UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
Departamento de Zoología y Antropología Física

TESIS DOCTORAL

Ecological and evolutionary drivers of chemical communication dynamics in lizards

Factores ecológicos y de evolutivos que afectan a la dinámica de la comunicación química en lagartos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Roberto García Roa

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Madrid, 2017

CHAPTERS:

Directores: José Martín Rueda - Pilar López Martínez

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Roberto García Roa

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Roberto García-Roa

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Memoria presentada por el licenciado Roberto García Roa para optar al grado de Doctor en Ciencias Biológicas, dirigida por el Doctor José Martín Rueda y la Doctora Pilar López Martínez del Departamento de Ecología Evolutiva del Museo Nacional de Ciencias Naturales- Consejo Superior de Investigaciones Científicas.

Madrid, 2016

Roberto García Roa

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Madrid, 2016

El doctorando

Roberto García Roa

V°B° del Director

José Martín

V°B° del Director

V°B° del Tutor

A todos los que creen que los sueños
se pueden perseguir despiertos.

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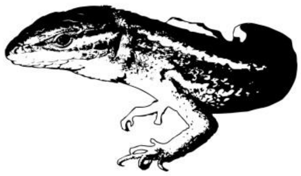

CHAPTERS:

AGRADECIMIENTOS /AGRADECIMIENTOS

Dicen que para no perder la perspectiva de un camino es importante recordar su inicio y los pasos que cambiaron el rumbo del mismo. Esto es especialmente importante cuando el camino fue, es o será complicado. Tengo la suerte de conocer cada uno de los pasos que cambiaron el rumbo en mi camino y recordar, sin lugar a dudas, las personas que me acompañaron con sus consejos, ayuda o apoyo en esos cambios de etapa. Seguramente si le preguntan a alguien que me conozca dirá que todo empezó cuando era niño, momento en el cual sólo me gustaban los juguetes de animales y como siempre me recuerdan "cuanto más feos mejor". Sin embargo, a nivel profesional, mi punto de inflexión fue Nicaragua. Allí tuve mi primer contacto con el trabajo de campo científico y seguramente es donde floreció (a nivel profesional) la parte más decidida de mí. Además, mi paso por Uruguay inundó mi cabeza de ciencia. Finalmente, mi segunda estancia en Nicaragua consolidó mi decisión mientras probaba mi fuerza de voluntad tanto física como mental.

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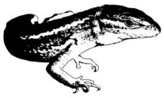
“*La diferencia entre intentarlo y conseguirlo es a menudo creer en lo que haces*”

Hay alas que no son fáciles de cortar, seguimos en la lucha.

SUMMARY

RESUMEN



ABSTRACT

Historically, despite of chemical communication is considered the oldest and widespread mode of conveying information, ecological and evolutionary hypothesis about animal life-history have chiefly been constructed on visual and acoustic interactions. However, the last decade has witnessed the important role that chemical signaling plays in animal communities and, thus, the number of studies investigating chemical signaling traits has increased significantly. In lizards, for instance, multiple works on chemical communication have revealed that chemical senses lead numerous processes underlying to their ecology and evolution. Even so, since most of studies have been focused on a small number of species, how and why this form of communication operates in lizards remains still poorly understood. In this context, the lower number of methodologies based on analytical chemistry approaches and tested in lizards might be a handicap to broad the spectrum of questions to be answered in the field of lizards´ chemical communication. Therefore, the description of alternative methodologies to those used hitherto might help to disentangle multiple questions that so far, remain unsolved. Concretely, we know remarkable little about how chemical signaling of lizards is influenced by ecological and evolutionary factors.

Lizards have been proven to be an useful model to understand visual and acoustic communication. Similarly, several researches have revealed that reptiles may also provide valuable information about how chemical signals may influence, for instance, the natural and sexual selection processes. On this basis, we used lizards as study model to investigate different questions regarding chemical communication by using different chemical, behavioral and evolutionary approaches. Thus, we first describe an alternative methodology for analyzing chemical secretions of lizards based on gas chromatography-mass spectrometry (GC-MS) for the semiquantification of the compounds identified in these secretions. Moreover, we studied a potential case of sexual dimorphism in chemical signaling through precloacal gland secretions in the lizard *Liolaemus wiegmanni*. We also investigated whether diet constraints may restrict

the expression of a potentially honest chemical sexual signal (vitamin E) in *Iberolacerta cyreni*. Further, we assessed chemical signal divergences between closely related species (*Podarcis bocagei* and *Podarcis carbonelli*) and between populations of the same species (*Gallotia galloti*), discussing whether the environment could be the driver of these compositional differences. Finally, we studied the diversification of the follicular epidermal glands and of chemical compounds of their secretions across squamates and lacertids, respectively.

We found that the proposed analytical methodology was able to detect physiological and environmental (temperature) effects on the composition of lizard chemical signals, which in turn may allow a deeper study of the different factors that could be shaping the chemical structure of these signals. Advances in chemical analyses techniques focused on lizards are needed to address the different factors that, as our works show, may influence the lizard chemical signals. In this context, we provide other example showing that females of some lizard species (e.g., *L. wiegmanni*) can convey information through follicular gland secretions and moreover, that this information is significantly different to that of males. Our results also reveal that supplementation of vitamin E in diet of *I. cyreni* males alters the chemical signaling and immune response of males, as well as the female responses through tongue flick rates. Remarkably, our study shows that the no identification of some compounds in chemical secretions does not necessarily imply the inability of a species to express them, since ecological factors may be hampering their higher expression. Furthermore, we found considerable differences between the chemical signals of two phylogenetically closer species (*P. bocagei* and *P. carbonelli*), which might be associated to environmental differences between both species. Also, we found compositional divergences of the femoral secretions among three populations of *Gallotia gallotia* (*G. g. eisentrauti*, *G. g. galloti* and *G. g. palmae*) that could have been driven by the differences in temperature and precipitation conditions where those populations inhabit. We also observed seasonal variations in chemical profiles of two of these populations (*G. g. eisentrauti* and *G. g. galloti*). On the other hand, our analyses studying the evolution of eight particular compounds in gland secretions reveal a heterogeneous tempo and mode of diversification of these chemicals across lizard species. With a similar approach, our results investigating the follicular epidermal glands diversification in squamates suggest the evolution of the number glands based on a stabilizing selection (Ornstein-Uhlenbeck) model of evolution with a moderate effect of phylogeny in their diversification. We obtained that the absence of these glands is the ancestral state in Squamata with a considerable effect of phylogeny in their anatomical location.

Gender, climatic conditions, diet and the phylogenetic relationships arise as influential factors in the final composition of lizard chemical signals and therefore, the study from multiple perspectives is the only way to finally achieve a deep knowledge about the role of the chemosensory mode in lizards' ecology and evolution.

RESUMEN

Históricamente, a pesar de que la comunicación química es considerada como la forma más antigua y extendida de transmitir información, las hipótesis ecológicas y evolutivas sobre la historia animal han sido principalmente construidas sobre interacciones visuales y acústicas. Sin embargo, la última década ha sido testigo del importante papel que juegan las señales químicas en las comunidades animales y de este modo, el número de estudios que investigan rasgos relacionados con la comunicación química se ha incrementado significativamente. En reptiles, por ejemplo, múltiples trabajos sobre comunicación química han revelado que el sistema químiosensorial influencia numerosos procesos subyacentes a su ecología y evolución. Aun así, debido a que la mayoría de los estudios han sido basados en un número reducido de especies, la comunicación química en reptiles continua siendo no del todo bien comprendida. Multitud de perspectivas deben ser tenidas en cuenta para entender totalmente cómo y por qué esta forma de comunicación opera en reptiles. Sin embargo, solo unas pocas metodologías basadas en técnicas químico-analíticas han sido testadas en lagartos. Por tanto, la descripción de metodologías analíticas alternativas a las usadas hasta ahora podría ayudar a responder múltiples preguntas que hasta ahora permanecen sin contestar. En concreto, sabemos muy poco sobre cómo la señalización química es influenciada por factores ecológicos y evolutivos.

Los lagartos han demostrado ser un modelo útil a la hora de entender la comunicación visual y acústica e, igualmente, varias investigaciones han revelado que los reptiles también pueden proporcionar valiosa información sobre cómo el uso de las señales químicas puede influir, por ejemplo, en los procesos de selección natural y sexual. Sobre esta base, hemos elegido los lagartos como modelo de estudio para investigar diferentes cuestiones referentes a la comunicación química, utilizando enfoques tanto químico, comportamental o evolutivo. Así, hemos descrito en primer lugar una metodología analítica alternativa para el estudio de las secreciones químicas de lagartos basada en el uso de cromatografía de gases acoplada a espectrómetría de masas (GC-MS) para la semicuantificación de los compuestos identificados

en estas secreciones. Además, hemos estudiado un caso potencial de dimorfismo sexual en la señalización química a través de secreciones de las glándulas precloacales enel lagarto *Liolaemus wiegmanni.* También investigamos si restricciones en la dieta pueden obstaculizar la expresión de una señal química sexual potencialmente honesta (vitamina E) en *Iberolacerta cyreni*. Además, evaluamos divergencias en la señalización química entre especies relacioandas (*Podarcis bocagei* y *Podarcis carbonelli*) y entre poblaciones de la misma especie (*Gallotia galloti*), discutiendo si el ambiente podría ser el causante de tales diferencias. Finalmente, estudiamos la diversificación de las glándulas foliculares epidérmicas y algunos de los compuestos identificados en las secreciones glandulares a lo largo de Squamata y lacértidos, respectivamente.

Hemos encontrado que la metodología analítica propuesta es capaz de detectar efectos fisiológicos y ambientales (temperatura) en la composición de las señales química de los lagartos, lo que permite un estudio más exacto sobre los diferentes factores que podrían estar modelando la estructura química de estas señales. Se necesitan avances en técnicas químico-analíticas enfocados a estos organismos para poder abordar el estudio de los diferentes factores que, como nuestros trabajos muestran, pueden influir en las señales químicas de los lagartos. En este contexto, ofrecemos otro ejemplo mostrando que las hembras de algunas especies de lagartos (e.j., *L. wiegmanni*) pueden transmitir información a través de las glándulas foliculares y además, esta información es significativamente distinta a la de los machos. Nuestros resultados también revelan que la suplementación en la dieta de vitamina E a los machos de *I. cyreni* alteró la señalización química y la respuesta inmune de los machos, así como la respuesta comportamental a través de tasas de protusiones linguales de las hembras. Remarcablemente, nuestro estudio muestra que la no identificación de algunos compuestos en las secreciones químicas no implica necesariamente la imposibilidad de las especies de hacerlo, dado que algunos factores ecológicos podrían estar impidiendo una mayor expresión. Además, encontramos considerables diferencias en las señales químicas de dos especies filogenéticamente muy cercanas (*P. bocagei* and *P. carbonelli*), lo que podría estar asociado a diferencias ambientes entre ambas especies. También, hemos encontrado divergencias en la composición de las secreciones femorales de tres poblaciones de *Gallotia gallotia* (*G. g. eisentrauti*, *G. g. galloti* and *G. g. palmae*), las cuales podrían estar provocadas por diferencias en las condiciones de temperatura y precipitación donde habita cada población. Además, observamos variación estacional en los perfiles químicos de dos de estas poblaciones (*G. g. eisentrauti* and *G. g. galloti*). Por otro lado, nuestros análisis sobre la evolución de ocho compuestos en particular de las secreciones químicas revelaron una diversificación heterogénea de los mismos en cuanto a modo y tiempo. Con un enfoque similar, nuestros resultados investigando la diversificación de las glándulas foliculares epidérmicas en Squamata sugieren que el número de estas sigue un modelo de evolución estabilizante, en el que habría un efecto moderado de la señal filogenética. Obtuvimos que la ausencia de estas glándulas sería el estado ancestral en Squamata con un considerable efecto de la filogenia en la localización anatómica de las mismas.

El sexo, las condiciones climáticas, la dieta y las relaciones filogenéticas se revelan como factores influyentes en la composición final de las señales químicas de los lagartos y por tanto, el estudio desde múltiples perspectivas es la única vía para conseguir finalmente un profundo conocimiento del papel que juega el sistema quimiosensorial en la ecología y evolución de los lagartos.

INTRODUCTION

INTRODUCCIÓN

Introduction

Researches have been long fascinated by communicative interactions among organisms. Animal communication has been the focus of multiples and lengthy studies across time (Bradbury & Vehrencamp, 2011). But, what is animal communication? The establishment of an agreed definition has become a major source of controversy (Carazo & Font, 2010; Font & Carazo, 2010). Some authors suggested that communication occurs when "senders" convey information to "receivers" and because of that, the behavior of the latter changes (Lindström & Kotiaho, 2002; Ruxton & Schaefer, 2011; Schaefer & Ruxton, 2015). This process is mediated by effective stimuli (often termed 'cues'). When a stimulus has coevolved in the sender and receiver given its influence on their behaviors, it is called "signal" (Carazo & Font, 2010; Ruxton & Schaefer, 2011). Selective forces underlying the communicative process operate in favor or against the signals and thereby trigger their loss or maintenance over time. Environmental conditions, physiological trade-offs, prey-predator interactions and conspecific competition are some factors that shape the structure, functionality and honesty of signals (Dawkins & Krebs, 1978; Bradbury & Vehrencamp, 2011).

Animal communication displays is based on a wide array of sensory modes across the tree of life. Animals may communicate through visual, acoustic, chemical and electric signals among others, and/or a combination of them. Depending on taxa, studies of each sensory channel have been often biased. For example, the role of colors and/or movements during courtship has been widely studied in many species of birds and lizards (Cornwallis & Uller, 2010; Seddon *et al.*, 2013). A growing body of literature also describes how acoustic emission affects the reproduction in anurans, insects and birds (Cocroft & Ryan, 1995; Wilkins *et al.*, 2013). In addition, researchers have shown an increased interest in understanding the influence of chemical communication on insect communities (Wyatt, 2003, 2009). But still, in an overall perspective, visual and acoustic traits have traditionally been the focus of the vast majority of research concerning to animal communication (Johnstone, 1996). Thus, this thesis is focused on one of the more unknown sensory channels, the chemical communication.

CHEMICAL SIGNALS

Chemical communication is the most ancient and widespread mode of communication (Muller-Schwarze, 2006; Wyatt, 2014). In animals, for example, chemical communication has been reported in multiples and diverse lineages (Wyatt, 2003, 2014). Although the term "pheromone" was proposed in 1959 for the first time (Karlson & Lüscher, 1959), previous evidences explicitly noted this type of communication in other animals. For instance, Rabelais (Rabelais, 1565) pointed that ancient Greeks knew that dog scents in heat were highly attractive for males. Subsequently, multiples authors mentioned, in one way or another, the chemical communication as a force underlying to organisms ecology and behavior. Indeed, Darwin (1871) highlighted that chemical signals might be acting together with visual and acoustic traits in sexual interactions of multiple organisms. Notwithstanding the foregoing, the progress in the field of chemical communication has been slow and fraught of controversy.

The "terms" of the controversy

Since Karlson and Lüscher (1959), based on insects, coined the term "pheromone" (*pherein* and hormōn: greek words meaning "to transfer" and "to excite" respectively) to those "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process", the study of chemical signaling has evolved significantly, raising doubts about the validity of the term in an overall view. The seeds of controversy had been sown.

Historically, investigations on chemical signaling in animals have often been skewed towards non vertebrate organisms, more particularly, arthropods (Symonds & Elgar, 2008).

Because of the complexity and diversity of chemical signals, the establishment of an overall and consensus concept became complicated. Thus, pheromone was originally described as a unique compound that elicits predictable behavioral and/or physiological responses in the receiver immediately after stimulus detection. In this context, both signal and response, as well as neural mechanisms mediating response would be genetically determined (Johnston & del Barco-Trillo, 2009). Against this idea, multiples alternatives emerged to broad the theoretical framework of the chemical signaling. Thus, Wyatt (2003, 2010, 2014) posited that a pheromone is a chemical with a particular function in the communication among individuals of the same species, whereas he proposed 'signature mixture' as a mixture of chemicals that shape the odor profile of a species. Elsewhere, Johnston and del Barco-Trillo (2009) proposed the classification of chemical signals according to the number of chemicals involved. They proposed the terms "pheromone" (one chemical that stimulates one or more responses), "pheromone blend" (small number of compounds in relatively specific proportions) and "mosaic signal" (multiples compounds and many of them are responsible of the receiver responses). Recently, Apps *et al*. (2015) refereed the term "chemical signal" for those chemicals or their mixture with elucidated structures that provoke similar responses in the receiver than expected in natural conditions. In this framework, given the broad diversity of animals and signaling modes, and the inherent complexity to establish a unique term and definition, from here on, we will use the term "chemical signals" to refer, in a broad sense, to those chemicals (also referred as "chemosignals" or "chemical compounds") or mixture of them that elicit behavioral or/and physiological responses in the receiver. Moreover, we will use the terms "sexual chemical signal" to define those particular chemical signals directly involved in sexual interactions.

The role of chemical signals

In a world where visible and acoustic stimuli flood almost everything, deciphering scents, although unnoticed most of the time for humans, present a common form of communication in many species. Indeed, a complex of 'multimodal signals' use to operate for providing the complete information to receiver (Rowe & Guilford, 1999; Dangles *et al.*, 2009). For instance, recent evidences revealed that *Anolis* lizards, which are widely studied because of their visual displays during social interactions (Ng *et al.*, 2013), might also detect chemical signals of conspecifics (Baeckens *et al.*, 2016) (figure 1). Both in an isolated way, as well as in a compendium of sensory modes, the chemosensory system often plays a key role in the reproduction and survival of many species.

Figure 1. Multiples species are able to convey information in a multimodal signal context. *Anolis carolinensis* are known by their colored dewlaps. However, recent papers reveal also evidences of chemical signaling in this species. Photo: R. García-Roa

Accordingly, scents in the environment provide helpful information to animals noticing the possible existence of closer resources (e.g., water) and environmental dangers (e.g., fire) (Ache & Young, 2005). Moreover, organisms often discriminate among odors from non-conspecific individuals [commonly referred as "allelochemicals"; (Whittaker & Feeny, 1971)], which have been exploited by multiple species to avoid predators, as well as for prey localization, pollination, and/or territorial marking (Labra & Niemeyer, 2004; Labra, 2007; Zimmermann *et al.*, 2009; Amo *et al.*, 2012; Weber *et al.*, 2016) (figure 2). Moreover, locating and recognizing conspecifics is crucial for sexual interactions (Adkins-Regan, 2005). In this sense, multiple studies have highlighted the use of chemical signals to differentiate between conspecific and closely related heterospecific individuals (Doty, 1973; Nevo *et al.*, 1976; Huck & Banks, 1980).

Figure 2: Plants have developed a broad spectrum of signals (color, morphology, scents) to attract insects, which unconsciously collaborate in the pollination of their pollen. Photo: R. García-Roa

In all social interactions, especially in those species that form long-term social groups and establish social networks, hierarchies, alliances, friendships, kin favoritism or pair bonding, the recognition among individuals becomes imperative (Goodson, 2005; Toth & Robinson, 2007). In this framework, chemical signals have been proved to act as identity signs in many species. As an example of this, the analyses of cuticular compounds in the butterfly *Bicyclus anynana* showed that the chemical composition differed among body parts, sex and age of studied individuals, which affected the courtship behavior between males and females (Heuskin *et al.*, 2014). Another point to consider is the establishment of hierarchies that may even occur in species without social clustering when individuals geographically overlap in the search of resources or/and mates. In either instances the dominant–subordinate relationship is often determined through fighting contests (Arnott & Elwood, 2009). Given that these confrontations are costly for individuals in terms of health and survival, some species use chemical signals (as well as other type of signals) to assess the "quality" of the opponent (Gosling & McKay, 1990), thus preventing further unnecessary fights. Moreover, the chemical discrimination between the dominant and subordinate individual may be remembered after fighting, even when the dominant individuals are not present. For example, experiments with golden hamsters (*Mesocricetus auratus*) showed how after fighting, loser individuals showed hesitancy to male-winner scents. This behavior repeated until one week after the initial interaction (Lai & Johnston, 2002). Moreover, chemical signals are also useful to territory scent marking. Experimental studies revealed that the allocation of chemical signals of an individual in neutral areas triggered a dominant behavior of that individual over the opponents when they were subsequently allocated in that areas (Gosling & McKay, 1990). This behavior has also been documented in females (Fischer & McQuiston, 1991). In addition, the role of the chemosensory system in sexual selection processes has been largely emphasized. In fact, as it was mentioned above, the influence of chemical signals in sexual attractiveness captured attention of the great naturalist that explored the animal behavior. For example, Darwin (1871) in *Descent of Man, and Selection in Relation to Sex* described "*The males are always the wooers; and they alone are armed with special weapons for fighting with their rivals. They are provided, either exclusively or in a much higher degree than the females, with odoriferous glands. There is another and more peaceful kind of context, in which the male endeavor to excite or allure the females by various charms. This may be affected by the powerful odors emitted by the males during the breeding season; the odoriferous gland having been acquired through sexual selection*". Therefore, odors engaging in sexual interactions could be one of the most insightful reasons for which scientists though that chemical signals could be deeply involved in the ecology and evolution of species. In this issue, the terminology has also been the subject of major disputes, plausibly due to the diverse nature of studied species and reproductive mechanisms. "Courtship pheromone", "aphrodisiac" or "sex pheromone", are some of the terms historically used. Recently, Johansson and Jones (2007) defined "sex pheromone" (SPh) as the substance released by an individual that changes the sexual behavior of conspecifics, which influences positively to the sender. They classified sex pheromones in “species recognition pheromones” (SPh useful to recognize conspecific individuals), “mate recognition pheromones” (SPh used to coordinate sexual behavior between males and females) and “mate assessment pheromones” (SPh that conspecifics use to differentiate between individual potential mates)(Johansson & Jones, 2007). However, based on the fact that the word “pheromone” is controversial *per se* (Nordlund & Lewis, 1976; Wyatt, 2009, 2010, 2014) (figure 3) and the scarce knowledge about the functionality of some chemicals in particular groups of animals, we will use the term “sexual chemical signals”.

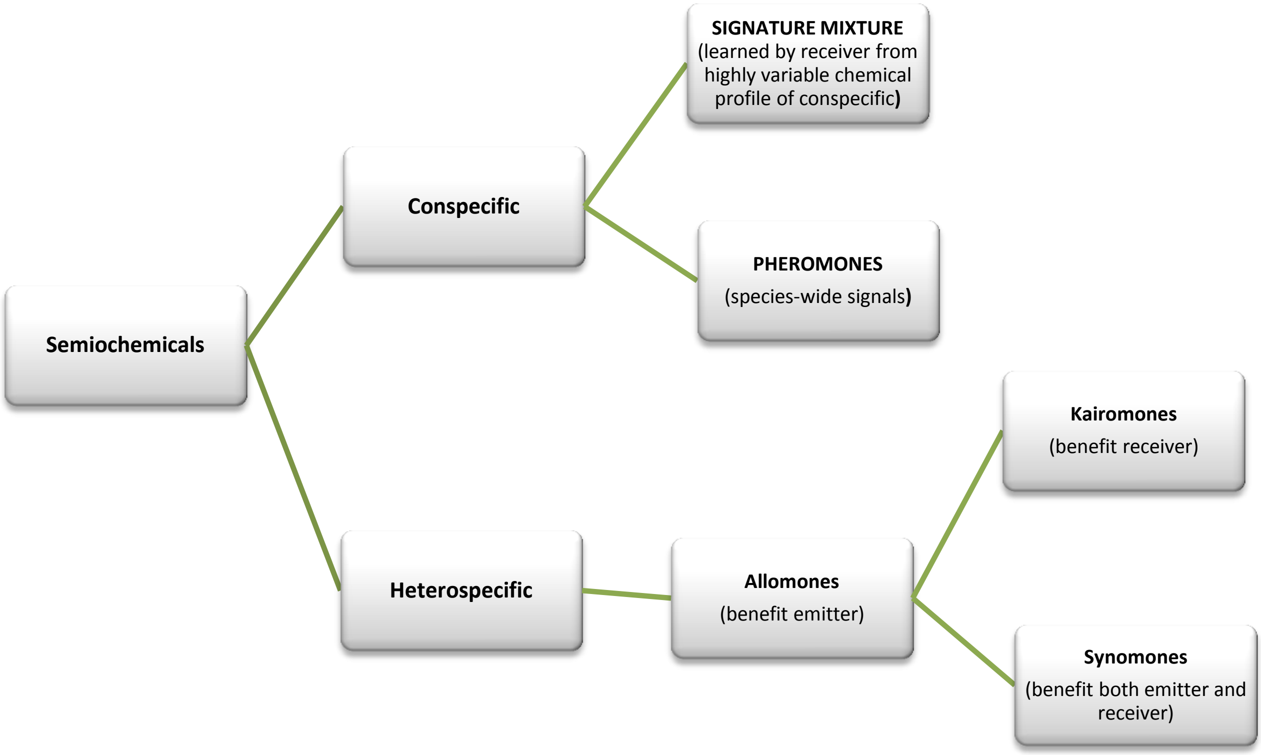


Figure 3: Diagram with different terms often used in studies of chemical communication. Modified from Wyatt (2014)

This term defines those chemicals, or the mixture of them, directly implicated in sexual communication among conspecific. There is accumulating empirical evidence showing the major facet that sexual chemical signals have in mate choice. In butterfly *Bicyclus anynana,* for instance, the composition of pheromones determined female mate choice (Nieberding *et al.*, 2012). However, despite of their relevancy in sexual interactions, the knowledge of the mechanisms underlying to mate-choice based on chemical signaling are currently poorly known (Johansson & Jones, 2007). Chemical signals are also essential in the discrimination among different groups of animals of the same species (herds, packs, breeding-groups, communities, etc.) (Johnston & del Barco-Trillo, 2009). In this level, the recognition occur among individuals of a group, and the discrimination of those individuals by other groups, which, in turn, may have another characteristic odor shared by the totality of its members, but with some differences among individuals (Mykytowycz, 1968; Pillay, 2000; Buesching *et al.*, 2003). Therefore, taking all the above into account, the diversity of chemical signals must be sufficiently wide to respond faithfully to the requirements of each specific ecological circumstance.

Composition and variation

Chemical signals are ubiquitous and diverse, being the result of multiple episodes of evolution inherent to each-species diversification (Zimmermann *et al.*, 2009; Arbuckle & Speed, 2015). To understand how chemosignals have evolved, the selective pressures exerted by abiotic and biotic factors (environment, predators, parasites, etc.), the ecological functionality of signals, as well as their chemical structure (e.g., type of compounds), should be taken into account (Alberts, 1992). In this respect, the information about the evolution of traits associated with chemical communication is still scarce and possibly, the evolutionary dynamics and mechanisms might differ depending on the type of chemical signals. Thus, in pheromones, some hypotheses have suggested that they could be the result of the evolution of some molecules that would have conferred selective advantage to their holders (Steiger *et al.*, 2010; Wyatt, 2010, 2014). This idea would explain, at least in part, the striking diversity of chemical signals found in animals. For example, the pheromones and other chemical signals used in underwater [amino acid L-kynurenine; a sex pheromone of masu salmon, *Oncorhynchus masou*

(Yambe *et al.*, 2006)] or terrestrial [unsaturated methyl ketones in female garter snakes, *Thamnophis sirtalis* (Mason & Parker, 2010)] conditions, as well as multiple insects pheromones such as the poly-unsaturated long-chain hydrocarbons [(3Z,6Z,9Z,12Z,15Z)-pentacosapentaene of the spruce coneworm, *Dioryctria abietella* (Löfstedt *et al.*, 2012)]. However, the establishment of evolutionary hypotheses becomes complicated when chemicals are not isolated, shaping a mixture of them, whose relationships among them in the evolutionary process is fundamentally unknown (Löfstedt *et al.*, 1991; Kather & Martin, 2015).

As occur in other sensory modes, it is expected that chemical signals have evolved to increase the efficiency of the information transmission (Bradbury & Vehrencamp, 2011). There are multiples factors drivers of signals diversification (Symonds & Elgar, 2008), such as avoiding hybridization with closely related sympatric species (McElfresh & Millar, 2001), predator and parasitoid eavesdropping (Aukema *et al.*, 2000), deception and mimicry (Brandt *et al.*, 2006), and/or environmental pressures (Van Oudenhove *et al.*, 2012). As a result, the composition of chemical signals is highly diverse (Weldon *et al.*, 2008; Wyatt, 2014; Apps *et al.*, 2015). It is common to find proteins, hydrocarbons, heteroatomic compounds and steroids, as well as aldehydes, carboxylic acids, esters, ketones, ketals and phenols. Apps *et al*. (2015) proposed the classification of chemical signals in respect to functional groups and core structures. Thus, they differentiated among “steroid nucleus, alcohol, amine, nitrogen, sulfur, nitrogen heterocycle, sulfur-heterocycle, cyclic, aromatic, lactone, ketone, aldehyde, unsaturated, ester, straight chain, oxygen-heterocycle, hydroxyl, and terpenoids”. On this basis, the composition may differ qualitatively or/and quantitatively among species depending on the type of chemical signal (i.e., pheromone *sensu stricto* –one compound–, or chemosignals or mixture signature – several compounds–) (figure 4). This variation in composition may differ depending on health condition, gender or the age of the sender, as well as the diet or the source of chemicals signals.

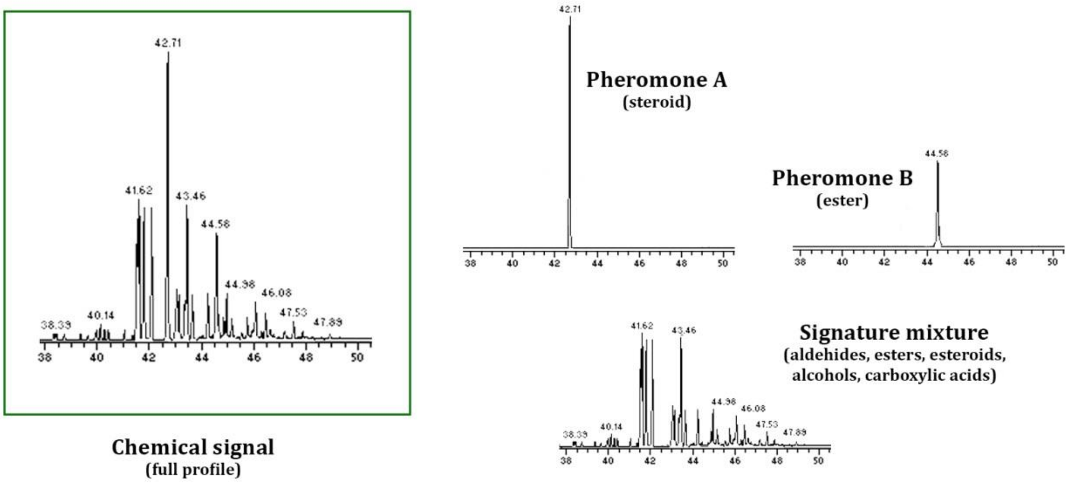


Figure 4: Chemical signals may differ depending on multiple factors as taxa, source of the signal, health state, age, gender or season among others. Moreover, chemical signals may be a complex compendium of multiple compounds. For example, the illustration shows that the full profile of the chemical signal is shaped by the Pheromone A and B, which are particular compounds (steroid and ester, respectively) and a signature mixture, which composition is a wide spectrum of other type of compounds

Reptiles

For centuries, reptiles have attracted considerable public attention, both within and outside of science. The early apparition of reptiles, approximately 250 millions of years ago (Reisz *et al.*, 2011), and their further diversification across time to current five orders, Testudines (turtles), Rhynchocephalia (tuatara), Squamata (amphisbaenians, lizards and snakes), Crocodylia (alligators and caimans, crocodiles, and gavials), and Aves (birds), have long fascinated to researchers around the world. Reptiles are the subject of a plentiful literature focused on different aspects of their ecology and evolution. They inhabit a wide spectrum of environments, where selection operates on their behavior, morphology and physiology. This fact has driven a broad range of adaptations at many different levels. For example, the communication of reptiles has diversified towards multiple sensory channels that encompass a combination of signals, such as visual, acoustic, tactile or chemical, which are often involved in their reproduction and survival (Pough *et al.*, 2016). However, most studies have tended to focus on visual stimuli (colors, movements), remaining the chemical signaling as a relatively neglected area.

The improvement of protocols and techniques in the field of analytical chemistry has allowed increasing the number of works focused on the chemical communication of reptiles (Houck, 2009; Mason & Parker, 2010). Although the first studies about chemical communication in reptiles date back to early in the 20th century (Baumann, 1927, 1929), this research field has grown rapidly in recent decades. Multiple works have evidenced that chemical communication is often a key pillar in reptilian life (Weldon *et al.*, 2008; Houck, 2009). Within reptiles, chemical communication has mostly been addressed in snakes and lizards (Weldon *et al.*, 2008; Houck, 2009; Mason & Parker, 2010). In the latter, chemical signals have been proved to be essential in the behavior and reproduction of many species (Martín & López, 2014, 2015). Even so, the use of chemical signals in reptiles remains still poorly understood.

Chemical communication in lizards

Multiple evidences have revealed the chemosensory abilities of lizards and their relevant role in survival and reproduction of many species. Lizards have a wide diversity of sources of chemical signals (feces, skin, epidermal glands, etc.) (Mason, 1992). Moreover, lizards own a well-developed chemosensory system for detection and discrimination of multiple scents from prey, predators or/and conspecifics (Mason & Parker, 2010).

a) Sources of chemical signals

The **skin** of lizards is shaped by a multilayered stratum corneum impregnated of lipids, which confer a transepidermal permeability barrier to avoid water loss and the penetration of substances from the environment through the skin. In addition, skin confers protection against pathogenic microorganisms, ectoparasites and predators (Pough *et al.*, 2016) and moreover, it is also involved in mate choice (Weldon *et al.*, 2008). Other sources of chemosignals are the **cloacal secretions** and **feces** (Cooper Jr & Trauth, 1992; Wilgers & Horne, 2009). Although often overlooked, both cloacal secretions and feces might be especially useful in species lacking other important sources of chemicals, such as femoral or precloacal glands (Mason, 1992). Different compounds might be expelled with the feces, which seem to have scent-marking and conspecific recognition properties (Moreira *et al.*, 2006; Wilgers & Horne, 2009). In addition, lizards own the holocrine glands. They are divided in follicular and generation glands. **Follicular glands** are tubular structures of the dermis anatomically located in different epidermal regions of the body: femoral, precloacal, antebrachial, inguinal, and/or dorsal (Mayerl *et al.*, 2015). There is not a clear agreement to name them in literature according to the anatomical position, but they are fundamentally classified as 'femoral' and 'precloacal' (or 'preanal') pores (Valdecantos *et al.*, 2014; Baeckens *et al.*, 2015; Mayerl *et al.*, 2015). Lizards may have femoral, precloacal, both or none pores. The morphology of them is specific dependent and also, it may slightly change among individuals (Mayerl *et al.*, 2015). These follicular glands protrude through pores a 'waxy' secretion whose composition is highly variable among species (Martín & López, 2014) but, furthermore, it can also change depending on population, sex, age or season (Alberts *et al.*, 1992; Martín & López, 2006b). The composition of secretions is mostly proteins and lipids (Weldon *et al.*, 2008). Some evidences revealed that proteins seem to be relatively stable among individuals (Alberts, 1990). The high variety of proteins was found among species (Alberts, 1990; Alberts *et al.*, 1993). Instead, the lipidic fraction has a high degree of molecular diversity. The chemical profile of secretions is metabolism and specific dependent. This fact together with the volatility of the lipid compounds suggest the lipidic fractions as an "identifying trait" of the holder. So far, the number of species whose chemical secretions have been described is very low (≈50 spp.) (Martín & López, 2014; Martín *et al.*, 2015b, 2016). In most cases steroids were the major type of compounds following by carboxylic or fatty acids. Also, alcohols, esters of carboxylic acids and alcohols (=waxy esters), squalene and other terpenoids, tocopherols, ketones, aldehydes, furanones, alkanes or amides can be found (Weldon *et al.*, 2008). Finally, **generation glands** are also suggested as sources of chemical signals. These glands are anatomically located in femoral, precloacal, antebrachial (forearm), and dorsal epidermal regions of the body. There is little information about the role of these glands as source of chemical signals. Louw *et al.* (2011) analyzed the generation glands of the lizard *Cordylus giganteus* and they found alkenes, carboxylic acids, alcohols, ketones, aldehydes, esters, amides, nitriles and steroids. The diversity and complexity associated to the chemosensory channel of communication has been subject of an increasing number of researches during the past decade. However, many questions about the ecological and evolutionary causes of this diversity remain unanswered.

b) Vomeronasal system and olfaction

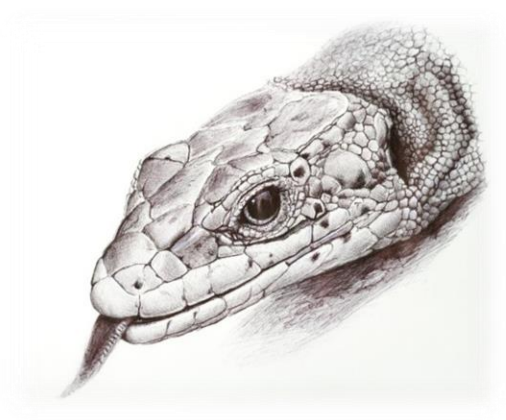
Lizards may detect multiple chemical stimuli because they own well-developed olfactory and vomeronasal organs. Scents, which are often perceived by nares, are processed by nasal organs. Then, lizards elicit tongue-flicks mediating vomerolfaction. In other cases, tongue-flick acts as an exploratory mechanism of the environment that takes chemicals to deliver them to the vomeronasal organ (Box 1). Then, a specific signal is sent to the central nervous system, whereupon accessory olfactory pathways begin their activation.

c) Functionality of chemical signals in lizards

While some groups of lizards are considered “visual oriented” [e.g., ‘Iguania’ clade; (Losos, 2009)], other linages are believed to rely chiefly on chemical signals [e.g., ‘Scleroglossa’ clade; (Schwenk, 1993)]. However, some evidences suggest that visual-oriented lizards are also able to detect and respond to scents (Labra & Niemeyer, 2004; Labra, 2007, 2011; Baeckens *et al.*, 2016). Therefore, to a greater or lesser degree, the use of chemosensory system might be influencing the behavior of many lizard species. For example, chemical senses can be used to detect prey and predators (Cooper Jr *et al.*, 2002), as for example in *Liolaemus lemniscatus* lizards, which showed in different experiments the ability to discriminate both prey and predator chemical cues (Labra & Niemeyer, 2004; Labra, 2007). Also, chemical signals from the skin allowed the recognition between hetero and conspecific individuals of some species. Thus, north American skinks of the genus *Plestiodon* showed aggressive responses against males of their own species but also, against those heterospecific males whose scents have been manipulated with conspecific odors (Cooper Jr & Vitt, 1987). Skin lipids were also decisive in the behavior of *Eublepharis macularius* males in response to the presence of conspecific scents. Thus, males responded aggressively to chemosignals from other males. Instead, scents from females in breeding condition elicited high TF rates (Brillet, 1990; Cooper Jr & Steele, 1997). Similar behaviors have been described in other lizard species [see (Mason & Parker, 2010)].

Without a doubt, most of the information available about chemosignals mediating social interactions of lizards comes from studies of femoral and precloacal secretions, or by the chemicals identified in them. Thus, empirical evidences show that the qualitative and quantitative differences in the chemical profiles of these secretions may significantly influence the behavioral response of the receiver. For example, sex recognition through scents has been proved in multiple species, even when additional traits, such as color for example, had been manipulated (López & Martín, 2001; López *et al.*, 2002). Also, during male-male interactions, some lizards seem to modulate the agonistic response on the base of the chemosignal profiles of the rival.

Box1: Tongue flicking (TF) is a common and widespread behavior of snakes and lizards. TF is a sampler mechanism with which snakes and lizards capture odors from their environment. Given their easy observation, TF rates have been used to measure the chemosensory discrimination abilities of these reptiles (Cooper, 1994). Usually, cotton swabs and papers imbued with the chemical scent are randomly presented to snake and lizards snout. According to the 'interest' of the individual, each scent elicits a different number of TF, which are counted during the time that scientist decides. TF experiments compare the scent stimulus with an odorless control (e.g., deionized water) to detect potential confounding effects. Significant differences of TF among samples reflect the ability of the snake or lizard to discriminate among them. A larger number of TF reflect a higher degree of interest in the stimulus (e.g., presence of prey predators or potential mates, as well as familiar vs. unfamiliar conspecific recognition). In addition, the time elapsed between the chemical stimulus presentation and until the first TF is often recorded. This is called as 'latency time'. Draw: TF of *Iberolacerta cyreni* (F. García García)



Experiments with Iberian rock lizards (*Iberolacerta monticola*) revealed that dominant males had higher levels of hexadecanol and octadecanol in their secretions. Subsequent test showed that males elicited higher TF rates to swabs with higher abundances of hexadecanol and octadecanol. Interestingly, only the swabs with hexadecanol provoked that lizards bit the swabs (Martín *et al.*, 2007). Different empirical evidences have also shown that males might assess the fighting potential of other males based on chemosignals (Carazo *et al.*, 2007). In fact, dominance status could be showed by the males both at close range (i.e., in male-male direct interactions) and by scent marking, which would be useful to establish dominance of territories (Aragón *et al.*, 2003). The discrimination of scents during male-male interactions allows lizards to avoid unnecessary fights, pre-identifying themselves as "dominant" or "subdominant" males (Martín *et al.*, 2007). Additionally, secretions are useful in the recognition of familiar vs. unfamiliar individuals. For instance, experiment carried out with Iberian wall lizards (*Podarcis hispanica s.l*) showed that the aggressive interactions decreased when males were truly familiar or whether unfamiliar male odors were manipulated to resemble familiar ones (Aragón *et al.*, 2003). Therefore, lizards might "remember" the chemical profile of other individuals, which would reduce agonistic interactions in successive encounters with those individuals. Moreover, recognition of familiar and unfamiliar individuals might contribute to stablish a stable social system reducing the number and intensity of aggressive interactions (Martín & López, 2015).

Finally, the chemosensory system plays a crucial role in intersexual interactions of some species of lizards. Concretely, traits associated with chemical signaling are suggested to be involved in female mate choice, both pores and secretions chemical profiles. Hence, females of *Iberolacerta cyreni* preferred scents from those males with lower levels of fluctuating asymmetry in their femoral pores. Authors suggested that chemical signals may indicate 'quality' of males (Martín & López, 2000). In the case of scent marking, females have been observed spending more time in areas scent-marked by males of higher "quality" (i.e., heightened immune response, lower parasite load, greater body condition, etc.) (Martín & López, 2006a; Kopena *et al.*, 2009, 2011, 2014b), or with better genetic compatibility (Olsson *et al.*, 2003). Indeed, the presence or/and higher levels of particular compounds in males' secretions is often correlated with higher preferences by females during mate choice. Thus, several studies have been carried out testing how female mate choice differed when the abundance of those compounds, isolated or in secretion, changed because of natural (e.g., age, sex) or experimental conditions (e.g., manipulation of the diet) (Martín & López, 2015). For instance, experiments of diet supplementation with cholesta-5,7-dien-3-ol (i.e., provitamin D_3) in *I. cyreni* revealed that females elicited higher TF rates to scents of males with high levels of this chemical. Further, females preferentially occupied scent-marked territories of these males (Martín & López, 2006a). Similar results were observed with α-tocopherol (i.e., vitamin E), ergosterol (=pro-vitamin D_2), or oleic acid [see (Weldon *et al.*, 2008; Mason & Parker, 2010; Martín & López, 2014, 2015]. Therefore, all these compounds have been suggested to be potential honest chemical signals.

Ecological and evolutionary drivers of chemical communication dynamics in lizards

As we have already noted, the presence and abundance of some chemical compounds is determinant in social interactions of many species. But, what factors are determining the quantitative and qualitative differences on chemical profiles of those species? Animal communication through other sensory modes (e.g., visual and acoustic) has been proved to be affected by different ecological factors (Forrest, 1994; Losos, 2009). Therefore, it would not be surprising that chemical signals are subject of similar pressures.

a) Gender

Chemical signaling in lizards have mainly been based on males. Perhaps, the territorial behavior, the striking expression of ornaments and/or the fact of being the subject of election during female mate choice are important factors for which the researches on this field have been biased to males (Martín & López, 2015). Moreover, the presence of well-developed follicular glands during reproductive season, through which their secretions are protruded, in many species have made it easier to study chemical communication based essentially in male chemosignals (Valdecantos *et al.*, 2014; Mayerl *et al.*, 2015). Conversely, in many species, females do not have follicular glands, or when present are often small and without secretions. Even, female lizards of the same species may develop different morphological strategies. For example, in some *Liolaemus* species, females of the same population may have or not follicular glands (own observations). In addition, females of many species are able to detect and discriminate chemical signals of males (Duvall, 1981; Cooper Jr & Trauth, 1992; López & Martín, 2005; Barbosa *et al.*, 2006). Therefore, although females have apparently less developed some structures associated to chemical communication (i.e., glands and their secretions in some species), they could convey information through this sensory channel. This framework raises ecological and evolutionary questions that remain overlooked. Since few studies have been focused on the study of chemical profiles of both sexes (Alberts, 1990; Khannoon *et al.*, 2011; Martín *et al.*, 2016), the information about chemical signals in females, as well as potential dimorphisms on chemical signalling is scarce. On this basis, studies analysing structures, signals and behaviours associated with chemical communication are needed.

b) Diet

Multiple compounds found in chemical secretions of lizards are only synthetized by microorganisms and plants (e.g., some steroids or tocopherols) (Weldon *et al.*, 2008), which compel lizards to acquire them from the diet. Often, these compounds are fundamental for metabolic mechanisms due to their key roles in physiological functions (Weldon *et al.*, 2008; Martín & López, 2015). Even so, some of these compounds are allocated in chemical signals (Martín & López, 2006a, 2010a). Lizards divert chemicals from metabolism to increase their abundances in chemical secretions (Weldon *et al.*, 2008). Therefore, physiological trade-offs are established to manage the allocation of these compounds to chemosignals and the requirements of metabolism.

In this context, some compounds might act as honest signals of "quality" because their high abundances in secretions mirror that the individuals are able to obtain sufficient amounts of these compounds from the diet to use them in both metabolism and chemical secretions (Martín & López, 2015). Hence, trophic resources are essential to these physiological trade-offs and thereby, it is expected that changes in diet may influence directly the chemical profile of lizard secretions. For example, experiments supplementing males of green lizards, *Lacerta viridis* and *L. schreiberi*, with vitamin E (i.e., α-tocopherol) revealed an increase of this vitamin in their secretions. Moreover, females preferred to occupy territories scent-marked by males with the highest levels of vitamin E in their secretions. Therefore, the availability of the vitamin in diet conditioned its allocation in male secretions and further, affected the female responses (Kopena *et al.*, 2011; Kopena *et al.*, 2014a). These results lead to suggest that α-tocopherol might function as a potential honest chemical signal (Martín & López, 2015).

c) Environment

Environment is one of the most relevant factors studied in animal communication (Ryan *et al.*, 1990; Osorio & Vorobyev, 2008). There is a considerable amount of literature showing how environmental conditions shape visual signals in lizards (Losos, 2009; Cole, 2013; Bohórquez-Alonso & Molina-Borja, 2014). The environmental conditions where species inhabit affect the emission, transmission and reception of signals. Therefore, the communication efficiency and efficacy are highly influenced for surrounding conditions (Endler, 2000). Therefore, environment acts as a selective force in the structure of signals and, therefore, the sensory systems of sender and receiver must be tuned with those environmental conditions where each species or population inhabit (Cole, 2013). This issue has also been raised in lizard chemical communication. For instance, since volatility of chemicals and substrate use is crucial to increase the efficiency of scent marking, it would be expected that the environment might influence the final configuration of lizard chemical profiles. In this framework, some recent works have studied the chemical profile of precloacal and femoral secretions from closer related species, or even of different populations of the same species (Escobar *et al.*, 2003; Runemark *et al.*, 2011; Gabirot *et al.*, 2012a; Gabirot *et al.*, 2012b; Martín *et al.*, 2013). In most of them, the hypothesis of potential effects of environment shaping those interspecific or interpopulation differences was suggested. However, there is currently no clear evidence supporting this idea. Therefore, the need to find patterns that link chemical composition of chemosignals and its surrounding environment remains.

In addition, when environmental conditions change in a short period of time, the efficiency of the signal and hence, the transmission and/or reception of information may be affected. Two recent works showed that changes in climatic conditions surrounding chemical secretions altered their efficiency to convey information (Martín & López, 2013; Martín *et al.*, 2015a). In the context of global warming, Martín and López (2013) showed that the efficacy (i.e., detectability and persistence) of substrate scent-mark decreased at high temperatures. Given the context of global change, in which temperatures are rapidly increasing, plastic responses, fast evolution or/and adaptation of chemosignals would be required to keep communicating. However, although intuitive due to the above mentioned results, there is no evidence of whether changes in temperature affect the chemical structure (i.e., composition) of the chemosignals.

d) Evolution

The study of evolutionary dynamics of signals responsible for animal communication has grown with the development of new phylogenetic approaches. However, the overwhelming majority of research studying evolution of signals has been focused on morphological and acoustic signals (Searcy & Nowicki, 2005; Ng *et al.*, 2013). However, despite the key role identified for chemical signals in natural and sexual selection processes across the animal world, the study of macro-evolutionary diversification of traits and signals engaging in chemical communication remains fundamentally neglected (Steiger *et al.*, 2010; Kather & Martin, 2015). In reptiles, the only studies exploring the global diversification of chemical communication have been based on morphological structures; in an attempt to elucidate potential ecological causes of follicular glands evolution, some authors studied the relationship between number of femoral and precloacal pores and surrounding environmental conditions (Escobar *et al.*, 2001; Pincheira-Donoso *et al.*, 2008; Baeckens *et al.*, 2015). In the genus *Liolaemus*, phylogenetic comparative analyses showed that the number of pores was explained by shared ancestry (Pincheira-Donoso *et al.*, 2008). In a recent work with lacertid species (Fam. Lacertidae) the results revealed that substrate use might affect the number of pores (Baeckens *et al.*, 2015). However, no studies have addressed how these glands have diversified across all squamates.

On the other hand, the information regarding the evolution of chemical signals is scarce (Symonds & Elgar, 2008; Swaney & Keverne, 2009; Schiestl, 2010; Steiger *et al.*, 2010; Kather & Martin, 2015). In the context of insect pheromones some authors suggest two possible dynamics of evolution. One case explains an evolutionary process of chemical compounds selection, in which some chemicals are selected and other deleted under selective pressures. The quantitative and qualitative changes are gradual and under a phylogenetic conservatism (Symonds & Elgar, 2008). The second model suggests 'saltational' shifts in chemical composition (Kather & Martin, 2015). In this latter case, the diversification of signals would be under strong stabilizing selection, which selects in favour of drastic adaptive changes. Thus, the chemical profile of signals would be highly different to precedent individuals. Given the extraordinary diversity of evolutionary dynamics described in lizards, the apparition of these models of evolution would not be surprising. However, nothing is known about how chemosignals diversify over time. The hypothesis of pre-existing sensory bias in females has been suggested to explain the evolution and maintenance of particular chemicals across time (Martín & López, 2008, 2014; Mason & Parker, 2010). Experimental studied revealed that females of *I. cyreni* elicited higher TF rates both to prey and male secretions stimuli (Martín & López, 2008). Subsequently, similar tests were conducted with cholesta-5,7- dien-3-ol (provitamin D_3), which also triggered higher TF rates of females (Martín & López, 2010b). Authors suggested that this steroid identified in both prey and male scents might be responsible (at least in part) of the higher responses by females in both cases. This approach was also tested with oleic acid (cis-9-octadecenoic acid) with similar results. Interestingly, females showed a negative an inverse response between oleic acid and cholesterol. Cholesterol was correlated to males´ body size eliciting selective responses of other males to this compound. Mason and Parker (2010) suggested that chemical signals in males can diversify under different selection pressures: for example, cholesterol as a dominance signal and oleic acid as an attractiveness signal to females. However, further work on the evolution of lizard chemosignals would help us to understand how and why chemical signals have evolved along lizards' diversification.

Objectives

This thesis examines some factors with potential influence in the chemical communication of lizards. Hence, the thesis is divided into seven chapters with the following specific objectives:

- **CHAPTER 1**: The development of an alternative analytical methodology that allows the detection of quantitative and qualitative modifications of chemical profiles after different treatments on individuals or samples.
- **CHAPTER 2**: Comparing sexual differences in chemical signalling of *Liolaemus wiegmannii* lizards through the description of chemical profiles of precloacal secretions in both genders.
- **CHAPTER 3**: Examining the influence that diet supplementation of a potentially honest compound (vitamin E) has on chemical signalling and mate choice of *Iberolacerta cyreni* lizards, taking into account that vitamin E had never been found before in the femoral secretions of this species.
- **CHAPTER 4**: Investigating potential interspecific differences in chemical profiles of femoral secretions between two closely related wall lizard species (*Podarcis bocagei* and *P. carbonelli*).
- **CHAPTER 5**: Assessing potential effects of climatic factors (temperature and precipitation) and seasonality on chemical profiles of femoral secretions in three subspecies of *Gallotia galloti* lizards (*G. g. eisentrauti*, *G. g. galloti* and *G. g. palmae*).
- **CHAPTER 6**: Investigating the evolutionary diversification of particular chemical compounds found in a set of lizard species belonging to Lacertoidea subclade.
- **CHAPTER 7**: Investigating the diversification of follicular epidermal glands used for chemical signalling in squamate reptiles.

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CHAPTER I

SEMI-QUANTITATIVE DETERMINATION OF SCENT COMPOUNDS TO ANALYSE CHEMICAL SIGNALS OF LIZARDS

CAPÍTULO I

DETERMINACIÓN SEMICUANTITATIVA DE LOS COMPUESTOS PRESENTES EN LAS SEÑALES OLOROSAS COMO MODO DE ANALIZAR LAS SEÑALES QUÍMICAS DE LAGARTOS

SEMI-QUANTITATIVE DETERMINATION OF SCENT COMPOUNDS TO ANALYSE CHEMICAL SIGNALS OF LIZARDS

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ABSTRACT

Our understanding of animal communication derives mostly from studies of acoustic and visual signals, while chemical signalling is still relatively poorly known. In lizards, in particular, compounds from femoral and precloacal chemical secretions may play key roles in sexual selection by means of mate-choice and intrasexual competition. However, the functionality of these compounds is not entirely clear. In this context, since some used methodologies may have inherent technical limitations, the development and implementation of alternative methodologies that allow analysing chemical signals more accurately may be useful to gain a thorough understanding of lizard´s ecology and evolution. Thus, we present a straightforward methodology to analyse lizard scents based on gas chromatography coupled to mass spectrometry (GC-MS). The approach uses an internal standard (n-heptadecane) for the semi-quantification of the compounds found in the scents. Two different experiments were designed in order to test the applicability of the methodology. The analytical procedure allowed revealing changes in the lizard chemical signals both when animals were previously subjected to vitamin E supplementation in the diet and when deposited chemical secretions were exposed to high temperatures. The presented methodology might be applied to the study of variations in the chemical profiles produced by the effects of different physiological and climatic factors, acting either on the production of secretions itself or on the deposited scent marks. This methodology allows the comparison of relative amounts of different compounds in secretions while, at the same time, confers statistical independence among compounds for obtaining their chemical semi-quantification. Moreover, this method allows comparing samples considering multiple chemical compounds simultaneously.

Keywords: Chemical signals; chemoreception; chromatography; mass spectrometry; scents

Introduction

Multiple modes of animal communication have been explored with the purpose of improving the knowledge of animal ecology and evolution (Bradbury & Vehrencamp, 2011; McGregor 2013; Smith, 2013; Troscianko & Stevens, 2015). However, our understanding of animal communication derives mostly from studies of acoustic and visual signals (Espmark *et al*. 2000; Hauser & Konishi 2003; Osorio & Vorobyev 2008), while chemical signals, for example, have historically received much less attention, in addition to having been chiefly studied in arthropods (Wyatt, 2009; Wyatt, 2010), remaining our knowledge rudimentary in other animals. Therefore, more studies on chemical communication within vertebrates must be performed to improve the knowledge around animal ecology and evolution.

Reptiles often own well-developed olfactory and vomeronasal organs that function in chemoreception (Halpern, 1992; Lutterschmidt *et al*., 2006) to detect and discriminate prey, predators and conspecifics (Houck 2009; Mason & Parker 2010b). Moreover, reptiles chemical signals may play key roles in sexual selection by means of mate-choice and intrasexual competition (Weldon *et al*., 2008; Parker & Mason, 2009; Martín & López, 2015). Lizards, in particular, may use different sources of chemical signalling (i.e., skin, feces, gland secretions) (Labra, 2011; Martín & López, 2014). However, researches addressing lizard´s chemical ecology have been chiefly based on the chemical signals contained in the follicular epidermal glands secretions and feces (Martín & López, 2014; Martín & López, 2015). In respect to the follicular glands, they are located in the femoral, or in the precloacal region or forming continuous rows between both areas (Valdecantos *et al*., 2014; Baeckens *et al*., 2015; Mayerl *et al., 2015)*, and produce waxy secretions that provide useful information in lizard social interactions (Mason & Parker, 2010). In these secretions, lipids are the main compounds together with proteins (Weldon *et al*., 2008; Martín & López, 2014). Several evidences have revealed that quantitative or qualitative variations of particular lipids may trigger behavioural changes during intraspecific interactions (Carazo *et al*., 2007; Kopena *et al*., 2011). Even so, the functionality of these and other lipids remain unclear and, moreover, the information available of this topic comes from a limited number of compounds (Weldon *et al.*, 2008; Martín & López, 2014).

So far, the methodologies used to study chemical signalling in lizards have differed depending on the aim and the research group. Thus, many works have been based on behavioural approaches, for example, studying differences in tongue flick rates or preferences for scent-marked areas (Cooper, 1994; Cooper Jr & Steele, 1997; Baird *et al.*, 2015). Moreover, studies using analytical techniques have also provided relevant information to understand lizard chemical communication. In this respect, gas chromatography coupled to mass spectrometry (GC-MS) is currently the most common analytical technique used to investigate chemical composition of lizard secretions (Louw *et al.*, 2007; Flachsbarth *et al.*, 2009; Khannoon *et al.*, 2011; Louw *et al.*, 2011; Heathcote *et al.*, 2014; Martín & López, 2015). However, procedures may vary in terms of the sample treatment and analysis. Frequently, the secretions are subjected to a solid-liquid extraction step by adding a certain solvent to the vial containing the secretion (Kopena *et al.*, 2009; Gabirot *et al.*, 2012). The extracted compounds can be subsequently derivatized by adding certain reagents to the sample, to replace the active points in the molecules by non-polar groups, in order to improve the detectability of some type of compounds, such as fatty acids, and to avoid the degradation of non-polar columns increasing their useful lives (Khannoon *et al.*, 2011). After separation of compounds in the GC, the mass spectra resulting from the MS analysis can be identified using mass spectra libraries (Stein, 2012) and by comparing the retention times and mass spectra with commercial standards available from chemicals supply companies (Louw *et al.*, 2007; Martín *et al.*, 2015a). However, not all the chemicals found in chemical secretions are commercially available nor have been described previously. For instance, some derivative compounds, such as many steroids or the unidentified tocopherol derivative found in chemical secretions of *Lacerta schreiberi* lizards (López & Martín, 2006). This chemical was later tentatively identified as 6-0-

methyl-α-tocopherol based on mass spectra (Kopena *et al*., 2009). Therefore, the tentative identification by using mass spectra libraries or the identification of the type (i.e., class) of some compounds may be the only resources. In addition, analyses on GC-MS use to operate the MS in scan mode to identify the higher number of compounds in the sample as possible (Kopena *et al*., 2009; Runemark *et al*., 2011; Martín *et al*., 2016). This mode of analyses can provide lists of tens of compounds and, therefore, the construction of a calibration curve for each of them is impractical, or even impossible when some particular standard is not available commercially. The final results show the relative abundance of each peak (i.e., compound) (Escobar *et al*., 2003; Runemark *et al*., 2011; Heathcote *et al*., 2014; Martín *et al*., 2015a) expressed as a percentage of the peak areas considered in the total ion chromatogram (TIC). Although accepted, this approach has certain limitations. Since the reference for the percentage value is based on the TIC, an increase in the area of a given peak will result in the decrease on the relative areas of the rest of the peaks. This non-independence of the compounds leads to the problem of the fluctuation in the proportions of compounds, although their amounts do not actually change. For example, four compounds are in similar proportions (0.25, 0.25, 0.25, 0.25 - the sum of the proportions of the chemicals is constant and always 1). If the amount of one compound increases and the signal recorded is doubled, this will change the proportions of the other three compounds, although their concentrations (signals recorded) remain constant (0.4, 0.2, 0.2, 0.2). In this context, the use of relative proportions might make difficult to statistically confirm the possible effects that different factors (e.g., environmental or physiological) may exert on specific compounds. For instance, in the example above, an external factor might have directly affected to the first compound, increasing its concentration. But at the same time, the other three compounds decreased their relative amounts, even if the external factor did not affect them. Thus, an erroneous interpretation of these results could conclude that the external factor negatively affected the concentrations of the last three compounds, while the truth is that their actual concentrations did not change.

The aim of this study is to provide an alternative methodology to investigate chemical signals of lizards, based on the semi-quantification of their chemical components. This method allows the semi-quantification of compounds by conferring quantitative independence among them, avoiding the constant-sum problem (the sum of the relative amounts must be always 1). To test the reliability of our protocol, two experiments were performed identifying variations in the chemical compositions of lizard secretions produced by the effects of physiological (dietary supplementation) and climatic (temperature) factors.

Materials and Methods

We analysed potential changes in the chemical composition of chemical secretions from two lizard species, *Iberolacerta cyreni* and *Psammodromus algirus*. For both experiments, all lizards were captured by noosing during the first week of May 2015.

The first experiment involved male Carpetan rock lizards (*I. cyreni*) captured at "Alto del Telégrafo" (40°47′N, 04°00′W, Sierra de Guadarrama, Madrid, Spain). They were divided in control males (n=19) and treated-males (n=19). The treated males were orally supplemented using plastic syringes with 5μL of vitamin E (i.e., α-tocopherol; from Sigma-Aldrich Chemicals Co.), whose composition is 97 % of synthetic vitamin E (approx. 1014 IU ml21) and 3% soybean oil (with approx. 0.32 IU ml21 of natural vitamin E, i.e., D-α-tocopherol) (Kopena *et al.*, 2011). The dietary supplementation was conducted every two days during three weeks in June 2015. A similar procedure was followed during the same period of time for control lizards, but supplementing them with 5μL of soybean oil alone. With this experiment we aimed to assess whether our methodology was able to detect potential changes in the relative abundances of the chemical components found in secretions, due to the effect of an external factor (diet supplementation) exerted on individuals. The diet supplementation may lead to physiological changes that may be partially expressed in qualitative and qualitative variations in the chemical composition of secretions (Martín & López, 2015). Given the high number of compounds found in femoral secretions of *I. cyreni*, we selected a set of these compounds that represent different types of chemicals, such as aldehydes (tetradecanal), steroids (cholesta-3, 5-diene, cholesterol, campesterol, lanost-8-en-3-ol and β-sitosterol) and α-tocopherol. Moreover, these compounds encompass different relative abundances, ranging from low (e.g., lanost-8-en-3-ol) to high (cholesterol) (López & Martín, 2005), ensuring that this method can be used to study a wide array of compounds, ranging from high to low relative abundances.

In the second experiment, secretions of 18 males of Algerian Psammodromus lizards (*P. algirus*) at "La Golondrina" oak forest near Navacerrada village (40°43´ N, 04°01´W; Sierra de Guadarrama, Madrid, Spain) were extracted and split in two halves. The first half of secretions was exposed to 28 °C in an incubator for 3 h prior its conservation at -20 °C in a freezer. The other half of secretion was kept directly in the freezer after extraction as a control. Since a high environmental temperature may alter the detectability of scent marks of lizards (Martín & López, 2013), we aimed to test whether our methodology allowed detecting potential changes in the chemical components of the chemical secretions of *P. algirus*, due to the effects of a temperature treatment directly applied over secretions after being deposited .We examined changes in some selected compounds (i.e., hexadecanoic acid, 9,12-octadecadienoic acid, octadecanoic acid, squalene, cholesta-3,5-diene, cholesterol, ergosterol, campesterol, stigmasterol and β-sitosterol). In this case, the compounds selected were also chosen because they represented a variety of different chemical classes. However, as in the previous experiment, other compounds might have been used in the analysis.

Secretion collection

All the materials used for the secretion extraction were cleaned with *n*-hexane (99 %, J.T. Baker; Deventer, The Netherlands) in order to remove organic compounds. Then, we extracted the secretions from live animals using sterile forceps pressing gently around the pores until collecting the sample in a vial (figure 1A). However, a direct extraction pressing the glass vial over the pores is also possible (figure 1B). Blank control vials were also prepared in order to compare with those containing samples and to exclude potential events of contamination from handling procedures during sampling and analysis processes. Extracted secretions were directly introduced into total recovery glass vials (1.1 mL, Análisis Vínicos S.L., Tomelloso, Spain, ref. V2275) closed with Teflon-lined stoppers. Vials were stored at -20 °C to avoid sample degradation because of volatility of some chemicals and potential bacterial activity that may change the composition of secretions (Canuel & Martens, 1996).

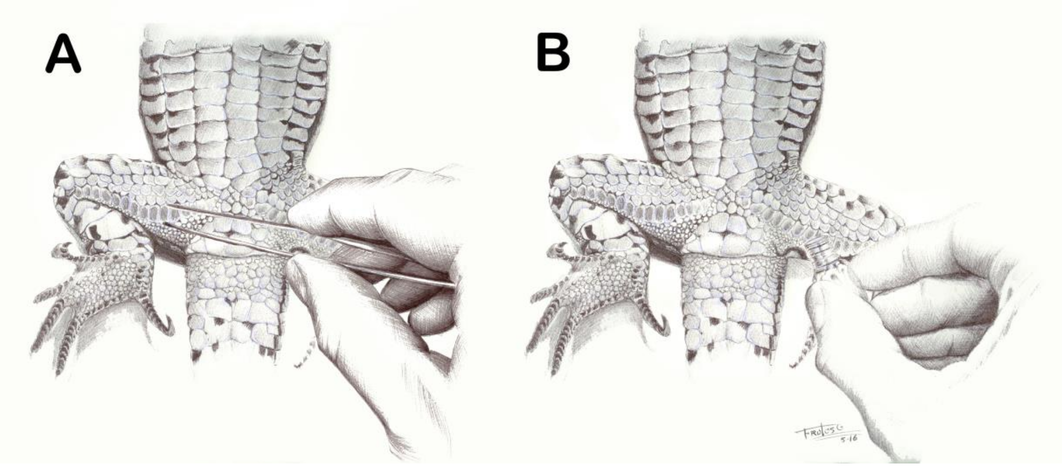

Figure 1: Extraction of chemical secretions of lizards. (A) Secretion collection after gently pressing with forceps around the lizard's femoral pores. (B) Direct collection of secretions by pressing glass vials against the lizard's femoral pores. Both methods may be also used for the extraction of precloacal pores secretions

Sample preparation

In the analytical laboratory, samples were removed from the vials using tweezers and dissecting needles and were weighed using a XP2U ultra-microbalance (Mettler Toledo; Columbus, OH. Accurate readability: 0.0001 mg). Disposable micro aluminium foil dishes with rims were used for weighing. During the weighing process, the temperature in the room was kept at 20 °C. All the laboratory supplies (tweezers, dissecting needles, etc.) were cleaned with *n*-hexane (99 %, J.T. Baker; Deventer, The Netherlands) before and after each weighing measurement. *n*-Hexane was also used as solvent for solid-liquid extraction. In addition, *n*-heptadecane (99 %, Sigma-Aldrich; St Louis, MO) was added to the samples as an internal standard (IS). For this, a solution of 50 ppm of *n*-heptadecane dissolved in *n*-hexane was prepared. Then, 1 μL of this solution was added to the sample per each 20 μg of femoral secretion. The mixture was vortex-mixed for 2 min and left it into the fridge (4 °C) for the precipitation of solid particles. Five minutes later, the liquid phase was collected with a micropipette and transferred to a total recovery glass vial suitable for GC analysis, which were closed with Teflon-lined stoppers, and this was called the sample. Finally, the samples were kept at -20 °C until their analysis.

Analysis of samples

Analyses were performed with a TRACE GC Ultra gas chromatograph coupled to a mass spectrometer (MS) equipped with a triple quadrupole analyzer (TSQ Quantum XLS, Thermo Fisher Scientific Inc.; Bremen, Germany) operated in electron impact ionization mode (EI, -70 eV of electron energy). The mass spectrometer was operated in scan detection mode. The current of the filament was 150 μA. A volume of 2 μL of each sample was injected in the analytical system. Injections were performed in a programmed temperature vaporization (PTV) injector in splitless mode. The split flow was 10 mL/min and the splitless time was 2 min. The injector was kept at 250 °C during the injection and transfer phases with a constant septum purge. We included a cleaning phase of the injector after the transfer phase consisting in an increase of temperature of the injector at 14.5 °C/s up to 350 °C. Then, the temperature was held at 350 °C for 5 min. The flow was also increased up to 50 mL/min in the cleaning phase. A capillary column HP-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness) purchased from Agilent Technologies (Palo Alto, CA, USA) was used for the separation. An initial temperature of 100 °C for 3 min was programmed in the GC oven and it was increased at 5 °C/min to 300 °C. The final temperature was held for 15 min. Helium was used as the carrier gas at a constant flow rate of 0.8 mL/min. The temperature of the transfer line and the MS source were set at 300 °C and 240 °C, respectively. Data recording was started 7 min after the separation began, as solvent delay and in order to increase the filament life.

We used Xcalibur™ 2.1.0.1140 software (Thermo Fischer Scientific Inc., San Jose, CA, USA) to record and process the acquired data. Finally, we identified the chemical components by comparing their mass spectra with those of compounds provided by the NIST/EPA/NIH (NIST 02) computerized mass spectral library. Ccommercial standards were compared with spectra and retention times of our compounds to confirm the identification. The areas of all the analytes (A_{anal}) were expressed as a percentage (*RA,* relative abundance) of the IS peak area (A_{stand}). That is, $RA = (A_{anal}/A_{stand}) \times 100$.

Statistical procedures

We conducted General Linear Models (GLM) to study potential variations occurred in the relative abundance of each compound because of the effect of the dietary supplementation of vitamin E in *I. cyreni*. We used repeated measures General Linear Models (GLM) to assess potential changes in the relative abundance in each of the selected compounds due to the effect of temperature in the deposited chemical secretions of *P. algirus*. We log-transformed the data resulting from semi-quantification of each compound to ensure normality (Shapiro- Wilk's test). The tests of homogeneity of variances (Levene's test) showed that heterogeneity of variances were not significant in all cases. All the statistical analyses were conducted with STATISTICA, version 8.0 and SPSS 20.0.0.

Results

The repeatability and intermediate precision of the analytical method were studied for the IS, since it is the unique compound present in all the samples at the same concentration. The repeatability was studied for five consecutive injections, being the relative standard deviation (RSD) 0.04 % for the retention times and 1.7 % (RSD) for peak areas. The intermediate precision was studied in four non-consecutive days over eight days and was 0.2 % (RSD) for the retention times and 6.7 % (RSD) for the peak areas.

Vitamin E supplementation

Overall, secretions of supplemented males showed significant differences compared to those of control males (GLM comparing relative abundances of seven compounds; $F_{7,30} = 39.31$, $P < 0.001$) (figure 2A). Even so, the effect of the vitamin E dietary supplementation differed among compounds. Thus, relative amounts of α-tocopherol ($F_{1,36} = 236.45$, $P < 0.001$), campesterol ($F_{1,36} = 18.27$, $P < 0.001$) and lanost-8-en-3-ol ($F_{1,36} = 22.79$, $P < 0.001$) were highly influenced by the supplementation, increasing their relative abundances in samples of treated males compared to control males. Also, β-sitosterol ($F_{1,36} = 10.20$, $P = 0.002$), cholesterol ($F_{1,36} = 11.07$, $P = 0.002$) and cholesta-3,5-diene ($F_{1,36} = 4.48$, $P = 0.04$) had significant higher relative amounts in the secretions of supplemented males than in control males. However, in the case of tetradecanal, the diet supplementation did not lead to significantly different changes in its relative abundance ($F_{1,36} = 0.02$, $P = 0.87$).

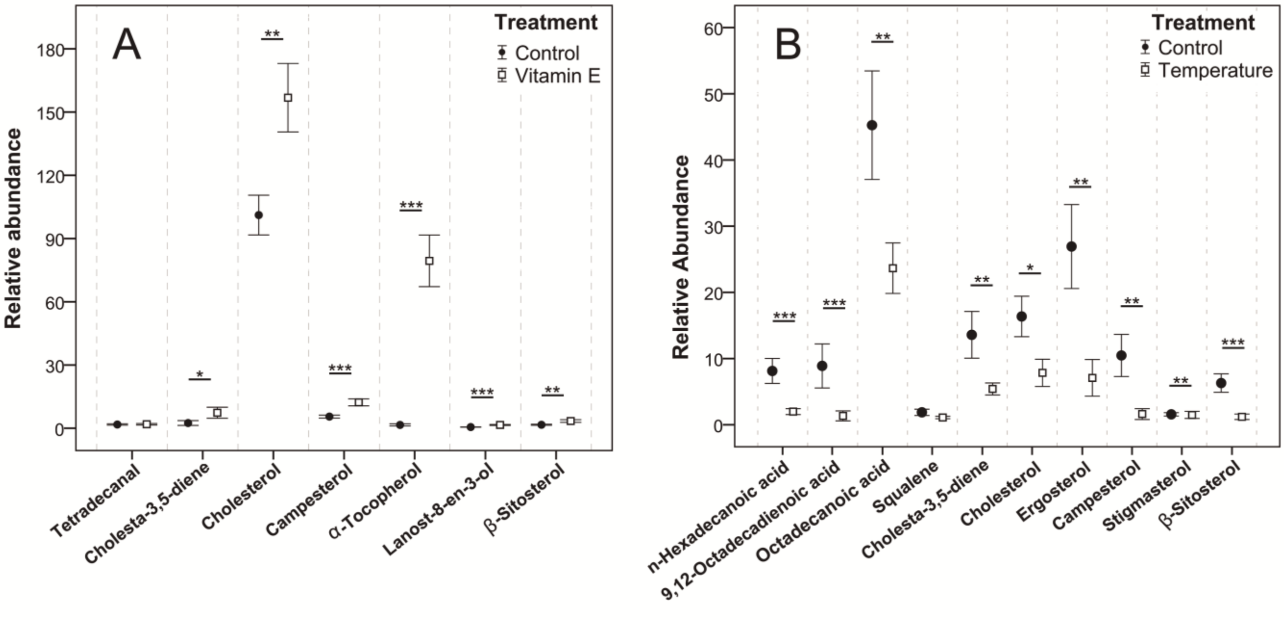

Figure 2: Effects of two different treatments on the chemical composition of lizard secretions. (A) Comparison of the relative abundances (mean ± SE) of seven particular compounds in secretions of control and vitamin E diet supplemented males of *Iberolacerta cyreni* lizards. (B) Comparison of the relative abundances (mean ± SE) of ten particular compounds in secretions of *Psammodromus algirus* male lizards after being deposited and exposed to a high temperature treatment. (***, P < 0.001; **, P < 0.01; *, P < 0.05)

Temperature effects on the secretions

Temperature-treatment effects differed among compounds (figure 2B). Thus, relative amount of *n*-hexadecanoic acid ($F_{1,16} = 23.14$, $P < 0.001$), 9,12-octadecadienoic acid ($F_{1,16} = 19.23$, $P < 0.001$), octadecanoic acid ($F_{1,16} = 9.24$, $P = 0.007$), cholesta-3,5-diene ($F_{1,16} = 9.89$, $P = 0.006$), cholesterol ($F_{1,16} = 7.34$, $P = 0.01$), ergosterol ($F_{1,16} = 12.65$, $P = 0.002$), campesterol ($F_{1,16} = 9.74$, $P = 0.009$), stigmasterol ($F_{1,16} = 29.38$, $P = 0.009$) and β-sitosterol ($F_{1,16} = 23.56$, $P < 0.001$) were affected by the temperature decreasing their relative abundances after the treatment. However, the temperature treatment did not significantly affect to the relative abundance of squalene ($F_{1,16} = 0.36$, $P = 0.149$).

Discussion

The development and implementation of alternative methodologies that allow analysing chemical signals more accurately is crucial to gain a thorough understanding of lizard´s ecology and evolution (Mason & Parker 2010; Martín & López 2015). We provide here an alternative methodology to study lizard chemical secretions. This methodology allows describing the profile of chemical secretions and moreover, the detection of potential variations in their chemical components produced by different factors (vitamin E dietary supplementation and environmental temperature).

Sample preparation

Multiple small-sized species or even females and subadults of medium and big lizards produce little secretions (Martín & López 2014). This fact may hinder analytical process, since slight signal peaks cannot be easily detected in GC-MS process. Probably, this could be one of the reasons why studies on subadults and females are scarce [see e.g., (Alberts, 1990; Khannoon *et al.,* 2011; Martín *et al.*, 2015, García-Roa *et al.*, 2016b)]. In this context, the development of methodologies with higher sensitivity to detect slight signals might extend the focus to the above-mentioned cases, which would avoid skewing the studies on chemical communication only to males of medium and big sizes. Also, the ability to analyse small portions of the total secretion allows lizards to continue carrying information (i.e., the rest of chemical secretion) for subsequent communicative events. In addition, the possibility to reliably analyse small samples also opens opportunities for studies of temporal changes in the compositions of secretions, using repeated sampling of the same individual. Even so, we are aware that our methodology may also be constrained by the minimum amount of sample required for the analyses, since for samples of less than 0.3 mg, the volume of solvent needed was too low for the preparation and the injection in the chromatographic system. However, the secretions used in this work weighed an average of 1.167 mg, which far exceed the minimum required.

For the semi-quantification of compounds we used *n*-heptadecane (i.e., IS) as reference to which relativize the other compounds in order to obtain their relative abundances. Given that all samples are spiked with the same concentration of IS, its area is always constant. Thus, different samples can be rigorously compared compound by compound, with total statistical independence among them, avoiding the problem of the constant-sum (TIC=1, see the introduction for an example of this case). The IS must be, ideally, of similar chemical characteristics to the analytes and moreover, the peak must not overlap with any of the analyte peaks. In these regard, *n*-heptadecane is a non-polar compound that does not overlap with any compound of interest. Instead, we previously tested *n*-hexadecane and *n*-octadecane, but they eluted close to other peaks of interest and were discarded.

Sample analysis

In this work we used a non-polar capillary GC column already used for the analysis of secretions of lizards with satisfactory results (Martín *et al*., 2013; Martín *et al*., 2015a; García-Roa *et al*., 2016a). Other columns with different stationary phases may also be used for the separation of specific compounds (e.g., steroids, alcohols, ethers) and, thus, will provide better separations of those compounds in terms of resolution and separation times. However, the type of column used in this work is more useful for the separation and identification of a larger number of different types of compounds and then, their semiquantification through the proposed approach.

The data recording was done in the scan mode, which in combination with the relatively long GC method, allow the description of the secretion chemical profiles, as was carried out in previous studies (López & Martín, 2005; Martín & López, 2006). Figure 3 shows the typical chromatogram for a sample of *P. algirus*, in which several peaks are detected.

From a practical point of view, the described methodology resulted very cheap since little volumes of reagents were needed; less than 100 µL of the IS solution in all the cases and only some drops of *n*-hexane for cleaning the lab supply. Considering that all the needed instrumentation and lab material is available in the laboratory (GC-MS system, micro-pippetes, etc.), the price for each analysis including all the disposable material (pipette tips, paper, aluminum foils, glass vials) and consumables (helium, solvents, etc.) was estimated in less than 0.3 € per sample. Overall, the sample preparation process spent less than 5 min per sample, which makes the process especially useful for the preparation of a large number of samples. Several samples can be prepared at the same time, while only one at the same time can be analyzed in the GC-MS system. The total time for each analysis is of around 1 hour, including the sample preparation.

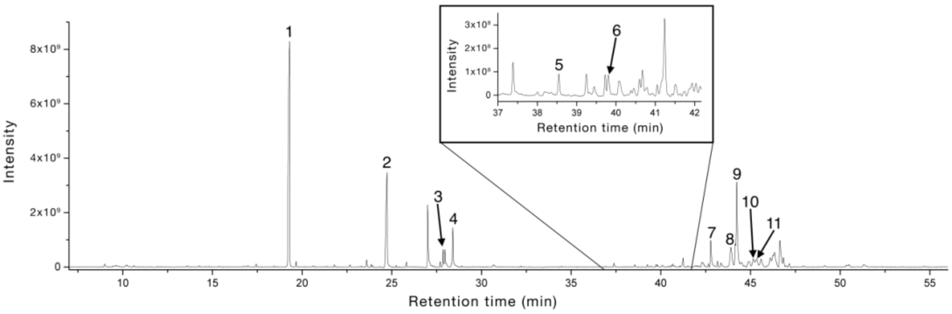

Figure 3. Chromatogram showing the separation of compounds present in secretions of a Psammodromus algirus male lizard (Fam. Lacertidae). 1 – n-heptadecane (internal standard); 2 – n-hexadecanoic acid; 3 – 9,12-octadecadienoic acid; 4 – octadecanoic acid; 5 – squalene; 6 – cholesta-3,5-diene; 7 – cholesterol; 8 – campesterol; 9 – ergosterol; 10 – stigmasterol; 11 – β-sitosterol

Application of the methodology

Two different studies were designed in order to test the validity of the analytical methodology. Concretely, the aim of the first experiment was to confirm the reliability of the analytical methodology to study the physiological effects produced by the dietary supplementation of vitamin E on the relative abundances of seven compounds. The results showed that the vitamin E supplementation had a direct effect on the composition of the chemical secretions. Thus, the concentrations of almost all studied compounds (all but tetradecanal) changed after the treatment. Although these seven compounds were used in this study to demonstrate that our protocol is able to detect compositional changes resulting from vitamin E supplementation, further experimental investigations needs to be performed to establish whether other chemical components might be affected by the supplementation of this vitamin.

The aim of the second experiment was to explore whether the analytical method allowed detecting the effect of temperature on secretions after being deposited (i.e., on scent marks). All the compounds studied, except squalene, showed a decrease in their concentration levels when the temperature was increased up to 28 °C. These changes might alter the quality of deposited scent-marks since, if their components are lost due to high temperatures, its efficacy as chemical signals may be also lost as was suggested in previous behavioural experiments with other lizard species *(Martín & López, 2013; Heathcote et al., 2014; Martín et al.*, 2015b). In view of the results, our methodology may be useful to study changes in the relative abundances of compounds produced by an increase in the temperature.

Further insights and implications

The methodology described here constitutes an alternative way to explore lizard chemosignals. We believe that the use of this methodology will allow expanding and enhancing the ecological and evolutionary knowledge of lizards chemical communication, since this approach is probed to be useful to study potential variations in the different compounds found in chemical secretions with a total analytical independence among compounds. This may facilitate the execution of new studies in the search of deeper knowledge on important topics, such as the effects of environmental changes (e.g., climate change) on the chemical communication in lizards. Only a few studies have examined the effect that environmental variables might exert on lizard chemical signals (Martín & López, 2013; Heathcote *et al.*, 2014; Martín & López, 2015). For example, the detectability and efficiency of scent marks decrease at high temperature in rock lizards (*I. cyreni*) (Martín & López, 2013) and wall lizards (*Podarcis hispanica*) (Martín *et al.*, 2015b). However, both studies were based on behavioural approaches, by comparing the chemosensory responses (i.e., tongue-flick rates) of female lizards when they were exposed to scent marks of males. The methodology presented in this work could provide new information about the chemical fundaments underlying these behaviours. This fact opens a wide spectrum of possibilities to explore how and why chemical compounds are affected by environmental variables.

Compounds present in chemical secretions may be directly synthesized by lizards or be acquired from the diet (Weldon *et al.*, 2008). Therefore, levels of these chemicals in secretions may be largely dependent on the condition of each individual (Martín & López, 2015). Multiple studies have found physiological dependence in the expression of several compounds such as cholesterol, cholesta-5,7-dien-3-ol, ergosterol, octadecenoic acid, hexadecanol or α-tocopherol [reviewed in (Weldon *et al.*, 2008; Martín & López 2014, 2015)]. Thus, the level of expression of these compounds seems to be under the effect of physiological trade-offs between metabolism requirements and the allocation of the compounds in secretions. However, a large amount of chemicals already found in chemical secretions remain unstudied. This is probably explained because it is difficult to detect patterns in compounds with lower abundances in chemical secretions using TIC proportions, as we explained in the Introduction example. Therefore, with the new methodology, each compound may be studied individually, as well as its effects on the lizard condition. Thus, the methodology presented here might help to deeply understand the relationship between these compounds and the condition of lizards. In this regards, the possibility of studying analytes independently of the rest of the compounds present in a sample allows exploring new physiological hypotheses about the ecological and evolutionary context surrounding chemical signalling. We further note that, although this methodology has been focused on lizard secretions, the approach could be specifically adapted to other vertebrates that own similar lipophilic gland secretions such as mammals, birds and other reptiles.

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CHAPTER II

GENDERS MATTERS: SEXUAL DIFFERENCES IN CHEMICAL SIGNALS OF *LIOLAEMUS WIEGMANNII* LIZARDS (IGUANIA, LIOLAEMIDAE)

CAPÍTULO II

EL GÉNERO IMPORTA: DIFERENCIAS SEXUALES EN LAS SEÑALES QUÍMICAS DEL LAGARTO *LIOLAEMUS WIEGMANNII*(IGUANIA, LIOLAEMIDAE)

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GENDERS MATTERS: SEXUAL DIFFERENCES IN CHEMICAL SIGNALS OF *LIOLAEMUS WIEGMANNII* LIZARDS (IGUANIA, LIOLAEMIDAE)

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ABSTRACT

Males have traditionally been the focus of study when analyzing traits involved in communication. However, intraspecific interactions often imply both sexes as signals senders. Researches have shown the relevant role that chemical communication plays in lizards. However, the knowledge about the female scents is scarce. The aim of this paper is to characterize, by using gas chromatography-mass spectrometry (GC-MS), the lipophilic fraction of precloacal gland secretions of adult males and females of the lizard *Liolaemus wiegmannii* and evaluate intersexual differences in composition and proportion of their chemical profiles. We identified 48 and 41 lipohilic compounds in males and females, respectively. The main compounds were steroids and waxy esters. We also found squalene, alcohols, aldehydes and carboxylic acids. Overall, we found significant differences between sexes with respect to the relative proportions of compounds. We also found differences in proportions of three of the five predominant compounds shared by both sexes (squalene, cholesta-3,5-dien and cholesta-2,4-dien). Our study describes that females of *L. wiegmannii* hold lipids in their precloacal secretions, which are further clearly different from those of males. Further researches are needed to understand the ecological implications of these sexual differences in precloacal secretion of *L. wiegmannii.*

Keywords: Chemoreception; female signals; lipids; lizards; Uruguay

Introduction

The understanding of animal ecology and evolution becomes complicated without attempting to elucidate ecological processes and mechanisms underlying to intraspecific interactions. Animal interactions are inherent to sexual communication and, intrinsically, to sexual selection (Andersson & Simmons, 2006). In this context, outsized ornaments, striking colorations and noteworthy acoustic behaviours of males have historically put the focus over males as the main protagonists in animal communication (Andersson & Simmons, 2006). However, intraspecific interactions often imply both sexes as signals senders, or even only one direction of signalling in which females act as signals senders (Clutton-Brock, 2009). For instance, females´ competition for obtaining better territories and/or resources during the reproductive season has been well documented (Clutton-Brock, 2007). Also, some species develop social behaviours, in which to maintain a dominant o cooperative hierarchy involve complex understanding processes within and between sexes (Sinn *et al.*, 2008). Additionally, females may compete for access to mates (i.e., male mate-choice) (Edward & Chapman, 2011). Therefore, signals sent by females may be involved in multiple ecological interactions. In this respect, visual and acoustic signals are the most commonly studied communications systems (Amundsen & Forsgren, 2001; Sueur & Aubin, 2004). .Unfortunately, alternative modes of females' communication remain relatively overlooked.

Recent researches have shown the relevant role that chemical communication plays in some living-organisms groups (Johansson & Jones, 2007; Symonds & Elgar, 2008). In lizards, chemoreception is determinant in intraspecific communication (Houck, 2009; Mason & Parker, 2010; Martín & López, 2015). Chemosignals engage in territoriality (Carazo *et al.*, 2008),as well as in sexual recognition and mate or rival assessment (Johansson & Jones, 2007; Martín & López, 2007). According to the latter, chemical signals are often directly involved in mate choice in some lizards' species (Martín & López, 2015). In this context, femoral and precloacal glands are important sources of lizards' chemosignals (Weldon *et al.*, 2008). These glands protrude secretions that embody informative signals in form of lipids and proteins (Weldon *et al.*, 2008; Martín & López, 2014). Indeed, testosterone and other reproductive hormones regulate the activity of these glands, underpinning the tenet that chemical secretions have relevant functions within sexual-selection and reproductive lizards' processes (Martín & López, 2015). The composition of secretions may differ between species, populations or even among individuals (Escobar *et al.*, 2003; Runemark *et al.*, 2011; García-Roa *et al.*, 2016). Over this basis, recent studies have analysed intersexual differences in scent composition through descriptive and behavioural approaches [e.g., (Khannoon *et al.*, 2011; Martín *et al.*, 2016)]. However, although the function of male chemical cues has been highlighted as relevant in both inter- and intraspecific interactions, the role of chemical signals of female lizards is still poorly understood. This is possibly ascribed in part to the lack of studies that analyse the chemical profiles of the scents of females (Alberts, 1990; Khannoon *et al.*, 2011; Martín *et al.*, 2015, 2016).

Unfortunately, chemical communication has been only explored in some particular groups of lizards (Weldon *et al.*, 2008; Martín & López, 2014). Concretely, the genus *Liolaemus* (Liolaemidae) has been the focus of multiple works in the context of chemical communication (Labra, 2011). Behavioural studies have shown the importance of chemoreception in the ecology of this genus (Labra & Niemeyer, 2004; Labra, 2007). Different sources of scents (i.e., feces, skin and/or precloacal glands) can be involved in *Liolaemus* interactions (Labra *et al.*, 2002). With respect to precloacal glands, adult males of some *Liolaemus* species have conspicuous precloacal pores (Pincheira-Donoso *et al.*, 2008a; Valdecantos *et al.*, 2014), which protruded secretions might yield information about the holders during the mating-season (Labra *et al.*, 2002; Martín & López, 2014). On the other hand, although the presence of precloacal glands in females has been described in a few *Liolaemus* species, there is also a wide range of interspecific phenotypic alternatives; species with females without pores, species with females with pores but without apparent secretion, or species with females with pores and abundant secretions. This fact reflects the complexity intrinsic to sexual communication in the *Liolaemus* genus and thus, the importance of addressing studies investigating the chemical communication of females. Therefore, given the high diversity of life-strategies and ecological requirements within the *Liolaemus* group, it might conform an insightful model to explore chemical communication in lizards (Labra, 2011). One of the horizontal pillars in the study of chemical ecology is based on improving the knowledge of the specific profile of chemical signals (Martín & López, 2014). However, there are only a few works that have analysed the diversity of compounds found in precloacal secretions of males of several *Liolaemus* species (Escobar *et al.*, 2001; Escobar *et al.*, 2003). Moreover, thus far, nothing is known about chemical composition of female scents and their potential intersexual differences in this genus. Hence, the aim of this paper is to characterize, by gas chromatography-mass spectrometry (GC-MS), the lipophilic fraction of precloacal gland secretions of adult males and females of *Liolaemus wiegmannii* during the reproductive season and evaluate intersexual differences in composition and proportion of their chemical compounds.

Material and methods

Species and study area

Field-work was carried out during springs of 2013 and 2014 in Solymar ("Costa de Oro"), Canelones department, Uruguay (34°50'23.10"S; 55°57'36.63"W). Adult males and females of *L. wiegmannii* were captured alive by noosing. This is a small terrestrial lizard species with preferences for coastal biotopes over 750 km across south of Uruguay. Usually, *L. wiegmannii* inhabits covered or semicovered dunes, where hiding under burrows and shrubs is used as predator defense (Carreira & Maneyro, 2013). This species is also broadly distributed across different geographic points of Argentina and Brazil with similar environmental characteristics (Avila *et al.*, 2009). The colour pattern is phenotypically cryptic, with orange-yellow throat and orange dorsal spots in some males. Females and juveniles have less contrasted pattern with brown as the predominant colour (Carreira *et al.*, 2005). The captured individuals measured between 45-60 cm (SVL; males) and 45-55 cm (SVL; females). All individual, males and females had precloacal pores.

Once lizards were captured, we extracted the waxy precloacal secretions by softly pressing of the glass vial around the pores. In the case of females, only in 22.2 % of them was not possible to extract secretions. Although we have no data about the exact weight of secretions, it was clear that male secretions were seemingly more abundant than those of females. We kept secretions into the vials and closed them with Teflon-lined stoppers. Samples were stored at -20 °C until analytical processed. Moreover, we used blank control vials (i.e., without secretion) by using the same methodology. Thus, we could compare controls with the secretion-samples for excluding potential contaminants as result of handling and analytical procedures.

Analyses of femoral gland secretions

Samples were analyzed using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl/ 95 % dimethylsiloxane) column (Supelco, Equity-5, 30 m length x 0.25 mm ID, 0.25- µm film thickness). We used a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. Sample injections (2 µl of each sample dissolved in *n*-hexane) were performed in splitless mode using helium as the carrier gas, with injector and detector temperatures at 250 °C and 280 °C, respectively. The temperature program was as follows: 50°C isothermal for 3 min, then increased to 300 °C at a rate of 5 °C/min, and then isothermal (300 °C) for 15 min. Mass spectral fragments below m/z = 46 were not recorded. We performed the initial identification of chemicals by comparison of mass spectra in the NIST/EPA/NIH (NIST 02) computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards (from Sigma–Aldrich Chemical Co.) when these were available. Impurities identified in the solvent and/or the control vial samples were not considered. We determined the relative amount of each compound using the percent of the total ion current (TIC).

The compositional analysis, consisting in logit transforming the proportion data by taking the natural logarithm of proportion ÷ (1 – proportion), was used to correct the problem of non-independence of proportions (Aebischer *et al.*, 1993). Thereafter, we calculated euclidean distances between every pair of samples to generate a resemblance matrix that provided the basis of the subsequent analyses. We conducted a permutational multivariate analysis of variance tests (PERMANOVA) (Anderson, 2001; Anderson *et al.*, 2008) based on the Euclidean resemblance matrix using 999 permutations to explore potential differences in chemical secretions between sexes. Furthermore, canonical analyses of principal coordinates (CAP analyses) were also used to investigate intersexual differences. We used the software PRIMER V6.1.13 implemented with the PERMANOVA+ V1.0.3 add-on package (Anderson & Willis, 2003).

Results

The maximum number of compounds identified in our study was 48 (Table 1). Levels of these compounds differed between sexes and among samples. Overall, steroids were the most abundant compounds (71.2 % of TIC), followed by waxy esters (20. 4%) and squalene (8 %). Some other types of compounds were found in low levels, such as alcohols (0.1%), aldehydes (0.1 %) and carboxylic acids (0.1 %). On average, cholesta-4,6-dien-3-ol (17.2 %) was the most abundant compound in secretions, followed by cholesta-3,5-dien (11.9 %), cholesta-2,4-dien (10.9%) and squalene (8 %). The proportions in the rest of compounds ranged from 5.2 % (cholest-4-en-3-one) to 0.01 % (hexadecanoic acid, 1-methyl ethyl ester and octadecanal).

Table 1:Lipophilic compounds found in precloacal secretions of *Liolaemus wiegmannii* females (n=7) and males (n=8)

RT	Compounds	Males (Mean±SD)	Females (Mean±SD)
33.30	1-Methylethyl ester- hexadecanoic acid	0.01 ± 0.02	
33.50	Octadecanal	0.02 ± 0.02	
36.56	Octadecanol	0.27 ± 0.26	
36.89	Nonadecanal	0.19 ± 0.40	
38.29	Eicosanol	1.00 ± 2.07	
38.65	9-Octadecenoic acid, ethyl ester	0.07 ± 0.06	
45.52	Squalene	4.53 ± 4.87	11.63 ± 3.57
45.87	4,6,8(14)-Cholestatrien	0.86 ± 1.22	4.41 ± 2.97
46.12	Cholesta-2,4-dien	16.40 ± 6.02	5.57 ± 4.74
46.40	Unid.Steroid (199,253,355,368,379)	0.65 ± 0.43	4.74 ± 4.79
46.52	Cholest-5-en-3-ol, derivative?	0.29 ± 0.54	2.10 ± 1.14
46.71	Cholesta-4,6-dien-3-ol	15.92 ± 5.39	18.60 ± 14.39
46.88	Cholest-5-en-3-ol, derivative?	0.62 ± 0.91	0.51 ± 0.56
46.98	Cholesta-3,5-dien	15.06 ± 6.46	8.89 ± 3.05
47.31	Unid.Steroid (155,197,251,350,365)	1.88 ± 0.82	2.04 ± 1.03
47.52	Cholesta-2,4-dien, metil derivative?	1.65 ± 0.82	0.44 ± 0.47
47.81	Cholesta-5,7-dien-3-ol, acetate	0.56 ± 0.29	0.63 ± 0.53
47.85	Unid.Steroid (155,197,251,350,365,378)	1.65 ± 1.08	1.64 ± 0.92
48.08	Cholesta-4,6-dien-3-ol, methyl derivate	0.60 ± 0.39	0.34 ± 0.57
48.29	Unid.Steroid (235,253,296,348,380)	0.20 ± 0.16	3.22 ± 3.50
48.36	Cholesta-3,5-dien, methyl derivative?	1.69 ± 0.62	0.46 ± 0.48
48.65	Cholesta-2,4-dien, ethyl derivative?	0.92 ± 0.91	0.56 ± 0.43
49.01	Unid.Steroid (155,197,251,364,375,390)	0.70 ± 0.54	0.50 ± 0.38
49.24	Cholesta-4,6-dien-3-ol, ethyl derivate?	0.48 ± 0.26	0.33 ± 0.25
49.47	Cholesta-3,5-dien, ethyl derivative?	2.81 ± 4.76	0.55 ± 0.34
49.75	Unid.Steroid (155,197,251,355,377,393)	0.95 ± 0.74	1.13 ± 0.39
49.94	Cholesterol	1.44 ± 0.70	0.33 ± 0.35
50.26	Unid.Steroid (143,158,183,195,211,237,253,378,395)	0.15 ± 0.12	0.28 ± 0.16
50.54	Cholestan-3-one	0.12 ± 0.28	0.16 ± 0.11
51.08	Cholesta-3,5-dien-7-one	0.78 ± 0.45	0.97 ± 0.90
51.51	Cholest-4-en-3-one	8.58 ± 6.11	1.95 ± 1.60
51.94	Cholesta-4,6-dien-3-one	0.29 ± 0.42	0.32 ± 0.25
52.13	Hexadecyl hexadecanoate	0.01 ± 0.01	0.44 ± 0.48
52.81	Cholest-5-en-3-one	0.88 ± 0.58	
53.04	Cholest-5-en-3-ol, derivative?	0.01 ± 0.01	3.65 ± 3.87
53.90	Stigmast-4-en-3-one	0.68 ± 0.31	0.26 ± 0.18
54.20	Hexadecyl 9-hexadecenoate	0.12 ± 0.29	0.06 ± 0.09
54.30	9-Hexadecenil 9-hexadecenoate	0.13 ± 0.16	0.14 ± 0.22
54.43	Octadecyl hexadecanoate	0.10 ± 0.08	0.35 ± 0.34
55.56	Hexadecyl 9-octadecenoate	1.42 ± 2.48	5.99 ± 7.39
56.32	Octadecyl octadecanoate	0.25 ± 0.59	7.92 ± 7.69
56.81	9-Octadecenyl 9-hexadecenoate	0.12 ± 0.17	0.09 ± 0.14

RT	Compounds	Males (Mean±SD)			Females (Mean±SD)		
57.13	Octadecyl 9-octadecenoate	0.18	±	0.23	0.65	±	0.43
57.43	Eicosyl hexadecanoate	0.38	±	0.33	0.29	±	0.37
61.17	Eicosyl 9-octadecenoate	1.85	±	1.59	2.17	±	2.55
61.53	Docosyl hexadecanoate	4.15	±	8.39	0.83	±	1.20
66.89	Docosyl 9-octadecenoate	7.09	±	4.58	4.12	±	5.39
67.34	Tetracosyl hexadecanoate	1.30	±	1.53	0.73	±	1.52

The relative abundance of each chemical was determined as the proportion of the total ion current (%TIC) and reported as the average (± 1SD). Characteristic ions (m/z) are reported for unidentified compounds. RT: Retention time

The number of chemicals identified was different among sexes. Hence, males of *L. wiegmannii* had seven compounds more than females, totalling 48 and 41 respectively (Table 1). We found that hexadecanoic acid 1-methyl ethyl ester, octadecanal, octadecanol, nonadecanal, eicosanol, 9-octadecenoic acid ethyl ester and cholest-5-en-3-one were found only in males but were absent in females' precloacal secretions. They represented the 14.5% of compounds and 2.4 % of TIC area in male secretions. However, all compounds found in females were also found in males. In both sexes steroids were the most abundant type of compounds (77.8 % of TIC in males and 64.6 % in females). However, while waxy esters (17.1 %), squalene (4.5 %), alcohols (0.3 %), aldehydes (0.2 %) and esters of carboxylic acids (0.1 %) were the subsequent most abundant types of compounds in males, we only found waxy esters (23.8 %) and squalene (11.6 %) in females. The proportions of the two more predominant types of chemicals in both sexes (i.e., steroids and waxy esters) did not show intersexual significant differences (ANOVA, steroids: $F_{1,13} = 0.01$, $P = 0.99$; waxy esters: $F_{1,13} = 0.24$, $P = 0.62$) (figure 1).

Moreover, we identified cholesta-2,4-dien (16.4 %), cholesta-4,6-dien-3-ol (15.9 %) and cholesta-3,5-dien (15 %) as predominant compounds in males, while in females the most abundant chemicals were cholesta-4,6-dien-3-ol (18.6 %), squalene (11.6 %) and cholesta-3,5-dien (8.8 %). In the rest of compounds, proportions in males ranging from 8.5% (cholest-4-en-3-one) to 0.01 % (hexadecanoic acid 1-methyl ethyl ester, octadecanal, hexadecyl hexadecanoate, cholest-5-en-3-ol derivative), and between 7.9 % (octadecyl octadecanoate) and 0.07 % (tetracosyl hexadecanoate) in females.

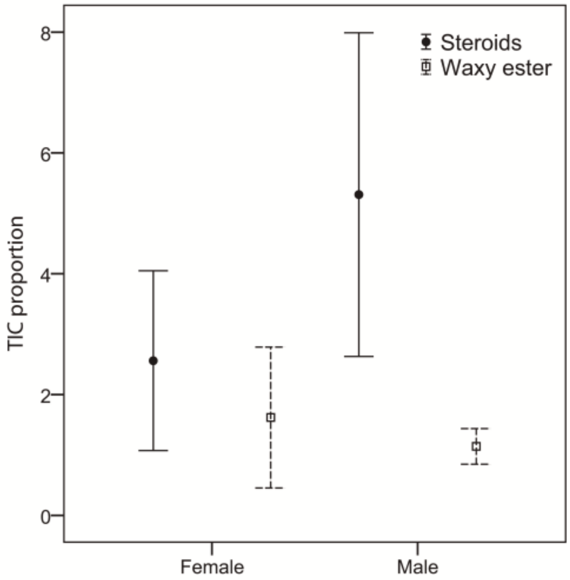

Figure 1: Comparison of the proportion (Mean±SE) of the two majoritarian types of compounds between sexes of *Liolaemus wiegmannii*

Our results comparing individuals of each sex by using the PERMANOVA analysis based on the resemblance matrix, revealed significant differences between the two sexes in the proportion of chemicals (pseudo $F_{1,13}$ = 13.11, P = 0.002). We further tested with a cluster analysis based on euclidean distances among secretions´ profiles (i.e., transformed TIC proportions of chemicals) whether individuals could be grouped per sex based on their chemical profiles alone. Definitively, our analyses with the CAP tool clustered 100 % of the samples into the correct sex (permutational test, δ_1^2 = 0.997, P = 0.002, using leave-one-out cross-validation and m = 2 axis).

Additionally, PERMANOVA analysis showed significant differences between both sexes (pseudo $F_{1,13}$ = 3.50, P = 0.027) by using only the five major compounds shared by both sexes (squalene, cholesta-4,6-dien-3-ol, cholesta-3,5-dien, cholesta-2,4-dien and docosyl 9-octadecenoate) (figure 2). Further analyses exploring potential differences for each chemical between sexes revealed that proportions of squalene were significantly greater in females (ANOVA: $F_{1,13}$ = 12.3, P = 0.003), whereas cholesta-3,5-dien (ANOVA: $F_{1,13}$ = 9.52, P = 0.008), and cholesta-2,4-dien (ANOVA: $F_{1,13} = 7.20$, $P = 0.018$) were found in proportions significantly higher in males. However, proportions of cholesta-4,6-dien-3-ol (ANOVA: $F_{1,13} = 3.08$, $P = 0.10$) and docosyl 9-octadecenoate (ANOVA: $F_{1,13} = 0.24$, $P = 0.62$) did not significantly differ between sexes.

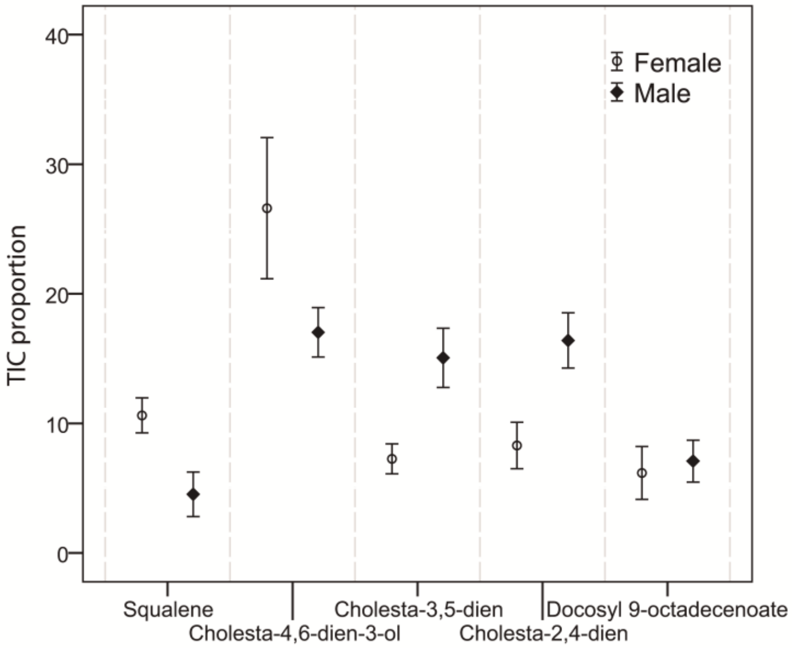

Figure 2: Mean (±SE) of the five compounds with highest proportions shared between male and females of *Liolaemus wiegmannii*

Discussion

In this work we describe for the first time the composition of precloacal secretions in females and males of *L. wiegmannii*. The lipophilic fraction in both sexes is fundamentally formed by steroids. This type of compounds has been commonly found as majoritarian in most of lizard species analysed hitherto (Escobar *et al.*, 2001; Martín & López, 2014). It has been suggested that high levels of particular steroids could be used as signals during intersexual encounters because of their association with the individual "quality", such as some vitamin precursors as cholesta-5,7- dien-3-ol (precursor of vitamin D_3) or ergosterol (provitamin D_2) (Weldon *et al.*, 2008; Martín & López, 2014). Moreover, some steroids, such as cholesterol, could have protecting functions within the secretions providing a protector matrix for other compounds (Weldon *et al.*, 2008; Martín & López, 2014). However, although *L. wiegmannii* chiefly hold steroids in their precloacal secretions, the level of expression of some of them is interestingly different with respect to other lizards. Overall, cholesta-4,6-dien-3-ol and cholesta-3,5-dien were the most abundant steroids, which differ of other lizard species in which cholesterol has the highest levels in femoral secretions (Martín & López, 2014). Multiples evidences have highlighted that some iguanian species might carry in chemical secretions other steroids as majoritarian (Alberts *et al.*, 1992; Martín *et al.*, 2013, 2016). However, studies analysing the precloacal secretion of several *Liolaemus* species showed that in some species, cholesterol was also the predominant compound (Escobar *et al.*, 2001, 2003). Our investigation reveals that the relative abundance of cholesterol is low in *L. wiegmannii*. In fact, numerous chemicals – fundamentally other steroids, waxy esters and squalene – overcome the proportion of cholesterol found in secretions. Although there is no information about the signalling function of most compounds identified in lizard scents, the description of alternative compounds as predominant in the secretion of different species outlines the extraordinary diversity of chemicals potentially employed as chemosignals in lizard communication. Indeed, the variation of the composition, especially the compounds with high predominance, in species phylogenetically close might in part be a reflection of the evolutionary trajectories underlying to the linages´ diversification. In this context, for example, the *Liolaemus* species whose secretions were described by Escobar *et al.* (2001, 2003) do not belong to the "wiegmannii" clade (Pincheira-Donoso *et al.*, 2008b). This phylogenetic distance with *L. wiegmanni* could be an important factor to partially explain the differences in chemical profiles.

Our study describes females of *L. wiegmannii* as carrier of lipids in their precloacal secretions, which are further clearly different from those of males. Female scents lack some chemicals identified in males but in addition, the proportions of some predominant chemicals are highly variable between sexes (Table 1 and figure 2). However, in contrast with recent studies in the lacertid lizard *Acanthodactylus boskianus* (Khannoon *et al.*, 2011) and the agamid lizard *Uromastyx aegyptia microlepis* (Martín *et al.*, 2016), the significant differences in majoritarian chemicals are not only explained because of the higher amount of these compoundsfound in males´ secretions. Our study shows that females of *L. wiegmanni* secrete higher levels of some majoritarian compounds (e.g., squalene, cholesta-4,6-dien-3-ol) than males. Moreover, we found larger abundances of some waxy esters (e.g., hexadecyl 9-octadecenoate or octadecyl octadecanoate) in females than in males. This variation in presence/absence, as well as in the relative abundance between sexes, would be coherent with the overarching belief that certain compounds might be performing signalling tasks of different relevance depending on the sex (Martín & López, 2015). For instance, some steroids have been proved as indicators of "quality" during female mate-choice (Martín & López, 2015). The no significant difference among the overall proportion of steroids and waxy esters between sexes (figure 1) yield evidences that the intersexual differentiation arise of selected compounds. As we mentioned above, the functions of multiple chemical components remain poorly studied. However, it would not be surprising that those compounds highly different between sexes and, further, with abundant proportions, may have specific behavioural roles characteristic of each sex.

The knowledge of chemical profiles from lizard precloacal or/and femoral secretions is an important step to understand the role that sexes play during intraspecific communication. Furthermore, the striking diversity of communicative traits embodied in *Liolaemus´* lineages provides an excellent model to assess chemical communication. However, no studies have previously explored intersexual differences of chemosignals in this genus. Therefore, further works on *Liolaemus*, and particularly in females, should be undertaken to understand the role that the gender plays in chemical communication.

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CHAPTER III

DIET CONSTRAINTS MAY RESTRICT THE EXPRESSION OF A POTENTIALLY HONEST CHEMICAL SEXUAL SIGNAL IN IBERIAN ROCK LIZARDS

CAPÍTULO III

LAS LIMITACIONES EN LA DIETA PUEDEN RESTRINGIR LA EXPRESIÓN DE UNA SEÑAL QUÍMICA SEXUAL POTENCIALMENTE HONESTA EN LA LAGARTIJA ROQUERA

DIET CAN CONSTRAIN THE EXPRESSION OF A POTENTIALLY HONEST CHEMICAL SIGNAL IN IBERIAN ROCK LIZARDS

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ABSTRACT

Multiple evidences suggest that those individuals able to afford the costs of elaboration and maintenance of handicap signals are favoured by selection since this reflects honestly the ‘quality’ of bearers to competitors and potential mates. But, might have a species restricted the expression of a particular honest chemical signal under the same assumption? A challenging area in chemical ecology is to confirm which factors underlie divergences in chemical profiles, even more when they are evolved through sexual selection. Vitamin E (α-tocopherol; VE), a compound of dietary origin and with metabolic relevancy, has been suggested as an honest chemical signal in some lizard species, although it has not been previously identified in the femoral secretions of Carpetan Rock lizards (*Iberolacerta cyreni*). Here, we investigated if diet constraints may restrict the expression of a potentially honest chemical sexual signal (VE) in this species. Surprisingly, our results of an experimental VE dietary supplementation show that VE expression in chemical secretions of *I. cyreni* may be the result of constraints in availability of this vitamin in the diet. In addition, VE supplementation also significantly influenced the immune response of males and, moreover, the female responses to male scent stimulus. Our results suggest that although several compounds have not yet been identified in chemosignals of lizards, this does not mean their inability to express them. These results are especially relevant if these compounds might have key roles during mate-choice, because external factors (e.g. temporal and spatial changes in availability in the diet) might alter the expression of chemicals and, therefore, the sexual selection processes.

Key words: chemical communication; chemoreception; sexual selection; signal reliability;

Introduction

The ‘handicap paradigm’ proposes that those individuals able to afford costs associated with ellaboration and maintenance of secondary sexual characters are favoured by selection since that display signals honestly the ‘quality’ of bearers to competitors and potential mates (Zahavi, 1975; Zahavi & Zahavi, 1999). In this context, the study of physiological mechanisms underlying the expression of honest signals have been mainly based in visual and acoustic traits (Searcy & Nowicki, 2005), but almost neglected in chemical signalling traits (Zala *et al.*, 2004; Foster & Johnson, 2011) in spite of the fundamental role that chemical communication plays in sexual selection and diversification of many species (Dussourd *et al.*, 1991; Bacquet *et al.*, 2015; Weber *et al.*, 2016).

In recent years there has been a growing interest in studying the chemical ecology of lizards as model organisms (Weldon *et al.*, 2008; Johnston & del Barco-Trillo, 2009; Mason & Parker, 2010; Martín & López, 2014). Several evidences revealed that chemical signals engage in natural and sexual selection processes of lizards (Martín & López, 2014, 2015), since the composition of these signals often determine the response of receivers and also the reproductive and survivorship success of the senders (Mason & Parker, 2010). Lizards are well known for the chemical activity of their femoral and precloacal glands, which secrete a waxy combination of lipids and proteins (Mayerl *et al.*, 2015). The composition and abundance of secreted lipophilic compounds vary among individuals, populations and species (Khannoon *et al.*, 2011; Martín & López, 2013b; García-Roa *et al.*, 2016) and this variation is used for social and sexual recognition (Carazo *et al.*, 2007, 2008; Kopena *et al.*, 2011). These differences are subject to physiological and endocrine regulation (Martín & López, 2015) in response to biotic (e.g., trophic resources, parasites) and abiotic (e.g., environmental conditions) factors that shape the final profiles of chemical signals. According to the handicap paradigm (Zahavi & Zahavi, 1999), mating success can override the costs of honest signalling only in high-quality mates (Polnaszek & Stephens, 2014; Roulin, 2015) and therefore, only these individuals may express honest compounds in their signals. However, might a species have restricted the expression of a particular honest chemical compound under the same assumption? A challenging area in chemical ecology is to identify which factors underlie species-level differences in chemical profiles (Symonds & Elgar, 2008; Zimmermann *et al.*, 2009; Steiger *et al.*, 2010; Weber *et al.*, 2016), especially when those profiles evolve through sexual selection (Blaustein, 1981).

In this regard, diet is a major driver of sexual ornamentation (Hebets *et al.*, 2008; Sorensen *et al.*, 2010) and particularly important in the chemical signalling of many organisms (Howard & Blomquist, 2005; Fedina *et al.*, 2012). However, how diet may influence the expression of honest chemical signalling is still unclear. This is particularly relevant in those compounds potentially honest that have a dietary origin such as vitamin E (VE), which presence in chemical secretions has been described in many lizard species (Martín & López, 2014; Martín *et al.*, 2016) (figure 1).

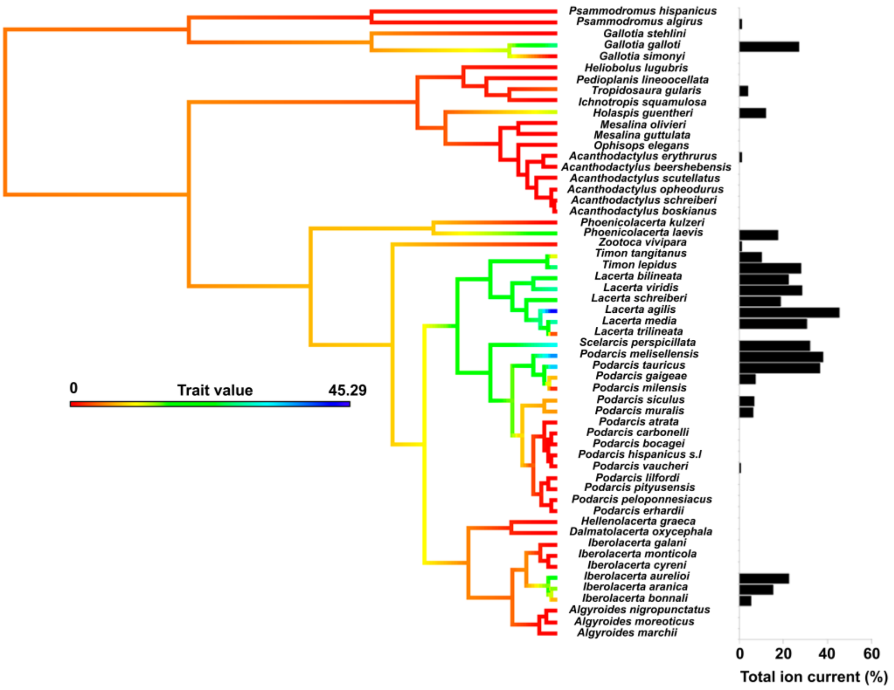

Figure 1: Relative abundances of vitamin E (α-tocopherol) in femoral secretions of several Lacertid lizard species (Fam. Lacertidae). Tree extracted from Pyron *et al.*'s squamates phylogeny (2013). The tree exhibits a maximum-likelihood phylogenetic reconstruction of ancestral proportion of vitamin E across linages (Revell & Freckleton, 2013). Black bars show the relative abundance (Total ion current; % TIC) of vitamin E found in each species

VE is a radical scavenger and antioxidant involved in cell membrane defence (Brigelius-Flohe & Traber, 1999; Traber & Atkinson, 2007), and its deficiency can trigger neurological disorders and physiological diseases (Mardones & Rigotti, 2004). This vitamin is only synthetized by plants or microorganisms and hence it must be acquired through the diet by other organisms (Weldon *et al.*, 2008). Although VE participates in many metabolic and physiological processes, lizards allocate much of this vitamin to their chemical secretions (Weldon *et al.*, 2008; Martín & López, 2010b; Kopena *et al.*, 2011). The magnitude of such allocation may reflect male "quality" and influence females' mating choice (Kopena *et al.*, 2011; Kopena *et al.*, 2014b; Martín & López, 2014). Moreover, this might be explained because high levels of VE in femoral secretions seem to be indicative of a heightened immune response (Martín & López, 2010b; Kopena *et al.*, 2014a). Further, females can show increasing preferences for scents of males secreting high VE proportions (Kopena *et al.*, 2011), probably due to a pre-existing sensory bias in females towards the search for this vitamin in their food (Ryan & Keddy-Hector, 1992; Martín & López, 2008).

In this paper, we investigated whether a species might have restricted the expression of a potentially honest chemical compound as a result of constrains in diet. We used the Iberian rock lizards (*Iberolacerta cyreni*), one of the reptile species which chemical ecology has been most heavily studied (see examples in (Weldon *et al.*, 2008; Martín & López, 2015)). Although previous studies did not identify VE in chemical secretions of *I. cyreni* (López & Martín, 2005; Martín & López, 2010a, 2013b), nor in phylogenetically closely related species such as *I. monticola* and *I. galani* (López *et al.*, 2009), recent explorations seem to reveal that other species of the same genus (i.e., *I. aurelioi, I. bonnali, I. martinezricae*) may divert VE to femoral secretions (unpublished data; figure 1). Alternatively, multiple quantitative and qualitative shifts in VE expression might have occurred during the diversification of lacertid lizards, so that the contrasting expression among species could be an adaptive or plastic response to the environment (see also figure 1). On this basis, we hypothesized that VE expression in chemical secretions of *I. cyreni* might be conditioned by ecological factors that hinder its expression. Therefore, we tested whether VE expression in chemical gland secretions of *I. cyreni* is promoted by diet availability. In addition, we hypothesized that VE dietary acquisition by males may improve their apparent "quality" (e.g., increase of immune response and/or attractive chemical signalling) and affects females' mate choice.

Material and Methods

(a) Field work, lizards care and maintenance

The Carpetan rock lizard (*I. cyreni*) is a medium sized diurnal lacertid lizard that inhabits rocky highlands (above 1700 m elevation) along the Sistema Central Spanish mountains, where this species is endemic. We collected by noosing live adult male (n=38) and female (n=13) lizards at 'Alto del Telégrafo' (23°10′12″N, 102°53′19″W, Sierra de Guadarrama, Madrid, Spain) during the first week of May 2015. Then, lizards were transferred to "El Ventorrillo" field station of the Museo Nacional de Ciencias Naturales (5 km from the capture area). During the course of the study, lizards were individually housed in outdoor 51×36×28 cm PVC terraria with coconut fiber as substratum, rocks for cover and water *ad libitum*. Lizards were fed mealworm larvae (*Tenebrio molitor*) and house crickets (*Acheta domesticus*) dusted with calcium powder. We released all animals to their capture sites at the end of the experiment (approx. five weeks after capture).

(b) Vitamin E supplementation

We randomly assigned males to two treatments, control lizards (C-males, n=19) and vitamin E dietary supplemented lizards (E-males, n=19). We administered to E-males a dietary dose of 5 μl of vitamin E supplement (synthetic (±)-α-tocopherol; purchased from Sigma-Aldrich Chemicals Co.) every other day, during 30 days. The vitamin supplement mainly contained synthetic vitamin E (97 %; approx. 1014 IU mL^{-1}) and also soybean oil (3 %; approx. 0.32 IU mL^{-1} of natural vitamin E, i.e., D-α-tocopherol). Therefore, we provided E-males with approximately 5.05 IU of vitamin E per dose, which is below the tolerable levels of ingestion (Bender, 2003; Divers & Mader, 2005). C-males were provided with 5 µl of soybean oil alone, once every two days during 30 days. We used sterile plastic syringes with a cannula to deliver slowly the solution into the mouth of lizards for ensuring that all individuals received the entire dose. After the supplementation period, the femoral gland secretions of each male were collected and preserved at -20 °C into glass vials closed with Teflon-lined stoppers. We also obtained blank control vials during the procedure, but without secretions. We conducted with these blank vials the same collection and analytical methodology to compare with vials that carried secretions and hence, to exclude potential contaminants.

(c) Chemical analyses of femoral gland secretions

The secretion of each male was weighted using a XP2U ultra-microbalance (±0.1 µg) in a room at controlled temperature (20 °C). All the laboratory supplies used for weighing were cleaned with n-hexane (95%) before and after the weighting of the secretions. We prepared a solution of 5 ppm of n-heptadecane in n-hexane to be used as an internal standard. Then, 1 µL of this solution was added per each 20 µg of femoral secretion and the mixture was preserved again in a new glass vial. The mixture was vortex-mixed for two minutes and left into the fridge for the precipitation of solid particles during a few minutes. Subsequently, the liquid phase was collected and transferred to a total recovery glass vial. The final samples were kept at -20 °C until further analysis.

For samples analyses, we used a TRACE GC Ultra gas chromatograph coupled to a TSQ Quantum XLS mass spectrometer settled by a triple quadrupole analyzer (Thermo Fisher Scientific Inc., Bremen, Germany) that operated in electron impact (EI, -70 eV of electron energy) scan detection mode. We set the current of the filament to 150 µA. Then, we injected 2 μL of each sample in the gas chromatograph using a programmed temperature vaporisation (PTV) injector in splitless mode. We used a split flow of 10 mL/min of helium and 2 min of splitless time. In addition, we kept the injector at 250 °C during the transfer phases with a constant septum purge. We also performed a cleaning phase of the injector after the transfer phase, increasing injector temperature from 14.5 °C/s up to 350 °C and hold at 350 °C for 5 min. Also, flow was enlarged up to 50 mL/min in the cleaning phase. We used a capillary column HP-5MS (30 m × 0.25 mm i.d., 0.25 μm film thicknesses) purchased to Agilent Technologies (Palo Alto, CA, USA) for the separation phase with an initial oven temperature of 100 °C (3 min) and posterior increment of 5 °C/min to 300 °C. The final temperature was held for 15 min. We used helium as the carrier gas at a constant flow rate of 0.8 mL/min. The temperature of the transfer line and the MS source were set at 300 °C and 240 °C, respectively.

For the chromatogram analyses we used the software Xcalibur™ 2.1.0.1140 (Thermo Fischer Scientific Inc., San Jose, CA, USA). Then, to identify the chemicals we firstly compared their mass spectra in the NIST/EPA/NIH (NIST 02) computerized mass spectral library. Subsequently, we compared the spectra and retention times of compounds with those of authentic standards (from Sigma– Aldrich Chemical Co.) when these were available. This step is useful to confirm the initial identification. We did not consider the impurities found in the solvent and/or the control vial samples. We determined the relative amount of each compound using the percent of the total ion current (TIC). In addition, once α- tocopherol was identified, its area in the chromatogram was relativized to the area of the internal standard compound (i.e., n-heptadecane), which concentration was previously know as we mentioned above. This allowed confirming potential variations in relative abundance of VE regardless of the other compounds in secretions.

Finally, we analyzed potential differences in relative abundances of chemicals between C- and E-males by using general linear models (GLMs) analyses.

(d) Immune response

We assessed the immune response of male lizards by conducting a delayed-type hypersensivity test −phytohematoglutinin injection (PHA) test− by means of a subcutaneous injection of a mitogen in the left hindlimb. Then, we measured the swelling occurred after 24 hours. We calculated the lizard immune responses subtracting pre- to post-injection measures (Martín & López, 2010b; Kopena *et al.*, 2014a). Some researchers have suggested that physiological PHA reaction could be a nonspecific complex inflammation related with infiltration of cells representing both adaptive and innate immunity (Vinkler *et al.*, 2012; Salaberria *et al.*, 2013). Final swelling may be result of a diverse index of cutaneous immune activity. Therefore, with the PHA test we aimed to assess a standardized index of immunocompetence, regardless of the types of the concerned immune cells. To assess potential differences in immune responses between males of both treatments we used general linear models (GLMs) analyses.

(e) Chemosensory responses of females to scent of males

Tongue-flicking (TF) behaviour has been widely associated with squamates chemoreception (Cooper, 1994). Lizards and snakes extrude tongues for sampling chemicals from the environment and other individuals (Cooper Jr & Burghardt, 1990; Cooper, 1994). It is thought that differential TF rates are the result of stimuli discrimination. Yet, a higher interest by a scent seemingly lead lizards to increase the number of elicited TF (Kopena *et al.*, 2011; López & Martín, 2012; Martín & López, 2012). Then, we measured the responses of females to scents (i.e., femoral secretions) from males of the two treatments (C-males and E-males). Thus, we compared TF rates of females to swabs impregnated with secretions of the two groups of males. These swabs were imbued with approximately the same amount of secretion (2 x 1mm of waxy secretion from each of two femoral pores of males). This avoided that females responded to sample-size variation instead of to differences in chemical composition. We also used water as odourless control stimulus. All females were exposed to the three stimuli (water vs. C-male vs. E-male) in a randomized order. We only performed one trial per day for each female with the purpose of preventing lizards´ stress. TF experiments were conducted in outdoor conditions during three days at the end of the supplementation experiments. Each female could bask at least 2 h before trials (Martín & López, 2006a). We exposed the secretion-imbued swab to 2 cm anterior to lizard´s snout. Then, we noted the number of TF directed to the swab along 1 min since the first TF. We also measured the latency, which is the time (s) that females took since the initial exposition of the swab to the first TF. We tested for potential differences in TF rates and latency of females using repeated measures ANOVAs with the type of scent (i.e., water vs. E-males vs. C-males) as a within factor.

All data were previously log-transformed to ensure normality (Shapiro-Wilk´s test), as well as the homogeneity of variances (Levene´s test).

Results

(a) Femoral secretions

Our results revealed the presence of VE in gland secretions of *I. cyreni* males at the end of the dietary supplementation (figure 2). In C-males, the main compounds in secretions were steroids (91.76 % of TIC), followed by carboxylic acids (2.56 %), alcohols (1.67 %), aldehydes (1.45 %), waxy esters (1.23 %), VE (1.21 %) and squalene (0.12 %). In E-males, we also found steroids as main compounds (69 % of TIC). However, we subsequently found VE (28.12 %) as the second more abundant compound, followed by waxy esters (0.88 %), aldehydes (0.81 %), alcohols (0.80 %), carboxylic acids and their esters (0.32 %) and squalene (0.06 %). Significant differences of relative TIC proportions between treatments were only evidenced in carboxylic acids and their esters ($F_{1,36}$ = 5.33, P = 0.02), squalene ($F_{1,36}$ = 5.37, P = 0.02) and VE ($F_{1,36}$ = 77.78, P < 0.001). However, we did not found significant differences between treatments for proportions of steroids ($F_{1,36}$ = 1.04, P = 0.31), alcohols ($F_{1,36}$ = 3.94, P = 0.054), waxy esters ($F_{1,36}$ = 0.51, P = 0.47) or aldehydes ($F_{1,36}$ = 1.68, P = 0.20).

Our analyses using the "injection standard" to explore potential differences in the relative abundance of VE between E-males (79.42±18.22) and C-males (1.64±0.36) confirmed that E-males secreted significantly higher amounts of VE than C-males ($F_{1,36}$ = 236.45, $P < 0.001$) (figure 2b).

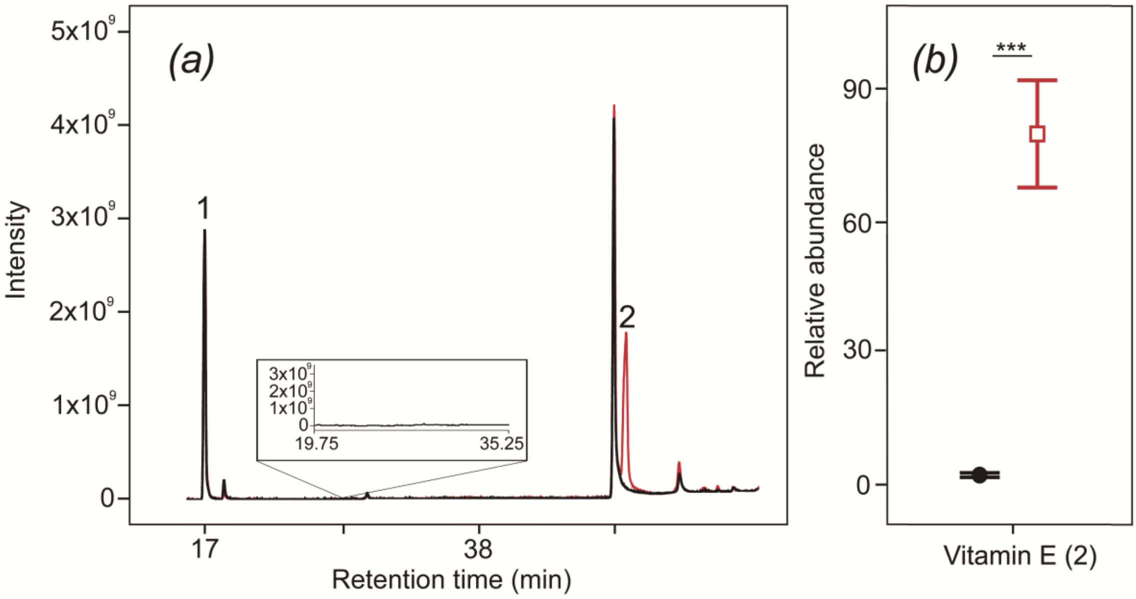

Figure 2: Chromatograms from femoral secretions of Iberolacerta cyreni male lizards. (a) Comparison of chromatograms from two different treatments: 'C-males' (black) were soybean oil supplemented and 'E-males' (red) were vitamin E supplemented. Compound (1) represents the internal standard chemical (n-heptadecane). Compound (2) represents the α-tocopherol peak of E-males. (b) Comparison of the relative abundances (mean ± SE) of α-tocopherol in secretions of C-males and E-males

(b) Relationships between chemical signals and the immune response

Vitamin-E supplementation affected the individual immune responses with E-males having significantly higher immune responses than C-males (One-way ANOVA; $F_{1,36}$ = 23.14, $P < 0.001$). However, the relationship between the relative abundance of VE and immune response was unclear (C-males: r^2 = 0.05, $F_{1,18}$ = 0.084 , P = 0.77; E-males: r^2 = 0.03, $F_{1,18}$ = 0.45 , P = 0.50).

(c) Chemosensory responses of females to scent of males

In all cases the females responded to the impregnated swabs by tongue flicking. Our results showed significant differences in latency among scent stimuli (repeated measures ANOVA; F2,22 = 20.77, P < 0.001). Although swabs with water had significantly longer latencies than swabs with males' scent (Tukey´s test: $P < 0.0001$ in both cases), there were no significant differences between latencies to secretions of E-males and C-males ($P = 0.11$) (figure 3). The overall rate of TFs over swabs differ significantly among the types of stimuli (repeated measures ANOVA; $F_{2,22} = 118.13$, $P < 0.001$; see figure 3b). Swabs with water triggered significantly lower rates of TFs than those with secretions of males (Tukey´s test: $P < 0.0001$ in both cases). Moreover, swabs with secretions of E-males received significantly higher TF responses than secretions of C-males ($P = 0.01$).

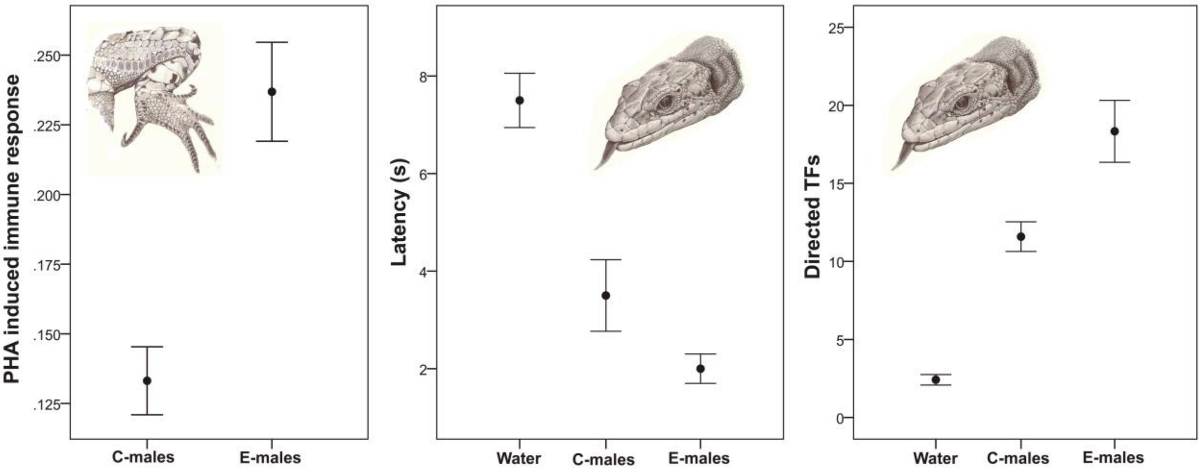

Figure 3: Effect of vitamin E dietary supplementation on the immune response of *Iberolacerta cyreni* males and chemosensory responses of females to scent of males. (A) Immune response measured in control and supplemented male lizards (C-males and E-males, respectively). (B) Latencies (mean ±SE in s) and (C) number (mean ±SE) of directed tongue-flicks (TF) elicited by female lizards to swabs with deionized water, scent from femoral secretions of control males (C-males) and vitamin E supplemented males (E-males)

Discussion

Our study shows that the expression of a potentially honest compound in chemical signals of a lizard can be significantly affected by diet constrains to the point of precluding its secretion. Our study also reveals that removal of male dietary constraints drives physiological changes that enhance immune responses of males and, moreover, conditioned female choice of potential mates.

(a) Chemical signaling profiles

The role of some secreted compounds in social and sexual behavior and therefore, in community assembling and speciation, has been supported for several species (Gabirot *et al.*, 2012; Martín & López, 2012, 2013a) . This is even more remarkable in those compounds suggested to be honest signals, such as VE. However, why this compound has been found in some species and not in others was unclear. In this study, we have detected VE in the femoral secretions of *I. cyreni* for the first time, but this VE secretion is stimulated by dietary supplementation, which could explain that this vitamin had not been detected before in wild individuals. Therefore, the lack of detection of particular secreted chemicals should not lead to conclude that a species is unable to express them. As in the case of *I. cyreni* and VE, striking divergences in chemical profiles of closer-related species might be unleashed by ecological factors. For example, VE has been suggested to have antioxidant and immunostimulatory properties (Wolf *et al.*, 1998; Brigelius-Flohe & Traber, 1999; García-Roa *et al.*, 2016) but their secretion is metabolically demanding, so individuals must tradeoff between associated costs and benefits, and such trade-off might be indicative of their "quality" (Weldon *et al.*, 2008; Martín & López, 2015). In addition, lizards mainly thermoregulate by exposing themselves to solar radiation, the ultraviolet fraction of which has oxidative effects in different ectotherms. At high altitudes, where thermal energy availability is low, lizards increase their sunbathing activity (Gvoždík, 2002), and this might force many species like *I. cyreni* to reduce VE in secretion as they need to increase the levels of this vitamin in metabolism to be used as antioxidant (Wolf *et al.*, 1998; Brigelius-Flohe & Traber, 1999). VE dietary supplementation could solve the former physiological trade-off, allowing individuals to allocate also higher amounts of VE in secretions. This hypothesis could be also applicable to closer relative species (i.e., *I. galani* and *I. monticola*) in which VE has not been found in their femoral gland secretions. But, however, *Iberolacerta* species from the Pyrenees (*I. aranica*, *I.aurelioi* and *I. bonnali*) do secrete this vitamin. This fact might be partially explained because of potential differences among this species in VE availability in their diets. In this respect, further work is needed to establish the relative role of biotic (e.g., diet, parasitism, predation) and abiotic (e.g., climate, microhabitat) factors in determining VE secretion patterns across species.

(b) Consequences for sexual selection

Recent studies have suggested that a range of compounds identified in lizard chemical secretions, including VE, can act as potential honest chemical signals in intersexual interactions (Martín & López, 2006b, 2010b; Kopena *et al.*, 2011). Since VE amounts in lizard secretions are directly associated with male "quality" (Martín & López, 2010b; Kopena *et al.*, 2011, 2014b), a high allocation of this vitamin to secretions could result in reproductive advantages that might override physiological trade-offs. On this basis, our study supports that, in the absence of VE dietary shortage, males improved their immune response and also changed their chemical signaling which in turn increased their scents´ attractiveness, corroborating, therefore, the potential role of VE as an honest chemical compound (Kopena *et al.*, 2011, 2014b, a).

Interestingly, although VE has not been previously identified in scents of male *I. cyreni*, females elicited higher TF rates to secretions of E-males. Since VE is needed in metabolism and must be acquired from dietary sources, female preferences could be chemically sensory biased. Similarly, behavioral experiments with this species showed that females increased chemosensory responses to stimuli from both invertebrate prey and femoral secretions of males, revealing cholesta-5,7-dien-3-ol (provitamin D3) as one of the compounds potentially responsible of eliciting these higher chemosensory responses.

Together, our results present VE as a ‘‘masked’’ potentially honest compound in *I. cyreni*, whose functionality is condition-dependent, i.e., quality of individuals to solve physiological trade-offs, and whose expression in chemical signals is highly dependent on diet constraints. Likewise, it would not be surprising that other compounds may be masked as a result of ecological pressures and physiological requirements. On this framework, ecological changes in populations of lizards could alter the physiological trade-offs and hence, the expression of certain compounds in the chemical signals. This would be particularly relevant in the case of honest chemical signals, which degree of expression may alter the mate recognition and preference in female mate-choice and hence, the sexual selection processes (Escobar *et al.*, 2003; Runemark *et al.*, 2011; Gabirot *et al.*, 2012).

Conclusions

The evidences from this study suggest that the expression of some honest chemical signals might be constrained because of ecological pressures. Hence, absence of some chemicals in lizard scents (Martín & López, 2014) does not mean that, in particular conditions, they can be expressed. Specifically, we have confirmed the ability of *I cyreni* to express VE through gland secretions in spite that this compound was not found in secretions of natural populations. Our research shows the importance that diet resources may have in the physiological processes underlying to honest chemical signaling. This influence may affect the relative abundances of compounds determining the female responses to potential mates. This study provides a cutting-edge perspective about mechanisms that underlie the expression of a potentially honest chemical signal. Since this research has raised the physiological and ecological dependence of chemical signaling in the expression of honest compounds, many questions remain in need of further investigation. Hence, future works should focus on improving the knowledge of what factors are engaged in the higher or lower expression of some compounds in chemical sexual signals.

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CHAPTER IV

INTERSPECIFIC DIFFERENCES IN CHEMICAL COMPOSITION OF FEMORAL GLAND SECRETIONS BETWEEN TWO CLOSELY RELATED WALL LIZARD SPECIES, *PODARCIS BOCAGEI* AND *PODARCIS CARBONELLI*

CAPÍTULO IV

DIFERENCIAS INTERESPECÍFICAS EN LA COMPOSICIÓN QUÍMICA DE LAS GLÁNDULAS FEMORALES ENTRE DOS ESPECIES CERCANAMENTE EMPARENTADAS, *PODARCIS BOCAGEI* Y *PODARCIS CARBONELLI*

This chapter reproduces entirely the manuscript:

García-Roa, R., Cabido, C., López, P. & Martín, J. (2016) Interspecific differences in chemical composition of femoral gland secretions between two closely related wall lizard species, *Podarcis bocagei* and *Podarcis carbonelli*. *Biochemical Systematics and Ecology*, **64**, 105-110.

INTERSPECIFIC DIFFERENCES IN CHEMICAL COMPOSITION OF FEMORAL GLAND SECRETIONS BETWEEN TWO CLOSELY RELATED WALL LIZARD SPECIES, *PODARCIS BOCAGEI* AND *P. CARBONELLI*

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ABSTRACT

Chemical signals play an important role in intraspecific communication and social organization of many animals, but they also may be useful in interspecific recognition. In lizards, chemical signals are often contained in femoral gland secretions, of which composition may vary between species and populations. This may be especially important in recognition and reproductive isolation between closely related species. We analyzed by gas chromatography–mass spectrometry (GC–MS) the lipophilic fraction of femoral gland secretions of two closely related wall lizard species, *Podarcis bocagei* and *P. carbonelli* to test for possible interspecific differences in chemical composition. We found 56 lipophilic compounds in femoral gland secretions of male *P. bocagei* and 60 in *P. carbonelli*. The main compounds were steroids and waxy esters, but we also found carboxylic acids and their esters, alcohols, amydes, aldehydes, squalene, ketones and furanones. There were significant differences between species with respect to the number and relative proportions of compounds. Differences in chemical composition might be a consequence of phylogenetic differences per se, but they could also be explained by ecological adaptation to different microclimatic conditions. These differences in chemical profiles may explain the known chemosensory interspecific recognition between these two lizards, contributing to their reproductive isolation.

Keywords: Chemical signals; chemoreception; *Podarcis* species complex; lizards; steroids; waxy esters

Introduction

Several groups of animals have chemosensory systems that are used to detect different chemical cues involved in prey and predator detection, social organization or mate choice (Mason, 1992; Wyatt, 2014) and also in species recognition (Mas & Jallon, 2005). Concretely, chemical ecology plays an important role in lizards because chemical signals may mediate in intra and interspecific communication (Cooper Jr *et al.*, 2002; Weldon *et al.*, 2008; Martín & López, 2011; Martín & López, 2014). Specific compounds secreted by lizards are essential in mate recognition and mate assessment (Martín & López, 2013) and territoriality (Martín & López, 2007). In addition to this, chemoreception may be especially relevant in lizard species recognition, acting sometimes as a prezygotic barrier that may preclude reproduction between syntopic species (Gabirot *et al.*, 2012).

In lizards, chemical cues are usually produced by males through secretions via femoral, preanal or/and postanal glands, secretion being particularly abundant during the reproductive season (Alberts *et al.*, 1992; Mason, 1992). Proteins and lipids are the main compounds in these gland secretions and may differ thoroughly intraspecific and interspecifically (Weldon *et al.*, 2008), probably due to environmental and phylogenetic factors (Martín & López, 2013). However, research on chemical composition of lizards' secretion and the potential information conveyed by these chemicals is relatively scarce and restricted to a few phylogentic groups (see Weldon *et al.*, 2008; Martín & López, 2014).

In spite of recent research focused on interspecific interactions in lizards, much of the studies on this topic have only been carried out on a small number of lizard groups, for example in *Eumeces* skinks (Cooper Jr & Vitt, 1987), tropidurid lizards of the genus *Liolaemus*, or lacertid lizards of the genus *Podarcis* (Gabirot *et al.*, 2012a). Recent molecular studies have demonstrated the complexity of the '*Podarcis hispanica*' lizard species complex (Kaliontzopoulou *et al.*, 2012; Geniez *et al.*, 2014), their phylogenetic relationship and underlying evolutionary processes still remaining unclear. Pinho *et al.* (2007) suggested that *P.*

bocagei, P. carbonelli, *P. hispanica* and *P. vaucheri* suffered a fast diversification in their evolutionary history. Concretely, the Bocage's wall lizard (*P. bocagei*) and the Carbonell´s wall lizard (*P. carbonelli*) are two closely related diurnal lizard species of medium size, inhabiting western parts of the Iberian Peninsula. Some years ago, *P. carbonelli* was considered a subspecies of *P. bocagei* (Mellado, 1980). Nevertheless, molecular studies based on morphologic and genetic data showed *P. carbonelli* as a distinct species supporting its specific status (Harris & Sá-Sousa, 2001; Harris & Sá-Sousa, 2002). Recent research provides phylogenetic relationships showing both species as not being sister taxa (Kaliontzopoulou *et al.*, 2012).

These two wall lizard species are distributed in sympatry with other *Podarcis spp*. e.g., *P. bocagei* with *P. hispanica* type 1 (i.e*., P. guadarramae*), and *P. carbonelli* with *P. hispanica* type 1 (*P. guadarramae*) and 2 (*P. virescens*) (Sá-Sousa, 2001; Harris & Sá-Sousa, 2002; Geniez *et al.*, 2014). Moreover, there are some contact zones between *P. bocagei* and *P. carbonelli* (Carretero *et al.*, 2002) and some cases of hybridization have been reported (Galán, 2002; Pinho *et al.*, 2007). In relation to this, the ability to discriminate conspecific from heterospecific chemical cues may be essential for lizards with syntopic and closely related species. Cooper and Pèrez-Mellado (2002) analyzed the existence of interspecific recognition in *P. hispanica (sensu lato)*, reporting the ability of males to distinguish among conspecific females and *P. carbonelli* sympatric females based on chemical cues alone. Subsequent behavioral studies demonstrated reciprocal chemosensory discrimination between *P. bocagei* and *P. carbonelli* (Barbosa *et al*., 2005) and between *P. bocagei* and *P. hispanica* type 1 (Barbosa *et al.*, 2006). However, no attention has been paid to chemical composition of lizards' secretions in these studies. Nevertheless, chemical characterizations of femoral secretions of other *Podarcis* lizard species have been described in recent works (Gabirot *et al*., 2012a) showing that steroids (mainly cholesterol) are predominant over other compounds such as fatty acids, alcohols, etc.

In the present paper, with the goal to understand the functions of chemical signals in interspecific communication and reproductive isolation in these closely related lizard species, we report the lipophilic compounds found in femoral gland secretions of male *P. bocagei* and *P. carbonelli* using gas chromatography–mass spectrometry (GC-MS). We specifically tested the existence of interspecific differences in chemical composition of secretions, which could explain the previously known chemosensory recognition between these species. We also compared composition of secretions with those of other related lizard species for which previous data is already known.

Material and methods

Study area and species

The Bocage's wall lizard (*P. bocagei*) and the Carbonell´s wall lizard (*P. carbonelli*) are two species widespread in western Iberian Península. *P. bocagei* is distributed in Asturias, Cantabria, Galicia, north of Castilla-León (Spain) and north of River Douro and some mountains sites of Tras-os-Montes (Portugal) (Salvador, *et al*. 2014). Populations of *P. carbonelli* inhabit mainly relict zones of Castilla León and Extremadura (Spain), and south of River Douro (Portugal) (Sá-Sousa, 2008; Galan, 2014). Both species are endemic of the Iberian Peninsula.

During field-work carried out in spring 2007 (May-June, corresponding to the species mating seasons), we captured focused on *P. bocagei* at “Louro” (Galicia, Spain; 42°44’22.30’’N, 9°04’46.07’’W) and P*. carbonelli* at “La Alberca” (Salamanca, Spain; 40°29’44.51’’N, 6°05’17.92’’W). The lizards were captured alive by noosing, and immediately after that, secretions were extracted from femoral glands by softly pressing around the femoral pores. We collected secretions into glass vials with glass inserts, and later closed them with Teflon-lined stoppers and stored at -20 °C until analyses. We also prepared blank control vials by using the same procedure, but without collecting secretion, and the same analytical methodology to compare with the secretion-samples and be able to exclude potential contaminants from the handling procedure, the solvent or laboratory equipment.

Analyses of femoral gland secretions

We analyzed the samples using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl/ 95 % dimethylsiloxane) column (Supelco, Equity-5, 30 m length x 0.25 mm ID, 0.25 µm film thickness) and a Finnigan- ThermoQuest Trace mass spectrometer (MS) as the detector. We performed splitless sample injections (2 µl of each sample dissolved in *n*-hexane) with helium as the carrier gas, and injector and detector temperatures at 250 °C and 280 °C, respectively. The oven temperature program started at 50 °C, was maintained isothermal for 3 min, then increased to 300 °C at a rate of 5 °C/min, and finally isothermal (300 °C) for 15 min. Mass spectral fragments below m/z = 46 were not recorded. Initial identification of secretion components was done by comparing their mass spectra with those in the NIST/EPA/NIH (NIST 02) computerized mass spectral library. We confirmed identifications by comparing spectra and retention times with those of authentic standards (fromSigma– Aldrich Chemical Co.) when these were available. Impurities identified in the control vial samples were not considered.

The relative amount of each compound was determined as the percent of the total ion current (TIC). To correct the problem of nonindependence of proportions, we used the compositional analysis, by logit transforming the proportion data taking the natural logarithm of proportion / (1–proportion) (Aebischer *et al.*, 1993). Then, we calculated Euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of further analyses. We used permutational multivariate analysis of variance tests [PERMANOVA; (Anderson, 2001; McArdle & Anderson, 2001)] based on the Euclidean resemblance matrix using 999 permutations to analyze whether the composition of the femoral secretions varied between species. We also used a canonical analysis of principal coordinates [CAP; (Anderson & Willis, 2003)] to investigate differences between species.

Calculations were made with the software PAST 3.05 (Hammer *et al.*, 2001) and PRIMER V6.1.13 (Clarke & Gorley, 2006) with the PERMANOVA+ V1.0.3 add-on package (Anderson *et al.*, 2008). We compared the relative abundance of the different types of compounds found in femoral secretions between species with one-way ANOVA tests (Sokal & Rohlf, 1995).

Results

We found 56 lipophilic compounds in femoral gland secretions of male *P. bocagei* (Table 1), which was a mixture of 26 steroids (61.8 % of TIC), 12 waxy esters (34.3 %), five carboxylic acids and their ethyl esters ranged between n-C_{16} and n-C_{18} (1.6 %), two amydes (0.8 %), squalene and other terpenoid (0.8 %), four aldehydes (0.4%), four alcohols between C_{14} and C_{20} (0.2 %) and a ketone (0.1 %). On average, the five most abundant chemicals of *P. bocagei*, which together comprised nearly 75% of the TIC area, were cholesterol (42.3 % of TIC), and four waxy esters: octadecyl 9-octadecenoate (9.4 %), eicosyl hexadecanoate (9.2 %), octadecyl hexadecanoate (8. 7%), and eicosyl 9-octadecenoate (4.8 %).

In addition, we found in the lipophilic fraction of femoral gland secretions of male *P. carbonelli* a mixture of 60 compounds (Table 1), including 16 waxy esters (55.5 % of TIC), 27 steroids (42.6 %), four alcohols between C_{14} and C_{20} (0.9 %), five carboxylic acids ranged between n-C_{10} and n-C_{18} and their ethyl esters (0.3 %), squalene (0.2 %), four aldehydes (0.2%), a ketone (0.1 %), a furanone (0.1 %) and one amyde (0.1 %). On average, the five most abundant chemicals, which comprised more than 60% of the TIC area, were cholesterol (20.2 %), and four waxy esters: hexadecyl 9-hexadecenoate (15.4 %), octadecyl hexadecanoate (9.4 %), octadecyl 9-octadecenoate (8.5 %) and eicosyl hexadecanoate (8.3 %).

There were clear differences in the presence/absence of some lipophilic compounds between *P. bocagei* and *P. carbonelli* (Table 1). Thus, *P. bocagei* had nineteen exclusive compounds (33.9 % of compounds; but only 3.1 % of the TIC area) that were not found in *P. carbonelli*, which had fifteen exclusive compounds (25 % of compounds; 22.4 % of TIC area).

Table 1: Lipophilic compounds found in male's femoral secretions of Bocage's wall lizard (Podarcis bocagei; n=9) and Carbonell's wall lizard (Podarcis carbonelli; n=6)

RT (min)	Compound	*P. bocagei*	*P. carbonelli*
		mean ± SD	mean ± SD
13.3	Nonanal	-	0.03 ± 0.04
18.5	Decanoic acid	-	0.01 ± 0.01
23.5	Tetradecanol	0.03 ± 0.04	0.04 ± 0.03
28.1	Hexadecenal	0.17 ± 0.10	0.09 ± 0.07
28.7	Pentadecanal	0.03 ± 0.02	0.04 ± 0.05
30.8	Hexadecanal	0.10 ± 0.08	0.01 ± 0.01
32.2	Hexadecanol	0.03 ± 0.02	0.05 ± 0.06
32.5	2-Heptadecanone	0.12 ± 0.08	0.12 ± 0.11
33.9	Hexadecanoic acid	0.82 ± 0.95	0.27 ± 0.17
34.3	Hexadecanoic acid, ethyl ester	0.02 ± 0.02	-
34.8	Octadecanal	0.06 ± 0.05	-
36.0	Octadecanol	0.07 ± 0.06	0.52 ± 0.66
36.56	5-Dodecyldihydro-2(3H)-furanone	-	0.06 ± 0.07
37.3	9,12-Octadecadienoic acid, ethyl ester	0.77 ± 2.14	-
37.4	9-Octadecenoic acid, ethyl ester	0.02 ± 0.02	-
37.7	Octadecanoic acid	0.01 ± 0.01	-
37.7	9,12-Octadecadienoic acid	-	0.01 ± 0.02
37.8	9-Octadecenoic acid	-	0.02 ± 0.03
37.9	Octadecanoic acid, ethyl ester	-	0.02 ± 0.04
39.8	Eicosanol	0.01 ± 0.01	0.31 ± 0.54
40.9	9-Octadecenamide	0.03 ± 0.02	0.05 ± 0.08
47.1	13-Docosenamide	0.82 ± 0.45	-
47.5	Squalene	0.75 ± 0.48	0.24 ± 0.23
47.7	Unid. Steroid (199,253,341,352,367)	0.03 ± 0.02	-
47.9	Unid. Terpenoid	0.05 ± 0.02	-
48.4	Cholesta-2,4-diene	0.05 ± 0.03	0.15 ± 0.16
48.7	Cholesta-4,6-dien-3-ol	0.14 ± 0.04	0.12 ± 0.10
48.6	Unid.Steroid(141,156,349,364)	1.52 ± 0.66	0.74 ± 0.43
48.9	Cholesta-3,5-diene	0.39 ± 0.24	0.67 ± 0.71
49.2	Unid.Steroid(197,251,349,365)	2.68 ± 0.63	1.67 ± 0.92
49.3	4,6,8(14)-Cholestatriene	-	0.12 ± 0.11
49.4	Unid.Steroid (195,209,251,349,365)	2.84 ± 1.12	1.63 ± 0.81

RT (min)	Compound	*P. bocagei*	*P. carbonelli*
		mean + SD	mean + SD
49.5	Unid.Steroid (251,349,365)	0.42 ± 0.11	0.35 ± 0.26
49.6	Cholesta-5,7-dien-3-ol, acetate	0.40 ± 0.13	0.48 ± 0.59
49.7	Unid.Steroid (210,237,350,365,389)	0.36 ± 0.11	0.26 ± 0.18
49.8	Unid.Steroid (251,347,364,377)	0.04 ± 0.02	0.16 ± 0.26
49.9	Unid. Ester of 9-Octadecenoic acid	-	0.46 ± 1.10
50.0	Unid.Steroid (141,156,209,364,379)	0.09 ± 0.04	-
50.2	Unid.Steroid (141,156,209,349,364,379,400)	0.28 ± 0.07	1.13 ± 2.17
50.4	Unid.Steroid (181,193,235,348)	-	3.06 ± 7.29
50.5	Unid.Steroid (197,251,363,379,387)	0.18 ± 0.06	-
50.6	Unid.Steroid (155,197,251,363,379)	-	0.65 ± 1.45
50.9	Unid.Steroid (195,209,363,379)	0.13 ± 0.07	-
51.0	Cholesterol methyl ether	0.38 ± 0.34	1.36 ± 1.44
51.1	Unid.Steroid (197,209,365,381)	0.08 ± 0.06	0.03 ± 0.02
51.9	Cholesterol	42.36 ± 9.80	20.20 ± 11.66
52.5	Cholesta-5,7-dien-3-ol	1.26 ± 0.93	2.27 ± 1.31
52.8	Cholesta-5,7-dien-3-ol , derivative?	-	0.02 ± 0.03
53.2	Campesterol	0.38 ± 0.42	-
53.5	Cholest-4-en-3-one	0.77 ± 0.42	2.69 ± 4.18
53.7	3-Ethoxy-methoxy-cholestane	0.42 ± 0.11	2.05 ± 1.44
54.0	Cholesta-4,6-dien-3-one	3.98 ± 2.05	1.21 ± 0.93
54.2	Hexadecyl hexadecanoate	-	0.68 ± 1.07
54.2	Octadecyl tetradecanoate	0.14 ± 0.08	-
54.3	Cholest-7-en-3-ol, acetate	-	0.46 ± 0.97
54.8	Unid.Steroid (214,267,366,381)	2.26 ± 0.98	0.90 ± 1.00
54.9	Hexadecanoic acid, ethenyl ester	-	0.88 ± 0.96
55.4	Cholest-5-en-3-one	-	0.06 ± 0.12
55.1	Unid.Steroid (253,341,353,380,414)	0.26 ± 0.47	-
55.7	Heptadecyl hexadecanoate	0.30 ± 0.12	0.25 ± 0.25
55.9	Stigmast-4-en-3-one	-	0.02 ± 0.02
56.6	Unid.Steroid (214,267,380,395)	0.10 ± 0.07	-
56.7	Tetradecyl 9-octadecenoate	0.79 ± 1.06	1.77 ± 3.39
57.1	Hexadecyl 9-octadecenoate	0.18 ± 0.16	0.94 ± 0.57
57.2	Hexadecyl 9-hexadecenoate	-	15.45 ± 34.81

RT (min)	Compound	*P. bocagei*	*P.carbonelli*
		mean + SD	mean ± SD
57.3	Octadecyl hexadecanoate	8.67 ± 3.82	9.39 ± 9.36
58.2	Octadecanoic acid, ethenyl ester	-	0.20 ± 0.21
59.1	Heptadecyl 9-octadecenoate	0.06 ± 0.11	0.08 ± 0.07
59.3	Nonadecyl hexadecanoate	0.31 ± 0.11	0.43 ± 0.34
61.4	Octadecyl 9-octadecenoate	9.43 ± 8.64	8.53 ± 5.97
61.8	Eicosyl hexadecanoate	9.20 ± 3.02	8.30 ± 8.61
62.9	Unid.Steroid (211,295)	-	0.15 ± 0.20
64.1	Nonadecyl 9-octadecenoate	0.21 ± 0.32	0.31 ± 0.22
64.6	Heneicosyl hexadecanoate	0.21 ± 0.10	0.17 ± 0.20
67.7	Eicosyl 9-octadecenoate	4.79 ± 3.28	7.70 ± 7.03

The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (± 1SD). Characteristic ions (m/z) are reported for unidentified compounds. RT: Retention time

The PERMANOVA analysis based on the resemblance matrix comparing individuals of each species showed that there were significant differences in the proportion of compounds between the two species (pseudo $F_{1,13}$ = 73.44, P = 0.001). Moreover, when individuals were classified by cluster analysis based on euclidean distances among their chemical profiles (i.e., relative proportions of compounds), outstanding differences were found between the two species, showing that individuals from the same species clustered close together and separated from individuals from the other species (figure 1). The CAP analysis assembled 100 % of the chemical profiles into the correct species using these euclidean distances (permutational test, δ_1^2 = 0.997, P = 0.001, using leave-one-out cross-validation and m = 2 axis). In contrast, a further PERMANOVA made with only the five major compounds shared by both species (cholesterol, octadecyl hexadecanoate, octadecyl 9-octadecenoate, eicosyl hexadecanoate, and eicosyl 9-octadecenoate) showed no significant differences between both species (pseudo $F_{1,13}$ = 1.08, P = 0.36).

Comparing between the two species the relative abundance of the different types of compounds, *P. bocagei* had significantly relatively higher proportions of amydes ($F_{1,13}$ = 17.88,

$P = 0.001$) and terpenoids ($F_{1,13} = 7.06$, $P = 0.019$) and lower proportions of waxy esters ($F_{1,13} = 4.68$, $P = 0.049$) and furanones ($F_{1,13} = 7.05$, $P = 0.019$) than *P. carbonelli*. Whereas there were not significant differences between species in proportions of steroids ($F_{1,13} = 4.25$, $P = 0.06$), carboxylic acids and their esters ($F_{1,13} = 1.37$, $P = 0.26$), alcohols ($F_{1,13} = 3.68$, $P = 0.077$), aldehydes ($F_{1,13} = 3.85$, $P = 0.071$) and ketones ($F_{1,13} = 0.005$, $P = 0.93$).

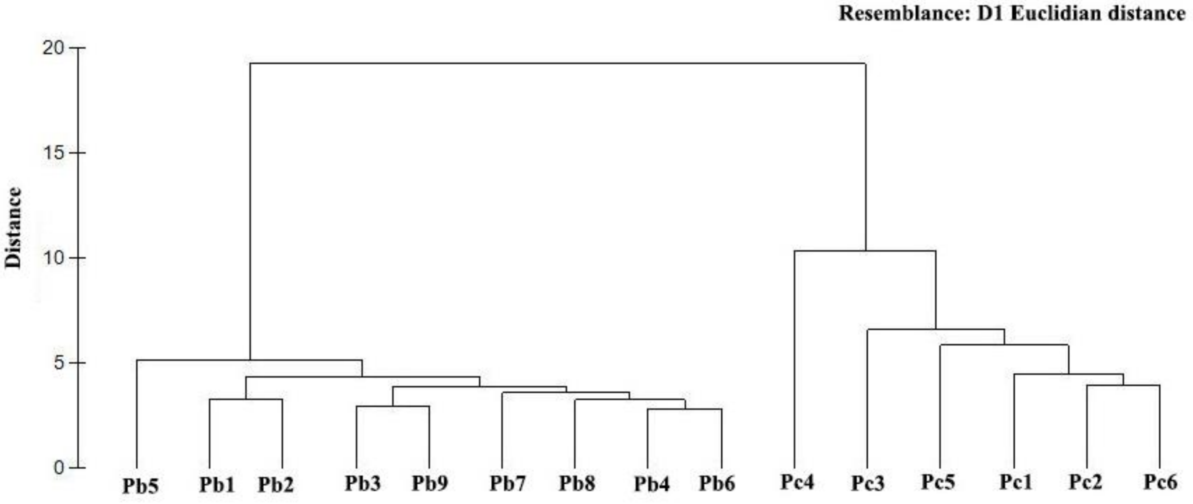

Figure 1: Dendrogram of cluster analysis for individual samples of femoral secretions of male lizards *Podarcis bocagei* (Pb1 to Pb9) and *P. carbonelli* (Pc1 to Pc6)

Discussion

The current study shows that lipophilic compounds found in femoral gland secretions of male lizards *P. bocagei* vary in composition and proportions from those of the related *P. carbonelli*. Steroids and waxy esters were the main types of compounds in both species. Cholesterol was the major compound of the secretions similarly to many other lacertid lizard species, specially other species within the genus *Podarcis* (Gabirot *et al.*, 2012a; reviewed in Weldon *et al.*, 2008; Martín & López, 2011, 2014). However, the secretions are also clearly different from other related species within the *Podarcis hispanicus* species complex (Gabirot *et al.*, 2012a). Thus, in comparison with other closely related, but more saxicolous-dweller, species of *Podarcis* lizards, such as *P. guadarrame* and *P. virescens* (Gabirot *et al.*, 2012a), the relative high proportion of waxy esters in the ground-dwelling *P. bocagei* and *P. carbonelli* is notorious. This may be an ecological adaptation to increase the persistence of scent marks under the microclimatic conditions experienced within the vegetated ground, in comparison to on exposed rocks, due to the more stable chemical characteristics of waxy esters (Alberts, 1992).

However, the different proportion of some groups of lipids (e.g., waxy esters are more abundant in *P. carbonelli*), together with the presence of minor compounds exclusive of each species could explain the significant differences in the chemical profiles between *P. bocagei* and *P. carbonelli*. A possible explanation for these differences might be the different climatic conditions in each sampled area, because waxy esters could protect scent marks from rapid evaporation in the more Mediterranean-xeric habitat of *P. carbonelli*. Likewise, the observed increase of squalene in *P. bocagei* could be attributed to the wetter conditions of its habitat in comparison with that of *P. carbonelli,* due to the antioxidant function of squalene, which will protect other compounds. However, these wall lizard species also inhabit other different western areas of the Iberian Peninsula, in which climatic conditions might change slightly, potentially affeting the composition of secretions in other areas (Martín *et al.*, 2013).

On the other hand, and according to previous research, these two lizard species may occur in sympatry with other *Podarcis* wall lizards (Sá-Sousa, 2001; Carretero *et al.*, 2002). In this case, species-specific differentiation in sexual signals is one of the most important facts for reproductive isolation between species, especially when hybridization events have been observed (Galán, 2002). In addition to color and behavior, chemoreception may play an important role in species recognition in these lizards. In this respect, the differences observed in chemical profiles of these two species seem to be consistent with the observation of chemosensory interspecific discrimination of some wall lizard species. Barbosa *et al.* (2005) reported the ability of *P. bocagei* and *P. carbonelli* males to distinguish conspecific and heterospecific females. This also agrees with further research, which showed that *P. bocagei* and *P. hispanica* (type 1) (i.e., *P. guadarramae*) males discriminated females of their own species (Barbosa *et al*., 2006). Whereas these previous studies tended to focus on intersexual recognition based on chemical cues, they overlooked male-male discrimination. In this context, conspecific male recognition in related sympatric species may be relevant for avoiding interspecific competition due to territoriality (Labra, 2011). In other lizard species, agonistic attacks have been reported to be directed to males of the same species due to the ability to recognize conspecific even at an individual level (Cooper & Vitt, 1987; Cooper & Pérez-Mellado, 2002).

Taking the above data into consideration, compounds which might facilitate long range detection by conspecifics may be aldehydes (Weldon *et al*., 2008; Martín & López, 2011). Curiously, in contrast to earlier research of the other sympatric wall lizard species (*P. hispanica* type 1 and 2; Gabirot *et al*. 2012a), we found evidence of three aldehydes shared by *P. bocagei* and *P. carbonelli* (hexadecenal, pentadecenal and hexadecanal), and two more exclusive of each species (octadecanal and nonanal respectively). However, these results need to be interpreted with caution, and more research on this topic needs to be undertaken before the association between aldehydes and species recognition can be more clearly understood.

Furthermore, female choice based on chemical cues plays an important role in sexual selection (Martín & López, 2011). Previous analysis observed that female wall lizards might discriminate chemical cues of males at an individual and/or conspecific level (Barbosa *et al*., 2005, 2006; Gabirot *et al*., 2012a), which may affect the females' mate choice decisions. Therefore, chemical differences between males of sympatric species, may affect female mate choice and be determinant to avoid hybridization.

Future studies on chemical characterization of lizard secretions in sympatric areas or contact zones are, therefore, recommended to asses more thoroughly the role of chemical signals in reproductive isolation of these wall lizard species.

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CHAPTER V

CLIMATIC FACTORS AS POTENTIAL DRIVERS OF INTERPOPULATIONAL DIVERGENCES IN CHEMICAL SIGNALS OF AN INSULAR LIZARD

CAPÍTULO V

FACTORES CLIMÁTICOS COMO POSIBLES CAUSANTES DE DIVERGENCIAS INTERPOBLACIONALES EN LAS SEÑALES QUÍMICAS DE UN LAGARTO INSULAR

CLIMATIC FACTORS AS POTENTIAL DRIVERS OF INTERPOPULATIONAL DIVERGENCES IN CHEMICAL SIGNALS OF AN INSULAR LIZARD

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ABSTRACT

Communication is crucial for animals´ ecology and evolution. During species diversification, environmental pressures have often acted as drivers of evolutionary shifts. Climatic factors may force the diversification of signaling traits involved in sexual and natural selection processes. Particularly, chemical signals of many species of lizards are determinant in mate choice and male-male competition and hence, in speciation processes. But, how and why chemical signals evolve is still unclear. Genetic drift and adaption to the environment are two potential factors that might shape chemical signals. However, there are no clear evidences to support this hypothesis. Here, we described differences in chemical signals of three subspecies of the insular Tenerife lizard (*Gallotia gallotia*) and showed that divergences (levels of cholesterol and α-tocopherol, i.e., vitamin E) were congruent with those expected under certain climatic conditions where each subspecies inhabits, given the potential functionality proposed for those chemicals in past studies. Particularly, we found higher vitamin E levels (compound with potential antioxidant properties) in the subspecies inhabiting wetter areas (*G. g. eisentrauti and G. g. palmae*) and higher cholesterol levels (compound with potential properties against dry conditions) in the subspecies of the warmest and driest area (*G. g. galloti*). These differences were contrary to expected by phylogenetic distances between subspecies. Moreover, we described seasonal changes in chemical profiles, as well as in cholesterol and vitamin E levels, which could be partially explained by physiological fluctuations throughout the year. We discuss the potential effects that climatic factors might be exerting on chemical signals, as well as the evolutionary consequences of subspecies differences in chemical communication.

Keywords: Adaptation, chemical signals, climate, divergence, island, steroids, tocopherol.

Introduction

Communication is crucial for animals´ ecology and evolution. Its intrinsic complexity lies in subsequent evolutionary processes of diversification that triggered a wide repertoire of signaling traits with a vast range of functions (Uetz *et al.*, 2009; McGregor, 2013). In this evolutionary scenario, environmental pressures have often acted as drivers of evolutionary shifts, as for example in *Anolis* lizards species (Losos, 2009). Thus, signaling traits are believed to evolve towards an improvement of signal efficacy, which can be reflected in a better transmission, durability or persistence of the signal (Searcy & Nowicki, 2005; Carazo & Font, 2010; Font & Carazo, 2010). In this context, population differences in the evolutionary optimization of signaling under unequal selective pressures may be so influential that important divergences involved in conspecific recognition may arise, laying the biological groundwork to potential speciation episodes (Ryan *et al.*, 1990; Seehausen *et al.*, 2008; Martín *et al.*, 2015a).

For many decades, most studies of animal communication and sexual selection have traditionally focused on acoustic and visual interactions (Touhara, 2013). Despite of Darwin (1859) already noted the importance of chemoreception in sexual selection, how and why chemical signals evolve is still unclear, especially in some vertebrate linages such as lizards. Several evidences noted that chemical senses are used by lizards to discriminate between conspecific features (e.g., sex, size age and familiarity), or to recognize potential prey and predators (Cooper, 1994; Martín *et al.*, 2007; Johnston & del Barco-Trillo, 2009; Mason & Parker, 2010). Lizards own different sources of chemical signals, such as feces, skin or gland secretions (Labra, 2011; Martín & López, 2014). With regard to the latter, femoral and precloacal follicular secretions have widely been studied during the last years. These secretions may provide information of the bearer condition (López *et al.*, 2006; Martín &

López, 2007) and influence the behavior in conspecific interactions, playing an important role in sexual selection through female mate-choice and male-male interactions (Carazo *et al.*, 2007; Martín *et al.*, 2007; López & Martín, 2011; Heathcote *et al.*, 2016). Secretions contain a set of lipids and proteins whose composition varies between species, populations and/or individuals (Escobar *et al.*, 2003; Martín *et al.*, 2013; García-Roa *et al.*, 2016a; García-Roa *et al.*, 2016b). Genetic drift and adaption to environmental conditions are two important factors that possibly shape the diversity of compounds found in lizard chemical signals (Martín & López, 2006b, 2013; García-Roa *et al.*, 2016a). For instance, Martín *et al.* (2015a) revealed that the efficacy of chemical signals (i.e., duration and detectability) of two different-altitude *Podarcis hispanicus* lizard populations differed under different conditions of temperature and humidity. These authors suggested that climatic conditions might be acting as selective forces, shaping the chemical profile of each-species secretions to improve their efficacy according to the conditions where each species inhabits (Martín & López, 2014). In fact, since some compounds have been suggested to have protecting properties of other compounds in the secretion, their presence and overall abundances might be an adaptive response to the surrounding climatic conditions. For example, cholesterol is believed to function as an apolar matrix that holds and protect other lipids in secretions from high temperatures and dryness, while α-tocopherol (i.e., Vitamin E) might serve as protector of other compounds in wet environments due to its antioxidant properties (Alberts *et al.*, 1992; Rosenau *et al.*, 1995; Wolf *et al.*, 1998; Escobar *et al.*, 2003; Traber & Atkinson, 2007; Weldon *et al.*, 2008; Martín & López, 2014).

On the other hand, most of species with femoral or precloacal glands usually produce chemical secretions only or mainly in the mating season (Martín & López, 2014; Martín & López, 2015). This reinforces the overarching hypothesis of the sexual-selection role of chemical signals. Due to this, there is a huge bias in the characterization of lizards' chemical profiles, which is mainly based on samples extracted in the reproductive season and, therefore, the analyses of secretions from other seasons are extraordinary scarce (Alberts, 1990; Martins *et al.*, 2006). However, some lizard species are active the whole year as a consequence of favorable and stable climatic conditions. Consequently, it would be expected that chemical communication might have functional roles along the year, but considering the balance between physiological costs of production and the pressure imposed by the requirements of each season (e.g., benefits expected during the reproductive period) (Alberts, 1990).

Within this background, we identified compositional divergences in the chemical signals of three subspecies of the insular Tenerife lizard, *Gallotia galloti*, and analyzed whether different proportions of the two most abundant compounds in scents (i.e., vitamin E and cholesterol) might be driven by local climatic conditions where lizards inhabit. The Tenerife lizard is a large lizard endemic to Canary Islands (Spain) divided in four subspecies: *G.g. eisentrauti* (*Gge*)*, G. g. galloti* (*Ggg*), *G. g. palmae* (*Ggp*) and *G. g. insulanagae (Ggi)*. They are native from Tenerife Island (*Gge* and *Ggg*), La Palma Island (*Ggp*), and the small islets of Roque de Anaga (*Ggi*) offshore Tenerife. There are considerable differences in habitat use among subspecies. For instance, *Gge* inhabits cloudy and wet densely vegetated forest areas in northern Tenerife, while *Ggg* lives in dry and sunny semidesert areas in the south and center of Tenerife (Salvador, 2015). Therefore, different ecological pressures might embody phenotypical divergences. Actually, pholidosis, coloration and body size are some morphological traits known to vary among these subspecies (Bohórquez-Alonso & Molina-Borja, 2014; Salvador, 2015). In this work, we investigated the chemical secretions of three *G. gallotia* subspecies (*Gge*, *Ggg* and *Ggp*). Hence, we addressed: (1) annual and (2) seasonal climatic differences (i.e., temperature and precipitation) between sampling points; (3) differences in chemical composition of femoral gland secretion among males of the three subspecies; (4) the potential role of cholesterol and vitamin E as an adaptive strategy to protect other compounds and thus, the efficiency of chemical communication under different climatic conditions. Moreover, (5) we analyzed the seasonal variation in chemical composition in *Gge* and *Ggg* subspecies, and discussed possible causes of changes in proportion of cholesterol and vitamin E between spring (reproductive season) and winter (non-reproductive season).

Material and methods

Study sites

We conducted our study in two islands (Tenerife and La Palma) of the Canary Archipelago, Spain. Because *G. galloti* lizards are active during the whole year, we considered two different and contrasted life-history periods, spring (reproductive season; R) and winter (non-reproductive season; NR) (Salvador, 2015). In Tenerife, we visited two populations of *G. galloti*, in April (R) and December (NR) 2013. The *Gge* population was located at El Pris in the North of the island (28°30' 46"N,16°25'4" W) and the *Ggg* population was in Malpaís de Güímar, in the Southeast of the island (28°18'3" N,16°23'49" W). The distribution of these two subspecies does not overlap in the populations sampled. Further, we studied a population of *Ggp* close to El Pedregal at La Palma Island (28°37'13" N, 17°54'24" W) in March 2013 (R). Due to logistic reasons we could not sample here in the non-reproductive season.

Climatic conditions

We characterized precipitation and temperature at the sampling points. All climatic data were downloaded from ´WorldClim´ (available at http://www.worldclim.org/). These environmental layers are the result of average monthly climate interpolation in the 1950-2000 year period (Hijmans *et al.*, 2005). Considering the scale of our analysis, we used a spatial resolution of 30 s (0.86 km at the equator). We firstly obtained the mean of the annual climatic data of temperature and precipitation for 17 random points within each of the sampled areas. Finally, we extracted mean climatic values restricted to the months when the peak of reproduction occurs in *G. gallotia* (March, April and May) and three winter months in the non-reproductive season (November, December and January). The spatial analysis and visualization were made using ArcGis v10.3 Software.

Sampling and analysis of femoral gland secretions

Field-work was carried out between 9:00 a.m. and 2:00 p.m. We captured lizards by using pitfall traps baited with banana and tomato (Oppliger *et al.*, 1999). Traps were hidden next to bushes and rocks to avoid exposure to direct sunlight. We checked traps every 15 min to avoid overheating of captured lizards. Due to the high temperatures in these islands all year round, lizards were transported within individual cotton bags to near enclosures where lizards were sampled in a fresh room. There, we collected the secretion of femoral pores from male lizards. Secretions were extracted by pressing gently pores and, then, secretions were introduced in glass vials with glass inserts and Teflon-lined stoppers. The large size of these lizards allowed us to do an easy extraction process. In order to obtain blank controls, we used the same procedure without collecting secretion. Samples were stored at -20 °C until analyses. Lizards were healthy and successfully released within the next 24 h in the same field-spot where they had been captured.

We analyzed secretion using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl/ 95 % dimethylsiloxane) column (Supelco, Equity-5, 30 m length x 0.25 mm ID, 0.25-μm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. We operated in splitless analysis mode sample injections. We analyzed 2 μl of each sample dissolved in 200 μl of n-hexane. The n-hexane (99 %) for organic residue analysis was obtained from J.T. Baker (Deventer, The Netherlands). We used helium as the carrier gas during the injection (270 °C) and detection (250 °C) phases with a constant septum purge. The analysis temperature program began at 50 °C isothermal for 10 min, increased to 280 °C at a rate of 5 °C/min, and then isothermal (280 °C) for 20 min. Data recording began 7 min after the separation initiated using the Software XcaliburTM 1.4 (Thermo Fischer Scientific Inc., San Jose, CA, USA). Impurities identified in the control vial samples were not considered.

For initial compounds identification, we used the NIST/EPA/NIH 2002 computerized mass spectral library, through comparison of mass spectraof compounds. When possible, identifications were confirmed by comparison of spectra and retention times with those of authentic standards (Sigma-Aldrich Chemical Co). Then, we calculated relative proportions of each compound determined as the percent of the total ion current (TIC) in secretions. For the comparison of chemical profiles, we corrected the problem of non-independence of proportions using logit transformation of the proportion data by taking the natural logarithm of proportion / (1–proportion) (Aebischer *et al.*, 1993).

Statistical procedures

We used General Linear Models (GLM) to examine climatic differences (i.e., annual and seasonal values of temperature and precipitation) among the three sampled areas. To assess chemical profile differences among subspecies, we used the software PRIMER v6.1.13 with the PERMANOVA+ v1.0.3 add-on package. We calculated the euclidean distances between every pair of individual samples to produce a resemblance matrix and then used a single factor permutational multivariate analysis of variance test (PERMANOVA) (McArdle & Anderson, 2001) based on the Euclidean resemblance matrix using 999 permutations. Possible differences were also analyzed with a canonical analysis of principal coordinates (CAP) (Anderson & Willis, 2003). To explore whether climatic conditions might be driving changes in proportions of two major compounds in secretions, we analyzed statistical differences in levels of cholesterol and vitamin E among the three subspecies in the reproductive season. Additionally, we tested for statistical differences between the reproductive and non-reproductive season in *Gge* and *Ggg*. These analyses were carried out by means of two-way PERMANOVA and General Linear Models (GLMs) taking into account the effects of subspecies and season and their interaction. We confirmed both normality of data (Shapiro- Wilk's test) and homogeneity of variances (Levene's test) in all cases. Pairwise comparisons were carried out using Tukey's tests. All the statistical analyses were performed with R 3.2.2, SPSS 20.0.0 and STATISTICA v8.0 Software.

Results

Climatic conditions

Annual mean values of climatic variables were significantly different among the three sampled areas (GLM; Temperature: $F_{2,50} = 147.56$, $P < 0.001$; Precipitation: $F_{2,50} = 205.58$, $P < 0.001$). All the pairwise comparisons for temperature and precipitation were significantly different (*Tukey's tests*, $P < 0.001$ for all) (figure 1). Similarly, in the context of the potential effect of climatic factors during the reproductive season on secretions, there were significant differences among the sampling areas of the three subspecies (*Gge*, *Ggg* and *Ggp*) in both, temperature (GLM; $F_{2,50} = 143.61$, $P < 0.001$) and precipitation data ($F_{2,50} = 220.01$, $P < 0.001$). The pairwise comparisons of temperature data between populations showed higher significant differences (*Tukey's* tests, $P < 0.001$ in all cases). The precipitation values were significantly different between B and C areas (Tukey's tests, $P < 0.001$), between A and C areas ($P < 0.001$) and between A and B areas ($P = 0.01$) (figure 2). Also during the non-reproductive season, we found significant differences in climatic factors among the three areas (GLM; Temperature: $F_{2,50} = 133.34$, $P < 0.001$; Precipitation: $F_{2,50} = 197.54$, $P < 0.001$) with pairwise comparisons being significantly different in all cases (Tukey's tests, $P < 0.001$). Therefore, our results show that *Gge* inhabits relatively cold areas with high humidity in accordance with temperature and precipitation values. Although in a lesser degree, something similar seemingly occurs for *Ggp.* Conversely, *Ggg* inhabits a warmer and dryer region than the other subspecies.

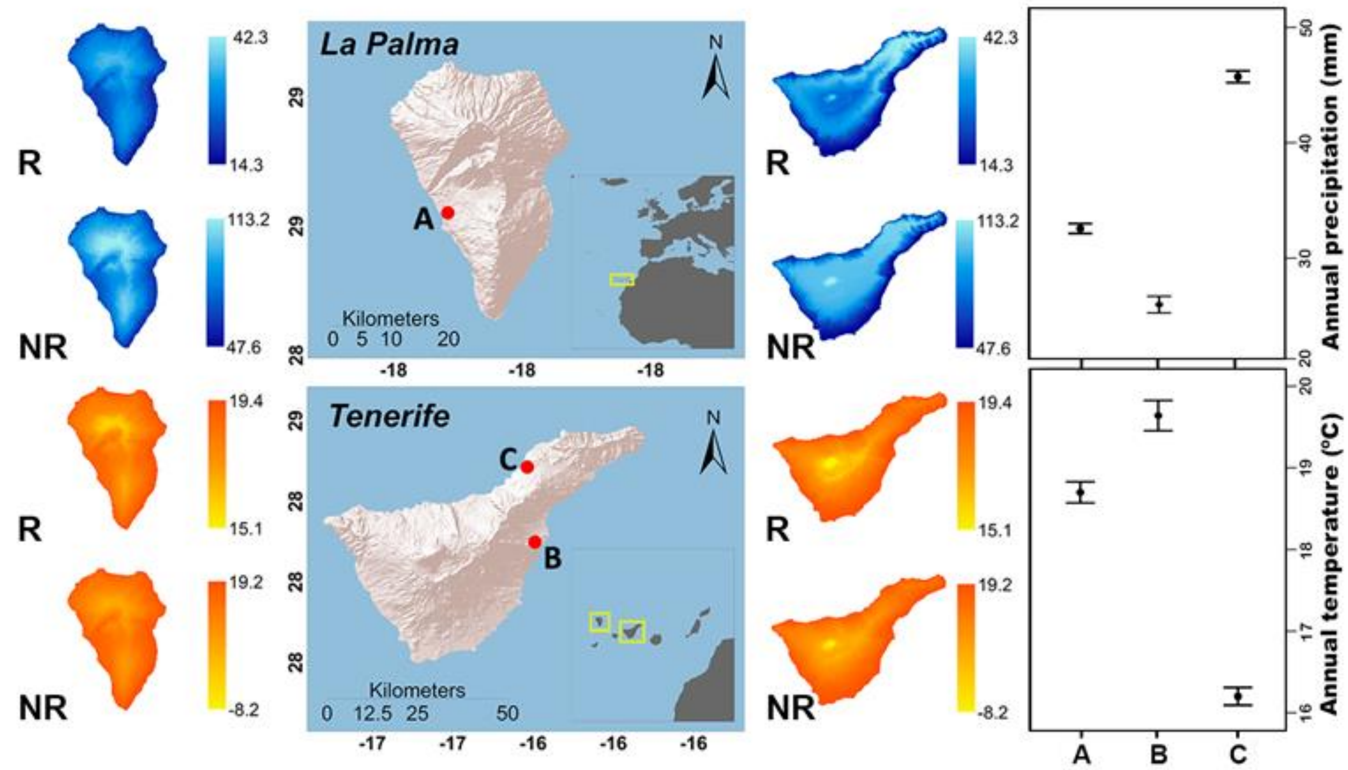

Figure 1: The islands of Tenerife and La Palma (Canary Islands, Spain). Marked with red points are the sampled areas of *Gallotia galloti* lizards (A: El Pedregal, *G. g. palmae*; B: Malpaís de Güimar, *G. g. galloti*; C: El Pris, *G. g. eisentrauti*). The four top maps show precipitation levels (blue gradient) and the four bottom maps show temperature levels (orange gradient levels) during the reproductive (R) and non-reproductive (NR) seasons. The right plots show mean (± SE) annual differences in precipitation (top) and temperature (bottom) among sampling points (A, B and C)

Chemical composition analysis

Overall qualitative compositions of femoral secretions on males of the three subspecies of *G. galloti* were similar for the most representative types of major compounds. Considering together, during the reproductive period, vitamin E (34.91 %) was the most abundant compound, closely followed by cholesterol (24.32 %). However, chemical profiles clearly differed among the three subspecies both in the number, abundance and presence-absence of some compounds (Table 1).

Table 1: List of compounds identified in femoral secretions of male lizad Gallotia galloti of three subspecies (i.e., G.g. eisentrauti, G.g. galloti and G.g. palmae)

		Gallotia galloti eisentrauti						*Gallotia galloti galloti*						*Gallotia galloti palmae*		
		NR season			R season			NR season			R season			R season		
RT (min)	Compound	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE
14.6	Nonanol	0.05	±	0.03	0.04	±	0.02	0.16	±	0.10	0.08	±	0.08	0.08	±	0.07
15.0	Decanal	0.01	±	0.05	0.01	±	0.01	0.02	±	0.02	0.02	±	0.03	–	–	–
17.4	2,4-Decadienal	0.01	±	0.01	0.02	±	0.02	–	–	–	–	–	–	0.01	–	0.02
19.6	Decanoic acid	0.01	±	0.01	0.02	±	0.02	–	–	–	–	–	–	–	–	–
20.0	Decanol	0.06	±	0.05	0.10	±	0.06	0.18	±	0.17	0.09	±	0.12	0.10	±	0.07
22.5	Undecanol	0.01	±	0.01	0.03	±	0.03	–	–	–	0.03	±	0.06	0.01	±	0.01
23.0	Dodecanal	0.01	±	0.01	0.01	±	0.01	–	–	–	–	–	–	–	–	–
24.3	Dodecanoic acid	–	–	–	0.07	±	0.09	–	–	–	–	–	–	–	–	–
24.8	Dodecanol	0.05	±	0.02	0.04	±	0.03	0.13	±	0.13	0.08	±	0.10	0.07	±	0.03
25.3	Tetradecanal	0.20	±	0.09	0.10	±	0.06	0.20	±	0.06	0.12	±	0.07	0.19	±	0.07
27.0	Tetradecanol	0.34	±	0.18	0.29	±	0.04	0.25	±	0.15	0.13	±	0.11	0.29	±	0.22
27.9	9-Hexadecenal	–	–	–	–	–	–	–	–	–	–	–	–	0.35	±	0.11
27.6	Pentadecanal	0.27	±	0.14	0.05	±	0.03	0.11	±	0.04	0.06	±	0.04	0.47	±	0.33
28.7	Tetradecanoic acid	0.01	±	0.01	0.19	±	0.14	–	–	–	0.04	±	0.04	–	–	–
29.2	Pentadecanol	0.17	±	0.03	0.04	±	0.02	0.13	±	0.10	0.08	±	0.12	–	–	–
29.7	Hexadecanal	1.23	±	0.61	0.75	±	0.38	0.62	±	0.24	0.27	±	0.23	1.28	±	0.94
30.3	6,10,14-Trimethyl-2-pentadecanone	0.01	±	0.01	0.03	±	0.02	0.01	±	0.01	0.02	±	0.03	–	–	–
30.7	Pentadecanoic acid	0.03	±	0.02	0.08	±	0.06	0.03	±	0.04	0.02	±	0.02	–	–	–
32.0	Hexadecanoic acid, methyl ester	–	–	–	–	–	–	–	–	–	–	–	–	0.78	+	0.40

		Gallotia galloti eisentrauti						*Gallotia galloti galloti*						*Gallotia galloti palmae*		
		NR season			R season			NR season			R season			R season		
RT	Compound	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE
31.2	Hexadecanol	1.24	±	0.72	0.39	±	0.24	1.12	±	0.80	0.44	±	0.34	–	–	–
31.8	Heptadecanal	0.23	±	0.08	0.03	±	0.03	0.11	±	0.07	0.08	±	0.15	–	–	–
32.3	9-Hexadecenoic acid	–	–	–	0.23	±	0.20	0.02	±	0.03	0.11	±	0.10	–	–	–
32.8	Hexadecanoic acid	0.44	±	0.78	4.06	±	1.20	0.79	±	1.46	2.96	±	2.04	0.89	±	0.76
33.2	Hexadecanoic acid, ethyl ester	0.08	±	0.07	0.11	±	0.11	0.04	±	0.04	0.77	±	1.58	0.09	±	0.07
33.5	Heptadecanol	0.01	±	0.01	0.07	±	0.07	–	–	–	0.02	±	0.03	–	–	–
33.7	Octadecanal	4.41	±	3.07	0.38	±	0.32	1.67	±	1.82	0.65	±	0.65	3.00	±	1.67
33.8	Hexadecanoic acid, 1-methylethyl ester	–	–	–	0.31	±	0.31	0.07	±	0.12	0.11	±	0.13	–	–	–
34.6	Heptadecanoic acid	–	–	–	0.08	±	0.03	–	–	–	0.06	±	0.06	–	–	–
35.0	Octadecanol	0.14	±	0.10	0.35	±	0.27	0.09	±	0.07	0.46	±	0.43	0.52	±	0.61
35.3	2-Nonadecanone	1.02	±	0.65	0.12	±	0.07	0.42	±	0.24	0.16	±	0.09	0.39	±	0.29
35.6	Nonadecanal	0.44	±	0.39	0.02	±	0.02	0.35	±	0.25	0.08	±	0.14	0.53	±	0.27
35.9	9,12-Octadecadienoic acid	0.01	±	0.01	1.49	±	1.11	–	–	–	1.62	±	1.38	0.02	±	0.04
36.0	9-Octadecenoic acid	0.10	±	0.18	2.33	±	1.08	–	–	–	4.48	±	7.91	0.19	±	0.50
36.3	9,12-Octadecadienoic acid, ethyl ester	–	–	–	0.27	±	0.50	–	–	–	0.37	±	0.50	–	–	–
36.4	Octadecanoic acid	0.29	±	0.52	2.00	±	1.10	0.03	±	0.05	2.29	±	1.35	0.61	±	1.12
36.8	Octadecanoic acid, ethyl ester	–	–	–	0.14	±	0.17	0.16	±	0.31	0.15	±	0.23	–	–	–
37.4	Eicosanal	0.18	±	0.14	0.30	±	0.16	0.16	±	0.13	0.10	±	0.12	0.27	±	0.23
38.7	Eicosanol	–	–	–	–	–	–	0.14	±	0.16	0.34	±	0.27	–	–	–
39.1	5,8,11,14-Eicosatetraenoic acid,ethyl ester	–	–	–	–	–	–	0.19	±	0.13	0.26	±	0.33	–	–	–
39.7	Eicosanoic acid	–	–	–	–	–	–	–	–	–	1.45	±	1.24	0.19	±	0.22
40.3	Eicosanoic acid, ethyl ester	–	–	–	–	–	–	–	–	–	0.65	±	0.79	–	–	–
46.7	Squalene	0.62	±	0.22	3.99	±	2.92	0.41	±	0.40	1.54	±	0.97	0.51	±	0.23
46.8	Cholesta-2,4-diene	0.24	±	0.25	0.32	±	0.36	0.16	±	0.23	0.02	±	0.03	0.06	±	0.07
47.0	Unidentified terpenoid	0.04	±	0.05	0.08	±	0.10	–	–	–	–	–	–	–	–	–

		Gallotia galloti eisentrauti						*Gallotia galloti galloti*						*Gallotia galloti palmae*		
		NR season			R season			NR season			R season			R season		
RT	Compound	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE
47.4	Cholesta-4,6-dien-3-ol	0.13	±	0.02	0.05	±	0.05	0.23	±	0.09	0.14	±	0.13	0.23	±	0.38
47.6	Cholesta-3,5-diene	0.34	±	0.16	0.49	±	0.34	0.33	±	0.20	0.31	±	0.29	0.32	±	0.27
49.5	γ-Tocopherol	–	–	–	0.01	±	0.01	–	–	–	0.01	±	0.01	0.03	±	0.08
50.2	Cholestanol	0.48	±	0.34	0.58	±	0.26	0.69	±	0.55	0.70	±	0.65	4.45	±	11.21
50.7	Cholesterol	51.92	±	14.07	19.35	±	6.65	54.21	±	6.25	41.98	±	13.32	11.65	±	4.39
50.8	α-Tocopherol	18.76	±	9.32	41.71	±	11.77	24.59	±	6.79	19.97	±	9.66	43.07	±	14.23
51.3	Cholestan-3-one	0.16	±	0.15	0.24	±	0.16	–	–	–	–	–	–	–	–	–
51.4	Ergosta-5,22-dien-3-ol	0.33	±	0.34	0.33	±	0.25	1.16	±	0.93	0.74	±	0.73	0.54	±	0.55
51.9	Ergosterol	0.16	±	0.12	0.19	±	0.26	–	–	–	–	–	–	1.10	±	1.93
52.1	Campesterol	7.71	±	2.21	4.58	±	2.83	3.52	±	1.63	4.84	±	2.40	12.38	±	17.31
52.3	Cholest-4-en-3-one	1.53	±	0.64	1.55	±	0.77	1.56	±	0.87	1.18	±	0.95	2.25	±	2.09
52.5	Stigmasterol	0.26	±	0.17	0.33	±	0.29	0.14	±	0.17	0.29	±	0.21	0.95	±	1.28
52.7	Cholesta-4,6-dien-3-one	0.33	±	0.06	0.30	±	0.21	0.57	±	0.28	0.29	±	0.22	0.36	±	0.30
53.0	Stigmasterol derivative?	0.05	±	0.10	0.13	±	0.22	–	–	–	–	–	–	0.82	±	1.59
53.2	Sitosterol	3.68	±	1.61	4.40	±	1.28	2.87	±	1.96	3.36	±	1.95	4.99	±	3.19
53.3	Stigmastanol	0.93	±	0.50	0.50	±	0.49	0.54	±	1.08	0.38	±	0.37	0.34	±	0.84
53.8	Unid. steroid(143,157,211,253,353,380,412)	0.01	±	0.02	0.28	±	0.52	–	–	–	–	–	–	–	–	–
53.8	Cholest-5-en-3-one	–	–	–	–	–	–	–	–	–	–	–	–	0.09	±	0.17
53.9	Hexadecanoic acid, ethenyl ester	0.13	±	0.08	1.13	±	0.30	0.16	±	0.10	0.26	±	0.24	0.15	±	0.24
54.1	Hexadecyl 9-hexadecenoate	0.06	±	0.07	0.28	±	0.22	–	–	–	–	–	–	–	–	–
55.3	Cholest-4-ene-3,6-dione	–	–	–	–	–	–	–	–	–	–	–	–	0.79	±	0.87
55.9	Octadecyl 9-hexadecenoate	–	–	–	0.44	±	0.37	0.02	±	0.04	0.16	±	0.15	–	–	–
56.2	Octadecyl hexadecanoate	0.04	±	0.57	1.12	±	1.52	0.48	±	0.48	2.05	±	3.48	–	–	–
56.4	9-Octadecenyl hexadecanoate	–	–	–	–	–	–	–	–	–	–	–	–	0.12	±	0.20
56.9	Octadecanoic acid, ethenyl ester	–	–	–	–	–	–	0.23	±	0.27	0.48	±	0.40	0.55	±	0.54
57.6	Eicosyl hexadecenoate	–	–	–	–	–	–	0.19	±	0.19	0.37	±	0.43	1.26	±	1.14

		Gallotia galloti eisentrauti						*Gallotia galloti galloti*						*Gallotia galloti palmae*		
		NR season			R season			NR season			R season			R season		
RT	Compound	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE	mean	±	SE
59.1	Unidentified waxy ester?	–	–	–	–	–	–	–	–	–	–	–	–	0.27	±	0.32
59.4	9-Octadecenyl 9-hexadecenoate	0.04	±	0.08	0.31	±	0.35	–	–	–	–	–	–	0.26	±	0.17
60.5	9-Octadecenyl 9-octadecenoate	–	–	–	–	–	–	0.24	±	0.28	1.16	±	1.40	–	–	–
60.7	9-Octadecenyl octadecanoate	–	–	–	–	–	–	–	–	–	–	–	–	0.62	±	1.02
60.9	Octadecyl octadecanoate	0.01	±	0.02	0.75	±	1.07	–	–	–	–	–	–	0.57	±	0.61
61.2	Unidentified waxy ester?	0.35	±	0.60	0.68	±	0.81	0.39	±	0.31	0.28	±	0.35	0.31	±	0.35
65.4	Unid. ester of 9-hexadecenoic acid	0.03	±	0.04	0.39	±	0.22	0.04	±	0.09	0.04	±	0.07	0.06	±	0.09
65.9	Octadecyl eicosanoate	0.03	±	0.05	0.81	±	0.56	–	–	–	0.79	±	0.91	0.36	±	0.51
67.2	Unidentified waxy ester?	0.36	±	0.14	0.07	±	0.10	–	–	–	–	–	–	–	–	–

The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (±1SD). Characteristic ions (m/z) are reported for unidentified compounds. RT: Retention time. (R) reproductive and (NR) non-reproductive seasons

In male *Gge* secretions, we found a mixture of 66 lipophilic compounds in the reproductive season (Table 1). We identified vitamin E as the main compound (41.71 %), followed by steroids (33.36 %), carboxylic acids and their esters (11.38 %), waxy esters (5.98 %), squalene and other terpenoids (4.07 %), aldehydes (1.67 %), alcohols (1.35 %), ketones (0.2 %) and γ-tocopherol (0.01 %). The number of compounds decreased during the non-reproductive season (Table 1). Concretely, we identified only 58 lipophilic compounds, which proportions changed in comparison with the reproductive season samples. Thus, we mainly found steroids (68.28 %), but also vitamin E (18.76 %), aldehydes (6.99 %), alcohols (2.07 %), ketones (1.23 %), waxy esters (1.04 %), carboxylic acids and their esters (0.97 %) and finally, squalene and other terpenoids (0.66 %).

The femoral gland secretions of male *Ggg* during the reproductive season were constituted by 60 lipophilic compounds (Table 1), represented by a mixture of steroids (54.23 %), vitamin E (19.97 %), carboxylic acids and their esters (15.32 %), waxy esters (5.58 %), alcohols (1.74 %), squalene and other terpenoids (1.54 %), aldehydes (1.37 %) and γ-tocopherol (0.01 %). Samples of the non-reproductive season contained only 49 compounds (Table 1). The main components were also steroids (65.98 %), followed by vitamin E (24.59 %), aldehydes (3.24 %), alcohols (2.19 %), waxy esters (1.76 %), carboxylic acids and their esters (1.32 %), ketones (0.5 %) and squalene and other terpenoids (0.41 %).

Finally, in male *Ggp* secretions, we found a total of 53 lipophilic compounds during the reproductive season (Table 1), identifying two large differentiated blocks, vitamin E (43.07 %) and steroids (41.33 %). Also, we found aldehydes (6.11 %), waxy esters (4.53 %), carboxylic acids and their esters (2.77 %), alcohols (1.09 %), ketones (0.57 %), squalene and other terpenoids (0.51 %) and γ-tocopherol (0.03 %).

INTER-SPECIES DIFFERENCES

We found significant overall differences among chemical profiles of the three subspecies during the reproductive-season in a PERMANOVA analysis (pseudo $F_{2,18} = 48.99$, $P = 0.001$). The pairwise comparisons showed significant differences in all cases (permutation tests; $6.20 < t < 7.34$, $P = 0.002$ for all). The CAP analysis classified 95.2 % of individuals within the correct subspecies in accordance with the chemical composition of their femoral secretions alone, using leave-one-out cross-validation and m = 2 axes ($\delta_1^2 = 0.98$, $P < 0.001$). Moreover, comparing the proportion of secreted cholesterol during the reproductive period, we found overall significant differences (GLM; $F_{2,18} = 22.30$, $P < 0.001$). However, the pairwise comparisons showed that *Ggg* differed significantly of *Gge* (Tukey's tests, $P < 0.01$) and *Ggp* ($P < 0.001$), but there were not significant differences between *Gge* and *Ggp* ($P = 0.07$) (figure 2). We observed a similar pattern in secreted proportions of vitamin E, which differed significantly among subspecies (GLM; $F_{2,18} = 8.51$, $P < 0.01$), but while *Ggg* showed significant differences with *Gge* (Tukey's tests, $P < 0.01$) and *Ggp* ($P < 0.01$), proportions of *Gge* and *Ggp* were not significantly different ($P = 0.98$).

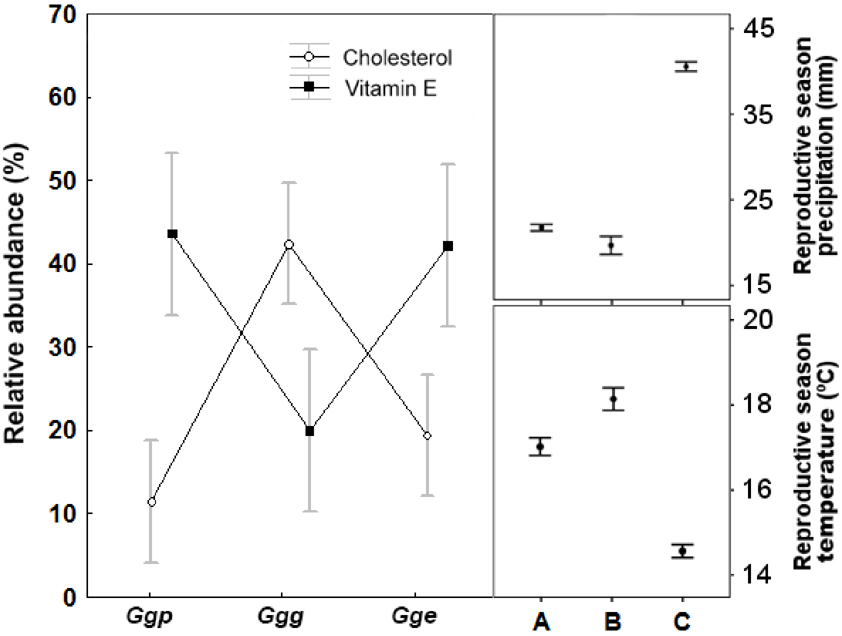

Figure 2: Proportions of cholesterol and vitamin E in femoral gland secretions of *Gallotia galloti palmae* (*Ggp*), *Gallotia galloti galloti* (*Ggg*) and *Gallotia galloti eisentrauti* (*Gge*) (left) in the reproductive season (left). Right plots show precipitation (top) and temperature (bottom) mean (± SE) values in the reproductive season of the three sampling points: A: El Pedregal, Ggp; B: Malpaís de Güimar, Ggg; C: El Pris, Gge)

INTER-SEASONAL DIFFERENCES

The inter-seasonal comparison of chemical profiles between *Gge* and *Ggg* showed significant differences related with the subspecies and season (two-way PERMANOVA; Subspecies: pseudo $F_{1,19} = 73.38$, $P < 0.001$; Season: pseudo $F_{1,19} = 9.73$, $P < 0.01$; Interaction: pseudo $F_{1,19} = 1.99$, $P = 0.13$). The CAP analysis classified 100 % of individuals within the correct category according to their chemical profiles both for subspecies ($\delta_1^2 = 0.99$, $P < 0.001$, m=2 axis) and season ($\delta_1^2 = 0.90$, $P < 0.001$).

In addition, we observed significant differences in cholesterol proportions among subspecies (GLM; $F_{1,19} = 7.6$, $P = 0.01$), and between the reproductive and non-reproductive season (GLM; $F_{1,19} = 18.01$, $P < 0.0001$), but the interaction was significant (subspecies x season: $F_{1,19} = 6.69$, $P = 0.01$) (figure 3).

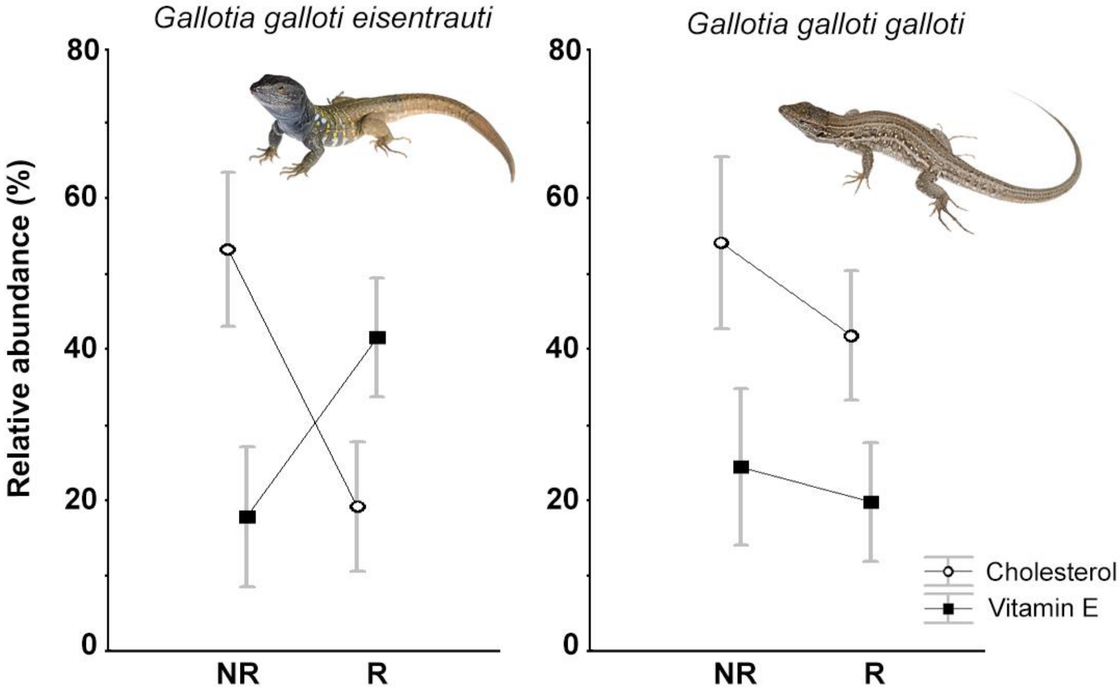

Figure 3: Comparison of relative abundances (% of TIC areas) of cholesterol and vitamin E (mean ± SD) found in femoral gland secretions of *Gallotia galloti galloti* and *Gallotia galloti eisentrauti* during the reproductive (R) and non-reproductive (NR) seasons

Pairwise comparisons showed that there were seasonal differences in *Gge* (Tukey's tests, $P < 0.001$), but not in *Ggg* ($P = 0.37$). Moreover, differences between *Gge* and *Ggg* were only significant during the reproductive season ($P < 0.01$), but not in the non-reproductive season ($P = 0.99$). Similarly, regarding to vitamin E proportions, there were not overall significant differences between subspecies or seasons (GLM; subspecies: $F_{1,19} = 1.95$, $P = 0.17$; season: $F_{1,19} = 3.80$, $P = 0.06$), but the interaction was significant (subspecies x season: $F_{1,19} = 11.15$, $P < 0.01$), showing that there were significant seasonal differences in *Gge* (Tukey's tests, $P < 0.01$) but not in *Ggg* ($P = 0.77$), and that differences between the two subspecies were only significant during the reproductive season ($P < 0.01$) but not in the non-reproductive season ($P = 0.60$) (figure 3).

Discussion

Our study offers a congruent parallelism between levels of particular compounds found in chemical signals of *G. gallotia* and those expected under certain climatic conditions (i.e., temperature and precipitation) where each subspecies inhabits, given the potential functionality proposed for those chemicals in past studies. In this framework, we also provide the composition of the chemical secretions of three subspecies *of G. galloti* lizards. These secretions were characterized by the same major types of compounds in all subspecies, although with clear quantitative and qualitative differences between some of them (see Table 1). Similarly to other lacertid lizards, steroids were the main compounds found in *G. galloti* (Martín & López, 2014). Within the genus *Gallotia*, chemical description of femoral secretions remained practically unknown. In fact, *Gallotia simonyi* is the only species whose secretions have been described and, steroids were also the most abundant type of compounds (Martín *et al.*, 2015b). However, unlike the case of *G. gallotia*, vitamin E was not found in *G. simonyi* secretions. Also noteworthy is the high proportion of campesterol in the three subspecies, which seems to be typical of lizard species belonging to subfamily Gallotinae, as described for

Psammodromus spp. or *G. simonyi* (Martín & López, 2006a; López & Martín, 2009; Martín & López, 2014; Martín *et al.*, 2015b).

Climatic factors as potential drivers of chemical changes

Recent studies carried out in some lizard species have noted inter-population differences in the chemical composition of gland secretions. For instance, Escobar *et al*. (2003) showed compositional divergences in secretions from two populations of *Liolaemus fabiani* lizards, which was suggested as a possible adaptation to environmental conditions. Recent studies have also described divergences in chemical signals from isolated populations of insular lizards species (Runemark *et al.*, 2011; Martín *et al.*, 2013).In these cases, the hypothesis that climatic pressures might model the chemical structure of the signals was always proposed. Owing to the lack of studies that directly study climatic conditions within a "chemical-compositional approach", there is a pressing need to find evidences that may relate the inter-population climatic-niche differences with their concomitant representation in the lizard chemical profiles. For example, cholesterol is the main compound in gland secretions of many lizards species, possibly due to its function as a matrix upon which are sustained the other components (Escobar *et al.*, 2001; Escobar *et al.*, 2003; Weldon *et al.*, 2008; Martín & López, 2014). In other species, vitamin E is secreted as a complement, or even replacing the cholesterol as the main compound (Kopena *et al.*, 2009, 2014a). The presence of this vitamin has been associated with its antioxidant properties (Rosenau *et al.*, 1995; Wolf *et al.*, 1998; Traber & Atkinson, 2007), and its higher abundance is suggested to be associated to wet environments (Weldon *et al.*, 2008; Martín & López, 2011, 2014). Nevertheless, the high relatives abundances of vitamin E observed in chemical secretions of species phylogenetically closer [e.g., *Lacerta schreiberi, L. viridis* or *Timon lepidus* (López & Martín, 2006; Kopena *et al.*, 2009; Martín & López, 2010)] raised doubts regarding to whether these high levels might be mainly triggered by genetic drift or as an adaptation to climatic conditions. On this basis, our results describe a clear parallelism between climatic differences in sampled areas and particular divergences in the chemical secretions of the three subspecies that inhabit them. Although the proportions of cholesterol and vitamin E could be affected by other external factors (e.g., trophic resources, metabolism), the relative abundances of both compounds in the three subspecies in relation with their climatic conditions are congruent to consider that climatic pressures may be influencing the signal composition. These evidences would support the hypothesis that climatic conditions might unleash divergences in the composition of chemical signals in lizards. Thus, the high levels of vitamin E in *Gge* and *Ggp* during the reproductive season might be explained by the high precipitation and low temperature values in their geographic ranges in comparison with the dry-warm areas occupied by *Ggg*. The higher levels of vitamin E could protect other compounds due to its antioxidant function. In contrast, the high levels of cholesterol in *Ggg* during the reproductive season might act protecting secretions against the dry and hot climatic conditions.

It is remarkable that despite of genetic distances being larger between Tenerife and La Palma *G. gallotia subspecies* (Richard & Thorpe, 2001), chemical profiles and particularly vitamin E and cholesterol levels of *Gge* of Tenerife show more similarities with *Ggp* of La Palma than with *Ggg* from Tenerife*,* which could be partially explained by the potential effect of the different climatic conditions. However, we are aware that phylogenetic relationships, not only environmental pressures, may also be exerting a shaping force. As noted above, *Gge* and *Ggg* from Tenerife are genetically closer than *Ggp* from La Palma (Richard & Thorpe, 2001) and therefore, this might also constraint the secretion of lizards to adapt their chemical signals to the environment. This could explain that although *Gge* secretions would theoretically need higher proportions of vitamin E – due to the wetter climatic conditions where they inhabit – than those of *Ggp*, the actual relative abundance of this compound is lower. Further investigations must be done to clarify whether the divergences in the chemical signaling revealed here are genetically fixed within *G. galloti* subspecies or, conversely, whether chemical differences have arisen as a result of phenotypic plasticity to the local environment.

Moreover, evidences have recently suggested that levels of secreted vitamin E are under a trade-off between metabolic requirements (antioxidant and immunostimulatory functions) and the need to protect and improve the chemical signaling through gland secretions, since high proportions of vitamin E influenced the female response to male secretions (Kopena *et al.*, 2011, 2014a, b; Martín & López, 2015). This trade-off may explain that the high levels of vitamin E in *Gge* during the reproductive season later decrease during the non-reproductive season. This suggests that lizards may use different "chemical" strategies depending on the season. Thus, *Gge* and *Ggp* might invest higher proportions of vitamin E than *Ggg* during the reproductive season to possibly protect the rest of compounds due to the wet climatic conditions, thus, ensuring a proper chemical communication when it has important reproductive consequences (Martín & López, 2015). However, during the non-reproductive season, keeping high levels of vitamin E can be unnecessarily costly. The overall list of compounds also showed seasonal changes in presence and absence of some of them in both of the two Tenerife subspecies (i.e., *Gge* and *Ggg*). Although the function of some of these compounds is not clear, their decreasing numbers during the non-reproductive season also suggest different physiological patterns of investment depending on the time of the year.

Summarizing, the variation in species chemical profiles may be directly related to the fact of inhabiting environments with particular climatic conditions, together with the underlying species phylogenetic-history and the physiological costs of allocating compounds to secretions. Therefore, among-population differences in chemical composition may be the result of a compromise between their phylogenetic relationships and adaptive mechanisms to improve the signal efficacy under different climatic conditions.

Long-term evolutionary consequences

Numerous cases of speciation have arisen with the evolution of barriers that prevent the genetic exchange among populations. This may be extrapolated to the chemosensory system, in which enough differences in chemical signals might lead to phenotypic divergences drivers of speciation episodes (Smadja & Butlin, 2009). This is more important when these differences involve compounds potentially honest, as have been suggested in vitamin E, for instance. However, our study reflects divergences in chemical profiles of secretions from three geographically distant populations with significant climatic differences and therefore, further work needs to be performed to establish whether such divergences appear at the same level in the contact areas of the two Tenerife subspecies. Furthermore, it would be interesting to explore whether chemical differences appear in other species, populations and/or subspecies of the genus *Gallotia*. Behavioral experiments under strictly controlled conditions are also advisable to confirm that the lizard secretions with higher levels of cholesterol and vitamin E significantly increase their efficiency under dry or wet environments respectively. In addition, although levels of cholesterol and vitamin E observed here are clearly different among populations, being congruent with those expected patterns under the climatic pressures where lizards inhabit, other non-controlled factors might also influence the chemical profiles (e.g., trophic resources, social stress, health status, etc.). Thus, further manipulative experiments are required to entrench the degree to which climatic and alternative factors are driving divergences in lizard chemical signals.

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Gallotia galloti galloti male o south of Tenerife island. Drawing: F. García

CHAPTER VI

HETEROGENEOUS TEMPO AND MODE OF EVOLUTIONARY DIVERSIFICATION OF CHEMICAL COMPOUNDS IN LIZARD CHEMICAL SIGNALS

CAPÍTULO VI

TIEMPO Y MODO HETEROGÉNEOS EN LA DIVERSIFICACIÓN EVOLUTIVA DE LOS COMPUESTOS QUÍMICOS PRESENTES EN LAS SEÑALES QUÍMICAS DE LOS LAGARTOS

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HETEROGENEOUS TEMPO AND MODE OF EVOLUTIONARY DIVERSIFICATION OF CHEMICAL COMPOUNDS IN LIZARD CHEMICAL SIGNALS

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ABSTRACT

Important part of the multivariate selection shaping social and interspecific interactions among and within animal species emerges from communication. Therefore, understanding the diversification of signals for animal communication is a central endeavor in evolutionary biology. Over the last decade, the rapid development of phylogenetic approaches has promoted a stream of studies investigating evolution of communication signals. However, comparative research has primarily focused on visual and acoustic signals, while the evolution of chemical signals remains largely unstudied. An increasing interest in understanding the evolution of chemical communication has been inspired by the realization that chemical signals underlie some of the major interaction channels in a wide range of organisms. In lizards, in particular, chemosignals play paramount roles in female choice and male-male competition, and during community assembly and speciation. Here, using phylogenetic macro-evolutionary modeling, we show for the very first time that multiple compounds of scents for communication in lizards have diversified following highly different evolutionary speeds and trajectories. Our results suggest that cholesterol, α-tocopherol and cholesta-5,7-dien-3-ol have been subject to stabilizing selection (OU model), whereas the remaining compounds are better described by Brownian motion modes of evolution. Additionally, the diversification of the individual compounds has accumulated substantial relative disparity over time. Thus, our study reveals that the chemical components of lizard chemosignals have proliferated across different species following compound-specific directions.

Key words: Animal communication, disparity, chemosensory, pheromones, lizards, sexual selection

Introduction

Animal communication influences the trajectories of social, ecological and phenotypic evolution across multiple levels of biodiversity, from the sexes to the complexity of assemblages (Smith, 2013). Not surprisingly then, the quantitative study of the drivers, rates and directions of diversification of signals employed by animals to engage in social and sexual communication has been the focus of an increasing stream of studies, which have flourished with the development of phylogenetic approaches designed for comparative analyses (Ratcliffe & Nydam, 2008; Chen *et al.*, 2012; Derryberry *et al.*, 2012; Mason *et al.*, 2014). As a result, the implementation of multiple programs of research investigating the adaptive evolution of signals across broad ranges of species varying extensively in their 'strategies' for production and delivery of signals, and in the environmental pressures (i.e., sources of selection) shaping them, has contributed to accelerated advances in our understanding of the evolutionary dynamics of animal communication at larger spatial and taxonomic scales

Given that animal species employ a broad diversity of phenotypic traits during communication, systems of production and delivery of signals are known to be shaped by multiple extrinsic (e.g., resource availability, population density, sex ratios) and intrinsic (e.g., phylogenetic inertia) factors. Indeed, both sexual and natural selection can often operate in coordination or antagonistically to shape the same signal. For example, while signal expression can positively correlate with the 'genetic quality' of the signaler, the expression of the signal itself can compromise the expression of other energetically costly traits with strong effects on fitness (Simmons & Emlen, 2006; Losos, 2009; Irschick *et al.*, 2014).

As a result of the accelerated development of phylogenetic methods for comparative analyses of trait evolution, a stream of studies has investigated the diversification history of signals in animals. However, the overwhelming majority of such studies have been focused on visual and acoustic signals (Gingras *et al.*, 2013; Santana *et al.*, 2013; Wilkins *et al.*, 2013;

Huang & Rabosky, 2014). In contrast, comparative studies of chemical signals remain fundamentally ignored in most groups of organism (Symonds & Elgar, 2008; Kather & Martin, 2015).This gap of knowledge could hinder the emergence of new ecological and evolutionary hypotheses in the context of multimodal communication (Faria *et al.*, 2014; Stacks & Salwen, 2014). Therefore, investigating the evolutionary tempo and mode of chemosignal diversification along the phylogenetic history of lineages that rely on these forms of communication is a major pending step to strengthen our overall understanding of the evolutionary dynamics of communication.

Research on chemical communication has highlighted the key role that chemosensory systems play in species interactions, niche adaptation, speciation and extinction (Amo *et al.*, 2008; Steiger *et al.*, 2010; Apps *et al.*, 2015; Bacquet *et al.*, 2015; Martín & López, 2015). However, techniques aimed to investigate communication at the chemical level are analytically demanding and thus, ongoing advances in this field have been slower than research on other signals, such as visual and acoustic (Touhara, 2013). Despite these difficulties, some accelerated improvements in the development of technologies and methodologies for chemical analyses have inspired an increasing interest in exploring an expanding range of questions around the ecology and evolution of chemical interactions (Symonds & Elgar, 2008; Johnston & del Barco-Trillo, 2009; Ding *et al.*, 2014; Wyatt, 2014; Martín & López, 2015; Baeckens *et al.*, 2016a). These advances have made it increasingly more feasible to explore in detail the evolution of chemical signals and their multiple compounds across different species, and across multiple individuals within species. However, studies investigating the macro-evolutionary diversification of the chemical components of communication remain fundamentally neglected (Symonds & Elgar, 2008; Steiger *et al.*, 2010).

In reptiles, in particular, chemosensory systems have been shown to play paramount roles in social and sexual interactions (Pincheira-Donoso *et al.*, 2008; Mason & Parker, 2010; Martín & López, 2015). In fact, phenomena as important as female mate choice mechanisms are thought to rely more heavily on chemical than on other forms of signaling among lizards (Martín *et al.*, 2007; Kopena *et al.*, 2011; López & Martín, 2012). In these reptiles, a number of studies have failed to identify evidence revealing a role for quantitative traits biasing mating success during female mate choice (which has consolidated the view that sexual selection in these animals takes place via male-male contests) (Olsson et al. 1998). In contrast, accumulating evidence suggests that this mechanism is fundamentally mediated by chemical signals (i.e., chemical compounds or/and a mixture of them) (Martín & López, 2014; Martín & López, 2015) from secretions produced by follicular femoral and precloacal glands (Cooper, 1994; Escobar *et al.*, 2003; Flachsbarth *et al.*, 2009; Martín & López, 2014; García-Roa *et al.*, 2016). Indeed, recent literature confirms that both natural and sexual selection are affected by these secretions (López & Martín, 2005; Martín & López, 2006c; Martín *et al.*, 2007; Martín *et al.*, 2015). For example, studies focused on global warming have shown that the effect of different climatic variables alters the efficacy of chemoreception in lizards, and consequently, the fundamental basis of communication underlying population stability (Martín & López, 2013; Martín *et al.*, 2015). Also, experiments conducted in males of European green lizards (*Lacerta viridis*) showed that females preferred to use areas scent-marked by males with high proportions of vitamin E (Kopena *et al.*, 2011). Similar female preferences for males producing "quality" secretions have also been reported in other species (Martín & López, 2015). Therefore, the study of chemosignal evolution has emerged as a vital perspective to push forward our understanding of species and trait diversification.

In this paper, we present the first empirical study investigating the macro-evolutionary diversification of chemical compounds found in femoral and precloacal secretions produced by lizards to engage in social communication. Among reptiles in general, species of the Superfamily Lacertoidea have offered classical model systems shaping our understanding of chemical communication, and thus, the chemical profiles of their secretions have been routinely described in the refereed literature (Martín & López, 2014). In fact, lacertoid lizards have been the subject of the greatest number of behavioral and chemical ecology experiments to date (Weldon *et al.*, 2008; Martín & López, 2014; Martín & López, 2015). Consequently, this lineage provides an ideal point of reference to quantitatively characterize evolutionary variation of chemical traits underlying communication. Specifically, we investigate the evolutionary trajectories and rates of diversification of particular chemical compounds over time, by employing phylogenetic modeling of the relative proportion of each compound measured in the secretions of each species.

Material and Methods

Study species

We gathered a comprehensive dataset encompassing 20 lacertoid species for which the detailed chemical composition of their male chemical secretions has been profiled [reviewed in (Martín & López, 2014)]. In this paper, we added information for the chemical composition of the secretions of other five species for which these data remained unavailable (Table S1). The total sample of species we have employed for this study encompasses a broad diversity of environments, which captures a range of areas where selection is expected to operate in contrasting ways as a result of variations in climate and in the intensity of interspecific competition arising from coexistence with other lizard species (Cox & Temple, 2009). For the preparation of our species-level dataset, we averaged values of relative amounts for chemical compounds taken from multiple populations per species if they were available (see references of Table S1).

Chemical compounds

We performed an exhaustive collection of data on the relative abundance of particular compounds found in femoral and precloacal secretions from the refereed literature as well as from samples directly collected and processed by ourselves. Lizard chemical secretions are highly complex and consist of multiple compounds. We focused on the following subset of chemicals given their identified role in ecological interactions and communication in these reptiles(Weldon *et al.*, 2008; Martín & López, 2014): (i) cholesterol, a steroid, usually the most abundant compound found in lizard secretions, which is thought to play a role in 'holding' and protecting other compounds (Escobar *et al.*, 2003; Weldon *et al.*, 2008). High levels of cholesterol have also been associated with dominance (Martín & López, 2007) and intersexual interactions (Martín & López, 2006d); (ii) campesterol, a relatively common steroid in lizard secretions, particularly dominant or highly common in some lineages (e.g., *Psammodromus* and *Gallotia*, respectively). High levels of campesterol have been associated with signal quality (Martín & López, 2006b; López & Martín, 2009); (iii) stigmasterol, a relatively common, but not abundant steroid that is believed to be acquired via ingestion of plants. This compound is associated with structural properties in secretions, as well as with healthy conditions (Othman & Moghadasian, 2011); (iv) ergosterol (i.e., provitamin D_2), a common steroid that acts as a metabolic precursor of vitamin D_2, and believed to offer a reliable indicator of male healthy condition. Therefore, this compound has been seen to play a key role in mate choice (Martín & López, 2006c; Martín & López, 2008), making in particularly interesting given the difficulties to demonstrate mate choice in lizards based on quantitative traits (Olsson *et al.*, 1998); (v) 9,12-Octadecadienoic acid (i.e., linoleic acid), is a unsaturated fatty acid, costly to obtain. It has been attributed important functions in metabolism and thus, it might act as an indicator of male "quality" (Weldon *et al.*, 2008; Martína *et al.*, 2011); (vi) α-Tocopherol (i.e., vitamin E), usually found in lizard species in high proportions. It is believed to have antioxidant properties, protecting other compounds in secretions (Wolf *et al.*, 1998; Brigelius-Flohe & Traber, 1999). Also, high levels of α-tocopherol are linked to the quality of lizards and therefore, it has been assigned an important role during competition over sexual mates (Kopena *et al.*, 2011); (vii) cholestanol, commonly found in lacertids, and thought to be related with healthy body condition (Weldon *et al.*, 2008); and (viii) cholesta-5,7-dien-3-ol, a steroid present in some lizard species, it is the precursor of vitamin D_3. It has also been related to male quality, acting as a potential indicator of health condition (López & Martín, 2005; Martín & López, 2006d).

Chemical analyses of secretions

We analyzed chemical secretions produced by femoral glands of males of the species shown in Supplementary Table S1. We employed traditional techniques based on gas chromatography (GC) methodology, by using a Finnigan-ThermoQuest Trace2000 GC fitted with a poly (5 % diphenyl/ 95 % dimethylsiloxane) column (Supelco, Equity-5, 30 m length x 0.25 mm ID, 0.25 µm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as the detector. We conducted splitless sample injections (2 µl of each sample dissolved in n-hexane) with helium as the carrier gas, and injector and detector temperatures at 250 °C and 280 °C, respectively. The GC process was programmed with an initial temperature at 50 °C (10 min), and posterior increase of temperature until 280 °C (at a rate of 5 °C/min), and kept finally at this temperature for 30 min. Mass spectral fragments below m/z = 46 were not recorded. Initially, we identified secretion compounds by comparing their mass spectra with those in the NIST/EPA/NIH (NIST 02) computerized mass spectral library. Then, the confirmation of identifications was done by comparing spectra and retention times with those of authentic standards (from Sigma– Aldrich Chemical Co.) when these were available. We did not consider impurities identified in the control vial samples.

The relative amount of each compound was determined as the percentage of the total ion current (TIC). Finally, we collated the compounds-of-interest amounts to generate the final data base.

Phylogenetic macro-evolutionary analyses

To quantify the evolutionary diversification of the selected compounds, we employed phylogenetic macro-evolutionary analyses based on a model-selection approach. These analyses were performed on a time-calibrated molecular phylogenetic tree for our focal lizards, extracted from Pyron et al.'s (Pyron *et al.*, 2013) supertree for squamate reptiles (lizards and snakes).

We compared the tempo and mode of evolutionary diversification of the individual chemical compounds along the phylogenetic tree against a range of models that describe the directionality and speed of trait evolution during a lineage's history. We first compared four evolutionary models: a traditional Brownian-motion model (BM), which describes a random walk of trait evolution along the branches in the phylogeny. This model describes increases in trait variance centered on the initial value at the root of the tree, and increasing with the distance from the tree root. An Ornstein-Uhlenbeck model (OU), which assumes that once traits have adaptively evolved, stabilizing selection pulls the trait values around an adaptive optimum for the trait. An early-burst or "niche-filling" model (EB), which describes exponentially increasing or decreasing rates of evolution over time based on the assumption that niches are saturated by accumulating species within a lineage, and therefore, describing scenarios where accumulated diversities play a role in the rates of lineage accumulations themselves. Finally, a Delta model, which describes a time-dependent model of trait evolution, where the effects that early versus late evolution in the tree have on the rates of trait diversification. This model returns a δ value which indicates whether recent evolution has been fast when $\delta>1$, or slow when $\delta<1$)(Hernández *et al.*, 2013; Astudillo-Clavijo *et al.*, 2015; Pincheira-Donoso *et al.*, 2015). To compare the goodness of fit of these alternative models we employed an Akaike Information Criterion (AIC) approach. We provide values reported as AICc (bias-corrected version of AIC) and ΔAICc (the difference between each model and the best model). The best-fitted model is determined by identifying the lowest AICc score, which equals 0 (Pincheira-Donoso *et al.*, 2015). All model analyses and fitting were performed with the R package 'geiger'(Harmon *et al.*, 2008).

We subsequently investigated if the chemical compounds have evolved around an optimum value (i.e., whether their diversification has been influenced by stabilizing selection promoting convergences of the traits around one or more peaks on a 'Simpsonian landscape'), by employing the R package 'surface' (Ingram *et al.*, 2013; Mahler *et al.*, 2013). The surface method fits an adaptive radiation model in which lineages on the studied phylogeny may experience convergent shifts towards adaptive optima on the abovementioned macro-evolutionary Simpsonian landscape. Importantly, this model does not assume whether some lineages correspond to particular optima. Based on an OU model in which all species are pulled against a single adaptive optimum in morphospace, surface employs a stepwise model selection approach based on AICc, which allows for identification of the best model and the numbers and positions of adaptive peaks (i.e., trait 'regimes'), and hence, for convergence towards these optima over evolutionary time (Ingram *et al.*, 2013; Pincheira-Donoso *et al.*, 2015).

Finally, we used the amount of each compound to model their relative disparity across linages. We performed disparity-through-time analyses (DTT). This analysis firstly calculates the average disparity for each trait over time (Slater *et al.*, 2010; Hipsley *et al.*, 2014; Ingram, 2015; Jonsson *et al.*, 2015; Pincheira-Donoso *et al.*, 2015). DTT analyses compare the observed disparity values with those expected under a Brownian-motion model after 10,000 simulations across phylogeny. Subsequently, the average body size disparity obtained from both the real and the simulated data are plotted against the age of the nodes to calculate the morphological disparity index (MDI). This index quantifies the overall difference in relative disparity for the studied trait among and within subclades (i.e., differences in the range of variation) compared with the expectation under the null Brownian motion model of evolution (Slater *et al.*, 2010). More specifically, negative MDI scores indicate lower than expected trait relative disparity under Brownian motion (i.e., low average subclade relative disparity), which indicates that the majority of disparity occurs among subclades, and thus, that they occupy smaller and more isolated areas of the morphospace. Positive MDI values indicate that relative disparity among subclades shows a stronger overlap in morphospace(Pincheira-Donoso *et al.*, 2015). We conducted DTT analyses using the R package 'geiger' (Harmon *et al.*, 2008). In addition, we used the R package 'phytools' (Revell, 2012) to project the phylogeny within morphospace defined by time on *x*-axis (My since the root) and the relative abundance of each compound on *y*-axis. Also, we reconstructed the relative abundance of each compound for ancestral species in the tree (Revell & Freckleton, 2013).

Results

Relative amount of species chemical compounds in the study

Our analyses reveal that cholesterol is the predominant compound in our species (73.61 %), followed by α-tocopherol (9.96 %), campesterol (7.61 %), cholestanol (3.98 %), cholesta-5,7-dien-3-ol (1.98 %), 9,12-octadecadienoic acid (1.28 %), ergosterol (1.19 %) and stigmasterol (0.39%; figure 1). All these values, however, vary in the overall chemical profile description of each species (see Table S1 for details).

Tempo and mode of compound diversification

Our analyses comparing the four models of evolution performed among chemical compounds revealed substantial variation in the evolutionary trajectories followed by each of them during the clade's phylogenetic history (Table 1).

While the analyses identified the stabilizing selection (OU model) as the best approximation to describe diversification for cholesterol, α-tocopherol and cholesta-5,7-dien-3-ol, the BM model best described the evolution of the remaining compounds. In addition, the three compounds for which the OU model was selected showed different numbers of local adaptive peaks on the Simpsonian landscape. More specifically, while we found a single optimum value for cholesterol (32.7 %), six optimal values for α-tocopherol (0.07, 6.91, 18.73, 28.2, 32.08 and 37.2 %) and cholesta-5,7-dien-3-ol (0.02, 1.26, 2.27, 4.48, 7.5 and 8.5 %) were identified by the surface analyses.

Table 1: Evolutionary diversification models of chemical compounds. Data values are based on comparing four evolutionary models. Fitted models are Brownian-motion (BM), Ornstein-Uhlenbeck (OU), Early-Burst (EB) and Delta. Best-fit of models based on (delta) bias corrected Akaike Information Criteria (AICc)

Linage	Model	Model parameters	β	LogL	AICc	ΔAICc
Cholesterol	BM	-	2187.89	-117.59	239.72	2.52
	OU	α=2.72	4000.40	-115.02	237.20	0.00
	EB	α=-0.00	2187.89	-117.58	242.32	5.12
	Delta	δ=2.99	941.34	-115.63	238.40	1.20
Campesterol	BM	-	67.53	-74.11	152.77	0.00
	OU	α=0.02	67.99	-74.11	155.37	2.60
	EB	α=-0.00	67.53	-74.11	155.37	2.60
	Delta	δ=1.63	47.35	-73.97	155.10	2.33
Stigmasterol	BM	-	0.57	-14.42	33.40	0.00
	OU	α=2.72	1.17	-13.30	33.75	0.34
	EB	α=-0.00	0.57	-14.42	36.00	2.60
	Delta	δ=2.99	0.26	-13.25	33.65	0.24
Ergosterol	BM	-	8.46	-48.14	100.84	0.00
	OU	α=0.00	8.46	-48.15	103.44	2.60
	EB	α=-0.21	10.12	-48.15	103.43	2.59
	Delta	δ=2.05	5.12	-47.91	102.97	2.13
9,12-Octadecanoic acid	BM	-	5.40	-42.53	89.61	0.00
	OU	α=0.00	5.40	-42.53	92.21	2.60
	EB	α=-4.79	230.55	-41.57	90.30	0.68
	Delta	δ=0.99	5.43	-42.53	92.21	2.60
Tocopherol	BM	-	417.73	-96.89	198.33	0.38
	OU	α=2.71	832.20	-95.40	197.95	0.00
	EB	α=-0.00	417.74	-96.89	200.93	2.98
	Delta	δ=2.99	187.65	-95.47	198.09	0.14
Cholestanol	BM	-	88.21	-77.45	159.45	0.00
	OU	α=2.71	179.63	-76.23	159.62	0.17
	EB	α=-0.00	88.21	-77.45	162.05	2.60
	Delta	δ=2.99	40.26	-76.23	159.61	0.16
Cholesta-5,7-dien-3-ol	BM	-	25.82	-62.09	128.74	5.37
	OU	α=2.71	42.13	-58.11	123.37	0.00
	EB	α=-0.00	25.82	-62.09	131.33	7.97
	Delta	δ=2.99	10.55	-59.49	126.13	2.76

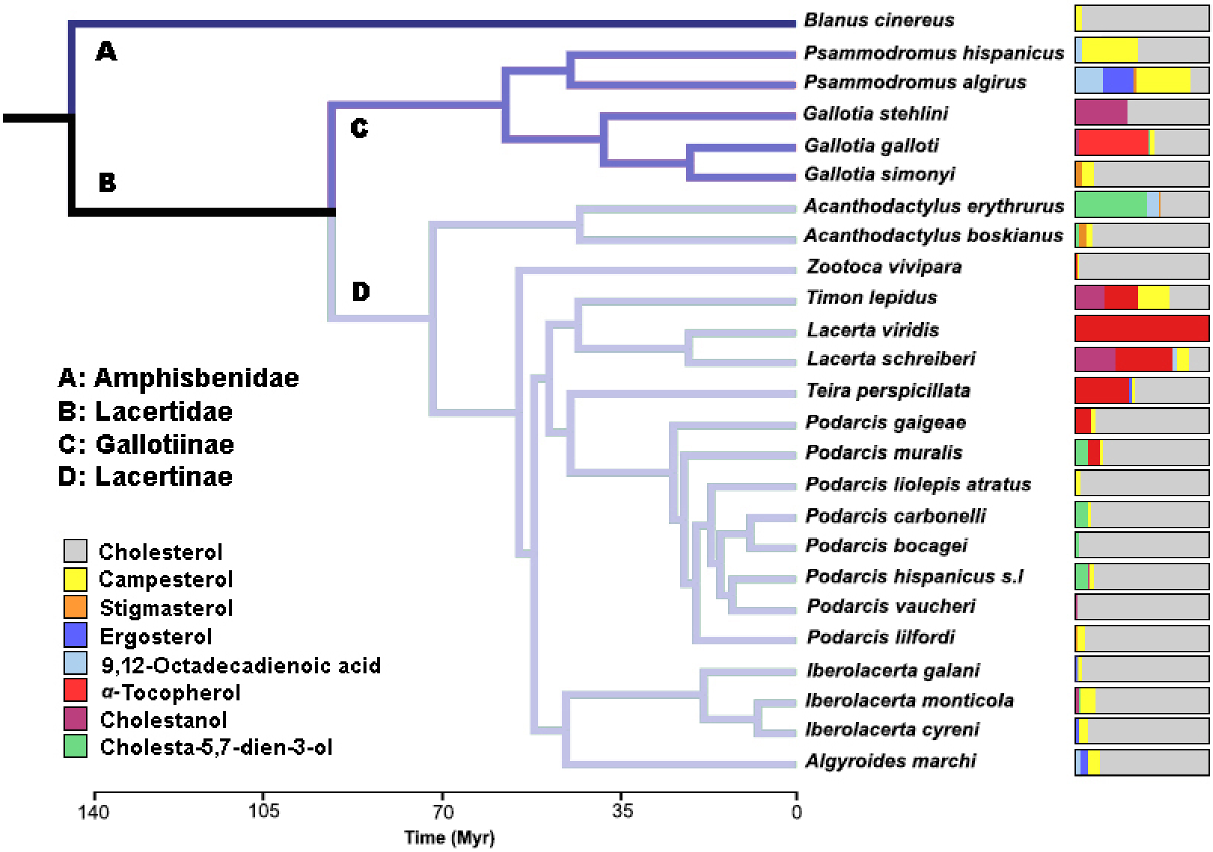

Figure 1: Phylogenetic relationship between analyzed species. Color bands show relative amounts of each compound with respect to the others for chemical secretions of the different analyzed species

The DTT analyses revealed positive MDI values in all compounds (i.e., higher values than expected under BM model). However, the evolutionary trajectories varied considerably among compounds. While campesterol (MDI = 0.31), stigmasterol (MDI= 0.84), ergosterol (MDI = 0.81), 9, 12-octadecadienoic acid (MDI = 0.51), α-tocopherol (MDI = 0.42) and cholestanol (MDI = 0.58) showed initial steep increases in relative disparity (in some cases slightly above the 95% CI), cholesterol (MDI = 0.04) and cholesta-5,7-dien-3-ol (MDI = 0.31) relative disparity decreased early during the clade's history (figure 2). In fact, the cholesterol DTT plot reflects an overall tendency to decrease over time. Only in the more recent segment of the clade's phylogenetic history (around. 6 Myr) relative disparity increases slightly above the upper limit of the 95% CI. Prominent increases and decreases are observed in the relative disparity of stigmasterol, ergosterol, α-tocopherol, cholestanol and cholesta-5,7-dien-3-ol plots, between 140-10 Mya, sometimes exceeding the 95 % CI (figure 2). Finally, diversification of each compound across the phylogeny show strong morphospace overlapping in the ancestral trajectories of their evolution (figure 3).

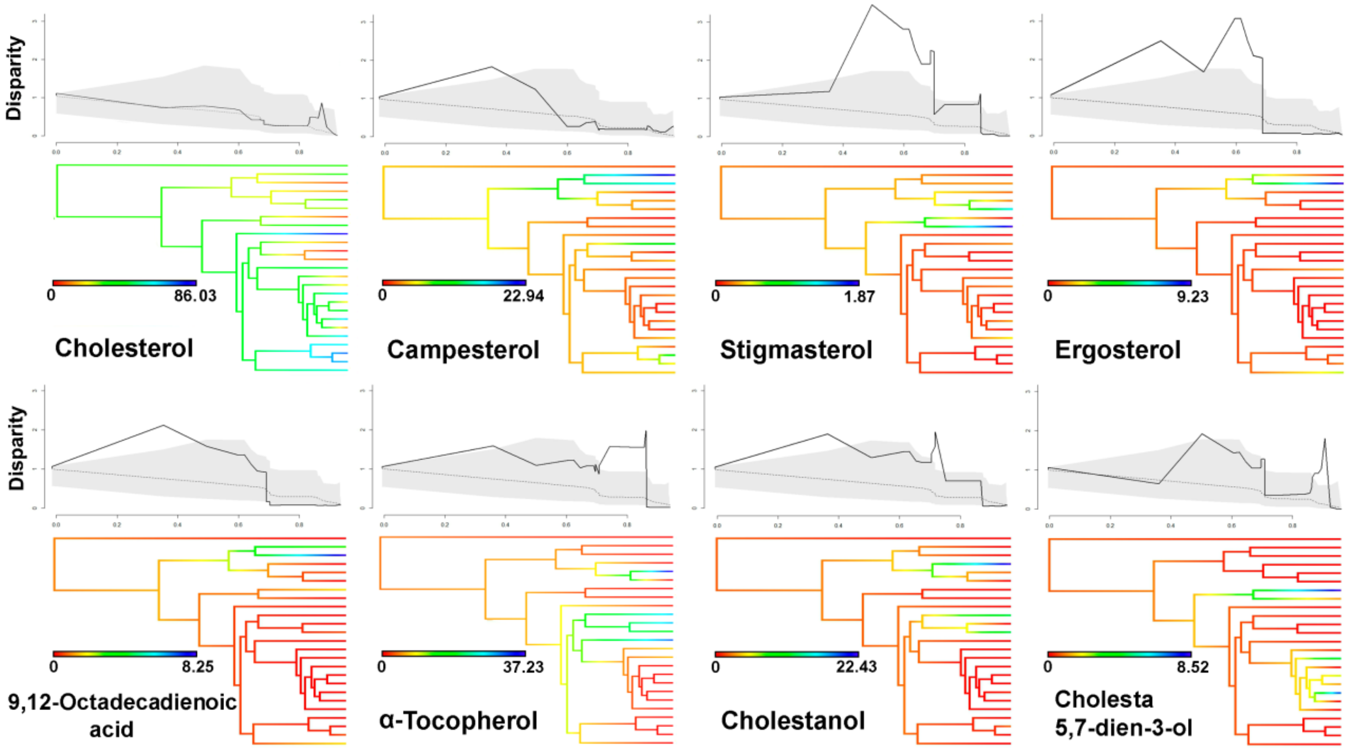

Figure 2: Tempo and mode of evolutionary diversification of proportions of chemical compounds in secretions of lizards. The top plot shows mean subclade disparity through time (DTT) showing proportion of time from taxon origin to present (X-axis) for lizards chemical compounds (lower solid line) compared with the median subclade DTT of phenotypic evolution under a Brownian motion model (dashed line). The grey band shows the 95% DTT range for the simulated data. Model is based on 10,000 simulations. The phylogenetic tree shows a maximum-likelihood ancestral trait reconstruction of each compound across phylogeny

Discussion

Our study provides the first analysis investigating the phylogenetic macro-evolutionary diversification dynamics of chemical signals employed by lizards during sexual and social communication. Our results reveal a clear pattern of heterogeneous tempo and mode of evolutionary diversification among different compounds within each species' chemosignals, and across species. That is, we show that the chemical compounds might follow a 'mosaic' (or ‘modular’) mode of evolutionary diversification where changes in some chemicals do not necessarily influence the others in coordination.

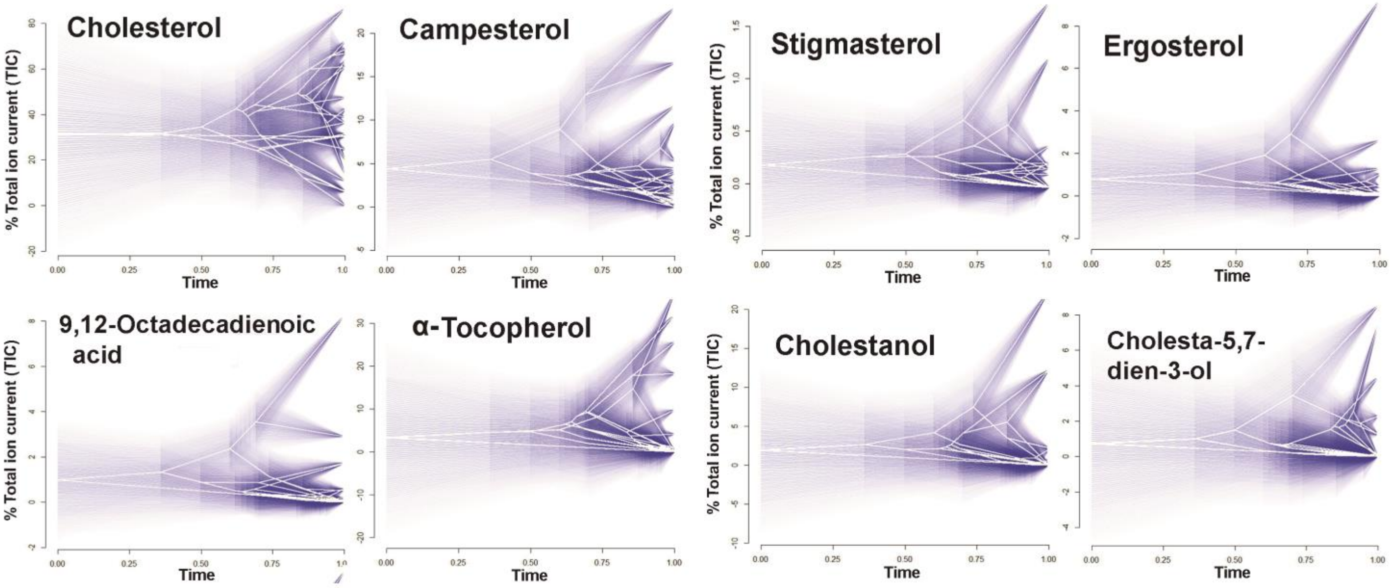

Figure 3: Chemical compounds evolution in lizards. The graph provides a morphospace projection of each chemical defined by relative time since the origin clade to present (X-axis) and compound proportions (Y-axis), which state has been estimated using likelihood approach. The degree of uncertainty is indicated by increasing transparency of the plotted blue lines around the point estimates with the entire range showing the 95 % confidence interval

Consequently, our findings have two major implications. Firstly, given that both the presence/absence, as well as the relative abundance of some compounds might diversify independent from the other components of the scents, we suggest that chemical signals could embody a complex network of elements with potentially high and dynamic evolutionary lability given the weak degree of “chemical correlation” observed among them. And therefore, second, we suggest that selection is likely to have shaped the overall conformation of the chemical scents by exerting asymmetric effects on each chemical compound, thus promoting asymmetric rates of diversification that make this complex mosaic pattern emerge. The effect of selection on compounds is expected to be associated with the functional or structural role that each of them play in signal efficiency in different environments (e.g., social, ecological or climatic) (Martín & López, 2015; Baeckens *et al.*, 2016b). Indeed, our ancestral reconstruction analyses reveal that multiple episodes of phenotypic shifts have occurred during different periods along the phylogeny (figure 2). Interestingly, our analyses studying different models of evolution show that the two major compounds, cholesterol and α-tocopherol, both of which have been assigned structural properties (Weldon *et al.*, 2008; Martín & López, 2014), were found to have been shaped by stabilizing selection (OU model). The evolutionary pattern of cholesterol proportions revealed by ancestral reconstruction analyses shows episodes in which some species experienced changes towards reduced proportions or even total disappearance of the compound. Given its structural function, the diversification of the relative abundance of cholesterol in chemical secretions might be subject to selective pressures exerted by environment. Intriguingly, the evolution of cholesterol seems to follow an inverted pattern with respect to α-tocopherol in some *Lacerta sensu lato* species (e.g., genus *Lacerta*, *Timon* and *Zootoca*) (figure 2). However, despite our results revealing heterogeneous trajectories of diversification across compounds and across species, we also observed that, as it would be expected, some of the compounds show a degree of coordinated evolution, revealing patterns of parallel evolution across lineages. This fact would be especially expected in components such as cholesterol, cholesta-5,7-dien-3-ol, ergosterol and α-tocopherol, given that their relative proportions in the scents are mediated by physiological trade-offs arising from the high costs involved in their production (Martín & López, 2006a, 2007; Kopena *et al.*, 2011; Martín & López, 2012; Martín & López, 2015). Therefore, physiological costs to allocate high abundances of some compounds to secretions could influence the allocation of high amounts of other chemicals and thus, leading to the emergence of the abovementioned trade-offs as the basis for some form of 'chemical conflict' among compounds.

Likewise, our model-selection analyses based on the DTT simulations reveal that the patterns and rates of evolutionary diversification among compounds differ substantially across species (figure 2 and 3). These finding lead us again to reinforce the hypothesis that the chemical network which all compounds are part of is evolutionarily labile given that different factors (i.e., different selection pressures) can target different compounds rather independently to shape the optimal relative proportion of the chemical components needed to make the signal efficient and as cost-effective as possible in each different environment. For example, multiples evidences have shown that chemical signal composition might vary in according to different climatic conditions where lizards inhabit as an adaptive response to maximize the efficiency of chemical signals (Escobar *et al.*, 2003; Martín *et al.*, 2013; Martín *et al.*, 2015). Likewise, it has been shown that the relative abundance of some compounds, such as cholesterol and α-tocopherol, can experience adaptive variations across species of lizards as a function of variation in the climatic conditions they are exposed to (Gabirot *et al.*, 2012). However, not only structural compounds play key roles in the efficiency of signal production and delivery in lizard. Some steroids (e.g., cholesterol, campesterol, stigmasterol, and cholestanol), as well as α-tocopherol and fatty acids (e.g., 9,12-Octadecadienoic acid) have been associated with lizard health conditions (Weldon *et al.*, 2008; Martín & López, 2014; Martín & López, 2015). Additionally, steroids that act as vitamin precursors (e.g., ergosterol of vitamin D_2 and cholesta-5,7-dien-3-ol of vitamin D_3) are also believed to play important roles in signalling the health condition of the sender, mostly males (Martín & López, 2015). Thus, these compounds that provide information about "quality" of the signaller have increasingly been suggested to generate variance in the chances of getting access to sexual mates among males during both male-male interactions (Martín *et al.*, 2007) and female mate choice (Martín & López, 2000, 2006a). Therefore, as suggested by previous studies (Symonds & Elgar, 2008), the combination between the facts that chemical compounds have a tendency to diversify independently from each other, that climatic factors can influence their adaptation, and the crucial roles that many of the components play in fitness-linked activities, such as competition over mates, reinforce our view that chemical signals are potentially highly evolutionarily label. Collectively, the findings presented in this paper combined with previous research investigating the signalling roles of scents, provides a series of lines of evidence highlighting the importance in increasing the impetus in investigating chemical signals not only in the traditional context of behavioural ecology, but also under a macro-evolutionary perspective.

Previous studies have shown the key role of animal signals during species diversification, which can operate as drivers influencing diversification, thus playing roles during the causes and the consequences of their evolution (Maynard Smith & Harper, 2003). Our study shows different evolutionary patterns in relevant compounds found in sexual chemical signals. To date, the evolutionary trajectories of the presence and abundance of these compounds in chemical signals have remained fundamentally neglected, and thus, our study provides a starting baseline to highlight the need to continue with studies of a similar nature, but replicated across other organisms. Ecological pressures responsible for natural selection operating on signal efficiency are likely to influence the abundance of chemical components

Our study is the first to investigate the macro-evolutionary diversification of the chemical signals and their specific components in an explicit comparative context, and thus, we are aware that our results may suffer from limitations. Especially given that we are making general inferences based on a limited number of species from the same clade and with a focus on some compounds chosen based on their known roles during signal production and delivery. However, until now, the numbers of species for which data on the chemical composition of their signals is available, as well as the compounds whose functionality has been studied, is highly limited and therefore, a rather intrinsic limitation for this type of studies. Further research with larger numbers of species and compounds is therefore an important need to expand our understanding of the evolution of this dimension of animal communication, especially in lineages like lizards, in which chemical signals have been suggested to replace and eclipse the role of quantitative traits that operate as efficient signals in other lineages. Despite the limitations of this study, our findings provide a first and replicated evolutionary overview that should be considered in developing future evolutionary and ecological hypotheses centred on chemical communication.

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Table S1: Relative amount of eight lipophilic compounds found in male's femoral secretions of 25 lacertoid species

Species	Cholesterol	Campesterol	Stigmasterol	Ergosterol	9,12-Octadecadienoic acid	α-Tocopherol	Cholestanol	Cholesta-5,7-dien-3-ol	Reference
Blanus cinereus	30.49	1.42	0.00	0.00	0.00	0.00	0.00	0.00	López and Martín 2005c
Psammodromus hispanicus	31.50	22.94	0.00	0.00	2.90	0.00	0.00	0.00	López and Martín 2009
Psammodromus algirus	6.02	16.57	0.24	9.23	8.25	0.17	0.00	0.04	Martín and López 2006a
Gallotia stehlini	11.54	0.00	0.18	0.00	0.00	0.00	22.43	0.00	*
Gallotia galloti	31.00	2.91	0.20	0.05	0.50	37.20	2.21	0.00	García-Roa *et al*. Submitted
Gallotia simonyi	24.94	2.41	1.31	0.00	0.00	0.00	0.00	0.00	Martín *et al*. 2015
Acanthodactylus erythrurus	6.00	0.06	0.00	0.00	1.73	0.04	0.00	8.52	López and Martín 2005a
Acanthodactylus boskianus	30.78	1.58	1.87	0.00	0.00	0.00	0.00	1.26	Khannoon *et al*. 2011
Zootoca vivipara	86.03	0.78	0.00	0.00	0.00	1.16	0.00	0.00	Gabirot *et al*. 2008
Timon lepidus	10.79	11.65	0.23	0.00	0.00	27.90	12.30	0.00	Martín and López 2010
Lacerta viridis	0.00	0.00	0.00	0.00	0.00	25.63	0.00	0.00	Kopena *et al*. 2009
Lacerta schreiberi	6.43	3.90	0.00	0.00	0.56	18.74	12.30	0.00	López and Martín 2006
Scelarcis perspicillata	47.42	0.50	0.00	1.29	0.00	32.08	0.00	0.05	*
Podarcis gaigeae	14.50	2.39	0.13	0.49	0.07	7.44	0.00	0.17	Runemark *et al*. 2011
Podarcis muralis	18.55	0.60	0.19	0.13	0.12	6.40	0.00	4.49	Martín et al. 2008; Pellitteri-Rosa *et al*. 2014
Podarcis liolepis	63.38	2.66	0.00	0.09	0.18	0.00	0.00	0.00	Gabirot *et al*. 2010
Podarcis carbonelli	20.20	0.38	0.00	0.00	0.01	0.00	0.00	2.27	García-Roa *et al*. 2016
Podarcis bocagei	42.36	0.00	0.00	0.00	0.00	0.00	0.00	1.26	García-Roa *et al*. 2016
Podarcis hispanicus	64.36	2.71	0.25	0.01	0.07	0.00	0.56	7.51	Martín and López 2006b; Gabirot *et al*. 2010; Gabirot *et al*. 2012
Podarcis vaucheri	13.43	0.00	0.00	0.00	0.00	0.04	0.00	0.00	*
Podarcis lilfordi	64.01	4.24	0.42	0.00	0.01	0.00	0.00	0.00	Martín *et al*. 2013
Iberolacerta galani	61.95	1.80	0.00	0.13	0.00	0.00	0.00	0.00	*
Iberolacerta monticola	73.64	9.68	0.01	0.43	0.00	0.00	2.02	0.00	López *et al*. 2009 and unpublished data*
Iberolacerta cyreni	67.01	5.33	0.00	1.02	0.58	0.00	0.00	0.14	López and Martín 2005b
Algyroides marchi	59.07	4.48	0.00	2.62	1.72	0.00	0.00	0.00	*

The relative amount of each component is determined as the percent of the total ion current (TIC) and reported as the average. (*) Species with no previous description of the eight chemical compounds. Samples are from adult males of the species *Gallotia stehlini* (27°44' N, 15°35' W; n=6), *Scelarcis perspicillata* (40°00' N, 03°52' E; n=5), *Podarcis vaucheri* (35°10’ N, 2°25’ W; n=8), *Iberolacerta galani* (42°24’ N, 6°24’ W; n=10), *I. monticola* (43°19’ N, 8°15 W; n=13) and *Algyroides marchi* (38°20’N, 1°38’ W; n=6).

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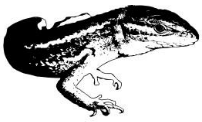
CHAPTER VII

THE NEGLECTED SCENT OF ANIMAL COMMUNICATION: A GLOBAL ANALYSIS OF THE DIVERSIFICATION OF GLANDS FOR CHEMICAL SIGNALING IN SQUAMATE REPTILES

CAPÍTULO VII

EL OLVIDADO OLOR DE LA COMUNICACIÓN ANIMAL: ANÁLISIS GLOBAL DE LA DIVERSIFICACIÓN DE GLÁNDULAS UTILIZADAS PARA LA SEÑALIZACIÓN QUÍMICA EN SQUAMATA



THE NEGLECTED SCENT OF ANIMAL COMMUNICATION: A GLOBAL ANALYSIS OF THE DIVERSIFICATION OF GLANDS FOR CHEMICAL SIGNALING IN SQUAMATE REPTILES

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ABSTRACT

Chemical communication is a crucial mode of social, sexual and ecological interactions among modern reptiles. However, how do traits associated with type of signaling evolve at a broad-scale and comparative level remains fundamentally unknown. In fact, most research investigating diversification and evolutionary shifts in traits used for chemical communication has mostly focused on arthropods, while vertebrates remain essentially neglected. Using a global dataset covering over 80% (7,905 species) of the world's diversity of lizards and snakes (squamates), we investigate the tempo, mode and phylogenetic patterns of diversification and distribution of the follicular glands (FG) used by these organisms to communicate chemically. We assembled a global-scale dataset on the number and location of FG for 7,905 species of squamates from the literature, and from field and museum specimens. Using a molecular phylogeny for 3533 squamates, we performed macroevolutionary phylogenetic model-selection analyses to investigate the diversification of these glands across the squamates world's diversity and separately for major lineages. FG are present in 13.66% of all squamates. Of these, 47.82% of species have precloacal glands, 36.49% femoral glands, and 15.69% have both. Gland location is strongly phylogenetically-determined, with the crown ancestor lacking them. Analyses identified an Ornstein-Uhlenbeck model as the best approximation describing gland diversification. Analyses also reveal consistently high rates of gland diversification. We provide the very first global-scale, comprehensive study investigating diversification in one of the major components of reptile communication. While an effect of phylogeny on trait expression has been identified, the vast diversification in FG suggests active episodes of evolutionary change and repeated patters of origin of numbers and locations of them along the squamate tree of life.

Key words: macroevolution, reptiles, chemical ecology, chemosignalling, convergence, epidermal glands

Introduction

The evolution of systems for communication in animals plays a fundamental role in shaping ecological, social and sexual interactions both within and among species (Smith, 2013). Therefore, the efficiency in the adaptive evolution of communication systems is underlain by complex interactions between natural and sexual selection operating simultaneously (Seddon *et al.*, 2013). Animals have developed a broad range of signaling modes (mostly visual, acoustic and chemical) (Bro-Jørgensen, 2010; Cornwallis & Uller, 2010). In lizards, for example, while some species exhibit striking-colored ornaments, such as the classical dewlap in *Anolis* (Ng *et al.*, 2013), others such as geckos have evolved the ability to produce sounds during social interactions (Yu *et al.*, 2011). However, despite the diversity of communication systems, most of research has traditionally centred around visual and acoustic signals (Bradbury & Vehrencamp, 2011; Smith, 2013). Chemical communication, in contrast, has only recently seen a rather steep increase in the interest in understanding how interactions via pheromones can underlie and strongly influence naturally- and sexually-selected animal interactions (Wyatt, 2010, 2014).

Following accelerated advances in the development of methodologies employed to analyze chemical signals (Touhara, 2013), the field of chemical ecology has rapidly thrived in recent years, consolidating as a central perspective to elucidate drivers behind population and community dynamics (Wyatt, 2014; Apps *et al.*, 2015). For instance, a number of studies conducted in invertebrates and vertebrates have revealed that the behavioural basis of multiple interactions dependent primarily on chemical signals (Weldon *et al.*, 2008; Johnston & del Barco-Trillo, 2009; Wyatt, 2014; Apps *et al.*, 2015). Importantly fact, one of the central contributions of research on chemical communication for understanding social dynamics among vertebrates is the realization that intra- and inter-sexually selected interactions in several reptiles importantly rely on chemical scents. In fact, despite the widespread evidence for female mate choice as a driver of fitness skews across the animal kingdom (Andersson, 1994), it has proven challenging to demonstrate its role during sexual selection in lizards (Olsson *et al.*, 1998) . Interestingly, in recent years it has been suggested that female choice does in fact operate on lizards, but primarily via chemical signals (Martín & López, 2000; López & Martín, 2005; Martín & López, 2006b). In addition, studies conducted in squamates have revealed the key role that chemical scents have in multiple social and sexual interactions in general (Houck, 2009; Mason & Parker, 2010). For example, territoriality (Carazo *et al.*, 2008), social recognition (Labra & Niemeyer, 1999) and conspecific assessment (Martín & López, 2006a; Carazo *et al.*, 2007), are mediated by chemical signals in multiple species.

Reptiles employ multiple ways to secrete chemical scents, such as the skin and feces (Weldon *et al.*, 2008), yet, the better-known source of chemical signals used by these organisms is follicular epidermal glands (FG) (Mason & Parker, 2010; Martín & López, 2014; Mayerl *et al.*, 2015). These glands are specialized tubular structures embedded in the dermis that protrude waxy secretions outside the body through epidermal pores, which have historically been used as an equivalent of the FG, since each gland is directly connected to one pore (Valdecantos *et al.*, 2014; Mayerl *et al.*, 2015). Although the numbers and locations of these FG have been linked to phylogenetic structure (Pincheira-Donoso *et al.*, 2008), they have evolved an astonishing range of variation across living squamates (Mayerl *et al.*, 2015). While the numbers of these FG vary from one to ~130, their location can range from the cloacae (precloacal), the ventral surface of the thighs (femoral) or both (Mayerl *et al.*, 2015). In a large diversity of species, FG are lacking. Although the drivers and patterns of evolution of FG remain fundamentally unknown, it has been hypothesized that the wide interspecific diversity of FG could be the result of selective mechanisms to increase the efficacy of the chemical signaling (Pincheira-Donoso *et al.*, 2008; Valdecantos *et al.*, 2014; Baeckens *et al.*, 2015). Given the investment associated to the development and functionality of these structures, selective pressures (e.g., allocation of energy, environmental factors) could shape adaptive variation of FG. On the other hand, a comparative study conducted in the hyper-diverse lizard genus *Liolaemus* revealed that, despite previous evidence suggesting an effect of natural selection emerging from climatic factors (Escobar *et al.*, 2001), the primary factor explaining patterns of FG numbers across lineages was shared ancestry (P-D et al 2008). Likewise, another study on lacertid lizards revealed a marginal environmental effect on FG number, although phylogenetic inertia remains the major factor (Baeckens *et al.*, 2015). However, and despite these phylogenetic effects, FG have extensively evolved along the squamate phylogenetic history. The lack of large-scale comparative studies investigating the actual rates and trajectories of FG during these reptiles phylogenetic history therefore remains a major pending challenge.

In this paper, we employ a global-scale dataset to investigate the tempo and mode of evolutionary diversification of FG across the Squamata tree of life. Using a 3533 species squamate phylogeny, we interrogate the competing hypotheses that the pace and degree of diversification of FG are limited and strongly constrained by phylogeny, and that FG have undergone active evolutionary patterns of diversification along the squamates history, which can be described by deterministic (rather than random walk) models of proliferation. To achieve this aim, we implement a model-selection approach based on our global-scale dataset, which therefore allows the very first comprehensive test of these hypotheses at the level of a representative proportion of the squamates global diversity.

Material and Methods

Data Collection

We assembled a global dataset on presence, number and location of FG for 7904 species of Squamata (amphisbaenians, lizards and snakes) from the literature. These data cover 94% of all squamate families and over 80% of all species (see Table S1). To guarantee a comprehensive account of the phylogenetic distribution of the variation of these glands, and to inform the phylogenetic models about where the trait exist and where they have been lost, our data include species with and without them. Likewise, given that females of many species lack FG, we focused on males only. For each species, we obtained the mean pore number (i.e. pores on both left and right thigh) calculated from the average of multiple samples or as the midpoint between the minimum and maximum number of pores. Numbers of average pores per species can vary among references – in these cases we averaged data provided by all published sources for a. FG were classified based on their location as 'precloacal' (pores located on the edge of the cloacae), 'femoral' (pores on the ventral surface of the thighs), both (when a continuous row of pores expands from one hind limb to the other through the cloacae area), or neither (pores are absent).

Comparative analyses

We based all phylogenetic analyses in a recent molecular tree for global Squamata presented by Pyron et al. (2013). Once data on FG were collected, the resulting tree has 3,533 of the 7,095 species for which FG data were available.

To assess evolutionary patterns in the number and location of FG in squamates, we first tested if the average number of FG differs depending on their anatomical location, using phylogenetic analyses of variance ('phylANOVA'). We then examined potential differences in FG number among the four main taxa where FG are known to be present (i.e., Gekkota, Iguania, Lacertoidea and Scincoidea) based on traditional GLMs. Dibamidae was not taking into account since this small family of squamates had only one species with FG. Pairwise comparisons were based on Tukey's HSD tests in all cases (Sokal & Rohlf, 1995). All statistical tests were performed using R 3.2.2 and SPSS 20.0.0. Software.

Phylogenetic signal and ancestral state

We estimated the phylogenetic signal of FG number and location in all squamates. Generally, phylogenetic signal is recognized to be the tendency of related species to resemble one another for a specific trait, and Pagel's λ and Blomberg K are two quantitative measure of this pattern (Pagel, 1999; Blomberg *et al.*, 2003a). A λ value close to 0 indicates no phylogenetic structure in the trait, whereas a λ value close to 1 corresponds with the original, untransformed branch lengths (Pagel, 1999). A K value lower than 1 implies that relatives resemble each other less than expected under Brownian motion evolution along the hypothesized tree, whereas K > 1 implies that closely related species are more similar than expected under Brownian motion evolution. The signal of the continuous variables was assessed using Pagel's λ and Blomberg's K (nsim=1000) and the 'phylosig' function in the 'phytools' package (Revell, 2012). Discrete variables were calculated only using Pagel's λ and the 'fitDiscrete function' in the 'geiger' package (Harmon *et al.*, 2008).

Additionally, we used ancestral character state reconstructions ('ace' function) to assess estimations of ancestral states of FG location at the root of the Squamata tree, and on specific internal nodes of interests (i.e. Sauria, Gekkota, Iguania, Lacertoidea and Scincoidea). Moreover, based on Revell and Freckleton (2013), we conducted ancestral reconstruction analyses to visualize the evolution of FG number along branches of the phylogenic tree. These analyses use a Maximum-Likelihood approach to reconstruct ancestors states (Felsenstein, 1985; Revell & Freckleton, 2013).

Modelling evolution of FG

To examine the diversification of FG number across Squamata we conducted two approaches aimed to elucidate both global and lineage-specific macro-evolutionary diversification dynamics. Thus, we firstly analyzed the whole global dataset and then, we focus on the three lizard lineages with the largest number of species with FG in our dataset (i.e., Gekkota, Iguania and Lacertoidea). We fitted four alternative evolutionary models that describe different regimes of phenotypic evolution: i) Brownian-motion model (BM) describes a phenotypic random walk dynamic across phylogeny (Hernández *et al.*, 2013;

Pan *et al.*, 2014) in which the variance of the trait is centered around the initial value at the root of the tree, increasing in proportion to the distance from the root; ii) Ornstein-Uhlenbeck model (OU) that is referred as the "stabilizing selection" and fits the evolutionary dynamic assuming traits are pulled to adaptive optimum values (Hansen, 1997); iii) Early-Burst model (EB) or "accelerating-decelerating model", which assumes that species evolve under a density-dependent availability of niche space, predicting rapid early diversification followed by decreases in evolutionary rates as a result of saturation of niche space over time (Blomberg *et al.*, 2003b; Harmon *et al.*, 2010; Paleo-López *et al.*, 2016); iv) Delta model describes the impact that different modes (early versus late) of evolution in the tree have in the rates of trait evolution. Hence, the model return a $\delta>1$ or $\delta<1$ when recent evolution has occurred fast or slow, respectively (Pagel, 1999; Pincheira-Donoso *et al.*, 2015). We perform the comparisons of goodness of fit for these four models based on Akaike Information Criterion (AIC) (Akaike, 1974, 1998). We use the bias-corrected version of AIC (AICc) to determine the ΔAICc values, which result from the difference between the lowest AICc and the AICc of each alternative model. Therefore, the best-fit model has ΔAICc=0 (Burnham & Anderson, 2002; Pincheira-Donoso *et al.*, 2015). All these analyses were implemented using 'geiger' package in R (Harmon *et al.*, 2008).

We further investigated the disparity of the FG number over time. We performed disparity-through-time analyses (DTT) which plots fluctuations in average of relative disparity over time (Harmon *et al.*, 2003; Frédérich *et al.*, 2013; Ingram, 2015). This analysis calculates the mean disparity of FG number to subsequently, compare it with the disparity expected under a null model of BM estimated by means of 10000 simulations. Finally, the average of these two values (i.e. FG number disparity from dataset and simulated data from BM) are plotted against node age to obtain the morphological disparity index (MDI) (Harmon *et al.*, 2003; Astudillo-Clavijo *et al.*, 2015; Pincheira-Donoso *et al.*, 2015), which quantify the differences on the trait disparity among and within subclades by comparing with the expectation under null hypothesis of BM model.

Thus, values below zero (i.e., those values lower than expected under BM model) describe that most of disparity is among subclades, which are distributed in smaller and isolated morphospace regions. Instead, MDI values above zero mean that disparity among subclades is highly overlapped in the morphospace (Pincheira-Donoso *et al.*, 2015). We carried out DTT analyses using R package 'geiger' (Harmon *et al.*, 2008). We finally built a "traitgram" plot to project the Squamata phylogeny onto the FG number morphospace over time since the root origin. The resulting projection is based on ancestral node estimations using maximum likelihood approaches (Ruta *et al.*, 2006). The analyses was performed using the R package 'phytools' (Revell, 2012).

Results

Diversity of follicular epidermal glands

We observed that FG are present in 13.66% of the 7,905 species in our dataset. Excluding snakes (which entirely lack FG), a total of 24.8% of the global squamates have FG. These proportions vary across clades, being present in 35.2% Gekkota species, 26.82% of Iguania and in 96.8% of Lacertoidea. In contrast, lower proportions of species with FG were found in Dibamidae (14.28%) and Scincoidae (1.11%) (figure 1 and Table 1). FG are entirely lacking in the clade Anguimorpha (173 species). *Mniarogekko chaoua* was the squamate species with the highest mean number of FG (95), while multiple species of different subclades presented only 1 or 2 FG (Table 1; request supplemental table). Regarding the phylogenetic distribution of the location of FG, we found that the 47.82 % of species have precloacal FG, 36.49 % femoral FG and 15.69 % both types.

Analyses conducted on the variation of FG across major squamates lineages (i.e, Gekkota, Iguania, Lacertoidea and Scincoidea) reveal that these structures differ significantly among groups (ANOVA, $F_{3,1074} = 21.68$, $P < 0.001$). Subsequent pairwise comparisons between clades showed that differences were significant between Iguania

Table 1: Summary of information about the presence and number of follicular epidermal glands (FG) in squamates. The number of FG is reported as the average (±SE) for Squamata clade and the lineages where the presence of FG has been described

	Squamates (n=7904)	Gekkota (n=841)	Iguania (n=1264)	Lacertoidea (n=437)	Scincoidea (n=1619)	Dibamidae (n=7)
Number of FG	19.53 ± 0.49	21.05 ± 1.05	13.92 ± 0.74	28.86 ± 0.75	22.72 ± 2.43	4
Number of precloacal FG	8.51 ± 0.45	13.57 ± 1.13	6.64 ± 0.35	3.89 ± 0.18	0	4
Number of femoral FG	29.98 ± 0.61	25.68 ± 3.87	32.74 ± 1.24	29.83 ± 0.73	22.72 ± 2.43	0
Number of both-location FG	28.81 ± 1.28	33.22 ± 1.61	23.84 ± 2.64	21.22 ± 2.27	0	0
Species with FG	1077	296	339	423	18	1
Species with precloacal FG	515	178	238	97	0	0
Species with femoral FG	392	14	84	276	18	0
Species with both-location FG	170	104	16	50	0	0

and Lacertoidea (*Tukey's* test: $P < 0.001$), but not between Iguania and Gekkota ($P = 0.46$), and Iguania and Scincoidea ($P = 0.09$). Likewise, no differences were found between Gekkota and Lacertoidea ($P = 0.46$), Gekkota and Scincoidea ($P = 0.97$), nor Lacertoidea and Scincoidea ($P = 0.99$).

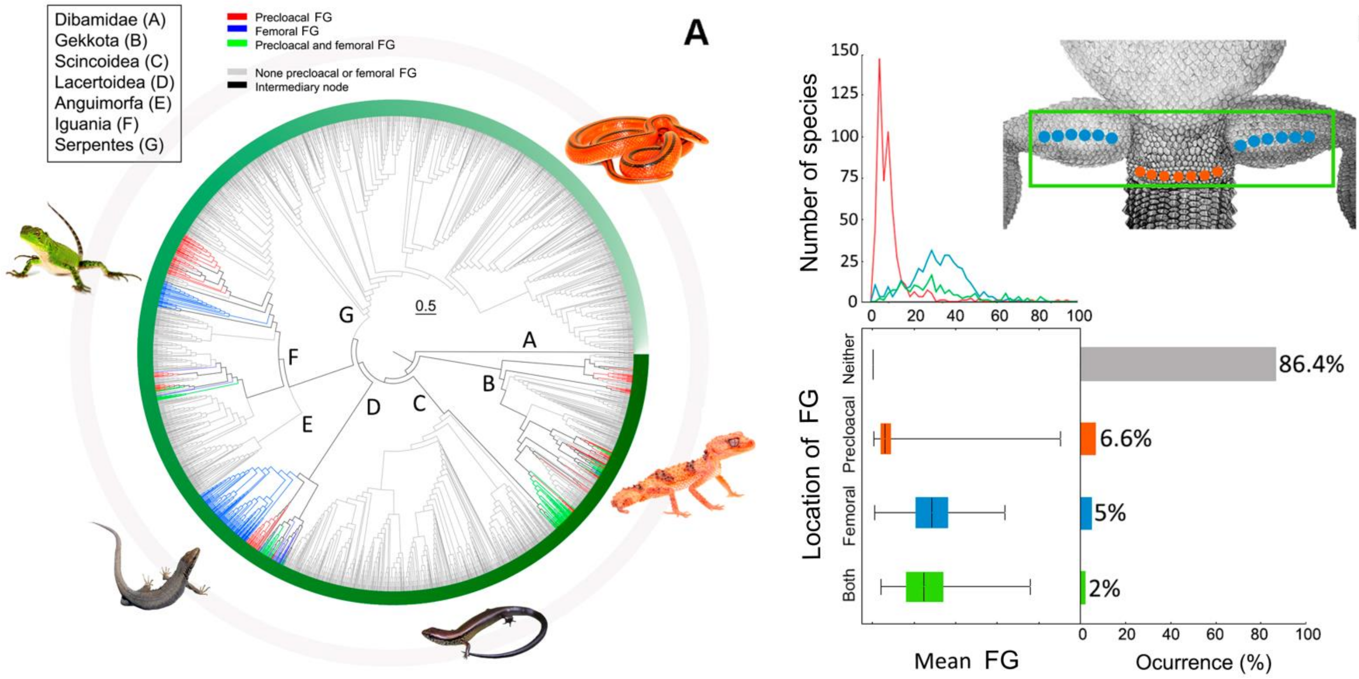

Figure 1: Overall distribution of number and location of follicular epidermal gland (FG) in Squamata. (A) Phylogenetic view of the FG number across Squamata phylogeny. Color branches show the absence or presence of FG, and their anatomical location (red: precloacal FG, blue: femoral FG, green: continue row of FG from femoral to precloacal region, grey: neither FG, black: intermediate branches). Surrounding green band shows ancestry direction. Sample sizes: 3533 species (B) Statistical summary of presence, number and location of FG in squamates. Top draw shows different location of FG. Left top graph provides frequency of the number of FG (mean; X-axis) in number of species (Y-axis) for each location. Box-plot graph illustrate different locations of FG (whiskers show max-min values). Histogram represents the percentages of each FG location. In all cases color defines location of FG (red, precloacal FG; blue, femoral pores; green, continue row of FG from femoral to precloacal region). Photos: Roberto García-Roa (*Iberoacerta bonnali* and *Sphenomorphus cherriei*), James D. Emerson (*Oreocryptophis porphyraceus* and *Nephrurus wheeleri*) and Santiago Ron (*Iguana iguana*)

A phylogenetic ANOVA performed to quantify whether the number of mean FG differs significantly depending on their anatomic location on a species' body revealed predictable differences among them (phylANOVA; $F_{2,605} = 172.58$ $P = 0.016$). Species with precloacal FG have on average a lower number of these glands (mean=8.51) than species with femoral FG (mean=29.98; P = 0.024), or with both combined (mean=28.81; P = 0.034). Finally, No significant difference was found in the number of FG between species with femoral FG or with both combined (P = 0.766).

Phylogenetic signal and ancestral state

Overall, the number of FG showed a moderate phylogenetic signal in Squamata, with a high λ and intermediate K (Table 2). A qualitatively similar extent of phylogenetic signal was observed in the subclades Gekkota and Lacertoidea. In contrast, Iguania and especially Scincoidea showed very high K values, meaning that species from those clades resemble each other more in their mean number of FG than expected under Brownian motion of evolution. All squamates, and lizards in particular, showed a high phylogenetic signal in their anatomical FG location (Table 2).

Ancestral reconstructions

Ancestral state reconstruction reveals that the basal ancestor of modern squamates lacked FG (likelihood, or LL, of 99.7%). This can also be said for the ancestral state of lizards (LL = 99.9%), and even for the subclade Gekkota in particular (LL = 99,78%). Deeper into the lizard tree we see a change at the Scincoidea root into a state with a continuous row of FG (LL = 79.2%), and one with femoral FG at the Lacertoidea root (LL = 89.4%). A transition towards ‘no FG’ as ancestral state is apparent at the Iguania root (LL = 87%).

Macro-evolutionary patterns and models

Our model-selection analyses investigating the FG diversification dynamics identified the OU model (i.e., stabilizing selection model suggesting diversification pulled against adaptive peaks) as the best approximation describing the tempo and mode of diversification of these traits in squamates and in Gekkota, Iguania and Lacertoidea

Table 2: Phylogenetical signals (Pagel's λ and Blomberg's K) and results of ancestral state reconstructions of follicular epidermal gland (FG) number and anatomical FG location, calculated for all squamates, and for lizards, and lizard subclades separately

	FG number				**Anatomical FG location**						
	Phylogenetic signal				Phylogenetic signal	Ancestral state reconstruction					
	Blomberg's K	P	Pagel λ	P	Pagel λ	Rate Index Estimate	SD	Scaled likelihoods at the root			
								Absent	Precloacal	Femoral	Both
Squamates	0.539	0.001	0.989	< 0.001	0.999	0.0813	0.0068	0.997	< 0.001	< 0.001	< 0.001
Lizards	0.572	0.001	0.978	< 0.001	0.999	0.1146	0.0096	0.999	< 0.001	< 0.001	< 0.001
Gekkota	0.407	0.001	0.998	< 0.001	0.999	0.1668	0.0218	0.978	0.0210	< 0.001	< 0.001
Scincoidae	8.156	0.001	0.999	< 0.001	0.999	0.0388	0.0195	0.207	0	0	0.792
Lacertoidea	0.44	0.001	0.846	< 0.001	0.999	0.2273	0.0375	0.045	0.053	0.894	0.007
Iguania	1.99	0.001	0.981	< 0.001	0.999	0.106	0.0173	0.87	0.002	0.126	0.003

analysed separately. The Delta, BM and EB models were ranked in decreasing order based on their AIC values after the OU model (Table 3).

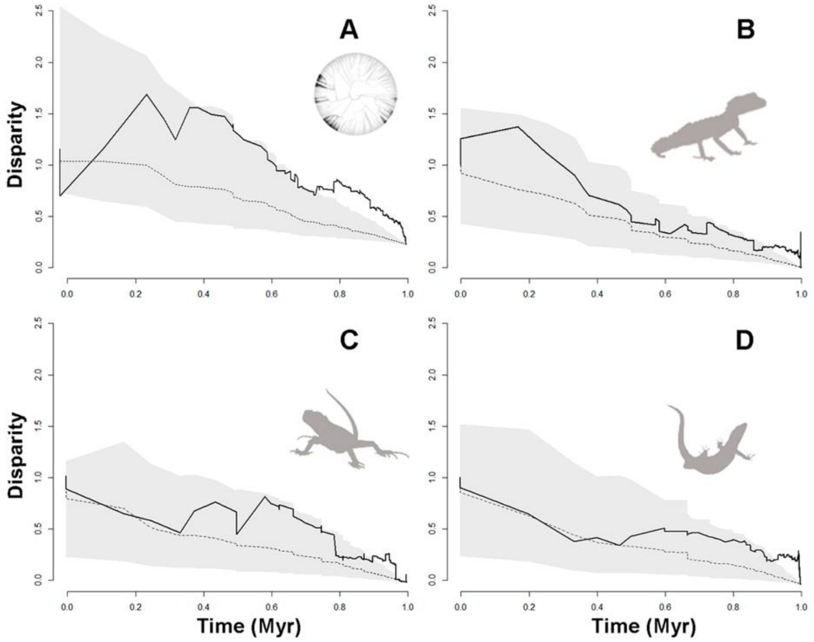

Figure 2: Mean disparity through time (DTT) in Squamata, Gekkota, Iguania and Lacertoidea follicular epidermal gland (FG) number (lower solid line). The solid line show the DTT FG number, while dotted line represents the expected values under Brownian Motion model of evolution based on 10 000 randomizations. The grey band shows the 95% confidence interval of DTT range

Finally, we obtained positive values of the DTT analyses in Squamata (MDI = 0.07; figure 2), indicatingthat rates of subclade-level relative disparity are higher than those simulated under the BM model of evolution. This suggests that the evolution of relative disparity within each individual clade has diversified in overlap on the FG morphospace with the evolution of disparity in other subclades in our phylogeny (figure 3). Higher values than those from BM model were also revealed by DTT analyses performed separately in Gekkota (MDI = 0.23), Iguania (MDI = 0.16) and Lacertoidea (MDI = 0.10). In all three lineages, the number of FG converged and overlapped across lineage’s morphospace, a finding that was particularly noticeable in Iguania and Lacertoidea (figure 4).

Table 3: Rates and modes of the follicular epidermal gland number diversification in overall Squamata, Gekkota, Iguania and Lacertoidea. Data values are based on comparisons of four fitted evolutionary models: Brownian-motion (BM), Ornstein-Uhlenbeck (OU), Early-Burst (EB) and Delta. They were best-fitted based on bias corrected Akaike Information Criteria (AICc)

Lineage	Model	Model parameters	β	LogL	AICc	ΔAICc
Squamata	BM	-	2075.41	-8861.71	17727.43	103.5
	OU	α=2.71	2132.26	-8808.96	17623.93	0
	EB	α=-0.00	2075.48	-8861.71	17729.43	105.5
	Delta	δ=2.99	698.39	-8835.35	17676.71	52.78
Gekkota	BM	-	8636.35	-1776.57	3557.17	58.39
	OU	α=2.71	8835.83	-1746.36	3498.78	0
	EB	α=-0.00	8636.24	-1776.57	3559.2	60.42
	Delta	δ=2.99	2911.82	-1758.08	3522.22	23.44
Iguania	BM	-	1296.99	-1897.94	3799.88	2.52
	OU	α=2.51	1347.21	-1895.66	3797.36	0
	EB	α=-0.00	1296.97	-1897.93	3801.9	4.54
	Delta	δ=2.01	661.82	-1896.04	3798.12	0.76
Lacertoidea	BM	-	9628.89	-1011.86	2027.77	19.35
	OU	α=2.71	9825.2	-1001.16	2008.42	0
	EB	α=-0.00	9628.57	-1011.86	2029.782	21.362
	Delta	δ=2.99	3261.19	-1002.27	2010.64	2.22

Discussion

Our study provides the first global-scale comparative analysis investigating the diversity, phylogenetic distribution and evolutionary diversification of the FG employed by squamate reptiles for chemical communication. Our results reveal that the degree of phylogenetic effect underlying variation in the number of FG is moderate across the squamate phylogeny. Yet, we also observed that the strength of this phylogenetic signal varies among subclades. In addition, evolutionary diversification in FG numbers across lineages is better explained by a stabilizing selection model of evolution (OU) in both

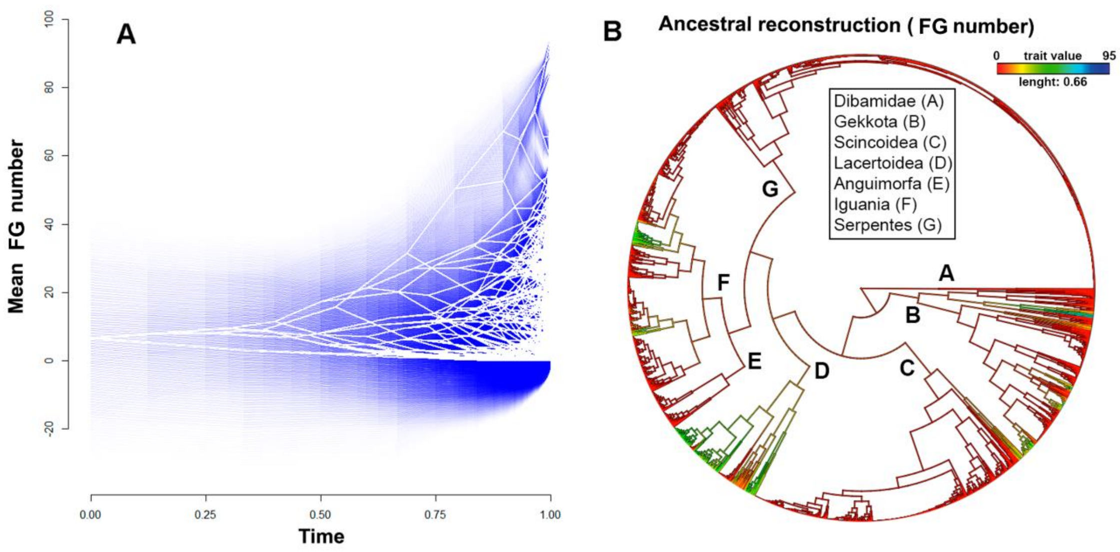

Figure 3: Diversification of follicular epidermal gland (FG) number across squamates. (A) Projection of the Squamata phylogeny into a morphospace. Projection of the Squamata phylogeny into a morphospace defined by relative time since the clades´ origin (X-axes) and FG number (Y-axis). Ancestral FG number is calculated using maximum likelihood. The increase of transparence of blue lines mirrors the degree of statistical uncertainty with 95 % confidence interval. (B) Ancestral character state estimation of FG number across Squamata phylogeny. The phylogenetic tree reveals a maximum-likelihood ancestral character state reconstruction of FG number across squamates

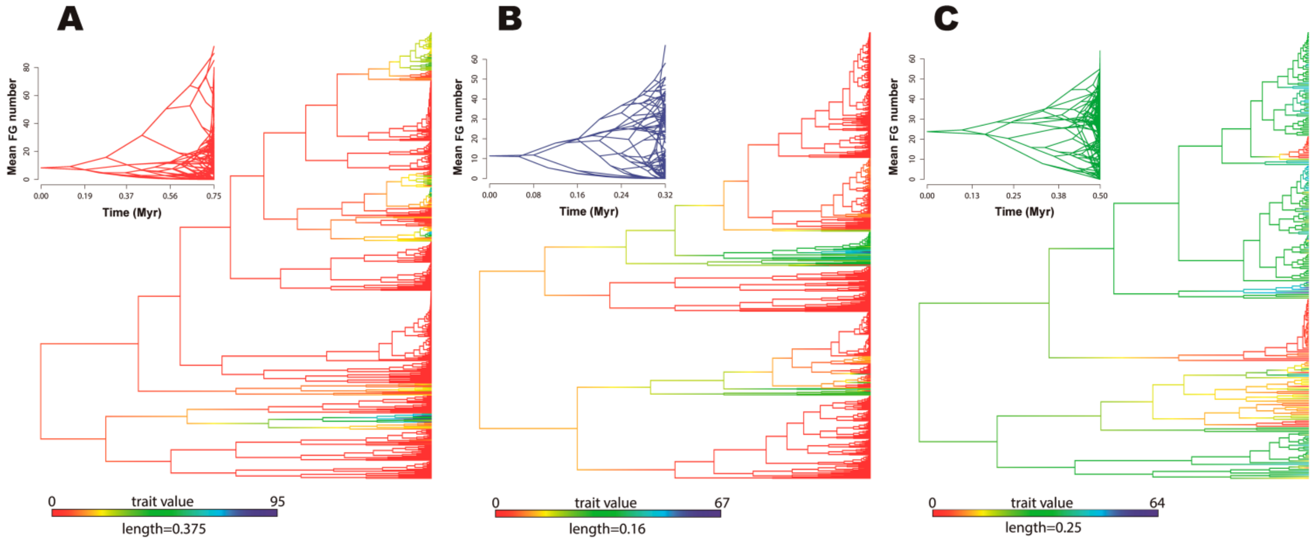

Figure 4: Ancestral character estimations and phenograms of the follicular epidermal gland (FG) in Gekkota, Iguania and Lacertoidea lineages. The phylogenetic trees reveal the maximum-likelihood phylogenetic ancestral character state reconstructions of FG number along the branches and nodes of the three lineages. Top tree of each phylogeny shows the morphospace´s projection defined by the relative time since the clades´ origin (X-axes) and pore number (Y-axis)

squamates in general and in specific lineages in particular. The patterns of FG relative disparity lays above the values expected from a Brownian motion model, which implies a prominent degree of subclade overlap on the ‘FG morphospace’. Finally, our results of ancestral state reconstruction coupled with diversification analyses suggest that despite phylogenetic signal, FG have evolved extensively, following a basal ancestor that was more likely to lack these glands, and from which they emerged and disappeared in repeated evolutionary episodes during the squamate evolutionary history.

Functional role of follicular glands

Femoral and precloacal glands play central roles in sexual and social interactions in squamates. Their relevance has been associated with their function as producers of chemical secretions, which have been shown to be crucial during conspecific and heterospecific interactions inmany species [e.g., see (Weldon *et al.*, 2008; Houck, 2009; Mason & Parker, 2010; Martín & López, 2014), for reviews]. However, the information available about the importance of these glands as pheromone producers comes primarily from lacertids and *Liolaemus* lizards (Pincheira-Donoso *et al.*, 2008; Labra, 2011; Martín & López, 2015), probably given that most species in these lineages have FG (in 96.8% of lacertids, in 92.14% of *Liolaemus*). However, it remains fundamentally unknown how predominant the role of FG is shaping and maintaining social interactions among sexes and species belonging to lineages where these glands are less common and low in numbers (e.g., in Scincoidae or Dibamidae). On the other hand, some hypotheses have suggested the divergence of sensory modes in squamates based on morphological (e.g., tongue shape, level of sensory cells in their vomeronasal organ) and behavioural (number of average tongue-flicks and foraging mode) data (Schwenk, 1993, 1994, 1995; Vidal & Hedges, 2009). Thus, Iguania was proposed as ‘visually-oriented' and the Scleroglossa (all other lineages) as ‘chemically-oriented' (Schwenk, 1993, 1995). In this sense, our results are not in line with this hypothesis, since we could not confirm clear differences in both groups in respect to the percentage of species with FG (Iguania, 26.82% and Scleroglossa, 11.11%). This finding is in agreement with previous evidences that revealed the importance of the chemosensory mode for Iguania species (Vidal & Hedges, 2009; Labra, 2011; Baeckens *et al.*, 2016). Moreover, it is remarkable that some diverse lineages within Scleroglossa (e.g., skinks and snakes) show a scarce or lack percentage of species with FG (1.11% and 0, respectively). In this framework, although the transference of information through FG did not show a clear differentiation between Iguania and Scleroglossa groups, it is important to point out that other sources of chemical signaling (e.g., skin and feces) might be simultaneously acting to convey information during social and sexual interactions.

In addition, FG have received much attention as systematic characters, due to their extraordinary diversity observed among species (Cole, 1966; Baeckens *et al.*, 2015; Mayerl *et al.*, 2015). Thus, although there may be intraspecific variations since females often lack FG, juveniles present immature FG, or the number of pores can vary slightly among individuals, remarkable interspecific differences have been described in the FG number (Pincheira-Donoso *et al.*, 2008; Baeckens *et al.*, 2015), anatomical location (femoral, precloacal or continue rows) (Mayerl *et al.*, 2015), and morphology (lobed, tubular, simple, branched, acinar or tubuloalveolar; see (Valdecantos *et al.*, 2014)). In this context, despite interspecific differences are present in some lineages, our results reveal a phylogenetic conservatism in FG diversification, especially strong in their anatomical location. However, we consider that a global data set with specific data of FG number and location is useful to further classical taxonomic studies in squamates.

Tempo and mode of epidermal glands evolution: Phylogenetic patterns

Our analyses showed that while the location of FG is strongly influenced by shared ancestry, the effect of phylogeny on FG number differs depending on the group. Thus, Scincoidea and Iguania lineages showed a clear conservatism of the FG number. This supports previous results of Pincheira-Donoso *et al.*´s study (2008), where the authors concluded that pore number in *Liolaemus* lizards was determined by shared ancestry, and not by adaptive responses to selective pressures (e.g., climatic factors). In Gekkota and Lacertoidea, however, the phylogenetic signal remains in moderate values. However, the extremely limited number of studies confronting the phylogenetic inertia hypothesis with the adaptive hypothesis of FG evolution demands the need to take existing findings cautiously. In fact, a recent study (Baeckens *et al.*, 2015) conducted on lacertid lizards revealed a moderate effect of phylogenetic ancestry shaping variation in FG, but also, a moderate predictability of occupation of substrate behind numbers of FG across species. Therefore, according to these authors' findings, it remains possible that an adaptive signals underlying FG variation exists, which would at least partly contribute to explain the fact that FG have extensively diversified across squamates, as shown by our analyses. Overall, squamates in general, and lizards in particular, also obtained moderate values of phylogenetic signal in the pore number. In addition, we further identified stabilizing selection as the model that better explained the diversification of FG number within squamates, which is in line with the strong subclade overlap in morphospace also revealed by the DTT analysis (figure 3 and 4). Therefore, and given that ancestry remains as an important factor underlying FG number, results suggest that the number of these glands might have evolved pulled around optimal values in both squamates as a whole and Gekkota, Iguania and Lacertoidea.

Additionally, the absence of FG arises as ancestral state in squamates (and in lizards too), although this differed among lineages. Table 3 shows the absence of FG as ancestral state in Gekkota and Iguania (although not significantly). This latter differs with previous results obtained by Mayerl *et al*. (2015), which may be caused by an increment in the number of species examined in the present study. The ancestral state of FG location in Lacertoidea is femoral; however it was not significant, as was found in previous evidences (Mayerl *et al*. 2015). Interestingly, contrary to Mayerl *et al*. (2015) our results suggest the presence of FG as ancestral state in Scincoidea, although no significantly. These differences in the ancestral origin of FG among lineages, as well as the different effect that ancestry has in some of them, reflect that FG could have appeared simultaneously at different temporal scales across Squamata. In view of the absence of FG is the ancestral state of the clade, the subsequent apparition of these glands might have arisen as a need to communicate by means of an alternative channel. Thus, given the striking diversity of signaling traits among squamates (sounds, colors, movements, chemicals, etc.), the different investment of each species in the chemosensory mode could condition the final expression (i.e., number, location and morphology) of the FG, even maximizing the use of alternative sources of chemical signals as skin or feces, as might occur in snakes, in which FG are absent but their chemical signaling is well known (Weldon *et al.*, 2008; Mason & Parker, 2010). In this sense, multiple evidences revealed the physiological costs associated to the production and maintenance of the chemosensory system in squamates (Houck, 2009; Mason & Parker, 2010; Martín & López, 2015). Therefore, species with multimodal signaling (acoustic, visual or/and chemical), might be more constrained in the production and evolutionary maintaining of these signaling traits than those that base their communication in only one mode of communication. In addition, we also consider important to highlight that FG are closely interrelated with their chemical secretions. Growing evidences describe the extraordinary diversity associated to these secretions, resulting from a wide spectrum of compounds and their relative abundances (Weldon *et al.*, 2008; Runemark *et al.*, 2011; Martín & López, 2014; García-Roa *et al.*, 2016). Therefore, the production and maintenance of chemical signaling structures that improve the efficiency of the chemosensory system do not only depend of the FG (number, locations, morphology, etc.), but also of the specific investment on chemical secretions. The fact that ancestry has considerable influence in FG diversification could lead one to surmise that the adaptive character to environmental conditions of the chemical signaling in squamates, might chiefly reside in the chemical signals and not in the FG.

The diversification of chemical signaling traits is poorly known. Some hypotheses mainly based on pheromones have proposed that progressive or abrupt changes could draw the evolutionary dynamic of chemical signaling traits (Symonds & Elgar, 2008). Our results suggest that both ways of evolution might have operated in the FG. Thus, gradual changes in the number FG can be observed across Squamata lineages (figure 3), especially evident in some geckos, iguanids and lacertids, which is also supported by the remarkable overlap in the FG-number morphospace (figure 4). In addition, "saltational" shifts in FG number are shown in different points of the clade. For example, while most of *Liolaemus* species (176 spp.) are equipped with precloacal FG, 15 species (some of them phylogenetically closer.) lack these glands. This also occurs in multiple lineages of gekkota (e.g., genus *Hemidactylus*, *Gekko*, *Cyrtodactylus*) and Lacertoidea (e.g., amphisbaenids) where the presence of FG is predominant, but some of their species do not present FG. While shared ancestry is revealed as an important factor underlying to overall FG number and location, the reasons behind particular cases (as above mentioned) of abrupt changes within clades are still unclear. Some authors have suggested the hypothesis of "between channel compensation" (Baeckens *et al.*, 2015) in which, given harsh environmental conditions, species might increase investment in complement or alternative signaling channels until in some cases invalidate the original sensory channel (e.g.,., FG) and finally loss the trait. However, further works are required to confirm the validity of this hypothesis based on chemical signaling traits in squamates. The design and development of studies focused on elucidate the evolutionary mechanisms underlying to these saltational changes in epidermal glands of particular lineages of Squamata remain therefore as pending area for future.

Conclusions

Our results show that FG extensively diverge across Squamata phylogeny. This variation is asymmetric, with some lineages having a high frequency of species with them while other lineages entirely lack them. Our analyses show a strong effect of phylogeny, as shown by previous studies. However, we show that this phylogenetic signal is also asymmetrically distributed across lineages as in some it is strong while in other it is not. Therefore, our results suggest that the apparition and loss of this trait have repeteadly occurred at different temporal scales along lineages diversification. We consider that in spite of phylogenetic relationships influence the evolution of these glands, further works must be done to confirm if phylogenetic independent adaptive events might be behind of FG evolution in particular groups of squamates. Moreover, alternatively to number and location of FG, further analyses focusing on the morphology of pores are needed to glimpse the complete FG life-history. Additionally, integrative analyses with chemical secretions profiles might provide a global view about the evolution of chemical signaling in squamates.

Acknowledgements

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DISCUSSION

DISCUSIÓN

Discussion

The present thesis includes seven chapters addressing the chemical communication of lizards from different perspectives. Hence, this thesis has been built under methodological (Chapters I), behavioral and ecological (Chapters II, III, IV and V) and evolutionary approaches (VI and VII). This reflects the great diversity of questions to consider in this field and, moreover, the need to comprehensively address the study of chemical communication in lizards from different points of view.

In this framework, we first provide an alternative methodology to analyze chemical secretions of lizards **(Chapter I)**. Several papers studying femoral and precloacal gland secretions obtained important achievements that improved the understanding of lizards´ chemical communication [see (Mason & Parker, 2010; Martín & López, 2014, 2015]. However, given that some of these methodologies were analytically limited, the establishment of alternative protocols might broaden the range of questions to address. For example, in the context of global warming, a behavioral study revealed that increases of temperature may alter the efficacy of chemical communication in Iberian rock lizards (*Iberolacerta cyreni*) (Martín & López, 2013). Moreover, a subsequent experiment with *Podarcis guadarramae* revealed that male chemical secretions had a lower efficacy (i.e., females respond with lower TF rates) when temperature and dryness increase, especially in highland populations (Martín *et al.*, 2015a). However, chemical approaches detecting compositional changes may be useful to understand how the environment really affects to the chemical signals. This is because the use of methodologies based on proportions of compounds alone to characterize chemical secretions may hinder detection of slight changes in these chemical profiles. In this respect, the proposed methodology is able to detect changes produced by physiological and environmental factors and, moreover, allows the analysis of variations of each compound separately. Thus, methodologies allowing the detection of changes in particular chemicals (even when these compounds are not predominant in chemical signals) under different circumstances might be useful to shed light on the role that these factors play in lizard chemical communication, which in turn is widely unknown to date (Martín & López, 2014). On the other hand, the number of lizard species whose chemosignals have been described is surprisingly low (≈50 until 2016) (Martín & López, 2014; Martín *et al.*, 2015b, 2016). Moreover, the number of studies assessing ecological and evolutionary questions regarding chemical signals is very scarce comparing with visual and acoustic signals, for instance. We do not consider that these facts are caused by a lack of interest of the scientific community in lizards´ chemical signaling, but rather by the inherent complexity of analytical procedures used to characterize chemical signals. Thus, the description of alternative and easy methodologies might be useful to increase the number of researchers interested in working in this field.

Sexual dimorphism in lizards has been described in multiple types of signals, mostly visuals (e.g., horns, colors, movements) (Shine, 1989) (figure 1). However, works studying sexual differences in chemical signaling traits are scarce (Alberts, 1990; Khannoon *et al.*, 2011; Martín *et al.*, 2016). The manuscript presented in **Chapter II** is one of the few studies exploring this issue in lizards. Thus, our work investigating differences in the composition of the chemical secretions in *Liolaemus wiegmanni* male and females illustrates a significant dimorphism between both sexes. This finding is in line with the few previous studies revealing sexual differences in the composition of chemosignal profiles (Alberts, 1990; Khannoon *et al.*, 2011; Martín *et al.*, 2016). In this context, and given that

Figure 1. Different species show striking sexual dimorphism, easily observable as color and anatomical traits. In this picture, it is showed the sexual dimorphism of female (left) and male (right) *Psammodromus algirus* observed in a population closer

Previous evidences have shown that some compounds are associated with particular functions (e.g., dominance, territoriality, sexual attractiveness) [see (Weldon *et al.*, 2008; Martín & López, 2014, 2015)]. Therefore, since the need and the purpose of signaling might vary between sexes, the chemical composition of the signal may consequently also differ. Moreover, on the basis that chemical profiles are hormone-dependent (Martín & López, 2015), the physiological differences during the reproductive season between both sexes could also increase the dimorphism in chemosignals. However, the proportions of the two more predominant types of chemicals (i.e., steroids and waxy esters) did not show overall significant differences between sexes. It is plausible that the use of a small number of individuals could hamper the emergence of some potential differences between sexes. For this reason, further studies increasing the number of individuals and populations, and additional signaling traits (e.g., size, color) are needed to establish a better perspective of the dimorphism in this species. Nevertheless, our results reveal another case of sexual dimorphism in lizard chemical signals, showing that females not only use the chemical communication as a channel of receive information from conspecifics. They can also produce chemosignals to convey information to conspecifics. In this sense, additional works focusing on male and female chemosignals would help us to understand the overall scenario over which lizard populations are organized.

In **Chapter III**, we assessed the potential effects that diet constraints may have on lizard chemical signaling. Our results revealed that restrictions in diet of a particular compound (vitamin E) may affect the physiological trade-offs to the point of changing the immune response of males and the female responses in *Iberolacerta cyreni*. Our evidences suggest that diet constraints might be hindering the expression of a potentially honest sexual chemical signal. In this sense, behavioral experiment performed with *Timon lepidus* (Martín & López, 2010), *Lacerta schreiberi* (Kopena *et al.*, 2014) and *Lacerta viridis* (Kopena *et al.*, 2011) showed the amount of vitamin E in chemical secretions as determinant in female mate choice. Interestingly, in spite of this vitamin had been not previously described in chemical secretions of *I. cyreni*, its abundance in chemical signals might be also crucial in female responses in this species, since females seemed to prefer scent of those males with higher levels of vitamin E in their femoral secretions.

On the other hand, it has been suggested that lizard species are able, or are not, to express certain compounds in their chemical secretions (Weldon *et al.*, 2008; Mason & Parker, 2010; Martín & López, 2014). This hypothesis seems to be obvious in an overall view but, because of the diversity of compounds identified in chemical secretions, this idea must be taken cautiously. In fact, the evidences revealing which factors could underlie to the quantitative or qualitative interspecific differences found in chemical signal composition are scarce. The results of our study suggest the availability of some trophic resources as a potential factor to explain, at least in part, those differences. Therefore, it would not be surprising that species phylogenetically closer with noteworthy differences in particular compounds are highly influenced by external factors (e.g., diet) that condition the higher o lesser expression of several compounds in chemical signals. The genus *Iberolacerta* is a clear example of this, since Pyrenean species (*I. aranica*, *I. aurelioi* and *I. bonnali*) own high proportions of vitamin E in their secretions, but in the rest of iberian *Iberolacerta* species (*I. cyreni, I. galani and I. monticola*) the presence of vitamin E had not been confirmed. On the basis of the results obtained, the non-confirmation of the vitamin E in chemical secretions does not necessarily mean the inability of lizards to express it. Therefore, external factors could be shaping the composition of lizard chemical profiles. In this context, experimental investigations are needed to clarify which factors, and in which species, might be constraining the expression of other chemical components. This is especially relevant for those chemicals that might form potentially honest signals, which degree of expression in chemical secretion could alter the sexual selection processes, as has been shown in the case of vitamin E and *I. cyreni*.

Environmental factors have been proved to be important factors shaping visual signals of lizards (e.g., colors and behaviors) (Losos, 2009). However, alternative sensory modes, such as chemical and acoustic ones remain poorly studied in lizards. In **Chapter IV** we focused on studying the compositional differences in the femoral secretions of two closely related lizard species (*Podarcis bocagei* and *P. carbonelli*). We found that, although some variations in chemical profiles might be a consequence of phylogeny *per se*, the different expression of several compounds (e.g., waxy esters, squalene) in each species could be explained by the environmental differences where both populations inhabit. Accordingly, we also analyzed if a case of divergence in the chemical signals among three populations of *Gallotia galloti* could be driven by certain climatic factors (i.e., temperature and precipitation) and, moreover, we investigated the seasonal effect on chemical signals in two of these populations (**Chapter V)**. Our results reveal coherent evidences to suggest that climatic factors (i.e., temperature, precipitation and seasonality) might influence the composition of the chemical signals in these populations. Since the focus of the study was on three particular populations, we are aware that the results must be treated with caution. However, clear patterns were observed among the three populations, especially in the relative abundances of cholesterol and vitamin E (α-tocopherol). Namely, higher levels of vitamin E were found in those populations that inhabiting wetter areas (i.e., *G. g. eisentrauti* and *G. g. palmae*), which fits with the hypothesis that vitamin E might have antioxidant structural properties (Brigelius-Flohe & Traber, 1999; Traber & Atkinson, 2007; Weldon *et al.*, 2008; Martín & López, 2014). However, although climatic factors arise as potential drivers of variations in the chemical profiles of these populations, we consider that collection of complementary data (e.g., diet, genetic) would be useful to explain the complete context in which chemosignals have been evolutionary shaped. In addition, the observed seasonal effects on chemical profiles may be partially explained by the fluctuations in the physiological and endocrine processes throughout the year (Alberts *et al.*, 1992; Martín & López, 2015). For example, in spring, when chemical signals may be especially useful for mate choice (Weldon *et al.*, 2008), *G. g. eisentrauti* allocated higher levels of vitamin E in their chemical secretions. Although physiologically costly for males, a higher diversion of this vitamin to chemical secretions from metabolism could increase their attractiveness and have consequences in mating success and inter-sexual selection processes, as it has been described in other lacertids, such as *L. viridis* and *T. lepidus* (Martín & López, 2010; Kopena *et al.*, 2011). In contrast, outside of the reproductive season, the relative abundance of vitamin E in secretions decreased, possibly to allow the use of this vitamin in metabolism (Brigelius-Flohe & Traber, 1999; Mardones & Rigotti, 2004). Seasonal variation in chemical signals was also evidenced in *Iguana iguana* males, whose femoral secretions had more lipids during the breeding than during the non-breeding season (Alberts *et al.*, 1992). Mainly, works studying lizard chemical communication are based on samples from the reproductive months, since many species do not bear secretions in other periods (Martín & López, 2014). However, some species are active and produce secretions during the whole year and, therefore, investigating the functionality of chemical signaling in the non-reproductive months could provide important keys to understand the role of chemosignals in both inter and intrasexual interactions.

Finally, we present here two studies investigating the evolution of some chemical signaling traits over time. Thus, we firstly studied the diversification of the follicular epidermal glands (FG) in squamates **(Chapter VI)**. In this work we provide the largest data base of presence/absence, number and location of FG in squamates hitherto. Despite of previous important contributions to this field (Pincheira-Donoso *et al.*, 2008; Valdecantos *et al.*, 2014; Baeckens *et al.*, 2015; Mayerl *et al.*, 2015), the above mentioned aspects remained understudied in the overall clade of Squamata. Our results describe the tempo and mode of follicular gland diversification in squamates and also in the three clades with major number of species with pores (Gekkota, Iguania and Lacertoidea) (figure 2). Previous macro-ecological studies attempting to elucidate the ecological causes in the number of pores conducted on certain lineages were not enlightening. While some authors did not find ecological causes behind the number of pores (Pincheira-Donoso *et al.*, 2008), others found the substrate use as possible driver of diversity in the number of pores (Mayerl *et al.*, 2015). However, these studies were performed with a limited set of lizard species and they did not investigate how have these glands diversified in squamates over time.

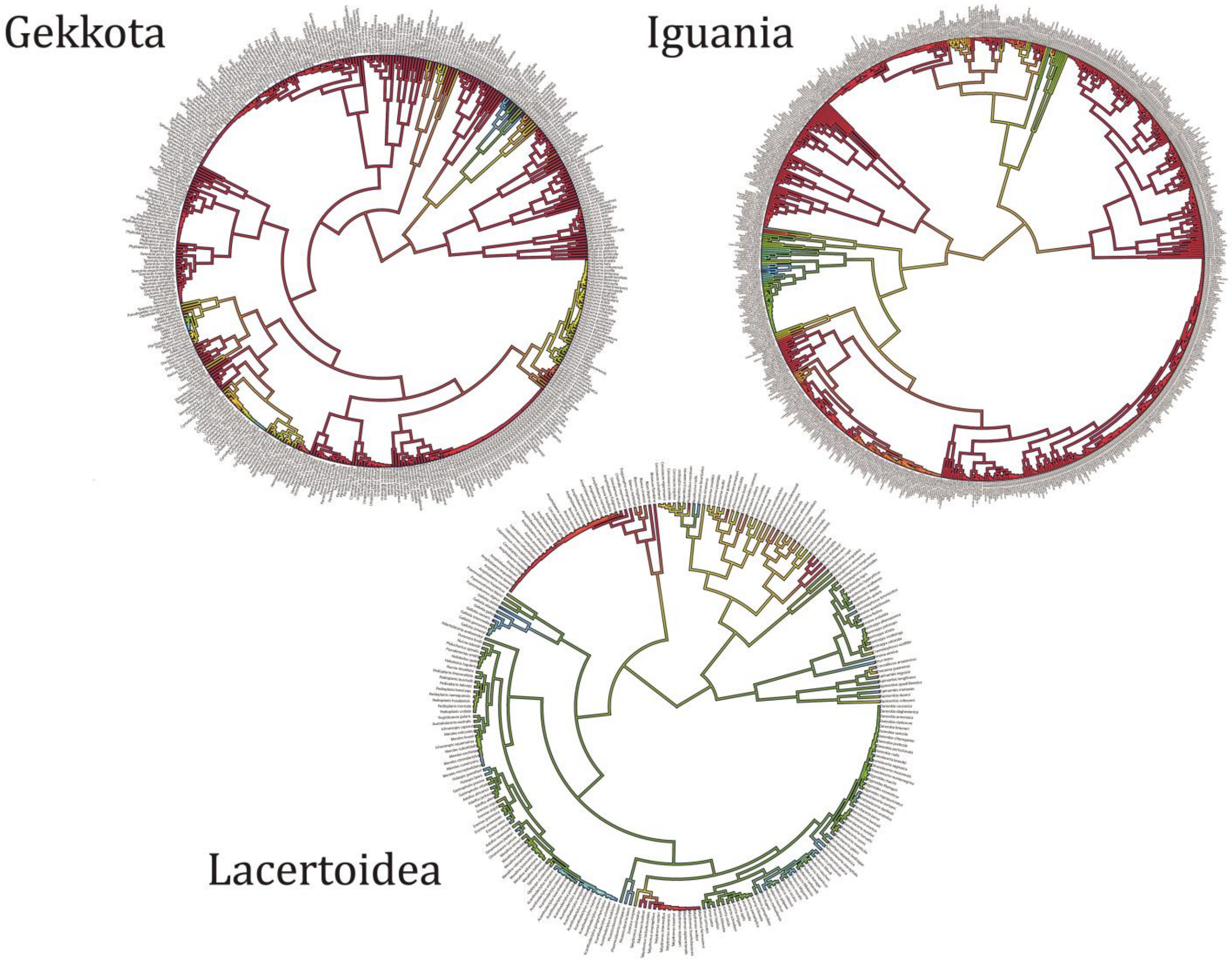

Figure 2. Ancestral state reconstruction of follicular gland number in three linages of Squamata (Gekkota, Iguania and Lacertoidea).

Our results suggest an evolutionary scenario in which the number of FG has evolve under a stabilizing selection with a prominent overlap in the morphospace. Moreover, since the absence of follicular glands is the ancestral state in squamates, these glands might have evolved throughout Squamata diversification as a needed to alternative signaling. Our study shows the phylogenetic relationships as an important factor underlying the evolution of FG. The apparition and loss of this trait could have occurred at different temporal scales along lineages diversification, suggesting repeated and simultaneous episodes of gland diversification at different temporal scale within lineages. In addition, phylogeny does not explain totally the diversification of these glands and therefore, ecological pressures might be shaping their presence/absence and number. In this context, further studies taking more ecological variables and the overall clade into account are needed to understand the diversification of this chemical signaling trait in lizards.

Additionally, with a similar approach, we studied the diversification of eight particular compounds found in chemical secretions of a group of Lacertoidea lizards **(Chapter VII).** Overall, our results revealed a pattern of heterogeneous tempo and mode of evolutionary diversification among the analyzed chemicals. Therefore, each compound might evolve under different pressures describing thereby different modes of evolution, such as Brownian motion and Ornstein-Uhlenbeck models. This finding provides an evolutionary framework in which chemical compounds might evolve regardless or not to the other chemicals, shaping a complex network of evolution underlying to the diversification of the overall chemical communication of lizards. However, because of the few species whose chemosignals have been described, our work is built over a short set of species. It would be advisable to study the evolution of a large number of compounds on the base of a major number of species also using environment to investigate not only how compounds evolve, but also why they did it.

FUTURE DIRECTIONS

The study of chemical communication in lizard has substantively increased during the past decades. Important findings have highlighted the paramount role that chemosignals play in processes of sexual and natural selection. Even so, there is still a lot of work to be done before reaching a real understanding of the scale in which chemical signals operate. This thesis presents some different examples of approaches that must be addressed. First and foremost, the description of an alternative methodology will allow us to expand the range of further studies investigating environmental and physiological effects on chemical signals. Higher temperature may negatively affects the chemical signals of particular species and thus their chemical communication (Martín & López, 2013; Heathcote *et al.*, 2014; Martín *et al.*, 2015a). For instance, in the context of climate change, the influence of the increasing temperature on chemical communication could have relevant consequences, since chemical signals are been proved to engage in mate choice and male-male competition and therefore, the degradation of these signals might alter conspecific recognition and assessment in some species. However, only behavioral experiments had been able to suggest the mentioned temperature effect hitherto. Since we have confirmed now that temperature may actually affect the composition of chemosignals, we hope that our findings serve as incentive to impulse further researches investigating the potential effects that climatic factors (temperature, dryness, humidity, light) exert in the chemical structure and composition of lizard chemical signals and may help to explore what species might have their chemical communication more threatened under the effects of global warming. Similarly, the improvement of analytical methodologies enables detection of slight changes in chemical profiles caused by physiological factors. Particular compounds are believed to be involved in both metabolism and chemical communication (Martín & López, 2015). However, this has been chiefly proved in a few predominant major compounds (e.g., α-tocopherol, oleic acid) (Weldon *et al.*, 2008). Thus, specific methodologies might shed light about the physiological dynamic and the functionality of minor compounds, which lower abundances, on the other hand, do not necessarily have to mean a lower importance in communication.

Remarkably, how and why chemical communication has evolved had remained historically understudied. Global patterns often provide new keys to understand different aspects of animal communication. The lack of knowledge about how traits linked to chemical communications evolve hinds the establishment of integrative hypotheses that may explain the high diversity of chemosignals in lizards, the presence/absence of some structures associated to them, or the predominance of this sensory mode over others in some lineages of lizards. The inclusion of evolutionary approaches in lizard chemical communication will be necessary to obtain the global history of this channel of communication in lizards. Hence, future works on this issue must assess not only the evolution of particular structures (follicular glands) and some chemical compounds, but the highest number of compounds as possible increasing the set of species and including environmental variables that could have shaped the chemical signaling in lizards over time.

Finally, as a cornerstone to understand the chemical communication of lizards, the number of species whose chemical signals are described must increase. So far, we know the composition of chemical secretion of almost 50 species. Given that there are more than 1000 species of squamates with follicular glands, the degree of knowledge about this form of communication is very poor. Indeed, other sources of chemical signals (e.g., skin, feces) might be conveying information but however, the understanding of these channels of information, as well as the composition of their chemical signals, has been almost overlooked. Therefore, if we want to understand the chemosensory system, alternative sources of chemical signaling must be also studied with the aim of implementing integrative studies that disentangle the evolution and the ecological role of chemical communication in lizards.

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Conclusions

1) Our described protocol based on GC-MS with an “injection standard” provide a fast and sensitive method that allows the detection of changes in profiles of chemical secretions, conferring statistical independence among compounds for obtaining their chemical semi-quantification, which may be used to detect physiological and environmental potential effects in the composition of lizard chemical signals.

2) The study investigating male and female chemical signals in *Liolaemus wiegmannii* lizards firstly revealed that females may convey information through precloacal glands. But also, we found a clear sexual dimorphism, mainly expressed as quantitative differences in the chemicals identified in the precloacal secretions.

3) Our study with dietary supplementation of vitamin E in males of *Iberolacerta cyreni* evidenced that this species is able to express high levels of vitamin E in their femoral secretions when there are no constraints of this compound in its diet. Moreover, a high level of vitamin E in diet may influence the chemical signaling, female mate choice and male immune response in the studied species. Our results corroborated previous studies highlighting the importance of diet for the chemical signaling in lizards.

4) In addition to phylogenetic relationships, environmental factors might be shaping the composition of chemical signals, as have been suggested in our studies analyzing potential differences between *Podarcis bocagei* and *Podarcis carbonelli*, as well as in our work investigating environmental conditions as potential drivers of chemical signals diversification in *Gallotia galloti*.

5) Although phylogenetically closer, different population of *G. galloti* differed in the chemical profile of their chemical signals possibly,in order to,increase the efficiency of the information transfer. Temperature, precipitation and seasonality might be potential drivers of compositional divergences in the chemical secretions of three populations of *G. galloti* (*G. g. eisentrauti*, *G. g. galloti* and *G. g. palmae*). Our results showed that vitamin E (α-tocopherol) is the predominant compound in populations of *G. galloti* (*G. g. eisentrauti*, and *G. g. palmae*) with high levels of precipitation and low temperature. Therefore, vitamin E might act as antioxidant protector of chemical secretions during the reproductive season of lizards.

6) Overall, our results showed that particular compounds of lizard chemical signals have diversified following different tempo and mode of evolutionary dynamics. Therefore, each component might evolve following heterogeneous multivariate directions.

7) The presence of follicular epidermal glands (precloacal, femoral or both) in squamates in low, since we found the presence of these glands only in 13.62 % of all studied species. However, the relevancy of this channel of information might differ among linages because of the different percentages of species that own the glands in each linage, ranging from 1.11 % in skinks to 96.8 % in lacertid lizards.

8) Our results suggest that the number of pores diversify following a stabilizing selection (Ornstein-Uhlenbeck model), overlapping regions of the lineage's morphospace. The ancestral state reconstruction analyses showed the absence of follicular glands as the ancestral state in squamates. Our analyses of phylogenetic signals revealed the shared ancestry as relevant factor underlying the diversification of these glands. The results of our study analyzing follicular gland

diversification suggested repeated and simultaneous episodes of gland diversification at different temporal scale within lineages.