


RESEARCH ARTICLE

Composition of a chemical signalling trait varies with phylogeny and precipitation across an Australian lizard radiation

Stephen M. Zozaya^{1,2}  | Luisa C. Teasdale^{1,3} | Craig Moritz¹ | Megan Higgin² | Conrad J. Hoskin²

¹Research School of Biology, Australian National University, Acton, Australian Capital Territory, Australia

²College of Science and Engineering, James Cook University, Townsville, Queensland, Australia

³Department of Molecular Biology, Max Planck Institute for Developmental Biology, Tübingen, Germany

Correspondence

Stephen M. Zozaya, Research School of Biology, Australian National University, Acton, ACT 2601, Australia.
Email: stephen.zozaya@anu.edu.au

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Abstract

The environment presents challenges to the transmission and detection of animal signalling systems, resulting in selective pressures that can drive signal divergence amongst populations in disparate environments. For chemical signals, climate is a potentially important selective force because factors such as temperature and moisture influence the persistence and detection of chemicals. We investigated an Australian lizard radiation (*Heteronotia*) to explore relationships between a sexually dimorphic chemical signalling trait (epidermal pore secretions) and two key climate variables: temperature and precipitation. We reconstructed the phylogeny of *Heteronotia* with exon capture phylogenomics, estimated phylogenetic signal in amongst-lineage chemical variation and assessed how chemical composition relates to temperature and precipitation using multivariate phylogenetic regressions. High estimates of phylogenetic signal indicate that the composition of epidermal pore secretions varies amongst lineages in a manner consistent with Brownian motion, although there are deviations to this, with stark divergences coinciding with two phylogenetic splits. Accounting for phylogenetic non-independence, we found that amongst-lineage chemical variation is associated with geographic variation in precipitation but not temperature. This contrasts somewhat with previous lizard studies, which have generally found an association between temperature and chemical composition. Our results suggest that geographic variation in precipitation can affect the evolution of chemical signalling traits, possibly influencing patterns of divergence amongst lineages and species.

KEYWORDS

chemical signal, climate, lizard, multivariate phylogenetic regression, mvGLS, pheromone

1 | INTRODUCTION

Signalling systems serve important functions in animals that influence survival and reproduction: finding and choosing mates; orientation; warning, detecting and assessing competitors; recognizing 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 fewer offspring. 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

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(Seehausen et al., 2008). The environment can, thus, exert selection pressure to optimize the efficacy of signals against the background environment ('sensory drive', Endler, 1992). When the traits in question are linked to assortative mating, this can influence reproductive isolation and speciation (Boughman, 2002; Endler & Basolo, 1998; Seehausen et al., 2008).

Chemical signalling is the most ubiquitous and ancient mode of signalling amongst organisms (Hildebrand & Shepherd, 1997). Despite this, there are few studies on the influence of the environment on the evolution of chemical signals (Symonds & Elgar, 2008; Yohe & Brand, 2018), in contrast to the well-studied influence of the environment on visual and acoustic signalling systems (reviewed in Cummings & Endler, 2018). Chemical signals must be perceived against a background of other chemicals, and abiotic factors such as temperature and humidity influence the signal, the background, and the sensory system (Bradbury & Vehrencamp, 2011; Yohe & Brand, 2018). Characterizing 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, high temperatures and humidity shorten the longevity of chemical signals by increasing rates of evaporation, diffusion, and degradation (e.g. oxidation) (Alberts, 1992; Bossert & Wilson, 1963; Regnier & Goodwin, 1977; Royer & McNeil, 1993; Wilder et al., 2005). To deal with this, some animals have evolved non-signalling compounds termed 'keeper substances' to increase the longevity of chemical signals (e.g. Hayes et al., 2003; Hurst et al., 1998; Regnier & Goodwin, 1977).

Lizards and snakes (squamates) are an important group to study the evolution of chemical signals. Squamates use chemical signals to mark territories, identify kin and rivals, deceive competitors, and attract and choose mates (Martín & López, 2014; Mason & Parker, 2010). Considering these functions, we should expect adaptations that optimize the persistence and detectability of chemical signals under local climatic conditions (Alberts, 1992), and there is already experimental and comparative evidence to suggest this in lizards. High temperatures negatively impact the persistence and detectability of chemical signals in the lizard *Iberolacerta cyreni*, with secretions treated to warm temperatures eliciting weaker behavioural responses (Martín & López, 2013). Furthermore, a comparison of two populations of the lizard *Podarcis guadarramae* (as *P. hispanicus*) showed that populations inhabiting disparate climates possess divergent chemical blends, with those from warmer, drier environments being more easily detectable than those from cool, wetter environments after being experimentally treated to warm conditions (Martín et al., 2015).

Comparative studies have identified some chemical classes potentially influencing such variation in persistence and detectability. A phylogenetic comparative study of 64 species and subspecies of lacertid lizards found that species inhabiting hotter and drier climates had higher proportions of stable fatty acids and high-molecular-weight alcohols (Baeckens et al., 2018). Another comparative study found that lower proportions of unsaturated fatty acids and higher

proportions of aldehydes correlate with increasing habitat temperature (but not precipitation) amongst 12 species of *Sceloporus* lizards (Campos et al., 2020). A limitation of these comparative studies, however, is that they are restricted to analysing variation amongst whole chemical classes, rather than individual compounds. Finding the putative compounds associated with climatic adaptation requires multivariate methods that can analyse dozens of individual components.

Phylogenetic comparative methods (PCMs) are useful for investigating trait evolution amongst large numbers of species or populations whilst accounting for the non-independence of observations due to shared evolutionary history (Felsenstein, 1985; Harvey & Pagel, 1991). Until relatively recently, the suitability of PCMs to analyse multivariate data has been limited (Adams & Collyer, 2018, 2019); hence, there are relatively few studies of chemical signals in a phylogenetic context (but see Baeckens et al., 2018; Campos et al., 2020; Symonds & Elgar, 2004; Symonds & Wertheim, 2005; Van Wilgenburg et al., 2011; Weber et al., 2016). Chemical signals can range from one to dozens or more compounds that can each vary in their relative proportions. In many cases, the number of variables approaches or exceeds the number of observations, making statistical analysis difficult. Studies of multivariate traits have often dealt with this issue via dimension reduction techniques, such as principal component analysis (PCA). The disadvantages of dimension reduction, however, are that some proportion of trait variation is inevitably discarded, it can give too much weight to very minor components (Martin & Drijfhout, 2009), and phylogenetic comparative analysis of dimension-reduced trait axes can yield spurious statistical conclusions (Uyeda et al., 2015). Recently, however, PCMs have been developed that are suited to highly multivariate data (Clavel et al., 2019; Clavel & Morlon, 2020; Collyer & Adams, 2018; Collyer et al., 2015). Although developed largely for morphometric data, these offer valuable and previously unused tools for investigating the evolution of multivariate chemical traits.

Here, we use a multivariate phylogenetic comparative approach to investigate how phylogenetic history and climate have shaped the chemical composition of epidermal pore secretions in Australia's *Heteronotia* lizard radiation. *Heteronotia* (Gekkonidae) is a genus of five recognized species (Wilson & Swan, 2017), two of which—*H. binoei* (Gray, 1845) and *H. planiceps* Storr, 1989—are complexes of deeply divergent genetic lineages, many representing undescribed 'cryptic species' (Fujita et al., 2010; Moritz et al., 2016; Oliver et al., 2017; Pepper et al., 2013; Riedel et al., 2021; Zozaya et al., 2019). Similar to many lizard groups (Mayerl et al., 2015), male *Heteronotia* have epidermal pores (Figure 1b) that exude waxy secretions with complex and variable chemistry. Previous work has shown that the secretions of deeply divergent genetic lineages (i.e. candidate species) within the *H. binoei* complex have chemical blends that are more variable between than within lineages (Zozaya et al., 2019). This chemical divergence, and the fact that epidermal pores are present only in males, suggests that these secretions are a sexually selected trait that potentially influences species discrimination, as

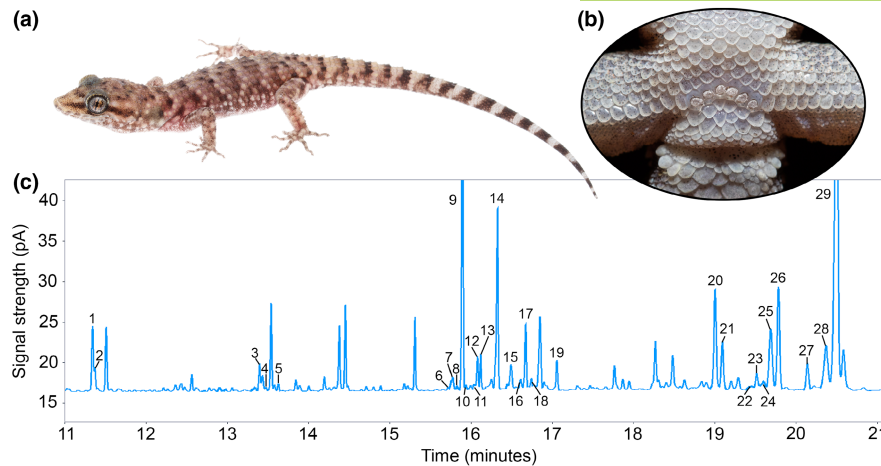


FIGURE 1 Characterizing epidermal pore secretions. (a) An example of *Heteronotia binoei* (EIU lineage), and (b) the pre-cloacal epidermal pores from which secretions were collected. (c) An example gas chromatograph trace of derivatized epidermal pore secretions from a male *Heteronotia planiceps* (plan-M lineage), which shows the 29 integrated peaks. Peaks without labels are those identified as possible contaminants or coinciding with contaminants. Note that some peaks are particularly small in this sample but can be larger in samples from other lineages

has been suggested for other lizard groups (Barbosa et al., 2006; Gabirot et al., 2012; Labra, 2011). If so, knowing the factors influencing chemical variation is necessary for understanding reproductive isolation in this system and in squamates more broadly (Wollenberg-Valero et al., 2019).

As we outline above, adaptation to local climatic conditions is one possible driver and/or constraint of chemical signal variation in squamates. The epidermal pores of *Heteronotia* and other lizards are positioned ventrally, where they deposit secretions onto substrates (Mayerl et al., 2015). Lizards appear to detect epidermal pore secretions only at close distances (Alberts, 1993). Therefore, secretions likely need to persist as long as possible, and we should expect epidermal pore secretions, and the substrate-borne secretions of other animals, to be adapted to prevailing environmental conditions (Alberts, 1992; Baeckens et al., 2018). *Heteronotia* occupies habitats from humid coastal forests to Australia's central deserts. This broad climatic breadth, high lineage diversity, and chemically variable secretions make *Heteronotia* an excellent radiation in which to study both phylogenetic signal and the influence of climate on the evolution of a sexually selected chemical trait. We collected and characterized epidermal pore secretions from 33 lineages of *Heteronotia* lineages across northern Australia to (1) assess phylogenetic signal in the chemical composition of epidermal pore secretions and (2) test if and how chemical variation amongst lineages is associated with two key climate variables: mean annual temperature and mean annual precipitation. Based on the results of Baeckens et al. (2018), we expected to find low levels of phylogenetic signal (i.e. high lability) in chemical composition. We also expected to find support for relationships between chemical composition and both temperature and precipitation, with relationships likely reflected by variation in just a few components—possible 'keeper components' or the signalling compounds themselves.

2 | MATERIALS AND METHODS

Broadly, our approach was to collect epidermal pore secretions from as many *Heteronotia* lineages as possible. We targeted genetic lineages as identified in previous and ongoing phylogenomic analyses. 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 mean annual temperature and mean annual precipitation on chemical composition using multivariate phylogenetic regression via penalized likelihood.

2.1 | Field sampling

We sampled epidermal pore secretions from *Heteronotia* geckos across northern Australia from September to December 2017 and September to November 2018; months coinciding with the mid to late reproductive season of these geckos in northern Australia (personal observation). We targeted 25 lineages of *H. binoei* and eight lineages of *H. planiceps* for a total of 33 divergent genetic lineages (see Section 3). We sampled 1–13 (mean = 8.2) adult male geckos from each lineage. Geckos were captured at night by hand and epidermal pore secretions were collected following the methods of Zozaya et al. (2019). 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. A control sample containing no epidermal pore secretions was collected at each site to identify contaminants introduced during sample collection and preparation (see Zozaya et al., 2019). Samples were stored in a dark car freezer ranging from -20°C to

–5°C for up to 35 days in the field, followed by laboratory storage at –22°C until characterized via gas chromatography (details below).

2.2 | mtDNA sequencing and phylogenetics

With very few exceptions, genetic analyses of *Heteronotia* have demonstrated high congruence between lineage identity inferred from mtDNA and multilocus nuclear gene sequencing (Fujita et al., 2010; Moritz et al., 2016; this study). We, thus, performed phylogenetic analysis of mtDNA sequence data to confirm the lineage membership of geckos sampled at sites for which no prior genetic data were available and for sites where more than one lineage may be present. 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, Zozaya et al., 2019, or Riedel et al., 2021) and all individuals at sites where more than one lineage occurs. DNA extraction and polymerase chain reaction (PCR) protocols followed the methods of Zozaya et al. (2019), which used the *ND2* primers ('tRNAI' and 'tRNAA') and PCR protocols of Fujita et al. (2010). Sequences were edited and aligned using GENEIOUS version, 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) and an additional 241 sequences from Moritz et al. (2016) and 20 sequences from Riedel et al. (2021), thus representing all major lineages of *H. binoei*, all lineages of *H. planiceps* sampled herein, and, as outgroups, two individuals of the *H. spelea* complex. We used this combined alignment of 661 *ND2* sequences (see Section 3) 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.

2.3 | Species tree inference

Using custom target capture, we generated sequence data for nuclear gene exons from 54 individuals representing 33 deeply divergent genetic lineages across four currently recognized species of *Heteronotia* (*H. binoei*, *H. planiceps*, *H. spelea* and *H. fasciolatus*; 1–2 samples per lineage; Tables S1 and S2). Details of exon capture and bioinformatics workflow methods are available in Appendix S1. We performed multispecies coalescent phylogenetic analysis using StarBEAST2 (version 0.13.1; Ogilvie et al., 2017) implemented in BEAST2 (Bouckaert et al., 2014). Although computationally intensive, and so only practical for a modest number of genes, the multispecies coalescent implemented in StarBEAST2 avoids overestimation of tip branch lengths that can plague concatenation approaches in recent radiations, such as *Heteronotia* (Ogilvie et al., 2017). 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 data sets, 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 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 exceeded 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.

2.4 | Combined phylogeny

We could not acquire exon capture data for three deeply divergent *H. binoei* mtDNA lineages that were sampled for epidermal pore secretions: CC, CQ and Paluma-W (Figure S1; Table S3). In order to include these lineages in our phylogenetic comparative analysis, we inserted these three lineages into the StarBEAST2 species tree based on their mtDNA relationships and relative levels of divergence. This was done manually 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.

2.5 | Chemical characterization and divergence

Epidermal pore secretions were characterized via gas chromatography (GC) using the methodology of Zozaya et al. (2019), but with three differences to improve the detection of compounds with longer retention times: first, the inlet temperature was set to 250°C (vs. 200°C); second, the oven was held at 325°C for 10 min (vs. 8 min) and finally, the flame ionization detector was set to 325°C (vs. 250°C). Gas chromatograms were integrated manually using Agilent OPENLAB software. Chemical peaks that appeared in the chromatograms of control samples, or that coincided with these, were regarded as contaminants and excluded from the integration and analysis of all samples. A total of 269 samples across 33 lineages for two currently recognized species of *Heteronotia* (*H. binoei* and *H. planiceps*) were usable for analysis following GC characterization and integration (1–13 samples per lineage; Table S3). All lineages except plan-I ($N = 1$) are represented by three or more samples (mean = 8.2). A total of 29 chemical peaks were consistently integrated across all chromatograms (Figure 1; Figure S2). 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) 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 logit-transformed values were then standardized, so that each peak had a mean of 0 and standard deviation of 1 across lineages, which were then used for all subsequent analyses. Standardization was done to scale variables so that variation in more prevalent compounds was not weighted more heavily than minor compounds (see Jolliffe et al., 2007 in the context of PCA).

2.6 | Assessing phylogenetic signal of chemical variation

We assessed multivariate phylogenetic signal using two methods: K_{mult} (Adams, 2014), an algebraic generalization of the K statistic (Blomberg et al., 2003); and Pagel's λ model (Pagel, 1999). The data set of 29 standardized logit-transformed peak proportions was used for all analyses. We calculated K_{mult} values using the 'physignal' function in the R package *geomorph* with 10 000 permutations for significance testing (Adams et al., 2019). We calculated λ by running a null (intercept only) model using the 'mvgls' function in the R package *mvMORPH* (Clavel et al., 2015) with the model specified as 'lambda' (Clavel et al., 2019). To assess the influence of manually adding the *H. binoei* 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} and λ on four data sets: (1) all 33 *Heteronotia* lineages; (2) only the 30 *Heteronotia* lineages represented in the StarBEAST2 tree; (3) all 25 *H. binoei* lineages and (4) only the 22 *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 analysis (Blomberg et al., 2003).

As stated above, multivariate phylogenetic signal was assessed on the 29 logit-transformed peak proportions. We then visualized amongst-lineage chemical secretion variation using phylomorphospace plots of principal component (PC) axes. Principal component analyses (PCA) were performed using the 'rda' function in the R package *vegan* (Oksanen et al., 2017) with a correlation matrix specified (Jolliffe et al., 2007). Our aim here was to visualize if and how the largest axes of chemical variation correspond to phylogenetic relationships. PC axes were not used in any subsequent analyses. We identified chromatogram peaks most strongly contributing to the respective PC axes using the '70% of absolute highest loading and higher' rule of thumb (Mardia et al., 1979). For example, if the highest loading was 0.8, then coefficients greater than 70% of that value (>0.56) would be regarded as strongly associated with the respective PC axis.

2.7 | Assessing associations between climate and chemical variation

We assessed the influence of two climate variables on amongst-lineage chemical signal variation: mean annual air temperature (T_{mean}) and mean annual precipitation (P_{mean}). These represent the averages

from 1970 to 2000 and were obtained from the WorldClim 2 database (Fick & Hijmans, 2017) at a 1 km² (30s) resolution for every site from which epidermal pore secretions were sampled (Table S1). We chose this data source because it is the highest spatial resolution currently available for Australia and represents a time frame long enough that we might reasonably expect to observe the signature of adaptation to local climates. We did not include factors such as seasonality, humidity, aridity, evaporation and elevation because these are highly collinear with T_{mean} and P_{mean} ; however, there is no correlation between T_{mean} and P_{mean} in our data (Figure S4). Both T_{mean} and P_{mean} were standardized to a mean of 0 and a standard deviation of 1 for subsequent analyses.

We performed multivariate phylogenetic regression to test the influence of T_{mean} and P_{mean} on chemical signal composition using the penalized-likelihood framework of Clavel and Morlon (2020). This method is appropriate for high-dimensional data sets, can account for phylogenetic non-independence amongst response and predictor variables and is more powerful than other current multivariate phylogenetic regressions when the response of interest does not lie in the first axis of variation (Clavel & Morlon, 2020). All analyses were first run as a type III regression with $T_{\text{mean}} \times P_{\text{mean}}$ as an interaction term; if not significant, the interaction term was removed (equivalent to a type II regression). Analyses were done using the 'mvgls' function in the R package *mvMORPH* (Clavel et al., 2015; Clavel & Morlon, 2020) with the default penalized likelihood method, the RidgeArch penalty specified, and using Pagel's λ phylogenetic model. We chose the λ model—as recommended by Clavel and Morlon (2020)—because it effectively models trait evolution via Brownian motion whilst allowing for the contribution of independent random noise to amongst-lineage trait variation (Clavel et al., 2019). Non-parametric hypothesis testing was performed on the models using the 'manova.gls' function with Pillai's trace and 10000 permutations. As for our analyses of phylogenetic signal, we repeated phylogenetic regressions on each of the four data sets described above: (1) all 33 *Heteronotia* lineages; (2) only *Heteronotia* lineages represented in the StarBEAST2 tree; (3) all *H. binoei* lineages and (4) only *H. binoei* lineages appearing in the StarBEAST2 tree. Again, this was done to assess the sensitivity of our results to the manual addition of the CC, CQ and Paluma-W lineages to the phylogeny based on mtDNA relationships and to compare results with and without including *H. planiceps*. Finally, we accounted for multiple testing (inflated Type I error) by applying false discovery rate correction (FDR; Benjamini & Hochberg, 1995) to the set of all p -values from these four analyses. To visualize multivariate relationships, we calculated canonical variate axes representing the linear combination of chemical variation most closely associated with each predictor variable (T_{mean} and P_{mean}) for the respective analysis. Response variables were weighted according to the coefficients of the respective *mvGLS* model. We again identified chromatogram peaks most strongly associated with the respective climate variables using the '70% of absolute highest loading and higher' rule-of-thumb (Mardia et al., 1979).

Finally, to explore if and how peaks strongly associated with climate may have also contributed to the largest axes of amongst-lineage

chemical variation, we compared individual chromatograph peak contributions between the PCA and *mvGLS* analyses. We did this by comparing the highest individual peak loadings in the PCAs with the highest individual peak coefficients from the *mvGLS* analyses.

3 | RESULTS

3.1 | mtDNA phylogenetics

We obtained 905–1041 bp *ND2* sequences from 69 new individuals of *H. binoei*. These, combined with an additional 592 *ND2* sequences from Zozaya et al. (2019), Moritz et al. (2016) and Riedel et al. (2021), yielded a final alignment of 661 *ND2* sequences. The RAxML analysis of these sequences confirms the lineage identity of geckos sampled for pheromones (Table S3). 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; Riedel et al., 2021; Zozaya et al., 2019), although deeper relationships vary and typically have lower support (Figure S1). Our phylogeny reveals two new deeply divergent *H. binoei* mtDNA lineages from central and southeastern Queensland: the Kroombit lineage from Kroombit Tops; and the nearby Biloela lineage, found in the vicinity of Biloela and the Blackdown Tableland sandstone plateau (Figure S1).

The rock-associated Kimberley endemic, *Heteronotia planiceps*, is revealed as a complex of deeply divergent allopatric populations with phylogenetic depths similar to that amongst lineages of the continent-wide, ecological generalist *H. binoei* (Figure S1; Oliver et al., 2017; Pepper et al., 2013). The phylogenetic relationships and biogeography of these species complex will be dealt with in detail elsewhere (S. M. Zozaya & C. Moritz, unpublished data), but the important result for our study here is that all populations sampled for epidermal pore secretions come from identified deeply divergent allopatric lineages.

3.2 | Species tree phylogenetics

The StarBEAST2 species tree based on 100 nuclear exons recovers the *H. binoei*, *H. planiceps* and *H. spelea* groups as three strongly supported major clades within *Heteronotia* (Figure 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. All but one of the deeply divergent mtDNA lineages are well-supported as separate lineages in the nuclear exon-based StarBEAST2 species tree (Figure 2). The exception is the *H. binoei* Biloela mtDNA lineage that—whilst deeply divergent for *ND2* sequences—is very similar to the geographically adjacent *H. binoei* Kroombit lineage for nuclear exon sequences, and we, hereafter, consider the two as a single lineage ('Kroombit'). With the exception of the *spelea* group (*H. spelea* and *H. fasciolatus*), *H. planiceps* plan-F, and *H. binoei* Biloela, all 30 remaining lineages

in this nuclear exon phylogeny were sampled for epidermal pore secretions.

We inserted the *H. binoei* CC, CQ and Paluma-W mtDNA lineages (Figure S1) into the StarBEAST2 exon tree based on the relationships and relative levels of divergence observed for these lineages in the RAxML *ND2* phylogeny (Figure 3a). *Heteronotia binoei* 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 (Figure 3a). Lineages from which we did not sample pheromones (*H. spelea*, *H. fasciolatus*, *H. planiceps* plan-F and *H. binoei* Biloela) were pruned from the phylogeny.

3.3 | Phylogenetic signal of chemical variation

The composition of epidermal pore secretions shows high levels of phylogenetic signal (all *Heteronotia* lineages: $K_{\text{mult}} = 1.19$, $p = 0.001$; $\lambda = 0.97$). Because K_{mult} and λ are close to one, this indicates that chemical composition closely follows a Brownian motion (BM) model of multivariate trait evolution (Blomberg et al., 2003; Pagel, 1999). Phylogenetic signal remains high when the data are restricted to only those lineages represented in the StarBEAST2 exon phylogeny (StarBEAST2 *Heteronotia* lineages: $K_{\text{mult}} = 1.16$, $p = 0.001$; $\lambda = 0.95$), indicating that the manual addition of the *H. binoei* CC, CQ and Paluma-W lineages based on mtDNA relationships does not strongly influence measures of phylogenetic signal. Phylogenetic signal is somewhat lower when *H. planiceps* lineages are excluded from analysis (all *H. binoei* lineages: $K_{\text{mult}} = 0.93$, $p = 0.001$; $\lambda = 0.89$), including when only those *H. binoei* lineages represented in the StarBEAST2 phylogeny are used (StarBEAST2 *H. binoei* lineages: $K_{\text{mult}} = 0.93$, $p = 0.001$; $\lambda = 0.92$).

Phylomorphospace plots of PC axes were used to visualize how the most variable axes of amongst-lineage chemical variation correspond to phylogenetic relationships and to identify the chromatogram peaks most strongly contributing to these axes. Loading coefficients for all PC axes appear in Table 1. When considering *H. binoei* and *H. planiceps* lineages together, our plots show that the largest differences in chemical composition are associated with just two nodes in the phylogeny (Figure 4). PC1 (42% of total variation) separates most *H. planiceps* lineages (plan-A–M) from *H. binoei* and *H. planiceps* plan-N1–plan-N3, whereas PC2 (19% of total variation) separates plan-N1–N3 from the rest of *H. planiceps* and *H. binoei* (Figure 4a). Notably, *H. planiceps* plan-N1, plan-N2 and plan-N3 have small geographic ranges in limestone karsts in the hot, semi-arid southern limit of the *H. planiceps* distribution (Oliver et al., 2017; see Discussion below). PC3 (9% of total variation) separates shallower lineages within *Heteronotia*—although the density of points and phylogenetic connections makes it difficult to interpret relationships.

When considering *H. binoei* alone, closely related lineages generally have similar chemical compositions (Figures 4c,d; Table 1.); the five lineages from inland northeastern Queensland clustering on PC1 (18% total variation) and PC2 (17% total variation), appearing in the top of Figure 4c, offer the clearest example. There are exceptions

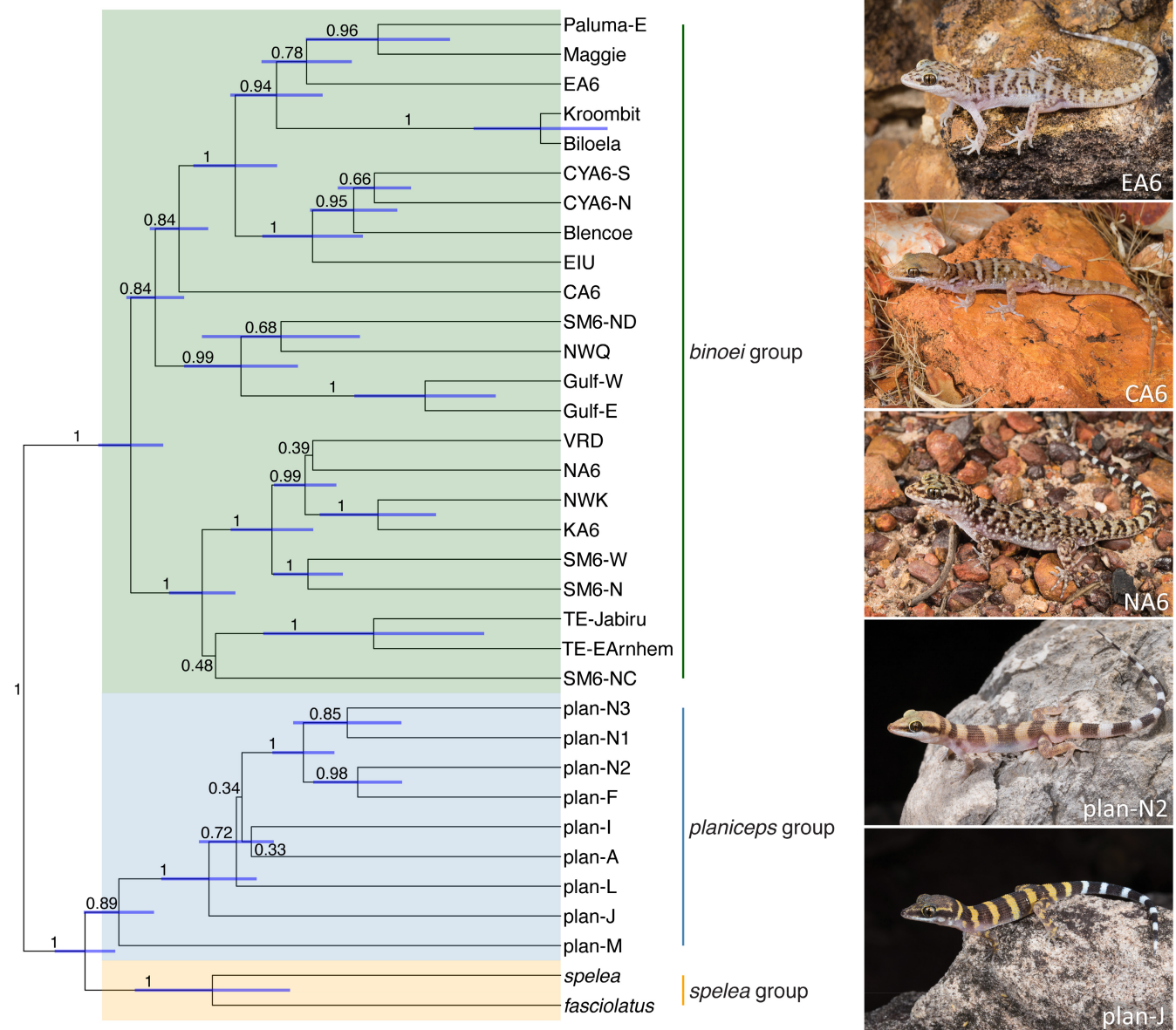


FIGURE 2 *Heteronotia* species tree phylogeny inferred from 100 nuclear exon sequences using StarBEAST2. Branch labels are posterior probabilities (PP) with node bars showing the 95% highest posterior density of node depth (absent for PP < 0.5). Photos of select lineages appear on the right

however three distantly related lineages (*H. binoei* CQ, SM6-NC and NWQ) are noticeably divergent from the rest of *H. binoei* on PC1 and PC2, appearing on the bottom and bottom left of Figure 4c. These, along with *H. binoei* SM6-ND and GULF-W, also cluster on PC2 and PC3 (15% total variation), appearing in the top left of Figure 4d. All five of these lineages have small geographic ranges in the hot and relatively dry southern Gulf of Carpentaria region (Figure 2), although the implications of this are unclear. Figure 4d, illustrating PC2 and PC3, 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. The *H. binoei* Kroombit lineage, which by far occupies the lowest average temperatures of our sampled lineages ($T_{\text{mean}} = 18.4^{\circ}\text{C}$), stands apart from the other eastern Queensland lineages (Figure 4d).

3.4 | Associations between climate and chemical variation

The interaction between T_{mean} and P_{mean} was not significant in any of the multivariate phylogenetic regression analyses on the four data sets (i.e. all 33 *Heteronotia* lineages; all 25 *H. binoei* lineages; all 30 *Heteronotia* lineages represented in the StarBEAST2 tree and all 22 *H. binoei* represented in the StarBEAST2 tree; Table S4). We, therefore, re-ran all models without the interaction term and report the results of those below.

Both T_{mean} and P_{mean} were significantly associated with chemical composition in the initial analysis of all 33 *Heteronotia* lineages (residual $\lambda = 0.956$; P_{mean} : Pillai's trace = 0.626, $p = 0.024$, Figure 5a; T_{mean} : Pillai's trace = 0.626, $p = 0.025$, Figure 5c), and when only

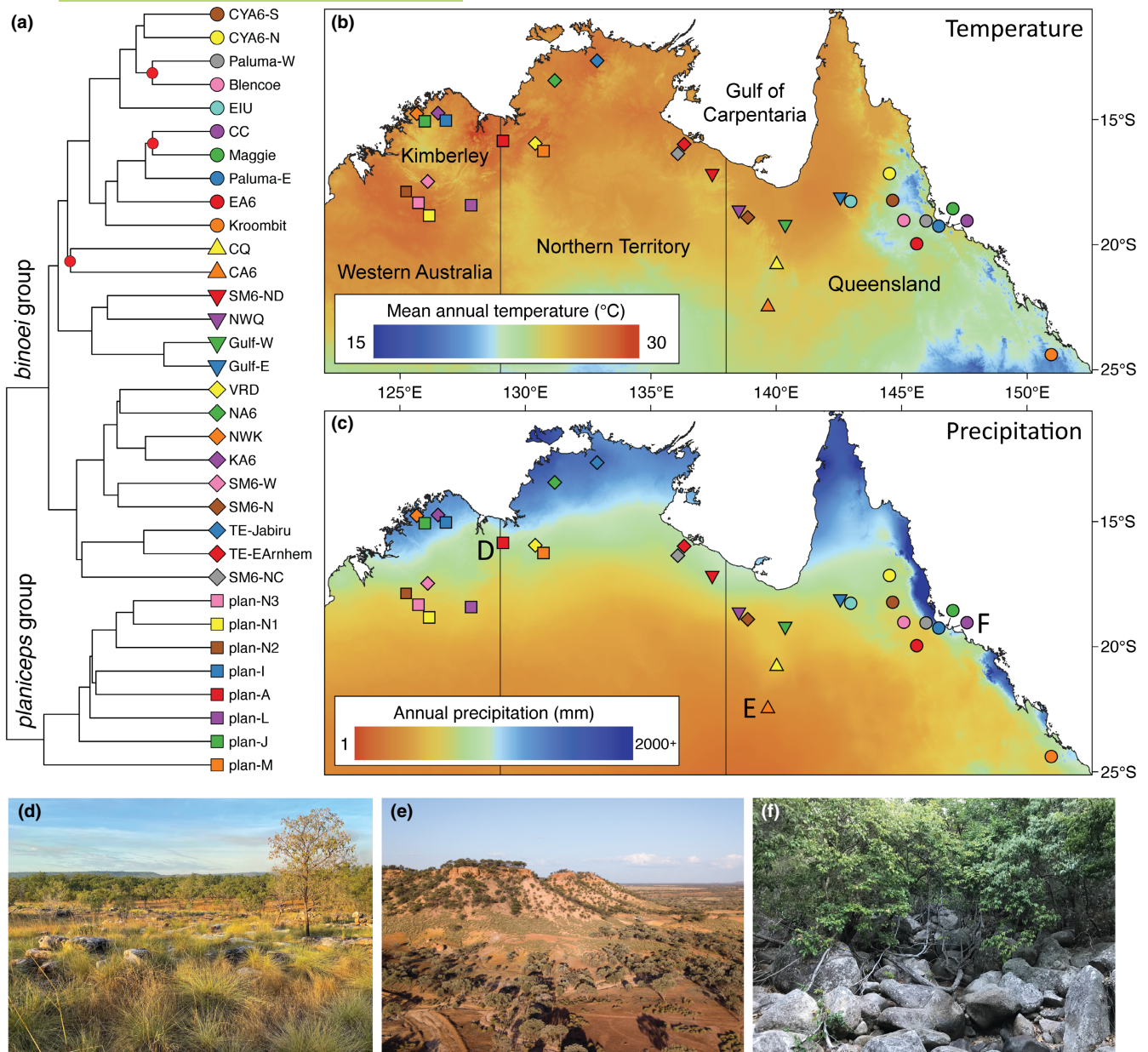


FIGURE 3 *Heteronotia* lineages and localities sampled for epidermal pore secretions. (a) The combined phylogeny with three additional mtDNA lineages (CC, CQ, Paluma-W) added to the StarBEAST2 species tree. Red dots show nodes created from the manual addition of these three lineages. Symbols are unique to each taxon and correspond to locations on adjacent maps. (b, c) Maps of sites where lineages were sampled: (b) variation in mean annual temperature (T_{mean}); (c) variation in annual precipitation (P_{mean}). Symbols at sympatric sites are slightly offset to prevent obscuring each other. Australian states, and geographic regions discussed in-text, are labelled in b. (d–f) Photos of three sampling sites illustrate the range of environmental variation, with photos corresponding to the respective sites labelled in c

the 25 lineages of *H. binoei* were included (residual $\lambda = 0.793$; P_{mean} : Pillai's trace = 0.731, $p = 0.011$, Figure 5b; T_{mean} : Pillai's trace = 0.723, $p = 0.024$, Figure 5d). However, Figure 5c,d revealed the *H. binoei* Kroombit lineage—occupying the coldest climate in our data set—to be an outlying point with high leverage in the relationship with T_{mean} (Figure 5a,c). We, therefore, excluded Kroombit and repeated these analyses as a post hoc sensitivity test. Whilst the overall results for P_{mean} were unaffected (all lineages: Pillai's trace = 0.637, $p = 0.023$; *H. binoei* lineages: Pillai's trace = 0.739, $p = 0.008$; Table S6, Figure S3), the relationship with T_{mean} was no longer statistically significant in both

the analysis of all lineages (residual $\lambda = 0.939$; Pillai's trace = 0.523, $p = 0.278$, Figure 5e) and when only *H. binoei* lineages were included (residual $\lambda = 0.715$; T_{mean} : Pillai's trace = 0.611, $p = 0.336$, Figure 5f). Inspection of Figure 5f reveals what looks like a relationship between chemical composition and temperature; however, this appears to be a spurious relationship due to phylogenetic clustering on both the x and y axes. Although the chemical composition of the Kroombit lineage could reflect the cool climate in which the lineage lives, we find no support for a relationship between chemical composition and temperature amongst the remaining *Heteronotia* lineages. Finally, the

TABLE 1 PCA loadings of chromatogram peaks for PC axes 1–3 for the PCA of all 33 *Heteronotia* lineages and for the PCA of only the 25 lineages of *Heteronotia binoei*

Peak	All lineages			<i>H. binoei</i> lineages only		
	PC1 (42%)	PC2 (19%)	PC3 (9%)	PC1 (18%)	PC2 (17%)	PC3 (15%)
1	-0.612	0.010	-0.541	-0.489	0.236	-0.328
2	-0.132	-0.140	-0.847	-0.641	-0.191	-0.182
3	0.703	0.244	-0.384	-0.385	0.622	-0.411
4	0.594	0.585	0.204	0.582	0.535	-0.059
5	0.797	0.228	-0.237	-0.119	0.730	-0.072
6	0.478	0.462	-0.252	0.054	0.182	-0.584
7	0.028	-0.836	-0.349	-0.738	-0.011	0.232
8	-0.371	-0.884	-0.117	-0.715	-0.298	0.330
9	0.737	0.226	-0.138	0.051	-0.730	-0.320
10	-0.249	-0.760	-0.079	-0.468	-0.541	0.150
11	0.744	-0.196	-0.124	-0.355	0.554	0.318
12	0.795	0.026	-0.251	-0.316	0.067	-0.421
13	0.085	-0.686	-0.270	-0.589	-0.121	0.415
14	0.758	-0.520	0.323	0.090	0.058	0.683
15	0.391	-0.627	0.384	0.196	0.116	0.683
16	0.418	-0.698	0.125	-0.212	0.475	0.093
17	0.643	-0.528	0.310	-0.027	0.268	0.435
18	0.481	-0.558	0.504	0.099	0.299	0.567
19	0.666	-0.068	-0.158	-0.264	0.739	0.079
20	-0.849	0.434	0.070	0.592	0.264	-0.409
21	-0.902	-0.111	0.024	-0.259	0.072	0.514
22	0.839	0.273	0.102	0.324	-0.338	0.485
23	0.676	-0.057	0.058	-0.067	-0.002	0.256
24	0.593	0.407	0.449	0.618	-0.163	0.274
25	-0.729	0.356	0.263	0.513	-0.418	0.291
26	-0.790	-0.398	0.126	0.075	0.569	0.184
27	0.927	0.175	-0.084	-0.251	0.104	0.439
28	-0.781	0.261	0.271	0.444	0.203	0.123
29	-0.961	0.006	0.141	0.474	0.279	0.210

Note: Values that are 70% or more of the highest loading for the respective PC axis are in bold. Values are rounded to three decimal places.

planned sensitivity analyses that included only those lineages represented in the StarBEAST2 phylogeny yielded similar highest weighted peak coefficients, although *p*-values vary, indicating that the manual addition of the CC, CQ and Paluma-W lineages to the phylogeny based on mtDNA relationships increased our power but did not skew the relationship (Tables S4 and S5).

Coefficients for P_{mean} from the multivariate phylogenetic regressions of all 33 *Heteronotia* lineages and only the 25 *H. binoei* lineages appear in Table 2 (however, coefficients for both P_{mean} and T_{mean} for these and all sensitivity analyses appear in Table S7). Because both response and predictor variables were standardized prior to analysis, coefficient values represent the standard deviation change in each chromatogram peak per standard deviation increase in P_{mean} , where $\text{sd}(P_{\text{mean}}) = 317$ mm across all 33 *Heteronotia* lineages and 304 mm across the 25 *H. binoei* lineages.

Five chromatogram peaks dominated the association with precipitation in analyses both with lineages of *H. planiceps* (all lineages: 14, 20, 21, 23, 28) and without lineages of *H. planiceps* (*H. binoei*: 23, 24, 26, 28, 29), although only two peaks (23 and 28) loaded highly in both analyses (Table 2). Intriguingly, many of the peaks strongly associated with P_{mean} (all lineages: 20, 21, 28; *H. binoei*: 23, 28, 29) appear in only small proportions in *H. binoei* and the three plan-N lineages but are abundant in most lineages of *H. planiceps* (plan-A–M; Table 1; Figure 4). This may explain the disparate responses of the *H. planiceps* plan-A–M lineages versus the remaining 28 *Heteronotia* lineages in the multivariate phylogenetic regressions (Figure 5). Many of these peaks coincide with those that loaded highly in the principal components analyses (Table 1). In the analyses of all 33 lineages, peaks 14, 20, 21, 23 and 28 were strongly associated with both P_{mean} and with PC1. In the analyses including only lineages of

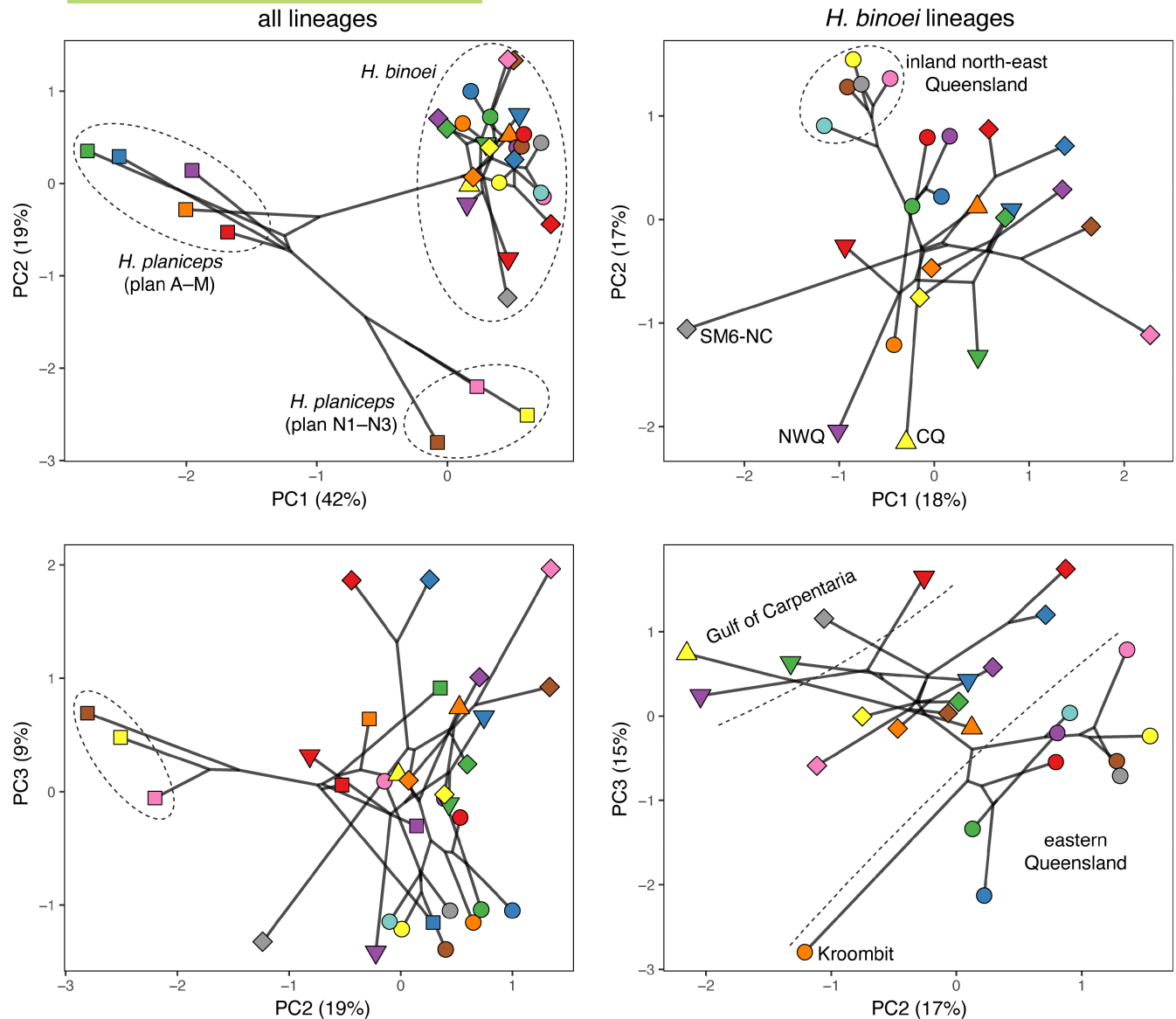


FIGURE 4 Phylomorphospace plots of principal component (PC) axes 1–3 of variation in chemical composition for all 33 sampled lineages of *Heteronotia* (left) and for only the 25 lineages of *Heteronotia binoei* (right). The top-left panel illustrates how the largest divergences in chemical composition are associated with just two phylogenetic splits, which separate the *Heteronotia planiceps* complex from the *H. binoei* complex, and then, the three *H. planiceps* plan-N lineages (plan-N1–plan-N3) from the rest of *H. planiceps*. Lineages, clades and geographic groups that are referred to in-text (see Section 3) are labelled

H. binoei, peak 24 was strongly associated with both P_{mean} and PC1, and peak 26 was strongly associated with both P_{mean} and PC2. Only peak 29 was strongly associated with P_{mean} but did not overlap with high-loading peaks in the PCA of *H. binoei* chemical variation (but does load highly in the PCA when *H. planiceps* are included). These results suggest that chemical variation associated with precipitation has contributed to some of the major divergences amongst lineages.

4 | DISCUSSION

Studies of animal chemical signals have generally found levels of phylogenetic signal lower than that expected under Brownian

motion (Symonds & Elgar, 2004; Symonds & Gitau-Clarke 2016; Weber et al., 2016), including in lizards (Baeckens et al., 2018). We found, however, that variation in the composition of *Heteronotia* epidermal pore secretions is generally consistent with a BM model of trait evolution—although with notable discrepancies at two nodes (discussed below). This is similar to the results of Symonds and Wertheim (2005), who found high phylogenetic signal in the aggregation pheromones of *Drosophila*. They suggested that this is because *Drosophila* aggregation pheromones do not influence reproductive isolation (unlike contact pheromones; Antony & Jallon, 1982) and thus are not under selection for species specificity; versus the aggregation pheromones of bark beetles, which do influence reproductive isolation and evolve in rapid shifts, possibly due to

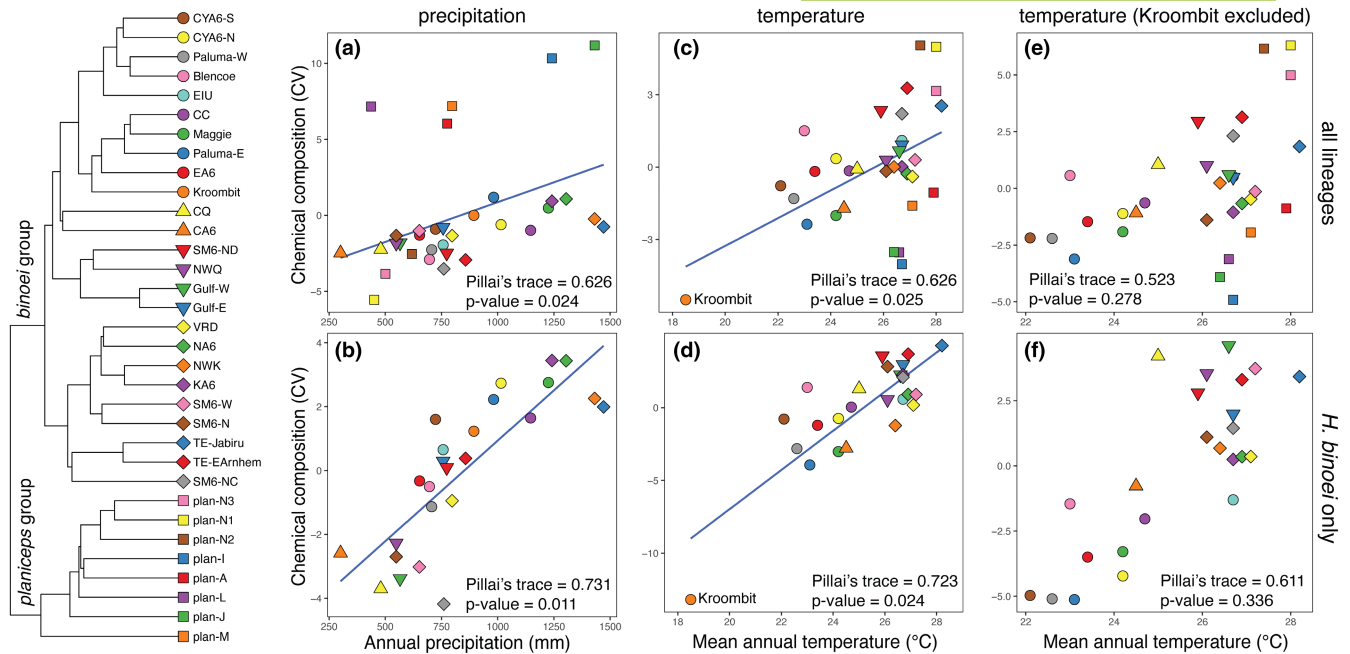


FIGURE 5 Amongst-lineage chemical variation in *Heteronotia* geckos is significantly associated with precipitation (a, b) but is only associated with temperature in models that include the outlying *Heteronotia binoei* Kroombit lineage (c, d vs. e, f without Kroombit), suggesting no overall association between chemical variation and temperature amongst lineages generally. Canonical variate (CV) axes represent the linear combination of chemical variation most closely associated with the respective predictor variable (see Section 2). A line of best fit is included for statistically significant relationships (the line is derived from the respective CV values and not the *mvGLS* model itself). The top panels (a, c, e) represent analyses that included lineages of both *H. binoei* and *Heteronotia planiceps*, whereas the bottom panels (b, d, f) represent analyses that included lineages of *H. binoei*. Symbols correspond to the phylogeny (left). Panel a illustrates the somewhat disparate responses of the *H. planiceps* plan-A–M lineages versus the remaining 28 *Heteronotia* lineages (including plan-N1–N3), possibly reflecting the phylogenetic structure in chemical variation (Figure 4a)

reinforcement and character displacement (Symonds & Elgar, 2004). 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 stabilizing and/or directional selection. Furthermore, the assumption that a signalling trait influencing reproductive isolation should deviate from BM might only be reasonably expected in systems with pervasive syntopy amongst closely related species (e.g. in the *Drosophila* and bark beetle examples above). For example, high phylogenetic signal has also been shown for components of frog calls (e.g. Escalona Sulbarán et al., 2019; Goicoechea et al., 2010), a multivariate acoustic trait with demonstrated strong links to mate choice and reproductive isolation (Gerhardt, 2010), and a trait for which reproductive character displacement is known to influence divergence amongst some populations and species (Hoskin et al., 2005; Lemmon, 2009). Similar results have been shown for bird vocalizations (e.g. Mejías et al., 2020). Depending on the perceptual capabilities of the receiver, even small changes in signalling traits could facilitate discrimination amongst populations and species, for example, in relative proportions of pheromone compounds in *Drosophila* (e.g. Higgin et al., 2000). Indeed, despite the stochastic mode of evolution we found across all *Heteronotia* lineages sampled here, even recently diverged lineages of *Heteronotia*

exhibit non-overlapping chemical blends in their secretions (Zozaya et al., 2019). If the epidermal pore secretions of *Heteronotia* do influence species discrimination—as has been suggested for other lizards (Barbosa et al., 2006; Gabriot et al., 2012; Labra, 2011)—then our results suggest that traits influencing behavioural isolation can also show patterns of high phylogenetic signal.

We also found strong support that the composition of epidermal pore secretions varies amongst *Heteronotia* lineages in association with geographic variation in precipitation but not temperature. These results add to the growing evidence that climate influences chemical signal composition in lizards (Baeckens et al., 2018; Campos et al., 2020; Kabir et al., 2020; Martín et al., 2015), although the prominent role of precipitation and absence of a role for temperature in our system conflicts somewhat with previous studies (discussed further below). Environmental factors are well known to influence the evolution of visual and acoustic cues (reviewed in Cummings & Endler, 2018), sometimes with consequences for signal divergence and behavioural isolation amongst closely related taxa (Boughman, 2002; Endler & Basolo, 1998; Seehausen et al., 2008). How the environment might similarly influence chemical signals is an area that, by comparison, has received little attention (Yohe & Brand, 2018). Our results are consistent with the hypothesis that—much like ambient light can influence the evolution of visual signals—geographic variation in precipitation can affect the evolution of

TABLE 2 Coefficients from the multivariate phylogenetic regressions of the chemical composition against annual precipitation (P_{mean}) for all 33 sampled *Heteronotia* lineages, as well as only the 25 lineages of *Heteronotia binoei*

Peak	P_{mean}	
	All lineages	<i>H. binoei</i>
1	0.146	0.053
2	-0.146	-0.145
3	-0.147	0.095
4	0.101	0.186
5	-0.085	0.190
6	0.082	0.069
7	-0.072	0.162
8	-0.117	0.044
9	-0.165	0.044
10	-0.182	-0.096
11	0.012	0.303
12	-0.148	0.185
13	-0.049	-0.040
14	-0.476	-0.393
15	-0.192	0.031
16	-0.159	0.279
17	-0.239	0.073
18	-0.275	-0.130
19	-0.153	-0.103
20	0.404	0.313
21	0.394	0.178
22	-0.234	-0.210
23	-0.515	-0.587
24	-0.360	-0.443
25	0.222	0.103
26	0.210	0.456
27	-0.220	-0.285
28	0.430	0.469
29	0.324	0.519

Note: Values represent the standard deviation (SD) change in the respective chromatogram peak per SD increase in P_{mean} (SD = 317 mm across all 33 *Heteronotia* lineages, or 304 mm across the 25 *H. binoei* lineages). Values are rounded to three decimal places, and those that are 70% or more of the highest weighted coefficient in the respective column are bolded.

chemical signals, driving signal divergence amongst populations and species. This is to be expected considering that high humidity and substrate moisture reduce the longevity of chemical cues and signals (Bossert & Wilson, 1963; Regnier & Goodwin, 1977; Royer & McNeil, 1993; Wilder et al., 2005). For polar compounds, this is likely driven by competition with water vapour for the same polar sites (i.e. on the keeper substance or substrate), causing faster evaporation (Regnier & Goodwin, 1977). Factors such as this might be

particularly relevant for driving chemical signal divergence in ecologically diverse radiations that occupy a broad range of climates.

High temperatures are known to shorten the longevity of animal chemical signals (Bossert & Wilson, 1963; Martín & López, 2013; Regnier & Goodwin, 1977), and studies of other lizard groups support an association between temperature and the composition of epidermal pore secretions (Baeckens et al., 2018; Campos et al., 2020; Martín et al., 2015). Why then did we find no association between temperature and amongst-lineage chemical variation in *Heteronotia*? One possibility is that our sampling did not include a sufficiently variable range of temperatures to detect an effect. We focused our sampling in tropical northern Australia, where the majority of *Heteronotia* diversity resides, but where temperature variation is modest. Another interesting possibility is that *Heteronotia*, and possibly other nocturnal lizards, are not as affected by the hotter temperatures experienced by diurnal lizards, such as the lacertid and *Sceloporus* lizards that are the focus of other studies. How such differences in behaviour and ecology might influence selection on chemical signals is an interesting direction for future study.

The relationship between precipitation and chemical composition in our study is dominated by variation in 5–8 chromatogram peaks, depending on whether lineages of *H. planiceps* were included in analyses. Although this represents a modest proportion of the total chemical blend (29 peaks analysed herein), the results of Martín et al. (2015) indicate that variation in just a few compounds could represent adaptation to disparate climatic environments in lizards. If some of the aforementioned compounds are linked to assortative mating, then pheromone divergence via local adaptation could influence behavioural isolation amongst lineages in secondary contact (Boughman, 2002; Endler & Basolo, 1998; Seehausen et al., 2008; Smadja & Butlin, 2009). Instead, if these compounds are keeper substances—or simply by-products of other physiological processes linked to climate (e.g. Heathcote et al., 2014; Slotsbo et al., 2016)—then such divergence may not be important for behavioural isolation unless the respective compounds are subsequently co-opted as signalling components (Endler & Basolo, 1998; Leonhardt et al., 2016). Epidermal pore secretions are released via holocrine glands, the product of which is produced by rupturing cells in the gland lining (Maderson & Chiu, 1970). Consequently, these secretions may contain by-products of cellular processes unrelated to chemical signalling. These considerations highlight the most difficult aspect of studying chemical signalling traits: identifying the functional components present within complex chemical blends.

Identifying functional components may change estimates of the phylogenetic signal discussed earlier. Because we do not know which components represent signalling compounds (i.e. pheromones), we may have under or overestimated the true magnitude of chemical signal divergence amongst lineages, and thus under or overestimated phylogenetic signal. Different functional components are likely under different selective pressures, meaning that estimates of phylogenetic signal for functional components will likely differ from secretions as a whole.

All chemical components associated with precipitation also overlapped with those most strongly contributing to the largest axes of chemical variation amongst lineages. This is consistent with the hypothesis that adaptation to climate has influenced amongst-lineage chemical variation. We hypothesize that adaptation to climate may have contributed to the stark divergences observed between *H. planiceps* and *H. binoei*, and between the *H. planiceps* plan-N lineages and the rest of *H. planiceps* (Figures 4a,b and 5a,b). Unlike the widespread *H. binoei* complex, *H. planiceps* are restricted to the Kimberley, a relatively moist region. Even within dryer, eastern parts of this region, *H. planiceps* are typically found in moist, sheltered landscapes, such as gorges and deeply dissected escarpments. This could account for the high proportions of certain peaks in *H. planiceps* (e.g. peaks 20, 21, 28, 29), peaks that are associated with increasing precipitation and that occur in smaller proportions in the three semi-arid limestone lineages of *H. planiceps* (plan-N1–N3), and the more widespread and habitat generalist lineages of *H. binoei*. Although circumstantial, these observations suggest that climate—particularly precipitation—might contribute to evolutionary shifts in the composition of chemical traits, whilst also constraining divergence amongst closely related lineages occupying climatically similar areas.

Animal chemical signals have long been of interest—particularly in mammals and insects (Symonds & Elgar, 2008)—but the difficulty of working with multivariate traits means that our understanding of chemical ecology and evolution remains in its early stages. Understanding the evolutionary drivers and consequences of chemical signal variation depends, amongst other things, on acquiring a more thorough understanding of the role of specific compounds. Doing so for multidimensional chemical traits involves detailed experimentation to test the bioactivity of dozens of compounds and an effectively infinite combination of compound ratios. Using multivariate regression, as we did here, can help to narrow down candidate compounds to inform subsequent manipulative experiments assessing the effects of compounds on behaviour. Whilst we have focused on understanding chemical signal variation in a cryptic species complex of Australian lizards, our approach may be valid for anyone studying chemical signals or other highly multivariate signalling systems.

AUTHOR CONTRIBUTIONS

S.M.Z. conceived the study with input on design and execution from all co-authors, acquired funding (in part), performed fieldwork, chemical characterization, statistical analysis, mtDNA phylogenetics, created all figures and wrote the initial draft; C.M. and L.C.T. provided exon capture data and performed the phylogenomic analysis; C.M. provided a vehicle for fieldwork; C.J.H. contributed to fieldwork; M.H. and C.J.H. provided additional funding and input on phenotypic analysis; all authors contributed to editing the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data associated with this study are available at the Dryad repository: <https://doi.org/10.5061/dryad.4j0zpc8dx>.

PEER REVIEW

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ORCID

Stephen M. Zozaya  <https://orcid.org/0000-0003-3451-3552>

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