



Original Article

Volatile fatty acid and aldehyde abundances evolve with behavior and habitat temperature in *Sceloporus* lizards

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Animal signals evolve by striking a balance between the need to convey information through particular habitats and the limitations of what types of signals can most easily be produced and perceived. Here, we present new results from field measures of undisturbed behavior and biochemical analyses of scent marks from 12 species of *Sceloporus* lizards to explore whether evolutionary changes in chemical composition are better predicted by measures of species behavior, particularly those associated with visual displays, chemoreception, and locomotion, or by measures of habitat climate (precipitation and temperature). We found that more active lizard species used fewer compounds in their volatile scent marks, perhaps conveying less specific information about individual and species identity. Scent marks from more active lizard species also had higher proportions of saturated fatty acids, and the evolution of these compounds has been tracking the phylogeny closely as we would expect for a metabolic byproduct. In contrast, the proportions of unsaturated fatty acids were better explained by evolutionary shifts in habitat temperature (and not precipitation), with species in warmer climates using almost no volatile unsaturated fatty acids. The proportion of aldehydes was explained by both behavior and environment, decreasing with behavioral activity and increasing with habitat temperature. Our results highlight the evolutionary flexibility of complex chemical signals, with different chemical compounds responding to different elements of the selective landscape over evolutionary time.

Key words: behavioral activity, chemical signal, femoral secretion, habitat temperature, *Sceloporus*, volatile organic compounds.

INTRODUCTION

Over evolutionary time, the structure of communicative signals is shaped both by the habitat through which signals must travel and by organismal constraints that limit the types of signals that can

be produced and received. Although many environmental and ecological factors have influenced the composition of animal chemical signals over time (reviewed in Alberts 1992; Endler 1992; Johansson and Jones 2007; Steiger et al. 2011; Wyatt 2014), selection often targets signal efficacy (Stuart-Fox et al. 2007; Steiger et al. 2011). Signal efficacy is directly enhanced or reduced by the physical characteristics of animal habitats (Alberts 1992; Endler 1993;

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Wyatt 2003). Additionally, different signal components may evolve to solve different efficacy issues (Hebets and Papaj 2005) or respond to different elements of a selective landscape such that trade-offs occur between signal modalities. This study aimed to describe the composition of chemical signals and behavioral use of scent marks in the diverse lizard genus, *Sceloporus* (Martins 1993a; Ossip-Klein et al. 2013; Wiens et al. 2013; Lawing et al. 2016), and to consider evolutionary relationships between chemical compounds, behavior, and climate.

Variations in environmental temperature or moisture impact the diffusion and persistence of animal scent marks (Alberts 1992; Endler 1992, 1993; Wyatt 2003), which diffuse into animal habitats at temperature- and humidity-dependent rates (reviewed in Wyatt 2014). Volatile compounds disperse faster in hot and wet habitats (Bossert and Wilson 1963; Regnier and Goodwin 1977) that increase evaporation rates and decrease signal persistence (Alberts 1992). Diffusion rates also depend, in part, on the physical properties of a compound as determined by its functional groups (see review Moore and Crimaldi 2004). To resist rapid signal diffusion and boost persistence, animals living in hot or wet habitats may evolve high proportions of such stable compounds (Alberts 1992; Scordato et al. 2007). Fatty acids, for example, may provide a stabilizing function and are common and abundant components of vertebrate signals (see reviews by Alberts 1992; Weldon et al. 2008; Apps et al. 2015; Mayerl et al. 2015). In rodents, saturated fatty acids could function as a waxy chemical matrix that holds onto other, more volatile signaling compounds, extending the persistence of scent marks (Hurst et al. 1998; Novotny et al. 1999; Wyatt 2014). In contrast, signals having high proportions of highly volatile compounds, such as aldehydes (Apps et al. 2015), may better suit species living in cool or dry habitats where these signals could last longer. Despite their high volatility and rapid diffusion, naturally pungent aldehydes are prevalent in social communication, being used in social signals of moths (reviewed in Byers 2006) and vertebrates, including birds, nonavian reptiles, and mammals (Burger 2005; Apps et al. 2015).

In addition, species social dynamics, such as the behavioral activities of signalers and receivers, likely also evolve with chemical signal composition. Receivers combat biological constraints on signal processing (Vickers 2000) by orienting chemosensory structures toward odor sources (Alberts 1992) and facilitate odor detection by using high rates of chemosensory behavior, such as antennal movements of crustaceans (Derby and Sorensen 2008) and insects (Hansson and Stensmyr 2011), sniffing in mammals (Catania 2006, 2013; Wachowiak 2011), and tongue flicking in squamates (Houck 2009; Mason and Parker 2010). Chemosensory and locomotory behavior that enhances signal transmission (Bossert and Wilson 1963) or detection can compensate for low proportions of important signaling compounds (Moore and Crimaldi 2004), especially when signals are less than optimally suited for their environments (Steiger et al. 2011). Furthermore, signalers can boost signal efficacy by emitting chemical signals at times, in locations, and under conditions that maximize the probability of detection as demonstrated in lemurs (Palagi and Norscia 2009) and goldfish (Appelt and Sorensen 2007) while minimizing other risks (e.g., predation; Endler 1992).

Behavioral thermoregulators rely on ambient temperatures to regulate physiological processes related to signal production and detection (Houck 2009; Mason and Parker 2010; Sinervo et al. 2010), making their chemical signals particularly susceptible to direct impacts of habitat temperature. Many lizards secrete waxy chemical plugs from femoral pores on their inner thighs (Cole

1966; Maderson 1972) and detect volatile compounds using olfaction and vomerolfaction (Duvall 1981; Mason and Parker 2010). In lizard chemical signals, fatty acids are energetically costly signals of quality (López et al. 2006; Martín and López 2010) and elicit strong chemosensory responses in conspecifics (Cooper et al. 2002). Some studies suggest that lizard chemical signals are shaped for stability in their environments (Alberts 1993; Baeckens et al. 2017a; Martín et al. 2017), being less behaviorally effective when presented under hot or dry conditions relative to native habitats (Martín and López 2013; Martín et al. 2015). In contrast, other studies show that lizard chemical signals are comprised of compounds that should rapidly volatilize (Baeckens et al. 2018) or be unstable in native habitats (Martín et al. 2016), suggesting that factors other than climate, such as species behavior, likely also impact chemical signal evolution. Species having longer activity periods can engage in more social communication, such as performing more visual or chemical signaling behavior, but the associated increase in fecundity is accompanied by increased predation and mortality risk during a given breeding season (Adolph and Porter 1993). Thus, chemical signal composition may reflect species differences in behavioral activity or behavior associated with social communication.

In this study, we used lizards of the genus *Sceloporus*, named for their secretory femoral pores. Femoral pore secretions mediate territorial interactions (Alberts and Werner 1993; Mason and Parker 2010; Campos et al. 2017), elicit chemical behavior (Hews et al. 2011; Pruett et al. 2016), and alter territorial space use (Campos et al. 2017). To explore the evolutionary history of lizard chemical signals, we examined how the volatile composition of femoral pore secretions has been changing with 1) climate (habitat temperature or precipitation) or 2) behavioral activity (including rates of chemosensory, locomotory, and visual displays) in a phylogenetic context. Using adult males across 12 species of *Sceloporus*, we filmed behavior in the field, collected and chemically analyzed secretions, and then added previously published data on climate and phylogeny. We focused on abundances of volatile saturated fatty acids, unsaturated fatty acids, and aldehydes but also tested whether chemical richness, defined here as the total number of different volatiles in species signals and often interpreted as complexity or conspicuousness (Slade et al. 2016; Baeckens et al. 2017a, 2018; Jänig et al. 2019), was associated with behavioral frequencies.

METHODS

We collected femoral pore secretions (plugs <1.0 mm diameter; Supplementary Figure S1) and recorded behavior of adult males between May 2012 and August 2016 at sites described in Table 1 as part of a more extensive study of the evolution of multimodal signals in *Sceloporus* lizards (Hews and Martins 2013; Martins et al. 2015; Pruett et al. 2016; Romero-Diaz et al. 2019). For each species, we collected behavioral and chemical data from the same population. Because breeding seasons vary in annual timing across species, we collected data at comparable points in their breeding seasons such that the intensity of male territorial aggression was at its highest (Rose 1981). We used several resources to determine the timing of breeding, including publications on populations within the regions of our study sites for each species, state-level field guides, or evaluation of animals in museum collections (i.e., reproductive status with collection dates and sites). A selection of publications we used is available in Table 1. Because reproductive timing can vary annually with environmental factors (Smith et al. 1995), we also confirmed mating activity through observed courtship and aggressive territorial displays by males. We first filmed a

Table 1
Species used in this study and brief descriptions of habitats, locations, and dates of data collection

<i>Sceloporus</i> species	Habitat	Location	Dates	Breeding	References
<i>S. graciosus</i>	High-elevation pine woodlands	San Gabriel Mountains of southern California, USA	May–June 2015	April–June	Goldberg (1975a, 1975b); Martins (1991, 1993a, 1993b)
<i>S. occidentalis</i>	Oak woodlands	Texas Hill Country, USA	May 2015	April–July	Goldberg (1973, 1975a)
<i>S. variabilis</i>				February–August	Benabib (1991, 1994); Granados-González et al. (2019)
<i>S. virgatus</i>	Oak-pine woodlands	Chiricahua Mountains, southeastern Arizona, USA	May 2016	May–July	Rose (1981); Ballinger and Ketels (1983); Smith et al. (1995)
<i>S. jarrovi</i>	Open mixed deciduous forest; cedar glades	Near Lake Monroe, IN, USA; Cedars of Lebanon State Forest, TN, USA	October 2013	September–October	Ballinger (1979); Moore (1986)
<i>S. undulatus</i>			June 2017; July–August 2016	Late April–June	Klukowski and Nelson (1998); Klukowski et al. (1998)
<i>S. megalepidurus</i>	Semiarid areas	Near San José Alchichica, Puebla, Mexico	September 2013	October–November	Vargas-García et al. (2019)
<i>S. grammicus</i>	Scrub forest	Nopala, Hidalgo, Mexico	October 2014	October–November	Vargas-García et al. (2019)
<i>S. merriami</i>	Rocky desert canyons	Big Bend Ranch State Park, TX, USA	May 2015	May–August	Grant and Dunham (1990)
<i>S. cozumelae</i>	Sandy beaches	Yucatan Peninsula near Puerto Morelos; Isla Blanca, Quintana Roo, Mexico	May 2013	May–September	Smith (1938); Maslin (1963); Fitch (1978); López González and González Romero (1997); Mendoza-Quijano et al. (1998); Ramírez-Bautista and Olvera-Becerril (2004)
<i>S. siniferus</i>	Tropical deciduous forest	Parque Nacional Huatulco, Oaxaca, Mexico	June–July 2012	May–August	Ramírez-Bautista et al. (2015)
<i>S. parvus</i>	Large boulder fields in the mountains	San Joaquin, Queretaro, Mexico	June 2013	April–June	García-Vázquez et al. (2014); Pruett (2014)

lizard and subsequently caught the lizard to collect its secretions, when possible.

Behavioral measures

We recorded the undisturbed behavior of 20–30 adult males per species during their respective breeding season, approaching individuals to an average distance of 4–5 m, and filming for up to 10 min during their daily activity period. Behavioral data from 4 of the 12 species (*Sceloporus parvus*, *Sceloporus cozumelae*, *Sceloporus merriami*, and *Sceloporus siniferus*) were also presented in Martins et al. (2015, 2018). We scored videos for chemical and visual displays and locomotion, then calculated species' averages for rates of behavior. *Chemical behavior*, involved in olfaction and vomerolfaction, included licks (tongue touch to a substrate), tongue flicks (tongue in the air), lip smacks (rapid opening and closing of the mouth), and chin wipes (jaw rub across a substrate). For contrast, we also counted the number of *headbob displays*, stereotyped and species-typical series of up-and-down movements of the head and torso used in social interactions. Many forms of chemical behavior occur as lizards move around their environments, so we also scored general activity levels in two ways. First, we counted the number of short *locomotion bouts* or episodes in which the subject moved distances <10 cm, which was often associated with chemical sensing or visual display behavior in this study. Second, we calculated the number of all behavioral acts as a measure of total *activity*, summing the total number of *short-distance locomotory movements* (<10 cm), *long-distance movements* (>10 cm), *chemical behavior* (tongue flicks plus jaw rubs, counted individually), *visual display behavior* (headbob displays plus shudder displays, counted

as *individual bouts*), *adjustments* (small motions of the head or torso that do not move the lizard from one place to another), *attacks*, and *tail wags*. Previous studies of social communication (Martins et al. 2015, 2018), reproduction, thermoregulation (Watters 2009), and survival (Grant 1990; Adolph and Porter 1993) use similar measures of total activity, allowing us to connect the present study to a broader body of literature. For each behavioral measure, we estimated the count of behavior per hour and used this frequency in statistical analyses.

Chemical analysis of secretions

We extracted secretions from lizard pores using alcohol-cleaned forceps and wearing nitrile gloves, and then stored the secretions in 2-mL glass vials with Teflon®-lined screw caps at –20 °C. At Indiana University's Institute for Pheromone Research, we used stir bar (Gerstel Twister® polydimethylsiloxane [PDMS]-coated magnetic stir bars) sorptive extraction to chemically analyze secretions (Soini et al. 2005; Pruett et al. 2016). For *Sceloporus graciosus*, *Sceloporus variabilis*, *Sceloporus occidentalis*, and *Sceloporus grammicus*, we analyzed samples from six individuals per species. Samples weighed 1.1–2.2 mg, 1.8–2.4 mg, 1.5–1.8 mg, and 2–2.7 mg for each species, respectively. For other species, we pooled samples from multiple individuals to obtain sufficient quantities for analysis. We combined secretions from 17 *Sceloporus virgatus*, 7 *Sceloporus jarrovi*, and 13 *Sceloporus megalepidurus* males to create six pooled samples per species weighing 1.6–2.4 mg, 1.8–2.5 mg, and 1.8–2.4 mg, respectively. We pooled secretions from 14 *Sceloporus undulatus* individuals into three samples, weighing 0.7–2.9 mg. For *S. cozumelae*, *S. parvus*, *S. siniferus*, and *S. merriami*, we used samples and measures previously described

in Pruett et al. (2016), which are two samples per species, each pooling secretions from 7–10 individuals and weighing 2 mg. We also added secretions from 13 *S. merriami*, pooled into four samples (2–4 males per sample, each weighing 1.5–2.0 mg), for a total of six pooled samples for this species.

We chemically analyzed samples using gas chromatography–mass spectrometry (GC–MS). We also processed reagent blanks (2 mL OmniSolv™ water [EMD Millipore Corporation, Billerica, MA] with 50 mg ammonium sulfate [Sigma-Aldrich, St. Louis, MO]) as a control. We added an internal standard (IS) of 8 ng of synthetic 7-tridecanone (Sigma-Aldrich, Saint Louis, MO) dissolved in 5 μ L methanol (Baker Analyzed, Mallinckrodt Baker Inc., Phillipsburg, NJ) to every sample. We analyzed volatile-embedded stir bars in a Thermal Desorption Autosampler with a Cooled Injection System (TDSA-CIS 4 from Gerstel GmbH) connected to an Agilent 6890 N gas chromatograph–5973iMSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE). Other equipment specifications, including oven program and temperature protocol details, are provided in Pruett et al. (2016).

We identified volatile compounds by comparing mass spectra and retention times against references, reference standard compounds when available, and the National Institute of Standards and Technology database. We used postrun selected ion currents and measured the peak area of each compound. To estimate the relative abundance of a compound, we normalized compound peak areas using the IS peak area and sample mass (compound peak area/IS peak area/sample mass). Then, we classified compounds into chemical classes based on functional groups, and calculated proportions of total volatiles made up of each chemical class. We calculated species' averages for chemical richness, or the total number of different volatiles, as a proxy for signal complexity (Hebets and Papaj 2005).

Climate measures

To test the hypothesis that habitat temperature or precipitation has been changing with species' volatile composition over evolutionary time, we used climate data deposited in the Dryad Digital Repository to support Lawing et al. (2016). Lawing et al. (2016) estimated mean climate profiles for each *Sceloporus* species by averaging bioclimate data (from WorldClim database) for each point at which that species has been reported (in the Global Biodiversity Information Facility). They found that BIO4 (temperature seasonality = standard deviation of temperature \times 100), BIO6 (lowest minimum temperature of the coldest month), BIO12 (mean annual precipitation), and BIO15 (precipitation seasonality = coefficient of variation) were especially important in describing changes in suitable *Sceloporus* habitat over evolutionary time. To these measures, we added BIO1 (mean annual temperature) in our analysis as a point of comparison to other studies. As the best available estimate of the climate experienced by the species as a whole, we used mean climate measures averaged across all recorded locations for each species. Although these measures may not accurately reflect the specific climates experienced by the lizards we sampled in the field, they are more appropriate for our phylogenetic comparative analyses of long-term evolutionary history. Furthermore, Parker and Mason (2009) demonstrated in snakes that annual periods of low-temperature dormancy (as occurs in many seasonal breeders) significantly boosts the synthesis and concentration of sex pheromones, suggesting that habitat temperatures during periods of behavioral inactivity can still be influential in shaping signal composition.

Phylogeny and statistical analyses

We fit separate phylogenetic generalized least squares regression models (PGLS: Martins and Hansen 1997) to estimate the inter-specific relationships between different types of behaviors or habitat measures (Xs) and the relative abundances of each major class of compound (Ys) while incorporating phylogeny (despite the large number of measures in our study, we did not use multivariate analyses because of the relatively small sample size in terms of the number of species). We used a recent phylogeny of *Sceloporus* (Wiens et al. 2013), pruned to include only the species relevant to this study, and fit models that reflect three sets of microevolutionary assumptions: 1) nonphylogenetic (TIIPS), assuming that species phenotypes are independent of one another, 2) PGLS-BM using Felsenstein's (1985) independent contrasts approach, applying a Brownian motion process to describe phenotypes that retain evidence of shared evolution along a phylogeny, and 3) PGLS-OU (Martins and Hansen 1997) using an Ornstein–Uhlenbeck process to model phenotypic evolution with some constraint, for example, due to stabilizing selection.

Despite the large number of measures in our study, our analyses were based on a relatively small sample size of 12 species, with the likely low statistical power being further reduced by the underlying phylogenetic relationships. Thus, we emphasized parameter estimation and model selection on a limited number of models with few parameters, instead of multivariate analyses or corrections for multiple comparisons. We used modules from *ape* (Paradis et al. 2004), *phytools* (Revell 2012), *phangorn* (Schliep 2011), *geiger* (Harmon et al. 2008), and *nlme* (Pinheiro et al. 2017) packages in R (R Development Core Team 2017) to estimate the parameters of these models, to compare fits in terms of Akaike information criteria (AIC), and to examine regression residuals to make sure they fit the assumptions of these analyses. We report results only for the best-fit models (as determined by lowest AIC value) and Pearson's product-moment correlations (r).

RESULTS

Sceloporus secretions are dominated by fatty acids

Fatty acids were abundant components of secretions produced by adult males of all 12 *Sceloporus* species (Figure 1). Over 76% of volatiles detected in five species were fatty acids (saturated and unsaturated combined), ranging from 94% (*S. grammicus*) to 76% (*S. undulatus*). Another five species had 42% (*S. merriami*) to 62% (*S. occidentalis*) fatty acids in secretions. Only *S. megalepidurus* and *S. cozumelae* had relatively low proportions (26%) of fatty acids. More specifically, saturated fatty acids made up 26% (*S. cozumelae* and *S. megalepidurus*) to 62% (*S. grammicus*) of total volatiles, whereas unsaturated fatty acids made up 0% (*S. megalepidurus*) to 58% (*S. graciosus*) of volatiles. Saturated fatty acids were more abundant than unsaturated for all species except *S. jarrovi* and *S. graciosus*. Two unsaturated fatty acids (oleic and linoleic acids) and nine saturated fatty acids (especially tetradecanoic, hexadecanoic, and octadecanoic acids) were particularly abundant. Aldehydes made up between 0% and 20% of total volatiles, averaging 6% across all species. Nonanal and decanal were the most abundant aldehydes.

We found a total of 47 volatile compounds representing 10 chemical classes (Figure 1). Average chemical richness ranged from 8 to 30 volatiles per species. Most species (7 of 12) produced between 25 and 30 compounds (*S. parvus*, *S. variabilis*, *S. cozumelae*, *S. siniferus*, *S. merriami*, *S. graciosus*, and *S. occidentalis*). Sister taxa,

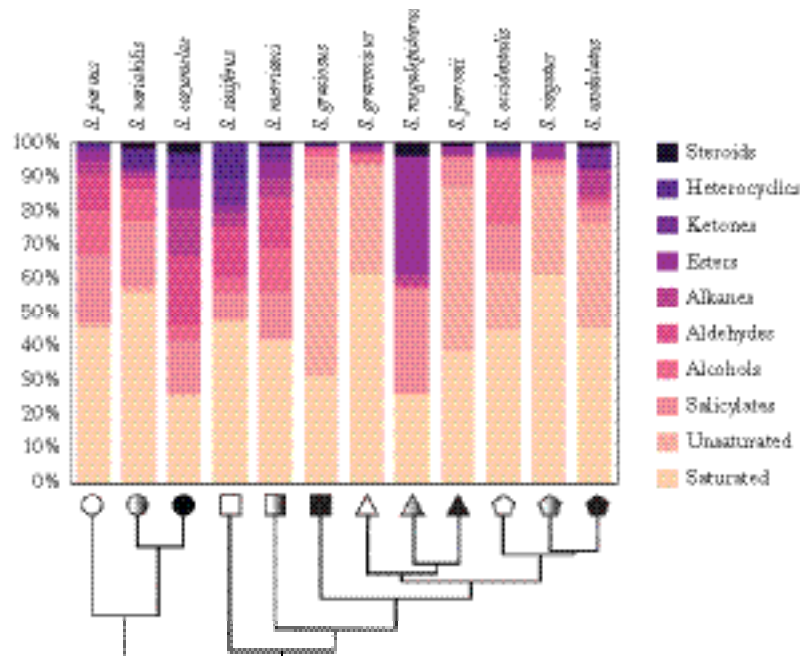


Figure 1

Percentage of total measured volatiles made up of different chemical classes in *Sceloporus* secretions. The phylogenetic tree on the x axis, pruned from over 90 species in Wiens et al. (2013) to the 12 used in this study, shows species relationships, and each unique symbol represents a different species and all remaining figures use these symbols.

S. undulatus and *S. virgatus*, produced fewer compounds (18 and 14, respectively), as did the three live-bearing species, *S. grammicus* (15), *S. jarrovi* (12), and *S. megalapidurus* (8).

Many species had two or three other classes making up large proportions of their secretions. Salicylates, alcohols, and aldehydes made up over 41% of volatiles in *S. parvus* and *S. merriami* secretions, whereas 50% of *S. cozumelae* secretions were made up of salicylates, aldehydes, and alkanes. *Sceloporus megalapidurus* secretions contained large proportions of salicylates (31%) and esters (35%), whereas secretions of *S. occidentalis* had large proportions of salicylates (14%) and alcohols (19%). Salicylates were also an abundant component of *S. jarrovi* (9%), *S. graciosus* (7%), and *S. variabilis* (20%). Secretions of *S. variabilis* also had 9% alcohols. Secretions of *S. siniferus* consisted of 15% aldehydes and 11% heterocyclic compounds (two novel volatile cyclic dipeptides, cyclo[L-Leu-L-Pro] and cyclo[L-Pro-L-Pro]; Romero-Diaz et al. 2020), whereas 15% of *S. undulatus* secretions were composed of alkanes and methyl ketones. Secretions of *S. grammicus* and *S. virgatus* were somewhat unusual in having all remaining classes of compounds comprising less than 4% of secretions.

The stir bar sorptive extraction and analysis method is classified as a solventless trace-analysis method suitable for biological and environmental samples (Baltussen et al. 2002; Soini et al. 2005, 2009, 2013). Detection levels of compounds in the *Sceloporus* lizard femoral pore secretions were about 0.02×10^6 peak area counts corresponding to about 0.1 ng of these compounds.

Does volatile composition evolve with habitat temperature or precipitation?

Here, we report only results for best-fitting phylogenetic comparative models, which are also summarized in Supplementary Table S1, including the model (nonphylogenetic TIPS, Ornstein–Uhlenbeck process PGLS-OU, or Felsenstein’s (1985) Brownian

Motion process PGLS-BM), *P*-values, and Pearson’s product-moment correlation *r*-values. Degrees of freedom (df) = 10 for all models in this study.

Proportions of unsaturated fatty acids vary with habitat temperature, not precipitation

Species living in colder habitats had secretions with greater proportions of unsaturated fatty acids. This relationship was strong and consistent for measures of BIO1, annual mean temperature, and BIO6, minimum temperature of the coldest month (BIO1: $r = -0.8$, $P < 0.01$; BIO6: $r = -0.8$, $P < 0.01$; Figure 2a). Proportions of unsaturated fatty acids increased with BIO4, temperature seasonality ($r = 0.8$, $P < 0.01$; Figure 2b). The TIPS model treating phylogenetic relatedness as unimportant was the best-fitting model for BIO1 based on the lowest AIC value, and PGLS-BM was best-fitting for BIO4 and BIO6, suggesting that these relationships retain evidence of shared evolution along a phylogeny. We found no significant relationships between habitat precipitation and species’ proportions of unsaturated fatty acids (BIO12: TIPS $r = -0.2$, $P = 0.5$; BIO15: PGLS-BM $r = -0.01$, $P = 0.6$).

Aldehyde proportions increase with habitat temperature, not precipitation or seasonality

Species’ proportions of aldehydes increased with habitat temperature (Figure 2c) based on mean annual temperature (BIO1: TIPS $r = 0.8$, $P < 0.01$) and minimum temperature of the coldest month (BIO6: TIPS $r = 0.8$, $P < 0.01$). Relationships between aldehyde proportions and minimum temperature (BIO4: TIPS $r = -0.4$, $P = 0.2$), precipitation seasonality (BIO15: TIPS $r = -0.02$, $P = 1$), and mean annual precipitation (BIO12: TIPS $r = 0.06$, $P = 0.9$) were weaker and not statistically significant. Phylogenetic relatedness explained very little of these relationships such that these best-fit estimates are from TIPS models.

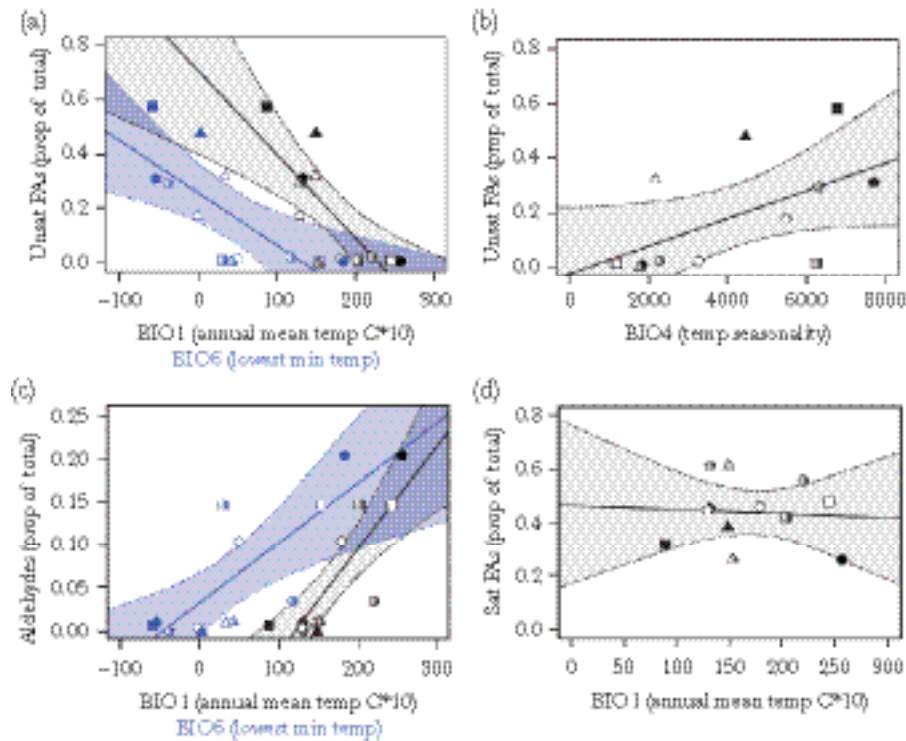


Figure 2

Relationships between habitat temperature and species' proportions of unsaturated fatty acids (a, b), aldehydes (c), or saturated fatty acids (d) in volatile signals. All regressions in figures come from TIPS models and shaded regions represent 95% confidence intervals on the slope of the regression. Symbols represent the same species as in Figure 1. (a) As annual mean temperature and the lowest minimum temperature of habitats increased, species' proportions of unsaturated fatty acids significantly decreased ($r = -0.8$ for both) and (c) aldehydes significantly increased ($r = 0.8$ for both). (b) Unsaturated fatty acid proportions also significantly increased with temperature seasonality ($r = 0.8$), but (d) proportions of saturated fatty acids did not change with mean temperature ($r = -0.07$).

Saturated fatty acids and chemical richness are not associated with climate

We found no evidence for relationships between the proportions of saturated fatty acids and any measures of habitat temperature (BIO1: TIPS $r = -0.07$, $P = 0.8$; Figure 2d; BIO4: TIPS $r = 0.07$, $P = 0.8$; BIO6: TIPS $r = -0.1$, $P = 0.7$) or precipitation (BIO12: TIPS $r = 0.2$, $P = 0.4$; BIO15: TIPS $r = 0.3$, $P = 0.4$). Phylogeny explained little of these relationships, and estimates are for best-fitting nonphylogenetic TIPS models.

Similarly, we found no association between the chemical richness of species' signals and any temperature (BIO1: TIPS $r = 0.4$, $P = 0.2$; BIO6: TIPS $r = 0.3$, $P = 0.3$) or precipitation measures (BIO12: TIPS $r = 0.06$, $P = 0.9$), including seasonality (BIO4: TIPS $r = 0.07$, $P = 0.8$; BIO15: PGLS-BM $r = -0.5$, $P = 0.9$). The TIPS model was best fitting for all except one of these relationships (BIO15), for which the PGLS-BM model was best fit, although the TIPS model yielded similar results.

Does volatile composition evolve with behavior?

Rates of chemical behavior were consistently lower than were rates of headbob displays and short bouts of locomotion (Figure 3). Headbob display rates were lower than short bouts of locomotion for only two species (*S. merriami* and *S. undulatus*), but both behavioral rates were very similar in *S. jarrovi*. As above, we report only the results for best-fitting phylogenetic comparative models and $df = 10$ for all models.

Saturated fatty acids, but not unsaturated, increase with activity and chemical behavior

Species that produced greater proportions of saturated fatty acids in secretions also performed higher rates of activity whether measured as overall activity levels (PGLS-BM $r = 0.9$, $P << 0.01$), short bouts of locomotion (PGLS-BM $r = 0.9$, $P << 0.01$), or chemical behavior (PGLS-BM $r = 0.9$, $P << 0.01$; Figure 4a,b). All models produced similar results, and PGLS-BM was the best-fitting model, suggesting that these relationships have evolved closely with the phylogeny. We found little evidence of an evolutionary relationship between saturated fatty acid proportions and the number of headbob displays (TIPS $r = 0.3$, $P = 0.4$).

Similarly, we found no relationship between unsaturated fatty acids and behavior, whether scored as rates of overall activity (TIPS $r = 0.4$, $P = 0.1$), chemical behavior (TIPS $r = 0.3$, $P = 0.4$), short bouts of locomotion (TIPS $r = 0.4$, $P = 0.2$), or headbob displays (TIPS $r = 0.5$, $P = 0.1$) based on the best-fit TIPS models, although all models yielded similar results.

Aldehyde proportions have been changing with rates of activity and locomotion

Species that used low aldehyde proportions (<5% of total measured volatiles) also displayed high levels of activity (115–367 behavioral acts per hour), while species with high aldehyde proportions (10–23% of volatiles) engaged in less measurable behavior (73–111 behavioral acts per hour) and resulted in a negative correlation between mean activity rates and aldehyde proportions (TIPS $r = -0.6$, $P < 0.05$; Figure 4c).

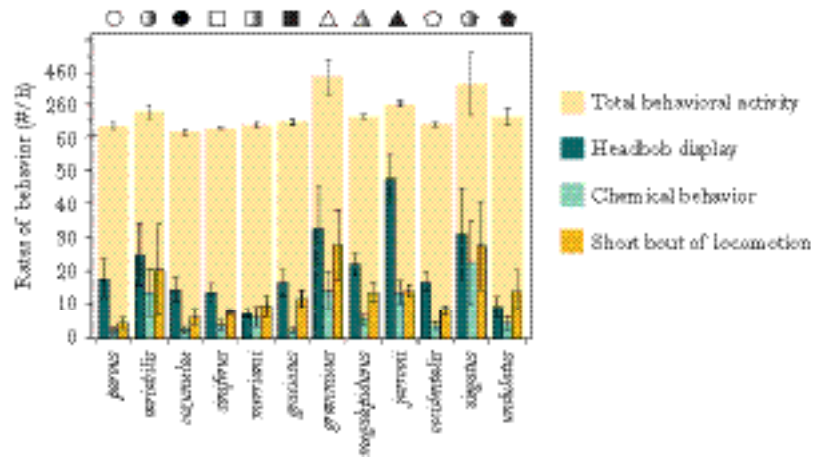


Figure 3

Mean rates of baseline behavior in numbers per hour (#/h) for *Sceloporus* species in this study. Parallel lines mark a shift in scale on the y axis. Bars represent standard errors.

We found a similar negative relationship between aldehyde proportions and short bouts of locomotion (TIPS $r = -0.6$, $P < 0.05$; Figure 4d) and only weak relationships with rates of chemical behavior (TIPS $r = -0.5$, $P = 0.1$) or headbob displays (TIPS $r = -0.5$, $P = 0.08$).

Chemical richness decreases as activity and headbob displays increase

We found a negative relationship between chemical richness and headbob display rates (TIPS $r = -0.6$, $P < 0.05$; Figure 4f), as well as activity rates (TIPS $r = -0.6$, $P < 0.05$; Figure 4e). We found only weak trends between chemical richness and short bouts of locomotion (TIPS $r = -0.5$, $P = 0.08$) or rates of chemical behavior (TIPS $r = -0.5$, $P = 0.1$).

DISCUSSION

Our results demonstrate that volatile composition has been changing with both habitat temperatures and behavioral activity during the evolution of *Sceloporus* lizards. We report three novel findings regarding the evolution of volatile signals in vertebrates: 1) proportions of unsaturated fatty acids have been decreasing and aldehydes increasing with habitat temperatures; 2) proportions of saturated fatty acids have been increasing and aldehydes have been decreasing with species rates of behavioral activity; and 3) less active species use more complex chemical signals.

Fatty (carboxylic) acids dominated volatile signals of *Sceloporus* males, consistent with results in other lizard taxa using precloacal and femoral gland secretions (Weldon et al. 1990; Alberts 1992; Escobar et al. 2001; Louw et al. 2007; Gabirot et al. 2008). In reptiles, fatty acids are stored primarily for reproductive purposes (Price 2017), being allocated toward egg and sperm production (Derickson 1976; Simandle et al. 2001) and transported to developing offspring in live-bearing species (Van Dyke et al. 2014). Thus, diverting fatty acids away from reproductive investment and toward chemical signals may be a costly process. Additionally, three of the four species that produced signals with the lowest richness are live-bearing (*S. megalepidurus*, *S. jarrovi*, and *S. grammicus* [Qualls and Andrews 1999; Andrews 2000; Shine 2004]), along with oviparous *S. virgatus*, suggesting a possible link between maternal investment in reproduction and male investment in volatile production. In contrast with our results, one study by Gabirot et al. (2008) found no

differences in chemical richness between oviparous and viviparous populations of *Lacerta vivipara* lizards, although the lizard strains in the 2008 study are still from the same species and capable of hybridization, which may explain the qualitative similarities in chemical signals. Associations between birth mode and chemical richness may be indirect or genus specific.

Compound abundances change with habitat temperature, not precipitation

Species in cool habitats use more unsaturated fatty acids

Functionally, unsaturated fatty acids might enhance signal efficacy in cooler habitats due to higher vapor pressures relative to saturated fatty acids. Species producing large proportions of unsaturated fatty acids also tended to live in habitats reaching lower temperatures based on low annual temperatures and greater temperature seasonality and produced little to no unsaturated fatty acids in habitats reaching annual mean temperatures above 15 °C in this study (Figure 2a). We found no evidence to support the hypothesis that proportions of saturated fatty acids have been changing with habitat temperature or precipitation. Unsaturated fatty acids remain in a liquid state at low temperatures and more readily volatilize relative to waxy saturated fatty acids, which potentially trap other important volatiles in a thickened chemical matrix. For example, linoleic acid, the most abundant unsaturated fatty acid on average in this study, has a vapor pressure over 20× greater than the saturated fatty acid octadecanoic acid (Kim et al. 2016; another compound found in *Sceloporus*) at behaviorally active temperatures for this genus (25 °C) despite both compounds having an 18-C chain skeleton.

Ectothermic physiology likely plays some role in chemical signal evolution such that compound abundances could arise from indirect selection on the physiological benefits these compounds provide to animals. Studies from insect ectotherms demonstrated changes in qualitative and quantitative aspects of chemical signals with body temperatures (reviewed in Boullis et al. 2016), and Heathcote et al. (2014) showed that the amount of time lizards spend basking (*Podarcis muralis*) significantly alters the composition of femoral pore secretions. Compounds may begin as byproducts of necessary metabolic processes (Henneken et al. 2017) before gaining communication function over evolutionary time (reviewed

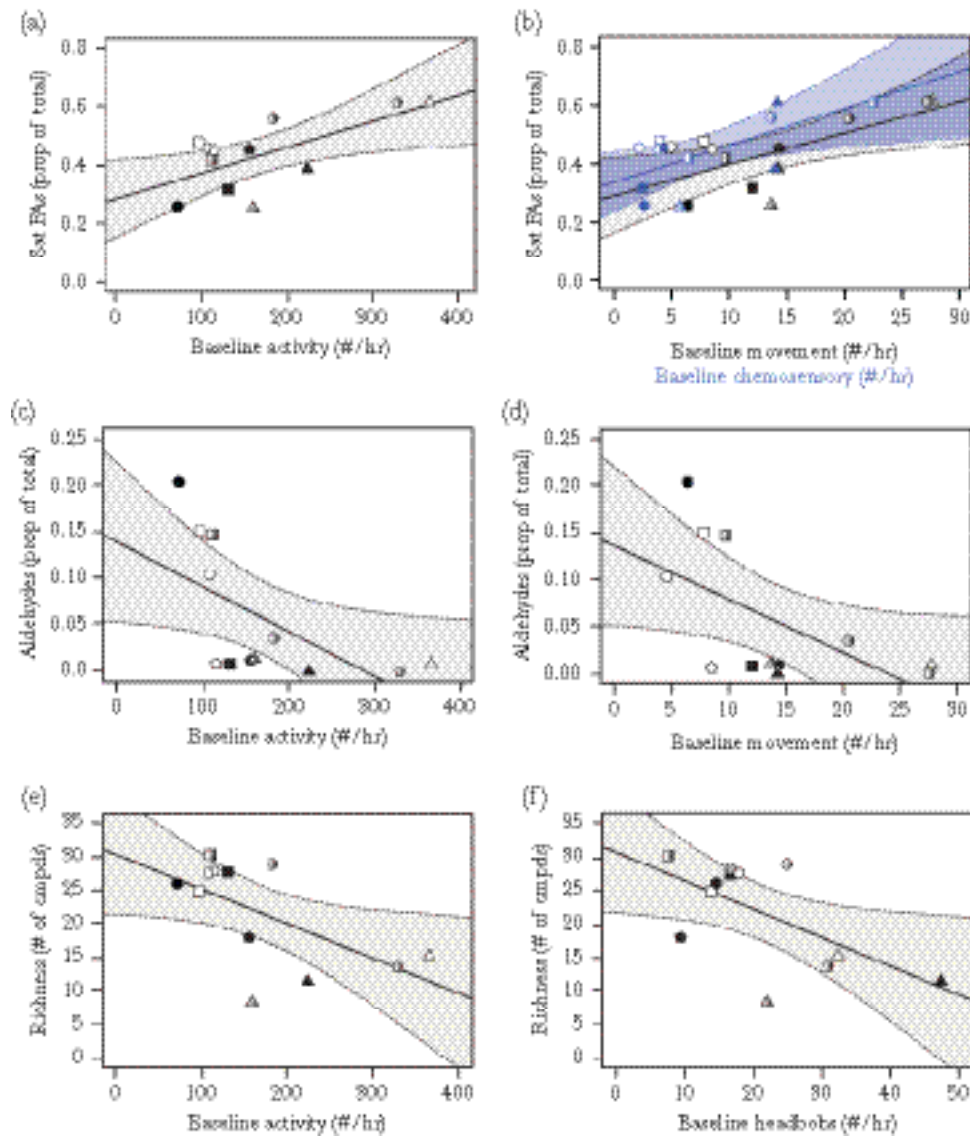


Figure 4

Significant relationships between species' rates of behavior and chemical signal composition. All regressions in figures come from TIPS models, and shaded regions represent 95% confidence intervals. Symbols represent the same species as in Figure 1. (a, b) Proportions of saturated fatty acids in species' secretions increased with rates of (a) behavioral activity and (b) short bouts of locomotion or chemical behavior (in blue; for a and b: PGLS-BM $r = 0.9$). (c, d) Species' proportions of aldehydes decreased as rates of (c) activity or (d) locomotion increased (for c and d: TIPS $r = -0.6$). (e, f) Chemical richness decreased as rates of (e) activity or (f) headbobs increased (for e and f: TIPS $r = -0.6$).

in Endler and Basolo 1998; Blomquist and Vogt 2003; Wyatt 2014; Leonhardt et al. 2016). Animals that have adapted to cold environments incorporate more unsaturated fatty acids into their membranes and tissues (Geiser and Kenagy 1987; Simandl et al. 2001; Slotsbo et al. 2016) and the same may be true for femoral gland cells such that compounds already present in gland cells incidentally become incorporated into secretions via regular turnover of glandular cells (Cole 1966; Maderson 1972).

This study, along with evidence from other studies, suggests possible interaction effects between the abundances of unsaturated fatty acids in lizard secretions, habitat temperatures, and body size. Our data link high proportions of unsaturated fatty acids to low habitat temperatures and do not include snout-vent-length (SVL) measures for each species. However, other researchers have shown that most *Sceloporus* species obey an inverse Bergmann's rule with smaller

animals inhabiting colder sites, enabling them to rapidly reach high enough body temperatures to meet metabolic demands (Ashton et al. 2003), suggesting that small-bodied species could also produce high abundances of unsaturated fatty acids. In further support of this interaction effect, one study demonstrated a negative relationship between body size based on SVL and abundance of unsaturated linoleic acid in glandular secretions using *Lacerta monticola* lizards (López et al. 2006). Future work using multifactor analyses are necessary to determine direct relationships involving all three factors.

Species in hot habitats use more aldehydes

High habitat temperatures, and not precipitation, were associated with high proportions of aldehydes in this study. This result was surprising because aldehydes are susceptible to oxidation (Apps et al. 2015) and should rapidly dissipate in hot environments, leading to a

short-lived signal. Lizards are forced to seek thermal refuges when diurnal temperatures become too hot, limiting the time for daily activities (Sinervo et al. 2010; Lawing et al. 2016). Thus, high proportions of potent aldehydes may be advantageous to species evolving in hot habitats by quickly transmitting information to receivers. Such signals would likely demand rapid receiver responses. This idea is consistent with the van Damme et al. (1990) finding that a lizard's tongue flick at higher rates in hot habitats. Our results are also partially consistent with the Baeckens et al. (2018) finding that high aldehyde proportions were associated with mesic (hot and wet) habitats in Lacertidae lizards but inconsistent with the Martín et al. (2017) finding that aldehyde proportions decreased with habitat temperatures and increased with precipitation in *Psammodromus algirus* lizards. Such differences may be partially explained by the types of aldehydes present, the inclusion of nonvolatile compounds in these other studies, or by compensation for rapid diffusion with other waxy compounds (e.g., waxy esters; Gabirot et al. 2008) or species behavior and highlight the importance of replicating evolutionary studies across different genera.

One major aldehyde component of *Sceloporus* secretions, nonanal, is attractive to mosquitoes (Syed and Leal 2009) and other blood-feeding insects (Guerenstein and Guerin 2001), which *Sceloporus* mainly prey on (Huey and Pianka 1981). If prey items are attracted to secretions, the high abundance of aldehydes may benefit species in hot habitats that have restricted feeding windows, especially because *Sceloporus* are sit-and-wait foragers. In contrast, attracting mosquito disease vectors could increase risks of malaria, impacting lizard metabolism, reducing energetic function, and altering social display rates (Jos and Sarni 1987).

Compound abundances change with behavioral activity

More active species produce more saturated fatty acids

We show, for the first time, to our knowledge, a link between lizard behavior and saturated fatty acid content of lizard secretions, finding that proportions of saturated fatty acids in lizard secretions increased with activity rates, a relationship that has evolved along a phylogeny. Because these results were consistent for measures of chemical behavior, locomotion, and total behavioral activity (Figure 4a,b), this suggests that general levels of behavioral activity may be an important behavioral predictor of saturated fatty acid abundances. Because very little data exist on evolutionary relationships between species behavior and chemical signal composition, this result is an important step toward understanding such relationships.

Several types of data suggest that saturated fatty acids may be key components of femoral gland secretions. Green iguanas tend to perform more tongue flicks toward the lipid fractions of femoral gland secretions than to the protein fractions (Alberts and Werner 1993), and rock lizards perform a tongue flick faster toward the saturated fatty acid hexadecanoic acid (a major component of *Sceloporus* secretions) than toward a pungency control (dichloromethane; Martín and López 2010). Similarly, high abundances of saturated fatty acids in *Sceloporus* secretions may elicit higher rates of chemosensory behavior because these components are more salient. In some *Sceloporus* species, tongue flicks directed at substrates often occur immediately following a translational movement (Cooper et al. 1994), especially in novel environments (Simon et al. 1981), and may explain why we found a similar relationship between saturated fatty acids and locomotion in our data. While higher rates of chemosensory behavior are performed after foraging or biting prey items

in active foragers (Cooper 1992), at least two species of *Sceloporus* do not use chemical cues to locate or identify prey items, to choose ambush sites for foraging (Cooper 1989, 2003), or to detect natural predators (Simon et al. 1981). Thus, rates of chemosensory sampling in *Sceloporus* may be more related to social communication than to foraging rates (but see Goldberg et al. 2019). While variation in population densities in this study may have impacted rates of chemosensory sampling, Simon et al. (1981) directly tested this hypothesis in *S. jarrovi* and found no significant differences in substrate licks between a low- and a high-density population.

The significant contribution of dietary fatty acids to whole-animal physiology suggests that species differences in diets could also impact the abundance of free fatty acids in secretions. Lizards alter the fatty acid content of their secretions either via diet, passive deposition into gland cells, or by selectively incorporating fatty acids into secretions (Geiser et al. 1992), and these possibilities are not mutually exclusive. Supplementing male diets with different vitamins alters female preferences for male scent (Martín and López 2006; Kopena et al. 2011), suggesting that subtle dietary differences might impact signal composition across species (but see Baeckens et al. 2017a). *Sceloporus* primarily consume different insects with opportunistic supplementation of diets with snails or lizard hatchlings or tails (Rose 1976; Ballinger and Ballinger 1979; Leyte-Manrique and Ramírez-Bautista 2010; Watters 2010; Hierlihy et al. 2013). Highly active species may consume more insects with higher proportions of saturated fatty acids, or already have a high content of saturated fatty acids in their tissues, incorporating these fatty acids into secretions. Fatty acid contents of lizard membranes (phospholipids) or fat stores (triacylglycerols) can reflect fatty acid proportions in the diets in desert iguanas (Simandle et al. 2001). Although food limitation can alter the fatty acid contents of lizard tissues (McCue 2008), such that food availability may impact fatty acids in secretions, in at least one of our study species, severe food limitation may not be likely (Ballinger 1981).

In addition to direct impacts of diet, other factors may also contribute to species differences in fatty acid content of femoral pore secretions. Diets high in unsaturated fatty acids lower preferred basking temperatures of lizards, whereas lizards with high saturated fatty acid diets select higher basking temperatures (Geiser and Learmonth 1994), suggesting broadly that dietary fatty acids impact thermoregulation and behavior, and may indirectly impact secretions. Furthermore, species differences in levels of circulating free fatty acids, which are impacted by testosterone, could be associated with differences in abundances of free saturated fatty acids in lizard secretions. Reproductive male *Urosaurus ornatus* lizards have significantly lower circulating free fatty acids than late reproductive males despite having similar rates of lipid storage and release, suggesting that differences in breeding activities between males in different reproductive stages may be explained by energy derived from circulating lipid substrates (see Lacy et al. 2002). Future studies should determine the primary factors that influence free fatty acid composition in lizard femoral pore secretions and the extent to which diet, metabolism, and behavioral activity impact fatty acids in secretions.

Less active species produce more odorous secretions

From an efficacy perspective, the odorous properties of aldehydes (Larrañaga et al. 2016) and complex composition of volatile-rich signals (lizards [Baeckens et al. 2018] and lemurs [delBarco-Trillo et al. 2012]) could enhance signal detectability (see Guilford and Dawkins 1991) in species that move around less or come into contact with conspecific secretions less frequently. We found that species

with more odorous secretions, based on high aldehyde proportions and volatile richness, were also less behaviorally active, and richness was negatively associated with locomotion, although this latter relationship only approached significance. Animal locomotor patterns are determined by available energy and costs of movement (Shepard et al. 2013) such that more fragrant secretions could benefit less active species, or species that move less, by inviting conspecific attention from greater distances (whether the odor is attractive or repulsive). It is also possible that a sedentary lifestyle allows for volatile-rich secretions by shifting energetic costs away from behavioral activities and toward the production of volatiles.

We might expect species producing signals with high chemical richness, which were also less active in this study, to live in denser populations such that rich signals allow for the identification of a greater number of different individuals in crowded habitats. For at least one species, *S. merriami*, populations with a low density of adults could extend home ranges into larger areas than populations with a high density (Ruby and Dunham 1987). Thus, high-density species confined to smaller home range areas may use richer chemical signals to allow conspecifics to identify a greater number of individuals based on odor cues. A similar pattern has been detected in primates such that multimale–multifemale species produce richer chemical signals compared to pair-bonded species (delBarco-Trillo and Drea 2014).

Many factors influence population density in lizards, such as predation, migration, congeneric, or conspecific competition (Schoener and Schoener 1980; Stamps et al. 1997). In our study, *S. jarrovi* overlapped with *S. virgatus*, and each produced signals with low richness (12 and 14, respectively), whereas *S. graciosus* overlapped with *S. occidentalis* (Martins 1991) and both species produced richer signals (28 each). Thus, congener overlap alone may not be a major factor contributing to variation in volatile richness. Complex signals have also been linked to high intrasexual competition in lizards (Ord and Garcia-Porta 2012) and, in primates, females from female-dominant species produce chemical signals with higher chemical richness compared to males (delBarco-Trillo and Drea 2014). Thus, *Sceloporus* species with higher male–male competition may have selected for chemical signals with higher richness to improve male assessment of competitors or to aid in individual recognition. A phylogenetic study found no relationship between interspecific variation in chemical signal complexity and intensity of male–male competition based on sexual dimorphism in shape and size across 60 species of Lacertidae lizards Baeckens et al. (2017b). These results suggest that chemical signal complexity is not associated with male–male competition or that other measures (e.g., proportion of activity period allocated to aggression) are more important. Female mate choice in lizards is rare (Olsson and Madsen 1995; Tokarz 1995; Lailvaux and Irschick 2006) and probably not a strong driver of male–male competition in *Sceloporus*. The link between signal composition and population densities or intraspecific male–male competition remains an interesting avenue for future research.

Trade-offs in chemical complexity

Species that used secretions with high chemical richness were also less active and performed headbob displays less frequently, revealing trade-offs between chemical complexity and rates of visual display behavior or total behavioral activity. In this study, *S. merriami* produced signals with the highest richness and performed the lowest rates of visual signals based on headbob behavior. Complex signals

can evolve with changes in social dynamics, such as increasing intrasexual competition in lizards (see section above) or social group sizes in primates (delBarco-Trillo et al. 2012). Our results advance this perspective by suggesting that chemical signal complexity is related to behavioral use of the signal. In animals that use multiple sensory channels, an evolutionary change in one signal component can potentially impact other behavioral and sensory aspects of senders and receivers (Hews and Martins 2013), and such trade-offs in chemical and visual display behavior have been documented in *Sceloporus* (Hews and Benard 2001; Thompson et al. 2008; Hews et al. 2011; Martins et al. 2018). Complex signals may be energetically costly for signalers to produce and for receivers to maintain the sensory machinery to detect such complexities (Niven and Laughlin 2008; Wilson et al. 2013). The message being conveyed by the chemical complexity of scent marks, which selection can act upon as a functional unit in animals (Hebets and Papaj 2005), may be the same or similar to the message conveyed in the visual modality (Martins et al. 2018) such that this redundancy in information is not favored by other ecological or social aspects of the species (Bro-Jørgensen 2010). Nonetheless, the specific environmental or ecological conditions that favor signal complexity in the chemical modality warrant further investigation.

We found 47 different compounds in this study and between 8 and 30 compounds per species. Our richness measures are comparable to those reported for glandular secretions in other lizard species using similar GC–MS methods (11–26 per *Liolaemus* species [Escobar et al. 2001]; 44 total [Escobar et al. 2003]). GC–MS methods target volatile organic compounds that are nonpolar and have low molecular weights; hence, different results may be obtained using different methods that target other nonvolatile (see Alberts et al. 1993), polar or high molecular weight compounds. As an example, Louw et al. (2007) identified 53 compounds in the femoral pore secretions of *Cordylus giganteus* lizards using GC–MS and 173 compounds using GC–FID (flame ionization detector) analysis. *Sceloporus* likely also produce proteins or other nonvolatile compounds having large molecular weights, for which conspecific detection requires direct contact with the secretion. Therefore, patterns reported here may differ using other chemical analysis techniques, outside of the breeding season when secretory production is lower in *Sceloporus* (Martins et al. 2006), or using scent glands other than femoral (Mayerl et al. 2015), such as preloacal (Escobar et al. 2001; Escobar et al. 2003; Pincheira-Donoso et al. 2008), cloacal, or generation glands or glandular scales (van Wyk and Mouton 1992).

CONCLUSION

Lizard investment in or reliance on the chemosensory modality is often estimated using numbers of scent glands, although the available data on how this measure relates to climate and behavior is conflicting (Escobar et al. 2001; Pincheira-Donoso et al. 2008; Iraeta et al. 2011; Baeckens et al. 2015; Jara et al. 2018). By including behavioral data in phylogenetic studies of animal chemical signals, we can test many of the hypotheses that large chemical and climate data sets have generated. Our study contributes behavioral and chemical data on a diverse lizard genus while also taking advantage of the available climate and phylogenetic data to address this behavioral gap in the evolution of chemical signals with climate and behavior. Although our results illustrate how environmental and ecological factors can act as complex selective regimes to influence signal composition, future research should investigate how these different factors interact to modify signal composition

over evolutionary time. Our study also illustrates the use of phylogenetic comparative analyses with relatively small numbers of taxa, striking a balance between an in-depth and nuanced view of each species with the insights possible from interspecific comparisons.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Campos et al. (2020).

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