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The Role of Multimodal Sensory Cues During Predator Detection by The Leopard Gecko (Eublepharis macularius)

Role multimodálních smyslových vjemů při detekci predátora gekončíkem nočním (Eublepharis macularius)

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 7. 8. 2024 David Hirschler

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Abstrakt

Cílem této práce bylo prozkoumat vliv multimodálních smyslových vjemů při detekci predátora gekončíkem nočním, *Eublepharis macularius*, ve srovnání s vjemy unimodálními. V průběhu pokusů jsme dále ověřili validitu používání svlečené hadí kůže jako chemického stimulu vyvolávajícího antipredační reakce. Rovněž jsme testovali rozdíly v reaktivitě mezi třemi ontogenetickými stádii gekončíků nočních, konkrétně v juvenilním, subadultním a adultním věku. Signifikantní antipredační reakce byly vyvolány pouze tehdy, když byl mezi předkládanými stimuly přítomen i stimulus chemický. Neobjevili jsme žádný signifikantní vliv vizuálních a akustických/mechanosensorických vjemů na rozsah či intenzitu antipredačních reakcí, což naznačuje, že chemická modalita je v tomto kontextu dominantní. Ontogenetická stádia se signifikantně lišila svou reaktivitou. Juvenilní a subadultní gekoni byli průměrně reaktivnější než dospělci s tím, že subadulti projevovali nejvyšší míru reaktivity. Gekončíci v žádném experimentu neprojevili průkaznou míru antipredačního chování v reakci na mikroten, což potvrzuje validitu jeho používání jako kontrolního stimulu.

Klíčová slova

antipredační chování, multimodalita, detekce predátora, šupinatí plazi, gekončík noční

Abstract

The aim of this thesis was to examine the effects of multimodal sensory inputs during predator detection by the leopard gecko, *Eublepharis macularius*, as compared to individual unimodal cues. Throughout the process we further validated the use of shed snakeskin as a chemical stimulus eliciting antipredator reactions. We also tested differences in reactivity between three ontogenetic stages of leopard geckos, namely juveniles, subadults and adults. We found that combined multimodal cues elicited significant antipredator response only when containing a chemical stimulus. Furthermore, we discerned no significant effect of visual or acoustic cues on the scope and intensity of antipredator reaction, therefore suggesting that chemical cues play a dominant role in this context. Ontogenetic stages significantly differed in their reactivity. On average, juveniles and subadults were shown to be more reactive than adults, with subadults displaying the highest degree of reactivity. Geckos in all experiments showed no significant amount of antipredator reaction in response to HDPE (high-density polyethylene), confirming the validity of its use as a control stimulus.

Key words

antipredator behavior, multimodality, predator detection, squamate reptiles, leopard gecko

Content

Introduction

The threat of predation is a nearly universal constant for most animals. This has caused immense selective pressure on organisms to develop characteristics designed to avoid predator detection, deter predators and detect threats. Being able to recognize predators can be especially important, since failing to initiate the appropriate antipredator response can have fatal consequences (Blanchard et al. 1990). Antipredator behavior is also quite costly, as it requires animals to expend energy (for instance during escape or active defense), or to reduce their activity, costing them time that could otherwise be spent foraging or mating (Labra & Niemeyer 2004, Farallo et al. 2010). These costs may be even more significant for ectothermic reptiles due to the restriction of basking time (Cooper 2000). The ability to detect and recognize predators is heavily dependent on the potential prey's sensory systems. Very commonly, animals depend on their visual abilities to detect predators (Cooper 2008, Pessoa et al. 2014). It is, however, not always possible to recognize predators visually. Predators could lay hidden waiting to strike, or both prey and predators may live under conditions where visual detection is either impossible or significantly hindered. Due to the prevalence of such situations, other means of detection are necessary. Chemical cues are another frequent avenue for predator detection, although they can usually only be used to recognize the near presence of a predator, rather than their exact location (Hartman & Abrahams 2000, Amo et al. 2004, Hickman et al. 2004). Mechanosensory/acoustic stimuli are also employed (Huang et al. 2011, Elmasri et al. 2012, Jung et al. 2020).

Given the complex nature of the environment organisms inhabit, it is only logical to assume, that combining these different modalities will allow animals to avoid predation more successfully, therefore increasing their chances of survival and reproduction. Even though there have been a fair number of studies devoted to the subject of multimodal stimuli during predator detection (e.g., Hartman and Abrahams 2000, Amo et al. 2006, Cliff et al. 2022), there are still many unanswered questions regarding the effectiveness of experimental methods as well as the actual importance of multimodality.

We set 4 main goals in this thesis:

1) To compare the antipredator responses of leopard geckos (*Eublepharis macularius*) when exposed to stimuli targeting one or multiple modalities.
2) To test the dominance of modalities during the process of predator detection while attempting to isolate the effect of individual modalities.

3) To assess and compare the reactivity of leopard geckos within three different ontogenetic stages when exposed to shed snakeskin.
4) To compare reactivity to shed snakeskin stored under disparate conditions.

We predict that leopard geckos will display antipredator responses more frequently when exposed to multimodal stimuli, than to stimuli targeting only one modality. We also expect that of the unimodal stimuli, chemical cues will prove the most effective at eliciting antipredator behavior. Furthermore, we anticipate (based on previous observations), that juvenile and subadult leopard geckos will display a greater degree of antipredator behavior, compared to that of adults. Finally, we believe that shed snakeskins stored under sub-optimal conditions (in an open container at room temperature) will elicit fewer antipredator responses than snakeskins stored under conditions, which were previously established to maintain scent effectively (in a sealed container under -28 °C).

1 Literature Review

1.1 Introduction to multimodality

The idea that animals communicate using complex multicomponent signals is nothing new. The thought was notably expressed by Charles Darwin as early as 1872 (Darwin 1872). However, the next 100 years saw relatively little research done on the topic of multimodality, until the 1990's came with the publication of high-impact papers (Rowe & Guildford 1996; Partan & Marler 1999), which spurred a new wave of researchers devoted to the subject in the following years (Higham & Hebets 2013).

Most definitions regarding multimodality refer to multimodal signals. It is important to underline the difference between two types of traits known as cues and signals. In a widely used and supported definition, signals differ from cues by the selective pressure under which their communicative function is formed. Cues may also carry specific information to other organisms, but they have not evolved specifically to do so (Maynard Smith & Harper 2003).

Multimodal signals can be defined as signals that are received through two or more sensory modalities by the receiving organism. This definition requires a sufficient understanding of the animal's sensory systems as well as the relevant signaling environment (Higham & Hebets 2013). Classification of multimodal signals is frequently achieved by comparing receiver responses to the complete combined stimulus and to individual isolated components (Partan & Marler 1999). Using this understanding of multimodal signals makes applying various principles to multimodal cues easier thanks to the emphasis on the perception of the receiver, rather than the intention of the sender. This framework has been further modified and expanded to include greater emphasis on the effect of varying receiver sensory systems (Ronald et al. 2012) and ecological context (Munoz & Blumstein 2012).

It is necessary to distinguish between multimodal signals and unimodal composite signals. While the former contains components that affect different sensory channels, the latter consists of components directed at a single sensory channel (Partan & Marler 2005). Visual composite signals may for example simultaneously display size, color and movement, with each component contributing to the elicitation of a specific response (Endler 1993).

There are various ways in which individual parts of a multimodal signal interact with each other and affect resulting receiver behavior. Multimodal signals may be "fixed", where components of the signal are always produced together. For instance, for a frog to vocalize it must inflate its vocal sac, thus simultaneously producing both an acoustic and a visual signal (Smith 1977). Signals that can be produced independently are referred to as “free” (Wickler 1978). We may also split the components of multimodal signals into “redundant” (conveying the same information) and “nonredundant” (conveying different information). We may then further categorize these based on the effect they have on receiver behavior when combined compared to individual components. “Redundant” components may be split into “equivalent” (individual components cause the same reaction as the combined signal) and “enhancing” (the combined signal elicits a stronger response than individual components). “Nonredundant” components are divided into “independent” (both components elicit their own different responses even when combined), “dominant” (when combined, one component overpowers the other), “modulating” (when combined, one component doesn’t elicit its own response, but rather increases the intensity of the response elicited by the other component), and “emerging” (the combined signal causes an entirely different response than individual components) (Partan & Marler 2005).

Multimodal cues have been shown to play a role in various interspecific and conspecific interactions of squamate reptiles. For example, multimodality influences the hunting behavior of Arafura file snakes (*Acrochordus arafurae*). During tests using artificial lures, females of the species showed a significantly higher tendency to attack lures that carried fish scent and were moving, thus combining chemical and visual cues to detect their prey. This study also showed interesting intraspecific differences. Males only showed increased hunting behavior toward lures carrying fish scent, regardless of movement. The study’s authors explain this disparity as the possible effect of foraging mode. While male file snakes are active foragers who search for their prey using scent, females are ambush predators who also depend on visual cues in order to strike at unsuspecting prey without revealing their own presence (Vincent et al. 2005). Males of the Coastal day gecko (*Cnemaspis littoralis*) possess both visual and chemical traits evolved for intraspecific communication, yellow gular patches and secreting ventral glands. When exposed to these conspecific stimuli unimodally, showed significantly fewer bouts of movement than when exposed to a multimodal stimulus combining visual and chemical cues (Kabir & Thaker 2021). When taking refuge from simulated predator attacks, wall lizards (*Podarcis muralis*) have been shown to exit refuges earlier when they contain visual cues of snakes, either alone or in combination with chemical cues. Chemical stimuli alone were not enough to significantly shorten time spent in refuges. However, the multimodal stimulus containing both chemical and visual cues was shown to have an even stronger effect than visual cues alone. This suggests an enhancing effect of individual modalities when combining to create a multimodal stimulus (Amo et al. 2004).

1.2 Leopard Gecko (*Eublepharis macularius*)

The leopard gecko, *Eublepharis macularius* (Blyth, 1854), is a reptile model species belonging to the family of Eublepharidae. It inhabits semi-humid rocky areas with scrubby vegetation throughout parts of Pakistan, India, Iran and Afghanistan (Seufer et al. 2005).

1.2.1 Morphology

Leopard geckos are relatively small (SVL 120-160 mm, tail length 89-90 mm) but robust reptiles with wide flat heads. As is the case with most geckos, the eye contains a vertical pupil. However, quite unlike the vast majority of Gekkotans, Eublepharids possess movable eyelids. The limbs also diverge from the family norm, each toe equipped with a sharp claw and missing the lamellar structure typical of geckos. The tail is large, ringed and very mobile, primarily serving as fat storage. Regeneration is imperfect, the regenerated appendage being shorter, more bulbous and lacking the ringed structure of the original tail. The skin consists of small granular scales separating larger round tubercules, making the surface rough and bumpy (Khan 2006).

1.2.2 Activity and Social System

Leopard geckos are nocturnal, spending nights foraging solitarily. Daytime is spent sleeping in rock crevices in groups of varying size. Group composition changes based on breeding activity. During the breeding season (March to July), reproductively active males disperse due to mutual aggression and maintain distance using scent marking. Males share shelter with several females in a breeding group. After a 10-to-20-day gestation period, females lay a fixed number of 2 relatively large eggs. Aside from careful egg placement, parental care is absent (Khan 2006). Sex determination is temperature-dependent (Viets et al. 1993).

1.2.3 Prey and Predators

Diet consists mainly of invertebrates. Adult leopard geckos occasionally prey on juvenile birds, snakes, rodents as well as other geckos. Adults are considered a serious predatory threat to conspecific juveniles. Other predators include owls, monitor lizards, foxes, jackals and, quite frequently, snakes (Khan 2006).

1.2.4 Antipredator Behavior

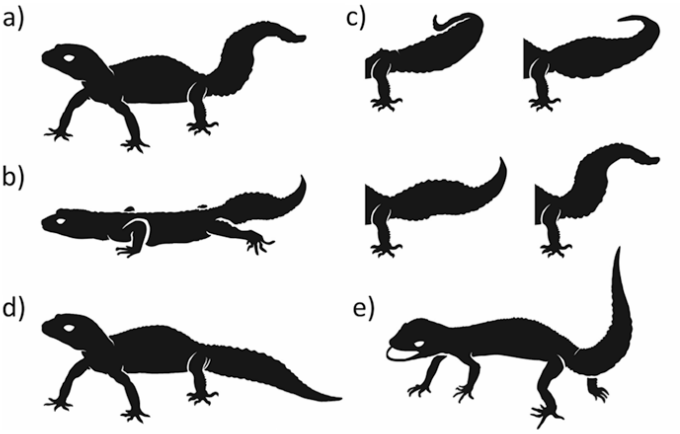

Figure 1: Illustration of different pose elements displayed by leopard geckos in antipredator reactions. (a) high posture with raised tail; (b) low posture with raised tail; (c) waving tail; (d) high posture without raised tail; (e) high posture with raised tail and mouth open (Drawing: Silvie Lišková; Taken from: Landová et al. 2023)

Strong predatory pressure has led leopard geckos to evolve several characteristic antipredator behaviors, making them an excellent model organism for the study of predator detection. These behaviors may be sorted into two categories, active and passive. Active antipredator behaviors are connected to threat and aggression. They include high posture on extended legs (Figure 1a, d), widely opening the mouth (Figure 1e) and biting. Passive antipredator behaviors are generally meant to distract and evade the predator. They include low posture (pressing the body flat against the ground, Figure 1b), waving the tail in the air (Figure 1c), freezing (remaining motionless for extended periods) and finally avoidance or escape from the predator (Landová et al. 2013). In extremely dangerous interactions with a predator, leopard geckos may autotomize their tail, which remains highly mobile for some time after detachment in an effort to distract predator attention (Bellairs & Bryant 1985). However, given the importance of the tail's function as a fat reserve, autotomy is quite costly (Russell et al. 2015).

1.2.4.1 Eliciting Methods

There are several well tested methods of eliciting antipredator responses in leopard geckos. Poking the gecko near the base of the tail has been shown to be a strongly aversive stimulus. Spraying water seems to be a weaker alternative to prodding, causing the same types of reactions, only with less intensity and reliability (Landová et al. 2013). Responses to live snakes vary in intensity based on snake species independently of sympatry. Therefore, *E. macularius* seems to possess a generalized aversion and antipredator reaction to all snakes. Active saurophagous foragers such as *H. ravergieri,* other members of the colubrid genera *Hemorrhois* (and to a lesser extent *Spalerosophis*) elicit the strongest responses with the most active defensive behavior. Other species such as the boid *Eryx johnii* tend to elicit more passive defensive behavior including low posture and staying motionless in order to avoid detection (Landová et al. 2016). Another proven method is the use of shed snakeskin as a chemical stimulus. The key odorant in eliciting antipredator responses in leopard geckos through chemical stimulation appears to be a non-polar molecule. This is supported by the significant reduction of antipredator behavior elicited in leopard geckos by snakeskins treated with non-polar solvents. Shed snakeskin was recently validated as a bioassay for predator scent in leopard geckos (Landová et al. 2023).

1.2.4.2 Ontogenetic Variation

Eublepharis macularius has been shown to possess varying arsenals of antipredator behavior based on the ontogenetic stage. Younger individuals (under 5 months of age) display frequent deterrent vocalizations when stimulated by light prodding with a stick or by spraying water, often followed by attacks towards the stimulus. The prevalence of this trait diminishes significantly with increasing age and is almost entirely absent among adults. Vocalizations in response to stick prodding seem to be gradually replaced by escape attempts, which represent an uncommon strategy among juveniles but become increasingly frequent with age up until subadulthood. Once past the age of 8-9 months, escape attempts gradually lose their prevalence, but remain quite common even among full adults. Defensive postures seem to play a prominent role in antipredation regardless of ontogenetic stage (Landová et al. 2013).

1.3 Sensory Perception

The way any organism interacts with its environment is dictated by its sensory apparatus. Without a clear understanding of how an animal perceives incoming information, accurately interpreting and predicting its behavior is difficult, if not impossible. This represents a vital issue for any behavioral study. Considering the subject of this thesis, this chapter will be centered around *Squamate* sensory perception with special focus devoted to *Gekkotans.*

1.3.1 Squamate Sensory Apparatus

As one would expect in such a large and successful group of vertebrates, Squamates possess a strong arsenal of sensory receptors. Aside from some specifically adapted clades, most squamates have well-developed vision. Unlike most vertebrates, Squamates lack a duplex retina consisting of both rods and cones (Baden & Osorio 2019). Instead, their retinas consist of a single type of receptor, which can be more cone-like in diurnal animals (Schott et al. 2016), or more rod-like in nocturnal animals (Yokoyama & Blow 2001). Despite this, many Squamate species can see within a broad spectrum of wavelengths, including UV. This is aided by the presence of oil droplets with varying properties which may improve photoreceptor spectral sensitivity and discrimination (Loew et al. 2002). The ability to detect wavelengths within the UV spectrum was first confirmed in anoles (Kawamura & Yokoyama 1998) and later in geckos

(Yokoyama & Blow 2001). Knowledge on the visual system of snakes is still quite limited. Most snakes do possess both UV-sensitive and long-wavelength-sensitive cones (Katti et al. 2018). However, the degree to which they are able to see color is uncertain. Some authors suggest that photoreceptor opponency likely causes snakes to be colorblind (Hemmi et al. 2002), while others believe that RH1-expressing photoreceptors provide at least a limited degree of color vision (Davies et al. 2009).

Chemoreception plays a vital role in conspecific communication as well as prey and predator detection. Squamates possess a series of synapomorphies associated with vomerolfaction. These include a direct connection between the vomeronasal organ and oral cavity, a bifurcated tongue tip, frequent protrusion of the tongue (tongue-flicking) and other modifications associated with tongue protrusion (Schwenk 1993). Distinguishing the relative importance of vomeronasal olfaction via Jacobson's organ and olfaction *sensu stricto* remains a constant challenge. The most commonly accepted explanation of this issue is the Cowles and Phelan hypothesis (Schwenk 1993), which states that olfaction serves to detect substances from longer distances, initiating specific responses to cues that require less discrimination or starting up vomerolfactory investigation for more precise analysis (Cowles & Phelan 1958). It has been suggested that there may be selection to reduce tongue-flicking in order to avoid detection by predators or alerting prey (Cooper 1994). Ambush predators and active foragers differ in their tendency to tongue-flick. Tested active foragers tended to frequently utilize the behavior to explore new stimuli and recognize prey, while ambush predators showed little to no tongue-flicking before attacking prey (Cooper & Habegger 2000).

Squamates have shown their impressive chemosensory capabilities within experiments many times. Diurnal geckos of the genus *Naultinus* have been shown to use chemical cues in a broad variety of contexts, including foraging, conspecific communication, and predator detection. Tests conducted on Marlborough green geckos showed that the scent of fruit increased geckos' foraging, exploratory behavior, whereas the fecal cues of a native predator (tuatara) elicited freezing behavior. Males showed increased activity and tongue-flicking in the presence of female scent. Showing that even squamates that have considerable adaptations toward visual orientation may maintain well-developed and functional chemosensory apparatus (Hoare et al. 2007). Squamates may use chemical cues to assess quite specific traits. Males of the lacertid *Podarcis hispanica* are capable of recognizing whether or not females are pregnant using only chemical cues (Cooper & Pérez-Mellado 2002). Territorial males of *Podarcis liolepis* use their neighbors' scent marks to gain information about their rivals' competitive potential and even to recognize specific individuals (Carazo et al. 2007, 2008). The fact that many squamates possess such a strongly developed chemosensory system may suggest a possible trend towards the domination of chemical cues over all other sensory inputs.

1.3.1.1 Gekkotan Sensory Specifics

Acoustic communication seems to play a more significant role among geckos than most other extant non-avian reptiles, as is evidenced by their ability to vocalize, a trait few other squamates possess (Russell & Bauer 2020). Gustation (chemical exploration using the taste buds) seems to be underdeveloped in geckos. Studied species have shown very low densities of taste buds (Schwenk 1985), therefore supporting the view that tongue-flicking is mainly used for vomerolfaction (Schwenk 1993). It has long been understood that geckos are equipped with substantial olfactory capabilities, supported by the presence of a very large vomeronasal organ, well-developed olfactory chamber, and an olfactory bulb of considerable size (Pratt 1948).

2 Materials and Methods

This thesis falls under the project: “Vývoj personality v průběhu ontogeneze u gekončíka nočního (*Eublepharis macularius*) a její souvislost s kognicí”, requested by Charles University, Ovocný trh 3/5, 166 36 Praha 1 – Staré Město, IČ: 00216208, issued by the Ministry of Education, Youth and Sports of the Czech Republic, Karmelitská 529/5, 118 12 Praha 1 under the reference number MSMT-28796/2021-3 in Prague on November 18th, 2021.

All experiments conducted as part of this thesis were performed by Bc. David Hirschler after acquiring a Certificate of professional competence for designing experiments and experimental projects under Section 15d (3) of Act No 246/1992 Coll., on the Protection of Animals against Cruelty, as amended, upon the successful completion of the Training Course for Acquiring Qualification and Professional Competence (Designing Experiments and Experimental Projects). The certificate was issued on March 24th 2023 under the registration number CZ 04770 by the Ministry of Agriculture of the Czech Republic, Těsnov 65/17, 110 00 Praha 1 – Nové Město.

2.1 Animals

We tested the antipredator reactions of juvenile, subadult and adult leopard geckos (*Eublepharis macularius*). All geckos were incubated and raised under controlled laboratory conditions. Adults represent the third filial generation descended from a wild-caught parental generation. Both subadults and juveniles represent their offspring (the fourth filial generation).

Adults (3-5 years of age at the time of testing) were kept individually on lignocel substrate in glass terraria measuring 30 x 30 x 70 cm. Geckos were allowed access to water and were fed with mealworms (*Tenebrio molitor*) dusted with vitamins (Kombisol AD_3E, Trouw Nutrition Biofaktory) and minerals (Roboran D, UniVIT).

Subadults were kept individually on lignocel substrate or paper towels in perforated plastic boxes measuring 20 x 20 x 15 cm. Juveniles were kept individually on paper towels in perforated plastic boxes measuring approximately 20 x 15 x 10 cm. Geckos were fed with crickets and had free access to water.

All generations were incubated at controlled temperatures. Adults were all incubated at 28.5 °C to ensure that animals of both male and female sex are produced with a female-biased sex ratio (Viets et al. 1993). Subadults and juveniles were incubated in groups at three different temperatures: 26.5, 28.5 and 30.5 °C in order to test the effect of incubation temperature on personality as part of a separate project.

Light was provided within the room for 12 hours per day simulating a day-night cycle. Lights turned on and off gradually to simulate the rising and setting of the Sun. Room temperature was maintained between 26 and 28 °C, preferred temperatures by wild leopard geckos (Bergmann & Irschick 2006), via the use of heating cables and air conditioning.

All geckos were weighed and measured prior to testing. Weighing took place using small digital scales in units of whole grams. Measurements were gained by first scanning individual animals and then using scans to measure snout-vent length, tail length and width, and head length and width (Jančúchová-Lásková et al. 2015).

During testing, we also utilized adult individuals of the diadem snake, *Spalerosophis diadema* (Schlegel, 1837), which were housed in a separate room, ensuring that leopard geckos were unfamiliar with them or their scent. This species inhabits semi-arid areas from western Sahel to the Indian peninsula (Schatti et al. 2010). Diadem snakes are aglyphous, oviparous, and diurnal. However, they tend to take shelter under rocks or vegetation during daily heat peaks. *S. diadema* can grow fairly large, with maximum total lengths (snout to tip of the tail) reaching nearly 1.5 m, allowing them to consume a wide variety of smaller rodents, birds and non-avian reptiles (Yadollahvandmiandoab et al. 2018). Our snakes were raised in captivity as the F1 generation descended from wild-caught individuals.

2.2 Materials

To elicit antipredator responses, we used shed snakeskin, which was collected from adult *Spalerosophis diadema* within 24 hours after being shed and stored according to specific experiment requirements (in most cases in a sealed glass container at temperatures of approximately –20 °C). As a control stimulus we utilized pieces of transparent HDPE (high-density polyethylene) . Sheds and were stored separately and handled with separate tools in order to avoid contamination. Both were cut into pieces (approximately 1 x 1 cm) and presented using either metal tweezers (approximately 5 cm between the researchers hand and the presented stimulus) or a telescopic fishing rod (approximately 40 cm from hand to stimulus). To prepare the rod, all guides were removed including the terminal guide at the tip of the rod. This preparation left us with a thin, bare rod with a small opening at the tip into which paperclips (with one bend straightened) could be inserted and subsequently used to affix and present pieces of snakeskin or HDPE .

A female adult diadem snake was placed into a small glass container (17.5 x 7 x 8.5 cm) immediately before testing each day during the Snake Multimodality Test and afterwards released back into its home terrarium. The container was sealed using clear cellulose tape to stop snake scent from penetrating outward and affecting gecko behavior. Another identical container was kept clean and empty for use as a control.

2.3 Methods

2.3.1 Experimental Procedure

Two days prior to testing, 30 x 30 cm polystyrene partitions were added to adults' home terraria to reduce space available to geckos down to the front 30 x 30 x 30 cm portion. This was done in order to make geckos accessible during testing and to ensure they were sufficiently well-lit to allow accurate evaluation of displayed behaviors. Partitions were perforated in order to allow adequate airflow. In the process, all hides (paper rolls, tree bark, boxes) were made inaccessible. Simultaneously, magnets, which are used to secure the door of each terrarium, were dampened by the application of several layers of aluminum foil. This was done as a means to reduce vibrations caused by opening terraria, which were previously suspected to cause increased reactivity in leopard geckos (Landová et al. 2023). Hides were also removed from subadult and juvenile housing two days prior to testing.

The basic procedure for all tests involved presenting either a 1 x 1 cm piece of HDPE or a chemical stimulus in the form of a 1 x 1 cm piece of snakeskin to leopard geckos (*Eublepharis macularius*), specifically to the tip of the snout. Gecko reactions were recorded for 90 seconds after first presentation using a mirrorless camera (Canon EOS M50).

2.3.1.1 Triple Modality Test

In order to test the validity of this experimental procedure, we conducted a control experiment. This included 40 adult leopard geckos, each of which was subjected to a sequence of 3 treatments in their home terraria (reduced space due to the addition of polystyrene partitions). Geckos were naïve and had no prior experience with shed snakeskin or tests of this nature. They had, however, previously

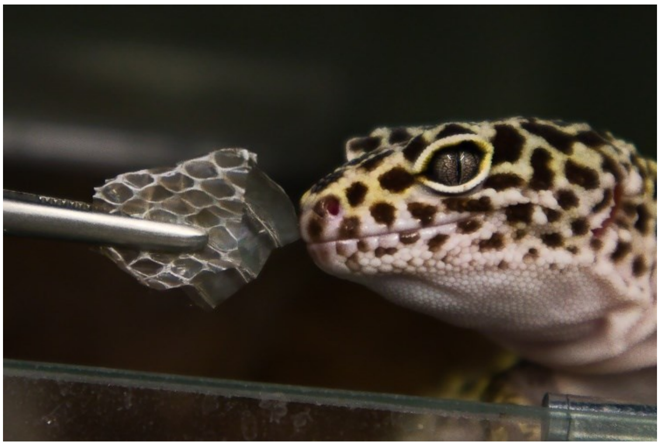

Figure 2: Adult leopard gecko having a piece of shed snakeskin presented to its snout with a pair of metal tweezers.

been subjected to multiple cognitive and personality tests, including reactivity tests which simulated a predator attack. Each treatment was repeated for all geckos before starting the next treatment. Firstly, a piece of HDPE was presented to the tip of the snout using metal tweezers. Subsequent behavior was recorded for 90 seconds. Secondly, a piece of snakeskin was presented in the same manner, while recording for 90 seconds (Figure 2). The third treatment was identical to the first. In between each treatment, geckos were given an approximately 60-minute rest period. Subsequent analysis of all video footage revealed a significant effect of treatment on antipredator response ($p < 0.001$). While treatments 1 and 3 (HDPE) elicited antipredator responses in just 1 gecko each, treatment 2 (snakeskin) caused 13 geckos to display antipredator behavior, results congruent with those of Landová et al. 2023. Thus, we confirmed the validity of using *S. diadema* sheds as chemical stimuli to elicit antipredator responses and of using HDPE as control stimuli.

The main test consisted of 8 different treatments designed to isolate the effect of individual cues and compare their significance when presented individually or when combined to create a multimodal stimulus. The experiment included 40 adult leopard geckos, each of which was subjected to all 8 treatments in a pseudo-randomized order in their home terraria. Polystyrene partitions and magnet dampeners were added, and hides were removed 2 days prior to testing to allow geckos to acclimate. Afterwards, tests took place over the course of 8 days to ensure that each animal was only tested once per day.

Treatments were designed to present a combination of stimuli targeting 3 different sensory modalities: chemical, visual and mechanosensory, as well as control stimuli, meant to elicit no antipredator response. The presented chemical stimulus was in the form of a 1 x 1 cm piece of shed snakeskin presented to the tip of the snout. As a chemically neutral control, we used a 1 x 1 cm piece of HDPE (Figure 3). Both were handled and stored carefully and separately to ensure that remained odorless. Snakeskins were stored in sealed containers at temperatures below 0 °C since their collection until the day they were used. Visual cues were determined by the method used to present chemical and control stimuli. When presented using metal tweezers, we considered the researchers hand as a visual stimulus (a

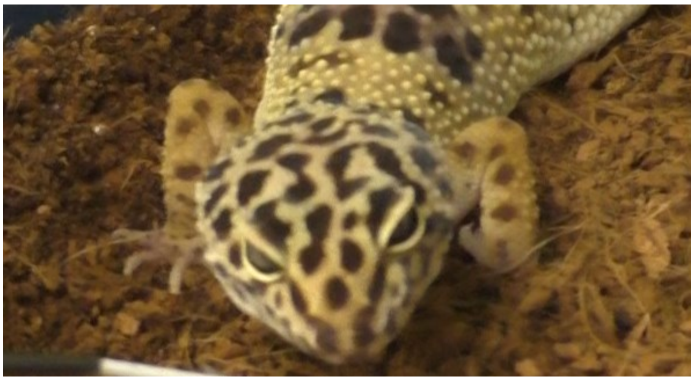

Figure 3: A 1 x 1 cm piece of HDPE being presented to the snout of an adult leopard gecko using a telescopic fishing rod with a paperclip inserted in the tip.

large moving object placed near the gecko). As a visual control, meant to minimize the effect of visual stimulation, we presented using a thin telescopic fishing rod, allowing the researcher to present chemical cues without placing their hand near the animal subject. Mechanosensory stimulation was achieved by strongly tapping the glass of the terrarium with metal tweezers immediately before presenting chemical cues. This caused vibrations of the terrarium itself as well as auditory vibrations, all meant to serve as an alert, increasing the reactivity of geckos. As an effective control for this stimulus, we not only didn't tap the glass, but, as mentioned earlier, prior to testing, we padded the magnetic closing mechanisms of all terraria using aluminum foil in order to avoid shaking while opening them.

Treatment 1 consisted of a piece of HDPE presented using a telescopic rod without a mechanosensory alert. Treatment 2 consisted of a piece of HDPE presented using a pair of metal tweezers without a mechanosensory alert. Treatment 3 contained a piece of shed snakeskin presented using a pair of metal tweezers without a mechanosensory alert. Treatment 4 was represented by a piece of shed snakeskin presented using a telescopic rod without a mechanosensory alert. Treatment 5 consisted of a piece of HDPE presented using a telescopic rod with a mechanosensory alert. Treatment 6 consisted of a piece of HDPE presented using a pair of metal tweezers with a mechanosensory alert. Treatment 7 consisted of a piece of shed snakeskin presented using a pair of metal tweezers with a mechanosensory alert. Treatment 8 was represented by a piece of shed snakeskin presented using a telescopic rod with a mechanosensory alert.

The order of presentation was pseudo-randomized. In practice, this meant that presentation order was truly randomized for each gecko and subsequently rearranged to ensure that no treatment appeared in one position disproportionately more often than any other when taking into all geckos into account. Treatments were switched until they all appeared in each position close to 5 times (e.g. treatment 4 was presented to 5 geckos as the 1st treatment they experienced during testing), thus creating a much more even spread than the truly randomized order.

Stimuli were presented to geckos and reactions were recorded for 90 seconds using a visual spectrum mirrorless camera for further analysis of displayed behaviors.

2.3.1.2 Snake Multimodality Test

For this experiment we tested 42 adult leopard geckos in only 2 modalities: visual and chemical. As a chemical stimulus, we once again used a 1 x 1 cm piece of shed snakeskin, with a 1 x 1 cm piece of HDPE serving as a control. Both were presented using a telescopic fishing rod to avoid the possibility of the researcher's hand affecting gecko behavior. During this test, we introduced a different type of visual stimulus. This took the form of a live adult diadem snake in a sealed glass container (Figure 4). As a control, we used an identical empty glass container. Snakeskin was stored identically to the Triple Modality Test.

Figure 4: Glass container with an adult Spalerosophis diadema as it was used in the Snake Multimodality Test.

Each gecko went through 4 treatments in a pseudo-random order over the course of 4 days (1 treatment per day). Treatment 1 consisted of an empty glass container being placed into one side of the terrarium and a piece of HDPE presented to the gecko's snout using a telescopic rod. Treatment 2 was a live snake in a sealed glass container placed into one side of the terrarium and a piece of HDPE presented to the gecko's snout using a telescopic rod. Treatment 3 consisted of a live snake in a sealed glass container being placed into one side of the terrarium and a piece of shed snakeskin presented to the gecko's snout using a telescopic rod. Treatment 4 included an empty glass container being placed into one side of the terrarium and a piece of shed snakeskin presented to the gecko's snout using a telescopic rod.

Stimuli were presented to geckos and reactions were recorded for 90 seconds for further analysis of displayed behaviors.

2.3.1.3 Scent Loss Test

For this experiment we had 2 different sets of shed snakeskin. Both were collected at the same time from the same diadem snake and stored for 2 months prior to testing. One was stored in a sealed container at temperatures around –20 °C in the same manner as described previously (from this point onwards simply: freezer snakeskin). The other was stored in an open container at room temperatures (from this point on: room temperature snakeskin). This was done in order to compare each snakeskin's ability to elicit antipredator responses in leopard geckos and by extension, assess the stability of snakeskin scent over time.

The test was performed on 42 adult geckos, who were exposed to 3 treatments each day of testing. The 1st and 3rd treatments of the sequence consisted of a 1 x 1 cm piece of HDPE presented to the snout using a pair of metal tweezers. The 2nd treatment involved a 1 x 1 cm piece of shed snakeskin presented to the snout using metal tweezers. Each gecko was subjected to the full sequence of treatments on 1 day, and again on the 2nd day of testing. Both sets of snakeskin were used during testing. Their use was split between both days, with half of tested geckos being exposed to freezer snakeskin on the 1st day and room temperature snakeskin on the 2nd day (vice versa for the other half of geckos).

Stimuli were presented to geckos and reactions were recorded for 90 seconds for further analysis of displayed behaviors.

2.3.1.4 Ontogenetic Comparison Test

Tests of the before mentioned sequence, consisting of 3 treatments (1st treatment HDPE, 2nd treatment snakeskin, 3rd treatment HDPE), were also performed on 71 juvenile and 87 subadult leopard geckos in order to compare reactivity between ontogenetic stages (Figure 5). Each gecko went through this sequence a total of 3 times (once per day), so that repeatability could be measured. All treatments were presented using metal tweezers, while making sure that HDPE and snakeskins were always handled with separate tools.

Figure 5: Juvenile leopard gecko having a piece of shed snakeskin presented to its snout with a pair of metal tweezers.

Since juveniles and subadults were housed individually in plastic boxes, no partitions were added, and preparation only involved removing hides 2 days prior to testing. During testing, boxes were handled slowly and carefully so as not to disturb geckos. That included opening box lids, immediately before testing, as carefully as possible to avoid shaking, and then gently replacing them afterwards. Every reaction was recorded for 90 seconds since the snakeskin was first placed by the snout. Recordings were used in further analysis.

Data from juvenile and subadult subjects was also compared to adult data gained from previous experiments.

2.3.2 Video Analysis

Utilizing Activities – Event Recorder ver. 2 (Vrba & Donát 1992), used to create records of coded behavior during observation, we categorized and marked displayed gecko behavior from the video footage we gathered throughout all experiments.

Creating an accurate record required a sufficient ethogram of leopard gecko behavior. For this we utilized the ethogram developed by Landová et al. over the course of previous years (as is described in Landová et al. 2013). This ethogram was briefly mentioned in the description of leopard gecko antipredator behavior in the Literature Review section of this thesis. What follows is a more detailed description of the behaviors we observed and marked, as well as behaviors that were added or omitted from our set with explanations of our reasoning.

Firstly, it seems appropriate to mention behaviors that are not specific only to antipredator responses (in some cases antagonistic interactions in general). One of the conveniences of studying the behavior of a squamate species, is their tendency to tongue-flick when exploring an object or area of interest, delivering chemical cues to the vomeronasal organ. We marked "tongue-flicks" directed toward presented stimuli as a measure of the gecko's interest. When disinterested, leopard geckos will either retain an unmoving leisurely position (sometimes even seemingly falling asleep). This behavior was not marked with any specific code, more so as the absence of codes following the start of recording or after a tongue-flick. A more active alternative is exploration of the surrounding environment, where geckos, rather than devoting their attention to presented stimuli, simply wander around their terrarium, drinking, digging, climbing, as though there was no disturbance happening. This behavior was marked as "ignoring". Some geckos also made attempts to eat the presented stimulus, prompting us to add "eating" as one of the coded behaviors. These attempts involved no prior threatening posture, nor were they made with rapid strikes, but rather involved one slow bite. These specific characteristics distinguish "eating" from defensive "biting" as described below.

When categorizing the specific antipredator behaviors of leopard geckos, we took mostly the same approach as described in Landová et al. 2013, splitting them into two main categories based on their role within the predator-prey interaction. The first category, active antipredator behavior, consists of behaviors connected to threat and aggression, where the gecko is attempting to intimidate or even injure the predator. In order to appear larger and more imposing, leopard geckos will raise themselves into a high posture, extending their legs, often hunching their back (Figure 6). During this posture, the tail is usually at least partially raised. This posture itself was coded simply as “high posture”. Often displayed together with this raised stance, is a wide opening of the mouth, revealing the contrasting pink interior along with the bright red tip of the gecko’s tongue (Figure 7). The mouth will usually be held open for extended periods. This behavior was coded as “mouth open threat”. The apparent climax of these defensive behaviors is a swift painful bite utilizing the geckos relatively strong jaws and many small but sharp conical teeth. Adult bites can puncture human skin. Geckos will either attack rapidly, quickly letting go before resuming a threatening stance, or they may latch on to the target for several seconds. Biting behavior directed towards the stimulus was coded as “biting”. While exhibiting these behaviors, geckos will frequently alternate between a lateral display, showing the predator the full length of their body and a direct stance, fully showing the open mouth and binocularly fixating on the target before striking (Figure 8). Although the effectiveness of these defensive behaviors has not been tested against live predators in a natural environment, one is inclined to


Figure 6: An adult leopard gecko displaying partially raised posture, beginning to hunch its back with tail raised.


Figure 7: An adult leopard gecko displaying fully raised posture with a raised tail, threatening with an open mouth.

believe that they represent a sufficient deterrent, seeing as they are a fixed, inherited, pattern of behavior, displayed since hatching until adulthood. One active antipredator behavior, that doesn't tend to last into adulthood, is warning vocalization directed at predators. This juvenile behavior takes the form of a loud chirp, usually in concert with threatening postures and biting (Landová et al. 2013). We did not code vocalizations simply because, during testing, we did not observe any such behavior among geckos of any of the three tested ontogenetic stages.

Figure 8: An adult leopard gecko displaying a direct threat posture, raised on extended legs with mouth open, binocularly fixated on the target, preparing to attack.

Passive antipredator behaviors represent a less confrontational alternative to active antipredator behaviors. As a way to reduce their immediate noticeability, leopard geckos may enter a low stance, pressing their body to the ground. This behavior was coded as "low posture". Once assuming this stance, geckos may significantly reduce movement, either remaining completely still or moving very slowly. We refer to such states of greatly reduced movement as "freezing", although, given the often-sluggish nature of leopard geckos, this behavior proved difficult to assess and record. One very common and characteristic behavior involves the leopard geckos specially developed tail. A threatened gecko may raise its tail into the air and wave it in a slow curving motion, partially furling and unfurling repeatedly (Figure 9). We refer to this display as "tail waving". As was previously mentioned, the leopard gecko's tail serves as a fat reserve, therefore presenting a rather large target for predators to strike, allowing the gecko to autotomize the appendage and escape. Although we most often observed it

Figure 9: An adult leopard gecko in a low stance waving its raised tail to distract predator attention.

performed in a low stance, it was also frequently seen displayed in higher postures. In the point of categorizing tail waving as a passive defensive behavior we differ from Landová et al. 2013, who categorized it as an active defensive behavior. Lastly, we coded avoidance and escape behaviors collectively as "avoidance". We describe avoidance behavior as hastened movement away from the presented stimulus, either by simply turning the head/body, or by moving to a different part of the enclosure. In order to allow leopard geckos to display such behavior, stimuli were not constantly pressed to subjects' snouts throughout the whole 90 second duration of each test. Instead, stimuli were presented to the snout and, whenever the subject moved away from the stimulus (avoiding or ignoring it), the researcher waited in the same position for approximately 2 seconds before moving the stimulus to the gecko's snout again. Aside from an estimated total of 5 instances, geckos made no attempt to escape their terraria or plastic boxes when exposed to snakeskin. Such small numbers were deemed too low to have any significance on their own and were instead counted as prolonged instances of "avoidance".

We added one additional behavior to this list, "creeping", which we describe as a slow cautious approach towards the presented stimulus, keeping a low stance and binocular fixation of the target (Figure 10). Due to the nature of the experiments we performed, there weren't many opportunities for leopard geckos to display this behavior.

Figure 10: An adult leopard gecko creeping towards the presented stimulus.

For statistical evaluation, we scored geckos' antipredator reactions in a binary fashion (1 – yes/0 – no). The presence of antipredator behavior was scored, if at least once per the trial, active (high posture, mouth open threat, biting) or passive (low posture, freezing, tail-waving, avoidance) antipredator behavior was displayed.

Using Activities, we created single letter codes for each of the previously described behaviors. We then went through each video, marking each displayed behavior, utilizing the programs capability to record not only the number of times a behavior was marked, but also the latencies and durations of each behavior. We also distinguish between event behavior and state behaviors. The former consists of brief actions that do not involve any prolonged process, therefore making the measurement of duration in these cases irrelevant. This includes tongue-flicks as well as eating and biting attempts. All other coded behaviors (e. g., postures or tail waving) were categorized as state behaviors. These are performed over a longer observable period of time, causing their duration to be of more value. The program's output were individual .prn observation files. Files from each experiment could then be separately converted using the "READER" function to a collective .prn summary file which could be imported as a spreadsheet into Microsoft Excel for further statistical analysis.

2.3.3 Statistical Analysis

Importing data spreadsheets into Microsoft Excel allowed us to organize data for further analysis within R project ver. 4.4.0 (R Core Team 2024). We performed GLM (Generalized Linear Model) tests using the 'geeglm' function under the Generalized Estimating Equation Package, 'geepack', ver. 1.3.10 (Højsgaard 2024), with geckos' antipredator response as a dependent variable with binomial distribution, and treatment as a categorical explanatory variable. The effect of gecko identity was controlled by adding an animal identity argument to the marginal model. We also performed tests of the same type to examine the effect of incubation temperature, age, and testing order on antipredator response. In order to analyze the number of tongue-flicks, we first calculate the square roots of tongue-flick numbers in order to achieve better distribution. We then performed LME (Linear Mixed-Effects) model tests using the 'lme' function under the Linear and Nonlinear Mixed Effects Models package, 'nlme', ver. 3.1-164 (R Core Team 2023), with the square root of tongue-flicks as a response variable, and treatment as a fixed effect predictor variable. The effect of gecko identity was controlled by adding identity as a random intercept to the model. We similarly tested for the effect of incubation temperature, age, and testing order on tongue-flicking. In order to test repeatability of behaviors for individual geckos during repeated trials, we utilized the 'rptBinary' function of the Repeatability Estimation for Gaussian and Non-Gaussian Data package, 'rptR' ver. 0.9.22 (Stoffel 2019).

We also employed PCA (Principal Component Analysis) of displayed behavior of adult leopard geckos to simplify the complexity in high-dimensional data.

Graphs were created primarily using STATISTICA 6.0 (StatSoft, Inc. 2001), as well as the NodeXL Basic plugin ver. 1.0.1.448 (The Social Media Research Foundation 2021) within Microsoft Excel, and Paint.NET ver. 5.0.13 (dotPDN LLC 2024).

3 Results and Discussion

3.1 Triple Modality Test

Throughout the course of the control test, out of the 78 trials involving a piece of HDPE bag, geckos displayed only 2 instances of high posture, the only antipredator behavior shown towards the plastic stimuli. In contrast, of the 39 trials consisting of a piece of shed snakeskin presented to the snout, 13 geckos showed a wide array of antipredator responses including both active and passive defensive behaviors. This result confirmed the validity of using HDPE bags as a control stimulus and shed snakeskin as a chemical stimulus triggering antipredator behavior. Leopard geckos showed significantly more (Estimate ± Standard Error) tongue-flicks towards snakeskin (1.382 ± 0.240, $p < 0.0001$) compared to the 1st control (Intercept: 1.364 ± 0.188). The 2nd control did not differ significantly from the 1st (-0.305 ± 0.240, $p = 0.207$).

In the Triple Modality Test, treatment was shown to have a highly significant effect on the number of geckos displaying antipredator behavior ($\chi2 = 47.6$, $p < 0.0001$) when compared to the empty control treatment (treatment 1, see Experimental Procedure for descriptions of treatments). All treatments that included a chemical stimulus elicited significantly more antipredator reactions (all $p < 0.01$) than those without a chemical stimulus (all $p > 0.1$). This effect is made even more clear when examining treatments that test just one modality. The treatment testing only chemical modality (treatment 4) was the only significant unimodal treatment (1.895 ± 0.603, $p = 0.002$), while visual (treatment 2, 0.251 ± 0.665, $p = 0.706$) and acoustic/mechanosensory (treatment 5, -0.747 ± 0.755, $p = 0.322$) elicited no significant change in behavior compared to the control (Intercept: -2.197 ± 0.527). When examining multimodal treatments combining different modalities, we still see that chemical stimulation is the main driving factor eliciting antipredator response, with the only non-chemical multimodal treatment (treatment 6) combining acoustic/mechanosensory and visual stimuli proving insufficient to elicit antipredator behavior in any significant measure (0.251 ± 0.665, $p = 0.706$). These results are shown in Figure 11 and Supplementary table S1.

When diving deeper and comparing treatments containing the chemical stimulus between each other, we see no significant effect of treatment on antipredator behavior ($\chi2 = 4.1013$, $p = 0.2507$). Post-hoc Tukey test showed no significant contrast between any of the 4 treatments ($p > 0.2$), confirming that none of the other presented stimuli caused a change in the degree of antipredator response to chemical cues. For more statistical data see Table S2 under Supplementary materials.

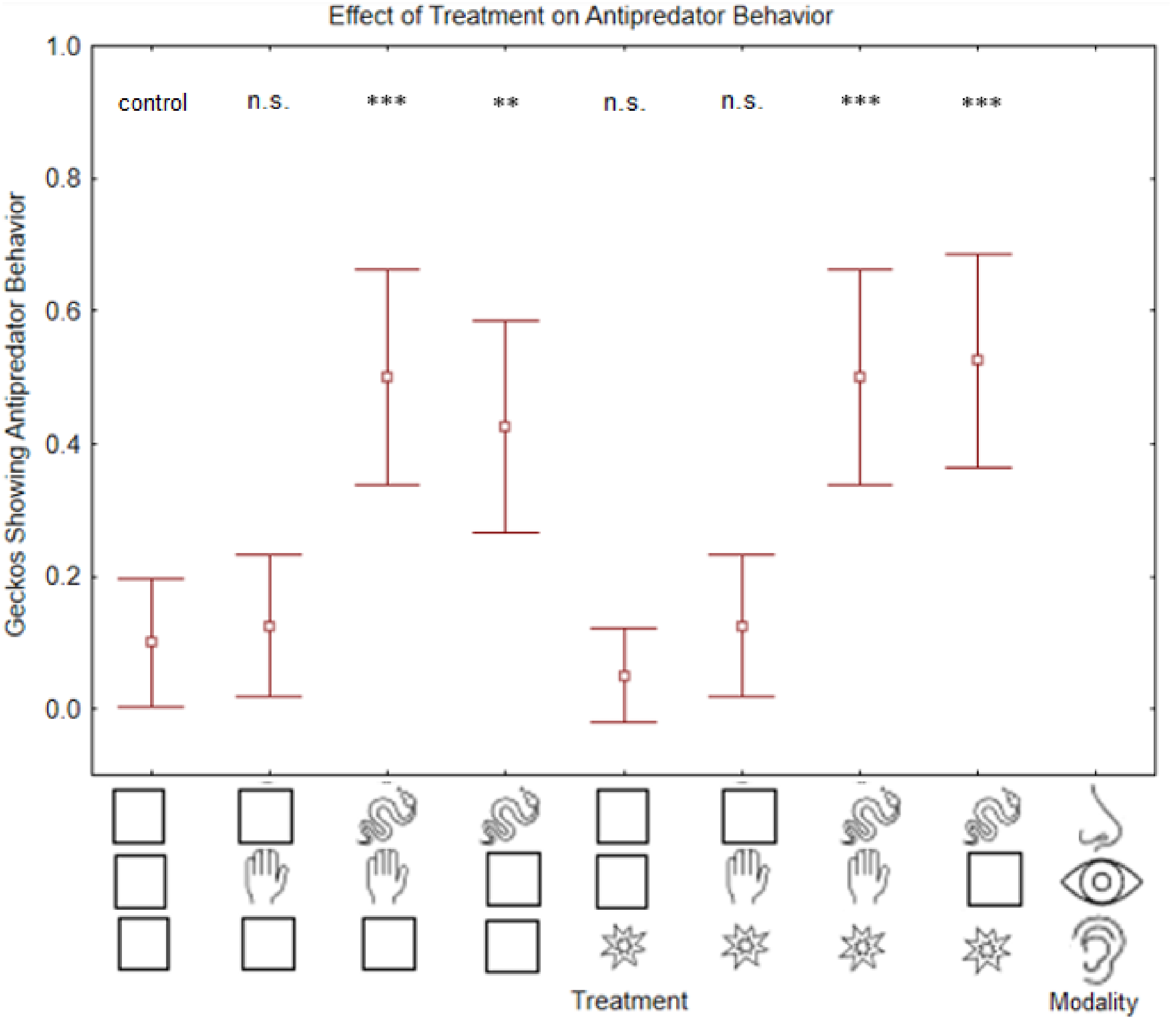

*Figure 11: The effect of treatment on antipredator response elicited in leopard geckos in the Triple Modality Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows 8 different treatments combining varying modalities. Each treatment is described by 3 rows of stimuli within 1 column. Stimuli are split into rows based on which sensory system they target, these are shown on the far right, from top to bottom: chemical, visual, acoustic/mechanosensory. Empty squares represent either a control, or no stimulus was presented in that modality. Chemical stimuli took the form of shed snakeskin, visual stimuli were represented by the researcher's hand holding tweezers and acoustic/mechanosensory stimuli were represented by tapping the glass of terraria immediately prior to presenting other stimuli. (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p > 0.05$)*

The effect of treatment on tongue-flicks was also shown to be significant (ANOVA: $F_{(7,273)} = 10.8692$, $p < 0.001$) when comparing treatments to the control. The visual stimulus wasn't shown to cause a significant increase of chemical exploration, i.e. tongue-flicking (0.391 ± 0.265, $p = 0.1408$) in comparison to the control (Intercept: 1.505 ± 0.190). Neither was the acoustic/mechanosensory stimulus (0.311 ± 0.265, $p = 0.2405$). Once again, the chemical stimulus proved to be the only significant elicitor of vomerolfactory exploration (1.135 ± 0.265, $p < 0.0001$). However, examining multimodal treatments didn't quite show the same dominant effect of the chemical stimulus. Although all treatments containing a chemical cue were shown to have a significant effect (all $p < 0.001$), the multimodal treatment combining visual cues with acoustic/mechanosensory stimuli also showed some significance (0.875 ± 0.265, $p = 0.0011$). These results are shown in Figure 12 and Table S3.

When comparing treatments containing the chemical stimulus between each other, we still see a significant effect of treatment on tongue-flicking (ANOVA: $F_{(3,117)} = 4.4578$, $p = 0.0053$). Post-hoc Tukey tests showed significant contrasts between treatments 4 (chemical) and 7 (chemical, visual and acoustic/mechanosensory) (0.267 ± 0.26, $p = 0.0278$), and between treatments 7 and 8 (chemical and acoustic/mechanosensory) (0.884 ± 0.26, $p = 0.005$). In each of these 2 cases geckos showed more tongue-flicks in the treatment presenting in more modalities, suggesting a greater interest in multimodal stimuli. For more statistical data see Table S4 under Supplementary Materials.

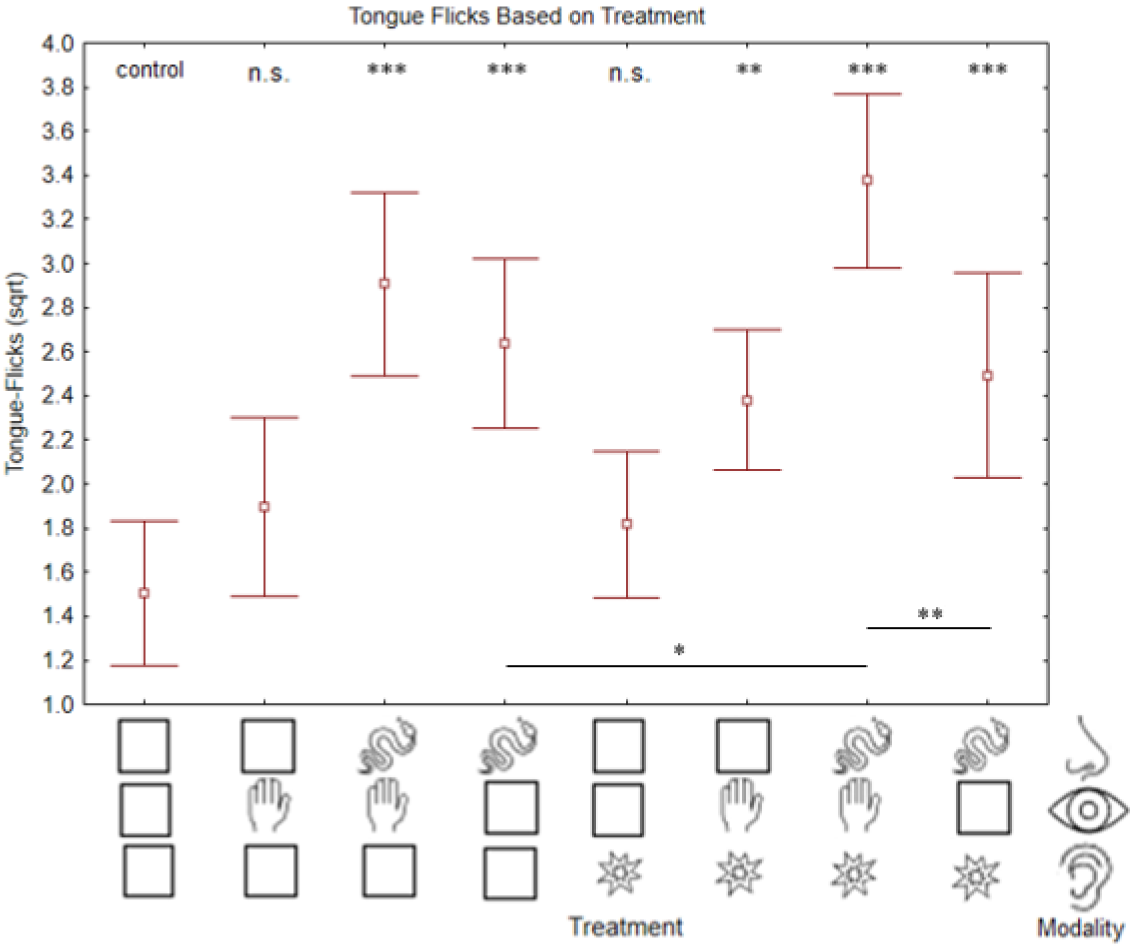

*Figure 6: The effect of treatment on tongue-flicks (sqrt) in the Triple Modality Test. Mean number of tongue-flicks (sqrt) is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows 8 different treatments combining varying modalities. Each treatment is described by 3 rows of stimuli within 1 column. Stimuli are split into rows based on which sensory system they target, these are shown on the far right, from top to bottom: chemical, visual, acoustic/mechanosensory. Empty squares represent either a control, or no stimulus was presented in that modality. Chemical stimuli took the form of shed snakeskin, visual stimuli were represented by the researcher's hand and acoustic/mechanosensory stimuli were represented by tapping the glass of terraria immediately prior to presenting other stimuli. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

We employed PCA to explore the behavior of geckos. Two main factors were extracted according to the eigenvalues (>1). PCA revealed a spread of behaviors primarily explained by Factor 1, which accounted for 30.49% of the variance in data. When expressed as a biplot along with Factor 2, which only explained 13.51% of variance (see Figure 13), active antipredator behaviors (biting, mouth open threat, high posture) clustered together, with mouth open threats and biting behavior showing an especially strong positive correlation. Avoidance behavior showed more positive correlation with active antipredator behaviors than with tail-waving and low posture, which correlated positively with each other but showed no correlation with active behaviors. Factor 1 seems to represent antipredator behavior, with all antipredator behaviors (especially active and avoidance) gaining positive values, in contrast to tongue-flicks and ignoring. Aside from avoidance, Factor 2 effectively splits active and passive antipredator behavior. This may suggest that avoidance would be best recategorized as an active antipredator behavior.

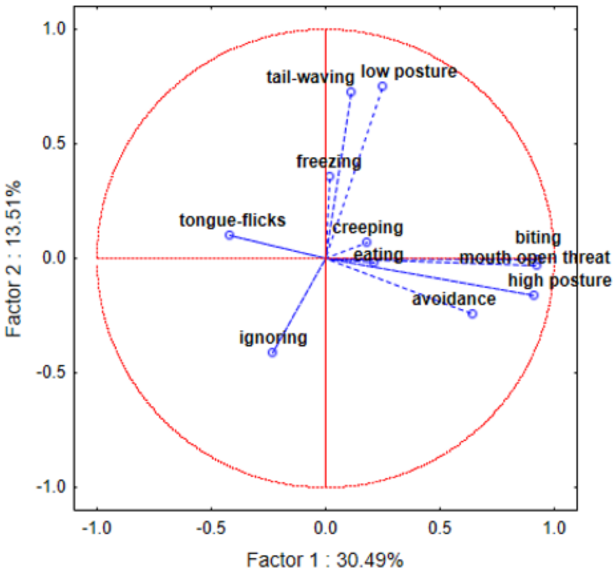

Figure 7: Principal Component Analysis biplot of Factors 1 and 2 showing variance of leopard gecko behavior.

Using reaction latencies gained from snakeskin treatments of the control test, the Triple Modality Test and the Scent Loss Test, we created an interaction plot examining the sequence of displayed behaviors. As can be seen in Figure 14, active antipredator behaviors: biting (B), mouth open threats (M) and high posture (HP) often followed in close succession

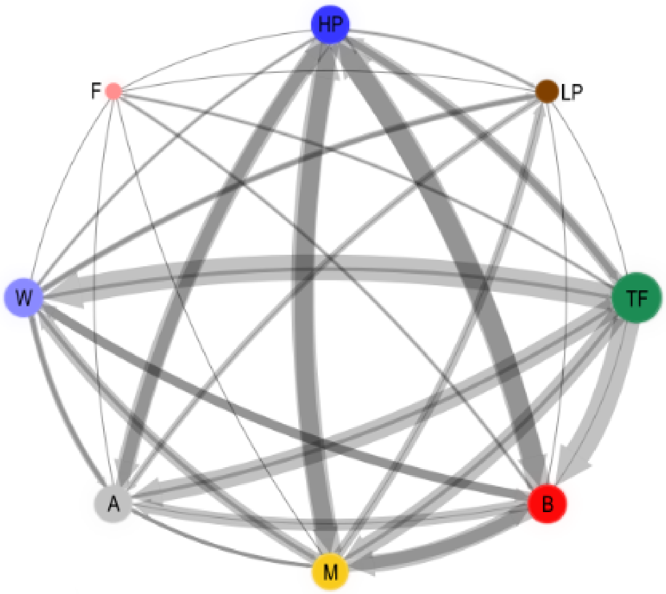

Figure 8: Interaction plot displaying the sequence relationships of individual behaviors: high posture (HP), low posture (LP), tongue-flicking (TF), biting (B), mouth open threat (M), avoidance (A), tail-waving (W), freezing (F). The sequence of behavior is indicated by arrows pointing from one behavior to another. The number of times the behavior is displayed is represented by the size of its circle.

in varying orders. We can also see that biting behavior was practically never followed by tongue-flicking (TF). What is further evident, is that biting, mouth open threats, avoidance (A) and tail-waving (W) were each often immediately preceded by tongue-flicking. This would suggest that, even in cases where treatments elicit antipredator behavior, vomerolfaction is often utilized to analyze chemical stimuli. Interestingly, high posture didn't follow immediately after tongue-flicks nearly as often as biting did, indicating that leopard geckos detecting predator odor may prefer to quickly strike at the threat rather than trying to appear larger. When one bite inevitably isn't enough, geckos will frequently alternate between high posture (usually with mouth open) and biting, employing more of their defensive arsenal in order to deter predators.

As can be seen by the relative sizes of the circles shown in Figure 14, tongue-flicking (TF) was the most common behavior. In contrast, low posture (LP) was shown very rarely. In the tests of Landová et al. 2016, where leopard geckos' antipredator behavior towards live snakes was recorded, subadult leopard geckos confronted with *Spalerosophis diadema* and *S. atriceps* displayed low postures to a significant degree in both cases. Adults were not exposed to *S. diadema*, but when reacting to *S. atriceps* they did not show a significant number of low postures. This may suggest that low postures are a less frequent antipredator behavior in adults than younger geckos.

Leopard geckos did not display any increase in antipredator behavior when exposed to multimodal stimuli as compared to unimodal chemical stimuli. Neither visual nor acoustic/mechanosensory stimuli were shown to have a significant effect on resulting antipredator behavior. Taking into account these results, we consider 2 possible explanations of the phenomena:

1) Chemical modality is dominant in the context of predator detection by leopard geckos, providing the most significant indication of the presence of a snake predator under conditions where the ability to use other sensory systems is hindered (silent, cryptic predators waiting in dark rock crevices).
2) Our choice of stimuli and animals may have proven to be a limitation of our study. Leopard geckos raised under laboratory conditions and utilized in different experiments may be entirely too accustomed to the presence of researchers, making noise, opening terraria, and handling geckos, therefore making the selected visual and acoustic/mechanosensory stimuli insufficient. This would suggest that multimodality cannot be fully ruled out and that a different visual stimulus could plausibly elicit stronger reactions. It may be suggested that wild-caught leopard geckos might show stronger reactions to visual stimuli in the form of researchers' hands than captive-raised individuals. It is also possible that visual and acoustic predator stimuli play a larger role when responding to other types of predators. For example, Elmasri et al. 2012 found that brown anoles increased locomotion rates in the presence of a visual cue in the form of a grackle (a common predator in the studied population) model, a response which was modulated by acoustic cues (recorded grackle vocalizations). The authors, however, concluded that anole responses to predators are likely dependent on previous experience, something that our leopard geckos lacked.

The effect of treatment on tongue-flicking diverged from antipredator behavior in several instances. One is represented by the bimodal visual and acoustic/mechanosensory stimulus, which, when presented, elicited significantly higher rates of tongue-flicking than the control. This suggests that these combined cues may increase chemosensory exploratory behavior, effectively alerting leopard geckos to the presence of something which should be investigated further. This may be further supported by 2 significant examples of increased tongue-flicking when comparing between treatments. More tongue-flicks were displayed in response to the full multimodal treatment containing chemical, visual and acoustic/mechanosensory cues, than toward the bimodal chemical and acoustic treatment and the unimodal chemical treatment.

From these results it would seem, that although visual and acoustic/mechanosensory cues do not increase antipredator behavior, they may serve to alert leopard geckos to the presence of something which should be further investigated.

In the control test, leopard geckos tongue-flicked significantly more towards the chemical stimulus than either of the controls. This presents an interesting contrast with Landová et al. 2023, where the 1st control was tongue-flicked more than the chemical stimulus. This could possibly be caused by differences in the way individual evaluators recorded tongue-flicks. Another likely affecting factor is that, while we used snakeskin from 1 species of snake in 1 trial, in the previous study each gecko was tested 6 times, reacting to snakeskin from a different species each time.

While preparing for the next experiment, we believed it more likely, that our choice of visual and acoustic/mechanosensory stimuli was inadequate. For the next set of trials, we chose a different visual stimulus and decided to dispense with the acoustic/mechanosensory stimulus entirely, as we could not feasibly produce a stronger vibration on one terrarium, without disturbing the inhabitants of surrounding terraria.

3.2 Snake Multimodality Test

During the Snake Multimodality Test, treatment was shown to have a slightly significant effect on antipredator behavior ($\chi2$ = 9.01, *p* = 0.029). Much like in the Triple Modality Test, the unimodal visual treatment did not elicit a significant number of antipredator responses (1.15 ± 1.20, *p* = 0.33778) when compared to the control (Intercept: -3.71 ± 1.01). The only significant treatments were those with chemical cues (2.41 ± 01.11, *p* = 0.02935). However, the significance was considerably diminished compared to the Triple Modality Test. As can be seen in the results depicted in Figure 15, relatively few leopard geckos displayed antipredator behavior throughout the experiment. As is also evident, the bimodal chemical and visual treatment elicited the same number of responses as the unimodal chemical treatment. For more statistical data see Table S5 under Supplementary Materials.

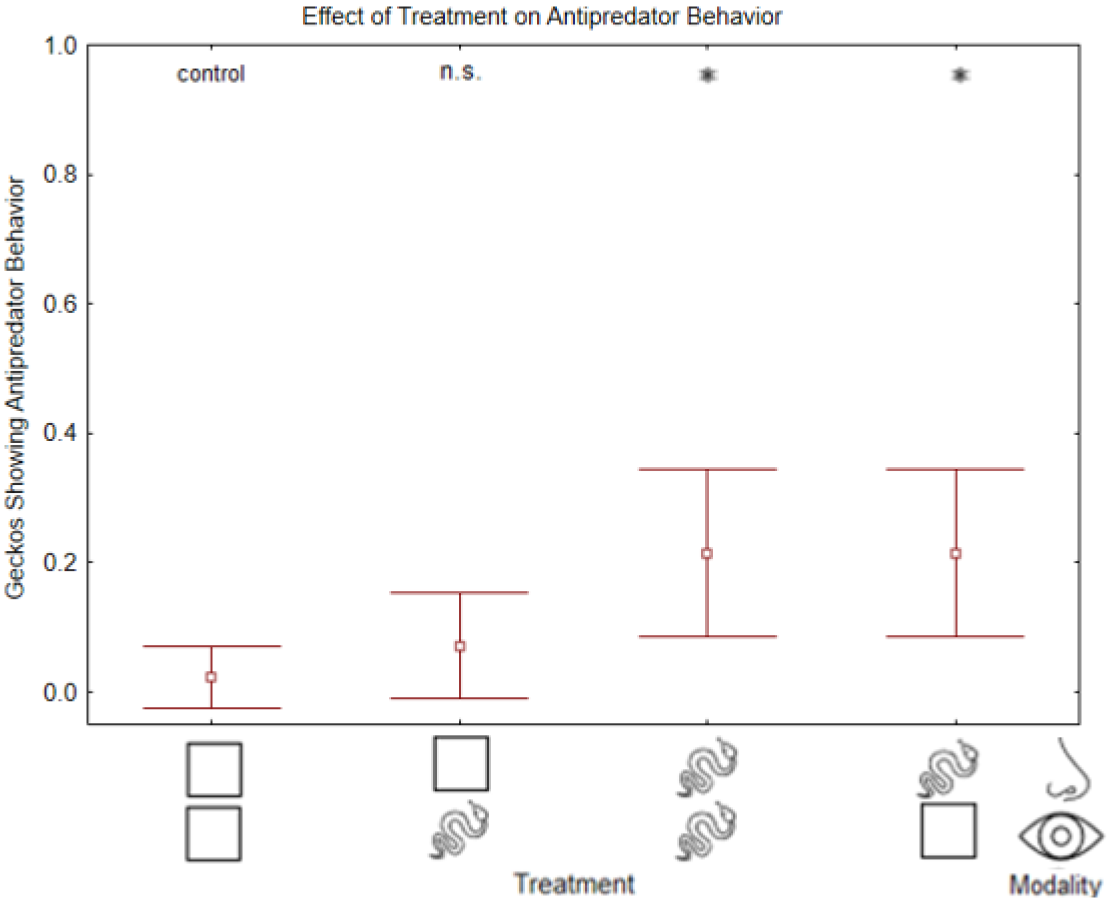

*Figure 9: The effect of treatment on antipredator response elicited in leopard geckos in the Snake Multimodality Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows 4 different treatments combining varying modalities. Each treatment is described by 2 rows of stimuli within 1 column. Stimuli are split into rows based on which sensory system they target, these are shown on the far right, from top to bottom: chemical, visual. Empty squares represent a control in that modality. Chemical stimuli took the form of shed snakeskin, visual stimuli were represented by a live snake in a sealed glass container. Pieces of HDPE and empty glass containers were used as controls in the chemical and visual modalities respectively. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

The effect of treatment on tongue-flicks in the Snake Multimodality Test was significant (ANOVA: $F_{(3,123)} = 4.6068$, $p = 0.0043$) when comparing to the control. Only treatments containing a chemical stimulus (treatment 3: 0.975 ± 0.268, $p = 0.0004$); treatment 4: 0.615 ± 0.268, $p = 0.0234$) elicited a significantly higher number of tongue-flicks than the control (Intercept: 2.192 ± 0.210). As is evident from Figure 16, the two treatments containing chemical cues did not differ enough for the visual stimulus to be significant. See Table S7 for statistical data.

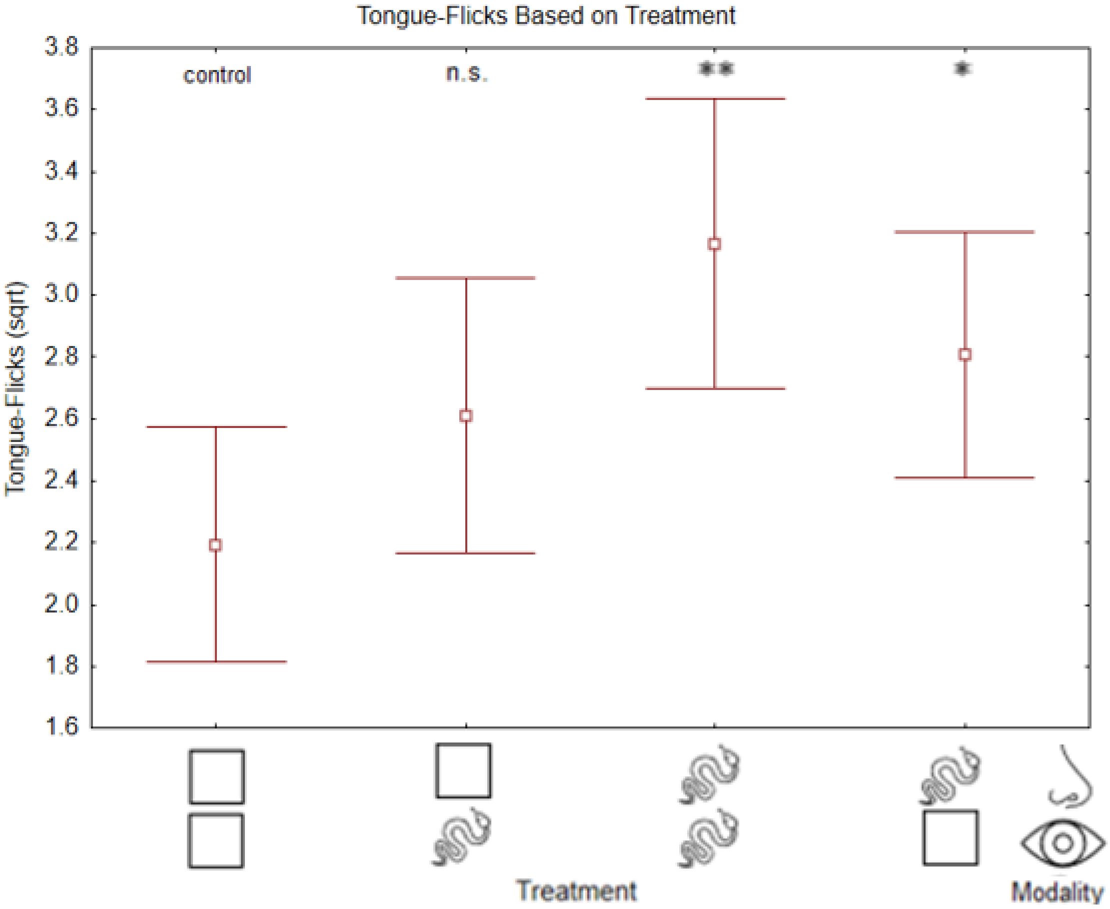

*Figure 10: The effect of treatment on tongue-flicks (sqrt) in the Snake Multimodality Test. Mean number of tongue-flicks (sqrt) is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows 4 different treatments combining varying modalities. Each treatment is described by 2 rows of stimuli within 1 column. Stimuli are split into rows based on which sensory system they target, these are shown on the far right, from top to bottom: chemical, visual. Empty squares represent a control in that modality. Chemical stimuli took the form of shed snakeskin, visual stimuli were represented by a live snake in a sealed glass container. Pieces of HDPE and empty glass containers were used as controls in the chemical and visual modalities respectively. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

The Snake Multimodality Test showed even less evidence for multimodality playing a role during predator detection in leopard geckos than the Triple Modality Test. Chemical cues seem to once again be the only dominant driving force eliciting antipredator behavior. The effect of the visual stimulus was entirely negligible. At this point, it would seem reasonable to conclude, that multimodal cues do not cause an increase in antipredator behavior compared to unimodal chemical cues. This result fully contradicts our predictions. In fact, it appears that our choice of visual cue and control, in this case, actually diminished the reactivity of geckos (9 reactions compared to 20 recorded in the bimodal visual and chemical treatment of the Triple Modality Test). We suspect this may have been caused by the novel effect of the glass container that was used to display a live snake and as a control. The placement of this previously unknown object into the leopard gecko's home terrarium could have had a disruptive effect on their resulting behavior. It is entirely plausible that geckos were too busy becoming familiar with the container itself to devote much more of their energy to try and detect predator cues. In future experiments, this approach could be improved by providing geckos with an acclimation period, placing an empty container into their terraria for several days before testing. As we only had 2 containers at our disposal and wanted to test at least 40 animals, our time constraints did not allow for such an approach. Treatments containing chemical cues elicited a higher number of tongue-flicks than the control, a result consistent with that of The Triple Modality Test. The fact that in these past 2 experiments, leopard geckos displayed more tongue-flicks towards the stimuli that elicited a higher number of antipredator reactions, does not entirely conform to the results of Landová et al. 2023, where geckos displayed the highest number of tongue-flicks towards a control stimulus.

Farallo et al. 2010 found that black spiny-tailed iguanas reduced foraging to a similar degree in the presence of a live snake and in the presence of indirect predator cues (snake feces). This may hint at the same dominant effect of chemical cues as we have found in our tests, where the difference between multimodal and unimodal chemical cues is insignificant. This is further supported by the findings of Miller & Gutzke 1995, who, when testing the importance of the vomeronasal organ in predator detection, demonstrated that pit vipers rely entirely on vomerolfaction to detect the presence of ophiophagous kingsnakes, showing no antipredator reaction when vomerolfaction was impeded.

Overall, the combined results from both previously described experiments refute our prediction, that multimodal cues will elicit stronger reactions than unimodal cues. They do, however fulfill our expectations as to the significance of the chemical stimulus overshadowing those of other stimuli.

3.3 Scent Loss Test

3.3.1 Freezer Snakeskin

The effect of treatment on antipredator behavior in the freezer snakeskin portion of the Scent Loss Test was significant ($\chi 2 = 30.322$, $p < 0.001$). Freezer snakeskin elicited antipredator behavior to a significantly higher degree (2.610 ± 0.707, $p < 0.001$) than the 1st control stimulus (Intercept: -2.996 ± 0.725). The amount of antipredator reactions displayed towards the control stimulus did not change after exposure to the snakeskin stimulus ($1.171 * 10^{-15} \pm 1.050$, $p = 1$). For results see Figure S1 and Table S9.

Treatment was also shown to have a significant effect on tongue-flicks (ANOVA: $F_{(2,82)} = 5.2$, $p = 0.0075$). However, the snakeskin treatment did not elicit significantly more tongue-flicks (0.189 ± 0.236, $p = 0.424$) than the 1st control (Intercept: 2.264 ± 0.184) while the 2nd control treatment elicited significantly fewer (-0.542 ± 0.236, $p = 0.024$), perhaps suggesting an exhaustion of chemosensory exploratory behavior by being exposed to 2 treatments previously in the same day. See Figure S2 and Table S10 for results and statistical data.

3.3.2 Room Temperature Snakeskin

Antipredator behavior in the room temperature snakeskin portion of the Scent Loss Test was significantly affected by treatment ($\chi 2 = 12.4$, $p = 0.002$). Room temperature snakeskin elicited antipredator behavior to a significantly higher degree (2.303 ± 0.694, $p < 0.001$) than the 1st control stimulus (Intercept: -2.996 ± 0.725). The amount of antipredator reactions displayed towards the control stimulus did not change significantly after exposure to the snakeskin stimulus (0.431 ± 0.429, $p = 0.315$). For results see Figure S3 and Table S11.

The effect of treatment on tongue-flicking appeared slightly significant (ANOVA: $F_{(2,82)} = 4$, $p = 0.023$). Room temperature snakeskin elicited tongue-flicks to a degree only bordering on significance (0.508 ± 0.257, $p = 0.052$) compared to the 1st control stimulus (Intercept: 2.199 ± 0.210). The amount of tongue-flicks displayed towards the control stimulus did not change significantly after exposure to the snakeskin stimulus (-0.194 ± 0.257, $p = 0.454$). For results see Figure S4 and Table S12.

3.3.3 Comparison

When comparing between the 2 presented snakeskins, we see that there is no significant difference in the degree of elicited antipredator behavior ($\chi 2 = 0.376$, $p = 0.54$). Even if we choose to look deeper, splitting reactions into active and passive antipredator behavior, we find the same result. Neither active antipredator behavior ($\chi 2 = 0.0539$, $p = 0.82$), nor passive antipredator behavior ($\chi 2 = 0.0451$, $p = 0.83$) showed significant contrasts between the 2 differently stored snakeskins. See results in Figure 17 and Tables S13-15.

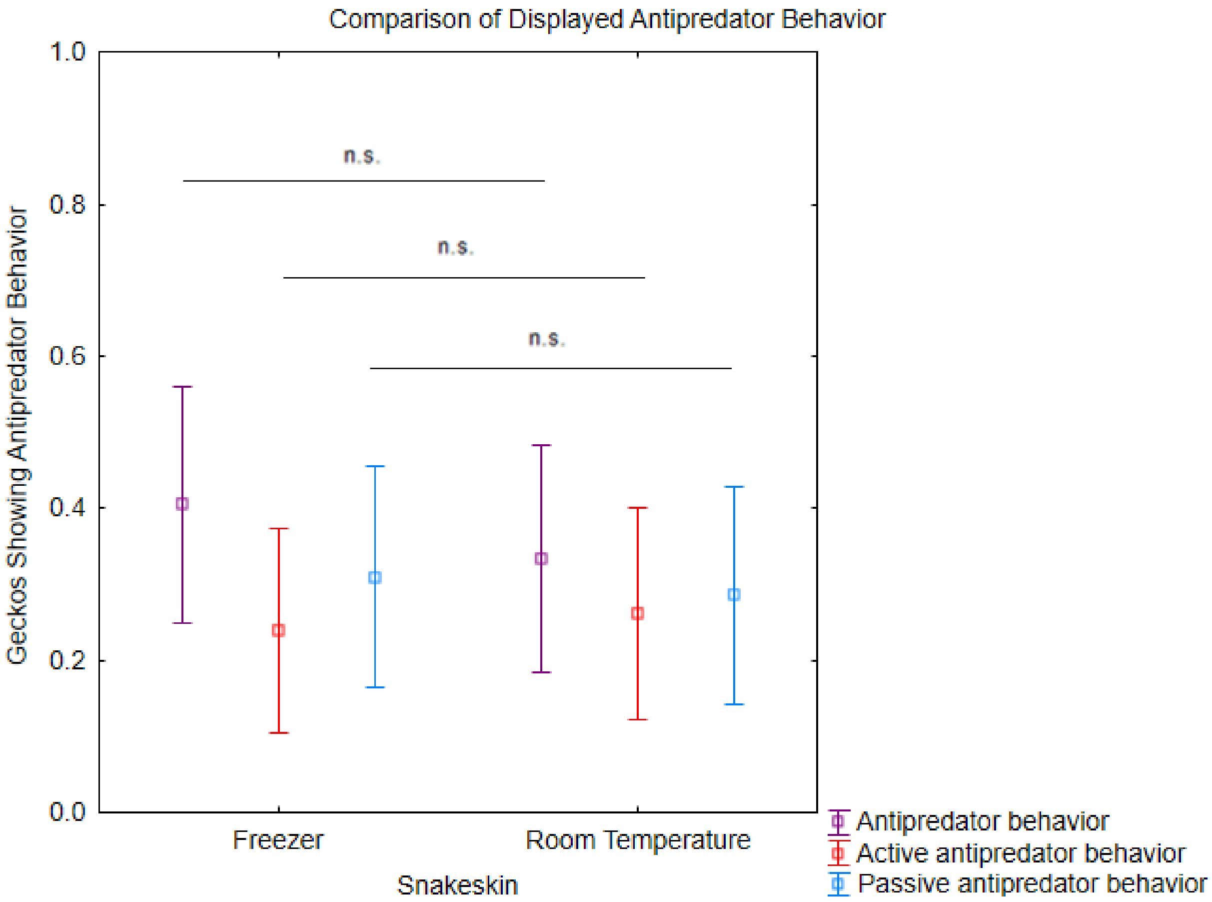

*Figure 17: Comparison of the reactivity of leopard geckos in the Scent Loss Test when exposed to snakeskins. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows two treatments differing in the way snakeskin was stored prior to testing. Freezer snakeskin was stored in a sealed glass container at temperatures < -28 °C. Room temperature snakeskin was stored in an open glass container at room temperature. (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p > 0.05$)*

Leopard geckos did not show any significant difference in tongue-flicking between the 2 snakeskins (-0.254 ± 0.311, $p = 0.419$).

After 2 months of being stored under disparate conditions both snakeskins elicited the same level of antipredator behavior in leopard geckos. This contradicts our prediction that snakeskin stored in a sealed container at -28 °C will elicit stronger reactions. This suggests that the key odorous compounds within snakeskin which cause antipredator reactions in leopard geckos are not very volatile. As is shown in Landová et al. 2023, snakeskins that were deodorized using a polar solvent (ethanol) still elicited a significant degree of antipredator behavior, whereas those treated with a non-polar solvent (petrol) did not, suggesting that the key compounds in question are also most likely non-polar. These represent useful steps towards understanding the mechanism by which antipredator behavior is elicited in leopard geckos when presented with shed snakeskin.

3.4 Ontogenetic Comparison Test

3.4.1 Juveniles

Juvenile antipredator behavior was strongly affected by treatment ($\chi2 = 86.6$, $p < 0.0001$). Snakeskin elicited antipredator behavior to a significantly higher degree (2.498 ± 0.350, $p < 0.0001$) than the 1st control stimulus (Intercept: -2.733 ± 0.313). The amount of antipredator reactions displayed towards the control stimulus did not change significantly after exposure to the snakeskin stimulus (0.351 ± 0.344, $p = 0.31$). For results see Figure 18 and Table S16.

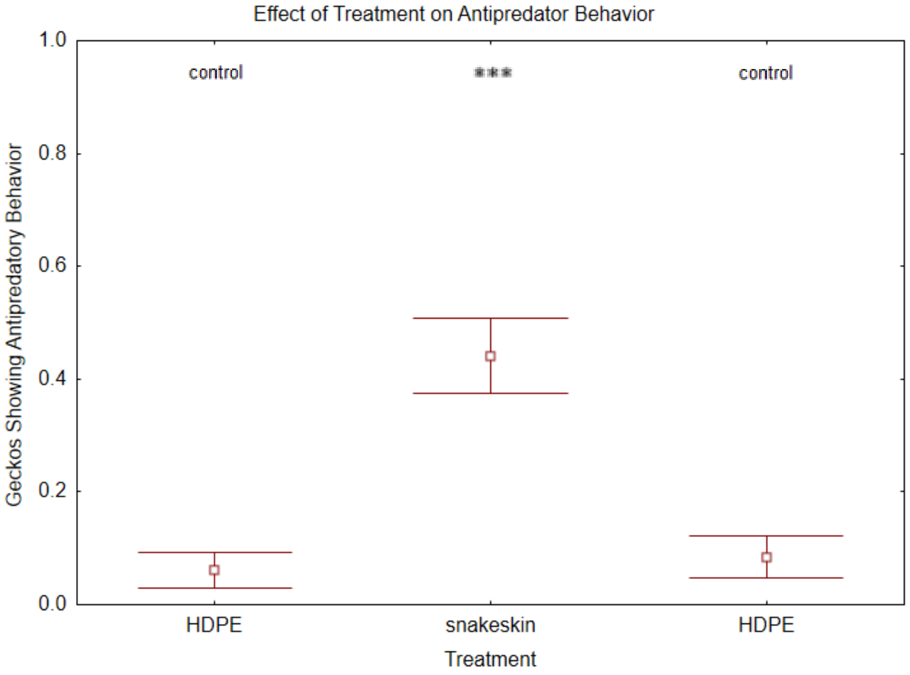

*Figure 18: The effect of treatment on antipredator response elicited in juvenile leopard geckos in the Ontogenetic Comparison Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

The effect of treatment on tongue-flicking also appeared significant (ANOVA: $F_{(2,566)} = 46.2283$, $p < 0.0001$). Snakeskin elicited tongue-flicks to a significantly higher degree (0.669 ± 0.090, $p < 0.0001$) compared to the 1st control stimulus (Intercept: 1.213 ± 0.068). The amount of tongue-flicks displayed towards the control stimulus did not change significantly after exposure to the snakeskin stimulus (-0.147 ± 0.090, $p = 0.1049$). For results see Figure 19 and Table S17.

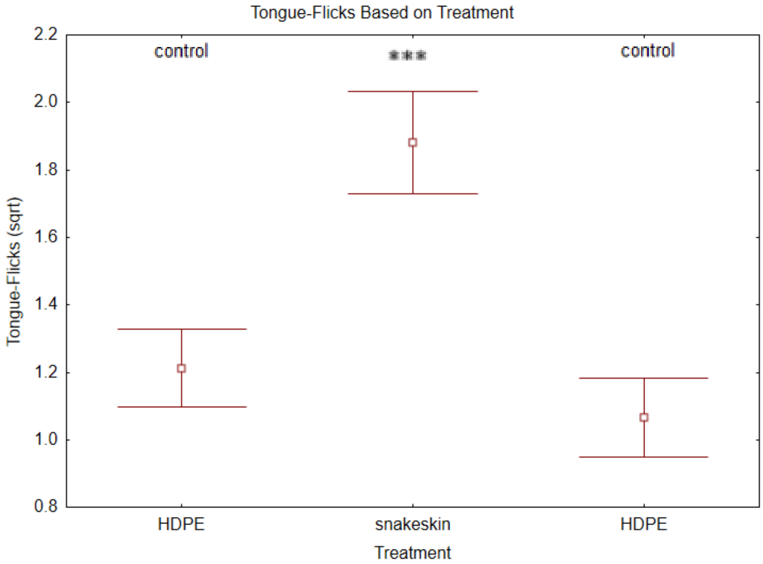

*Figure 19: The effect treatment on tongue-flicks elicited in juvenile leopard geckos in the Ontogenetic Comparison Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

3.4.2 Subadults

Subadult antipredator behavior was strongly affected by treatment ($\chi 2 = 140$, $p < 0.0001$). Snakeskin elicited antipredator behavior to a significantly higher degree (2.359 ± 0.227, $p < 0.0001$) than the 1st control stimulus (Intercept: -1.899 ± 0.221). The amount of antipredator reactions displayed towards the control stimulus did not change significantly after exposure to the snakeskin stimulus (0.330 ± 0.203, $p = 0.1$). For results see Figure 20 and Table S18.

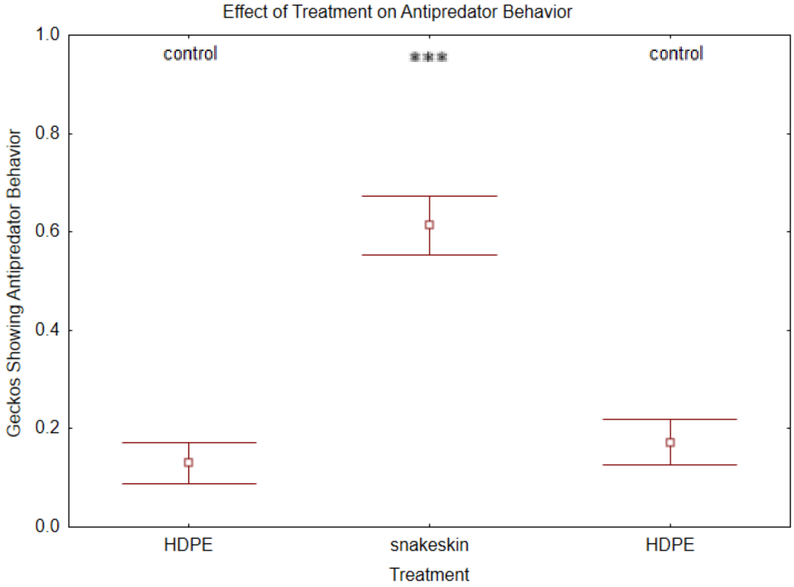

*Figure 20: The effect of treatment on antipredator response elicited in subadult leopard geckos in the Ontogenetic Comparison Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

The effect of treatment on tongue-flicking also appeared significant (ANOVA: $F_{(2,694)} = 22$, p < 0.0001). However, snakeskin did not elicit tongue-flicks to a significantly different degree (-0.053 ± 0.098, $p = 0.587$) than the 1st control stimulus (Intercept: 2.033 ± 0.080). The amount of tongue-flicks displayed towards the control stimulus in fact decreased significantly after exposure to the snakeskin stimulus (-0.590 ± 0.098, $p < 0.001$). For results see Figure 21 and Table S19.

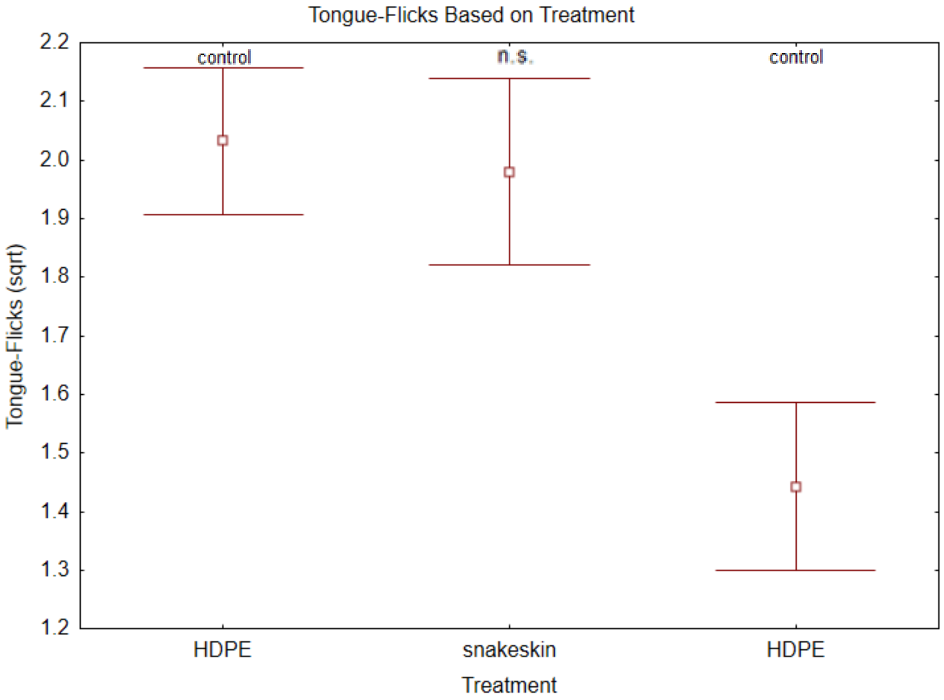

*Figure 21: The effect treatment on tongue-flicks elicited in juvenile leopard geckos in the Ontogenetic Comparison Test. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

3.4.1 Comparison

When comparing data gathered from the first time each ontogenetic stage was exposed to the HDPE-snakeskin-HDPE sequence, we see some pronounced differences in their reactivity. Adults reacted less frequently than juveniles (-1.56 ± 0.428, $p < 0.001$) and subadults (-2.73 ± 0.478, $p < 0.0001$). Subadults were shown to be the most reactive even when compared to juveniles (-1.17 ± 0.425, $p = 0.016$). Significantly fewer adults showed active antipredator behavior than juveniles (-1.320 ± 0.438, $p = 0.007$) and subadults (-1.973 ± 0.437, $p < 0.0001$). However, juveniles and subadults did not differ significantly (-0.653 ± 0.337, $p = 0.128$). Passive antipredator behavior was once again more frequently displayed by juveniles (-1.01 ± 0.421, $p = 0.044$) and subadults (-1.16 ± 0.410, $p = 0.013$) when compared to adults. Just as with active antipredator behavior, juveniles and subadults did not show significant differences when contrasted with each other (-0.15 ± 0.323, $p = 0.887$). For results and statistical data see Figure 22 and Tables S20-22.

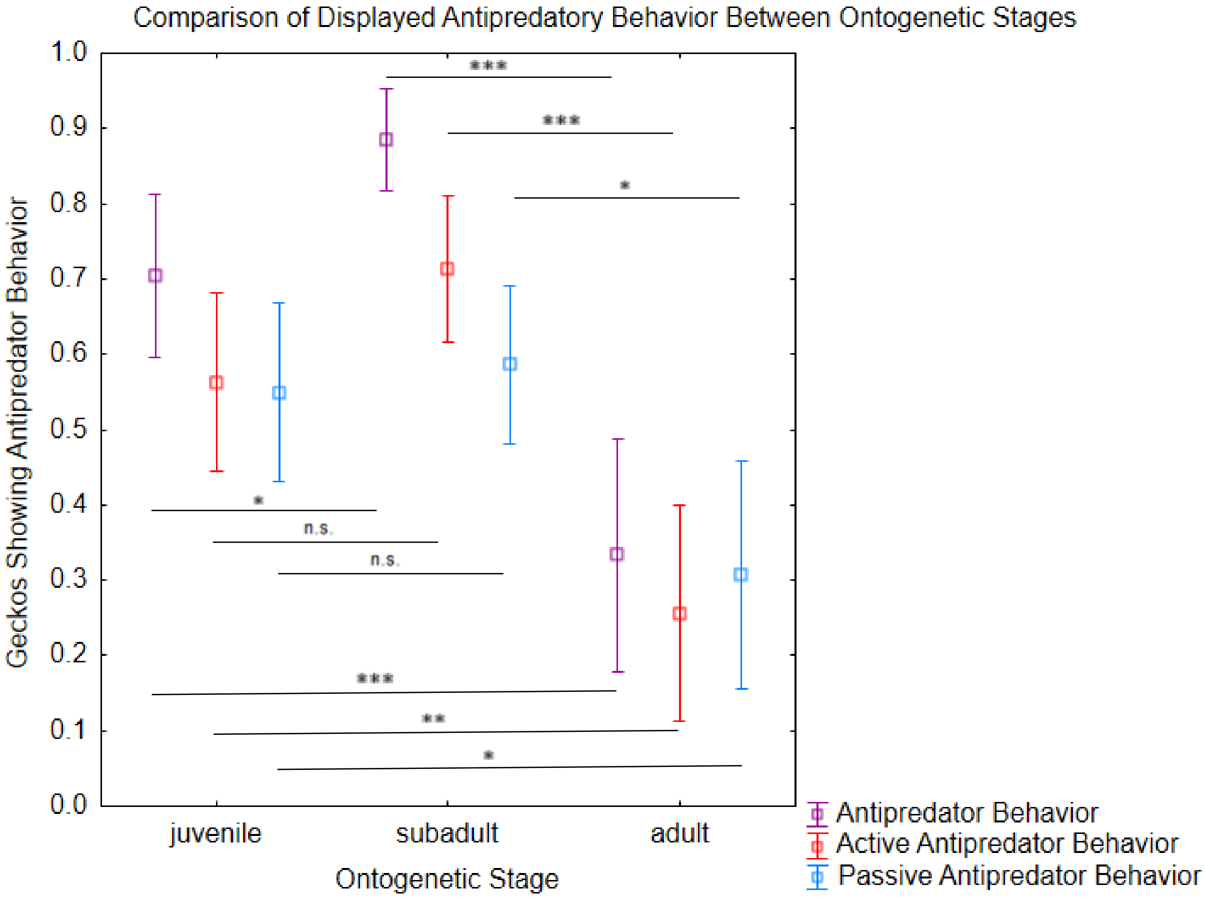

*Figure 22: Comparison of the reactivity of 3 different ontogenetic stages of leopard geckos in the Ontogenetic Comparison Test when exposed to snakeskins. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows ontogenetic stage. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

Tongue-flicks showed a similar trend. Adults displayed significantly fewer tongue-flicks than juveniles (1.370 ± 0.229, $p < 0.0001$) and subadults (1.572 ± 0.221, $p < 0.0001$). Juveniles and subadults did not differ (0.202 ± 0.183, $p = 0.516$). See Table S23.

In all cases, adult leopard geckos were shown to be the least reactive. Both juveniles and subadults showed a great degree of reactivity, with approximately 70 % of juveniles and nearly 90 % of subadults showing antipredator behavior when presented with a piece of shed snakeskin, making subadults the most reactive ontogenetic stage overall. Active and passive antipredator behaviors were also more prominent among younger geckos. When comparing these results to those of Landová et al. 2013, we see that both studies saw an increase in antipredator behavior from juveniles to subadults. However, our adult subjects were considerably less reactive and did not attempt to escape from presented stimuli. This contrast may have been partially caused by the fact, that our tested adult leopard geckos had been raised under laboratory conditions and had been subjected to several previous behavioral experiments and were therefore more accustomed to the near presence of researchers.

The findings of Martín et al. 2015 may also provide an interesting insight into predator detection through chemoreception. The study found that naïve Iberian wall lizard hatchlings are less discriminate towards predators and show escape responses equally when reacting to chemical cues of sympatric and allopatric snake species. In contrast, some populations of adults showed escape behavior significantly more when exposed to sympatric snake predator cues, indicating that predator response elicited by chemical cues is experience dependent. It is not unreasonable to think that a similar phenomenon may be present in leopard geckos, with juvenile and subadult geckos responding to snake chemical cues indiscriminately by intense antipredator behavior, and adults incorporating previous experience with specific predators into the process of assessing threat and the necessity of initiating antipredator behavior. This may be a way to help adults avoid wasting resources on unnecessary defensive responses, instead investing time and energy into other important activities, such as foraging or mating. Landová et al. 2016 hypothesized leopard geckos may be able to chemically recognize whether a predator has been preying on leopard geckos, an ability that might affect antipredator behavior. Such discrimination based on predator diet has been shown in European house crickets (Hoefler et al. 2012), striped mice (Pillay et al. 2003), and agile frogs (Hettyey et al. 2010). Abramjan et al. 2020 theorized that the pale coloration of subadult leopard geckos renders them the most conspicuous on dark backgrounds of all ontogenetic stages. This vulnerability could be a possible explaining factor for subadults' heightened reactivity to predator cues even when compared to juveniles.

3.4.2 Repeatability

When it came to antipredator behavior, leopard geckos fell mostly within expected repeatability scores for ectotherms under link-scale approximation (Bell et al. 2009). Juveniles had the highest repeatability (R = 0.184, CI = 0.006 - 0.312, $p = 0.007$) followed by subadults (R = 0.158, CI = 0.004 - 0.282, $p = 0.009$) and lastly adults, who did not show a significant degree of repeatability (R = 0.123, CI = 0 - 0.328, $p = 0.095$). For active antipredator behavior, adults showed very high repeatability scores (R = 0.452, CI = 0.12 - 0.919, $p < 0.001$), whereas subadults scored quite low (R = 0.077, CI = 0 - 0.179, $p = 0.095$). On the other hand, subadults showed high repeatability in passive antipredator behavior (R = 0.28, CI = 0.061 - 0.403, $p < 0.0001$), while adults did not show significant repeatability (R = 0.032, CI = 0 - 0.209, $p = 0.346$). Juveniles achieved low, but still significant repeatability scores in both active (R = 0.125, CI = 0 - 0.262, $p = 0.037$) and passive antipredator behavior (R = 0.127, CI = 0 - 0.267, $p = 0.037$). No ontogenetic stage showed significant repeatability in tongue-flicking. For statistical data see table S24.

In all cases, low repeatability could possibly be an example of habituation. For adults, this may be compounded by the fact that their data was taken from 3 different experiments which spanned nearly a year with several months in between tests. The fact that tongue-flicks were not repeatable for any age group is interesting when we compare it to another squamate, the northern common boa (*Boa imperator*), which showed significant repeatability for tongue-flicking behavior when reacting to a simulated predator attack (Šimková et al. 2017).

4 Conclusions

Half of our predictions were shown to be accurate, and half were refuted by our results. Leopard geckos did not display an increase in antipredator behavior when presented with multimodal stimuli than when exposed to only unimodal stimuli. Not only was the effect of the chemical stimulus stronger than those of visual and acoustic/mechanosensory stimuli, but it was also the only significant elicitor of antipredator behavior among all presented stimuli. Our prediction about the effect of snakeskin storage prior to testing was also inaccurate. Snakeskins stored under suboptimal conditions elicited antipredator behavior to roughly the same degree as those stored under more optimized conditions. Adults were shown to be less responsive to chemical cues than juveniles and especially subadults, who were the most reactive overall.

Although our results do not support the possibility of multimodality playing a role in snake predator detection by leopard geckos, it should not yet be ruled out entirely. With certain methodical improvements and a different choice of visual cues, a multimodal effect may still be revealed. Our findings do, however, further validate the use of shed snakeskin as a chemical stimulus utilized to elicit antipredator behavior in leopard geckos, as well as the use of pieces of HDPE bags as a control stimulus.

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Supplementary Materials

Table S1: Statistical data from the Triple Modality Test, ANOVA, elicited antipredator behavior compared to control.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	**S. E.**	**Wald**	**p**
(Intercept)	-2.197	0.527	17.38	< 0.0001
TREATMENT2	0.251	0.665	0.14	0.7057
TREATMENT3	2.197	0.568	14.98	0.0001
TREATMENT4	1.895	0.603	9.89	0.0017
TREATMENT5	-0.747	0.755	0.98	0.3224
TREATMENT6	0.251	0.665	0.14	0.7057
TREATMENT7	2.197	0.615	12.78	0.0004
TREATMENT8	2.297	0.573	16.1	< 0.0001

Table S2: Statistical data from the Triple Modality Test, post-hoc Tukey test, comparison of elicited antipredator behavior between all chemical treatments.

m=geeglm(Antipredatory.behaviour~TREATMENT,id=ID,family=binomial)

contrast	**Estimate**	**S. E.**	**z**	**p**
TREATMENT4-TREATMENT3	-0.667	0.342	-1.953	0.2063
TREATMENT4-TREATMENT8	-0.122	0.405	-0.302	0.9905
TREATMENT4-TREATMENT7	-0.35	0.348	-1.008	0.745
TREATMENT3-TREATMENT8	0.545	0.447	1.22	0.6138
TREATMENT3-TREATMENT7	0.317	0.379	0.836	0.8374
TREATMENT8-TREATMENT7	-0.228	0.36	-0.634	0.921

Table S3. Statistical data from the Triple Modality Test, ANOVA, elicited tongue-flicking compared to control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	**S. E.**	**DF**	**t**	**p**
(Intercept)	1.5048	0.1898	273	7.9303	< 0.0001
TREATMENT2	0.3911	0.2648	273	1.4771	0.1408
TREATMENT3	1.4022	0.2648	273	5.2961	< 0.0001
TREATMENT4	1.1351	0.2648	273	4.2875	< 0.0001
TREATMENT5	0.3114	0.2648	273	1.1763	0.2405
TREATMENT6	0.8748	0.2648	273	3.3041	0.0011
TREATMENT7	1.8706	0.2648	273	7.0653	< 0.0001
TREATMENT8	0.9867	0.2648	273	3.7270	0.0002

Table S4: Statistical data from the Triple Modality Test, post-hoc Tukey test, comparison of elicited tongue-flicking between all chemical treatments.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

Contrast	**Estimate**	**S. E.**	**DF**	**z**	**p**
TREATMENT3-TREATMENT4	0.267	0.26	117	1.027	0.7338
TREATMENT3-TREATMENT7	-0.468	0.26	117	-1.802	0.2777
TREATMENT3-TREATMENT8	0.415	0.26	117	1.598	0.3837
TREATMENT4-TREATMENT7	-0.735	0.26	117	-2.829	0.0278
TREATMENT4-TREATMENT8	0.148	0.26	117	0.571	0.9405
TREATMENT7-TREATMENT8	0.884	0.26	117	3.4	0.005

Table S5: Statistical data from the Snake Multimodality Test, ANOVA, elicited antipredator behavior compared to control.

geeglm(formula = Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-3.71	1.01	13.46	0.0002
TREATMENT2	1.15	1.20	0.92	0.3378
TREATMENT3	2.41	1.11	4.75	0.0294
TREATMENT4	2.41	1.11	4.75	0.0294

Table S6: Statistical data from the Snake Multimodality Test, ANOVA, comparison of elicited antipredator behavior between chemical treatments.

geeglm(Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-1.30	0.376	11.9	0.0006
TREATMENT 4	$-3.67*10^{-16}$	0.346	0.0	1.0000

Table S7. Statistical data from the Snake Multimodality Test, ANOVA, elicited tongue-flicking compared to control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	2.1921	0.2101	123	10.4356	0.0000
TREATMENT2	0.4182	0.2677	123	1.5623	0.1208
TREATMENT3	0.9748	0.2677	123	3.6413	0.0004
TREATMENT4	0.6146	0.2677	123	2.2957	0.0234

Table S8. Statistical data from the Snake Multimodality Test, ANOVA, comparison of elicited antipredator behavior between chemical treatments.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	3.17	0.215	41	14.70	0.000
TREATMENT4	-0.36	0.253	41	-1.42	0.162

Table S9: Statistical data from the freezer snakeskin portion of the Scent Loss Test, ANOVA, elicited antipredator behavior compared to the 1st control.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-2.996	0.7246	17.09	$3.56*10^{-5}$
TREATMENT2	2.610	0.7073	13.62	0.0002
TREATMENT4	1.171	1.050	0.00	1.0000

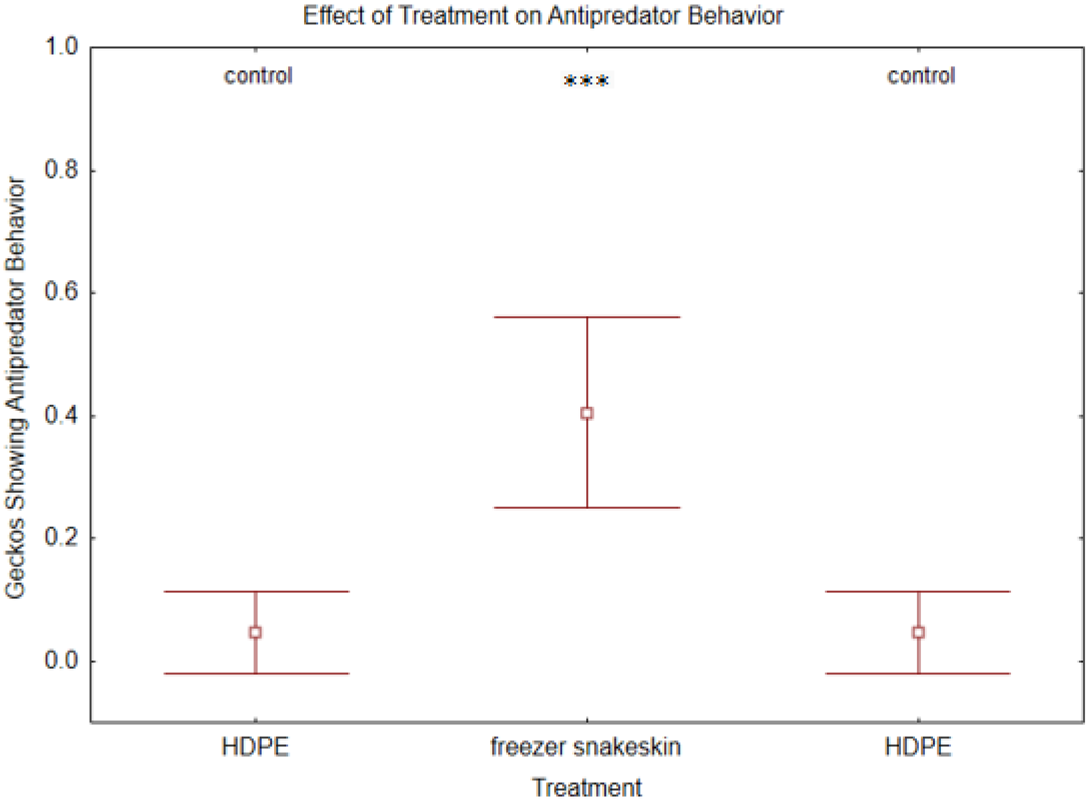

*Figure S1: The effect of treatment on antipredator response elicited in leopard geckos in the Scent Loss Test when using freezer snakeskin as a chemical stimulus. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

Table S10. Statistical data from the freezer snakeskin portion of the Scent Loss Test, ANOVA, elicited tongue-flicking compared to the 1st control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	2.264	0.184	82	12.3	0.0000
TREATMENT2	0.189	0.236	82	0.8	0.4238
TREATMENT4	-0.542	0.236	82	-2.3	0.0239

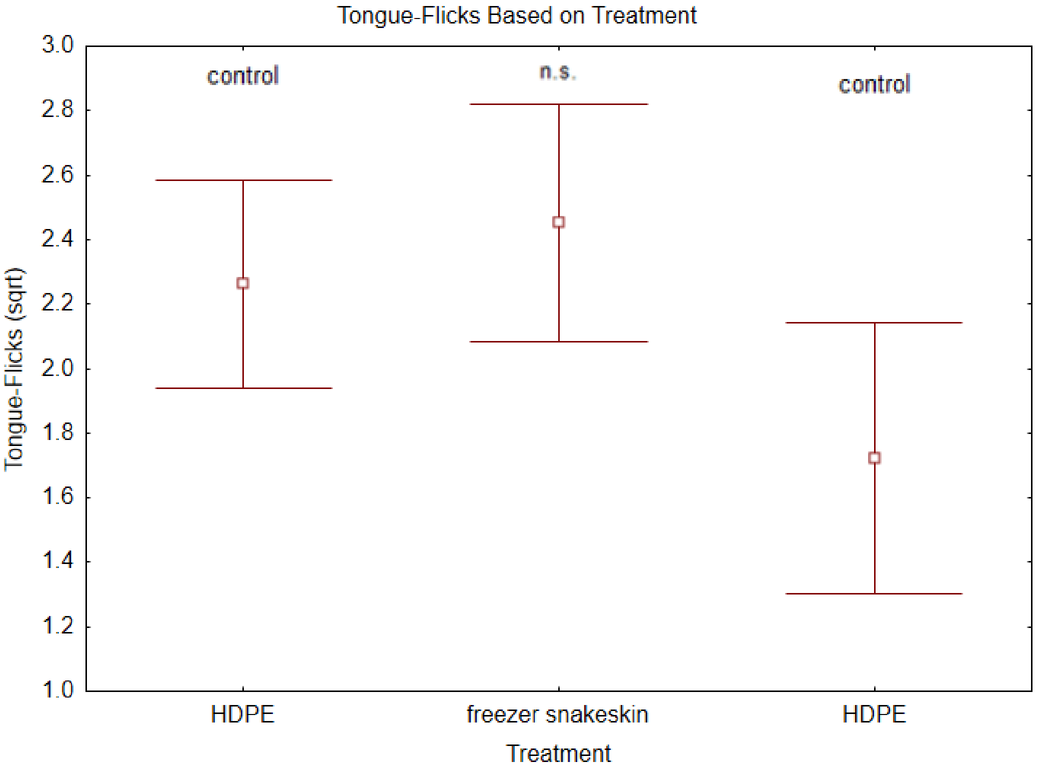

*Figure S2: The effect of treatment on tongue-flicks in the Scent Loss Test when using freezer snakeskin as a chemical stimulus. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p > 0.05$)*

Table S11: Statistical data from the room temperature snakeskin portion of the Scent Loss Test, ANOVA, elicited antipredator behavior compared to the 1st control.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-2.996	0.725	17.09	$3.6*10^{-5}$
TREATMENT3	2.303	0.694	11.00	0.0009
TREATMENT4	0.431	0.429	1.01	0.3152

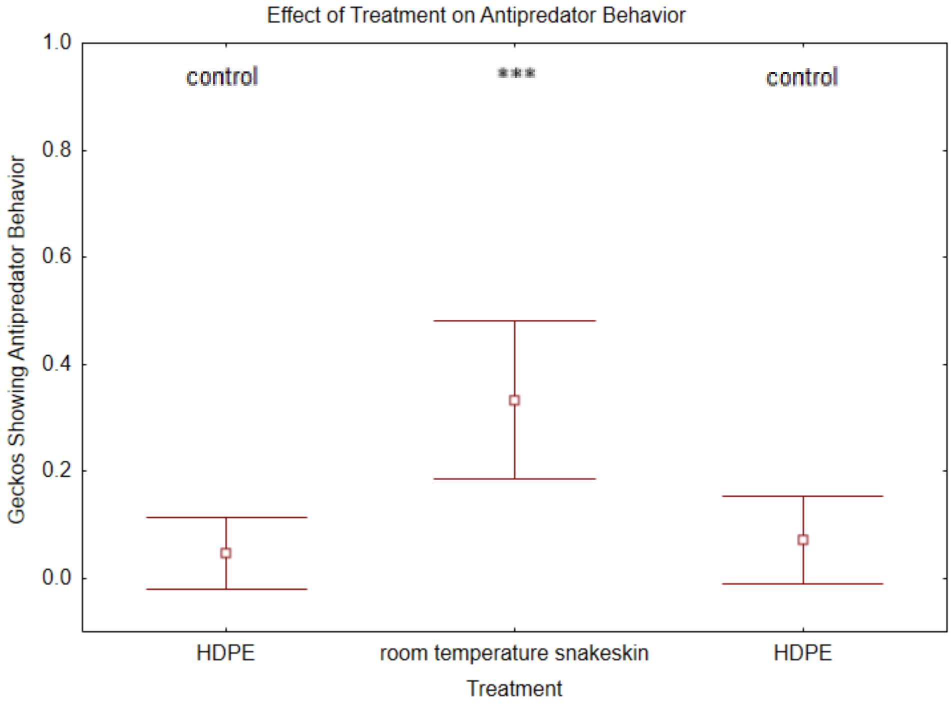

*Figure S3: The effect of treatment on antipredator response elicited in leopard geckos in the Scent Loss Test when using room temperature snakeskin as a chemical stimulus. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: p < 0.001, **: p < 0.01, *: p <0.05, n.s.: p > 0.05)*

Table S12. Statistical data from the room temperature snakeskin portion of the Scent Loss Test, ANOVA, elicited tongue-flicking compared to the 1st control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	2.199	0.210	82	10.49	0.0000
TREATMENT3	0.508	0.257	82	1.97	0.0517
TREATMENT4	-0.194	0.257	82	-0.75	0.4538

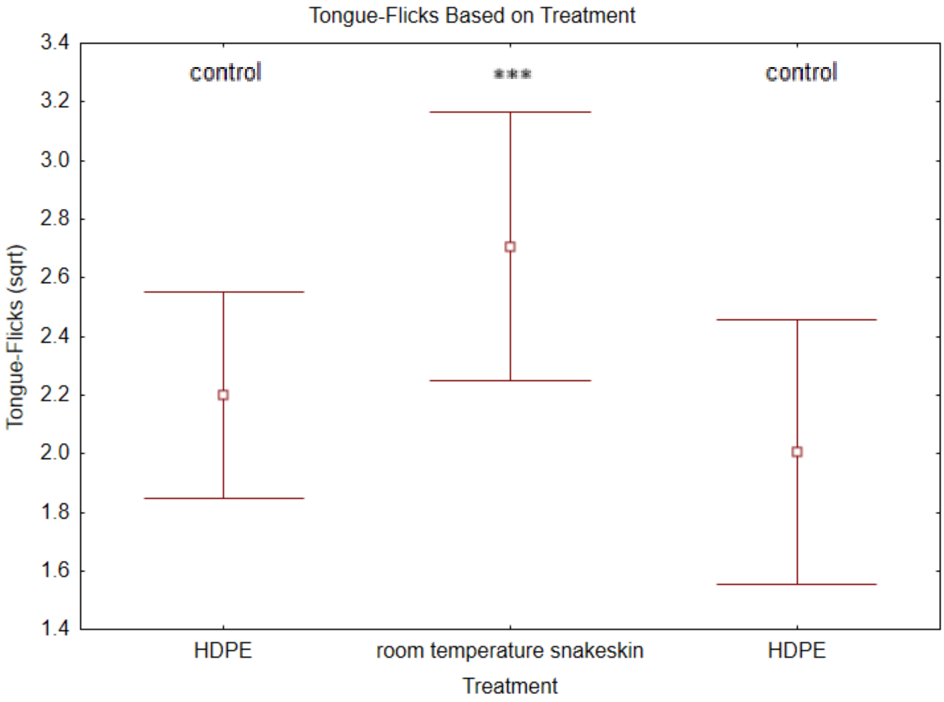

*Figure S4: The effect of treatment on tongue-flicks in the Scent Loss Test when using room temperature snakeskin as a chemical stimulus. Mean portion of leopard geckos displaying antipredator behavior is displayed on the y-axis with ± 0.95 confidence intervals. The x-axis shows treatments form left to right in the sequence they were presented. Each treatment contained one of two stimuli: HDPE (a piece of transparent high-density polyethylene) or a piece of shed snakeskin of a diadem snake. (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p > 0.05$)*

Table S13: Statistical data from the snakeskin comparison portion of the Scent Loss Test, ANOVA, antipredator behavior.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-0.386	0.360	1.15	0.28
TREATMENT3	-0.307	0.502	0.38	0.54

Table S14: Statistical data from the snakeskin comparison portion of the Scent Loss Test, ANOVA, active antipredator behavior.

geeglm(formula=Active.defensive.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-1.163	0.399	8.50	0.0035
TREATMENT3	0.127	0.547	0.05	0.8163

Table S15: Statistical data from the snakeskin comparison portion of the Scent Loss Test, ANOVA, passive antipredator behavior.

geeglm(formula=Passive.defensive.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-0.802	0.369	4.74	0.03
TREATMENT3	-0.114	0.536	0.05	0.83

Table S16: Statistical data from the Juvenile portion of the Ontogenetic Comparison Test, ANOVA, elicited antipredator behavior compared to the 1st control.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-2.733	0.313	76.15	$< 2*10^{-16}$
TREATMENT2	2.498	0.350	50.99	$9.3*10^{-13}$
TREATMENT3	0.351	0.344	1.04	0.31

Table S17. Statistical data from the Juvenile portion of the Ontogenetic Comparison Test, ANOVA, elicited tongue-flicking compared to the 1st control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	1.2126	0.0680	566	17.8308	0.0000
TREATMENT2	0.6689	0.0904	566	7.3956	0.0000
TREATMENT3	-0.1469	0.0904	566	-1.6239	0.1049

Table S18: Statistical data from the Subadult portion of the Ontogenetic Comparison Test, ANOVA, elicited antipredator behavior compared to the 1st control.

geeglm(formula=Antipredatory.behaviour ~ TREATMENT, family = binomial, id = ID)

	Estimate	S. E.	Wald	p
(Intercept)	-1.899	0.221	73.80	$< 2*10^{-16}$
TREATMENT2	2.359	0.227	107.80	$< 2*10^{-16}$
TREATMENT3	0.330	0.203	2.63	0.1

Table S19. Statistical data from the Subadult portion of the Ontogenetic Comparison Test, ANOVA, elicited tongue-flicking compared to the 1st control.

m=lme(sqrtTF~TREATMENT, random=~1|ID)

	Estimate	S. E.	DF	t	p
(Intercept)	2.033	0.0798	694	25.48	0.000
TREATMENT2	-0.053	0.0976	694	-0.54	0.587
TREATMENT3	-0.590	0.0976	694	-6.05	0.000

Table S20: Statistical data from the Comparison portion of the Ontogenetic Comparison Test, post-hoc Tukey test, comparison of elicited antipredator behavior between all ontogenetic stages.

m=geeglm(Antipredatory.behaviour~AGE,id=ID,family=binomial)

Contrast	**Estimate**	**S. E.**	**df**	**z**	**p**
Adult - juvenile	-1.56	0.428	Inf	-3.648	0.0008
Adult - subadult	-2.73	0.478	Inf	-5.722	<.0001
Juvenile - subadult	-1.17	0.425	Inf	-2.762	0.0159

Table S21: Statistical data from the Comparison portion of the Ontogenetic Comparison Test, post-hoc Tukey test, comparison of elicited active antipredator behavior between all ontogenetic stages.

m=geeglm(Active.defensive.behaviour~AGE,id=ID,family=binomial)

Contrast	**Estimate**	**S. E.**	**df**	**z**	**p**
Adult - juvenile	-1.320	0.438	Inf	-3.014	0.0073
Adult - subadult	-1.973	0.437	Inf	-4.519	<.0001
Juvenile - subadult	-0.653	0.337	Inf	-1.940	0.1275

Table S22: Statistical data from the Comparison portion of the Ontogenetic Comparison Test, post-hoc Tukey test, comparison of elicited passive antipredator behavior between all ontogenetic stages.

m=geeglm(Passive.defensive.behaviour~AGE,id=ID,family=binomial)

Contrast	**Estimate**	**S. E.**	**df**	**z**	**p**
Adult - juvenile	-1.01	0.421	Inf	-2.396	0.0437
Adult - subadult	-1.16	0.410	Inf	-2.830	0.0129
Juvenile - subadult	-0.15	0.323	Inf	-0.466	0.8872

Table S23: Statistical data from the Comparison portion of the Ontogenetic Comparison Test, post-hoc Tukey test, comparison of elicited tongue-flicks (sqrt) between all ontogenetic stages.

m=lme(sqrtTF~AGE, random=~1|ID)

Contrast	**Estimate**	**S. E.**	**df**	**t**	**p**
Adult - juvenile	1.370	0.229	194	5.995	< 0.0001
Adult - subadult	1.572	0.221	194	7.114	< 0.0001
Juvenile - subadult	0.202	0.183	194	1.100	0.5155

Table S23: Statistical data from the Repeatability portion of the Ontogenetic Comparison Test, Link-scale approximation, comparison of repeatability of different behaviors between all ontogenetic stages.

rptBinary(Antipredatory.behaviour~1+(1|ID), grname="ID",data=data)

rptBinary(Active.defensive.behaviour~1+(1|ID), grname="ID",data=data)

rptBinary(Passive.defensive.behaviour~1+(1|ID), grname="ID",data=data)

rptGaussian(sqrtTF~1+(1|ID), grname="ID",data=data)

Life stage	**Behaviour**	**R**	**CI**	**p**
Juveniles	Antipredatory behaviour	0.184	0.006 - 0.312	0.00739
	Active defensive behaviour	0.125	0 - 0.262	0.0373
	Passive defensive behaviour	0.127	0 - 0.267	0.0372
	sqrt Tongue flicking	0.068	0 - 0.201	0.183
Subadults	Antipredatory behaviour	0.158	0.004 - 0.282	0.00854
	Active defensive behaviour	0.077	0 - 0.179	0.0952
	Passive defensive behaviour	0.28	0.061 - 0.403	< 0.0001
	sqrt Tongue flicking	0.102	0 - 0.235	0.0625
Adults	Antipredatory behaviour	0.123	0 - 0.328	0.0945
	Active defensive behaviour	0.452	0.12 - 0.919	< 0.001
	Passive defensive behaviour	0.032	0 - 0.209	0.346
	sqrt Tongue flicking	0.053	0 - 0.258	0.318