Using Pheromones to Understand Cryptic Lizard Diversity

Thesis submitted by

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Front cover: Composite of 16 individual Bynoe's geckos (*Heteronotia binoei*) representing four deeply divergent genetic lineages within this cryptic species complex.

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Publications associated with this thesis—published, submitted, or in preparation—are listed below. A statement of author contributions is given for each.

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Abstract

Animals use mating traits to attract and choose mates. This involves two components: a mating signal in one sex, and an associated preference for that signal in the opposite sex. Because mating traits influence mate choice, the divergence of signals and preferences between populations can result in or reinforce reproductive isolation—that is, speciation. The importance of divergence in mating traits versus other phenotypes is made evident by the observation that, across diverse animals groups, many closely related species differ primarily or only in their mating traits. When the mating traits in question are outside the sphere of human perception, the result can be evolutionarily distinct but seemingly identical 'cryptic species' that we humans cannot tell apart. Knowledge of mating traits is thus important for understanding and resolving animal diversity.

Lizards (including snakes, which are derived lizards) are the most species-rich group of terrestrial vertebrates; but are also a group for which our knowledge of mating traits and reproductive isolation is relatively poor. Furthermore, phylogenetic studies reveal that many recognised 'species' are actually comprised of multiple genetically divergent but morphologically similar cryptic species. Even among animals in general, lizards appear to contain disproportionately high levels of cryptic species diversity. Therefore, identifying the mating traits in cryptic lizard groups will be key to understanding and resolving this diversity. Considering that many of these cryptic species are—by definition—visually similar, chemical signals (pheromones) are a good candidate for investigation as a mating trait. In this thesis I test whether pheromones are a mating trait influencing reproductive isolation in a widespread cryptic species complex of Australian lizards, the Bynoe's gecko (*Heteronotia binoei*).

Chapter 1 is a general introduction reviewing the main themes of this thesis: cryptic species; mating traits and their influence on reproductive isolation; and the role of pheromones as a mating trait in lizards. I introduce *Heteronotia* geckos as my study system and outline the research aims addressed by subsequent chapters.

Chapter 2 tests whether pheromones have diverged among genetically distinct lineages (candidate species) within the *H. binoei* cryptic species complex, and then tests whether pheromones are more divergent than morphology. To do this, I sampled and characterised male pheromones from 10 lineages of *H. binoei*. I also took morphometric measurements from preserved specimens representing these 10 lineages. I show that pheromones have diverged among all but two of these 10 lineages, and that there is limited

overlap in pheromone composition among co-occurring lineages. In contrast, morphology has diverged among only some lineages, and there is considerable overlap in morphological variation even among co-occurring lineages. Finally, I use estimates of trait overlap and trait distance between lineages to show that pheromones are more divergent than morphology in this species complex. These results suggest that pheromones have been important in the diversification of this morphologically conservative lizard radiation, and that pheromones might be an informative character to integrate into lizard systematics and taxonomy.

Chapter 3 tests whether divergent pheromones influence female discrimination among lineages. To do this, I first collected geckos of three co-occurring lineages from three sites representing each pairwise combination of sympatry between these lineages. To test the influence of pheromone secretions on female discrimination without other confounding influences (such as male dominance), I isolated male pheromone secretions on cotton swabs and presented them to females in both simultaneous and sequential choice experiments. Female geckos sniffed conspecific male pheromones longer than unscented controls, and two of the three lineages sniffed conspecific pheromones longer than heterospecific pheromones. However, the order that scent treatments were encountered or presented also influenced female sniffing duration, highlighting the importance of considering treatment order when designing and analysing experiments such as these. I conclude that male pheromones are a mating trait influencing female lineage discrimination and hence reproductive isolation in *Heteronotia* geckos.

Chapter 4 begins teasing apart the factors shaping pheromone divergence. I use a phylogenetic comparative approach to first assess phylogenetic signal in pheromone divergence, and then to test if and how pheromone variation correlates with two key climatic variables: temperature and precipitation. To do this, I sampled pheromones from 25 lineages of the *H. binoei* complex and 8 lineages of the *H. planiceps* complex for a total of 33 lineages across northern Australia. The phylogeny of these lineages was reconstructed using exon capture sequencing and multispecies coalescent phylogenetics, supplemented with mtDNA data for three lineages lacking exon data. I first show that pheromone divergence is closely associated with shared evolutionary history—although there have been 'leaps' in pheromone composition associated with two phylogenetic splitting events. Accounting for phylogenetic relationships, I then show that pheromone variation correlates with temperature in lineages of *H. binoei* but not *H. planiceps*, and that pheromone variation correlates with precipitation in both species complexes. However, pheromone

composition in the two groups respond to precipitation in subtly different ways. Because temperature and moisture can influence the rate that chemical signals evaporate, and thus the efficacy of those signals, these relationships likely reflect adaptation of pheromones to different climatic conditions. Finally, I identify individual pheromone components strongly associated with climate, to guide further research.

Chapter 5 summarises and synthesises the results of my three data chapters, highlights various caveats and challenges, and suggests promising future research directions leading on from my results.

This thesis combines analytical chemistry, morphometrics, behavioural experiments, and phylogenetic comparative analyses to understand the function and evolution of pheromones in a cryptic species complex of Australian lizards. In these chapters I present data from more than 800 individual lizards, 720 hours of behavioural footage, and more than 20,000 kilometres worth of fieldwork. I show that pheromones function as a mating trait influencing behavioural isolation in *Heteronotia* geckos, indicating that pheromones will be important for understanding mate choice, reproductive isolation, and diversification in lizards, the most speciose group of terrestrial vertebrates.

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CHAPTER 1 — Introduction: cryptic species, mating traits, and lizard diversity

Cryptic species

How many species are there? Now with cheap and easy DNA sequencing we know only that we're really not sure. It is now commonplace that a phylogenetic study done on a taxon reveals that what was before considered one species is actually several deeply divergent genetic lineages that warrant recognition as individual species. These species often look so similar to us that we cannot tell them apart without genetic analysis, which is why most have remained hidden from taxonomists and are revealed only after phylogenetic screening. These are commonly referred to as 'cryptic species'—or sometimes 'sibling species', particularly in earlier literature (Mayr 1963; Bickford et al. 2007). Bickford and colleagues (2007) define cryptic species as "…two or more distinct species that are erroneously classified (and hidden) under one species name". Cryptic species are a hot topic in systematics, taxonomy, and conservation biology, and pose several challenges to these fields, as well as to studies of ecology and even issues of agriculture, fisheries, and human health (reviewed in Bickford et al. 2007).

But are cryptic species real in a biological sense? Considering the disagreements surrounding the way species themselves are defined (e.g., Mayr 1976; Simpson 1951; Mayden 1997; Coyne & Orr 2004; de Quieroz 2007), it's unsurprising that the term cryptic species is contentious. Some claim the term is applied too broadly and should be defined more objectively (Struck et al. 2018), while others suggest the term is inherently subjective (Heethoff 2018). Below I outline eight broad categories that can result in populations or lineages being regarded as cryptic species. These include both biological phenomena as well as the biases of we humans who study them; and while these points have been broken up into discrete categories, the concepts bleed into one another. Other authors have made some of these points. For example, Fišer et al. (2018) review three of the biological processes listed below (recent divergence, niche conservatism, and convergence), particularly in respect to cryptic amphipod diversity; and Bickford et al. (2007) cover morphological constraints in extreme environments and human perception. I am unaware,

however, of any previous works that explicitly attempt to summarise and discuss the biological and anthropic factors that interact to produce what we call cryptic species.

Niche conservatism

Many if not most taxa regarded as cryptic species are genetically divergent but morphologically similar populations that are mostly or entirely geographically isolated (allopatric or parapatric) from each other. Geographic isolation is considered important for the initiation of lineage divergence and speciation (Mayr 1963; Futuyma 1987; Turelli et al. 2001; Coyne & Orr 2004; Harvey et al. 2019). If populations experience similar environments, and morphology is tied closely to the ecological niche, then there are few reasons to expect morphological divergence to accompany genetic divergence in absence of other selective forces. Wiens (2004) proposed that the tendency for species to retain similar ecological niches over evolutionary time scales (phylogenetic niche conservatism, PNC; Ricklefs & Latham 1992; Peterson et al. 1999) is key to fostering the geographic isolation of populations and thus initiating speciation. There is substantial support for this hypothesis (Kozak & Wiens 2010; Pyron et al. 2015; Wiens & Graham 2005), indicating that phenotypically conservative allopatric divergence is likely the norm. Although research has often focussed on how ecological divergence, which is associated with phenotypic divergence, promotes speciation, conservatism—whether through developmental constraints or interactions with other species—can promote speciation by increasing the likelihood of population fragmentation and isolation (Bell et al. 2010; Hoskin et al. 2011; Singhal et al. 2019).

Recent divergence

Authors have suggested that, in some cases, cryptic species are similar simply because they have diverged recently (Fišer et al. 2018; Struck et al. 2018). The simplest evolutionary models predict that morphological disparity increases due to stochastic processes alone (Adams et al. 2009). If so, then morphological differences accrue gradually over time such that young sister species might be expected to have few if any phenotypic differences. Fišer et al. (2018), however, found that recent divergence predicted morphological similarity in only a small fraction of amphipods. Considering that evolution can occur rapidly (e.g., Herrel et al. 2008; de Amorim et al. 2017), recently diverged populations also likely fall into the above category of niche conservatism—allopatric divergence in similar environments is

the most typical starting point for speciation, and thus recently diverged lineages are likely allopatric populations in similar habitats. Fišer et al. (2018) claim that recent divergence is a selectively neutral mechanism (although I contend it is not a mechanism but simply a reference point in time) and that it should predominate in areas where climatic perturbations fragment the ranges of ancestral species. They then state that PNC is a selection-based mechanism that is stronger in areas of strong directional selection. The two are tied, however, because the fact that populations are fragmented in the first place, and stay fragmented long enough to diverge, is because of selection maintaining the niche (Wiens 2004; Pyron et al. 2015). Nevertheless, characters that are selectively neutral might be more likely to diverge via drift and become fixed within a population given enough time. Such characters might be useful for diagnosing species—for example, subtle scale differences in many lizards, which are then no longer considered cryptic—but the characters themselves are unlikely to be relevant to the process of speciation.

Convergence

Just as closely related species in similar environments are likely to be morphologically similar, more distantly related species experiencing similar conditions can converge on similar phenotypes (e.g., Bravo et al. 2014; Esquerré & Keogh 2016). Sometimes convergent phenotypes are so similar that it results in taxonomic confusion. For example, an analyses of amphipod diversity showed that 26% of cryptic amphipods were not sister groups, particularly in subterranean habitats (Fišer et al. 2018). However, convergence appears to be an uncommon mechanism that produces cryptic species, but it does sometimes occur (Henry et al. 1999; Vrijenhoek 2009; Heideman et al. 2011). Struck et al. (2018) also highlighted the role of convergence in generating cryptic species diversity, but considered convergent evolution and parallel evolution separately. The distinction between the two, however, is an arbitrary divide based on the underlying genes involved, and so I consider both under the term convergence (see Arendt & Reznick 2008 for discussion).

Mimicry

Some organisms have evolved to be phenotypically similar to another species with the function of being mistaken for that species. This is mimicry, and it can be classified into two mechanisms: Batesian mimicry, where a harmless species mimics the appearance of a dangerous species (the model, which is often poisonous or venomous); and Müllerian

mimicry, where two harmful species evolve to be similar to each other, with the advantage that predators need form only a single search image (Ruxton et al. 2019). This can create taxonomic confusion when mimics, co-mimics, and models are sufficiently similar (Schaefer et al. 2002; Rabemananjara et al. 2007; Boppré et al. 2017). Mimicry can also produce cryptic species through convergence (see above). For example, when different harmless species mimic the same model, the mimics also become similar to each other (Chiocchio et al. 2019).

Intraspecific variation: local adaptation, polymorphism, and plasticity

Variation within a species is sometimes so great that there are few if any reliable diagnostic characters between it and other closely related species. Local adaptation can create high levels of site-to-site variation within a species such that individuals from disparate localities may appear starkly different despite high levels of gene flow between them (Boratynski et al. 2014; Rabosky et al. 2014). The effect can be that individuals from one site resemble another closely related species more than they resemble members from other conspecific populations (perhaps because of convergence; Anderson et al. 2014). Distinguishing among species can also be difficult when one or several of the respective species exhibit high levels of among-individual variation within a single population, for example, because of phenotypic plasticity (e.g., plants; Vrijenhoek 2009).

Polyploid speciation

Polyploid speciation occurs when an increase in the number of chromosome sets results in reproductive isolation, a process that can be instantaneous (Coyne & Orr 2004). This can happen both during a reproductive event between members of the same species (autopolyploidy) or during a hybridisation event between members of different species (allopolyploidy). Autopolyploids are phenotypically identical to the parent species in most respects (Levin 1983). Allopolyploids vary in their similarity to their parental species, but there are several examples where they are indistinguishable (to taxonomists, at least) from one of the parental species (e.g., in lizards; Moritz 1983; Adams et al. 2003). However, polyploids are often larger than their parental species because of the increase in cell size associated with larger genomes (Levin 2002). Polyploidy is most common in plants (Levin 2002; Coyne & Orr 2004), but it does often occur in animals—particularly fishes, squamate

reptiles, and salamanders—often resulting in clonally reproducing all-female (parthenogenetic) lineages (Dowling & Secor 1997; Fujita & Moritz 2009).

Species concepts and species delimitation

What we call cryptic species will necessarily depend on how we conceptualise and define species themselves (Heethoff 2018). Estimates of biodiversity—including cryptic diversity—are affected by the species concept one adopts (Agapow et al. 2004). There are more than twenty species concepts, and debate over their utility and objectivity has gone on for the better part of a century (Mayr 1963, 1976; de Quieroz 1998, 2007; Mayden 1997; Coyne & Orr 2004; Zachos 2018*a*, 2018*b*). Disagreement stems in part from a mismatch between conceptual and operative species concepts; the species concept adopted by those studying the process of speciation (conceptual) often differs from those who describe and name species (operative). And the debate becomes more complicated by the fact that life consists of both sexually and asexually reproducing organisms. A species concept that is useful for studying population and lineage divergence in sexually reproducing organisms will not necessarily be useful for understanding the diversification of asexually reproducing organisms (Frost & Hillis 1990). Considering this, it's unsurprising that researchers sometimes disagree on whether certain populations do or do not warrant classification as distinct species (e.g., ungulates; Zachos 2018*b*; Gippoliti 2019). Despite disagreements such as these, those adhering to different species concepts often agree on a case-by-case basis because all widely used species concepts emphasise the importance of species as independently evolving lineages (de Quieroz 2007; Hillis 2019). But conceptualising species is one matter, deciding whether populations fit the criteria of the respective species concept is another (de Quieroz 2007).

Species delimitation practices aim to resolve species boundaries through empirical analysis of one or more types of data, which can include genetics, morphology, ecology, behaviour, and physiology (de Quieroz 2007; Wiens 2007; Carstens et al. 2013). It is now common for studies to combine different data types in what is now referred to as 'integrative taxonomy' (Dayrat 2005; Padial et al. 2010); however, even ostensibly integrative taxonomic studies often place more emphasis on one data source—usually genetics (Carstens et al. 2013). The analytical toolkit for species delimitation is broad and continuously growing, and different approaches have different strengths and weaknesses (see Singhal et al. 2018 for synopsis). Many approaches can overestimate the number of species when assumptions are not met, thus overestimating the number of (cryptic) species

(Sukumaran & Knowles 2017). For example, Chambers and Hillis (2019) showed that multispecies coalescent methods can over-split geographically widespread taxa when sampling is insufficiently dense, resulting in arbitrarily defined species boundaries largely determined by *a priori* designation of hypothesised species boundaries. Indeed, geographically structured genetic divergence is frequently treated as synonymous with cryptic species diversity without any additional analysis or data indicating that populations are reproductively isolated where they meet (Carstens et al. 2013). In cases where divergent populations do not meet (i.e., they are allopatric), deciding whether they do or do not represent distinct species is more difficult and will usually be more subjective, and consequently more likely to cause disagreement. Hillis (2019) provides a perspicuous and detailed overview of species delimitation that, although focussed on reptiles and amphibians, is relevant to species delimitation in all sexually reproducing organisms.

Cryptic phenotypes and the limits of human perception

Cryptic species are generally regarded as such because we humans are unable to differentiate them without the aid of genetic analysis. In some cases, *post hoc* analysis of putative cryptic species does reveal subtle morphological differences, and researchers have proposed terms such as 'pseudo-cryptic species' and others to differentiate these from 'truly' cryptic species (Sáez et al. 2003; Achurra et al. 2015). While this seems arbitrary, it illustrates the focus on morphology implicit or explicit in all discussions of cryptic species. But morphology is only one aspect of an organism's phenotype. Although morphology is often useful for we visually oriented humans who wish to tell species apart, there are many other phenotypes that are important—sometimes more important—for reproductive isolation and ecological coexistence among species. Thus, some cryptic species might be regarded as such only because important phenotypic differences fall outside the limits of what we humans can perceive, a point that has been raised by several authors (Mayr 1963; Knowlton 1993; Henry 1994). Indeed, non-morphological phenotypes that we humans *can* perceive have been important for resolving the taxonomy of many groups, for example, mating calls in frogs (e.g., Channing et al. 2002). Detailed studies have demonstrated that some animal species differ in mating signals that we cannot detect without the aid of technology: pheromones in moths (Löfstedt et al. 1991); ultrasonic signals in bats and some insects (Henry 1994; Jones 1997); and electrical signal in some fish (Hopkins & Bass 1981). Furthermore, ecological divergence and coexistence among species can involve the divergence of non-morphological traits, such as physiology and behaviour. For example,

differences in metabolite profiles were found between two cryptic earthworm species (Liebeke et al. 2014), possibly reflecting subtle ecological differences; and deeply divergent but morphologically identical lineages of *Tubifex* worms differed in their resistance to high levels of cadmium (Sturmbauer et al. 1999). These examples highlight that—in many or perhaps most cases—cryptic species are simply an artefact of the limits of human perception.

Why are there cryptic species?

Here I aimed to briefly summarise the biological and anthropic factors that influence what we call cryptic species. While I have broken these factors into categories, these categories are not discrete, and two or more interact in most real scenarios—and there will usually be some aspect of human bias. For example, niche conservatism might explain why two deeply divergent allopatric populations are morphologically similar, but how we conceptualise and delimit species will influence whether or not we call those populations distinct species. Two co-mimic butterflies might be visually identical yet possess distinct pheromones that influence mate choice (González-Rojas et al. 2019)—that they are regarded as cryptic is only because we humans are relatively good at seeing but not at smelling. In the introduction to their review, Fišer et al. (2018) wrote: "Over the last two decades, increased evidence emerged for speciation governed by entirely different [non-ecological] mechanisms, leading to the so-called sibling or cryptic species." This statement reflects the sentiment of many who view cryptic species as the outcome of distinct and perhaps only recently appreciated evolutionary processes (e.g., cryptic speciation). But the distinction between what we call species and what we call cryptic species lies in the perceptual, methodological, and cognitive biases of we humans who study them. I included a sentence similar to the previous in a (rejected) manuscript submission for what became Chapter 2 (Zozaya et al. 2019*a*), to which a reviewer commented, "Please drop the references to human perception. This is irrelevant to nature." Perhaps. But what we choose to study, how we study it, and how we interpret the results of our studies are all influenced by our human biases (Kokko 2017). In recognising this, cryptic species become a lens through which our own limitations and biases—and their influence on how we study and understand nature—come into focus.

Mating traits

The sight of a feather in a peacock's tail, whenever I gaze at it, makes me sick

— Charles Darwin

The peacock's tail flew in the face of Darwin's theory of natural selection. At first he could not reconcile how a feature that would both attract predators and impede movement could benefit the survival of an animal. This and other observations contributed to the formation of his theory of sexual selection, as he came to realise that some traits exist with the primary function of winning mates. He called these traits 'secondary sexual characters' (Darwin 1871). While primary sexual characters are traits directly involved in the act of reproduction (e.g., genitalia), secondary sexual characters are not involved in reproduction itself but are typically exhibited in only one sex of a species with the function of attracting and/or defending mates. Some secondary sexual characters are used in intrasexual conflict to combat rivals for access to mates (Andersson 1994; Andersson & Iwasa 1996), such as the antlers of deer or the horns of rhinoceros beetles. Other secondary sexual characters function as mating signals that are used to attract members of the opposite sex. For example, the chirps of male crickets and the orange spots of male guppies are used to attract females of the respective species (Houde 1997; Wagner & Reiser 2000). For mating signals to be effective there must be a corresponding preference for that signal in members of the opposite sex, and that preference must manifest itself through mate choice (Kirkpatrick 1982; West-Eberhard 1983; Andersson 1994; Kirkpatrick & Ryan 1991; Ryan 1997). I refer to the combination of mating signals and their associated preferences as 'mating traits'. It is important to note that some traits can influence both intrasexual conflict and mate choice (e.g., Callander et al. 2013), and can also contribute to other functions (e.g., Chung & Carroll 2015). Herein, however, I specifically focus on mating traits and their role in the evolution of reproductive isolation and speciation.

The coevolution of mating signals and their associated preferences can lead to the divergence of mating traits between populations to the effect that members of one population are less attracted to members of the other. This reduces hybrid mating attempts, thus reducing gene flow and consequently leading to premating reproductive isolation via divergent mate choice—a mechanism termed 'behavioural isolation' (Coyne & Orr 2004). Comparative, experimental, and theoretical studies offer compelling evidence supporting the role of behavioural isolation in the speciation process (Lande 1981, 1982; Turelli et al. 2001; Boughman 2002; Ritchie 2007). Several evolutionary processes can result in the divergence of mating traits among populations, which I briefly summarise below.

Natural selection under disparate environments can drive adaptation to maximise the efficacy of signals and the sensory system, a process termed 'sensory drive' (Endler 1992; Seehausen et al. 2008; Fuller & Endler 2018; see Chapter 4 for a more detailed discussion). Mating traits can also vary as a by-product of natural selection on other traits, possibly because of pleiotropy or linkage disequilibrium between the genes involved in selection (Maynard Smith 1966; Rundle & Nosil 2005; Servedio 2009), or when mating signals are physically linked to other functions (e.g., cuticular hydrocarbons in insects; Chung et al. 2014). In contrast to natural selection, Fisherian sexual selection involves the evolution of mating traits in arbitrary directions because fitness depends on the mate preferences of the choosing sex (Fisher 1930; West-Eberhard 1983). This generally requires that the genes influencing the respective mating signals in one sex and the associated preferences in the opposite sex be closely linked, potentially leading to runaway coevolution of signals and preferences (Kirkpatrick & Hall 2004; Anderson & Simmons 2006). This mechanism has been favoured as an explanation for many of the more extravagant mating signals observed in animals (Pomiankowski & Iwasa 1998). Mutation and drift can also influence mating trait divergence (Kirkpatrick & Hall 2004; Mendelson et al. 2014), as well as interacting with Fisherian sexual selection (Pomiankowski & Iwasa 1998).

When mating is likely to occur between closely related sympatric species, and where this results in a loss of fitness, there is expected to be strong selection for the evolution of behavioural isolation, usually through reproductive character displacement (hereafter called RCD; Howard 1993). This can occur when courtship or mating between two species results in reduced reproductive success, or when the resulting hybrids suffer reduced fitness (i.e., reinforcement; Howard 1993; Servedio & Noor 2003). In such cases, RCD is expected to strengthen behavioural isolation where the respective species are sympatric. This can also lead to behavioural isolation between allopatric populations of the same species when only some population experience sympatry (Higgie et al. 2000; Hoskin et al. 2005). The effects of RCD can be driven by many types of species interactions and is not limited to cases of attempted hybridisation (Hoskin & Higgie 2010). For example, if a predator or parasite uses the prey/host's mating signals to locate it, there may be selection for a shift in the signal to reduce the likelihood of being detected and located (Zuk & Kolluru 1998). It can also occur because of competition in signal space when two species have overlapping, and thus interfering, mating signals (Amezquita et al. 2006). This can lead to hampered search effort for the receiver and wasted signalling effort for the signaller, resulting in selection for signal divergence. Similar to RCD by reinforcement, this

can result in premating isolation in allopatric populations of a single species when only some populations experience overlap (Hoskin & Higgie 2010).

The preceding paragraphs highlight how mating trait divergence can both initiate speciation and be a consequence of speciation by other processes when the respective species subsequently co-occur. Because of this, mating traits—specifically mating signals—are often good proxies to infer species boundaries and are useful for resolving the taxonomy of many animal groups. This is because many otherwise morphologically similar species differ primarily in their mating signals, as was observed by Darwin (1871). Examples include wing colouration in butterflies, the song and plumage of birds, and the songs of frogs and singing insects (Ptacek 2000). Taxonomically describing species in groups such as these is made easier because their mating signals are easily perceived and differentiated by we humans who study them. However, as I outlined in the previous section on cryptic species, when the mating signals in question fall outside the sphere of human perception, the result can be reproductively isolated but otherwise visually identical cryptic species. Technology has facilitated the characterisation of many such signals, which has enhanced our understanding of the diversity and biology of some such groups. In lacewing insects, for example, ultrasonic courtship songs are the only observable phenotypic difference among many widely sympatric but reproductively isolated species (Wells & Henry 1998). Understanding mating traits is not only important for understanding speciation and the origins of biodiversity but also crucial for resolving the taxonomy in many otherwise cryptic groups. Comprehensively understanding biodiversity, how it has evolved, and how it interacts requires accurate taxonomic resolution, for which cryptic species pose a problem. That knowledge will remain incomplete until we understand the traits the animals themselves use to tell each other apart—their mating traits.

Lizards

There are a lot of them. If you include snakes and amphisbaenians—both of which are simply morphologically derived limb-reduced lizards—then there are over 10,400 lizard species currently recognised, making them the most species-rich group of terrestrial vertebrates (Uetz et al. 2019). And the number is growing. Several new lizard species are described every week. Many of these represent novel discoveries (e.g., Appendix I and Appendix II), but most are cryptic species identified following phylogenetic analysis (e.g., Doughty et al. 2015, 2018). Although the quality of species delimitation studies varies, there is evidence that many visually identical but closely related cryptic species co-occur without

any contemporary gene flow (Pinto et al. 2019; Singhal et al. 2018). A recent meta-analysis of cryptic species diversity across animals showed that, when research effort is accounted for, reptiles are comprised of relatively high levels of cryptic species diversity. And the research attention received by lizards is indeed enormous: for example, they're models of adaptive radiation (Losos 2009), biogeography (Losos 2009; Bell et al. 2010; Werneck et al. 2012), sex chromosome evolution (Ezaz et al. 2005; Gamble et al. 2014, 2015), the evolution of sociality (While et al. 2009; Davis et al. 2010), biomechanics (Bels et al. 1994; Lailvaux & Irschick 2007), and even the inspiration of new technologies (Sethi et al. 2008). Despite this enormous attention, we have a poor understanding of the role of mating traits and mate choice in lizard speciation (Wollenberg Valero et al. 2019).

This is not necessarily from lack of trying, though, and there are exceptions. Visual signals in lizards have, unsurprisingly, received the most attention. Lizards such as *Anolis* and *Draco* have extensible, often colourful dewlaps that are typically unique to each species within an area and facilitate species recognition (Losos 1985, 2009; Klomp et al. 2017). When a lizard displays it often combines such signals with stereotypical movements, such as push-ups and head-bobs (Martins 1993; Ramos & Peters 2017). Such displays play a role in territory defence and intrasexual selection (Cooper & Burns 1987; Thompson & Moore 1991; Losos 2009; Peters et al. 2016), but few studies have demonstrated any role of visual signals in mate choice, perhaps with the exception of UV colour badges in European *Lacerta* (Olsson et al. 2011). Body size has been shown to influence mate choice in both male and female lizards (Cooper & Vitt 1993; Olsson 1993), and while clearly important, it is unclear whether this results in behavioural isolation among species. But considering their extremely similar appearances, visual signals are unlikely candidates as mating signals in cryptic species complexes—although as we've seen, ultraviolet signals are possible, but probably not for nocturnal and fossorial species (a considerable proportion of squamate diversity). While some lizards do indeed use vocal signals (i.e., geckos), there is only evidence for a role in territoriality but not for mate choice (although this remains understudied).

A promising avenue for further research is the study of pheromones—chemical signals that elicit a behavioural or physiological response in individuals of the same species (Karlson & Lüscher 1959). Although there are relatively few studies of reptile pheromones compared to other animal groups (< 1% of studies, reviewed in Symonds & Elgar 2008), pheromones are known to play a role in the social behaviour of squamates (reviewed in Mason & Parker 2010; Martín & López 2014). Pheromones are used to identify rival males

and mark territories (Aragón et al. 2001; Martín et al. 2007*a*; Khannoon et al. 2011), and to identify kin and familiars (Bull et al. 1999; Carazo et al. 2008). Most importantly here, there is evidence that pheromones influence mate choice (López et al. 2002; Martín & López 2006*a*, 2008, 2011, 2014; Kopena et al. 2011). Pheromones in squamates can be produced from several sources that vary among taxa. Below I introduce the known sources and summarise evidence for the role of each in mediating behavioural isolation.

Pheromones from the skin

The skin of squamates produces lipids that are thought to function in reducing water loss (Roberts & Lillywhite 1980). These compounds can occur in unique combinations that are usually species or population-specific, and there is unequivocal evidence that they influence mate choice and behavioural isolation in some snakes. The males of sympatric species of North American garter snakes (*Thamnophis* spp.) strongly prefer the scent of females of their own species to those of other species (Ford 1982; Ford & Schofield 1984); however, some allopatric species do not appear to discriminate between each other (Ford 1982), while other allopatric species do (Ford & O'Bleness 1986). A study of *T. sirtalis parietalis* in Canada showed that male snakes from hibernation sites in eastern Manitoba preferred the courtship pheromones of females from their own population over those from hibernation sites in western Manitoba, while snakes from the western population exhibited no preference. This asymmetrical isolation was associated with differences in the female sex pheromones of the two populations (LeMaster & Mason 2003). More recently, a study of two morphologically similar species of sea krait (*Laticauda* spp.) from Vanuatu demonstrated that males of the two species are able to discriminate between conspecific and heterospecific females by scent alone, and preferentially initiated courtship behaviour toward the scent of conspecifics (Shine et al. 2002). The behavioural influence of skin-borne lipids is more poorly known in squamates other than snakes. The leopard gecko (*Eublepharis macularius*) is also known to produce and perceive skin lipids (Mason & Gutzke 1990; Cooper & Steele 1997), although there is no evidence of its role in a mating context.

Pheromones from the cloaca

Very little is known about the glands found in the cloacae of squamates. Pheromone-producing cloacal glands are known from skinks (Scincidae; Cooper & Grastka 1987), plated lizards (Gerrhosauridae; Cooper & Trauth 1992), and snakes (Siegel et al. 2014).

There is strong evidence that the product of cloacal glands influence male mate choice and behavioural isolation in North American skinks. Cooper and Vitt (1986) showed that three morphologically similar *Plestiodon* species (previously *Eumeces*) were able to discriminate among females of each species using only scent cues from the cloaca. They later showed that males from two of the three species initiated courtship *only* with females of their own species (Cooper & Vitt 1987), but one of these (*P. inexpectatus*) would initiate courtship with a heterospecific female (*P. fasciatus*) if that female had been treated with the cloacal scent of a conspecific female. Subsequent behavioural assays suggest the pheromones in question originate from the urodaeal glands (Cooper & Grastka 1987; Trauth et al. 1987).

Another study investigating two allopatric, genetically divergent, and morphologically identical populations of the Australian skink *Lampropholis similis* (as *L. coggeri*) showed evidence that males prefer the scent of females originating from their own population, although the effect was small (Scott et al. 2015). Scent was presented on cotton swabs that had been rubbed along the skink's posterior body surface near the cloaca, hindlimbs, and tail, making it uncertain whether pheromones originated from skin-borne secretions, the cloaca, or both. I include this study here considering that swabbing focussed on the cloacal region.

Pheromones are also known from the scats of some lizards, which are likely produced from glands in the cloaca and deposited onto the surface of the faeces (Martín & López 2014). Pheromones from scats facilitate the identification of kin and familiars in some skinks (Bull et al. 1999), but so far there is no evidence that faeces-based pheromones are involved in mate choice (Martín & López 2011).

Pheromones from epidermal holocrine glands

Although originating from within the skin, epidermal glands are specialised scent-depositing structures that, given the research effort they've received, warrant separate treatment from the skin-borne lipids discussed above. Epidermal glands are usually separated into two types: generation glands and follicular glands. Both are holocrine glands that yield waxy secretions via the rupturing of entire cells (Mayerl et al. 2015). Generation glands are simply modified scales that produce layers of glandular material as new epidermal layers are generated (Maderson 1972; Van Wyk & Mouton 1992; Mouton et al. 2010, 2014). These glands can occur on various parts of a lizard's body, but are most commonly positioned anterior to the cloaca or on the ventral surface of the hindlimbs (Mayerl et al. 2015). In contrast, follicular glands are tubular organs—possibly derived from

generation glands—that are embedded in the skin and secrete waxy plugs through pores set within or between scales (Cole 1966; Mayerl et al. 2015). Follicular glands are always found positioned anterior to the cloaca, on the ventral surface of the hindlimbs, or both (Cole 1966; Mayerl et al. 2015). This has earned them names such as 'femoral pores', 'pre-anal pores', 'pre-cloacal pores', and various other combinations; but for simplicity I follow Mayerl et al. (2015) and will hereafter refer to follicular glands as 'epidermal pores'. Epidermal holocrine glands are common among lizard families (Cole 1966; Mayerl et al. 2015; García-Roa et al. 2017), but are notably absent in snakes and skinks, which were discussed above. When they are present, these glands are usually better developed or only present in males (Cole 1966; Mayerl et al. 2015).

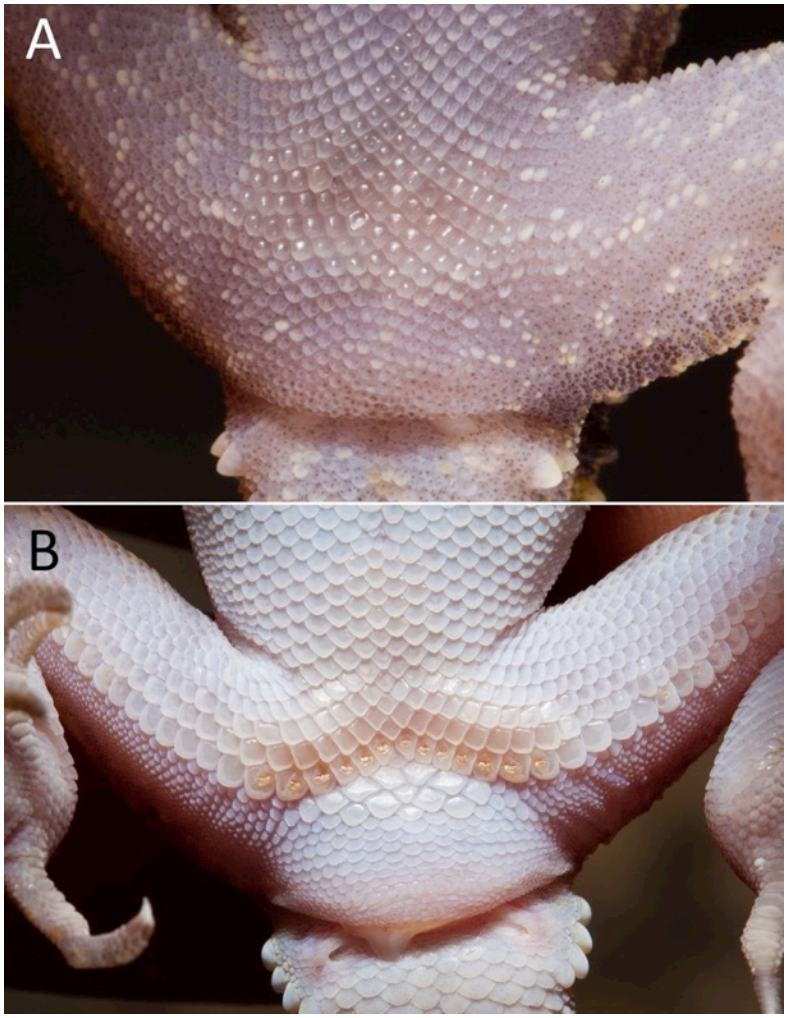

Figure 1.1: Examples of epidermal holocrine glands in geckos. Generation glands (A) are the shiny patch of scales centred anterior to the cloaca between the legs in this *Nactus cheverti*. Epidermal pores (B) are present in a wide V-shaped row anterior to the cloaca in this *Cyrtodactylus mcdonaldi*. Epidermal pore secretions are visible as yellowish spots.

Epidermal pores have received much more research attention than other sources of pheromones, including generation glands, likely because they are conspicuous, sexually dimorphic, and in many species it's relatively easy to collect the secretions of these pores (Martín & López 2014; Mayerl et al. 2015). The influence of these secretions on mate choice, however, has been investigated in only a few groups, and studies in the context of behavioural isolation are even fewer. Research on the composition and behavioural activity of epidermal pore secretions has overwhelmingly focussed on lizards of the family Lacertidae (Martín & López 2004, 2014). The females of several lacertid species appear to use epidermal pore secretions to assess the quality of conspecific males, with secretions conveying information such as: age (López et al. 2003); health and immune response (Martín et al. 2007*b*; Martín & López 2015); basking opportunities (Heathcote et al. 2014); levels of vitamin D (Martín & López 2006*a*), vitamin E (Kopena et al. 2011), ergosterol (Martín & López 2012), and oleic acid (Martín & López 2010); and fluctuating asymmetry (López et al. 2002).

Although epidermal pore secretions clearly influence female behaviour, studies of pheromone-mediated behavioural isolation via female choice have produced mixed results. Martín and López (2006*b*) demonstrated that the epidermal pore secretions of male *Podarcis hispanica* differed between two genetic lineages, and that males were able to discriminate between lineages using scent; females, however, did not appear to discriminate between males of the two lineages. A similar study showed that female *P. hispanica* did not respond differently to males from their own population versus males from other populations (Gabirot et al. 2013); however, when more populations of *P. hispanica* were considered, lizards from geographically distant populations did discriminate between the scents of their own and allopatric populations (Gabirot et al. 2012). Male *P. bocagei* and *P. hispanica* responded more strongly to the scent of females of their own species, but females did not differ in their response to males (Barbosa et al. 2006). Results were similar for a study including the island-dwelling *P. atrata* and adjacent mainland *P. hispanica* (Gabirot et al. 2010). In-depth study of a contact zone between two hybridising lineages of *P. muralis* with divergent pheromones and morphology show little role for female mate choice in reproductive isolation, with assortative mating driven largely by male interactions (While & Uller 2017; MacGregor et al. 2017). These results suggest that—while pheromones do influence female behaviour—differences in female pheromones, rather than males, might mediate behavioural isolation in lacertid lizards, a group in which both males and females possess epidermal pores.

Studies investigating the role of epidermal pores on mate choice and behavioural isolation in other lizard groups are few. A study on South American *Liolaemus* lizards by Labra (2011) found that individuals showed a stronger response to the secretions of conspecifics versus heterospecifics; however, sample sizes and analysis in that study were limited, and the results do not clearly indicate whether discrimination is driven by intrasexual or intersexual interactions. Although not explicitly testing mate choice, an investigation of multi-modal signalling in diurnal *Cnemaspis* geckos showed that female geckos respond more strongly to the epidermal pore secretions of males than to sexually dichromatic male colouration (Kabir et al. 2019), suggesting that epidermal pore secretions influence female behaviour in these geckos.

Summary: pheromones and behavioural isolation in squamates

These studies demonstrate that several squamate groups use pheromones when choosing mates, and that pheromones and preferences can vary among populations and species. Even though relatively few species have been studied, these taxa—snakes, skinks, lacertids, iguanians—are spread across the phylogeny of squamates. This suggests that, although the sources of pheromones and their influence on mate choice vary among taxa, mating pheromones are likely a common feature across squamates. Pheromone-mediated behavioural isolation appears to be driven by male mate choice in those groups so far investigated. Evidence for the role of female mate choice in behavioural isolation is less clear, although it is certainly important in intraspecific mate assessment. However, the field of pheromone research in squamates is still in its infancy. Research effort has focussed on a few conspicuous diurnal species that are easy to observe, many of which possess sexually dimorphic colour patterns that vary among populations and species (e.g., most lacertids). To my knowledge, no studies have focused on the influence of pheromones on mate choice in nocturnal or fossorial species—a considerable proportion of squamate diversity—or on species that do not differ morphologically (see Chapter 3). Because they are otherwise so morphologically similar, cryptic species might offer valuable systems to study how pheromones influence mate choice and behavioural isolation in the absence of morphological divergence.

Study system: Bynoe's geckos

I have outlined how cryptic species can often be viewed as an artefact of the limits of human perception, how knowledge of mating traits has been crucial for understanding diversity and resolving the taxonomy of many animal groups, and how lizards contain disproportionately high cryptic species diversity and are a group for which we have a relatively poor understanding of mating traits. I then speculated that—given that cryptic species are, by definition, visually similar—pheromones are a promising candidate mating trait in cryptic lizard groups. If so, pheromone data will be crucial for understanding mate choice and speciation in lizards, and perhaps useful for resolving the taxonomy of morphologically conservative groups. In this study I assess the divergence, function, and evolution of pheromones in a diverse and problematic cryptic species complex of Australian lizards: the Bynoe's gecko *Heteronotia binoei* (Gray 1845).

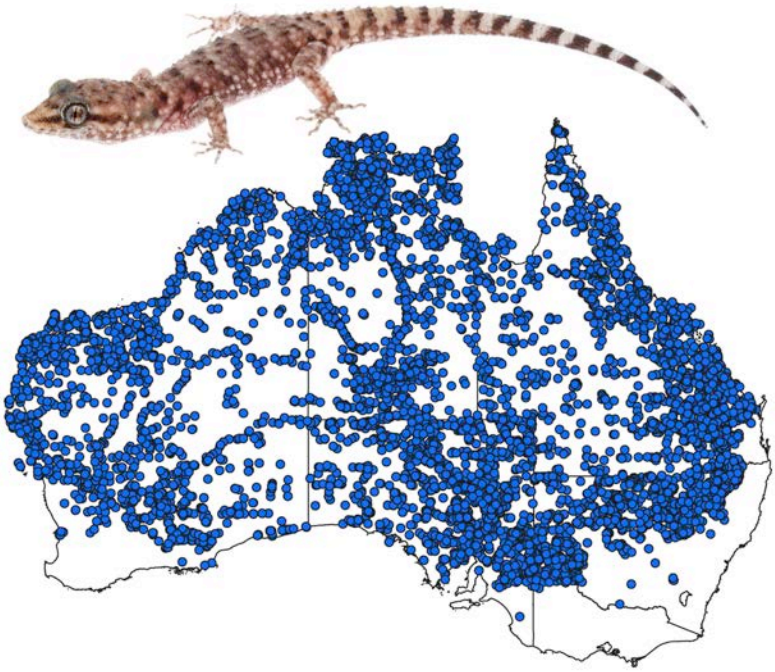

Figure 1.2: An example of *Heteronotia binoei* (top) and occurrence records for the species as it's currently recognised. Records obtained from the Atlas of Living Australia.

Bynoe's geckos are small (80–120 mm nose-to-tail), nocturnal lizards of the family Gekkonidae (Wilson & Swan 2017). Named for Sir Benjamin Bynoe, Charles Darwin's assistant-surgeon on *The Beagle*, these geckos occur across nearly all of Australia (fig. 1.2) in a wide variety of habitats. The genus contains four other currently recognised species that

inhabit rocky regions in central and north-western Australia: *H. atra*, *H. fasciolatus*, *H. planiceps*, and *H. spelea* (Wilson & Swan 2017). Although currently classified as a single species, *H. binoei* has long been recognised as a complex of genetically distinct undescribed species. Cytological work conducted by Craig Moritz in the 1980s revealed seven chromosome races within *H. binoei*, and two additional, clonally reproducing all-female (parthenogenetic) races of hybrid origin (Moritz 1983, 1984). Commenting on this work, the eminent Australian herpetologist Allen Greer (1989) wrote, "The biological significance of all this cytogenetic variation remains to be revealed. But one thing is certain, taxonomically it is a waking nightmare." Taxonomic revision of *H. binoei* was then and still is—more than 30 years later—hampered by a lack of diagnostic characters among candidate species.

But the nightmare got worse. More recent phylogenetic work using mtDNA and multi-locus nDNA sequence data revealed even more candidate species within *H. binoei* (Fujita et al. 2010; Moritz et al. 2016). To illustrate the state of the problem when I started this project, I present the mtDNA chronogram from Moritz et al. (2016) in fig. 1.3, with an arbitrary cut-off of 10% sequence divergence applied as a rough means to identify candidate species ('candidate' being the key word). This yields 23 sexually reproducing candidate species within *H. binoei* (fig. 1.3). The work I present in this thesis reveals even more (Chapters 2 & 4); and there's the added issue of the asexual races (Moritz 1983, 1984). Some candidate species within *H. binoei* occur in sympatry and appear genetically distinct, offering compelling evidence that such lineages represent biological species. Many candidate species, however, are parapatric or allopatric, and divergence among lineages encompasses a broad spectrum, making it difficult to infer which lineages should be considered distinct species and which simply reflect intraspecific structure among interbreeding populations. Using cut-offs of divergence like I did in figure 1.3, while commonly done, is ultimately arbitrary without additional data, and offers little insight into contemporary gene flow and the process of speciation. And while individuals of *H. binoei* from different localities sometimes appear different (at least in terms of colour-pattern), there is high within-lineage variation both between and within populations such that fixed interspecific differences are few or non-existent (fig. 1.4). Phylogenetics alone has not, and likely cannot, resolve the evolutionary history and taxonomy of Bynoe's geckos.

While the *H. binoei* species complex is problematic, the nature of the problem makes this a promising system for studying the role of pheromones as a mating trait in cryptic lizard species. Furthermore, *Heteronotia* possess epidermal pores that are unique to males

(versus present in both sexes in the lacertids and *Liolaemus* lizards discussed earlier). This sexual dimorphism suggests a role in sexual selection, and is a promising trait to study in terms of female mate choice. The presence of epidermal pores also makes extracting pheromone secretions relatively easy and non-lethal to the animal (Chapter 2). Considering these points, I chose *Heteronotia* geckos as a system to address the aims of this study.

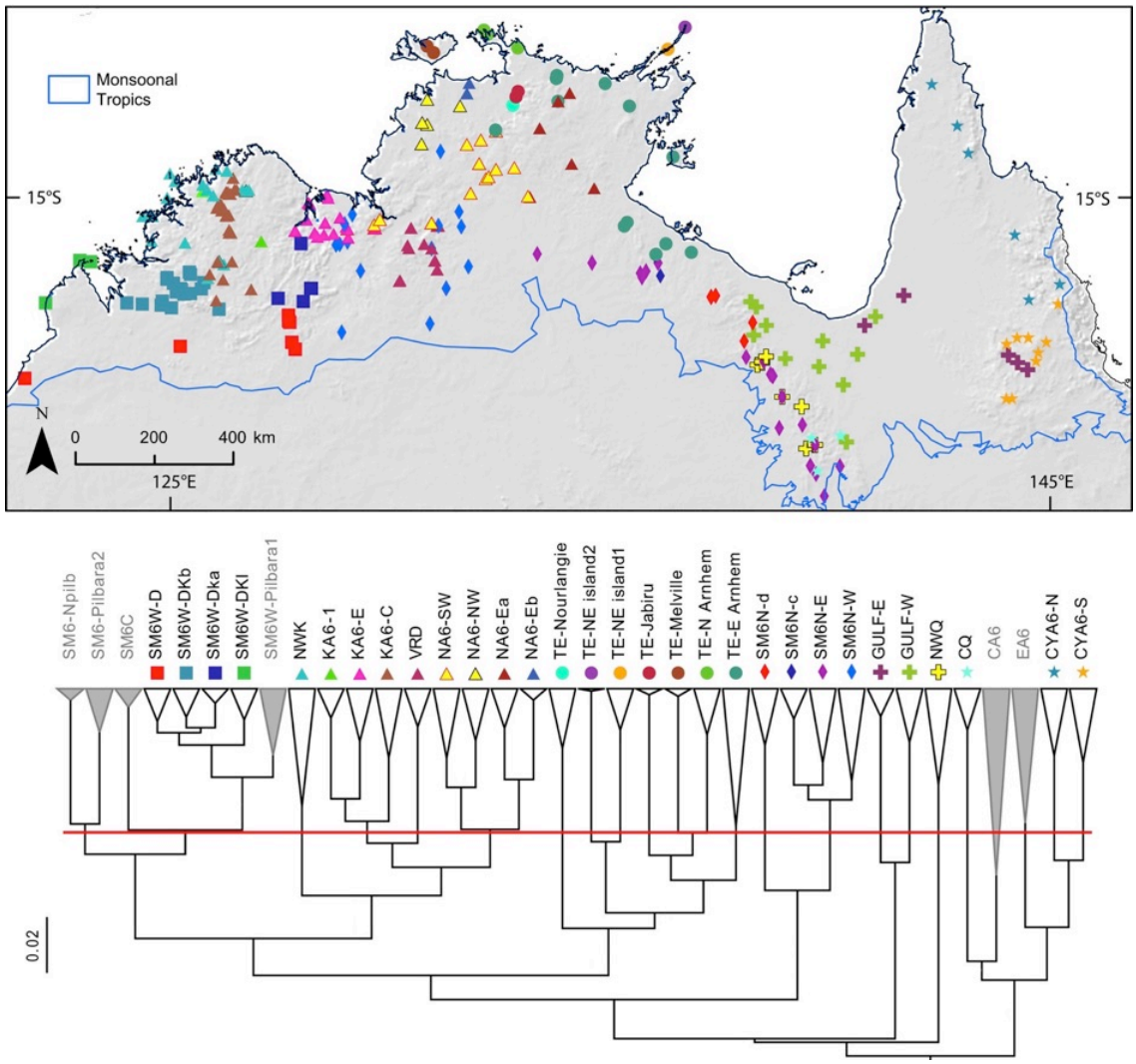

Figure 1.3: A modified version of figure 2 from Moritz et al. (2016) showing mitochondrial lineages of *Heteronotia binoei* and their distributions (only lineages occurring in the Australian monsoonal tropics are shown on the map—additional lineages occur across the remainder of the extensive range shown in fig. 1.2). I've applied an arbitrary cut-off of 10% sequence divergence (shown by the red line) to the mtDNA chronogram to identify candidate species within *H. binoei*, which yields 23 mtDNA lineages as candidate species.

Figure 1.4: Dorsal photos of *Heteronotia binoei*, with each row showing two individuals of the same lineage to illustrate the extremely high levels of colour-pattern variation within lineages. This variation obscures any obvious visual differences between lineages. Lineages are (from top to bottom): EA6, CYA6-S, EIU, and GULF-E (see Chapter 2 for more detail on these lineages).

Aims and outline

The overall aim of this study was to test whether pheromones function as a mating trait influencing behavioural isolation in morphologically conservative lizards and, in doing so, assess the utility of pheromone data as a character for resolving the taxonomy of cryptic lizard groups. If evidence indicated that pheromones do function as a mating trait, my aim was then to make progress toward understanding the evolutionary processes that influence pheromone variation. I use an integrative approach to address these aims, uniting phylogenetics, analytical chemistry, morphometric analysis, behavioural assays, and phylogenetic comparative methods. More specifically, in this thesis I use this approach to answer the following three research questions related to my aims:

1 – Have male pheromones diverged among candidate species?

Chapter 2 tests whether pheromones have diverged among deeply divergent genetic lineages (candidate species) of *H. binoei*. To accomplish this, I collected epidermal pore secretions from individuals of 10 candidate species in north-eastern Australia and characterised pheromone composition using gas chromatography, allowing me to assess multivariate pheromone divergence. Assessing pheromone divergence was important for building the foundation of my research because a lack of divergence among lineages would suggest that pheromones are unlikely to influence behavioural isolation. I also collected morphometric data for these lineages to test whether they are actually morphologically cryptic, and to assess whether pheromones have diverged more than morphology among lineages. If pheromones have diverged among lineages, and are more divergent than morphology, then pheromones may play an important role in speciation in these groups and might be useful in taxonomic studies of cryptic lizards.

2 – Do pheromones influence reproductive isolation via female mate choice?

Chapter 3 tests whether the pattern of pheromone divergence observed in Chapter 2 actually matters for mate choice and behavioural isolation among candidate species. I did this by collecting 240 individual geckos of three deeply divergent genetic lineages that co-occur in north-eastern Australia. I then used behavioural experiments to test whether (1) female geckos detect and care about the pheromones of males, and (2) whether female geckos discriminate between the pheromones of conspecific and sympatric heterospecific males. If females do discriminate among conspecific and heterospecific male pheromones, this would suggest a role in mate choice and behavioural isolation.

3 – What factors influence pheromone evolution?

Chapter 4 begins to investigate this broad question by focussing on the role phylogenetic history and climate play in shaping among-lineage pheromone divergence in *Heteronotia*. To accomplish this, I sampled pheromones from 33 divergent lineages of *Heteronotia*, including 25 lineages of *H. binoei* and eight lineages of *H. planiceps*. The phylogeny of these lineages was then reconstructed using exon capture sequencing and multi-species coalescent phylogenetics, supplemented with mtDNA sequence data for three lineages lacking exon data. I then used this phylogeny to (1) assess phylogenetic signal in pheromone divergence,

and (2) test if and how two major climatic variables (temperature and precipitation) influence among-lineage pheromone variation using multivariate phylogenetic regression.

Thesis structure

This thesis consists of this introduction (Chapter 1), three data chapters (Chapters 2–4), and a general discussion (Chapter 5). The first section of this introduction on cryptic species is being prepared as a perspective piece for publication. Each of the three data chapters have been prepared as standalone manuscripts that are either published (Chapter 2), submitted for publication (Chapter 3), or in the final stages of preparation for publication (Chapter 4). Consequently, there is some unavoidable repetition of material through these chapters, mainly in the introductions. Because the three data chapters include several co-authors, in these chapters I use personal pronouns that are plural, whereas in this introduction and the general discussion (Chapter 5) I have used singular personal pronouns. Supplementary material appears after the respective chapter. Finally, I have included two additional manuscripts completed during my candidature as appendices that—while not related to pheromones—are related to resolving and understanding lizard diversity. These are published papers that each describe and name a new species of lizard, one for which I am the lead author (Appendix I; Zozaya et al. 2019*b*), and one for which I am a co-author (Appendix II; Hoskin et al. 2018).

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CHAPTER 2 — Are pheromones key to unlocking cryptic lizard diversity?

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Abstract

Animals use mating traits to compete for, attract, and choose mates. Because mating traits influence mate choice, the divergence of mating traits between populations can result in reproductive isolation. This can occur without associated morphological divergence, producing reproductively isolated 'cryptic species' that are visually indistinguishable. Thus, identifying the mating traits in morphologically conservative groups is key to resolving diversity and speciation processes. Lizards contain many such groups, with phylogeographic studies often revealing highly divergent but morphologically cryptic lineages within species. Considering that cryptic lizard species can be sympatric but morphologically indistinguishable, we hypothesise that candidate species will exhibit divergent pheromones and that pheromones will have typically diverged more than morphology. To test this, we used gas chromatography to characterise pheromones (epidermal pore secretions) from 10 genetically divergent lineages of the Bynoe's gecko (*Heteronotia binoei*) species complex in northern Australia. Multivariate analyses of pheromone blends and morphology indicate that pheromones are lineage-specific and have diverged relatively more than morphology. Such specificity suggests that pheromones influence behavioural isolation in this morphologically conservative lizard radiation. These results suggest that pheromone data may unlock the tremendous 'cryptic' diversity currently being uncovered in many lizard groups.

Introduction

Phylogeographic studies reveal that many 'species' are actually complexes of morphologically similar but deeply divergent genetic lineages. With further investigation, some of these are found to be undescribed species, often referred to as 'cryptic species'. Resolving cryptic species is important because they compose a significant and largely undescribed proportion of global biodiversity (Bickford et al. 2007). Cryptic species often reflect species classification biased by the limits of human perception. We rarely find cryptic species in birds, a group that has diversified in colour and vocalisation, but instead find them more often in groups such as reptiles, crustaceans, and onycophorans (Pérez-Ponce de León & Poulin 2016). Many cryptic species represent genetically divergent allopatric populations; however, some cryptic lineages exhibit little or no gene flow when in sympatry or secondary contact (e.g., Stuart et al. 2006; Amato et al. 2007; Singhal et al. 2018; Pinto et al. 2019). Considering this, these species are unlikely to be cryptic to each other. More likely, the traits that facilitate behavioural isolation in these groups are difficult to observe. Indeed, the diversity of many groups has been better resolved by technologies that facilitate analysis of 'cryptic' signalling traits, such as pheromones in ermine moths (Löfstedt et al. 1991), ultrasonic communication in lacewing insects (Wells & Henry 1998), and weak electrical signals in mormyrid fishes (Hopkins & Bass 1981). Understanding cryptic species diversity therefore requires knowledge of the traits the animals themselves use to discriminate each other.

Animals use mating traits to compete for, attract, and choose mates, which involves both a mating signal and an associated preference for that signal (Endler 1989; Andersson 1994). The divergence of signals and preferences among populations can result in premating isolation, involving reduced attraction between members of different populations or species that prevents courtship or mating (Mayr 1963; Ptacek 2000; Hoskin et al. 2005; Ritchie 2007). When the respective signals are not visual, mating traits can diverge between populations without any associated morphological changes, resulting in genetically isolated but visually similar species that differ primarily in their mating signals (Panhuis et al. 2001). Considering this, we expect cryptic species to be more common in groups that do not typically exhibit visual or auditory signalling. This is not a new idea: Ernst Mayr postulated that cryptic species (referred to by Mayr as 'sibling species') would be more common in groups in which chemical cues are more highly developed than the sense of vision (Mayr 1963). We predict this is true not just of chemical signals but of any signalling trait not easily perceived by humans. Conversely, one might expect groups

already known to harbour substantial morphologically cryptic diversity to employ non-visual (cryptic) signals as mating traits.

Squamate reptiles (lizards and snakes) are the most speciose clade of terrestrial vertebrates, with over 10,000 described species worldwide (Uetz & Hošek 2018). In many taxa there is strong sexual dimorphism for breeding colours or ornamentation, with these traits often differing between species (e.g., *Anolis*; Losos 2009). But many other taxa lack such obvious traits, and for these taxa genetic studies often reveal high levels of cryptic diversity. Lizards and snakes are a standout group among vertebrates in terms of the number of cryptic species; and, when corrected for research effort, are ranked highly for cryptic diversity among all animal groups (Pérez-Ponce de León and Poulin 2016). Geckos appear to harbour especially high levels of cryptic diversity (Werneck et al. 2012; Domingos et al. 2014; Garcia-Porta et al. 2017; Moritz et al. 2018). Other than in some systems with obvious visual sexual dimorphism, behavioural isolation is less thoroughly studied in lizards and snakes compared to many other animal taxa; hence our understanding of speciation in this species-rich group is incomplete. Considering that these cryptic species complexes are, by definition, extremely similar in morphology, visual cues are unlikely to serve as mating signals. Chemical communication (i.e., pheromones), however, is important in the social interactions of many lizards and snakes (Mason & Parker 2010; Martín and López 2014). Pheromones influence intrasexual aggression (e.g., Martín et al. 2007; Khannoon et al. 2011), kin identification (e.g., Bordogna et al. 2016), and mate choice (e.g., Cooper & Vitt 1986; Shine et al. 2002; Martín and López 2004, 2006; Scott et al. 2015). In several other animal groups, divergent pheromones influence premating reproductive isolation, particularly among insects such as moths and drosophilid flies, but also in vertebrates such as rodents, fishes, birds, and reptiles (reviewed in Smadja & Butlin 2009). This is unsurprising given that olfaction appears to be the most ubiquitous sensory system among animals (Ache & Young 2005).

We test the hypothesis that pheromones have diverged among a cryptic species complex of Australian geckos. The Bynoe's Gecko *Heteronotia binoei* (Gray 1845) is a small, terrestrial, nocturnal lizard distributed widely across Australia (Wilson & Swan 2017). Although currently classified as a single species, *H. binoei* has long been recognised as a complex of several morphologically similar species (Moritz et al. 1990; Fujita et al. 2010). More recent multi-locus sequencing across the range of *H. binoei* reveals that it comprises a dozen or more morphologically conservative but genetically distinct candidate species (Moritz et al. 2016). Species boundaries remain uncertain because genetic divergence

between lineages varies across a broad spectrum, and morphological characters (such as scalation and colour-pattern) are extremely variable within populations. Several genetic lineages of *H. binoei* are known to occur in sympatry or at parapatric contact zones with little or no genetic introgression (Moritz et al. 2016 and unpublished data; fig. 2.1A). Accordingly, we expect that premating isolating barriers will be present between overlapping lineages. Similar to many other lizards (García-Roa et al. 2017), *Heteronotia* possess glands that produce a waxy secretion via epidermal pores anterior to the cloaca (fig. 2.1B inset). In *Heteronotia* these epidermal pores are present only in males. Several studies in other lizards provide evidence that female lizards prefer to associate with the secretions of conspecific versus heterospecific males, suggesting a role for epidermal pore secretions in mate choice and behavioural isolation (reviewed in Martín & López 2011).

Considering these points, and that many *H. binoei* lineages are visually indistinguishable, we predict that pheromones influence behavioural isolation in these lizards. If so, we expect the chemical blends of epidermal pore secretions to have diverged between lineages. We also predict that pheromones will have diverged to a higher degree than morphology, which would inform us on the speciation process and suggest that pheromones might be a useful trait to integrate into species delimitation. To test these hypotheses we characterised morphology and the lipophilic fraction of epidermal pore secretions from 10 lineages of *H. binoei* occurring in three regions of sympatry in north-east Australia, where the deepest phylogenetic structure occurs (Moritz et al. 2016). We then compared both the degree of pairwise overlap and mean pairwise distances for morphology and pheromone traits between lineages to test whether pheromones are more divergent than morphology. We hence demonstrate how this methodology could unlock cryptic species diversity in many lizard and snake groups.

Material and Methods

Field sampling

Ten genetically divergent mtDNA lineages (candidate species) of *H. binoei* were sampled across northern Queensland (fig. 2.1A) in November 2015 and November 2016, coinciding with the reproductive season of these geckos (authors' unpub. data). The distribution of lineages through this region was largely already known from a combination of geographically extensive screening of mtDNA and eight nDNA introns (Fujita et al. 2010; Moritz et al. 2016). For this geographic region in particular, there is strong concordance in

major lineages detected by mtDNA and nDNA introns, and divergence among the candidate species we sampled is deep (ca. 10–20% for mtDNA) (Moritz et al. 2016; see results below). Our sampling focuses on three regions of contact where several candidate species co-occur in a mosaic of widely sympatric or narrowly overlapping lineages (fig. 2.1A). Here we briefly introduce each region:

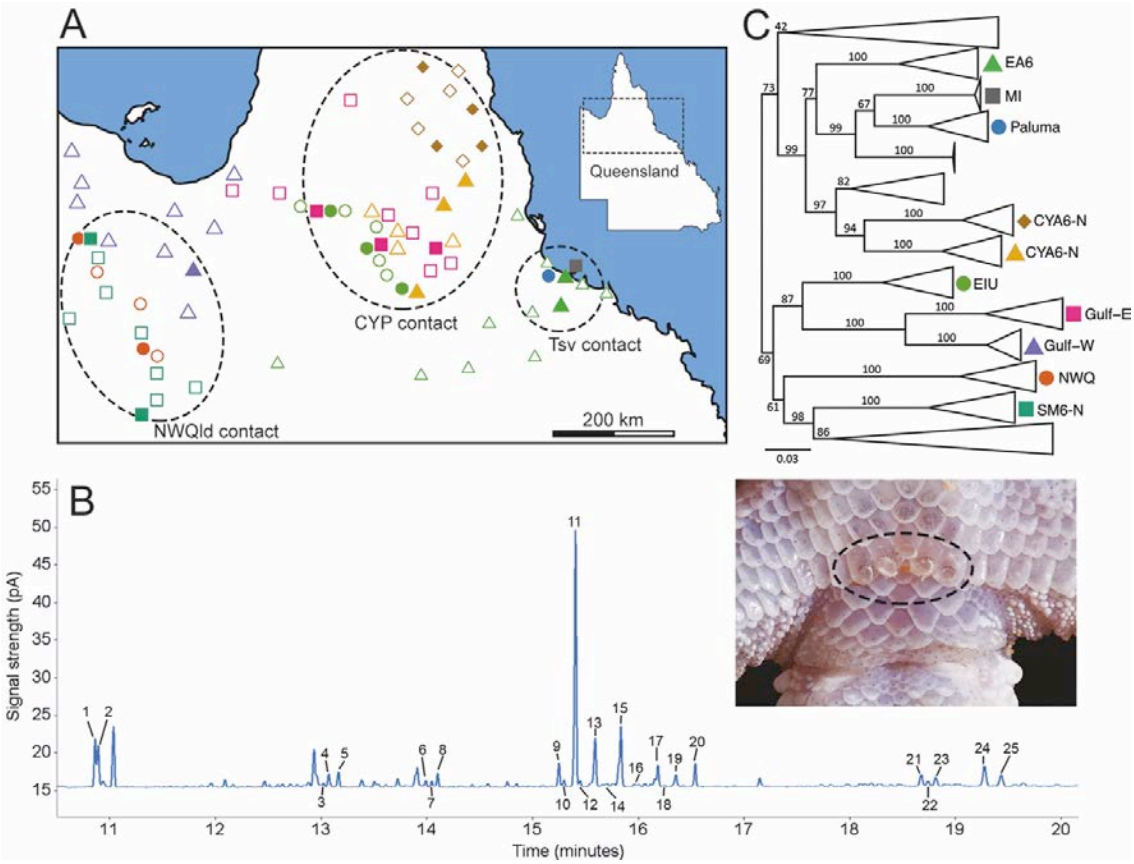

Figure 2.1: (A) Distributions and pheromone sampling sites for the 10 lineages of *Heteronotia binoei* sampled in this study. Colours and shapes correspond to the symbols appearing next to the respective lineage in the phylogeny. Solid symbols represent sites where the respective lineage was sampled for pheromones, whereas open symbols show the known distributions for each lineage based on mtDNA sequencing. Symbols are slightly offset at sites of sympatry so as not to obscure each other. Dashed circles depict the three regions of contact referred to throughout the text. (B) Gas chromatograph (GC) trace of pheromones from the epidermal pores (inset) of a single male *Heteronotia binoei*. Individual peaks represent single compounds or groups of similar compounds and, taken together, form a pheromone blend. Numbered peaks are those used to assess multivariate pheromone divergence among lineages. Note that peaks 3, 14, 16, and 18 are small in this sample. Prominent peaks lacking numbers are those identified as possible contaminants. (C) Relationships among major mtDNA (*ND2* lineages of *H. binoei* inferred using RAxML,

with numbers showing bootstrap support values (%) for major nodes. The lineages sampled in this study are labelled, and those not sampled are collapsed where possible.

North-west Queensland region (NWQld contact). Here we sampled three candidate species: the rock specialist NWQ, and the two habitat generalists SM6-N and GULF-W (fig. 2.1A). Although somewhat separated by habitat, NWQ is often syntopic with both SM6-N and GULF-W, sometimes being found within the same retreat sites (authors pers. obs.). The SM6-N and GULF-W lineages appear to have narrowly overlapping parapatric distributions.

Southern Cape York Peninsula region (CYP contact). Here we sampled four candidate species: CYA6-N, CYA6-S, EIU, and GULF-E (fig. 2.1A). CYA6-N and CYA6-S are parapatric habitat generalists. EIU is mostly rock-dwelling and is sympatric with the habitat generalists CYA6-S in the south and GULF-E in the north of its distribution, although all three have been recorded within the same retreat sites near Forsayth, Queensland. CYA6-S is sympatric with GULF-E at several sites.

Townsville region (Tsv contact). A smaller area (~50 km radius) with three candidate species: EA6, Paluma, and MI (fig. 2.1A). EA6 is a widespread habitat generalist, occurring across eastern and southern Australia, whereas Paluma and MI are sister lineages restricted to the Paluma Range and Magnetic Island, respectively. Notably, MI appears to be the most strongly rock-dwelling lineage of *H. binoei.* EA6 overlaps with the Paluma lineage along the eastern foothills of the Paluma Range. MI is currently isolated on Magnetic Island, but likely occurred in contact with EA6 during lower sea levels.

We sampled 1–5 sites for each lineage depending on the number of known localities for each (some lineages are known from a few or single localities; e.g., MI and Paluma). We sampled a minimum of five male geckos at each site, and up to 10 males at sites where the geckos were abundant. Geckos were captured at night and processed as follows: Tweezers were used to gently apply pressure around the epidermal pores to exude the waxy secretions, which were then collected with a clean stainless steel probe and deposited into an individual glass gas chromatography microvial (fig. S2.1). Tweezers and probes were cleaned with analytical grade isopropanol and wiped with a new Kimwipe before and after sampling a gecko. Each vial was sealed with a PTFE-lined cap and stored in a -20°C freezer until analysed. A control was collected at each site by following the same protocols as above but not collecting any sample from a gecko, allowing us to identify contaminants incurred during sampling. Finally, a small section of tail-tip was collected for mtDNA sequencing.

Lineage identification and relationships

For each site in this study, at least three individuals were sequenced for *ND2* (NADH dehydrogenase subunit 2) to confirm the identification of the lineages present. If, however, the site was known to or found to have more than a single lineage, all individuals were sequenced. DNA was extracted using the salting-out method of Sunnucks and Hales (1996) and amplified using the PCR primers tRNAI and tRNAA from Strasburg and Kearney (2005). We followed the PCR protocols of Fujita et al. (2010). The resulting sequences were cleaned and aligned in Geneious version 6.1.8 (Drummond et al. 2008). The alignment was visually checked and verified by translating the *ND2* coding region into amino acids. These sequences were then aligned with a subset of the *ND2* alignment from Moritz et al. (2016) that included 136 sequences representing all major lineages of *H. binoei*, as well as six sequences of *Heteronotia planiceps* to serve as an outgroup. We used this combined alignment (231sequences; see results) to infer a phylogenetic tree using maximum-likelihood with RAxML version 8.2.11 (Stamatakis 2014). We applied the GTRCAT approximation of rate heterogeneity and performed a rapid bootstrap analysis with 100 bootstrap replicates for statistical support. Sequences are deposited in GenBank (accession numbers MK521075–MK521169).

Most of the mtDNA lineages have been found to be distinct for nuclear genes in previous multi-locus analyses with extensive sampling of individuals (Moritz et al. 2016) and so represent candidate species. For three newly discovered mtDNA lineages from north-east Queensland (EIU, MI, and Paluma), our current sampling of individuals for nDNA is limited to 1–2 individuals per lineage, but this does confirm their phylogenetic distinctiveness (see Chapter 4). Given their substantial mtDNA divergence and this preliminary nDNA data, we include these as candidate species here.

Morphological data

For morphological analyses we used measurements from 136 genetically identified (*ND2* sequenced) preserved specimens of the 10 lineages of *H. binoei* held at the Australian National University and James Cook University (table S2.1). Specimens were not the same individuals that were sampled for pheromones but were collected from the same regions. All available adult specimens for each lineage were measured to the nearest 0.01 mm for eight linear morphological traits using Mitutoyo digital callipers. The traits were chosen as they have been shown to correlate with how lizards use their environment (Losos 2009). The traits were: snout-to-vent length (SVL), inter-limb length, head length, head width,

snout length, eye diameter, hindlimb length, and forelimb length. See table S2.1 for details of how traits were measured and raw data.

Pheromone characterisation

Epidermal pore and control samples were characterised by gas chromatography in a randomised order. Each vial had 50 μL of *n*-Hexane and 50 μL N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) added. Hexane serves as the solvent whereas BSTFA derivatizes the sample, allowing polar compounds to mix with the solvent. New caps with PTFE wafers were then used to seal each vial. Vials were then mixed on a vortex for 30 s and placed on a 70°C heat block for 60 min to promote derivatization. Vials were then mixed with a vortex for an additional 30 s following removal from the heat block. Samples were then immediately characterised on an Agilent 6890N gas chromatograph using an Agilent HP-5 30 m column with a 0.25 mm diameter. An Agilent 7683 autosampler was used to inject 1 μL of sample into a 200°C pulsed-pressure splitless inlet. Due to a software error, only 0.5 μL was injected for those samples collected in November 2016 (see table S2.2); however, since we analyse pheromone composition as proportional data, this does not affect our analyses. The oven temperature program started at 50°C, then ramped at 25°C/min to 125°C, then at 15°C/min to 325°C and held for 8 min, for a total run time of 24.5 min. The flame ionisation detector was set at 250°C. Before and after sample injection, the autosampler syringe was cleaned with three washes of analytical grade isopropanol followed by three washes of analytical grade *n*-Hexane. Chromatograms were manually integrated using Agilent OpenLab software. Chromatogram peaks that appeared in controls were considered to be contaminants and were excluded from integration of all samples. Pheromone data are deposited in the Dryad Digital Repository (Zozaya et al. 2019).

Phenotypic trait analysis

We compared divergence of morphology and pheromone composition among the 10 lineages of *H. binoei* sampled. Our approach was to first compare these traits across all lineages and then repeat the analyses on subdivided datasets representing the three regions of contact (fig. 2.1A) to better detect fine-scale patterns of divergence among co-occurring lineages. Pairwise contrasts were carried out between lineages within each contact zone. We did not employ phylogenetic comparative methods here for two reasons. First,

phylogenetic comparisons of trait evolution with only 10 taxa are not statistically sound (Blomberg et al. 2003). Second, such analysis is not important for answering our core question: Are pheromone blends lineage-specific, and are pheromone blends more divergent than morphology between candidate species? We recognise that phylogenetic comparative analyses are important for understanding the evolution of these traits and work is underway incorporating data for over 30 lineages across the *Heteronotia* radiation to investigate phenotypic trait evolution (see Chapter 4).

Morphological Traits. We performed a principal component analysis (PCA) on the eight morphological traits and retained the minimum number of PCs needed to account for approximately 100% of body size variation (PC1) and approximately 85% of the remaining body shape variation (table S2.3). All PCAs were performed using the 'rda' function in the R package *vegan* using a correlation matrix (Oksanen et al. 2017). This was done for the complete dataset of all 10 lineages and then repeated for individual datasets representing the three contact zones. 'Sex' was initially included as a main effect and an interaction with lineage in the analysis of body shape outlined below, but both effects were not significant. Thus we excluded 'sex' and included both males and females in analyses of body shape to increase sample sizes.

Pheromone Traits. We successfully characterised pheromone samples from a total of 128 individual geckos representing the 10 genetically divergent lineages (table S2.2). A total of 25 peaks were integrated for all pheromone samples (fig. 2.1B; table S2.4). Each peak represents either a single compound or two or more compounds with similar retention times. When similar compounds could not be reliably separated they were considered as a single trait. Proportions were calculated using the total of all 25 integrated peaks as the divisor. We log-contrast transformed each peak to account for the unit-sum constraint of proportional data using log10(proportion peak *n*/proportion denominator peak) (Aitchison 1986). Peak 8 was selected as the denominator of the log-contrast transformation because it varies the least among lineages, resulting in 24 log-contrast pheromone traits. We corrected for variation in chemical characterisation of pheromone composition between years by taking the residuals of a linear regression of each log-contrast trait against year (2015 or 2016). We used PCA (methodology as above) to reduce the 24 log-contrast transformed traits for multivariate analysis, keeping the minimum number of PC axes to account for approximately 85% of trait variation (table S2.5). This was done for the complete dataset of all lineages and as well as for each dataset representing the three contact zones. For each contact zone comparison, however, we did not correct for year-to-

year variation and instead used data collected only within the same field season (2015 for CYP and Tsv contacts; 2016 for NWQld contact). To ensure that our results were not an artefact of the log-contrast transformation method, we also re-ran all analyses with logit transformed pheromone data (Warton & Hui 2011), which did not change our results (see table S2.6 for details and results of these analyses).

Testing for phenotypic trait divergence among lineages

To test whether phenotypes differed significantly among lineages, the following dependent variables were included in the multivariate analyses: for the overall comparison of all 10 lineages, PC1–5 representing 98% of morphological variation (~100% body size variation and 85% body shape variation) and PC1–9 representing 85% of pheromone variation were retained; for the NWQld contact, PC1–5 representing 97% of morphological variation and PC1–6 representing 82% of pheromone variation were retained; for the CYP contact, PC1–5 representing 96% of morphological variation and PC1–6 representing 84% of pheromone variation were retained; for the Tsv contact, PC1–4 representing 98% of morphological variation and PC1–5 representing 85% of pheromone variation were retained.

To test the fixed effect of genetic lineage on morphology and pheromone traits, we carried out multivariate analyses using the MIXED procedure in SAS version 9.2. We carried out the formal significance testing using the MIXED procedure due to our unbalanced sampling design and to correctly specify the variance-covariance structure amongst variables. To obtain a multivariate significance test in the MIXED procedure, a likelihood ratio test is used to compare two models whose parameters are estimated using maximum likelihood: the full model containing lineage (the lineage-by-trait model) vs. the null model without lineage (the trait-only model) (full details are in Wright 1998; e.g., Hoskin et al. 2011). We selected the variance-covariance structure for each analysis by running every full (lineage-by-trait) model with each of eight covariance structures available in the MIXED procedure (Kincaid 2007). We then compared the AICc scores of the full models and selected the model that yielded the lowest AICc score (table S2.7) (Fernandez 2007). Both full and null models were run using denominator degrees of freedom estimated using the Kenward-Rogers method due to unequal sample sizes among lineages (SAS Institute 2010). To obtain the likelihood ratio test (LRT), the difference in the -2 log likelihoods between the full and null models was tested against a X^2 distribution with degrees of freedom being the difference in number of parameters between the two models

(Wright 1998). If the effect of lineage is found to be significant using the LRT, then a model with planned contrasts comparing between lineages can be assessed. In MIXED, significance testing for planned multivariate contrasts is done using the full model, and with parameters calculated using restricted maximum likelihood (REML) rather than maximum likelihood (Wright 1998). This then provides multivariate *F*-tests for pairwise comparisons between lineages for both morphology and pheromones within each contact region. All *P*-values were globally adjusted for multiple comparisons using the False Discovery Rate (FDR) method (Benjamini & Hochberg 1995).

To visualise the above formal statistical analyses, we calculated the first two canonical variates (CVs) from a multivariate linear model, which represent the linear combination of traits that differ most among the respective lineages (fig. 2.2, table S2.8). The models included the same parameters as each respective multivariate analysis above but used the GLM procedure rather than the MIXED procedure in SAS, which cannot produce canonical variates. The first two CVs of the overall morphology analysis accounted for 57.17% (CV1) and 22.46% (CV2) of variation included in the model (total 79.63%); and the first two CVs of the overall pheromone analysis accounted for 46.25% (CV1) and 24.42% (CV2) of variation included in the model (total 70.63%). The percentage of variation included in the contact zone analyses are presented in table S2.8. To interpret which morphology and pheromone traits contributed to the divergence amongst lineages, we used the rule of thumb where traits contribute significantly to a CV if they have a loading of at least 70% of the absolute highest loading variable (Mardia et al. 1979; e.g., Blows & Higgie 2003, Hoskin et al. 2011). This identified which PCs contributed significantly to each CV (table S2.8). We then used the same approach to identify which traits contributed significantly to each relevant PC (morphology: table S2.3; pheromones: table S2.5).

Comparison of trait divergence

To test whether lineages differ relatively more in morphology or pheromones we compared both pairwise trait overlap and mean pairwise trait distances for the two trait types. To compare trait overlap we carried out three steps. First, we calculated the minimum convex hull polygons of these two CVs for each lineage from the overall analysis that included all 10 lineages. Second, the overlap area and union area (the area of the two respective polygons combined) were then calculated for each pairwise lineage comparison using the 'gArea' and 'gUnion' functions in the R package *rgeos* (Bivand et al. 2014). Finally, the

proportional area of overlap for each lineage comparison was calculated by dividing the overlap area by the union area. Because of a large number of zero values (due to no polygon overlap between many lineages) we tested whether the degree of overlap among all 10 lineages differed significantly between traits via a non-parametric Kruskal-Wallis one-way ANOVA by ranks, with proportion of overlap as the response variable and trait type (morphology versus pheromones) as the explanatory variable.

To compare trait distances we took the average pairwise Euclidean distance between each lineage pair for both morphology and pheromones. For morphology, each variable was corrected for body size by taking the residuals of a linear regression of each trait against SVL, yielding seven size-independent measures of body shape. The eight morphological traits (SVL and seven size-corrected shape variables) were then standardised to a mean of 0 and standard deviation of 1 to scale the two trait types. The 24 log-contrast pheromone variables were also standardised before analysis. For both morphology and pheromones, we then used the R package *vegan* to first calculate a Euclidean distance matrix between all observations with the function 'vegdist', followed by calculating mean pairwise distances between lineages with the function 'meandist' (Oksanen et al. 2017). We used a t-test to test if mean pairwise distances among the 10 lineages differed significantly for the two trait types (morphology versus pheromones). To be conservative, we also re-ran this t-test comparison but with mean pairwise distances calculated with PC1–6 for each morphology (99% variation) and pheromones (76% variation) so that the same number of dimensions represented each trait type. This was in case the far higher number of dimensions for pheromones biased the result.

Results

Lineage identification and relationships

We successfully obtained ~919 bp sequences of *ND2* from 95 individuals of *H. binoei* sampled in this study. These, combined with the ~1,041 bp sequences from 136 individuals taken from Moritz et al. (2016), yielded an alignment of 231 sequences. Phylogenetic analysis of these sequences confirms the lineage membership of geckos from which pheromones were sampled. All 10 candidate species sampled herein are deeply divergent mtDNA lineages with ML bootstrap support values of 100% (fig. 2.1C). The major lineages recovered in this phylogeny are consistent with those of previous studies (Fujita et al. 2010; Moritz et al. 2016), although deep relationships vary. Additionally, our phylogeny

includes three newly discovered divergent lineages from north-east Queensland that are not in previous studies: EIU, MI, and Paluma.

Phenotypic divergence among lineages

Overall lineage comparison. Morphology and pheromone composition differed significantly among the 10 lineages of *H. binoei* (table 2.1). Figure 2.2 is a visual representation of phenotypic divergence among lineages, with the first column of panels representing the overall lineage comparison and subsequent columns representing each region of contact. Inspection of PCA and CV loadings (tables S2.3, S2.8) reveals that morphological divergence in the overall comparison is largely driven by body size (CV1) and eye diameter (CV2). For pheromone data, CV axes are influenced by many peaks (12 out of 25 peaks), the majority of which have early retention times (tables S2.5, S2.8).

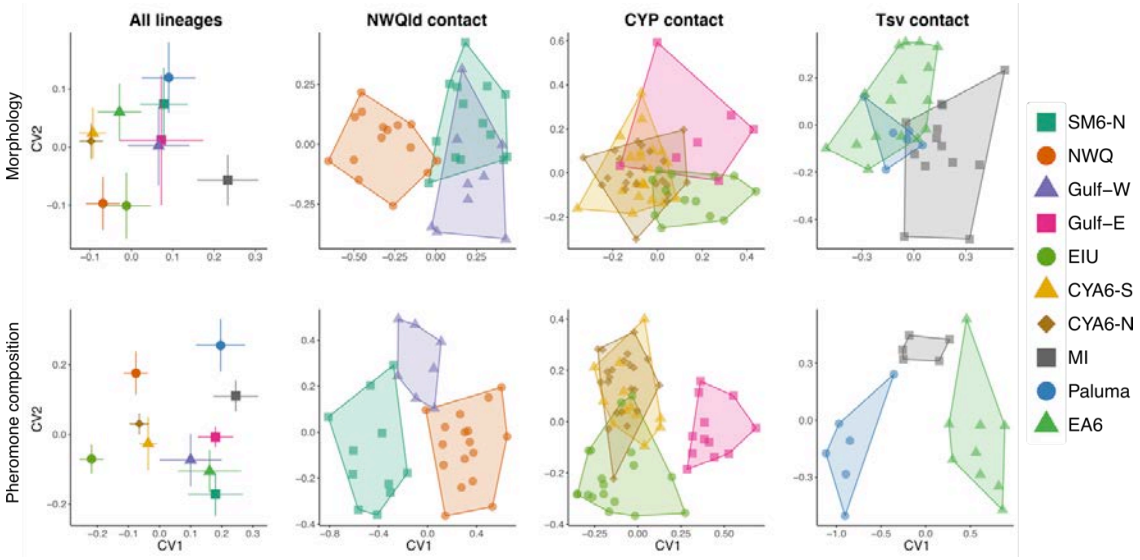

Figure 2.2: Morphology and pheromone blends show variation within and between the 10 candidate species of *Heteronotia binoei* examined here. Colours and symbols match Figure 2.1. The second and third rows are trait space representations of the first two canonical variate axes (CV1 and CV2) of morphology (first row) and pheromone composition (second row). The first column shows trait values for all 10 lineages; symbols are centroids representing the mean of CV1 and CV2 for each lineage, with whiskers representing 95% confidence intervals. Subsequent columns represent the three regions of contact with corresponding labels appearing above each column; symbols represent the trait values for individuals and polygons representing minimum convex hulls for each lineage.

Table 2.1: Results of multivariate analysis of morphological and pheromone divergence among 10 candidate species of *Heteronotia binoei* in north-east Australia

		MANOVA		
Sites (regions of contact), traits, and lineages	X^2	*F*	*df*	*P*
All lineages				
Morphology	234	…	45	<0.001
Pheromone composition	450	…	81	<0.001
NWQld contact (GULF-W, NWQ, SM6-N):				
Morphology	40	…	10	<0.001
GULF-W *vs.* NWQ	…	4.53	5,175	<0.001
GULF-W *vs.* SM6-N	…	2.03	5,175	0.076
NWQ *vs.* SM6-N	…	5.45	5,175	<0.001
Pheromone composition	49	…	12	<0.001
GULF-W *vs.* NWQ	…	3.45	6,192	0.002
GULF-W *vs.* SM6-N	…	3.39	6,192	0.003
NWQ *vs.* SM6-N	…	5.34	6,192	<0.001
CYP contact (CYA6-N, CYA6-S, EIU, GULF-E):				
Morphology	46	…	15	<0.001
CYA6-N *vs.* CYA6-S	…	0.15	5,295	0.980
CYA6-N *vs.* EIU	…	4.08	5,295	0.001
CYA6-N *vs.* GULF-E	…	4.14	5,295	0.001
CYA6-S *vs.* EIU	…	4.14	5,295	0.001
CYA6-S *vs.* GULF-E	…	3.87	5,295	0.002
EIU *vs.* GULF-E	…	3.62	5,295	0.003
Pheromone composition	132	…	18	<0.001
CYA6-N *vs.* CYA6-S	…	1.46	6,140	0.196
CYA6-N *vs.* EIU	…	12.90	6,140	<0.001
CYA6-N *vs.* GULF-E	…	16.04	6,140	<0.001
CYA6-S *vs.* EIU	…	6.65	6,140	<0.001
CYA6-S *vs.* GULF-E	…	10.51	6,140	<0.001
EIU *vs.* GULF-E	…	16.18	6,140	<0.001
Tsv contact (EA6, MI, Paluma):				
Morphology	35	…	8	<0.001
EA6 *vs.* MI	…	6.98	4,128	<0.001
EA6 *vs.* Paluma	…	2.06	4,128	0.090
MI *vs.* Paluma	…	3.73	4,128	0.006
Pheromone composition	32	…	10	<0.001
EA6 *vs.* MI	…	2.61	5,85	0.030
EA6 *vs.* Paluma	…	4.13	5,85	0.002
MI *vs.* Paluma	…	2.70	5,85	0.026

Note: Results show the overall analysis of all lineages, as well as analysis for each of three regions of contact with pairwise comparisons. A likelihood ratio test is used to test for overall multivariate divergence amongst lineages. The likelihood ratio X^2 value is the difference between the -2 log-likelihoods of the full (lineage-by-trait) model and the null (trait-only) model. For the pairwise planned contrasts, significance testing is done within the full model using *F*-tests. The statistical significance for all *P*-values was unchanged by False Discovery Rate (FDR) adjustment. The un-adjusted *P*-values are reported here.

NWQld contact. Both morphology and pheromone composition differed significantly among lineages at this contact zone, with overall body size and eye diameter being the main contributors to the divergence (tables S2.3, S2.8). Pairwise contrasts offer more detail regarding patterns of divergence (table 2.1; fig. 2.2): the rock-dwelling NWQ is smaller and has relatively larger eyes in comparison to both GULF-W and SM6-N, while these two lineages are not significantly morphologically divergent from each other. Pheromone

composition, however, differed significantly for all three pairwise contrasts, with eight peaks predominantly contributing to the divergence (tables S2.5, S2.8). All eight peaks had early or moderate retention times.

CYP contact. Morphology and pheromone composition differed significantly among lineages (table 2.1; fig. 2.2). Morphology was significantly divergent between all lineages except for the comparison between CYA6-N and CYA6-S, with overall body size being the main contributor to the divergence (tables S2.3, S2.8). Similarly, pheromone composition differed significantly between all pairwise comparisons except between CYA6-N and CYA6-S. Ten peaks contributed to the divergence amongst lineages, with eight of those having early retention times (tables S2.5, S2.8). Notably, the closely related and parapatric CYA6-N and CYA6-S lineages do not differ in any of the phenotypic traits measured.

Tsv contact. Morphology and pheromone composition differed significantly among lineages at this contact zone (table 2.1; fig. 2.2), with overall body size contributing most to the divergence (tables S2.3, S2.8). The large-bodied MI lineage is morphologically divergent from both EA6 and Paluma, although EA6 and Paluma are not significantly divergent from each other (table S2.3 and S2.8). Pheromone composition, however, differs strongly and significantly for all pairwise contrasts. Interestingly, only five compounds contribute strongly to divergence at this contact (tables S2.5, S2.8).

In summary, the pattern of morphological divergence is mostly consistent across analyses, with size being important in CV1 in the overall comparison and at all contacts and eye diameter being important in CV2 in the overall comparison and contributing at two of the three contacts (NWQld and Tsv but not CYP). The only common pattern in pheromone divergence is that many peaks are divergent amongst lineages (overall comparison: 12 peaks; NWQld: 8 peaks; CYP: 10 peaks), with earlier retention times being generally more involved in divergence than those with later retention times. The exception is the Tsv contact, where only five peaks appear to be involved in the divergence among lineages and with no pattern of early or late retention times.

Comparison of morphological versus pheromone divergence

Pheromone composition overlaps significantly less than morphology among the 10 lineages (Kruskal-Wallis test, $X^2 = 18.75$, $df = 1$, $P < 0.001$) (fig. 2.3A). Similarly, mean pairwise distances among the 10 lineages are significantly greater than morphological distances, both when all variation is included (t-test, $t = -13.37$, $df = 83$, $P < 0.001$) and when the more

conservative approach with PC axes is performed (t-test, t = -7.34, df = 80, $P < 0.001$) (fig. 2.3B).

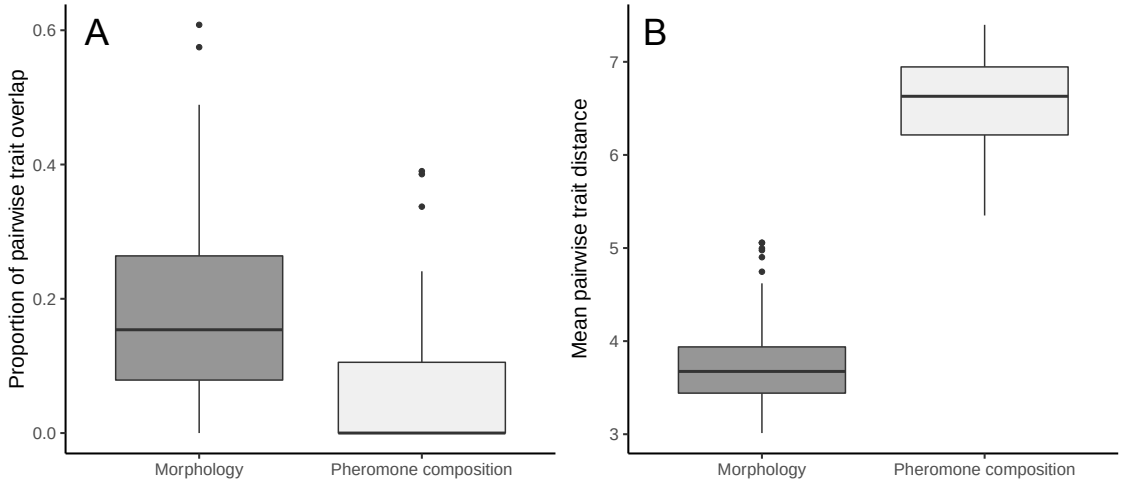

Figure 2.3: Pheromone blends have diverged relatively more than morphology among the 10 candidate species of *Heteronotia binoei* examined here, with less overlap in trait space (A) and greater distance between lineage means (B). (A) Overlap analysis: Boxplots illustrate the proportion of pairwise trait overlap among lineages of *H. binoei*. The more different these traits are between lineages, the lower the value of overlap. (B) Distance analysis: Boxplots illustrate the mean pairwise Euclidean trait distance among lineages of *H. binoei*. The more different these traits are between lineages, the higher the distance value. Boxplots show medians as horizontal black lines, interquartile ranges (IQR) around the medians as boxes, non-outlier ranges (within 1.5 x IQR) as whiskers, and outliers (greater than 1.5 x IQR) as individual data points.

Discussion

Comparing phenotypic divergence among closely related species can reveal the traits influencing coexistence and reproductive isolation. While pheromones are known to be important in social interactions in many squamates, they have rarely been analysed in terms of divergence between cryptic vertebrate lineages. This is because the collection of secretions and the analysis and interpretation of chemical signals they contain is difficult in comparison to morphology or other signalling modalities, such as vision or acoustics. A particular issue is the potentially enormous dimensionality of chemical signals, where a signal can range from a single to hundreds of compounds. Here we provide a methodology

for field collection and subsequent analysis of complex pheromone blends, and a framework for comparing divergence in pheromones and other traits. We addressed the issue of dimensionality by performing data reduction via PCA and by using a conservative approach that biases comparisons of trait divergence in favour of morphology over pheromones. Further, we analysed divergence across multiple regions of contact to assess consistency in our results.

We found substantial pheromone divergence between genetically divergent lineages of *H. binoei*. On average, overlap in pheromone composition between lineages is less than morphological overlap and mean pairwise phenotypic distances between lineages are greater for pheromones than morphology (figs. 2.2, 2.3). Further, while morphological divergence is idiosyncratic, pheromone blends are consistently divergent among all pairwise comparisons except between CYA6-N and CYA6-S. Importantly, at all three contact regions we found very little overlap in pheromones between lineages. The observation of lineage-specific pheromone blends, particularly in areas of overlap, and that pheromones have diverged more than morphology, meet our prediction that pheromones influence behavioural isolation in this cryptic species complex.

Although less divergent than pheromones, there is significant morphological divergence among lineages. Morphological differences are known to reflect ecological divergence in lizards, such as structural habitat use and prey type (Losos 2009; Moritz et al. 2018). Such divergence can reduce competition and facilitate coexistence (Schluter 2000), and may directly play a role in mate choice and reproductive isolation (e.g., Richmond & Jockusch 2007). Morphological divergence in the *Heteronotia* lineages examined here is difficult to interpret. First, morphology is sometimes divergent but with considerable overlap between lineages. Pairwise contrasts show that morphological divergence is most often driven by body size. We see no clear interpretation of body size divergence (e.g., rock-dwelling lineages are both large and small) but our study was not designed to test this in detail. The other morphological trait contributing to lineage divergence in some analyses was larger eyes associated with rock-dwelling lineages (NWQ, EIU, and MI). This is interesting because a tendency for larger eyes has been observed in other rock-dwelling geckos from north-eastern Australia (e.g., Hoskin & Couper 2013). Further research is necessary to assess whether morphological variation reflects ecological divergence in *H. binoei*.

The importance of pheromone divergence and behavioural isolation has been long recognised among invertebrate biologists (Smadja & Butlin 2009). For example, the

pheromones of some closely related moths share the same major chemical components but differ in the blend of these components, contributing to the maintenance of reproductive isolation (Linn & Roelofs 1995). Similarly, differences in the blend of cuticular hydrocarbons influence premating isolation in various drosophilid flies (e.g., Blows and Allan 1998; Higgie et al. 2000). It is now evident that pheromones influence reproductive isolation in many vertebrate groups as well (Smadja & Butlin 2009), including lizards. Behavioural studies suggest that several lizard and snake groups use pheromones when choosing mates, and that signals and preferences can vary among populations within and between species (Barbosa et al. 2006; Gabirot et al. 2012; García-Roa et al. 2016). Even though relatively few species have been studied, these taxa—snakes (Mason et al. 1989; Shine et al. 2002), skinks (Cooper & Vitt 1986, 1987; Scott et al. 2015), iguanians (Escobar et al. 2003; Labra 2011), and lacertids (Martín & López 2004)—are distributed across the clade of squamate reptiles, suggesting that pheromones are a common signalling trait across this group.

Of particular interest are lizard groups that possess epidermal pores. These are absent in snakes and skinks but present in lizard groups such as lacertids, tropidurids, and gekkonids (the gecko family containing *Heteronotia*). Behavioural studies of lacertid and tropidurid lizards suggest that the lipophilic fraction of epidermal pore secretions function both in a mate choice context and in male–male interactions (reviewed in Martín & López 2014). Epidermal pore secretions contain dozens of compounds, which makes teasing apart the influence of individual compounds difficult and potentially irrelevant, given that it is likely that combinations of compounds are perceived in animals (Firestein 2001). Behavioural assays suggest that cholesta-5,7-dien-3-ol, ergosterol, and oleic acid influence female choice in lacertid lizards (Martín & López 2006, 2010) and that cholesterol, hexadecanol and other long chain alcohols influence male-male interactions (Martín & López 2007, 2008; Khannoon et al. 2011). Several of these or similar compounds are present in the epidermal pore secretions of most lizard species so far characterised (Weldon et al. 2008; Martín & López 2014), including geckos (Khannoon 2012). Preliminary analysis confirms that some of these compounds—such as cholesterol (peak 11, fig. 2.1B)—are present in the secretions of *H. binoei*; however, full characterisation has been hampered because *H. binoei* produce very small volumes of secretion (Zozaya et al. in progress; see Chapter 5 for further discussion). Nevertheless, our results show that candidate species within *H. binoei* differ in the relative proportion of shared compounds. Considering this and that epidermal pore secretions influence mate choice and intrasexual

competition in other lizards, we predict that behavioural experiments will reveal similar roles in *Heteronotia*. Our results here suggest that it is a combination of many pheromone compounds—including both minor and major compounds—that differ amongst lineages; therefore, it is likely that a blend of compounds mediates behavioural isolation amongst these cryptic lineages.

Interpreting the significance of pheromone divergence is difficult without an understanding of how different compounds influence behaviour, and the selection pressures acting on the evolution of pheromone blends. Given the hugely multidimensional nature of chemical signals, determining the specific influences of individual compounds on behaviour might often be impossible; nevertheless, identifying the processes influencing pheromone evolution is possible (Yohe & Brand 2018). Pheromone divergence among populations and species can result from several processes, including: divergent natural selection to different local environments (Endler 1992; Boughman 2002); selection to reduce maladaptive hybridization between sympatric species (i.e., reinforcement; Howard 1993; Higgie et al. 2000; Hoskin et al. 2005; Dyer et al. 2014), as well as other types of reproductive interference (Hoskin & Higgie 2010); divergent sexual selection, possibly due to female preference, male-male competition, or both (Andersson 1994; Nosil et al. 2007); and stochastic processes, such as drift and founder effects (Lande 1981). Divergent natural selection under local environmental conditions can play a role in the evolution and divergence of pheromones (Yohe & Brand 2018). Pheromones are a chemical signal, and a signal must be transmitted effectively in its environment if it is to be reliably detected by the intended receiver (Endler 1992). Thus, environmental conditions can exert selection pressure to optimise signal efficacy against background conditions (e.g., sensory drive; Boughman 2002). For example, Martín et al. (2015) provide evidence that differences in pheromone blends between two populations of the Iberian wall lizard (*Podarcis hispanicus*) reflect adaptation to local environments. A substantial influence of climate on the evolution of epidermal pore secretions is further supported by a comparative analysis of 64 species of lacertid lizards (Baeckens et al. 2018), which showed that the pheromone secretions of species from more arid areas have higher proportions of stable fatty acid esters and high molecular weight alcohols. While our study does not address the influence of environment on pheromone composition (but see Chapter 4), our results show that pheromones have diverged even among sympatric lineages of *H. binoei*, suggesting that abiotic factors alone cannot account for the degree of pheromone divergence observed here. Detailed behavioural data and phylogenetic

comparative analyses will be key for interpreting the full significance of pheromone variation and divergence among populations and candidate species within this complex (Chapters 3 & 4).

Understanding cryptic species diversity

Cryptic species pose particular challenges to taxonomists and therefore to biodiversity and extinction estimates. Knowledge of sexually selected signals has been crucial for understanding species boundaries and diversification in many morphologically conservative groups. Taxonomic analyses of orthopteran insects (e.g., Nityananda & Balakrishnan 2006), frogs (e.g., Padial et al. 2008), mormyrid fish (e.g., Crawford & Hopkins 1989), and many other groups have relied heavily on the analysis of signalling traits. Analyses of these traits have provided information regarding reproductive isolation and the processes that drive it, and have facilitated the description of many otherwise indistinguishable species. Without knowledge of these signals the taxonomy of many groups would be poorly resolved.

Our results contribute to the understanding of signalling traits in squamate reptiles, the most speciose group of terrestrial vertebrates. Studies of lizard signals have focused heavily on visual ornaments and displays, such as sexually dimorphic colouration, and stereotypical movements (Pough et al. 2015). These studies have been important for understanding diversification and behaviour in many lizard groups (e.g., *Anolis*; Losos 2009). We suspect the study of pheromones will contribute similarly to our understanding of lizard groups lacking such visual displays, such as the *H. binoei* complex examined herein. Taxonomic resolution of the *H. binoei* complex is difficult because morphology in this radiation is mostly uninformative, colouration is variable and overlapping among most lineages, and genetic divergences vary across a broad spectrum (fig. 2.1C; Fujita et al. 2010; Moritz et al. 2016). Genetic analyses can be valuable for estimating whether sympatric or parapatric populations are reproductively isolated, but are more ambiguous for those that are allopatric (Singhal et al. 2018). For *H. binoei*, nearly all lineages so far examined with multilocus sequence data are statistically supported as separately evolving lineages (Fujita et al. 2010; Moritz et al. 2016; unpub. data). Our chemical analysis indicates that pheromones are typically divergent among candidate species, even some that are relatively closely related (e.g., EA6, MI, Paluma). With further research and methodological development (outlined below; see Chapter 5), pheromone data can be integrated into a taxonomic framework to better infer species boundaries, particularly when morphology is highly conservative (Padial et al. 2010). Explicitly testing for reproductive isolation is arduous and often impractical,

but analysis of mating traits—particularly those influencing reproductive isolation—can be a more practical alternative to infer species boundaries.

Future directions

Pheromone divergence needs to be further linked to reproductive isolation in this system, and there are several ways to test this. First, behavioural studies can test the expectation that pheromones will be involved in mate choice and/or male competition (see Chapter 3). Second, the degree of pheromone divergence and contemporary gene flow can be assessed across the many pairwise contacts between lineages, with the expectation that greater pheromone divergence will correlate with lesser gene flow even after controlling for genetic distance. Third, assessing relative pheromone divergence in sympatric versus allopatric populations would test for an expected pattern of reproductive character displacement where lineages overlap. Finally, phylogenetic comparative methods can be used to test the expectation of more rapid divergence of pheromones compared to other traits, such as morphology, across the 30+ lineages of the *Heteronotia* radiation across Australia.

The other avenue for further research in this system is investigating the genetic and environmental contributions to pheromone variation. As outlined above, our discovery of pheromone differences between lineages that are in sympatry and sampled at the same time suggests a strong genetic component. However, pheromone blends in lizards can be influenced by factors such as diet and thermal environment (Martín & López 2014; Heathcote et al. 2014). Resolving the role of plasticity due to the environment could involve correlating within-lineage variation in pheromone blends with abiotic variables, phylogenetic comparative analyses including abiotic variables (Chapter 4), and estimating pheromone heritability using lab-reared families.

Beyond this system, studies are needed across more taxa to understand the importance of pheromones in population divergence and speciation (Symonds & Elgar 2008; Smadja & Butlin 2009). Chemical signals are particularly complex to measure, analyse, and interpret; thus, pheromones have been studied relatively less than visual or acoustic signals in vertebrates (Symonds & Elgar 2008; Smadja & Butlin 2009). This in part explains why major groups of vertebrate diversity (e.g., many lizard clades) remain poorly resolved. Pheromones may also play a significant role in animal groups that rely primarily on visual or acoustic communication (e.g., frogs; Byrne & Keogh 2007) and future research may find an important role of pheromones in reproductive isolation in these groups. Although studying pheromones is difficult, they offer exciting opportunities for further

resolving vertebrate diversity and the underlying processes generating it (Smadja & Butlin 2009).

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An individual of the NWQ lineage photographed at Lawn Hill (Boodjamulla) National Park, Queensland.

Chapter 2 – Supplementary material

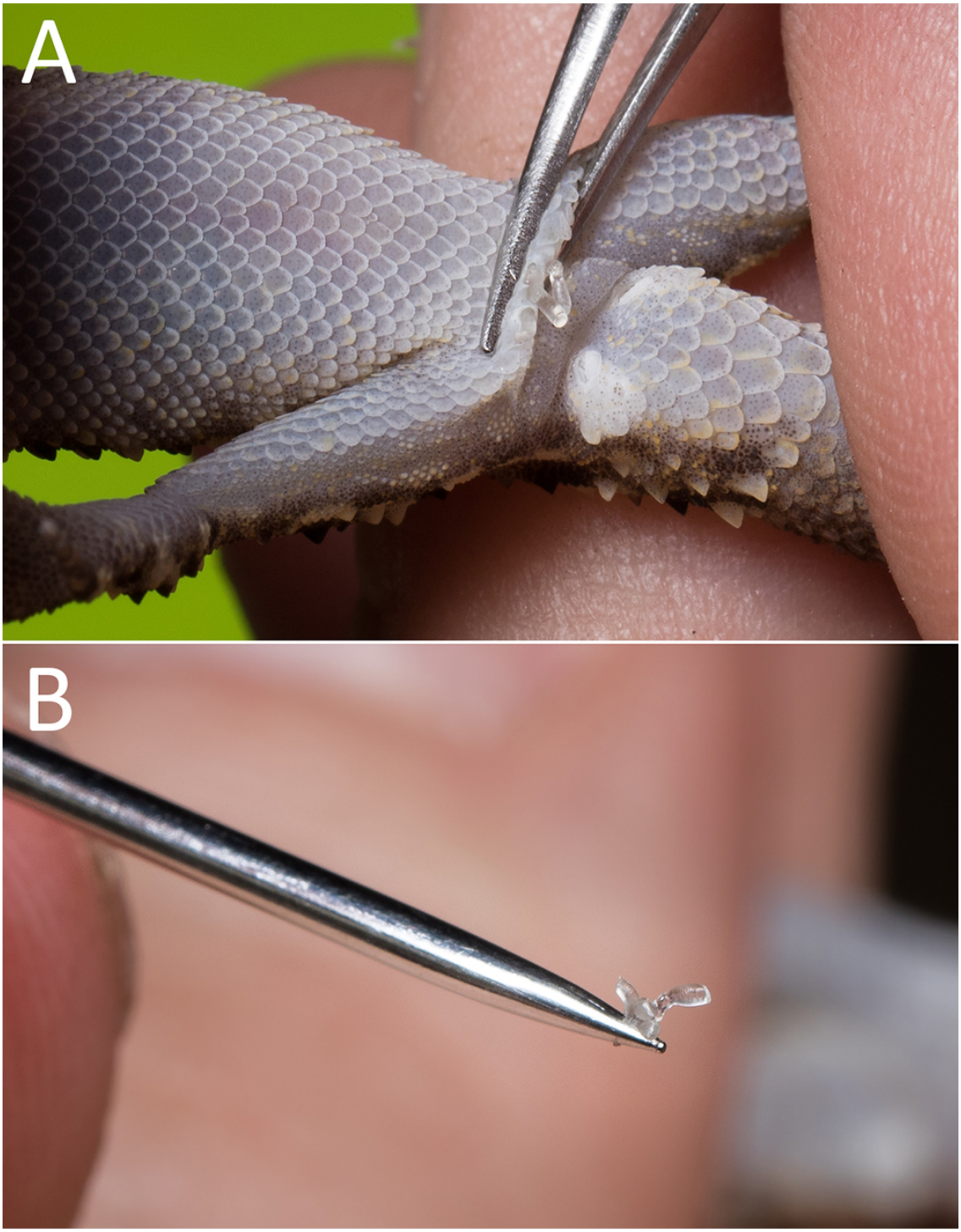

Figure S2.1: Sampling epidermal pore secretions from a male *Heteronotia binoei*. (A) Gently applying pressure around the male's epidermal pores to exude the waxy secretions, (B) which can then be collected using a stainless steel probe.

Table S2.1: Individual *Heteronotia binoei* specimens measured for morphological analysis. All measurements are reported in millimetres (mm). The traits are: snout-to-vent length (SVL), anterior tip of the rostral scale to the posterior margin of the cloaca; inter-limb length (ILL), posterior insertion of the forelimb to the anterior insertion of the hindlimb; head length (HL), anterior tip of the rostral scale to the anterior margin of the ear; head width (HW), widest point on the head, just anterior to the ears; eye diameter (EyeD), measured horizontally from the anterior to the posterior margins of the orbit; snout length (SL), anterior tip of the rostral scale to the anterior margin of the orbit; hindlimb length (HLL), knee to the ankle with the upper leg and ankle held at right angles to the lower leg; and forelimb length (FLL), elbow to the wrist with the upper arm and wrists held at right angles to the forearm.

Field ID	Lineage	Sex	Latitude	Longitude	SVL	ILL	HL	HW	EyeD	SL	HLL	FLL
CCM5225	CYA6-N	male	-17.36	144.69	40	16.3	11.19	8.33	2.96	4.53	6.75	5.57
CCM5226	CYA6-N	female	-17.36	144.69	42	16.65	11.22	8.81	2.63	4.81	7.63	5.47
CCM5230	CYA6-N	female	-16.89	144.21	43	18.42	11.47	7.85	2.93	4.54	7.17	5.47
CCM5231	CYA6-N	female	-16.89	144.21	39	17.29	10.21	7.64	2.3	4.36	6.59	5.44
CCM5232	CYA6-N	female	-16.37	143.98	40	17.24	10.83	8.51	2.83	4.25	6.8	5.34
CCM5233	CYA6-N	male	-16.37	143.98	37	14.81	10.35	8.26	2.61	4.27	6.59	5.29
CCM5243	CYA6-N	male	-16.00	144.08	39	15.63	10.68	8.05	2.74	4.21	6.84	5.99
CCM5244	CYA6-N	male	-16.00	144.08	40	17.63	10.9	8.54	2.56	4.93	7.28	5.66
CCM5249	CYA6-N	female	-15.75	144.05	35	15.32	9.5	7.07	2.2	4.26	6.76	5.09
CCM5250	CYA6-N	male	-15.75	144.05	37	15.23	10.33	8.07	2.8	4.01	6.81	5.03
CCM5258	CYA6-N	female	-13.47	142.97	42	18.06	11.23	8.41	2.55	4.46	7.07	5.5
CCM5264	CYA6-N	female	-17.05	144.35	42	17.44	11.26	8.39	2.79	4.49	7.2	5.73
CCM5318	CYA6-N	male	-13.92	143.19	37	14.47	9.52	8.25	2.26	3.98	6.16	4.79
CCM5321	CYA6-N	male	-14.32	143.27	39	16.24	10.36	8.64	2.3	4.41	6.98	5.39
CCM5332	CYA6-N	male	-14.63	143.46	40	16.19	11.15	8.57	2.59	4.6	7.36	5.56
CCM5340	CYA6-N	female	-14.79	143.40	36	16.44	9.19	7.24	2.09	3.86	6.51	4.6
CCM5347	CYA6-N	male	-15.91	144.84	36	14.95	10.28	7.72	2.27	3.98	6.25	5.33
CCM5348	CYA6-N	male	-15.91	144.84	38	16.48	10.58	8.09	2.06	4.49	6.68	5.28
CCM5352	CYA6-N	female	-16.22	144.71	40	17.61	11.04	7.96	2.53	4.7	6.96	5.47
CCM5353	CYA6-N	male	-16.22	144.71	38	16.45	10.44	8.5	2.71	4.2	6.23	4.7
SMZ0651	CYA6-N	female	-17.16	144.52	45	21.04	11.39	8.85	3.24	4.84	6.99	5.25
SMZ0655	CYA6-N	male	-17.16	144.52	41	17.09	11.28	9.12	2.83	4.6	6.8	5.16
CCM0106	CYA6-S	female	-18.15	144.57	38	17.51	10.46	8.22	2.69	4.77	6.99	5.28
CCM0107	CYA6-S	male	-18.15	144.57	41	17.39	10.54	8.63	2.54	4.28	6.69	4.88
CCM0114	CYA6-S	female	-19.54	144.20	41	17.56	10.68	8.46	2.72	4.45	6.66	5.19
CCM0115	CYA6-S	male	-19.54	144.20	39	17.28	10.51	8.11	2.45	4.75	6.71	5.31
CCM0118	CYA6-S	male	-19.53	144.06	37	15.89	9.96	7.63	2.23	4.42	6.62	5.12
CCM0119	CYA6-S	female	-19.53	144.06	40	17.69	10.42	8.45	2.64	4.64	6.97	5.52
CCM5194	CYA6-S	male	-18.57	143.57	39	15.49	10.54	7.94	2.47	4.39	6.65	5.22
CCM5195	CYA6-S	male	-18.57	143.57	32	13.5	8.91	6.98	2.15	3.58	5.71	4.19
CCM5200	CYA6-S	female	-17.64	145.06	44	18.25	11.11	8.7	2.38	4.72	7.36	5.72
CCM5201	CYA6-S	male	-17.64	145.06	43	16.66	11.67	8.97	2.77	4.67	7.67	5.7
CCM5211	CYA6-S	female	-17.38	144.96	38	17.09	9.95	7.64	2.68	4.21	6.83	5.16
CCM5212	CYA6-S	female	-17.38	144.96	39	16.54	10.62	8.39	2.56	4.28	6.85	5.4
CCM5254	CYA6-S	male	-17.42	145.10	39	15.73	10.32	8.16	2.59	4.29	7.35	5.34
CCM5255	CYA6-S	male	-17.42	145.10	40	16.17	11.11	8.14	3.05	4.58	6.98	5.57
CCM5256	CYA6-S	female	-17.42	145.10	39	16.12	10.44	7.86	2.44	4.22	6.94	5.22
CCM5259	CYA6-S	male	-13.47	142.97	39	15.66	10.59	8.07	2.61	4.27	6.99	5.32
CCM5263	CYA6-S	female	-17.05	144.35	42	17.75	11.17	8.55	2.87	4.48	7.26	5.63
CCM5317	CYA6-S	female	-13.92	143.19	43	19.55	11.15	8.71	2.47	4.58	6.85	5.89
CCM5322	CYA6-S	male	-14.32	143.27	37	15.19	9.71	7.82	2.54	4.12	6.63	4.87
CCM5333	CYA6-S	female	-14.63	143.46	40	16.87	10.57	8.13	2.61	4.23	6.99	5.64
SMZ0548	CYA6-S	female	-19.54	144.20	44	21.4	11.04	8.92	2.76	4.51	6.78	5.16
CCM5374	EA6	female	-19.36	146.81	42	18.38	10.78	7.88	2.61	4.29	7.36	5.41
CCM5375	EA6	male	-19.36	146.81	41	16.87	10.56	8.16	2.43	4.26	6.92	5.35
CCM5381	EA6	male	-19.46	147.48	36	13.44	10.1	6.93	2.13	4.17	6.92	4.72
CCM5382	EA6	female	-19.46	147.48	43	18.54	11.01	8.84	2.4	4.35	6.99	5.12
conx5358	EA6	female	-19.38	146.47	48	22.98	12.44	8.69	3.28	5.19	7.93	6.04
conx5360	EA6	female	-19.26	146.81	43	19.83	11.34	8.72	2.6	4.66	7.1	5.51
conx5361	EA6	male	-19.26	146.81	44	19.12	11.41	9.05	2.93	4.47	7.04	5.77
conx5363	EA6	male	-19.20	146.77	48	21.92	12.46	9.41	2.94	5.12	7.72	6.14
conx5364	EA6	male	-19.20	146.77	42	19.5	10.91	8.23	3.03	4.49	7.12	5.35
conx5365	EA6	male	-19.20	146.77	45	19.59	11.74	9.1	3.11	4.93	6.93	5.66
conx5366	EA6	male	-19.44	146.97	38	17.02	10.08	7.96	2.78	4.18	6.42	5.04
SMZ0842	EA6	female	-18.61	146.49	45	18.77	11.86	9.65	3.38	4.8	7.39	5.57
SMZ0843	EA6	male	-18.61	146.49	40	18.75	10.19	7.64	2.8	4.15	6.32	4.92
SMZ0865	EA6	male	-19.51	146.88	46	21.53	12.1	8.65	3.23	4.96	7.65	5.7
SMZ0867	EA6	female	-21.55	148.23	43	19.7	10.61	8.02	2.7	4.31	6.57	5.18

Field ID	Lineage	Sex	Latitude	Longitude	SVL	ILL	HL	HW	EyeD	SL	HLL	FLL
SMZ0883	EA6	male	-19.33	146.76	38	17.08	10.09	8.04	2.73	4.03	6.37	4.93
CCM0086	EIU	female	-18.82	143.41	48	18.89	13.12	9.57	3.44	5.81	8.13	6.81
CCM0087	EIU	male	-18.82	143.41	42	17.42	11.29	8.2	2.81	4.84	7.18	5.66
CCM0096	EIU	female	-18.79	143.45	38	16.08	10.57	7.86	2.5	4.61	6.77	5.42
CCM0103	EIU	female	-18.80	143.44	45	20.13	11.83	8.98	2.75	5.89	7.53	5.75
CCM0104	EIU	female	-18.80	143.44	43	18.83	11.6	8.11	2.73	4.97	7.28	5.6
CCM0117	EIU	male	-19.59	144.02	39	16.37	10.56	7.62	2.87	4.53	6.34	4.17
CCM5164	EIU	male	-18.80	143.42	40	18.33	10.84	7.89	2.98	4.5	6.73	5.77
CCM5165	EIU	female	-18.80	143.42	39	17.03	10.38	7.96	2.36	4.47	7.36	6.34
CCM5170	EIU	female	-18.80	143.44	39	17.52	10.75	8.29	2.8	4.39	7.4	5.51
CCM5171	EIU	female	-18.80	143.44	45	19.9	12.17	9.01	2.89	5.31	8.02	6.21
CCM5173	EIU	male	-18.81	143.40	39	16.06	10.7	7.96	3.1	4.33	6.91	5.41
CCM5174	EIU	female	-18.81	143.40	42	17.65	11.86	8.13	2.99	4.89	7.68	5.68
CCM5175	EIU	female	-18.81	143.40	45	18.78	12.53	9.1	3.27	5.07	8.88	6.46
CCM5122	GULF-E	female	-17.93	141.76	42	18.39	11.26	8.45	2.93	4.52	7.61	5.75
CCM5126	GULF-E	male	-17.93	141.76	42	18.91	12.29	8.82	3.35	5.02	7.79	6.13
CCM5127	GULF-E	male	-17.93	141.76	46	20	12.19	9.05	3.02	4.97	7.71	5.88
CCM5130	GULF-E	female	-18.19	142.90	39	16.31	10.49	8.32	2.24	4.39	6.62	5.43
CCM5177	GULF-E	female	-18.95	143.61	50	22.18	13.19	9.25	3.67	5.21	8.42	6.43
SMZ0872	GULF-E	male	-17.95	144.41	47	21.2	12.4	11.04	3.21	5.1	7.51	5.96
SMZ0960	GULF-E	male	-18.25	142.52	51	23.3	12.96	9.92	3.25	5.71	7.85	6.26
CCM0320	GULF-W	female	-18.09	138.30	48	20.14	12.24	8.87	2.9	5.09	8.99	6.52
CCM0321	GULF-W	male	-18.09	138.30	47	21.78	12.34	9.39	2.95	5.58	8.24	6.33
CCM5109	GULF-W	female	-18.52	140.67	45	18.45	12.32	8.77	2.82	4.95	8.12	5.82
CCM5110	GULF-W	female	-18.52	140.67	49	20.78	13.33	10.06	3.38	5.53	8.83	6.58
CCM5111	GULF-W	female	-18.52	140.67	46	19.17	11.78	9.24	3.09	4.74	7.59	6.16
CCM5137	GULF-W	female	-18.19	140.69	47	21.63	12.18	8.84	2.98	4.83	7.37	5.99
CCM5138	GULF-W	female	-18.19	140.69	42	19.11	11.04	8.1	2.57	4.66	7.43	6.1
CCM5139	GULF-W	male	-18.19	140.69	44	17.78	11.49	8.69	3.08	4.75	8.4	6.05
CCM5140	GULF-W	male	-18.19	140.69	38	15.56	10.08	7.92	2.5	4.21	7.1	5.5
CCM0045	MI	male	-19.14	146.85	48	20.16	12.47	9.47	3.12	5.17	8.18	6.5
CCM0046	MI	male	-19.14	146.85	54	23.1	14.4	11.11	3.94	6.05	8.6	6.44
CCM0047	MI	male	-19.13	146.87	44	19.53	11.75	8.6	2.9	4.99	7.28	5.65
CCM0048	MI	female	-19.13	146.87	54	22.13	14.23	11.19	3.32	5.9	9.39	7.05
CCM0049	MI	male	-19.13	146.87	52	21.46	14.73	10.11	3.4	6.16	9.52	7.17
CCM0050	MI	female	-19.13	146.87	55	22.49	14.34	11.18	3.82	5.77	9.12	7.23
CCM0051	MI	male	-19.15	146.84	53	24.82	14.05	10.45	3.78	5.72	8.62	6.87
CCM0052	MI	female	-19.15	146.84	51	22.79	13.31	9.95	3.54	5.55	8.6	6.57
CCM0053	MI	male	-19.16	146.84	44	19.37	11.86	8.57	3.1	5.03	7.47	5.78
CCM0097	MI	female	-19.16	146.84	48	20.45	13.14	9.72	3.17	5.39	8.06	6.19
conx5369	MI	female	-19.14	146.85	55	24.42	14.37	10.65	3.95	6.15	9.44	7.22
SMZ0727	MI	female	-19.14	146.85	59	26.75	14.96	11.87	4.27	6.43	9.12	7.25
SMZ0832	MI	male	-19.29	147.03	47	22.08	12.87	10.22	3.89	5.35	8.49	6.75
CCM0185	NWQ	female	-20.68	139.50	44	19.64	11.26	8.34	3.12	4.52	7.44	6
CCM0245	NWQ	male	-19.72	139.39	41	17.88	11.4	8.45	3.09	4.5	7.03	5.65
CCM0246	NWQ	male	-19.72	139.39	41	18.16	11.01	8.27	3.18	4.56	6.98	5.13
CCM0251	NWQ	female	-20.60	139.68	43	18.33	11.06	8.33	2.84	4.47	7.15	6.12
CCM0256	NWQ	female	-19.50	138.95	42	16.67	11.26	8.29	3.18	4.66	6.93	5.05
CCM0257	NWQ	male	-19.50	138.95	41	16.27	11.43	8.97	3.01	4.6	6.88	5.04
CCM0261	NWQ	female	-18.70	138.49	41	19.34	11.1	8.04	3.11	4.58	7.3	5.8
CCM0262	NWQ	female	-18.70	138.49	43	18.95	11.26	8.12	2.99	4.53	7.76	5.48
CCM0274	NWQ	female	-18.70	138.48	42	18.49	11.47	7.74	2.96	4.79	7.17	5.55
CCM0276	NWQ	male	-18.70	138.48	39	15.63	11.33	8.02	2.77	4.6	7.04	5.49
CCM0278	NWQ	female	-18.70	138.48	44	18.02	11.95	8.06	3.38	4.84	7.41	6.14
CCM0286	NWQ	female	-18.58	138.58	38	16.73	10.62	7.82	2.85	4.29	6.61	4.74
CCM0287	NWQ	male	-18.58	138.58	41	17.5	11.69	8.54	2.8	4.86	7.33	5.54
CCM0293	NWQ	male	-18.99	138.69	35	15.09	9.66	7.05	2.55	4.09	6.34	4.99
CCM0319	NWQ	female	-18.73	138.50	34	14.48	9.81	7.02	2.65	4.03	6.23	4.55
conx5370	Paluma	female	-19.01	146.27	44	19.75	11.3	8.14	2.62	4.7	7.36	5.91
conx5641	Paluma	male	-19.24	146.42	46	20.11	12.02	8.73	3.02	4.79	7.72	6.24
conx5642	Paluma	male	-19.24	146.42	45	20.4	11.82	9.35	2.96	4.98	7.6	5.9
conx5691	Paluma	female	-19.01	146.27	47	23.74	12.13	9.1	3.23	4.71	8.08	6.19
SMZ0888	Paluma	female	-19.24	146.44	49	23.13	12.83	9.8	3.34	5.38	7.92	6.54
SMZ0889	Paluma	female	-19.24	146.44	46	21.4	11.61	9.15	3.01	4.73	6.98	5.58
CCM0176	SM6-N	female	-21.26	139.72	47	21.88	11.96	9.05	2.77	4.96	7.53	6.17
CCM0177	SM6-N	male	-20.76	139.72	48	20.62	12.54	9.82	3.5	5.25	8.06	7.17
CCM0178	SM6-N	female	-21.07	139.59	46	21.66	11.56	8.67	2.75	4.68	7.36	6.02
CCM0179	SM6-N	male	-21.07	139.59	44	20.03	10.91	8.75	2.7	4.58	7.59	5.58
CCM0180	SM6-N	female	-21.07	139.59	45	18.43	11.78	8.83	2.86	4.66	7.49	5.95
CCM0181	SM6-N	female	-21.08	140.28	49	23.28	13.04	9.24	3.36	5.28	8.44	6.27
CCM0182	SM6-N	male	-21.08	140.28	41	19.49	10.94	8.68	2.47	4.56	7.45	5.28
CCM0235	SM6-N	male	-20.14	139.41	49	21.06	12.81	9.87	3.4	5.12	8.03	6.37
CCM0255	SM6-N	male	-19.50	138.95	49	21.67	13.13	9.82	3.13	5.6	8.74	6.34
CCM0408	SM6-N	male	-16.64	135.85	41	17.45	11	8.23	2.59	4.53	6.95	5.15
CCM0413	SM6-N	female	-16.64	135.85	50	20.58	12.88	9.34	3	5.08	8.07	6.69
CCM0414	SM6-N	female	-16.64	135.85	45	19.16	12.43	8.42	3.02	5.28	7.67	6.41
CCM0415	SM6-N	female	-16.64	135.85	48	22.81	12.77	9.18	3.33	5.25	8.02	6.41
SMZ0790	SM6-N	female	-21.71	139.49	40	18.78	9.94	7.91	2.14	4.24	6.01	4.41

Table S2.2: Individual adult male *Heteronotia binoei* sampled for pheromones. The 'Pheromone data' column indicates whether pheromones were both collected *and* successfully characterised for the respective individual.

Field ID	Lineage	Latitude	Longitude	Year sampled	Pheromone data
SMZ0162	CYA6-N	-17.15	144.52	2015	yes
SMZ0164	CYA6-N	-17.15	144.52	2015	yes
SMZ0165	CYA6-N	-17.15	144.52	2015	yes
SMZ0166	CYA6-N	-17.15	144.52	2015	yes
SMZ0168	CYA6-N	-17.15	144.52	2015	no
SMZ0177	CYA6-N	-17.15	144.52	2015	no
SMZ0178	CYA6-N	-17.17	144.51	2015	yes
SMZ0181	CYA6-N	-17.17	144.51	2015	yes
SMZ0188	CYA6-N	-17.17	144.51	2015	yes
SMZ0193	CYA6-N	-17.17	144.51	2015	yes
SMZ0194	CYA6-N	-17.17	144.51	2015	yes
SMZ0196	CYA6-N	-15.79	144.27	2015	yes
SMZ0204	CYA6-N	-15.79	144.27	2015	yes
SMZ0205	CYA6-N	-15.79	144.27	2015	yes
SMZ0213	CYA6-N	-15.79	144.27	2015	yes
SMZ0288	CYA6-N	-17.04	145.35	2015	yes
SMZ0289	CYA6-N	-17.04	145.35	2015	yes
SMZ0290	CYA6-N	-17.04	145.35	2015	yes
SMZ0291	CYA6-N	-17.04	145.35	2015	yes
SMZ0298	CYA6-N	-16.53	145.14	2015	yes
SMZ0299	CYA6-N	-16.53	145.14	2015	yes
SMZ0300	CYA6-N	-16.53	145.14	2015	yes
SMZ0301	CYA6-N	-16.53	145.14	2015	yes
SMZ0302	CYA6-N	-16.53	145.14	2015	yes
SMZ0143	CYA6-S	-19.54	144.20	2015	yes
SMZ0144	CYA6-S	-19.54	144.20	2015	yes
SMZ0145	CYA6-S	-19.54	144.20	2015	yes
SMZ0146	CYA6-S	-19.54	144.20	2015	yes
SMZ0147	CYA6-S	-19.54	144.20	2015	yes
SMZ0248	CYA6-S	-18.21	144.57	2015	yes
SMZ0251	CYA6-S	-18.21	144.57	2015	yes
SMZ0252	CYA6-S	-18.21	144.57	2015	yes
SMZ0254	CYA6-S	-18.21	144.57	2015	yes
SMZ0255	CYA6-S	-18.21	144.57	2015	yes
SMZ0314	CYA6-S	-17.74	145.03	2015	yes
SMZ0317	CYA6-S	-17.74	145.03	2015	yes
SMZ0322	EA6	-19.19	146.77	2015	yes
SMZ0323	EA6	-19.19	146.77	2015	yes
SMZ0325	EA6	-19.19	146.77	2015	yes
SMZ0326	EA6	-19.19	146.77	2015	yes
SMZ0327	EA6	-19.19	146.77	2015	yes
SMZ0351	EA6	-19.89	146.64	2015	yes
SMZ0352	EA6	-19.89	146.64	2015	yes
SMZ0353	EA6	-19.89	146.64	2015	yes
SMZ0355	EA6	-19.89	146.64	2015	yes
SMZ0356	EA6	-19.89	146.64	2015	no
SMZ0359	EA6	-19.89	146.64	2015	no
SMZ0360	EA6	-19.89	146.64	2015	no
SMZ0361	EA6	-20.31	146.88	2015	no
SMZ0829	EA6	-19.19	146.77	2016	yes
SMZ0106	EIU	-18.82	143.41	2015	yes
SMZ0107	EIU	-18.82	143.41	2015	yes
SMZ0111	EIU	-18.82	143.41	2015	no
SMZ0112	EIU	-18.82	143.41	2015	yes
SMZ0113	EIU	-18.82	143.41	2015	yes
SMZ0114	EIU	-18.82	143.41	2015	yes
SMZ0119	EIU	-18.82	143.41	2015	yes
SMZ0124	EIU	-18.82	143.41	2015	yes
SMZ0125	EIU	-19.55	143.90	2015	yes
SMZ0126	EIU	-19.55	143.90	2015	yes
SMZ0127	EIU	-19.55	143.90	2015	yes

Field ID	**Lineage**	**Latitude**	**Longitude**	**Year sampled**	**Pheromone data**
SMZ0128	EIU	-19.55	143.90	2015	yes
SMZ0132	EIU	-19.55	143.90	2015	yes
SMZ0134	EIU	-19.55	143.90	2015	yes
SMZ0262	EIU	-18.19	142.27	2015	yes
SMZ0280	EIU	-18.19	142.27	2015	yes
SMZ0281	EIU	-18.19	142.27	2015	no
SMZ0282	EIU	-18.19	142.27	2015	yes
SMZ0283	EIU	-18.19	142.27	2015	yes
SMZ0284	EIU	-18.19	142.27	2015	yes
SMZ0683	EIU	-18.27	142.62	2016	yes
SMZ0685	EIU	-18.27	142.62	2016	yes
SMZ0686	EIU	-18.27	142.62	2016	yes
SMZ0688	EIU	-18.27	142.62	2016	yes
SMZ0689	EIU	-18.27	142.62	2016	yes
SMZ0690	EIU	-18.27	142.62	2016	yes
SMZ0694	EIU	-18.27	142.62	2016	yes
SMZ0695	EIU	-18.27	142.62	2016	yes
SMZ0118	GULF-E	-18.80	143.41	2015	yes
SMZ0233	GULF-E	-18.88	144.54	2015	yes
SMZ0234	GULF-E	-18.88	144.54	2015	yes
SMZ0236	GULF-E	-18.88	144.54	2015	yes
SMZ0237	GULF-E	-18.88	144.54	2015	yes
SMZ0238	GULF-E	-18.88	144.54	2015	yes
SMZ0242	GULF-E	-18.88	144.54	2015	no
SMZ0270	GULF-E	-18.27	142.62	2015	yes
SMZ0271	GULF-E	-18.27	142.62	2015	yes
SMZ0275	GULF-E	-18.27	142.62	2015	yes
SMZ0276	GULF-E	-18.27	142.62	2015	yes
SMZ0277	GULF-E	-18.27	142.62	2015	yes
SMZ0278	GULF-E	-18.27	142.62	2015	yes
SMZ0821	GULF-W	-19.23	140.35	2016	yes
SMZ0822	GULF-W	-19.23	140.35	2016	yes
SMZ0823	GULF-W	-19.23	140.35	2016	yes
SMZ0824	GULF-W	-19.23	140.35	2016	yes
SMZ0825	GULF-W	-19.23	140.35	2016	yes
SMZ0826	GULF-W	-19.23	140.35	2016	yes
SMZ0827	GULF-W	-19.23	140.35	2016	yes
SMZ0102	MI	-19.15	146.84	2015	no
SMZ0103	MI	-19.15	146.84	2015	no
SMZ0223	MI	-19.15	146.84	2015	yes
SMZ0224	MI	-19.15	146.84	2015	yes
SMZ0225	MI	-19.15	146.84	2015	yes
SMZ0226	MI	-19.15	146.84	2015	yes
SMZ0227	MI	-19.15	146.84	2015	yes
SMZ0746	NWQ	-18.70	138.48	2016	yes
SMZ0750	NWQ	-18.70	138.48	2016	yes
SMZ0751	NWQ	-18.70	138.48	2016	yes
SMZ0756	NWQ	-18.70	138.48	2016	yes
SMZ0758	NWQ	-18.70	138.48	2016	yes
SMZ0772	NWQ	-18.70	138.48	2016	yes
SMZ0801	NWQ	-20.58	139.57	2016	yes
SMZ0802	NWQ	-20.58	139.58	2016	yes
SMZ0805	NWQ	-20.58	139.58	2016	yes
SMZ0807	NWQ	-20.58	139.58	2016	no
SMZ0808	NWQ	-20.58	139.58	2016	yes
SMZ0809	NWQ	-20.58	139.58	2016	yes
SMZ0810	NWQ	-20.58	139.58	2016	yes
SMZ0811	NWQ	-20.58	139.58	2016	yes
SMZ0812	NWQ	-20.58	139.58	2016	yes
SMZ0813	NWQ	-20.58	139.58	2016	yes
SMZ0814	NWQ	-20.58	139.58	2016	yes
SMZ0815	NWQ	-20.58	139.58	2016	yes
SMZ0215	Paluma	-19.24	146.44	2015	yes
SMZ0216	Paluma	-19.24	146.44	2015	yes
SMZ0217	Paluma	-19.24	146.44	2015	yes
SMZ0219	Paluma	-19.24	146.44	2015	yes
SMZ0220	Paluma	-19.24	146.44	2015	yes
SMZ0221	Paluma	-19.24	146.44	2015	yes

Field ID	**Lineage**	**Latitude**	**Longitude**	**Year sampled**	**Pheromone data**
SMZ0754	SM6-N	-18.69	138.53	2016	yes
SMZ0761	SM6-N	-18.69	138.53	2016	yes
SMZ0762	SM6-N	-18.69	138.53	2016	yes
SMZ0764	SM6-N	-18.69	138.53	2016	yes
SMZ0768	SM6-N	-18.69	138.53	2016	yes
SMZ0769	SM6-N	-18.69	138.53	2016	yes
SMZ0770	SM6-N	-18.69	138.53	2016	yes
SMZ0771	SM6-N	-18.69	138.53	2016	yes
SMZ0788	SM6-N	-21.71	139.49	2016	yes
SMZ0791	SM6-N	-21.71	139.49	2016	yes
SMZ0797	SM6-N	-21.71	139.49	2016	yes

Table S2.3: The proportion of variation explained, eigenvalue, and loadings of each morphological trait for each PC axis from the Principle Components Analyses of morphological data. Only those PC axes included in respective multivariate analyses are included. For the PCs that significantly contribute to divergence in each analysis (from the CV loadings in Table S8), we have bolded the trait loadings that significantly contribute to those respective PCs (using the '70% of absolute highest loading and higher' rule of thumb, with the highest loadings also underlined; Mardia et al. 1979, e.g., Blows and Higgie 2003, Hoskin et al. 2011).

All lineages	PC1	PC2	PC3	PC4	PC5
Proportion explained	0.855	0.044	0.036	0.026	0.018
Eigenvalue	6.841	0.354	0.285	0.208	0.145
SVL	**1.975**	-0.180	0.253	-0.041	0.041
ILL	**1.800**	-0.580	0.557	-0.443	-0.005
HL	**1.988**	0.051	-0.108	0.099	-0.149
HW	**1.852**	-0.223	0.132	0.691	0.348
EyeD	**1.749**	-0.503	**-0.871**	-0.173	0.035
SL	**1.907**	0.166	0.071	0.219	-0.562
HLL	**1.869**	0.605	-0.069	-0.107	-0.045
FLL	**1.841**	0.622	-0.008	-0.279	0.366

NWQld contact	PC1	PC2	PC3	PC4	PC5
Proportion explained	0.792	0.081	0.041	0.034	0.023
Eigenvalue	6.339	0.646	0.331	0.270	0.181
SVL	**1.413**	-0.218	0.131	-0.061	0.104
ILL	**1.219**	-0.557	**0.496**	-0.244	-0.146
HL	**1.412**	0.138	-0.005	0.180	-0.147
HW	**1.327**	-0.136	0.092	0.418	0.417
EyeD	**1.022**	**0.995**	0.300	-0.099	0.005
SL	**1.374**	-0.011	-0.121	0.243	-0.349
HLL	**1.320**	-0.056	**-0.499**	-0.010	-0.064
FLL	**1.311**	0.034	-0.296	-0.492	0.188

CYP contact	PC1	PC2	PC3	PC4	PC5
Proportion explained	0.772	0.074	0.055	0.039	0.027
Eigenvalue	6.174	0.593	0.441	0.310	0.217
SVL	**1.601**	-0.276	-0.109	-0.075	0.124
ILL	**1.432**	-0.554	-0.259	-0.429	0.380
HL	**1.621**	0.038	0.145	0.020	-0.115
HW	**1.390**	-0.487	-0.201	**0.747**	-0.059
EyeD	**1.339**	-0.139	0.970	-0.050	-0.019
SL	**1.486**	0.030	-0.330	-0.308	**-0.583**
HLL	**1.456**	0.628	0.033	0.005	0.009
FLL	**1.376**	**0.784**	-0.194	0.132	0.293

(*Table S2.3 continued*)

Tsv contact	PC1	PC2	PC3	PC4
Proportion explained	0.906	0.042	0.022	0.014
Eigenvalue	7.246	0.334	0.176	0.115
SVL	**1.413**	0.035	-0.082	0.173
ILL	**1.278**	0.547	**-0.351**	0.030
HL	**1.417**	-0.167	0.014	0.037
HW	**1.365**	0.019	**0.287**	0.297
EyeD	**1.314**	0.375	**0.336**	-0.282
SL	**1.397**	-0.167	0.048	-0.006
HLL	**1.355**	-0.392	-0.119	-0.162
FLL	**1.386**	-0.192	-0.142	-0.103

Table S2.4: Retention times for peaks used in multivariate analysis of pheromone blends.

Peak #	Retention time (mins)
1	10.86
2	10.90
3	13.02
4	13.08
5	13.16
6	13.99
7	14.05
8	14.10
9	15.25
10	15.30
11	15.41
12	15.45
13	15.59
14	15.66
15	15.84
16	15.96
17	16.16
18	16.25
19	16.36
20	16.54
21	18.68
22	18.74
23	18.81
24	19.27
25	19.43

Table S2.5: The proportion of variation explained, eigenvalue, and loadings of each log-contrast transformed pheromone peak for each PC axis from the Principle Components Analyses of pheromone data. Only those PC axes included in respective multivariate analyses are included. For the PCs that significantly contribute to divergence in each analysis (from the CV loadings in Table S8), we have bolded the trait loadings that significantly contribute to those respective PCs (using the '70% of absolute highest loading and higher' rule of thumb, with the highest loadings also underlined; Mardia et al. 1979, e.g., Blows and Higgie 2003, Hoskin et al. 2011).

All lineages	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Proportion explained	0.410	0.108	0.099	0.059	0.048	0.042	0.035	0.028	0.027
Eigenvalue	9.849	2.589	2.378	1.408	1.140	1.008	0.847	0.680	0.657
log-contrast peak 1	-0.819	0.740	-0.603	0.435	-0.416	0.071	-0.097	0.286	-0.124
log-contrast peak 2	-0.684	0.725	-0.630	**0.494**	-0.533	0.223	0.062	0.341	-0.045
log-contrast peak 3	-0.714	0.684	**0.753**	0.057	0.178	0.134	0.057	0.052	0.274
log-contrast peak 4	-1.056	0.127	-0.402	**-0.568**	0.165	0.045	-0.095	0.225	0.558
log-contrast peak 5	-0.505	0.918	-0.078	-0.306	0.658	-0.381	0.042	0.087	-0.619
log-contrast peak 6	-1.051	-0.130	-0.431	**-0.509**	0.182	0.212	-0.160	0.366	0.398
log-contrast peak 7	-0.775	0.905	0.051	**-0.478**	0.357	-0.259	0.208	-0.171	0.109
log-contrast peak 9	-0.585	-0.658	0.160	**0.499**	-0.105	-0.939	-0.354	-0.020	0.236
log-contrast peak 10	-0.774	0.183	**-0.947**	0.316	0.199	0.095	-0.133	-0.559	-0.082
log-contrast peak 11	-0.738	0.594	0.594	**0.517**	-0.393	-0.117	0.341	0.023	0.218
log-contrast peak 12	-0.809	-0.517	-0.578	-0.250	-0.397	-0.050	0.573	0.055	-0.168
log-contrast peak 13	-1.109	-0.164	-0.140	-0.174	-0.152	-0.721	-0.026	0.334	-0.234
log-contrast peak 14	-0.774	-0.670	**-0.801**	-0.062	0.133	-0.170	0.217	-0.181	0.074
log-contrast peak 15	-1.147	-0.112	0.602	-0.239	-0.339	-0.221	0.081	-0.115	-0.171
log-contrast peak 16	-1.228	-0.150	0.210	-0.352	-0.334	0.087	-0.240	-0.203	-0.066
log-contrast peak 17	-1.241	-0.135	0.275	-0.145	-0.244	-0.031	0.356	-0.223	0.174
log-contrast peak 18	-1.059	0.152	0.284	**-0.464**	-0.505	0.294	-0.209	-0.527	-0.189
log-contrast peak 19	-1.024	0.435	-0.049	0.158	0.012	-0.084	**-0.823**	-0.133	0.138
log-contrast peak 20	-0.924	0.155	-0.195	**0.660**	0.500	-0.082	0.459	-0.382	0.277
log-contrast peak 21	-1.186	-0.303	0.399	0.153	0.140	0.370	0.082	0.143	-0.089
log-contrast peak 22	-1.179	-0.468	-0.463	0.106	0.194	0.308	-0.108	0.063	-0.240
log-contrast peak 23	-0.957	-0.673	0.423	0.390	0.359	0.340	-0.166	0.120	-0.081
log-contrast peak 24	-1.216	-0.219	0.455	-0.025	0.247	0.069	0.201	0.302	-0.054
log-contrast peak 25	-1.175	-0.235	0.394	0.348	0.267	0.147	-0.100	0.079	-0.293

NWQld contact	PC1	PC2	PC3	PC4	PC5	PC6
Proportion explained	0.436	0.119	0.092	0.069	0.057	0.048
Eigenvalue	10.468	2.859	2.210	1.645	1.373	1.149
log-contrast peak 1	-0.503	-0.102	0.516	**-0.701**	0.270	-0.047
log-contrast peak 2	-0.379	0.026	0.547	**-0.685**	0.236	0.346
log-contrast peak 3	-0.629	-0.532	0.228	0.055	-0.007	-0.078
log-contrast peak 4	-0.683	0.446	0.425	0.334	0.098	0.158
log-contrast peak 5	-0.099	0.321	0.510	0.015	**-0.644**	**-0.540**
log-contrast peak 6	-0.333	0.571	0.469	0.462	-0.061	**0.452**
log-contrast peak 7	-0.423	0.333	0.283	-0.063	**-0.617**	0.364
log-contrast peak 9	-0.707	-0.033	-0.676	-0.125	-0.108	0.151
log-contrast peak 10	-0.648	0.054	-0.422	-0.402	-0.439	0.224
log-contrast peak 11	-0.826	-0.265	-0.034	-0.239	0.101	0.118
log-contrast peak 12	-0.179	**0.862**	-0.206	-0.286	0.094	-0.103
log-contrast peak 13	-0.630	**0.668**	0.034	-0.053	0.112	-0.278
log-contrast peak 14	-0.090	**0.601**	-0.658	-0.084	-0.105	0.174
log-contrast peak 15	-0.910	0.187	-0.152	0.144	0.395	0.008
log-contrast peak 16	-1.009	0.074	-0.010	0.000	-0.021	-0.067
log-contrast peak 17	-0.962	0.097	-0.170	0.194	0.223	-0.020
log-contrast peak 18	-0.905	0.024	0.073	0.256	0.186	0.115
log-contrast peak 19	-0.814	-0.439	0.176	0.224	-0.195	0.319
log-contrast peak 20	-0.887	-0.239	-0.198	-0.219	-0.196	-0.009
log-contrast peak 21	-0.847	-0.249	-0.144	0.261	0.061	-0.032
log-contrast peak 22	-0.919	0.172	0.023	-0.144	-0.118	-0.298
log-contrast peak 23	-0.858	-0.524	-0.064	0.124	-0.209	-0.073
log-contrast peak 24	-0.864	0.198	0.001	0.110	0.240	-0.266
log-contrast peak 25	-0.880	-0.196	0.071	0.038	-0.117	-0.308

CYP contact	PC1	PC2	PC3	PC4	PC5	PC6
Proportion explained	0.438	0.157	0.100	0.065	0.045	0.033
Eigenvalue	10.507	3.777	2.401	1.571	1.081	0.801

CYP contact (continued)	PC1	PC2	PC3	PC4	PC5	PC6
log-contrast peak 1	-0.649	0.619	**-0.686**	0.326	-0.306	0.006
log-contrast peak 2	-0.493	0.626	**-0.563**	0.543	-0.467	-0.152
log-contrast peak 3	-0.470	**0.786**	**0.626**	0.008	0.039	-0.170
log-contrast peak 4	-0.946	0.132	-0.350	-0.258	0.430	0.062
log-contrast peak 5	-0.431	**0.960**	0.202	-0.166	0.424	-0.152
log-contrast peak 6	-1.101	-0.161	-0.069	-0.135	0.078	-0.086
log-contrast peak 7	-0.614	**0.907**	0.199	-0.222	0.361	-0.021
log-contrast peak 9	-0.504	**-0.689**	-0.037	0.225	0.057	-0.692
log-contrast peak 10	-0.767	0.222	**-0.789**	0.049	0.250	-0.059
log-contrast peak 11	-0.384	**0.786**	0.389	0.523	-0.410	-0.078
log-contrast peak 12	-0.865	-0.530	-0.273	0.108	-0.275	0.239
log-contrast peak 13	-1.071	-0.134	-0.198	-0.015	0.091	-0.188
log-contrast peak 14	-0.886	-0.594	-0.368	0.061	0.185	0.099
log-contrast peak 15	-0.949	0.044	0.475	-0.257	-0.270	0.165
log-contrast peak 16	-0.976	-0.104	-0.002	-0.662	-0.272	-0.081
log-contrast peak 17	-1.043	-0.023	0.109	0.188	0.047	0.524
log-contrast peak 18	-0.845	0.272	-0.047	-0.595	-0.402	0.304
log-contrast peak 19	-0.896	0.409	-0.414	-0.358	-0.086	-0.291
log-contrast peak 20	-0.755	0.177	0.094	0.725	0.466	0.321
log-contrast peak 21	-0.995	-0.199	0.408	0.146	-0.063	-0.082
log-contrast peak 22	-1.001	-0.523	-0.252	0.058	0.142	0.044
log-contrast peak 23	-0.812	**-0.694**	0.476	0.160	-0.059	-0.127
log-contrast peak 24	-0.991	-0.106	**0.621**	0.057	0.071	-0.068
log-contrast peak 25	-1.065	-0.234	0.465	0.201	-0.128	-0.056

Tsv contact	PC1	PC2	PC3	PC4	PC5
Proportion explained	0.545	0.133	0.080	0.051	0.040
Eigenvalue	13.076	3.191	1.928	1.233	0.971
log-contrast peak 1	-0.869	0.129	-0.078	0.033	-0.069
log-contrast peak 2	-0.751	0.139	-0.143	0.240	0.049
log-contrast peak 3	-0.723	-0.416	-0.013	0.097	0.162
log-contrast peak 4	-0.626	-0.291	-0.245	-0.089	-0.475
log-contrast peak 5	-0.658	**0.533**	0.199	-0.010	-0.025
log-contrast peak 6	-0.749	-0.013	**-0.444**	-0.042	-0.263
log-contrast peak 7	-0.755	-0.221	-0.183	0.091	-0.255
log-contrast peak 9	-0.404	-0.205	-0.090	-0.688	0.187
log-contrast peak 10	-0.299	0.703	-0.382	0.205	0.215
log-contrast peak 11	-0.573	-0.369	-0.016	0.322	-0.067
log-contrast peak 12	-0.713	-0.370	-0.091	-0.051	0.035
log-contrast peak 13	-0.772	-0.108	0.200	-0.369	-0.030
log-contrast peak 14	-0.701	-0.418	-0.279	0.069	0.071
log-contrast peak 15	-0.753	0.036	0.417	-0.199	0.146
log-contrast peak 16	-0.826	-0.267	0.105	0.091	0.233
log-contrast peak 17	-0.827	-0.246	0.233	0.223	-0.005
log-contrast peak 18	-0.645	-0.101	**0.557**	0.231	0.105
log-contrast peak 19	-0.784	0.091	-0.072	-0.272	-0.019
log-contrast peak 20	-0.441	0.222	**-0.603**	0.036	0.401
log-contrast peak 21	-0.826	0.149	0.170	0.004	0.136
log-contrast peak 22	-0.774	0.445	-0.032	0.010	-0.187
log-contrast peak 23	-0.468	**0.742**	-0.009	-0.136	-0.093
log-contrast peak 24	-0.814	0.006	-0.011	0.014	0.154
log-contrast peak 25	-0.583	0.497	0.360	0.056	-0.207

Table S2.6: Comparisons of results for log-contrast and logit transformed pheromone data. Because the individual PC axes produced using logit transformed variables tend to account for less variation than those produced using log-contrast variables, only the minimum number of PCs that account for at least 70% of the data are kept for analysis via the MIXED procedure and to calculate pairwise pheromone overlap. This was done to maintain statistical power; however, we note that all PCs with eigenvalues greater than 1 were kept in doing so. Considering this, the results of the analyses performed on logit transformed pheromone traits can be considered more conservative. Values given for the comparisons of trait overlap are the results of a Kuskal-Wallis one-way ANOVA by ranks; the values for comparisons of mean trait distances are the results of a t-test (for these t-values are given instead of X^2 values).

Dataset & transformation method	X^2	*df*	*P*
All lineages			
log-contrast (PC1–9 = 85%)	450	81	<0.001
logit (PC1–8 = 73%)	437	72	<0.001
NWQld contact (GULF-W, NWQ, SM6-N)			
log-contrast (PC1–6 = 82%)	49	12	<0.001
logit (PC1–6 = 74%)	72	12	<0.001
CYP contact (CYA6-N, CYA6-S, EIU, GULF-E)			
log-contrast (PC1–6 = 84%)	138	18	<0.001
logit (PC1-5 = 70%)	121	15	<0.001
Tsv contact (EA6, MI, Paluma)			
log-contrast (PC1–5 = 85%)	32	10	<0.001
logit (PC1–5 = 70%)	44	10	<0.001
Pheromone overlap *vs.* morphological overlap			
Pheromone data log-contrast transformed	18.75	1	<0.001
Pheromone data logit transformed	5.44	1	0.0196
Pheromone distance *vs.* morphological distance			
Pheromone data log-contrast transformed	t = -13.37	83	<0.001
Pheromone data logit transformed	t = -25.79	88	<0.001

Table S2.7: Model selection of covariance structures for MIXED analysis in SAS. Below are the AICc scores for each trait analysis with each of eight variance-covariance structures available in MIXED (Kincaid 2007). Selected models are in bold.

	AICc							
Analysis	UN	VC	CS	AR(1)	TOEP	CSH	ARH(1)	TOEPH
All lineages morphology	868.0	861.9	863.8	864.2	866.7	853.2	**852.5**	855.3
All lineages pheromones	2109.7	2105.2	2106.3	2102.8	2105.4	2073.4	**2069.8**	2071.2
NWQld contact morphology	402.0	**383.6**	385.2	384.8	391.1	391.4	390.7	397.3
NWQld contact pheromones	573.0	**546.4**	547.1	548.9	555.4	551.9	554.7	560.4
CYP contact morphology	592.1	**565.1**	567.3	567.2	572.8	573.0	572.9	578.8
CYP contact pheromones	855.6	849.6	851.3	851.9	860.2	**834.2**	835.2	843.4
Tsv contact morphology	295.4	285.8	287.5	286.6	290.1	286.7	**285.5**	288.6
Tsv contact pheromones	321.0	**296.9**	298.2	297.9	306.4	299.3	299.1	307.5

Table S2.8: Details for the first two canonical variates (CVs) produced via proc GLM in SAS for each trait analysis. We have bolded the PC loadings that significantly contribute to each CV (using the '0.7 of absolute highest loading and higher' rule of thumb, with the highest loadings also underlined; Mardia et al. 1979). The 'Percentage explained' column gives per cent variation explained by lineage for the respective analysis, rather than the per cent of total variation explained by the model (which SAS output does not provide). However, for the 'All lineages' analyses the number of groups (10) was greater than the number of PC axes included in the analysis. This means that the CVs produced (which are equal to the number of PC variables) account for 100% of variation. Thus, the values given for the 'All lineages' analyses do represent the actual percentage of variation included in the respective model; and we note that these are the same CVs included in the comparison of trait overlap.

		Loadings								
	Percentage explained	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
All lineages										
Morphology CV1	57.13	**0.268**	0.001	0.032	-0.009	-0.004				
Morphology CV2	22.56	-0.020	-0.061	**0.174**	-0.022	0.110				
Pheromones CV1	46.26	0.007	-0.002	**0.257**	0.024	-0.028	-0.050	-0.033	0.041	0.024
Pheromones CV2	24.42	0.023	0.011	-0.013	**-0.121**	-0.055	-0.067	**0.112**	0.079	0.075
NWQld contact										
Morphology CV1	89.28	**0.293**	**-0.327**	-0.070	0.035	0.088				
Morphology CV2	10.72	0.004	-0.055	**0.269**	0.018	-0.014				
Pheromones CV1	82.84	0.202	**0.338**	0.004	0.025	0.030	0.125			
Pheromones CV2	17.16	0.057	-0.073	0.007	**0.153**	**-0.141**	**0.108**			
CYP contact										
Morphology CV1	65.26	**0.246**	0.061	0.046	-0.089	-0.047				
Morphology CV2	33.80	**0.105**	**-0.132**	-0.020	**0.117**	**0.137**				
Pheromones CV1	63.83	0.036	-0.118	**0.261**	-0.006	-0.085	-0.053			
Pheromones CV2	33.59	0.029	**0.227**	0.084	-0.017	-0.024	-0.035			
Tsv contact										
Morphology CV1	90.96	**0.413**	-0.138	0.081	-0.073					
Morphology CV2	9.04	-0.079	-0.079	**0.248**	-0.021					
Pheromones CV1	88.86	0.150	**0.536**	0.278	-0.023	0.110				
Pheromones CV2	11.14	0.007	-0.101	**0.243**	0.096	-0.112				

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CHAPTER 3 — Female discrimination of divergent male pheromones in a cryptic species complex of lizards

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Abstract

Knowledge of mating signals and their influence on mate choice is important for understanding reproductive isolation in animals. Squamates (lizards and snakes) are the most speciose clade of terrestrial vertebrates and receive considerable research attention, yet our knowledge of mating signals for this group is relatively poor. Furthermore, phylogenetic studies reveal that many recognised species are comprised of multiple genetically divergent but morphologically similar 'cryptic species', indicating that species diversity is currently underestimated. Considering the morphological similarity within cryptic squamate groups, chemical cues (pheromones) are a good candidate for investigation as a mating signal. Here we test whether divergent male pheromones influence female discrimination among sympatric genetic lineages (candidate species) of the Australian Bynoe's gecko (*Heteronotia binoei*) species complex. We collected individuals of three genetic lineages at three sites representing each pairwise combination of sympatry between these lineages. We isolated male pheromone secretions and presented them to females in both simultaneous and sequential choice experiments. Female geckos sniffed conspecific male scents longer than unscented controls, and two of the three lineages also sniffed conspecific male scents longer than heterospecific male scents, suggesting that pheromones influence female discrimination in these lineages. The order that scent treatments were encountered also influenced female response, highlighting the importance of considering novelty when designing behavioural experiments for lizards. We conclude that pheromones play a role in female discrimination and hence reproductive isolation in this cryptic species complex, and therefore propose that pheromones will be important for understanding diversification in morphologically conservative squamate radiations.

Introduction

In the formation of his theory of sexual selection, Darwin (1971) noted that many closely related animal species differ primarily or solely in their secondary sexual characters. This observation led Darwin to suggest that sexual selection influences species formation. Key to this was the idea that these characters (referred herein as 'mating signals') influence how individuals of the opposite sex choose their mates. Examples of mating signals include the vocalisations of frogs, the plumage and displays of birds, and the colour patterns of fishes and butterflies (Ptacek 2000). Empirical and theoretical works have since supported the role of mate choice and mating signals in premating reproductive isolation (e.g., Lande 1981, 1982; Wiernasz & Kingsolver 1992; Ryan & Rand 1993), a mechanism that is now termed behavioural (sexual) isolation (Mayr 1963; Coyne & Orr 2004; Ritchie 2007).

Behavioural isolation occurs when the coevolution of mating signals and their associated preferences (together referred herein as 'mating traits') results in divergence between populations, rendering individuals of one population less attractive to members of the other (Endler 1989; Andersson 1994; Boughman 2002; Coyne & Orr 2004). This can initiate speciation when mating traits diverge among allopatric populations as a result of selection, mutation, or drift, resulting in some degree of reproductive isolation upon secondary contact (Lande 1981; Kirkpatrick & Ryan 1991; Nei et al. 1983; Boughman 2002). Behavioural isolation can also evolve after other isolating barriers have arisen, for example, as a consequence of natural selection to reduce reproductive interference or maladaptive hybridisation where some degree of post-mating isolation is already present (e.g., reproductive character displacement and reinforcement; Howard 1993; Butlin 1987; Noor 1999; Higgie et al. 2000; Hoskin et al. 2005; Gröning & Honchkirch 2008; Hoskin & Higgie 2010). Mating traits are therefore subject to both sexual selection and natural selection (Andersson 1994). Knowledge of these traits is crucial for understanding phenotypic diversity and speciation in sexually reproducing animals.

One can infer the presence of behavioural isolation when two or more species co-occur and breed at the same time but rarely or never mate with each other (Coyne & Orr 2004); but identifying the individual traits that influence behavioural isolation is often very difficult. Comparative studies that compare closely related sympatric species offer a good starting point to identify candidate mating signals (Mayr 1963). This is a relatively easy and useful method, but ultimately these signals must be tested using behavioural experiments to assess their influence on mate choice. How this is executed depends on the modality of the signal (e.g., acoustic, visual, chemical) and the taxa in question, but studies generally aim to

isolate the signal of interest (usually from a male, e.g., frog call recordings) to assess discrimination by the other sex (usually female) (e.g., Littlejohn et al. 1960; Ryan & Rand 1993; Gerhardt & Huber 2002). By isolating the signal from the whole animal, however, such study designs exclude the possibility of actual mating. Thus, if discrimination for divergent mating signals is detected, inference is then required to assess the degree to which this likely translates to premating isolation. The advantage of doing this, however, is that one can be sure that it is the trait of interest that is influencing behaviour, rather than the confounding influence of other traits (e.g., intrasexual competition; Shackleton et al. 2005).

Research on mating signals has largely focused on visual and acoustic traits—signals that are often easily perceived by people and grab our attention (Ptacek 2000). Thus, animal groups that use these traits have formed the bulk of studies on mating signals, and are relatively well resolved because taxonomists often use these traits to diagnose and describe species. When the mating signals in question are not readily detected by human senses (including visual and acoustic signals outside of human perception), signal divergence can occur without any perceivable phenotypic divergence. This results in 'cryptic species', currently a hot topic in systematics and phylogeography (Bickford et al. 2007; Pérez-Ponce de León & Poulin 2016; Fišer et al. 2018). Many species of frogs and crickets are morphologically indistinguishable but exhibit divergent acoustic signals that allowed taxonomists to distinguish them (e.g., Littlejohn 1959). But things become more difficult when signals are entirely outside the sphere of human perception. For example, ultrasonic calls, pheromones, and electrical signals are important in many groups (Hopkins & Bass 1981; Lofstedt et al. 1991; Henry 1994) but have been studied relatively infrequently compared to visual and acoustic traits within human perceptual limits. Genetic studies often reveal high levels of undescribed cryptic diversity in groups that employ these 'cryptic signals', for example in bats (Mayer & von Helversen 2001) and electric fishes (Feulner et al. 2006). Knowledge of mating traits in cryptic groups is generally key to resolving taxonomy and understanding the diversification of these groups, especially in conjunction with genetic data.

Squamate reptiles (lizards and snakes) are estimated to harbour a disproportionally high level of cryptic species diversity (Pérez-Ponce de León & Poulin 2016). This is also a group for which we have a poor understanding of mating traits and behavioural isolation. Visual traits appear to serve as mating signals in some lizards, primarily those with bright display colours (e.g., Losos 2009; Bajer 2010; Olsson et al. 2011). However, considering the

visual similarity of species in most reptile genera, cryptic groups are unlikely to employ visual signals. Vocalisations appear rare among lizards, with the exception of geckos, which are known to use vocal signals for territoriality (Hibbitts et al. 2007; Rohtla et al. 2019). Chemical communication (via pheromones), however, appears to be widespread among lizards and snakes (Mason & Parker 2010; Martín & López 2014; Mayerl et al. 2015). Pheromones mediate lizard social interactions (Mason and Parker 2010; Martín and López 2014) and have been shown to influence male mate choice in North American *Plestiodon* skinks (Cooper & Vitt 1986), *Thamnophis* garter snakes (Mason et al. 1989; Shine et al. 2004), and *Laticauda* sea kraits (Shine et al. 2002). The role of pheromones in female choice, however, has been more contentious. Some studies have supported the role of pheromones in female choice in squamates (López et al. 2002**,** 2003; Martín & López 2000, 2006a, 2010, 2013; Kopena et al. 2011; Bordogna et al. 2016) while others have not (Olsson et al. 2001; Olsson & Madsen 1995; Barbosa et al. 2006; Martin & Lopez 2006b, 2006c; Gabirot et al. 2010; Martín et al. 2016; Heathcote at al. 2016; While & Uller 2017; MacGregor et al. 2017). Notably, nearly all of the taxa so far investigated are territorial, diurnal lizard species that often exhibit sexual dimorphism in colouration. At this time, no studies have explicitly investigated pheromone-mediated mate choice in nocturnal or fossorial species.

The Bynoe's gecko (*Heteronotia binoei*) is a small, nocturnal species of gekkonid lizard that occurs across most of Australia (Wilson & Swan 2017). Phylogenetic studies suggest that *H. binoei* is a cryptic species complex comprised of 10–20 independently evolving lineages (i.e., undescribed candidate species), many of which occur in sympatry or at parapatric contact zones (Fujita et al. 2010; Moritz et al. 2016; Zozaya et al. 2019; Chapter 2). Similar to many other lizards, male *H. binoei* possess epidermal pores anterior to the cloaca that produce a waxy secretion. These secretions contain pheromones that are known to influence social interactions in other lizards (Mayerl et al. 2015). In a previous study we showed that the chemical compositions of these pheromone secretions differ among divergent genetic lineages of *H. binoei* in north-eastern Australia, including between lineages in sympatry (Zozaya et al. 2019; Chapter 2). Divergence in pheromones is significantly greater than divergence in morphology (i.e., size and shape), suggesting that they play a role in the diversification of this group. This makes *H. binoei* a good system to study the role of pheromones on mate choice and behavioural isolation in lizards.

We hypothesise that epidermal pore secretions function as a mating signal influencing female mate choice in the *Heteronotia binoei* cryptic species complex and tested

this using behavioural experiments. To do this we used adult individuals from three sympatric genetic lineages (undescribed candidate species) of *H. binoei* that we collected from three sites representing each pairwise combination of sympatry between these lineages. We first performed simultaneous choice experiments to test whether females discriminate between an unscented control and the scent of a conspecific male. We then used a simultaneous choice design to test whether females discriminate between the scent of conspecific versus heterospecific males. Finally, because we found a significant novelty effect in the simultaneous conspecific versus heterospecific scent trials (i.e., due to which swab was encountered first), we also performed sequential choice experiments to minimise the influence of novelty.

Material and methods

Sampling and captive care

We collected three genetically divergent lineages of the *H. binoei* species complex: CYA6-S, EIU, and GULF-E (lineage names from Moritz et al. 2016 and Zozaya et al. 2019; Chapter 2). These three lineages co-occur in a mosaic of sympatry and parapatry in north-eastern Australia (fig. 3.1). They are all morphologically very similar except for variation in maximum body size (Zozaya et al. 2019; Chapter 2). There are some habitat differences among the lineages: CYA6-S is habitat generalist that occurs in open grasslands, woodlands, and rock outcrops; EIU is largely restricted to rock outcrops but also occurs on fallen timber in dry thicket forest; and GULF-E is largely restricted to grassland and is rarely found on large rock outcrops. Despite these broad ecological differences, the three candidate species have been found in various combinations of sympatry (within a meter of each other) at several sites.

Three collection sites were chosen that represent each pairwise combination of sympatry between these three lineages. Figure 3.1 illustrates the collection sites of the three genetic lineages. Geckos were collected from mid-winter to early spring from the Croydon area (Site 1, from 18–20 July 2016), Gorge Creek Station (Site 2, from 11–14 August 2016), and near the Lynd Junction (Site 3, from 6–8 September 2016). We captured 20 males and 20 females of each lineage from each site for a total of 240 individuals.

Geckos were maintained individually in white opaque plastic containers (length 30 cm, width 11.5 cm, height 12 cm) with a fiberglass mesh lid. Washed play sand (Richgro, Jandakot, WA) was provided as a substrate and a ceramic tile served as a hide. One end of

the enclosure rested on a heating element that brought surface sand temperatures to 32–35˚C from 11:00–17:00 each day; ambient air temperatures otherwise ranged between 20–28˚C depending on the time of day and season. All geckos were maintained in the same room and thus experienced identical thermal regimes. Ceiling mounted fluorescent lights provided light for the entire room on a day-night cycle matching that of Townsville, Queensland. Geckos were fed 2–4 crickets (depending on cricket size) twice weekly during the first six months of captivity, and then once weekly thereafter because geckos often refused crickets when fed too frequently. Crickets were dusted with calcium (Repcal, Rep-Cal Research Labs, Los Gatos, CA) and multivitamin (Herptivite, Rep-Cal) supplements once per week. Enclosures were sprayed with water before each feeding to provide humidity to assist with skin shedding and to provide water droplets for the geckos to drink.

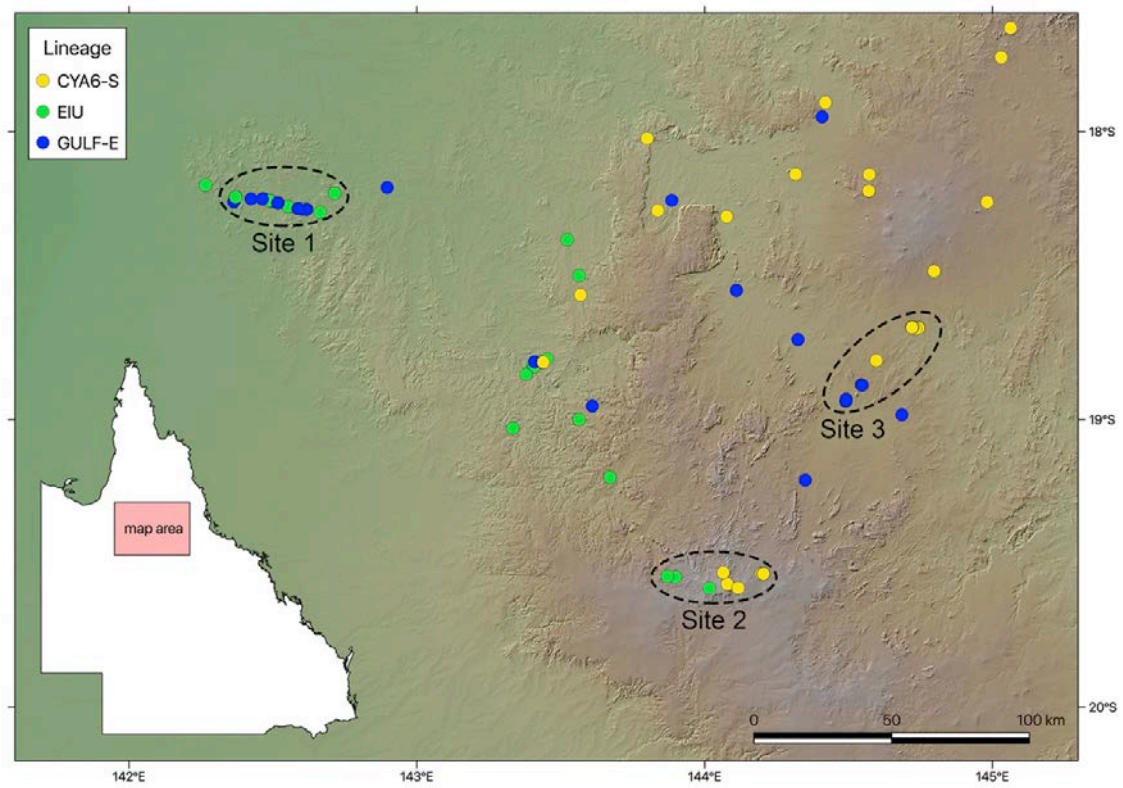

Figure 3.1: Map of the study region showing known localities for each of the three focal lineages of *Heteronotia binoei*. Dashed ellipses enclose collection sites with site labels below. The inset depicts Queensland, Australia.

Approximately half of all females were gravid upon collection or became gravid within 1–3 weeks after collection, indicating that these geckos begin breeding in early to mid-winter in north-eastern Australia. Females were provided with deep sand substrate to facilitate egg laying and monitored twice weekly to detect when eggs had been laid. Females

laid between 1–3 clutches between October–December 2016. Eggs were incubated and at least one egg from every clutch successfully hatched. All females were housed in isolation, thus these observations show that female *H. binoei* can store sperm for at least four months. To control for prior mating and sperm storage on reproductive status, we maintained all geckos in isolation for approximately one year to ensure that no further egg laying occurred. No eggs were laid after December 2016, suggesting that sperm stores had been exhausted.

Scent detection trials

Scent detection trials were conducted in August 2017, coinciding with the breeding season approximately one year following collection. Trials were conducted with a total of 120 female geckos (20 per lineage per site). Each female was simultaneously presented with two cotton swabs: one scented by the epidermal pores of a randomly assigned male from her own site and lineage, and the other an unscented control (fig. 3.2A). Scent was collected by gently restraining the respective male while rotating the cotton swab across the male's epidermal pores 30 times. Swabs were mounted on a holder made of an inverted plastic deli cup and placed at the opposite end of the enclosure to the female's hide (fig. 3.2A). Treatments were assigned to either the left or right side of the holder by tossing an Australian ten-cent coin. The enclosure was illuminated by an infrared lamp and filmed from above for 90 min. Trials were conducted at night between 18:00–04:00, coinciding with the activity times of these geckos in the wild. Ambient temperatures were always between 22–23°C during experiments. We recorded the swab first encountered by the female and the number of seconds spent sniffing each swab the first time each swab was encountered. Sniffing was scored when the female's snout was within 5 mm of, and oriented to, the cotton swab tip (see Results). We used seconds spent sniffing rather than the number of tongue-flicks for two reasons: first, geckos are thought to rely primarily on olfaction, and vomerolfaction (scent detection via tongue-flicking) to a lesser degree (Schwenk 1993); second, because experiments were recorded under low infrared light conditions, and because the geckos are small, it was not always possible to detect and record each tongue-flick. Under circumstances where a female gecko never emerged from its hide during the experiment, the trial was repeated on a different night. If a female gecko did not encounter both swabs, or never emerged from its hide for both the original and repeated trial, the trials for that gecko were excluded from analysis.

Simultaneous choice trials

Simultaneous female choice trials were conducted in September 2017. Female discrimination of conspecific and heterospecific male scent was tested exactly as per the scent detection trials except that females were presented simultaneously with both the scent of a randomly selected conspecific male of her own site and lineage and the scent of a randomly selected heterospecific male of her own site but a different lineage (fig. 3.3A). All other details of the methodology are identical to the scent detection trials. We did not size-match the males whose scents were presented to each female, as is often done in other studies of female mate choice, because we are broadly interested in how female choice of male scents might influence reproductive isolation in the wild, rather than intra-sexual selection. Additionally, because the lineages differ in body size, size matching would have been impossible to do consistently across trials.

Sequential choice trials

Female discrimination was subsequently assessed using a sequential choice design in an attempt to reduce the effect of order of swab encounter on female response (see Results). Trials were conducted in June–July 2018, during the breeding season the year after sequential choice trials were conducted. Each female was presented with a single treatment swab (conspecific or heterospecific scent) on one night and then presented with the alternate treatment two nights later (fig. 3.4A). The order that treatments were presented was alternated between each female. Ambient temperatures were always between 25–26°C for all trials. The methods were otherwise identical to the simultaneous choice trials.

Statistical analyses

Our analytical approach was two-fold. First, we assessed which swab was approached first, which can only be assessed using a paired-choice design (i.e., the scent detection trials and simultaneous choice trials). Second, we assessed the length of time spent sniffing each swab, which can be assessed for both paired-choice trials and the sequential trial. For the paired-choice designs (scent detection trials and simultaneous choice trials), we tested whether female geckos from each lineage approached one swab treatment (e.g., conspecific male scent) more often than the other, which would suggest that airborne (volatile) pheromone compounds influence female behaviour. To do this we performed binomial tests via the R function 'binom.test' (R Core Team 2018) with the number of 'successes'

being the number of trials where the female first visited the conspecific scented swab.

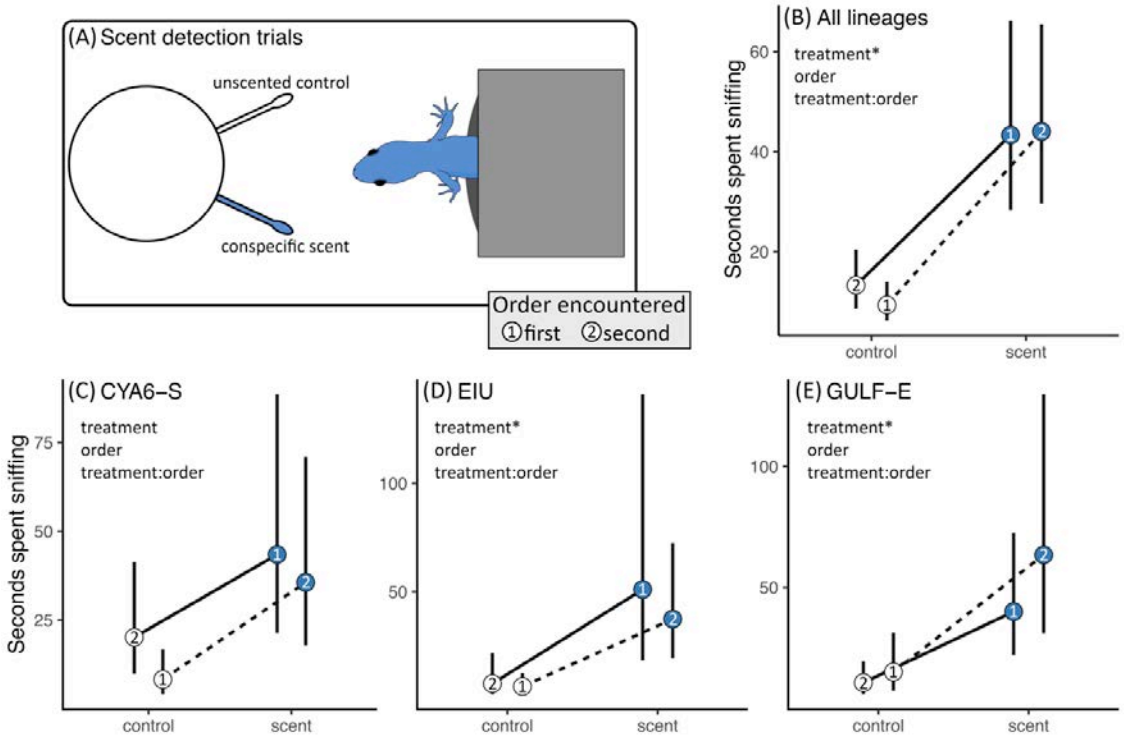

Figure 3.2: Scent detection trials. (A) Female *Heteronotia binoei* are simultaneously presented with an unscented control swab and a swab scented by the epidermal pores of a conspecific male from the female's site of origin. Grey squares represent the female's hide, circles represent swab holders, and projections from the circles represent cotton swabs. Panel B shows the results for the analysis of all lineages together, with the remaining panels (C–D) showing the results for analyses of individual lineages (labels top-left of panels). Circles are the means of the negative binomial distribution; whiskers represent 95% confidence intervals. Numbers within circles show the order swabs were encountered by the respective females, which is also conveyed by the lines connecting circles. Fixed effects are shown in the top-left of each panel, with statistically significant effects marked with an asterisk (*). Female *H. binoei* sniff conspecific male scented swabs longer than unscented control swabs, regardless of the order those swabs are encountered.

For all trials, we then used generalised linear mixed-effects models to test whether female geckos sniffed scented swabs longer than control swabs (scent detection trials), and whether they sniffed conspecific male scents longer than heterospecific male scents (simultaneous choice trials and sequential choice trials). For each experiment we first performed an analysis including all three lineages together and then performed separate

individual analyses for each of the three lineages. The number of seconds spent sniffing was the response variable, and treatment (scented versus control, or conspecific scent versus heterospecific scent) and order (the treatment first encountered/presented) were included with an interaction term as fixed effects. Observations were nested within individual, site, and lineage (but only in the all-lineages models) as a random effect (random intercept model). A negative binomial distribution was used to account for overdispersion in the female response data (fig. S3.1). Analyses were done in R using the 'glmer.nb' function from the package *lme4* (Bates et al. 2018; R Core Team 2018), which fits the model by maximum likelihood via Laplace approximation. Effect sizes were calculated by taking the exponent of the slope estimate, which yields the factor difference among levels within a fixed effect (see table 3.1).

Results

Scent detection trials

A total of 88 scent detection trials were useable for analysis: 29 for CYA6-S (N = 15 Site 2, N = 14 Site 3); 27 for EIU (N = 10 Site 1, N = 17 Site 2); 32 for GULF-E (N = 13 Site 1, N = 19 Site 3). Female geckos did not first visit the scented swab or the control swab significantly more often than the other, regardless of the female's lineage (CYA6-S: scented swabs encountered first 15/29, P = 1; EIU: scented swabs encountered first 19/27, P = 0.052; GULF-E: scented swabs encountered first 13/32, P = 0.377). Sniffing behaviour was conspicuous and involved the gecko's snout touching the respective swab or being within 5 mm of it, and was associated with short side-to-side or forward-to-back head movements, tongue-flicking, and occasional vocalisations (1–3 short chirps). Our observations suggest that geckos must be very close to or in contact with a swab before sniffing behaviour initiates.

There was substantial among-female variation in sniffing duration, even within a given treatment/order combination, although there was much greater variation among females for scented treatments versus controls (fig. S3.1). Nevertheless, female geckos sniffed conspecific scented swabs 3.25 times longer on average than unscented control swabs when all lineages were considered together (all lineages treatment: Z = 3.85, df = 168, P < 0.001, see table 3.1; fig. 3.2). Neither the order swabs were encountered nor the interaction between order and swab type affect the length of time females sniffed swabs (table 3.1). Treatment was the only significant effect for the individual analyses of both

EIU (Z = 2.62, df = 47, P = 0.009) and GULF-E (Z = 3.13, df = 57, P = 0.002), with EIU sniffing scented swabs 6.68 times longer than controls and GULF-E sniffing scented swabs 3.76 times longer than controls (table 3.1; fig. 3.2). Even though the CYA6-S lineage sniffed conspecific swabs on average longer than control swabs, this difference was not significant and nor were any of the other fixed effects for this lineage (table 3.1; fig. 3.2).

Table 3.1: Results of the negative binomial GLMM analyses of female sniffing duration. *P*-values less than 0.05 are in bold.

		Treatment			Order			Treatment*Order		
	df	Slope[1]	*Z*	*P*	Slope[1]	*Z*	*P*	Slope[1]	*Z*	*P*
Scent detection										
All lineages	168	1.181	3.85	**<0.001**	-0.350	-1.17	0.241	0.366	0.87	0.382
CYA6-S	51	0.768	1.52	0.127	-0.890	-1.77	0.076	0.691	0.98	0.326
EIU	47	1.900	2.62	**0.009**	-1.884	-0.30	0.761	-0.122	-0.14	0.887
GULF-E	57	1.325	3.13	**0.002**	0.349	0.74	0.460	0.112	0.17	0.866
Simultaneous choice										
All lineages	164	0.953	3.20	**0.001**	-0.745	-2.48	**0.013**	-1.076	-2.54	**0.011**
CYA6-S	33	1.147	1.68	0.093	-0.747	-1.16	0.244	-1.563	-1.67	0.093
EIU	49	0.906	2.01	**0.044**	-5.630	-1.11	0.266	-0.700	-0.97	0.331
GULF-E	69	0.940	2.12	**0.034**	-0.788	-1.68	0.094	-1.296	-2.11	**0.035**
Sequential choice										
All lineages	184	1.032	5.80	**<0.001**	-0.440	-2.08	**0.037**	-0.760	-2.93	**0.003**
CYA6-S	53	1.054	3.92	**<0.001**	-1.134	-2.63	**0.008**	-1.369	-3.42	**<0.001**
EIU	49	1.000	2.97	**0.003**	0.000	0.00	0.999	-0.582	-1.15	0.249
GULF-E	69	0.995	3.86	**<0.001**	-0.260	-1.00	0.316	-0.460	-1.28	0.201

[1] Values represent the raw slope estimates from the respective negative binomial model output. Effect sizes (reported in-text for significant effects) are calculated by taking the exponent of the slope estimate, which yields the factor difference among levels within a fixed effect. For example, a slope of 0.906 for Treatment means that heterospecific scents were sniffed only 0.4 times as long as conspecific scents; or, conversely, conspecific scents were sniffed 2.47 times longer than heterospecific scents.

Simultaneous choice trials

A total of 86 trials were useable for analysis: 20 for CYA6-S (N = 10 Site 2, N = 10 Site 3); 28 for EIU (N = 9 Site 1, N = 19 Site 2); 38 for GULF-E (N = 19 Site 1, N = 19 Site 3). Similar to the scent detection trials, female geckos did not first visit the conspecific swab or the heterospecific swab significantly more often than the other, regardless of the female's lineage (CYA6-S: conspecific swabs visited first 9/20, P = 0.823; EIU: conspecific swabs visited first 17/28, P = 0.344; GULF-E: conspecific swabs visited first 17/38, P = 0.627).

Similar to the scent detection trials, there was substantial among-female variation in female sniffing duration (fig. S3.2). Treatment, order, and the interaction between the two

were all significant when all three lineages were analysed together (table 3.1; fig. 3.3). Results for the EIU lineage show a significant effect of treatment only (Z = -2.01, df = 49, P = 0.044), with females sniffing conspecific scented swabs on average 2.47 times longer than heterospecifics (table 3.1; fig. 3.3). Results for the GULF-E lineage show a significant effect of treatment (Z = -2.12, df = 69, P = 0.034) as well as the interaction between treatment and order (Z = 2.11, df = 69, P = 0.035) (table 3.1; fig. 3.3). Results for the CYA6-S again show no significant fixed effects, possibly because of low power (only half of all trials were usable for analysis); however, figure 3.3 suggests there is an interaction between treatment and order for this lineage (albeit non-significant; see table 3.1).

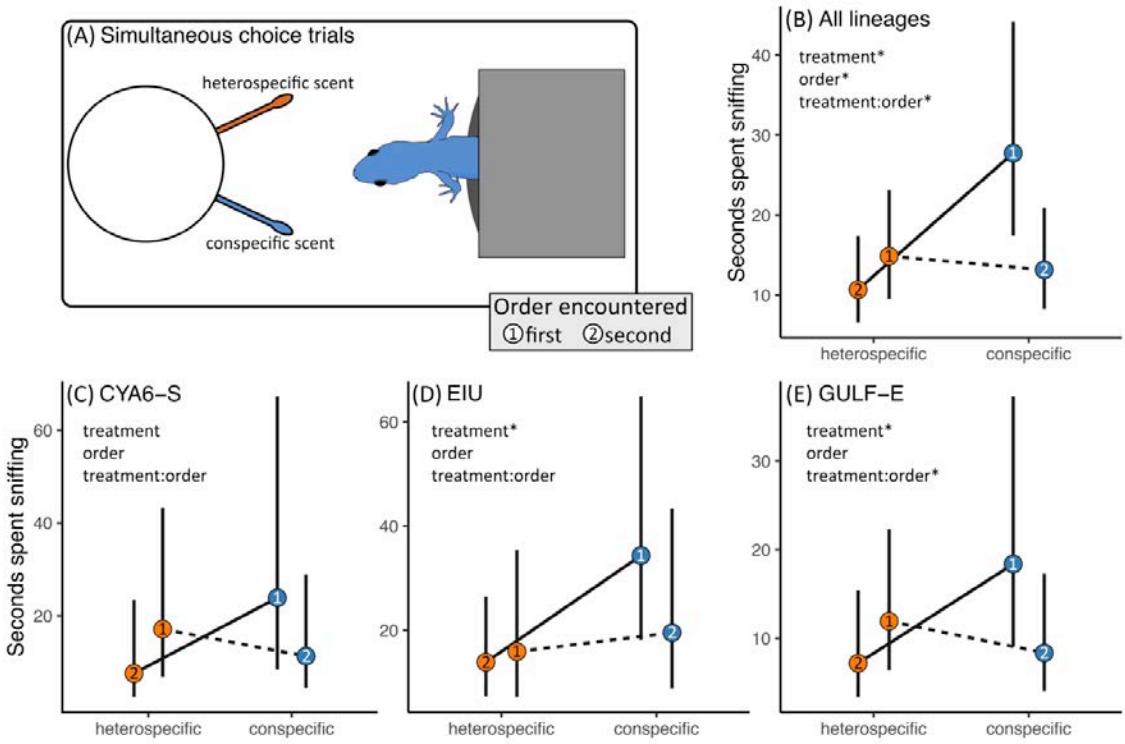

Figure 3.3: Simultaneous choice trials. (A) Female *Heteronotia binoei* are simultaneously presented with a swab scented by the epidermal pores of a conspecific male and another scented by a heterospecific male, both from the female's site of origin. Panel B shows the results for the analysis of all lineages together, with the remaining panels (C–D) showing the results for analyses of individual lineages. Figure layout is identical to figure 3.2. The plots illustrate that an interaction between treatment and the order swabs are encountered influence female sniffing duration, although only scent treatment was significant in the analysis of the EIU lineage (D). In all cases, conspecific swabs that are encountered first are sniffed longest, whereas heterospecific swabs that are encountered second are sniffed least.

These results indicate a significant novelty effect. Females generally sniff conspecific scented swabs that are encountered first the longest, and heterospecific scented swabs encountered second the least. When the heterospecific swab is sniffed first there does not appear to be a considerable difference in female response between the two treatments. Thus, scent treatment does influence female response, with conspecific-scented swabs being sniffed longer, but the magnitude of the response is impossible to assess given the significant interaction with order. We therefore carried out sequential choice trials.

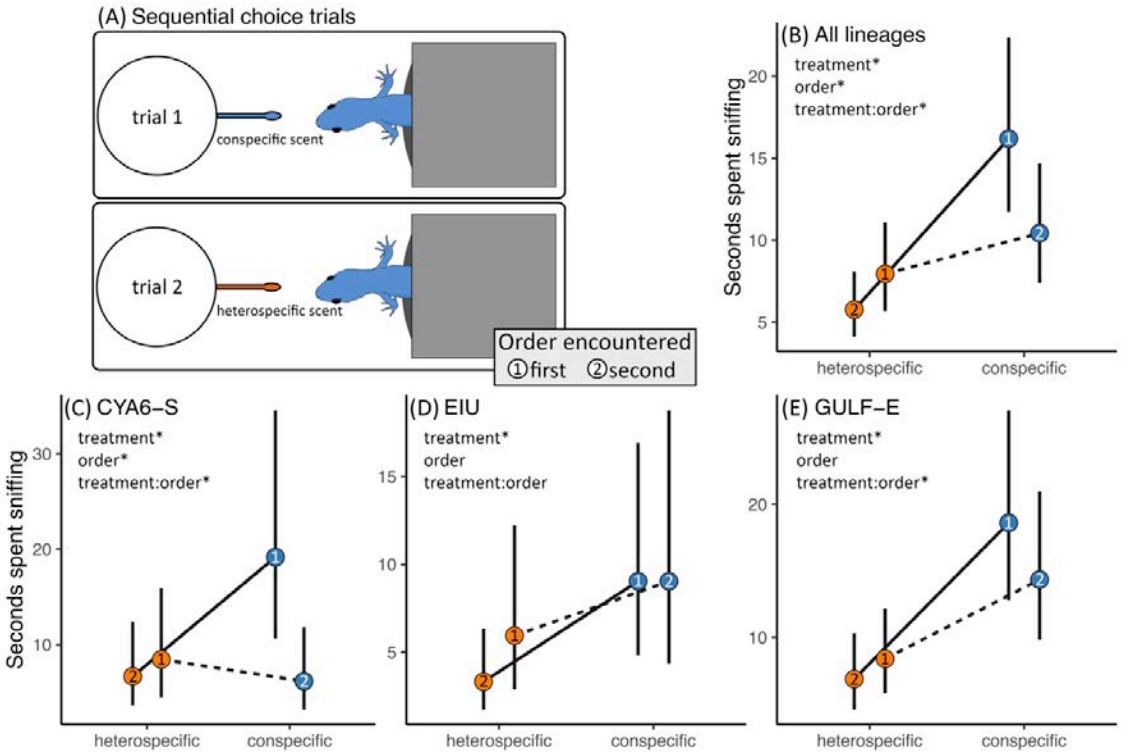

Figure 3.4: Sequential choice trials. (A) Design as for the paired-choice trials, except that female *Heteronotia binoei* are presented one treatment on the first night (here shown as a conspecific scent; top) and the second treatment two nights later (here shown as a heterospecific scent; bottom). Panel B shows the results for the analysis of all lineages together, with the remaining panels (C–D) showing the results for analyses of individual lineages. Figure layout is otherwise identical to figure 3.2. The plots illustrate that an interaction between treatment and the order swabs are presented influences female response (seconds spent sniffing) for the analysis of all lineages (B); however, this appears driven largely by the CYA6-S lineage (C). In contrast, females of the EIU (D) and GULF-E (E) lineages sniffed conspecific scents significantly longer than heterospecific scents, regardless of the order those swabs were encountered.

Sequential choice trials

Trials from a total of 96 geckos were useable for analysis: 30 for CYA6-S (N = 14 Site 2, N = 16 Site 3); 28 for EIU (N = 10 Site 1, N = 18 Site 2); 38 for GULF-E (N = 20 Site 1, N = 18 Site 3). There was again substantial among-female variation in sniffing duration (fig. S3.3). All fixed effects were significant in the overall analysis, including the interaction between treatment and order, although the slope of the interaction is less than for the simultaneous choice trials (table 3.1; fig. 3.4). Thus, the sequential choice method did not fully remove the influence of novelty on female response, but it did reduce its effect (table 3.1). The analyses of individual lineages, however, show that the interaction is largely driven by the CYA6-S lineage (treatment x order interaction: Z = 3.42, df = 53, $P < 0.001$; table 3.1; fig. 3.4). In contrast, treatment is the only significant effect for both EIU (Z = 2.11, df = 69, P = 0.035) and GULF-E (Z = 2.11, df = 69, P = 0.035), both of which sniffed conspecific scents on average 2.7 times longer than heterospecific scents (table 3.1; fig. 3.4). This demonstrates that female geckos from these two lineages discriminate between the pheromone secretions of conspecific and heterospecific males, and spend more time investigating the scent of conspecific males.

Discussion

The divergence of mating signals among closely related populations can influence reproductive isolation (Mayr 1963; Wells & Henry 1998; Boughman 2001; Coyne & Orr 2004). Considering this and that many animal species differ almost entirely in their mating signals, knowledge of these signals is crucial for understanding animal diversity. Here we used behavioural experiments to test if divergent male pheromones influence female discrimination of lineages in a morphologically cryptic species complex. First, we found that female geckos spent significantly longer sniffing swabs scented by male pheromone secretions than unscented control swabs. Second, we found that female geckos spent significantly longer sniffing the scent of conspecific males than the scent of sympatric heterospecific males, demonstrating that there is female discrimination of male pheromone secretions in these lizards. We found these results over and above a significant effect of novelty (treatment order), which is discussed below. The combined evidence of a sexually dimorphic male trait, significant pheromone divergence among candidate species (Zozaya et al. 2019; Chapter 2), and female discrimination of conspecific over heterospecific scent support our hypothesis that pheromones serve as a mating signal in Bynoe's geckos. Our

study is the first to show that interspecific variation of pheromones influences female discrimination in lizards, and the first to investigate pheromone-mediated female choice among sympatric cryptic lizard species.

While we found female discrimination of pheromones based sniffing duration, we did not detect differences in which swab females visited first. For example, in the scent detection trials female geckos did not visit scented swabs first more often than controls but they spent more time sniffing the scented swabs when they encountered them. Sniffing behaviour, such as side-to-side head movements and occasional tongue-flicking, were observed only once the female had detected the swab at very close range. These observations suggest that volatile pheromone compounds do not strongly influence female behaviour, at least not at distances of more than a few centimetres. Indeed, gas chromatography characterisation of epidermal pore secretions shows that most compounds have high retention times and are relatively heavy (Zozaya et al. 2019; Chapter 2), and hence are not very volatile. This makes sense considering that the pheromone excreting pores are near the cloaca, where secretions will be passively deposited on substrates, possibly across the male's home range. In pygmy blue-tongue lizards (*Tiliqua adelaidensis*), males follow paths intentionally laid down by females that direct them towards her burrow (Ebrahimi et al. 2015). Considering that *Heteronotia* geckos are small, nocturnal, and live in complex environments where visual cues are limited, deposition of scent might play a similar role but instead for females to find males of their own species.

The degree of pheromone discrimination was not the same across the three lineages in our study, with females of the CYA6-S lineage showing weaker discrimination. No fixed effects were significant in either the scent detection trials or the simultaneous choice trials for this lineage. Visualisation of the scent detection trials (fig. 3.2C) shows a trend that females sniff scented swabs longer, suggesting we may have lacked power to detect a significant effect in these trials. Nevertheless, CYA6-S showed a significant interaction between treatment and order in the sequential choice trials. Although we cannot be certain here, this lack of significant discrimination may be similar to the results of Kwiatkowski and Sullivan (2002) who found that of three populations of chuckwalla lizards in Arizona, one population demonstrated reduced female choice of male colouration. Many factors will contribute to the degree of discrimination in each lineage, including the relative divergence in mating traits and the degree (if any) of reproductive character displacement and gene flow in areas of secondary contact. Incorporating more detailed pheromone sampling across lineage distributions, ancestral range reconstruction, and population genomics would

be useful to determine the history and outcomes of interaction between the lineages in the wild.

Most studies of pheromone choice in reptiles have been conducted within species, and the results have been highly variable. There is compelling evidence for pheromone-based male choice of females in lizards and snakes (e.g., Cooper & Vitt 1986; Mason et al. 1989; Shine et al. 2004*;* Shine et al. 2002) but evidence for pheromone-based female choice of males is limited. Some studies have inferred female discrimination of male pheromones (e.g., López et al. 2002, 2003; Martín & López 2000, 2006a, 2010, 2013; Kopena et al. 2011; Bordogna et al. 2016) but others have found no such evidence (e.g., Martin & Lopez 2006b, 2006c; Martín et al. 2016; Heathcote at al. 2016; While & Uller 2017; MacGregor et al. 2017). Very few studies have investigated the effect of pheromone divergence on choice between lineages. Investigations of a hybrid zone between two lineages of the Common Wall Lizard (*Podarcis muralis*) in southern Europe concluded that pheromone divergence does not influence female choice (Heathcote at al. 2016; While & Uller 2017; MacGregor et al. 2017). In contrast, our results indicate that male pheromones influence female discrimination in Bynoe's geckos.

There are several potential reasons why results vary so much among studies of female mate choice in lizards and snakes. First, phylogenetic splits among the major squamate groups are very old. A recent study placed gekkonids (the group to which *H. binoei* belongs) as sister to all other squamates, having possibly diverged as early as the Triassic (Simões et al. 2018). We should expect the presence, strength, and mode of female choice to vary hugely across lizard groups considering these ancient divergences, especially given that female choice appears to vary even between populations of the same species (Cooper & Vitt 1987; Kwiatkowski & Sullivan 2002; Shine et al. 2004; this paper). Second, mate choice studies in lizards have overwhelmingly focused on a few diurnal taxa, many of which exhibit conspicuous sexual dichromatism (e.g., lacertids). Few studies have investigated the mating traits of nocturnal lizards (such as Bynoe's geckos) and, to our knowledge, none have investigated fossorial species, which comprise a considerable proportion of lizard and snake diversity, and for which the opportunity to employ visual signals would be rare or non-existent. Third, sperm storage appears common in reptiles (Olsson & Madsen 1998; Sever & Hamlet 2002), suggesting a potential role for cryptic female choice exercised after matings.

Finally, the experimental set-up will greatly influence the results, requiring careful consideration of what makes a biologically relevant experiment. Mate choice studies in

frogs generally consist of playing two or more recorded calls in an arena to a female (Gerhardt & Huber 2002). Simultaneous choice experiments are biologically relevant for frogs because multiple calls can be broadcast over sufficient distance to a choosing female and because in the wild females of most species choose among males in a chorus. Female choice is then scored as movement to one of the speakers (e.g., Ryan 1980; Hoskin et al. 2005). In contrast, simultaneous pheromone choice experiments may be of limited utility in lizards because, as discussed above, pheromones in lizards are probably not broadcast over large distances and most lizard species probably assess mates over sequential encounters rather than simultaneously. Our use of both simultaneous and sequential pheromone choice trials highlights the confounding effect of novelty; i.e., the importance of considering the order treatments are presented or encountered when designing and analysing experiments such as these. Our sequential choice design reduced the magnitude of the novelty effect observed in the simultaneous choice trials. In Bynoe's geckos, and probably most lizards that use pheromones, sequential choice designs more accurately reflect conditions in the wild where a female encounters males sequentially over the course of hours or days. As has been outlined elsewhere, experimental designs should focus on simulating conditions in the wild (Stapley 2008; Dougherty & Shuker 2015). Nevertheless, even in our sequential choice experiments there was still a significant interaction of order and treatment, which emphasises the need to account for order in statistical models of experiments such as these (Reading & Backwell 2007; Wong & Svensson 2009).

Here we isolated pheromones on swabs for both simultaneous and sequential pheromone choice trials. Isolating the pheromones from the geckos was important to determine whether the trait in question serves as a mating signal; however, this removes the trait from the context of the whole animal and hence overlooks the potential role of multimodal signalling (including calling behaviour) that may be used for some social interactions (e.g., Hankison & Morris 2003; Vicente & Halloy 2017; Kabir et al. 2019). It also means male competition and cryptic female choice among stored sperm from multiple matings cannot be assessed. These are all valuable avenues for further research in this system.

There was substantial variation in the amount of time individual females spent sniffing a given treatment. The novelty effect discussed above accounts for some of this, but considerable variation in female sniffing time persists even within a given treatment/order combination. A study by Kabir et al. (2019) on diurnal *Cnemaspis* geckos observed variation in female response to male scent and considered this to be because of

variation in male quality. We did not control for male body size and condition, factors that are known to influence pheromone composition and female response (Martín & López 2015). Although this may be seen as a limitation of our study it is also a more relevant design to answer our main question: Do pheromones function as a mating trait influencing behavioural isolation? Our design more accurately reflects conditions in the wild where a female would encounter males of varying quality and lineage/species membership. Size matching is crucial for understanding sexually selected traits within a species, but if the traits in question play a role in premating isolation, we expect that female discrimination among lineages/species will persist despite variation in male quality (Pfennig 1998, 2000). Furthermore, there was also considerable variation in female response to unscented control swabs, even when those controls were encountered first. This observation suggests that there is variation in female propensity to investigate a stimulus, possibly interpretable as variation in curiosity or boldness, which can affect measures of mate choice (David & Cézilly 2011). There might also be variation in the strength of female preference among individuals (e.g., Ryan et al. 2019). Variation in the strength of female preference can be influenced by hormone levels and/or reproductive condition (e.g., Lynch et al. 2006), factors that are difficult to control for. That we have shown female discrimination of lineages despite these potentially confounding factors strengthens our conclusion that pheromones function as a mating signal in Bynoe's geckos.

Investigations of pheromones in reptiles account for only 1% of all pheromone studies conducted (Symonds & Elgar 2008). We previously suggested that pheromone data might be useful for resolving cryptic lizard diversity (Zozaya et al. 2019; Chapter 2) but highlighted that more evidence is needed to link pheromone divergence with reproductive isolation. This study bridges that gap in knowledge, providing strong evidence that pheromone divergence translates to lineage discrimination. We infer that pheromone divergence and associated female choice for these pheromones plays a role in premating reproductive isolation in this system, and hence in many other cryptic reptile groups. This not only contributes to our understanding of the diversification processes in these systems, but also that pheromones are a valuable trait for taxonomy (Padial et al. 2010). As is done for frog calls and mating signals in other groups, pheromone data could be integrated with other types of data (e.g., morphology, genetics) to resolve particularly complex groups. More work in this system and others—particularly across phylogenetically and ecologically disparate reptile groups—will be valuable for understanding the importance of pheromones in mate choice and speciation in reptiles and other vertebrates.

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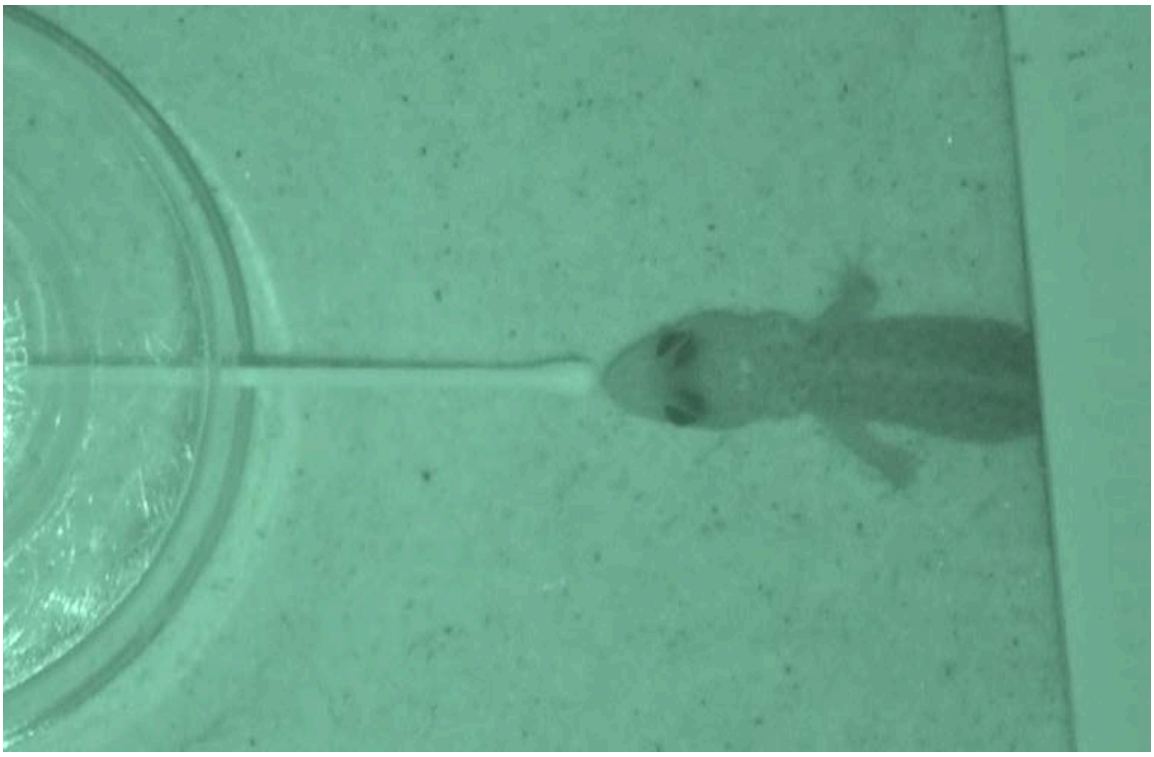

A female *Heteronotia binoei* (CYA6-S) sniffing a swab during the sequential choice experiments. Note how cute she is.

Chapter 3 – Supplementary material

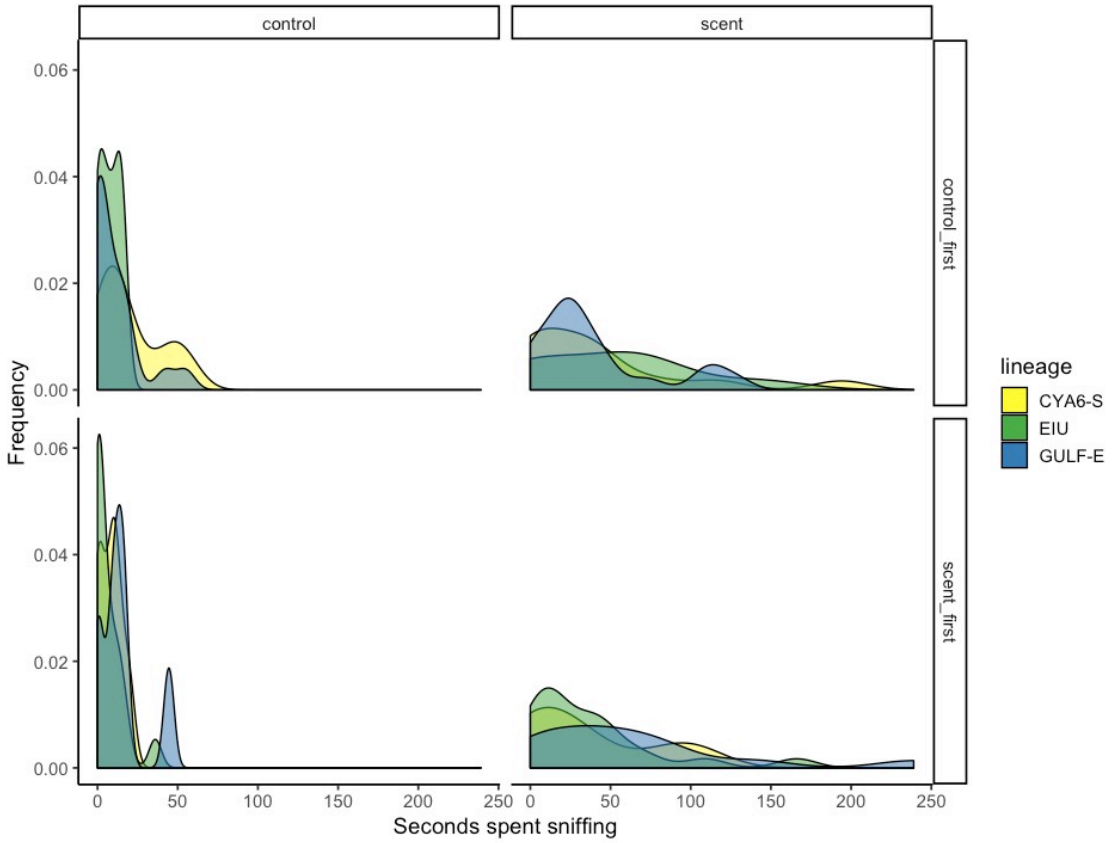

Figure S3.1: Distributions of female responses (seconds spent sniffing) for the scent detection trials. Each panel represents a different treatment/order combination to illustrate among-female variation in sniffing time.

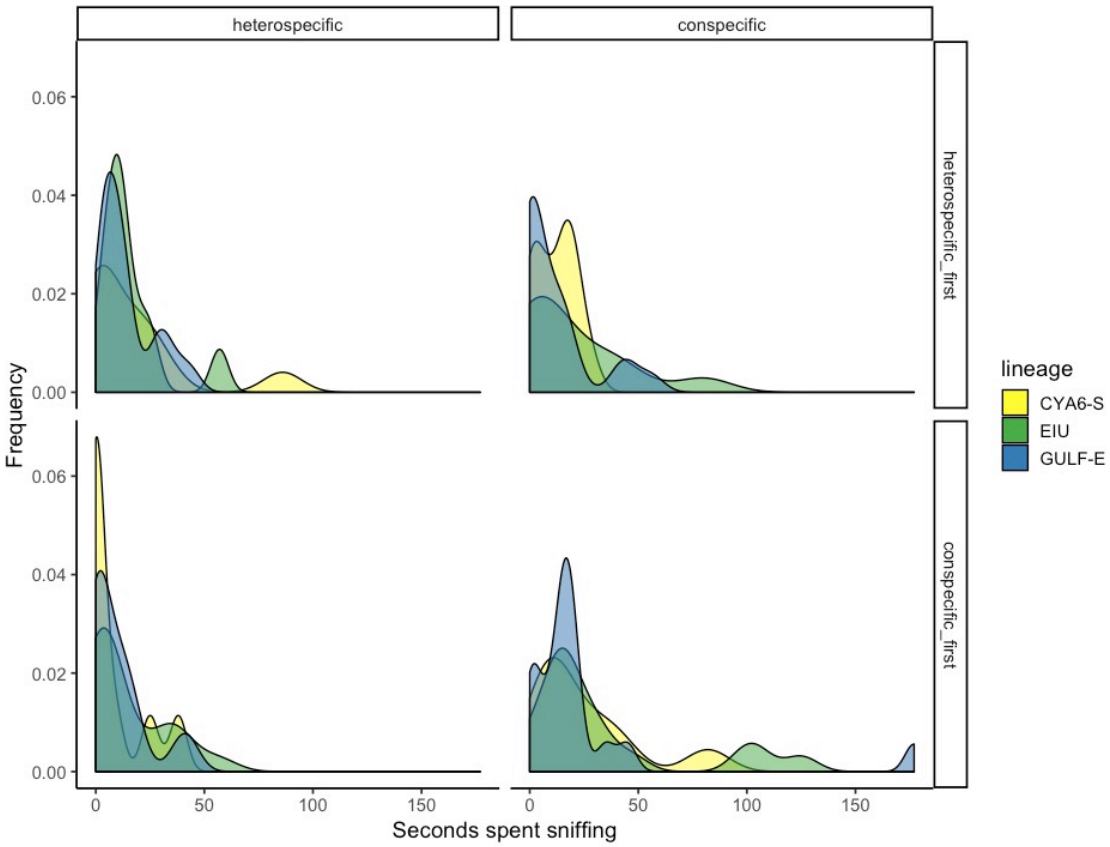

Figure S3.2: Distributions of female responses (seconds spent sniffing) for the simultaneous choice trials. Each panel represents a different treatment/order combination to illustrate among-female variation in sniffing time.

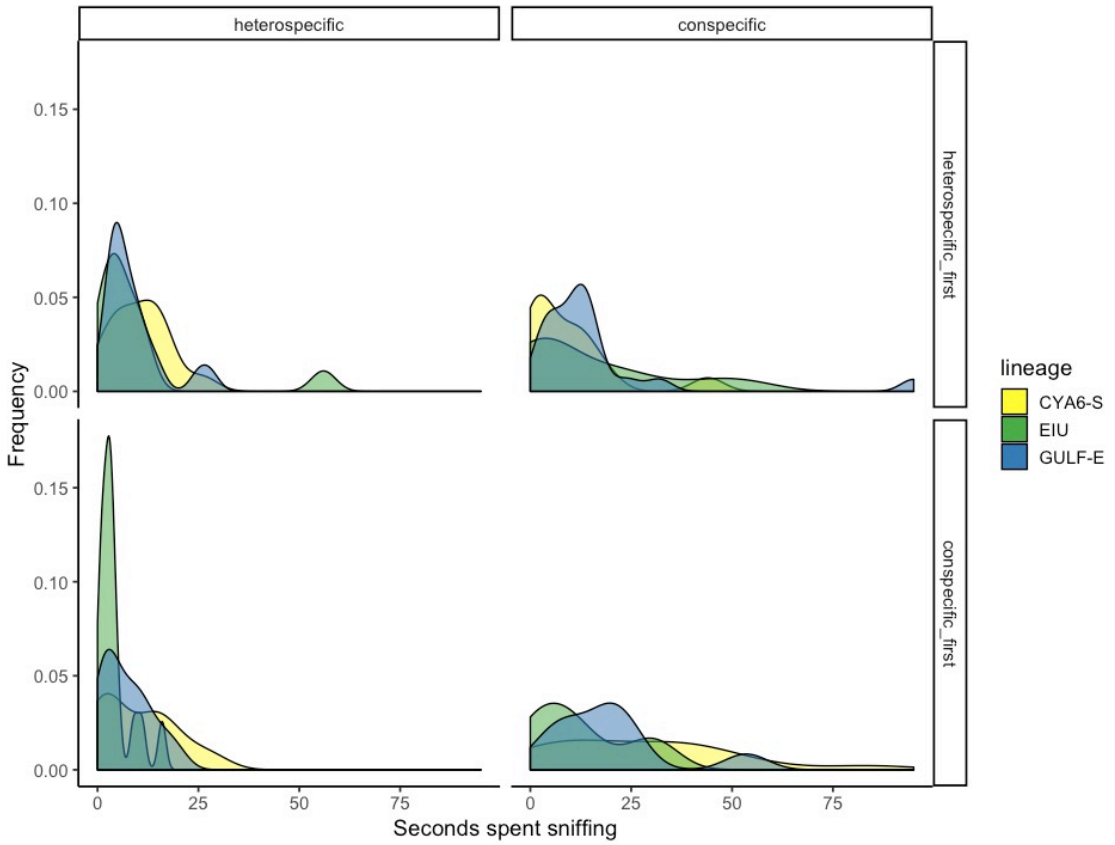

Figure S3.3: Distributions of female responses (seconds spent sniffing) for the sequential choice trials. Each panel represents a different treatment/order combination to illustrate among-female variation in sniffing.

CHAPTER 4 — Pheromone divergence is associated with climate and phylogeny in an Australian lizard radiation

Stephen M. Zozaya, Luisa C. Teasdale, Craig Moritz, Megan Higgie, and Conrad J. Hoskin

Abstract

In animals, signalling systems serve important functions related to survival and reproduction. The environment can present challenges to the transmission and detection of these signals, resulting in selective pressures that drive signal divergence among populations in disparate environments. The challenges posed by the environment depend on the modality of the signal itself. For chemical signals, such as pheromones, climate is potentially important because factors such as temperature and moisture influence how quickly chemical signals evaporate and thus how easily they are detected. Considering this, we should expect chemical signals to be adapted to prevailing climatic conditions. Here we focus on a widespread Australian lizard radiation (*Heteronotia*) to test if and how pheromone divergence among 33 lineages is correlated with two key climate variables: temperature and precipitation. We reconstructed the phylogeny of these lineages with exon capture phylogenomics and used a phylogenetic comparative approach to first assess phylogenetic signal in pheromone data, and then to perform a multivariate regression of pheromones against temperature and precipitation while accounting for phylogenetic relationships. Phylogenetic signal was high, indicating that pheromone composition is closely associated with shared evolutionary history. Accounting for this, we show that temperature correlates with pheromone divergence in the *H. binoei* complex but not the *H. planiceps* complex, and that precipitation correlates with pheromone divergence in both species complexes, although the response of pheromones to precipitation differs subtly between the two. These results likely reflect adaptation of pheromones to disparate climatic conditions, and we identify pheromone components strongly associated with these relationships to guide further research.

Introduction

Signalling systems serve important functions in animals that are vital for survival and reproduction: finding and choosing mates; orientation; warning, detecting, and assessing competitors; recognising kin and familiars; deceiving rivals, prey, and predators; and alerting others of—and being alerted to—danger (Bradbury & Vehrencamp 2011). These functions influence the fitness of both signallers and receivers; an individual that cannot attract a mate or that does not heed a warning call in the presence of a predator will have few offspring—if any. To function effectively, a signal must be transmitted through, and perceived against, its background environment (Endler 1992). For example, blue colouration is an ineffective signal for fish inhabiting murky water where blue wavelength light is scarce (Seehausen et al. 2008). The environment can thus exert selection pressure leading to the co-adaptation of signalling traits and the sensory system to optimise their efficacy against the background environment, a process termed 'sensory drive' (Endler 1992). When the traits in question are linked to mate choice, sensory drive can influence reproductive isolation and subsequently lead to speciation (Endler & Basolo 1998; Boughman 2002; Seehausen et al. 2008).

The precise manner that the environment influences signals and the sensory system depends on the modality of the signal. For example, wind exerts challenges for movement-based signals performed against wind-blown vegetation (Ramos & Peters 2017). Visual signals involving colour and pattern depend on ambient light environments, such as the murky water fish example given above. And acoustic signals must be perceptible against background noise, such as the mating calls of frogs advertising beside thunderous torrents and waterfalls (Feng et al 2006). Examples are myriad and illustrate the environmental pressures exerted on signalling systems. Understanding adaptation to environmental pressures is important for understanding the evolution of signalling traits and their diversity.

Many studies have demonstrated the influence of environmental factors on the evolution of visual and acoustic signalling systems (reviewed in Cummings & Endler 2018). In contrast, there have been few studies on the influence of the environment on chemical signals (Symonds & Elgar 2008; Yohe & Brand 2018). Chemical signalling is the most ubiquitous and ancient mode of signalling among animals (Hildebrand & Shepherd 1997), but it is also the most difficult to study. Chemical signals can be complex; the potential combination of chemical compounds and their relative proportions is effectively infinite. Collecting and characterising chemical signals poses one challenge, determining the

biological activity of specific compounds within chemical blends poses another. Then there is the challenge of characterising the chemical signalling environment. Chemical signals must be perceived against a background of other chemicals, and the signal, the background, and the sensory system are influenced by abiotic factors, such as temperature and humidity (Bradbury & Vehrencamp 2011; Yohe & Brand 2018).

Characterising the chemical background is not easy (Riffell et al. 2008), but investigating the role abiotic factors play in the evolution of chemical signals is simpler and somewhat better understood. For example, animals evolve to produce signalling compounds that function best under the temperatures they experience (Alberts 1993). High humidity and rainfall shorten the longevity of chemical signals by increasing evaporation and washing away scent marks (Alberts 1992; Royer & McNeil 1993; Wilder et al. 2005). But because of this increased evaporation, moisture also makes it easier for the receiver to detect these signals (e.g., in rodents; Vander Wall 1998). To face these challenges some animals have evolved 'keeper substances', non-signalling compounds that increase the longevity of the scent mark (Hurst et al. 1998; Hayes et al. 2003). Yohe and Brand (2018) provide a valuable review of the challenges of sensory drive research on chemical signalling systems and what we already know about this process. Despite much recent progress, our understanding of the role the environment plays in shaping chemical signals is limited.

Lizards and snakes (squamates) are a good group to study the evolution of chemical signals. Squamates use chemical signals (pheromones) to mark territories, identify kin and rivals, deceive competitors, and to attract and choose mates (Mason & Parker 2010; Martín & López 2014; Chapter 3). Considering these important functions we should expect strong selective pressures acting on their efficacy, and there is already limited experimental and comparative evidence to suggest this. High temperatures negatively impact the persistence and detectability of pheromone secretions in the lizard *Iberolacerta cyreni*, with secretions kept under warm temperatures receiving reduced chemosensory investigation via tongue-flicking (Martín & López 2013). Furthermore, a comparison of the composition of pheromone secretions between two populations of the lizard *Podarcis guadarramae* (as *P. hispanicus*) showed that populations inhabiting disparate climates possess divergent pheromone blends, with the pheromones of those from warmer, drier environments being more easily detectible than those from cool, wetter environments after being experimentally treated to warm conditions (Martín et al. 2015). Finally, a phylogenetic comparative study of 64 species and subspecies of lacertid lizards by Baeckens et al. (2018)

found that species inhabiting drier climates had higher proportions of stable fatty acids and high molecular weight alcohols. Although the number of studies is limited, these results highlight the potential role of climate in shaping the chemical signals of squamates.

Many squamate groups are extremely adaptable, with related species spanning environments from mountain rainforests to treeless deserts, making them excellent systems for studying adaptive evolution under disparate ecological conditions (e.g., Australian agamids; Gray et al. 2019). Phylogenetic comparative methods (PCMs) offer a useful set of tools for investigating trait evolution among large numbers of species or populations while accounting for the non-independence of observations due to shared evolutionary history (Felsenstein 1985; Harvey & Pagel 1991). Studies comparing a few species or populations are valuable but are limited in their ability to identify common evolutionary trends. Until relatively recently, however, the suitability of PCMs to analyse multivariate data has been extremely limited (Adams & Collyer 2018, 2019); thus, studies of multivariate chemical signals in a phylogenetic context are few (but see Symonds & Elgar 2004; Symonds & Wertheim 2005; Weber et al. 2016; Baeckens et al. 2018).

Chemical signals can range from one to dozens or more compounds that vary in their relative proportions. In many cases, the number of variables approaches or exceeds the number of observations, making statistical analysis difficult (Zuur et al. 2007). Studies of multivariate traits have often dealt with this issue via dimension reduction techniques, such as principal components analysis (PCA; e.g., Arnegard et al. 2010; Chapter 2). The disadvantage of dimension-reduction, however, is that some proportion of trait variation is inevitably discarded, and phylogenetic comparative analysis of dimension-reduced trait axes can sometimes yield spurious statistical conclusions (Uyeda et al. 2015). But now thanks to the ballooning field of geometric morphometrics, new PCMs using residual randomisation procedures have been developed that are suited to data with more variables than observations (Collyer et al. 2015). Although developed largely for morphometric data, this offers a valuable and hitherto unused tool for investigating the evolution of multivariate chemical signals.

Here we use a multivariate phylogenetic comparative approach to investigate how phylogeny and climate shape the pheromone blends of the *Heteronotia* lizard radiation across northern Australia. *Heteronotia* (Gekkonidae) is a genus of five recognised species (Wilson & Swan 2017), two of which (*H. binoei* and *H. planiceps*) are complexes of deeply divergent genetic lineages that together comprise 20 or more undescribed 'cryptic species' (Fujita et al. 2010; Moritz et al. 2016; Oliver et al. 2017; Zozaya et al. 2019; Chapter 2;

Moritz unpublished data). We have previously shown that deeply divergent genetic lineages (i.e., candidate species) within the *H. binoei* complex have divergent male pheromone blends (Zozaya et al. 2019; Chapter 2), and that female *H. binoei* discriminate between the pheromones of conspecific and heterospecific males in regions of sympatry (Chapter 3), suggesting that pheromones influence female mate choice and reproductive isolation in these lizards. Furthermore, *Heteronotia* are a complex mosaic of allopatric, parapatric, and sympatric lineages distributed across most of Australia, occupying habitats from humid coastal forests to the continent's central deserts. This wide climatic breadth, high lineage diversity, and the important role of pheromones make *Heteronotia* an excellent radiation to study the influence of climate on the evolution of a chemical signalling trait. Here we collect and characterise pheromones from *Heteronotia* across northern Australia to: (1) assess the strength of phylogenetic signal in pheromone divergence; and (2) test if and how climate influences pheromone variation among lineages.

Material and methods

Broadly, our approach was to collect pheromones from as many lineages of *Heteronotia binoei* and *H. planiceps* as possible. We targeted lineages presented in published phylogenies and also sampled new regions to add new lineages. We constructed a species tree for most of the lineages using exon capture phylogenomics, and added three lineages lacking exon capture data to this tree based on their relationships in a comprehensive mtDNA phylogeny. We then used these phylogenies to assess phylogenetic signal and to test the influence of two key climate variables (mean annual temperature and mean annual precipitation) on pheromone composition using multivariate phylogenetic regression.

Field sampling

We sampled pheromones from *Heteronotia* geckos across northern Australia from September–December 2017 and September–November 2018; months coinciding with the mid- to late reproductive season of these geckos in northern Australia (Chapter 3; SMZ pers. obs.). We targeted 25 lineages of *H. binoei* and eight lineages of *H. planiceps* for a total of 33 divergent genetic lineages (see Results). We sampled 1–12 adult male geckos for each lineage from each site (only males possess pheromone-secreting pores). Geckos were captured at night by hand and epidermal pore secretions were collected from individuals following the methods of Zozaya et al. (2019; Chapter 2). A small section of tail-tip was

collected from each gecko for mtDNA sequencing to confirm lineage identity at sites not sampled in previous studies or where more than one lineage co-occurs. Finally, a control sample was collected at each site to identify any contaminants incurred during pheromone collection (see Zozaya et al. 2019; Chapter 2). Samples were stored in a car freezer that maintained temperatures ranging from -20°C to -5°C for up to 35 days in the field, and then they were returned to the laboratory and stored at -20°C until characterised via gas chromatography (details below).

mtDNA sequencing and phylogenetics

We performed phylogenetic analysis of mtDNA sequence data to confirm the lineage membership of geckos sampled at sites for which no prior genetic data was available, and for sites where more than one lineage may be present. To do this we sequenced the gene NADH dehydrogenase subunit 2 (*ND2*) for at least three individuals from sites not previously sampled (i.e., not represented in Moritz et al. 2016 or Zozaya et al. 2019; Chapter 2), and all individuals at sites where more than one lineage occurs. DNA extraction and PCR protocols followed the methods of Zozaya et al. (2019; Chapter 2), which used the *ND2* primers tRNAI and tRNAA from Strasburg and Kearney (2005) and the PCR protocols of Fujita et al. (2010). Sequences were edited and aligned using Geneious 2019.1.3 (2019) and then visually inspected and verified by translating the *ND2* coding region into amino acids. The resulting sequences were then combined with the *ND2* alignment from Zozaya et al. (2019; Chapter 2) and an additional 241 sequences from the alignment of Moritz et al. (2016), thus representing all major lineages of *H. binoei*, all populations of *H. planiceps* sampled for pheromones, and two individuals of the *H. spelea* group (which were not sampled for pheromones). We used this combined alignment of 661 *ND2* sequences (see Results) for phylogenetic analysis via maximum-likelihood with RAxML version 8.2.11 (Stamatakis 2014). We used the GTRCAT approximation of rate heterogeneity without codon partitions, and performed a rapid bootstrap analysis of 100 replicates for statistical support. This mtDNA phylogeny was used to test lineage membership of individuals sampled for pheromones and to identify any new lineages that were discovered after the exon data was completed (below).

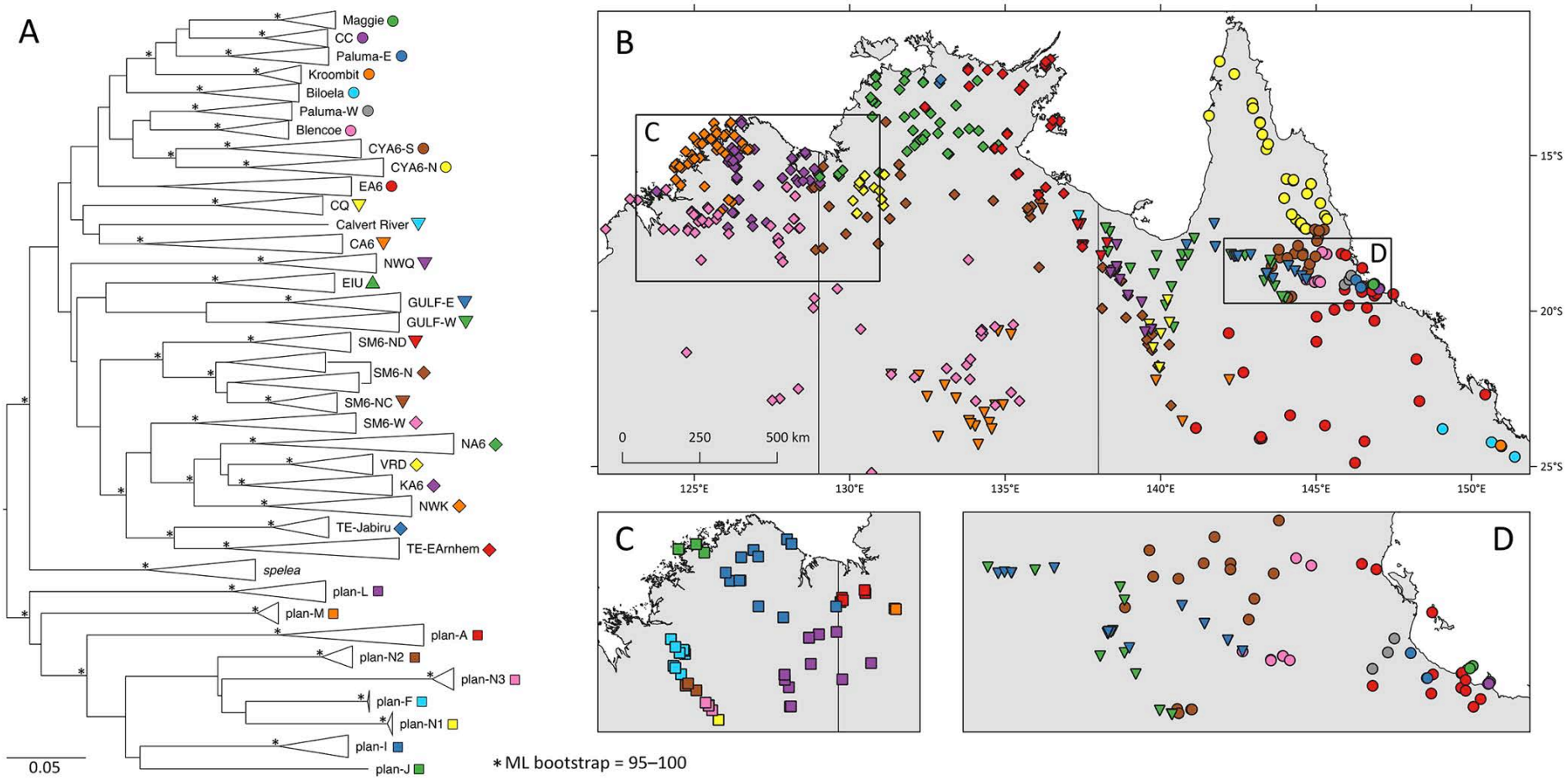

Figure 4.1: Mitochondrial phylogeny and geographic distributions for the *Heteronotia* lineages included in this study. (A) Maximum likelihood phylogeny inferred with RAxML using 1,041 bp *ND2* sequences. Lineages are collapsed for graphical purposes, with coloured symbols corresponding to the adjacent maps. (B) Localities for the respective lineages of *H. binoei* across northern Australia (based on Moritz et al. 2016; Zozaya et al. 2019; this study). (C) Localities for the respective lineages of *H. planiceps*. (D) Zoomed-in view of the Townsville and Einasleigh Uplands regions to better show the distributions of lineages in this area. Note that the SM6-NC lineage is nested within SM6-N for mtDNA (A).

Exon capture, bioinformatics workflow, and species tree inference

We generated exon capture data for 54 individuals representing 33 candidate lineages across four nominal species of *Heteronotia* (1–2 samples per lineage; table S4.1). Tissues for some newly discovered mtDNA lineages (Calvert River, CC, Paluma-W; see Results) were not available at the time, and exon capture failed for the CQ lineage. Exon capture probes were developed from liver transcriptomes from four individuals representing four lineages of *Heteronotia* (3 *H. binoei*; 1 *H. planiceps*; table S4.2). Methods for transcriptome assembly followed Singhal et al. (2013). Following the protocols outlined in Bragg et al. (2016) and Bi et al. (2012), we targeted 4,406 protein-coding exons greater than 200 bp that were identified based on a reciprocal BLAST hit (Altschul et al. 1990) to an exon from the *Anolis* genome (Alfoldi et al. 2011). DNA preparation, preparation of Illumina sequencing libraries, and hybrid enrichment for targeted exons all followed the procedures of Bragg et al. (2016) (similarly followed by Moritz et al. 2018, Ashman et al. 2018, and Oliver et al. 2019).

We processed the raw sequencing reads following the pipeline presented in Bragg et al. (2016). Briefly, this involved using a workflow developed by Singhal (2013) to first remove duplicate, low complexity, and contaminant reads, and then performing adaptor trimming using Trimmomatic (version 0.22; Bolger et al. 2014). We assembled the clean reads for each sample separately using Velvet (K values 31, 41, 51, 61, 71, and 81; version 1.2.08; Zerbino & Birney 2008). Contigs representing the targeted loci were then identified using a BLASTX search against the reference protein sequences the probes were designed from. Where multiple contigs were identified, the most likely contig was selected using a reciprocal best-hit criterion. Introns were then removed using Exonerate (version 2.2.0; Slater & Birney 2005) and sequences that represented less than 65% of the target exon were discarded. We then identified heterozygous sites by mapping the cleaned reads back to the trimmed contigs using BOWTIE 2 (version 2.2.2; Langmead & Salzberg 2012) and then calling variants using GATK (release gb82c674; McKenna et al. 2010). We then performed read-backed phasing using GATK resulting in two predicted haplotype sequences for each contig. The sequences were then collated such that we had a single fasta file per exon containing the relevant sequence for each sample.

Finally, we performed multispecies coalescent phylogenetic analysis using StarBEAST2 (version 0.13.1; Ogilvie et al. 2017) implemented in BEAST2 (Bouckaert et al. 2014). Sequences were aligned using MACSE (Ranwez et al. 2011), which produces alignments in the correct reading frame. We aligned one haplotype per sample and

removed all sequences that were less than 50% of the alignment length. We then selected all exons that were represented in 80% or more of the 54 samples. We then ranked these exons by the number of variable sites, followed by visually checking those with the highest number of variable sites as this can indicate misalignment, contamination, or paralogous sequences. As StarBEAST2 can only handle small datasets, for analysis we selected the 100 exons that are most completely represented across the respective taxa. The StarBEAST2 analysis was run with a partition for each exon, constant populations, and GTR+Γ site model for each exon with four Γ categories and using empirical rate frequencies. We used a strict clock model and a birth death tree prior. We ran two independent instances of the analysis until all ESS values were greater than 200 (just over 2 billion generations) and checked for convergence between the two runs in Tracer (version 1.7; Rambaut et al. 2018). We then built a Maximum Clade Credibility tree using TreeAnnotator with common ancestor node heights and a 10% burn-in.

Combined phylogeny

We could not acquire exon capture data for three deeply divergent mtDNA lineages that were sampled for pheromones: CC, CQ, and Paluma-W (fig. 4.1; table S4.3). In order to include these lineages in our phylogenetic comparative analysis of pheromone divergence, we inserted these three lineages into the StarBEAST2 species tree based on their mtDNA relationships and relative levels of divergence. This was done using the 'bind.tip' function in the R package *phytools* (Revell 2012), followed by forcing the tree to be ultrametric using the 'force.ultrametric' function in the same package.

Pheromone characterisation and divergence

Pheromone characterisation was accomplished via gas chromatography (GC) using the methodology of Zozaya et al. (2019; Chapter 2), but with three differences to improve detection of heavier compounds: first, the inlet temperature was set to 250°C (versus 200°C); second, the oven was held at 325°C for 10 min (versus 8 min); and finally, the flame ionisation detector was set to 325°C (versus 250°C). All other aspects of GC characterisation were identical to Zozaya et al. (2019; Chapter 2). GC chromatograms were integrated manually using Agilent OpenLab software. Chemical peaks that appeared in the chromatograms of control samples were regarded as contaminants and excluded from the integration and analysis of all samples. A total of 29 chemical peaks were consistently

integrated across all GC chromatograms. Relative proportions for each peak were calculated by dividing the area under a peak by the sum of the area under all peaks (total ion current; TIC) for the respective sample. We logit-transformed each peak to account for the unit-sum constraint of proportions (Aitchison 1986; Warton & Hui 2011) and calculated the mean logit-transformed value for each lineage. The mean logit-transformed values were then standardised so that each peak had a mean of 0 and standard deviation of 1 across lineages, which were used for all subsequent phylogenetic comparative analyses.

Based on the results of Chapter 2 (Zozaya et al. 2019) we know that pheromone composition differs more between than within lineages. Nevertheless, because we include many new *Heteronotia* lineages here, we assessed whether pheromone variation is greater between than within lineages using a non-phylogenetic permutational MANOVA (perMANOVA) via the residual randomisation permutational procedure (RRPP; Collyer et al. 2015). All lineages sampled for pheromones were included except for plan-I, for which we had only a single sample. We assessed among-lineage divergence in the data set of 29 standardised logit-transformed pheromone peaks using the 'lm.rrpp' function in the R package *RRPP* (Collyer & Adams 2018), with 10,000 permutations for significance testing.

Phylogenetic signal

Phylogenetic signal is the tendency for traits in closely related taxa (phylogenetic 'tips') to be more similar to each other than to taxa sampled randomly from a phylogeny (Blomberg et al. 2003). We assessed multivariate phylogenetic signal using K_{mult} (Adams 2014), an algebraic generalisation of the *K* statistic (Blomberg et al. 2003). K_{mult} values of 1.0 indicate that trait divergence exactly matches a Brownian motion (BM) model of trait evolution; values less than 1.0 indicate that traits have diverged more than is expected under purely BM evolution; values greater than 1.0 indicate that traits are more similar than expected under purely BM evolution. While there are methods for testing and estimating phylogenetic signal under evolutionary processes other than Brownian motion (e.g., Ornstein-Uhlenbeck, early burst), these are currently unsuitable for analysing highly multivariate data (Adams & Collyer 2018, 2019). K_{mult} values for the multivariate data set of 29 standardised logit-transformed peak proportions were calculated using the 'physignal' function in the R package *geomorph* with 10,000 permutations for significance testing (Adams et al. 2018). To assess the influence of manually adding the CC, CQ, and Paluma-W lineages to the StarBEAST2 tree based on mtDNA relationships, and to compare phylogenetic signal both with and without including lineages of *H. planiceps*, we also

estimated K_{mult} for four subsets of pheromone data: (1) all 33 *Heteronotia* lineages; (2) only those *Heteronotia* lineages represented in the StarBEAST2 tree; (3) all *H. binoei* lineages; and (4) only those *H. binoei* lineages appearing in the StarBEAST2 tree. We did not analyse a subset that included only *H. planiceps* because eight lineages are too few for meaningful statistical analysis (Blomberg et al. 2003). We then visualised phylogenetic structure in among-lineage pheromone variation using phylomorphospace plots of principal component (PC) axes. Principal component analyses (PCA) to produce these plots were performed using the 'rda' function in the R package *vegan* (Oksanen et al. 2019).

Phylogenetic multivariate regression

We assessed the influence of two climate variables on among-lineage pheromone variation: mean annual air temperature (T_{mean}), and mean annual precipitation (P_{mean}). These represent the annual averages from 1970–2000 and were obtained from the Worldclim 2 database (Fick & Hijmans 2017) for every site that pheromones were sampled from (fig. 4.3B). We also obtained aridity index data for Australia simply to visualise climatic variation among sites (Williams et al. 2010). This aridity index represents the ratio of precipitation to potential evaporation, illustrating aspects of both T_{mean} (via its influence on evaporation) and P_{mean}. We did not include aridity in our statistical analysis—or factors such as humidity, evaporation, and elevation—because of high levels of co-linearity with T_{mean} and P_{mean} that would violate the assumptions of our models below.

We tested the influence of T_{mean} and P_{mean} on pheromone blends by performing a multivariate phylogenetic regression via RRPP, which can account for phylogenetic non-independence and is insensitive to the number of trait dimensions (i.e., variables can exceed the number of observations) (Collyer et al. 2015). This was done using the 'lm.rrpp' function in the R package *RRPP* with 10,000 permutations for significance testing (Collyer & Adams 2018). Effect sizes (Z) are calculated from the standard deviate of the respective F distribution. As for our analyses of phylogenetic signal, we repeated the RRPP regressions on the four subsets of pheromone data described above. This was done to compare results with and without *H. planiceps* lineages (because of considerable differences in the pheromone blends between the two species complexes; see Results), and to ensure that the manual addition of the CC, CQ, and Paluma-W lineages did not change the nature of our results. We used the phylogenetic variance-covariance matrix from the respective tree to account for phylogenetic non-independence among observations. The analysis was first run as a type III regression with $T_{mean}*P_{mean}$ as an interaction term. If not significant, the

interaction term was removed with just T_{mean} and P_{mean} as predictor variables (equivalent to a type II regression). Multivariate relationships were visualised by plotting regression scores (Drake & Klingenberg 2008) against the respective climate variable. This regression score is the trait axis associated with the multivariate trait changes predicted by the respective regression model, which also includes residual trait variation in the respective direction of multivariate trait space. Regression scores thus offer a means to visualise the strength of multivariate relationships (see Drake & Klingenberg 2008 for further details).

Results

mtDNA phylogenetics

We obtained 905–1,041 bp *ND2* sequences from 89 new individuals of *H. binoei*. These, combined with an additional 572 *ND2* sequences from Zozaya et al. (2019; Chapter 2) and Moritz et al. (2016), yielded a final alignment of 661 *ND2* sequences. The RAxML analysis of these sequences confirms the lineage identity of geckos and sites sampled for pheromones (table S4.3). There is strong ML bootstrap support for nearly all terminal lineages (i.e., candidate species), consistent with mtDNA phylogenies produced in previous studies (Fujita et al. 2010; Moritz et al. 2016 Zozaya et al. 2019; Chapter 2), although deeper relationships vary and typically have lower support (fig. 4.1A). Our phylogeny, however, has more extensive geographic sampling in north-eastern Australia than do previous studies, and we identify several new deeply divergent genetic lineages. The Blencoe and Paluma-W lineages are two newly discovered sister lineages from north-eastern Australia that together are sister to the lineage containing CYA6-N and CYA6-S (fig. 4.1). A new rock-dwelling lineage from Cape Cleveland (CC lineage) is allied to the Magnetic Island (Maggie) and Paluma-E (as Paluma in Zozaya et al. 2019; Chapter 2) lineages (fig. 4.1A). We also discovered two new lineages from central Queensland: the Kroombit lineage from Kroombit Tops; and the nearby Biloela lineage, found in the vicinity of Biloela and the Blackdown Tableland sandstone plateau (fig. 4.1A–B). Finally, a single sample from the Calvert River region in the Gulf of Carpentaria region of the Northern Territory is identified as a new deeply divergent lineage (Calvert River), allied to the widespread arid zone lineage CA6 (fig. 4.1A–B).

Heteronotia planiceps is revealed as a complex of deeply divergent allopatric populations (fig. 4.1). The phylogenetic relationships and biogeography of this species complex will be more fully dealt with elsewhere (Moritz et al. in prep); however, the important thing here is

that all populations sampled for pheromones come from identified deeply divergent allopatric lineages.

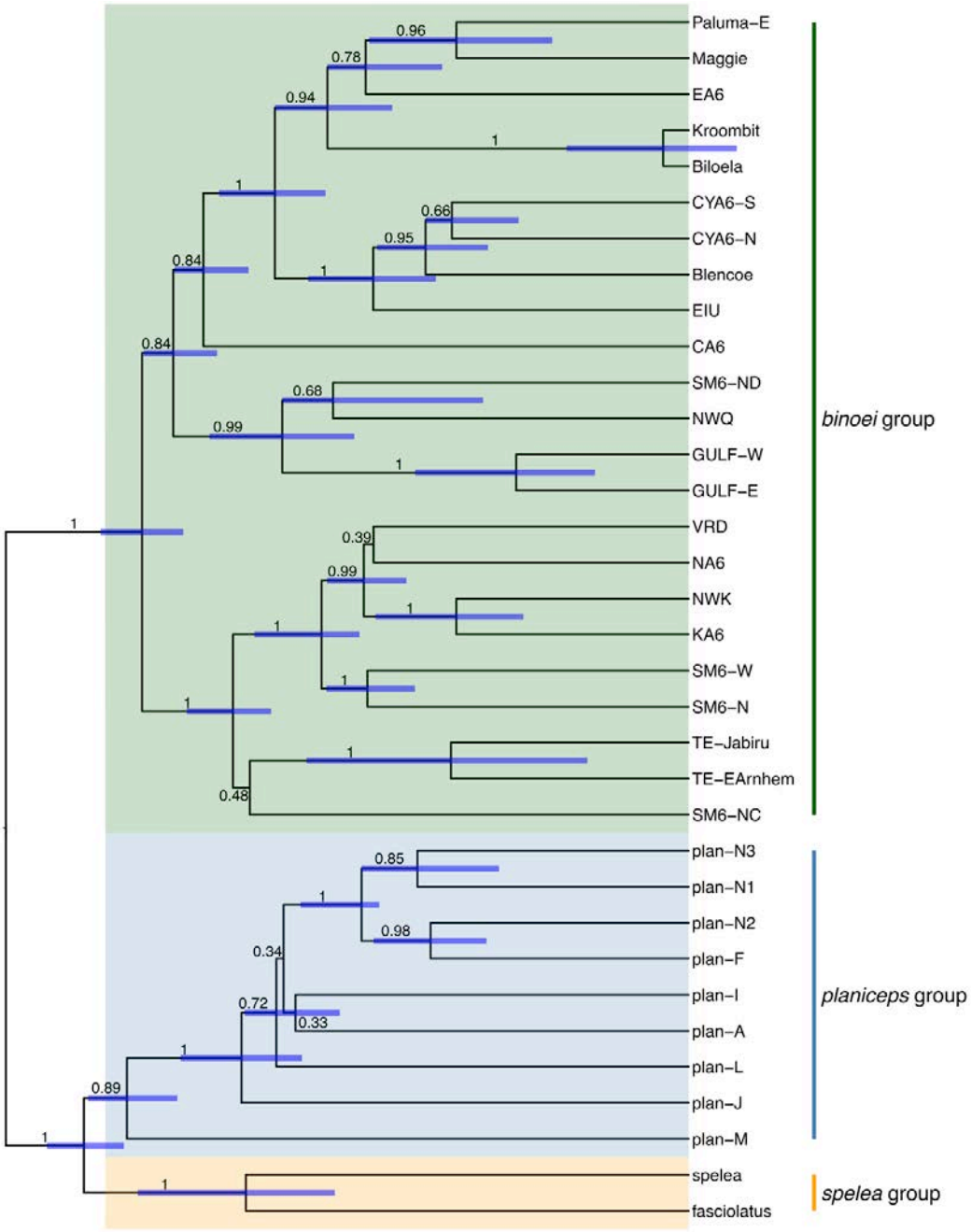

Figure 4.2: *Heteronotia* species tree phylogeny inferred from 100 exon sequences using StarBEAST2. Branch labels are posterior probabilities with node bars showing the 95% highest posterior density (HPD) of node depth (absent for PP<0.5). Major species groups are highlighted and labelled.

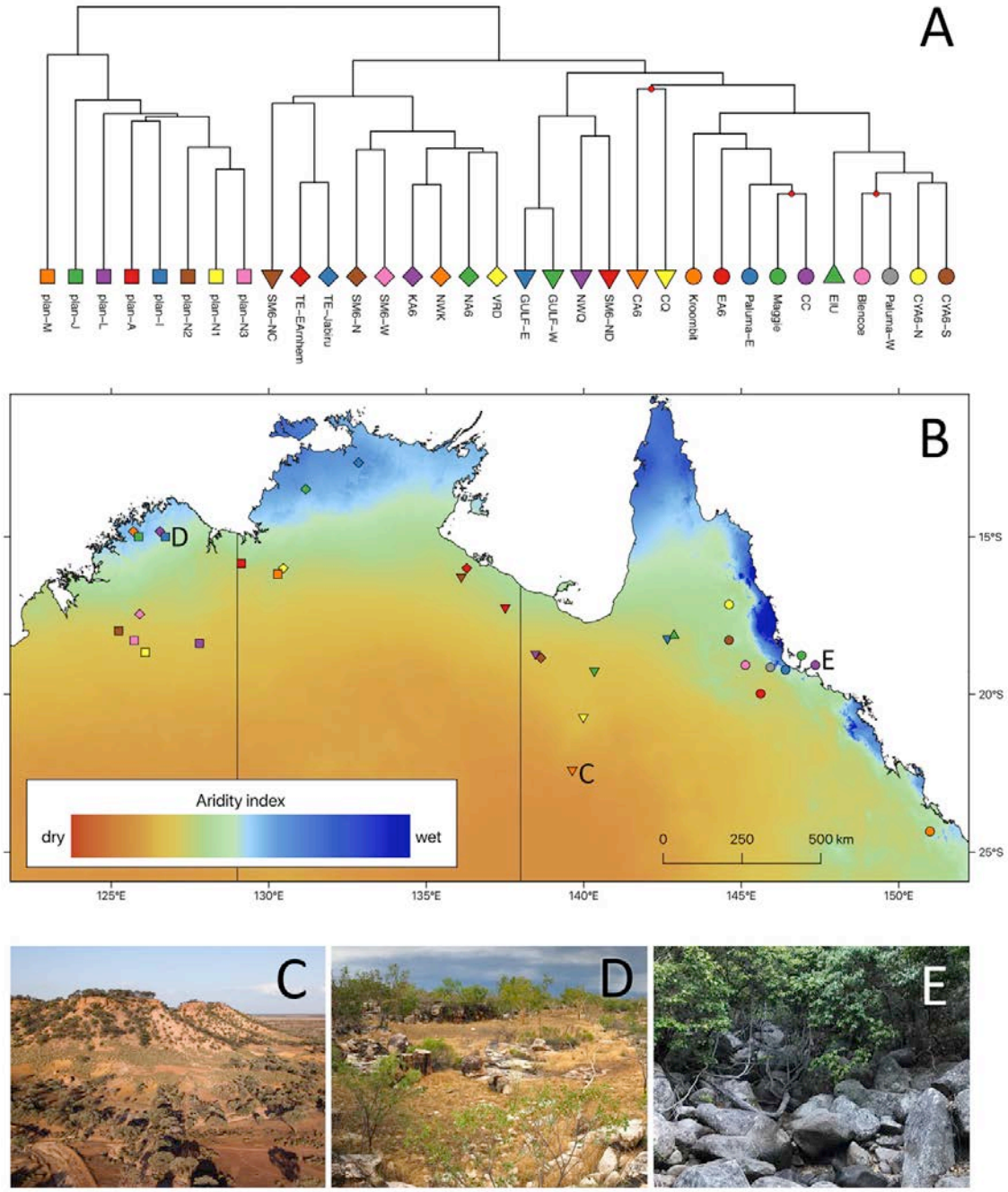

Figure 4.3: *Heteronotia* lineages and localities sampled for pheromones. (A) The combined phylogeny with three additional mtDNA lineages (CC, CQ, Paluma-W) added to the SarBEAST2 species tree. Red dots show nodes created from the addition of these three lineages. (B) Map of sites where lineages were sampled for pheromones, with symbols corresponding to the respective lineages in A. The map background displays aridity to illustrate aspects of both temperature and precipitation. Symbols at sympatric sites are slightly offset to prevent obscuring each other. (C–E) Photos of three sampling sites showing environmental differences along the aridity gradient, with photos corresponding to the respective sites labelled in B.

Species tree phylogenetics

The StarBEAST2 species tree recovers *H. binoei*, *H. planiceps*, and the *H. spelea* group as three strongly supported major clades within *Heteronotia* (fig. 4.2). There is strong support for a sister relationship between the *H. planiceps* group and the *H. spelea* group, which together form a clade occupying the rocky ranges of central and western Australia. Nearly all deeply divergent mtDNA lineages for which we had exon data are well-supported lineages in the StarBEAST2 species tree (fig. 4.2). The exception is the Biloela lineage that—while deeply divergent for *ND2* sequences—is extremely similar to the Kroombit lineage for exon capture data, and we hereafter consider the two as a single lineage ('Kroombit'). With the exception of the *spelea* group (*H. spelea* and *H. fasciolatus*), plan-F, and Biloela, all lineages in this phylogeny were successfully sampled for pheromones.

Combined tree

We inserted the CC, CQ, and Paluma-W lineages (see mtDNA phylogeny, fig. 4.1A) into the StarBEAST2 exon tree based on the relationships and relative levels of divergence observed for these lineages in the RAxML *ND2* phylogeny (fig. 4.3A). CC was placed as sister to the Maggie lineage, CQ was placed as a deeply divergent sister lineage to CA6, and Paluma-W was placed as sister to Blencoe (fig. 4.3A). The exon phylogeny, with these three lineages inserted, forms the basis of all subsequent analysis. However, we also ran all comparative analyses without these lineages to ensure that the strength and direction of our results were not influenced by the insertion of the three lineages.

Pheromone characterisation and divergence

We successfully sampled pheromones from 25 lineages of *H. binoei* and 8 lineages of *H. planiceps* for a total of 33 lineages (all shown in the combined tree; fig. 4.3A). A total of 274 pheromone samples were usable for analysis following GC characterisation and integration (1–13 samples per lineage; mean 8.3; table S4.3). All lineages except plan-I ($N = 1$) are represented by three or more samples. The 29 gas chromatogram peaks integrated across all samples are shown in figure 4.4. These include most of the 25 peaks integrated in Zozaya et al. (2019; Chapter 2), but peak numbers here are not always identical to the peak numbers in Chapter 2 (table S4.4 gives further details and shows how peaks correspond between these chapters).

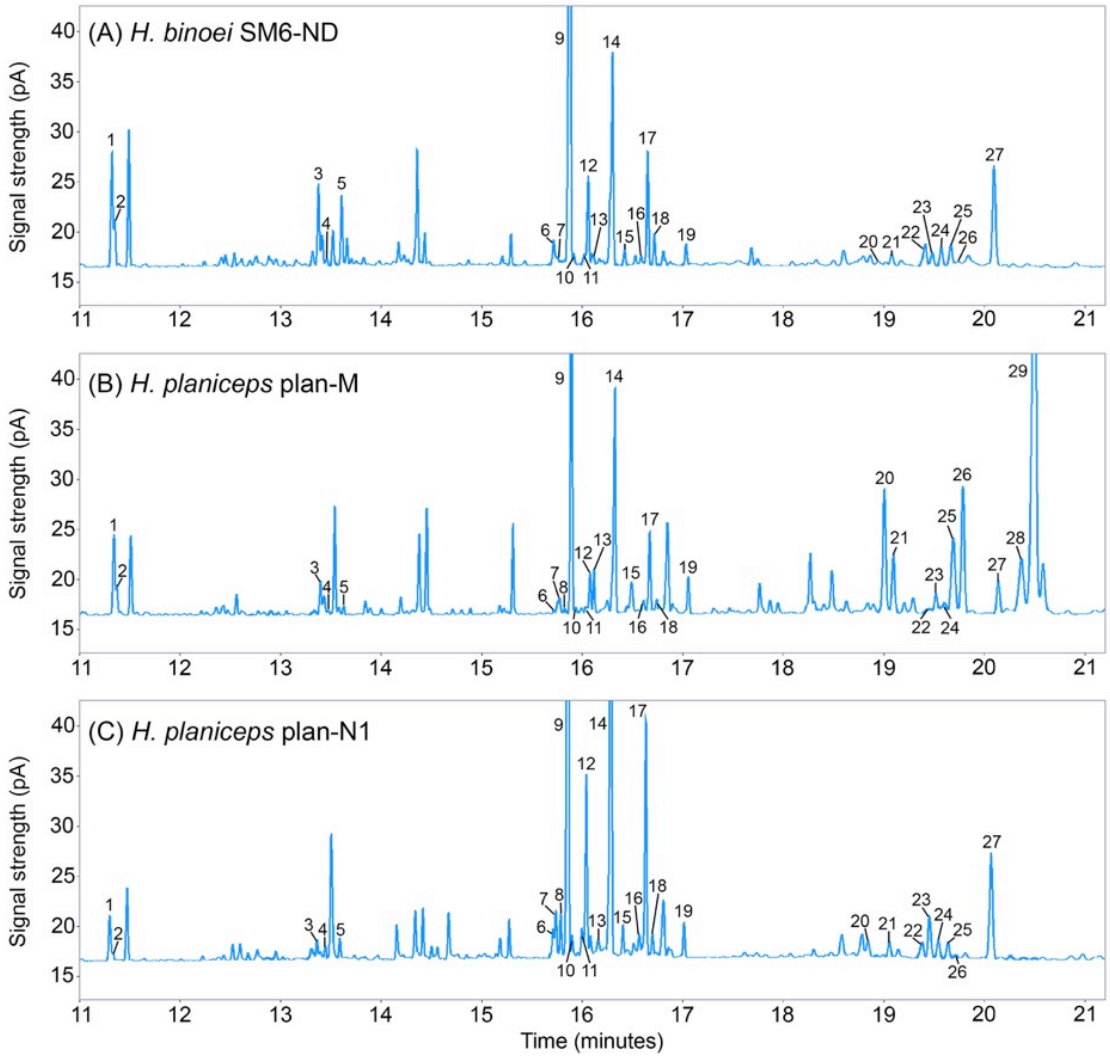

Figure 4.4: Gas chromatograph traces of epidermal pore secretions from three male *Heteronotia* representing (A) *H. binoei* SM6-ND lineage, (B) *H. planiceps* plan-M lineage, and (C) *H. planiceps* plan-N1 lineage. Together these plots show all 29 integrated peaks. Peaks without labels are those identified as possible contaminants or coinciding with contaminants.

We were able to assess whether among-lineage pheromone variation is greater than within-lineage variation for 273 individuals across 32 lineages (plan-I was represented by only a single sample and was thus excluded). Pheromone variation in *Heteronotia* is greater among lineages than it is within lineages (RRPP perMANOVA: $df = 31/241$, $F = 12.70$, $Z = 26.36$, $p < 0.001$). The heatmap in figure 4.5 illustrates among-lineage variation in standardised logit-transformed peak proportions. Visual inspection reveals noticeable differences between the pheromone blends of *H. binoei* and *H. planiceps* lineages. Peaks 20, 21, 25, 26, 28, and 29 occur in high proportions in lineages of *H. planiceps*, with the

exception of the three plan-N lineages, that—although nested well within *H. planiceps*—have very different pheromone blends, with generally higher proportions in the middle peaks (peaks 7, 8, 10, 14, 15, 16, 17, 18) (fig. 4.5).

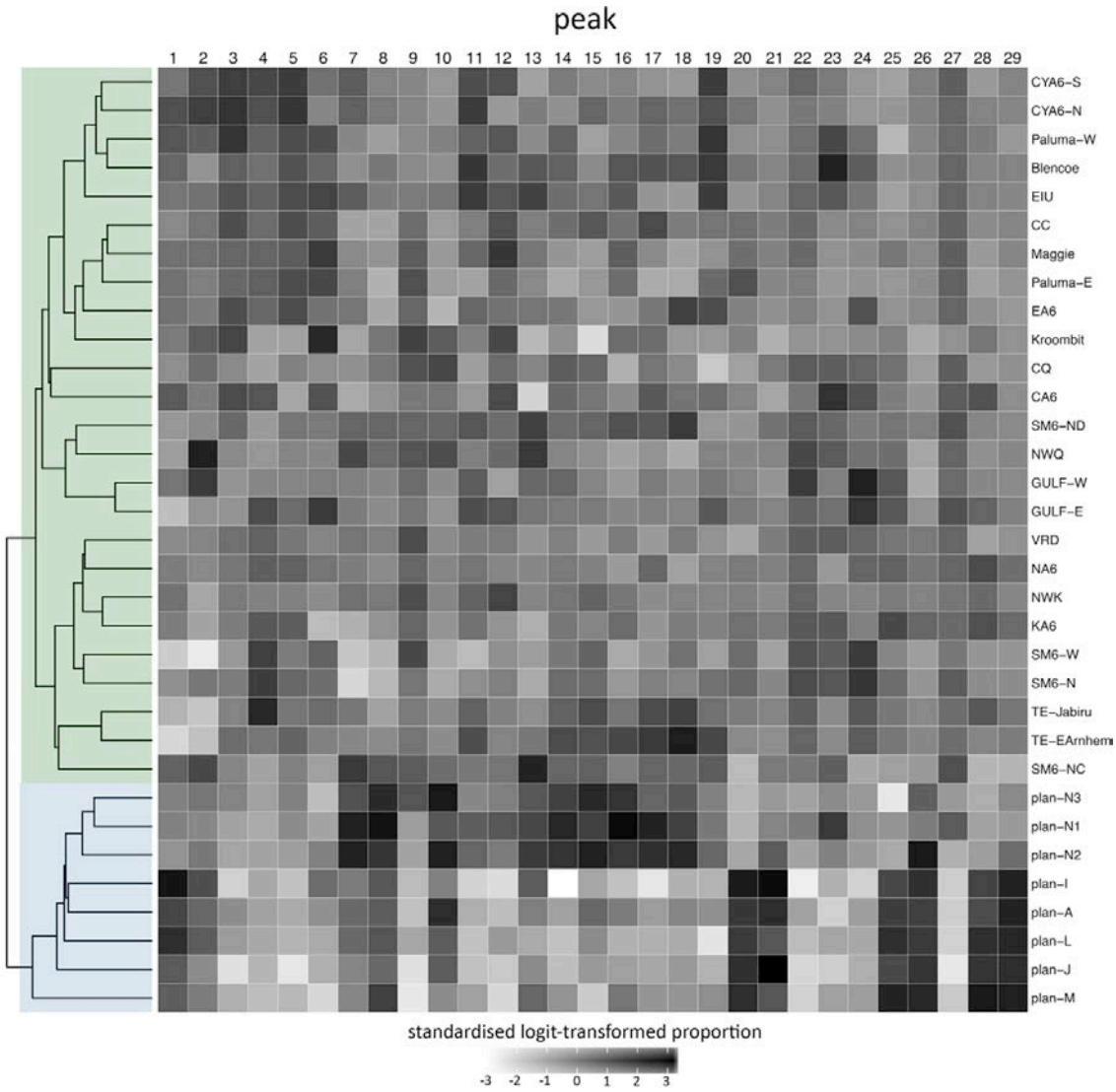

Figure 4.5: Heatmap illustrating among-lineage pheromone variation in *Heteronotia* for the 29 integrated pheromone peaks. Grey-scale shading shows standardised logit-transformed pheromone proportions to illustrate variation for each peak across lineages. Shading on the combined phylogeny (left) highlights the two species complexes: *H. binoei* (green) and *H. planiceps* (blue).

Phylogenetic signal

Pheromone composition shows high levels of phylogenetic signal (all *Heteronotia* lineages: K_{mult} = 1.18, P = 0.001). Because K_{mult} is greater than one, this indicates that pheromones

are more similar among closely related lineages than is expected given a purely BM model of multivariate trait evolution. Phylogenetic signal remains high when the data are restricted to only those lineages represented in the StarBEAST2 phylogeny (StarBEAST2 *Heteronotia* lineages: K_{mult} = 1.16, P = 0.001, versus K_{mult} = 1.18 for all lineages), indicating that the manual addition of the CC, CQ, and Paluma-W lineages based on mtDNA relationships does not strongly influence measures of phylogenetic signal. Signal is lower, however, when *H. planiceps* lineages are excluded from analysis (all *H. binoei* lineages: K_{mult} = 0.93, P = 0.001), including when only those *H. binoei* lineages represented in the StarBEAST2 phylogeny are used (StarBEAST2 *H. binoei* lineages: K_{mult} = 0.93, P = 0.001), indicating that—although phylogenetic signal is still high—pheromone disparity among closely related *H. binoei* lineages is somewhat greater than is expected given a purely BM model of multivariate trait evolution.

Phylomorphospace plots illustrate phylogenetic signal in pheromone divergence (fig. 4.6). Pheromone peak loadings for these PC axes appear in table S4.5. The plot of PC1 and PC2 for all 33 *Heteronotia* lineages (fig. 4.6A) shows that the largest differences in pheromones are associated with just two phylogenetic splits: the split between *H. binoei* and *H. planiceps*, and the split of the clade within *H. planiceps* containing plan-N1, N2, and N3. This is consistent with the major among-clade differences in peak proportions observed in figure. 4.5. Figure 4.6B illustrates that the plan-N lineages stand apart from the rest on PC2, and that PC3 accounts for pheromone variation separating shallower lineages within *Heteronotia*—although the density of points and phylogenetic connections makes it difficult to interpret relationships. Figures 4.6C, D show PC axes for only the *H. binoei* lineages and better illustrate variation and phylogenetic signal within this species complex. Figure 4.6C shows that closely related lineages of *H. binoei* generally have similar pheromone blends (the five lineages from southern Cape York Peninsula clustered in the top of fig. 4.6C offer the clearest example). Three distantly related lineages (CQ, SM6-NC, NWQ; all represented by inverted triangles) are notably divergent from the rest of *H. binoei* and appear on the bottom and bottom-left of the plot. Notably, all three of these lineages occur in the southern Gulf of Carpentaria region (fig. 4.1 & 4.3). Figure 4.6D shows a divide between a monophyletic group of lineages from eastern Queensland on the bottom-right and the remainder of *H. binoei* on the top-left. Similar to the plots including *H. planiceps*, these plots show that the starkest differences in pheromone blends are often associated with just a few phylogenetic splits.

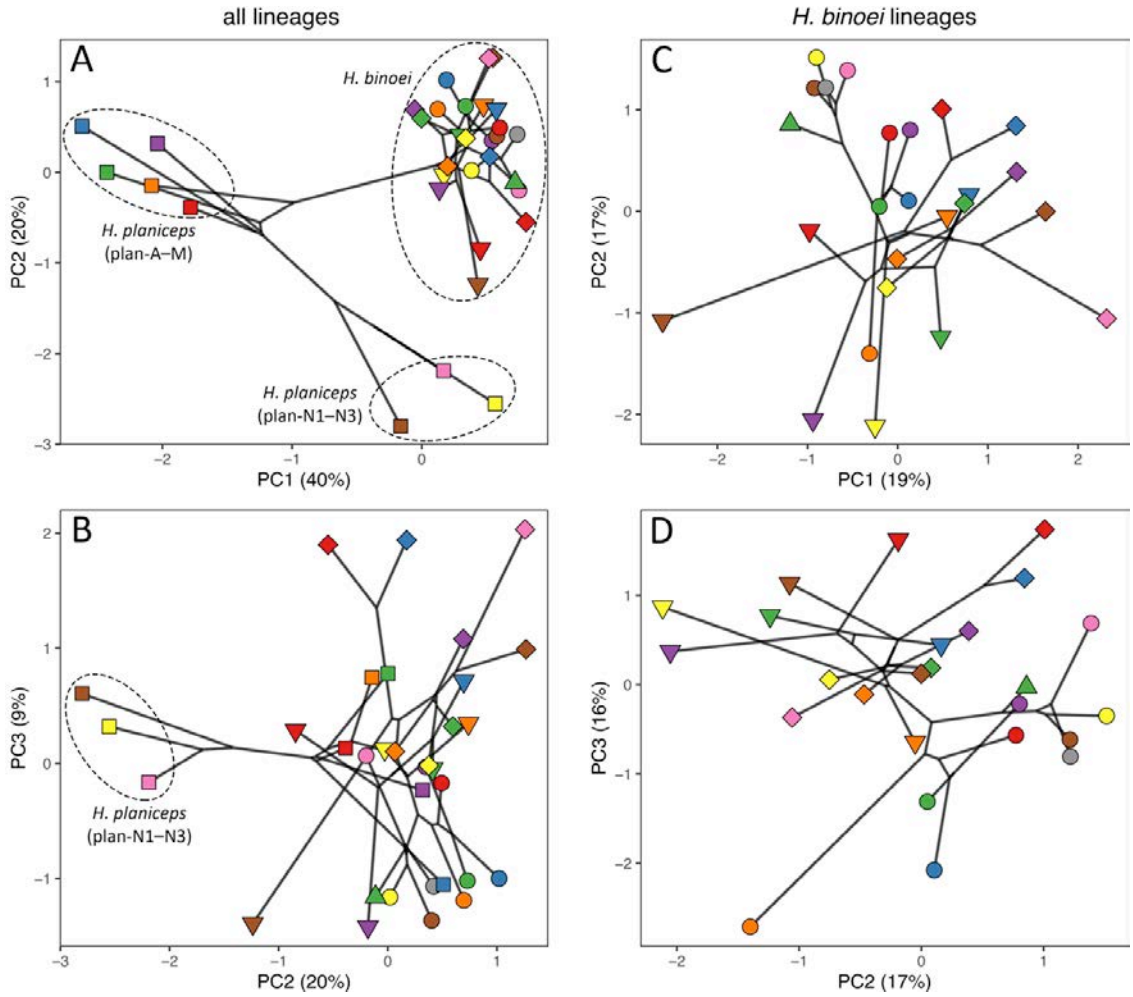

Figure 4.6: Phylomorphospace plots of principal component (PC) axes 1–3 of pheromone variation for all 33 sampled lineages of *Heteronotia* (A–B) and for only the 25 lineages of *H. binoei* (C–D). Panel A illustrates how the largest divergences in pheromone variation are associated with just two phylogenetic splits, which neatly separate the *H. planiceps* complex from the *H. binoei* complex, and then the three plan-N lineages (N1, N2, N3) from the rest of *H. planiceps,* in which they are deeply nested. Dashed ellipses enclose each of these three pheromone groups in panel A.

Climatic influence on pheromones

The interaction of T_{mean} and P_{mean} was not significant in any of the analyses on the four subsets of pheromone data (i.e., all 33 *Heteronotia* lineages; all *H. binoei* lineages; all *Heteronotia* lineages represented in the StarBEAST2 tree; and all *H. binoei* represented in the StarBEAST2 tree; table S4.6). We therefore re-ran all models without the interaction term and report the results of those (table 4.1; fig. 4.7). In the analysis including all 33 *Heteronotia* lineages, T_{mean} was not significant (df = 1/30, F = 1.515, Z = 1.127, R^2 = 0.045, P = 0.131;

fig. 4.7A); however, P_{mean} was significantly correlated with pheromone composition (*df* =1/30, F = 2.438, Z = 2.127, R^2 = 0.072, P = 0.023; fig. 4.7B), accounting for 7.2% of pheromone variation. In contrast, when only the 25 lineages of *H. binoei* were analysed, T_{mean} was significantly correlated with pheromone composition (*df* =1/22, F = 1.824, Z = 1.717, R^2 = 0.072, P = 0.045; fig. 4.7C) and also accounted for 7.2% of pheromone variation. Mean annual precipitation was borderline significant (*df* =1/22, F = 1.650, Z = 1.565, R^2 = 0.065, P = 0.054; fig. 4.7D) and accounted for 6.5% of pheromone variation. Analyses of only those lineages represented in the StarBEAST2 tree yield very similar relationships and R^2 values, although *P*-values vary, indicating that the manual addition of the CC, CQ, and Paluma-W lineages did not skew our results (table S4.7–8).

Table 4.1: Results of the multivariate phylogenetic regression of pheromone composition against T_{mean} and P_{mean} for all 33 *Heteronotia* lineages and for only the 25 lineages of *H. binoei*. Significant *P*-values of 0.05 or less appear in bold.

	df	SS	MS	R^2	F	Z	P
All lineages							
T_{mean}	1	9331	9330.7	0.045	1.515	1.127	0.131
P_{mean}	1	15018	15018.2	0.072	2.438	2.127	**0.023**
Residuals	30	184821	6160.7	0.886			
Total	32	208717					
H. binoei only							
T_{mean}	1	15495	15495	0.072	1.824	1.717	**0.045**
P_{mean}	1	14020	14019.8	0.065	1.650	1.565	**0.054**
Residuals	22	186859	8593.6	0.864			
Total	24	216264					

Coefficient loadings for pheromone peaks for T_{mean} and P_{mean} appear in table 4.2. The peak coefficients that are most strongly influenced by the respective climate variables are identified using the '70% of absolute highest loading and higher' rule-of-thumb (Mardia et al. 1979; e.g., Chapter 2 table S2.3). As T_{mean} increases, peak 15 increases while peak 3 decreases in both the analysis of all 33 lineages and when only *H. binoei* are considered. When *H. planiceps* are included, however, an increase in T_{mean} is also associated with a decrease in peak 6 (although this model is not statistically significant), but when only *H. binoei* lineages are analysed an increase in T_{mean} is also associated with an increase in peak 22. As P_{mean} increases, peak 23 decreases for both the analyses, but there are again differences depending on whether or not lineages of *H. planiceps* are included. When all 33 lineages are included, increasing P_{mean} is also associated with an increase in peak 20 and a decrease in peak 14 (campesterol; table S4.4. But when only *H. binoei* lineages are included, increasing

P_{mean} is associated with increases in peaks 26 and 29. Curiously, these two peaks are present in only small proportions in *H. binoei* but are large in most lineages of *H. planiceps*. Regression score plots illustrate how lineages of *H. planiceps* do not follow the same relationship as *H. binoei* for T_{mean} (fig. 4.7A); and, while the trajectory of the relationship is similar, five of the eight lineages of *H. planiceps* appear to respond somewhat differently to P_{mean} than do the remaining lineages (fig. 4.7B).

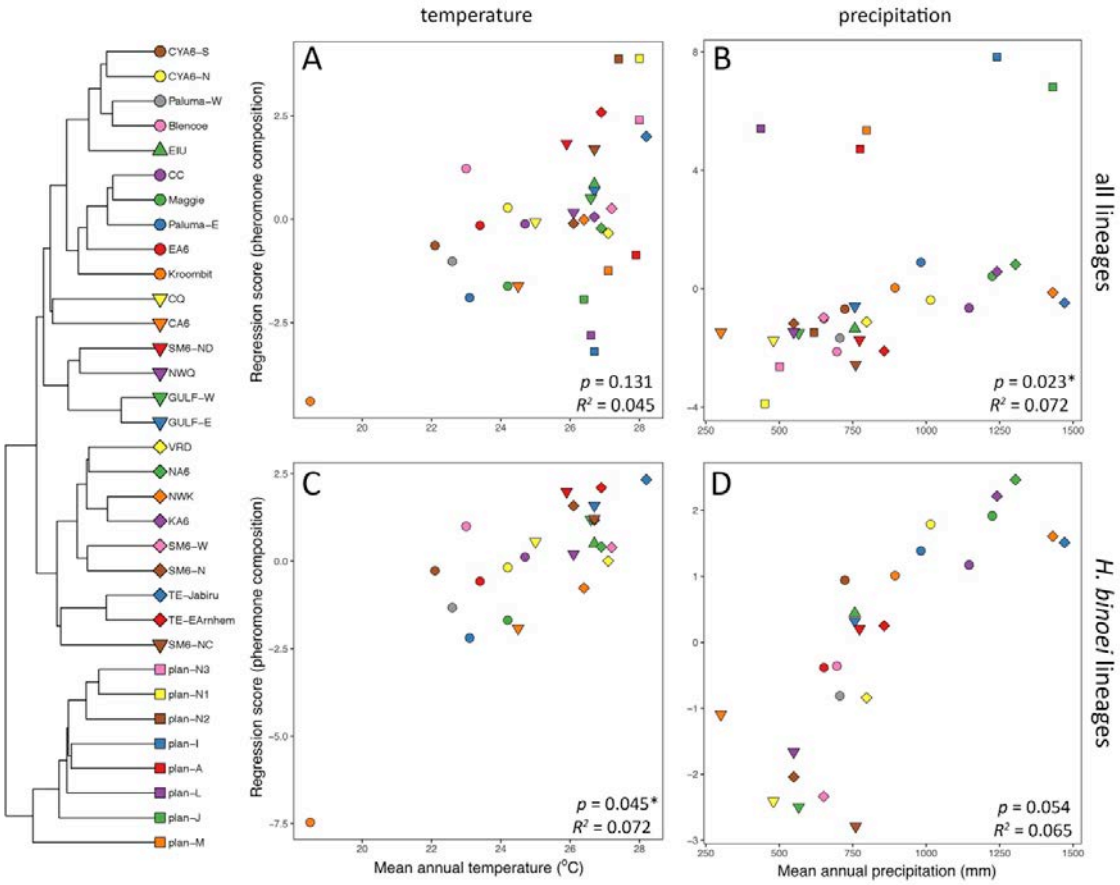

Figure 4.7: Regression scores illustrating multivariate relationships for the phylogenetic regression of pheromone composition against mean annual temperature (A, C) and mean annual precipitation (B, D) for all 33 lineages of *Heteronotia* (A–B) and for only the 25 lineages of *H. binoei* (C–D). Symbols correspond to the phylogeny (left), which represents the combined tree of all lineages sampled for pheromones. Pheromone composition is significantly correlated with temperature among lineages of *H. binoei* (C), but not when lineages of *H. planiceps* are included (A). Pheromone composition is correlated with precipitation among all *Heteronotia* lineages (B) as well as among only *H. binoei* lineages (D); however, pheromones of the two species complexes respond to precipitation in subtly different ways (note squares versus other symbols in B).

Table 4.2: Pheromone peak loadings for T_{mean} and P_{mean} for all 33 *Heteronotia* lineages as well as only the 25 lineages of *H. binoei*. Values that are 70% or more of the highest loading are in bold.

	All lineages		*H. binoei* only	
Peak	T_{mean}	P_{mean}	T_{mean}	P_{mean}
1	-0.170	0.073	-0.499	0.054
2	-0.271	-0.271	-0.316	-0.288
3	**-0.661**	-0.078	**-0.716**	0.107
4	-0.131	0.174	0.205	0.179
5	-0.337	0.049	-0.182	0.271
6	**-0.565**	0.077	-0.484	0.010
7	0.301	-0.182	0.100	0.053
8	0.375	-0.266	0.009	-0.096
9	-0.264	-0.047	-0.002	0.102
10	0.277	-0.312	-0.045	-0.237
11	-0.178	0.002	-0.052	0.194
12	-0.452	-0.127	-0.486	0.120
13	0.351	-0.127	0.297	-0.117
14	0.180	**-0.396**	0.432	-0.313
15	**0.561**	-0.184	**0.698**	0.025
16	0.115	-0.107	-0.188	0.400
17	0.030	-0.215	-0.006	0.059
18	0.231	-0.187	0.235	-0.044
19	-0.256	-0.080	-0.159	-0.070
20	0.022	**0.361**	-0.196	0.340
21	0.326	0.312	0.387	0.200
22	-0.064	-0.132	**0.700**	-0.281
23	-0.101	**-0.480**	0.159	**-0.606**
24	0.039	-0.282	0.461	-0.473
25	0.258	0.200	0.445	0.052
26	0.375	0.149	0.073	**0.536**
27	-0.216	-0.139	0.394	-0.339
28	0.177	0.326	0.110	0.350
29	0.305	0.235	0.168	**0.471**

Discussion

Teasing apart sources of variation is important for understanding the evolution of chemical signalling traits and the function of the constitutive components. Here we used a multivariate phylogenetic comparative approach to assess phylogenetic signal and to test if and how the climatic environment influences pheromone variation among 33 genetically divergent lineages of *Heteronotia* geckos across northern Australia. We constructed a comprehensive phylogeny to assess phylogenetic signal in pheromone divergence, and to perform a multivariate phylogenetic regression of pheromone composition against two key climatic variables: mean annual temperature and mean annual precipitation. This is one of few phylogenetic comparative studies of pheromones (see Symonds & Elgar 2008 for a review of earlier studies; Weber et al. 2016), only the second study to use PCMs to study

how climate shapes pheromone composition (Baeckens et al. 2018), and the first study to employ multivariate phylogenetic regression via RRPP for comparative analysis of a chemical signalling trait.

Pheromone divergence in *Heteronotia* is strongly associated with shared evolutionary history. Assessment of phylogenetic signal indicates that pheromones have diverged somewhat less among closely related lineages than is expected under a Brownian motion model of trait evolution. Phylogenetic signal was lower, however, when only *H. binoei* lineages were analysed—although it was still high overall. But interpreting the biological significance of phylogenetic signal is not straightforward because several processes can produce a particular pattern of phylogenetic signal (Blomberg et al. 2003; Revell et al. 2008), such as varying evolutionary rates through time, developmental constraints, and stabilising and/or directional selection. The phylomorphospace plot in figure 4.6A shows that the greatest divergences in pheromone blends coincide with just two phylogenetic splits: the split of *H. binoei* and *H. planiceps*; and the split of the plan–N lineages (N1, N2, N3) within *H. planiceps*. Although pheromone evolution in these geckos appears to be gradual in general, it is possible that an increase in evolutionary rate associated with these splitting events (e.g., 'saltational shifts'; Baker 2002), followed by more gradual change within the resulting lineages, accounts for some of the phylogenetic signal observed in our study. Ultimately—and as we outline more fully in the following paragraphs—our limited understanding of the role and production of individual compounds makes understanding the significance of phylogenetic signal and the evolutionary processes that drive it difficult. Perhaps measures of phylogenetic signal would differ if the precise compounds influencing behaviour could be identified and isolated for analysis. Regardless of these speculations, our results clearly show that accounting for phylogenetic relationships is crucial when investigating the evolution of pheromone blends among lizards.

Contrary to our results, Baeckens et al. (2018) found low levels of phylogenetic signal in the pheromones of lacertid lizards. However, that study binned individual compounds into chemical classes (e.g., steroids, fatty acids, alcohols), and it was the relative proportions of these chemical classes that were compared and analysed. This is very different to our approach, which treated the proportions of 29 individual compounds as a single multivariate data set, thus precluding a meaningful comparison of these disparate results.

We found that pheromone variation among lineages of *Heteronotia* is correlated with climate, contributing to the limited but compelling evidence indicating that climate influences pheromone composition in lizards (Martín et al. 2015; Baeckens et al. 2018).

Temperature is important for pheromone variation within *H. binoei* but not within *H. planiceps*, whereas precipitation is important across both *H. binoei* and *H. planiceps* (although the nature of the relationship between precipitation and specific compounds within the pheromone blends differs between the two species complexes; fig. 4.7B; table 4.2). Thus, the climatic environment likely contributes to shaping pheromone divergence in *Heteronotia* geckos, providing insight into how these highly multivariate signalling traits have evolved.

Temperature and moisture influence the persistence and detectability of chemical signals. Rainfall can simply wash away secretions deposited onto substrates, and temperature, humidity, and substrate moisture increase the rate that chemicals evaporate (Bossert & Wilson 1963; Regnier & Goodwin 1977; Royer & McNeil 1993; Wilder et al. 2005). This reduces the amount of time that the respective signals persist, but can also increase the ease with which they are detected (Vander Wall 1998). The pheromone-secreting epidermal pores of *Heteronotia* and other lizards are positioned on the ventral surface, where they deposit secretions onto substrates (Mayerl et al. 2015). Our observations of female sniffing behaviour in Chapter 3 indicate that geckos detect secretions only at very close distances (within a few centimetres), as is the case in other lizards (Alberts 1993). Considering these points, it's probably important that these secretions endure in the environment for as long as possible, and thus we should expect pheromones to be adapted to local environmental conditions.

Although the proportion of pheromone variation accounted for by temperature (4.5–7.2%; table 4.1) and precipitation (6.5–7.2%; table 4.1) appears relatively small in our study, this pattern of pheromone variation could represent adaptation to optimise the efficacy of pheromone secretions in different environments. For example, the results of Martín et al. (2015) highlight that even very small differences in pheromone composition significantly affects the persistence of secretions under different environmental conditions. The study found subtle but significant differences in the pheromone compositions of two populations of the European lacertid *Podarcis guadarramae* (as *P. hispanicus*) inhabiting disparate climates: a warm and dry lowland environment, and a cool and humid highland environment. The authors experimentally treated the secretions of male lizards from each population to warm and dry conditions, followed by behavioural assays to assess detectability by females. Exposure to warm, dry conditions reduced the ability of females to detect the secretions from both populations; however, secretions originating from the population inhabiting cool, humid conditions suffered the most severe decrease in detectability.

Our analyses identified several pheromone peaks that are associated with variation in temperature and precipitation (table 4.2). Variation in mean annual temperature influences pheromone variation in *H. binoei* (but apparently not *H. planiceps*), with those lineages from hotter areas having higher proportions of peaks 15 and 22, and lower proportions of peak 3. Variation in mean annual precipitation also influences pheromone variation across all 33 lineages of *Heteronotia* sampled herein, with increasing precipitation being associated with higher proportions of peak 20 and lower proportions of peaks 14 and 23. When only lineages of *H. binoei* are analysed, however, increasing precipitation is still associated with lower proportions of peak 23 but higher proportions of peaks 26 and 29. Curiously, peaks 26 and 29 are present in only small proportions in *H. binoei* but occur very high proportions in five of the eight lineages of *H. planiceps* (fig. 4.5). But while variation in these peaks is associated with climatic factors, the full significance of this variation is unclear because we do not yet know the precise function of specific compounds, or even their chemical identities (although see table S4.4). For example, these compounds might directly influence behaviour (e.g., mate choice; Chapter 3), in which case pheromone divergence via sensory drive could directly influence reproductive isolation among populations. Or these compounds might instead form a matrix of 'keeper substances' that simply enhances the efficacy of the signalling compounds themselves (e.g., Hurst et al. 1998; Hayes et al. 2003). There are dozens of compounds present in the epidermal pore secretions of lizards, but we know the subtle behavioural influences of only a few (Martín & Lopez 2014; Mayerl et al. 2015). Furthermore, these secretions are released via epidermal holocrine glands, the product of which is created by the rupturing of cells in the gland lining (Maderson & Chiu 1970; see Chapter 1). As a consequence, these secretions likely contain a host of compounds originating from those cells, some of which might represent by-products of cellular processes unrelated to chemical signalling. If true, this might also contribute to the high levels of phylogenetic signal we observed in pheromone variation.

Understanding the full significance of our results will depend on acquiring a more thorough understanding of the role of specific compounds, the biochemical pathways that produce them, and the various processes that influence variation. It remains to be tested whether our results reflect adaptation to maximise the efficacy of pheromones under different climatic conditions, but we have identified promising components to guide further study. Determining the chemical identity of these peaks will be crucial to addressing these knowledge gaps and is currently underway (see Chapter 5 for further discussion). Regarding the influence of climate, a promising next step would be to investigate

pheromone variation among climatically disparate populations within one or more of the very widespread *Heteronotia* lineages (e.g., EA6) to see if similar patterns of climate-driven pheromone variation persist among interbreeding populations. This could be combined with behavioural assays to test if and how these specific compounds influence behaviours such as mate choice and male–male interactions, and whether the persistence and detectability of these compounds vary under different climatic conditions. Considering microhabitat might also be very important for understanding pheromone evolution among populations and lineages but is a relatively understudied aspect of sensory drive (Cummings & Endler 2018). In desert-dwelling *Uromastyx* lizards, for example, pheromone secretions are largely comprised of compounds that are not stable in the hot, arid conditions in which they occur (Martín et al. 2016); however, *Uromastyx* spend considerable time in burrows, and pheromone secretions might be adapted to function in these cooler microhabitats. More detailed investigation of microhabitats might help explain why the pheromones of *H. binoei*, which are typically habitat generalists, respond differently to the pheromones of *H. planiceps*, which are exclusively rock-dwelling. Understanding the influence of climate on pheromone composition, and the consequences for behaviour and reproductive isolation, requires more experimental and comparative work.

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Heteronotia planiceps (plan-J lineage) from the Mitchell Plateau, Western Australia.

Chapter 4 – Supplementary material

Table S4.1: Lineage and locality data for the 54 samples from which exon capture sequences were obtained.

Sample ID	Species	Lineage	Latitude	Longitude
ABTC76887	*H. binoei*	Biloela	-24.2233	150.6447
CCM5361	*H. binoei*	Blencoe	-18.0994	145.1941
CCM2782	*H. binoei*	CA6	-20.6463	134.7734
ABTC32337	*H. binoei*	CA6	-25.2833	116.2000
CCM5347	*H. binoei*	CYA6-N	-15.9090	144.8384
CCM5332	*H. binoei*	CYA6-N	-14.6260	143.4582
CCM0119	*H. binoei*	CYA6-S	-19.5324	144.0649
CCM5188	*H. binoei*	CYA6-S	-18.2741	143.8369
conx5360	*H. binoei*	EA6	-19.2570	146.8044
ABTC12125	*H. binoei*	EA6	-29.8167	138.1667
CCM5171	*H. binoei*	EIU	-18.8015	143.4363
CCM5099	*H. binoei*	GULF-E	-17.9346	141.7589
CCM5177	*H. binoei*	GULF-E	-18.9533	143.6097
CCM5107	*H. binoei*	GULF-W	-19.8225	140.1627
CCM5140	*H. binoei*	GULF-W	-18.1901	140.6907
CCM3454	*H. binoei*	KA6	-15.0562	126.4369
ABTC76941	*H. binoei*	Kroombit	-24.3258	150.9403
CCM0050	*H. binoei*	Maggie	-19.1313	146.8695
CCM2300	*H. binoei*	NA6	-13.9438	132.9037
TE005/Gko774	*H. binoei*	NA6	-12.3538	131.8102
CCM0246	*H. binoei*	NWK	-19.7214	139.3919
CCM1221	*H. binoei*	NWK	-14.8211	125.7211
CCM0246	*H. binoei*	NWQ	-19.7214	139.3919
conx5641	*H. binoei*	Paluma-E	-19.2390	146.4361
CCM1837	*H. binoei*	SM6-N	-15.6236	131.6334
CCM0121	*H. binoei*	SM6-N	-20.9220	139.5565
CCM2654	*H. binoei*	SM6-NC	-16.2743	136.0816
CCM0370	*H. binoei*	SM6-ND	-17.1979	137.4343
WAMR170596	*H. binoei*	SM6-W	-21.8983	115.7040
ABTC31254	*H. binoei*	SM6-W	-25.6570	125.5560
ABTC31236	*H. binoei*	TE-EArnhem	-15.6167	135.3500
ABTC11135	*H. binoei*	TE-Jabiru	-12.5500	132.9300
CCM0546	*H. binoei*	VRD	-15.6062	131.0800
CMNT059	*H. binoei*	VRD	-16.0503	130.4021
CCM1898	*H. fasciolatus*	*fasciolatus*	-23.7253	133.3432
ABTC93248	*H. planiceps*	plan-A	-15.8753	129.0513
CCM3065	*H. planiceps*	plan-A	-15.8336	129.1102
D77012	*H. planiceps*	plan-F	-17.4825	125.0290
D77052	*H. planiceps*	plan-F	-16.7812	124.9206
R172838	*H. planiceps*	plan-I	-15.9839	127.0539
R164964	*H. planiceps*	plan-I	-14.7666	127.0475
R168898	*H. planiceps*	plan-J	-14.5867	125.1033
R167811	*H. planiceps*	plan-J	-14.6733	125.7322
CCM1823	*H. planiceps*	plan-L	-18.4213	127.8455
CCM3032	*H. planiceps*	plan-L	-18.4252	127.8197
CCM3000	*H. planiceps*	plan-M	-16.0503	130.4021
CCM0246	*H. planiceps*	plan-M	-19.7214	139.3919
D77034	*H. planiceps*	plan-N1	-18.7510	126.0800
PMO228	*H. planiceps*	plan-N1	-18.7510	126.0800
PMO219	*H. planiceps*	plan-N2	-17.9145	125.2816
Z29024	*H. planiceps*	plan-N2	-17.9125	125.2827
CCM3332	*H. planiceps*	plan-N3	-18.5149	125.9256
WAMR135010	*H. spelea*	*spelea*	-23.3861	119.6294
WAMR160145	*H. spelea*	*spelea*	-21.3219	121.0020

Table S4.2: Exon capture probes were developed from the transcriptomes of the following four individuals (Moritz et al. unpublished).

Sample ID	Species	Lineage	Locality	Latitude	Longitude
CMWA02	*H. binoei*	NWK	Mt Elizabeth Stn. (dump)	-16.4202	126.1043
CMNT159	*H. binoei*	VRD	Victoria River Research Stn.	-16.1265	130.9536
CMNT160	*H. binoei*	SM6-N	Victoria River Research Stn.	-16.1265	130.9536
CMNT48	*H. planiceps*	Plan-A	Bullo River	-15.6576	129.6593

Table S4.3: The number of pheromone samples successfully characterised for each lineage (*N*) and their respective sampling localities. The last column (right) shows the studies that sequenced individuals from each locality.

Lineage	*N*	Locality	Latitude	Longitude	mtDNA sequenced
Blencoe	13	Greenvale	145.070	-19.028	this study
CA6	12	70 km N. Boulia	139.663	-22.412	this study
CC	9	Cape Cleveland	147.026	-19.291	this study
CQ	7	Mary Kathleen	140.008	-20.737	this study
CYA6-N	8	Chillagoe	144.516	-17.164	Chapter 2, Moritz et al. 2016
CYA6-S	8	Mt Surprise	144.571	-18.206	Chapter 2, Moritz et al. 2016
EA6	9	Toomba Stn	145.591	-19.958	this study, Moritz et al. 2016
EIU	7	Croydon	142.614	-18.270	Chapter 2
GULF-E	10	Croydon	142.614	-18.270	Chapter 2, Moritz et al. 2016
GULF-W	11	Four-ways	140.348	-19.227	Chapter 2, Moritz et al. 2016
KA6	11	Theda Stn	126.520	-14.785	this study, Moritz et al. 2016
Kroombit	3	Kroombit Tops	150.953	-24.343	this study
Maggie	9	Magnetic Island	146.845	-19.143	Chapter 2
NA6	11	Adelaide River	131.186	-13.495	this study, Moritz et al. 2016
NWK	6	Mitchell River NP	125.716	-14.823	this study, Moritz et al. 2016
NWQ	10	Lawn Hill NP	138.481	-18.704	this study, Moritz et al. 2016
Paluma-E	9	Bluewater Range	146.444	-19.240	Chapter 2
Paluma-W	7	Zigzag Stn	145.919	-19.150	this study
SM6-N	7	Lawn Hill NP	138.481	-18.704	Chapter 2, Moritz et al. 2016
SM6-NC	8	Caranbirini	136.082	-16.275	this study, Moritz et al. 2016
SM6-ND	10	Calvert Hills	137.435	-17.197	this study, Moritz et al. 2016
SM6-W	10	Mornington	126.110	-17.512	this study, Moritz et al. 2016
TE-EArnhem	11	Boroloola	136.303	-16.020	this study, Moritz et al. 2016
TE-Jabiru	9	Jabiru	132.852	-12.654	this study, Moritz et al. 2016
VRD	10	Gregory NP	130.471	-15.993	this study, Moritz et al. 2016
plan-A	8	Keep River NP	129.050	-15.872	C. Moritz et al. in prep.
plan-I	1	Theda Stn	126.520	-14.785	C. Moritz et al. in prep.
plan-J	6	Mitchell River NP	125.716	-14.823	C. Moritz et al. in prep.
plan-L	6	Sawpit Gorge	127.821	-18.425	C. Moritz et al. in prep.
plan-M	10	Gregory NP	130.471	-15.993	C. Moritz et al. in prep.
plan-N1	6	Ngumban Cliffs	126.081	-18.750	C. Moritz et al. in prep.
plan-N2	7	Oscar Range	125.302	-17.916	C. Moritz et al. in prep.

Table S4.4: This table shows how the 29 gas chromatogram peaks integrated in this study (first column) correspond to the 25 peaks integrated in Chapter 2 (second column). Each row represents a different compound, with the numbers simply showing the peak number that represents that compound in each of the two chapters. For example, peak 1 is the same compound in both studies, but peak 9 in this study is the compound represented as peak 11 in Chapter 2. Integrated peaks differ between these studies for two reasons: first, this study includes more peaks, largely because of the presence of peaks in *Heteronotia planiceps* (not included in Chapter 2) that are present in only miniscule proportions in *H. binoei*; second, those compounds that are peaks 3 and 6–8 in Chapter 2 are excluded in this study because of possible contaminants in some samples that coincide with these peaks (rows highlighted in red). Chemical identities for those compounds that are known are shown in the third column.

Peak number		Chemical ID
This study	Chapter 2	
1	1	
2	2	
3	-	
-	3	
4	4	
5	5	
-	6	
-	7	
-	8	
6	9	9,12,15-Octadecatrienoic acid
7	10	
8	-	
9	11	Cholesterol
10	12	
11	-	
12	13	Stigmasterol
13		
14	15	Campesterol
15	16	
16	-	
17	17	β-Sitosterol
18	18	
19	20	cholest-5-en-7-one
20	-	
21	-	
22	-	
23	21	
24	22	
25	23	
26	-	
27	24	
28	25	
29	-	

Table S4.5: Pheromone peak loadings for PC axes 1–3 for the PCA of all 33 *Heteronotia* lineages and for the PCA of only the 25 lineages of *H. binoei*. Values that are 70% or more of the highest loading are in bold.

	All lineages			*H. binoei* lineages only		
Peak	PC1 (40%)	PC2 (20%)	PC3 (9%)	PC1 (19%)	PC2 (17%)	PC3 (16%)
1	**0.731**	0.229	-0.129	-0.507	0.191	-0.394
2	-0.415	**-0.856**	-0.141	**-0.622**	-0.224	-0.194
3	-0.034	**-0.828**	-0.360	-0.383	**0.568**	-0.480
4	0.457	0.477	-0.253	**0.571**	**0.548**	-0.084
5	**0.785**	0.220	-0.205	-0.149	**0.734**	-0.080
6	0.604	0.570	0.234	0.062	0.140	**-0.606**
7	**0.690**	0.261	-0.386	**-0.745**	-0.016	0.225
8	**-0.956**	0.031	0.160	**-0.713**	-0.303	0.321
9	**-0.754**	0.290	0.278	0.091	**-0.749**	-0.257
10	**0.928**	0.158	-0.095	-0.460	**-0.562**	0.154
11	**-0.814**	-0.370	0.141	-0.388	**0.579**	0.291
12	**-0.700**	0.380	0.290	-0.303	0.017	-0.460
13	0.606	0.380	0.458	**-0.608**	-0.104	0.444
14	**0.682**	-0.071	0.008	0.053	0.097	**0.704**
15	**0.850**	0.251	0.119	0.168	0.164	**0.681**
16	**-0.893**	-0.123	0.021	-0.245	0.468	0.066
17	**-0.821**	0.470	0.097	-0.060	0.292	0.407
18	-0.189	-0.083	**-0.834**	0.067	0.335	**0.546**
19	0.648	-0.082	-0.150	-0.287	**0.733**	0.021
20	0.432	**-0.599**	0.497	**0.595**	0.265	-0.397
21	0.595	-0.580	0.291	-0.280	0.100	**0.513**
22	0.362	**-0.726**	0.093	0.339	-0.293	**0.550**
23	0.327	**-0.660**	0.377	-0.087	-0.010	0.233
24	**0.717**	-0.581	0.310	**0.615**	-0.125	0.292
25	0.038	**-0.698**	-0.260	0.517	-0.376	0.331
26	**0.779**	0.023	-0.283	0.054	**0.592**	0.155
27	**0.736**	-0.221	-0.114	-0.276	0.117	0.415
28	-0.307	**-0.738**	-0.113	0.441	0.218	0.083
29	-0.647	0.059	-0.559	0.457	0.314	0.209

Table S4.6: Results of multivariate phylogenetic regressions via RRPP that included the interaction of T_{mean} and P_{mean} for the four subsets of pheromone data.

	df	SS	MS	R^2	*F*	*Z*	*P*
All 33 lineages							
T_{mean}	1	7738	7738.2	0.03708	1.2615	0.70037	0.2272
P_{mean}	1	6569	6568.7	0.03147	1.0708	0.34863	0.3448
T_{mean}*P_{mean}	1	6931	6931.5	0.03321	1.13	0.46503	0.3052
Residuals	29	177890	6134.1	0.8523			
Total	32	208717					
All 25 *H. binoei* lineages							
T_{mean}	1	7304	7304.2	0.03377	0.8609	-0.25268	0.6052
P_{mean}	1	8710	8710.4	0.04028	1.0266	0.22246	0.4235
T_{mean}*P_{mean}	1	8689	8689	0.04018	1.0241	0.21545	0.4257
Residuals	21	178170	8484.3	0.82385			
Total	24	216264					
All lineages in StarBEAST2 tree							
T_{mean}	1	6892	6892.2	0.03664	1.1212	0.45994	0.3142
P_{mean}	1	6290	6290.4	0.03344	1.0233	0.26894	0.3855
T_{mean}*P_{mean}	1	6614	6614	0.03516	1.0759	0.37615	0.3464
Residuals	26	159827	6147.2	0.84965			
Total	29	188110					
H. binoei lineages in StarBEAST2 tree							
T_{mean}	1	6767	6767.1	0.03619	0.7946	-0.42735	0.6694
P_{mean}	1	8610	8609.7	0.04604	1.011	0.1913	0.4286
T_{mean}*P_{mean}	1	8555	8554.8	0.04575	1.0045	0.17475	0.4347
Residuals	18	153292	8516.2	0.8198			
Total	21	186988					

Table S4.7: Results of multivariate phylogenetic regressions via RRPP of only those lineages represented in the StarBEAST2 species tree (excluding the T_{mean}*P_{mean} interaction).

	df	SS	MS	R^2	*F*	*Z*	*P*
All lineages							
T_{mean}	1	7635	7635.2	0.04059	1.2386	0.68314	0.2411
P_{mean}	1	14337	14337.4	0.07622	2.3258	1.96481	0.0335
Residuals	27	166441	6164.5	0.88481			
Total	29	188110					
H. binoei only							
T_{mean}	1	13008	13008	0.06957	1.5271	1.2558	0.1049
P_{mean}	1	12395	12395.1	0.06629	1.4551	1.2086	0.1126
Residuals	19	161846	8518.2	0.86555			
Total	21	186988					

Table S4.8: Pheromone peak loadings for T_{mean} and P_{mean} for the analyses of all four subsets of pheromone data (excluding the T_{mean} * P_{mean} interaction). Values that are 70% or more of the highest loading are in bold.

	All lineages		All *H. binoei*		All StarBEAST2 lineages		*H. binoei* StarBEAST2 lineages	
Peak	T_{mean}	P_{mean}	T_{mean}	P_{mean}	T_{mean}	P_{mean}	T_{mean}	P_{mean}
1	-0.170	0.073	-0.499	0.054	-0.149	0.079	-0.467	0.062
2	-0.271	-0.271	-0.316	-0.288	-0.254	-0.273	-0.298	-0.293
3	**-0.661**	-0.078	**-0.716**	0.107	**-0.636**	-0.129	**-0.708**	0.037
4	-0.131	0.174	0.205	0.179	-0.116	0.141	0.241	0.113
5	-0.337	0.049	-0.182	0.271	-0.290	0.018	-0.122	0.223
6	**-0.565**	0.077	-0.484	0.010	**-0.547**	0.046	-0.463	-0.057
7	0.301	-0.182	0.100	0.053	0.274	-0.182	0.079	0.076
8	0.375	-0.266	0.009	-0.096	0.336	-0.255	-0.054	-0.024
9	-0.264	-0.047	-0.002	0.102	-0.287	-0.033	-0.099	0.166
10	0.277	-0.312	-0.045	-0.237	0.284	-0.260	-0.068	-0.097
11	-0.178	0.002	-0.052	0.194	-0.174	-0.021	-0.044	0.163
12	-0.452	-0.127	-0.486	0.120	-0.421	-0.154	-0.458	0.091
13	0.351	-0.127	0.297	-0.117	0.337	-0.140	0.291	-0.136
14	0.180	**-0.396**	0.432	-0.313	0.225	**-0.389**	**0.524**	-0.274
15	**0.561**	-0.184	**0.698**	0.025	**0.572**	-0.211	**0.735**	0.003
16	0.115	-0.107	-0.188	0.400	0.091	-0.170	-0.215	0.328
17	0.030	-0.215	-0.006	0.059	0.065	-0.258	0.039	0.003
18	0.231	-0.187	0.235	-0.044	0.227	-0.204	0.238	-0.066
19	-0.256	-0.080	-0.159	-0.070	-0.225	-0.151	-0.093	-0.210
20	0.022	**0.361**	-0.196	0.340	-0.004	**0.335**	-0.224	0.254
21	0.326	0.312	0.387	0.200	0.319	**0.327**	0.408	0.246
22	-0.064	-0.132	**0.700**	-0.281	-0.044	-0.122	**0.697**	-0.262
23	-0.101	**-0.480**	0.159	**-0.606**	-0.054	**-0.465**	0.216	**-0.592**
24	0.039	-0.282	0.461	-0.473	0.049	-0.265	0.459	**-0.464**
25	0.258	0.200	0.445	0.052	0.199	0.228	0.382	0.102
26	0.375	0.149	0.073	**0.536**	0.368	0.135	0.114	**0.511**
27	-0.216	-0.139	0.394	-0.339	-0.196	-0.138	0.393	-0.338
28	0.177	0.326	0.110	0.350	0.168	**0.331**	0.120	0.352
29	0.305	0.235	0.168	**0.471**	0.276	0.233	0.132	**0.465**

CHAPTER 5 — Summary and synthesis

Summary

The first aim of this study was to determine whether pheromones function as a mating trait influencing reproductive isolation in the *Heteronotia binoei* cryptic species complex. Once that question was addressed, the second aim was to begin teasing apart the factors shaping pheromone variation. To address these aims I focussed on answering three research questions, each of which corresponds to one of my data chapters. I used both a comparative and experimental approach to answer these questions, integrating genetics, analytical chemistry, morphometrics, behavioural assays, and phylogenetic comparative methods. Below I summarise the core findings from each of my three data chapters.

Pheromones have diverged more than morphology among lineages of Heteronotia

In Chapter 2 I tested whether pheromone blends have diverged among divergent genetic lineages (candidate species) of *H. binoei*, and then tested whether pheromones have diverged more than morphology. Using pheromone samples from 128 individual geckos representing 10 genetically divergent lineages of *H. binoei*, I showed that pheromone blends have diverged among all but two lineages (CYA6-N and CYA6-S), and that, with the exception of these two, there is limited overlap in pheromone composition among co-occurring lineages. Morphology has diverged among some but not all lineages, and is largely associated with body size, but there is often considerable overlap in morphological variation even among co-occurring lineages. Using estimates of pairwise trait overlap and pairwise trait distance to directly compare levels of trait divergence among lineages, I then showed that pheromone blends have indeed diverged significantly more than morphology in this cryptic species complex. My results suggest that pheromones have been important in the diversification of this morphologically conservative lizard group and warrant further investigation to test their role as a mating trait. Furthermore, because pheromones are more divergent than morphology, pheromones may be a useful character to integrate into taxonomic studies.

Divergent male pheromones influence female discrimination of sympatric lineages

Chapter 3 followed on to test whether divergent male pheromones actually influence the ability of female geckos to discriminate among males of different lineages—an important step to assess whether pheromones are a mating trait influencing reproductive isolation. To accomplish this, I captured wild adult geckos from three divergent lineages (CYA6-S, EIU, GULF-E) that co-occur in a mosaic of sympatry and parapatry in north-eastern Australia. Using experiments where I simultaneously presented females with a cotton swab scented by the epidermal pores of a conspecific and an unscented control swab, I showed that female geckos detect male pheromones and sniff pheromone secretions significantly longer than unscented controls. Female geckos, however, did not tend to visit one swab treatment first more often than the other, and sniffing behaviour commenced only at very short distances, suggesting that volatile (airborne) compounds from male pheromone secretions are not influencing female behaviour, at lest not at distances of more than a few centimetres.

I then used similar experiments to determine whether female geckos can discriminate between the pheromone secretions of sympatric conspecific and heterospecific males. Although these experiments did show that females tend to sniff conspecific male pheromones longer, there was a significant interaction between treatment and the order females encountered a treatment. This indicated the presence of a significant novelty effect, whereby females tend to sniff whichever treatment they encounter first longer than they otherwise would.

In an attempt to reduce the influence of novelty, I repeated experiments one year later using a sequential choice design. These experiments were similar to the previous but, instead of having scent treatments presented to females simultaneously, treatments were presented to females individually and on separate nights. This experimental design still showed a significant, albeit weaker, influence of novelty; however, this appeared to be driven largely by the CYA6-S lineage. Swab treatment was the only significant effect for both the EIU and GULF-E lineages, which both tended to sniff conspecific scented swabs 2.6 times longer than heterospecific scented swabs.

That female geckos discriminate among lineages using male pheromones and tend to spend longer sniffing the pheromones of conspecific over heterospecific males indicates that pheromones are a mating trait influencing reproductive isolation in the *H. binoei* cryptic species complex. These results also highlight the need to account for novelty when designing and analysing behavioural experiments such as these.

Pheromone divergence is associated with shared evolutionary history and climate

Having established the role of pheromones as a mating trait, in Chapter 4 I began teasing apart the factors shaping pheromone divergence, focussing on the role of shared evolutionary history and climate. I used a phylogenetic comparative approach to accomplish this, sampling pheromones from 25 lineages of the *H. binoei* complex as well as 8 lineages of the *H. planiceps* complex, for a total of 33 divergent lineages across northern Australia. The phylogeny of these lineages was reconstructed using exon capture sequencing and multi-species coalescent phylogenetic inference, supplemented with mtDNA sequence data for three lineages lacking exon data. Using these pheromone and phylogenetic data I showed that there is high phylogenetic signal in pheromone divergence, indicating that pheromones have evolved gradually in a manner similar to Brownian motion, with more closely related lineages being more similar to each other—although there is evidence of 'leaps' in pheromone composition associated with two phylogenetic splits: the split between *H. binoei* and *H. planiceps*, and then the split within *H. planiceps* of the plan-N1, N2, and N3 lineages.

I then used multivariate phylogenetic regression to test if and how mean annual temperature and mean annual precipitation are correlated with pheromone variation among lineages. Temperature is correlated with pheromone variation in *H. binoei*, accounting for about 7% of pheromone variation, but not in *H. planiceps*. Precipitation is correlated with pheromone variation across both *H. binoei* and *H. planiceps*, also accounting for approximately 7% of pheromone variation, although the two species complexes appear to have responded to precipitation in subtly different ways (in terms of the way individual compounds to respond to precipitation). Using this analysis I was able to identify the individual pheromone components that are most strongly associated with these climate variables, providing candidate compounds to guide further research.

Synthesis and significance

I have shown that the chemical compositions of epidermal pore secretions have diverged among genetic lineages of *Heteronotia* geckos, that pheromones from these secretions influence the ability of female geckos to discriminate among males of different lineages, and that females display greater interest in conspecific male pheromones. These results signify that pheromones are a mating trait influencing reproductive isolation in this cryptic species complex of Australian lizards. Although my research focussed on *Heteronotia*, the

implications of my results are potentially broad. Epidermal pores are present in numerous clades spanning the phylogeny of lizards (Mayerl et al. 2015), suggesting that pheromones will be important for understanding mate choice, reproductive isolation, and thus diversification in many squamates—the most species-rich group of terrestrial vertebrates (Uetz et al. 2019). I predict that pheromones—whether from epidermal pores or other sources—will be especially important for understanding reproductive isolation in cryptic species complexes that do not differ discernably in other traits.

To my knowledge, Chapter 3 is the first study to show that female lizards can use epidermal pore secretions for interspecific discrimination. My findings are in contrast to studies on European lacertid lizards, which have shown weak or negative support that pheromones influence female discrimination and reproductive isolation (e.g., MacGregor et al. 2017; While & Uller 2017; see Chapter 3 for further discussion). However, lacertid lizards are diurnal, and most species—especially those investigated—have sexually dimorphic colour patterns. My study represents the first to assess pheromones and reproductive isolation in a nocturnal lizard group. Further, my study is the first to investigate pheromones across sympatric cryptic lizard species. Phylogenetic splits within lizards are ancient (Simões et al. 2018; Karin et al. 2019), and the importance of pheromones will no doubt vary even among closely related groups. With only 1% of all pheromone studies having been on squamates (Symonds & Elgar 2008), our knowledge of pheromone signalling in lizards is minimal. My study contributes to addressing a large gap in our knowledge.

Although not originally an aim of my study, in Chapter 3 I show that the order treatments are encountered or presented can have a significant effect on female response. My results highlight the need to consider the influence of treatment order both in the design of choice experiments and subsequent data analysis (Reading & Backwell 2007; Wong & Svensson 2009).

To my knowledge, Chapter 4 is the first study to use the residual randomisation permutational procedure (RRPP; Collyer & Adams 2018) for analysis of a chemical trait, is only the second study to use phylogenetic comparative methods to test how climate can influence pheromone divergence (Baeckens et al. 2018), and is one of few phylogenetic comparative studies of pheromones in general (see Symonds & Elgar 2007 for review; Weber et al. 2016; Baeckens et al. 2018). Chemical signals are notoriously difficult to study because, among other reasons, they are often highly multivariate. Analyses developed for high-dimensional morphological data, such as RRPP, provide a new and promising avenue

for the study of chemical signals. Using multivariate phylogenetic regression via RRPP I show that pheromone divergence is correlated with temperature and precipitation. This was an important step to begin teasing apart sources of variation in these highly multivariate pheromone blends, and I identified candidate compounds to guide further investigation. In doing this I also uncovered a promising system for studying how the environment influences chemical signals, a neglected aspect of chemical signalling and sensory drive research in general (Yohe & Brand 2018). Considering that *Heteronotia* occur across a range of environments, from dry coastal rainforest to the continent's most arid regions, they provide an excellent system to further investigate how the environment can shape the composition of chemical signalling traits.

Pheromone data may be a useful character to integrate into taxonomic studies of reptiles. Delimiting and describing species is a major challenge. While genomic analysis, if sufficiently detailed, can be used to measure whether populations in contact (either parapatry or sympatry) are reproductively isolated (e.g., Singhal et al. 2018; Pinto et al. 2019), doing so for every single population is expensive, time consuming, and ultimately unfeasible. Furthermore, while detailed genomic analysis can reveal whether certain populations represent reproductively isolated species, only rarely will such analyses offer insight into the mechanisms of reproductive isolation that have created and maintained species boundaries. With further development (see Future directions), integrating pheromone data into reptile systematics and taxonomy may provide a useful and biologically meaningful framework for resolving and describing cryptic lizard diversity. I expect the same will be true for other sexually reproducing animal groups for which we have a poor understanding of mating traits—although the mating signals in question will vary.

Although work remains to be done, my thesis has also contributed toward resolving the taxonomy of *Heteronotia binoei*, a species complex that has evaded taxonomic resolution for more than 30 years (Moritz 1983, 1984). Through the course of my research I sampled several new localities, some of which revealed new deeply divergent genetic lineages of *H. binoei* (see Chapters 2 and 4). Considering that many of the lineages I sampled are genetically and phenotypically distinct even in sympatry, this is good evidence that such populations represent reproductively isolated species. For example, CYA6-S, EIU, and GULF-E all co-occur, have divergent pheromones, and females discriminate between lineages based on these pheromones. Thus, these three lineages are almost certainly reproductively isolated species. The challenge, however, is to determine the species status

of parapatric and allopatric lineages (e.g., CYA6-S vs. CYA6-N; GULF-E vs. GULF-W), and to find morphological characters—if any exist—to help non-specialists identify these animals in the field (as it stands, pheromones and genetics are not suitable for immediate identification in the field).

Finally, in the course of my PhD research I also discovered and described a new species of *Gehyra* gecko (*Gehyra electrum*; Appendix I; Zozaya et al. 2019), and co-authored the description of a new *Oedura* velvet gecko (*Oedura argentea*; Appendix II; Hoskin et al. 2018). Although not directly related to my research on pheromones, these papers nevertheless contribute to our understanding of lizard diversity, and so I include them here as appendices.

Caveats, challenges, and reflections

An expert is a person who has found out by [their] own painful experience all the mistakes that one can make in a very narrow field — Niels Bohr

My PhD was marked with challenges, at least in part because it was an exercise in system building. We knew that *Heteronotia* are small, nocturnal, largely terrestrial lizards; we knew that they are a cryptic species complex; and we knew they had epidermal pores. Putting these together, we figured they were likely to be a good group in which to investigate the role of pheromones as a mating trait. Everything else I figured out as I stumbled along. I loved it. But I also gained an appreciation for how difficult and frustrating it is to create a new system, especially one that involves a large behavioural component. I made many mistakes, I discovered several dead ends, and my results have several caveats.

When my PhD began, I sought to assess pheromone divergence and its consequences not only in *Heteronotia* but also in several other gecko genera and some skinks. But I quickly learned that studying chemical signalling traits is difficult, and that acquiring even modest sample sizes for a single study species was stressful enough. In narrowing my focus, I was able to gain a clearer picture of the divergence and role of pheromones in *Heteronotia*, but was unable to explore the implications of my results in lizards more broadly. I did, however, co-supervise an Honours student on a project testing pheromone divergence in *Oedura* velvet geckos. That study has also revealed significant differences in pheromones among lineages, and the preliminary results are discussed in more detail below.

Of all the aspects of my PhD, the behavioural experiments of Chapter 3 suffer the most caveats and caused me the most setbacks. A large part of this was simply because of limited natural history knowledge for *H. binoei*. I set out to collect geckos in mid-winter 2016, assuming that breeding would have not yet started, meaning I could then begin behavioural experiments in spring. But the first gecko I captured on my first night of collecting in July 2016 was an adult female gravid with eggs. I learned that night that, at least in tropical northern Australia, *H. binoei* begin breeding in winter (and continue well into summer). I even found individuals mating under rocks when air temperatures were only 5°C! Once animals were brought back to the lab, I learned another thing: females store sperm. About 30% of the females I collected laid up to three clutches of two eggs, with each clutch 3–5 weeks apart, even though they had been maintained in isolation since capture. All clutches produced offspring. From all this I learned that female *H. binoei* can store viable sperm for at least four months. A consequence of this natural history lesson, however, was that I was delayed an entire year—until the following breeding season—before I could begin my behavioural experiments.

The next major setback was a result in itself: that females tend to sniff whichever scent treatment they encounter first longer than they otherwise would. This novelty effect made interpreting female discrimination more difficult. In an attempt to reduce the influence of novelty, I again waited an entire year, performing sequential choice experiments during the following breeding season. So, going from capturing geckos to acquiring all the data presented in Chapter 3 took two years. A consequence of this was that I never got the opportunity to perform some of the other experiments I was also interested in. For example, one reason I originally collected geckos from three different sites (two sites per lineage) was because I wanted to assess female discrimination between both males of different lineages (between-lineage discrimination) as well as between males from different sites within the same lineage (between-population discrimination). This may have provided additional insight into behavioural isolation among populations and lineages. I had also intended to investigate the role of pheromones in male–male interactions. In the end I simply didn't have time for these experiments and had to focus on answering my core question regarding female discrimination between lineages. Had I been prescient and known all this in advance, I might have focussed on sampling three times as many individuals from only a single site rather than three. Doing so would have increased the power of my analyses since I would have no longer needed to nest sites within lineages as a random effect—although, there is something to be said about having demonstrated female

discrimination across more sites and lineages. Regardless, Chapter 3 was a surprisingly enormous effort and I am proud of the advances it makes. I gained a new appreciation for the difficulties of studying animal behaviour and the value of even the most basic natural history knowledge.

My behavioural experiments also suffer from an important caveat that I discussed in Chapter 3: while I showed that female geckos discriminate among males of different lineages using pheromones, I did not demonstrate mate choice per se. My aim was to assess the influence of epidermal pore secretions alone, which necessarily meant isolating pheromone secretions from other confounding influences, including male–male interactions and forced copulations, and using a proxy (sniffing duration) to measure female response. The advantage of this was that I could indeed demonstrate that pheromones alone influence female discrimination; however, exactly how pheromones influence real courtship encounters and reproductive isolation (including cryptic female choice, considering they store sperm), and how pheromones might interact with other traits in a multi-modal context, remains to be studied (see Future directions). Establishing the link between mate choice and pheromone variation is not straightforward, even in the most well studied systems (Smadja & Butlin 2009).

An obvious gap in my thesis is that I failed to identify the chemical identity of the compounds in *Heteronotia* pheromone secretions. I had very limited access to a gas chromatograph-mass spectrometer (GC-MS) during my project; and when I did first get access to a GC-MS, I discovered that—with the methods I was using—the quantities of most compounds were so small that identification was possible only for the largest peaks (which are mostly sterols; table S4.4). I am now again in the process of identifying these compounds via GC-MS using new methods, but because of mechanical issues this has not been finished in time for submission of this thesis. The lack of chemical identification does not limit the interpretation of the results from Chapters 2 and 3; however, identifying these compounds will be valuable for interpreting the significance of the pheromone compounds associated with climate shown in Chapter 4. Identifying the compounds in *Heteronotia* pheromone secretions will be crucial for future research testing how different compounds influences behaviour, reproductive isolation, and the persistence of pheromones in the environment. The present lack of knowledge in this area also makes it difficult to interpret and understand the high levels of phylogenetic signal in pheromone divergence observed in Chapter 4. Identifying compounds and testing their functions will facilitate more meaningful comparative analyses in the future (see Future directions).

My analyses in Chapter 4 would have benefited from more fine scale climate data. For example, considering these geckos are nocturnal, it would be interesting to see whether nighttime temperatures better predict pheromone composition. I pursued this avenue but have so far been unable to find these data at a sufficiently high spatial resolution. Furthermore, this study was relatively coarse in using only two climate variables: mean annual temperature and mean annual precipitation. I did this because many other potentially interesting variables, such as humidity and aridity, are strongly correlated with these variables. Phylogenetic canonical correlation analysis (PCCA; Revell & Harrison 2008) would have provided a means to include many correlated variables (e.g., Harrison et al. 2015; Baeckens et al. 2018), but at the cost of hindering interpretation of how individual climatic variables relate to aspects of pheromone blends. In the end I pursued a simpler analysis to identify important components to guide future study. Advances in multivariate phylogenetic comparative methods, including those that integrate evolutionary models other than Brownian motion (e.g., Ornstein-Uhlenbeck and early burst), will facilitate more rigorous and informative analyses in the future.

Future directions

Pheromones are difficult traits to study. The component chemicals of a pheromone can consist of only one or two compounds up to dozens or more (Wyatt 2003; Bradbury & Vehrencamp 2011)—and variation in the presence, absence, and proportions of different compounds mean that the potential combination of pheromone blends is effectively infinite (Wyatt 2003; Yohe & Brand 2018). This multivariate nature makes it difficult to tease apart how components interact to influence behaviour. Complicating this further is that, sometimes, certain compounds can influence several aspects of behaviour (e.g., mate choice and intrasexual interactions), as well as survival in general (Wyatt 2003; Bradbury & Vehrencamp 2011). For example, in *Drosophila* fruit flies, cuticular hydrocarbons (CHCs) influence both mate choice and male–male interactions, but are also part of a waxy cuticle that prevents desiccation and is costly to produce (Higgie et al. 2000; Blows 2002; Rouault et al. 2004; Ferveur 2005). Furthermore, the functionality of pheromones depends on the sensory and nervous systems of the receiver. Although my thesis has focussed on pheromones as a signal, variation in chemical receptors, sensory processing, and cognition are important for understanding the role and evolution of pheromones. After all, it's the sensory system that first detects a pheromone, and then the nervous system that decides whether or not it's attractive (or represents a competitor, etc.). Thus, various processes

associated with natural and sexual selection can interact and conflict to shape the co-evolution of pheromones and the sensory system. Combine the difficulty of studying multivariate chemical traits with the difficulty of understanding their influence on behaviour (itself a multivariate and labile set of traits), one is left with a crucial piece of biology that is nevertheless a serious challenge to study. But the factors that make studying pheromones difficult are also the factors that make pheromones fascinating.

Here I have built a system for studying the role of pheromones as a mating trait in lizards. In doing so I have addressed a few core questions, but many more must be answered to understand the broader significance of my results, both in the context of lizard diversification and the role of pheromones in speciation more generally. Below I list some promising future research directions. Some of these are already in progress, some I planned to address but failed to for reasons discussed above, and others are important questions leading on from my results.

As I highlighted in the section above, identifying the compounds in pheromone secretions is crucial for further study (and is currently in progress). Having accomplished that, the next step is to determine the biological activity of the many chemical constituents within these secretions. This will involve answering many questions:

- Which compounds influence mate choice, both within and between populations and species?
- Is there a role for pheromone discrimination in cryptic female mate choice (i.e., sperm allocation)?
- Do any compounds influence male–male interactions, and if so, are any of these the same compounds that influence mate choice?
- To what degree is variation in compounds heritable, and to what degree is variation plastic?
- What are the biochemical pathways that produce these pheromone compounds?
- What compounds influence the efficacy of pheromones under different climates and microhabitats; and are these compounds also influencing behaviour, or are they simply 'keeper substances' that optimise the signalling compounds?
- Are any compounds simply by-products of cellular processes with no function as signals or keeper components?
- Are pheromones combined with other signalling traits (e.g., vocalisations; movements) in multi-modal signalling?

- What is the relationship between the sensory system (chemical receptors and cognition), pheromone composition, and the potential roles in behaviour mentioned above?

Answering these questions will be difficult but necessary to understand how natural and sexual selection interact to shape the evolution of pheromones and the sensory system, and how these processes subsequently influence speciation (Smadja & Butlin 2009).

In addition to answering these fine-scale questions, assessing the link between pheromone divergence and reproductive isolation between populations and lineages can be explored using macro-evolutionary approaches. My collaborators and I have already begun work that will use genome-wide SNP and pheromone data to investigate how the degree of pheromone divergence predicts gene flow (or lack thereof) among lineages of *H. binoei* in sympatry and parapatry. This will provide insight into the role of pheromones in the diversification of these lizards, including the potential role of sympatry in driving pheromone divergence via reproductive character displacement (Butlin 1987; Noor 1999; Hoskin & Higgie 2010). Furthermore, if the relationship between species boundaries and pheromone divergence is clear, this will lead into developing a framework for integrating pheromone data into species delimitation and taxonomy (and revising the taxonomy of *Heteronotia*).

Finally, do pheromones from epidermal pore secretions function similarly in other squamate groups? The full implications of my results will become clear only as pheromones are studied in more taxa. Similar to my results for *H. binoei* in Chapter 2, an on-going study of Australian velvet geckos (*Oedura* spp.) is revealing significant pheromone divergence among divergent lineages and species within this group (fig. 5.1; Macor, Zozaya, Higgie & Hoskin, unpublished), including among some that are morphologically cryptic. While both *Heteronotia* and *Oedura* are geckos, the two belong to different families (Gekkonidae and Diplodactylidae, respectively) that last shared a common ancestor more than 100 million years ago (Karin et al. 2019), suggesting that pheromones might function similarly even among distantly related taxa. Squamates are the most speciose group of terrestrial vertebrates (Uetz et al. 2019) and—even among animals in general—contain disproportionately high levels of cryptic species diversity (Pérez-Ponce de Leon & Poulin 2016). Even with the on-going taxonomic resolution of cryptic groups using genetics, and the enormous research attention squamates receive in general, our understanding of reproductive isolating mechanisms in squamates remains relatively poor (Wollenberg Valero et al. 2019). My results suggest that studying pheromones and their role in

reproductive isolation will be key to unlocking our understanding of cryptic lizard diversity and, in doing so, will better our understanding of the role pheromones—and other mating traits—have played in shaping animal diversity.

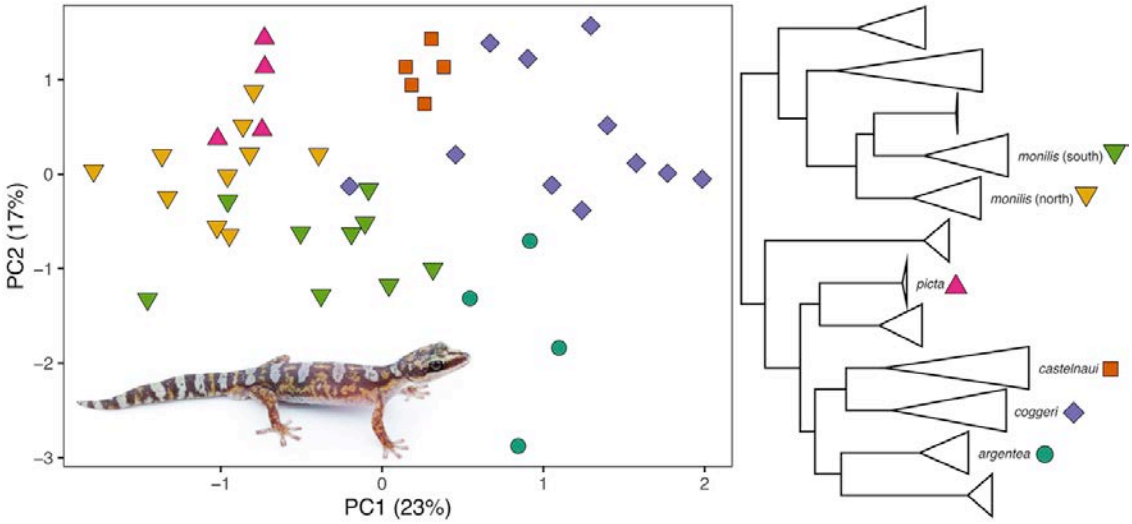

Figure 5.1: Pheromone divergence among six species/lineages of *Oedura* velvet gecko in north-eastern Australia. The scatterplot on the left shows the first two principal component axes of pheromone variation, with the simplified mtDNA phylogeny on the right showing relationships for the respective lineages/species (Macor, Zozaya, Higgie & Hoskin, unpublished). An example of one of the lineages (*O. monilis* north) appears within the plot. Pheromone samples for the remaining lineages/species have been collected and are currently being analysed.

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Opportunistic herping before an evening of pheromone sampling at Theda Station in the northern Kimberley, Western Australia, October 2017. From left to right: Brendan Schembri, Aaron Fenner, Stephen Zozaya, Conrad Hoskin, Chris Jolly, Stewart Macdonald. A storm drove us all to cover in a sandstone cave shortly after this was taken. Photo: Alana de Laive.

APPENDIX I

A new species of rock-dwelling gecko (Gekkonidae: *Gehyra*) from the Mt Surprise region of northern Queensland, Australia

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A new species of rock-dwelling gecko (Gekkonidae: *Gehyra*) from the Mt Surprise region of northern Queensland, Australia

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Abstract

We describe a new species of rock-dwelling *Gehyra* Gray, 1834 (Gekkonidae) from the Einasleigh Uplands of inland north Queensland, Australia. Morphological, ecological, and molecular data clearly support the new species as distinct and place it within the '*australis* group'. *Gehyra electrum* **sp. nov.** is distinguished from congeners by a combination of medium adult size (SVL 46–50 mm), an orange-brown to pinkish-orange background colouration with a pattern of distinct whitish spots and irregular black to purple-brown blotches or bars, possessing 7–8 undivided subdigital lamellae on the expanded portion of the fourth toe, and a wedge-shaped mental scale that separates the inner-postmental scales along 40% or more of their length. *Gehyra electrum* **sp. nov.** is a rock specialist currently known only from granite outcrops of the Mt Surprise region, Queensland. This is the second recently described *Gehyra* from the Einasleigh Uplands and adds to the growing number of endemic reptiles recognised in the region.

Key words: Einasleigh Uplands, endemism, *Gehyra catenata*, *Gehyra dubia*, *Gehyra einasleighensis*, *Gehyra electrum* **sp. nov.**, sympatry

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APPENDIX II

A new species of velvet gecko (Diplodactylidae: *Oedura*) from sandstone habitats of inland north Queensland, Australia

Conrad J. Hoskin, Stephen M. Zozaya, and Eric Vanderduys

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A new species of velvet gecko (Diplodactylidae: *Oedura*) from sandstone habitats of inland north Queensland, Australia

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Abstract

We describe a new species of velvet gecko (Diplodactylidae: *Oedura*) from the sandstone ranges of central-north Queensland, Australia. *Oedura argentea* **sp. nov.** is a medium-sized (SVL 61–80 mm) gecko that is distinguished from its congeners by a combination of its relatively small size, a pattern of 5–6 dark-edged pale transverse bands from neck to pelvis, a silvery iris, a slender tail, a single cloacal spur, and in possessing 14–22 pre-cloacal pores in males. *Oedura argentea* **sp. nov.** is a sandstone specialist currently known only from the Gregory Range and nearby sandstone outcropping at Bulleringa National Park. Further surveys are required to determine the limits of distribution through this region. *Oedura argentea* **sp. nov.** is the fifth described species of *Oedura* in north-eastern Queensland. We also assess the name *O. fracticolor* De Vis, 1884 because it is an unresolved name pertaining to this general region. Based on colour-pattern and locality in the original description, we conclude that *O. fracticolor* is a senior synonym of *O. castelnaui* (Thominot, 1889); however, we propose that priority be overturned under Articles 23.9.1.1 and 23.9.1.2 of the ICZN (1999) and that the name *O. fracticolor* be regarded as *nomen oblitum* and *O. castelnaui* a *nomen protectum*.

Key words: *Oedura argentea* sp. nov., *Oedura castelnaui*, *Oedura fracticolor*

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