed little reaction towards chemicals of saurophagous snakes (even those species that occur in sympatry). Using μCT scans of a limited number of individuals, I found that island lizards tend to have smaller accessory olfactory bulbs (the region in the brain involved in processing vomerolfactory information) than mainland specimens – which would agree with reduced investment in chemosensation. I hypothesise that insular conditions (limited resource availability and predator relaxation) select against allocating energy to the development and maintenance of the expensive neural tissues required for chemosensation.

In the second part of the thesis, I used a well-known study system (recognition of adder, *Vipera berus*, chemicals by common lizards, *Zootoca vivipara*) to test the usefulness of two techniques for the identification of kairomones. Ignorance on the specific nature of the kairomones triggering lizard anti-predatory behaviour has long time limited advances in the field. I found that neutral lipids, extracted with n-hexane from adder skin, provoked the typical fear-response in common lizards. Subsequent Gas Chromatography – Mass Spectrometry analysis of the viper skin washes revealed a complex cocktail of 165 different molecules, several of which are likely candidate-kairomones. In a subsequent study, I tested a recently developed technique (Proton-Transfer Reaction Time-of-Flight Mass Spectrometry, PTR-TOF MS) that allows real-time capture and extremely accurate mass annotation of highly volatile molecules. Bio-assays showed that common lizards can detect the presence of adders based on such volatile molecules only – provided the scent had been deposited recently. My results show that predator scent may be a dynamic source of information, revealing not only whether a predator has passed by, but also how long ago.

Voor veel dieren is het vermogen om roofdieren te detecteren en herkennen cruciaal om te overleven. Als gevolg hebben soorten verschillende zintuigen ontwikkeld die hen waarschuwen voor aankomend gevaar. Een van deze zintuigen, het reukvermogen, is waarschijnlijk het oudste en meest wijdverspreid, maar ook het minst begrepen. Technologische restricties, en de neiging van onze eigen soort om vooral te vertrouwen op visuele en auditieve signalen, hebben lang het onderzoek naar chemische signalen belemmerd. Dit heeft als resultaat dat we maar een beperkte kennis hebben over het belang van en de mechanismen achter de herkenning van predatoren via chemische signalen en de factoren die evolutie hierin veroorzaken.

Lacertide hagedissen (Lacertidae, Squamata, Reptilia) vormen goede studiemodellen om hier verandering in te brengen. Deze dieren gebruiken chemische moleculen in veel situaties; onder andere bij het detecteren en herkennen van roofdieren. Wanneer zij geconfronteerd worden met gevaarindicerende kairomonen zullen deze hagedissen onmiddellijk de frequentie verhogen waarmee ze met hun tong de omgeving aftasten naar chemische moleculen, en gaan ze over tot het vertonen van heel stereotypisch gedrag, beide elementen die gemakkelijk kunnen gekwantificeerd en geïnterpreteerd worden in een experimentele setting. Bovendien zijn lacertide soorten abundant aanwezig in een grote diversiteit aan habitatten die verschillen in de compositie en densiteit van de roofdiergemeenschap.

Via de toepassing van twee verschillende onderzoeksstrategieën, draagt deze thesis bij tot het verrijken van onze kennis rond de mogelijkheden van hagedissen om chemische signalen van roofdieren te detecteren. Allereerst heb ik gedragsexperimenten uitgevoerd waarbij ik twee soorten van lacertide hagedissen in contact bracht met geur van verschillende roofdieren; dit, om het effect te testen van het predator type (zoogdier of slang), de origine van de predator (inheems, invasief of allopatrisch), alsook die van de prooi (vastelands- of eilandpopulaties van de hagedis) op chemische predatorherkenning. Ten tweede, heb ik enkele recent ontwikkelde technieken toegepast die het onderzoeksveld van de chemische ecologie zouden kunnen vooruit stuwen.

Studies op chemische predatorherkenning bij lacertiden hebben tot dusver gefocust op het vermogen tot het detecteren en identificeren van slangen. Intrigerend genoeg heeft niemand zoogdieren overwogen. Aangespoord door de alarmerende uitbreiding van het leefgebied van een invasieve mangoeste

(*Herpestes auropunctatus*, een berucht jager van reptielen) in de Balkan, vatte ik onderzoek aan om na te gaan of lacertide hagedissen chemische signalen van deze mangoeste kunnen detecteren en herkennen. Tot mijn verbazing vertoonden langstaarthagedissen (*Takydromus sexlineatus*), een soort die voorkomt in de natuurlijke range van de mangoeste, geen tekenen van stress of openlijk antipredator gedrag wanneer ze geconfronteerd werden met geur van de mangoeste. Nochtans uitten deze hagedissen gedragingen die typisch zijn voor lacertiden in gevaar wanneer hen geur werd aangeboden van een sympatrische slangensoort (*Ahaetulla prasina*). Nog verrassender was de observatie dat muurhagedissen (*Podarcis melisellensis*) van het Kroatische vasteland wel het typische antipredator gedrag vertoonden wanneer zij in contact kwamen met mangoestegeur, terwijl muurhagedissen van eilanden dit niet deden. Dit, ongeacht of de mangoeste was geïntroduceerd op de eilanden of niet. Deze muurhagedissen van de Kroatisch eilanden vertoonden zelfs tekenen van een algemeen minder ontwikkeld reukvermogen. Ze reageerden namelijk ook niet op gevaarlijke slangen (zelfs niet die soorten die in sympatrie leven). Via µCT scanning bij een beperkt aantal individuen vond ik dat de hersenregio's die instaan voor het verwerken van chemische signalen ontvangen via het Jacobson orgaan gemiddeld kleiner zijn bij eilandhagedissen dan bij hagedissen van het vasteland. Mijn hypothese is dat dit veroorzaakt wordt door bepaalde eilandcondities (beperkte aanwezigheid van voedsel en verminderde predatiedruk).

In de tweede helft van de thesis gebruikte ik een welgekend studiesysteem (herkenning van adder, *Vipera berus*, moleculen door levendbarende hagedissen, *Zootoca vivipara*) om de toepasbaarheid te achterhalen van twee technieken in het identificeren van kairomonen. Onze onwetendheid over de chemische identiteit van kairomonen, die antipredator gedrag in hagedissen triggeren, heeft gedurende lange tijd de vorderingen in het veld van de chemische ecologie beperkt. Ik vond dat non-polaire lipiden, geëxtraheerd met n-hexaan van addervellen, de typische angstreactie in levendbarende hagedissen veroorzaken. Chemische analyses met Gas Chromatografie – Massa Spectrometrie legden een diverse cocktail van 165 verschillende componenten bloot. Ten minste één van deze componenten is het effectieve kairomoon. In een vervolgstudie testte ik een recent ontwikkelde techniek (Proton-Transfer Reactie Time-of-Flight Massa Spectrometrie, PTR-TOF-MS) die real-time metingen toelaat van extreem vluchtige bestanddelen. Bioassays tonen ons dat levendbarende hagedissen de aanwezigheid van adders

kunnen bepalen aan de hand van enkel deze moleculen – tenminste, wanneer geur heel recent werd afgezet. Deze resultaten beklemtonen de complexiteit van informatie vervat in predatorgeur en gebruikt door prooidieren.

Chapter 1

General introduction

The ecology of sensing

From tundra to tropics, deep sea trenches to alpine meadows, the earth's environments change incessantly. Day becomes night, sunshine turns into rain, rivers flood and trees fall. An animal's worst predator, its favourite prey or a potential mate may appear at any time. In this landscape of uncertainty, animals move and behave in ways that benefit their survival and reproductive success. They escape predation and find food and shelter to survive, and locate suitable partners to reproduce. To that purpose, all living organisms have evolved multiple and often sophisticated ways of sensing features of their surroundings. Prokaryotes and unicellular eukaryotes may move along gradients of light intensity (Jékely 2009), or concentrations of chemicals (Swaney et al. 2010; Wuichet and Zhulin 2010), perform basic behavioural responses to mechanical stress (Hara and Asai 1980), or navigate along the earth's magnetic (Monteil and Lefevre 2020) and gravitational fields (Krause and Bräucker 2009). Even seemingly static life forms such as plants have sensors that help them direct themselves towards the light to optimise photosynthesis (e.g. Losi and Gärtner 2012). The most advanced sensory systems (and subsequent stimulus-driven behaviours), however, arose in the animal kingdom along with the evolution of neurones (Jékely 2011). The amplification of sensory stimuli through neuronal action potentials enables animals to distinguish more details in their perceived environment. Furthermore, neuronal networks allow the rapid transfer of information and fast coordinated responses in a multicellular body (Monk and Paulin 2014).

The most widely-known 'big five' of animal senses are vision, hearing, taste, smell and touch. Besides, species belonging to disparate taxa have evolved ways to detect, for instance, changes in electric fields (e.g. rays and sharks, monotremes; Pettigrew 1999; Newton et al. 2019), feel the Earth's magnetic field (e.g. migratory birds, honey bees; Wiltschko and Wiltschko 2005; Liang et al. 2016), or sense differences in infrared energy (e.g. pythons, boids and vampire bats; Campbell et al. 2002; Gracheva et al. 2011). Despite this plethora of possibilities for retrieving information from the environment, species usually only deploy a limited subset of sensory systems. Some species simply have not evolved certain senses due to phylogenetic constraints. For instance, among birds, violet-sensitive vision is only present in a few lineages (galliforms, musophagiforms andcharadriiforms); in these lineages, it has enabled the development of colourful porphyrin plumage pigments that are important for intraspecific communication (Bleiweiss 2015). In

contrast, senses that were ancestrally present may be secondarily lost. Extreme examples are fossorial creatures (e.g. blind mole rats, *Spalax ehrenbergi*; David-Gray et al. 1998) and cave-dwelling species (e.g. populations of Mexican tetras, *Astyanax mexicauns*; Wilkens 2007), that are **deprived** of their ability to see.

Several mechanisms may lead to the depletion or loss of a sensory system (Rétaux and Casane 2013). When a sense falls into disuse, deleterious mutations can accumulate in the genetic domains that build the organ (Futuyma 2010). Random genetic drift may fixate one or more of these mutations. For instance, trichromatic colour vision in primates is thought to be adaptive for foraging on reddish food. On Madagascar, the dull colouration of fruits may have relaxed selection on such a trait and allowed the reduction of colour vision from trichromacy to dichromacy in the lemur *Eulemur rubriventer* through genetic drift (Jacobs et al. 2019). Such neutral effects are expected to be more pronounced following a genetic bottleneck event and through founder effects that occur, for instance, after animals have newly colonised an island (Raine et al. 2006). Alternatively, the loss may be actively selected for. Both at rest and whilst signalling do sensory neurons cause a high energy expenditure. Therefore, it can be expected that animals will re-allocate that energy towards other structures whenever a sense becomes redundant (Niven and Laughlin 2008; Moran et al. 2015). The strength of the selective pressure will depend critically upon the precise environmental circumstances in which a specific animal finds itself. For instance, energy-limited habitats, such as caves or islands, increase the need for energy saving or divert attention away from tasks not related to foraging (Endler 1993). A functional trade-off may occur at the level of the brain, where areas responsible for the processing of one sensory modus could be recruited by another modus, as suggested by cross-modal reorganisation following the loss of one sense in humans and animals (Merabet and Pascual-Leone 2010). Also, it has been suggested that underused sensors may be selected against because they are prone to injury or can function as an entry point of infection (Krishnan and Rohner 2017). The latter is illustrated by the way in which the SARS-Cov-2 virus enters mammalian bodies through the eye (Seah and Agrawal 2020). Finally, selection for another trait, negatively linked to a particular sense, would indirectly lead to reduced performance of that sense. In cave fish, for instance, altered embryonic expression of the sonic hedgehog gene promotes the development of taste buds, but at the same time reduces eye size (Yamamoto et al. 2009).

Since both the costs and the benefits of particular sensory modalities depend on the prevailing ecological circumstances, sensory systems can be expected to differ among species, populations and even among individuals within populations (Dangles et al. 2009). For a subset of sensory modalities (mostly vision) and taxa, there is indeed evidence for adaptive evolution – at least on a wide taxonomic scale. Teleost fish, for instance, exhibit a great diversity of visual pigments and colour vision, depending on their lifestyles (review in Bowmaker 1995). The peak sensitivity of cones in hymenopterans (Chittka and Menzel 1992) and primates (Regan et al. 2001) has evolved in concert with the colouration of flowers and fruits, respectively. The ease at which signals from specific modalities travel through a certain type of habitat may lead to the favouring of one modality, while others are disfavoured. Across mammals, arboreality selects for larger eyes but smaller noses; and aquatic or semi-aquatic carnivores are less olfactory oriented than their terrestrial counterparts (Nummela et al. 2013).

With a number of fascinating exceptions (e.g. Jacobs 1984; Larmuseau et al. 2009; Ronald et al. 2017; reviewed in Dangles et al. 2009) few studies have examined the variation in sensorial systems resulting from differing ecologies and contextual environments at lower taxonomical level - i.e. comparing closely related species, populations within species or individuals within a population. As a consequence, we know very little on how quickly animals can adjust their sensorial systems when evolutionary pressures change. Furthermore, especially for sensory modalities other than vision and hearing, we know little on how the environment can influence the signalling, per se – i.e. the ability to send, transmit or receive a sensory cue (Yohe and Brand 2018). In a world in which both settings (see box 1) and ecological interactions themselves (box 2) are changing at an unprecedented pace due to anthropogenic activities, this lack of knowledge jeopardises the rollout of effective conservation measures.

Perceiving the world through chemicals

The chemical senses may constitute the oldest and most widespread mode of information gathering, being deployed by even the simplest of extant life forms (i.e. bacteria, slime moulds and protists; Ache and Young 2005). Yet, due to the technical difficulties accompanying the investigation of something as obscure as a chemical molecule, the importance of chemical communication has for a long time

Box 1

Sensory pollution and climate change

Today's human-dominated world is severely challenging the sensory systems of organisms. Firstly, sensory pollution (i.e. the rising levels of artificial light, acoustic noise and the changing composition of chemicals in the air due to anthropogenic activities) impacts the detectability of sensory cues, hindering adequate behavioural responses (Dominoni et al. 2020). For instance, in ovenbirds (*Seiurus aurocapilla)*, anthropogenic noise reduces the maximal travelling distance and reliability of male vocalisations (as an indicator of the male's quality) (Habib et al. 2007). In general, the rise in auditory noise around human settlements and centres of high traffic is thought to severely impact vocal signalling in many bird species (Slabbekoorn and Ripmeester 2008). Conservationists and ornithologists have seen this claim substantiated by observed positive effects on bird survival and fitness after levels of auditory noise dropped due to the corona crisis (Schouppe 2020; Schuster 2020; Wei-Haas 2020). A further illustration of the negative impact of sensory pollution on the different steps of signalling can be found in plant-insect interactions (Jürgens and Bischoff 2017). Flowering plants send out chemicals to attract pollinators. Elevated ozone (O_3) concentrations due to anthropogenic activities have been shown to impact the physiology of plants (e.g. Gimeno et al. 2004) and, therefore, have equal potential to alter the plant's chemical emissions. Furthermore, because of its reactivity, ozone can further degrade compounds of the floral scent (e.g. terpenoids, Bonn and Moortgat 2003), potentially altering the contained message. Finally, there are indications of ozone directly affecting the pollinator's chemical senses. Namely, in western honey bees (*Apis mellifera*), ozone exposure altered the antennal response to floral scent compounds (Dötterl 2016).

Secondly, sensory pollution is known to impact the reliability of sensory cues. When this change in reliability of the cue is not recognised by receiver animals, they may be lured into an evolutionary trap (Schlaepfer et al. 2002; Gilroy and Sutherland 2007). Evolutionary traps involve a dissociation between cues that organisms use to make any behavioural or life-history decision and outcomes normally associated with that decision (Schlaepfer et al. 2002). Sea turtles, for instance, such as the green sea turtle (*Chelonia mydas*) and the loggerhead sea turtle (*Caretta caretta*), use visual cues in the form of water-reflected moonlight to orient themselves towards the ocean after hatching on the beach (Mrosovsky 1972). Artificial light at the beach front has a disorienting effect, causing the animals to move in the opposite direction. The resulting exhaustion, dehydration and capture by predators has lead to massive mortalities. Using the acquired theoretical knowledge on turtle visual perception and orientation, management practices have been set up, reducing the wattage of beachfront luminaires and aiming these downwards and away from the beach (Witherington and Martin 2000).

Box 1 (continued)

Although rarely investigated, climate change may also disrupt natural communication systems. A convincing example is the reduced efficacy (i.e. detectability and persistence) of scent marks used in mate acquisition by the mountain lizard (*Iberolacerta cyreni*) under predicted future temperature conditions (Martín and López 2013). Ambient temperatures affect vapour pressures of scent-constituting chemicals more or less depending on their physical and chemical properties (Müller-Schwarze 2006). Also chemoreception in itself may be affected. For instance, electrical responses to amyl acetate delivered to olfactory receptors of a tortoise (*Gopherus polyphemus*) deteriorated significantly towards extremes up to +35 ◦C and down to +10 ◦C (Tucker 1963). Thus, climate change may affect various sorts of chemical interactions, be it intraspecific, predator-prey related, or other (Draper and Weissburg 2019). More research is needed on this topic to better inform conservationists of the consequences of global temperature changes (Manning et al. 2004; Van Dyck 2012).

remained under-appreciated and understudied (Symonds and Elgar 2008). Until the second half of the 20th century, biologists inferred the use of smell in animal interactions primarily through the elimination of other potential modalities. Jean-Henri Fabre (1911; Figure 1), for instance, described how male great peacock moths (*Saturnia pyri*) flocked around a female placed behind wire gauze, but ignored females visible through sealed glass. It was not until 1959, when Adolf Butenandt and his team had reared and milked thousands of silk moths (*Bombyx mori*), that the first sex pheromone (bombykol) could be chemically identified (Butenandt et al. 1961). In the period following this discovery, many insect pheromones have been described (Symonds and Elgar 2008). Particularly the development of Gas Chromatography – Mass Spectrometry (GC-MS) in 1959, which was not at hand during the chemical analysis of bombykol, ushered a new era of chemical cue discovery (Hummel and Miller 1984). More recently, driven by the seminal work of Linda Buck and Richard Axel (1991), the olfactory receptors binding odorous ligands are being discovered through genetic techniques (Dulac and Axel 1995; Herrada and Dulac 1997; Ryba et al. 1997; Matsunami and Buck 1997; Liberles and Buck 2006). The merit of research on the workings of olfaction for the broad scientific community was formally acknowledged when Buck and Axel were awarded the Nobel Prize in Physiology or Medicine in 2004 (Nobel Media 2020).

In order to create a better understanding of chemical interactions and to enable useful generalisations and predictions, cue categories were defined

according to the message that is being conveyed, and depending on who sends and receives that message (Figure 2). The word 'semiochemical' was first postulated by Law and Regnier (1971) and is an overarching term describing all chemicals involved in interactions of organisms belonging to the same species (i.e. pheromones and signature mixtures), as well as, different species (allelochemicals). Pheromones (Karlson and Lüscher 1959; Wyatt 2010) famously carry messages between the sexes of a species and thus facilitate mate location, mate choice and mate guarding. But other pheromones cause aggregation behaviour or trailing, alarm other individuals of danger (e.g. a predator), or cause them to disperse or mature. Typically, responses to pheromones are innate (though responses can be conditional on development as well as context, experience, and internal state; Wyatt 2010). Signature mixtures, on the other hand, are variable subsets of molecules of an animal's chemical profile which are learnt by conspecifics, allowing them to distinguish individuals or colonies. Allelochemicals (Whittaker 1970b; Whittaker 1970a; Dicke and Sabelis 1988) are divided into subcategories according to who benefits from the interaction. Synomones (Nordlund and Lewis 1976; Dicke and Sabelis 1988) are chemicals that benefit both emitter and receiver. Two examples are the fragrant floral scents used in plant-pollinator interactions and the attractants of predatory insects emitted by plants that are under attack by herbivorous insects (Schiestl 2015). Allomones (Nordlund and Lewis 1976; Dicke and Sabelis 1988), on the other hand, benefit the emitter but not the receiver. For instance, the odour of waterbucks (*Kobus defassa*) and zebras (*Eguus quagga*)

Figure 1 Left: Jean-Henri Fabre (1823-1915), French naturalist and a pioneer of chemical ecology, wondering how female peacock moths (*Saturnia pyri*) attract males. Right: *Saturnia pyri* on a branch in Hérens, Switzerland. © Paul Cools

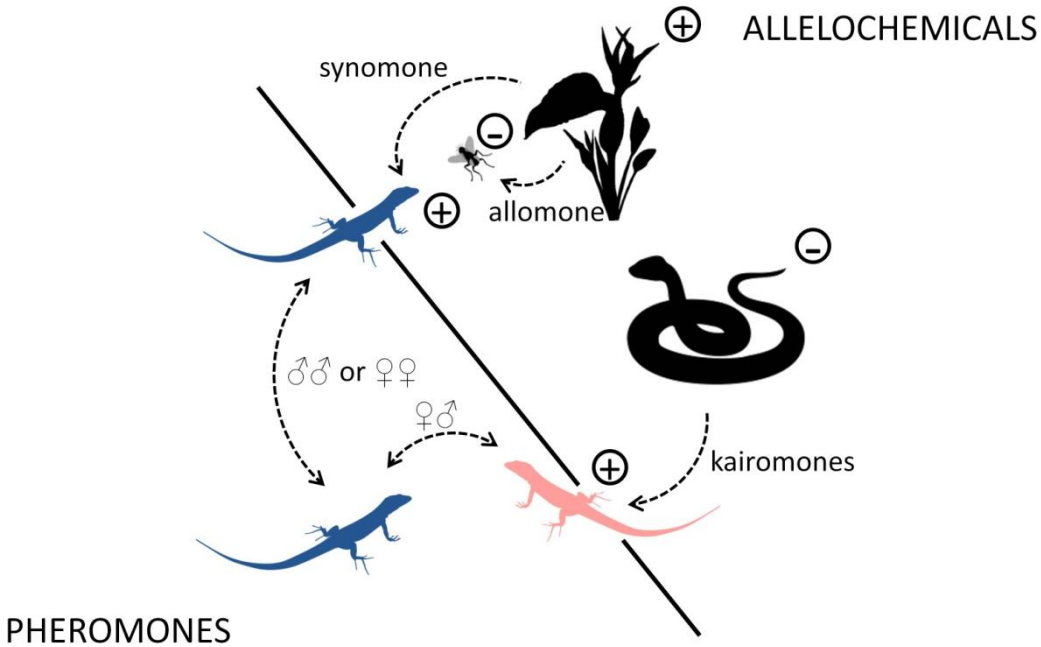

Figure 2 Schematic overview of chemicals used in intraspecfic (= pheromones) and interspecific (= allelochemicals) ecological interactions. Pheromones can carry information between individuals of the same or opposite sex. Allelochemicals are classified according to who benefits from the chemical interaction. Allomones are beneficial for the emitter but not the receiver. For instance, with its scent of rotting meat, dead horse arums deceive blowflies, which normally oviposit in carcasses, into pollinating the flowers (Pérez-Cembranos et al. 2018). Synomones benefit emitter and receiver, such as when lizards are attracted to the stench of arums to locate areas of high insect prey density. The lizards are thought to also disperse the seeds of the dead horse arum. A kairomone benefits the receiver but not the emitter. Examples are plentiful in predator-prey interactions where prey evade predator scent and predators are attracted by the prey's odour.

repels tsetse flies (*Glossina* sp.; Masiga et al. 2014; Olaide et al. 2019). Finally, kairomones (Nordlund and Lewis 1976; Dicke and Sabelis 1988) are allelochemicals that are exploited by the receiver, causing a disadvantage for the emitter. Examples are plentiful in predator-prey interactions, where predators either locate their prey through its scent, or the prey escapes predation by evading a predator's scent (reviewed in Kats and Dill 1998; Conover 2007). Evidently, these categories are not mutually exclusive. For instance, insectivorous tit species, such as the great tit (*Parus major*) and blue tit (*Cyanistes caeruleus*), use the pheromones emitted by the winter moth (*Operophtera brumata*) as kairomones to locate and eat them (Saavedra and Amo 2018).

In vertebrates, our understanding of chemosensory interactions and the true nature of semiochemicals is lagging behind (Symonds and Elgar 2008; Apps 2013; Müller-Schwarze 2016). This results from the behaviour of vertebrates being often complex (i.e. having an elaborate behavioural repertoire) and not that straightforward to interpret (Chittka and Niven 2009; Grant 2016). Furthermore, it is much more difficult to offer a chemical molecule in a bio-assay than to playback a sound or visual stimulus through video or an MP3 player (Wyatt 2011). It complicates the set-up and interpretation of such assays, which are used to understand the role of a specific odour or assess the relevance of specific candidate semiochemicals selected from this odour (Wyatt 2014). Also, vertebrate odours exhibit greater chemical complexity and interindividual variability than invertebrate chemical exudates (with the possible exception of the signals of eusocial insects; Slessor et al. 2005). It makes the isolation of candidate compounds generally more difficult. Nevertheless, the refinement of existing chemo-analytical tools and the development of better sampling techniques have greatly advanced the field.

Predator avoidance and the chemical senses

Because of the direct impact on survival, predator-prey interactions are a fundamental part of a species' ecology (Kats and Dill 1998). As such, predation is thought to be a main evolutionary driver of the morphology, physiology and behaviour of prey species. At the same time, but perhaps to a lesser extent, anti-predatory adaptations in prey species may necessitate counter-adaptations in the predator species (Dawkins and Krebs 1979). As such, predation may well have been the main driver behind the development of neurones approximately 550 million years ago and, subsequently, the evolution of sophisticated senses and the complexity in behaviour we see today throughout the animal kingdom (Moroz 2009; Monk and Paulin 2014; Monk et al. 2015).

The senses play a critical role in predator-prey interactions as they allow both parties to eavesdrop on each other. Eavesdropping is defined by Peake (2005) as 'the use of information in signals by individuals other than the primary target', but may also include the detection of cues that are not involved in purposeful communication of the eavesdropped animal (e.g. visual cues evoked by generic movements; Bradbury and Vehrencamp 1998). In a predator-prey context, signals travelling through the wide set of sensory modalities have the potential to

be picked up, as long as the eavesdropper has the appropriate machinery to do so. Prey animals have been found to eavesdrop on their predators' visual (e.g. Atkins et al. 2016), auditory (e.g. Cantwell and Forrest 2013), chemical (reviewed in Kats and Dill 1998), and vibrational cues (e.g. Sitvarin et al. 2016). Through these various modalities, prey can make a more or less accurate assessment of the distribution of predators across space, i.e. the so-called landscape of fear (Laundré et al. 2014; Jordan and Ryan 2015). However, as vigilance in perceived risky patches in this landscape of fear diverts attention away from other ecological tasks, prey should carefully interpret the predator's cues and weigh the costs against the benefits of responding.

Olfaction (including vomerolfaction) has a number of features that make it especially useful in the context of predator avoidance (Müller-Schwarze 2006). In contrast to visual cues, chemical cues work in darkness and in highly cluttered habitats, and may allow overcoming predatory tactics such as (visual) crypsis and stealth foraging. Volatile compounds emitted from a predator or its cues may readily become airborne and, therefore, may expose a predator's presence at a distance. Especially in a predator-prey context where prey aim to stay concealed from predators, this feature seems desirable. Substrate-borne compounds, on the other hand, last longer than auditory, visual or tactile stimuli, permitting the prey to detect areas frequented by the predator, even in its absence. Therefore, these also indicate areas of heightened risk without the need of close contact. Finally, odours (and vomodours) can carry information about the predator that may be relevant to the prey, but not easy to read from other cues, e.g. on its hunger status (Licht 1989; Scherer and Smee 2016) or dietary habits (Hirvonen et al. 2000).

Although the role of kairomones in vertebrate predator-prey interactions is evident (reviewed in Müller-Schwarze 2006), it remains a daunting task to resolve which chemicals carry what message to the prey. As for semiochemicals in general, the most effective way of identifying kairomones is through a response-guided strategy. In this approach, the behavioural or neuronal response of focal animals to ever smaller subsets of a chemical blend are assessed, eventually reducing the odour to its active kairomonal fraction. As the repetitive nature of the procedure requires a considerable sample size and correct interpretation of the results is only possible with an exhaustive knowledge on the (behavioural or neuronal) response towards predator odours (Mackintosh 1985), such experiments

Box 2

Prey naivety and sensory ecology

Prey naivety is defined as the existence of ineffective anti-predator defences owing to the lack of (evolutionary) experience with a given predator (Cox and Lima 2006). Four different levels of naivety exist (Banks and Dickman 2007). Level 1 naive prey show no recognition of the alien as a predatory risk and, hence, adopt no anti-predator behaviour. This is either caused by prey animals not having the sensory equipment for detecting predator-derived cues, or, if they do, lacking the ability to associate the cue with danger. Level 2 naivety occurs when prey recognise the predator as dangerous but adopt the wrong anti-predator response. Level 3 naivety occurs when prey recognise the predator as dangerous, have appropriate anti-predator defences that are suited for that predator, but are simply 'outgunned' by the superior hunting tactics of the alien species. Finally, level 4 naive prey suffer excessive sublethal costs of predation by 'over-responding' to an exotic predator (Carthey and Banks 2014). Knowing the level of naivety of a prey is important for the design of conservation practices. However, much uncertainty remains on the relative occurrence of each level in naive prey, worldwide. Level 1 naivety is considered to be the most damaging form of naivety. It is also the level that I will focus on in several chapters of this thesis (see This thesis).

The factors determining whether or not a prey overcomes naivety appear to be highly complex. They remain a topic of rigorous study, due to their relevance in conservation biology. The following list of key hypotheses is derived from Carthey and Blumstein (2018) recent paper which describes a framework for predicting predator recognition in a changing world based on the prey's eco-evolutionary experience.

Adaptation: with sufficient time and heritable variation, prey will adapt to contemporary threats and ultimately be able to discriminate predators from non-predators (sensu Darwin 1859).

Multipredator hypothesis: this predicts that prey will retain evolved abilities to respond to extinct predators as long as they retain other predators. An implicit assumption of this hypothesis is that antipredator behaviours are genetically correlated or linked because it would be disadvantageous for the underlying traits to independently assort. This is because a prey species that was able to respond to one of its predators, but not to another predator, would be selectively disadvantaged compared to one that was able to respond to both. Thus, selection is expected to create correlated antipredator systems that should be somewhat resilient to the loss of a specific predator (Blumstein 2006).

Box 2 (continued)

Recoverable templates: the key idea underlying recoverable templates is that recognition templates exist but are not activated without experience. Experience might include specific exposure to predators (e.g. when fish have one-trial olfactory learning that permits predator identification; Brown et al. 2011) or could include priming (which is seen when a non-specific stimulus is required for the later proper performance of a behaviour).

Relaxed selection: the assumption under a relaxed selection model is that if recognition and/or discrimination abilities are no longer selected, and if there are any costs to maintaining them, these abilities will be lost (Lahti et al. 2009). Costs could be energetic (maintaining unnecessary sensory organs or brain tissue is expensive) or opportunity costs (responding to a predator when not present would reallocate time from important activities to an unimportant activity).

Archetypes: the archetypes hypothesis proposes that prey will recognize and respond to introduced predators that are of the same 'archetype' as familiar local predators. Distinctions at the taxonomic level of family are the proposed proxy for a practical interpretation of 'archetypes', but an archetype can also be defined as 'the set of predators against which a given suite of antipredator adaptations is effective' (Cox and Lima 2006).

Labeling: a group of hypotheses suggest that prey use general features common to predators to 'label' a novel animal as being predatory, irrespective of prior experience with that particular predator species. For example, prey might recognise a chemical leitmotif (Stoddart 1980; Epple et al. 1993) or sulfurous chemical compounds resulting from meat digestion (Dickman and Doncaster 1984) in the olfactory products of a novel predator. General fear towards novel stimuli, objects, or environments, also called 'neophobia' (Barnett 1958; Ferrari et al. 2014), is another way in which prey might label all novelty as indicative of danger. These features can all be considered predator 'labels'.

Naiveté: naiveté theory predicts that native prey will not recognize or respond to a novel predator because of a lack of experience (Diamond and Case 1986). More recent formulations posit that there are in fact multiple levels of naiveté, through which prey might progress with time and experience (Banks and Dickman 2007; Carthey and Banks 2014).

Rapid change: when faced with a novel predator, prey might rapidly develop antipredator behavior via plasticity, learning, and/or rapid evolution (Griffin et al. 2000; Cox 2004; Brown and Chivers 2005; Brookes and Rochette 2007).

have been carried out on a small set of vertebrates only, mostly lab-reared rodents (rats and mice). We now know of more than a dozen different kairomone chemicals, present in mammal carnivore urine or faeces, or emitted from their glands, that elicit fearful behaviour in rondents (e.g. 2-phenylethylamine, Ferrero et al. 2011; Mup13 and Feld4, Papes et al. 2010; three pyrazines, Osada et al. 2013; three pyridines, Brechbühl et al. 2015).

In spite of the research done on model rodents (see box 3 on rodents as chemosensory models), several outstanding questions remain that hamper a full understanding of chemosensation. One important question is whether only a single or few molecules out of their chemical context cover the full kairomonal content of a predator's odour (Apfelbach et al. 2015). With increasing evidence of the complexity of behavioural anti-predator responses observed in natural systems, it seems hard to believe that a single compound would be sufficient for simultaneously conveying information on, for instance, a predator's social (Gese 1999), and hunger status (Licht 1989), its diet (Murray and Jenkins 1999), and the recentness of the predator's visits (Bytheway et al. 2013). Furthermore, it is unclear whether the identified kairomones found to date are also interpreted by non-rodent and non-mammalian prey. And do non-mammalian predators exude the same or similar chemicals than has been found with mammalian predators? A deeper knowledge on the subject would be extremely helpful in assessing, for instance, the stability of the many new predator-prey interactions which now occur due to human-aided introductions (box 2).

Lacertids as models in chemosensory predator detection

In an attempt to break through today's near-sighted, rodent-biased view of chemosensory predator recognition, I have turned to lacertid lizards, for a number of reasons which I like to mention here briefly.

First, like many other squamates (e.g. colubrid snakes, Burger 1989; iguanid lizards, Labra and Hoare 2015; skinks, Downes 2002; and geckos, Webb et al. 2009), lacertid lizards are capable of recognising predators by chemical cues alone (Thoen et al. 1986; Van Damme et al. 1995; Van Damme and Castilla 1996; Van Damme and Quick 2001; Amo et al. 2005; Durand et al. 2012; Mencía et al. 2016; Mencía et al. 2017; Ortega et al. 2017; Ortega et al. 2018). In fact, they are known to use chemosensory information in a variety of important daily activities, including prey detection and recognition (Cooper 1991; Desfilis et al. 2003) mate assessment

Box 3

Chemical senses in vertebrates

Overall, most tetrapods, including rodents, possess two olfactory organs (Figure 3): (1) the 'main' olfactory organ, and (2) the 'accessory' olfactory organ or vomeronasal organ (VNO), also called the Jacobson organ. The main olfactory organ is widely present in vertebrates, ranging from jawless and jawed fishes to amphibians, reptiles, birds and mammals. In all these animals, the olfactory epithelia are located in the nasal cavities, and mainly process airborne cues. The vomeronasal organ is also shared by many vertebrates and may have evolved already in early jawless vertebrates (Ubeda-Bañon et al. 2011). It further developed in fish (González et al. 2010; Ferrando and Gallus 2013) and early tetrapods and is still important in most amphibians and reptiles (Eisthen and Polese 2007) and many mammalian lineages (Grus et al. 2005), but was secondarily lost in birds and several mammalian groups (Ubeda-Bañon et al. 2011). The ducts leading to the vomeronasal epithelia either open in the nasal cavities (e.g. in rodents) or the oral cavity (in felids, ungulates and some reptilian lineages). In the reptilian lineage, in particular, a peculiar and unique way of sampling chemicals from the environment for processing in the VNO has developed which differs fundamentally from the rodent nose. Many lizards and snakes (Squamata, Reptilia) use their tongue to sample chemicals from the environment. The tongue collects scent molecules and delivers them to the vomeronasal fenestrae in the roof of the mouth, by means of an incompletely understood, possibly hydraulic mechanism (Filoramo and Schwenk 2009). The tongue of these squamate reptiles can thus be seen as a component of the vomerolfactory system which, besides to volatiles, gives access to substrate-borne molecules.

The rate at which squamate reptiles protrude their tongue to the external environment is elevated when specific odours are presented that relate to food items (Kubie 1978; Cooper and Alberts 1991; Cooper et al. 2001), mates (Mason et al. 1990; Shine et al. 2002), rivals (Aragón et al. 2000), kin (Pernetta et al. 2009), heterospecific competitors (Barbosa et al. 2006; Williams et al. 2020) and predators (Thoen et al. 1986; López and Martín 2001; Bealor and O'Neil Krekorian 2006). Therefore, tongue-flick count has been a convenient measure for the chemosensory interest of squamates in ecologically relevant scents (Mason and Parker 2010). However, tongue flicking is also influenced by body temperature (higher frequences at higher body temperatures; Van Damme et al. 1990). Also personality has been found to play a role in the number of performed tongue flicks. For instance, garter snakes (*Thamnophis elegans*) from fast-living ecotypes perform more tongue flicks over a set period of time compared to slow-living ecotypes (Ganglo et al. 2017). These factors need to be accounted for in behavioural assays with squamates, e.g. by keeping the body temperatures of focal squamates within a narrow temperature range and using repeated measures designs in which individuals are tested with both control and treatment odours.

Box 3 (continued)

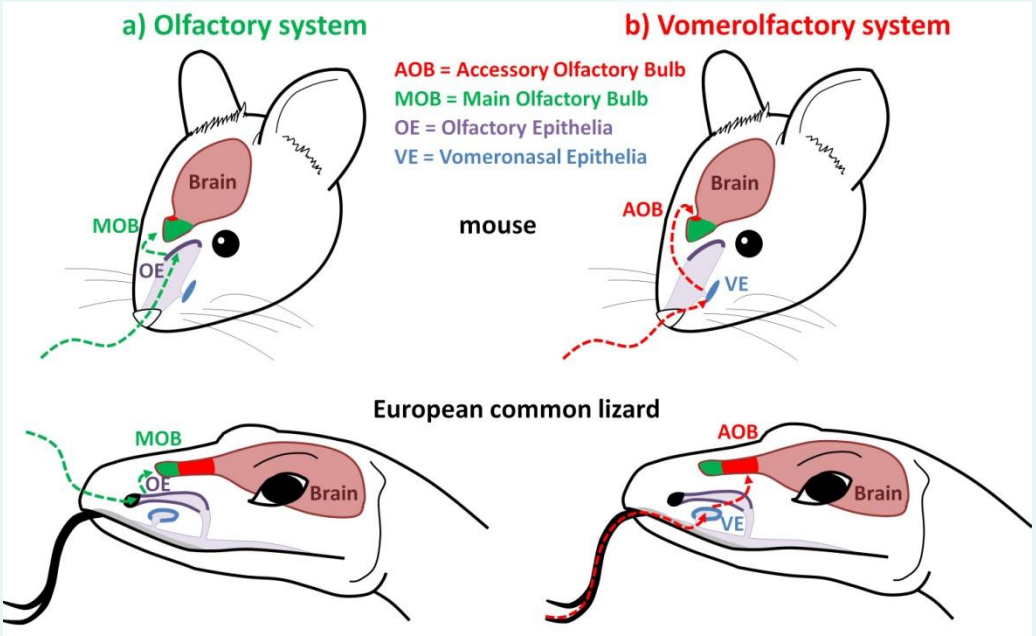

Figure 3 The two main chemical senses of rodents and squamate lizards. a) Olfaction in both mouse and lizard functions through the detection of chemicals through the nose. b) The vomeronasal systems of mice and lizards have their openings at different locations. In the mouse, the vomerolfactory organ is also located in the nose. However, in the vomerolfactory system of squamates, the tongue delivers chemical particles to paired ducts that open in the palate and connect to the chemosensitive Jacobson's organ.

(Cooper and Pèrez-Mellado 2002; Martín and López 2008), territory inspection (Carazo et al. 2011), sex recognition (Barbosa et al. 2006) and species recognition (Barbosa et al. 2006; Williams et al. 2020). The observation that many lacertids are highly 'chemically-oriented' animals, is of obvious importance in this context.

Second, most conveniently, lacertids flick their tongues. They use rapid, repeated extrusions of the tongue to sample the chemical environment. The beauty of this is that tongue flick rate, the frequency with which the tongue is extended and retracted can be used as a direct, easy-to-measure index of the lizard's ability to detect a chemical cue, and its interest in that cue. In most other animals, including the rodents, gauging the responsiveness to individuals towards chemical information requires invasive or complex techniques such as the

implanting of electrophysiological probes or fixing the animals' head in a scanning apparatus.

While an increased tongue-flick rate strongly indicates a lizards' interest in a particular odour, it does not convey the nature of that interest. Chemical cues of prey, sexual partners and rivals might elicit increased tongue flick rates just like predatory clues do. A third asset of lacertid lizards as model organisms in the study of chemosensory predator recognition is that they will mount a highly typical behavioural response when confronted with predator cues. This response includes elements used by many other animals under threatening situations (e.g. reduced activity), but also a number of stereotypic stress-indicating behaviours that have been described in species across the lacertid family (Verbeek 1972; Thoen et al. 1986; Van Damme and Quick 2001; Font et al. 2012b). For instance, lacertids confronted with predator cues will typically wriggle their tail in the sagittal plain, possibly in an attempt to divert the predator's attention towards that autotomisable and expendable body part. Lacertids under predatory threat will also exhibit rapid movements of the hands and feet ('foot shakes', Verbeek 1972), move about in a very slow, cautious way ('slow motion', Thoen et al. 1986) or pull sudden, rapid sprints ('startles', Thoen et al. 1986). The purpose and efficacy of these behaviours has not been studied in detail, but they are likely to aid avoiding detection, to distract or startle the predator, or to deter pursuit. For sure, they can be quantified and used as a bio-assay for predator chemical recognition.

Fourth, despite sharing many aspects of their general biology (body plan, behavioural repertoire, diet, activity rhythm, thermoregulatory strategy, …) lacertid lizard species are ecologically versatile and occur in a wide variety of habitats and microhabitats (Arnold et al. 2007). They thrive in ecosystems ranging from arctic tundra and alpine meadows, over Mediterranean scrub and deserts to tropical forests. They inhabit land-bridge and oceanic islands where they often reach high densities and constitute the main vertebrate predator. This versatility offers interesting opportunities to study evolutionary aspects of vomerolfactory predator recognition, e.g. how (well) the perceptive system adapts to local variation in the predator community (e.g. Van Damme and Castilla 1996; Durand et al. 2012; Mencía et al. 2016; Mencía et al. 2017; Ortega et al. 2017; Ortega et al. 2018).

In addition to these specific reasons, lacertids are just generally rewarding study organisms because they often occur in large densities, can be caught fairly easily, and do remarkably well in captive conditions. Within minutes of being put

in a terrarium, lizards will assume their natural behaviour of basking and foraging, and exhibit the typical responses to predator cues described above.

Finally, the investigation of lacertid lizards has great value from a conservation point-of-view. More than 320 lacertid species are known to be distributed over most parts of the Old World (Arnold 1989; Uetz et al. 2020). In these areas, they take in a central position in the ecosystem. Lacertids prey upon an enormous variety of small invertebrates, besides the occasional consumption of vertebrates and plant matter. In turn, these lizards are preyed on by certain birds, carnivorous mammals, snakes, and sometimes even other lacertids (Castilla et al. 1991; Schleich et al. 1996; Barbadillo et al. 1999; Maslak and Pasko 1999; Corti and Lo Cascio 2002; Baeckens and Briesen 2017). Consequently, lacertid lizards are important mediators in the transfer of matter and energy throughout the food web (Carretero 2004). Furthermore, lacertid lizards have been shown to be important pollinators and seed dispersers (Fuster and Traveset 2019) and when these lizards are removed from the landscape due to alien predation, severe alteration of the vegetative habitat occur (Traveset 2002). Therefore, it is highly alarming that particularly these animals belong to one of the world's most threatened taxa, with a disproportionally high proportion of lizard species being threatened by climate change, habitat loss and degradation, overharvesting and (of particular importance here), the introduction of alien predators and competitors (Gibbons et al. 2000; Spatz et al. 2017; Howard et al. 2020). This thesis will help understand whether, when and how lacertids can adjust to changes in their (predatory) environment – allowing more insightful conservation and restoration plans.

State-of-the-art: current knowledge on chemosensorial predator recognition in lacertid lizards

The suitability of lacertid lizards as models for the study of chemosensory predator recognition has not entirely escaped the attention of scientists. Since Thoen et al. (1986) first described the ability of common lizards (*Zootoca vivipara*) to distinguish between the chemical cues left behind by saurophagous snakes (adders, *Vipera berus*, and smooth snakes, *Coronella austriaca*) from control odours or cues of non-saurophagous snakes (grass snake, *Natrix natrix*), authors have demonstrated similar skills in other lacertid species (Van Damme and Castilla 1996; Van Damme and Quick 2001; Downes and Bauwens 2002; Amo et al. 2005;

Durand et al. 2012; Mencía et al. 2016; Mencía et al. 2017; Ortega et al. 2017; Ortega et al. 2018). Moreover, Van Damme et al. (1995) showed that predator chemical cue recognition in *Z. vivipara* is innate. Additionally, Martín et al. (2015) found that individual lizards, over their lifetime, become more apt in discriminating different snake species.

Taking advantage of the fact that many lacertid species occur both on islands and on the mainland, several studies have examined the evolutionary flexibility of their chemosensory predation recognition abilities. The results of these studies are disparate. On Columbretes Islands (Spain), individuals of *Podarcis hispanica atrata* still respond fearfully to chemicals of its historical predator *Vipera latastei*, although this snake has been eradicated from the islands over a century ago (Van Damme and Castilla 1996). However, after 7000 years of isolation on a French Atlantic island, *Podarcis muralis* fail to react adequately to chemicals of three saurophagous snakes (Durand et al. 2012). Specimens of *Podarcis siculus* on Menorca seem to have acquired the ability to recognise chemical cues of a saurophagous snake (*Macrorotodon mauriticanus*) that was introduced onto the Balearic Islands in historical (possibly Roman) times (Mencía et al. 2017). In contrast, *Scelarcis perspicillata* and *Podarcis lilfordi* have failed to develop the capacity of recognising chemical cues of that same snake species (Mencía et al. 2017). Then again, Ortega et al. (2017) report that *Podarcis pityusensis* of Ibiza can identify chemical cues of another saurophagous snake, *Hemorrhois hippocrepis*, that was introduced onto the island a mere 11 years ago. Exactly what drives the geographical and temporal variation in chemical predator recognition remains unclear.

Although this short assessment (a more extensive literature review can be found in the general discussion) indicates that the subject has received some attention in the previous decades, our knowledge of lacertid predator chemical recognition is still showing several painful gaps. For instance, little is known on the (neuro-)morphology of the lacertid vomeronasal system. Judged from their behaviour, some populations, and species of lacertids seem more adept in chemosensory predator recognition than others, but whether this is reflected in the size, number or performance of their receptors, neural pathways and processing areas in the brain has never been assessed. Also virtually nothing is known on the nature of the molecules that elicit fearful behaviour in these animals (i.e., the kairomones). We do not know whether they are specific to species, genera

or family of snakes, or (terrestrial) predators in general. Prior to this work, nobody had looked at the responses of lacertids to odours of mammalian predators. Information on the chemical nature of the molecules involved (e.g. their molecular weight, volatility) would also benefit our understanding of their real-life relevance: How long do kairomones deposited by predators remain lingering in the habitat? Does the concentration or composition of kairomones convey information on the imminence of the danger?

This thesis

The aim of my PhD work was to fill in some of these gaps. Apart from this introduction and a general discussion, this thesis contains six manuscripts that are either published, submitted or in preparation for submission for publication. In the first three chapters (2, 3 and 4), I use established techniques (mostly, behavioural observations) to further examine the ability of lacertid lizards to detect predator chemical cues, focusing on predator-prey combinations that sparked my interest for reasons detailed below. The last three chapters (5, 6 and 7) are more methodologically oriented. In these, I test and explore a number of techniques that may open new avenues for answering outstanding questions on chemosensory predator recognition in lizards (and other animals). More specifically, these techniques could be used to characterise the kairomones involved in predator recognition.

The first part of this thesis features an unusual predator: the small Indian mongoose (*Herpestes auropunctatus*). I chose this predator because it is a mammal with omnivorous and highly opportunistic food habits that will readily eat reptiles in its native range (Mahmood and Adil 2017) and elsewhere (Berentsen et al. 2018). Previous studies on lacertid predator recognition have exclusively considered snake predators, so I considered it worthwhile checking whether lizards could recognise a mammalian predator. Such a distantly related predator may deploy a very different hunting strategy against which chemosensory anti-predatory responses may be more or less well suited. In addition, in almost all ecosystems in which they have been introduced, mongooses have caused drastic declines and even extinctions of the native populations of birds, mammals and especially reptiles (Hays and Conant 2007). Indeed, in many areas of the world, including some of our study sites in Croatia, mongooses have been introduced to eradicate venomous snake populations, with collateral effects on other native

reptile populations (Tvrtković and Kryštufek 1990; Barun et al. 2011b). Variation in responses to mongoose scent is, therefore, relevant from an evolutionary perspective ([how fast] do prey species learn to recognise alien predator cues) and from a conservation biology point of view.

In Chapter 2, I test whether mongoose scent is picked up and used by members of a lacertid species home to the native range of *Herpestes auropunctatus*, the Asian grass lizard (*Takydromus sexlineatus*). I compare the lizards' tongue flick rates and behaviour when experimentally confronted with mongoose scent, the scent of a local snake predator (the Oriental whip snake, *Ahaetulla prasina*), and odourless and pungency controls.

In Chapter 3, I perform similar tests, but now using Dalmatian wall lizards, *Podarcis melisellensis,* coming from study sites inside and outside the area into which the mongoose has been introduced in the 1920s. Again, I compared the lizards' response to mongoose odours to their behaviour in the presence of chemicals of local saurophagous snakes (the Balkan whip snake, *Hierophis gemonensis*, and the eastern Montpellier snake, *Malpolon insignitus*), and in two control situations.

The results obtained in Chapter 3 point strongly towards an effect of insularity on overall chemosensory prowess in *Podarcis melisellensis*. Therefore, in Chapter 4, I try to connect reduced tongue flicking rates in island lizards to changes in the relative size of the main and accessory olfactory bulbs, brain areas involved in the processing of chemosensorial information.

The second, methodological part of the thesis starts with Chapter 5, in which I return to the cradle of lacertid chemosensory ecology: the recognition of adder (*Vipera berus*) scent by common lizards (*Zootoca vivipara*). Using bio-assays, I test whether the kairomones that elicit the stereotyped response in the lizards can be extracted from the viper with n-hexane. I use Gas Chromatography – Mass Spectrometry to explore the contents of adder chemical cues as a first step in identifying candidate kairomone molecules.

In Chapter 6, I explore the usefulness of a new technique, Proton-Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-TOF-MS) for studying the volatile emissions of vertebrates, including lacertids and vipers. As far as I know, this is the first time that the method is used in vertebrate chemical ecology. A promising asset of PTR-TOF-MS is that it allows real-time monitoring of volatile

organic compounds, even in semi-natural conditions. This should open new avenues in the study of more volatile chemical messages of vertebrates.

As a first application of PTR-TOF-MS in the chemical ecology of reptiles, I study how the chemical composition of adder scent, following deposition onto the substrate, changes over time. I examine which volatile compounds evaporate first into the surroundings, and which linger on. By simultaneously performing bio-assays on common lizards, I explore whether the changes in the composition of the predator's scent can influence the prey's behaviour.

Finally, in the general discussion (Chapter 8), I integrate results from the respective chapters and compare them to findings in the literature. I also elaborate on a number of outstanding questions on 1) the chemical nature of kairomones and the implications of resolving these and other semiochemical identities for various fundamental and applied scientific fields, 2) the genetic underpinnings of chemosensory predator recognition and the usefulness of this knowledge to understand results derived from my study systems, and 3) the relative role of chemosensation compared to other sensory systems in mammal and snake detection. I highlight several approaches that may aid in advancing the field of vertebrate chemical ecology.

Part I

Chapter 2

The Asian grass lizard (*Takydromus sexlineatus*) does not respond to the scent of a native mammalian predator

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Abstract

Lacertid lizards use chemical cues emitted by saurophagous snakes to evade predation. Whether these lizards can detect and respond to the chemical cues of predatory mammals has not been studied. As many mammals carry distinct body odours and/or use chemical cues for intraspecific communication, lizards can be expected to use these chemicals as early warning cues. To test this idea, we observed the behaviour of Asian grass lizards (*Takydromus sexlineatus*) that had been transferred to an unfamiliar test arena containing one of four scent treatments. No particular scent was applied to the arena in the control situation. Diluted aftershave served as a pungency control. In the snake treatment, scent of the Oriental whip snake (*Ahaetulla prasina*) was applied. We included this treatment to learn how Asian grass lizards react to predator chemical cues. Finally, in the mongoose treatment, the lizards were confronted with scent cues of several small Indian mongooses (*Herpestes auropunctatus*). Snake scent elicited foot shakes, startles and tail vibrations. These are behaviours that in lacertid lizards are associated with stressful situations such as predatory encounters. Surprisingly, lizards confronted with mongoose scent exhibited none of these stress-indicating behaviours. In fact, their behaviour did not differ from that of lizards subjected to an odourless control treatment. These results raise concern. Mongooses are rapidly invading ecosystems worldwide. If lizards that have co-evolved with mongooses are unable to detect these predators' presence through chemical cues, it seems highly unlikely that evolutionary naïve lizards will develop this ability rapidly.

Introduction

Predation is considered a major selective force shaping the morphology, physiology, behaviour and life history of prey animals (reviewed in Lima and Dill 1990; Kats and Dill 1998). Rapid detection and accurate identification of the predator are often key to surviving a predatory encounter (Cox and Lima 2006; Banks and Dickman 2007). Accordingly, many prey species have evolved sense organs capable of detecting predatory cues (Derby and Sorensen 2008; Takahashi 2014; Pereira and Moita 2016; Kotrschal et al. 2017).

Lacertid lizards are known to have keen chemical senses: (a) the olfactory system with the sensory epithelium situated in the nose and (b) the vomerolfactory system in which scent molecules are transported via the tongue to the Jacobson's organ in the roof of the mouth (Halpern and Kubie 1984; Halpern 1992; Cooper 1996). Studies on a variety of lacertid species have shown that these senses assist in the detection of saurophagous snakes (e.g. Thoen et al. 1986; Amo et al. 2004a; Mencía et al. 2016). However, it is unclear whether lizards also use their chemoreceptive systems to detect and identify mammalian predators (Weldon 1990). Many mammalian predators of lacertid lizards carry distinctive body odours (Gorman 1976; May et al. 2012), produce scented urine or excrements (Fendt et al. 2005; Burnham et al. 2008; Greene et al. 2016) and/or scent mark their territory (Gorman and Trowbridge 1989). One could imagine lizards exploiting these scents as early warning cues. However, whether lizards can actually detect and recognise mammal scents has, to our knowledge, never been examined.

A handful of studies have been performed focussing on other lizard taxa. New Zealand skinks and geckos exhibited no response to the chemical cues of introduced ship rats (*Rattus rattus*). However, cues of a native reptile predator also failed to evoke a behavioural response, suggesting that the lizard species involved in that study had inadequate chemical senses to begin with (Monks et al. 2019). In New Caledonia, endemic skinks (*Caledoniscincus austrocaledonicus*) avoid refuges scented with the odours of *Rattus* sp. and feral cats (*Felis catus*), while endemic geckos (*Bavayia septuiclavis*) only avoided the scent of *R. exulans*, the predator with which they have been coexisting longest (i.e. 3,000 years; Gérard et al. 2014; Gérard et al. 2016). These studies may also paint a partial picture because they consider responses to non-native mammalian predators that were only fairly recently introduced into the lizards' habitat. Perhaps these lizards have had

insufficient time to evolve a proper identification-response system towards these mammals in particular. Webster et al. (2018) found that Boulenger's skinks (*Morethia boulengeri*) and southern marbled geckos (*Christinus marmoratus*) stopped foraging in response to the scents of native quolls (*Dasyurus maculatus*) and dingoes (*Canis lupus dingo*).

Here, we investigate the ability of a lacertid lizard (the Asian grass lizard, *Takydromus sexlineatus*) to detect chemical cues of a native mammalian predator, the small Indian mongoose (*Herpestes auropunctatus*). For comparison, we used scent of the native Oriental whip snake (*Ahaetulla prasina*) to evoke a baseline anti-predatory reaction in the lizards. Information on how a lizard responds to mongoose chemical cues is particularly relevant as the small Indian mongoose and the Javan mongoose (*H. javanicus*), which were formerly considered as being one species (Veron et al. 2007), have been intentionally introduced into ecosystems worldwide to control rats and snakes. In many cases, they also preyed considerably on native mammals, birds and other reptiles, sometimes even leading to local extinctions (reviewed in Hays and Conant 2007). For this reason, mongooses are considered one of the hundred most dangerous invasive species in the world (Lowe et al. 2000).

Material & methods

Study species

The Asian grass lizard is a small lacertid lizard with well-developed chemical senses (Cooper et al. 2000b; Baeckens et al. 2017a) that lives in grassland habitats, agricultural sites and near human settlements (Pauwels 2000) throughout its distributional range in Southeast Asia (from southern China to the Indonesian islands of Sumatra and Java and the island of Borneo; Zhao and Adler 1993). Habitats occupied by this lizard are frequented by native generalist predators, including the small Indian mongoose (Nellis 1989; Chutipong et al. 2016) and the Oriental whip snake (Sharma 2019). Both the small Indian mongoose and Oriental whip snake are highly opportunistic feeders with more or less overlapping diets. Mongooses eat reptiles, small rodents and birds, besides invertebrates and plant material (Nellis 1989; Hays and Conant 2007; Lewis et al. 2010). The Oriental whip snake has also been observed in the wild feeding on various kinds of lizards, other snakes, (amphibious) fish, birds and small mammals (Pauwels 2000; Dunbar and

Dunbar 2015; Vogrinc et al. 2016). Therefore, mongooses and snakes are expected to be equally relevant predators of the Asian grass lizard.

Lizard housing conditions

We obtained twenty adult male Asian grass lizards (average snout-vent length: 56.71 mm, range 52.69–63.45 mm; average body mass: 4.03 g, range 2.68–6.24 g) via the pet trade (Amfibia BVBA). The animals had been caught in the wild on Java, Indonesia. Individuals were housed in groups of three to five in terraria measuring 100 × 50 × 50 cm (length × width × height). The floors of the terraria were covered with paper towels, which were changed weekly. We added branches and stones as environmental enrichment. A 60 Watt incandescent lamp suspended at one side of the terrarium provided a temperature gradient of between 23 and 32°C, allowing the lizards to thermoregulate and maintain body temperatures to near-optimal levels (Zhang and Ji 2004). Additionally, a UVB lamp was suspended inside the terrarium to prevent a vitamin D deficiency (Adkins et al. 2003). Lighting maintained a 12:12 hr light:dark circadian rhythm. Water was available ad libitum. Furthermore, we sprayed the terraria daily to guarantee an optimal humidity. Lizards were fed vitamin E-dusted crickets (*Acheta domesticus*) twice a week.

Scent collection

Mongoose scent was collected from eight males, caught on Korčula island in Croatia and housed individually at the research facility of IDT Biologika GmbH in Dessau-Roßlau, Germany. Scent from the Oriental whip snake was obtained from one wild-caught Indonesian male obtained via the pet trade (La Ferme Tropical) and housed at the laboratory in Antwerp. The use of only one individual as a donor of the treatment stimulus is not considered best practice and is discouraged by Kroodsma et al. (2001) and Hurlbert (1984). However, our purpose was merely to use the snake's scent in generating a baseline anti-predatory response by grass lizards to compare mongoose-evoked behaviour with. We deemed this treatment appropriate as such. Paper towels were placed in the home cages of the predators for a period of 5 days. We used clean tweezers at all times while handling the paper towels. Human contact with the animals during scent collection was limited, and the paper towels were left untouched by carers. These precautions ensured that contamination with human scent was avoided. After the scent collection period, the paper towels were taken from the home cages, cut into 5 × 5 cm pieces with clean scissors and either placed into double plastic bags for storage in a

freezer at −20°C or immediately used in focal observations. If stored, the paper towels were kept for maximum one month. Mongoose chemical samples were transported on dry ice to the laboratory in Antwerp. Freezing prevents the scent from ageing before use in focal observations (Bytheway et al. 2013). Preliminary experiments showed that the described storage and handling of the samples does not prevent a response by two other lacertid lizards (*Zootoca vivipara* and *Podarcis melisellensis*) to predator odours. Frozen scent samples reached room temperature within a minute after taking them out of the freezer (confirmed by an unpublished pilot study), after which a focal observation could start.

In addition to the predator scent, we also prepared an odourless control by clipping 5 × 5 cm pieces out of clean paper towels. A pungency control was prepared by administering one drop of diluted aftershave (one volume of Mennen Skin Bracer to 9 volumes of deionised water) onto a 5 × 5 cm piece of clean paper towel. This control represents scent that is not predator related (Mennen Skin Bracer contains only plant-based castor oil as opposed to animal-derived castoreum sometimes found in cosmetics) and is unknown to the lizards (Cooper et al. 2003). Therefore, any reaction by the lizards towards this treatment should not be due to fear, but the consequence of general chemosensory and explorative behaviour.

Focal observations

The procedure for the focal observations was adapted from Thoen et al. (1986) and is commonly used for testing predator cue recognition in lacertid lizards (Van Damme and Quick 2001; Downes and Bauwens 2002; Ortega et al. 2017). We observed the lizards in a closed test arena measuring 50 × 40 × 40 cm (l × w × h). One of the arena's walls was coated with a dark window film (Norauto), which allowed us to observe the lizard without disturbing it. A 60 Watt incandescent lamp, installed centrally in the roof of the arena, provided an optimal temperature for lizard activity. A few seconds before every behavioural test, the observer rubbed a paper towel piece comprising one of the four scent treatments across the floor and thereafter placed it in a randomly chosen corner of the test arena. Three additional towel pieces, arbitrarily selected from among all towels treated with the same scent as the first one, were placed in the remaining corners of the arena. We chose not to subject every individual to all treatments, because previous studies found that lizards tend to become indolent after repeated testing (Gérard et al. 2014; Van Moorleghem et al. 2020). Instead, we assigned each lizard to one of

two experiments. In Experiment A, eleven lizards were observed in the two control situations (odourless and pungency control) and in the *A. prasina* ('snake') treatment. In Experiment B, the remaining nine lizards were observed in the odourless control situation and in the *H. auropunctatus* ('mongoose') treatment. In both experiments, the different scent treatments were presented according to a balanced test design. A period of approximately 24 hr was left between consecutive trials for the same lizard individual.

During a time period of 10 min, which began shortly after the lizard was placed in the centre of the test arena, the following behaviours were scored: the time spent Walking, Not-moving, Basking, Nudging and Standing up-right against one of the test arena's walls, and the amount of Tongue flicks (indicative of chemical sampling; Graves and Halpern 1990), Labial licks, Head rubs (the lizard rubs its head sideways over the substrate), Tail vibrations, Startles and Foot shakes. The latter three are considered to be indicative of stress or linked to predator-escape strategies in lizards (Mori 1990; Van Damme and Quick 2001; Font et al. 2012b). See Thoen et al. (1986) and Monks et al. (2019) for a detailed description of all aforementioned behaviours. After each observation, the lizard was placed back in its home terrarium. The test arena was cleaned with 70% ethanol and left to dry before the next observation could begin.

Statistical analysis

Statistics were performed using R version 3.3.0 (R Core Team, 2016). We ran a Factor Analysis of Mixed Data (FAMD; Lê et al. 2008) on the behavioural variables for each experiment. Data points for the behavioural variables were transformed to improve normality (Table 1) prior to FAMD analyses. Some of the behavioural variables exhibited highly skewed distributions with an excess of zeros. Because transformations did not help, we recoded these variables into binomial quantities (Table 1), with 0 indicating that the focal lizard did not perform the behaviour and 1 indicating that it did. We used linear mixed-effect models (LMM; lme4 package; Bates et al. 2015) to test the effect of Treatment (either the odourless control, pungency control, snake or mongoose scent) on the scores of each observation on the main dimensions produced by the FAMD. Besides Treatment, Trial and Treatment x Trial were also entered as explanatory variables into these models. The variable Trial takes on a value equal to the number of times that the lizard had been tested before the current observation and therefore indexes possible habituation effects. Predictor weights (=the summarised Akaike weights of all

candidate models in which an explanatory variable appears) were used to estimate the probability that a certain variable is a component of the best model (Symonds and Moussalli 2011).

To study Treatment effects in more detail, we also ran mixed-effect models on individual behavioural variables. LMMs were used for normally distributed variables (Table S1). For the full models that included the amount of performed Tongue flicks as a dependent variable, we added the time the lizards spent Walking (transformed to reach normality) as a covariate, as well as all two-way interactions with Treatment and Trial. This is necessary as it corrects for the positive correlation between Tongue-flicking and Walking (Thoen et al. 1986; Van Damme et al. 1995; Schulterbrandt et al. 2008). The binomial variables describing whether lizards had been seen Basking, Nudging and Standing up-right were analysed using generalised linear mixed-effect models (GLMM; lme4 package) with a binomial fit and a logit link function. The effect of Treatment and Trial on the number of Foot shakes, Head rubs and Startles was also examined using GLMMs. Depending on which best fitted the data, a Poisson or negative binomial fit and a log link function were used (Table S1). The proportion of observations in which Tail vibrations were performed was analysed using a Fisher's exact test instead of mixed models, as this behaviour was completely absent for some scent treatments.

Lizard identity was entered into all LMMs and GLMMs as a random effect to account for the repeated use of the same lizard (Figure S1). Assumptions regarding normality of residuals (for LMMs), homoscedasticity and linearity were met and the data were checked for overdispersion (in the case of GLMMs). Models were compared using the second-order Akaike Information Criterion (AIC_c) as well as their Akaike weights (w_i) (Symonds and Moussalli 2011). The significance of pair-wise differences in behaviour over scent treatments was assessed using the Bonferroni correction for multiple testing in the lsmeans package in R (Lenth 2016).

Results

Experiment A: snake recognition

The FAMD resulted in two new composite variables that jointly accounted for approximately 64% of the behavioural variation lizards exhibited in Experiment A (snake vs. controls; Table 1a). The first dimension represented a gradient in

a)

		Dimension 1			√Dimension 2		
	eigenvalue	4.91			2.09		
	variance (%)	44.65			19.02		
	cumulative variance (%)	44.65			63.68		
Mixed-effect Models	explanatory variables	Treatment	Trial	Treatment x Trial	Treatment	Trial	Treatment x Trial
	importance values	0.83	0.15	0	*0.98*	0.3	0
Behavioural variables	No-move†	**15.08**			0.32		
	Walk†	**17.17**			1.80		
	√Tongue flick	**14.96**			0.00		
	√Labial lick	3.60			0.78		
	Foot shake^{bin}	8.97			7.50		
	Startle^{bin}	0.56			**32.15**		
	Head rub^{bin}	5.30			7.18		
	Bask^{bin}	9.23			7.44		
	Nudge^{bin}	12.31			0.06		
	Stand-up^{bin}	12.65			8.32		
	Tail vibration^{bin}	0.16			**34.45**		

b)

		Dimension 1²			Dimension 2		
	eigenvalue	4.38			1.29		
	variance (%)	54.75			16.09		
	cumulative variance (%)	54.75			70.85		
Mixed-effect Models	explanatory variables	Treatment	Trial	Treatment x Trial	Treatment	Trial	Treatment x Trial
	importance values	0.25	0.15	0	0.14	0.4	0.02
Behavioural variables	No-move	0.02			0.36		
	√Walk	**0.21**			0.00		
	3√Tongue flick	**0.20**			0.00		
	Foot shake^{bin}	0.12			0.00		
	Bask^{bin}	0.00			**0.59**		
	Nudge^{bin}	**0.18**			0.01		
	Stand-up^{bin}	0.14			0.01		
	Labial lick^{bin}	**0.13**			0.02		

√ square-root transformed; 3√ third-root transformed; † Box-Cox transformed; bin coded into a binomial quantity; ² squared

Table 1 (left) The main dimensions (i.e. with eigenvalues greater than 1) retrieved from the FAMD analyses of Asian grass lizard behaviour, with associated LMM results, as well as, contributions of the relevant behavioural variables for (A) Experiment A and (B) Experiment B

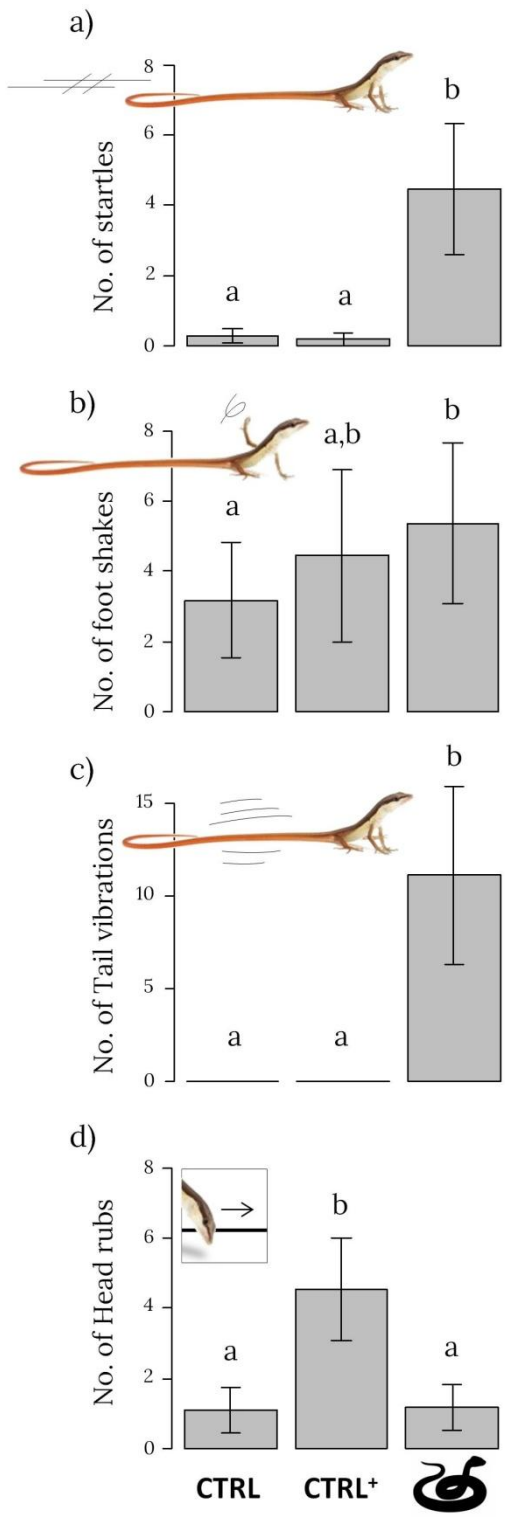

Figure 1 (right) Behavioural responses of the Asian grass lizard to three different scent treatments. Group means for behaviours performed during the odourless control (CTRL), pungency control ($CTRL^{+}$) and Oriental whip snake scent treatment (snake silhouette) are given, with the error bars being representative of the standard error. Letters above the bars indicate whether two group means are significantly different ($p < .05$) from each other (indicated with a different letter) or not (indicated with the same letter). Post-hoc multiple comparisons with Bonferroni correction were used for comparison of means, with the exception of the data for Tail vibrations, in which the Fisher's exact test was used. Insets are a visual representation of each behaviour which is also noted along the y-axis. The original picture was taken by Mickael Leger

Table 2 Behaviours observed in Experiments A and B and their mean (range) values over Treatment

	Experiment A			Experiment B	
	odourless control	**pungency control**	***A. prasina***	**odourless control**	***H. auropunctatus***
No-move	513.75 (357.77-600)	472.22 (366.32-600)	541.14 (385.04-600)	335.63 (0-600)	320.5 (2.05-600)
Walk	31.22 (0-78.2)	68.89 (0-178.73)	29.61 (0-85.07)	40.67 (0-145.74)	82.15 (0-253.17)
Tongue flick	109.55 (6-258)	197.55 (0-340)	94.27 (4-415)	64 (0-221)	222.89 (0-853)
Labial lick	66.82 (28-168)	76.36 (21-183)	46.91 (2-116)	6.44 (0-24)	9.89 (0-31)
Bask	45.92 (0-232.04)	35.9 (0-137.06)	20.98 (0-133.85)	123.19 (0-576.04)	155.06 (0-597.95)
Nudge	4.55 (0-26.65)	12.04 (0-93.1)	0.27 (0-1.78)	2.29 (0-11.73)	10.15 (0-41.61)
Stand-up	4.25 (0-21.69)	7.85 (0-32.18)	7.97 (0-42.5)	78.44 (0-600)	30.26 (0-112.34)
Foot shake	3.18 (0-15)	4.45 (0-26)	5.36 (0-23)	2.56 (0-13)	4.22 (0-26)
Startle	0.27 (0-2)	0.18 (0-2)	4.45 (0-22)	0 (0-0)	0.11 (0-1)
Head rub	1.09 (0-6)	4.55 (0-16)	1.18 (0-6)	-	-
Tail vibration	0 (0-0)	0 (0-0)	11.09 (0-52)	-	-

Note: The values of the timed variables No-move, Walk, Bask, Nudge and Stand-up are presented in seconds, whereas those for Tongue flick, Labial lick, Foot shake, Startle, Head rub and Tail vibration are shown as counts. A hyphen indicates combinations of behaviours and scent treatments for which data were insufficient to calculate means.

explorative behaviour, with high scores for lizards that exhibited long bouts of Walking (factor loading = +0.92) and elevated Tongue flick rates (+0.86) but little No-move behaviour (−0.86). We found little evidence that either Treatment, Trial or their interaction induced this variation (Table 1a). The second FAMD dimension correlated strongly with the incidence of Startle behaviour (+1.57) and Tail vibrations (+2.61) and can, therefore, be considered a stress-gradient. Lizards observed in the snake treatment scored significantly higher on this second dimension (with more likely instances of Startling and Tail vibrations) than lizards in both control situations ($t_{24.2}$ = −3.31, P = 0.0089 and $t_{24.2}$ = −3.78, P = 0.0027 when compared to the odourless and pungency control, respectively; Table 1a). Behaviour in both controls did not differ significantly from each other ($t_{24.2}$ = 0.48, P = 1.00).

Experiment B: mongoose recognition

The first two dimensions of the FAMD explained approximately 71% of the total behavioural variance. As in Experiment A, the first FAMD dimension indicated the level of explorative behaviour, characterised by Walking (+0.95) and Tongue-flicking (+0.94), and now also Nudging behaviour (+2.08) and Labial licks (+1.12). Neither Treatment nor Trial, or a combination of both explained variation along this axis (Table 1b). The second dimension reflected variation in the duration of Basking behaviour primarily (−0.70). Again, neither scent treatment nor trial number influenced the scores on this axis (Table 1b). Additionally, not a single Tail vibration and only one Startle was seen across all behavioural trials in Experiment B (Table 2). None of the tests on individual behavioural variables revealed an effect of Treatment (Table S1b and Figure S1). It proved impossible to run a GLMM for the binomial variable Basking, because the random effect term (Lizard individual) caused the model to become overfit. A Fisher's exact test revealed no Treatment effect (P = 1.00).

Discussion

Asian grass lizards in our experiments changed their behaviour when confronted with snake chemicals. Scent of the Oriental whip snake elicited Startles, Foot shakes and Tail vibrations, indicative of stress. This suggests that Asian grass lizards, like other lacertids previously studied (e.g. Van Damme and Quick 2001; Amo et al. 2004a; Mencía et al. 2016), can detect the odour of saurophagous snakes and relate it to increased predation risk, even in the absence of visual cues.

Surprisingly, however, the scent of the mongoose did not evoke any notable changes in the lizards' behaviour. We discuss a number of non-mutually exclusive hypotheses to explain this discrepancy.

First, lizards may not react to mongoose odour simply because they have not evolved the necessary odorant receptors. We have no information on the nature of mongoose kairomones, nor on the kind of receptors available in the lizards' epithelia (Silva and Antunes 2017), so we cannot test this explanation directly. Both Oriental whip snakes and mongooses are genuine predators of the Asian grass lizard, and they have both coexisted with it for a long time. As a small caveat, the lizards in our experiments originated from Java and have, therefore, coexisted with the Javan mongoose rather than with the Indian mongoose. However, it seems unlikely that this could explain the lizards' lack of response to mongoose scent. The two *Herpestes* species have diverged only recently (5 Mya) and are still interbreeding (Veron et al. 2007; Patou et al. 2009). Moreover, several studies have shown that scents of closely related mammals tend to be highly similar (Bininda-Emonds et al. 2001; Carthey et al. 2017).

Therefore, it is difficult to see why natural selection would bestow lizards with odorant receptors for snake but not mongoose scent. Evolutionary constraint could be one explanation—perhaps it is easier for lizards to evolve receptors for reptilian rather than for mammalian odours. For instance, lizards may have evolved chemoreceptors for the detection of conspecific cues in a social or reproductive context. Co-opting such receptors for predatory recognition may be more likely for phylogenetically related predators that perhaps emit more similar chemicals (snakes) than for distantly related ones (mammals). Alternatively, being able to detect mongoose scent may not be selected for. Kats and Dill (1998) have argued that the benefits of chemosensory recognition (i.e. early warning) must be traded-off with its costs regarding the energy and time spent by responding. Perhaps the scent of a snake in our experiments is more informative than that of mongooses. Asian vine snakes (*Ahaetulla*) are well-camouflaged ambush predators that pass much of their time waiting motionless for passing prey (Chowdhury et al. 2017; Kartik 2018). The scent of a vine snake is therefore a reliable (and, due to its concealment, possibly the only) cue for its proximity. In sharp contrast, mongooses are active hunters that forage over large distances and maintain wide home ranges (Pitt et al. 2015). The scent of a mongoose may not be very

informative about its whereabouts. Visual cues may be more reliable signs of mongoose menace (Brock et al. 2015).

A second possible reason for our lizards' failure to respond to mongoose scent is that the response is learned, and therefore, requires prior exposure to the stimulus. We judge this explanation to be highly unlikely for three reasons. First, our animals were wildcaught in Java, Indonesia, shortly before the experiments. Both mongooses and whip snakes are abundant in the area (Thy et al. 2012; Chutipong et al. 2016), so our study animals may have been exposed to scents of the predators before experimentation. Also, chemosensory predator recognition in other lizard species is innate rather than learned (Martín et al. 2015), so lizards probably do not need prior exposure with the stimulus to mount an anti-predatory response. Finally, it is difficult to see why lizards would need prior exposure to mongoose scent, but not snake scent to mount an anti-predator response.

In the previously suggested explanations, the lack of response towards mongoose scent was thought to result from the lizard's inability to detect the chemical cues. A second possibility is that the lizards detect and recognise the scent, but 'choose' not to exhibit Foot shakes, Tail vibrations and Startles. Font et al. (2012b) have argued that these behaviours (seen in many lacertids) work as pursuit-deterrent signals— the prey notifying the predator that it has been detected and that any further attack will be pointless. Pursuit-deterring signals are more likely to work with ambush predators than with active foragers. Oriental whip snakes rely on concealment and will launch fast, unexpected attacks on unwary prey, often within the vegetation over-heading the prey. However, as these snakes are rather slow when moving over ground (Sharma 2019), it seems unlikely that they will engage in the pursuit of an alarmed lizard. Mongooses, on the other hand, are fast and agile hunters that will actively pursue lizards (Lewis et al. 2010). Foot shakes, Tail vibrations and short Startles are probably more likely to draw the attention of a mongoose than to discourage it from attacking (Conover 2007). In addition, these behaviours require the lizard to stop moving, which could be the wrong strategy when threatened by a fast-moving predator. With this reasoning, the lizards' lack of (visible) response towards mongoose scent can be considered adaptive.

It should be noted that our experimental set-up might have precluded certain types of anti-predator behaviour. For instance, we did not provide lizards with hiding places or climbing structures that they might use to escape from

predators, perhaps mammal predators in particular. It would be interesting to repeat the tests in more natural conditions. On the other hand, a lizard sensing a dangerous odour while in an unfamiliar, open environment can be expected to behave differently compared to a lizard in the same setting, but without dangerous cues at hand. This was not the case for animals in the mongoose treatment.

A third explanation of the lizards' apparent apathy towards mongoose chemical cues could be that the individuals used in Experiment B were, for some unknown reason, generally less responsive than the individuals in Experiment A. The fact that the control treatments of both experiments differ (Table 2) may hint in that direction. On the other hand, the difference in overall responsiveness between lizards in the control situation of Experiments A vs. B could be due to differential carry-over and/or habituation effects. Indeed, the effect of treatment history differed between Experiments A and B (Figure S2). In Experiment A, lizards that were first tested in a snake-scented environment exhibited a stronger response in the control environment than lizards that had no previous experience with snake scent either because they were tested for the first time, or had only been tested before in the pungency control environment. This suggests that the former lizards perceived the new environment as potentially dangerous on the basis of their previous experience. In Experiment B, lizards that were first tested in the mongoose-scented environment exhibited less stress responses in the control environment than lizards in the control treatment that were tested for the first time. This may reflect habituation to a new, but apparently safe, environment and reinforces the idea that mongoose scent is not detected or perceived as dangerous.

Studying anti-predator strategies that are efficient against the small Indian mongoose (i.e. those employed by prey in the mongoose's native range) is relevant in the light of the multiple introductions to ecosystems worldwide and the resulting predatory pressures on local prey (Hays and Conant 2007). If other squamate species, like the Asian grass lizard, fail to mount an anti-predator response when smelling mongoose-derived cues, this may help explain why the introduction of these carnivorans can have such disastrous effects on the local herpetofauna.

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Chapter 3

Chemosensory deficiency may render island-dwelling lizards more vulnerable to invasive predators

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Abstract

Newly introduced predators constitute a major threat to prey populations worldwide. Insular prey animals in particular often do not succeed in overcoming their naivety towards alien predators, making them specifically vulnerable. Why this is the case remains incompletely understood. Here, we investigate how the ability to detect and respond to predator chemical cues varies among populations of the Dalmatian wall lizard, *Podarcis melisellensis*. Lizards were sampled from five locations in south-eastern Croatia (one mainland location and four islands) that varied in the composition of their predator community. We observed the lizards' behaviour in response to chemical cues of native saurophagous snakes (the Balkan whip snake, *Hierophis gemonensis*, and eastern Montpellier snake, *Malpolon insignitus*) and an introduced mammalian predator (the small Indian mongoose, *Herpestes auropunctatus* – a species held responsible for the loss of numerous insular reptile populations worldwide). Mainland lizards showed elevated tongue-flick rates (indicative of scent detection) as well as behaviours associated with distress in response to scents of both native and introduced predators. In sharp contrast, island lizards did not alter their behaviour when confronted with any of the predator cues. Alarmingly, even lizards from islands with native predators (both snakes and mammals) and from an island on which mongooses were introduced during the 1920s were non-responsive. This suggests that insular populations are chemosensorily deprived. As failure at the predator-detection level is often seen as the most damaging form of naivety, these results provide further insight into the mechanisms that render insular-living animals vulnerable to invasive species.

Introduction

Invasive species constitute one of the primary causes of indigenous species endangerment and extinction (Lowe et al. 2000). In particular, the arrival of a novel apex predator can have dramatic effects on local prey populations (Courchamp et al. 2003; Salo et al. 2007). The introduction of placental mammalian predators in Australia, for example, has resulted in the loss of dozens of species over the past 200 years (e.g. Strahan 1995). Furthermore, the impact on local fauna is often not restricted to single prey species, as cascading effects leave their mark further on along the food web (Schoener et al. 2002; Maron et al. 2006; Willson 2017), sometimes even hampering the functioning of the entire ecosystem (Zavaleta et al. 2009).

The reason why invasive predators have such a tremendous impact is often related to naivety of the local prey species (Cox and Lima 2006; Salo et al. 2007). In a stable ecosystem, predation risk imposed by indigenous predators is a great driving force that shapes prey behaviour, morphology, physiology and certain life-history traits (Lima and Dill 1990; Kats and Dill 1998). The co-occurrence of prey and predator species can consequently be seen as the outcome of long series of co-evolutionary events, in which the prey has countered all consecutive adaptations that improved the predator's hunting skills (Downes and Shine 1998; Banks and Dickman 2007). Alien predators reaching untrodden ground can rapidly attain high population densities (Courchamp et al. 2003), leaving insufficient time for the prey species to genetically adapt to the novel risk situation. Some prey species may escape extinction because their behavioural repertoire happens to include elements fit to evade the novel predator – perhaps because they evolved in sympatry with ecologically similar native predators (Cox and Lima 2006). However, this does not always suffice. For instance, many Australian prey species have suffered from the introduction of placental predators (foxes, cats, dingoes) despite their historical contact with indigenous marsupial predators that hunt in a very similar way (Banks and Dickman 2007). Why some native prey species can, and others cannot, cope with alien predators remains poorly understood (Ehlman et al. 2019).

Island communities tend to be more vulnerable to alien introductions than their mainland counterparts (Lowe et al. 2000; Courchamp et al. 2003; Yoshida 2008). Due to their isolated nature and limited surface area, many islands have a depauperate predator community (MacArthur and Wilson 1967; Terborgh 2010).

Under these relaxed conditions, redundant anti-predatory adaptations may be lost through genetic erosion, or because the energy used to build and maintain them is reallocated into more useful structures (Niven and Laughlin 2008). The latter seems most likely on islands that are poor in resources. Re-evolving adequate anti-predatory behaviour may not be feasible on an ecological timescale (Blumstein 2002). The loss and gain of predator recognition in insular prey has received some attention in the past (Blumstein 2006; Carthey and Blumstein 2018). However, the results are disparate, even between closely related species. The lizard *Podarcis hispanica atrata* from the Columbretes Islands (Spain) has retained the ability to recognise chemical cues of the predatory snake *Vipera latastei* although the snake was eradicated from the island more than 100 years ago (Van Damme and Castilla 1996). However, after 7000 years of insularity, individuals of the related species *Podarcis muralis* from islands off the French Atlantic coast no longer react adequately to chemicals of three saurophagous snakes (Durand et al. 2012). Balearic specimens of *Podarcis siculus* seem to have acquired the ability to recognise chemical cues of *Macroprotodon mauritanicus*, a saurophagous snake that has been introduced on Menorca in historical (possibly Roman) times (Mencía et al. 2017). In contrast, *Podarcis lilfordi* and *Scelarcis perspicillata* on the Balearic Islands have failed to develop the capacity to distinguish chemical cues from the latter snake. Then again Ortega et al. (2017) found that *Podarcis pityusensis* of Ibiza readily recognised chemical cues of *Hemorrhois hippocrepis*, a saurophagous snake that was introduced onto the island 11 years before the study. These results seem to suggest that the speed at which chemoreceptive predator recognition is lost, or gained, in these insular lizards may depend on the species, on the predator in question or on the (historical) context.

To better predict the outcome of alien predator introductions on insular fauna, more knowledge is needed on individual effects of the aforementioned factors on the loss and gain of anti-predator behaviour. Here, we test whether anti-predator tactics of the Dalmatian wall lizard, *Podarcis melisellensis*, differ among study sites varying in predator community composition (i.e. the context-effect on trait loss). We compare lizards from a high-risk mainland locality to lizards from large and small islands (intermediate and low risk). We examine the lizard's responses to cues of local snake species, as well as the small Indian mongoose (*Herpestes auropunctatus*), a mammalian predator that was recently

introduced into the area (i.e. context-effect on trait gain) (Tvrtković and Kryštufek 1990; Barun et al. 2008).

As it is the initial step in predator avoidance, the lack of recognition of novel predators is expected to be the most damaging form of naivety (Cox and Lima 2006; Banks and Dickman 2007). We therefore focus on the adaptability of the chemical senses in the Dalmatian wall lizard. Lacertid lizards obtain chemical information on their environment (including the presence of predators) through their nose (olfaction) or via the tongue that delivers molecules to the Jacobson's organ in the roof of their mouth (vomerolfaction) (Bertmar 1981; Cooper 1996). Activity of the latter can easily be gauged by counting the number of tongue flicks these lizards perform (Graves and Halpern 1990). Moreover, *P. melisellensis* is preyed upon by a variety of animals, including native mammals (e.g. garden dormouse, *Eliomys quercinus dalmaticus*; I. Budinski, pers. comm.), the invasive small Indian mongoose (Barun et al. 2010), birds (e.g. hooded crow, *Corvus cornix*; Baeckens and Briesen 2017) and snakes (e.g. Balkan whip snake, *Hierophis gemonensis*; De Meester et al. 2018), possibly allowing for the development of highly discriminative chemical anti-predator senses (Helfman 1989). It is consequently an appropriate species to tackle the following research questions: (1) Does the Dalmatian wall lizard use its chemical senses to detect native predators?; (2) Are these lizards able to discriminate between scents of closely related native predators which pose varying threats? (3) Do they lose the ability to chemically detect predators when living on islands or under a relaxed predatory pressure? (4) Does this impact their ability to recognise invasive predators?

Material & Methods

Study sites & species

The Dalmatian wall lizard occurs in Mediterranean and sub-Mediterranean zones from extreme north-eastern Italy to north-western Albania (Ajtic et al. 2009). Adult lizards were caught by noose during May on the Croatian mainland near the village of Majkovi (21♂, 15♀) and on the islands Brusnik (7♂, 5♀), Mali Barjak (7♂, 6♀), Vis (12♂, 9♀) and Korčula (10♂, 10♀) (Fig. 1). Average snout–vent length of all lizards studied was 58.1 mm (±0.8 mm SE), average body mass 4.88 g (±0.23 g). Sampling occurred over a period of 2 years. The island populations were sampled and tested in 2015, the mainland population in 2016. In both years, lizards were

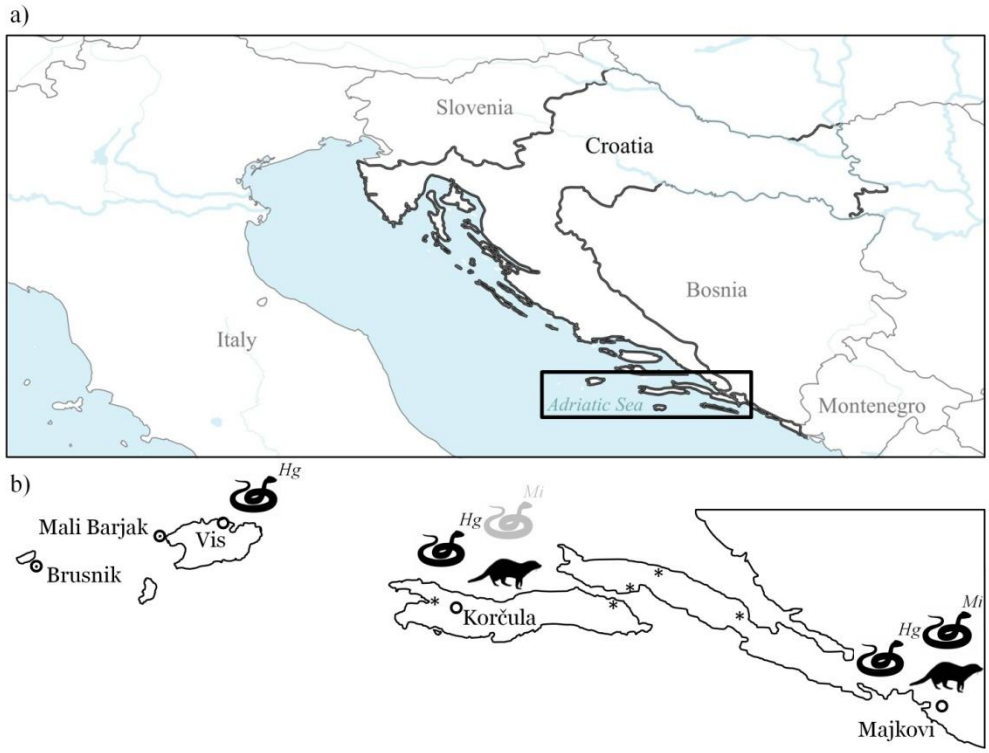


Figure 1 Map of the study area. Map b) is a downscaled representation of the outlined area in map a). Localities at which the Dalmatian wall lizard (*Podarcis melisellensis*), was sampled are indicated with a circle. An asterisk indicates places at which the small Indian mongoose (*Herpestes auropunctatus*) was introduced between 1921 and 1927 (Tvrtković and Kryštufek 1990). Animal silhouettes indicate the presence at our study location of one of the three predators: Balkan whip snake (*Hierophis* gemonensis; snake silhouette with Hg superscript), eastern Montpellier snake (*Malpolon insignitus*; snake silhouette with Mi superscript) and the small Indian mongoose (mongoose silhouette). A light-grey silhouette indicates that the predator is present at the sample location, but we did not use its scent as a treatment in focal observations of lizards (see text for further elaboration)

released at the site of capture at the end of the experiment, which lasted not more than 1 month.

The populations sampled within this study experience different predator pressures (Table 1). The study site of Majkovi harbours the most diverse predator community, followed by Korčula island, Vis island and the two islets Mali Barjak and Brusnik where (non-avian) predators are absent (Kryštufek and Kletečki 2007; Jelić et al. 2009; Barun et al. 2015). This allows for the investigation of a potential influence of predatory pressures on chemosensory anti-predator behaviour towards native and introduced predators.

	Brusnik	Mali	Vis	Korčula	Majkovi
Snakes					
Hierophis gemonensis			x	x	x
Elaphe quatorlineata			x	x	x
Coronella austriaca					x
Malpolon insignitus				x	x
Telescopus fallax			x	x	x
Platyceps najadum					x
Vipera ammodytes				(x)	x
Zamenis situla				x	x
Lizards					
Pseudopus apodus			x	x	x
Mammals					
Rattus rattus			x	x	x
Eliomus quercinus			x	x	x
Erinaceus roumanicus			x	x	x
Felis catus			x	x	x
Vulpes vulpes					x
Sus scrofa				x	x
Canis aureus				x	x
Canis lupus familiaris			x	x	x
Martes foina			x	x	x
Herpestes				x	x

Table 1 Table presenting all potentially relevant predators of the Dalmatian wall lizard, *Podarcis melisellensis*, on the various locations considered during our study. Information was retrieved from literature (Kryštufek and Kletečki 2007; Barun et al. 2010; Žagar et al. 2013; Barun et al. 2015; Eko-monitoring d.o.o. 2017), through personal observations and communications with I. Budinski, B. Lauš, A. Barun and Z. tadić. An (x) indicates a historical record; now probably extinct (Barun et al. 2010)

The Balkan whip snake is widespread among the Adriatic islands and mainland (Kryštufek and Kletečki 2007). In addition to small lizards it also feeds on mice and arthropods, but it is usually the most abundant snake species and therefore a relevant predator of the Dalmatian wall lizard (De Meester et al., 2018). In our study area the snake occurs in sympatry with the latter near Majkovi on the mainland, on Korčula island and on Vis island (Fig. 1).

The eastern Montpellier snake (*Malpolon insignitus*) is thought to be more specialised than the Balkan whip snake in lizard prey and very often preys upon

the study species on the mainland (B. Lauš, pers. comm.; Beshkow and Gerasimow 1980; Pleguezuelos 1998; De Haan 1999). The eastern Montpellier snake is only present on Korčula of the studied island localities, which makes the species inappropriate for testing scent- evoked anti-predator responses by lizards over island populations. However, using its scent in mainland trials alongside that of the Balkan whip snake allowed us to verify the relevance of the latter as a scent donor in all our studied populations. Moreover, it offered the opportunity to test whether mainland lizards have developed the skills to differentiate between scents of relatively closely related predator species that differ in their level of posed risk (Helfman 1989).

The generalist small Indian mongoose, which is native to south-east Asia, is present in our study system as well. This mammalian predator has been intentionally introduced to numerous island systems worldwide to control rat and snake populations. In many cases, this has led to the rapid extinction of local prey species (Hays and Conant 2007), resulting in the mongoose being considered as one of the hundred most dangerous invasive species in the world (Lowe et al. 2000). It was introduced onto Korčula between 1921 and 1927 near the village of Vela Luka to control venomous nose-horned vipers (*Vipera ammodytes*) (Tvrtković and Kryštufek 1990). This is near our sampling location (Fig. 1). The mongoose was also released on the Pelješac peninsula at localities ~40 km distant (as the crow flies) from the sampled mainland population near the village of Majkovi. On both Korčula island and the southern Croatian mainland it rapidly established viable populations and further spread over land to become an often-sighted species (Barun et al. 2008). On Brusnik and Mali Barjak no snake or mammal predators have ever been present.

Lizard housing conditions

Dalmatian wall lizards captured on the islands of Brusnik, Mali Barjak and Vis were transported to and housed on the island of Vis in the town of Komiža. Individuals from the island of Korčula were taken to the city of Vela Luka on the island of Korčula. Mainland lizards were housed at a location close to the village of Slano. Lizards were transported in individual cloth bags. Individuals from the same population were housed together in nylon mesh portable terrariums (Exo Terra) of 122 × 76 × 42 cm (length × width × height). Lizards were individually marked by colour codes that we applied to the head with non-toxic markers. This ensured quick and easy identification of one lizard among the others in the home

terrarium. As colour codes may fade, we additionally toe-clipped lizards to enable remarking (Langkilde and Shine 2006). The terrariums were enriched with moss, stones and bark. The lizards were fed daily with commercially obtained mealworms (*Tenebrio molitor*) or wild-caught invertebrates. Water was present ad libitum in a ceramic bowl, and additionally vaporised in each terrarium on a daily basis. A 60-W incandescent lamp, suspended on one side of the terrarium, provided a temperature gradient between 32 and 23 °C in which the lizards could optimally regulate their body temperatures. The lamps were switched on for 14 h each day, mimicking the natural light–dark regime for our study sites during May.

Predator housing & scent collection

In this section we describe the procedure of scent collection on sterile cotton gauzes (Multipharma, Brussels, Belgium). The effectiveness of this procedure for predator scent collection was confirmed by unpublished preliminary research with another lacertid lizard (*Zootoca vivipara*) and its snake predator (*Vipera berus*). When handling the gauzes, clean tweezers were used at all times. During scent collection, human contact with the animals was limited and the cotton gauzes were left untouched by caretakers. These precautions ensured that there was no contamination of the gauzes with human scent. Gauzes were left for 4–5 days in a place where contact with the animal over this time period was ensured (animal-specific details are given below). Afterwards, samples were stored no longer than 3 weeks in a freezer at −10 °C until use. All scent-donating animals were housed under a natural light–dark regime at our own field station (Balkan whip snake in 2015) or at partnering facilities (see also Ethical Note below).

Mongoose scent was collected from adult males caught with baited cage traps on the island of Korčula. The mongooses were held in the trapper's facilities in Sinj, Croatia (2015, N = 3), or at the research centre of IDT-Biologika GmbH in Dessau-Roßlau, Germany (2016, N = 15). The animals were fed daily (poultry and beef) and had access to water at all times. Sterile cotton gauzes were divided among the mongoose cages, placed on the bottom of each cage and covered by a blanket or bedding. This allowed the scent of the animal to penetrate into the gauzes while visual cues, such as hair and excrement, were captured on the blanket or bedding.

Scent of the two species of native saurophagous snakes was collected on gauzes distributed over the snake's housings. We made sure that the snakes contacted the pieces of cotton multiple times, to guarantee transfer of chemical

cues. All snakes used for scent donation were hand caught in Croatia. Balkan whip snake individuals were obtained from Vis (2015, 1♂), Pag (2016, 1♂) and Sinj (2016, 1♀). Eastern Montpellier snake individuals came from Vir (2016, 1♂) and Split (2016, 2♀). Snakes were not fed during the scent collection sessions.

Both a positive baseline and a negative control were offered during the experiment. A negative control was presented in the form of cotton gauzes which came directly out of the package and were therefore odourless. We rubbed cut ginger roots on a cotton gauze to produce a positive baseline control. Ginger should be an unknown odour for the lizards with no association with a predatory context.

Focal observations

At least 24 h was left between the time of capture and the start of the first experimental trial in order to let the lizards feel at ease in the home terrariums. We employed a repeated measures experimental design in which each lizard was observed multiple times, each time when confronted with a different scent treatment. Island lizards (N = 66; for more detailed population sizes, see previous section on study sites and species) were presented with Balkan whip snake scent, mongoose scent and both control treatments in a randomised order (66 individuals × 4 treatments = 264 observations in 2015). The mainland lizards (N = 36) were additionally offered scent of the eastern Montpellier snake in a randomised way with the other scents (36 individuals × 5 treatments = 180 observations in 2016). To minimise carry-over effects, ~24 h was left between trials of the same individual. This resulted in focal observations lasting 4 days for island lizards and 5 days for mainland lizards. All lizards were observed during May. The exact dates depended on when a lizard had been captured.

The observations were performed between 9 a.m. and 6 p.m. in a separate observation room using a test arena of 50 × 40 × 40 cm (l × w × h). This arena was open at the top in order to approximate the situation of scent encounter by a lizard in the field. One of the walls was coated with a dark window film (norauto), which enabled the observer to note behaviours without disturbing the observed lizard. We used the event- recording software JWatcher v.1.0 (Blumstein and Daniel 2007) for this purpose. A 60-W incandescent lamp was suspended above the arena. Before each observation the test arena was cleaned with ethanol (Bauwens et al. 1987) and the bottom was covered with fresh sand to avoid scent contamination by a previous lizard. One clean stone was placed in each corner.

Subsequently, each of the four stones was rubbed with a gauze containing one of the scent treatments and the gauze was then placed at the stone on the left-hand side and closest to the observer. The body temperature (cloacal) of the lizards was measured immediately before each observation and was on average (±SE) 29.3 ± 0.1 °C. The individual was placed in the middle of the test arena, shortly after which a 5-min observation period was started. Lacertid lizards show the most pronounced response in the 5 min following detection of a predator chemical cue, after which the effect fades away (Thoen et al. 1986). We counted the number of Tongue-flicks, Tail vibrations, Foot shakes and Startles. The latter three are considered to be indicative of stress or linked to predator-escape strategies in lizards (Mori 1990; Van Damme and Quick 2001; Font et al. 2012b). We also noted the time (seconds) that the lizard spend Walking, Digging, Standing-up, performing Slow- motion behaviour or No-move at all (see Thoen et al. 1986 for a detailed description of behaviours). After each observation the lizard was placed in a terrarium other than its home terrarium until all observations for the day were completed. We did this to prevent an influence of the observed lizard's behaviour on non-observed individuals.

Statistical analysis

Statistics were done in R v.3.3.0 (R Core Team, 2016). As some of the timed variables were correlated and constrained by the total amount of time of the trials, we initially analysed Walk, No-move, Dig and Stand-up simultaneously with a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) using the adonis function contained within the vegan package (Oksanen et al. 2016). The models were set to calculate Euclidian distances using 9999 permutations. To examine separate behaviours in more detail, we additionally performed univariate analyses. We ran linear mixed-effect models (LMMs; lme4 package; Bates et al. 2015) for the time variables Walk (square-root transformed) and No-move. Despite being a count-variable, the number of observed Tongue flicks was normally distributed, rather than Poisson-distributed. We therefore opted to run LMMs for this variable as well. The time spent Digging, Standing-up and performing Slow-motion behaviour exhibited a highly skewed distribution with an excess of zeros. Because transforming did not help, we recoded these variables into binomial quantities, with 0 indicating that the focal lizard did not perform the behaviour and 1 indicating that it did. Binomial variables were analysed using generalised linear mixed effect models (GLMMs; lme4 package)

with a binomial fit and a logit link function. For the count data on the number of Foot shakes, Tail vibrations and Startles, GLMMs were used as well with a Poisson fit and log link function. In all mixed models, the individual (nested within population) was included as a random effect to account for repeated measures.

Explanatory variables used in both multivariate and univariate models were the scent treatment, the population from which the lizards originated and the sex of the lizard (hereafter Treatment, Population and Sex, respectively). For the models that included Tongue flicks as a dependent variable we added the time the lizards spent Walking as a covariate. This is necessary because lizards tend to flick their tongue more often when walking around (Thoen et al. 1986; Van Damme et al. 1995; Schulterbrandt et al. 2008). Furthermore, due to the repeated measures design, the history of treatment presentation for a specific lizard could have an impact on how this lizard responds to the next treatment. Including the Trial number as a fixed effect accounts for the variation in behaviour due to the repetition of the experiment. In this case it is assumed that lizards change their behaviour just as a result of the number of tests to which they were subjected, and that these effects are additive. However, when a lizard recognised the scent of a predator as a threat in one of the previous trials, this could have a larger impact on the behaviour in the following trials compared to the situation wherein this lizard was solely confronted with the control treatments. To include a varying effect of the scent treatments, a Cumulative variable is necessary. We calculated this variable by assigning a separate value to each scent treatment. The experimental history is then equal to the sum of all values of treatments already presented to the lizard in previous trials. In our case we assigned a value of '1' to all predator scent treatments and a value of '0' to both the controls. We created two sets of models containing one of both experience-based variables (Trial or Cumulative variable) for each observed behaviour. Full models included main effects as well as all two-way interactions between the fixed effects. The dispersion of our data points in multivariate space was approximately equal over all factorial levels. Assumptions regarding normality of residuals (for LMM), homoscedasticity and linearity were met. The GLMMs were checked for overdispersion.

We selected the best PERMANOVA model using the second-order Akaike information criterion (AIC_c). For univariate models, to estimate the likelihood that a certain fixed effect accurately explains the observed variation in a behavioural variable we applied a method described by Symonds and Moussalli (2011). First, we

built a 99.9% confidence set of models that are the most likely to be the best approximating model (based on the AIC_c) for each behavioural variable (Burnham and Anderson 2002) using the AICcmodavg package in R (Mazerolle 2016). Models containing Trial and Cumulative variable as the experience-based variable were considered simultaneously in the analyses. From the 99.9% confidence set we extracted the importance value of each explanatory variable by summing the Akaike weights (w_i) of the models that include the variable. We performed multiple comparisons with a Bonferroni correction using the lsmeans package in R (Lenth 2016) on behavioural variables for which Treatment (or any interaction containing this variable) was weighted more than 90%.

Ethical note

All methods and experimental protocols as described above were in accordance with the policies and requirements of the Ethical Committee for Animal Experiments of the University of Antwerp. This research did not require any authorization under Belgian law (Art. 2.6 of the Belgian Law of 4 May 1995; Annex VII, Belgian Law of 29 May 2013) because fieldwork was conducted abroad. Animals caught and housed by our own research team (i.e. Dalmatian wall lizards in both years, and a Balkan whip snake in 2015) were handled with permission from the national Croatian Ministry of Environment and Nature (licence numbers 517-07-1-1-1-15-3 and 517-07-1-1-1-1-16-4) and released at the site of capture at the end of the experiment. Scent from individuals of both snake species, housed at the Zoo of Zagreb, and the mongoose, housed at the research facilities of IDT-Biologika GmbH and Association BIOM, was obtained through a partnership with the licensed facilities. It was ensured that all animals were kept according to the prevailing local and European regulations. Appropriate TRACES documents were acquired by IDT-Biologika GmbH for the transport of mongoose individuals from Croatia to the research facility in Germany

Results

The effect of Treatment on Tongue flick rate varied considerably among populations (i.e. after accounting for the covariates Sex, Walk and the Cumulative variable; see Table 2). Interestingly, the largest difference was between mainland (Majkovi) and island (Brusnik, Mali Barjak, Vis and Korčula) lizards. Mainland lizards exhibited an increase in Tongue flick rate (compared to the negative control) when confronted with chemical cues of both the Balkan whip snake ($t_{384.79} = 3.76$, $P <$

Table 2 Statistical output of mixed-effect models, used to analyse behaviours performed by Dalmatian wall lizards from Croatian islands (Brusnik, Mali Barjak, Vis and Korčula) and the mainland (Majkovi) when confronted with various scent treatments (a negative control, positive baseline control, and scent from the Balkan whip snake, the eastern Montpellier snake and the small Indian mongoose). Pop = population of origin; Treat = the offered scent treatment; Sex = the sex of the focal lizard; Trial = the trial number; CVar = the cumulative variable

				experience		**Pop x**	**Pop x**	**Pop x experience**		**Treat x**	**Treat x experience**		**Sex x experience**	
	Pop	**Treat**	**Sex**	**Trial**	**CVar**	**Treat**	**Sex**	**Trial**	**CVar**	**Sex**	**Trial**	**CVar**	**Trial**	**CVar**
√Walk	**1.00**	0.09	0.65	**1.00**	0.00	0.00	0.03	**1.00**	0.00	0.00	0.00	0.00	0.11	0.00
No-move	**1.00**	0.08	0.62	**1.00**	0.00	0.00	0.00	**1.00**	0.00	0.01	0.00	0.00	0.12	0.00
Stand-up[bin]	**1.00**	0.88	0.34	0.87	0.13	0.02	0.04	0.00	0.03	0.02	0.00	0.00	0.00	0.02
Dig[bin]	**1.00**	0.31	0.62	0.00	**1.00**	0.00	0.01	0.00	**0.99**	0.10	0.00	0.00	0.00	0.16
Startle	**1.00**	–	–	–	–	–	–	–	–	–	–	–	–	–
Startle[k]	–	0.76	0.27	0.40	0.19	–	–	–	–	0.00	0.00	0.02	0.00	0.05
Startle[m]	–	**0.99**	0.46	0.03	**0.96**	–	–	–	–	0.03	0.00	**0.95**	0.01	0.14
Foot shake[m]	–	**1.00**	0.28	0.01	0.27	–	–	–	–	–	–	–	–	–
Tongue flick[†]	**1.00**	**1.00**	**0.98**	0.00	**1.00**	**0.99**	0.01	0.00	**1.00**	0.13	0.00	0.28	0.00	0.26

Importance values are given of the independent variables (Population, Treatment, Sex, Trial and the Cumulative variable) and their two-way interactions for each behavioural response variable.
Values > 0.90 are indicated in bold type. A dash (–) indicates explanatory variables for which it proved impossible to run models.
[a] Variable coded into a binomial quantity.
[b] model containing only the data from Korčula island lizards;
[c] model containing data from mainland Majkovi lizards.
†Explanatory variable √Walk, as well as all two-way interactions with the remaining explanatory variables, were included in the analysis, but left out of the table for clarity.

0.001) and the mongoose ($t_{382.28}$ = 3.49, P = 0.0016). In contrast, ginger (the positive baseline control) did not elicit increased Tongue flick rates ($t_{383.15}$ = 0.40, P = 1.00; Fig. 2A). In island lizards, Tongue flick rates were similar in all scent treatments (all P > 0.05; Fig. 3). Overall, mainland lizards Tongue flicked more (87.69 ± 5.65 tongue flicks per 5 min; mean ± SE in the control treatment) than all of the island lizards (50.35 ± 4.55).

Dalmatian wall lizards expressed stress primarily by performing Startles and Foot shakes in our experimental set-up. Only on rare occasions did we see Tail vibrations or Slow-motion behaviour (four and three times out of the total 444 observations, respectively). Furthermore, signs of stress were typically observed in mainland lizards (Figs 2, 3). In fact, because of the near-absence of the Startle and Foot-shake behaviour in some of our island populations, it proved impossible to run full mixed-effect models to test Population and Treatment effects simultaneously. Nevertheless, by splitting the dataset according to Population we were able to analyse Treatment effects on Startle and Foot- shake behaviour for mainland lizards as well as the Startling behaviour for Korčula island lizards. We emphasise that the rare occurrence of behaviours indicative of stress in island lizards subjected to our experimental set-up is in itself a noteworthy result.

Startles were seen hardly at all in lizards from the islets (Brusnik – Majkovi: Z = – 3.24, P = 0.012; Mali Barjak – Majkovi: Z = 2.83, P = 0.046) and the island of Vis (Vis – Majkovi: Z = 2.92, P = 0.035) (Table 2; Fig. 3). The lizards from Korčula island displayed Startles at intermediate frequencies and did not differ significantly from any of the other populations in this behaviour (all P > 0.05). Therefore, we opted to test for Treatment effects in populations separately and only looked at those populations for which Startling behaviour had been performed at relevant frequencies, namely in Korčula and Majkovi lizards. Only mainland lizards exhibited Startles more often when confronted with chemical cues of the snake (Z = 2.54, P = 0.033) and mongoose (Z = 3.23, P = 0.0037) than in the negative control situation. The experimental history of the animals affected their way of responding to the Treatment (Table 2). Mainland lizards that had been confronted with predator scent at least twice in previous trials (i.e. Cumulative variable > 1) no longer exhibited elevated Startle frequencies. In Figure 2B we eliminate this habituation effect for clarity reasons by only showing results for values of the Cumulative variable <2. Lizards from Korčula island did not show significant differences in Startling behaviour among treatments (Table 2; Fig. 3) even when

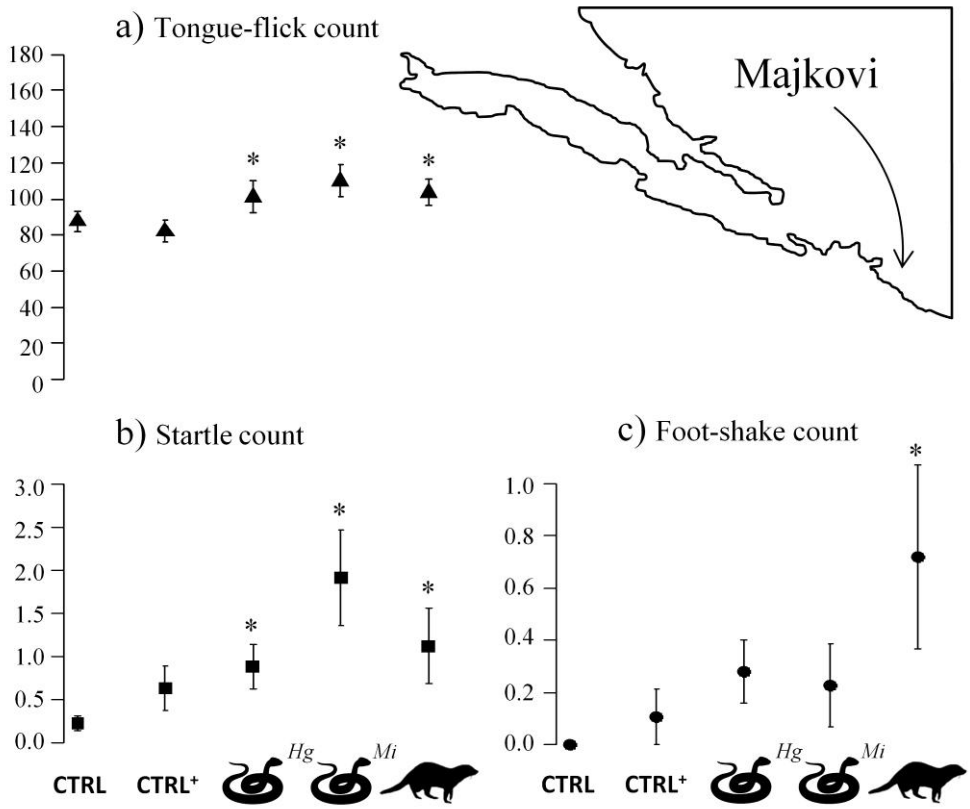
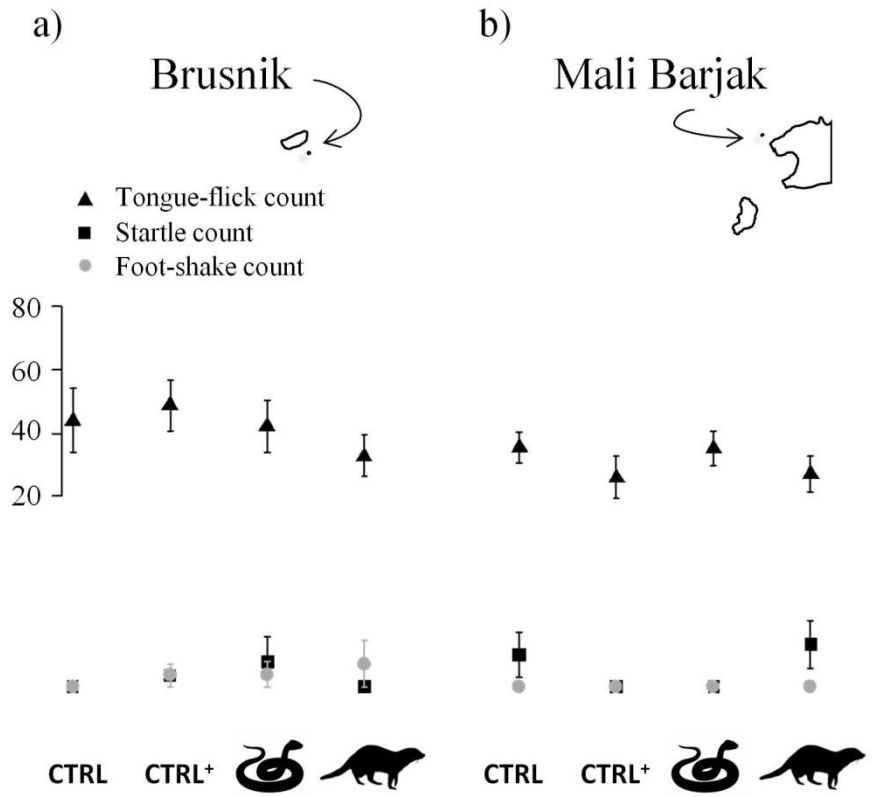


Figure 2 Behavioural responses of mainland Dalmatian wall lizards towards five different scent treatments. Means are given in separate panels for the number of performed Tongue flicks (a), Foot shakes (b) and Startles (c). Error bars represent the standard errors. An asterisk indicates a significant departure from the value in the negative control situation. Symbols on the x-axes depict the different scent treatments to which the lizards were subjected, namely a negative control (CTRL), ginger scent as a positive baseline control ($CTRL^{+}$), Balkan whip snake scent (snake silhouette with Hg superscript), eastern Montpellier snake scent (snake silhouette with Mi superscript) and small Indian mongoose scent (mongoose silhouette). The inset map provides an indication of the sampling location

taking the lizard's experimental history into account. Foot shakes were only seen occasionally, which made it impossible to model variance over populations with mixed models for this variable. A chi-squared test showed that, similarly to the Startle behaviour, the populations differed significantly in the occurrence of Foot shakes performed by the lizards ($\chi^2_4 = 9.45$, $P = 0.049$). Mainland lizards exhibited

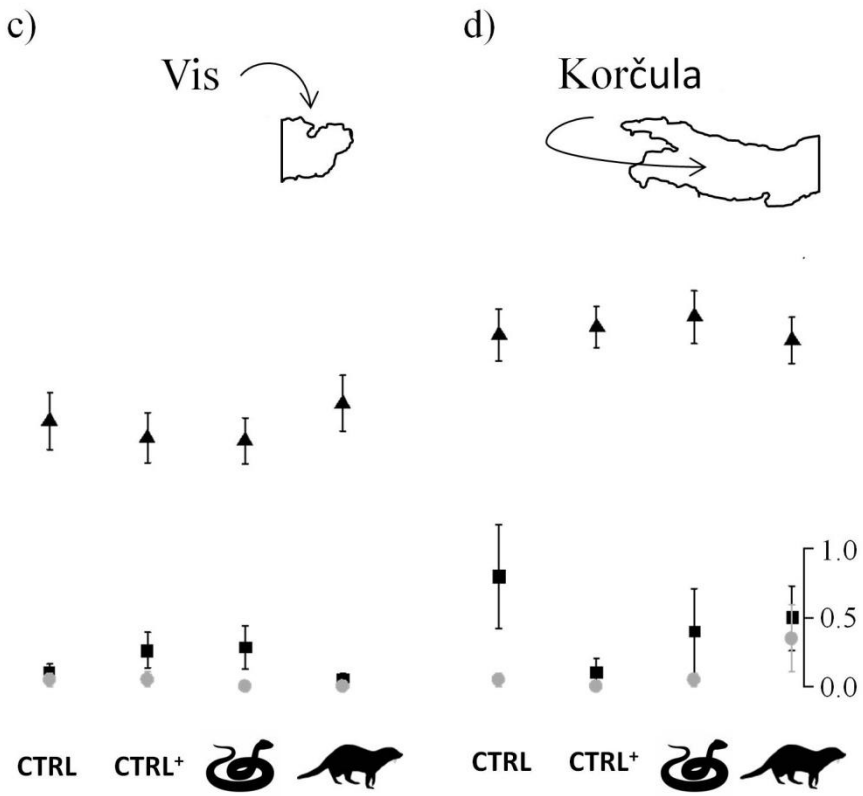
Foot shakes in 15 out of 144 observations (10%). Island lizards rarely performed the behaviour (Brusnik: 3/48 focal observations, 6%; Mali Barjak 0/39, 0%; Vis 2/84, 2%; Korčula 4/80, 5%). Mainland lizards exhibited Foot shakes more often when confronted with mongoose chemicals (Z = 2.94, P = 0.0099; based on candidate models only including main effects, see Table 2) compared to the negative control (Fig. 2C). We did not find significant differences between the number of Foot shakes performed if confronted with scent of the Balkan whip snake scent (behaviour observed in six out of 36 observations with this scent treatment, which is equal to the mongoose-scent treatment) and the negative control situation (two out of 36 observations). However, the power of the test was low given the small numbers. For island lizards, the behaviour was not observed a sufficient number of times to perform statistics. Finally, a permanova on the timed behaviours only revealed an effect of one of the experience indexes that depended on the population being examined (Cumulative variable × Population: $F_{4,297}$ = 7.91, P < 0.001). This was also reflected in the output from univariate analyses (Table 2). Treatment did not affect the time that lizards spent Walking or Not-moving nor



did it affect the occurrence of Digging or Stand-up behaviour in any of the studied populations.

Mainland lizards from Majkovi were offered scent of the eastern Montpellier snake, besides that of the Balkan whip snake. Chemicals of the former evoked an increase in Tongue flick count (compared to the negative control; $t_{158.37} = -5.41$, $P < 0.001$), which was similar to what was seen in response to Balkan whip snake and mongoose chemicals (all $P > 0.05$; Fig. 2A). Furthermore, the Startle count was significantly higher compared to the negative control ($Z = 3.64$, $P =$

Figure 3 (left and below) Behavioural responses of insular Dalmatian wall lizards towards four different scent treatments. Separate panels represent results for lizards originating from (a) Brusnik, (b) Mali Barjak, (c) Vis and (d) Korčula. Each sampling location is additionally indicated on an inset. Means are given with error bars representing the standard error. There were no significant departures from the negative control value for any of the behaviours and in any of the populations. Symbols on the x-axes depict the different scent treatments to which the lizards were subjected, namely a negative control (CTRL), ginger scent as a positive baseline control (CTRL$^+$), Balkan whip snake scent (snake silhouette) and small Indian mongoose scent (mongoose silhouette)



0.0011; Fig. 2B). Again, the effect was similar for all predatory scent treatments (all $P > 0.05$). As for the Balkan whip snake, scent of the eastern Montpellier snake did not have a significant effect (compared to the negative control situation) on the number of observed Foot shakes by mainland lizards. However, the behaviour was observed in six out of 36 observations (equal to the mongoose scent treatment and contrasting with the two out of 36 observations for the negative control) and non-significance could again be a result of a too low power of the test due to the limited amount of times the behaviour was performed (Fig. 2C). The time that lizards spend Walking and Not-moving or the occurrence of Digging or Standing-up behaviour during the observation was not affected by the eastern Montpellier snake scent treatment (Table 2). Although a PERMANOVA on the timed variables performed by mainland lizards revealed a Treatment effect ($F_{4,139} = 2.94$, $P = 0.004$) besides an effect of the Cumulative variable ($F_{1,139} = 5.90$, $P = 0.003$), post-hoc pairwise comparisons with a Bonferroni correction revealed no behavioural differences when comparing responses to scent of the eastern Montpellier snake with the negative control situation ($F_{1,139} = 3.07$, $P = 0.18$).

Discussion

Our observations show that mainland Dalmatian wall lizards can detect chemical cues of both native saurophagous snakes and introduced mongooses, and recognise them as dangerous. That lizards can identify odours of predatory snakes is unsurprising (Kats and Dill 1998; Mason and Parker 2010), but the response towards mongoose chemicals is noteworthy for two reasons. First, relatively few studies have documented that lizards can detect and recognise mammalian scents (Weldon 1990; Kats and Dill 1998; Monks et al. 2019). In a notable exception, Webster et al. (2018) recently showed that Boulenger's skink (*Morethia boulengeri*) and southern marbled geckos (*Christinus marmoratus*) reduce their foraging activity in the presence of scent deposited by native quoll (*Dasyurus viverrinus*) and dingo (*Canis lupus dingo*). Second, the mongoose has been introduced into the lizards' habitat less than a century ago (Tvrtković and Kryštufek 1990; Barun et al. 2008), making it a relatively new threat. How fast lizards can acquire the ability to chemically recognise newly introduced snake predators is debated (Mencía et al. 2017; Ortega et al. 2017; see Introduction). Only a handful of studies have investigated whether lizards can develop the means to process chemical cues of introduced mammalian predators, and their results are disparate. Webster et al.

(2018) found that olfactory cues of red foxes (*Vulpes vulpes*) and domestic cats (*Felis catus*), that invaded their study area about 150 years ago, elicited the same anti-predatory behaviour in Boulenger's skinks and southern marbled geckos as did cues from native predators. New Caledonian skinks (*Coledoniscincus austrocaledonicus*) avoid refuges scented by Pacific rats (*Rattus exculens*, introduced ~3000 years ago), ship rats (*Rattus rattus*, 150 years ago) and feral cats (150 years ago); but Caledonian geckos (*Bavayia septuiclavis*) are only repelled by Pacific rat cues (Gérard et al. 2014). Monks et al. (2019) found no evidence that New Zealand skinks (*Oligosoma polysoma* and *O. infrapunctatum*) and geckos (*Woodworthia maculata* and *Naultinus manukanus*) had learned to recognise olfactory cues of ship rats that became established in the area after 1860. The only conclusion that can be drawn from these contrasting results is that some lizard taxa easily develop the ability to perceive ominous mammalian odours, while others do not. Our results, which to our knowledge are the first on lacertid lizards, indicate that Dalmatian wall lizards fall in the first category.

Why some lizard species have learned to respond to the scent of alien mammals (and other species have not) remains an open question. First, aspects of the prey species' ecology could be important: lizards that run a higher risk of being predated, or that rely strongly on olfaction for predator avoidance, may be more likely to quickly evolve or learn to recognise the odours of new predators (Gérard et al. 2014). We have no exact measures of mortality due to predation in Dalmatian wall lizards, but the species is an active hunter and often basks in exposed areas; it is therefore a likely target of many predators. Dalmatian wall lizards tongue-flick frequently while foraging and during encounters with conspecifics and predators and thus can be classified as chemically-oriented. Second, time since introduction has been considered an element in loss of naivety (Banks and Dickman 2007; Gérard et al. 2014). Indian mongooses have now coexisted with mainland Dalmatian wall lizards for about 100 years (~50 generations). At least for some species of lizards, this seems sufficient to acquire an aversion for predator chemical cues (Ortega et al. 2017; Webster et al. 2018); but see Gérard et al. 2016; Monks et al. 2019). Note that at present we do not know whether the chemical recognition of particular scents is passed on from one generation to another (which would probably require fast genetic adaptation or maternal effects) (Bourdeau et al. 2013), or whether each generation of Dalmatian wall lizards needs to learn which cues indicate danger (Griffin 2004; Hollis et al. 2017). Previous

research on other lacertid lizards suggests that chemical predator recognition is largely innate (Van Damme et al. 1995; Martín et al. 2015); see also Mori and Hasegawa 1999; Downes and Adams 2001), but this research used long-established predator–prey models; whether naïve lizards can also recognise the cues of recently arrived predators has never been tested. The level of 'eco-evolutionary experience' (Saul et al. 2013; Gérard et al. 2016) is considered a third factor that might influence the likelihood of prey acquiring a defence against new predators. According to this principle, prey would more rapidly respond to new predator cues if they are already familiar with the predator's 'archetype' (Cox and Lima 2006). In our study system, mainland Dalmatian wall lizards have been predated upon for centuries by other mammals, such as beech martens (*Martes foina*), red foxes and feral cats (Serafini and Lovari 1993; Bertolino and Dore 1995; Sheng et al. 2011; Ferrero et al. 2011; Lanszki et al. 2016), which might share features of their scent with Indian mongooses. Interestingly, Gérard et al. (2016) found that New Caledonian skinks do not recognise scent of the unfamiliar Indian mongoose as dangerous, although they have been exposed to feral cats for over 150 years. The authors conclude that cats and mongooses do not belong to the same predator 'archetype' (despite both being small, carnivorous mammals), and attribute this to the distant divergence time between the two species (~37 Mya). If phylogenetic relatedness genuinely predicts scent similarity, it seems unlikely that familiarity with beech martens or red foxes would help Dalmatian wall lizards recognise mongooses, since both have diverged even longer ago from mongooses (~54 Mya) (see also Carthey et al. 2017). However, perhaps similarity in scent reflects likeness of diet, rather than phylogenetic relatedness (Nolte et al. 1994; Wallace and Rosen 2000; Ferrero et al. 2011; Pereira and Moita 2016).

In sharp contrast to their mainland conspecifics, Dalmatian wall lizards sampled from island populations did not exhibit increased Tongue flick rates or stress behaviours when confronted with chemical predator cues. This result was somewhat anticipated for lizards of Mali Barjak and Brusnik: these tiny islands are free of both snake and mammalian predators and thus the gradual loss of predator recognition or anti- predatory behaviour could be expected (Kats and Dill 1998; Blumstein 2002). However, Balkan whip snakes and other saurophagous snakes thrive on the larger islands of Vis and Korčula, as do several generalist mammalian predators (Table 1). Moreover, the small Indian mongoose was introduced onto Korčula around the 1920s and is still present on the island at high densities (Barun

et al. 2011a). The lack of response towards predator chemical cues in lizards from Vis and Korčula was therefore surprising. One possible explanation is that despite the presence of some ophidian and mammalian predator species, the islands are safe compared to the mainland. This could be due to the relative poorness of island predator communities (Blumstein 2002; Lawlor et al. 2002; Sarà and Morand 2002; Brock et al. 2015); see Table 1 for our study system) or because insular predator populations tend to be less dense than mainland populations (Oksanen and Oksanen 2000; Labra and Niemeyer 2004; Kryštufek and Kletečki 2007; Hollings et al. 2015). Lizard-specialists, such as smooth snakes (*Coronella austriaca*) and Dahl's whip snakes (*Platyceps najadum*), occur on the mainland but are lacking from all the islands in our study system (Jelić et al. 2009); perhaps they are the prime drivers of chemosensory predator recognition in Dalmatian wall lizards. An alternative explanation for the lack of response to predator chemical cues in our island lizards is that insular environments select for reduced chemosensory sensation (Durand et al. 2012; Monks et al. 2019). This idea is corroborated by the fact that lizards from islands in our study exhibited relatively low Tongue flick rates (compared to mainland lizards), in all experimental treatments. Lizards on islands must typically cope with poor or strongly fluctuating dietary resources (Brown and Perez-Mellado 1994; Sagonas et al. 2015) and have evolved behavioural, morphological and physiological adaptations to do so (e.g. Brown and Perez-Mellado 1994; Van Damme 1999; Pafilis et al. 2007). In particular, island populations of several lacertid lizards have evolved longer and more elaborated gastrointestinal tracts that allow them to digest food more efficiently (Pafilis et al. 2007; Herrel et al. 2008; Vervust et al. 2010; Sagonas et al. 2015). However, according to the expensive tissue hypothesis (Aiello and Wheeler 1995), investment in expensive gut tissue might well come at the expense of other expensive tissue, such as the brain. This constraint on the central nervous system can lead to less developed senses (Niven and Laughlin 2008). We are currently performing brain scans on specimens of Dalmatian wall lizards collected from Croatian landbridge islands and mainland locations to test this hypothesis. Alternatively, the overall reduction in chemosensory activity observed in island lizards could be sparked by a dearth of 'chemical challenges' (Lahti et al. 2009). For instance, in the simplified prey community of islands, distinguishing between palatable and non-palatable prey species may not require investing in expensive chemosensory equipment. Islands also tend to house much denser populations of

lizards (Novosolov et al. 2016), which may facilitate mate finding to an extent that chemosensory cues are no longer required.

In conclusion, the good news emerging from our observations is that mainland Dalmatian lizards have acquired the ability to recognise an introduced mammalian predator within an ecological time frame. The bad news is that island populations of the same lizard species do not respond to predator chemicals. Whatever the exact cause of the diminished chemosensory faculty in island lizards, it is likely to jeopardise their survival when faced with alien predators. This should be taken in consideration during management practices.

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Chapter 4

Insularity reduces lizard vomerolfactory activity and the volume of the brain regions that support it

Van Moorleghem, C., Goyens, J., Herrel, A., Tadić, Z., Huyghe, K., Van Damme, R. Manuscript in preparation. Insularity reduces lizard vomerolfactory activity and the volume of the brain regions that support it

Abstract

In response to the peculiarities of insular environmental settings, island-dwelling animals tend to depart from their mainland conspecifics in diverse aspects of their biology. For instance, island tameness, in which island animals lose their wariness of potential predators, is thought to reflect reduced predatory risk and/or the limited availability of resources on insular environments. Such tameness may be the result of the dulling of senses in insular animals. Whether insularity evokes sensory deprivation has rarely been investigated. Here, we provide evidence that individuals of a lizard species (*Podarcis melisellensis*) living on islands exhibit reduced vomerolingual sampling, i.e. they tongue-flick less than their mainland conspecifics when exploring new environments. In lacertids, tongue flicking behaviour is indicative for chemosensorial activity. Remarkably, reductions occur on islands of different type (*De novo* or landbridge island) and size (from 0.01 to 90 km²), constituting varying ecological landscapes (none to several terrestrial predator and competitor species). Furthermore, analyses of µCT scans show that island specimens also have reduced accessory olfactory bulbs (AOBs) compared to their mainland conspecifics. Therefore, 'ecological naiveté' of island-dwelling animals may result from morphological reorganisation at the level of the brain. Given the high importance of the chemical senses in the ecology of squamate reptiles, chemosensory deprivation on islands may lead to an elevated vulnerability towards ecological disruption.

Introduction

Islands comprise special environments that may stimulate extraordinary trait development in their inhabitants (MacArthur and Wilson 1967; Clegg 2010). First, island ecosystems tend to harbour fewer species (MacArthur and Wilson 1967). This results in fewer interspecific interactions and the relaxation of natural selection acting on the traits involved in such interactions. For instance, the paucity of (larger) predators in island ecosystems (Buckley and Jetz 2007) has prompted the loss of wariness in many insular prey species, a phenomenon referred to as 'island tameness' (Darwin 1839). Island animals experimentally confronted with potential predators typically fail to recognise the danger and do not react at all, or respond inadequately (e.g. Humphrey et al. 1987; Blázquez et al. 1997; Cooper et al. 2014). Second, resulting from the relative rarity of heterospecific competitors on islands, density compensation may occur in insular populations. Because of the ecological release from heterospecifics, island populations attain much higher densities than conspecific or related populations on the mainland (Buckley and Jetz 2007). At least in some species, the unusually high numbers of conspecifics may disrupt normal social behaviour and structuring, including the abandoning of territoriality if defending home ranges becomes uneconomic (e.g. Evans 1951; Gray and Hurst 1998; Taborsky and Taborsky 1999). Third, in comparison with mainland habitats, islands tend to be poor in dietary resources (Terborgh 2010). This is certainly the case for mesopredators, like lizards, that prey primarily on terrestrial arthropods (Taverne et al. 2019). In contrast to lizards, arthropod populations generally do not exhibit density compensation. This results in a significant reduction of the total amount of prey biomass available per mesopredator (Olesen and Jordano 2002; Olesen and Valido 2003). Furthermore, the diversity of arthropods on islands is typically smaller than on the mainland due to geographic isolation and the structural simplicity of island habitats (Janzen 1973; Gillespie and Roderick 2002). This may force mesopredators to forage on the available prey species which may be of low biomass or energetic quality (Pérez-Mellado and Corti 1993; Pérez-Mellado et al. 2008).

Relatively free from heterospecific predators and competitors, but at the same time having to cope with limited resources, island animals can be expected to downsize expensive brain tissue in favour of digestive tissue (the brain-guts hypothesis, Aiello and Wheeler 1995). Accordingly, in endothermic animals

(mammals and birds), several studies have documented island-driven reduction in overall brain size (Mace and Eisenberg 1982; Weston and Lister 2009; Csiki-Sava et al. 2018; Sayol et al. 2018) or a reorganisation of neural substructures (Köhler and Moyà-Solà 2004; Csiki-Sava et al. 2018). In particular, because the variety in ecological interactions is often considerably reduced, sensory organs (and the neural circuits that support them) seem unnecessary luxury in island environments and thus prone to economisation (Niven and Laughlin 2008). For instance, the brain of *Myotragus balearicus*, an extinct goat-antelope that used to live on the islands of Majorca and Menorca, was characterised by a reduced visual cortex and unusually small olfactory bulbs (Dechaseaux 1961; Köhler and Moyà-Solà 2004). However, there are also cases that seem to contradict such pattern. For example, the kogaionid *Litovoi tholocephalos*, an extinct inhabitant of Madagascar, is thought to have had exceptionally keen senses in an insular environment, as deduced from its neural anatomy (Csiki-Sava et al. 2018). In this specific case, the effect of insularity may have been overridden by a strong selective pressure for the preservation of particular senses resulting from this species' nocturnal lifestyle. This is supported by the observed strong reduction in overall brain size of *Litovoi*.

Evidence of sensory deprivation in extant insular animals is rare. Nevertheless, knowledge on whether the economisation on animals' sensory systems is a recurring event on many types of islands and what drives such trends would be extremely useful, for instance, in assessing the vulnerability of insular populations to the introduction of alien species. Increased investment in digestive tissue has been documented in several island lizards (Pafilis et al. 2007; Herrel et al. 2008; Vervust et al. 2010; Sagonas et al. 2015). Moreover, in an earlier study (Van Moorleghem et al. 2020/Chapter 3), we observed that insular populations of the Dalmatian wall lizard (*Podarcis melisellensis*) fail to detect odour cues derived from both native and introduced predators while their mainland counterparts were perfectly able to do so. Whether these observations were indeed the consequence of an overall deterioration of the chemosensory systems due to insularity requires formal testing. Here, we further investigate behavioural, as well as, neurological features of the chemosensory organ in a mainland population and three island populations of Dalmatian wall lizards.

Material & Methods

Study species

The Dalmatian wall lizard has a wide Mediterranean and sub-Mediterranean distributional range, stretching from northeastern Italy to western Albania on the mainland, but also including many islands in the Adriatic (Ajtic et al. 2009). As other lacertids, this lizard relies on its chemical senses when searching for food and mates, or when evading predators (Van Damme and Quick 2001; Huyghe et al. 2012; Pérez-Cembranos et al. 2018; Van Moorleghem et al. 2020). Dalmatian wall lizards were caught by noose in May 2017 on the Croatian mainland near the Cetina river in the village of Bajagić, which is part of the Sinj municipality (15 ♂, 12 ♀), and on the islets of Mali Barjak (12 ♂, 13 ♀) and Brusnik (12 ♂, 16 ♀), and the island of Vis (11 ♂, 9 ♀). The islands were selected to represent a varied set of insular systems: 1) a *De novo* island of volcanic origin, colonised by lizards after formation (Brusnik), and 2) two landbridge islands on which communities are fragments of the adjacent mainland (Vis and Mali Barjak). On the islets of Brusnik and Mali Barjak, Dalmatian wall lizards have no terrestrial predators. On Brusnik, we found a gecko (*Hemidactylus turcicus*; pers. obs.). However, we do not expect this nocturnal gecko to form a significant competitor to the Dalmatian wall lizard, as is also evidenced by the latter's extreme density on the islet. On the larger island of Vis, lizards experience interspecific competition and predation (Kryštufek and Kletečki 2007). Our samples contained only adult individuals having an average snout-vent length (SVL) of 58.3 mm (± 0.5 mm SE) (Brecko et al. 2008). Lizards were transported in cloth bags to our field station in Komiža on the island of Vis. Individuals from the same population were kept separately in a nylon mesh portable terrarium (Exo Terra) of 122 cm x 76 cm x 42 cm (length x width x height), which we enriched with stones, branches and bark. Two 60-Watt incandescent lamps were suspended at one side of each terrarium to provide an optimal temperature gradient. The lamps were switched on for 14 hours each day, mimicking the natural light-dark regime for our study sites during the month of May. Clean water was available at libitum in stone bowls and environmental humidity was kept relatively high by daily vaporising water inside the terrariums. The lizards were given mealworms (*Tenebrio molitor*) each day as a food source. They were set free, within a week's time, at the exact site of capture.

Behavioural observations

In many squamates, chemical particles are sampled from the environment and delivered to the vomeronasal organ (VNO) by the tongue. The characteristic lingual movements that are associated with chemical sampling in these animals are called 'Tongue flicks', and the number of tongue flicks per unit time is a convenient index of the level of the animal's chemosensory activity (Burghardt 1967). We noted the number of Tongue flicks and the time spent moving around (Walking) for individual lizards during a ten minute time period in a test arena of 50 × 40 × 40 cm (l × w × h). The test arena had opaque walls except for one transparent side which we coated with a dark window film (norauto) to enable observing the lizard without disturbing it. Before each observation the test arena was cleaned with ethanol (Bauwens et al. 1987) and the bottom covered with fresh sand to avoid scent contamination by a previous lizard. A 60-Watt incandescent lamp was suspended above the test arena to stimulate lizard activity. Before each observation, we measured the focal lizard's body temperature (cloacal) to be sure it approximated the preferred temperature of the species (Huyghe et al. 2007). The individual was then placed in the middle of the test arena and the behavioural observation was started. Behaviours were scored using JWatcher v1.0 (Blumstein and Daniel 2007).

Olfactory bulb measurements

Squamates are equipped with two olfactory systems: 1) the main olfactory system, working through the chemosensory epithelia in the nose with nerve fibers projecting to the Main Olfactory Bulb (MOB), and 2) the vomerolfactory system, of which the sensors are situated in a cavity in the roof of the mouth (also called the Jacobson organ) and signal to the Accessory Olfactory Bulb (AOB) (fig. 1). According to the principle of proper mass (Jerison 1973), the size of a neural structure is proportional to the complexity of the behaviours that it serves (Wylie et al. 2015). The size of the MOB and AOB should, therefore, reflect how strongly particular lizards rely on each chemosensory subsystem for sampling their chemical environment (Halpern 1992; Font et al. 2012a).

Male specimens from the islet of Mali Barjak (5) and the Sinj mainland area (5), preserved in 70 % ethanol, were acquired from the private collection of A. Herrel (Muséum National d'Histoire Naturelle in Paris, France). These lizards had been collected during the first two weeks of September 2016. Upon capture by

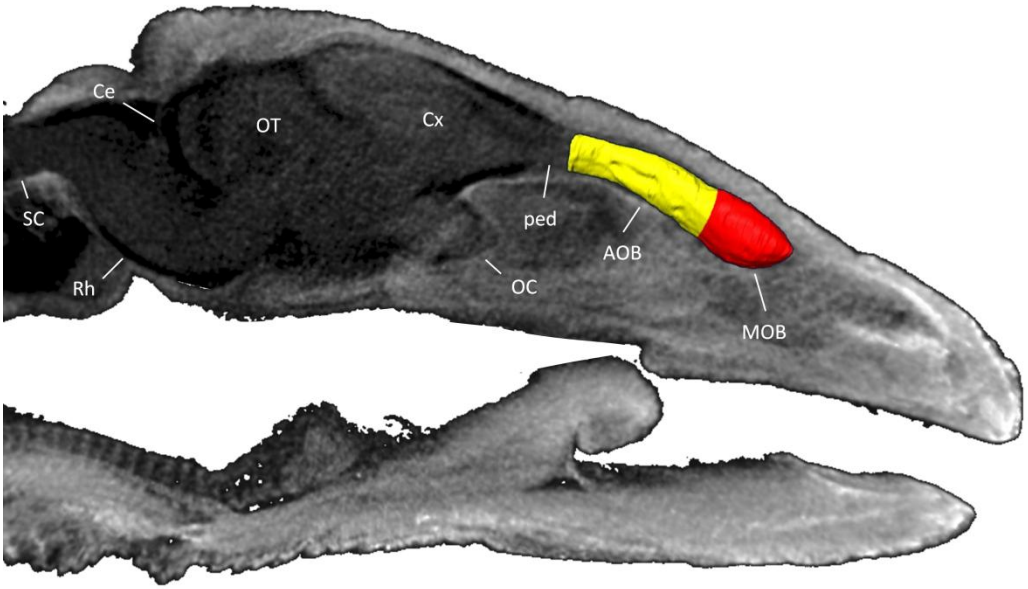


Figure 1 Median plane µCT image of the head of the Dalmatian wall lizard with volumetric rendering of the right olfactory bulb. Abbreviations of broad brain structures are indicated. SC: spinal cord, Rh: rhombencephalon, Ce: cerebellum, OT: optic tectum, Cx: cerebral cortex, OC: optic chiasm, ped: olfactory peduncle, AOB: accessory olfactory bulb (yellow), MOB: main olfactory bulb (red)

noose, measurements of Body mass and SVL had been taken. Subsequently, lizards had been euthanised according to European ethical guidelines and regulations through an intramuscular injection with 0.3 mL pentobarbital. Whole specimens were stained in 2.5 % phosphotungstic acid (PTA, Merck, Darmstadt, Germany) solution in 70 % ethanol for four weeks. We mounted each specimen separately in a Plexiglas container using a 0.8 % agar solution. The samples were scanned with a Skyscan 1172 high resolution µCT scanner (Bruker microCT, Kontich, Belgium), using a source voltage of 70 kV, a source current of 140 µA, a sample rotation angle of 0.65°, an aluminum filter of 0.5 mm thickness, a camera exposure time of 650 ms, and a frame averaging of 5. The scans lasted 24 minutes and resulted in a pixel size of 14 µm.

Next, we imported the scans in the 3D image manipulation software Amira (Amira 5.4.4; 64-bit version, FEI, Hillsboro, OR, USA). We selected the voxels belonging to the right olfactory bulb of each individual using a combination of automatic thresholding, based on the greyscale value, and manual corrections in the three orthogonal views. Similar to Sampedro et al. (2008), we used a

constriction in cross-sectional areas along the olfactory bulb to visually separate the rostral, ellipsoid MOB from the caudal, elongated AOB. The AOB, in turn, is delimited caudally by a transversal constriction at the beginning of the olfactory peduncles. Hence, a 3D surface model of the olfactory bulbs was created (Fig. 1). We extracted the volume of this model for each specimen in order to perform statistical analyses. Brain length was defined as the distance comprised between the caudal boundary of the AOB and the posterior edge of the cerebellum (Sampedro et al. 2008).

Statistical analyses

We ran linear models using R version 3.3.0 (R Core Team 2016) to analyse the data from behavioural observations and olfactory bulb measurements. We made sure assumptions regarding linearity, normality of residuals and homoscedasticity were met when running both behavioural and brain models.

Population of origin, Sex and the duration of Walking were used as explanatory variables in candidate models describing Tongue flick rates. Walking was included because lizards tend to perform more chemosensory sampling when walking around, and our focus is on assessing variation in chemosensory behaviour rather than a lizard's state of activity. Full models were further supplemented with the interactions between explanatory variables.

To meet the requirement of normality, we took the reciprocal of the third-root of MOB measurements. The measurements of the AOBs were squared. Population of origin, total Brain length and Body condition were introduced as explanatory variables in the candidate models of MOB and AOB volumes. We included Brain length as a fixed effect in the full model to resolve potential relative differences in olfactory bulb volumes (see e.g. Sampedro et al. 2008). The variable 'Body condition' was created by taking the residuals of a regression between the natural logarithm of Body mass on the natural logarithm of SVL (Bonnet and Naulleau 1994; Schulte-Hostedde et al. 2005). In the final full model, we included all possible interactions.

We selected the best-fitting models for both Tongue-flick data and volumetric measurements of olfactory bulbs by applying the information theory-based method described in Symonds and Moussalli (2011). This method compares candidate models using the second-order Akaike Information Criterion (AIC_c) and builds a 99.9 % confidence set (Burnham and Anderson 2002). The AICcmodavg package was used to this purpose (Mazerolle 2016). From the confidence set we

extracted the relative importance of each explanatory variable by summing the Akaike weights (w_i) of the models that include the variable (= the predictor weight). Based on a model including only fixed effects with predictor weights greater than 0.9, post-hoc comparisons with a Bonferroni correction were done to assess pairwise differences between levels of explanatory variables both in tongue flick rates and volumes of brain areas regulating olfaction.

Results

Behavioural observations

In the models describing variation in tongue flick rates, the interaction between Population and Walking had the highest predictor weight (= 0.97; Table S1). This combination of fixed effects explained 64 % (R^2) of the total variation among individuals. As expected, lizards with high walking durations performed more tongue flicks. The relationship was steeper in mainland lizards (Sinj, slope = 1.15) than in island lizards (Brusnik, slope = 0.49; Mali Barjak, slope = 0.66; Vis, slope = 0.56). Additionally, regardless of their activity level, lizards on the mainland performed considerably more tongue flicks than lizards on the islands (Sinj vs. Mali Barjak: t_{77} = 5.24, P < 0.0001; Sinj vs. Brusnik: t_{77} = 3.70, P = 0.0024; Sinj vs. Vis: t_{77} = 5.127, P < 0.0001; Fig. 2). Lizards from the three island populations did not differ in tongue flick rates (all P = 1.00). Sex had a predictor weight of 0.40 and is, therefore, not likely to drive variation in the number of tongue flicks.

Olfactory bulb measurements

Lizards from the island Mali Barjak and the Mainland (Sinj) did not differ in their average SVL (t_8 = -0.82, P = 0.44), Body condition (t_8 = 1.33, P = 0.22) or Brain length (t_8 = 0.62, P = 0.55). However, AOB volume was strongly affected by Population (predictor weight = 0.98; R^2 = 0.74). Post-hoc comparisons revealed that lizards from the mainland Sinj area have significantly larger AOBs than lizards from Mali Barjak (t_8 =4.83, P = 0.0013; Fig. 3b). No (interaction with a) morphometric variable had a predictor weight above 0.33 (Table S2). None of the explanatory variables explained a noteworthy amount of variation in MOB volume (all predictor weights < 0.25; Fig. 3a; Table S3). The null model was ranked first in a 99.9 % confidence set with an Akaike weight of 0.54.

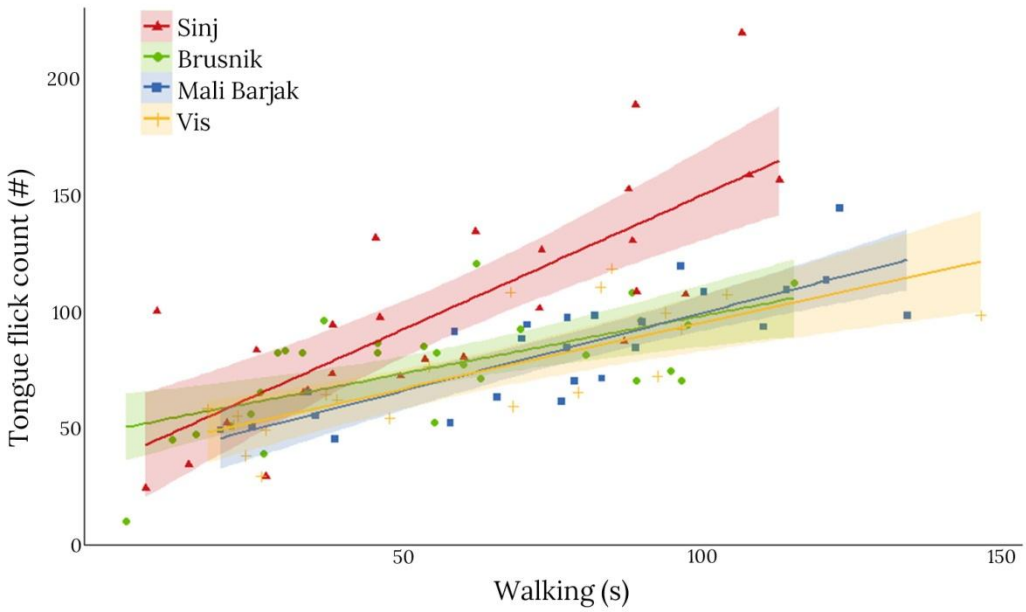


Figure 2 Scatterplot illustrating the relationship between the number of Tongue flicks performed by Dalmatian wall lizards belonging to different populations and the time they spend Walking (in seconds) during 10-minute trials

Discussion

We find that Dalmatian wall lizards from island populations have reduced tongue flick rates and smaller accessory olfactory bulbs (the latter at least in Mali Barjak individuals) than conspecifics on the mainland. In other words, they seem to rely less on vomerolfaction and to have reduced investment in the neural tissue supporting it. This reduction occurs on islands of different type (*De novo* or landbridge island) and size (from 0.01 to 90 km²), and which constitute varying ecological landscapes (none to several terrestrial predator and competitor species, and potentially variable resources). The differences between mainland and island lizards were not due to differences in body size, condition or overall brain size. Rather, it indicates an island-characteristic shift in the balance between the benefits and costs of using the VNO. Which aspects of insularity would evoke such a shift is less clear.

A first possible explanation is that in island environments, the chemical cues that can be picked up by the VNO have become redundant, or at least less relevant. Lizards of the genus *Podarcis* use vomerolfaction in predator detection

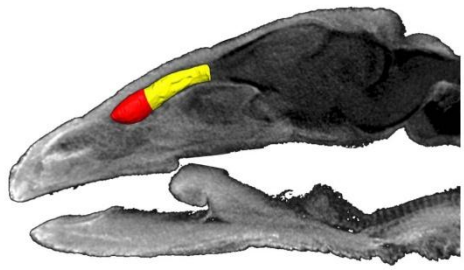
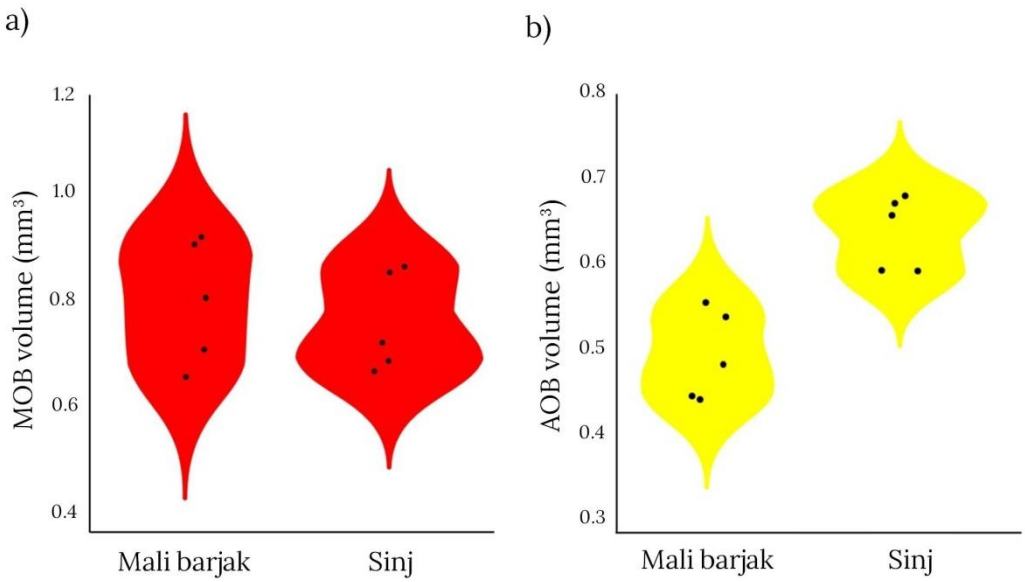


Figure 3 Violin plots of individual volumes of a) the Main Olfactory bulbs (MOB; red) and b) the Accessory Olfactory Bulbs (AOB; yellow) of Dalmatian wall lizards originating from a mainland (Sinj) and an island (Mali Barjak) population. The inset on the right illustrates the position of these two brain regions in the head of the lizard (see Figure 1 for further details).



(Van Damme and Quick 2001; Downes and Bauwens 2002; Cerini et al. 2020; Van Moorleghem et al. 2020/Chapter 3), species recognition (Barbosa et al. 2005, 2006), prey detection (Cooper 1991) and in intraspecific interactions such as territorial scent marking (Edsman 1990; Font et al. 2012a), sex recognition (López and Martín 2001), mate choice (Carazo et al. 2011) and rival assessment (Carazo et al. 2007). Changes in the relevance of any of these functions, or in the role that chemicals play in those functions, could result in relaxed selection on vomerolfactory abilities. This would allow neutral evolutionary processes to act on the VNO (Futuyma 2010); deleterious mutations can accumulate in the genetic domains that build the organ, and drift may erode the genetic architecture supporting chemosensation. the latter effect can be strengthened even more by the occurrence of genetic bottlenecks or founder effects. The lizard populations on landbridge islands Vis and Mali Barjak are fragments of a formerly contiguous

land mass which became separated due to rising sea levels after the most recent glacial period (Wurm, 18,000 ya; Podnar et al. 2004). Therefore, lizard populations on these islands might be considered going through a sustained genetic bottleneck whose magnitude is a function of island area and whose duration is a function of island age (Hurston et al. 2009). Brusnik was colonised by Dalmatian wall lizards in more recent times. As a result, lizards from Brusnik might descend from colonisers which may, by chance, have been chemosensory deprived. Although, an earlier study on *P. melisellensis* in the Adriatic did not find conclusive evidence that bottleneck events severely impact the genetics of our studied populations (Gorman et al. 1975).

Absence of or low predation pressure on islands seems a likely reason for economising on vomerolfactory behaviour and equipment (Monk and Paulin 2014). Indeed, a few studies have associated sensory deprivation on islands with relaxed predation. The ears of noctuid moths endemic to Tahiti, for instance, have become functionally vestigial due to the absence of bats (Fullard 1994). Flightless birds that evolved on predator-poor islands, such as the elephant birds of Madagascar and the kiwi kakapo and moas of New Zealand, tend to have decreased visual capacity (Torres and Clarke 2018). Our observations provide mixed evidence for the importance of predator relaxation. Vomerolfactory activity in lizards from Mali Barjak and Brusnik is low (compared to the mainland), as would be expected on the basis of the complete absence of terrestrial predators on these islets. However, lizards of Vis exhibit equally low tongue flick rates, while this larger island harbours a relatively rich predator community of saurophagous snakes (Balkan whip snake, *Hierophis gemonensis*; cat snake, *Telescopus fallax*; four-lined snake, *Elaphe quatuorlineata*) and mammals (rat, *Rattus rattus*; cat, *Felis catus*) that may feed opportunistically on lizards (Kryštufek and Kletečki 2007; see also Table 1 in Van Moorleghem et al. 2020/Chapter 3). In a previous study, we documented how lizards from Vis fail to react to kairomones of the Balkan whip snake, where conspecifics from the mainland mount a typical stress response (Van Moorleghem et al. 2020/Chapter 3). The apparent indifference of lizards from the Vis population towards (predator) chemical cues is puzzling. Observations on lizards from a larger series of islands, with varied predator communities, are required to properly test the role of predator relaxation in chemosensory deprivation.

Living on islands may also relax the need for a competent species recognition system. Signal intensity (Doutrelant et al. 2016) and complexity (Ord

and Martins 2006; Vanhooydonck et al. 2009; Morinay et al. 2013) tends to be lower on islands and other habitats with few closely related sympatric species. For instance, island birds tend to have duller and simpler colouration patterns (Doutrelant et al. 2016) and songs (Morinay et al. 2013) than mainland relatives. If signals become less pertinent in insular environments, then so might the systems to perceive and interpret them. The Dalmatian wall lizard is the only lacertid lizard on the islets, but we have repeatedly seen Mediterranean house geckos (*Hemidactylus turcicus*) on Brusnik. Given the phylogenetic distance and the strong difference in morphology and activity between the species, it seems unlikely that *P. meliselensis* would require an elaborate species recognition system to avoid mistaken interactions with this gecko species. The island of Vis harbours a considerable population of sharp-snouted rock lizards (*Dalmatolacerta oxycephala*), but this lacertid also differs considerably in microhabitat use and coloration (Lailvaux et al. 2012). On the mainland, the Dalmatian wall lizard could come in contact with several other lacertids (Dalmatian algyroides, *Algyroides nigropunctatus*; Horvath's rock lizard, *Lacerta horvathi*; Mosor rock lizard, *Lacerta mosorensis*; sharp-snouted rock lizard, *Dalmatolacerta oxycephala*; tree-lined lizard, *Lacerta trilineata*; green lizard, *Lacerta viridis*), including several congenerics (common wall lizard, *Podarcis muralis*; Italian wall lizard, *Podarcis siculus*; Jelić et al. 2009). It would be worthwhile to observe how respective populations of Dalmatian wall lizards react to chemical cues produced by these species.

Insularity may also influence selective pressure on sensory systems through changes in the intensity of sexual selection. Although seldom addressed in lizards, general theory predicts lower fitness benefits of mate choice in islands, due to lower parasite pressure (Ishtiaq et al. 2012; Loiseau et al. 2017) and reduced genetic diversity (Frankham 1997). This might relax selection on the production of chemical signals that in lacertids convey information on male social status, condition and competitive ability (Aragón et al. 2001; López and Martín 2002; Carazo et al. 2007; Martín et al. 2007; Martín and López 2007; Gabirot et al. 2013), and on the neural structures required to detect and process such information. In addition, density compensation on islands may lead to such a high number of trespassing floaters (individuals that cannot acuire or maintain a territory) that territoriality becomes uneconomical (Stamps and Buechner 1985). If a territorial social structure is abandoned for a hierarchical system (as documented in

iguanids, Evans 1951, rodents, Gray and Hurst 1998, and flightless birds, Taborsky and Taborsky 1999), producing and detecting scent marks may become obsolete. Alas, we lack the necessary information on social organisation and chemical cue production required to check these ideas.

Many lacertid lizards, including members of the genus *Podarcis*, use chemical information to locate and identify arthropod or plant food items (Cooper 1991; Cooper et al. 2001; Cooper and Pérez-Mellado 2001; Cooper et al. 2002; Baeckens et al. 2017b) and to avoid toxic invertebrates (Gregorovičová and Černíková 2015). It could be argued that island lizards have no or less use of such abilities, because their menu is simplified due to the reduced arthropod richness on islands. Or they may have become less picky as a consequence of increased intraspecific dietary competition (Vicente et al. 1995; Sagonas et al. 2014; Runemark et al. 2015; Sagonas et al. 2015) and therefore no longer have discriminative powers. Indeed, Castilla et al. (2008) have reported the inclusion of venomous yellow scorpions (*Buthus occitanus*) into the diet of a wall lizard (*Podarcis atrata*) endemic to the Columbretes Islands. We suggest staging food preference and discrimination tests to insular and continental lizard populations to evaluate the likeliness of this scenario.

The explanations offered in the previous paragraphs assume that vomerolfaction has lost ecological relevance in island conditions, allowing deterioration of its genetic underpinnings (e.g., due to genetic drift). Alternatively, or in addition, investing in vomerolfaction could be selected against if the behaviour and the neural circuits supporting it are costly (as suggested by Niven and Laughlin 2008). Data on the diet of insular Dalmatian wall lizards shows instances of cannibalism and occasional consumption of vegetal matter and low-nutrient foods (e.g. Formicidae) on Brusnik, Mali Barjak and Vis (Gelineo and Gelineo 1963; Pérez-Mellado et al. 2008), indicating that all three islands are resource-limited. *Podarcis* lizards that are confronted with low-quality foods on islands have been shown to invest in a more efficient digestive system (Pafilis et al. 2007) which must be traded off with expenses in brain tissue (Aiello and Wheeler 1995). Alternatively, a rapid response to resource limitation on islands may occur through plasticity. The lizard brain stands out as one of the best examples of structural plasticity in vertebrates (Font et al. 2001). Although this is most apparent at the level of neuronal cell proliferation it might also apply to the overall

sizes of brain structures. The likeliness of this explanation for our results could easily be tested through common garden experiments.

The fact that island lizards in our study do not have smaller MOBs may seem at odds with the idea that low food availability is causing a reduction in neural circuitry. However, at least in mammals, the relative sizes of different brain components seem to have evolved quite independently (Barton and Harvey 2000), allowing species to economise on particular areas first. For instance, rapid mosaic evolution of the nasal and vomeronasal system has been described in Caribbean bats (Yohe et al. 2017; Yohe et al. 2018). We can only speculate why island lizards would economise on AOBs and not on MOBs. An interesting hypothesis on the relative roles of the main and accessory olfactory systems is that suggested by Cowles & Phelan in the 1950's. These authors proposed that nasal olfaction may be more of a 'quantitative' distance sensing system that iniates further investigation via the tongue-Jacobson's organ system (Cowles and Phelan 1958). Perhaps, a broadly tuned olfactory system provides a chemosensory solution to insular live. Some experimental evidence is available on the Cowles & Phelan hypothesis. For instance, a neuronal connection between motor centers that control the tongue musculature (i.e. the hypoglossal nucleus) and the medial amygdale, which receives direct and indirect vomeronasal and indirect olfactory inputs, reinforces the idea that both kinds of chemosensory information modulate tongue-flick rates (Martínez-Marcos et al. 2001). Furthermore, Duvall (1981) saw Cowles and Phelan's claimes substantiated by his observation that western fence lizards (*Sceloporus occidentalis*) responded with significantly shorter tongue flick latencies (i.e. the time until the first performed tongue flick) when any chemical cue other than distilled water was presented; however, only conspecifically (and not cologne) marked paper sheets elicited raised lingual investigation, suggesting that the vomeronasal system was capable of discriminating between chemical cues while the nasal system was not. Further investigation of the main olfactory organ's role in reptile ecology is necessary to assess whether MOB volumes remain the same on islands because 1) the organ is under similar selective pressure in these habitats compared to the mainland, 2) it was already at its functional minimum on the mainland, or 3) it compensates for the partial loss in functionality of the VNO (Yohe et al. 2018).

Sensory depletion is not necessarily disadvantageous to insular lizards. On the contrary, optimal energy-use in such systems may benefit lizard fitness; at

least, as long as insular conditions remain the same. However, recent decades have seen severe human-induced changes to both insular and mainland environments. An appropriate response to such changes may require the functionality of particular senses. For instance, a recent study showed that Dalmatian wall lizards from Vis, Mali Barjak and Brusnik do not respond to chemical cues from predators (Van Moorleghem et al. 2020). In contrast, lizards from the adjacent mainland do perform anti-predator behaviours which are accompanied by a rise in the number of Tongue flicks. As the latter indicates vomeronasal sampling (Filoramo and Schwenk 2009), this strongly suggests that the VNO steers chemosensory predator detection in these lizards. A behavioural and neurological deprivation of this sensory system (current study) may prevent chemical detection of the invasive small Indian mongoose (*Herpestes auropunctatus*), as seen on the neighbouring island Korčula (Van Moorleghem et al. 2020).

There is a disproportional decline in insular populations and the disappearance of island-dwelling endemics (Spatz et al. 2017). Concern is raised that the same insular conditions to which island-dwelling animals have adjusted, predispose them for extinction under rapid ecological and environmental change (Howard et al. 2020). Our results may have uncovered a mechanism through which this occurs. Therefore, we strongly encourage further investigations in other, widely divergent taxa to assess whether sensory deficiency is occurring more frequently on islands, and provokes heightened vulnerability to environmental change in island inhabitants.

Acknowledgements

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Part II

Chapter 5

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.

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: Jędrzejewski 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 2014), 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. 2000a), 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. (2000a). 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 and 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. 2012b); 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 randomised 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 generalised linear mixed-effect models (GLMM; lme4 package, Bates et al. 2015, 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

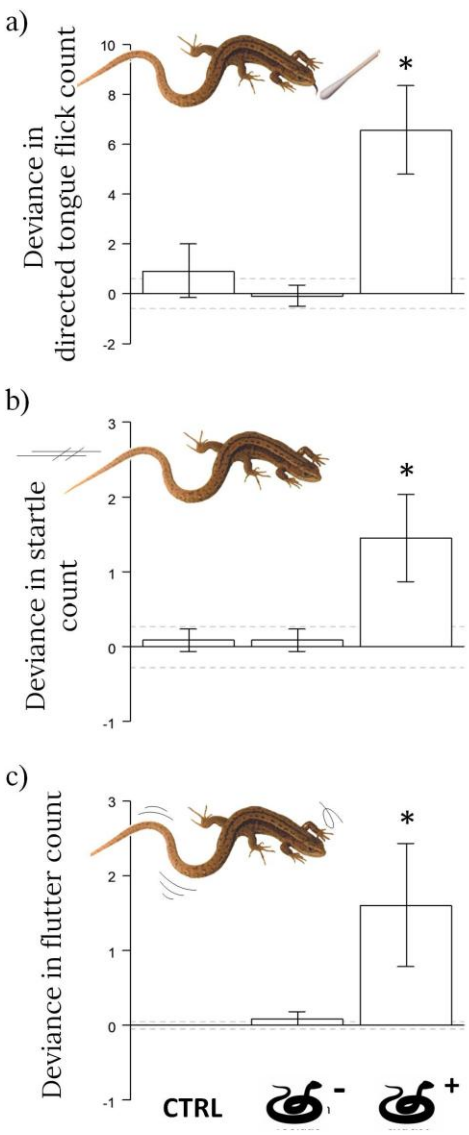

Figure 1 Deviance from the positive baseline control in counts of (a) Directed tongue flicks, (b) Startles, and (c) Flutters (i.e. Tail waving and Foot shakes) in Experiment A. The solid horizontal line represents the mean of the respective behaviour when the positive baseline control was offered, with 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.

balance: linoleic acid (Elias et al. 1980), long-chained alkanes (Lillywhite and 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.

Acknowledgements

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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], *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]

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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 ♂)	✓	✓	✓		✓	
38.6	Octadecanoic acid, ethyl ester *	0.01 ± 0.01	3 (1 ♀, 1 ♂)	✓	✓	✓		✓	
39.7	5,8,11,14-Eicosatetraenoic acid, methyl	+	2 (1 ♀, 1 ♂)	✓		✓			

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
	ester								
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 ♂)						

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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 ♂)	✓				✓	
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 ♂)						

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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]
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 ♂)						

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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 ♂)						
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 ♂)	✓				✓	

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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 ♂)	✓		✓		✓	
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]

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
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

RT	Compound	Proportion	*V. berus*	Ar	Am	Li	Sn	Ma	Snake genera
51.5	γ-Tocopherol *	+	1 (0 ♀, 1 ♂)	✓		✓			
52.0	D-α-Tocopherol *	0.01 ± 0.01	1 (0 ♀, 1 ♂)	✓		✓		✓	
WAXY ESTERS									
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 and Downing 1974; [14] Ball 2004; [15] Weldon et al. 1991; [16] Weldon et al. 1990; [17] Roberts and Lillywhite 1980; [18] Schell and 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	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

Chapter 6

Proton - transfer - reaction time – of - flight mass spectrometry (PTR-TOF-MS) as a tool for studying animal volatile organic compound (VOC) emissions

Portillo-Estrada, M., Van Moorleghem, C., Janssenswillen, S., Cooper, R. J., Birkemeyer, C., Roelants, K., Van Damme, R. Submitted. Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) as a tool for studying animal volatile organic compound (VOC) emissions

Abstract

Chemical sensing in vertebrates is crucial, and efforts are undertaken towards deciphering their chemical language. Volatile organic compounds (VOCs) are a group of chemicals believed to play an essential role in a wide variety of animal interactions. Therefore, understanding what animals sense and untangling the ecological role of their volatile cues can be accomplished by analysing VOC emissions. A Proton-Transfer Reaction Time-of-Flight Mass Spectrometer (PTR-TOF-MS) is an instrument that measures VOCs in real-time in an air sample. Since this technique acts as a hyper-sensitive 'nose', it has a similar potential in deciphering the chemical language of vertebrates.

Here, we validate the use of PTR-TOF-MS as a tool to help resolve vertebrate interactions through VOCs. The instrument monitors and records the full spectrum of emitted VOCs with a high accuracy and low detection limit, including transient VOC emissions. We propose and test diverse measuring configurations that allow for measurement of VOC emissions from different vertebrates and their exudates: full body, specific parts of the body, urine and femoral pores. In addition, we test configurations for detecting sudden and short-lasting VOC outburst, such as during adder skin shedding and upon mechanical and physiological stimulation of amphibia. Our configurations work in tandem with Gas Chromatography Mass Spectrometry (GC-MS) to allow compound structure verification.

We discuss the configurations and methodologies used and conclude with recommendations for further studies, such as the choice of chamber size and flow. We also report the results of the measurements on vertebrates —that are novel to science— and discuss their ecological meaning. We argue that PTR-TOF-MS has a high potential to resolve important unanswered questions in vertebrate chemical ecology. If combined with a structure verification tool, such as GC-MS, the creative deployment of PTR-TOF-MS in various future study designs will lead to the identification of ecologically relevant VOCs.

Introduction

Due to the technical difficulties accompanying the investigation of something as obscure as a chemical molecule, we have for long been unaware of the plethora of chemical messages going around in natural ecosystems. There are numerous proven situations in which animals interpret chemicals from the environment in a wide array of contexts, e.g., for vigilance against predators (Kats and Dill 1998), eavesdropping of potential prey (Conover 2007), when socially interacting among conspecifics (Wyatt 2010), or during host-finding by parasites (Chaisson and Hallem 2012). However, despite improvements in chemo-analytical methods, the true nature of informative compounds often remains unexplored. This is particularly the case in vertebrates that often send out highly complex blends of chemical compounds (Wyatt 2014). A better knowledge on the composition of chemical signals is key to attain a deeper understanding of the ecological interactions of animals. This will not only aid in fundamental research domains such as vertebrate chemical ecology, sensory ecology and ecology in general, but also in applied domains such as conservation biology (e.g. pheromone-mediated enhancement of captive breeding with endangered animals; Wilson et al. 2020) and bio-control (e.g. through the use of predator-derived kairomones as repellents of pests; Clarke et al. 2016; Sorensen and Johnson 2016).

Proton-Transfer Reaction Time-Of-Flight Mass Spectrometry (PTR-TOF-MS) (Graus et al. 2010) is a technique developed in the 2000s decade to measure volatile organic compounds (VOCs) in the air in real-time. It has been successfully used to control the quality and trace the origin of food and beverages (Deuscher et al. 2019), assessing toxic and polluting VOCs that impact human health (Huang et al. 2016), analysing biogeochemical fluxes from ecosystems (Portillo-Estrada et al. 2018) and their direct impact on atmosphere (Portillo-Estrada et al. 2020) and ozone formation (Zenone et al. 2016), in studies on plant (Portillo-Estrada et al. 2015) and lichen chemical ecophysiology (García-Plazaola et al. 2017), and to assess the pollutant-degrading potential of bacteria strains (Imperato et al. 2019). Since this technique acts as a hyper-sensitive 'nose', it has an equal potential in deciphering the chemical language of vertebrates. However, never before has PTR-TOF-MS been applied to this end.

Nowadays, more traditional methods combining compound separation with detection, like gas chromatography mass spectrometry (GC-MS) are believed to have more power than PTR-TOF-MS in resolving compound structures.

However, the results obtained by GC-MS VOC analysis strongly depend on the type of adsorbent that is used to sample the VOCs. Though recent developments in GC-MS such as solid phase microextraction (Brunetti et al. 2015) are suggested to improve the sampling technique, adsorbents for GC-MS are still likely to seize larger molecules (>C_3). Moreover, the offline coupling of sampling and analysis using a harsh ionization technique would prevent from detecting non-covalently linked scent molecules such as water clusters and similar assemblies. In addition, sampling for GC-MS analysis usually takes more than 10 minutes to yield a single chromatogram, making it suitable only for steady-state measurements or VOC emissions with a very slow dynamic. As behavioural interactions between animals are often short-lasting and unexpected, GC-MS on its own may not be capable of discriminating the informative chemicals. PTR-TOF-MS on the other hand monitors and records data in real-time at a high time resolution (up to 10 Hz) and with a low detection limit (tens of particles per trillion (ppt)). It uses a soft ionization method that avoids the fragmentation of long molecules (e.g. hydrocarbons, molecules with radicals, etc.) and it generates high-resolution spectra with a broad mass range (spectra of 1-500 Da with 0.0001 Da resolution). Furthermore, PTR-TOF-MS analyses does not require an intermediate step of chemical adherence to a cartridge (thermal desorption) or extraction fiber (SPME). Rather, it allows direct sampling of the animal's VOCs or, otherwise, the analysis of molecules derived from a field situation which have been sampled in a non-selective way using a hand pump and Teflon bag. It is an incredibly suitable tool for exploratory VOC emission analysis, because the whole spectrum of VOCs is recorded during the measurements and no decision on targeting any particular VOC (e.g. by choice of adsorbent) must be made beforehand.

To validate the implementation of PTR-TOF-MS in chemical ecology we design and test various configurations adapted to measure VOC emissions of various vertebrate animals during rest or during a dynamic process. These varying contexts of cue emission require different chamber setups, and benefit from real-time monitoring of VOC emissions. The tested configurations can be applied to address different ecological or behavioural research questions. They include the monitoring of: (1) VOCs released from the entire body of small vertebrates in rest: a predatory adder and prey lizard species that may detect each other's proximity by scent (Durand et al. 2012); (2) VOCs released by small vertebrates undergoing a natural process: adders shedding their skin, a process that is thought to discharge

volatile pheromones and triggers male-male competition (Andrén 1982); (3) VOCs released by small vertebrates undergoing an induced process with a dynamic response: poison secretion by amphibians after inducing physiological (hormone injection) or (4) mechanical stress (manual massage), a response that simulates an antipredator chemical defence mechanism (Toledo et al. 2011); (5) VOCs released by a specific signalling structure in the body of a small animal: the femoral pores of a lizard, known to be involved in intraspecific communication (Mayerl et al. 2015); (6) VOCs released by a specific body part of a large animal: the vulva of dogs, potentially releasing chemical information regarding the reproductive status (Dzięcioł et al. 2012); and (7) finally, we apply PTR-TOF-MS for assessing VOCs released from the exudates of a large animal, namely dog urine, potentially conveying individual information to conspecifics (Jezierski et al. 2019).

Some of the above measurements are complemented with thermal desorption GC-MS to verify compound structures and demonstrate the complementary use of both methods in experiments. These novel techniques provide unprecedented insight in the VOC emission of a wide range of vertebrates and could function as an adaptable method for future ecological research questions on specific behaviours, ecological conditions or animal body parts.

Material and methods

Analysis of VOCs with PTR-TOF-MS and thermal desorption GC-MS

The volatilome (the composition of a VOC blend) and the VOC emission rates of several experimental animals and their exudates (Table 1) were measured by sampling the air exiting from experimental chambers to a proton-transfer-reaction time-of-flight mass spectrometer (PTR-TOF-MS 8000, Ionicon Analytik GmbH, Innsbruck, Austria). The instrument's functioning, the analysis of spectra, calibration and calculation of concentrations are explained in detail in the Supplementary Information (S1). The volatilome of the adders and lizards was also measured with thermal desorption GC-MS with cartridges filled with Tenax TA. This allowed us to verify the compound structures measured by PTR-TOF-MS. More details are given in the Supplementary Information (S2). The data processing, calculation of VOC emission rates, integration of VOC emission peaks, and calculation of VOCs emitted in closed vials is found in the Supplementary Information (S3).

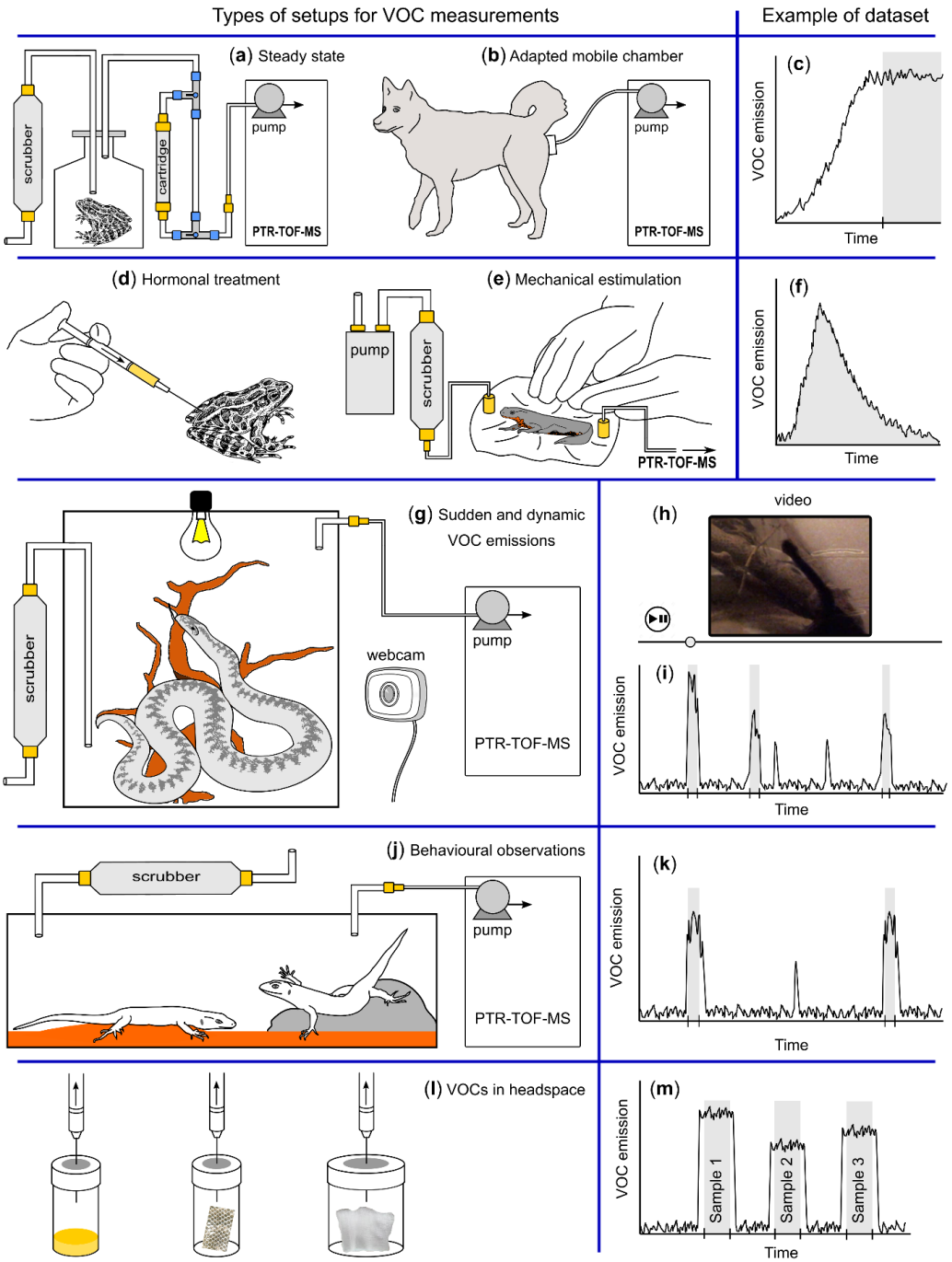
Table 1 List of species, anatomical characteristics (avg ± sd), and number of individuals used in each study. More information in the Supplementary Information (S.3.)

Species name	Family	Common name	Weight (g)	Snout-vent length (cm)	Experiment	Instrument (number of individuals)
Bombina orientalis (Boulenger, 1890)	Bombinatoridae	Fire-bellied toad	4.9 ± 0.7	4.06 ± 0.20	Skin secretions (norepinephrine)	PTR-TOF-MS (6)
Cynops pyrrhogaster (Boie, 1826)	Salamandridae	Fire belly newt	4.7 ± 0.9	5.66 ± 0.13	Skin secretions (mechanical)	PTR-TOF-MS (3)
Podarcis muralis (Laurenti, 1768)	Lacertidae	Common wall lizard	4.4 ± 0.8	6.20 ± 0.19	Full body scent	PTR-TOF-MS (3 ♂) + GC-MS (5 ♂)
Zootoca vivipara (Lichtenstein, 1823)	Lacertidae	Common lizard	2.97 ± 0.14	4.8 ± 0.9	Femoral pores scent	PTR-TOF-MS (3 ♂)
Vipera berus (Linnaeus, 1758)	Viperidae	European adder	35.0 ± 2.8	35.5 ± 2.1	Full body scent	PTR-TOF-MS (3) + GC-MS (2)
			38.7 ± 3.3	39.0 ± 4.2	Skin shedding	PTR-TOF-MS (4)
Canis familiaris (Linnaeus, 1758)	Canidae	Dog	Large differences		Urine VOCs	PTR-TOF-MS (6 ♀)

VOC sampling set-ups

Several configurations to measure VOCs in real-time adapted to different types of samples and animal sizes are explained below and summarised in a conceptual figure (Fig. 1).

Figure 1 (right) Scheme of diverse experimental setups to measure volatile organic compounds (VOCs) from animals and the type of results expected. (a) Setup for steady state measurements where a small animal (e.g. a frog) is enclosed in a glass bottle. The flow-through chamber is supplied with VOC-free air generated by passing room air through a scrubber (filled with activated carbon). The air is pulled through the chamber via a built-in pump coupled with a flow controller in the Proton-Transfer Reaction Time-of-Flight Mass Spectrometer (PTR-TOF-MS). Three-way stopcock valves control the flow direction: either directly to the PTR-TOF-MS or to pass a cartridge that traps VOCs for further analysis with a gas chromatography mass spectrometer (GC-MS). More information about the PTR-TOF-MS and the GC-MS is found in the Supplementary Information, S.1. and S.2., respectively. (b) Mobile chamber to enclose specific body parts or surfaces of large animals (e.g. a dog's vulva) and real-time monitoring of VOC emissions. In the setups of (a) and (b), the VOC concentrations recorded by the PTR-TOF-MS (c) are expected to increase to reach a steady level. The data (grey area) can be averaged to produce a single value. The setup in (a) can also be used to investigate VOC emission by a small animal after induction of physiological stress, e.g. through subcutaneous injection of a hormone in a toad to induce skin secretion (d). Alternatively, a stress response can be induced mechanically after inserting a small animal (e.g., a newt) into a PVDF (polyvinylidene difluoride) or Teflon bag. The bag is inflated by pumping in VOC-free air and when the VOC emissions are stable, the animal is massaged through the bag to provoke body secretions. The setups of (d) and (e) are expected to result into a (f) peak of VOC emission that can be integrated to calculate total VOCs emitted upon the application of stress.
A terrarium (g) can be used as an adapted version of the flow-through chamber (a) to monitor VOC emission during sudden and spontaneous natural processes like skin shedding in an adder. This setup allows the inclusion of elements to sustain a longer monitoring (e.g. lamp that controls photoperiod, water, wood sticks to promote skin shedding in adders). The animal can additionally be video-monitored to record its behaviour. The video (h) allows matching the VOC emission peaks (grey areas) (i) to specific shedding events. Similarly, (j, k) other configurations can be recreated to promote behaviours in other animals, like the courtship in lizards.
The VOCs emitted by body exudates (l) like urine, skin, or scented gauzes can be measured by enclosing the sample in a glass jar equipped with a Teflon septum. The sample is kept for a period of time inside the jar at a stable temperature to promote the steady concentrations in the gas phase of the pot's headspace. A glass syringe is used to sample the headspace and the sample is transferred to the PTR-TOF-MS. The time series of VOC analysis with the real-time PTR-TOF-MS (m) displays a clear difference between the background air and the sample air injected with the syringe.


Types of setups for VOC measurements
Example of dataset
(a) Steady state
(b) Adapted mobile chamber
(c)
scrubber
cartridge
pump
PTR-TOF-MS
VOC emission
Time
(d) Hormonal treatment
(e) Mechanical estimulation
(f)
(g) Sudden and dynamic VOC emissions
webcam
(h)
video
(i)
(j) Behavioural observations
(k)
(l) VOCs in headspace
(m)
Sample 1
Sample 2
Sample 3

Steady state measurements: Full body scent VOCs

Lacertid lizards (such as wall lizards) fall prey to saurophagous snakes (such as vipers) when sharing a habitat (Van Damme and Castilla 1996). During reptilian predator-prey interactions, it has been shown that chemodetection plays an important role. Snakes follow scent trails of their prey (Parker et al. 2017) and lizards avoid snake-exuded chemicals (Durand et al. 2012). A flow-through chamber setup was used for these measurements (Fig. 1a). In short: a European adder (N = 3) or common wall lizard (N = 3) is enclosed in a sampling chamber. The chamber is equipped with an inlet tube and an outlet tube, the latter being connected to the PTR-TOF-MS which also serves as an air pump. In the chamber, the clean air gets in contact with the animal individual and it becomes enriched with the VOCs that the individual emits (Figure 1a).

For each individual sampling, a clean 250-mL glass sample bottle was used as sampling chamber. The bottles were sealed with a ground glass stopper, that was perforated with a diamond bit to pass an inlet and outlet 1/8-inch (outer diameter, O.D.) PTFE (polytetrafluoroethylene) tube. The inlet tube was connected to a charcoal scrubber (Supelpure HC hydrocarbon trap 22445U, Sigma-Aldrich, St. Louis, MO, USA). It generated VOC-free air from the room air, thus reducing the inlet air VOC concentrations to a minimum. The built-in pump in the PTR-TOF-MS withdrew the clean air through the system at a flow rate of 250 mL min^{-1} (170 µmol s^{-1}), which resulted in an average air residence time in the chamber of 1 min. The air exiting the chamber could be either diverted to pass a GC-MS cartridge (more information on the GC-MS settings in Supplementary Information S.2.) or to reach the PTR-TOF-MS via a 1/16-inch O.D. PEEK (polyether ether ketone) capillary inlet. The VOC concentrations exiting the chamber were monitored with the PTR-TOF-MS, and when the concentration levels reached a steady value (Fig. 1c), a time series of stable data was recorded. Subsequently, the GC-MS cartridge was coupled to the sampling set-up and 5 L of air was passed through. Afterwards, the VOC emission rates of the individual were calculated from PTR-TOF-MS data (see Supplementary Information S.3.). Further details on the animal individuals, their provenance, housing, and the ethical statement of the experiments is found in the Supplementary Information (S.4.).

Dynamic response to stimulus: VOC emissions during physiologically-induced stress

The VOC emissions from the skin secretion of six fire-bellied toads individuals were recorded in the aforementioned flow-through chamber (Section 2.2.1.). The toads (see Supplementary Information S.3. for more information) were used to investigate the VOCs emitted by their skin secretions. Granular glands, found within the skin of many amphibians, are known to store a of cocktail of molecules, which may include alkaloids, amines, peptides and proteins (Daly et al. 2005; König et al. 2015). Because these molecules are secreted upon inducing stress, many of these molecules are considered antipredator toxins (Toledo et al. 2011). The secretion of low-molecular weight volatiles is far less understood. Skin secretion in the *B. orientalis* frogs was stimulated by subcutaneous injection of norepinephrine (80 nmol/g body weight) (Fig. 1d). Norepinephrine is a catecholamine that triggers contraction of myoepithelial cells which encircle the serous glands, causing the contents within to be secreted (Nosi et al. 2002). After injection, each frog was quickly transferred to the chamber. The VOC concentrations peaked after the injection (Fig. 1f), and the emission peak was corrected by the pre-injection VOC concentrations. Two parameters were extracted from each emission peak: the maximum emission rate and the total emission (10 min period); both parameters were further converted relative parameters by incorporating animal weight (see Supplementary Information S.3.).

Dynamic response to stimulus: A flexible chamber for mechanical stimulation

Three fire belly newts (see Supplementary Information S.3. for more information) were used to test the VOC emissions of their defensive secretions upon a simulated predator attack. In this case, the flow-through chamber consisted of a transparent PVDF (polyvinylidene difluoride) bag (model 30284-U Supelco, Eighty-Four, PA, USA) equipped with a screw cap valve and a Thermogreen septum (Fig. 1e). The septum was used as air inlet and the other exit of the screw cap was used as air outlet. The flexibility of the bag permitted manipulation of the newts from outside the bag, allow manual massage, and the application of light pressure with the fingers to approximate a predator's grasping. At all times, we ensured that the animals were not hurt or injured. In order to insert an individual into the bag, a hole was made with a razor and it was closed with tape once the individual was

inserted. Subsequently the bag was inflated through the inlet tube by a hand pump passing a VOC scrubber. The inlet flow was set to the same rate than the sampling rate of the PTR-TOF-MS inlet capillary so the bag would stay inflated. Once the VOC signals that were monitored with the PTR-TOF-MS were stable (body scent VOC emissions during rest), the individual was massaged by the researcher's hand from the outside. The VOCs excreted during the defensive reaction were measured in real-time with the PTR-TOF-MS and the peak emissions integrated.

VOCs from animal excretions and secretions

Many vertebrates are well-known for a specific behaviour, called scent marking (Thompson et al. 2020). By depositing excretions (urine, faeces) and/or secretions from specific glands (anal glands, metatarsal glands, etc.), individuals are believed to establish their territory (Asa et al. 1985; Hurst and Rich 1999), or to signal their personal identity (Burgener et al. 2009), gender, hierarchic status (Lisberg and Snowdon 2009) and sexual status (Pal 2003). By adjusting the PTR-TOF-MS sampling set-up, we can analyse the emission of such scent marks.

Urine samples from six female dogs in anestrous were taken by placement of a polypropylene flask underneath the dog's vulva. Samples were transferred to silanised glass vials with a PTFE septum in the cap (Fig. 1l). The vials were kept at −20 °C until use. The vials were brought to room temperature (25 °C), and 1000 µL were spiked and transferred to 20 mL crimp cap glass vials that were closed with a PTFE septum. The vials were left to reach liquid-gas phase equilibrium during two hours before the VOC measurements. Headspace air was withdrawn with a glass syringe and transferred to the PTR-TOF-MS through the 1/16 inch PEEK capillary.

Lacertid lizards, such as the European common lizard, possess a series of pores on their inner thighs that secret waxy substances to the environment. Femoral pore secretions deposited on natural substrates are thought to signal territoriality, mate quality, and serve in interspecific communication (Mayerl et al. 2015). The VOCs emitted by the wax secretions from femoral pores of three male common lizards were measured. Sterile gauzes were placed in an oven at 80 °C overnight to guarantee the evaporation of any interfering chemical. Subsequently, gauzes were rubbed onto the femoral pores to collect the waxy substances. As a control, gauzes were rubbed over the ventral side of the lizards in order to find compounds unique to femoral pore scent. The gauzes were incubated in glass jars at 25 °C for a few minutes before the measurements of VOCs in the headspace (Fig. 1l) to allow building of a solid-gas phase equilibrium for the VOCs. The PTR-

TOF-MS capillary was then connected to the glass jar to measure the steady level concentrations of VOCs in each jar.

Test arena for sudden and short-lasting processes: adder skin shedding

Similar as for many snake species, the annual spring moulting of European adders initiates their reproductive season (Andrén 1982). VOCs released during this shedding event seem to play an important role in triggering competitive and sexual interactions. The continuous and real-time monitoring via the current set-up allows the detection of such unexpected and short-lived VOC outbursts.

The test arena (Fig. 1g) had a flow-through setup similar to the chambers previously mentioned, but was bigger in volume (l×w×h): 40×40×40 cm (64 L). The floor was covered with a substrate of sand and several dried branches. This substrate was needed to provide abrasive surfaces to help the adder shed its skin. Drinking water was available in a ceramic bowl. A 60-W incandescent lamp was placed in the roof of the terrarium to provide an optimal temperature. A 16/8-h day-and-night cycle was upheld to stimulate the shedding process. The adders were placed individually until they shed the skin (usually from 2 to 5 days in the terrarium). VOC emission data was recorded continuously using the built-in automation tool (see appendix of Portillo-Estrada et al. 2018) in files of one hour duration. The animals were video recorded to aid the interpretation of the VOC emission time series (Video V1, https://youtu.be/-arpezUudik).

Results

The VOC measuring setups proposed produced high-quality results, with a total amount of 132 different VOC ions identified and quantified across all experiments (Table 2). The lowest emissions recorded were 1.33 ± 0.38 fmol g^{-1} s^{-1} from the fire belly newt's skin secretions, and the highest emissions 145 ± 31 fmol g^{-1} s^{-1} from the body scent of adder.

Steady state measurements: Full body scent VOCs

Although the full-body VOC emissions of adders and wall lizards were similarly diverse (20 VOCs), the emissions of both species showed little overlap, sharing only six VOCs (hydrogen chloride, acetaldehyde, ethanol, isoprene, $C_4H_6O_2$, and C_9H_{10}) (Table 2). The emissions were also quantitatively different, amounting to 400 ± 70 and 2.94 ± 0.25 pmol g^{-1} s^{-1} in adders and wall lizards, respectively. Eight compound structures were verified by GC-MS for the adder (C_2H_4O, acetaldehyde;

C_2H_6O, ethanol; C_3H_6O, acetone; C_5H_8, isoprene, $C_{10}H_{16}$, D-Limonene; $C_8H_{16}O_2$, tetrahydro-2,5-dimethyl-2H-pyranmethanol, octanoic acid, 3-cis-methoxy-5-trans-methyl-1R-cyclohexanol), and five for wall lizards (ethanol, isoprene, $C_{16}H_{34}$, 5-ethyl-5-propyl-undecane; C_8H_8O, acetophenone; $C_8H_{16}O$, 2-octanone;). The emissions were dominated by oxygenated VOCs (i.e. ketones, organic acids, alcohols, aldehydes): acetone, acetic acid, and ethanol in adders, and acetaldehyde and ethanol in lizards.

Dynamic response to stimulus:

Injection of norepinephrine to fire-bellied toads: Epinephrine triggered the defensive response of a skin secretion that would be irritating to its predator (pers. obs.). The secretions generated a peak of VOC emissions (Fig. 2) of about 10

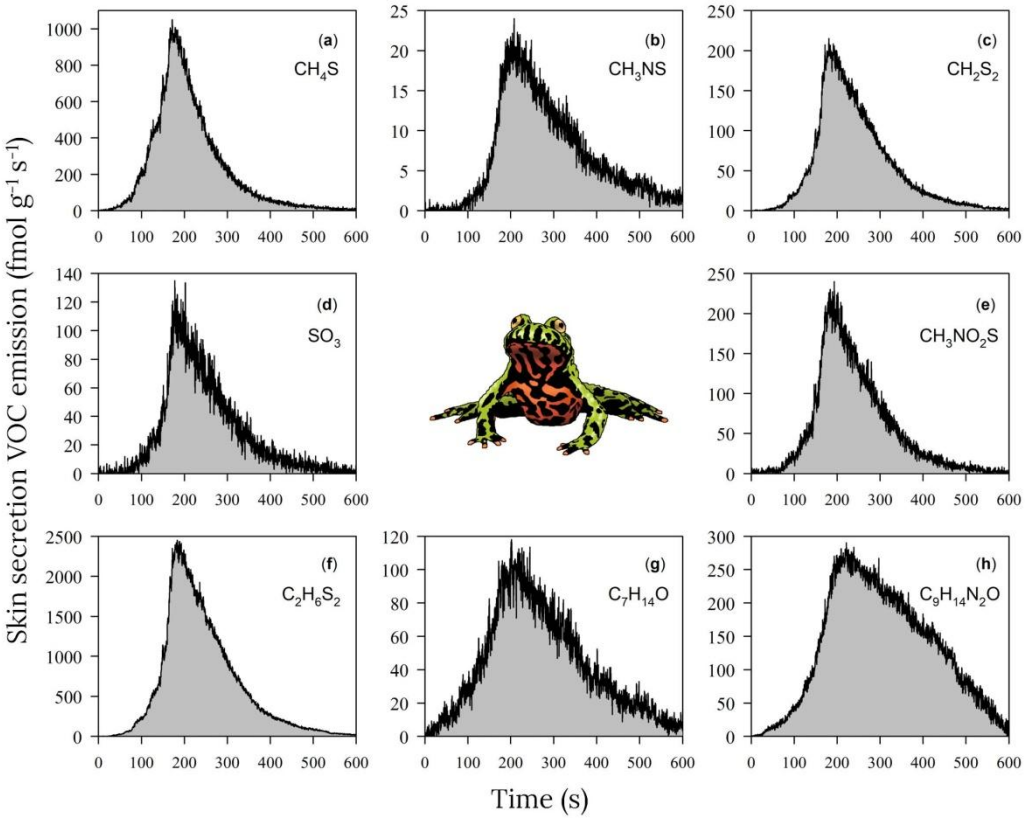


Figure 2 VOC peak emissions of defensive skin secretions after subcutaneous injection of epinephrine to the fire-bellied toad, *Bombina orientalis*. Data monitored with a PTR-TOF-MS and corrected by the pre-injection emission levels. The maximum emission rate of the peak (fmol g^{-1} s^{-1}) are reported in Table 2, as well as the total emissions (pmol g^{-1}) by integrating the area under the peak (grey).

minutes, reaching a maximum at about three minutes after the start of the peak. Out of the 18 VOCs detected in the emission blend, ten contained sulphur (94% of total emissions), and seven contained nitrogen (17% of total emissions) (Table 2). The most abundant VOCs were $C_2H_6S_2$, CH_4S, $C_9H_{14}N_2O$, $C_7H_{14}O$, and CH_2S_2. The odour of the secretions smelled similar to asparagus, cabbage, moist soil, mushrooms, pond weed, potato peels, roots, or wet leaves, as perceived by several colleagues without previous knowledge of the nature of the scent.

Mechanical stimulation of fire belly newts in a flexible chamber: The animal activated skin secretion and a peak emission similar to *B. orientalis* toads (Fig. 2). It was composed of nine VOCs, that accounted for of 26 ± 7 pmol g^{-1} (Table 2). The most abundant compound was C_4H_7N, and the rest of the blend was composed of alkenes, cycloalkenes, ketones, and also some methylated forms.

Test arena for sudden and short-lasting processes: adder skin shedding

The analysis of VOC emission peaks related to skin shedding events revealed a consistent blend of VOCs throughout the peaks and individuals. The skin shedding happened in steps (Video V1) and so did the associated VOC outbursts, which were spaced by several minutes (Fig. 3a). Each outburst lasted only for a few seconds (Fig. 3b,c), making them unpredictable and short-lasting. The VOC blend was composed of 31 VOCs, amounting to 71 ± 14 nmol g^{-1} (Table 2). The emissions were dominated by C_4H_8O, C_3H_4O, and $C_2H_4N_2$. Nine compound structures could be verified using the measurements of steady-state body scent ($C_2H_4O_2$, acetic acid; CH_3NO_2, methyl nitrite; $C_3H_6O_2$, propanoic acid; C_7H_8, toluene; $C_6H_{10}O$, 3-hexen-2-one; $C_6H_{12}O$, hexanal and 3-methylene 2-pentanone; C_7H_6O, benzaldehyde; C_8H_{10}, p-xylene).

VOCs from exudates preserved in a vial

The volatilome of dog urine samples was composed of 95 VOCs amounting to 5100 ± 800 ppb in air (Table 2). It was rich in nitrogen-containing VOCs (23) and sulphur-containing VOCs (6). Among the most abundant VOCs, there were methanol (CH_4O), acetonitrile (C_2H_3N), acetaldehyde (C_2H_4O), methanethiol (CH_4S), ethanol (C_2H_6O), acetone (C_3H_6O), acetic acid ($C_2H_4O_2$), 1,3-diaminourea (CH_6N_4O), and C_8H_8.

The gauzes impregnated in common lizard femoral pore secretions revealed the presence of 30 VOCs (Table 2). The volatilome was dominated in

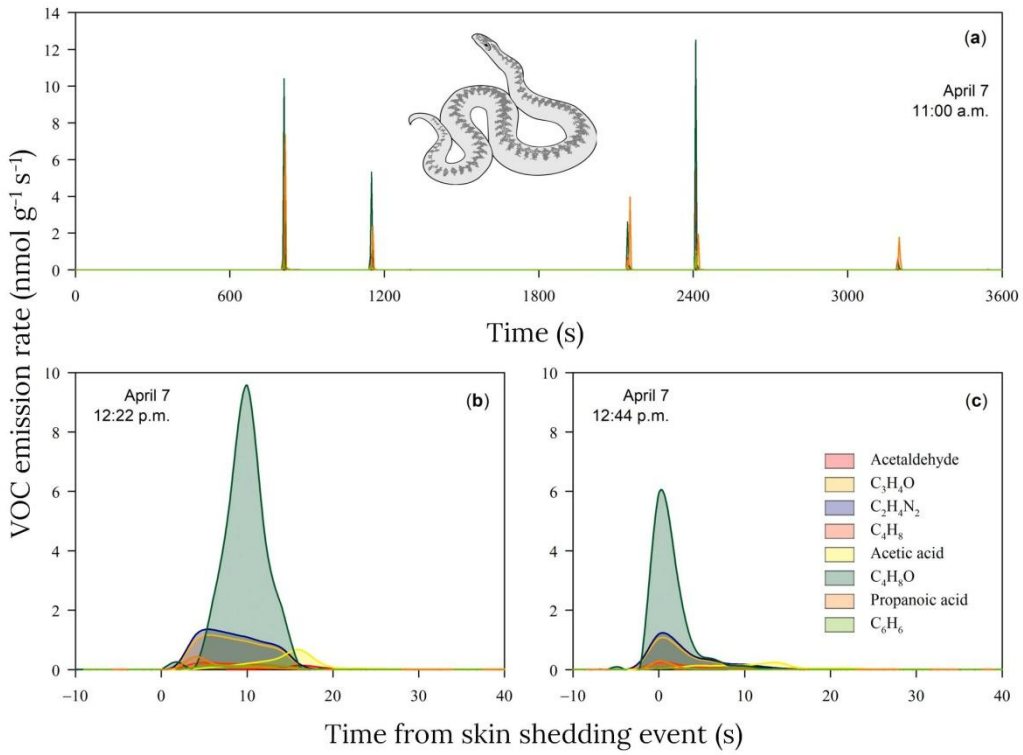


Figure 3 Time series (a) and detail of peak dynamics (b, c) of VOC emissions during skin shedding steps of Vipera berus. The VOCs selected in (b) and (c) amounted to > 1 nmol after the integration of the emission peak —other VOCs not shown, but listed in Table 2—. The total VOCs emitted during the events in (b) and (c) amounted for 105 and 79 nmol VOCs, respectively. The skin shedding event shown in (b) be seen in Video V1.

concentration by aldehydes, alcohols, and acids (68, 14, and 11 %, respectively). Nineteen VOCs in the volatilome were shared with the VOCs measured from the ventrum of the animal and with a similar proportional abundance in both cases. The newly detected compounds in the femoral pores had a concentration of 0.04 to 4.9 ppb in the incubator.

Discussion

Benefits of real-time VOC measurements

PTR-TOF-MS makes it possible to analyse and monitor the emission of VOCs in real-time. Here we expose three important benefits of real-time monitoring: (1) Real-time monitoring with high temporal resolution (≈ 1 s) can measure transient chemical messages, as shown in adder skin shedding monitoring. The

measurement of short time spans such as these outburst-like emissions is a challenge for current, more conventional techniques like GC-MS (one measurement every several minutes). (2) Tracking VOC emissions in real-time reveals the moment when the steady-state emission values are reached, as shown in the full body scent monitoring. It is important to match the timing of subsidiary measurements (e.g. cartridge filling for GC-MS, animal behaviour observation upon stimulus, CO_2 gas exchange rate, exudate sampling, etc.) with the moment when the chamber flow and animal VOC emissions are in equilibrium. (3) The tracking of emission dynamics allows to trace over time a peak emission (e.g. skin secretions of newts and toads), sudden outburst of emissions, fade-out speed of a signal, or the influence of a contemporary stimulus on a certain animal.

Using PTR-TOF-MS in chemical ecology

The PTR-TOF-MS instrument proved suitable for untargeted analysis of VOC emissions. This feature is particularly useful for a first scanning of VOC emissions when the molecules of interest are not yet known. Furthermore, analysing the full range of chemicals composing a scent may be useful for several reasons. Most obviously, an informative signal may be composed of more than a single compound. Moreover, if multiple compounds are important for delivering information, their relative abundance may be of significance too. For example, it is suggested that females of Iberian rock lizards (*Iberolacerta monticola*), may use the variance in male chemicals to distinguish male morphs and steer their mate choice (López et al. 2009). PTR-TOF-MS, in particular, is well-suited for measuring absolute abundances once the proton transfer rate constant (kPTR; Supplementary Information S.1.) is known for each molecule. It is necessary to note here that, despite most of the common VOCs (typical VOCs in plant research and atmospheric research) have a defined kPTR, further research may be needed on values of compounds acting in vertebrate chemical interactions.

There are other specific VOC sampling techniques that combine with GC-MS, such as thermal desorption of activated carbon cartridges (Kännaste et al. 2014) and solid phase microextraction (Brunetti et al. 2015). However, the sampling of VOCs depends on adsorption to a matrix of specific characteristics. Therefore, it is complex to perform an untargeted VOC analysis if the VOC measurement depends on the selectivity of the adsorption matrix in the sample cartridge. As evident from our tests, this narrows the spectrum of molecular mass range and discriminates VOC chemical classes. Consequently, our results with Tenax TA

matrix as adsorbent, despite being widely accepted and used, were somewhat unsatisfactory as compared with the wider spectrum of the PTR-TOF-MS —only a few compound structures could be verified with GC-MS in the steady state measurement—. PTR-TOF-MS scans a very wide range of molecules thanks to the proton-transfer reaction from hydronium —most VOCs have a higher proton affinity than water—. This feature allows overcoming the restriction of matrix affinity. Therefore, more than one type of matrix would be needed to identify all the ions measured by PTR-TOF-MS while GC-MS on the other hand is amenable to compounds that do not ionise by the latter. As a conclusion, we see great potential in combining both MS methods in chemo-ecological studies as follows: First, an exhaustive sampling can be performed using PTR-TOF-MS to search for candidate semiochemicals. In this phase, the high mass resolution of PTR-TOF-MS (to the nearest 0.0001 m/z) allows the accurate determination of the molecular formulae of sampled molecules. Second, targeted resampling can be performed with active carbon cartridges and analysed using GC-MS, which would clarify which isomers of the candidate molecules are present in the chemical mixture. Final testing of authentic standards through bio-assays would confirm or reject the presence of semiochemical properties in identified candidate molecules. If complemented with GC-MS and its power of structure confirmation, they both create a perfect tandem with an extensive potential in the field of animal chemo-ecology.

On the chamber size and flow

The chamber size, flow rate, and the expected VOC emission rate is a trinomial that should remain in concordance for the correct detection of VOC emissions:

The chamber size has a direct effect on the concentrations measured that later will be used to calculate the VOC emission rates. This is because the VOCs emitted dilute into the chamber air before exiting to the analyser. Generally, smaller chamber volumes are preferred over large to avoid the dilution of the VOCs emitted, that hinders the detection of small VOC emissions.

The chamber flow rate is inversely proportional to the VOC concentrations measured (see Equation 1). Thus, the smaller the chamber and the higher the flow rate, the higher the responsiveness (shorter air residence time) to changes in VOC emissions. However, a fast flow decreases the sample air concentration in flow-through chambers and the sample air VOC concentration may eventually fall under the threshold of detection of the analytical instrument. Opposite to this, in

large chambers with lower flow rates and long air residence time, the VOC emission dynamics become 'smeared' over time. This can affect to the point of neglecting the existence of VOC dynamics (e.g. outbursts). Unluckily, sometimes it is not possible to reduce the chamber size, e.g. if the animal needs a certain setup inside the chamber in observational experiments. As an example, we targeted in the steady-state measurements a residence time of 1 minute, i.e. 250 mL min^{-1} flow rate when using a 250 mL chamber.

The level of emissions expected is as important as the two previous factors for obvious reasons. Therefore, it is suggested to ponder the setups case by case to enclose the individuals in a chamber that is as small as possible without compromising the needs of the experiment.

VOC measurements focalised on crucial emitting sources

Targeting parts of an animal's body involved in scent production are important to correctly replicate what animals themselves sense when sniffing each other, like targeting a dog's vulva. It is important to remember that this circumstance works at every scale, no matter the size of the animal. Therefore, efforts must be made to overcome the problem of sensing with an instrument small targets in small animals, as it is targeting the VOC emissions of femoral pores. Our results on lizard femoral pores confirmed that targeting the crucial VOC-emitting regions was rewarding, because whole body measurements did not reveal all the VOCs measured from the femoral pores. Femoral pores are small emitting sources and their VOC emissions were certainly too diluted in the 250 mL chamber, making the concentrations non detectable. The gauze impregnation and incubation approach proved suitable to measure the emissions. As a further advantage to this approach, the gauzes may be used to transfer scent from animal to animal for behavioural experiments on olfaction and decision making.

In the case of a bigger animal like a dog, a focalised measurement could be possible by making a chamber that adapts to the region of interest. As an example, a chamber adapted to measure vulvar VOC emissions would be suitable to detect directly the volatilome that dogs smell when sniffing each other (Fig. 1b). In contrast to that focalised measurement, a bigger chamber of ≈0.5 m^3 to enclose the whole animal would necessitate a high flow rate, requiring an additional pumping system than of the PTR-TOF-MS. Moreover, the vulvar VOC emissions would dilute into a too big volume, probably trespassing the low threshold for detection in some VOCs. And finally, vulvar emission would mix with other

emitting sources (fur, mouth, anal secretions, etc.), making it impossible to disentangle from the rest. We therefore encourage researchers to investigate the expected VOC emission levels and adapt the chamber size and flow rate for an optimal measurement. We invite researchers to make focalised VOC emission measurements as possible, sometimes needing to extract exudates to be incubated in order to reach detectable concentrations.

The volatilome of lizards and European adder

Many species within the Squamata (an order within reptiles that comprises all lizards and snakes) are known for their highly developed and specialised chemical senses. Furthermore, clear indications of their ability to respond to airborne chemicals exist (Bull et al. 1993). For example, members of a monogamous pair of sleepy lizards (*Tiliqua rugosa*) were able to find one another after experimental separation, even in the absence of substrate-bound scent trails (Bull et al. 1993). European adders and wall lizards did not share that many VOCs in their body volatilome. Due to their predator-prey relationship and their highly developed chemical senses, they are expected to notice each other's presence by their volatilome. Furthermore, when shedding, a remarkable rise in VOC emissions was observed upon which competing adders can react (Andrén 1982). Further research on that topic is needed to gain insights in such interactions.

The most abundant VOCs in common lizard femoral pore volatilome, low molecular weight aldehydes and alcohols, have been measured in the femoral pores of lizard 'mesic' species. These are suggested to be involved in long-distance airborne communication within and even between species of the same ecological guild (Baeckens et al. 2018). Such molecules could be used by conspecifics in sexual and competitive interactions or, similar as suggested above, eavesdropped by predators or arthropod prey (Dicke and Grostal 2001).

The volatilome of defensive skin secretions

Amphibian skin secretions are widely accepted to play an important role in antipredator defence. Literally thousands of alkaloids, steroids, peptides, and proteins have been characterised from hundreds of species and taxa. Yet, although many species are well known for producing a distinct herbaceous or aromatic smell when stressed, VOCs have remained particularly understudied component of amphibian skin secretions and have been characterised in only a handful of species (e.g., Brunetti et al. 2015). To our knowledge, the present study is the first that

identifies VOCs emitted by a salamander. In addition, although *Bombina orientalis* skin secretion is one of the best-documented amphibian poisons (Xu and Lai 2015), its volatilome has so far never been investigated. Alongside a diverse arsenal of bioactive peptides, the skin secretion is found to emit a notable repertoire of sulphur and nitrogen-containing VOCs. Interestingly, it is the first time that CH_2S_2 (likely dithioformic acid) is reported as a secreted compound. And more research would be needed to elucidate the function of CH_3S and CH_3OS (likely methanethiolate and sulfenatomethane, respectively), that are common intermediates for the oxidation of different organosulphur compounds.

Sulphur-containing compounds are notorious for their pungent odours. They are, for instance, the major constituent of the skunk's defensive spray (Wood 1999). Among these compounds, methanethiol (CH_4S) is also known as a by-product of the metabolism of asparagus (Richer et al. 1989), which could explain the herbaceous smell released by *B. orientalis* during the experiments as described by colleagues. Similarly, $C_2H_6S_2$ and C_2H_6S (likely 1,2-dimethyldisulphane and ethanethiol, respectively) have a vegetable-like sulphide odour or garlic-like smell. These compounds usually have a low detection threshold (e.g. 0.001 ppb for ethanethiol in humans (Leonardos et al. 1969), that was largely trespassed in the measurements. Altogether, these odorous molecules could act as an aposematic signal to predators, advertising the toad's toxicity caused by co-secreted bioactive peptides. While hypothesised functions of amphibian volatile secretions include odorous aposematism (Yoshimura and Kasuya 2013; Brunetti et al. 2015), alarm signalling (Smith et al. 2004), kin recognition (Starnberger et al. 2013), sexual selection (Poth et al. 2012), insect repellence (Williams et al. 2006) and direct toxicity (Smith et al. 2003), currently only the use as predator aversion (Yoshimura and Kasuya 2013) and insect repellence (Williams et al. 2006) has been validated. Given the presence of a strong odour when both species were handled, it points towards at least some of the compounds in the volatilome being used for predator avoidance, in line with research conducted by (Yoshimura and Kasuya 2013).

The volatilome of dog urine

Urine samples are high-emitting sources, and the vial size and sample amount (1 mL) proved suitable to detect a large number of VOCs. In carnivores/dogs, urine scent marks are highly attractive to conspecifics, suggesting the presence of attractant molecules in the volatilome. The untargeted analysis carried out by PTR-TOF-MS confirmed the presence of many molecules that were previously

identified in the urine of other carnivores: hexanal (Canidae) (Preti et al. 1976; Raymer et al. 1985), ethenylbenzene (Iberian wolf) (Martín et al. 2010), 1-phenylethanone (red fox) (Jorgenson et al. 1978), benzoic acid, 2-octen-1-ol and 2-pentenylfuran (grey wolf) (Raymer et al. 1985), 1-aminourea (mammal urine), and methyl isopentenyl disulphide (coyote) (Schultz et al. 1988). Furthermore, acetic acid, isovaleric acid, 4-methylpentanoic acid, and isobutyric acid were previously found in dog anal secretion (Preti et al. 1976). Because urine scent is likely crucial for intra-specific individual identification, sexual predisposition during estrous cycle, territory marking, etc., it would be interesting to compare urine samples of individuals with differences in gender, hierarchic state, and estrous cycle. In addition, PTR-TOF-MS could act as a fast analyser for rapid detection of specific VOCs in the urine, a field still to be explored.

Conclusion

PTR-TOF-MS has proven to be a versatile tool to monitor the emission of vertebrate VOCs, when applied in a suitable experimental setup (i.e. correct chamber type, size, and flow rate). PTR-TOF-MS technique can successfully monitor VOC dynamics and short-lasting VOC emission; a feature made possible by the device´s fast and real-time analytical power. Furthermore, in the lowest mass range, the technique detected compounds that the widely used Tenax TA cartridges could not adsorb. Therefore, PTR-TOF-MS is a valuable tool for untargeted VOC emission analysis when there is no a priori knowledge on the nature of the relevant compounds involved in a process. If combined with a structure verification tool, such as GC-MS, the creative deployment of PTR-TOF-MS in various future study designs will lead to the identification of ecologically relevant VOCs.

In this proof-of-concept paper, we have shown distinct experimental setups that can be used, adapted and improved to measure VOC emissions from vertebrate and their exudates. We encourage researchers to use PTR-TOF-MS technique to answer ecological questions that include dynamics or short-lasting events; questions that, due to the lack of analytical time resolution of other well-established techniques, could not be answered in the past.

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Table 2 List of VOCs which emission rates (mean ± SE among individuals; Table 1) were monitored with a PTR-TOF-MS in different animals and experimental conditions. The molecular formula deduced from the protonated molecular mass recorded by PTR-TOF-MS, and the compound name of the original compound are given (an asterisk denotes the verification of the compound structure via GC-MS analysis, otherwise the most likely compound name is given based on an extensive literature research of articles of similar taxa or when a single structure was possible). See Tables S1 and S2 (Supplementary Information) for the full results of GC-MS analysis for lizards and adders.

Molecular formula	Protonated molecular mass	Compound name	*Bombina orientalis*		*Cynops pyrrhogaster*		*Podarcis muralis*	*Zootoca vivipara*		*Vipera berus*		*Canis familiaris*
			Skin secretion (body corrected)		Mechanical (body corrected)		Body	Ventrum	Femoral pores	Body	Skin shedding	Urine
			Maximum emission fmol g^{-1} s^{-1}	Total pmol g^{-1}	Maximum emission fmol g^{-1} s^{-1}	Total pmol g^{-1}	Emission rate fmol g^{-1} s^{-1}	Headspace ppb	Incubated ppb	Emission rate pmol g^{-1} s^{-1}	VOCs emitted nmol g^{-1}	VOCs headspace ppb
CH_2O	31.0178	Formaldehyde	–	–	–	–	–	–	–	–	–	22.6 ± 1.9
CH_4O	33.0335	Methanol	–	–	–	–	–	–	–	10.5 ± 1.5	–	1600 ± 430
HCl	36.9840	Hydrogen chloride	–	–	–	–	99 ± 7	2.0 ± 0.6	2.7 ± 1.2	41 ± 10	–	–
C_3H_4	41.0386	Propyne	–	–	–	–	–	–	–	0.63 ± 0.09	–	61 ± 11
C_2H_3N	42.0338	Acetonitrile	–	–	–	–	–	–	–	0.069 ± 0.014	–	550 ± 32
C_3H_6	43.0542	Propene	–	–	–	–	–	–	–	7.5 ± 1.2	0.101 ± 0.023	88 ± 14
C_2H_4O	45.0335	Acetaldehyde *	–	–	–	–	2430 ± 210	61.0 ± 9.0	49.0 ± 8.0	112 ± 22	0.76 ± 0.25	240 ± 70
CH_2S	46.9950	Thioformaldehyde	–	–	–	–	–	–	–	–	–	3.3 ± 1.2
CH_2O_2	47.0128	Formic acid	–	–	–	–	–	–	–	–	0.39 ± 010	–
C_2H_6O	47.0491	Ethanol *	–	–	–	–	182 ± 15	3.2 ± 3.9	10.0 ± 7.0	145 ± 31	–	108 ± 37
CH_3S	48.0029	Methanethiolate	220 ± 60	18 ± 5	–	–	–	–	–	–	–	22 ± 7
CH_4S	49.0107	Methanethiol	1000 ± 180	103 ± 19	–	–	–	–	–	–	–	225 ± 22
CH_6O_2	51.0441	Methanol hydrate	–	–	–	–	–	–	–	–	–	31 ± 9
C_3HN	52.0519	Cyanoacetylene	–	–	–	–	–	–	–	–	–	0.43 ± 0.13
C_4H_6	55.0542	Butadiene	–	–	–	–	–	–	–	–	–	55 ± 4
C_4H_7	56.0621		–	–	–	–	–	–	–	–	–	10.0 ± 2.6
C_3H_4O	57.0335	2-Propenal	–	–	–	–	–	–	0.13 ± 0.09	0.38 ± 0.09	9.4 ± 1.9	4.4 ± 0.9
$C_2H_4N_2$	57.0447		–	–	–	–	–	–	–	–	10.8 ± 2.2	–
C_4H_8	57.0699	Isobutylene	–	–	–	–	18.1 ± 2.2	0.40 ± 0.14	0.77 ± 0.09	–	1.98 ± 0.29	62 ± 8
C_3H_5O	58.0413		–	–	–	–	–	–	–	–	0.177 ± 0.032	4.4 ± 0.9
C_3H_7N	58.0651		–	–	–	–	–	–	–	–	0.162 ± 0.030	6.2 ± 1.4
$C_2H_2O_2$	59.0128	Glyoxal	–	–	–	–	–	–	–	–	–	16 ± 3.1

C_3H_6O	59.0491	Acetone *	–	–	–	–	–	–	–	75 ± 16	–	2000 ± 800
HN_3O	60.0192	Hydroxy azide	–	–	–	–	–	–	–	–	–	3.4 ± 0.8
C_3H_7O	60.0570		–	–	–	–	–	–	–	–	0.124 ± 0.033	40 ± 12
$C_2H_4O_2$	61.0284	Acetic acid *	–	–	–	–	–	–	4.9 ± 1.2	–	4.9 ± 1.1	156 ± 31
CH_3NO_2	62.0237	Methyl nitrite *	–	–	–	–	–	–	–	–	0.063 ± 0.022	3.9 ± 0.6
C_2H_6S	63.0263	Ethanethiol	23.5 ± 42	2.03 ± 0.35	–	–	–	–	–	–	–	–
$C_2H_6O_2$	63.0441	Ethanediol	–	–	–	–	–	–	1.6 ± 0.9	1.70 ± 0.27	0.11 ± 0.08	–
CH_3OS	63.9977	Sulfenatomethane	21.5 ± 6	2.8 ± 0.8	–	–	–	–	–	–	–	–
CH_4OS	65.0056		14.3 ± 4.5	0.78 ± 0.24	–	–	–	–	–	–	–	–
C_5H_6	67.0542		–	–	–	–	–	–	–	–	–	1.32 ± 0.24
C_4H_5N	68.0495	Allyl cyanide	–	–	–	–	–	–	–	–	–	4.0 ± 0.7
C_4H_4O	69.0335	Furan	–	–	–	–	–	–	–	–	–	2.8 ± 0.6
C_5H_8	69.0699	Isoprene *	54 ± 15	2.2 ± 0.6	–	–	29.1 ± 3.6	0.45 ± 0.14	0.25 ± 0.17	1.34 ± 0.27	0.110 ± 0.027	15.3 ± 1.6
C_4H_7N	70.0651		–	–	67 ± 19	10.9 ± 2.4	–	–	–	–	–	9.2 ± 2.5
C_4H_6O	71.0491	Methyl vinyl ketone	–	–	–	–	–	–	–	–	–	4.0 ± 1.1
C_5H_{10}	71.0855		–	–	19 ± 5	3.0 ± 0.7	10.6 ± 1.0	0.146 ± 0.048	0.217 ± 0.026	–	–	8.9 ± 1.7
C_3H_5NO	72.0444	Acrylamide	–	–	–	–	–	–	–	–	–	1.00 ± 0.32
C_4H_8O	73.0648	Cyclopropylcarbinol	–	–	–	–	–	–	–	0.47 ± 0.10	36 ± 10	99 ± 24
CH_3NS	74.0059	Thioformamide	43.5 ± 7	6.4 ± 0.9	–	–	–	–	–	–	–	–
C_3H_7NO	74.0600	Acetone oxime	–	–	–	–	–	–	–	–	–	4.9 ± 1.2
$C_3H_6O_2$	75.0441	Propanoic acid *	–	–	–	–	–	–	–	–	1.63 ± 0.43	19 ± 6
$C_3H_6O_2$	75.0441	Methyl acetate	–	–	–	–	–	–	0.23 ± 0.12	–	–	–
$C_2H_5NO_2$	76.0393	Glycine	–	–	–	–	–	–	–	–	–	1.4 ± 0.5
$C_3H_8O_2$	77.0597	Propanediol	–	–	–	–	–	–	–	0.102 ± 0.019	0.145 ± 0.035	3.2 ± 0.8
CH_2S_2	78.9671	Dithioformic acid	200 ± 60	18 ± 6	–	–	–	–	–	–	–	1.79 ± 0.39
C_6H_6	79.0542	Benzene	–	–	–	–	–	–	–	–	0.92 ± 0.42	2.9 ± 0.7
SO_3	80.9641	Sulfur trioxide	115 ± 35	12.9 ± 4.1	–	–	–	–	–	–	–	–
C_6H_8	81.0699	Cyclohexadiene	–	–	–	–	–	–	0.161 ± 0.032	0.245 ± 0.047	0.15 ± 0.06	1.10 ± 0.11
C_5H_7N	82.0651	Uric acid (fragment)	–	–	–	–	–	–	–	–	–	1.78 ± 0.29
H_3PO_3	82.9893	Phosphorous acid	–	–	–	–	4.7 ± 0.8	0.088 ± 0.022	0.19 ± 0.11	–	–	–
C_6H_{10}	83.0855	4-methyl-1,3-	–	–	8.0 ± 2.3	1.24 ± 0.31	10.2 ± 0.4	0.19 ± 0.15	–	–	–	6.1 ± 1.0

		pentadiene									
C_5H_9N	84.0808	Pentanenitrile	–	–	–	–	–	–	–	–	–	1.21 ± 0.12
C_5H_8O	85.0648	3-Penten-2-one	–	–	4.8 ± 1.3	0.73 ± 0.18	–	–	–	–	–	4.4 ± 0.6
C_6H_{12}	85.1012	Cyclohexane	–	–	–	–	–	–	–	–	–	4.2 ± 0.5
C_4H_7NO	86.0600	Pyrrolidin-2-one	–	–	–	–	–	–	–	–	–	0.71 ± 0.14
$C_4H_6O_2$	87.0441	2,3-Butanedione	–	–	–	–	64 ± 10	1.1 ± 0.5	1.77 ± 0.29	1.17 ± 0.21	0.196 ± 0.026	–
		Dihydrofuran-2(3H)-one										
$C_5H_{10}O$	87.0804	3-Methylbutanal	–	–	–	–	–	–	0.26 ± 0.11	–	–	–
		2-Methyl-3-buten-2-ol										
$C_5H_{10}O$	87.0804	2-Pentanone	–	–	–	–	–	–	–	–	–	46 ± 15
C_4H_9NO	88.0757	Butyramide	–	–	–	–	–	–	–	–	–	3.1 ± 0.7
$C_3H_4O_3$	89.0233	2-Oxopropanoic acid	–	–	–	–	3.7 ± 0.5	0.067 ± 0.010	0.117 ± 0.016	–	–	2.48 ± 0.41
$C_4H_8O_2$	89.0597	Ethyl acetate	–	–	–	–	–	–	0.179 ± 0.009	0.260 ± 0.042	0.114 ± 0.027	–
$C_4H_8O_2$	89.0597	2-Methylpropanoic acid (i.e. isobutyric acid)	–	–	–	–	–	–	–	–	–	5.3 ± 1.2
$C_3H_7NO_2$	90.0550	Aminopropanoic acid	–	–	–	–	–	–	–	–	–	3.0 ± 0.9
CH_6N_4O	91.0614	1,3-Diaminourea	–	–	–	–	–	–	–	–	–	216 ± 43
$C_2H_7N_2O_2$	92.0580		–	–	–	–	–	–	–	–	–	12.9 ± 2.8
C_7H_8	93.0699	Toluene *	–	–	–	–	–	–	–	–	0.142 ± 0.053	29 ± 9
CH_3NO_2S	93.9957		220 ± 70	24 ± 8	–	–	–	–	–	–	–	–
C_6H_5O	94.0413	Phenolate	–	–	–	–	–	–	–	–	–	3.1 ± 1.2
$C_2H_6S_2$	94.9984	1,2-Dimethyldisulfane	2430 ± 450	330 ± 52	–	–	–	–	–	–	–	–
C_6H_6O	95.0491	Phenol	–	–	–	–	–	–	–	–	0.472 ± 0.012	7.5 ± 2.4
C_7H_{10}	95.0855		–	–	–	–	–	–	–	–	0.147 ± 0.014	2.8 ± 0.6
C_7H_{12}	97.1012	2,3-Heptanediene	–	–	–	–	–	–	–	–	–	1.90 ± 0.25
$C_6H_{10}O$	99.0804	3-Hexen-2-one *	–	–	–	–	–	–	–	–	0.065 ± 0.019	2.80 ± 0.38
$C_5H_8O_2$	101.0597	5-Methyldihydrofura	–	–	–	–	–	–	–	–	0.225 ± 0.031	3.1 ± 0.6

		n-2(3H)-one (E)-2-Methyl-2-butenoic acid									
$C_6H_{12}O$	101.0961	Hexanal *	–	–	–	–	–	–	–	–	0.081 ± 0.009	3.06 ± 0.64
$C_5H_{10}O_2$	103.0754	3-Methylene 2-pentanone * 2,2-dimethyl-propanoic acid Methylbutanoic acid (i.e. isovaleric acid)	–	–	–	–	–	–	0.13 ± 0.06	1.17 ± 0.21	–	4.4 ± 0.9
$C_6H_{14}O$	103.1117	Dipropyl ether Butyl ethyl ether	–	–	–	–	–	–	–	–	–	0.32 ± 0.05
$C_3H_7N_2O_2$	104.0580		–	–	–	–	–	–	–	–	–	2.08 ± 0.32
$C_4H_8O_3$	105.0546		–	–	–	–	17.9 ± 2.0	0.256 ± 0.036	0.350 ± 0.024	–	–	–
C_8H_8	105.0699	Ethenylbenzene	–	–	–	–	–	–	–	–	–	121 ± 38
C_7H_6O	107.0491	Benzaldehyde *	–	–	–	–	–	–	–	–	0.139 ± 0.045	7.1 ± 1.8
C_8H_{10}	107.0855	Ethylbenzene *	–	–	–	–	–	–	0.103 ± 0.049	–	–	8.2 ± 1.9
C_8H_{10}	107.0855	*p*-Xylene *	–	–	–	–	–	–	–	–	0.131 ± 0.043	–
C_7H_9N	108.0808	*N*-Methylbenzenamine 2,6-Dimethylpyridine	–	–	–	–	–	–	–	–	–	1.02 ± 0.30
C_8H_{12}	109.1012	1,2-Cyclooctadiene	–	–	2.2 ± 0.6	0.31 ± 0.10	–	–	–	–	–	–
$C_7H_{10}O$	111.0804	2,4-Heptadienal	–	–	–	–	–	–	–	–	0.063 ± 0.018	2.58 ± 0.49
C_8H_{14}	111.1168	Cyclooctene	–	–	9.1 ± 2.6	1.22 ± 0.47	–	–	–	–	–	–
$C_5H_4O_3$	113.0233		–	–	–	–	–	–	–	–	–	0.60 ± 0.11
$C_6H_8O_2$	113.0597	1,2-Cyclohexanedione	–	–	–	–	–	–	0.060 ± 0.028	–	0.077 ± 0.018	–
$C_7H_{12}O$	113.0961	2-Heptenal	–	–	–	–	–	–	–	–	–	5.5 ± 1.0
$C_6H_{10}O_2$	115.0754	4-Methyl-3-Pentenoic acid	–	–	–	–	2.62 ± 0.22	0.044 ± 0.019	0.091 ± 0.007	–	0.097 ± 0.023	–
$C_7H_{14}O$	115.1117	Heptanal	110 ± 40	39 ± 13	–	–	–	–	–	–	–	6.7 ± 1.7
$C_6H_{12}O_2$	117.0910	4-	–	–	–	–	–	–	–	–	–	0.93 ± 0.19

		Methylpentanoic acid									
$C_6H_{12}O_2$	117.0910	Methyl isobutyl ketone	–	–	–	–	2.40 ± 0.41	0.042 ± 0.020	0.035 ± 0.013	–	–	–
		Methyl pivalate										
		3-Hydroxy-2-methyl-pentanal										
		Butyl acetate										
C_9H_{10}	119.0855	2,3-Dihydro-1H-indene	–	–	–	–	54 ± 8	0.89 ± 0.20	0.83 ± 0.033	0.058 ± 0.015	–	3.8 ± 0.9
C_9H_{11}	120.0934		–	–	–	–		–	–	–	–	0.38 ± 0.06
C_8H_8O	121.0648	Acetophenone *	–	–	–	–	5.2 ± 0.7	0.09 ± 0.06	0.096 ± 020	–	–	–
C_8H_8O	121.0648	1-Phenylethanone	–	–	–	–	–	–	–	–	–	15.3 ± 6.4
$C_7H_6O_2$	123.0441	Benzoic acid	–	–	–	–	–	–	–	–	–	0.482 ± 0.023
CH_2OS_3	126.9341		–	–	–	–	–	–	–	–	–	0.92 ± 0.14
$C_8H_{14}O$	127.1117	6-Methyl-5-hepten-2-one	–	–	5.9 ± 1.7	0.84 ±0.25	–	–	–	–	–	1.24 ± 0.28
$C_3H_4N_4O_2$	129.0407		–	–	–	–	–	–	–	–	–	0.55 ± 0.08
$C_{16}H_{34}$	129.0699	5-ethyl-5-propyl-undecane *	–	–	–	–	2.85 ± 0.26	0.0459 ± 0.0030	0.045 ± 0.015	–	–	–
$C_8H_{16}O$	129.1274	2-Octanone *	–	–	59 ± 17	8.0 ± 2.9	2.43 ± 0.23	0.0372 ± 0.0041	0.047 ± 0.010	–	–	–
$C_8H_{16}O$	129.1274	2-Octen-1-ol	–	–	–	–	–	–	–	–	–	1.21 ± 0.22
$C_{10}H_{10}$	131.0855	3-Methyl indene	–	–	–	–	–	–	–	–	–	0.228 ± 0.018
$C_7H_{14}O_2$	131.1066	2-Pentanol acetate	–	–	–	–	–	–	0.0401 ± 0.0023	–	–	–
		Heptanoic acid										
$C_{10}H_{12}$	133.1012	1-Phenyl-1-butene	–	–	–	–	–	–	–	–	–	2.2 ± 0.6
$C_2H_7N_5O_2$	134.0673	1-Aminobiurea	–	–	–	–	–	–	–	–	–	3.3 ± 1.1
$C_{10}H_{14}$	135.1168	1,4-Diethylbenzene	–	–	–	–	–	–	–	–	–	20 ± 7
$C_2H_9N_5O_2$	136.0829		–	–	–	–	–	–	–	–	–	2.8 ± 0.9
$C_8H_8O_2$	137.0597	Benzoic acid methyl ester	–	–	–	–	–	–	–	–	–	0.57 ± 0.07
$C_{10}H_{16}$	137.1325	*D*-Limonene *	–	–	–	–	–	–	–	0.084 ± 0.019	–	0.43 ± 0.07
$C_8H_{11}NO$	138.0913	4-(2-Aminoethyl)-phenol	9.2 ± 1.1	1.55 ± 0.22	–	–	–	–	–	–	–	–

$C_9H_{14}O$	139.1117	2-Pentylfuran	-	-	-	-	-	-	-	-	-	0.64 ± 0.11
$C_8H_{14}O_2$	143.1067	5-Butyldihydro-2-furanone	-	-	-	-	-	-	-	-	-	0.76 ± 0.10
$C_8H_{16}O_2$	145.1223	Tetrahydro-2,5-dimethyl-2H-pyranmethanol * Octanoic acid * 3-cis-Methoxy-5-trans-methyl-1R-cyclohexanol *	-	-	-	-	-	-	-	0.0187 ± 0.0029	-	0.149 ± 0.022
$C_6H_{12}S_2$	149.0453	Methyl isopentenyl disulfide	-	-	-	-	-	-	-	-	-	0.99 ± 0.34
$C_{11}H_{16}$	149.1325	2-Decyldodecyl-benzene	-	-	-	-	-	-	-	-	-	1.11 ± 0.29
$C_9H_{12}NO$	151.0992		5.7 ± 1.1	0.35 ± 0.07	-	-	-	-	-	-	-	-
$C_8H_{12}N_2O$	153.1022	2-Isopropyl-6-methyl-4-pyrimidone	24 ± 6	3.7 ± 0.8	-	-	-	-	-	-	-	-
$C_{10}H_{20}O$	157.1586	Decanal *	-	-	-	-	3.6 ± 0.5	0.068 ± 0.045	0.067 ± 0.012	-	-	-
$C_{10}H_{22}O$	159.1743	Decanol	-	-	-	-	1.65 ± 0.22	0.029 ± 0.010	0.049 ± 0.007	-	-	-
$C_{11}H_{15}N$	162.1277	Phenylpiperidine	-	-	-	-	2.47 ± 0.44	0.041 ± 0.016	0.053 ± 0.014	-	-	-
$C_{11}H_{16}O$	165.1274	1-Isopropyl-2-methoxy-4-methylbenzene	7.7 ± 2.0	0.99 ± 0.20	-	-	-	-	-	-	-	-
$C_{12}H_{16}O$	167.0492	2-ethylbutyl ester benzoic acid	-	-	-	-	-	-	-	-	-	0.87 ± 0.29
$C_9H_{14}N_2O$	167.1179	2-Isobutyl-3-methoxypyrazine	280 ± 80	58 ± 19	-	-	-	-	-	-	-	-
$C_8H_{18}N_2O_2$	175.1441		-	-	-	-	-	-	-	-	-	2.2 ± 0.7
$C_{15}H_{24}$	205.1951	Sesquiterpenes	-	-	1.33 ± 0.38	0.244 ± 0.046	-	-	-	-	-	-
		SUM =	4800 ± 1100	560 ± 130	175 ± 50	26 ± 7	2940 ± 250	70 ± 6	74 ± 7	400 ± 70	71 ± 14	5100 ± 800

Chapter 7

The smell of danger passing by: How long does it linger?

Van Moorleghem, C., Portillo-Estrada, M., Lambreghts, Y., Van Damme, R. Manuscript in preparation. The smell of danger passing by: How long does it linger?

Abstract

For many animals, chemical compounds left behind in the environment by other creatures constitute a primary source of information. In vertebrates, the correct interpretation of predator-derived molecules is crucial for their survival. Therefore, these animals may convert the complex and dynamic nature of predator scent into nuanced messages, rather than using a single molecule as an on/off switch for anti-predatory responses. Through the use of Proton-Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-TOF-MS), we looked into the complexity of the volatile aspect of the scent of a predatory snake (the European adder, *Vipera berus*) and how it changes over time. During behavioural experiments, a lizard (the European common lizard, *Zootoca vivipara*) reduced the time spend walking around in a test arena when subjected to this volatile aspect, but only if derived from the freshest cues (opposed to cues that had faded over two and twelve hours). When brought in contact with substrate-bound chemicals, lizards performed a more complete array of anti-predatory behaviours which implied a transition from a 'cryptic escape' to a true 'flight escape' as the scent cue faded over time. Therefore, these animals are able to interpret various aspects - i.e. volatile vs. substrate-borne, as well as, temporal change - of a complex predator scent cue.

Introduction

The chemical senses are essential in the lives of many species due to their role in predator detection. Animals as widely divergent as, for instance, damselflies (Chivers et al. 1996), songbirds (Amo et al. 2008), reptiles (Thoen et al. 1986), and mammals (Russell and Banks 2007) persist under predatory threat because they increase alertness, anxiety and/or initiate the performance of anti-predatory behaviours when smelling a predator (Kats and Dill 1998; Downes 2002). Although essential for survival, when performing such behaviours in vain, they prevent the prey from carrying out other ecological tasks (e.g. feeding and mate searching). Consequently, a correct interpretation of the often complex blend of predator-derived chemicals is crucial.

Predator odours consist of molecules belonging to a wide variety of chemical classes. Still, prey seem remarkably capable of interpreting many of these. For instance, the most exhaustively studied laboratory mouse avoids highly volatile pyrazine analogues in wolf urine (Osada et al. 2013), but equally well heavy-weight proteins, such as Feld4 and Mup13 from, respectively, cat saliva and rat urine (Papes et al. 2010). Compounds that possess such varying chemical properties may inform the prey in different ways. Volatile compounds will readily become airborne and, therefore, may expose a predator's presence at a distance, at least up-wind. On the other hand, when a prey arrives at a location replete with high-mass, substrate-bound compounds, predator cue detection may signify that the danger is (or was) in close proximity. In principle, prey could even judge the age of a predator's scent (and hence, its information content) on the basis of the relative density of volatile and non-volatile elements; the latter tend to linger in the environment for longer periods. Whether animals indeed interpret predator-derived cues to such extent, rather than using them simply as an on/off switch for anti-predatory responses, is incompletely understood (Bytheway et al. 2013; Van Buskirk et al. 2014).

In Squamates, the use of the chemical senses in detecting a predator's presence is well appreciated (Thoen et al. 1986; Gutzke et al. 1993; Van Damme and Quick 2001; Bealor and O'Neil Krekorian 2006; Webb et al. 2010b). Which kind of molecules induce anti-predatory behaviour and how they are read is, however, rarely studied. With their composite chemosensory system, lizards and snakes seem good model organisms to straighten out the roles of volatile and non-volatile chemical cues in predator detection. The squamate nasal epithelium is sensitive to

airborne stimuli that can be taken in by inhaling or sniffing with the nose. Their vomeronasal mucosa are generally thought to be stimulated by heavier molecules. However, lizards and snakes use their tongue to sample both the air ('air licking', 'lingual air sampling') and the substrate ('tongue touching', 'substrate tongue flicking'), so the environmental molecules that trigger the vomeronasal apparatus may be both airborne and substrate-deposited. Particularly lizards are convenient subjects for ethological studies as, upon detection of predator chemicals, they perform a varied range of conspicuous anti-predator behaviours (e.g. startling, tail-waving, and slow-motion behaviour; Thoen et al. 1986).

In this study, we analyse through behavioural assays what information may be conveyed to the European common lizard (*Zootoca vivipara*) through volatile cues, as well as, substrate-bound compounds derived from its predator, the European adder (*Vipera berus*). We assess the temporal change in composition of chemical traces left behind by adders, and whether this affects the response of the lizard. Chemical cues of varied age (time since deposition) are analysed with Proton-Transfer-Reaction Time-of-Flight Mass Spectrometry (PTR-TOF-MS), a technique developed for real-time measurement of volatile organic compounds (Jordan et al. 2009), and only recently introduced in the research domain of vertebrate chemical ecology (Chapter 6).

Materials & Methods

Study animals

Twenty-two adult male common lizards and one adult male European adder were captured in March 2016, in the nature reserve Marum (Brecht, Belgium). This area, dominated by moist heathland, harbours a dense population of common lizards and one of Western Europe's largest adder populations (Bauwens et al. 2016). All animals were transported in cotton bags to the laboratory at the University of Antwerp where they were housed during the course of the experiment. Bodyweights and snout-vent lengths (SVL) of the sampled lizards were 2.97 ± 0.14 g and 47.9 ± 0.9 mm (mean ± SE), respectively. The adder had an SVL of 420 mm and weighed 47 grams.

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 their natural environment. The sides were covered with paper in order to prevent lizards from interacting and exchanging behavioural cues, which could

impact focal observation results. Above one side of the terrarium, a 60-Watt incandescent lamp was suspended to provide an optimal temperature gradient and a 12:12 hour light:dark circadian rhythm. At noon, lamps were switched off for half an hour to prevent overheating. Water was available ad libitum and lizards were fed vitamin E dusted crickets (*Acheta domesticus*) twice a week and wax moth larvae (*Galleria mellonella*) once a week. Water was vaporised inside the terrariums daily to guarantee a humid environment.

The adder was housed in a separate room under lighting, heating and watering conditions similar to that of the lizards. The animal was not fed during its stay in our lab. This limits a potential effect of the adder's diet on the lizard's response. The adder used in the experiments was recaptured within the context of a monitoring program three months after we released it back into the field. It was found to be in good health, suggesting that the study did not instigate any lasting nuisance (Bauwens D, pers. comm.). Animal capturing and housing was conducted with permission of the Nature and Forest Agency of Belgium (permit reference number: ANB/ BL/FF-V16-00012) and all experiments were approved by the ethical committee of the University of Antwerp (2015-34). After the experiment, the animals were released at the location of capture.

Scent collection and preparation

We are interested in how lizards pick up chemical cues left behind by a predator on the substrate or on elements in the environment. The ability to recognise such 'indirect' signals seems especially valuable in a predation context, where direct contact with the original source of the cue is likely to be fatal. We therefore use sterile cotton gauzes (Multipharma, Brussels, Belgium) as a substrate to collect scent for presenting in focal observations and to use in chemical analyses. The gauzes were commercial-standard size of 5 × 5 cm, made up of a folded 19 × 12 cm tissue of 40 g/m^2 (8 layers). Prior to use, the gauzes were incubated overnight in a drying oven at 60 °C to cleanse out any volatiles characteristic of the gauzes. They were handled with clean tweezers and vinyl gloves to prevent contamination with human scent. Subsequently, these gauzes were either used as experimental controls or subjected to an odour treatment as described hereafter.

Fresh predator scent was collected by repeatedly rubbing a gauze over the adder's vent in a rostro-caudal direction. To analyse how the chemical composition of deposited adder scent changes over time and to prepare aged scent for focal observations, we placed freshly scented gauzes into 120 mL open-

top glass jars with a clean one-layer gauze tissue over the top. We choose to not close the glass jars to allow a natural diffusion of molecules from the gauze into the air. Temperature within the jars was kept at 25 °C. The standardization protocol prevented mimicking natural conditions (i.e. wind and other environmental influences), but made it possible to have comparably aged scent treatments for use in both chemical analyses and focal observations.

For the chemical analyses, scented tissues were left in the jars for a total of three days. Volatiles emanating from them were sampled with a PTR-TOF-MS (see below) at the following points in time: 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4.5 h, 6 h, 12 h, 24 h and 72 h after initial collection. To analyse how scent aging impacts the lizards' anti-predatory response, freshly scented tissues and tissues aged 2 and 12 hours (hereafter named 2 h and 12 h) were used in the bio-essay (see below). The latter two ageing treatments were selected based on results from chemical analyses and in such a way that the scent chemical composition differed considerably among them (see Result section).

Analysis of volatile compounds.

Volatile compounds emanating from tissues of different 'age' (fresh, and 0.5 hours – 3 days after scent collection) were analysed with a PTR-TOF-MS instrument (model 8000, Ionicon Analytik GmbH, Innsbruck, Austria) operated using H_3O^+ as primary ion. The spectrometer's PEEK capillary sampler was inserted into the jar's headspace and withdrew the air at a flow rate of 120 mL min^{-1} (82 µmol s^{-1}). We analysed the headspace air of four jars containing adder-scented tissues and in three control jars containing non-scented gauzes. The operational conditions of the drift tube were 600 V of electric potential, a temperature of 80 °C, and 2.3 mbar of pressure, affording a field density ratio of ~140 Townsend (1 Td = 10^{-17} cm^2 V^{-1} s^{-1}). The spectral sampling rate was set to 31250 spectra s^{-1} ranging from mass m/z 1 to 315. Further information about operational settings can be found in Portillo-Estrada et al. (2015). Once the online monitoring of volatile chemical concentrations in the jars revealed stable values (typically within 5 seconds), the mass spectra were recorded for 30 seconds. From these we selected the central ten seconds for further data analysis (one measure per second; these ten measurements were, subsequently, pooled together). Spectra were analysed with PTR-MS Viewer v3.2 (Tofwerk AG, Switzerland). As described in Portillo-Estrada et al. (2018) we calculated the instrument's transmission curve factors with a standard gas mixture, calibrated spectrum mass range, did multi-peak

discrimination analysis, chose the coefficient of reaction, and calculated molecule concentrations (see also the article's Supplementary Information). Molecular formulae were assessed by targeting unique atomic compositions at a compound mass resolution of 0.0001 atomic mass unit. Molecules with the lowest masses related to a single naturally occurring configuration. For compound structure verification of molecules with higher masses we referred to our previous study in which we analysed the body scent of adders using gas chromatography mass spectrometry (GC-MS; Chapter 6).

The concentrations of volatiles in all jars were measured at several time-points: 0 h (i.e. fresh scent), 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4.5 h, 6 h, 12 h, 24 h, and 72 hours. We evaluated the presence/absence of specific compounds by comparing the data of scented gauzes to control gauzes using a Student's *t*-test at $P < 0.05$ significance level. Reported concentrations are the average (n = 4) signals of the scented gauzes at every given time-point after subtracting the average signal of the control gauzes.

Focal observations

Freshly scented gauzes, and gauzes aged 2 hours and 12 hours were transferred to a freezer at −30 °C until their use in the bio-essay. Freezing effectively conserves the chemical composition of body odours (Lenochova et al. 2009). Gauzes of different scent ages were kept separately and were marked with a coloured thread according to the applied age treatment to prevent mix-ups. During the focal observation, these threads were buried under sand to prevent varying interests in cotton gauzes due to a visual bias (Putman et al. 2017; Figure 1). Gauzes were never frozen for more than 24 hours. Shortly prior to the start of an observation, four gauzes relevant to the assessed treatment (i.e. control, fresh, 2 h or 12 h) were removed from the freezer and left to thaw for 1 minute. Preliminary testing had revealed that the gauzes reached room temperature within this short time interval. The defrosted tissues were then placed in each of the four corners of an observation arena (50 x 40 x 40 cm) of which the bottom had been covered with clean sand. One of the arena's walls was coated with a dark window film (Norauto), allowing observation of the lizard without disturbing it. A 60-Watt incandescent lamp suspended above the arena provided heat to stimulate lizard activity.

We measured the body temperature (cloacal) of each lizard to ensure it was within the preferred temperature range of the species (i.e. 26.7-34.8 °C; Van

Figure 1 set-up of the observation arena. The gauzes containing the odour treatment are present in each corner of the test arena. Below on the left, a focal lizard is exploring the arena

Damme et al. 1986; Gvozdík 2002). The individual was then placed in the centre of the test arena and during ten minutes its behaviour was scored using the software JWatcher v1.0 (Blumstein & Daniel 2007). We counted the number of Tongue flicks. We also noted the time until the first cue-contacting tongue flick (hereafter: Latency). Upon touching one of the gauzes with their tongue, lizards typically performed several tongue flicks before changing position. We noted the number of these Source-directed tongue flicks. Furthermore, we recorded the number of Foot shakes, Tail vibrations, Startles, Bites and timed slow-motion behaviour all of which are considered indicator of stress in lizards (Ruxton et al. 2004; Mori 1990; Telemeco et al. 2011; Font et al. 2012b; see Verbeek 1972 and Thoen et al. 1986 for detailed descriptions). As we saw very few of any of these behaviours before the first cue-contacting tongue flick, we decided to collapse these variables into a general Stress variable by summing them (slow-motion behaviour was included as the number of bouts). More common behaviour, such as Walking, Sitting and Basking, were timed (in seconds).

The order of treatment presentation was randomised (random repeated measures design). Each observation of the same individual was done at the same time of the day to limit variability due to general diurnal activity pattern changes. There was a time period of 48 hours between each observation to prevent carry-over effects between trials. It took one day to complete one trial in which all individuals were observed once. After each observation the focal lizard was placed back in its home terrarium and the sand covering the bottom of the observation arena was replaced.

Statistical analyses of focal observations

Statistics were done in R version 3.3.0 (R Core Team 2016). We summarised lizard behaviour for two consecutive time slots: (1) between the start of the observation

and the moment at which the lizard's tongue touched a gauze for the first time; and (2) from then until the end of the observation period. We will assume that lizards in the former period (hereafter: 'volatiles-only phase') were in contact with the volatile, but not with substrate-bound predator molecules; while lizards could be informed by both types of molecules in the latter time slot (hereafter: 'full-scent phase'). Linear mixed-effect models (LMM; lme4 package, Bates et al. 2015) were used for normally distributed time variables. When raw data was not normally distributed, normality was achieved using the log-transformation. The duration of slow motion exhibited a highly skewed distribution with an excess of zeros. Because transformations did not help, we recoded this variable into a binomial quantity taking '0' when the behaviour had not been observed in the pertaining observation period and '1' when it did occur. Consequently, we analysed the recoded Slow-motion variable using generalised linear mixed-effect models (GLMM) with a binomial fit and logit link function. For the count data, GLMMs with a Poisson fit or a negative binomial fit (depending on which distribution fitted the data best) and log link function were used.

Full models included main effects, as well as, all two-way interactions between fixed effects. Besides the main variable of focus, which is treatment, other variables were included in the full models to account for possible variation not due to Treatment. For all models that analysed Tongue flicks as a dependent variable we included Walking as a covariate. This is necessary because tongue flicking mainly occurs when the lizard is actively exploring the arena (Thoen et al. 1986; Van Damme et al. 1995; Schulterbrandt et al. 2008). Latency was added to the model when not yet present as a dependent variable. It was not included in models that already contained Walking (i.e. the models explaining variance in Tongue flicks) as both variables are highly correlated. The covariate Latency accounts for the difference in timing of the first cue-contacting tongue flick. Additionally, lizard identity was entered into all LMMs and GLMMs as a random effect to account for the repeated use of the same lizard.

Due to the repeated measures design, all lizards were presented with adder scent four times. Although these scents differed in scent age, the history of treatment presentation could have an impact on how a lizard responds to the next treatment. In such case, lizards may change their behaviour merely as a result of the number of tests to which they were subjected and these effects are additive. This is accounted for by including the trial number (hereafter Trial) as a fixed

effect. However, a lizard's behaviour may be more strongly affected by the freshest scent treatments opposed to the older scents and the odourless control. In such case, a Cumulative variable is more appropriate. This value was obtained by summing predefined percentages according to the scent treatments that were given in previous observation rounds. The percentage assigned to a certain scent treatment is equivalent to its impact on further observations. These were chosen by selecting the Cumulative variable that resulted in models with the lowest AICs. Because no behavioural variable acts the same way, the percentages generating the most optimal model can differ between response variables. By way of example, the Cumulative variable values applied to Walking in the pre-directed tongue flick period are given in Table S1.

Assumptions regarding normality of residuals (for LMM), homoscedasticity and linearity were met. Overdispersion in Poisson-models was accounted for by including an observation-level random effect (Harrison 2014). Models were compared using the second-order Akaike Information Criterion (AIC_c) as well as their Akaike weights (w_i) (Symonds and Moussalli 2011) by applying the confset function from the AICcmodavg package (Mazerolle 2016). Predictor weights (= the summarised Akaike weights of all candidate models in which an explanatory variable appears) were used to estimate the probability that a certain variable is a component of the best model. To test the differences between the means over treatments, multiple comparisons were done with a Bonferroni correction using the lsmeans package (Lenth 2016). Two out of the twenty-two lizards fled to one of the treated gauzes before the observation was started during the control observation. There was therefore no data on the behaviour before the first directed tongue flick in the control treatment. These individuals were left out of the pre-directed tongue flick analyses.

Results

Adder scent composition

The PTR-TOF-MS measurements revealed a wide variety of volatiles emitted by the European adder. The airborne scent cue was composed of 35 ions with concentrations from tens of parts per trillion (ppt) to tens of parts per billion(ppb). Molecular masses ranged from 32.042 (methanol) to 161.243 g/mol ($C_{11}H_{15}N$) (Table 1). The mass of seven ions could unambiguously be linked to specific

Molecular formula of the protonated compound	Molecular Mass	Compound name	Initial concentration (ppb)
$(CH_4O)H^+$	33.0335	Methanol [B]	26.9 ± 1.5
$(HCl)H^+$	36.9840	Hydrogen chloride [B]	7.3 ± 1.2
$(C_3H_4)H^+$	41.0386	Propyne [B]	2.43 ± 0.33
$(C_2H_3N)H^+$	42.0338	Acetonitrile [B]	0.095 ± 0.019
$(C_2H_2O)H^+$	43.0178	Ethenone [B]	3.64 ± 0.48
$(C_3H_6)H^+$	43.0542	Propene [B]	4.5 ± 1.4
$(C_2H_6O)H^+$	47.0491	Ethanol [A,B]	16.8 ± 2.4
$(C_3H_4O)H^+$	57.0335		1.46 ± 0.09
$(C_3H_6O)H^+$	59.0491	Acetone [A]	12.883 ± 0.047
$(C_2H_4O_2)H^+$	61.0284	Acetic acid [A,1]	4.94 ± 0.27
$(C_5H_8)H^+$	69.0699	Isoprene [A]	2.43 ± 0.08
$(C_4H_6O)H^+$	71.0491		0.873 ± 0.046
$(C_5H_{10})H^+$	71.0855		0.42 ± 0.05
$(C_4H_8O)H^+$	73.0648		3.7 ± 0.9
$(C_3H_6O_2)H^+$	75.0441	Propanoic acid [A,1,2]	1.50 ± 0.09
$(C_6H_8)H^+$	81.0699		1.86 ± 0.15
$(C_5H_6O)H^+$	83.0491		0.501 ± 0.017
$(C_6H_{10})H^+$	83.0855		1.56 ± 0.10
$(C_4H_6O_2)H^+$	87.0441		0.32 ± 0.10
$(C_5H_{10}O)H^+$	87.0804	3-Methylbutanal [1]	0.241 ± 0.017
$(C_3H_4O_3)H^+$	89.0233		0.046 ± 0.015
$(C_4H_8O_2)H^+$	89.0597	2-Methylpropanoic acid [1,2] Butanoic acid [1,2,3] 1,4-Dioxane [3]	0.480 ± 0.011
$(C_6H_8O)H^+$	97.0648		0.328 ± 0.020
$(C_5H_{10}O_2)H^+$	103.0754	Methylbutanoic acid [1,2,3] Pentanoic acid [3]	0.31 ± 0.07
$(C_4H_8O_3)H^+$	105.0546		0.332 ± 0.034
$(C_6H_8O_2)H^+$	113.0597		0.348 ± 0.007
$(C_7H_{12}O)H^+$	113.0961		0.230 ± 0.017
$(C_6H_{10}O_2)H^+$	115.0754		0.118 ± 0.008
$(C_9H_8)H^+$	117.0699		0.072 ± 0.011
$(C_6H_{12}O_2)H^+$	117.0910	3-Methylene-2-pentanone [A] (Hexanoic acid [3] Methyl pentanoic acid [3])	0.086 ± 0.018
$(C_9H_{10})H^+$	119.0855		1.40 ± 0.19
$(C_8H_8O)H^+$	121.0648		1.40 ± 0.08
$(C_{10}H_{12}O)H^+$	149.0961		0.071 ± 0.008
$(C_{10}H_{16}O)H^+$	153.1274		0.0599 ± 0.0046
$(C_{11}H_{15}N)H^+$	162.1277		0.063 ± 0.010
Total sum			99.7 ± 4.4

[A] verified with GC-MS (Chapter 6); [B] only one likely structure that is naturally occuring; verified in other snake species: [1] Wood et al. 1995, [2] Simpson et al. 1993, [3] Chunfu et al. 2019.

Table 1 (left) List of volatile organic compounds detected with PTR-TOF-MS emitted by the adder. Animal scent was collected by gauzes (in four replicates) and transferred to 120 mL chambers. Scent concentrations were measured in headspaces during 10 seconds with PTR-TOF-MS. The compounds reported have a significantly (t-test, *P*-value < 0.05) higher concentration than in control gauzes and the values reported are corrected by the concentration in control gauzes. Compounds with capital letters in subscript were verified by us. If compounds were found in other snake species this is indicated with a number in superscript and references are found underneath the table.

naturally occurring molecular structures. Five compounds of higher molecular mass were verified by GC-MS analyses (see Chapter 6). The structures of the remaining 23 ions could not be determined with certainty. We could resolve the presence of at least 35 volatile compounds in the adder's scent deposits.

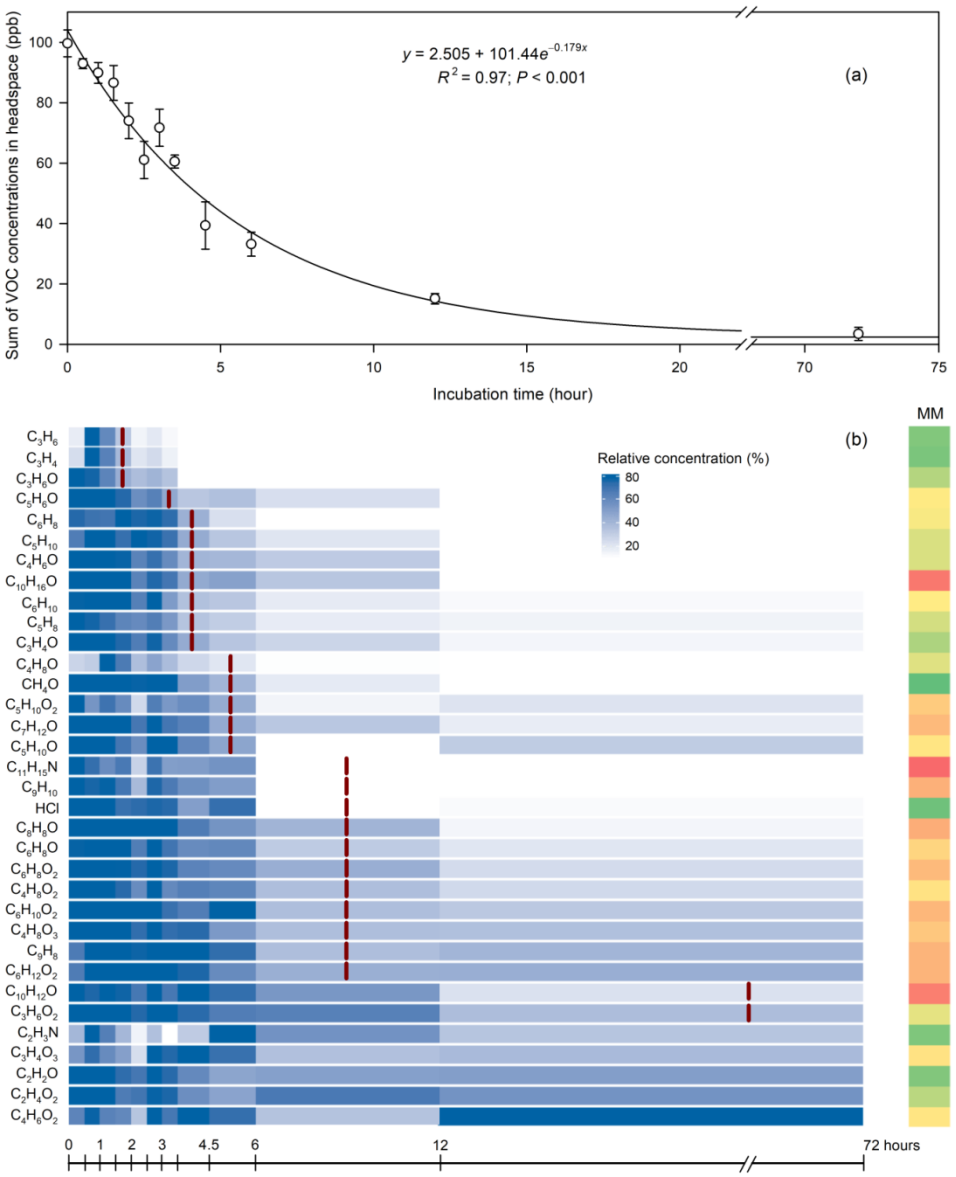
The cue was dominated by methanol, followed by ethanol and acetone. The combined concentration of volatile emissions decreased exponentially over time, declining one quarter of its initial magnitude after two hours of exposure to ambient air (Fig. 2a). After twelve hours of fade-out, the total VOC emissions had lowered to 15 % of the initial concentration. Beyond this time, the emissions became asymptotic and stabilised at approximately 3 % of the initial levels. The most stable chemicals that showed only limited change in concentrations over the different scent ages are found at the bottom of Figure 2b. Those that decreased the most in concentration over the first few hours are situated at the top of the same figure.

Focal observations

Although Treatment was intermediately supported in models describing variance in Latency (predictor weight = 70 %; Table 2), post-hoc testing did not reveal significant differences between the scent-age treatments and the odourless control (fresh scent – control: $t_{62.66}$ = - 2.26, P = 0.082; 2h – control: $t_{62.54}$ = - 0.54, P = 1.00; 12 h – control = $t_{62.49}$ = - 2.22, P = 0.091). In this 'volatiles-only' phase, lizards did curtail the proportion of time spent Walking when gauzes with fresh snake odour were present compared to the odourless control situation ($t_{67.42}$ = -2.65, P = 0.030; Fig. 3a). This was not the case when they experienced two- ($t_{66.24}$ = - 1.83, P = 0.22) or twelve-hours old snake scent ($t_{66.06}$ = - 0.34, P = 1.00). We found no indication for an effect of Treatment on the number of Tongue flicks or Stress-related behaviours for this phase (Table 2).

In the 'full-scent' phase, adder scent of all ages caused significant changes

Figure 2 Fading of the European adder's scent deposits during three days of incubation in 120 mL glass jars. (a) Fade-out of the total scent represented by the decrease in the summarised concentrations of all VOCs identified. Circles represent means with error flags depicting the SE (n = 4). (b) Proportional fade-out of individual compounds. A dark tone reflects the highest concentration of the specific compound indicated with their molecular formula on the left hand side of the figure. Lighter tones indicate proportional decreases relative to this highest concentration. Red lines represent the approximated point of 50 % fade-out. On the right hand side of the figure we have illustrated the molecular weight of the specific compounds. Green tones indicate light chemical structures, whereas red tones indicate heavier structures.



in lizard behaviour compared to the odourless control. However, the nature of the behavioural response differed across scent ages. The number of Source-directed tongue flicks was higher compared to the odourless control when the scent was fresh (Z = 3.49, P = 0.0014) or two hours old (Z = 5.17, P < 0.0001), but not when it had dissipated for twelve hours (Z = 2.34, P = 0.057; Table 2 and Fig. 3b). Lizards confronted with fresh scent were more inclined towards Slow-motion behaviour compared to lizards in the control situation (Z = 2.55, P = 0.032; Table 2 and Fig. 3d). No such tendency was seen when tissues had been exposed to the air for two (Z = 2.21, P = 0.081) or twelve hours (Z = 1.46, P = 0.44). On the other hand, in comparison with the control situation, lizards exhibited an increased number of Startles when confronted with two- (Z = 2.53, P = 0.034) and twelve-hour-old adder scents (Z = 2.72, P = 0.020), but not when presented with fresh scent (Z = 1.42, P = 0.47; Table 2 and Fig. 3c). No significant differences were found for other variables. Foot shakes and Bites were only seen occasionally, which made it impossible to model variance over populations with mixed models for this variable. A Chi-squared test on the Foot shakes and Bites data, showed no significant differences between any of the treatments for both variables (χ^2_3 = 5.26, P = 0.15

Table 2 Predictor weights for each variable included in mixed-effect models describing variance in common lizard behaviour. Individual behaviours are analysed for the pre- and post-directed tongue flick period separately. Treat = the offered scent treatment; Exp = the experience index; Lat = Latency until the first substrate flick

	Treat	**Exp**	**Lat**	**Treat x Exp**	**Lat x Exp**	**Treat x Lat**
Volatiles-only phase						
Latency	0.70	0.47	-	0.02	-	-
Tongue flick	0.23	0.44	-	0.01	-	-
Walk (s)	**0.86**	0.66	**1.00**	0.42	0.20	0.02
Stress	0.13	**0.80**	**0.95**	0.01	0.20	0.01
Full-scent phase						
Tongue flick	0.29	**1.00**	-	0.17	-	-
Source flick	**1.00**	**1.00**	-	0.12	-	-
Walk	0.73	**0.99**	**1.00**	0.38	0.25	0.67
Startles	**0.95**	0.40	0.34	0.01	0.04	0.04
Tail vibrations	0.28	0.67	0.28	0.01	0.02	0.02
Slow motion	**0.96**	**1.00**	0.37	0.00	0.14	0.06

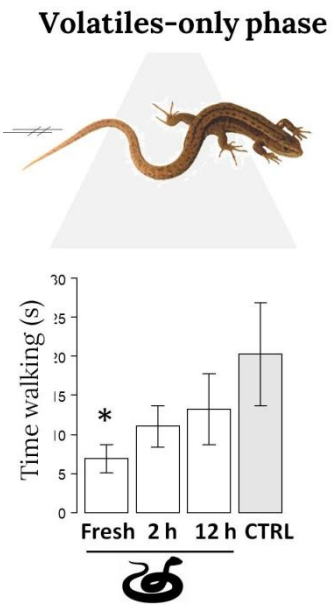


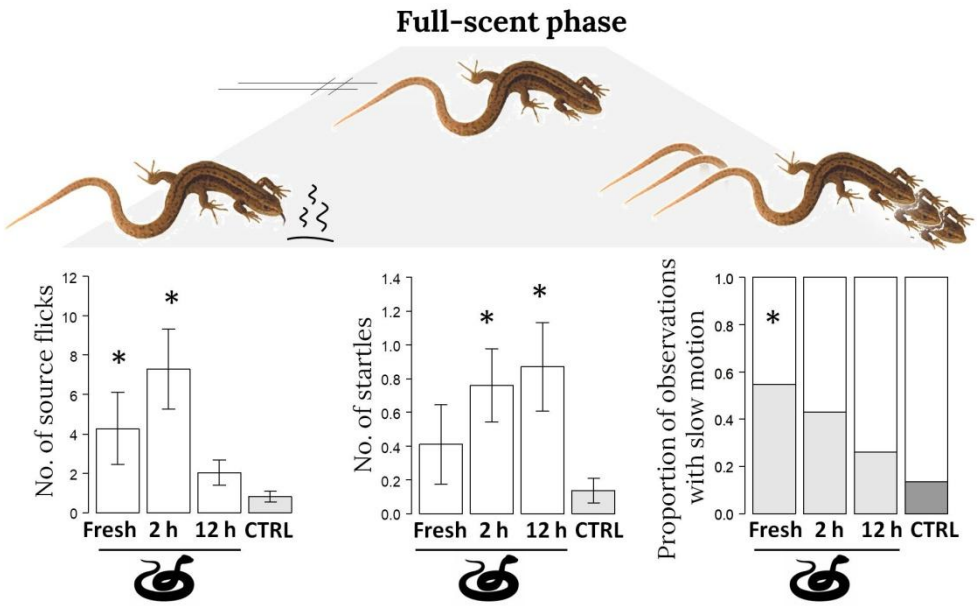
Figure 3 (right and continuous on next page) Behaviour of lizards in the 'volatiles-only' and 'full-scent' phase when smelling adder scent of increasing age (from fresh to twelve hours old) or an odourless control. For all graphs besides the one showing Slow-motion (which is depicted in proportions), means are given with the error bars representing the standard error. An asterisk indicates significant differences ($P > 0.05$) with the control treatment. *P*-values were calculated through post-hoc multiple comparisons based on the best models presented in Table 2.

and χ^2_3 = 3.49, P = 0.32, respectively). Nevertheless, neither of these behaviours were performed by lizards during control essays in contrast to occasional observations in the various adder scent treatments.

Discussion

PTR-TOF-MS revealed a plethora of low-weight molecules emanating from adder chemical deposits. Most of the compounds found in our earlier study, which directly assessed adder body emissions (Chapter 6), were found in the current analyses. This verifies the appropriateness of sampling with cotton gauzes. More than half of the adder cue's volume is composed of only three compounds, namely, methanol, ethanol, and acetone. These are commonly occurring metabolites, for instance, present in the breath (e.g. Turner et al. 2006) and excrements (e.g. Andersen and Vulpius 1999; Kwak et al. 2013) of vertebrates, exuded from the glands of invertebrates (e.g. Cammaerts et al. 1981) and derived from plant materials (e.g. Janzen 1977; Portillo-estrada 2018). The compounds are not known to be involved in chemical interactions between vertebrates (O'Connell et al. 1978), and due to their omnipresence may seem inappropriate as such. Although, methanol and ethanol exuded in excess by fermenting fruits and nectar evoke a behavioural response in some animals (e.g. beetles, Dowd and Bartelt 1991; treeshrews, Wiens et al. 2008; primates, Gochman et al. 2016). Acetone attracts various kinds of arthropod parasites to their vertebrate hosts (Jones et al. 1976; Bhasin et al. 2001; Kline et al. 2012).

The remainder of a minimum of 35 different molecules are present in much lower quantities, mostly trace levels. Reptilians have been shown to be



capable of detecting minor compounds in complex mixtures (Romero-Diaz et al. 2020). Therefore, such molecules may, equally well, be bioactive compounds. In male mammals, short-chained carboxylic acids such as acetic acid, propanoic acid and 2-methylpropanoic acid, evoke behaviours of sexual arousal (Michael et al. 1971; Nielsen et al. 2011).

More germane to our current question is that the PTR-TOF-MS instrument's high time resolution and sensitivity allowed for in-detail tracking of temporal dynamics in volatile discharges from aging adder cues. Due to the chemically divers nature of the cue, we see some molecules disappearing from the mixture already after two to three hours and others lingering for at least three days. Over this time span, concentrations of single compounds decreased at varying rates causing ratios between compounds to change. Whether a certain molecule is more or less prone to evaporate is dependent on several factors. Firstly, we observed that the components initially leaving the adder's cue are relatively non-polar, low-weight molecules, such as ethanol, propene, propyne, and acetone. Structures with similar molecular weights but higher polarities, such as methanol, hydrogen chloride, acetonitrile, and ethenone, are more under the influence of other polar molecules in the chemical matrix (Müller-Schwarze 2006). This may be the reason why they are retained substantially longer in the cue, when compared to their non-polar counterparts. Already before, has the

physicochemistry of the embedding chemical mixture been proven to exert an important effect on the persistence of chemical cues and, consequently, on how these are perceived by other animals (Regnier and Goodwin 1977; Müller-Schwarze 2006). For instance, when a European rabbit (*Oryctolagus cuniculus*) becomes dominant, a new compound appears in its secretion (2-phenoxyethanol) which slows the release rate of other signalling compounds (Hayes et al. 2003). It is suggested that, consequently, the rabbit will dominate the olfactory environment in much the same way as it does the physical environment. Note that in our analyses acetic and propanoic acid, which in other animals exude pheromonal effects (see previous paragraph), also belong to the most persistent fraction of the adder's cue, lasting at least three days. Perhaps, in these snakes, such molecules are also retained by the chemical matrix to signal information to conspecifics.

For a lizard, long-lasting adder-derived volatiles seem unfavourable as kairomones. Any anti-predatory response diverts the lizard's attention and energy away from other ecologically important tasks, such as mate-finding and foraging (Kats and Dill 1998). Therefore, anti-predatory behaviours may only be beneficial if an odour cue unambiguously signals a predator's imminent attack. This is less likely to be the case with old odour cues (Conover 2007; Bytheway et al. 2013). For this reason it is not surprising that, during focal observations, lizards reduced Walking when smelling airborne chemicals emanating from only the freshest adder scent. After this period, the concentration of adder volatiles may become too minute to be detectable, or lizards choose to ignore adder chemicals within an 'old-scent' configuration.

It remains difficult to select kairomone candidates. We know only little about how sensitive chemoreceptors in the olfactory and vomerolfactory epithelia of lizards are, and whether lizards rather focus on proportional changes or concentrations of specific molecules. As far as we can observe, compounds that show considerable change in the first few hours are isoprene (C_5H_8), C_5H_6O, C_4H_6O, $C_{10}H_{16}O$, $C_{11}H_{15}N$, and $C_5H_{10}O_2$, besides the previously discussed generally occurring neutral, low-weight molecules. Apart from isoprene, these molecules structural features remain to be assessed. The molecule with chemical formula $C_5H_{10}O_2$ could be methylbutanoic acid, pentanoic acid, or both. These have been identified in gland secretions and sloughs of a wide variety of predatory snakes (Simpson et al. 1993; Wood et al. 1995; Chunfu et al. 2019) and other vertebrate and invertebrate species (El-Sayed 2020).

Adder scent emanates airborne molecules that seem to alarm lizards to some extent. However, it is not until they perceive substrate-born predator chemicals, in addition, that lizards explore the cue through a rise in the number of Source-directed tongue flicks, and subsequently exhibit the full array of stress-indicating behaviours. This corroborates earlier work that demonstrated that the main adder kairomones are extractable with classical washing techniques (Chapter 5). In the current experiment, we additionally show that the nature of the behavioural response changes with the age of the adder's scent. When running across freshly deposited scent, lizards start to move in slow-motion style. When confronted with aged adder scent, lizards showed more startling behaviours. This seems to suggest a strategy switch in lizard anti-predator tactics from a 'cryptic escape' towards a 'flight escape' when adder scent ages. Possibly, fresh scent relays predator proximity, but no further information on the exact location or awareness of the adder towards the lizard (Bytheway et al. 2013). It is then better to stay unnoticed by performing slow movements (Van Damme and Quick 2001) while trying to attain visual contact of the predator. When detecting aged scent, chances are higher that the predator is already out of the range of detection. Consequently, it may be safer to perform more conspicuous behaviours, such as Startles, in an attempt to flee (Bauwens and Thoen 1981; Van Damme and Quick 2001).

A change in escape tactics over scent age has seldom been investigated before (but see e.g. Peacor 2006; Cavaggioni et al. 2008; Bytheway et al. 2013). However, other factors have been shown to affect a lizard's choice in anti-predatory behaviour. Bauwens and Thoen (1981) observed a switch in strategy between gravid and non-gravid common lizard females, where gravid lizards employed a cryptic escape and trusted on their camouflage to remain undetected by a predator (Heatwole 1968; Lima and Dill 1990), whereas non-gravid ones laid their chances more in a true flight escape. This seems to be caused by an effect of reproductive state on locomotor performance (Bauwens and Thoen 1981). Together with our results, it demonstrates the flexibility of anti-predator tactics within this species and the influence of both in- and external factors in decision-making.

In conclusion, we revealed that the European common lizard can interpret different facets of European adder deposits; responding to both volatile and substrate-bound molecules, as well as, fresh and aged scent cues. Moreover, the lizard is able to adjust its anti-predatory strategy towards these various aspects, suggesting differing contained messages. PTR-TOF-MS allowed us to look into the complexity of the airborne aspect of adder scent and how it varies over time. We

strongly encourage the use of this device in the field of vertebrate chemical ecology.

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Chapter 8

General discussion

I hope that this thesis will contribute to the field of chemical ecology in two ways. First, by adding new data to a growing body of literature on chemosensory predator recognition in lizards, information that both advances and broadens our understanding of this matter. Second, by exploring and applying new methods (in combination with more established ones) that may offer interesting avenues. Below, I first reiterate what I think are the major results that emerge from the conceptual-experimental part of the thesis (Chapters 2-4), then frame them against earlier findings in the field, as available in the literature. Next, I discuss how the methods explored in the second part (Chapters 5-7) could be used in future research in chemical ecology, using a number of examples that may be biased by my personal interests.

Two prey species, four predator species, two continents, two habitats, some answers and more questions

This thesis was initially triggered by concerns about the advance of the small Indian mongoose on the Balkan mainland and several islands in the Adriatic (Barun et al. 2008). Mongooses have been introduced into a multitude of habitats around the world to control rodent or snake populations, but rapidly gained a reputation for the collateral damage they inflicted upon native non-target populations of birds and reptiles (Berentsen et al. 2018). In the Balkan, the Dalmatian wall lizard (*Podarcis melisellensis*) is a dominant member of the herpetofauna, especially on the many islands in the Adriatic Sea. This lacertid lizard was naïve to mongooses until their introduction in the 1920s. Stronger, populations of the species on smaller islands have lived in isolation from any mammal predator since the last glacial, i.e. approximately 10.000 years ago (Kryštufek and Kletečki 2007). How would *P. melisellensis* (and other lacertid lizards) respond to this novel threat? Chemosensory predator detection constitutes an important first line defence in lizards – would Dalmatian wall lizards be able to recognise mongoose scent and respond to it with an appropriate anti-predator behaviour? Would the response be general, or would some populations do better than others? Would prior experience matter, or particularities of the environment (insularity, presence/absence of other mammal predators)? Because the literature was completely silent on whether lacertids could pick up mongoose scent (and mammalian scent in general), it seemed prudent to first test this in a lacertid species naturally occurring in sympatry with mongooses (Chapter 2). Surprisingly,

the Asian grass lizard (*Takydromus sexlineatus*), at home in the original distributional range of the mongoose, did not respond to chemicals left behind by the predatory mammal – although it did mount the typical anti-predatory behavioural response when confronted with scent of a local snake predator. Even more puzzling was the finding that at least some Dalmatian wall lizards, despite having evolved in allopatry with the mongoose, do seem capable of identifying the predator's odour as dangerous (Chapter 3). This apparent paradox can have several possible explanations. One of these explanations is that it may be the result of stronger selection on mongoose recognition in *P. melisellensis* than in *T. sexlineatus*. Mongooses may develop a particular preference for preying on lizards in areas into which they are introduced. Alternatively, the mongoose may experience ecological release in its range of introduction (Salo et al. 2007; Paolucci et al. 2013). In Asia, small Indian mongooses are subjected to competitive pressures from larger congeners *H. edwardsii* and *H. smithii*, as well as other larger carnivores (Simberloff et al. 2000). Studies on morphological characteristics, such as body size, skull length, and canine tooth diameter, have shown consistent patterns with ecological release from such competitors on many invaded islands. However, mongooses in Croatia have to compete with beach martens (*Martes foina*) and, consequently, do not show character release (Barun et al. 2015). Therefore, ecological release of these mongooses in Croatia is unlikely.

Otherwise, the adopted behavioural strategy may be maladaptive in *P. melisellensis*. It has been argued in Chapter 2 that the scent of snakes may indicate their whereabouts more reliably compared to the scent of a mongoose due to differences in their level of mobility. Especially ambushing snakes tend to stay at the location of scent deposition for long periods of time (Chowdhury et al. 2017; Kartik 2018). As vigilance leads focus away from other ecological tasks, this would explain an absent response in *T. sexlineatus* towards the supposed inadicate indicator of mongoose presence. Consequently, if mongoose scent in a Croatian habitat is equally informative as it is in Indonesia, *P. melisellensis* may present a case of level 4 naivety (sensu Banks and Dickman, 2007) in which it 'over-responds' to the scent of the alien mongoose. However, using the chemical senses in the detection of actively foraging predators is likely adaptive as the Dalmatian wall lizard responds to scent of two native actively foraging snakes, i.e. *Hierophis gemonensis* and *Malpolon insignitus*. Despite data being scarce on the mobility patterns of snakes, there are indications that actively hunting snakes may cover

ranges that tend towards those of the mongoose in size (Carfagno and Weatherhead 2008; Lelièvre et al. 2012; Pitt et al. 2015). Therefore, I do not expect that a presumed difference in activity level between snakes and mongooses alone explains my results.

Alternatively, the lack of response in *T. sexlineatus* may not reflect an inability to detect mongoose chemical cues, but constitute adaptive low-key behaviour. In this scenario, the behavioural response of *P. melisellensis* would make up an example of an inadequate reaction to alien mongoose cues (Sih et al. 2010). The startles and foot shakes performed by *P. melisellensis* when confronted with predator scent are meant to confuse or discourage attack (Font et al. 2012b). However, mongooses may have such superior hunting strategies that predation is highly likely to be successful upon prey discovery. Therefore, mongooses might be difficult to mislead or dissuade from attacking. These mammals may, for instance, have keener eye-sight compared to snakes that have undergone lineage specific modifications to their sense of vision resulting from a likely nocturnal or fossorial ancestor (Perry et al. 2018).

The results obtained in Chapter 3 hinted strongly at an overall effect of insularity on chemosensorial abilities. Lizards from island populations seem to make less use of their vomeronasal system, even when living in sympatry with terrestrial predators. This seems in line with the Expensive Tissue Hypothesis (Aiello and Wheeler 1995) stating that animals living in environments that are poor in energy resources (such as islands) should invest in gut tissue, rather than in brain tissue. This instigated the (preliminary) study of relative brain area size in Chapter 4, which confirmed that island dwelling *P. melisellensis* tend to have relatively small accessory olfactory bulbs (the brain region involved in processing chemical information obtained through vomerolfaction) than mainland lizards. Whether these differences reflect genetic adaptation or phenotypic plasticity, and which exact factors have driven these modifications, remains a question open to investigation.

A disadvantage of using model systems to study complex eco-evolutionary questions is that one may end up with a lack of degrees of freedom. The model species or populations, study sites, habitats, ecological circumstances will differ in more aspects than just the one(s) that are tested. I realise that this is also the case in my study. It is evident that factors such as the taxonomic identity of the predator (e.g. mammal versus snake, phylogenetic distance to the prey species,

phylogenetic distance to other predators), its origin (native or introduced), its foraging style (ambush or widely foraging), particularities of the environment (e.g. island versus mainland, presence of other prey or predator species) could all influence the question of whether a prey will be able to recognise a predator. Isolating the effects of all these factors would require a much larger set of experiments and a difficult selection of models. In an attempt to replace such careful dissection by a more 'fuzzy' approach, I will gauge the importance of some of these factors by combining my results with evidence from other studies.

A broader view of lizard chemosensory predator recognition

Since the seminal paper by Thoen et al. (1986), 45 studies have reported on the chemosensorial predator recognition abilities of lizard species (Table 1). A total of 110 predator-prey species pairs were studied. The majority of these 110 pairs (n=78; 71%) featured snakes as the predator, 17 (15%) starred mammals, 11 (10%) saurophagous lizards and 4 (4%) spiders or centipedes. Overall, in 83 of the 110 pairs (75%) considered, the prey proved capable of recognising the presence of the predator on the basis of chemical cues. A failure to recognise it was reported for 26 pairs (24%); in one pair, the outcome was unclear. These numbers underscore the importance of chemoreception in lizard predator recognition in general, although publication bias towards positive results may be an issue.

Different types of predators

The results of Chapter 2-3 suggest that at least some species of lacertid lizards can identify snake predator chemicals, but not mammalian predators. Table 1 suggests that this is generally true for lizards. In 68 of the 78 experiments (87%) that used snakes as predators, lizards exhibited signs of recognition; in the 17 experiments using mammalian predators, only 8 did (47%). These proportions differ significantly (χ^2=14.04, P=0.0002). It is unclear what makes mammal odours more difficult to detect or identify to lizards. In theory, mammals could simply produce less kairomones than snakes. This seems highly unlikely, because many mammals use chemicals in intraspecific communication (Johnson 1973; Liberles 2014) and there is ample evidence that mammal prey species can pick up these odours (review in Apfelbach et al. 2005). Alternatively, mammals and snakes may be depositing different chemicals, and (some) lizards may not be equipped to detect mammal chemicals. This makes sense, given the difference in phylogenetic distance between lizards and snakes on the one hand, and lizards and mammals on

the other. Perhaps lizard chemoreceptors initially involved for intraspecific communication purposes (e.g. mate and rival assessment, species recognition) and therefore became specialised in 'reptilian' odour detection. Such a system might later be co-opted for the recognition of snake predators, but may well have fallen short when it came to picking up more divergent, mammalian molecules. Alas, as argued in the thesis, virtually nothing is known on the nature of kairomones produced by animals that prey on lizards, or on the chemoreceptors of those lizards, so at the moment it is impossible to evaluate these assertions.

A snake's specialisation in prey items and its foraging strategy may affect its (chemical) detectability and the level of threat it poses. According to the Threat-sensitivity Hypothesis (Helfman 1989), prey individuals should trade-off predator avoidance against other activities by altering their avoidance responses in a manner that reflects the magnitude of the predatory threat. Several studies on lizard-predator interactions have corroborated this idea by showing that lizards can distinguish between odours of saurophagous (dangerous) and non-saurophagous (innocent) snake species, and respond to them proportionately. For instance, individuals of *Zootoca vivipara* and several *Podarcis* species respond vehemently to chemicals left behind by snakes that frequently consume lizards (e.g. *Coronella austriaca*, *Hierophis viridiflavus*), but less so to those produced by snakes that only occasionally prey on lizards (e.g. *Natrix* species; Thoen et al. 1986; Van Damme and Quick 2001; Ortega et al. 2018). Also, given the choice, mountain log skinks (*Pseudomoia entrecasteauxii*) will prefer shelters labelled with chemicals of the red-bellied black snake (*Pseudechis porphyriacus*, an occasional saurophage) above those holding odours of the white-lipped snake (*Drysdalia coronoides*, a lizard-specialist) (Stapley 2003). My own results from Chapter 2 and 3 do not suggest that lizards' ability to detect snake chemicals depends on the predators hunting habits. Both odours of species that pursue an actively foraging strategy (e.g. the Montpellier snake, *Malpolon insignitus*) and adopt an ambushing tactic (e.g. the Asian vine snake, *Ahaetula prasina*) elicited the typical anti-predator behaviour in our model lizard species. This is confirmed by several studies listed in Table 1. For instance, hatchlings of the rock monitor, *Varanus albigularis*, avoid chemicals of both spitting cobras (*Naja nigricollis*, a widely foraging snake) and those of horned adders (*Bitis caudalis*, a sit-and-wait predator) (Phillips and Alberts 1992). Scent of both actively hunting marsh snakes (*Hemiaspis signata*) and ambushing broad-headed snakes (*Hoplocephalus bungaroides*) elicit anti-predator

Table 1 Literature review of chemosensory lizard – predator interactions. From left to right columns indicate the broader taxon to which prey lizards belong, the Latin species name of lizard and predator, the broad group to which predators belong and whether they are native to the focal lizards habitat or not, and if not how long ago were they introduced. Finally, the table indicates whether the focal lizard originated from mainland or island habitats, whether it effectively responded to predator scent with stress-indicating behaviours, and the reference in which the interaction was first investigated.

Lizard taxon	Lizard species	Predator species	Predator type	Predator origin	Time since introduction	Prey origin	Discrimination	Reference
Amphisbaenia	*Anguis fragilis*	*Coronella austriaca*	snake	native	-	mainland	yes	(Cabido et al. 2004)
Amphisbaenia	*Blanus cinereus*	*Coronella girondica*	snake	native	-	mainland	yes	(López and Martín 2001)
Anguimorpha	*Heloderma horridum*	*Agkistrodon bilineatus*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Boa constrictor*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Charina trivirgata*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Crotalus basiliscus*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Crotalus molossus*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Crotalus triseriatus*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Drymarchon corais*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Loxocemus bicolor*	snake	native	-	mainland	yes	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Masticophis*	snake	native	-	mainland	yes	(Balderas-Valdivia and

		mentovarius						Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Pituophis deppei*	snake	native	-	mainland	no	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*H. horridum*	*Pseudoelaphe flaviruf*	snake	native	-	mainland	no	(Balderas-Valdivia and Ramírez-Bautista 2005)
Anguimorpha	*Varanus albigularis*	*Bitis caudalis*	snake	native	-	mainland	yes	(Phillips and Alberts 1992)
Anguimorpha	*V. albigularis*	*Naja nigricollis*	snake	native	-	mainland	yes	(Phillips and Alberts 1992)
Gekkota	*Amalosia lesueurii*	*Cryptophis nigrescens*	snake	native	-	mainland	yes	(Webb et al. 2009)
Gekkota	*A. lesueurii*	*Demansia psammophis*	snake	native	-	mainland	yes	(Webb et al. 2009)
Gekkota	*A. lesueurii*	*Acanthophis antarcticus*	snake	native	-	mainland	yes	(Webb et al. 2009)
Gekkota	*A. lesueurii*	*Cacophis squamulosus*	snake	native	-	mainland	yes	(Webb et al. 2010a)
Gekkota	*A. lesueurii*	*Hemiaspis signata*	snake	native	-	mainland	yes	(Webb et al. 2010a)
Gekkota	*A. lesueurii*	*Hoplocephalus bungaroides*	snake	native	-	mainland	yes	(Downes and Shine 1998)
Gekkota	*Bavayia septuiclavis*	*Rattus exulans*	mammal	introduced	ca. 3000 ya	island	yes	(Gérard et al. 2014)
Gekkota	*B. septuiclavis*	*Rattus rattus*	mammal	introduced	ca. 200 ya	island	no	(Gérard et al. 2014)
Gekkota	*B. septuiclavis*	*Felis catus*	mammal	introduced	ca. 200 ya	island	no	(Gérard et al. 2014)
Gekkota	*Coleonyx brevis*	*Hypsiglena torquata*	snake	native	-	mainland	yes	(Dial and Schwenk 1996)
Gekkota	*C. variegatus*	*Phyllorhynchus decurtatus*	snake	native	-	mainland	yes	(Dial et al. 1989)
Gekkota	*Naultinus manukanus*	*Sphenodon punctatus*	lizard	native	-	island	yes	(Hoare et al. 2007)
Gekkota	*N. manukanus*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	yes	(Monks et al. 2019)
Gekkota	*Woodworthia maculata*	*Sphenodon punctatus*	lizard	native	-	island	yes	(Monks et al. 2019)

Gekkota	*W. maculata*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	yes	(Monks et al. 2019)
Iguania	*Ctenosaura similis*	*Boa constrictor*	snake	native	-	mainland	yes	(Farallo et al. 2010)
Iguania	*Dipsosaurus dorsalis*	*Lampropeltis getula californiae*	snake	native	-	mainland	yes	(Bealor and Krekorian 2002)
Iguania	*Liolaemus chiliensis*	*Philodryas chamissonis*	snake	native	-	mainland	yes	(Labra and Hoare 2015)
Iguania	*L. fitzgeraldi*	*Philodryas chamissonis*	snake	native	-	mainland	no	(Labra and Niemeyer 2004)
Iguania	*L. lemniscatus*	*Philodryas chamissonis*	snake	native	-	mainland	yes	(Labra and Niemeyer 2004)
Iguania	*L. nigroviridis*	*Philodryas chamissonis*	snake	native	-	mainland	yes	(Labra and Niemeyer 2004)
Iguania	*Sceloporus jarrovii*	*Lampropeltis pyromelana*	snake	native	-	mainland	no	(Simon et al. 1981)
Lacertidae	*Archaeolacerta bedriagae*	*Hierophis viridiflavus*	snake	native	-	island	yes	(Van Damme and Quick 2001)
Lacertidae	*Iberolacerta cyreni*	*Coronella austriaca*	snake	native	-	mainland	yes	(Ortega et al. 2018)
Lacertidae	*I. cyreni*	*Vipera latastei*	snake	native	-	mainland	yes	(Ortega et al. 2018)
Lacertidae	*I. galani*	*Coronella austriaca*	snake	native	-	mainland	yes	(Mencía et al. 2016)
Lacertidae	*I. galani*	*Vipera seoanei*	snake	native	-	mainland	yes	(Mencía et al. 2016)
Lacertidae	*I. horvathi*	*Coronella austriaca*	snake	native	-	mainland	yes	(Žagar et al. 2015)
Lacertidae	*Podarcis guadarramae*	*Coronella austriaca*	snake	native	-	mainland	yes	(Martín et al. 2015)
Lacertidae	*P. guadarramae*	*Coronella girondica*	snake	native	-	mainland	yes	(Martín et al. 2015)
Lacertidae	*P. hispanicus*	*Vipera latastei*	snake	native	-	mainland	yes	(Van Damme and Castilla 1996)

Lacertidae	*P. hispanicus*	*Vipera latastei*	snake	native	-	island	yes	(Van Damme and Castilla 1996)
Lacertidae	*P. lilfordi*	*Macroprotodon mauritanicus*	snake	introduced	> 5000 ya	island	no	(Mencía et al. 2017)
Lacertidae	*P. melisellensis*	*Hierophis gemonensis*	snake	native		mainland	yes	Chapter 3
Lacertidae	*P. melisellensis*	*Malpolon monspessulanus*	snake	native		mainland	yes	Chapter 3
Lacertidae	*P. melisellensis*	*Herpestes auropunctatus*	mammal	introduced	ca. 100 ya	mainland	yes	Chapter 3
Lacertidae	*P. melisellensis*	*Hierophis gemonensis*	snake	native	-	island	no	Chapter 3
Lacertidae	*P. melisellensis*	*Malpolon monspessulanus*	snake	native	-	island	no	Chapter 3
Lacertidae	*P. melisellensis*	*Herpestes auropunctatus*	mammal	introduced	ca. 100 ya	island	no	Chapter 3
Lacertidae	*P. muralis*	*Coronella austriaca*	snake	native	-	mainland	yes	(Amo et al. 2004b; 2005)
Lacertidae	*P. muralis*	*Coronella austriaca*	snake	native	-	mainland	yes	(Durand et al. 2012)
Lacertidae	*P. muralis*	*Hierophis viridiflavus*	snake	native	-	mainland	yes	(Durand et al. 2012)
Lacertidae	*P. muralis*	*Hierophis viridiflavus*	snake	native	-	island	no	(Durand et al. 2012)
Lacertidae	*P. muralis*	*Vipera aspis*	snake	native	-	mainland	yes	(Durand et al. 2012)
Lacertidae	*P. muralis*	*Vipera aspis*	snake	native	-	island	yes/no	(Durand et al. 2012)
Lacertidae	*P. pityusensis*	*Hemorrhois hippocrepis*	snake	introduced	11 ya	island	yes	(Ortega et al. 2017)
Lacertidae	*P. siculus*	*Coronella austriaca*	snake	native	-	mainland	yes	(Downes and Bauwens 2002)
Lacertidae	*P. siculus*	*Hierophis viridiflavus*	snake	native	-	island	yes	(Van Damme and Quick 2001)

Lacertidae	*P. siculus*	*Macroprotodon mauritanicus*	snake	introduced	> 5000 ya	island	yes	(Mencía et al. 2017)
Lacertidae	*P. tiliguerta*	*Hierophis viridiflavus*	snake	native	-	island	yes	(Van Damme and Quick 2001)
Lacertidae	*Scelarcis perspicillata*	*Macroprotodon mauritanicus*	snake	introduced	> 5000 ya	island	no	(Mencía et al. 2017)
Lacertidae	*Takydromus sexlineatus*	*Ahaetula prasina*	snake	native	-	island	yes	Chapter 2
Lacertidae	*T. sexlineatus*	*Herpestes auropunctatus*	mammal	native	-	island	no	Chapter 2
Lacertidae	*Zootoca vivipara*	*Coronella austriaca*	snake	native	-	mainland	yes	(Thoen et al. 1986)
Lacertidae	*Z. vivipara*	*Hierophis viridiflavus*	snake	native	-	mainland	yes	(Bestion et al. 2014; Teyssier et al. 2014)
Lacertidae	*Z. vivipara*	*Vipera berus*	snake	native	-	mainland	yes	(Thoen et al. 1986)
Scincidae	*Caledoniscincus austrocaledonicus*	*Rattus exulans*	mammal	introduced	ca. 3000 ya	island	yes	(Gérard et al. 2014)
Scincidae	*C. austrocaledonicus*	*Rattus rattus*	mammal	introduced	ca. 200 ya	island	yes	(Gérard et al. 2014)
Scincidae	*C. austrocaledonicus*	*Felix catus*	mammal	introduced	ca. 200 ya	island	yes	(Gérard et al. 2014)
Scincidae	*Carlia rostralis*	*Varanus tristis*	lizard	native	-	mainland /island	yes	(Lloyd et al. 2009)
Scincidae	*C. rostralis*	*Varanus varius*	lizard	native	-	mainland /island	no	(Lloyd et al. 2009)
Scincidae	*C. rubrigularis*	*Varanus tristis*	lizard	native	-	mainland /island	no	(Lloyd et al. 2009)
Scincidae	*C. rubrigularis*	*Varanus varius*	lizard	native	-	mainland	no	(Lloyd et al. 2009)

						/island		
Scincidae	*C. storri*	*Varanus tristis*	lizard	native	-	island	yes	(Lloyd et al. 2009)
Scincidae	*C. storri*	*Varanus varius*	lizard	native	-	island	no	(Lloyd et al. 2009)
Scincidae	*Ctenotus taeniolatus*	*Cormocephalus* sp.	centipede	native	-	mainland	no	(Goldsbrough et al. 2006)
Scincidae	*Eulamprus tympanum*	*Pseudechis porphyriacus*	snake	native	-	mainland	yes	(Robert and Thompson 2007)
Scincidae	*E. heatwolei*	*Pseudechis porphyriacus*	snake	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*E. heatwolei*	*Drysdalia coronoides*	snake	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*E. heatwolei*	*Rhinoplocephalus nigrescens*	snake	native	-	mainland	no	(Head et al. 2002)
Scincidae	*E. heatwolei*	*Liasis maculosus*	snake	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*E. heatwolei*	*Hadronyche* sp.	spider	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*E. heatwolei*	Sparrasidae	spider	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*E. heatwolei*	Scolopendromorpha	centipede	native	-	mainland	yes	(Head et al. 2002)
Scincidae	*Lampropholis delicata*	*Drysdalia coronoides*	snake	native	-	mainland	yes	(Downes and Hoefer 2004)
Scincidae	*L. delicata*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	yes	(Monks et al. 2019)
Scincidae	*L. guichenoti*	*Demansia psammophis*	snake	native	-	mainland	yes	(Downes 2001)
Scincidae	*L. guichenoti*	*Rhinoplocephalus nigrescens*	snake	native	-	mainland	yes	(Downes and Shine 2001)
Scincidae	*Oligosoma infrapunctatum*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	no	(Monks et al. 2019)
Scincidae	*O. infrapunctatum*	*Sphenodon punctatus*	lizard	native	-	island	no	(Monks et al. 2019)
Scincidae	*O. polychroma*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	no	(Monks et al. 2019)

Scincidae	*O. polychroma*	*Sphenodon punctatus*	lizard	native	-	island	no	(Monks et al. 2019)
Scincidae	*O. polychroma*	*Erinaceus europaeus*	mammal	introduced	ca. 160 ya	island	no	(Dumont 2015)
Scincidae	*O. zelandicum*	*Rattus rattus*	mammal	introduced	ca. 160 ya	island	no	(Dumont 2015)
Scincidae	*O. zelandicum*	*Erinaceus europaeus*	mammal	introduced	ca. 160 ya	island	no	(Dumont 2015)
Scincidae	*Plestiodon laticeps*	*Lampropeltis getula getulus*	snake	native	-	mainland /island	yes	(Cooper 1990)
Scincidae	*P. laticeps*	*Lampropeltis triangulum elapsoides*	snake	native	-	mainland /island	yes	(Cooper 1990)
Scincidae	*Pseudemoia entrecasteauxii*	*Drysdalia coronoides*	snake	native	-	mainland	yes	(Stapley 2003)
Scincidae	*P. entrecasteauxii*	*Pseudechis porphyriacus*	snake	native	-	mainland	yes	(Stapley 2003)
Scincidae	*P. entrecasteauxii*	*Rhinoplocephalus nigrescens*	snake	native	-	mainland	yes	(Stapley 2003)
Teiidae	*Aspidoscelis dixoni*	*Masticophis flagellum*	snake	native	-	mainland	yes	(Punzo 2007)
Teiidae	*A. dixoni*	*Masticophis taeniatus*	snake	native	-	mainland	yes	(Punzo 2007)
Teiidae	*A. dixoni*	*Arizona elegans*	snake	native	-	mainland	yes	(Punzo 2007)
Teiidae	*A. marmorata*	*Crotaphytus collaris*	lizard	native	-	mainland	yes	(Punzo 2008)
Xantusiidae	*Xantusia henshawi*	*Trimorphodon lyrophanes*	snake	native	-	mainland	yes	(Kabes and Clark 2016)

behaviour in velvet geckos (*Amalosia lesueurii*) (Downes and Shine 1998; Webb et al. 2010a). It would be interesting to test whether odours of actively foraging, or ambushing snakes species are more or less often recognised by lizards in Table 1, but alas the foraging strategies of snakes are often not known or reported.

Ecological and evolutionary acquaintance

How does prey naivety affect chemosensory predator recognition ability? My own studies returned surprising results in this respect: the Asian grass lizard failed to respond to the scent of a predator with which it had co-evolved, whereas mainland Dalmatian wall lizards clearly responded to the chemicals of that same predator, although their distributional ranges only very recently came to overlap (Chapters 2 & 3). The literature data in Table 1 suggest that overall, lizards are more likely to respond to chemicals originating from 'native' predators: of 90 experiments, 73 (81%) of the prey lizards exhibited anti-predator behaviour. In 20 experiments with 'alien' predators, only 10 (50%) lizard populations seemed to recognise the cues. The difference is significant (χ^2=9.21, P=0.002) and raises conservation concerns (Gurevitch and Padilla 2004; Cox and Lima 2006; Salo et al. 2007).

An optimistic biologist might point out that species tend to be resilient, and will adjust to changes in their predator community through phenotypic plasticity (learning, see Martín et al. 2015 for an example featuring a lacertid lizard), or -given enough time- through genetic adaptation. My own results provide support for this optimism, albeit only for mainland populations of the Dalmatian wall lizard (Chapter 3). The literature offers both examples of lizard populations that developed predator recognition in the blink of an eye (10s of years, e.g. *Podarcis pituysensis,* Ortega et al. 2017) and populations that did not do so in the course of thousands of years (e.g. *Scelarcis perspicillata*, Mencía et al. 2017). If we compare responses to snakes introduced recently (11-200 years ago) to those introduced in historical times (>2000 years ago), we find no significant difference in the proportion of lizard species that respond to the snakes (χ^2=0.27, P=0.61). Chemicals of recently introduced snakes were recognised in 7 out of 15 cases, those of long time introduced snakes in 3 out of 5 cases (Table 1).

The island effect(s)

The results of Chapters 2 and 3 of this thesis fed my intuition of an 'island factor' in this equation. Island tameness is a well-known component of the so-called island

syndrome. Darwin himself commented on the tameness of birds on the Galapagos islands in 1839, noting, that “they did not even understand what was meant by stones being thrown at them; and quite regardless of us, they approached so close that any number might have been killed with a stick” and “a gun is here almost superfluous; for with the muzzle of one I pushed a hawk off the branch of a tree” (Darwin 1839). Island tameness is typically associated with the dearth of predators in insular communities, and the relaxed selection on anti-predator behaviour that comes with that. Naivety on islands could simply reflect unfamiliarity with a specific new predator, but may be further nurtured by the lack of predator ‘archetypes’, i.e. predators related or resembling that new predator (Cox and Lima 2006). Mainland habitats are more likely than islands to harbour such archetypes, predisposing mainland lizards to adaptive behaviour when confronted with alien (but similar) predators.

However, my results suggest that other particularities of the island environment (e.g., low dietary resources) may contribute to reduced wariness. Some of the island study sites of Chapters 2 and 3 do house terrestrial predators, but the local lizards nevertheless exhibited no response to their chemical cues. I suggest that poor island conditions may select for a reduction in the size of the brain, or particular areas thereof, perhaps to the benefit of an increased development of the gut (Herrel et al. 2008; Vervust et al. 2010; Sagonas et al. 2015). The data in Table 1 support the importance of an island effect. Of the 34 studies conducted on island-dwelling lizards, 17 (50%) described how lizards failed to respond to predator chemical cues. Of the 72 mainland lizard populations studied, only 6 (8%) could not recognise predator chemicals. This difference is highly significant (χ^2= 23.60, P<0.0001). The reasons why island lizards tend to score badly in chemosensory predator recognition remain incompletely understood and may vary between species, but these results make it painfully clear that they are extremely vulnerable to any changes in their predatory environment. This may well have contributed to the disproportionately high loss of reptile species on islands around the world (Foufopoulos and Ives 1999; Böhm et al. 2013). Conceptual frameworks designed to guide conservation plans in the light of alien predation risk (e.g. Carthey and Blumstein 2018) have elegantly incorporated a number of potentially important factors discussed above, such as the role of prior ecological and evolutionary experience, presence/absence of archetypes and selection pressure, but seem to have missed the fact that some populations may fail to

recognise predator cues because local environmental circumstances have deprived their sensorial functions. I think this might often be the case for island populations.

Although this analysis of literature data reveals a number of obvious trends, none of those are absolute. Predicting which lizards in which conditions will be able to respond adequately to chemical cues of particular types of predators remains difficult. To a large extend, this is due to a lack of knowledge concerning the mechanisms of chemosensory predator recognition. In the second part of this thesis (Chapters 5 to 7), I have explored a number of methods that could help fill in this knowledge gap.

Identifying ecologically relevant chemicals

A major factor thwarting our understanding of chemical predator recognition in lizards is that we hardly know anything on the identity of molecules involved. From Chapters 5 and 7, we learn that both the volatile and substrate-bound aspect of adder scent plays a role in predation risk assessment by common lizards. Compounds that possess such varying chemical properties may inform the prey in different ways. Volatile compounds will readily become airborne and, therefore, may expose a predator's presence at a distance, at least up-wind. In cases involving ambushing predators, such as *Vipera berus,* that strike from hide-outs occupied for relatively long periods of time this seems highly useful information for lizards to have. On the other hand, when a prey arrives at a location replete with high-mass, substrate-bound compounds, predator cue detection may signify that the danger is (or was) in close proximity. As *V. berus* often remains at the same location, such compounds would indicate high risk areas. Hence, this would explain the stronger response of *Z. vivipara* to these type of molecules.

Additionally, the finding that some adder kairomones are relatively non-polar compounds of high molecular weight coincides with the presumption from earlier literature that snake kairomones are detected through the vomeronasal organ (VNO). Namely, heavy-weight molecules are not likely to travel through the air into the nasal cavities to the olfactory system. On the contrary, these chemicals probably require a tongue-mediated delivery to the VNO for evoking a behavioural response. The suspicion of an involvement of the VNO has been put forward in earlier research in which lacertids increased tongue flicking in response to predator odours (e.g. Van Damme and Quick 2001; Downes and Bauwens 2002;

Martín et al. 2015). Therefore, kairomones may have a heavy-weight chemical nature in many lacertid-snake systems. This would explain why a reduction in the accessory but not the main olfactory bulbs (Chapter 4) may have led to absent chemosensory anti-predator tactics in insular Dalmatian wall lizards.

The identification of semiochemicals is a tedious process requiring repeated subsetting of candidate chemicals followed by an assessment of their bioactivity through behavioural assays (Baedke et al. 2019). Although I was unable to complete all steps of this process in the second part of the thesis, a solid base is set for further research. Through the application of traditional and novel techniques, I have uncovered a plethora of mainly adder-associated chemicals which can now be assessed for their roles in kairomone signalling. Furthermore, these can be tested for potential functions in a broader chemo-ecological context. For instance, some substances, such as 9-octadecenamide and 13-docosenamide, which we found were present on the shed skins of adders (Chapter 5), have been shown to exert inhibitory effects on Gram-positive (*Staphylococcus epidermidis* and *S.* aureus) and Gram-negative bacteria (*Enterobacter aerogenes* and *Klebsiella pneumonia*), as well as on harmful fungi (*Candida krusei*; Medeiros dos Reis et al. 2019). This finding has prompted me to set-up new experiments, which will start January 2021 at the Mason lab (Oregon State University), in which I will assess whether skin-derived chemicals may serve defensive purposes against bacterial infections and fungal disease. A primary focus will be to elude the potency of skin-derived chemicals to protect U.S. snakes against snake fungal disease (Allender et al. 2015).

It is my belief, and that of established experts (Schneider et al. 2013; Müller-Schwarze 2016), that studies on the nature of semio-chemicals and other ecologically relevant molecules will play an increasingly important role in various scientific fields. Deciphering the language of scent will help answer fundamental questions on the nature and evolution of chemosensory behaviour. As an example, a study by Ferrero et al. 2011) found the kairomone 2-phenylethylamine, which is used by rodents for predator avoidance, to be present in a wide variety of carnivorous mammals, suggesting generalised anti-predator behaviour. A pre-existing sensory bias of female Iberian rock lizards (*Iberolacerta cyreni*) for essential food nutrients may lay at the base of the evolution of cholesta-5,7-dien-3-ol (provitamin D3) into a pheromone that attracts females to well-fed males (Martín and López 2008). Additionally, a broadened knowledge on vertebrate

semiochemicals will aid in assessing the impact of chemical pollution and rapid climate change on ecological interactions. For instance, increased concentrations of humic acids (a byproduct of degrading organic matter and current ubiquitous pollutant) adsorb the steroid pheromones of goldfish, making them unavailable for intraspecific communication (Mesquita et al. 2003). Warming may affect the aging of chemical cues (Regnier and Goodwin 1977; Müller-Schwarze 2006). As, in Chapter 7, we have seen that the age of an adder's scent cue determines which behavioural anti-predator strategy is adopted by common lizards, future changes in global weather patterns have the potential to alter the nature of such interactions.

Furthermore, not knowing the true nature of vertebrate-derived chemicals has caused us to miss out on a tremendous source of biologically relevant and potent natural products. Applications have the potential to find their ways into widely divergent fields. In conservation biology, chemical fingerprints could be used to detect invasive species in cargo holds of airplanes and freighters (e.g. the brown tree snake, *Boiga irregularis*; Nielsen et al. 2004), or pheromones could be used to enhance captive breeding in endangered animals (e.g. giant panda, *Ailuropoda melanoleuca*; Wilson et al. 2020). The application of odours as environmental enrichment to animal housing facilities could facilitate stress-management in zoo and laboratory animals (e.g. odours have been found to increase behavioural diversity and activity levels in captive black-footed cats, *Felis nigripes*; Wells and Egli 2004), and targeted semiochemical control agents could be found to manage invasive pest species (e.g. pheromones that suppress larval development in cane toads, *Rhinella marina*, but not in native anurans; Clarke et al. 2016). Finally, predator kairomones may be particularly potent in diverting animals away from agricultural fields and human settlements and, therefore, may prevent human-wildlife conflict (e.g. scent from dingos discourages southern hairy-nosed wombats, *Lasiorhinus latifrons*, from burrowing in agricultural fields; Sparrow et al. 2016). This, to name a few of the plethora of possibilities (Nielsen et al. 2015; Jones et al. 2016; Müller-Schwarze 2016; Bombail 2019).

Neurological signalling

In order to fully comprehend a vertebrate's behaviour towards predators and harmless animals, native versus introduced predators, or mammals versus snakes, we need to uncover the neuronal signalling pathways underlying this behaviour

(Martínez-García et al. 2002). Through a collaboration with the Bio-Imaging lab at the University of Antwerp, we have attempted to trace the neural signal resulting from a predator-derived chemical cue in the Asian grass lizard through Manganese Enhanced – Magnetic Resonance Imaging (ME-MRI). Manganese (Mn^{2+}) is a contrast fluid with a high chemical similarity to calcium (Ca^{2+}) and enters excited neurones through voltage-gated calcium channels and the Na^{+}/Ca^{2+} exchanger (Massaad and Pautler 2010). Because Mn^{2+} uptake after systemic injections takes place over an extended period of time in awake and freely moving animals, sensory stimulus presentation can occur in relatively natural circumstances and can be accompanied by behavioural observations. Consequently, results from the latter can help interpret neuronal patterns found through MR imaging (Malheiros et al. 2015). We had hoped to optimise this technique to be able to apply it to, for example, our study system on Dalmatian wall lizards to see potential differences between the processing of cues from invasive and introduced predators. However, there were issues with determining an optimal Mn^{2+} dose, and a small-sized lacertid's neurological structures were on the edge of what is detectable with a 9.4T BioSpec (Bruker, Germany) MR-scanner. Perhaps a repetition of the experiment with a large lacertid species, such as *Timon lepidus* or *Lacerta trilineata,* could give more fruitful results.

Genetics

A major black horse in sensory biology, is the genetic underpinning of chemosensory predator recognition. Trait heritability is essential to adaptive evolution, but little to nothing is known on this subject in lizards. Genetics underlie opportunities of signal detection, processing and subsequent behavioural responses (Wang et al. 2018). Knowing the genes involved in predator detection, would allow determining whether the differences between island and mainland lizards in chemosensory abilities (i.e. Chapter 3 and 4) were due to plasticity or evolutionary processes; in the case of the latter, was there neutral evolution involved or did adaptation drive chemosensory deprivation? Furthermore, assessing whether lizards have specific kairomone receptors that function in the detection of a certain invasive predator would allow predicting whether these lizards are likely to show level 1 naivety or not. Such information may be critical for the conservation of native populations.

Only a few genes have been linked to predator detection. In rodents, Taar4 is the primary receptor of 2-phenylethylamine, a compound ubiquitous in carnivore urine (Ferrero et al. 2011). However, this receptor is not a part of the genome of the green anole (*Anolis carolinensis*; Eyun et al. 2016); an iguanid lizard which was the first squamate to have its genome sequenced in 2011 (Alföldi et al. 2011). The presence of Taar4 in other reptiles has not yet been assessed. Another receptor in rodents called Trpa1 binds the excessively studied 2,5-dihydro-2,4,5-trimethyl-thiazoline (TMT) from fox faeces (Wang et al. 2018). The gene's presence is confirmed in the genome of both lizards and snakes and shows some overlapping functions with that of mammals in that it is activated by heat and noxious chemicals (Saito et al. 2012). To our knowledge, it has never been tested whether reptiles are actually responsive to fox faeces or TMT.

Recent advances in genetic and genomic research have enabled researchers to trace broad-scaled patterns of evolution in certain classes of genes underpinning chemoreception in vertebrates. For instance, the pseudogenisation of a gene called TRPC2, which is crucial in neuronal signalling in the VNO (Yildirim and Birnbaumer 2007; Young et al. 2010), has been connected to the absence of vomeronasal communication in birds and Old World monkeys (Grus and Zhang 2006; Shi and Zhang 2007). A remarkable expansion of one type of vomeronasal receptor genes (i.e. vomeronasal type 2 receptors, V2Rs) is apparent in some species of squamates and seems to be linked with the appearance of a lingual-vomeronasal system (Brykczynska et al. 2013; Lind et al. 2019). Through studies on the functional role of the genes and gene products associated with (vomer)olfaction, predominantly in laboratory-reared rodents, researchers are now attempting to give ecological meaning to patterns of genetic variation throughout the animal kingdom. This type of research has great potential for understanding squamate chemosensation, as well. In the past decade, squamate genomes have become available (e.g. Castoe et al. 2013; Liu et al. 2015), a few being high-quality chromosome-level assemblies (Medeiros dos Reis et al. 2019; Yurchenko et al. 2019). Currently, splendid work is being performed to lift reptilian genetic research to the same level as that of mammals. Although genomic data remains unevenly distributed across phylogenetic groups, initiatives as there are the Reptilian Transcriptomes project (www.reptilian-transcriptomes.org; Tzika et al. 2015) are stirring invaluable progress in the field.

Multimodality in sensory ecology

My thesis is an illustration of how information retrieved through a single sensory modality can have a far-reaching impact on a species' ecology. Nevertheless, in a natural environment, lizards integrate sensory input through multiple senses to ensure an efficient and economic response to the situation at hand (Pereira and Moita 2016). For instance, wall lizards (*Podarcis muralis*) are known to combine chemical and visual cues of ambushing smooth snakes (*Coronella austriaca*) to avoid overestimating risk inside refuges (Amo et al. 2004c). Recent work has put forward the importance of multimodal sensory perception (Halfwerk and Slabbekoorn 2015). When using cues travelling through different modalities, animals can increase their chances of detecting important environmental events, enhance the processing of cues, or retrieve excess information (Halfwerk and Slabbekoorn 2015 and references therein). The survival of Asian grass lizards under native mammalian predatory pressures without the use of chemosensory anti-predatory tactics suggests that, instead, they use other sensory modalities to evade predation. On the other hand, for detecting snakes, the chemical cues in isolation were sufficient for evoking anti-predator behaviour, implying a main deployment of these senses in the detection of highly cryptic predators that rely on stealth during hunt. These observations do not exclude an elusive role of chemodetection in native mammal perception, or the use of vision or another sense when a snake leaves its hiding place.

Further research should investigate the relative role of sensory modalities to acquire a complete understanding of predator-prey interactions. Ethorobotics is a promising new technique that allows the parallel presentation of cues through multiple sensory modalities. By comparing responses to multimodal cues with responses to cues in only one or few modalities, the relative role of different sensory systems in various contexts can be assessed (Partan et al. 2009). A nice example of ethorobotics put into practice involves an experiment on defensive signalling by California ground squirrels (*Spermophilus beecheyi*) towards infrared-sensitive rattlesnakes (*Crotalus oreganus*; Rundus et al. 2007). Researchers found that a visual display in ground squirrels called 'tail-flagging' elicited cautious behaviour in rattlesnakes, which was more pronounced when an infrared component was added to the display. In a similar way, ethorobotics can be applied to my study system in Part I of the thesis to understand what entails an optimal response to mongoose cues (i.e. How does *T. sexlineatus* react to

mongoose cues travelling through different modalities?). By also applying the set-up to *P. melisellensis*, this may finally allow us to determine the adaptiveness of its response.

Appendix

Chapter 2: The Asian grass lizard (*Takydromus sexlineatus*) does not respond to the scent of a native mammalian predator

Table S1 Most probable models, as shown by the second-order Akaike Information Criterion (AIC_c) and Akaike weights (w_i), for explaining the variance in behaviour performed by *T. sexlineatus* in (A) Experiment A and (B) Experiment B. Models in which Treatment had a predictor weight greater than 90 % are given in bold.

A.

	model	*k*	AIC_c	ΔAIC_c	w_i	acc w_i
No-move†	Treatment	5	1707.57	0	0.52	0.52
	1	3	1708.37	0.80	0.35	0.87
	Trial	5	1711.55	3.99	0.07	0.94
Walk†	Treatment	5	327.84	0	0.52	0.52
	1	3	328.89	1.06	0.31	0.83
	Treatment + Trial	7	331.40	3.56	0.09	0.92
√Tongue flick	Walk†	4	162.95	0	0.66	0.66
	Treatment + Walk†	6	165.96	3.01	0.15	0.81
	Trial + Walk†	6	166.22	3.27	0.13	0.94
√Labial lick	Treatment	5	148.62	0	0.68	0.68
	Treatment + Trial	7	150.97	2.35	0.21	0.89
	1	3	152.67	4.05	0.09	0.98
Foot shake	**Treatment + Trial**	**6**	**194.17**	**0**	**1.00**	**1.00**
Startle	**Treatment**	**4**	**88.96**	**0**	**0.90**	**0.90**
Head rub	**Treatment**	**4**	**135.64**	**0**	**0.61**	**0.61**
	Treatment + Trial	**6**	**136.55**	**0.91**	**0.39**	**1.00**
$Bask^{bin}$	Treatment	4	47.57	0	0.65	0.65
	1	2	49.40	1.83	0.26	0.91
$Nudge^{bin}$	1	2	50.12	0	0.53	0.53
	Treatment	4	50.37	0.25	0.47	1.00
$Stand\text{-}up^{bin}$	Trial	4	45.45	0	0.64	0.64
	1	2	47.88	2.43	0.19	0.83
	Treatment + Trial	6	48.72	3.27	0.12	0.95

B.

	model	*k*	AIC_c	ΔAIC_c	w_i	acc w_i
No-move	1	3	496.59	0	0.70	0.70
	Trial	4	499.85	3.26	0.14	0.84
	Treatment	4	499.93	3.33	0.13	0.97
√Walk	1	3	241.51	0	0.51	0.51
	Trial	4	243.43	1.92	0.19	0.70
	Treatment	4	243.56	2.05	0.18	0.88
	Treatment * Trial	6	245.68	4.17	0.06	0.94
3√Tongue flick	Treatment + √Walk	5	39.83	0	0.52	0.52
	√Walk	4	41.90	2.07	0.19	0.71
	Treatment * √Walk	6	42.47	2.64	0.14	0.85
	Trial + Treatment + √Walk	6	43.39	3.56	0.09	0.94
Foot shake	Trial	4	82.50	0	0.51	0.51
	1	3	83.40	0.90	0.32	0.83
	Treatment + Trial	5	86.01	3.51	0.09	0.91
Labial lick	1	3	117.66	0	0.69	0.69
	Treatment	4	120.78	3.13	0.14	0.83
	Trial	4	120.91	3.26	0.14	0.97
$Nudge^{bin}$	Trial	3	28.71	0	0.46	0.46
	1	2	29.53	0.82	0.31	0.77
	Treatment + Trial	4	31.38	2.67	0.12	0.89
	Treatment	3	31.54	2.83	0.11	1.00
$Stand\text{-}up^{bin}$	Trial	3	25.44	0	0.67	0.67
	1	2	28.35	2.92	0.16	0.83
	Treatment + Trial	4	28.63	3.19	0.14	0.97

√ square-root transformed; 3√ third-root transformed; † Box-Cox transformed; ^{bin} coded into a binomial quantity.

Figure S1 Plots showing individual variation over Treatment for the main behaviours performed by Asian grass lizards in Experiments A and B. Behaviours are either timed in seconds (s) or given as counts (#). Connected data points having the same colour represent values for a single individual Asian grass lizard. The numbers that mark each value represent the trial number (either trial 1, 2, or 3) for which the value was noted. Symbols on the x-axes depict the different scent treatments to which the lizards were subjected, namely an odourless control (CTRL), diluted Mennen Skin Bracer aftershave as a pungency control ($CTRL^{+}$), Oriental whip snake scent (snake silhouette), and small Indian mongoose scent (mongoose silhouette). Statistical models describing variance in lizard behaviours can be found in Table S1.

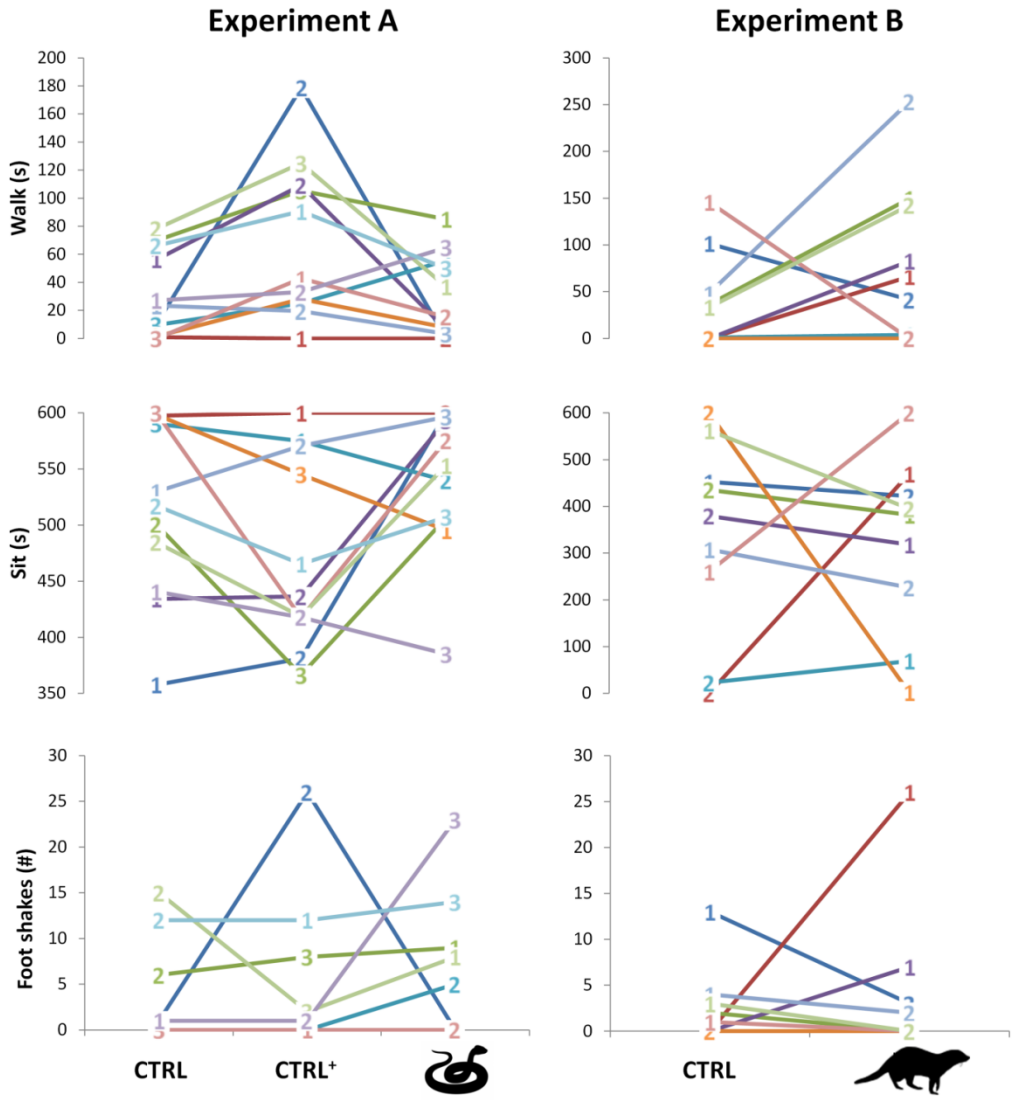


Figure S1 - continued

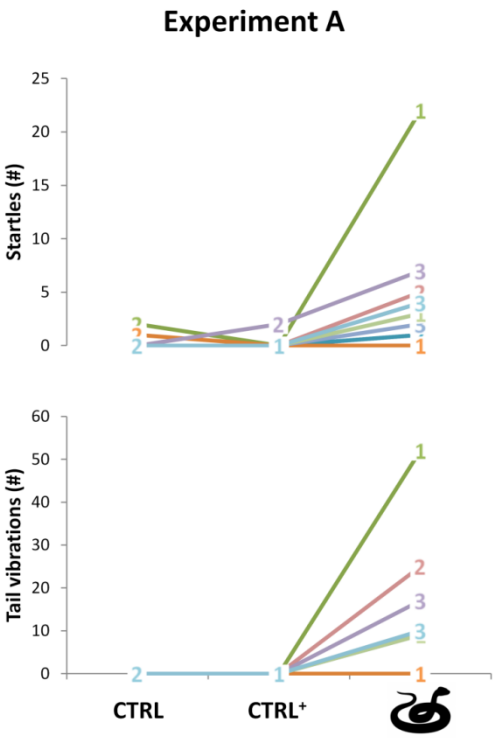
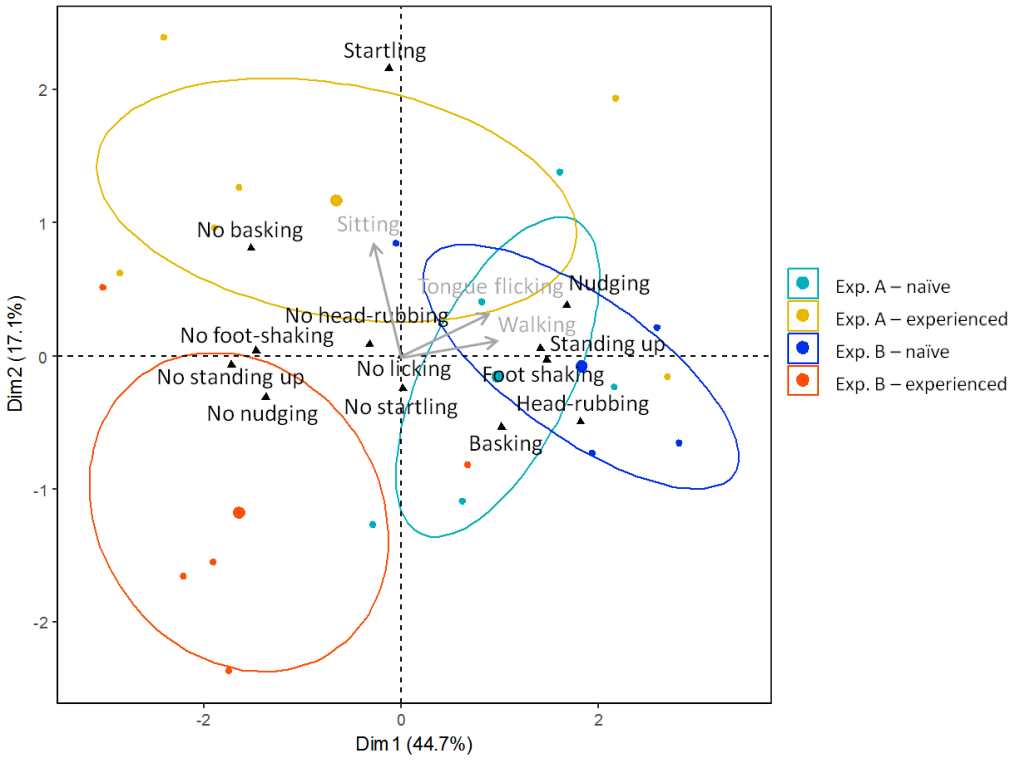


Figure S2 A graphical representation of the output from a Factor Analysis of Mixed Data (FAMD) on the behaviours performed by Asian grass lizards in the odourless control treatment. The first two dimensions of the FAMD are shown with the percentage of the explained variance of each dimension between brackets. Data points of individual observations are given by a small dot which is coloured according to the experiment it was performed in, as well as, whether the lizard had been introduced to a predator scented environment prior to the current control trial (experienced) or not (naïve). For each of these groups, 95 % confidence ellipses are drawn around the group mean, which is represented by a large dot. Grey arrows show continuous behavioural variables; black triangles represent the levels of binomial behavioural variables (see also Material & Methods for further information on each behavioural variable).

The behavioural variance seen along the first dimension can be best explained by looking only at the Treatment history, as deduced from a linear model containing only this variable ($F_{1,18}$ = 10.74, P = 0.0042). This probably represents the anticipated habituation effect accompanying repeated measures. A habituation effect has been dealt with by randomising the order of presentation of scent treatments to the lizards. Variance along the second dimension could best be explained by a model containing an interaction between Experiment and Treatment history ($F_{1,16}$ = 7.41, P = 0.015). This effect is driven by what may be interpreted as a carry-over effect, that is, a significant change in behaviour according to the lizards' Treatment history, which was observed in Experiment A, but not in Experiment B (Experiment A – naïve vs. experienced: t_{16} = -2.23, P = 0.040; Experiment B – naïve vs. experienced: t_{16} = 1.66, P = 0.12).



Chapter 4: Insularity reduces lizard vomerolfactory activity and the brain regions that support it

Table S1 99.9 % confidence set of best-ranked linear models examining the effect of a lizard's Sex, Population and the time it spend Walking during a focal observation on the number of performed Tongue flicks during that same observation. k = the number of fitted parameters (including the intercept) in the model; AIC_c = the second-order Akaike Information Criterion; Δi = the difference between the AIC_c value of the best and current model; w_i = the Akaike weight; acc w_i = cumulative Akaike weight which is ≤ 0.999

	Candidate models	k	AIC_c	Δi	w_i	acc w_i
1	Population*Walking	9	906.245	0	0.588	
2	Sex+Population*Walking	10	908.282	2.037	0.213	0.801
3	Sex*Population+Population*Walking	13	910.014	3.768	0.089	0.890
4	Sex*Walking+Population*Walking	11	910.656	4.410	0.065	0.955
5	Sex*Population+Sex*Walking +Population*Walking	14	912.649	6.403	0.024	0.979
6	Population+Walking	6	914.614	8.369	0.009	0.988
7	Sex+Population+Walking	7	914.996	8.750	0.007	0.995
8	Population+Sex*Walking	8	917.040	10.794	0.003	0.998
9	Walking+Sex*Population	10	919.205	12.960	0.001	0.999

Table S2 99.9 % confidence set of best-ranked linear models examining the effect of a lizard's Population, Body condition, and overall Brain length on the volume of its AOB. Table headings are explained in Table S1. A value of '1' under candidate models indicates the null model.

	Candidate models	k	AIC_c	Δi	w_i	acc w_i
1	Population	3	-2.144	0	0.597	
2	Population+Body condition	4	-0.882	1.262	0.317	0.914
3	Population+Brain length	4	2.382	4.526	0.062	0.976
4	Body condition	3	6.343	8.487	0.009	0.985
5	1	2	7.229	9.373	0.006	0.990
6	Population+Brain length+ Body condition	5	7.919	10.063	0.004	0.994
7	Population*Body condition	5	8.115	10.260	0.004	0.998
8	Brain length	3	10.072	12.216	0.001	0.999

Table S3 99.9 % confidence set of best-ranked linear models examining the effect of a lizard's Population, Body condition, and overall Brain length on the volume of its MOB. Table headings are explained in Table S1. A value of '1' under candidate models indicates the null model.

	Candidate models	***k***	**AIC_c**	**Δi**	**w_i**	**acc w_i**
1	1	2	-50.280	0	0.542	
2	Body condition	3	-48.232	2.048	0.195	0.737
3	Brain length	3	-47.353	2.927	0.126	0.863
4	Population	3	-46.431	3.849	0.079	0.942
5	Population + Body condition	4	-44.815	5.465	0.035	0.977
6	Brain length+Body condition	4	-42.562	7.717	0.011	0.989
7	Population+Brain length	4	-42.361	7.919	0.010	0.999

Chapter 6: Proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) as a tool for studying animal volatile organic compound (VOC) emissions

S1. Analytical procedure of the proton-transfer-reaction time-of-flight mass spectrometer (PTR-TOF-MS)

The instrument functioning is as follows: a discharge cathode generates hydronium ions (H_3O^+) from pure water (H_2O), and the hydronium ions meet the sample air containing VOCs for the proton-transfer reaction (PTR):

$$H_3O^+ + VOC \rightarrow H_2O + VOCH^+. \quad (1)$$

VOCs that have a higher proton affinity than water (697 kJ/mol) receive a proton (H^+) from hydronium ions, resulting into protonated VOCs ($VOCH^+$). The kinetic of this reaction is given by the proton transfer reaction rate (k_{PTR}), that is dependent on each molecule's properties. Subsequently, the protonated VOCs are driven through a drift tube by means of voltage acceleration to the time-of-flight (TOF) region. The drift tube was operated at 600 V drift voltage, 2.3 mbar pressure, and 60 °C temperature, resulting in a field density ratio (E/N) of ≈130 Td (1 Townsend = 10^{-17} V cm^2), where E is electric field and N is the gas density.

The protonated VOCs undergo a separation by their mass (mass spectrometry) thanks to a repellent electromagnetic pulse and a high vacuum environment (chamber pressure ≈2.5×10^{-7} mbar). The protonated VOCs —that have only one charge (q)— receive the same kinetic energy (U) that make them travel a known distance (d) to the detector at a different speed depending on their mass (m):

$$t_{\text{flight}} = \frac{d}{\sqrt{2U}}\sqrt{\frac{m}{q}}; \quad (2)$$

therefore the time of flight (t_{flight}; from the pulser to the detector) of all the protonated VOCs only depend on the only variable parameter, that is their mass (m). The instrument does not distinguish isomeric compounds (compounds with the same chemical formula but different molecular structure, e.g. different monoterpene structures) because these have the exact same mass. However, isobaric compounds have different chemical formula but the same nominal mass, e.g. $(C_4H_6O)H^+$ and $(C_5H_{10})H^+$ with adjacent peak centres at m/z 71.0491 and 71.0855,

respectively. They can be distinguished due to the high time resolution, that translates into a high resolution in the spectrum (to the nearest 0.0001 Da). Isobaric compounds create adjacent peaks in the spectrum and often multi-peak systems that can be resolved using the 'Multipeak' built-in tool in the software PTR-MS Viewer v3.2 (Ionicon, Innsbruck, Austria) (Portillo-Estrada et al. 2018).

The detection is done by a microchannel plate (MCP) detector, that amplifies the ion hits at the end of the TOF region, and couples the data with a time-to-digital converter (TDC) (Burle Industries Inc., Lancaster, PA), USA. The PTR-TOF-MS allows the rapid detection (up to 100 ms time resolution) of VOCs in real-time measurements with high sensitivity up to tens of cps/ppb (counts per second per parts per billion) and low detection limit up to ppt (parts per trillion) range. The protonated ions were pulsed in the TOF region every 32 μs, generating 31250 spectra with a mass range of 1-316 m/z every second. Water impurities were kept on average below 7 %, NO^+ was kept on average below 0.5% and oxygen impurities were kept below 6% relative to the primary ion H_3O^+, by fine-tuning the voltages of the instrument. The VOCs corresponding to the spectrum peaks were identified by their time of flight, that is proportional to the molecular mass. The peak centre (with a mass resolution of 0.0001 Da) was compared to a unique combination of atoms that corresponded to a unique molecular mass. The spectrum mass range was calibrated with compounds of known mass: i.e. NO^+ = 29.99744 Da, and 1,3-diiodobenzene fragment $(C_6H_4I)H^+$ = 203.94305 Da, that was permeated from a built-in internal mass calibration unit.

A calibration curve (known as transmission curve) was generated with a multi-component gas calibration mix. It contained eight pure compounds (methanol, acetaldehyde, acetone, isoprene, methyl vinyl ketone, benzene, toluene, t-2-hexen-1-al, c-3-hexen-1-ol, and α-pinene) ranging from m/z 33 to 137 with known concentrations (Apel Riemer, USA). The transmission curve served to inter- and extrapolate the level of discrimination at different molecular masses due to the sampling duty cycle in the orthogonally positioned TOF region (Warneke et al. 2015). The concentration of each compound was calculated by integrating the peak area in each spectrum, referring it to the concentration of H_3O^+ (using the isotopomer $H_3{}^{18}O^+$, m/z 21.0221, that is 500 times less abundant than hydronium ion but has a measurable peak area), the transmission value of the given molecular mass, and the proton transfer rate constant (k_{PTR}) of the molecule. The k_{PTR} was either calculated via direct calibration from the gas standard mixture

for these specific components, it was retrieved from the literature (e.g. Cappellin et al. 2012)— Supplementary Material), or otherwise a value of 2×10^{-9} cm^3 s^{-1} was assumed. The raw data generated by the PTR-TOF-MS was acquired by TofDaq software (Tofwerk AG, Switzerland) and the post-processing of raw data was done using PTR-MS Viewer v3.1 (Tofwerk AG, Switzerland).

S2. Compound structure verification with gas chromatography mass spectrometry (GC-MS)

The VOC emissions of five *Podarcis muralis* and two *Vipera berus* were measured also with GC-MS to compare the results with of PTR-TOF-MS and verify the compound structures. The individuals were enclosed individually in glass chambers in standard conditions. The chamber was equipped with a 3-mm (O.D.) PTFE inlet and outlet. The flow-through chamber was supplied with VOC-free sterile air that was filtered via an activated charcoal scrubber. The air outlet containing the VOCs emitted by the animal individual passed through a stainless steel cartridge filled with adsorbent Tenax TA (Supelco, Bellefonte, PA, USA) before reaching the pump, that pulled the air through the system at a flow rate of 500 mL min^{-1} during 10 min (5 L of air). Tenax TA is a porous polymer (2,6-diphenylene oxide) commonly used for trapping volatile and semi-volatile compounds from C_7 to C_{26}, however it can also trap smaller molecules too.

Air sample volatiles adsorbed into the Tenax TA matrix were analysed with a thermal desorption system *TD-20* coupled to a *GC-2010 Plus* and a *TQ-8040* triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The desorption unit was set to 60 mL min^{-1} purge flow rate and the following temperatures: 260 °C, trap cool –19 °C, trap heat 250 °C, interface 260 °C, transfer line 260 °C, and block 250 °C.

Compounds were separated on two GC columns connected with each other: *Rxi-1 ms*, 30 m × 0.25 mm I.D. (inner diameter), 0.25 µm df, and SGE Analytical Science BPX50, 2 m × 0.15 mm I.D., 0.15 µm df (Restek GmbH, Bad Homburg vor der Höhe, Germany) were used for chromatographic separation. Helium 5.0 was used as carrier gas with a flow of 1.55 mL min^{-1} (linear velocity of 35 cm/s) and a split ratio of 10. The GC oven was set to the following program: initial temperature 50 °C for 0.5 min, an increment of 10 °C min^{-1} until 250 °C during 19.5 min, and finally, the maximum temperature 250 °C for 5 min; the interface was set

to 250 °C. The MS ion source operated at 200 °C and 70 eV. The acquisition mode was Q3 scan in an m/z range between 30 and 300 Da and at a scan speed of 1428 Da/s. Mass spectra and VOC retention indices (RIs) were compared to the mass spectra and RIs published in the NIST library (National Institute of Standards and Technology).

S3. Data processing

—To determine the absence/presence of a VOC emitted by an animal or sample, data series of concentrations were compared to the background air data series (VOC-free air entering the chamber; Fig. 1**a**) or the pre-event or pre-treatment time in the case of skin secretions (Fig 1**d**, **e**). Significant difference in emission of a VOC were identified using Student t-tests at $p < 0.05$ significance level. This test revealed which VOCs were relevant to the analysis. The average of the background data (C_0) was subtracted to the measured data (C) to obtain the VOC concentration values of interest. The emission rate (E, nmol g^{-1} s^{-1}) for each VOC using the PTR-TOF-MS instrument is calculated as follows:

$$E = \frac{(C - C_0) \times F}{m}; \quad (3)$$

where C and C_0 are the VOC concentrations (mol compound/mol air, in ppb) recorded from the chamber outlet tube during the period of measurements (C) and during the control period (C_0). F is the chamber molar flow (mol s^{-1}), and m is the animal mass (g). If the flow is known in volumetric units (e.g. L min^{-1}), it must be converted into molar flow through the ideal gas law ($PV = nRT$). Depending on the research interest, m can be substituted by animal's snout-vent length (L_{SV}, mm) or surface area (A, cm^2). In these cases, E would be described as emission rate per L_{SV} (nmol cm^{-1} s^{-1}) or per area unit (nmol cm^{-2} s^{-1}), respectively.

In order to calculate the total amount of VOCs emitted (E_{peak}, mol g^{-1}) during an event (e.g. after a physiological treatment, Fig. 1**d**, **e**), the peak area (Fig. 1**f**) is integrated as:

$$E_{peak} = \sum_{t=i}^{j} E_t; \quad (4)$$

where E_t is the emission rate at a given time (t), i is the time at the start of the peak and j the time at the end. Special care must be taken when the data is not recorded at 1 s intervals and a time transformation is needed to adapt E_t so its data series can be summed up. For example, if the data series is recorded at 0.5 s intervals, an

average every two data values must be taken before the sum. Otherwise 0.5-s values can be summed up but the resulting E_{peak} divided by two.

The amount of VOC emitted (E_{sample}, mol g^{-1}) by a solid or liquid sample in a closed vial can be calculated as:

$$E_{\text{sample}} = \frac{(C_{\text{h}} - C_{\text{empty}}) \times n_{\text{h}}}{m}, \quad (5)$$

where C is the concentration in the headspace (typically in parts per billion; 10^{-9} mol VOC/mol air), C_{empty} is the concentration in the empty vial, n_h (mol) is the molar volume of the vial headspace, and m (g) the sample mass. n_h is calculated by transforming the volume of the headspace ($V_h = V_{vial} - V_{sample}$) into mol through the ideal gas law ($PV = nRT$). In a solid sample, V_{sample} can be estimated by dividing m by its density (mass per volume). We also propose a modification to the equation by substituting m by V_{sample} (mL), that will denote E_{sample} to mL of liquid sample (mol mL^{-1}). Special attention must be paid on keeping a constant temperature of the vials and reporting it. This is because the liquid-gas and solid-gas equilibrium will depend on the temperature.

S4. Animal housing and ethical statement

This research has been approved by the Ethical Committee for Animal Experiments, and all experiments have been performed accordingly. The housing of animals and sampling procedures follow the European convention (European Convention for the protection of Vertebrate animals used for experimental and other scientific purposes; CETS #123), Belgian law (Art. 2.6 of the Belgian Law of May 4th 1995; Annex VII, Belgian Law of May 29th 2013), and institutional regulation. After the experiment, all wild animals were released at the location of capture.

Six adult individuals of *Bombina orientalis* were purchased from an animal shop, De Kameleon (Tilburg, the Netherlands), and three *Cynops pyrrhogaster* individuals were purchased from Squama (Herent, Belgium). The animals were housed in glass terraria of 45×45×60 cm (l×w×h) and kept in an acclimatised animalarium with a 12/12-h day-and-night cycle. The frogs were fed weekly with powdered crickets *ad libitum* and their health and welfare was checked daily. Experiments involving amphibians were approved by the Ethical Committee of Animal Experimentation of the Vrije Universiteit Brussel (Permit nos. EC16-334-1 and EC15-AAA-2).

The adders and lizards were captured in March 2018 in the nature reserve Het Marum (Brecht, Belgium; 51.378010 N, 4.615180 E, 24 m a.s.l.) and near the train station of Muizen (Belgium; 51.009550 N, 4.512445 E), respectively. The capture of adders took place before the annual skin shedding. Adders and lizards were housed in separate rooms. Adders were held individually in terraria of 100×50×50 cm (l×w×h) with a 60-W incandescent lamp suspended above one side to provide an optimal temperature gradient. Lizards were housed in separate terraria, as well, measuring 41×41×71 cm. In these terraria, heat was provided by a 42-W incandescent lamp. The floors of all terraria were covered with river sand, pebbles, and moss to mimic a natural environment. A 12:12 hour light:dark circadian rhythm was upheld. Water was sprayed inside each terrarium daily to guarantee optimal humidity. At noon, lamps were switched off for half an hour to prevent overheating. Water was available *ad libitum* and lizards were fed vitamin E-dusted crickets (*Acheta domesticus*) twice a week and wax moth (*Galleria mellonella*) larvae once a week. The adders were not fed during their stay in the lab. The individuals were released to their provenance location in the wilderness as soon as the experiments ended.

Reptile capturing and housing was conducted with permission of the Nature and Forest Agency of Belgium (permit reference number for *Z. vivipara*, *P. muralis* and *V. berus* are ANB/ BL/FF-V16-00012, ANB/BL-FF/V18-00030, and ANB/BL-FF/V18-00029, respectively). All experiments with reptiles were approved by the ethical committee of the University of Antwerp (2015-34).

Although collection of dog urine does not include any animal experiments according to Belgian (Art. 2.6 of the Belgian Law of May 4th 1995; Annex VII, Belgian Law of May 29th 2013) and European legislation (European Convention for the protection of Vertebrate animals used for experimental and other scientific purposes), sampling has been conducted in agreement with the Ethical Committee of Animal Experiments of Vrije Universiteit Brussel (Project 16-634-3).

Table S1 List of compounds identified by thermal desorption GC-MS (Gas Chromatography Mass Spectrometry) in at least one individual of the European adders (*Vipera berus*) and not found in the control samples. The compounds were identified by comparing the mass spectra of volatiles with the spectra present in the NIST library (National Institute of Standards and Technology).

Retention time (min)	Peak area	Molecular formula	Compound name
1.997	125,490	N_2O	Nitrous oxide
2.166	181,981	CH_3NO_2	Methyl nitrite
2.272	574,259	C2H4O	Acetaldehye
2.320	309,442	CH_4S	Methanethiol
2.407	261,940	C_2H_6O	Ethanol
2.479	5,616,167	C_3H_6O	Acetone
2.756	509,880	C_3H_8O	1-Propanol
2.943	1,927,246	$C_2H_4O_2$	Acetic acid
3.539	355,789	$C_4H_{10}O$	1-Butanol
3.983	181,932	$C_3H_6O_2$	Propanoic acid
4.120	188,741	$C_5H_{10}O_3$	Ethyl (S)-(-)-Lactate
5.290	107,654	$C_4H_{10}O_2$	2,3-Butanediol
5.451	1,064,730	$C_6H_{12}O$	Hexanal
7.159	172,047	C_8H_{10}	p-Xylene
7.315	169,788	C_8H_{16}	1-Octene
7.555	12,304,658	C_8H_8	Styrene
7.559	2,792,853	C_8H_8	1,3,5,7-Cyclooctatetraene
7.638	758,355	$C_3H_6O_3$	Lactic acid
8.184	100,517	C_9H_{20}	Nonane
9.238	117,686	$C_{22}H_{40}O_2$	2H-Pyran, 2-(7-heptadecynyloxy)tetrahydro-
9.435	105,543	$C_8H_{16}O_2$	2H-Pyranmethanol, tetrahydro-2,5-dimethyl-
9.703	92,096	$C_5H_{12}O_3$	1,3-Propanediol, 2-(hydroxymethyl)-2-methyl-
9.834	2,548,204	$C_8H_{14}O$	5-Hepten-2-one, 6-methyl-
9.979	329,492	$C_8H_{16}O$	2-Octanone
9.988	296,575	$C_9H_{18}O$	Heptyl methyl ketone
10.159	402,904	C_7H_9NO	4-Cyanocyclohexene
10.172	719,911	$C_{10}H_{22}$	Hexane, 2,2,3,3-tetramethyl-
10.558	112,418	$C_{12}H_{26}$	Decane, 2,2-dimethyl-
10.645	291,230	$C_6H_{12}O$	2-Pentanone, 3-methylene-
10.652	253,882	$C_6H_{10}O$	3-Hexen-2-one
10.727	401,711	$C_7H_{10}O_2$	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-

10.737	361,616	$C_{12}H_{22}O_3$	Carbonic acid, nonyl vinyl ester
10.828	409,374	$C_5H_{12}O_2$	Neopentyl glycol
10.834	193,322	$C_5H_{11}ClO$	3-Chloro-2,2-dimethyl-1-propanol
11.008	384,590	$C_9H_{18}O_2$	Pentanoic acid, 1,1-dimethylpropyl ester
11.083	3,589,128	$C_8H_{18}O$	Hexanol <2-ethyl->
11.248	499,803	$C_{10}H_{16}$	D-Limonene
11.256	634,641	$C_{13}H_{22}O_2$	Geranyl propionate
11.331	1,446,749	$C_{10}H_{22}$	Heptane, 2,5,5-trimethyl-
11.430	185,763	$C_8H_{14}O_3$	Octanoic acid, 7-oxo-
11.438	204,030	$C_{16}H_{30}O$	Cyclopropane, 1-(1-hydroxy-1-heptyl)-2-methylene-3-pentyl-
11.787	149,748	$C_{10}H_{12}$	Benzene, (1-methylenepropyl)-
11.802	165,133	$C_6H_{11}O_2$	Ethyl 2-methylpentyl carbonate
11.974	75,974	$C_9H_{18}O$	Nonanal
12.073	78,534	$C_7H_{12}O_2$	2(3H)-Furanone, 5-ethyldihydro-5-methyl-
12.082	256,950	$C_{13}H_{28}$	Nonane, 5-(2-methylpropyl)-
12.133	443,335	$C_8H_{18}O$	1-Octanol
12.267	5,712,634	$C_{11}H_{24}$	Octane, 5-ethyl-2-methyl-
12.553	563,983	$C_9H_{18}O$	2-Nonanone
13.259	208,929	$C_{11}H_{24}$	Undecane
13.269	429,633	$C_{11}H_{24}$	Undecane <n->
13.372	1,009,899	$C_{12}H_{26}$	Decane, 3,7-dimethyl-
13.565	113,446	$C_{12}H_{24}$	2-Heptanone, 5-methyl-
13.577	252,228	$C_{22}H_{26}O_2$	2,9-Decanedione
13.622	380,377	$C_5H_8O_4$	Succinic acid, tridec-2-yn-1-yl pentafluorophenyl ester
13.724	146,104	$C_{12}H_{24}$	3-Dodecene, (E)-
13.918	134,838	$C_{10}H_{10}O$	1-(2-Vinylphenyl)ethanone
14.003	499,603	$C_{10}H_{10}$	1H-Indene, 3-methyl-
14.101	2,389,686	$C_9H_{10}O$	Benzaldehyde, 4-ethyl-
14.111	2,607,243	$C_9H_{10}O$	Benzaldehyde <para-ethyl->
14.242	492,952	$C_{10}H_{18}O_4$	3-t-Butyl-hexanedioic acid
14.551	13,702,745	$C_7H_6O_2$	Benzoic acid
14.693	760,551	$C_8H_{16}O_2$	Octanoic acid
14.767	421,451	$C_8H_{16}O_2$	3-cis-Methoxy-5-trans-methyl-1R-cyclohexanol
14.868	1,025,968	$C_{13}H_{28}$	Undecane, 2-methyl-
14.920	451,213	$C_{11}H_{14}$	Benzene, (2-methyl-1-butenyl)-
15.248	279,830	$C_{11}H_{18}$	2,2-Dimethyl-3-vinyl-bicyclo[2.2.1]heptane
15.251	502,533	$C_{11}H_{12}$	Benzene,2-cyclopenten-1-yl-
15.363	6,714,945	$C_{10}H_{20}O$	Decanal
15.533	261,984	C_7H_5NS	Benzothiazole

15.610	238,748	$C_{10}H_{12}O_2$	Benzaldehyde, 4-(1-methylethyl)-
15.723	1,439,355	$C_{12}H_{26}$	Dodecane
15.805	125,126	$C_{18}H_{24}O$	1-(1-Adamantyl)-1-phenylethanol
15.820	263,223	$C_9H_{10}O$	2-Propen-1-ol, 3-phenyl-, (E)-
15.943	932,161	$C_{14}H_{30}$	Tetradecane
16.029	671,454	$C_{21}H_{44}O_3S$	Sulfurous acid, hexyl pentadecyl ester
16.093	162,687	$C_8H_{14}O_2$	2(3H)-Furanone, 5-butyldihydro-
16.105	1,043,863	$C_{13}H_{28}$	Undecane, 2,5-dimethyl-
16.348	218,129	$C_9H_{10}O_2$	Phenethyl formate
16.654	320,019	$C_{10}H_{18}O$	2-Decenal, (Z)-
16.786	142,234	$C_9H_{10}S$	Cyclopropyl phenyl sulphide
17.083	1,344,893	$C_{16}H_{30}O_2$	Benzenepropanoic acid, 10-undecenyl ester
17.102	2,273,001	$C_{14}H_{30}$	Decane, 2,3,5,8-tetramethyl-
17.304	1,490,030	$C_{15}H_{32}$	Tetradecane, 5-methyl-
17.478	367,330	$C_{11}H_{22}O$	Undecan-2-one
17.579	411,353	$C_{25}H_{52}$	2-Methyltetracosane
17.689	299,283	$C_{11}H_{24}$	Octane, 2,6,6-trimethyl-
17.716	3,183,705	$C_{14}H_{30}$	Dodecane, 4,6-dimethyl-
17.771	1,640,189	$C_{11}H_{22}O$	Undecanal
17.862	202,295	$C_{10}H_{22}$	4,4-Dimethyl octane
17.913	496,860	$C_{13}H_{28}$	Nonane, 5-butyl-
18.052	131,569	$C_{13}H_{28}$	Tridecane <n->
18.232	536,419	$C_{16}H_{34}$	Hexadecane
18.301	370,674	C_8H_{18}	Hexane, 3,3-dimethyl-
18.525	168,865	$C_{10}H_{18}O_2$	Dodecalactone <gamma->
18.602	260,651	$C_{20}H_{42}$	Hexadecane, 2,6,11,15-tetramethyl-
18.856	226,183	$C_{16}H_{30}O_4$	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate
18.993	249,759	$C_{10}H_{18}O$	2-Decenal, (E)-
19.170	235,406	$C_{15}H_{32}$	Tetradecane, 4-methyl-
19.193	76,893	$C_{12}H_{22}O_2$	4-tert-Butylcyclohexyl acetate
19.280	422,220	$C_{12}H_{24}O_3$	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester
19.336	244,978	$C_{13}H_{28}$	Nonane, 5-methyl-5-propyl-
19.403	766,367	$C_{15}H_{32}O_2$	8,10-Dioxaheptadecane
19.506	247,557	$C_{14}H_{30}$	Tridecane, 2-methyl-
19.653	62,609	$C_{13}H_{28}$	Dodecane, 3-methyl-
19.734	212,817	$C_{12}H_{24}O$	Decyl methyl ketone
19.795	197,651	$C_{10}H_{20}$	3-Ethyl-2-pentadecanone
19.830	63,615	$C_{15}H_{32}$	Dodecane, 2,6,10-trimethyl-
20.046	1,093,562	$C_{12}H_{24}O$	Dodecanal <n->
20.194	156,011	$C_9H_{18}O$	3-Ethyl-2-undecanone
20.365	512,973	$C_{12}H_{16}$	Benzene, cyclohexyl-

20.393	373,488	$C_{12}H_{16}$	5,6,7,8,9,10-Hexahydrobenzocyclooctene
20.433	98,544	$C_{11}H_{24}O_3S$	Sulfurous acid, isohexyl 2-pentyl ester
20.579	310,653	$C_{15}H_{32}O$	Ether, dodecyl isopropyl
20.645	167,515	$C_{14}H_{22}$	syn-Tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-hexamethyl-
20.668	347,340	$C_{27}H_{55}Cl$	Heptacosane, 1-chloro-
20.920	739,783	$C_{13}H_{22}O$	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-
21.169	110,991	$C_{18}H_{38}$	Octadecane <n->
21.268	568,984	$C_{14}H_{20}O_2$	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-
21.356	170,641	$C_{10}H_{22}O_2$	Propane, 1,1'-[ethylidenebis(oxy)]bis[2-methyl-
21.456	333,051	$C_{17}H_{36}$	Heptadecane
21.512	1,083,323	$C_{12}H_{26}O$	1-Dodecanol
21.702	404,724	$C_{13}H_{28}$	Eicosane, 2,4-dimethyl-
21.886	126,844	$C_{13}H_{26}O$	Tridecan-2-one
22.100	51,663	$C_{10}H_{22}$	3-Ethyl-3-methylheptane
22.173	981,453	$C_{13}H_{26}O$	Tridecanal <n->
22.263	354,034	$C_{16}H_{34}$	Hexadecane <n->
22.597	139,102	$C_{12}H_{24}O_3$	Dodecanoic acid, 3-hydroxy-
22.725	279,227	$C_{20}H_{42}$	Eicosane
23.221	675,537	$C_{12}H_{17}NO$	Diethyltoluamide (DEET)
23.279	343,166	$C_{32}H_{66}$	Dotriacontane <n->
23.377	320,694	$C_{13}H_{24}O_4$	Glutaric acid, di(isobutyl) ester
23.444	352,976	$C_{12}H_{14}O_4$	Diethyl Phthalate
23.644	115,502	$C_{13}H_{28}$	Dodecane, 2-methyl-
23.955	384,344	$C_{15}H_{30}O$	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate
24.417	88,878	$C_{17}H_{34}O_2$	Tridecanoic acid, 4,8,12-trimethyl-, methyl ester
24.611	587,035	$C_{15}H_{30}O_2$	Dodecanoic acid, 1-methylethyl ester
24.738	176,900	$C_{37}H_{76}O$	1-Heptatriacotanol
24.807	132,133	$C_{16}H_{26}$	Benzene, (1-butylheptyl)-
25.021	184,489	$C_{16}H_{18}$	Ethane, 1-(o-ethylphenyl)-1-phenyl-
25.117	47,548	$C_{15}H_{24}O_2$	Murolan-3,9(11)-diene-10-peroxy
25.233	114,842	$C_{15}H_{32}$	Dodecane, 2,6,11-trimethyl-
25.362	484,476	$C_{18}H_{34}O_2$	Heptadecafluorononanoic acid, pentadecyl ester
25.511	817,522	$C_{16}H_{20}$	1,7-di-iso-propylnaphthalene
25.751	736,153	$C_{17}H_{32}O_2$	2-Propenoic acid, tridecyl ester
25.778	365,154	$C_{18}H_{34}O_2$	2-Propenoic acid, pentadecyl ester
25.879	237,824	$C_{18}H_{36}O$	2-Pentadecanone, 6,10,14-trimethyl-

26.004	95,440	$C_{15}H_{22}O_2$	Benzoic acid, 2-ethylhexyl ester
26.057	251,577	$C_{17}H_{34}O_3$	Malonic acid, 2-heptyl tetradecyl ester
26.134	799,071	$C_{16}H_{20}$	2,6-Diisopropylnaphthalene
26.233	1,199,717	$C_{18}H_{38}$	Heptadecane, 2-methyl-
26.328	136,487	$C_{16}H_{20}$	1,3-di-iso-propylnaphthalene
26.654	272,646	$C_{16}H_{18}$	2-(p-Tolylmethyl)-p-xylene
26.725	92,861	$C_{18}H_{38}$	Heptadecane, 8-methyl-
26.850	141,390	$C_{11}H_{16}$	Benzene, (2-decyldodecyl)-
26.875	158,440	$C_9H_9N_3O_3$	Benzene, (1-propylheptadecyl)-
26.943	59,178	$C_{13}H_{28}$	Tridecane
27.047	91,916	$C_{11}H_{24}O$	2-Isopropyl-5-methyl-1-heptanol
27.257	224,772	$C_{18}H_{30}$	Benzene, (1-ethyldecyl)-
27.460	225,446	$C_{34}H_{70}$	Tetratriacontane <n->
27.788	112,046	$C_{15}H_{22}O_3$	Salicylate <2-ethylhexyl->
27.840	31,675	$C_{18}H_{34}O$	8-Octadecenal
27.913	105,031	$C_{12}H_{14}N_2O_2$	Benzene, (1,3,3-trimethylnonyl)-
28.240	376,029	$C_{27}H_{56}$	2-Methylhexacosane
28.307	82,577	$C_{31}H_{56}$	Pentacosane, 13-phenyl-
28.422	190,874	$C_{18}H_{30}$	Benzene, (3,3-dimethyldecyl)-
28.506	620,849	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
28.755	161,356	$C_{15}H_{26}O_2$	Isocalamendiol
30.022	75,749	$C_{22}H_{34}O_4$	1,2-Benzenedicarboxylic acid, butyl decyl ester
30.357	1,580,876	$C_{16}H_{32}O_2$	Hexadecanoic acid <n->
31.533	57,602	$C_{19}H_{38}$	7-Octadecyne, 2-methyl-
32.090	69,380	$C_{12}H_{22}$	2,4-Dodecadiene, (E,Z)-
32.730	254,504	$C_{20}H_{40}O_2$	Dodecanoic acid, n.-octyl ester
33.484	561,662	$C_{18}H_{36}O_2$	Stearic acid
34.423	63,006	$C_{22}H_{42}O_2$	Phytol acetate
39.711	716,935	$C_{26}H_{54}$	Hexacosane <n->
39.757	122,960	$C_{15}H_{28}$	1-Pentadecyne
40.813	79,759	$C_{15}H_{30}O$	Pentadecanal-
41.621	273,083	$C_{27}H_{46}O$	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate
42.387	70,185	$C_{27}H_{44}$	Cholesta-2,4-diene
42.692	127,275	$C_{27}H_{44}$	Cholesta-3,5-diene
42.814	231,443	$C_{27}H_{44}O$	Cholesta-4,6-dien-3-ol, (3.beta.)-
44.236	1,006,821	$C_{27}H_{44}O$	Cholesta-5,7-dien-3.beta.-ol, 3,5-dinitrobenzoate
46.240	550,486	$C_{27}H_{42}O$	Cholesta-3,5-dien-7-one

Table S2 List of compounds identified by thermal desorption GC-MS (Gas Chromatography Mass Spectrometry) in at least one individual of wall lizard (*Podarcis muralis*) and not found in the control samples. The compounds were identified by comparing the mass spectra of volatiles with the spectra present in the NIST library (National Institute of Standards and Technology).

Retention time (min)	Peak area	Molecular formula	Compound name
2.137	244,964	CH_3NO_2	Methyl nitrite
2.214	23,994,000	CH_3Cl	Chloromethane
2.397	781,325	C_2H_6O	Ethanol
2.888	745,209	$C_6H_{14}O$	1-Pentanol, 2-methyl-
2.943	596,891	$C_2H_4O_2$	Acetic acid
3.551	137,036	$C_5H_9ClO_2$	Acetic acid, chloro-, isobutyl ester
3.960	178,346	C_7H_{14}	1-Hexene, 4-methyl-
4.010	506,139	$C_6H_{12}O$	2-Butanone, 3,3-dimethyl-
4.100	316,250	$C_9H_{20}O$	Pentane, 1-methoxy-
4.105	373,027	$C_5H_{12}O_3$	2-Propanol, 1,3-dimethoxy-
4.868	579,391	$C_6H_{14}O$	2-Pentanol, 4-methyl-
5.014	286,420	C_7H_8	Toluene
5.212	206,527	C_9H_{20}	Heptane, 3,4-dimethyl-
5.233	163,536	$C_8H_{18}O$	1-Hexanol, 2-ethyl-
5.236	248,818	$C_8H_{18}O$	1-Pentanol, 2-ethyl-4-methyl-
5.927	232,802	$C_5H_{10}O_3$	Butanoic acid, 3,3-dimethyl-, methyl ester
6.353	67,415	$C_{11}H_{24}$	Hexane, 2,3,4-trimethyl-
6.360	78,231	C_8H_{18}	Hexane, 2,4-dimethyl-
6.953	160,103	C_8H_{10}	Ethylbenzene
7.307	267,719	$C_6H_{10}O$	Cyclohexanone
7.708	124,994	$C_7H_{14}O$	Heptanal <n->
7.807	98,459	$C_8H_{16}O_2$	1-Pentanol, 2-methyl-, acetate
8.188	223,285	$C_{10}H_{22}$	Decane
8.913	152,429	$C_{10}H_{16}$	Pinene <alpha->
8.993	279,597	C_7H_6O	Benzaldehyde
9.752	62,774	C_9H_{20}	Heptane, 3-ethyl-
9.851	942,304	$C_{10}H_{11}NO$	2,6-Dimethyl-6-phenyl-5,6-dihydro(4H)-1,3-oxazine
10.160	1,170,936	$C_6H_{12}O$	4-Penten-1-ol, 2-methylene-
10.548	80,092	$C_{12}H_{26}$	Heptane, 2,2,4,6,6-pentamethyl-
10.552	306,668	C_9H_{20}	Heptane, 2,2,4,6,6-pentamethyl-
10.648	125,398	$C_8H_{16}O_2$	Acetic acid, hexyl ester
10.716	90,723	$C_{10}H_{22}$	Decane <n->
10.807	148,608	$C_{10}H_{16}$	3-Carene
10.817	56,709	$C_{10}H_{16}$	.beta.-Ocimene
10.995	96,135	$C_{10}H_{22}$	Octane, 3,3-dimethyl-

11.101	357,345	$C_8H_{18}O$	2-Ethyl-1-hexanol
11.115	1,478,573	$C_{11}H_{24}$	Undecane
11.250	813,194	$C_{10}H_{16}$	Limonene
11.254	473,813	$C_{11}H_{24}$	Nonane, 2,5-dimethyl-
11.326	1,409,101	$C_{11}H_{24}$	Decane, 4-methyl-
11.972	82,063	$C_9H_{18}O$	Nonanal <n->
12.074	286,238	$C_{13}H_{28}$	Nonane, 5-(2-methylpropyl)-
12.170	48,765	$C_8H_{18}O$	Octanol <n->
12.226	542,575	$C_{10}H_{12}$	(E)-1-Phenyl-1-butene
12.262	511,431	$C_{13}H_{28}$	Undecane, 4,7-dimethyl-
12.527	132,797,689	$C_8H_8O_2$	Benzoic acid, methyl ester
12.563	29,209	$C_{12}H_{24}O$	2-Dodecanone
13.663	452,049	$C_{11}H_{14}$	Benzene, 1-pentenyl-
13.895	153,551	$C_{11}H_{14}$	Benzene, (3-methyl-2-butenyl)-
13.902	198,267	$C_{11}H_{14}$	Benzene, 2-ethenyl-1,3,5-trimethyl-
13.904	199,374	$C_{11}H_{14}$	Benzene, (1-ethyl-1-propenyl)-
13.963	154,719	$C_8H_8O_3$	3,4-Dihydroxyacetophenone
14.007	538,178	$C_{10}H_{10}$	Benzene, (1-methyl-2-cyclopropen-1-yl)-
14.393	101,822	$C_6H_{10}O_3$	Hexanoic acid, 4-oxo-, methyl ester
14.465	186,129	$C_{10}H_{18}O$	Dec-(2E)-enal
14.506	1,282,376	$C_9H_{10}O$	Phenol, 4-(2-propenyl)-
14.558	1,194,628	C_8H_6	Bicyclo[4.2.0]octa-1,3,5-triene, 7-isopropyl-
14.607	314,764	$C_6H_3Cl_3$	Benzene, 1,2,3-trichloro-
14.633	471,741	$C_{10}H_{22}$	Heptane, 4-(1-methylethyl)-
14.678	834,363	$C_{12}H_{24}O$	Oxirane, decyl-
14.760	521,196	$C_{12}H_{26}$	Undecane, 4-methyl-
14.782	570,234	$C_7H_{12}O_2$	Cyclohexanone, 4-hydroxy-4-methyl-
14.873	137,383	$C_{11}H_{24}$	Nonane, 3,7-dimethyl-
14.873	155,614	$C_{12}H_{26}$	Undecane, 3,4-dimethyl-
15.167	250,991	$C_{12}H_{26}$	Undecane, 4,4-dimethyl-
15.365	1,684,180	$C_{10}H_{20}O$	Decanal
15.460	85,738	$C_{12}H_{24}$	6-Dodecene, (E)-
15.725	627,632	$C_{10}H_{22}$	Dodecane <n->
16.015	1,738,118	$C_8H_{12}O_2$	2,4-Hexadienoic acid, ethyl ester
16.109	666,846	$C_{13}H_{28}$	Decane, 2,6,7-trimethyl-
16.112	135,766	$C_{13}H_{28}$	Undecane, 4,6-dimethyl-
16.196	300,123	$C_{12}H_{26}$	Decane, 3,6-dimethyl-
16.760	109,708	$C_{10}H_{10}O$	Cinnamaldehyde <alpha-methyl->
17.105	1,049,014	$C_{12}H_{25}Cl$	Dodecane, 1-chloro-
17.404	5,107,699	$C_{10}H_{12}O_2$	Benzoic acid, 4-ethyl-, methyl ester
17.752	2,629,929	$C_{10}H_{12}O_2$	Benzoic acid, 4-ethyl-, methyl ester
18.077	122,830	$C_{13}H_{28}$	Tridecane

18.165	171,029	$C_{10}H_{10}O_2$	Cinnamate <methyl-, (Z)->
19.017	42,530	$C_8H_{17}Cl$	Octane, 2-chloro-
19.237	72,187	$C_{19}H_{40}$	Octadecane, 6-methyl-
19.287	132,528	$C_{12}H_{24}O_3$	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester
19.508	148,690	$C_{21}H_{44}$	Eicosane, 10-methyl-
19.746	146,102	$C_{16}H_{34}$	Undecane, 5-ethyl-5-propyl-
19.758	243,067	$C_{12}H_{10}O$	Biphenyl oxide
19.843	54,430	$C_{13}H_{28}$	Undecane, 3,8-dimethyl-
20.362	49,503	C_9H_{20}	Octane, 2-methyl-
20.519	154,591	$C_{16}H_{33}Cl$	Hexadecane, 1-chloro-
20.527	200,998	$C_{13}H_{28}$	Nonane, 5-(1-methylpropyl)-
20.580	137,800	$C_8H_{18}O$	1-Hexanol, 2,2-dimethyl-
20.674	814,921	$C_{12}H_{16}O_2$	Benzoic acid, 2-ethylbutyl ester
20.877	154,209	$C_{21}H_{44}$	Heneicosane
20.931	158,608	$C_{13}H_{22}O$	5,9-Undecadien-2-one, 6,10-dimethyl-
21.525	335,816	$C_{40}H_{82}O_2$	Hexadecane, 1,1-bis(dodecyloxy)-
21.580	147,165	$C_{12}H_{26}$	Decane, 3,8-dimethyl-
21.593	77,279	C_9H_{20}	Octane, 3-methyl-
22.157	91,217	$C_{15}H_{32}O$	n-Pentadecanol
22.373	204,999	$C_{13}H_{28}$	Decane, 6-ethyl-2-methyl-
22.383	71,445	$C_{19}H_{30}O_3$	Carbonic acid, decyl vinyl ester
22.605	269,479	$C_{18}H_{38}$	Hexadecane, 7,9-dimethyl-
22.732	39,080	$C_{13}H_{28}$	Decane, 5-ethyl-5-methyl-
23.093	79,123	$C_9H_{10}O_2$	Octane, 2,3,6,7-tetramethyl-
23.390	35,497	$C_{21}H_{42}O_2$	1,3-Dioxocane, 2-pentadecyl-
23.496	143,933	$C_{34}H_{58}O_4$	Bis(tridecyl) phthalate
24.160	352,799	$C_{17}H_{34}$	1-Heptadecene
24.241	196,474	$C_{12}H_{24}O$	Dodec-2-en-1-ol <trans->
24.447	57,776	$C_9H_{10}O_2$	4-Ethylbenzoic acid, 4-methylpentyl ester
24.517	31,331	$C_{16}H_{34}O_3S$	Sulfurous acid, pentyl undecyl ester
24.530	60,608	$C_8H_{18}O$	Pentyl tetradecyl ether
24.814	79,601	$C_{26}H_{46}$	Benzene, (1-butylhexadecyl)-
25.233	132,464	$C_{12}H_{16}O_4$	2-(2-Methoxyethoxy)ethyl benzoate
25.350	246,463	$C_{18}H_{28}$	Carbonic acid, dodecyl vinyl ester
25.429	250,039	$C_{28}H_{58}O$	Ditetradecyl ether
25.470	55,063	$C_{10}H_{14}O$	Benzene, 2-methoxy-1,3,5-trimethyl-4-nitro-
25.785	102,059	$C_{30}H_{50}O_4$	1,2-Benzenedicarboxylic acid, diundecyl ester
26.101	604,774	$C_{15}H_{15}N$	1,2-Diphenyl-1-isocyanoethane
26.620	317,209	$C_{16}H_{14}$	Benzene, 1,1'-(1,2-cyclobutanediyl)bis-,

			trans-
27.296	669,859	$C_{16}H_{14}$	Benzene, 1,1'-(1,3-butadienylidene)bis-
27.787	94,093	$C_{15}H_{22}O_3$	2-Ethylhexyl salicylate
27.995	559,928	$C_{15}H_{26}O_2$	Oxacyclohexadec-(12E)-en-2-one
28.118	123,852	C_9H_{12}	Spiro[4.4]nona-1,6-diene, (S)-
28.407	417,857	$C_{16}H_{28}O$	5-Cyclohexadecen-1-one
28.430	481,186	$C_{16}H_{12}$	Anthracene, 9-ethenyl-
29.707	132,005	$C_{20}H_{42}$	10-Methylnonadecane
29.875	1,045,357	$C_{17}H_{34}O_2$	Hexadecanoate <methyl->
30.460	599,392	$C_{16}H_{14}O$	1(2H)-Naphthalenone, 3,4-dihydro-4-phenyl-
31.031	424,727	$C_{15}H_{26}O_4$	Ethylene brassylate
32.742	53,855	$C_{20}H_{40}O_2$	Dodecanoic acid, isooctyl ester
41.620	139,161	$C_{28}H_{46}O_2$	Cholesteryl formate
41.637	92,060	$C_{34}H_{50}O_2$	Cholesteryl benzoate

Chapter 7: The smell of danger passing by: How long does it linger?

Table S1 Cumulative variable values for the variable walking (s) in the pre-directed tongue flick period. From these values it can be said that presenting the lizard with fresh smell has a big impact on how it responds by walking (in the pre-directed tongue flick period) to further treatments. This is in contrast with scents at fade-out points of two and twelve hours, that have only a limited impact on further trials. Unscented control gauzes do not have an impact on further trials, or at least not of the magnitude compared to the scented treatments.

Scent treatment	Value (%)
Fresh	84
2 hours	8
12 hours	8
No scent (control)	0

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