

https://dspace.org/

Scents and scentability: an investigation into the chemical crypsis of the Puff Adder (Bitis arietans)

Miller, Ashadee Kay

2022
https://hdl.handle.net/10539/34478

Downloaded from a DSpace repository. https://dspace.org/

Scents and Scentability: An Investigation into the Chemical Crypsis of the Puff Adder (*Bitis arietans*)

Ashadee Kay Miller



A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, South Africa

6 June 2022

All the secrets in the world worth knowing are hiding in plain sight

Robin Sloane

CANDIDATE'S DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



_____

Ashadee Kay Miller

6 June 2022

ABSTRACT

Few documented examples of chemical crypsis exist. This is in part due to the long-standing dogma that no organism can be truly chemically cryptic due to associated, odorous metabolic by-products, and in part due to our own limitations as vision-orientated organisms. Visual crypsis, in comparison, is a widely documented phenomenon. This adaptation is driven by selection pressures inherent in predator-prey arms races between prey and their vision-orientated predators. Yet, despite the prevalence of macrosmatic, scent-orientated predator species and their associated ability to exert similar selection pressures, the potential for chemical crypsis among species has and continues to be largely dismissed. The aim of this thesis was to more closely examine the chemical crypsis of the puff adder (*Bitis arietans*) and in so doing, contribute to an area of chemical ecology that is in its infancy. Currently, this extreme ambush predator serves as the only documented case of a chemically cryptic terrestrial vertebrate and the mechanisms driving this crypsis remain elusive. As such, I was afforded the novel opportunity to investigate not only the extent to which the puff adder's chemical crypsis applies, but to also assess two potential mechanisms driving their chemical crypsis, namely the down-regulation of associated microbiota and metabolic suppression as a means of controlling associated, odorous metabolic by-products. By using two considered biosensor species, the African elephant (*Loxodonta Africana*) and the snouted cobra (*Naja annulifera*), I demonstrate, using scent matching and y-maze techniques respectively, that the puff adder's chemical crypsis is broadly applicable and in clear defiance of previously accepted dogma of chemical crypsis being an impossibility. In addition, I demonstrate using culture-dependent techniques and DNA sequencing that the puff adder does not downregulate its associated microbiota as a means of achieving chemical crypsis, but rather, that several microbial species closely associated with their trachea and dorsal scales may indeed directly contribute towards their chemical crypsis via their own odour degradation abilities. Furthermore, the notion that this extreme ambushing snake achieves chemical crypsis by means of metabolic suppression is rejected. Using closed-system respirometry, I demonstrate that the puff adder's standard metabolic rate is not lower than those of several other species of snake, including those known to be odorous. Lastly, solid-phase micro-extraction and gas chromatography-mass spectrometry provide important insights into the puff adder's volatilome, demonstrating that it is predominated by non-odorous alkanes and that the presence of a known odour-absorbing, waxy carboxylic acid found on the surfaces of the adder's scale may serve to trap odorous substances. Though I have been unsuccessful in elucidating a specific mechanism underlying the puff adder's chemical crypsis, this thesis provides the groundwork for more targeted research questions that may achieve such ends.

ACKNOWLEDGEMENTS

To my mentor, Graham Alexander – thank you for trusting me with your idea of chemical crypsis and for allowing me the space to make this work my own. It has been a great adventure.

To my parents, thank you for your unwavering support during your own very trying times. I am finally done :)

To Craig, my love, thank you. Your support has been endless and deeply appreciated.

A ridiculous number of people made this work possible through their generous contributions of time, knowledge and access to facilities:

Sean and Jenna Hensman made available their incredible herd of elephants at Adventures With Elephants and allowed me the opportunity to train and be trained by these incredible animals. Their dedicated team of knowledgeable handlers assisted tirelessly throughout the many months of training and trials.

Michael Hensman generously provided accommodation during elephant training and trials.

Johan Marais, Clive Reed and Leonie Erasmus are thanked for the loan of their snouted cobras. BEAST is thanked for repeatedly reminding us of safe handling practises and is definitely not missed.

Alexander Whaley and LECO made available their equipment for headspace analyses and for (mostly) welcoming the snakes into their offices.

Lindelo Mguni held my hand through microbial culturing and PCR analyses, often staying till the early hours of the morning to assist. Justin Popescu assisted with microbial culturing.

Marc Humphries' willingness to assist in the wake of a disappearing analytical chemist is greatly appreciated.

Xavier Glaudas and Ishvara Singh are thanked for supplying supplemental videography and scanning electron micrographs of wild puff adders and puff adder scales respectively.

Kip Schultz is thanked for his kind donation of Lock & Lock™ containers used during metabolic analyses.

Alexandra Evans, Wade and Nicole Stanton-Jones, Jonathan Perry and Jude Callaghan are thanked for their assistance during elephant scent trials.

Rick Matthews made available his incredible photos of the puff adders and elephants.

Finally, my dear dog Zuri is greatly loved for her contribution and for being an ever willing participant in our scent-matching trials. She provided proof of concept for our training protocols and for the notion that the puff adders may be chemically cryptic, which ultimately resulted in this work being funded. Her suicide attempt just weeks prior to submission however was greatly not appreciated.

Stephen Lee is thanked for not only considering my request for funding only moments after meeting, but for setting in motion the necessary actions to make it so.

This work was funded by the United States' Army Research Office and U.S. Research, Development and Engineering Command under grant number: W911NF-16-1-03-27.

All animal procedures were cleared by the Animal Ethics Committee of the University of the Witwatersrand under permits: 2011/26/2A; 2014/02/B; 2014/29/B; 2014/30/B and 2017/06/40B.

CONTENTS

CANDIDATE'S DECLARATION	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
CONTENTS	v
LIST OF FIGURES	vi
LIST OF TABLES	viii
CHAPTER 1: INTRODUCTION	**1**
1.1. INTRODUCTION	1
1.2. REFERENCES	6
CHAPTER 2: THE ECOLOGICAL EXTENT OF THE PUFF ADDER'S (*BITIS ARIETANS*) CHEMICAL CRYPSIS	**10**
2.1. ABSTRACT	11
2.2. INTRODUCTION	11
2.3. MATERIALS AND METHODS	14
2.4. RESULTS	19
2.5. DISCUSSION	24
2.6. REFERENCES	30
CHAPTER 3: ASSOCIATED DORSAL AND TRACHEAL MICROBIAL ASSEMBLAGES OF THE PUFF ADDER (*BITIS ARIETANS*)	**37**
3.1. ABSTRACT	38
3.2. INTRODUCTION	38
3.3. MATERIALS AND METHODS	40
3.4. RESULTS	43
3.5. DISCUSSION	50
3.6. REFERENCES	55
CHAPTER 4: METABOLIC RATE AND ASSOCIATED METABOLIC BY-PRODUCTS OF THE PUFF ADDER (*BITIS ARIETANS*)	**65**
4.1. ABSTRACT	66
4.2. INTRODUCTION	66
4.3. MATERIALS AND METHODS	68
4.4. RESULTS	72
4.5. DISCUSSION	79
4.6. REFERENCES	83
CHAPTER 5: CONCLUSION	**89**
5.1. INTRODUCTION	90
5.2. REFERENCES	94

LIST OF FIGURES

Figure 2.1. Fan-assisted Y-maze design. Arrows indicate the airflow direction, and snake side-bias is offset through a rounded acrylic crawl-way trough. Access to the maze, but not the airflow, was initially restricted via a perforated screen (A) which afforded the test snake a five-minute acclimation period, after which the screen is removed to facilitate entry into the maze. Once the snake's head passed a decision point (B) in either of the terminal arms, a decision was considered made. Insert depicts how terminal boxes for live puff adder trials differed. **16**

Figure 2.2. The sequential process involved in batch-preparing scent cloths. Half of the blanks (clean, unscented cotton cloths) are added to cleaned scent-tub positioned on a heating mat (32 °C; 1) and sealed for a period of 6 hours (2). Thereafter, half of these cloths, now controls, are removed and stored in a dedicated glass jar (3). To the remainder, a snake scent-donor is added to the tub (4), the cloths repositioned such that they are in direct contact with the snake (5), and the tub sealed for a further 6 hours (6). Finally, all the cloths, now targets, are stored in another dedicated glass jar (7). **17**

Figure 2.3. Scent canister components (left) and an example of successive stages of the scent-matching line-up (right). The scent-matching elephant was presented with the reference odour at the start mat (A) which was reinforced with the reward of pellets after each sniff. After three sniffs, the elephant walked down the line-up, sniffing each canister consecutively (right). A match was indicated via a 'salute' posture (B), and a non-match was moved passed. If the salute was correct, the elephant was rewarded with oranges at the indicated canister after which he/she continued down the line-up. The run was concluded when the elephant made contact with the end mat (C) where it was rewarded with oranges. **18**

Figure 2.4. The percentage of correct choices made by individual snouted cobras (Naja annulifera). Black bars indicate live puff adder (Bitis arietans) trials and white, puff adder shed skin trials (positive control). Asterisks indicate trails that positively differ from chance (dashed red line) and triangles those trials that had to be excluded from $\chi 2$ analyses due to high numbers of non-start events (nBEAST = 7 ; nCaramel = 5). Zoolander's inability to reliably identify a positive control (55.6% accuracy) and his strong side bias resulted in the exclusion of his data from further analyses. **20**

Figure 2.5. Kaplan-Meier curves comparing the time taken for snouted cobras (*Naja annulifera*) to start Y-mazes (i.e., Latency) in live puff adder (*Bitis arietans*; solid line) and puff adder shed skin (dashed line). Complete measures (♦) indicate trials where cobras ran the maze. Censored measures (+) indicate trials where non-starts occurred (i.e., BEAST only ran 30% of her live puff adder trials). Inverse cumulative proportions indicates the percentage of trials that at that point in time, the event had not yet occurred. For example, at five minutes, Fred has not yet entered the maze during puff adder shed skin trials 40% of the time, but had 100% of the time by the 18th minute. **21**

Figure 2.6. Kaplan-Meier curves comparing the time taken for snouted cobras (*Naja annulifera*) to make a decision in live puff adder (*Bitis arietans*; solid line) and puff adder shed skin (dashed line) Y-maze trials. Complete measures (♦) indicate trials where cobras ran the maze. Censored measures (+) indicate trials where *non-starts* occurred (i.e., BEAST made a decision in only 90% of her puff adder shed skin trials). Inverse cumulative proportions indicates the percentage of trials that at that point in time, the event had not yet occurred. For example, Fred made 90% of his decisions in less than 5 minutes, but took more than 30 minutes to make one decision in his live puff adder trials. **22**

Figure 2.7. Tongue flick rate (flicks.sec.$^{-1}$) for snouted cobras (*Naja annulifera*) exposed to live puff adder (*Bitis arietans*; ●) and puff adder shed skin (◦) during Y-maze trials. Error bars represent standard error. TF rates did not differ between trials for any cobra, shown via Tukey HSD posthoc tests. **23**

Figure 2.8. Percent of encountered scent cloths (target, control, and blank) indicated by scent-matching African elephants (*Loxodonta africana*) during puff adder (*Bitis arietans*; left) and brown house snake (*Boaedon capensis*; middle) trials. Elephant biosensor performance for each snake species included (right). **24**

Figure 3.1. A tubed puff adder (Bitis arietans) being anaesthetised using isoflourethane and kept warm on an heated beanbag to promote breathing and the onset of anaesthesia (left). Swabbing of an anaesthetized puff adder's glottis (centre) and dorsal scales (right) using sterile ∑-Transwabs (Medical Wire, UK) pre-dipped in distilled water. **41**

Figure 3.2. Examples of whole-colony chromatic change as a result of the selective chromogenic medium within the Rapid'E-coli and Rapid'Salmonella agar plates (Bio-Rad Laboratories, CA, USA). **41**

Figure 3.3. Bacterial species genetically identified from tracheal (pink) and dorsal (blue) samples taken from puff adders (*Bitis arietans*; n = 20) and brown house snakes (*Boaedon capensis*; n = 11). Colonies that were identified to genus-level only are denoted as 'spp.'. Dot position indicates which species of snake the bacterial species/genus/family was found on (i.e., puff adder = left, brown house snake = right) and the number of dots indicates the number of individual snakes on which the associated bacterial species/genus/family was positively identified. Superscript numbering indicates the associated bacterial families and Venn diagrams indicate where commonalities across all three levels of taxonomic resolution occur. Chromatic bars indicate taxonomic richness for species, genera and family levels for both snake species. **46**

Figure 3.4. Fungal species genetically identified from tracheal (pink) and dorsal (blue) samples taken from puff adders (*Bitis arietans;* n = 20) and brown house snakes (*Boaedon capensis*; n = 11). Colonies that were identified to genus-level only are denoted as 'spp.' and samples with family-level agreement are listed as such. Dot position indicates which species of snake the fungal species/genus/family was found on (i.e., puff adder = left, brown house snake = right) and the number of dots indicates the number of individual snakes on which the associated fungal species/genus/family was positively identified. Superscript numbering indicates the associated fungal families and Venn diagrams indicate where commonalities across all three levels of taxonomic resolution occur. Chromatic bars indicate taxonomic richness for species, genera and family levels confirmed via DNA sequencing for both snake species. **47**

Figure 3.5. Potential involvement of puff adder-associated (*Bitis arietans*) microbial species in ß-keratin degradation into its less odorous constituents. **52**

Figure 3.6. Scanning electron micrographs showing the micro-ornamentation of the dorsal scales of *Bitis arietans* (left) and *Bitis rhinoceros* (centre, right; Spinner et al. 2013). Potential fungal and bacterial spores (A-E), unidentified organic matter (F) and a lepidopteran wing scale (G). **53**

Figure 4.1 Temperature-acclimated puff adders (*Bitis arietans*) contained within airtight Lock & Lock containers (i.e., closed system) within an incubator. Samples (30 ml each) were drawn via three-way Luer locks (white) at the beginning and end of temperature treatments of known duration to assess standard metabolic rates (SMR) via changes in oxygen concentration. **70**

Figure 4.2. Oxygen consumption measures were made using closed-system respirometry: environmental air is pumped through an airline at 100 ml/min (controlled via an Alicat Scientific Flow Metre; A); scrubbed of water via a Drierite scrubber (B) and of carbon dioxide (CO_2) via a soda lime-filled scrubber (C). Samples are injected one at a time via luer lock (1-5) into the H_20- and CO_2-free air line, and scrubbed again for CO_2 (D) before having the flow-rate corrected once more by another Alicat Scientific Flow Metre (E). Thereafter injected samples are carried via the airline into a Sable Systems Oxygen Respirometer (F) to measure oxygen concentration. Thereafter data are processed via an accompanying computer (G). **70**

Figure 4.3. Left: A brown house snake (*Boaedon capensis*) temporarily contained within a purpose-built desiccator through which filtered air is being pumped at 0.5 $l.hr^{-1}$ (A). Volatile collection is achieved through an exposed tri-phase, grey, solid-phase microextraction fibre (B) positioned within the outlet pipe. Top right: A close up of a puff adder (Bitis arietans) temporarily contained within a species-specific, purpose-built desiccator. Bottom right: desiccator set-ups were placed within water baths for assessment of volatile production across a thermal range. Warm water temperatures were controlled via the use of two 200W Dophin fish tank heaters (C). **72**

Figure 4.4. Standard resting metabolic oxygen consumption (ml $g^{-1} hr^{-1}$) for puff adders (*Bitis arietans*) measured over a temperature gradient. Black squares indicate mean measures; grey triangles represent individual data. Trend line represents a power regression fitted for individual data with the equation $y = 0.0002x^{1.7823}$; $R^2 = 0.77$; n = 33; $p < 0.01$. **73**

LIST OF TABLES

Table 2.1. Average Y-maze time measures for latency and decision by snouted cobras (*Naja annulifera*) when exposed live puff adders (*Bitis* arietans) and puff adder shed skin trials, assessed using Time-to-event analyses. Asterisks indicate significance. **21**

Table 3.1. Thermal cycling conditions used for PCR. **43**

Table 3.2. Bray-Curtis similarity percentages based on presence/absence data for bacterial and fungal species assemblages genetically identified from tracheal and dorsal environments of puff adders (*Bitis arietans*) and brown house snakes (*Boaedon capensis*). Instances where the microbial species were present in both anatomical regions within a snake species, the microbial species would be scored as two whereas a score of one would indicate that the species was found in only one region. **48**

Table 3.3. An assessment of known anti-microbial activity associated with the culture-dependent microbial species identified from the trachea and dorsal scales of the puff adder (Bitis arietans) and brown house snake (Boaedon capensis). **48**

Table 4.1. Previously reported temperature-dependent oxygen consumption ($ml.g^{-1}\ hr^{-1}$) for various snake species and mass-adjusted ($ml.g^{-1}\ hr^{-1}/g$) comparisons against our recorded puff adder (Bitis arietans) data. Values highlighted in blue, yellow, and red indicates SMRs lower, around the same and higher than those of the puff adder. Values with no highlight indicate values for temperatures outside of our tested range. **73**

Table 4.2. Volatile organic compound (>75% NIST matches) profiles for the puff adder (Bitis arietans) and the brown house snake (Boaedon capensis), assessed via solid-phase microextraction (SPME) Gas Chromatography-Mass Spectrometry (GC-MS).* indicates compounds previously identified in reptilian femoral secretions (refs), • indicates compounds whose isomers have been identified in reptilian femoral secretions and † indicates compounds (or their isomers) that have been previously identified as pheromones in other taxa. **74**

CHAPTER 1: INTRODUCTION

Predator-prey dynamics are driven by a constantly evolving biological arms race (Abrams 1986). The selection pressures associated with this evolutionary duel are thought to be one of the driving forces behind the wide diversity of species in existence today (Abrams 2000). Indeed, these selection pressures have given rise to some of the most spectacular adaptations in the natural world, for example, the spider-tailed horned viper (*Pseudocerastes urarachnoides*) that deceptively lures avian prey within striking distance using its arachnoid caudal lure (Rastegar-Pouyani et al. 2015) and the cinereous mourner (*Laniocera hypopyrra*) whose chicks behaviourally and visually mimic the toxic caterpillars of flannel moths to deter nest predation (Londoño et al. 2015)**.** While the predator-prey arms race is a two-way affair, the direction in which it operates is presumed to be asymmetrical, with greater selection occurring within prey organisms (Vermeij 1994)**.** This is simply because the associated costs for prey are far more acute and consequential than those for predators. Quite simply, an inability to detect the presence of a predator or the failure to evade capture, for example, can be fatal. In comparison, a predator's failure to detect or capture prey simply results in a missed feeding opportunity. Of course, if predators consistently fail to feed over time, associated costs can be equivalent, but several authors have argued, somewhat idealistically, that prey responses to decreased predation rates could nullify these effects (see Abrams 2000), for example through increased prey density resulting in increased encounter rates. Regardless of the directionality, biological arms races operate on many aspects of life, but can be broadly categorized into two main groups. One, adaptations that hinder detection (i.e., behavioural and physical crypsis) and two, adaptations that increase survival once detection has occurred (i.e., athletic ability, armour and weaponry, toxins, and signalling). This thesis focuses on one particular adaptation that falls within the first group in its attempt to reduce detection, and which up until recently was considered impossible (Conover 2007) – chemical crypsis.

Chemical crypsis results in an organism being undetectable via a chemical cue. Unlike a *chemical signal*, a *chemical cue* is unintentionally emitted by the sender, and when recognized by a receiver, results in either a neutral or detrimental effect on the sender (e.g., a kairomone). A *chemical signal*, on the other hand, is emitted intentionally and when detected by a receiver, advantages the sender (e.g., mimesis; Steiger et al. 2011). Thus, a chemically cryptic species differs from a chemical mimic in one fundamental way: A mimic is chemically conspicuous via intentional, but dishonest signalling. Few examples of chemically crypsis are known (e.g., Rödel et al. 2013; Brooker et al. 2015; Miller et al. 2015a; Resetarits et al. 2022), of which only one has been documented in a terrestrial vertebrate – the puff adder (*Bitis arietans*; Miller et al. 2015a). This snake species is considered to be

an extreme ambush forager, lying in wait of approaching prey for days at a time. This foraging strategy is closely associated with concealment tactics to capture prey, and more importantly, avoid predation. Of the 42 known puff adder predator species, 15 are considered macrosmatic (Miller et al. 2015a). The implications of such a vast array of both macrosmatic and vision-orientated predators are that selection pressures acting on this snake should be great, and no doubt underpin both its chemical (Miller et al. 2015a) and visual crypsis (Greene 1997; Stevens and Merilaita 2009).

Due to the inconsistent application of the terminology surrounding chemical crypsis within the literature, in this Thesis, I consider only species that do not rely on dishonest signalling as a means of detection evasion to be truly chemically cryptic. Thus, species that mimic or cloak (i.e., emit compounds that mask otherwise tell-tale chemical signatures that would identify a species; Stevens and Merilaita 2009) are considered chemically camouflaged, but are not cryptic. Within these parameters, only two examples are known, the puff adder and the pirate perch (*Aphredoderus sayanus*; Resetarits et al. 2022). Like the puff adder, the pirate perch is an ambush forager that is largely undetectable to several of its prey species, yet in both instances the underlying mechanism remains elusive. Previously, the possibility of true chemical crypsis was dismissed due to the inevitable production of odorous by-products associated with metabolism (Conover 2007). Yet, now there are two species that seemingly defy this restriction, suggesting that the dearth of chemically cryptic examples may simply be a function of research focus, particularly given the widespread prevalence of macrosmatic predators as selective agents.

Chemosensation is the oldest and most widespread form of communication in the natural world (Wyatt 2003). And yet it remains one of the most poorly understood of them all (Steiger et al. 2011). Unlike that of vision and sound, whose measurements are predictably bound within a few physical properties such as waves, wavelength, amplitude, electromagnetism, for example, the complexity associated with chemicals and their detection is vast (Steiger et al. 2011). Chemical cues and signals can range from a single compound to a bouquet of several that can vary in a multitude of ways including concentration and functional group, to the point that the chemical environment in so complex in its dimensions that it becomes almost limitless (Yohe and Brand 2018). Added to this, the chemical environment is greatly influenced by a host of often-interconnected, abiotic factors that are themselves constantly changing (Cetin et al. 2003), making the study of *in-situ* chemical ecology particularly challenging. Over and above these complexities are our own biases towards modalities that we intrinsically understand, such as vision (Ruxton 2009; Miller et al. 2015a), which further compounds the appearance of rarity when it comes to traits such as chemical crypsis. In an endeavour

to counteract these biases and contribute towards what little we know of chemical crypsis, I further examine the chemically cryptic case of the puff adder.

Currently, the puff adder is the sole example of a chemically cryptic, terrestrial vertebrate species. Neither of the two natural predators (i.e., dog and meerkat) used by Miller et al. (2015a) were able to detect this snake via airborne chemical cue. Through the use of both wild and laboratory populations of puff adders tested against background odour, and by virtue of using scent-matching techniques, whereby biosensors were rewarded to indicate any odour associated with the adder, regardless of intended signal, reliance on adaptive resemblance by the puff adder is highly unlikely (Miller et al. 2015a). Though the underlying mechanism of the adder's chemical crypsis remains elusive, two have been posited as possibilities, namely microbial manipulation and metabolic suppression.

Microbial Suppression – Microbial communities responsible for the production of odours may be suppressed or modified by the puff adders. Species odour can be greatly influenced, if not determined by, odour-producing bacteria and fungi, and in particular by those associated with the integument and respiratory tract of the animal (Archie and Theis 2011). Thus, any form of microbial suppression by the puff adder or by the microbial community itself could result in specific and intentional control of the snake's associated odour. Examples of host-mediation of skin-level microbiota are known (McKenzie et al. 2012), typically due to antimicrobial peptides (AMPs) being produced by the host (e.g., van Hoek 2014; Walke et al. 2014) but also via mechanical means. For example, microbe-penetrating nanostructures associated with an organism's dorsal surface have been shown to be a highly effective innate antimicrobial solution (Li et al. 2016). Alternatively, microbial suppression could be achieved via acquired means. Indeed, mutualistic relationships between host species and other antimicrobial-producing organisms have to date been well documented in several taxa such as sloths, birds, and amphibians (Soler et al. 2008; Ruiz-Rodríguez et al. 2009; Martín-Vivaldi et al. 2010; 2014; Higgimbotham et al. 2014; Kearns et al. 2017; Hill et al. 2018). Thus, given the well-known role of associated microbial species as body odour contributors and the array of documented antimicrobial solutions already known to science among vertebrate species, I consider this theory suitably worthy of investigation and specifically addressed in Chapter 3.

Metabolic Suppression – the standard metabolic rates of the puff adder may be low enough to suppress any associated, odorous by-products below detectable thresholds (Miller et al. 2015a). Ambush foragers in general have lower energetic requirements than their actively foraging counterparts (Norberg 1977). Indeed, several species of ambushing viperids are known to have field

metabolic rates that are lower than what is typical of actively foraging snakes (Secor and Diamond 1998; Dupoué et al. 2017; Stuginski et al. 2018), as well as comparatively low haematocrits (Lourdais et al. 2014). Given that haematocrit levels are typically intimately connected with the oxygen demands of a species, and the fact that the lowest snake haematocrit levels are found in boids and vipers (Brischoux et al. 2011), two clades in which ambush foragers predominate, it is reasonable to suspect that the puff adder's metabolic rate may indeed be low enough so as to suppress associated airborne chemical cues. As such, Chapter 4 deals with measures of the puff adder's standard metabolic rate (via close-system respirometry) and associated volatile production (via gas-chromatography, mass spectrometry) as a means of assessing metabolic suppression as the underlying mechanism.

But first, it is important to consider to what extent the puff adder's chemical crypsis is applicable. Though Miller et al. (2015a) biosensor selection of two natural puff adder predators (dogs and meerkats) was ecologically appropriate, the possibility exists that these snakes are chemically conspicuous to other predators. Olfactory ability among species is greatly varied and depends not only on effective odour collection and delivery mechanisms, but also on the presence of appropriate olfactory receptors. Receptor repertoire is genetically determined and species-specific (Niimura et al. 2014). Thus, in an effort to rigorously assess the extent of the puff adder's chemical crypsis, two additional biosensors were enlisted – the African elephant (*Loxodonta africana*) and the snouted cobra (*Naja annulifera*). I chose these two biosensor species for very specific and distinct different reasons. First, the olfactory acuity of an African elephant is presumed to be one of the greatest among species due to its ~ 2000 olfactory receptor (OR) genes. This incredibly high number of OR genes is the highest of any mammal tested to date (Niimura et al. 2014). Indeed, that olfactory acuity among African and Asian elephants (*Elaphas maximus*) demonstrated to date (i.e., Rizvanovic et al. 2013; Bates et al. 2007; Plotnik et al. 2019) is suggestive of an olfactory ability that likely exceeds that of other species (Miller et al., 2015b). As such, I felt it a suitable candidate as a scent-matching biosensor with which to assess the extent of the puff adder's chemical crypsis in spite of it not being a predator of puff adders. Second, snouted cobras are among the top puff adder predators, responsible for the removal of 25% of the adult population annually (Graham Alexander, pers. obs. 2020). This may in part be due to the fact that the vomeronasal system of snakes, which is responsible for the detection of molecules with high molecular weight and therefore low volatility (Baxi *et al.* 2006) is the most developed among all vertebrate lineages (Brykczynska et al. 2013). These cobras demonstrate clear successes in locating and consuming puff adders (Maritz et al. 2019) and although the manner in which this achieved is has not been untested, it reasonable to assume that it may in part be due to their highly specialized VNS. Detection by African elephants would indicate that the adder's crypsis depends largely on a receiver's receptor repertoire, and detection by snouted cobras would indicate that odour

molecules associated with the puff adder are likely to have such low volatility that detection via olfaction is greatly limited. Thus, through the application of these two additional biosensors, addressed in Chapter 2, I assess the extent to which the puff adder's chemical crypsis applies.

Finally, in Chapter 5, I provide a stand back overview of my findings within the context of chemical ecology and future research.

REFERENCES

Abrams, P.A. 1986. Is predator-prey coevolution an Arms Race? *Trends in Ecology and Evolution* 1, no. 4: 108–110.

———. 2000. The evolution of predator-prey interactions: Theory and Evidence. *Annual Review of Ecology and Systematics* 31: 79–105.

Archie, E.A., and K.R. Theis. 2011. Animal behaviour meets microbial ecology. *Animal Behaviour* 82, no. 3: 425–436.

Bates, L.A., K.N. Sayialel, N.W. Njiraini, C.J. Moss, J.H. Poole, and R.W. Byrne. 2007. Elephants classify human ethnic groups by odor and garment color. *Current Biology* 17, no. 22: 1938–1942.

Baxi, K.N., K.M. Dorries, and H.L. Eisthen. 2006. Is the vomeronasal system really specialized for detecting pheromones? *Trends in Neurosciences* 29, no. 1: 1–7.

Brischoux, F., G.E.A. Gartner, T. Garland, and X. Bonnet. 2011. Is aquatic life correlated with an increased hematocrit in snakes? *PLoS ONE* 6, no. 2.

Brooker, R.M., P.L. Munday, D.P. Chivers, and G.P. Jones. 2015. You are what you eat: diet-induced chemical crypsis in a coral reef fish. *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1799: 1–7.

Brykczynska, U., Tzika, A.C., Rodriguez, I., and M.C. Milinkovitch. 2013. Contrasted evolution of the vomeronasal receptor repertoires in mammals and squamate reptiles. *Genome Biology and Evolution* 5, no. 2: 389–401.

Cetin, E., M. Odabasi, and R. Seyfioglu. 2003. Ambient volatile organic compound (VOC) concentrations around a petrochemical complex and a petroleum refinery. *Science of the Total Environment* 312, no. 1–3: 103–112.

Conover, M. 2007. *Predator-Prey Dynamics: The Role of Olfaction*. Boca Raton, FL.: Taylor and Francis.

Dupoué, A., F. Brischoux, and O. Lourdais. 2017. Climate and foraging mode explain interspecific variation in snake metabolic rates. *Proceedings of the Royal Society B: Biological Sciences* 284, no. 1867.

Greene, H.W. 1997. *Snakes: the Evolution of Mystery in Nature*. Berkley and Los Angeles, CA: University of California Press.

Higginbotham, S., W.R. Wong, R.G. Linington, C. Spadafora, L. Iturrado, and A.E. Arnold. 2014. Sloth hair as a novel source of fungi and potent anti-parasitic, anti-cancer and anti-bacterial bioactivity. *Plos One* 9, no. 1:e84549.

Hill, A.J., J.E., Leys, D. Bryan, F.M. Erdmand, K.S. Malone, G.N. Russell, R.D. Applegate, H. Fenton, K. Niedringhaus, A.N. Miller, M.C. Allender and D.M. Walker. 2018. Common cutaneous bacteria isolated from snakes inhibit growth of *Ophidimyces ophiodiicola. EcoHealth* 15, 109–120.

Kearns, P.J., S. Fischer, S. Fernández-Beaskoetxea, C.R. Gabor, J. Bosch, J.L. Bowen, M.F. Tlusty and D.C. Woodhams. 2017. Fight fungi with fungi: Antigungal properties of the amphibian mycobiome. *Frontiers in Microbiology* 8: 2494.

Li, X., G.S. Cheung, G.S. Watson, J.A. Watson, S. Lin, L. Schwarzkopf, and D.W. Green. 2016. The nanotipped hairs of gecko skin and biotemplated replicas impair and/or kill pathogenic bacteria with high efficiency. *Nanoscale* 8, no. 45: 18860–18869.

Londoño, G.A., D.A. García, and M.A. Sánchez Martínez. 2015. Morphological and behavioral evidence of batesian mimicry in nestlings of a lowland amazonian bird. *American Naturalist* 185, no. 1: 135–141.

Lourdais, O., G.E.A. Gartner, and F. Brischoux. 2014. Ambush or active life: foraging mode influences haematocrit levels in snakes. *Biological Journal of the Linnean Society* 111: 636–645.

Maritz, B., G.J. Alexander, and R.A. Maritz. 2019. The underappreciated extent of cannibalism and ophiophagy in African cobras. *Ecology* 100, no. 2: e0252210.1002/ecy.2522.

Martín-Vivaldi, M., A. Peña, J.M. Peralta-Sánchez, L. Sánchez, S. Ananou, M. Ruiz-Rodríguez, and J.J. Soler. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society B: Biological Sciences* 277: 123–130.

Martín-Vivaldi, M., J.J. Soler, J.M. Peralta-Sánchez, L. Arco, A.M. Martín-Platero, M. Martínez-Beuno, M. Ruiz-Rodríguez, and E. Valdivia. 2014. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. *Journal of Animal Ecology* 83: 1289–1301.

McKenzie, V.J., R.M. Bowers, N. Fierer, R. Knight, and C.L. Lauber. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. The *ISME Journal* 6, no. 3: 588–596.

Miller, A.K., B. Maritz, S. McKay, X. Glaudas, and G.J. Alexander. 2015a. An ambusher's arsenal: chemical crypsis in the puff adder (*Bitis arietans*). *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1821.

Miller, A.K., M.C. Hensman, S. Hensman, K. Schultz, P. Reid, M. Shore, J. Brown, K. G. Furton, and S. Lee. 2015b. African elephants (*Loxodonta africana*) can detect TNT using olfation: Implications for biosensor application. *Applied Animal Behaviour Science* 171: 177–183.

Niimura, Y., A. Matsui, and K. Touhara. 2014. Extreme expansion of the olfactory receptor gene repertoire in african elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome Research* 24, no. 9: 1485–1496.

Norberg, R.A. 1977. An Ecological Theory on Foraging Time and Energetics and Choice of Optimal Food-Searching Method. *Journal of Animal Ecology* 46, no. 2: 511–529.

Plotnik, J.M., D.L. Brubaker, R. Dale, L.N. Tiller, and H.S. Mumby. 2019. Elephants have a nose for quantity. *Proceedings of the National Acadamey of Sciences*: 1–6.

Rastegar-Pouyani, N., F. Amiri, B. Fathinia, F. Todehdehghan, and E. Rastegar-Pouyani. 2015. Avian deception using an elaborate caudal lure in *Pseudocerastes urarachnoides* (Serpentes: Viperidae). *Amphibia Reptilia* 36, no. 3: 223–231.

Resetarits, W.J., T.M. Breech, J.R. Bohenek, and M.R. Pintar. 2022. Cue reduction or general cue masking do not underlie generalized chemical camouflage in pirate perch. *Ecology*: 1–14.

Rizvanovic, A., M. Amundin, and M. Laska. 2013. Olfactory discrimination ability of Asian elephants (*Elephas maximus*) for structurally related odorants. *Chemical Senses* 38: 107–118.

Rödel, M-O., C. Brede, M. Hirschfeld, T. Schmitt, P. Favreau, R. Stöcklin, C. Wunder, and D. Mebs. 2013. Chemical camouflage – A frog's strategy to co-exist with aggressive ants. *Plos One* 8, no. 12: 1–7.

Ruiz-Rodriguez, M., E. Valdivia, J.J. Soler, M. Martin-Vivaldi, A.M. Martín-Platero, and M. Martínez-Bueno. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology* 212, no. 22: 3621–3626.

Ruxton, G.D. 2009. Non-visual crypsis: a review of the empirical evidence for camouflage to senses other than vision. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 364: 549–557.

Secor, S.M., and J. Diamond. 1998. A vertebrate model of extreme physiological regulation. *Nature* 395, no. 6703: 659–662.

Soler, J.J., M. Martín-Vivaldi, M. Ruiz-Rodriguez, E. Valdivia, A.M. Martín-Platero, M. Martínez-Bueno, J.M. Perlata-Sánchez, and M. Méndez. 2008. Symbiotic association between hoopoes and antibiotic-producing bacteria that line in their uropygial gland. *Functional Ecology* 22: 864–871.

Steiger, S., T. Schmitt, and H.M. Schaefer. 2011. The origin and dynamic evolution of chemical information transfer. *Proceedings of the Royal Society B: Biological Sciences* 278, no. 1708: 970–979.

Stevens, M., and S. Merilaita. 2009. Animal Camouflage Mechanisms and Function. Cambridge University Press.

Stuginski, D.R., C.A. Navas, F.C. de Barros, A. Camacho, J.E.P.W. Bicudo, K.F. Grego, and J.E. de Carvalho. 2018. Phylogenetic Analysis of Standard Metabolic Rate of Snakes: A New Proposal for the Understanding of Interspecific Variation in Feeding Behavior. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 188, no. 2: 315–323.

van Hoek, M.L. 2014. Antimicrobial Peptides in Reptiles. *Pharmaceuticals* 7, no. 6: 723–753.

Vermeij, G.J. 1994. The evolutionary interaction among species: selection, escalation, and coevolution. *Annual Review of Ecology and Systematics* Vol. 25: 219–236.

Walke, J.B., M.H. Becker, S. C. Loftus, L.L. House, G. Cormier, R.V., and L.K. Belden. 2014. Amphibian skin may select for rare environmental microbes. *The ISME Journal* 8: 2207 – 2217.

Wyatt, T.D. 2003. *Pheromones and Animal Behaviour : Communication by Smell and Taste*. Cambridge University Press.

Yohe, L.R., and P. Brand. 2018. Evolutionary ecology of chemosensation and its role in sensory drive. *Current Zoology* 64, no. 4: 525–533.

CHAPTER 2: THE ECOLOGICAL EXTENT OF THE PUFF ADDER'S (*BITIS ARIETANS*) CHEMICAL CRYPSIS

2.1. ABSTRACT

Crypsis is achieved when the receiver fails to detect and discriminate a cue from background noise. This is usually a result of limitations within the receiver, be it behavioural, anatomical, physiological, genetic, or a combination of these factors. Puff adders (*Bitis arietans*) have previously been shown to be chemically cryptic to at least two known predator species – dogs (*Canis lupus familiaris*) and meerkats (*Suricata suricatta*). Their inability to detect these snakes may be driven by limitations within their respective olfactory and vomeronasal systems, or due to puff adders being chemically cryptic. To further examine the extent of the puff adder's chemical crypsis, we used two additional, stand-out biosensors – the snouted cobra (*Naja annulifera*) and the African elephant (*Loxodonta africana*) – to assess whether one of the top puff adder predators and a mammal with the highest known number of intact olfactory receptor genes respectively are able to detect puff adders via airbone chemical cues. For elephants, scent-matching protocols were used and for cobras, Y-maze tests were conducted. Despite vast differences in these biosensors' olfactory and vomeronasal systems, both cobras and elephants failed to detect these snakes but demonstrated no difficulty in detecting controls. The chemically cryptic status of puff adders is thus supported, challenging long-standing dogma that this form of chemical crypsis cannot be achieved by any living organism due to associated odorous, metabolic by-products. This remains the only demonstrated example of chemical crypsis among terrestrial vertebrates but is unlikely to be unique. Rather, c may be commonplace, especially among species that share similar life histories in areas where risk from olfactory-orientated predators is high. By considering its potential in other biological systems, we may fundamentally change our understanding of chemical subterfuge among predator and prey species and cast new light onto the landscape of chemical ecology.

2.2. INTRODUCTION

The puff adder (*Bitis arietans*) is a widespread, highly camouflaged, pan-African species of adder (Phelps 2010). Not only are puff adders visually cryptic, they are also chemically cryptic to at least two species of natural predator (Miller et al. 2015b). This ability to remain hidden from macrosmatic, scent-orientated predators is remarkable, but despite this, puff adders experience low annual survival rates. In some years, close to 50% of the adult population is removed by predators (Alexander and Maritz 2011) compared to an average of ~30% across all snake families (n_{spp} = 48) and 33% within the Viperidae (n_{spp} = 6; King et al. 2018). The majority of puff adder deaths can be attributed to predation and within the puff adder's swathe of > 40 predator species (Miller et al. 2015a), one of the most impactful are the cobras (Maritz et al. 2019). As a group, cobras consume snakes on a regular basis, accounting for up to 43% of detectable prey species within their diet. Of the six species of *Naja* Maritz et al. (2019) evaluated, three stood out for showing a particular preference for puff adders: *Naja nivea* (cape cobra), *Naja nigricinta* (zebra spitting cobra), and *Naja annulifera* (snouted cobra); comprising of 33, 42 and 43% of the snake meals consumed respectively. In a three-year study of a population of puff adders in Dinokeng, South Africa, *N. annulifera* were singlehandedly responsible for at least 50%

of the deaths, totalling around 25% of the adult population annually (Graham Alexander, pers. obs. 2020). Given that puff adders have been shown to be chemically cryptic to mammalian predators such as dogs and meerkats (Miller et al. 2015a) and that cobras appear adept at finding these snakes, it is possible that cobras can detect these adders chemically due to the sensitive reptilian olfactory and vomeronasal systems.

As in all tetrapods, the vomeronasal system (VNS) of mammals and reptiles is discrete from the olfactory system (Eisthen and Polese 2007; Eisthen 2002,1997) and comprises a vomeronasal (VNO) or Jacobson's organ and an accessory olfactory bulb. The VNS has been implicated in the detection of molecules with high molecular weight (Baxi et al. 2006) and is involved in various functions such as prey detection and discrimination (Halpern and Martinez-Macros 2003) and homo- and heterospecific scent trailing (Halpern 1992; Schwenk 1995). Although the components of the olfactory and vomeronasal systems are common to all the tetrapods (see Eisthen and Polese 2007 for a review), anatomical and physiological distinctions exist between mammalian and reptilian lineages. These include (but are not limited to) morphological differences in olfactory receptor cells (Eisthen 1992), the types of receptors (Brykczynska et al. 2013), variability of G-proteins associated to receptor sites (Luo et al. 1994) and in particular, differences in the physical connectivity of the VNS to respiratory airways (Eisthen and Polese 2007). In squamates, uniquely, the VNS is excluded from the nasal cavity, and instead, opens directly into the oral cavity, and in snakes and some lizard species, chemicals collected via tongue-flick are directly deposited onto the surrounding VNO epithelium (Graves and Halpern 1989;Schwenk 1994) facilitating environmental surveying that can have extreme fitness benefits. It is therefore not surprising that snakes not only have very large VNOs (Dawley 1998), but that their VNOs are the most developed among all vertebrate lineages (Brykczynska et al. 2013).

Snake behaviour is more greatly influenced by their VNS than by their olfactory system (Gabe and Saint Girons 1976). Indeed their highly developed VNO contains more sensory neurons than their olfactory epithelium (Gabe and Saint Girons 1976) which, in contrast to mammals', is predominated by water-soluble chemical receptors (V2Rs) and not airborne ones (V1Rs; Brykczynska et al. 2013). The high prevalence of VR2s within their VNO is not surprising given the highly specialized behaviour of tongue flicking common to all snake and some lizard species. This behaviour facilitates the collection of volatile and semi-volatile molecules (Baxi et al. 2006) from the environment for direct delivery to VNO epithelium within the palate (Graves and Halpern 1989;Schwenk 1994). Tongue flicking across odour plumes allows for sequential comparison of odour intensity (known as klinotaxis), but when the tongue is forked (which is true for all snakes), a simultaneous two-point comparison within the same plume (known as tropotaxis; Schwenk 1994) can be achieved. A snake's tropotaxic ability results in highly effective scent trailing (Burghardt 1970; Mason 1992), especially among active foragers such as

cobras (Schwenk, 1994). As such, snouted cobras are likely to be prime biosensor candidates with which to assess the extent of the puff adder's chemical crypsis.

Successful odour detection is reliant on not only effective odour collection and delivery mechanisms, as mentioned above, but also on the presence of the appropriate, associated receptors. The diversity and abundance of these receptors are genetically determined and are species-specific (Niimura et al. 2014). Few studies have investigated the olfactory and vomeronasal genes of snakes (e.g., Kishida and Hikida 2010; Dehara et al. 2012; Kishida and Hikida 2010) and only one has investigated a cobra species – the monocled cobra (*Naja kaouthia*; Kishida and Hikida 2010). In comparison, mammals have been widely researched (e.g., Vanderhaeghen et al. 1997; Niimura and Nei 2007; Rouquier and Giorgi 2007; Niimura et al. 2014), and African elephants (*Loxodonta africana*) possess the highest known number of olfactory receptor genes (~2000) of any mammal tested to date (Niimura et al. 2014). This is more than five times the number found in humans (~400), more than double the number found in dogs (~811), ~750 more than in rats (~1200; Niimura et al. 2014) and 100 times the number found in the monocled cobra (20 OR genes). Though the relationship between the number of olfactory receptor genes, olfactory receptors, and the repertoire of detectable odour molecules is not linear (Malnic et al. 1999), the level of olfactory acuity among both African and Asian elephants (*Elaphas maximus*) demonstrated thus far (Rizvanovic et al. 2013; Bates et al. 2007; Plotnik et al. 2019) is suggestive of a superior olfactory ability that likely exceeds that of other species (Miller et al. 2015a), including that of dogs and meerkats. Indeed, African elephants outperform dogs (Gazit et al. 2005) on trinitrotoluene (TNT) detection tasks with regard to specificity (99.7% versus 93.7%), and if given the identical task of finding puff adders using olfaction, could demonstrate some level of odour recognition not shown by Miller et al.'s (2015a) dogs or meerkats. Such a result would indicate that the puff adder's chemical crypsis is not a reflection of true scentlessness, but rather factors involved in the receiver's detection capacity.

In an effort to better understand the extent of the puff adder's chemical crypsis, using species-appropriate methods, both snouted cobras and African elephants were enlisted as biosensors. Potential detection by snouted cobras was assessed using standard Y-frame trials where several metrics (i.e., latency, time-to-decision, tongue-flick rate, and accuracy) were made. Proof of concept was demonstrated using fresh puff adder shed skins as the odour source, since these skins are easily detectable (Miller et al. 2015b). African elephants were asked to scent-match puff adder scent proxies within a line-up of controls and blanks, and their overall performance was assessed using two known biosensor metrics, sensitivity and specificity. Proof of concept for the elephant trials was established

through the use of brown house snake (*Boaedon capensis*) scent proxies. Data collected from both species were assessed against random and their respective within-trial controls.

2.3. MATERIALS AND METHODS

2.3.1. *Animal husbandry*

Puff adders and snouted cobras were maintained in single-species, temperature-controlled rooms (maintained at 28 °C), as to avoid odour desensitization or acclimation. Snakes were individually housed in glass-fronted terraria that were lined with newspaper and fitted with a hide, water dish and rocks. Water was provided *ad libitum*. Snakes were maintained on a diet of dead, purpose-bred laboratory mice, which were provided on a fortnightly basis. All scent samples were taken from snakes that had last fed at least five days prior to odour collection as to reduce the possibility of odour contamination. Puff adders were lab-borne to wild mothers and at the age of one year did not differ in chemical crypsis from their wild counterparts (Miller et al. 2015b); only puff adders older than one year were used in trials. Snouted cobras were wild caught across Gauteng, South Africa and their prior exposure to puff adders was thus unknown. Cobras were subjected to Y-mazed trials within a month of capture. Food was withheld for at least five days prior to Y-maze trials to ensure that cobras would be interested in feeding. Adventures With Elephants fulfilled all husbandry requirements for the elephants as outlined in The National Norms and Standards for the Management of Elephants in South Africa (GN30833 of 2008) in terms of section 9 of the National Environmental Management: Biodiversity Act, 2004 (Act No. 10 of 2004).

2.3.2. *Snouted Cobra Trials*

Snouted cobra (*n* = 7) responses to puff adder and puff adder shed skin were individually tested using an airflow-assisted Y-maze (Fig. 2.1; adapted from Lillywhite et al. 2015) Y-frame design, originally based on Quadango and Banks 1970). White, acrylic sheeting formed a rounded crawlway trough (large enough for all experimental snakes) throughout the base of the maze to prevent snakes from demonstrating an unintentional side-bias, and parallel ethylene-vinyl acetate (EVA) ridges for improved grip were added along the sheeting using a standard glue gun. A clear acrylic sheet, cut to precisely fit the shape of the Y, formed a secure lid that sealed the maze. This ensured that odours associated with experiments were held within the maze and that cobras could not escape the maze. Scent samples were placed into darkened, terminal boxes (one per terminal box) and cobras were allowed to individually navigate the self-contained maze at leisure once the trial begun for a period of up to 40 minutes. All maze components were thoroughly washed using bleach (NaClO) and wiped clean using 70% ethanol (C_2H_5OH) for scent-removal purposes between trials. Cobras were placed in the temporarily darkened start box (covered with newspaper) and allowed to settle for 5 minutes

before the start box was uncovered and the perforated screen (A, Fig. 2.1) removed to allow access into the maze, thereby beginning the trial. Trials were recorded using a GoPro Hero 4 Black positioned above the maze, and conducted in the absence of observers in a secure room, maintained at 25 °C. No other snakes aside from those immediately involved in testing were kept within this room at any time. Each cobra (n = 6) was subjected to ten runs per odour trial (live puff adder and fresh puff adder shed skin, not older than 5 days old) and subjected to a maximum of two trials in any one day that were run several hours apart between 08:00 and 18:00. Puff adder shed skin is easily detectable by dogs (Miller et al. 2015) and thus served as a positive control. Shed skin also served as a more appropriate control than a live, active forager such as a brown house snake would have given the propensity of active foragers to move frequently within terminal boxes could have skewed our results. Live puff adders, on the other hand seldom moved in these control boxes. For all trials, live puff adders and shed skins were randomly assigned to terminal boxes (i.e., left of right) and multiple puff adders across both sexes served as scent donors (live – n_{female}: 6; n_{male} 6; shed skin – n_{female}: 2; n_{female}: 4). Unassigned boxes were left empty. Snouted cobra responses to puff adders and their shed skins were assessed in separate trials, thus during any one trial, a cobra would be faced with either a live puff adder in one terminal box and an empty (or blank) terminal box, or an empty terminal box and a freshly shed puff adder skin in the other. Metrics collected via videography were: time-to-start, time-to-decision, tongue-flick rate, and decision. A decision was considered made once the cobra's head passed either of the predetermined points in the respective terminal arms (B; Fig. 2.1). If a cobra did not enter the maze after a period of 40 minutes, the run was considered a 'non-start' (NS) and the snake was returned to its terrarium. Tongue flicks were measured for a period where the snake was continuously visible until such time a decision was made. Tongue flick rate was calculated by dividing the number of tongue flicks recorded per second. Only data from cobras that were able to correctly identify controls (sheds) at rates significantly better than chance were considered during analyses.

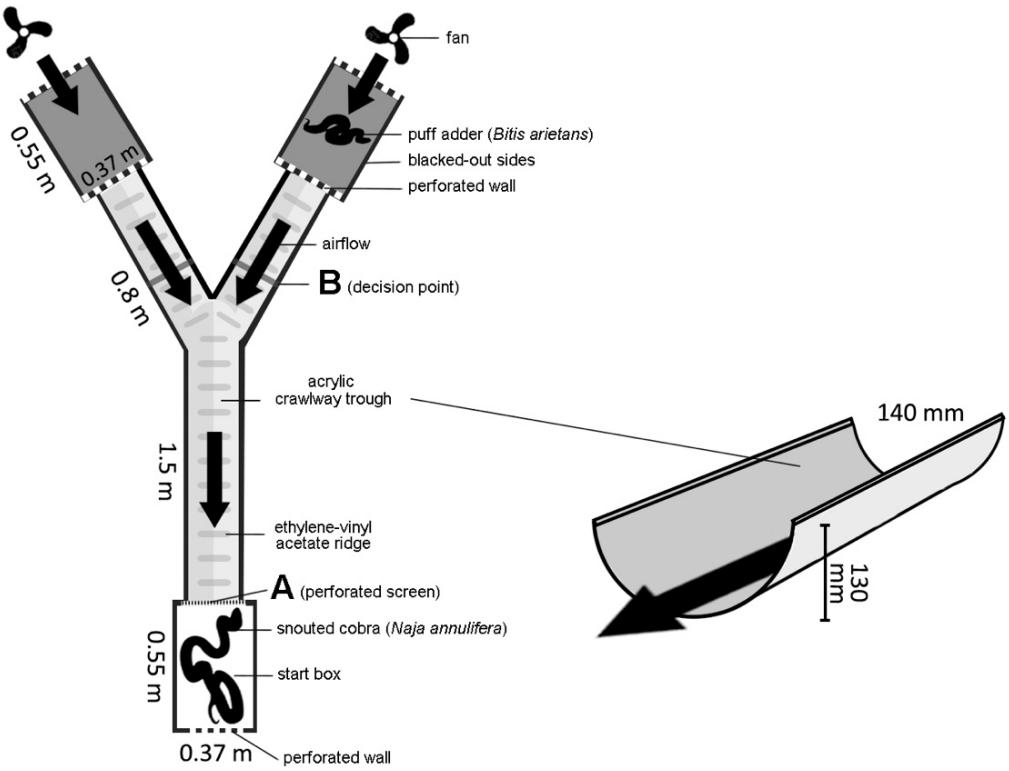


Figure 2.1. Fan-assisted Y-maze design. Arrows indicate the airflow direction, and snake side-bias is offset through a rounded acrylic crawl-way trough. Access to the maze, but not the airflow, was initially restricted via a perforated screen (A) which afforded the test snake a five-minute acclimation period, after which the screen is removed to facilitate entry into the maze. Once the snake's head passed a decision point (B) in either of the terminal arms, a decision was considered made. Insert depicts how terminal boxes for live puff adder trials differed.

2.3.3. *African Elephant Trials*

a. Scent Collection

Cotton scent cloths used in the elephant trials were prepared in batches, using a sequential process (Fig. 2.2). Briefly, cleaned, tumble-dried and unscented cotton cloths (blanks; Miller et al. 2015a) were stored in glass jars until such time as a new batch was required for trials. From this blank repository, half of the cloths were added to a plastic scent-tub, that had been cleaned using bleach (NaClO), then boiling water, and wiped with 90% ethanol (Fig. 2.2; 1). Cloths were left within the sealed scent-tub positioned on a 32 °C heat mat for 6 hours (Fig. 2.2; 2). After 6 hours, half the cloths, now controls, were removed from the scent-tub and stored in a clean glass jar (Fig. 2.2; 3). The scent-donor snake (i.e., a puff adder or a brown house snake) was then added to the scent-tub (Fig. 2.2; 4), and the remaining cloths were placed on top of the snake (5). The tub was again sealed for a further 6 hours at 32 °C (Fig. 2.2; 5), after which all the cloths, now targets, were removed and stored in a dedicated glass jar (7).

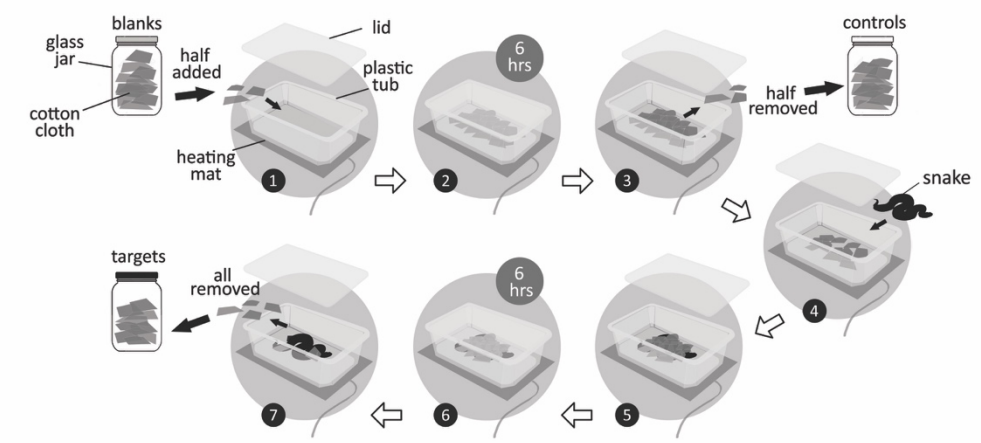


Figure 2.2. The sequential process involved in batch-preparing scent cloths. Half of the blanks (clean, unscented cotton cloths) are added to cleaned scent-tub positioned on a heating mat (32 °C; 1) and sealed for a period of 6 hours (2). Thereafter, half of these cloths, now controls, are removed and stored in a dedicated glass jar (3). To the remainder, a snake scent-donor is added to the tub (4), the cloths repositioned such that they are in direct contact with the snake (5), and the tub sealed for a further 6 hours (6). Finally, all the cloths, now targets, are stored in another dedicated glass jar (7).

b. Scent Matching Protocol

Two previously-trained African elephants (Mussina: 17 yo female; Chova: 23 yo male) owned by Adventures With Elephants in Bela Bela, South Africa, were used to test two snake odours (puff adder and brown house snake) using a scent-matching design. These elephants were selected specifically due to their previous scent-matching experience and all trials were performed outside at Adventures with Elephants, Bela-Bela, South Africa. All trials were double blind. Elephants were rewarded for correctly identifying and indicating target odours using two food rewards – BOSKOS^{TM} (WESenterprises PTY Ltd, Thabazimbi, South Africa) and diced oranges in the necessary amounts required to keep the elephants motivated. To start a trial, an elephant would be instructed to position itself on the start mat, where it would be exposed to the target odour via a reference scent canister loaded with a target cloth (Fig. 2.3.). At this stage, the target odour was reinforced by allowing the elephant to sniff the reference canister three times (food-rewarded after each sniff; supporting information, video 1) and immediately instructed to commence the trial by walking the line-up and sniffing each of the five cannisters. A match was indicated by means of a salute (Fig. 2.3; B) and non-matches were walked passed. Only correct indications were rewarded. At the end of each line-up, the elephant would position itself on the end mat (Fig. 2.3; C) where it would be rewarded for doing so. The process would be repeated once the entire set of canisters were replaced with a clean set; positions for target, control, and blank canisters were randomised between setups. The stainless steel cannisters were thoroughly washed with bleach (NaClO), rinsed with water, sprayed with 90% ethanol (C_2H_5OH) and allowed to air dry between trials and all handling of canisters were performed using nitrile gloves. Fifty trials each of two target scents (puff adder and brown house snake) and the appropriate controls were conducted. Throughout trials, zero-target (where no targets were present, i.e., control, blank, blank, control, blank), as well as double-target line-ups (i.e., blank, control, target,

target, blank) were included to reduce predictability and reinforce 'honest' indicating. For each target snake species, several individuals representing both sexes (n_{pa} = 10; n_{bhs} = 8) served as scent donors. During any one line-up, only species-specific target and control cloths were used.

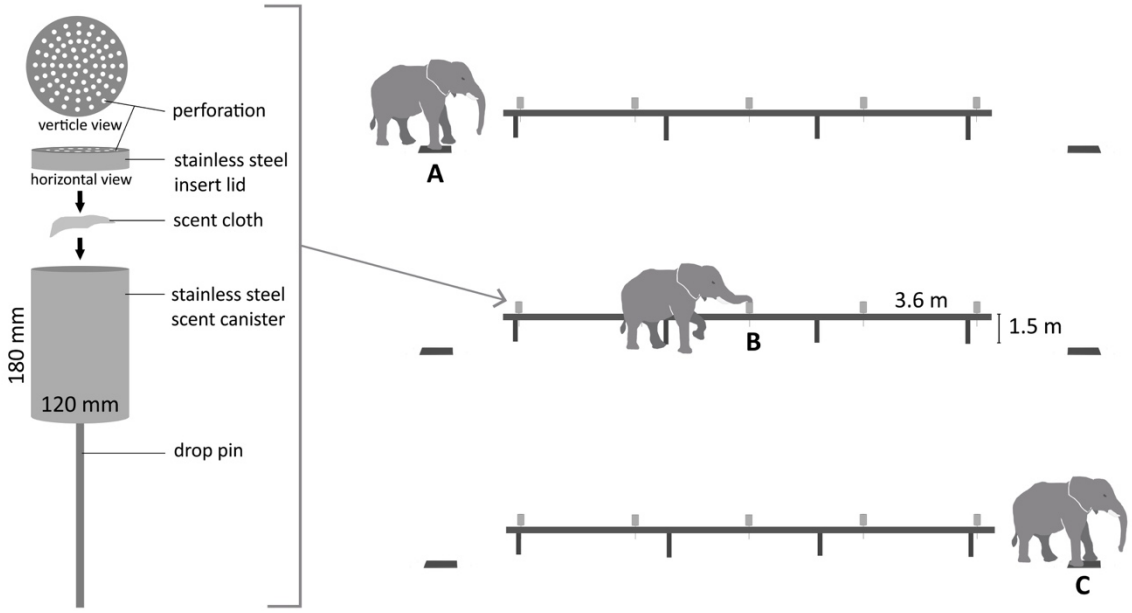


Figure 2.3. Scent canister components (left) and an example of successive stages of the scent-matching line-up (right). The scent-matching elephant was presented with the reference odour at the start mat (A) which was reinforced with the reward of pellets after each sniff. After three sniffs, the elephant walked down the line-up, sniffing each canister consecutively (right). A match was indicated via a 'salute' posture (B), and a non-match was moved passed. If the salute was correct, the elephant was rewarded with oranges at the indicated canister after which he/she continued down the line-up. The run was concluded when the elephant made contact with the end mat (C) where it was rewarded with oranges.

c. Elephant Detection Accuracy

Elephant accuracy was assessed using two standard detector metrics, sensitivity (Eq. 1) and specificity (Eq. 2; Helton 2009), which together are useful in differentiating between good and bad biosensors and simultaneously accommodates variations in the number of targets (i.e., 0 – 2) present within a given line-up. A biosensor that indicates *target* accurately and repeatedly may also indicate *non-targets* at high rates, making it a biosensor with high sensitivity but low specificity. A biosensor may also however only indicate *targets* but at a low frequency, making it a highly specific biosensor with low sensitivity. Good biosensors are therefore ones that have both high sensitivity and specificity scores, reflecting a detector that reliably indicates *target* odours when they are present and no other (Helton 2009; *sensu* Miller 2015b).

$$\text{Sensitivity} = \frac{\text{true positives}}{\text{true positives + false negatives}} \quad (1)$$

$$\text{Specificity} = \frac{\text{true negatives}}{\text{all false alerts + true negatives}} \quad (2)$$

2.3.4. *Statistical Analyses*

a. Snouted Cobra Trials

Cobra biosensor performance, based on their ability to detect puff adder shed skin (i.e., the positive control) was assessed using χ^2 (goodness of fit). This allowed us to disqualify any cobra that did not demonstrate a clear ability to detect the positive control. *Non-start* events were considered informative, since cobras that failed to start live puff adder mazes would participate in shed skin mazes immediately after. Thus, time-to-event analyses (i.e., Kaplan-Meier) were conducted where *non-starts* were coded as an incomplete response. Tongue flick rates between trials (live puff adder vs shed skin) were assessed using repeated-measure ANOVAs, coded for individual, and significant differences between trials were assessed using Tukey HSD post-hoc tests. All statistical analyses were performed using STATISTICA v. 8 (STATISTICA Data Analysis Software System, http://www.statsoft.com, 2001).

b. African Elephant Trials

Elephant indication rates for targets and controls were assessed against chance using χ^2 and differences in biosensor performance metrics were assessed using independent t-tests. All statistical analyses were performed using STATISTICA v. 8 (STATISTICA Data Analysis Software System, http://www.statsoft.com, 2001).

2.4. RESULTS

2.4.1. *Snouted Cobras Trials*

One cobra failed to demonstrate an ability to detect the positive control (shed skin was correctly selected only 55.6% of the time). This individual also showed a strong side bias, hence the name

Zoolander, and was thus excluded from the trials. The remaining five cobras were able to detect puff adder shed skin and chose the side of the maze with the skin at rates greater than chance (accuracy: 77.6% ± 8.3; χ^2 = 105.39, *df* = 5, *p* < 0.001; Fig. 2.4) but none were able to detect live puff adders (accuracy: 47.0% ± 9.4; χ^2 = 1.81, *df* = 3, *p* = 0.61; Fig. 2.4). On average, cobras took longer to enter the maze, if at all, when live puff adders were present than they did when presented with puff adder shed skin (510 seconds ± 538 vs 460 seconds ± 407 respectively) but these differences were not significant. When cobras did enter the maze, they made decisions relatively quickly (puff adder: 211 seconds ± 323; shed: 296 seconds ± 402). Latency and time to decision measures differed significantly between trials only for individuals that were frequent *non-starters* (Fig. 2.5 and 2.6, Table 1). Tongue-flick rates did not differ between trials (shed skin: 1.51 ± 0.36 flicks.sec^{-1}; live puff adder: 1.50 ± 0.31 flicks.sec^{-1}; Fig. 2.7).

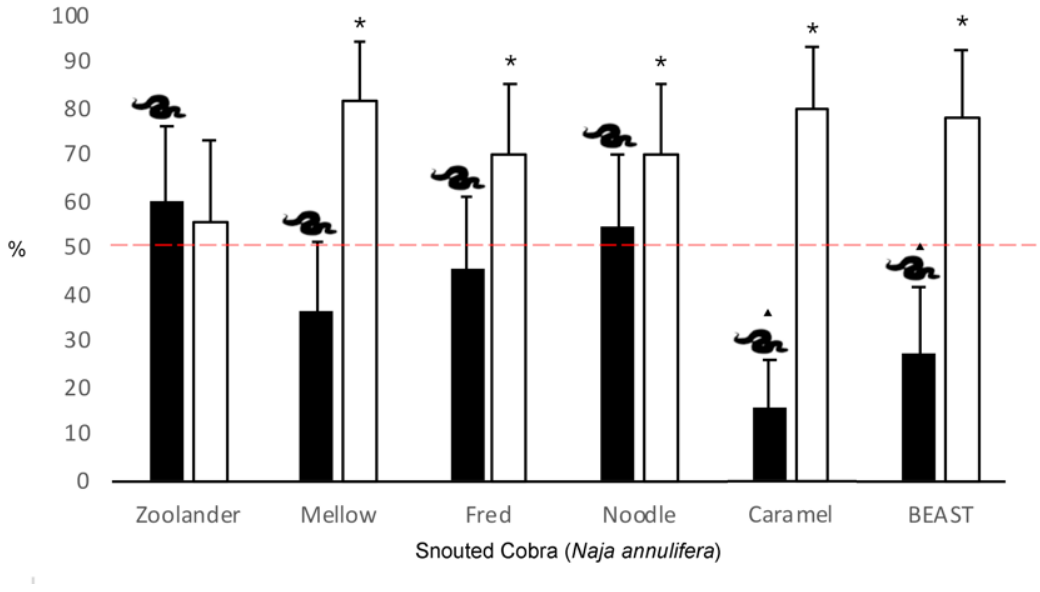


Figure 2.4. The percentage of correct choices made by individual snouted cobras (*Naja annulifera*). Black bars indicate live puff adder (*Bitis arietans*) trials and white, puff adder shed skin trials (positive control). Asterisks indicate trails that positively differ from chance (dashed red line) and triangles those trials that had to be excluded from χ^2 analyses due to high numbers of *non-start* events (n_{BEAST} = 7 ; $n_{Caramel}$ = 5). Zoolander's inability to reliably identify a positive control (55.6% accuracy) and his strong side bias resulted in the exclusion of his data from further analyses.

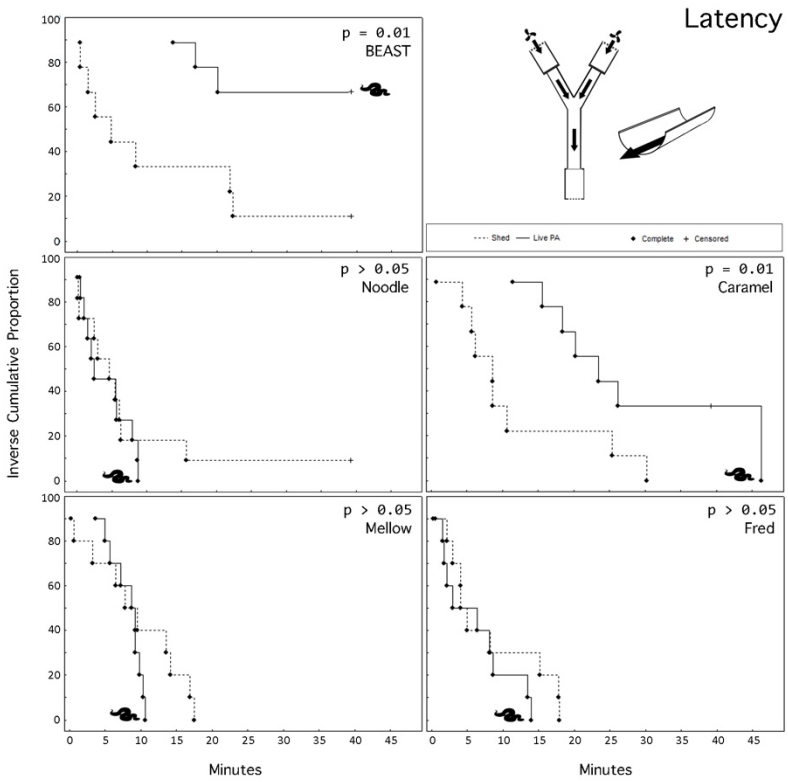


Figure 2.5. Kaplan-Meier curves comparing the time taken for snouted cobras (*Naja annulifera*) to start Y-mazes (i.e., Latency) in live puff adder (*Bitis arietans*; solid line) and puff adder shed skin (dashed line). Complete measures (♦) indicate trials where cobras ran the maze. Censored measures (+) indicate trials where non-starts occurred (i.e., BEAST only ran 30% of her live puff adder trials). Inverse cumulative proportions indicates the percentage of trials that at that point in time, the event had not yet occurred. For example, at five minutes, Fred has not yet entered the maze during puff adder shed skin trials 40% of the time, but had 100% of the time by the 18th minute.

Table 2.4. Average Y-maze time measures for latency and decision by snouted cobras (*Naja annulifera*) when exposed live puff adders (*Bitis* arietans) and puff adder shed skin trials, assessed using Time-to-event analyses. Asterisks indicate significance.

Snouted cobra (*Naja annulifera*)	**Trial** (total; *non-starts*)	**Latency** (seconds ± Std dev.)	**Latency** Gehan's Wilcoxon (*w*, *p*)	**Decision** (seconds ± Std dev.)	**Decision** Gehan's Wilcoxon (*w*, *p*)
Mellow ♀ Adult	Shed skin (10; 0)	550 ± 392	-8.00, 0.79	165 ± 105	26.00, 0.34
	Live Puff adder (10; 0)	485 ± 149		198 ± 213	
Fred ♂ Sub-adult	Shed skin (10; 0)	476 ± 409	-22.00, 0.43	362 ± 307	30.00, 0.27
	Live Puff adder (10; 0)	117 ± 94		269 ± 533	
Noodle	Shed skin (11; 1)	296 ± 268	-3.00, 0.95	157 ± 204	-19.00, 0.53

♀ Sub-adult	Live Puff adder (11; 0)	271 ± 205		229 ± 237	
Caramel ♀ Adult	Shed skin (9;0)	692 ± 597	59, 0.01*	867 ± 620	-36.00, 0.33
	Live Puff adder (9; 2)	1412 ± 601		321 ± 394	
BEAST ♀ Adult	Shed skin (9; 1)	475 ± 564	57.00, 0.01*	279 ± 334	-70.00, 0.005*
	Live Puff adder (9; 6)	1035 ± 279		317 ± 478	

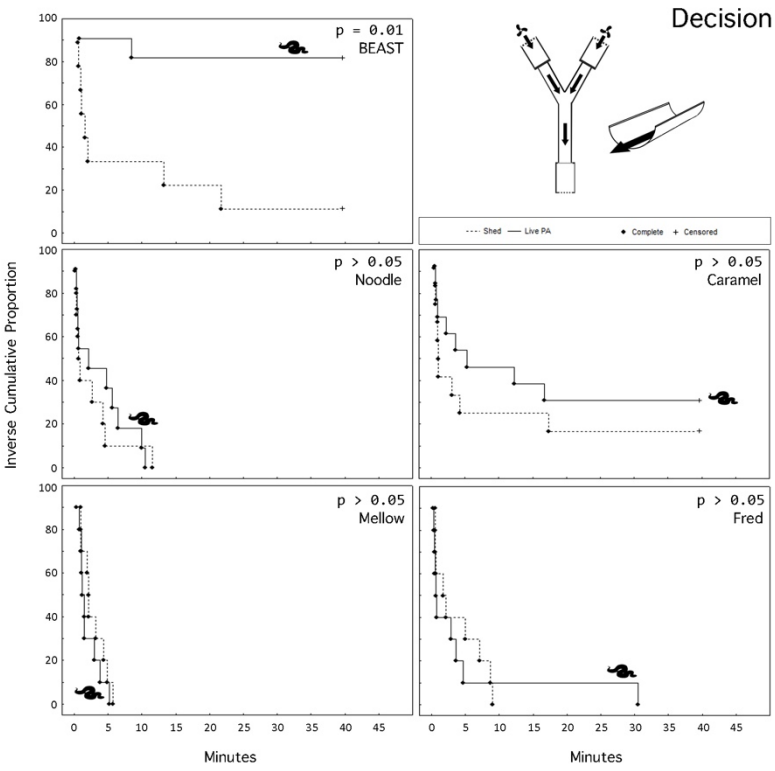


Figure 2.6. Kaplan-Meier curves comparing the time taken for snouted cobras (*Naja annulifera*) to make a decision in live puff adder (*Bitis arietans*; solid line) and puff adder shed skin (dashed line) Y-maze trials. Complete measures (♦) indicate trials where cobras ran the maze. Censored measures (+) indicate trials where *non-starts* occurred (i.e., BEAST made a decision in only 90% of her puff adder shed skin trials). Inverse cumulative proportions indicates the percentage of trials that at that point in time, the event had not yet occurred. For example, Fred made 90% of his decisions in less than 5 minutes, but took more than 30 minutes to make one decision in his live puff adder trials.

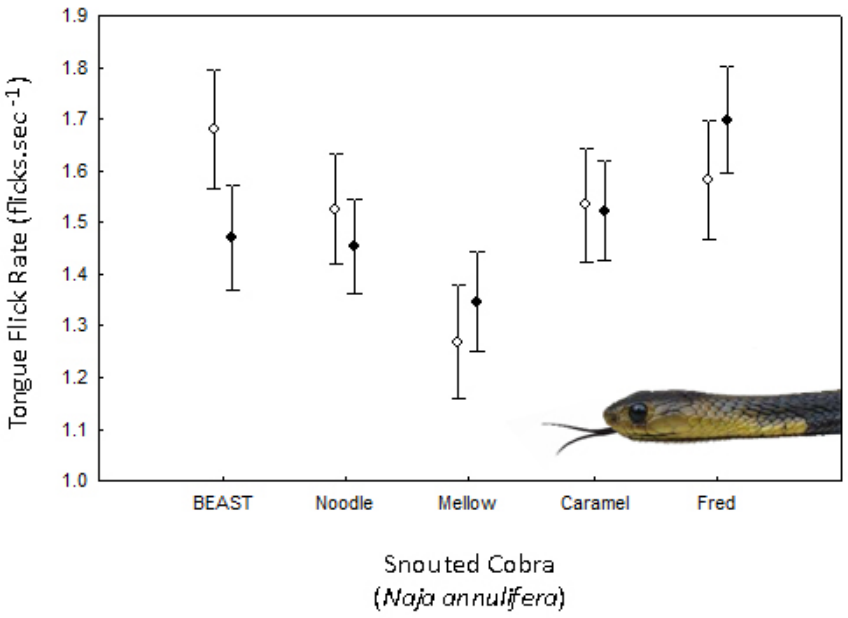


Figure 2.7. Tongue flick rate (flicks.sec.$^{-1}$) for snouted cobras (*Naja annulifera*) exposed to live puff adder (*Bitis arietans*; •) and puff adder shed skin (◦) during Y-maze trials. Error bars represent standard error. Tongue flick rates did not differ between trials for any cobra, shown via Tukey HSD posthoc tests.

2.4.1. *African Elephant Trials*

Elephants were unable to discriminate puff adder targets from their respective controls but could easily do so with respect to brown house snake targets and their associated controls. Although the elephants indicated all 50 puff adder targets, their high rate of indications on the puff adder controls (43 of the 46) demonstrates that to the elephants the targets did not differ from controls in odour, but that target and control cloths were chemically distinguishable from blank cloths since only four of the 154 blanks were indicated (Fig. 2.4). In comparison, brown house snake targets were distinctive from both their controls and blanks since all 50 targets were indicated, but only one control (n_{total} = 50) and one blank (n_{total} = 150). The rates at which both puff adder targets and puff adder controls were indicated were significantly greater than those of chance (targets: χ^2 = 5415, *df* = 54, $p < 0.001$; controls: χ^2 = 4666.67, *df* = 54, $p < 0.001$), meaning that elephants were actively choosing to indicate these (i.e., elephants could not distinguish between control and target). Indications on brown house snake targets were also significantly greater than those expected by chance (χ^2 = 14260, *df* = 45, $p < 0.001$) but indications of brown house snake controls were significantly lower than expected by chance (χ^2 = 958.03, *df* = 45, $p < 0.001$), demonstrating active avoidance (i.e., elephants could distinguish between control and target).

Chova's and Mussina's reliability in correctly indicating all target cloths results in a 100% sensitivity across both snake species, but their specificity (i.e., their tendency to indicate only targets) in trials differed significantly between snake species (brown house snake: 99.2%; puff adder: 77.1%; t_{99} = -11.82, p < 0.001), but not between themselves (brown house snake: Chova: 98.4%; Mussina: 100%; t_{44} = 1.32, p = 0.193; puff adder: Chova: 76.9%; Mussina: 77.3%; t_{53} = 0.105, p = 0917; Fig. 2.4: right). Thus, all comparisons across snake species were done using their pooled data. The overall drop in specificity from 99.2% for brown house snake to 76.0% for puff adder reflects the elephants' continued indications on puff adder control cloths (occurring at 18.4% frequency within the scent cloth population) despite these indications going unrewarded. If, however specificity and sensitivity are recalculated within the framework of puff adders being chemically cryptic, thereby considering indications on controls as honest, specificity during trials increases to 96.0% and sensitivity decreases from 100 to 96.9%, since 6.5% of controls were missed. These adjusted performance metrics are no longer different from those recorded during brown house snake trials (sensitivity: t_{99} = 1.84, p = 0.07; specificity: t_{99} = -1.82; p = 0.07).

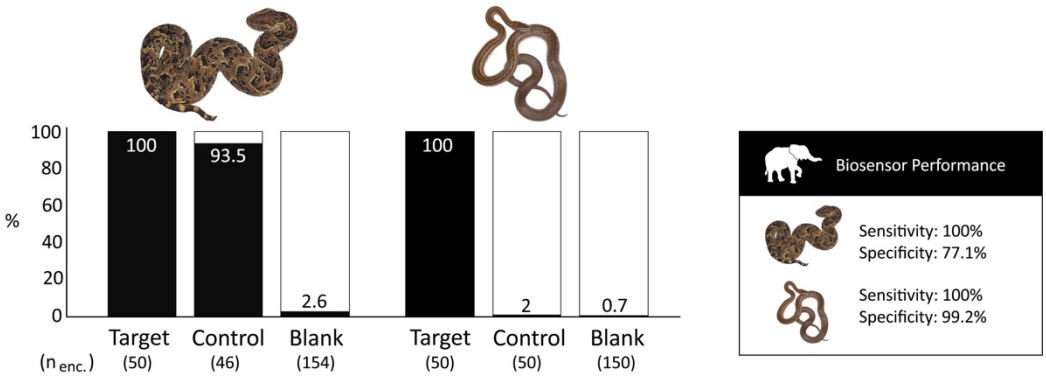


Figure 2.8. Percent of encountered scent cloths (target, control, and blank) indicated by scent-matching African elephants (*Loxodonta africana*) during puff adder (*Bitis arietans*; left) and brown house snake (*Boaedon capensis*; middle) trials. Elephant biosensor performance for each snake species included (right).

2.5. DISCUSSION

Both snouted cobras and African elephants failed to chemically discriminate puff adders and their respective controls (i.e., blanks for cobras and control cloths for elephants), but were easily able to chemically discriminate puff adder shed skin (i.e., the control odour used in the cobra trials) and brown house snakes (i.e., the control snake used in the elephant trials). These results demonstrate that if

there are odours associated with the puff adder, they are difficult to detect by even macrosmatic species such as dogs, meerkats (Miller et al. 2015a), African elephants, and snouted cobras. These findings support the hypothesis that puff adders are chemically cryptic, challenging previously held dogma that no metabolizing organism can achieve chemical crypsis due to associated odorous metabolic byproducts (Conover 2007).

Snouted cobras are generalist active foragers that rely primarily on chemoreception to find food (Cooper 1994). Responsiveness to prey cues, among generalists in particular, can be shaped by previous experience (Fuchs and Burghardt 1971; Arnold 1978;) and in snouted cobras, diet is additionally affected by body size but not by sex (Shine et al., 2007). Despite their unknown prey-exposure history, our wild-caught cobras, ranging in size from subadults to large adults, responded to puff adder shed skin at rates greater than chance, but continually failed to detect live puff adders. Neither individual nor treatment had any effect on toungue flick rates, which were also not significantly different from those measured in actively foraging, free-roaming Cape cobras. This strongly suggests that, irrespective of treatment, our cobras were actively foraging during all mazes they started. Latency and decision times were only significantly different across treatments for individuals who were frequent *non-starters* and eighty percent of *non-start* events were recorded in live puff adder trials. In the absence of prey detection via chemical cue (as would be the case for the cobras when presented with a chemically cryptic puff adder), other snakes showed a lack of motivation to traverse Y-mazes (Water, 1993), and thus we consider these non-starter events to be highly informative however, increasing sample size of Y-maze tests would improve our understanding of these behavioural responses.

Interestingly, it appears that snouted cobras are not the only cobra species to fail at chemically detecting puff adders. Videography of a foraging Mozambique spitting cobra (*Naja mossambica*), another known puff adder predator (Shine et al. 2007), reveals it not responding to the presence of a puff adder despite directly tongue-flicking the site where the puff adder had been only moments before, and despite the adder being only centimetres away during the entire interaction (supporting information, video 2). Even more interestingly, videography of an actively foraging Herald snake (*Crotaphopeltis hotamboeia*) shows the snake approaching a puff adder in ambush (supporting information, video 3). Puff adders are ophiophagus (Branch 1998), but only upon what appears to be direct lingual contact with the adder's body, does the Herald flee rapidly. Whether this contact may have elicited a response due to chemical detection of non-volatiles associated directly with the adder's body or through texture alone, is unknown, but may shed some light as to why tongue flick rates remain unchanged across our treatments.

Patterns of tongue flick rates among snakes vary greatly. Increases have been reported in response to several factors such as prey chemical concentration (Kubie and Halpern 1979), prey preference (Downes 1999; Aubret et al. 2006), proximity and prior prey exposure (Burger et al. 1991), including tactile interactions (Cooper et al. 1989), ontogeny (Saviola et al. 2012), and in response to predator cues (Weldon, 1982). But measurements of this metric have also yielded no differences (Brown and MacLean 1983; Kubie and Halpern 1979), even in species where increases have been detected using the same prey items (Kubie and Halpern 1979). Relative to tongue flick measures of other snake species, snouted cobras' are high (e.g., 1.5 flicks.sec^{-1} versus *Thamnophis elegans* - 0.7 flicks.sec^{-1}, Weldon 1982; *Pituophis melanoleucus* - 1.2 flicks.sec^{-1}, Burger 1988; 0.5 *Crotalus* spp., Chiszar and Radcliffe 1976). The maintenance of high toungue flick rates during foraging activities, even in the absence of prey odour, could be a behaviour shaped specifically by the stimulus ambiguity (Leavell and Bernal 2019) associated with cryptic prey. Since high toungue flick rates would increase the odds of direct-lingual contact with adders (and thus detection), the associated energy expenditure of maintaining high toungue flick rates would most likely be greatly offset by the opportunistic find of such a sizeable meal (i.e., a puff adder). Interestingly, the puff adder's tongue flick rates do not differ from those of actively foraging, free-roaming Cape cobras (*Naja nivea*; 1.54 $\pm$ 0.58; ANOVA: $F_{2,12}$ = 0.02, p = 0.98, Thilo Beck, unpublished data), another impactful puff adder predator (Maritz et al. 2019). This comparison suggests that when the snouted cobras did indeed enter the maze, they were actively foraging. Indeed, the application of Random Walk models (see Codling et al. 2008 for a review) based on telemetry data for both puff adders and snouted cobras could provide insight into whether this foraging method translates into observed predation rates.

Exposure to ophiophagus snake cues can elicit behaviour change, even in naïve snakes (Weldon 1982). Such behaviours may include head-hiding (Carpenter and Gillingham 1973), striking (Klauber 1972), increased toungue flick rates (Weldon 1982), body bridging (Weldon and Burghardt 1979), freezing, and rapid escape behaviour (Marchisin 1980; Weldon 1982), such as that seen in the Herald snake video (supporting information, video 3). Our lab-born, predator-naïve puff adders generally remained motionless during trials, but would quickly transition from this state to one of escape if a cobra repeatedly investigated the puff adder terminal box during the same trial, and in one notable instance, when a cobra defecated near the adder within the maze (supporting information, video 4). Whether this is an innate reaction to an ophiophagus snake, or simply a reaction to an unfamiliar odour (Chiszar et al. 1978) is unknown, but does mirror wild puff adder behaviour of fleeing odorous situations that carry potential risk (Miller et al. 2015a). Such scenarios would include when defecation has occurred, parturition sites, and due to the high detectability of shed skin by predators (e.g., dogs and snouted cobras), sites of ecdysis.

Puff adders are likely to be at increased risk when their chemical crypsis is compromised. Warner (2010) reports two postpartum puff adder predation events by otherwise well-fed Gaboon adders (*Bitis gabonica*), one within hours of parturition and the other, two weeks postpartum. One of these puff adders had until this predation event peacefully cohabited with the Gaboon in question in an outdoor, semi-natural enclosure for more than a year. In further support of Warner's findings, three out of only four recorded predations events on telemetered female puff adders tracked in Dinokeng, South Africa, occurred within two weeks postpartum (Xavier Glaudas, pers comms, 2020). Though Warner (2010) postulates that there may be pheromone involvement, contamination with odorous birthing fluids (see Butler et al. 1995) may compromise mother puff adders' chemical crypsis for periods that extends beyond the actual birthing event. This would represent additional breeding costs for the puff adder (of which there are many; see (Glaudas et al. 2020a,b), that have not previously been considered. Additionally, evidence of increased aggression among puff adders in captivity during confinement with shed skin and/or faeces (van Rensburg, unpublished Honours thesis) also highlights that remaining in close proximity to these odour sources is potentially stress-inducing and should be considered during the husbandry of chemically cryptic species. This observation contrasts previous findings whereby members of various snake families (i.e., colubrids, viperids, pythonids, elapids, and boids) exhibit increased defecation immediately post cage-cleaning, and is viewed as an attempt to promptly re-establish familiar odours (Chizsar et al. 1980; Stow and Shine 1993). Careful consideration of behavioural repertoires such as these for snake species under captive conditions may potentially serve to identify other chemically cryptic species and the value of such observations should not be underestimated.

African elephants are phenomenal scenters (Rizvanovic et al. 2013; Plotnik et al. 2019; Nevo et al. 2020) and problem solvers (Bates et al. 2007), but they were unable to locate puff adders by olfaction. Although there is clearly no predator-prey relationship between African elephants and either of the snake species tested – elephants are herbivores and are too large to be consumed by any snake – but their accurate (99.2% specificity) and reliable (100% sensitivity) detection of brown house snakes demonstrate that this relationship is not a prerequisite for detection. Additionally, during trials, elephants mistook blanks for targets fewer than a handful of times across treatments, despite collectively encountering them more than 300 times. This low indication frequency on blanks overall for both puff adder and brown house snake treatments, together with their high specificity and sensitivity scores for brown house snake odour demonstrate that the elephants clearly understood the task at hand. We therefore consider the application of African elephants as biosensors within this role appropriate.

During training it became evident that when confronted with the problem of finding puff adder "scent", Chova in particular would attempt to problem solve by "*cheating"*. This was achieved by Chova marking target canisters by rubbing his trunk across their tops several times (supporting information, video 5). Mussina would quickly lose interest in performing puff adder trials since reward ratios would drop from 100% to ~50% when targets changed from brown house snakes to puff adders – indications on controls were never rewarded despite these accounting for ~50% of indications during puff adder trials. *Cheating* during training was eventually prevented through access-restriction to cannisters post-reward, but during the testing phase, cannisters were entirely replaced between trials making cheating an impossibility. *Cheating* attempts were never detected during brown house snake training or testing phases. Mussina's interest in working also did not wane during brown house snake trials, and she regained and maintained her interest during puff adder trials only once we increased her reward pay out by an order of magnitude. Given that both the cheating attempts and disinterest occurred during puff adder trials only, in addition to growing support for the puff adder's chemical crypsis, the elephants' adjusted sensitivity (96.9%) and specificity scores (96.0%) for puff adder trials most likely reflect the elephants' true performance parameters.

Encounters between African elephants and puff adders are inevitable given their sympatry. Handlers of the habituated herd that includes the two elephants used in this work report an encounter between one of their females and a puff adder whereby a scuffle ensued, supposedly resulting in the elephant's leg being marginally swollen and irritated the following day (Joseph Kamupambe and David Mupupu, pers. comms 2018). Although several witnesses have confirmed these events, envenomation was not directly observed. Whether puff adder fangs are able to puncture elephant skin is unknown but, in comparison to fangs of other species, are highly effective at penetration (Crofts et al. 2019). Records of snakebite in Asian elephants (*Elephas maximus*) do exist (e.g., Malayan pit viper - *Calloselasma rhodostoma*; Boonsart et al. 2016) and several snakebite fatalities (by unknown spp.) have also been reported (Oo 2012). In African elephants, snakebite has been a suspected cause of death in at least one instance (Geraï et al. 2004) and snake-murder by African elephants has also been witnessed (*Psammophis* spp., Francis Kamupambe pers. comms 2018). It is thus not far-fetched to assume that elephants may view snakes as a threat, perhaps justifiably so, and that an ability to detect snakes, despite being neither prey nor predator, may still be beneficial.

The puff adder's chemical crypsis has been thoroughly tested within an ecological framework and is upheld. Although the mechanism that results in chemically crypsis remains elusive, it appears the puff adder's chemical crypsis is independent of receiver, suggesting an absence of airborne chemical cue, rather than a failing of detection. Further investigation by means of mechanical

assessment are required and approaches that assess associated microbiota (the primary source of body odour: Archie and Theis 2011), metabolic rates and associated volatile production may provide insight into the underlying mechanism. Investigations into the extent of the puff adder's chemical crypsis from the perspective of their prey would also be of value. In predator-prey arms races, selection pressure is more greatly exerted by the action of a species' predators than it is by their prey (Vermeij 1994). Thus, it may be possible that although the puff adder's chemical crypsis is more likely to have been driven by the effects of its predators than of its prey, knock-on selection pressures exerted by the puff on its prey may however have driven the evolution of the chemical receptors necessary for puff adder-detection within these prey. Puff adders currently remain the sole example of a chemically cryptic, terrestrial vertebrate, but anecdotal observations suggest that at least *B. gabonica* shares similar chemical crypsis (see Miller et al. 2015a for commentary). Until such time that the potential for chemical crypsis within ecological settings is more widely considered and appropriately and rigorously tested, our overall view of predator-prey dynamics stands to be greatly disadvantaged.

2.6. REFERENCES

Alexander, G.J., and B. Maritz. 2011. Predation risk in relation to behaviour in *Bitis arietans*: does it pay to keep your head down? 10th Herpetological Association of Africa Conference; Cape town, 11–14 January, 2011.

Archie, E.A., and K.R. Theis. 2011. Animal behaviour meets microbial ecology. *Animal Behaviour* 82: 425–436.

Arnold, S.J. 1978. Some effects of early experience on feeding responses in the common garter snake, *Thamnophis sirtalis*. *Animal Behaviour* 26: 455–462.

Aubret, F., G.M. Burghardt, S. Maumelat, X. Bonnet, and D. Bradshaw. 2006. Feeding preferences in 2 disjunct populations of tiger snakes, *Notechis scutatus* (Elapidae). *Behavioural Ecology* 17: 716–725.

Bates, L.A., K.N. Sayialel, N.W. Njiraini, C.J. Moss, J.H. Poole, and R.W. Byrne. 2007. Elephants classify human ethnic groups by odor and garment color. *Current Biology* 17: 1938–1942.

Baxi, K.N., K.M. Dorries, and H.L. Eisthen. 2006. Is the vomeronasal system really specialized for detecting pheromones ? *Trends in Neuroscience* 29: 1–7.

Boonsart, B., V. Thanapatcharoen, W. Pintaworng, and P. Charoenphan. 2016. Care report: the venomous snakebite treatment in an Asian elephant (*Elaphas maximus*) by the Malayan Pit Viper (*Calloselasma rhodostoma*). *Journal of Kasetsart Veterinarians* 26, no. 2: 111–117 ref. 12.

Branch, W.R. 1998. *Field Guide to Snakes and Other Reptiles of Southern Africa*. Cape Town, Struik Publishers.

Brown, W., and F. MacLean. 1983. Conspecific scent-trailing by newborn timber rattlesnakes, *Crotalus horridus*. *Herpetologica* 39: 430–436.

Brykczynska, U., A.C. Tzika, I. Rodriguez, M.C. Milinkovitch. 2013. Contrasted evolution of the vomeronasal receptor repertoires in mammals and squamate reptiles. *Genome Biology and Evolution* 5: 389–401.

Burger, J. 1989. Following of conspecific and avoidance of predator chemical cues by pine snakes (*Pituophis melanoleucus*). *Journal of Chemical Ecology* 15, no. 3: 799–806.

Burger, J., M. Gochfeld, and B.G. Murray. 1991. Role of a predator's eye size in risk perception by basking black iguana, *Ctenosaura similis*. *Animal Behaviour* 42, 471–476.

Burghardt, G.M. 1970. Chemical perception in reptiles. In: Johnston, J.W., D.G. Moulton, M. Turk, editors. *Communication by Chemical Signals*. New York: Appleton-Century-Crofts. p 241–308.

Burghardt, G.M., and P. Chmura. 1993. Strike-induced chemosensory searching by ingestively naive Gartner Snakes. *Journal of Comparative Psychology* 107, no. 1: 116–121.

Butler, J.A., Hull, T.W. and R. Franz. 1995. Neonate aggregations and maternal attendance of young in the eastern diamondback rattlesnake, *Crotalus adamanteus*. *Copeia*: 196–198.

Carpenter, C.C., and J.C. Gillingham. 1975. Postural responses to king snakes by crotaline snakes. *Herpetologica* 31: 293–302.

Chiszar, D., and C.W. Radcliffe. 1976. Rate of tongue flicking by rattlesnakes during successive stages of feeding on rodent prey. *Bulletin of the Psychonomic Society* 7, no. 5: 485–486.

Chiszar, D., K. Scudder, and L. Knight. 1976. Rate of tongue flicking by garter snakes (*Thamnophis radix haydeni*) and Rattlesnakes (*Crotalus v. viridis, Sistrurus catenatus tergeminus,* and *Sistrurus catenatus edwardsi*) during prolonged exposure to food odors. *Behavioral Biology* 18, no. 2: 273–283.

Chiszar, D., K. Scudder, L. Knight, and H.M. Smith. 1978. Exploratory behavior in prairie rattlesnakes (*Crotalis viridis*) and water moccasins (*Agkistrodon piscivorus*). *Journal of Herpetology* 20: 269–272.

Chiszar, D., S. Wellborn, M.A. Wand, and K.M. Scudder, and H.M. Smith. 1980. Investigatory behavior in snakes, II. Cage cleaning and the induction of defecation in snakes. *Animal Learning and Behaviour* 8, 505–10.

Codling, E.A., M.J. Plank., and S. Benhamou. 2008. Random walk models in biology. *Journal of the Royal Society Interface* 5: 813–834.

Conover, M. 2007. *Predator-Prey Dynamics: The Role of Olfaction*. Boca Raton, FL.: Taylor and Francis

Cooper, W.E. Jr. 1994. Chemical discrimintation by tongue-flicking in lizards: A revew with hypotheses on its origin and its ecological and phylogenetic relationships. *Journal of Chemical Ecology* 20, no. 2: 439–487.

Cooper, W.E. Jr, S.G. Mcdowell, and J. Ruffer. 1989. Strike-induced chemosensory searching in the colubrid snakes *Elaphe g. guttata* and *Thamnophis sirtalis*. *Ethology* 81: 19–28.

Crofts, S.B., Y. Lai, Y. Hu, and P.S.L. Anderson. 2019. How do morphological sharpness measures relate to puncture perfomance in viperid snake fangs? *Biology Letters* 15: 20180905

Dawley, E.M. 1998. Species, sex, and seasonal differences in VNO size. *Microscopy Research and Technique* 41: 506–518.

Dehara, Y., Y. Hashiguchi, K. Matsubara, T. Yanai, M. Kubo, and Y. Kumazawa. 2012. Characterization of squamate olfactory receptor genes and their transcripts by the high-throughput sequencing approach. *Genome Biology and Evolution* 4: 602–616.

Downes, S. 1999. Prey odor influences retreat-site selection by naive broad-headed snakes (*Hoplocephalus bungaroides*). *Journal of Herpetology* 33: 156–159.

Eisthen, H.L. 1992. Phylogeny of the vomeronasal system and of receptor cell types in the olfactory and vomeronasal epithelia of vertebrates. *Microscopy Research and Technique* 23, no. 1:, 1–21.

Eisthen, H.L. 1997. Evolution of vertebrate olfactory systems. *Brain, Behavior and Evolution* 50: 222–233.

Eisthen, H.L. 2002. Why are olfactory systems of different animals so similar? *Brain, Behavior and Evolution*, 59: 273–293.

Eisthen, H.L., and G. Polese. 2007. Evolution of vertebrate olfactory subsystems. In: Kaas JH, Bullock TH, editors. *Evolution of Nervous Systems: A Comprehensive Reference, Vol. 2*. *Nonmammalian vertebrates*. Amsterdam: Academic Press: 355–406.

Fuchs, J.L., and G.M. Burghardt. 1971. Effects of early feeding experience on the responses of garter snakes to food chemicals. *Learning and Motivation* 2: 271-279.

Gabe, M., and H. Saint Girons. 1976. Contribution à la morphologie comparée des fosses nasales et de leurs annexes chez les lépidosoriens. Me´m. Mus. Natn. Hist. Nat. Paris A98, 1–87.

Gazit, I., A. Goldblatt, and J. Terkel. 2005. The role of context specificity in learning: the effects of training context on explosives detection in dogs. *Animal Cognition* 8: 143–150.

Geraï, M.E., R. Slotow, R.D. Carr, and B. Reilly. 2004. Elephant reintroductions to small fenced reserves in South Africa. *Pachyderm* 37: 28–36.

Graves, B.M., and M. Halpern. 1989. Chemical access to the vomeronasal organs of the lizard *Chalcides ocellatus*. *Journal of Experimental Zoology* 249: 150-157.

Glaudas, X., S.E. Rice, R.W. Clark, and G.J. Alexander. 2020a. Male energy reserves, mate-searching activities, and reproductive success: alternative resource use strategies in a presumed capital breeder. *Oecologia* 194: 415–425.

Glaudas X, S.E. Rice, R.W. Clark, and G.J. Alexander. 2020b. The intensity of sexual selection, body size and and reproductive success: alternative resource use strategies in a presumed capital breeder. *Oikos* 129 no. 7: 998–1011.

Halpern, M. 1992. Nasal chemical senses in reptiles: strcuture and fucntion. In: Gans C, Crews D, editors. *Hormones, Brain and Behavior, Biology of the Reptili*a, Vol. 18. Chicago: University of Chicago Press. p. 423–523.

Halpern, M., and A. Martinez-Marcos. 2003. Structure and function of the vomeronasal system: an update. *Progress in Neurobiology* 70: 245–318.

Helton, W.S. 2009. *Canine Ergonomics: The Science of Working Dogs*. CRC Press.

King, R.B., K.M. Stanford, and P.C. Jones. 2018. Sunning themselves in heaps, knots, and snarls: The extraordinary abundance and demography of island watersnakes. *Ecology and Evolution* 8: 7500–7521.

Kishida, T., and T. Hikida. 2010. Degeneration patterns of the olfactory receptor genes in sea snakes. *Journal of Evolutionary Biology* 23: 302–310.

Klauber, L.M. 1972. *Rattlesnakes, Their Habits, Life Histories and Influence on Mankind,* Vol. 2. University of California Press.

Kubie, J.L., and M. Halpern. 1979. Chemical senses involved in garter snake prey trailing. *Journal of Comparative and Physiological Psychology* 93, no. 4: 648–667.

Leavell, B.C., and X.E. Bernal. 2019. The cognitive ecology of stimulus ambiguity : a predator – prey perspective. *Trends in Ecology and Evolution*: 1–13.

Lilywhite, H.B., J.B., Pfaller, and C.M. Sheehy III.Feeding preferences and responses to prey in insular neonatal Florida cottonmouth snakes. *Journal of Zoology* 297, no. 2: 156–163.

Luo, Y., S. Lu, P. Chen, D. Wang, D., and M. Halpern. 1994. Identification of chemoattractant receptors and G-proteins in the vomeronasal system of garter snakes. Journal of Biological Chemistry 269: 16867–16877.

Malnic, B., J. Hirono, T. Sato, and L.B. Buck. 1999. Combinatorial Receptor Codes for Odors. *Cell* 96: 713–723.

Marchisin, A. 1980. Predator-prey interactions between snake-eating snakes. Unpublished PhD dissertation, Rutgers University, New Brunswick, New Jersey.

Maritz, B., G.J. Alexander, and R.A. Maritz. 2019. The underappreciated extent of cannibalism and ophiophagy in African cobras. *Ecology* 100, no. 2: e02522.

Mason, R.T. 1992. Reptilian pheromones. In: Gans C, Crews D, editors. *Biology Of The Reptilia: Hormones, Brain, And Behavior*. Chicago, University of Chicago Press. p. 114–128.

Miller, A.K., M.C. Hensman, S. Hensman, K. Schultz, P. Reid, M. Shore, J. Brown, K.G. Furton, and S. Lee. 2015a. African elephants (*Loxodonta africana*) can detect TNT using olfaction: Implications for biosensor application. *Applied Animal Behaviour Science* 171: 177–183.

Miller, A.K., B. Maritz, S. McKay, X. Glaudas, G.J. Alexander. 2015b. An ambusher's arsenal: Chemical crypsis in the puff adder (*Bitis arietans*). *Proceedings of the Royal Society B, Biological Sciences*, 282, no. 1821.

Nevo, O., M.H. Schmitt, M. Ayasse, and K. Valenta. 2020. Sweet tooth: Elephants detect fruit sugar levels based on scent alone. *Ecology and Evolution* 10, no. 20: 11399–11407.

Niimura, Y., A. Matsui, and K. Touhara. 2014. Extreme expansion of the olfactory receptor gene repertoire in African elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome Research* 24: 1485–1496.

Niimura, Y., and M. Nei. 2007. Extensive gains and losses of olfactory receptor genes in mammalian evolution. *Plos One* 2, no. 8: e708.

Oo, Z.M. 2012. Health Issues of Captie Asain Elephants in Myanmar. *Gajah* 36: 21–22.

Phelps T. 2010. Old World Vipers, a natural history of the azemiopinae and viperinare. Frankfurt, Edition Chimaira.

Plotnik, J.M., D.L. Brubaker, R. Dale, R.N. Tiller, and H.S. Mumby. 2019. Elephants have a nose for quantity. *Proceedings of the National Academy of Sciences of the United States of America* 116, no. 25: 12566–12571.

Rizvanovic, A., M. Amundin, and M. Laska. 2013. Olfactory discrimination ability of asian elephants (*elephas maximus*) for structurally related odorants. *Chemical Senses* 38, no. 2: 107–118.

Rouquier, S., and D. Giorgi. 2007. Olfactory receptor gene repertoires in mammals. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 616: 95–102.

Saviola, A.J., D. Chiszar, and S.P. Mackessy. 2012. Ontogenetic shift in response to prey-derived chemical cues in prairie rattlesnakes *Crotalus viridis viridis*. *Current Zoology* 58: 549–555.

Schwenk, K. 1994. Why snakes have forked tongues. *Science* 263, no. 5153: 1573–1577.

Schwenk, K. 1995. Of tongues and noses: chemoreception in lizards and snakes. *Trends in Ecology and Evolution* 10: 7–12.

Shine, R., W.R. Branch, J.K. Webb, P.S. Harlow, T. Shine, and J.S. Keogh. 2007. Ecology of cobras from southern Africa. *Journal of Zoology* 272: 183–193.

Stow, A., and R. Shine. 1993. Cage cleaning, investigatory behaviour and the induction of defecation in elapid snakes. *Herpetology in Australia*: 311–314.

Vanderhaeghen, P., S. Schurmans, G. Vassart, and M. Parmentier. 1997. Specific repertoire of olfactory receptor genes in the male germ cells of several mammalian species. *Genomics* 39: 239–246.

Vermeij, G.J. 1994. The evolutionary interaction among species: selection, escalation, and coevolution. *Annual Review of Ecology and Systematics* Vol. 25: 219–236.

Warner, J.K., and K.J., Kyle. 2010. *Bitis gabonica gabonica* (Duméril, Bibron, and Duméril, 1854) Diet. *African Herp News* 51: 13–15.

Water, R.M. 1993. Odorized air current trailing by garter snakes, *Thamnophis sirtalis*. *Brain, Behavior and Evolution* 41: 219–223.

Weldon, P.J. 1982. Responses to ophiophagus snakes by snakes of the genus Thamnophis. *Copeia*: 788–794.

Weldon, P.J., and G.M. Burghardt. 1979. The ophiophage defensive response in crotaline snakes: Extension to new taxa. *Journal of Chemical Ecology* 5: 141–151.

CHAPTER 3: ASSOCIATED DORSAL AND TRACHEAL MICROBIAL ASSEMBLAGES OF THE PUFF ADDER (*BITIS ARIETANS*)

3.1. ABSTRACT

Associated microbiota can drive odour signatures in species. In particular, microbiota found within the trachea and on the integument of an organism are considered the dominant odour contributors. But what about in a species that is odourless? The puff adder (*Bitis arietans*) is a chemically cryptic, ambushing snake species with a pan-African distribution. Its success is no doubt in part shaped by its remarkable visual and chemically camouflage but given that currently no other example of a truly chemically cryptic, terrestrial vertebrate exists, little is known about this phenomenon. It has been suggested that the puff adder's chemical crypsis may be driven by an absence of associated microbiota. Given the recognised role of microbiota in animal odour and the propensity for antimicrobial mechanisms to exist in various forms within the natural world, we assessed the microbiota of the puff adder using culture-dependent techniques. Both the tracheal and dorsal environment were assessed, and microbial species were identified using Sanger sequences and compared against those sampled from the equivalent anatomical sites of a sympatric snake species known to be odourous, the brown house snake (*Boaedon capensis*). Puff adders were shown to have higher species richness than their odourous counterpart, clearly demonstrating that their chemically crypsis is not driven by an absence of microbiota. Rather it appears through the presence of at least two known odour-reducing species, *Bacillus subtilis* and *Acinetobacter radioresistens* and several other species of interest, *Cladosporium* spp. and *Enterococcus faecalis*, that their associated microbial assemblage may exist in catabolic synergy with one another in a manner that could directly assist in odour reduction.

3.2. INTRODUCTION

Body odour is largely determined by the microbial communities associated with an organism's integument and respiratory tract (Archie and Theis 2011). These communities can be host-determined (McKenzie et al. 2012; Walke et al. 2014) meaning that even species that share the same immediate environment can support vastly different microbial communities and produce different odours (Walke et al. 2014). Reptiles in general are considered to host a wide variety of bacterial and fungal species (e.g., Ferreira Junior et al. 2009; Colston 2017; Kohl et al. 2017; Pereira et al. 2017), including etiological agents such as *Salmonella* and *Escherichia coli* (Zancolli et al. 2015; Pereira et al. 2017) and indeed, many reptile species are known to be odorous (Miller et al. 2015). However one extraordinary example exists of an ambushing snake species that is chemically cryptic. This snake, the puff adder (*Bitis arietans*), is undetectable via olfactory cue by at least two predators (Miller et al. 2015) but the mechanism driving this crypsis is currently unknown. One leading theory posits that the adder may be down-regulating associated microbiota as a means of controlling body odour (Miller et al. 2015). Since antimicrobial mechanisms among species are widely documented, the potential for one to serve as a driver for the adder's chemical crypsis is plausible and worthy of investigation.

Natural antimicrobial activities include both innate and acquired mechanisms and these are fundamentally different from one another. Innate mechanisms are genetically determined and

therefore self-produced, while acquired mechanisms rely on a partnership with another antimicrobial-producing organism, typically itself a microbe. The most widely documented innate mechanism utilises antimicrobial peptides (AMPs) that are produced by the host organism and provide immunity of varying degree against bacteria, fungi, enveloped viruses and protozoa (Nicolas and Mor 2015). Some even act as anticarcinoma agents (Simmaco et al. 1998). AMPs are prolific in nature (Boparai and Sharma 2019) serving as integral components of an organism's immune system (Huan et al. 2020). Not only can they provide internal protection via the gut (Bevins and Salzman 2011) and saliva (Grant et al. 2019), as well as being found within venoms (Garcia et al. 2013), they can also act externally. AMPs found within the mucoid skin secretions of several species of amphibian (Simmaco et al. 1998), fish (Masso-Silva and Diamond 2014) and molluscs (Cheng-Hua et al. 2009) create an antimicrobial barrier amidst high risk, aquatic environments. Other innate, non-AMP approaches also exist and may rely on purely mechanical actions. For example, bactericidal nanopillars on invertebrate wing surfaces (Jenkins et al. 2020) and gecko skin (Li et al. 2016) have been shown to fatally penetrate bacteria, serving as a purely self-sufficient, mechanical antimicrobial solution. In contrast, the antimicrobial activity of one species can be conferred to another in a mutualistic relationship, resulting in acquired antimicrobial protection. This type of mutualism is not without precedent in birds (Soler et al. 2014; Ruiz-Rodriguez et al. 2009; Martín-Vivaldi et al. 2010; Martín-Vivaldi et al. 2014), mammals (Higginbotham et al. 2014), amphibians (Kearns et al. 2017), and reptiles such as the timber rattlesnake (*Crotolus horridus*; Hill et al. 2018) – a new world ecomorph of the puff adder in the sense that this highly venomous, north American snake is not only an ambush forager, but also visually cryptic. It is therefore not far-fetched that a similar situation may exist in the puff adder.

Studies on cutaneous microbiota of healthy snakes are rare but are slowly increasing. This is in part due to bioprospecting for alternatives to antibiotics (Alves and Albuquerque 2013) and in the growing concern for snake fungal disease (caused by the fungus *Ophidiomyces ophidiicola*) among wild populations (e.g., Guthrie et al. 2016). Most microbial studies on snakes have to date been driven by disease diagnostics on compromised individuals or are confined to the microbiota of their mouths and cloacae due to concerns associated with secondary infection related to snakebite (see Abrahamian and Goldstein 2011 for a review; Dehghani et al. 2016; Krishnankutty et al. 2018; Résière et al. 2018) and for improving husbandry practices (Hoppmann 2007) respectively. Southern African snakes however remain underrepresented and only one study has assessed the bacteria of puff adder mouths (e.g., Blaylock 2001), in which fewer than ten bacterial species were identified. The microbial assemblages associated with their scales and trachea however were, up until now, completely unknown. Although the bacteria from the mouth may provide some insight into to the potential microbial species within the adder's trachea due to anatomical proximity, it may not necessarily be

representative and differences in microbial assemblages have been detected over small spatial scales within the same organism (Smith et al. 2021). As such, to gauge whether the adder's chemical crypsis is a function of microbiota-downregulation we assessed the culturable microbiota of the dorsal integument and trachea of 20 puff adders using a swab and culture technique. Cultured microbiota were identified either through chromatic change of selected plating media or through DNA analyses. We also assessed the tracheal and dorsal microbiota of eight brown house snakes (*Boaedon capensis*) to serve as a comparison. This snake species is sympatric with puff adders and is known to be odorous (Miller et al. 2015).

3.3. MATERIALS & METHODS

3.3.1. Sample Collection

A laboratory population of twenty adult puff adders and eight adult brown house snakes were used for sampling. Nineteen of the puff adders were lab-borne to wild mothers and from the Dinokeng region of South Africa and one was a wild-caught, adult female from the same area. Lab-borne adders were exposed postpartum to their mothers for at least one day prior to their mothers' release and at one year of age were shown to be equivalent to their wild counterparts with regards to their chemical crypsis (Miller et al. 2015). All eight of the brown house snakes were wild-caught in KwaZulu-Natal, South Africa. Snakes were kept in species-specific rooms maintained at 28 °C and were individually housed in glass-fronted terraria that were lined with newspaper and fitted with a hide, water dish and rocks. Water was provided *ad libitum*. Snakes were maintained on a diet of dead, purpose-bred laboratory mice, which were provided on a fortnightly basis. Microbiota were collected via sterile ∑-Transwabs (Medical Wire, UK) rubbed either across the snakes' dorsal scales or within their trachea. Prior to swabbing, snakes were tubed and anaesthetized via exposure to isoflourethane-soaked cotton balls (Fig. 3.1; left.). To promote breathing, snakes were kept warm on heated beanbags and considered anaesthetized once muscle tone was lost and self-righting was no longer possible. Anaesthetized snakes were then removed from the glass tube, their mouths propped open using wooden depressors and their glottises exposed (Fig. 3.1, centre) which were then swabbed using sterile, Mini ∑-Transwabs (Medical Wire, UK), pre-dipped in distilled water. Swabs were immediately sealed in their provided liquid Amies medium that supports both aerobes and anaerobes, and labelled for later culturing. Due to two of the brown house snakes' trachea being too small, only six of the eight house snakes tracheas were swabbed. Dorsal samples were collected using two types of sterile ∑-Transwabs, one containing a charcoal gel Amies medium, for the promotion of fastidious microbiota, and one without. Swabs were dipped in distilled water and then rubbed vigorously along the snakes' dorsal scales (Fig. 3.1; right) and immediately sealed and labelled for later culturing. Post-swabbing,

snakes were returned to their home terraria where they typically recovered from the anaesthesia within twenty-minutes.



Figure 3.1. A tubed puff adder (Bitis arietans) being anaesthetised using isoflourethane and kept warm on an heated beanbag to promote breathing and the onset of anaesthesia (left). Swabbing of an anaesthetized puff adder's glottis (centre) and dorsal scales (right) using sterile ∑-Transwabs (Medical Wire, UK) pre-dipped in distilled water.

3.3.2. Sample Culturing

Five types of culturing media were used: Rapid'E-coli, Rapid'Salmonella (Bio-Rad Laboratories, CA, USA), Luria-Bertani, nutrient agar and potato dextrose. Prior to plating, samples were first incubated either in Luria-Bertani (LB) broth or in peptone water. For LB-incubated samples, swabs were individually transferred to sterile falcon tubes containing 15 ml of LB broth, vigorously shaken and then incubated at 37 °C on a shaker for 48 hours. Thereafter, the inoculated LB broth was streaked onto three of the media types, namely Rapid'E-coli, Rapid'Salmonella and LB agar plates using standard streaking procedures. Plates were incubated at 37 °C for 21 (Rapid'E-coli), 24 (Rapid'Salmonella), and 48 hours (LB agar). Control plates of all three media were also made and incubated accordingly to ensure that all plating media and techniques were aseptic. For Rapid media, colonies were identified through chromatic changes in the colonies themselves (Fig. 3.2), but for those grown on LB agar, PCR and genetic analyses were performed.

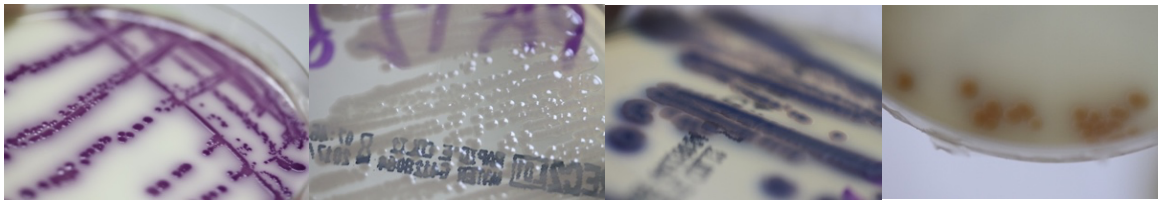


Figure 3.2. Examples of whole-colony chromatic change as a result of the selective chromogenic medium within the Rapid'E-coli and Rapid'Salmonella agar plates (Bio-Rad Laboratories, CA, USA).

For peptone water-incubated samples, dilutions of all swab samples were created using peptone water. For tracheal swabs, where the associated Amies medium was liquid, a 1:100 Amies-peptone dilution was made (total volume: 1 ml). For the dorsal samples where gel Amies were used, samples were first vigorously exposed to 2 ml of peptone water and thereafter, 100 µl inoculums were added to 900 µl of peptone water (total volume 1 ml). All dilutions were further incubated on a shaker

for 24 hours at 37 °C. After incubation, 100 µl of the each inoculated peptone water sample was used to make individual spread plates using sterilized, glass beads. All handling of samples and their subsequent plating was performed within a laminar flow hood under aseptic conditions. The plating medium for bacterial cultures was nutrient agar (NA) and for fungal samples, chloramphenicol-containing potato dextrose agar (PDA). Chloramphenicol is a bactericide and its inclusion in growth media facilitates fungal growth that may otherwise be downregulated by bacterial colonies. PDA and NA plates were incubated at 25 °C and 37 °C respectively for 48 hours and then inspected. Plates that did not show signs of growth were incubated for a further 24 hours. If no growth occurred after 72 hours, plates were considered sterile. Control plates of all media were made and incubated accordingly to ensure that all media and techniques were aseptic. All plates were photographed and colonies were categorized using a simple morphological key prior to any genetic sequencing to serve as a rudimentary failsafe in the event PCR failure. Rapid' plate colonies were identified via chromatic change.

3.3.3. PCR Amplification and DNA Analyses

Amplification was carried out in a 25 µL reaction volume containing 12.5 µL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystem, Boston, Massachusetts, USA), 1 µL magnesium chloride ($MgCl_2$) and 1.25 µL of each primer set. For bacterial samples, two universal primers, 8F (5′ – AGAGTTTGATCCTGGCTCAG – 3′; Turner et al. 1999) and 1492R (5′ – GGTTACCTTGTTACGACTT – 3′; Lane et al. 1991) were used to amplify the 16s rRNA gene. For fungal samples, two universal fungal primers, NL4 (5′ – GGTCCGTGTTTCAAGACGG – 3′) and NL1 (5′ – GCATATCAATAAGCGGAGGAAAAG – 3′), were used to amplify the D1/D2 region of 26s rDNA (White et al. 1990). PCRs were performed at the recommended thermal cycling conditions (Table 3.1) and product amplification was verified by electrophoresis using a 1% standard agarose gel, run at 100 volts for ~90 minutes. All unpurified PCR products that showed clear bands were then sequenced by Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, South Africa).

Table 3.1. Thermal cycling conditions used for PCR.

Step	Temperature (°C)	Duration
1	95	3 minutes
2	95	15 seconds
3	55	15 seconds
4	72	1 minute
5	Repeat Steps 2 – 4 (40 times)	
6	72	3 minutes
7	12	∞ (until samples removed)

Basic Local Alignment Search Tool (BLAST) software (Altschul et al. 1990) was used to identify trimmed sequences using NCBI GenBank's database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for bacterial species and UNITE (https://unite.ut.ee) for fungal species. Taxonomic thresholds were as follows: species – 98.7%; genus - > 94.5%; family > 86.5%; order – 82% (Yarza et al. 2014).

3.3.4. Statistical *Analyses*

Presence-absence species-level data were Jaccard-transformed and Bray-Curtis similarity matrixes for dorsal and tracheal assemblages between and within species were constructed using PRIMER7+ (Quest Research Limited, Auckland, New Zealand). Thereafter similarities were analysed using a reduced-model, two-factor permutational multivariate analysis of variance (PERMANOVA; 999 permutations) whereby snake species and anatomical region served as the two factors. Significant differences were further investigated using pairwise PERMANOVA comparisons. For overall comparisons, presence/absence data across anatomical regions were summed within snake species, such that a microbial species that was found in both dorsal and tracheal samples would be scored as two, but for those that were found in only one of these locations would be scored as one. P values < 0.05 were considered significant.

3.4. RESULTS

3.4.1. Bacteria

Puff adders were found to have the highest bacterial richness across all three taxonomic resolutions with a total of 20 species, 14 genera and ten families having been identified. In comparison, only sixteen species, 9 genera and 9 families were identified from the brown house snakes (Fig. 3.3). Twelve species were exclusively found on the adders, eight on the brown house snakes and eight were common to both snake species, resulting in overall similarity of 38.3% between the snakes (Table 3.2). No significant differences in the bacterial assemblages between the snake species were detected when both factors (i.e., snake species and anatomical region) were considered (pseudo-F = 1.158; p > 0.05). However, the standalone effect of anatomical region was highly significant (pseudo-F = 2.011;

$p < 0.01$). Tracheal samples across snake species demonstrated the highest similarity (58.8%) of all within and between species comparisons, followed by the 37.0% similarity for dorsal and tracheal bacterial assemblages within the puff adder. Only one pairwise comparison was shown to be significant, which was not surprising since there was no species overlap between the dorsal and tracheal assemblages of the brown house snake ($t = 1.333$, $p < 0.05$). All similarities were all supported statistically with no significant differences being found in pairwise comparisons (tracheal: $t = 1.211$, $p > 0.05$; dorsal: $t = 0.866$, $p > 0.05$; within-puff adder: $t = 1.110$, $p > 0.05$).

3.4.2. Fungi

In many of the fungal samples, targeted amplicons could not facilitate differentiation of closely related species despite their high scores (i.e., 100% pairwise match with 100% query cover). In these instances, we present genus-level results only. In total, 11 families, 12 genera and 13 species of fungi were genetically identified from our snake samples, of which all except two species were found on the adders (Fig. 3.4). In comparison, only four families, four genera and three species were found on the brown house snakes, resulting in a low overall similarity of 12.5% across the snake species (Table 3.2). In general, fungal species appear to be much more closely associated with the individual snake species when compared to the bacterial assemblages. Due to low taxonomic resolution, statistical assessments on the standalone fungal assemblages could not be performed, and these data are thus only statistically considered in the overall microbial assemblage assessments.

3.4.3. Bacteria and Fungi Combined

Bacterial and fungal assemblages do not exist in isolation, and similarities in overall microbial assemblages (i.e., both bacterial and fungal) across the snake species are thus likely to provide the greatest insight. This allowed us the opportunity to statistically consider the influence of the snakes' fungal assemblages on their overall microbial signature since the poor taxonomic resolution of our fungal data prevented standalone statistical assessments. Overall, microbial assemblages were significantly different between species when both factors were simultaneously considered (i.e., anatomical region and snake species; pseudo-$F = 1.731$, $p < 0.05$), resulting in an overall similarity of 30% across the snake species, but not when either snake species (pseudo-$F = 0.914$, $p > 0.05$) or anatomical regions (pseudo-$F = 1.569$, $p > 0.05$) were assessed individually. Tracheal assemblages however were not significantly different across snake species ($t = 1.00$; $p > 0.05$), demonstrating the highest similarity (41.7%; Table 3.2). Again, the next highest similarity (30.8%, Table 3.2) was found between the tracheal and dorsal assemblages of puff adder ($t = 1.239$, $p > 0.05$) and the least for the dorsal and tracheal assemblages of the brown house snake ($t = 1.312$, $p < 0.01$) with zero overlap in

microbial species (Table 3.2). Across-species dorsal assemblages were only marginally similar (26% similarity; $t = 1.275$; $p = 0.06$).

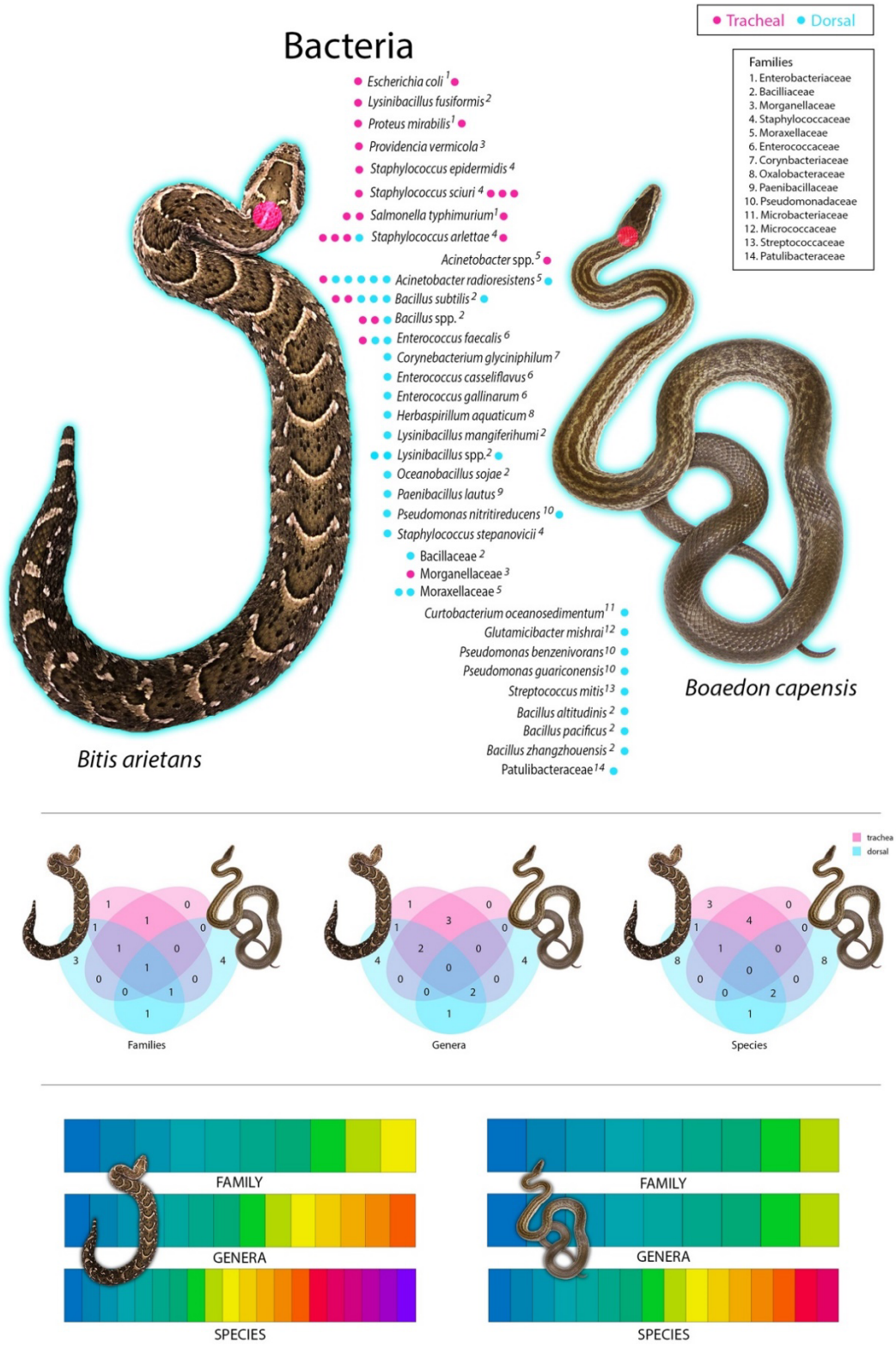


Figure 3.3. Bacterial species genetically identified from tracheal (pink) and dorsal (blue) samples taken from puff adders (*Bitis arietans*; n = 20) and brown house snakes (*Boaedon capensis*; n = 11). Colonies that were identified to genus-level only are denoted as 'spp.'. Dot position indicates which species of snake the bacterial species/genus/family was found on (i.e., puff adder = left, brown house snake = right) and the number of dots indicates the number of individual snakes on which the associated bacterial species/genus/family was positively identified. Superscript numbering indicates the associated bacterial families and Venn diagrams indicate where commonalities across all three levels of taxonomic resolution occur. Chromatic bars indicate taxonomic richness for species, genera and family levels for both snake species.

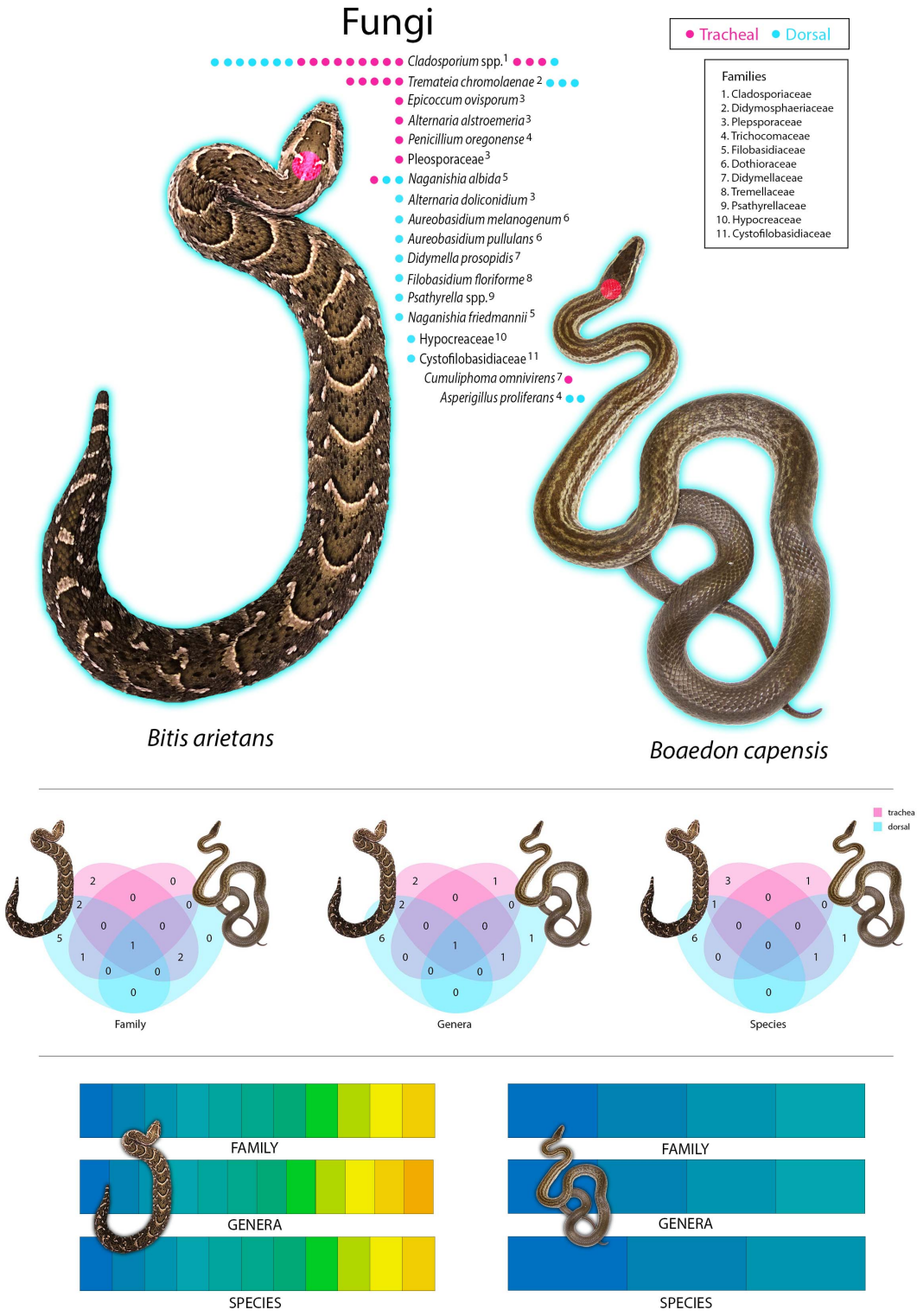


Figure 3.4. Fungal species genetically identified from tracheal (pink) and dorsal (blue) samples taken from puff adders (*Bitis arietans;* n = 20) and brown house snakes (*Boaedon capensis*; n = 11). Colonies that were identified to genus-level only are denoted as 'spp.' and samples with family-level agreement are listed as such. Dot position indicates which species of snake the fungal species/genus/family was found on (i.e., puff adder = left, brown house snake = right) and the number of dots indicates the number of individual snakes on which the associated fungal species/genus/family was positively identified. Superscript numbering indicates the associated fungal families and Venn diagrams indicate where commonalities across all three levels of taxonomic resolution occur. Chromatic bars indicate taxonomic richness for species, genera and family levels confirmed via DNA sequencing for both snake species.

Table 3.5. Bray-Curtis similarity percentages based on presence/absence data for bacterial and fungal species assemblages genetically identified from tracheal and dorsal environments of puff adders (*Bitis arietans*) and brown house snakes (*Boaedon capensis*). Instances where the microbial species were present in both anatomical regions within a snake species, the microbial species would be scored as two whereas a score of one would indicate that the species was found in only one region.

			Puff Adder		Brown House Snake
			Tracheal	Dorsal	Tracheal
Bacteria	Puff Adder	Tracheal			
		Dorsal	37.0		
	Brown House Snake	Tracheal	58.8	9.1	
		Dorsal	16.7	27.6	0
	Overall			**38.3**	
Fungi	Puff Adder	Tracheal			
		Dorsal	15.4		
	Brown House Snake	Tracheal	0	0	
		Dorsal	28.6	0	0
	Overall			**12.5%**	
Both	Puff Adder	Tracheal			
		Dorsal	30.8		
	Brown House Snake	Tracheal	41.7	7.4	
		Dorsal	23.5	21.6	0
	Overall			**30%**	

3.4.4. Potential Microbial Interactions

Although interactions among the detected microbiota species were not specifically assessed, none of the cultured fungal species were able to grow in the absence of chloramphenicol. Although this could be specific to the culture environment, it could also suggest that some of the microbial species present on the snakes may naturally inhibit the growth of other microbes. Indeed, many of the microbial species present are known to possess antimicrobial properties (Table 4.3).

Table 3.6. An assessment of known anti-microbial activity associated with the culture-dependent microbial species identified from the trachea and dorsal scales of the puff adder (Bitis arietans) and brown house snake (Boaedon capensis).

Bacterial Species (gram-negative: -; gram-positive: +)	**Associated Snake Species**	*Antimicrobial Potential (confirmed: +; unknown: ?)*	**Reference**
Escherichia coli (-)	both	+	(Gale and Sandoval 1956)
Lysinibacillus fusiformis (+)	puff adder	+	(Passera et al. 2021)
Providencia vermicola (-)	puff adder	?	-
Proteus mirabilis (-)	both	+	(Kart et al. 2020)
Salmonella typhimurium	both	?	-
Staphylococcus epidermis (+)	puff adder	+	(Cogen et al. 2010)
Staphylococcus sciuri (+)	both	+	(Nazipi et al. 2021)
Staphylococcus typhimurium (+)	both	+	(Kim & Mylonakis 2011)

Staphylococcus arlettae (+)	both	? but + for some members of the genus	(Amaresan et al. 2020; Cogen et al. 2010; Kim & Mylonakis 2011; Nazipi et al. 2021)
Acinetobacter spp. (-)	brown house snake	+	(Liu et al. 2007; Mujumdar et al. 2014)
Acinetobacter radioresistens (-)	both	? but + for some members of the genus	(Liu et al. 2007; Mujumdar, Bashetti, and Chopade 2014)
Bacillus spp. (+)	puff adder	+	(Sumi et al. 2015)
Enterococcus faecalis (+)	puff adder	+	(Gergolet Diaz et al. 2021)
Enterococcus casseliflavus (+)	puff adder	+	(Gergolet Diaz et al. 2021)
Enterococcus gallinarum (+)	puff adder	+	(Niederle et al. 2019)
Corynebacterium glycinophilum (+)	puff adder	?	-
Herbaspirillum aquaticum	puff adder	?	-
Lysinibacillus mangiferihumi† (+)	puff adder	+ for *Lysinibacillus sphaericus*	(Sakthivel et al. 2018; Shabanamol et al. 2021)
Lysinibacillus spp. (+)	both	+ for some members of the genus	(Ahmad et al. 2014)
Oceanobacillus sojae (+)	puff adder	+	(de Senna and Lathrop 2017)
Paenibacillus lautus (+)	puff adder	? but + for some members of the genus	(Aktuganov et al. 2008; Naing et al. 2014)
Pseudomonas nitritireducens (-)	puff adder	? but + for some members of the genus	(Amaresan et al. 2020)
Staphylococcus stepanovicii (+)	puff adder	? but + for some members of the genus	(Amaresan et al. 2020; Cogen et al. 2010; Kim & Mylonakis 2011; Nazipi et al. 2021)
Curtobacterium oceanosedimentum (+)	brown house snake	+	(Das et al. 2017)
Glutamicibacter mishrai (+)	brown house snake	?	-
Pseudomonas benzenivorans	brown house snake	? but + for some members of the genus	(Amaresan et al. 2020)
Pseudomonas guariconensis	brown house snake	? but + for some members of the genus	(Amaresan et al., 2020)
Streptococcus mitis (+)	brown house snake	+	(Walsh and Howe 2004)
Bacillus altitudinis (+)	brown house snake	? but + for some of the genus	(Sumi et al. 2015)
Bacillus pacificus (+)	brown house snake	? but + for some of the genus	(Sumi et al. 2015)
Bacillus zhangzhouensis (+)	brown house snake	? but + for some of the genus	(Sumi et al. 2015)

Table 3.3. Continued…

Fungal Species	Associated Snake Species	Antimicrobial Potential (confirmed: +; unknown: ?)	Reference
Cladosporium spp.	both	+ for several members of the genus	(Ding et al. 2008; Wang et al. 2013; Sugijanto and Dorra 2016)
Trematheia chromolaenae	both	?	-
Naganishia albida	puff adder	?	-
Epicoccum ovisporum	puff adder	? but + for at least one member of the genus	(Qader et al. 2021)
Alternaria alstroemeria	puff adder	?	-
Alternaria doliconidium	puff adder	?	-
Penicillium spp.	puff adder	+	(Larsen & Knøchel 1997)
Asperigillus proliferans	brown house snake	? but + for at least one member of the genus	(Darling et al. 1963)
Aureobasidium melanogenum	puff adder	+	(Bischoff et al. 2015)
Aureobasidium pullulans	puff adder	+	(Yalage Don et al. 2021)
Psathyrella spp.	puff adder	+	(Suay et al. 2000)

†Lysinibacillus mangiferihumi is a heterotypic synonym for Lysinibacillus sphaericus (Dunlap 2019).

3.5. DISCUSSION

Puff adder was found to have higher microbial richness than that of the chemically conspicuous brown house snake, which suggests that their chemically crypsis is not the result of microbial suppression. Overall microbial assemblages associated to each snake species were significantly different when anatomical region was considered. Several species of microbiota were specific to the puff adder, with the bacterial majority of these being located on the dorsal integument and the fungal majority being common to both anatomical regions. Despite these differences, the highest similarity in species assemblages was between the tracheal assemblages (58.8%) while the dorsal assemblages were only marginally different between species (21.6%; $p = 0.06$). Few previous studies have assessed the puff adder's microbiota, with only seven bacterial species having been isolated from the buccal cavities of puff adders (Blaylock 2001). Of these, two were common to our samples (*Proteus mirabilis* and *E-coli*), a further four were represented at the genus-level (*Salmonella*, *Pseudomonas*, *Proteus* and *Staphylococcus*) and one was entirely absent (*Citrobacter freundii*). No apparent prior studies have assessed the fungal assemblage of puff adders. A compliment of 18 additional bacterial species and

11 fungal species were isolated from the adders in our study. Not only does the adder support a high number of microbial species relative to the brown house snake (21 versus 19 species), several microbial species prominently found on the adder and seldomly associated with the brown house snake, if at all, are implicated in the breakdown of odorous molecules. Thus, contrary to previous thinking, the adder's microbial assemblage may play an active role in the snake's chemical crypsis.

Sixty-five percent of the identified bacterial species were gram-positive. This is in line with the propensity of bacterial assemblages in healthy snakes being more heavily constituted by gram-positive bacterial species (Hilf et al. 1990; Soveri et al. 1986). However, several gram-negative species were also detected, some of which are medical important (i.e., *Salmonella* and *E-coli*). One, *Acinetobacter radioresistens*, is especially noteworthy. Although this species was also found on a single brown house snake sample, it was the most commonly cultured bacterial species from the puff adders, prevalent mostly on their dorsal scales. This bacterium is resistant to radiation (Nishimura et al. 1988), extreme desiccation (Jawad et al. 1998) and most importantly, is able to breakdown aromatic compounds via rapid phenol catabolism (Mazzoli et al. 2007). *Acinetobacter radioresistens* can therefore survive on phenols, which are common in the natural world (Rappoport 2003), as its sole carbon source (Pessione et al. 1999). Phenols are odorous and a by-product of tyrosine metabolism for which lactic acid bacteria (LAB) are known (Garner et al. 2007). Tyrosine is a major component of the outer ß-keratin (Parry 2021) layer of *Bitis* scales (Ripamonti et al. 2009) and therefore the presence of a tyrosine-metabolising LAB species within the puff adder's microbial assemblage could easily support and maintain the presence of *A. radioresistens* through phenol production. Indeed, a known LAB species, *Enterococcus faecalis*, is present within the adder's dorsal and tracheal microbial assemblages. Typically, this bacterium is ubiquitous within the gastro-intestinal-tracts of animals and humans (Klein 2003), but importantly has also been found in close association to keratinoid environments (Ruiz-Rodriguez et al. 2009). Although LAB species are known for their tyrosine metabolism (and hence phenol production), *E. faecalis* also actively decreases the potency of keratinases from keratinolytic microbiota and thus can be considered highly beneficial to animals with keratinoid integuments, such as birds and reptiles, since compromised feathers and scales could result in a swathe of adverse and potentially fatal effects.

The puff adder appears to host a consortium of microbiota that are involved with odour breakdown. Specifically, it appears that several of the associated microbial species are implicated in the breakdown of ß-keratin into its non-odorous constituents. Two species of keratin-degrading microbiota were identified on the puff adder, *Bacillus subtilis* (Li 2019) and *Cladosporium* (Călin et al. 2017). *Bacillus subtilis*, the third most commonly cultured bacterial species from the puff adder, is a

highly effective keratinolytic bacterium, now used in industrial waste management (Q Li 2019), and a noteworthy phenol-cataboliser (Balasubramaniyan and Swaminathan 2000) whose spores are ubiquitous in soil (Bastos et al. 2020). *Bacillus subtilis* has been found to dramatically reduce odour in animal faeces (Bastos et al. 2020), as well as enzymatically reducing ammonia production during odorous fermentation processes (Abriba et al. 2012). *Cladosporium*, the predominant fungal species in both snake species, is abundant in both indoor and outdoor air (Bensch et al. 2018) and previously detected in buccal cavities and cloacae of snakes (Lukač et al. 2017). Since none of the control plates harboured any microbial growth throughout our culturing period, we are confident that the *Cladosporium* cultures are of snake-origin and not a reflectance of its aerosol nature. It is not surprising that both these keratinolytic species are common in the keratin-rich environment of puff adder scales, but it is certainly interesting that within the same microbial assemblage there exists companion microbial species known to reduce the efficacy of keratinases and ones that are able to metabolise multistage, downstream by-products associated with keratin-degradation known to be odorous (Fig. 3.5). Indeed, it appears the interplay among species within the puff adder's microbial assemblage is intricate and complex, but the presence of known odour-reducing species suggests that the puff adder's chemical crypsis may in some way be aided by their associated microbiota, in contrast to previously suggested ideas.

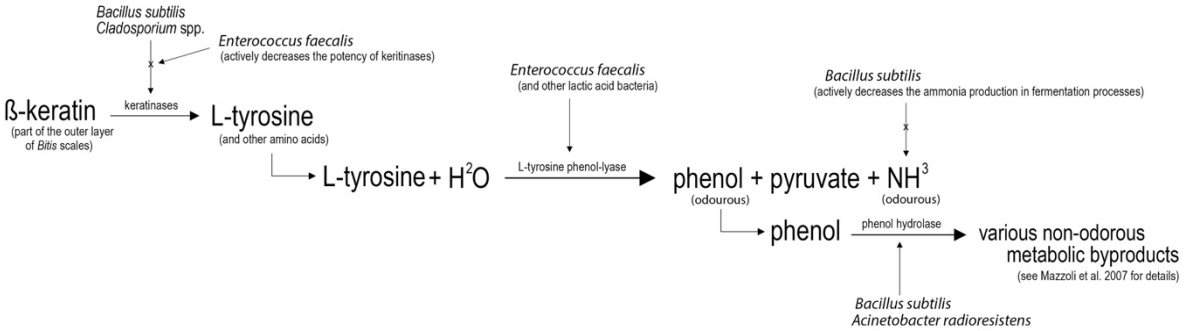


Figure 3.5. Potential involvement of puff adder-associated (*Bitis arietans*) microbial species in ß-keratin degradation into its less odorous constituents.

The puff adder's dorsal scales also possess micro-ornamentation that, unlike the needle-like, bactericidal nanostructures of some gecko skin and insect wings (Jenkins et al. 2020), are blunt and broadly edged (Fig. 3.6, left; Singh 2018). Similar structures are also found in a closely related species, the West African Gaboon adder (*Bitis rhinoceros*), a species also thought to be chemically cryptic (Miller et al. 2015). This micro-ornamentation contributes to visual camouflage through increasing the light absorption of black scales (Spinner et al. 2013; Singh 2018). While it appears that the primary function of these structures is to augment visual crypsis, it is possible that additional benefits are

simultaneously conferred. For example, the micro-ornamentation found on the adder's dorsal scales may also provide structural refugia for associated microbiota since the size range of both bacterial (0.5 – 2 µm; Driks, 1999; Ugwuodo & Nwagu 2020) and fungal spores (2 – 50 µm; di Filippo et al., 2013; Patel et al., 2018) and bacteria themselves (0.2 – 10 µm; Frosbisher et al. 1974) is small enough to fit in between the nano structures of the scales. Indeed, potential microbiota spores and trapped organic matter have been documented via scanning electron micrograph of *B. rhinoceros* shed skin (Fig. 3.6), indicating that the idea for a similar situation occurring in the puff adder is not far-fetched.

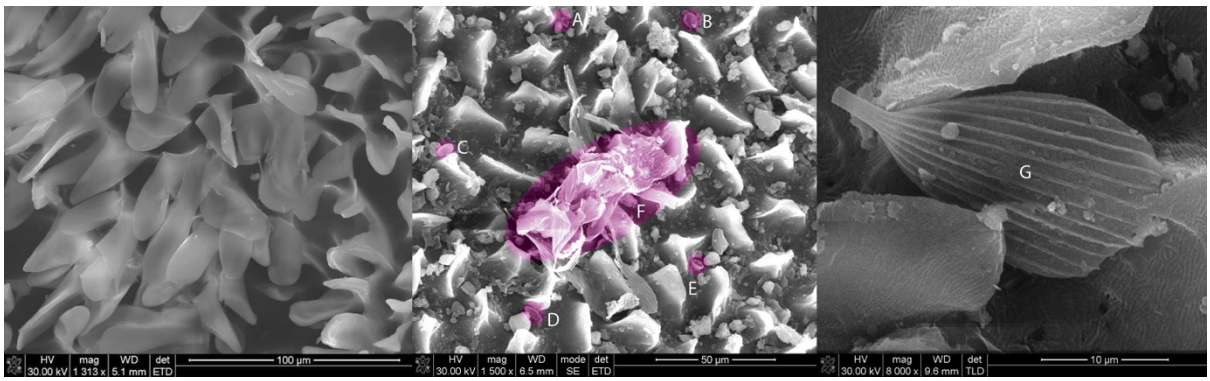


Figure 3.6. Scanning electron micrographs showing the micro-ornamentation of the dorsal scales of *Bitis arietans* (left) and *Bitis rhinoceros* (centre, right; Spinner et al. 2013). Potential fungal and bacterial spores (A-E), unidentified organic matter (F) and a lepidopteran wing scale (G).

The similarity in tracheal assemblages between the two snake species investigated may be reflective of diet. Previously, it has been suggested that the microbiota of prey and their faeces (Goldstein et al. 1979) may directly affect the buccal microbial assemblage of reptiles (Abrahamian & Goldstein, 2011; Fry et al. 2009). Our snakes were maintained under laboratory conditions during this study and fed purpose-bred, dead, laboratory rodents originating from the same source. It is therefore possible that tracheal assemblages may have been repeatedly inoculated (via the buccal cavity) with microbiota associated with this food source and that species suited to the moist conditions of the tracheal environment simply persisted. Several studies have however shown no relationship between the microflora of the snake buccal cavities and their diet (Blaylock 2001; Zancolli et al. 2015), arguing instead that antimicrobial properties of reptile saliva and venom is likely to restrict such microbial invasion of the buccal cavity (Avila et al. 2009). Hill et al. (2018) however found no differences in microbial assemblages between two sympatric species of snakes (*Crotalus horridus* and *Coluber constrictor*) that, such as in our study, represented one ambush- and one active-foraging species. Although we attempted to control for diet-influences through swabbing two weeks post the snakes' last feed, the influence of diet on our data however remains unknown. Two additional, potentially compounding factors should also be considered regarding the high tracheal similarity. First, the low sample size of brown house snake tracheal swabs (some tracheae were simply too small to swab) may have limited our ability to adequately represent the microflora of this species, and second, culture-

dependent bias in our samples may have reduced our ability to detect innate differences. Another important factor to consider is that the tracheal environment between the two snake species is likely to be less anatomically and physiologically different than that of the dorsal integument. While the puff adder dorsal scales are rough and strongly keeled, brown house snake scales are smooth and without keeling. The smoothness of the brown house snake scales is likely to make adherence by microbiota more difficult which could directly impact their associated microbial assemblages. Though increasing the sample size of brown house snakes to match puff adder numbers would certainly reveal whether species richness differences between these snake species is genuine or an artifact of this difference.

Culture-dependent techniques can be limiting (Al-Awadhi et al. 2013). Culture success depends on a host of variables such adequate nutrient availability, suitable temperature ranges and an environment where species are not outcompeted by others within the same sample, either through resource limitations or via active antimicrobial activities, for which almost all the detected microbial species are known (see Table 3.3). Culture-independent techniques, such as metagenomics and high-throughput sequencing, are therefore rapidly growing in popularity but the associated costs can be prohibitive. Culture-dependent techniques are however not entirely without value and similarities between culture-dependent and -independent assemblage data can still exist (see Smith et al. 2021). Assessments of associated microbiota using culture-independent techniques however would certainly strengthen our understanding of the microbial assemblages associated with the puff adder and is worthy of future investigation. Despite our approach being potentially restricted by culture-dependent techniques, we clearly demonstrate that the puff adder's chemical crypsis is not in any way driven by an absence of associated microbiota, but rather that their associated microbial assemblage is vast and complex. Certainly, the presence of numerous odour-reducing microbiota within the adder's microbial assemblage has highlighted the potential that the puff adder's extraordinary chemical crypsis may, at least in part, be driven by a previously unrecognised mutualism between snake and microbe.

3.6. REFERENCES

Abrahamian, F.M., and E.J.C. Goldstein. 2011. Microbiology of Animal Bite Wound Infections. *Clinical Microbiology Reviews*.

Abriba, C., E.E. Henshaw, J. Lenox, M. Eja, I.S. Ikpoh, and B.E. Agbor. *Microbial Succession and Odour Reduction during the Controlled Fermentation of Cassava Tubers for the Production of "Foofoo", a Staple Food Consumed Popularly in Nigeria*. *Journal of Microbiology and Biotechnology Research Scholars Research Library J. Microbiol. Biotech. Res*. Vol. 2012. http://scholarsresearchlibrary.com/archive.html.

Ahmad, V., A.N. Muhammad Zafar Iqbal, M. Haseeb, and M.S. Khan. 2014. Antimicrobial Potential of Bacteriocin Producing Lysinibacillus Jx416856 against Foodborne Bacterial and Fungal Pathogens, Isolated from Fruits and Vegetable Waste. *Anaerobe* 27: 87–95.

Aktuganov G., A. Melentjev, N. Galimzianova, E. Khalikova, T. Korpela and P. Susi. Wide-range antifungal antagonism of *Paenibacillus ehimensis* IB-X-b and its dependence on chitinase and beta-1,3-glucanase production. Can J Microbiol. 2008 Jul;54(7):577-87. doi: 10.1139/w08-043. PMID: 18641704.

Al-Awadhi, H., N. Dashti, M. Khanafer, D. Al-Mailem, N. Ali, and S. Radwan. 2013. *Bias Problems in Culture-Independent Analysis of Environmental Bacterial Communities: A Representative Study on Hydrocarbonoclastic Bacteria*. http://www.springerplus.com/content/2/1/369.

Alves, R.R.N., and U.P. Albuquerque. 2013. Animals as a Source of Drugs: Bioprospecting and Biodiversity Conservation. In *Animals in Traditional Folk Medicine: Implications for Conservation*, 67–89. Springer-Verlag Berlin Heidelberg.

Altschul S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman. 1990. Basic local alignment search tool. *J Mol Biol*, 215(3):403– 410.

Amaresan, N., M. Senthil Kumar, K. Annapurna, K. Kumar, and A. Sankaranarayanan. 2020a. *Beneficial Microbes in Agro-Ecology: Bacteria and Fungi*.

———. 2020b. *Beneficial Microbes in Agro-Ecology: Bacteria and Fungi*.

Archie, E.A., and K.R. Theis. 2011. Animal Behaviour Meets Microbial Ecology. *Animal Behaviour* 82, no. 3: 425–436.

Avila M., Ojcius D.M. and Ö Yilmaz. 2009. The oral microbiota: living with a permanent guest. *DNA Cell Biol*. 28:405–411.

Balasubramaniyan S, and M. Swaminathan. 2000. Bacterial degradation of phenols by *Bacillus subtilis*. *Indian Journal of Environmental Protection* 20, no. 2: 134–137

Bastos, T.S., D.C. de Lima, C.M.M. Souza, A. Maiorka, S.G. de Oliveira, L.C. Bittencourt, and A.P. Félix. 2020. *Bacillus subtilis* and *Bacillus licheniformis* reduce faecal protein catabolites concentration and odour in dogs. *BMC Veterinary Research* 16, no. 116: 1–8.

Bensch, K., J.Z. Groenewald, M. Meijer, J. Dijksterhuis, Jurjević, B. Andersen, J. Houbraken, P.W. Crous, and R.A. Samson. 2018. *Cladosporium* species in indoor environments. *Studies in Mycology* 89: 177–301.

Bevins, C.L., and N.H. Salzman. 2011. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nature Reviews Microbiology* 9: 356 – 368.

Bischoff, K.M., T.D. Leathers, N.P. Price, and P. Manitchotpisit. 2015. Liamocin oil from *Aureobasidium pullulans* has antibacterial activity with specificity for species of *Streptococcus*. *Journal of Antibiotics* 68, no. 10: 642–645.

Blaylock, R.S. 2001. Normal oral bacterial flora from some southern African snakes. *The Onderstepoort Journal of Veterinary Research* 68: 175–182.

Boparai, J.K., and P.K. Sharma. 2019. Mini review on antimicrobial peptides, sources, mechanism and recent applications. *Protein & Peptide Letters* 27, no. 1: 4–16.

Călin, M., D. Constantinescu-Aruxandei, E. Alexandrescu, I. Răut, M.B. Doni, M.L. Arsene, F. Oancea, L. Jecu, and V. Lazăr. 2017. Degradation of keratin substrates by keratinolytic fungi. *Electronic Journal of Biotechnology* 28: 101–112.

Cheng-Hua, L.I., Z. Jian-Min, and L.-S. Song. 2009. Molluscan research, a review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture. *Molluscan Research* 29, no. 1: 17–26

Cogen, A.L., K. Yamasaki, J. Muto, K.M. Sanchez, L.C. Alexander, J. Tanios, Y. Lai, J.E. Kim, V. Nizet, and R.L. Gallo. 2010. *Staphylococcus epidermidis* antimicrobial δ-toxin (phenol-soluble modulin-γ) cooperates with host antimicrobial peptides to kill group a *Streptococcus*. *PLoS ONE* 5, no. 1.

Colston, T.C. 2017. *The Reptile Gut Microbiome: Its Role In Host Evolution And Community Assembly*. PhD Thesis, University of Mississippi.

Darling, W.M., P.J. Campbell, and M. Mcardle. 1963. *Antibiotics from Aspergillus amstelodami*. *The Journal of General Microbiology* Vol. 33: 191 – 204.

Das, G., J.K. Patra, J. Choi, and K.-H. Baek. 2017. Anticandidal effect of endophytic bacteria isolated from *Equisetum arvense L*. against *Candida albicans* and *Candida glabrata*. *Brazilian Archives of Biology and Technology* 60: 1 – 16.

De Senna, A., and A. Lathrop. 2017. Antifungal screening of bioprotective isolates against *Botrytis cinerea*, *Fusarium pallidoroseum* and *Fusarium moniliforme*. *Fermentation* 3, no. 53

Dehghani, R., A. Sharif, M.A. Assadi, H. Haddad Kashani, and M.R. Sharif. 2016. fungal flora in the mouth of venomous and non-venomous snakes. *Comparative Clinical Pathology* 25, no. 6: 1207–1211.

i Filippo, P., D. Pomata, C. Riccardi, F. Buiarelli, and C. Perrino. 2013. fungal contribution to size-segregated aerosol measured through biomarkers. *Atmospheric Environment* 64: 132–140.

Ding, L., S. Qin, F. Li, X. Chi, and H. Laatsch. 2008. Isolation, Antimicrobial Activity, and Metabolites of Fungus Cladosporium Sp. Associated with Red Alga Porphyra Yezoensis. *Current Microbiology* 56, no. 3 (March): 229–235.

Driks, A. 1999. *Bacillus Subtilis* spore coat. *Microbiology and Molecular Biology Reviews* 63, no. 1: 1 – 20.

Dunlap, C.A. 2019. *Lysinibacillus mangiferihumi, Lysinibacillus tabacifolii* and *Lysinibacillus varians* are later heterotypic synonyms of *Lysinibacillus sphaericus*. *International Journal of Systematic and Evolutionary Microbiology* 69, no. 9: 2958–2962.

Ferreira Junior, R.S., A.K. Siqueira, M. v. Campagner, T. Salerno, T.C.S. Soares, S.B. Lucheis, A.C. Paes, and B. Barraviera. 2009. Comparison of Wildlife and Captivity Rattlesnakes (*Crotalus durissus terrificus*) Microbiota. *Pesquisa Veterinaria Brasileira* 29, no. 12: 999–1003..

Frobisher, M., R. D. Hinsdill, K. T. Crabtree and C. R. Goodheart. 1974. Fundamentals of Microbiology, PA, Philadelphia: W.B. Saunders Company.

Fry B.G., S. Wroe, W. Teeuwisse, M.J. van Osch, K. Moreno, J. Ingle, C. McHenry, T. Ferrara, P. Clausen, H. Scheib, K.L. Winter, L. Greisman, K. Roelants, L. van der Weerd, C.J. Clemente, E. Giannakis, W.C.

Hodgson, S. Luz, P. Martelli, K. Krishnasamy, E. Kochva, H.F. Kwok, D. Scanlon, J. Karas, D.M. Citron, E.J. Goldstein, J.E. McNaughtan, J.A. Norman. A central role for venom in predation by *Varanus komodoensis* (Komodo Dragon) and the extinct giant *Varanus* (Megalania) *priscus*. *Proceedings of the National Academy of Sciences of the United States of America* 106, no. 22:8969-74.

Gale, D., and B. Sandoval. 1956. Response of mice to the inoculations of both *candida albicans escherichia coli i*. The enhancement phenomenon. *Journal of Bacteriology* 73, no. 5: 616 – 624

Garcia, F., E. Villegas, G.P. Espino-Solis, A. Rodriguez, J.F. Paniagua-Solis, G. Sandoval-Lopez, L.D. Possani, and G. Corzo. 2013. Antimicrobial peptides from arachnid venoms and their microbicidal activity in the presence of commercial antibiotics. *Journal of Antibiotics* 66, no. 1: 3–10.

Garner, C.E., S. Smith, B. Lacy Costello, P. White, R. Spencer, C.S.J. Probert, and N.M. Ratcliffem. 2007. Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *The FASEB Journal* 21, no. 8: 1675–1688.

Gergolet Diaz, D.G., R.P. Pizzolitto, C. Vázquez, V.L. Usseglio, M.P. Zunino, J.S. Dambolena, J.A. Zygadlo, and C. Merlo. 2021. Effects of the volatile organic compounds produced by *Enterococcus* spp. strains isolated from maize grain silos on *Fusarium verticillioides* growth and *Fumonisin* b1 production. *Journal of Stored Products Research* 93: 101825

Goldstein, E.J., D.M. Citron, H. Gonzalez, F.E. Russell, S.M. Finegold. 1979, Bacteriology of rattlesnake venom and implications for therapy. *Jounral of Infectious Diseases* 140: 818–821

Grant, M., O. Kilsgård, S. Åkerman, B. Klinge, R.T. Demmer, J. Malmström, and D. Jönsson. 2019. the human salivary antimicrobial peptide profile according to the oral microbiota in health, periodontitis and smoking. *Journal of Innate Immunity* 11, no. 5: 432–443.

Guthrie, A., S. Knowles, A. Ballman, and J. Lorch. 2016. Detection of snake fungal disease due to *Ophidiomyces o*phiodiicola in Virginia, USA. *Journal of Wildlife Diseases* 52, no. 1: 57–69.

Higginbotham, S., W.R. Wong, R.G. Linington, C. Spadafora, L. Iturrado, and A.E. Arnold. 2014. sloth hair as a novel source of fungi with potent anti-parasitic, anti-cancer and anti-bacterial bioactivity. *PLoS ONE* 9, no. 1.

Hilf M., R.A. Wagner and V.L. YU. 1990. A Prospective Study of Upper Airway Flora in Healthy Boid Snakes and Snakes with Pneumonia. *Journal of Zoo and Wildlife Medicine* 21, no. 3: 318–325.

Hill, A.J., J.E. Leys, D. Bryan, F.M. Erdman, K.S. Malone, G.N. Russell, R.D. Applegate, et al. 2018. Common cutaneous bacteria isolated from snakes inhibit growth of *Ophidiomyces Ophiodiicola*. *EcoHealth* 15, no. 1: 109–120.

Hoppmann, E. 2007. Dermatology in Reptiles. *Topics in Medicine and Surgery* 16, no. 4: 210–224.

Huan, Y., Q. Kong, H. Mou, and H. Yi. 2020. Antimicrobial peptides: Classification, design, application and research progress in multiple fields. *Frontiers in Microbiology*. Frontiers Media S.A.

Jawad, A., A.M. Snelling, J. Heritage, and P. M. Hawkey. 1998. Exceptional desiccation tolerance of *Acinetobacter Rudioresistens*. *Journal of Hospital Infection* 39: 235–240.

Jenkins, J., J. Mantell, C. Neal, A. Gholinia, P. Verkade, A.H. Nobbs, and B. Su. 2020. Antibacterial effects of nanopillar surfaces are mediated by cell impedance, penetration and induction of oxidative stress. *Nature Communications* 11, no. 1: 1626.

Kart, D., S. Yabanoglu Ciftci, and E. Nemutlu. 2020. Altered metabolomic profile of dual-species biofilm: interactions between *Proteus mirabilis* and *Candida albicans*. *Microbiological Research* 230: 126346.

Kearns, P.J., S. Fischer, S. Fernández-Beaskoetxea, C.R. Gabor, J. Bosch, J.L. Bowen, M.F. Tlusty, and D.C. Woodhams. 2017. Fight fungi with fungi: antifungal properties of the amphibian mycobiome. *Frontiers in Microbiology* 8: 02494

Kim, Y., and E. Mylonakis. 2011. killing of *Candida albicans* filaments by *Salmonella enterica serovar typhimurium* is mediated by sopb effectors, parts of a type iii secretion system. *Eukaryotic Cell* 10: 782–790.

Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiology* 88, no. 2–3: 123–131.

Kohl, K.D., A. Brun, M. Magallanes, J. Brinkerhoff, A. Laspiur, J.C. Acosta, E. Caviedes-Vidal, and S.R. Bordenstein. 2017. Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. *Molecular Ecology* 26, no. 4: 1175–1189.

Krishnankutty, S.P., M. Muraleedharan, R.C. Perumal, S. Michael, J. Benny, B. Balan, P. Kumar, et al. 2018. Next-Generation Sequencing Analysis Reveals High Bacterial Diversity in Wild Venomous and Non-Venomous Snakes from India. *Journal of Venomous Animals and Toxins Including Tropical Diseases* 24, no. 41.

Larsen, A.G., and S. Kno Âchel. 1997. Antimicrobial activity of food-related *Penicillium Sp.* against pathogenic bacteria in laboratory media and a cheese model system. *Journal of Applied Microbiology*. Vol. 83: 111 – 119.

Li, Q. 2019. Progress in Microbial Degradation of Feather Waste. *Frontiers in Microbiology* 10: 2717.

Li, X., G.S. Cheung, G.S. Watson, J.A. Watson, S. Lin, L. Schwarzkopf, and D.W. Green. 2016. The nanotipped hairs of gecko skin and biotemplated replicas impair and/or kill pathogenic bacteria with high efficiency. *Nanoscale* 8, no. 45: 18860–18869.

Liu, C.H., X. Chen, T.T. Liu, B. Lian, Y. Gu, V. Caer, Y.R. Xue, and B.T. Wang. 2007. Study of the antifungal activity of *Acinetobacter baumannii* lch001 in vitro and identification of its antifungal components. *Applied Microbiology and Biotechnology* 76, no. 2: 459–466.

Lukač, M., D.H. Tomić, Z. Mandac, S. Mihoković, and E. Prukner-Radovčić. 2017. Oral and cloacal aerobic bacterial and fungal flora of free-living four-lined snakes (*Elaphe quatuorlineata*) from Croatia. *Veterinarski Arhiv* 87, no. 3: 351–361.

Martín-Vivaldi, M., A. Peña, J.M. Peralta-Sánchez, L. Sánchez, S. Ananou, M. Ruiz-Rodríguez, and J.J. Soler. 2010. Antimicrobial Chemicals in Hoopoe Preen Secretions Are Produced by Symbiotic Bacteria. *Proceedings of the Royal Society B: Biological Sciences* 277, no. 1678 (January 7): 123–130.

Martín-Vivaldi, M., J.J. Soler, J.M. Peralta-Sánchez, L. Arco, A.M. Martín-Platero, M. Martínez-Bueno, M. Ruiz-Rodríguez, and E. Valdivia. 2014. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. *Journal of Animal Ecology* 83, no. 6: 1289–1301.

Masso-Silva, J.A., and G. Diamond. 2014. Antimicrobial peptides from fish. *Pharmaceuticals* 7: 265 – 310.

Mazzoli, R., E. Pessione, M.G. Giuffrida, P. Fattori, C. Barello, C. Giunta, and N.D. Lindley. 2007. Degradation of aromatic compounds by *Acinetobacter radioresistens* s13: growth characteristics on single substrates and mixtures. *Archives of Microbiology* 188, no. 1: 55–68.

McKenzie, V.J., R.M. Bowers, N. Fierer, R. Knight, and C.L. Lauber. 2012. Co-Habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME Journal* 6, no. 3: 588–596.

Miller, A.K., B. Maritz, S. McKay, X. Glaudas, and G.J. Alexander. 2015. An Ambusher's Arsenal: Chemical Crypsis in the Puff Adder (Bitis Arietans). *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1821.

Mujumdar, S.S., S.P. Bashetti, and B.A. Chopade. 2014. Plasmid pupi126-encoded pyrrolnitrin production by *Acinetobacter haemolyticus* a19 isolated from the rhizosphere of wheat. *World Journal of Microbiology and Biotechnology* 30, no. 2: 495–505.

Naing, K.W., M. Anees, S.J. Kim, Y. Nam, Y.C. Kim, and K.Y. Kim. 2014. Characterization of antifungal activity of *Paenibacillus ehimensis* KWN38 against soilborne phytopathogenic fungi belonging to various taxonomic groups. *Annals of Microbiology* 64, no. 1: 55–63.

Nazipi, S., S.G. Vangkilde-Pedersen, M.M. Busck, D.K. Lund, I.P.G. Marshall, T. Bilde, M.B. Lund, and A. Schramm. 2021. An antimicrobial *Staphylococcus sciuri* with broad temperature and salt spectrum isolated from the surface of the African social spider, stegodyphus dumicola. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 114, no. 3: 325–335.

Nicolas, P., and A. Mor. 2014. Peptides as weapons against microorganisms in the chemical defence system of vertebrates. *Annual Review of Microbiology* 49: 227 – 304.

Niederle, M. v., J. Bosch, C.E. Ale, M.E. Nader-Macías, C. Aristimuño Ficoseco, L.F. Toledo, A. Valenzuela-Sánchez, C. Soto-Azat, and S.E. Pasteris. 2019. Skin-associated lactic acid bacteria from north American bullfrogs as potential control agents of *Batrachochytrium dendrobatidis*. *PLoS ONE* 14, no. 9.

Nishimura, Y., T. Ino, and H. Iizuka. 1988. *Acinetobacter radioresistens* Sp. Nov. isolated from cotton and soil. *International Journal of Systematic Bacteriology* 38, no. 2: 209–211.

Parry, D.A.D. 2021. Structures of the ss-keratin filaments and keratin intermediate filaments in the epidermal appendages of birds and reptiles (Sauropsids). *Ecosphere* 7, no. 8: e01439

Passera, A., M. Rossato, J.S. Oliver, G. Battelli, G.I.R. Shahzad, E. Cosentino, J.M. Sage, et al. 2021. Characterization of *Lysinibacillus fusiformis* Strain S4C11: in vitro, in planta, and in silico analyses reveal a plant-beneficial microbe. *Microbiological Research* 244: 126665.

Patel, T.Y., M. Buttner, D. Rivas, C. Cross, D.A. Bazylinski, and J. Seggev. 2018. variation in airborne fungal spore concentrations among five monitoring locations in a desert urban environment. *Environmental Monitoring and Assessment* 190: 634.

Pereira, H.C., D.O. Gomes, L.Q.L. Hirano, A.L.Q. Santos, and A.M.C. Lima. 2017. Oral microbiota in healthy *Bothrops atrox* (Serpentes: Viperidae) and in snakes with stomatitis. *Acta Veterinaria Brasilica* 11, no. 3: 180–183.

Pessione, E., S. Divari, E. Griva, M. Cavaletto, G.L. Rossi, G. Gilardi, and C. Giunta. 1999. Phenol hydroxylase from *Acinetobacter radioresistensis* is a multicomponent enzyme: Purification and characterization of the reductase moiety. *European Journal of Biochem*. Vol. 265. 549 – 555.

Qader, M.M., A.A. Hamed, S. Soldatou, M. Abdelraof, M.E. Elawady, A.S.I. Hassane, L. Belbahri, R. Ebel, and M.E. Rateb. 2021. Antimicrobial and antibiofilm activities of the fungal metabolites isolated from the marine endophytes *Epicoccum nigrum* M13 and *Alternaria alternata* 13a. *Marine Drugs* 19, no. 232.

Rappoport, Z. 2003. *The Chemistry of Phenols*. John Wiley & Sons, England.

Résière, D., C. Olive, H. Kallel, A. Cabié, R. Névière, B. Mégarbane, J.M. Gutiérrez, and H. Mehdaoui. 2018. Oral Microbiota of the Snake Bothrops Lanceolatus in Martinique. *International Journal of Environmental Research and Public Health* 15: 2122

Ripamonti, A., L. Alibardi, G. Falini, S. Fermani, and M. Gazzano. 2009. Keratin-Lipid structural organization in the corneous layer of snake. *Biopolymers - Peptide Science Section* 91, no. 12: 1172–1181.

Ruiz-Rodriguez, M., E. Valdivia, J.J. Soler, M. Martin-Vivaldi, A.M. Martín-Platero, and M. Martínez-Bueno. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology* 212, no. 22: 3621–3626.

Sakthivel, K., K. Manigundan, R.K. Gautam, P.K. Singh, Yogeshwari, T. Subramani, S.K. Sharma, and R. Khande. 2018. Bioefficacy of antimicrobial peptide biosynthesis-gene-linked antagonistic *Lysinibacillus sphaericus* strains for management of bacterial plant diseases in Andaman and Nicobar Islands, India. *Journal of Environmental Biology* 39, no. 4 (July 1): 517–521.

Shabanamol, S., M. Thampi, P. Sajana, S. Varghese, S. Karthika, T.K. George, and M.S. Jisha. 2021. Characterization of the major antifungal extrolite from rice endophyte *Lysinibacillus sphaericus* against *Rhizoctonia solani*. *Archives of Microbiology* 203, no. 5: 2605–2613.

Simmaco, M., G. Mignogna, and D. Barra. 1998. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 47, no. 6: 435–450.

Singh, I. 2018. *Microornamentation on snake scales*. Masters of Science dissertation. University of the Witwatersrand.

Smith, S.N., T.J. Colston, and C.D. Siler. 2021. Venomous Snakes Reveal Ecological and Phylogenetic Factors Influencing Variation in Gut and Oral Microbiomes. *Frontiers in Microbiology* 12: 657754.

Soler, J.J., M. Martin-Vivaldi, J.M. Peralta-Sanchez, and M. Ruiz-Rodriguez. 2014. Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. *The Open Ornithology Journal* 3, no. 1: 93–100.

Soveri, T., E. -Riitta, and S. Soveri. 1986. Aerobic oral bacteria in healthy captive snakes. *Acta Vet. Scand*. Vol. 27: 172–181.

Spinner, M., A. Kovalev, S.N. Gorb, and G. Westhoff. 2013. Colouration in *Bitis rhinoceros*: 1–9.

Suay, I., F. Arenal, F.J. Asensio, A. Basilio, M.A. Cabello, M.T. Díez, J.B. García, et al. 2000. Screening of basidiomycetes for antimicrobial activities. *Antonie van Leeuwenhoek*. Vol. 78: 129 – 139.

Sugijanto, N.E., and B.L. Dorra. 2016. Antimicrobial activity of *Cladosporium oxysporum* endophytic fungus extract isolated from *Aglaia odorata lour*. *Indonesian Journal of Medicine* 01, no. 02: 108–115.

Sumi, C.D., B.W. Yang, I.C. Yeo, and Y.T. Hahm. 2015. Antimicrobial Peptides of the Genus Bacillus: A New Era for Antibiotics. *Canadian Journal of Microbiology* 61, no. 2: 93–103.

Ugwuodo, C.J., and T.N. Nwagu. 2020. Stabilizing enzymes by immobilization on bacterial spores: a review of literature. *International Journal of Biological Macromolecules* 166: 238–250.

Walke, J.B., M.H. Becker, S.C. Loftus, L.L. House, G. Cormier, R. v. Jensen, and L.K. Belden. 2014. Amphibian skin may select for rare environmental microbes. *ISME Journal* 8, no. 11: 2207–2217.

Walsh, T.R., and R.A. Howe. 2004. Antimicrobial Agents. *World Intellectual Property Organization International Bureau* WO 2004/072093, no. A2..

Wang, X., M.M. Radwan, A.H. Taráwneh, J. Gao, D.E. Wedge, L.H. Rosa, H.G. Cutler, and S.J. Cutler. 2013. Antifungal activity against plant pathogens of metabolites from the endophytic fungus *Cladosporium* cladosporioides. *Journal of Agricultural and Food Chemistry* 61, no. 19: 4551–4555.

Yalage Don, S.M., L.M. Schmidtke, J.M. Gambetta, and C.C. Steel. 2021. Volatile organic compounds produced by *Aureobasidium pullulans* induce electrolyte loss and oxidative stress in *Botrytis cinerea* and *Alternaria alternata*. *Research in Microbiology* 172: 103788.

Yarza, P., P. Yilmaz, E. Pruesse, F.O. Glöckner, W. Ludwig, K.H. Schleifer, W.B. Whitman, J. Euzéby, R. Amann, and R. Rosselló-Móra. 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S RRNA Gene Sequences. *Nature Reviews Microbiology* 12, no. 9: 635–645.

Zancolli, G., D. Mahsberg, W. Sickel, and A. Keller. 2015. Reptiles as reservoirs of bacterial infections: real threat or methodological bias? *Microbial Ecology*: 579–584.

CHAPTER 4: METABOLIC RATE AND ASSOCIATED METABOLIC BY-PRODUCTS OF THE PUFF ADDER (*BITIS ARIETANS*)

4.1. ABSTRACT

The assumption that all species produce odorous metabolic by-products has resulted in the dogmatic opinion that no species can be truly chemically cryptic. As such, one leading theory posited that the chemically cryptic puff adder (*Bitis arietans*) may have such a low standard metabolic rate that the associated odorous by-product are maintained below a detectability threshold. Alternatively, the puff adder may be producing metabolic by-products that are simply undetectable within an ecological framework; receiver species may simply not possess the necessary olfactory receptor cells that would facilitate detection. To evaluate these two theories, I measured the standard metabolic rate of the puff adder using closed-system respirometry and assessed the puff adder's volatilome using solid-phase microextraction and gas-chromatography mass-spectrometry. The puff adder does not have a low standard metabolic rate compared to other snake species. Indeed, its metabolic rate is higher than that of several, chemically conspicuous species. Additionally, I found that the puff adder's volatilome is predominantly comprised of alkanes known to odourless, as well as a known odour-absorbing, volatility-reducing carboxylic acid on the surfaces its dorsal scales. Many of the associated odorous alkanes are also recognised as secondary metabolites (i.e., produced by microbiota), suggesting that the puff adder's microbial assemblage may be directly involved in shaping this ambusher's volatilome. As such, the puff adder's chemical crypsis may be driven by several mechanisms working in concert to produce odourless metabolic by-products, and by waxy compounds on the dorsal scales that trap otherwise odorous volatiles.

4.2. INTRODUCTION

Metabolic by-products are typically odorous (Conover 2007) and in ectotherms, metabolite production can be greatly affected by several factors. First, given the thermal effects on enzymatic function and their role in biochemical reactions (Hochachka and Somero 1984), temperature therefore greatly influences metabolic rates (Hawkins 1995; Gillooly et al. 2001; White et al. 2006). In addition, metabolic rates have been shown to be closely aligned to life history strategies (Beaupre and Montgomery 2007). For example, active foragers require extended periods of sustained energy with which to search for and pursue prey. As a result, active foragers typically have high metabolic rates (Dupoué, et al 2017), coupled with high levels of haematocrit for improved oxygen delivery (Lourdais et al. 2014), resulting in overall high levels of endurance capacity (Clemente et al. 2009). In contrast, ambush foragers require short-burst energy for prey-strike and are thus considered low energy specialists (Bedford and Christian 1998) with low metabolic rates and low levels of haematocrit (Brischoux et al. 2011). Indeed, the lowest haematocrit levels among snakes on average belong to two of the most extreme ambushing clades, viperids (e.g., the puff adder) and boids (Brischoux et al. 2011), suggesting that, for snakes, viperid and boid metabolic rates may too be among the lowest.

The biggest factor affecting metabolic rates however is mass. Allometry alone accounts for > 90% of the variation among species (White 2011) since larger animals simply have higher energy requirements than do smaller ones. Additionally, thermal physiology further compounds this mass

effect since endothermy is simply more energetically costly than is ectothermy. For example, a mammal's field metabolic rate may be 12 times higher than that of an equally sized reptilian counterpart, which in turn, may have a field metabolic rate 20 times lower than that its equally-sized avian counterpart (Nagy 2005). The metabolic difference between thermal physiologies persists even when body temperature is consistent (~15 % differential between equally sized mammals and reptiles; Kingsbury 1995). Thus, the associated energy requirements of an extreme ambush-foraging reptile, such as the puff adder, could be so low that it simultaneously confers a highly beneficial exaptation – chemical crypsis. With a suitably low metabolic rate, by-product production could potentially be maintained below a biologically detectable threshold. Certainly, viperid haematocrit levels (Brischoux et al. 2011) are suggestive of very low metabolic rates among this snake family and as such, the puff adder may meet this potential threshold condition.

Odorous metabolic by-products beyond those generated by the species itself also need to be considered when it comes to a species' odour profile. For example, by-products associated with a species' microbiome may be of significance. As molecular techniques improve, the role of microbiota in animal chemical signalling is slowly being revealed (Ezenwa and Williams 2014). This phenomenon is most widely known in mammals and insects due to research focus but is not without precedent in reptiles. For example, Theis et al. (2013) show that shifts in associated microbial assemblages covary with sex and reproductive state in the spotted hyena (*Crocuta crocuta*), resulting in differences in associated odour profiles used in chemical signalling. Additionally, bacteria within the anal pocket of Indian mongoose (*Herpestes auropunctatus*) contribute volatile metabolites that determine individual chemical signatures (Gorman et al. 1974; Gorman 1976). For insects, metabolites of gut bacteria are the main constituent of the aggregation pheromone in desert locusts (*Schistocerca gregaria*; Dillon et al. 2000). For reptiles, femoral secretions involved in social communication in the Algerian sand racer (*Psammodromus algirus*), a lacertid, may in part be comprised of microbial metabolites (Martín and López 2006). Though much of this research has not shown causality between microbial metabolites and their specific role in animal chemical signalling, one has. Li et al. (2013) demonstrated in laboratory mice (*Mus musculus*) that by limiting the precursor to the highly volatile bacterial metabolite trimethylamine (TMA) commonly found in mice urine, or indeed by removing the bacteria responsible for its production via antibiotic treatment, mice produced TMA-depleted urine that was much less attractive to conspecifics. As discussed previously (see Chapter 3), the puff adder is host to a wide range of microbial species, including known odour-producing and known odour-reducing species. It would therefore be naïve to disregard the potential contribution of associated microbial metabolites within its chemical signature.

The puff adder is chemically cryptic within an ecological framework (Miller et al. 2015). Dogs, meerkats, elephants and snouted cobras are all unable to locate the puff adder using airborne chemical cues (Miller et al. 2015; chapter 2). However, within the constraints of ecology, for an organism to be chemically detectable, several conditions need to be met. First, compounds associated with the species must be volatile. For a compound to be volatile, it must typically have a molecular weight < 300 and a vapour pressure above 0.01 mmHg at ambient temperatures (Rawson 2000). Second, the receiver must possess the ability to detect the odours by means of their olfactory receptor cells, which is governed via the number of olfactory receptor genes the receiver has (of which African elephants have the highest number of any mammal tested to date; Niimura et al. 2014). Third, detected compounds must have biological relevance so as to establish and maintain cue-recognition by the detector species. Dogs, meerkats and snouted cobras are all natural predators of the puff adder (Miller et al. 2015, chapter 2) and should therefore naturally associate any puff adder specific-odours with a potential feeding opportunity. Or, in the case of the elephants, a trained positive association can be established through conditioning if any elephant-detectable volatiles are present. While we can be sure that conditions two and three were not met for any of our selected biosensors, condition one cannot be ruled out without specifically assessing the puff adder's associated volatiles outside of an ecological framework using mechanical techniques such as solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS).

To assess whether puff adders produce any volatile compounds, biologically-detectable or not, I conducted headspace analyses of puff adders using SPME and GC-MS techniques. Headspace analyses were also performed for a sympatric, chemically conspicuous species, the brown house snake (*Boaedon capensis*) to serve as a control. I assessed compounds directly associated with the puff adder's dorsal scales via directly swabbing theses surfaces with sterile swabs dipped in distilled water. Swab-collected compounds were assessed using GC-MS and compared to those collected via SPME. Additionally, I assessed whether the puff adder's volatile production was reduced as a result of low standard metabolic rates (as suggested by their cladistically low haematocrit levels; Brischoux et al. 2011). This was achieved by measuring the adder's oxygen consumption over a range of temperatures using closed-system respirometry.

4.3. MATERIALS AND METHODS

4.3.1. *Animal Husbandry*

A laboratory population of 10 adult puff adders and eight brown house snakes (*Boaedon capensis*; used in GC-MS trials) were used for sampling. Nine of the puff adders were lab-borne to wild mothers from the Dinokeng region of South Africa and one was a wild-caught, adult female from the same area. Between trials, snakes were individually housed within terraria that were lined with newspaper and

equipped with rocks and hide-out. Water was provided *ad libitum.* Terraria were maintained in species-specific, temperature controlled rooms and snakes were fed fortnightly on purpose-bred, dead mice. Snakes were however starved for two-weeks prior to any experimental protocols.

4.3.2. *Standard Metabolic Rates*

Lock & Lock™ containers were fitted with Argyle™ Universal Green Bubble Tubing and three-way Luer locks. Thereafter, the internal volume of each container (tubing included) was measured using the respective water volumes. A suitable animal density measure was calculated using mean mass and volume measures made on dead puff adder specimens (n = 5). Snake volume was calculated using water displacement techniques and the subsequent puff adder-specific density value (0.74 gcm^{-3}) was found to be lower than the recommended standard (0.98 gcm^{-3}; Reginald et al. 2014). As such all our calculations involving density, 0.74 gcm^{-3} was used. Standard metabolic rates were measured using closed-system respirometry (Fig 4.2). Snakes (n = 10) were placed individually inside the adapted Lock & Lock containers and temperature-acclimated for a period of one hour inside a dark incubator (Fig. 4.1). During acclimation, lure locks remained open. After the acclimation period, the container air was circulated by repeatedly drawing 100 ml into an empty syringe and pumping this volume back into the container several times (i.e., in a pump action). Immediately after, a 30 ml baseline sample of each container air was drawn, after which all lure locks were closed. Snakes were left for a known period inside the incubator until such time the sampling process (including air circulation) was repeated once again for the final sample. Measures of oxygen consumption was made using a Sable Systems FC-1B O_2 Analyzer and all data were recorded, transformed, and analysed using Expedata v.1.9.13 (Sable Systems International, USA). Measures of oxygen consumption were made at 7, 10, 20, 25 and 30 °C.

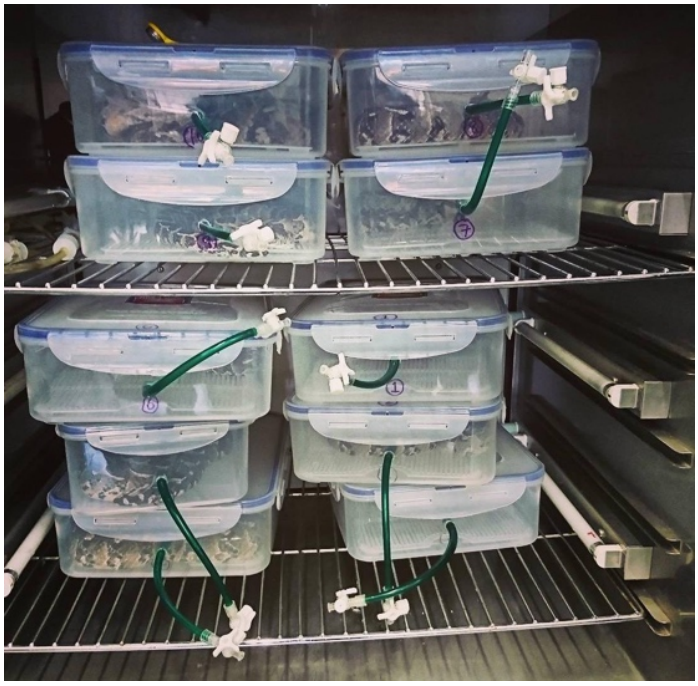


Figure 4.1 Temperature-acclimated puff adders (*Bitis arietans*) contained within airtight Lock & Lock containers (i.e., closed system) within an incubator. Samples (30 ml each) were drawn via three-way Luer locks (white) at the beginning and end of temperature treatments of known duration to assess standard metabolic rates (SMR) via changes in oxygen concentration.

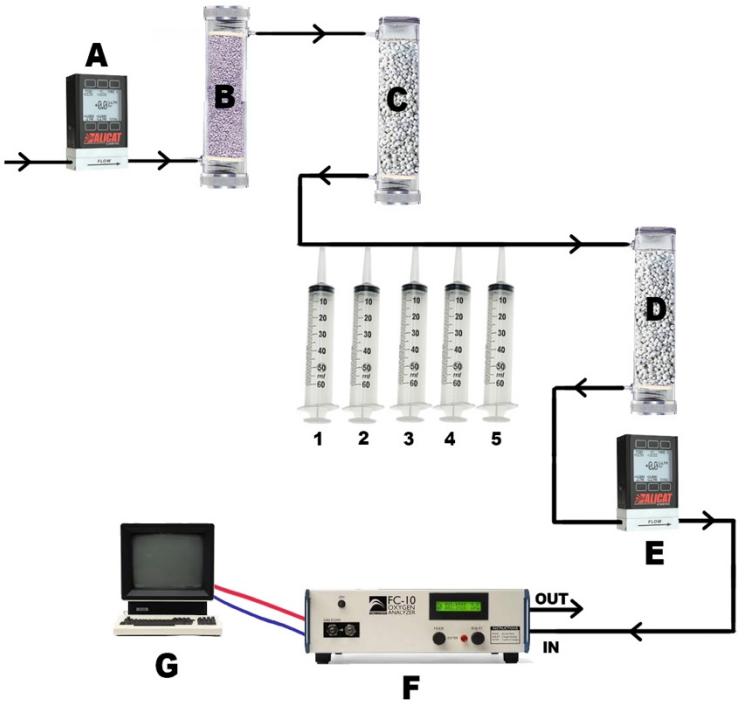


Figure 4.2. Oxygen consumption measures were made using closed-system respirometry: environmental air is pumped through an air line at 100 ml/min (controlled via an Alicat Scientific Flow Meter; A); scrubbed of water via a Drierite scrubber (B) and of carbon dioxide (CO_2) via a soda lime-filled scrubber (C). Samples are injected one at a time via luer lock (1-5) into the H_20- and CO_2-free air line, and scrubbed again for CO_2 (D) before having the flow-rate corrected once more by another Alicat Scientific Flow Meter (E). Thereafter injected samples are carried via the air line into a Sable Systems Oxygen Respirometer (F) to measure oxygen concentration. Thereafter data are processed via an accompanying computer (G).

4.3.3. *Solid-Phase Microextraction (SPME) Gas Chromatography-Mass Spectrometry (GC-MS)*

Puff adders (n = 8) and brown house snakes (n = 6) were individually placed in purpose-built glass desiccators through which filtered air was pumped at 0.5 $l.hr^{-1}$ (Fig. 4.3). Temperature of the desiccators were controlled using a water bath (Fig. 4.3, bottom right), whereby warm temperatures were achieved via the use of two, 200 W Dophyn fish tank heaters and cold temperatures via the use of icepacks. Snake volatiles were assessed by placing cool snakes into sterile desiccators and allowed to warm to 36 °C over a period of 100 minutes, during which the tri-phase (divinylbenzene/carboxen/polydimethylsiloxane) grey fibre positioned inside the airflow outlet (Fig. 4.3, B) was continuously exposed. This continuous exposure allowed the capture of any associated volatiles released during both the warming and thermal set-point periods. Solid-phase microextraction (SPME) of the fibre's contents were performed on a LECO Pegasus BT (SPME) at LECO Africa (Johannesburg, South Africa) using the following methods:

- GC method: Agilent 7890A; RXI-5MS column, 35°C – 300°C @ 10°C/min
- MS method: 10 s/s, source: 250°C, transfer line: 280°C, solvent delay: 30s
- Data Processing method: S/N 10, National Institute of Standards and Technology (NIST) 2015 library.

Fibre conditioning and appropriate background samples were performed prior to any experimental sampling to eliminate noise. In addition to headspace analyses, sterile ∑-Transwabs (Medical Wire, UK), dipped in either distilled water or hexane (C_6H_{14}), were rubbed vigorously over the dorsal scales of individual puff adders and immediately transferred to sterile glass containers for later analyses via GC-MS using the above GC-MS methods. Only peaks there identified by the NIST library were analysed and given that we are interested in species-specific and not individual odour profiles, compounds common to all individuals of a species are reported.



Figure 4.3. Left: A brown house snake (*Boaedon capensis*) temporarily contained within a purpose-built desiccator through which filtered air is being pumped at 0.5 $l.hr^{-1}$ (A). Volatile collection is achieved through an exposed tri-phase, grey, solid-phase microextraction fibre (B) positioned within the outlet pipe. Top right: A close up of a puff adder (*Bitis arietans*) temporarily contained within a species-specific, purpose-built desiccator. Bottom right: desiccator set-ups were placed within water baths for assessment of volatile production across a thermal range. Warm water temperatures were controlled via the use of two 200W Dophin fish tank heaters (C).

4.4. RESULTS

4.4.1. Standard Metabolic Rates

Temperature significantly affected puff adder SMR ($R^2 = 0.77$; $n = 33$; $p < 0.01$; Fig. 4.4). For intraspecific comparisons of SMR, data were mass-adjusted since SMRs are significantly affected by body mass (White et al 2006; Table 4.1). Overall, the puff adder was found to have a higher SMR than 14 other snake species, similar to the SMRs of two colubrids, the Brazilian false coral snake (*Oxyrhopus trigeminus*) and the Serra snake (*Tropidodryas serra*), and lower than those of 30 other species. For comparing the potential scope of odour-production however, absolute SMR values (i.e., not weight-adjusted values) provide better insight since the total potential volume of odour is more important than that of odour volume relative to the snake's body size. Thus, when considering absolute values, the puff adder has a higher SMR than that of 18 other snake species, including several species known to be odorous such as our frequently selected control species, the brown house snake (*B. capensis*), and several python species (e.g., *Python bivittatus progschai*, *P. molurus*).

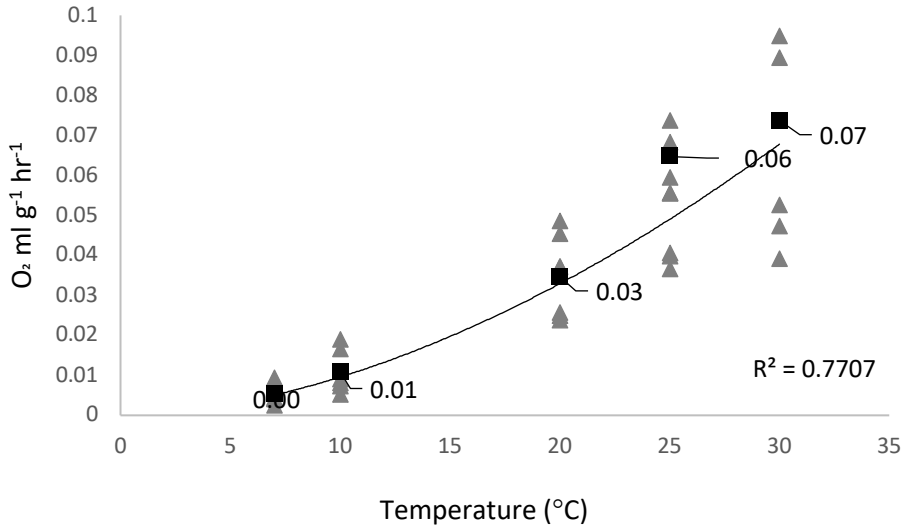


Figure 4.4. Standard resting metabolic oxygen consumption (ml g^{-1} hr^{-1}) for puff adders (Bitis arietans) measured over a temperature gradient. Black squares indicate mean measures; grey triangles represent individual data. Trend line represents a power regression fitted for individual data with the equation $y = 0.0002x^{1.7823}$; $R^2 = 0.77$; n = 33; $p < 0.01$.

Table 4.1. Previously reported temperature-dependent oxygen consumption (ml.g^{-1} hr^{-1}) for various snake species and mass-adjusted (ml.g^{-1} hr^{-1}/g) comparisons against our recorded puff adder (Bitis arietans) data. Values highlighted in blue, yellow, and red indicates SMRs lower, around the same and higher than those of the puff adder. Values with no highlight indicate values for temperatures outside of our tested range.

Species (reported as)	Mass (g)	Body Temp. (°C)	O_2 consumption (ml g^{-1} hr^{-1})	Mass-adjusted O_2 consumption (ml g^{-1} hr^{-1})	References
Bitis arietans	624	7	0.005	0.801^{E-4}	This study
		10	0.011	0.160^{E-4}	
		20	0.035	0.481^{E-4}	
		25	0.065	0.962^{E-4}	
		30	0.074	1.112^{E-4}	
Boa constrictor amarali	3270	20	0.018	0.056^{E-4}	Galvao et al. 1965
	4000 – 13000	21	0.009	0.010^{E-4}	Benedict 1932
		28	0.019	0.022^{E-4}	
		32	0.030	0.036^{E-4}	
Boaedon capensis (*Lamphroiphis fulihinosis*)	16.7 ± 0.3	25	0.041	24.371^{E-4}	Roe et al. 2004
Cerastes cerastes (*Cerastes cerastes gasperettti*)	121	20	0.024	1.983^{E-4}	Al-Sadoon 1991
		30	0.071	5.868^{E-4}	

Chironius quadricarinatus	61	20	0.034	5.607 E-4	Galvao et al. 1965
Crotalus atrox	2000 – 5000	17	0.008	0.022 E-4	Benedict 1932
		22	0.016	0.045 E-4	
		29	0.031	0.089 E-4	
		36	0.061	0.174 E-4	
		43	0.109	0.311 E-4	
Crotalus viridis	301	35	0.080	2.658 E-4	Ruben 1976
Vipera berus	63	25	0.090	14.286 E-4	Johansen and Lykkeboe 1979
Hydrodynastes gigas (*Cyclagras gigas*)	2680	20	0.032	0.120 E-4	Galvao et al. 1965
Dipsas albifrons	22	20	0.036	16.364 E-4	Galvao et al. 1965
Palusophis bifossatus (*Dryadophis bifossatus bifossatus*)	735	20	0.047	0.634 E-4	Galvao et al. 1965
Drymarchon corais	2000 - 3000	17	0.021	0.082 E-4	Benedict 1932
		22	0.021	0.082 E-4	
		29	0.036	0.142 E-4	
		30	0.052	0.206 E-4	
Epicrates angulifer	11000 - 13000	16	0.004	0.004 E-4	Benedict 1932
		21	0.009	0.007 E-4	
		28	0.016	0.013 E-4	
		31	0.030	0.025 E-4	
		32	0.020	0.017 E-4	
Eunectes murinus	11300	20	0.021	0.018 E-4	Galvao et al. 1965
Eunectes notaeus	14400	20	0.015	0.010 E-4	Galvao et al. 1965
Lampropeltis californiae (*Lampropeltis getula*)	221-319	20	0.081	< 0.001 E-4	Baldwin 1928
	1217	26	0.028	0.230 E-4	Davies 1982
Lampropeltis miliaris	401	20	0.034	0.848 E-4	Abe and Mendes 1980
		25	0.048	1.197 E-4	
		30	0.078	1.945 E-4	

Erythrolamprus poecilogyrus schotti (*Leimadophis poecilogyrus schotti*)	42	20	0.039	9.286 E-4	Galvao et al. 1965
Liophis miliaris	55	20	0.024	4.291 E-4	Galvao et al. 1965
Zamenis situla (Coluber natrix; Tripodonotus natrix)	84	11	0.040	4.714 E-4	Hill 1911
		16	0.071	8.429 E-4	
		24	0.115	13.690 E-4	
	113	18	0.031	2.708 E-4	
Oxyrhopus trigeminus	98	20	0.035	3.592 E-4	Galvao et al. 1965
Philodryas olfersii latirostris (*Philodryas olfersii*)	176	20	0.039	2.216 E-4	Galvao et al. 1965
Philodryas patagoniensis	388	20	0.055	1.410 E-4	Galvao et al. 1965
Tropidodryas serra (*Philodryas serra*)	145	20	0.035	2.414 E-4	Galvao et al. 1965
Pituophis melanoleucus mugitus (*Pituophis melanoleucus*)	40 – 584	20	0.038	1.218 E-4	Galvao et al. 1965
	422-822	25	0.058	0.936 E-4	
Python molurus	5000	18	0.007	0.014 E-4	Benedict 1932
		25	0.016	0.032 E-4	
		31	0.027	0.054 E-4	
		37	0.040	0.079 E-4	
Python bivittatus progschai (*Python molurus bivittatus*)	12370	27	0.011	0.009 E-4	Hutchison et al. 1966
		30	0.020	0.016 E-4	
		33	0.028	0.023 E-4	
Malayopython reticulatus saputrai (*Python reticulatus*)	32000	17	0.008	0.002 E-4	Benedict 1932
		22	0.011	0.004 E-4	
		24	0.012	0.004 E-4	
		26	0.016	0.005 E-4	
		28	0.026	0.008 E-4	
		30	0.029	0.009 E-4	
Malpolon moilensis	145	20	0.038	2.621 E-4	Al-Sadoon 1991
		30	0.079	5.448 E-4	
Natrix natrix	84	20	0.090	10.714 E-4	Hill 1911
Nerodia rhombifera	238	20	0.069	2.521 E-4	

		30	0.095	3.992 E-4	Jacobson & Whitford 1970
Dipsas mikania (*Sibynomorphus mikanii*)	11	20	0.113	102.727 E-4	Galvao et al. 1965
Thamnodynastes strigatus	55	20	0.041	7.455 E-4	Galvao et al. 1965
Vipera berus	70	20	0.064	9.143 E-4	Al-Sadoon 1991
		30	0.135	19.286 E-4	
Xenodon guentheri	50	30	0.135	27.000 E-4	Galvao et al. 1965
Xenodon merremii	502	20	0.043	0.857 E-4	Galvao et al. 1965
Xenodon neuwiedii	53	20	0.037	6.981 E-4	Galvao et al. 1965
Diadophis punctatus	5	20	0.056	0.011	Buikema & Armitage 1969
	4.4	30	0.132	0.030	
Elaphe gutata	800	25	0.038	0.038 E-4	Smith 1976
Helicops modestus	196	20	0.035	1.786 E-4	Abe & Mendes 1980
		25	0.040	2.041 E-4	
		35	0.055	2.806 E-4	
Sphalerosophis diadema (*Sphalerosophis cliffordii*)	351	30	0.136	3.875 E-4	Dmi'el and Bout 1972
Storeia dekayi	7.2	20	0.088	122.222 E-4	Clausen 1936
		30	0.163	225.695 E-4	
Salvadora hexalepis	65	20	0.046	7.077 E-4	Jacobson and Whitford 1971
		30	0.107	16.450 E-4	
Thamnophis butleri	19	25	0.048	25.474 E-4	Kamel and Gatten 1983
Thamnophis proximus	31	20	0.075	21.142 E-4	Jacobson and Whitford 1971
		30	0.150	48.387 E-4	
Thamnophis sirtalis	25	20	0.070	28.000 E-4	Aleksiuk 1976
	38		0.040	10.526 E-4	
	200	29	0.033	1.650 E-4	Taylor & Davies 1981

Hydrophis platurus (*Pelamis platurus*)	116	30	0.06	5.172^{E-4}	Graham 1974

4.4.2. Solid-Phase Microextraction (SPME) Gas Chromatography-Mass Spectrometry (GC-MS)

Species-wide volatile organic compound (VOC) profiles did not overlap at all between the puff adders and the brown house snakes. Thus no statistical analyses were necessary. The puff adder's VOC profile, like that of the brown house snake, was predominated by alkanes, which as a group are commonly referred to as 'odourless' in chemistry textbooks. For the puff adders, only three compounds, one phenol and two alkanes, were common to both headspace and swab samples (Table 4.2.). Of these, octadecane and 2,4-di-tert-butylphenol (2,4 DTBP) are known secondary metabolites (Pelyuntha et al. 2020; Zhao et al. 2020; Prakash and Arora 2021) that are produced mostly via microbial metabolism. Heptadecane, 2,6,10,14-tetramethyl-, nonane, 2,2,4,4,6,8,8-hetpamethyl- and dodecan,1-iodo are respective isomers of heneicosane, heptadecane and 1-iodo-2-methylundecane, all of which have roles in chemical signalling in various taxa (Achiraman et al. 2010; Khannoon et al. 2011). The sole carboxylic acid detected in the puff adder's VOC profile was octadecanoic acid was detected only in swab samples. This saturated fatty acid, reported to have a mild odour (see: https://pubchem.ncbi.nlm.nih.gov/compound/Stearic-acid), is solid at temperatures below 69.3 °C and is unlikely to contribute any odour cues to the adder's odour profile. In comparison, the brown house snakes' VOC profile was predominately made up of 2,2-diethylbutyraldehyde, 1-Iodo-2-methylundecane, n,n-dimethylundecane and n-methylundecane isomers. Although not every individual shared the same isomers, all individual brown house snake VOC profiles possessed at least one isomer of n-methylundecane and n,n-dimethylundecane each, and thus they are reported as species-wide compounds.

Table 4.. Volatile organic compound (>75% NIST matches) profiles for the puff adder (Bitis arietans) and the brown house snake (Boaedon capensis), assessed via solid-phase microextraction (SPME) Gas Chromatography-Mass Spectrometry (GC-MS).* indicates compounds previously identified in reptilian femoral secretions (refs), • indicates compounds whose isomers have been identified in reptilian femoral secretions and † indicates compounds (or their isomers) that have been previously identified as pheromones in other taxa.

Species	Compound (sample source)	Chemical Formula	Chemical Class	Retention Time	Reported odour
Bitis arietans	**2,4-Di-tert-butylphenol** (headspace & swab)	$C_{14}H_{22}O$	Phenol	(H) 12:44.6; (S) 14:08.2	-
	Octadecane* (headspace & swab)	$C_{18}H_{38}$	Alkane	(H) 10:44.0; (S) 23:12.7	Odourless
	Heptadecane* (headspace & swab)	$C_{17}H_{36}$	Alkane	(H) 10:23.5; (S) 27:52.4	Odourless
	Heptadecane, 2,6,10,14-tetramethyl-†[1] (headspace)	$C_{21}H_{44}$	Alkane	7:21.7	-
	Nonane, 2,2,4,4,6,8,8-heptamethyl- •[2] (headspace)	$C_{16}H_{34}$	Alkane	7:59.6	-
	Dodecane, 1-iodo†[3] (headspace)	$C_{12}H_{25}I$	Alkane	10:05.8	Odourless
	Pentadecane, 2,6,10,14-tetramethyl-•[4] (swab)	$C_{19}H_{40}$	Alkane	27:51.0	-
	Heptacosane* (swab)	$C_{27}H_{56}$	Alkane	22:56.1	-
	Octadecanoic acid* (swab)	$C_{18}H_{36}O_2$	Carboxylic acid	20:58.7	Pungent, oily

Boaedon capensis	**2,2-Diethylbutyraldehyde**	$C_8H_{16}O$	Aldehyde	8:33.1	-
	1-Iodo-2-methylundecane†[5]	$C_{12}H_{25}I$	Alkane	15:52.6	-
	Undecane, 2-methyl-	$C_{12}H_{26}$	Alkane	7:21.8	-
	Undecane, 3-methyl-			9:38.2	
	Undecane, 6-methyl-			5:36.1	
	Undecane, 2,4-dimethyl-	$C_{13}H_{28}$	Alkane	10:52.3	-
	Undecane, 3,8-dimethyl-			17:59.3	
	Undecane, 5,5-dimethyl-			10:02.1	
	Undecane, 5-ethyl-			21:40.4	
	Undecane, 6,6-dimethyl			3:33.5	

1 - isomer of heneicosane, a known termite pheromone; 2 - isomer of hexadecane; $C_{19}H_{40}$; 3 - isomer of 1-iodo-2-methylundecane; 4 -isomer of nonadecane; 5 - pheromone found in mice urine.

4.5. DISCUSSION

Puff adders do not rely on low standard metabolic rates as a means of reducing odour. Rather it appears that their species-specific VOC profile is comprised mainly of compounds that are reportedly odourless. In comparison to the 46 other snake species for which SMR data are available, the puff adder's SMR is higher than ~30% of the species when data are weight-adjusted and 38% of these when the data are absolute. Not only does the chemically cryptic puff adder have a SMR higher than nearly 40% of the reviewed snake species, several of these are commonly known to be chemically conspicuous such as pythons (e.g., *Python bivittatus progschai, Python bivittatus progschai*) and water snakes (e.g., *Nerodia rhombifera*), as well as the species I used as an odorous control, the brown house snake. In addition, the VOC profile of the puff adder is not only mainly comprised of compounds known to be odourless, but is also very different from that of the brown house snake, which is predominantly comprised of short-chain (i.e., more volatile) undecane derivatives that have been reported to have odours that vary from *fuel-like* to odourless, as well as a mouse pheromone (1-iodo-2-methylundecane; Achiraman et al. 2010) presumably transferred during constriction of mouse meals. These findings strongly suggest that while a low SMR may provide an added benefit in terms of controlling odour production, a low SMR in itself is not a requirement if the metabolic byproducts being produced are in any event, odourless over the body temperature range experienced by the puff adder.

Alkanes are frequently reported as being odourless. Seventy seven percent of the compounds found in the puff adders VOC are alkanes, three of the which are specifically listed as odourless in several chemical databases (see: Fisher Scientific; https://www.fishersci.se). Two of these, heptadecane and octadecane, were found on both the dorsal scales and within the headspace of puff adders. Both are solid, waxy substances at room temperature, confirmed bacterial metabolites of

Bacillus spp., and have fungicidal capabilities (Rajaofera et al. 2019). Octadecane is also an intermediate in the microbial breakdown of *n*-eicosane (Hadibarata et al. 2007) – a recognised reptile pheromone detected in several, but not all of the puff adders sampled in this study (and thus not reported in the species-specific VOC). Like *n*-eicosane, heptadecane has also been implicated as a reptile pheromone (Khannoon et al. 2011), and along with octadecane, can be further degraded by some bacterial species such as *Pseudomonas* (Shafeeq et al. 1989). Bacterial degradation of alkanes is not uncommon and as a class, alkanes are considered the most degradable of the hydrocarbons (Ehrhardt and Blumer 1972; Gilbert and Higgins 1978), while, interestingly, aromatic compounds are considered the least (Hadibarata et al. 2007). Thus, despite both being identified as bacterial metabolites, heptadecane and octadecane may secondarily provide a suitable growth medium for several species of bacteria and possibly some fungi associated with the puff adder's microbial assemblage, suggesting that there may possibly be an intricate, multi-layered involvement in the puff adder's chemical crypsis.

Further evidence within the puff adder VOC profile of a thriving microbial population (as reviewed in Chapter 3) is the presence of 2,4-di-tert-butyl-phenol (2,4 DTBP). Like heptadecane and octadecane, 2,4 DTBP was identified in both the puff adder's headspace and swab samples, indicating that it is ubiquitously associated with this species. This compound is of particular interest given its swathe of associated properties. It is recognized microbial metabolite with antioxidant and anti-inflammatory properties (Pelyuntha et al. 2020; Zhao et al. 2020). Additionally, it is also an antimicrobial to the point of autotoxicity for the producing species (Zhao et al., 2020) and due to its ability to disrupt biofilms, it can be particularly effective against antibiotic resistant species such as *Pseudomonas* and *Staphylococcus aureus* (Aissaoui et al. 2019), of which only one and zero samples were identified within the puff adder's microbiota respectively. 2,4 DTBP has been associated with 169 species to date, including 16 species of bacteria and 11 species of fungi, some of which are found within the puff adder's microbial assemblage such as *Bacillus* and *Naganishia* (as *Cryptococcus*; Viszwapriya et al. 2016; Aissaoui et al. 2019). 2,4 DTBP was also detected in a single brown house individual that is also coincidentally the only individual in which *Acinetobacter radioresistens* was found. Though *A. radioresistens* is not currently listed as a bacterial species known to produce this compound, it may be worthwhile assessing its potential as either a 2,4 DTBP producer (via metabolism) or indeed a 2,4 DTBP consumer, given that *A. radioresistens* is also known to breakdown phenols (Mazzoli et al. 2007; see chapter 3) such as 2,4 DTBP, which are known to be odorous. The presence of this compound may therefore benefit the puff adder in several ways, such as finely influencing its microbial assemblage through downregulation of certain species, odour breakdown, or

as an energy source for beneficial bacteria. However, further investigation is required to elucidate such potential roles in the adder's chemical crypsis.

The only compound found within the puff adder's VOC profile confirmed via several databases to be odorous is octadecanoic acid. This carboxylic acid, whose parent hydride is octadecane, is a saturated fatty acid that is, like octadecane and heptadecane, a waxy solid at room temperature (https://pubchem.ncbi.nlm.nih.gov). It is reported to have a mild odour (see: https://pubchem.ncbi.nlm.nih.gov), but due to its relatively high melting point (69.3 °C), is unlikely, in its association with the puff adders, to enter the air plume given that puff adders typically select target temperatures of ~32 °C (Alexander 2016). This is corroborated by fact that it was solely detected in the dorsal swab samples and not the headspace samples even when snakes were warmed to 36 °C (i.e., above their selected body temperature). Octadecanoic acid has however also been shown to serve as an absorbent for other odorous compounds such as amines (Boraphech and Thiravetyan 2015) and interestingly, materials modified with octadecanoic acid demonstrate decreased rates of volatile transmission in comparison to their non-modified counterparts (Su et al. 2019). As such, as opposed to potentially risking the puff adder's chemical crypsis through its own odorous contribution, octadecanoic acid may in fact be directly assisting in odour reduction through its entrapment of other odorous volatiles associated with the adders.

Puff adders may however be chemically conspicuous at temperatures not too far above their target temperatures. At the end of a 35 °C metabolic rate trial, a noticeably pungent, almost fishy odour, not unlike that of puff adder freshly shed skin, was evident in four of the adders' metabolic chambers. Unfortunately, due to almost consistent movement by the adders during this trial, the associated data were discarded from SMR analyses but the resultant discovery of this odour does provide two particularly interesting points of insight. First, at this temperature adders are noticeably uncomfortable and attempt to escape almost continuously despite 35 °C being considerably lower than that of a lethal limit (i.e.,~40 - 42 °C; Lueth 1941). Second, there appears to be particularly odorous compounds associated with these adders that are volatile at temperatures above their target temperatures. An evolutionary shift towards the use of more heavily-weighted compounds would result in such a situation and examples of this are not without precedent in other taxa. During the breeding season, ground nesting birds are at increased risk of predation as a result of incubation requirements and as such, shifts in the composition of preening wax from monoesters to more heavily-weighted diesters have been documented in several bird species (e.g., Reneerkens et al. 2007). This shift directly reduces associated volatiles, aiding in the birds' chemical crypsis. It appears that a similar system may be at play with regards to the chemically cryptic puff adder, which would explain why

individuals react so aversely when their body temperatures are higher than their target (i.e., 35 °C), despite there be little to no associated physiological risk of death. This potential mechanism additionally highlights a previously unconsidered cost of climate warming for this species of ambushing snake, whereby fewer opportunities to maintain body temperatures at or below target may adversely affect their ability to remain undetected.

Although not reported in the puff adder's VOC profile, 1-decanamine, N, N, dimethyl ($C_{14}H_{31}N$) was detected in several of the puff adders' headspace samples, including two of the adders associated with the pungent metabolic chambers at 35 °C. This particular amine is listed as having a '*fish-like*' odour on the National Institute of Standards and Technology (NIST) database and may contribute towards the pungent odour detected at 35 °C. It is worthwhile noting that only two of the adders associated with the odorous metabolic chambers were used in the VOC component of this work, and it remains unknown whether this amine is also present on the remaining two. Indeed, while this connection is not yet demonstrated, it does lend support to the notion that puff adders may indeed be producing odorous compounds, but that due to several mechanisms in place, these compounds are either restricted from entering the air plume via interference (i.e., via absorption into waxy compounds such as octadecanoic acid, broken down by associated bacteria/fungi) or via physical constraints such as associated molecular weight and vapour pressure. Certainly, from videography of puff adders captured in the wild, interactions with both prey and predator species are strongly suggestive that while they are unable to detect any puff adder via associated volatiles, several species may well be able to 'taste' them (discussed in Chapter 2). This would align with heavily weighted molecule explanation, as well as with the notion that otherwise-volatile compounds are simply trapped within their associated waxy layer of octadecanoic acid. Additionally, micro-ornamentation associated with the puff adder scales (discussed in Chapter 3) may, like octadecanoic acid (Su et al. 2019), serve to further reduce volatile transmission via mechanical obstruction. Though the mechanism underlying the puff adder's chemical crypsis has yet to be confirmed, scrutiny of potential mechanism to date strongly places their associated microbial assemblages as a mechanistic forerunner. It is however important to note that within the scope of this thesis, I was only interested in the volatiles associated with the species as a whole (which is also true for the brown house snake). This approach may have resulted in important volatiles being missed, which may naturally be present on most but not all snakes within a species and those which may be sex dependent. It would be worthwhile reassessing associated volatiles on an individual basis, which would require a much larger sample size of snakes.

4.6. REFERENCES

Abes, A.S. and E.G. Mendes. 1980. Effect of body size and temperature on oxygen uptake in the water snakes *Helicops modestus* and *Liophus miliaris* (Colubridae). *Comp. Biochem. Physiol.* 65a:367–370 .

Achiraman, S., G. Archunan, P. Ponmanickam, K. Rameshkumar, S. Kannan, and G. John. 2010. 1-Iodo-2 methylundecane [1I2MU]: An estrogen-dependent urinary sex pheromone of female mice. *Theriogenology* 74, no. 3: 345–353.

Aissaoui, N., M. Mahjoubi, F. Nas, O. Mghirbi, M. Arab, Y. Souissi, A. Hoceini, et al. 2019. Antibacterial potential of 2,4-di-tert-butylphenol and calixarene-based prodrugs from thermophilic *Bacillus licheniformis* Isolated in Algerian Hot Spring. *Geomicrobiology Journal* 36, no. 1: 53–62.

Al-Sadoon, M. 1991. Metabolic rate-temperature curves of the horned viper, *Cerastes cerastes gasperetti*, the moila snake, *Malpolon moilensis*, and the adder, *Vipera berus. Comparative Biochemistry and Physiology, Volume* 99A, no. 1/2: 119–122.

Aleksiuk, M. 1976. Reptilian hibernation: evidence of adaptive strategies in *Thamnophis sitarlis parietalis*. *Copeia* 1976: 170 – 178.

Alexander, G. 2016. Are ambush-foraging and thermoregulation mutually exclusive. A test using Puff Adders (*Bitis arietans*). *African Herp News* 63: 26.

Beaupre, S.J., and C.E. Montgomery. 2007. The meaning and consequences of foraging mode in snakes. In *Lizard Ecology: The Evolutionary Consequences of Foraging Mode*, 334–368. Cambridge University Press.

Baldwin, F.M. 1928. Oxygen consumption at 20° in certain snakes *Pituophis sayi* and *Lampropeltis getulus holbrooki* with some notes on size and *Lampropeltis getulus holbrooki* with some notes on size and seasonal difference seasonal difference. *Proceedings of the Iowa Academy of Science* 35, no. 75:313 – 318.

Bedford, G.S., and K.A. Christian. 1998. Standard metabolic rate and preferred body temperatures in some australian pythons. *Australian Journal of Zoology* 46, 317–328.

Benedict, F.G. 1932. The Physiology Of Large Reptiles. *Carnegie Inst. Wash. Publ. No. 425. 539pp.*

Boraphech, P., and P. Thiravetyan. 2015. Trimethylamine (Fishy Odor) adsorption by biomaterials: effect of fatty acids, alkanes, and aromatic compounds in waxes. *Journal of Hazardous Materials* 284: 269–277.

Brischoux, F., G.E.A. Gartner, T. Garland, and X. Bonnet. 2011. Is aquatic life correlated with an increased hematocrit in snakes? *PLoS ONE* 6, no. 2.

Buikema, A.L. Jr. and K.B. Armitage. 1969. The effects of temperature on the metabolism of the prairie ringneck snake, *Diadophis punctatus arnyi* Kennicott. *Herpetologica* 25:194–206.

Clausen H.J. 1936. The effect of aggregation on the respiratory metabolism of the brown snake *Storeria dekayi*. *Journal of Cell. and Comparative Physiology.* 8: 376 – 386.

Clemente, C.J., P.C. Withers, and G.G. Thompson. 2009. *metabolic* rate and endurance capacity in Australian varanid lizards (Squamata: Varanidae: Varanus). *Biological Journal of the Linnean Society* 97: 664–676.

Conover, M. 2007. *Predator-Prey Dynamics: The Role of Olfaction*. Boca Raton, FL.: Taylor and Francis.

Davies, P.M.C. 1982. The ontogenetic weight dependence of metabolism in the Florida king snake, *Lampropeltis getulus foridana*. *Comparative Biochemistry and Physiology Part A: Physiology* 73A: 291 – 295.

Dillon, R., C. Vennard, and A. Charnley. 2000. Exploitation of Gut Bacteria in the Locust. *Nature* 403: 851.

Dmi'el, R., and R. Borut. 1972. Thermal behaviour, heat exchange, and metabolism in the desert snake *Spalerosophis cliffordi*. *Physiological Zoology* 45, 1: 78–94.

Dupoué, A., F. Brischoux, and O. Lourdais. 2017. Climate and Foraging Mode Explain Interspecific Variation in Snake Metabolic Rates. *Proceedings of the Royal Society B: Biological Sciences* 284, no. 1867.

Ehrhardt, M., and M. Blumer. 1972. The source identification of marine hydrocarbons by gas chromatography. *Environmental Pollution* 3, no. 3: 179–194.

Ezenwa, V.O., and A.E. Williams. 2014. Microbes and animal olfactory communication: where do we go from here? *BioEssays* 36, no. 9: 847–854.

Galvão, P.E., J. Tarasantchi, and P. Guertzenstein. 1965. Heat production of tropical snakes in relation to body weight and body surface. The *American Journal of Physiology* 209, no. 3: 501–506.

Gilbert, P.D., and I.J. Higgins. 1978. The microbial degradation of crude mineral oils at sea. *Journal of General Microbiology* 108: 63–70.

Gillooly, J.F., J.H. Brown, G.B. West, V.M. Savage, and E.L. Charnov. 2001. Effects of size and temperature on metabolic rate. *Science, New Series* 293, no. 5538: 2248 – 2251.

Gorman, M., D. Nedwell, and R. Smith. 1974. An Analysis of the Contents of the Anal Scent Pockets of Herpestes Uuropunctatus (Carnivora : Viverridae). *J. Zool* 172: 389–399.

Gorman, M.L. 1976. A mechanism for individual recognition by odour in *Herpestes auropunctatus* (carnivora: viverridae). *Animal Behaviour* Vol. 24: 141 – 145.

Graham, J.B. 1974. Aquatic respiration in the sea snake *Pelamis platurus*. *Respiration Physiology* 21, no. 1: 1–7.

Hadibarata, T., S. Tachibana, and K. Itoh. 2007. Biodegradation of n-eicosane by fungi screened from nature. *Pakistan Journal of Biological Sciences* 10, no. 11: 1804–1810.

Hawkins, A.J.S. 1995. Effects of temperature change on ectotherm metabolism and evolution: metabolic and physiological interrelations underlying the superiority of multi-locus heterozygotes in heterogeneous environments. *Journal of Thermal Biology*. Vol. 20: 23 – 33.

Hill, A.V. 1911. The total energy exchanges of intact coldblooded animals at rest. *Journal of Physiology*. 43: 379 – 393.

Hochachka, P., and G. Somero. 1984. Peter W. Hochachka, George N. Somero - *Biochemical Adaptation: Mechanism and Process in Physiological Evolution-Oxford University Press (2002)-2*. Vol. 1. New York: Oxford University Press.

Hutchinson, V., H. Dowling, and A. Vinegar. 1966. Thermoregulation in a brooding female Indian python, *Python molurus bivittatus*. *Science* 151, no. 3711: 694–696.

Jacobson, E.R. and W.G. Whitford. 1970. The effects of acclimation on physiological responses to temperature in snakes *Thamnophis proximus* and *Natrix rhombifera. Comp. Biochem. Physiol. (A)* 35:439 – 449.

Johansen, K., and G. Lykkeboe. 1979. Thermal acclimation of aerobic metabolism and O_2-Hb binding in the snake, *Vipera berus. Journal of Comparative Physiology B*. 130: 293–300.

Kamel, S., and R.E. Gatten Jr. 1983. Aerobic and anaerobic activity metabolism of limbless and fossorial reptiles. *Physiological Zoology* 56, No. 3: 419–429.

Khannoon, E.R., B. Flachsbarth, A. El-Gendy, K. Mazik, J.D. Hardege, and S. Schulz. 2011. New compounds, sexual differences, and age-related variations in the femoral gland secretions of the lacertid lizard *Acanthodactylus Boskianus*. *Biochemical Systematics and Ecology* 39, no. 2: 95–101.

Kingsbury, B.A. 1995. Field metabolic rates of a eurythermic lizard. *Herpetologica* 51, no. 2: 155–159.

Li, Q., W.J. Korzan, D.M. Ferrero, R.B. Chang, D.S. Roy, M. Buchi, J.K. Lemon, et al. 2013. Synchronous evolution of an odor biosynthesis pathway and behavioral response. *Current Biology* 23, no. 1: 11–20.

Lighton, J.B. 2008. *Measuring Metabolic Rates : A Manual for Scientists*. Oxford University Press, Inc.

Lourdais, O., G.E.A. Gartner, and F. Brischoux. 2014. Ambush or active life: foraging mode influences haematocrit levels in snakes. *Biological Journal of the Linnean Society* 111: 636 – 645.

Lueth, F.X. 1941. Effects of temperature on snakes. *Copeia* 1941, no. 3: 125 – 132.

Martín, J., and P. López. 2006. Age-related variation in lipophilic chemical compounds from femoral gland secretions of male lizards *Psammodromus algirus*. *Biochemical Systematics and Ecology* 34, no. 9: 691–697.

Mazzoli, R., E. Pessione, M.G. Giuffrida, P. Fattori, C. Barello, C. Giunta, and N.D. Lindley. 2007. Degradation of aromatic compounds by *Acinetobacter radioresistens* S13: growth characteristics on single substrates and mixtures. *Archives of Microbiology* 188, no. 1: 55–68.

Miller, A.K., B. Maritz, S. McKay, X. Glaudas, and G.J. Alexander. 2015. An Ambusher's arsenal: chemical crypsis in the puff adder (*Bitis arietans*). *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1821.

Nagy, K.A. 2005. Field Metabolic Rate and Body Size. *Journal of Experimental Biology* 208: 1621 – 1625.

Niimura, Y., A. Matsui, and K. Touhara. 2014. Extreme expansion of the olfactory receptor gene repertoire in African elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome Research* 24, no. 9: 1485–1496.

Pelyuntha, W., C. Chaiyasut, D. Kantachote, and S. Sirilun. 2020. Organic acids and 2,4-di-tert-butylphenol: major compounds of *Weissella confusa* WM36 Cell-Free supernatant against growth, survival and virulence of *Salmonella typhi*. *PeerJ* 8: e8410.

Prakash, J., and N.K. Arora. 2021. Novel metabolites from *Bacillus safensis* and their antifungal property against *Alternaria alternata*. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 114, no. 8: 1245–1258.

Rajaofera, M.J.N., Y. Wang, G.Y. Dahar, P. Jin, L. Fan, L. Xu, W. Liu, and W. Miao. 2019. Volatile organic compounds of *Bacillus atrophaeus* HAB-5 Inhibit the growth of *Colletotrichum* gloeosporioides. *Pesticide Biochemistry and Physiology* 156: 170–176.

Rawson, N.E. 2000 Human olfaction. InTheneurobiology of taste and smell, 2nd edn (eds TE Finger, WL Silver, D Restrepo), pp. 257–284

Reneerkens, J., J.B. Almeida, D.B. Lank, J. Jukema, R.B. Lanctot, R.I.G. Morrison, W.I.C. Rijpstra, et al. 2007. Parental Role Division Predicts Avian Preen Wax Cycles. *Ibis* 149, no. 4 (October): 721–729.

Roe, J.H., W.A. Hopkins, J.W. Snodgrass, and J.D. Congdon. 2004. The influence of circadian rhythms on pre- and post-prandial metabolism in the snake *Lamprophis fuliginosus*. *Comparative Biochemistry and Physiology, Part A* 139: 159–168.

Ruben, J.A. 1976. Aerobic and anaerobic metabolism during activity in snakes. *Journal of Comparative Physiology B* 109: 147 – 157.

Shafeeq, M., D. Kokub, Z.M. Khalid, A.M. Khan, and K.A. Malik. 1989. Degradation of different hydrocarbons and production of biosurfactant by *Pseudomonas aeruginosa* isolated from coastal waters *t*. *MIRCEN Journal* 5: 505 – 510.

Smith, G.C. 1976. Ecological energetics of three species of ectothermic vertebrates. *Ecology* 75, no.2: 252–264.

Su, C., H. Zhao, H. Yang, and R. Chen. 2019. Stearic acid-modified starch/chitosan composite sponge with asymmetric and gradient wettability for wound dressing. *ACS Applied Bio Materials* 2, no. 1: 171–181.

Taylor, B. M., and P.M.C. Davies. 1981. Changes in the weight dependence of metabolism during the sloughing cycle of the snake *Thamnophis sitarlis parietalis. Comparative Biochemistry and Physiology Part A* 69: 113–119.

Theis, K.R., A. Venkataraman, J.A. Dycus, K.D. Koonter, E.N. Schmitt-Matzen, A.P. Wagner, K.E. Holekamp, and T.M. Schmidt. 2013. Symbiotic bacteria appear to mediate hyena social odors. *Proceedings of the National Academy of Sciences of the United States of America* 110, no. 49: 19832–19837.

Viszwapriya, D., U. Prithika, S. Deebika, K. Balamurugan, and S.K. Pandian. 2016. In vitro and in vivo antibiofilm potential of 2,4-di-tert-butylphenol from seaweed surface associated bacterium *Bacillus* subtilis against Group A *Streptococcus*. *Microbiological Research* 191: 19–31.

White, C.R. 2011. Allometric estimation of metabolic rates in animals. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 158, no. 3: 346 – 357.

White, C.R., N.F. Phillips, and R.S. Seymour. 2006. The scaling and temperature dependence of vertebrate metabolism. *Biology Letters* 2, no. 1: 125–127.

Zhao, F., P. Wang, R.D. Lucardi, Z. Su, and S. Li. 2020. Natural sources and bioactivities of 2,4-di-tert-butylphenol and its analogs. *Toxins* 12, no. 35:

Chapter 5: CONCLUSION

In this thesis, I set out to assess two potential mechanisms driving the puff adder's chemical crypsis, namely microbial and metabolic suppression, as well as to assess the extent to which the adder's chemical crypsis applies. In Chapter 2, I demonstrate, that despite the incredible olfactory receptor repertoire of African elephants and the highly effective vomeronasal system of the snouted cobra, neither biosensor species was able to detect the puff adder via chemical cues. This, together with numerous interactions between wild puff adders and several additional predator and prey species collected via *in-situ* videography (discussed in Chapter 2) confirms that the puff adder's chemical crypsis is generalized. Generalized chemical crypsis in combination with highly effective visual crypsis is no doubt the epitome of successful subterfuge among ambushing species, and in hindsight, seems obvious that crypsis within these two modal dimensions (i.e., vision and olfaction) should evolve in concert in systems where both vision-orientated and macrosmatic predators abound. It is indeed highly unlikely that this evolutionary adaptation is limited to this isolated example among terrestrial vertebrate species.

In Chapter 3, I established that the microbial assemblages associated with the puff adder are vast in comparison to those associated with the chemically conspicuous brown house snake. I further showed that the puff adder's assemblage includes microbial species implicated in odour breakdown, as well as those known to possess antimicrobial properties that may serve to selectively manage assemblage species. Although I have not conclusively demonstrated the role of microbiota in the puff adder's chemical crypsis, I have laid the foundation for more targeted research which is beyond the scope of my thesis.

Several specific tests could be conducted to establish whether the adder's microbial assemblage underpins its chemical crypsis. In particular, puff adders could be treated with broad-spectrum antibiotics and thereafter assessed using both ecologically- and mechanically-relevant protocols (i.e., biosensor application and gas chromatography-mass spectrometry). Additionally, the role of the associated bacterial metabolite, 2,4-di-tert-butylphenol (2,4 DTBP), should be of particular focus given its ubiquitous presence within the puff adder's volatilome. This compound is commercially available, making the assessment of its antimicrobial and antifungal extent particularly accessible. Furthermore, it would serve to establish whether *Acinetobacter radioresistens*, a known odour-reducer (Mazzoli et al. 2007)

either produces or consumes 2,4 DTBP, due to the correlation detected between the presence of both *A. radioresistens* and 2,4 DTBP among individual snakes (highlighted in Chapter 4), and the fact that 2,4 DTBP is both a known bacterial metabolite (Pelyuntha et al. 2020)and a phenol; *A. radioresistans* is a known phenol-cataboliser (Mazzoli et al. 2007).

Additionally, given that 2,4 DTBP is presumed to be odorous by virtue of being a phenol, it would be worthwhile assessing whether this standalone compound is detectable to any of the biosensor species implemented to date. This would elucidate whether its detection is reliant on factors such as purity and concentration, and if further tested in combination with octadecanoic acid (i.e., in the form of an octadecanoic acid-based, 2,4 DTBP ointment), would reveal whether this carboxylic acid, ubiquitous among the puff adders, serves to suppress 2,4 DTBP's volatility in manner that maintains the adder's crypsis. Octadecanoic acid has, after all, been shown to absorb odourants (Boraphech and Thiravetyan 2015) and reduce volatile transmission (Su et al., 2019; discussed in Chapter 4).

Despite the low haematocrits of vipers and low field metabolic rates of some ambushing snake species, the puff adder's standard metabolic rate is not lower than those of several other snake species, including some that are known to be odorous (see Chapter 4). This dispels the *metabolic suppression* hypothesis as the mechanism driving the puff adder's chemical crypsis. However, in Chapter 4, solid-phase micro-extraction and gas chromatography mass spectrometry reveal that the puff adder's metabolic by-products may, on the whole, be either non-odorous or non-volatile. And, as mentioned above, those that are both odorous and volatile at temperatures that fall within the adder's selected thermal range, may be broken down by odour-reducing microbes found within the adder's microbial assemblage, or removed from the headspace via associated compound-involvement (i.e., volatility reduction by octadecanoic acid).

The gap in the scientific literature of chemically cryptic examples highlights the need for more focused research within the field of chemical ecology. Failure to consider the impact of chemical crypsis within predator-prey dynamics stands to impoverish our understanding of the field. Chemosensation is, after all, the oldest form of communication (Wyatt 2003), facilitating some of the most basic requirements of life, such as prey location, mate recognition and predator avoidance (Yohe and Brand 2018). Indeed, the chemical

environment is so vast and subject to such rapid change, that the olfactory receptor (OR) gene families among taxa commandeer considerably large portions of a species genome (Hayden et al. 2010; Y. Niimura 2010; Niimura et al. 2014).

Additionally, OR genes are the fastest evolving genes known to science (Yohe et al. 2020); each mutation in the OR gene can directly result in olfactory refinement (Pellegrino et al. 2011; Leary et al. 2012; McBride et al. 2014), allowing species to rapidly adapt to their chemical environments. Given the vast number of ways in which the chemical environment can vary across space and time, geographically dependent selection pressures could give rise to regional differences in scent patterns (Tregenza et al. 2000) that can drive speciation events (ref: 76). For example, shifts in pheromone recognition and thus mating preference between populations can result in reproductive isolation and eventual speciation (Mullen, Mendelson, and Shaw 2007) .Thus, the evolutionary pace at which OR genes evolve reiterates not only the importance of chemical communication among species and the degree to which the chemical environment can change, but also that the chemical arms race is unrelenting.

The corollary of such rapid establishment of chemical cues and signals is that overall chemical turnover, and thus signal and cue loss, among species must be equally high. As Miller et al. (2015) hypothesize, incremental changes in associated odourants as a result of predator-driven selection pressures may indeed have driven the adder's shift towards chemical crypsis. I see two possible avenues of exaptation within this species that would easily have served to facilitate its odour reduction. First, the prevalence of octadecanoic acid ubiquitously associated with the dorsal scales of the puff adder may have originally served as an anti-desiccation solution (given its solid, waxy consistency) that became exapted to function as a volatile reducer. Exaptations of anti-desiccation agents into chemical communicative roles have been previously documented (Steinmetz et al 2003; Howard and Blomquist 2005). Thus, an exaptation into another chemical communicative role, albeit subtractive, is not an entirely far-fetched idea. Second, necessary mutualism between the adder and various species of keratinase-degrading microbiota may, in their original role as scale-protectors, have simultaneously conferred odour-reduction properties, either directly or via up- or downstream roles of chemical breakdown (see Chapter 3).

Post-partum ecdysis is known to establish water barriers in neonate snakes (Tu et al. 2002). Puff adder neonates shed within the first few hours of being born (A.K. Miller *pers. obs*.), which immediately reduces their chemical conspicuity to scent-matching dogs from 89.1% to 56.6% (A. K. Miller, unpublished data). This is no doubt due to the immediate sloughing of skin contaminated with odorous birthing fluids but may in part be due to lipid inclusions (Tu et al. 2002; Ball 2004; Augugliaro and Reinert 2005) within the newly emerged dermal layer, for example octadecanoic acid. Certainly, research into the evolutionary role of rapid postpartum ecdysis (considered the exception) among snake species may reveal chemically cryptic underpinnings that could serve to reveal other chemically cryptic candidate species.

Finally, I consider investigations in the thermal constraints of the adder's chemical crypsis particularly worthwhile given the acrid odour associated with several of the puff adders' metabolic chambers during the 36 °C trial (see Chapter 4). A thermally dependent crypsis has far reaching implications for this species under climate change and highlights potentially overlooked mechanistic aspects in risk evaluations for this and other species. In summary, chemical ecology should not be considered tangential to our understanding of the natural world simply because of its innate complexities and our personal limitations as non-macrosmatic species, but rather recognized for its integral role in almost all aspects of life.

REFERENCES

Agugliaro, J., and H.K. Reinert. 2005. Comparative skin permeability of neonatal and adult timber rattlesnakes (*Crotalus horridus*). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* 141, no. 1: 70–75.

Ball, J.C. 2004. The first shed skin of neonate corn snakes is chemically different from adult shed skins. *Journal of Herpetology* 38, no. 1: 124–127.

Boraphech, P., and P. Thiravetyan. 2015. Trimethylamine (fishy odor) adsorption by biomaterials: effect of fatty acids, alkanes, and aromatic compounds in waxes. *Journal of Hazardous Materials* 284: 269–277.

Hayden, S., M. Bekaert, T.A. Crider, S. Mariani, W.J. Murphy, and E.C. Teeling. 2010. Ecological adaptation determines functional mammalian olfactory subgenomes. *Genome Research* 20, no. 1: 1–9.

Howard, R.W., and G.J. Blomquist. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology* 50: 371 – 93.

Leary, G.P., J.E. Allen, P.L. Bunger, J.B. Luginbill, C.E. Linn, I.E. Macallister, M.P. Kavanaugh, and K.W. Wanner. 2012. Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proceedings of the National Academy of Sciences of the United States of America* 109, no. 35: 14081–14086.

Mazzoli, R., E. Pessione, M.G. Giuffrida, P. Fattori, C. Barello, C. Giunta, and N.D. Lindley. 2007. Degradation of aromatic compounds by *Acinetobacter radioresistens* S13: growth characteristics on single substrates and mixtures. *Archives of Microbiology* 188, no. 1: 55–68.

McBride, C.S., F. Baier, A.B. Omondi, S.A. Spitzer, J. Lutomiah, R. Sang, R. Ignell, and L.B. Vosshall. 2014. Evolution of mosquito preference for humans linked to an odorant receptor. *Nature* 515, no. 7526: 222–227.

Mullen, S.P., T.C. Mendelson, and K.L. Shaw. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse hawaiian crickets (Gryllidae: Trigonidiinae: Laupala). *Evolution* 61, no. 1: 223–231.

Niimura, Y. 2010. On the origin and evolution of vertebrate olfactory receptor genes: comparative genome analysis among 23 chordate species. *Genome Biology and Evolution* 1: 34–44.

Niimura, Yoshihito, A. Matsui, and K. Touhara. 2014. Extreme expansion of the olfactory receptor gene repertoire in african elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome Research* 24, no. 9: 1485–1496.

Pellegrino, M., N. Steinbach, M.C. Stensmyr, B.S. Hansson, and L.B. Vosshall. 2011. A natural polymorphism alters odour and deet sensitivity in an insect odorant receptor. *Nature* 478, no. 7370: 511–514.

Pelyuntha, W., C. Chaiyasut, D. Kantachote, and S. Sirilun. 2020. Organic acids and 2,4-di-tert-butylphenol: major compounds of *Weissella confusa* WM36 cell-free supernatant against growth, survival and virulence of Salmonella Typhi. *PeerJ* 2020, no. 1.

Steinmetz, I., E. Schmolz, and J. Ruther. 2003. Cuticular Lipids as Trail Pheromone in a Social Wasp. *Proceedings of the Royal Society B: Biological Sciences* 270, no. 1513: 385–391.

Su, C., H. Zhao, H. Yang, and R. Chen. 2019. Stearic acid-modified starch/chitosan composite sponge with asymmetric and gradient wettability for wound dressing. *ACS Applied Bio Materials* 2, no. 1: 171–181.

Tregenza, T., S.H. Buckley, V.L. Pritchard, and R.K. Butlin. 2000. Inter- and intrapopulation effects of sex and age on epicuticular composition of meadow grasshopper, *Chorthippus parallelus*. *Journal of Chemical Ecology* 26, no. 1: 257–278.

Tu, M.C., H.B. Lillywhite, J.G. Menon, and G.K. Menon. 2002. Postnatal ecdysis establishes the permeability barrier in snake skin : new insights into barrier lipid structures. *Journal of Experimental Biology* 205: 3019–3030.

Wyatt, T.D. 2003. *Pheromones and Animal Behaviour : Communication by Smell and Taste*. Cambridge University Press.

Yohe, L.R., and P. Brand. 2018. Evolutionary ecology of chemosensation and its role in sensory drive. *Current Zoology* 64, no. 4: 525–533.

Yohe, L.R., M. Fabbri, M. Hanson, and B.-A.S. Bhullar. 2020. Olfactory receptor gene evolution is unusually rapid across Tetrapoda and outpaces chemosensory phenotypic change. *Current Zoology* 66, no. 5: 505 – 514.