VARIACIONES POBLACIONALES EN LAS SEÑALES QUÍMICAS Y SU PAPEL EN LOS PROCESOS DE AISLAMIENTO REPRODUCTIVO Y ESPECIACIÓN EN EL "COMPLEJO DE ESPECIES" DE LA LAGARTIJA IBÉRICA (*Podarcis hispanica*)

Marianne Gabirot

Tesis doctoral 2010

Depto. de Psicobiología
Facultad de Psicología
Universidad Complutense de Madrid

Depto. Ecología Evolutiva
Museo Nacional de Ciencias Naturales
Centro Superior de Investigaciones Científicas

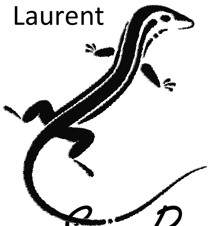
Depto. de Psicobiologia
Facultad de Psicología
Universidad Complutense de Madrid

Variaciones poblacionales en las señales químicas y sus papeles en los procesos de aislamiento reproductivo y especiación en el "complejo de especies" de la lagartija ibérica (*Podarcis hispanica*)

Memoria presentada por la Licenciada Marianne Gabirot para optar al grado
de Doctor en ciencias Biológicas, dirigida por el Dr. José Martin Rueda del
Museo Nacional de Ciencias Naturales – CSIC.
Madrid, Febrero 2010.

La Doctoranda	V°B° del Director
Marianne Gabirot	José Martin Rueda

La passion de comprendre, le désir d’accroitre les connaissances et surtout les surprises de la vérification expérimentale, sont les éléments stimulants de la vie de celles et ceux qui se consacrent à la recherche. B. Laurent



Sea Realista, Pida lo imposible. E. C. Guevara

Agradecimientos

Todo esta aventura empezó hace unos seis años cuando después de descubrir el mundo latinoamericano vine a hacer unas prácticas a la estación de El Ventorrillo para acabar mi año universitario. Después de esta visita de unos meses aprendiendo lo que es hacer experimentos y el concepto de la investigación pensé que tal vez no estaría mal seguir por este camino. Así que después de acabar mi Master en Paris tuve la oportunidad de poder pedir una beca española predoctoral, concretamente la de El Ventorrillo

Una noche mientras estaba de celebración en casa de unos amigos, me informaron por teléfono la posibilidad de obtener la beca, pero debía venir rápidamente a Madrid para arreglar los últimos papeles. Después de una carrera contra reloj para los trámites administrativos, en marzo del 2006 empecé mi tesis. Ahí comenzó una nueva etapa donde pasé de ser una simple estudiante, que sólo entregaba trabajos, a una “mitad estudiante mitad trabajadora”, en castellano sería una estudiante-trabajadora a tiempo completo y eso en un nuevo país.

En primer lugar quisiera agradecer a mis directores de tesis, Pilar López y José Martin, sin ellos esto no haría sido posible. Me enseñaron cómo plantear experimentos, analizar los datos y tener paciencia con la cromatografía. También aprendí con ellos el trabajo de campo en Madrid, cómo capturar a este maravilloso bicho y sobrevivir en este nuevo mundo laboral. José, gracias por haberme dejado suficiente independencia y libertad, pero siempre con un ojo encima por si acaso. También, por subirme la moral cuando al final de estos cuatros años mis nervios estaban muy sensibles.

A mi llegada, no sé lo que haría hecho sin la presencia de Nino. Me acogió y me cuidó como nadie, la verdad es que nunca podré agradecerle todas las cosas positivas que me ha aportado; yo, una francesita lejos de su familia, ahí en la mitad de la montaña madrileña, encontré un abuelo adoptivo que me enseñó un poco de la cultura española, entre toros y cañas. Gracias por soportar mis quejas y picos de nervios cuando la temporada de campo no iba como quisiera y siempre echarme una mano (el ‘mac giver’ serrano). Como cuidador profesional de lagartijas (mi niñera personal), no hay nadie mejor que Nino.

A mis compañeros, colegas, amigos encontrados durante esta beca y estancia en El Ventorrillo y Museo, qué deciros, gracias por estar acá, soportar mis bromas, mis expresiones en mal castellano (creo que reinventé un poco a vuestro idioma) y las charlas muy largas sobre todo y nada. Así que gracias Gema, Diego, Camila, Josué, Sara, Juan, Oscar, Elisa, Isabel, Conchi, Montse, Juan, Juanto, Natalia, Nuria etc. y supongo que me olvido de algunos. Más que una ayuda profesional, panas, menos mal que cada uno a su manera estuvo ahí para motivarme, ayudarme, guiarme y por supuesto divertirnos. ¡Gracias!

También quería denotar que esta beca me permitió hacer varias estancias en laboratorios y países extranjeros, lo que me dio la oportunidad de encontrar gente diferente e interesante. En primer lugar, gracias a mis amigos de Quito, donde regresé por segunda vez en este país maravilloso, para trabajar en comunicación química con reptiles un poco más grandes. Gracias chicos por su amistad y paciencia, Pablo sin ti no haría sido posible. No sólo me has enseñado el interés por los reptiles pero también a ver la vida de manera más positiva. ¡Sois todos chéveres!

Then, I would like to thank other laboratory, the Sweden lab' of Staffan Bensch, where I met very open and rigorous scientists people, especially thanks to Anna Runemark who help me to understand and learn genetically techniques, thank you for your patience and open Swedish mind.

Finally, the laboratory of Sinervo, where I was surprising about the facilities that Barry give to the person to demonstrate that knows and could made. Here I learnt to be independent and made my own tests and adapt to another lab and another lizards/system. Of course in same way I learn to speak Span-Glish, and I discovered the Californian life.

Maintenant un peu de langue maternelle, pour remercier ceux qui me sont proches et qui ont suivi la thèse et mes looping émotionnels de prés comme de loin. Je pense que je devrais remercier en premier celui qui a inventé internet et Messenger car je pense que sans lui j'aurais une note de téléphone à craquer, ainsi que les coups de blues auraient été plus durs. J'aurais une pensée donc à tous ceux de Poitiers qui sont toujours là et sur qui je peux compter en cas de coup de faisan ou pour me ressourcer en cas de manque de camembert. En résumé Jonathan, Sandrine, Bea, Loïc, Aurélie, Claire, Mickael, etc. Merci. Un petit coucou à Loulou, avec toi ca change tout.

Un grand et petit mot pour Benoît ; j'aurais bien copié ce que tu as écris dans ta thèse, mais je vais essayer de faire un peu plus orignal. Qui aurait pensé que 9 ans après on serait toujours là, on n'aurait jamais imaginé que un simple : « tu veux t'assoir à coté de moi, comme sera on ne sera pas seul dans notre coin » aurait conduit à cette amitié, au fait que l'on passe très peu de jours sans se donner des nouvelles et que malgré les kms on soit toujours aussi « in » compatible, pardon inséparable! Merci Benoît d'être là, pour nos conversation à 2 francs mais toujours très naturelles, simplement Merci d'être là, toi tout simplement toi.

Et je termine par la famille, la mienne est grande je vais donc compresser. Je sais que le fait d'avoir un thésard dans sa famille c'est un peu difficile à expliquer ou comprendre ; les questions qui reviennent : mais alors tu es encore étudiante ? C'est quand que tu as un vrai boulot ? Après ce capte vient celui de : pourquoi tu étudies les lézards, quelle étrange idée ? Merci à tous : frérot, oncles, tantes, cousins, cousines, pièces rapportées, pour tous ces moments familiaux si inoubliables et géniaux, surtout pour ne pas poser ces questions. Mais je dois l'avouer le fait d'être un peu plus loin que prévu fait que l'on voit sa famille d'un autre angle et l'on a qu'une envie c'est de profiter d'eux. Finalement, je ne pouvais finir sans remercier mes parents qui m'ont toujours permis de faire ce qui me plaisait, qui m'ont permis de poursuivre des études, et de longues études, de me laisser partir à l'étranger même si je sais que ca été dur pour eux comme pour moi. Si je suis comme je suis maintenant c'est grâce à comment vous m'avez modelé, et les principes que vous m'avez donné ; surtout à la liberté que vous m'avez laissé. Je sais que je ne suis pas forcement la fille imaginée avec une vie stable ni un travail facile et rangé, mais pour le moment c'est le risque que je prends et je vous remercie de me soutenir dans mes choix. Je suis fière d'avoir des parents comme vous, et je sais que je le dis que très peu, mais vous me manquez souvent, rien que de penser à un dimanche en famille les yeux s'humidifient ; simplement : Je vous aime.

Índice

- Parte 1 - Morfología y señales químicas de las lagartijas

- Parte 2 - Comunicación química y comportamiento interpoblacional

- Parte 3 - Reconocimiento de compuestos de las señales químicas

- Parte 4 - Relación entre el sistema inmune y las señales químicas

- Parte 5 - Comunicación química entre dos especies muy cercanas del complejo

INTRODUCCIÓN

Esta tesis se enmarca en el campo de la Ecología Evolutiva y su objetivo general es el de aumentar el conocimiento sobre las señales químicas en reptiles y específicamente en lagartijas. En concreto, intenté estudiar el papel de la comunicación química dentro del complejo de especies de la lagartija Ibérica, *Podarcis hispanica*, para ampliar la información sobre este tipo de señales y examinar si juegan un papel importante en la evolución y en los procesos adaptativos.

> *«Les chemins de la découverte sont ouverts, c'est le propre de l'homme de les parcourir et il n'en aura jamais terminé. » F. Comte (2009)*

La ciencia ha demostrado más allá de la duda razonable que los organismos vivos evolucionan y se diversifican a lo largo del tiempo, y que sus rasgos son frutos de un proceso, la selección natural, que explica este «diseño» (Ayala 2007). Darwin y otros biólogos del siglo XIX hallaron pruebas contundentes de la evolución biológica en el estudio comparado de los organismos vivos, en la distribución geográfica, y en los restos fósiles de organismos extintos.

Según Lamarck, las especies animales se transforman fundamentalmente a todos los niveles y logran ser especies más o menos evolucionadas; se adaptan. Esta transformación explicaría la diversidad de las especies vivas; las especies serían sólo relativas y temporales. El tiempo de vida de un hombre es demasiado corto para poder entrever las mutaciones considerables establecidas después de mucho tiempo. Es un hecho importante que el hilo conductor de toda la evolución y su efecto no se determina con una sola generación sino con todas las siguientes y esto casi hasta el infinito; unos millones de generaciones, con modificación lenta, muy lenta, de las especies y tal vez con mucho tiempo, la creación de nuevas variaciones, y después quizás de especies nuevas. No se sabe, ni se puede medir el número de variaciones que han existido, unas desde la aparición del individuo, otras durante el desarrollo y el contacto con el medio ambiente, y unas últimas con la desaparición de las especies que las tenían. Lo que es importante es que algunas fueron mantenidas, mejoradas hasta un óptimo, y que los individuos que las poseían pudieron multiplicarse. Lamarck ha apostado sobre la transformación interna de los seres vivos, adaptándose a las condiciones de la vida; Darwin habla de irreductibilidad de situaciones que descartan, eliminan sin remordimiento todo lo que no está ajustado: la selección natural como una eliminación del no apto.

SELECCIÓN NATURAL - lucha por la existencia

"¿Se puede, entonces, considerar improbable, al ver que variaciones útiles en cierto modo a cada ser en la gran y compleja batalla de la vida, debían a veces producirse en el curso de miles de generaciones? Si tal cosa sucede, ¿Podemos dudar (recordando que nacen más individuos de los que tienen posibilidad de sobrevivir) que los individuos que poseen alguna ventaja sobre otros, por leve que sea, tendrían la mayor probabilidad de sobrevivir y procrear entre los de su clase? Por otra parte, podríamos estar seguros de que cualquier variación que fuese perjudicial en menor grado sería inflexiblemente destruida. A esta preservación de las variaciones favorables y al rechazo de las variaciones perjudiciales, yo lo llamo Selección Natural." Darwin (1859)

Los elementos de la teoría de Darwin sobre la selección natural son (1) la existencia de una variación considerable entre los individuos de una misma especie de cualquier población, (2) una gran parte de esta variación es heredada (por vía genética) y (3) en cada generación, la supervivencia y fertilidad aumentan (o se estabilizan).

Se podría concluir que diferentes individuos no tienen la misma supervivencia. Los que tienen características más adaptadas al medio ambiente son los más susceptibles de sobrevivir y transmitir sus caracteres favorables a la generación siguiente. A través de sucesivas generaciones, las variaciones beneficiosas se conservan, se multiplicaran y se unirán; las variaciones perjudiciales o menos beneficiosas serán eliminadas (Ayala 2007). Darwin se daba cuenta de que las similitudes entre las especies eran a veces (no siempre) debidas a un ancestro común, mientras que las diferencias entre especies vecinas eran debidas a la adaptación por evolución a medios ambientes distintos.

Los principales tipos de interacciones entre los animales de diferentes especies son la depredación y la competición. Alimentándose cada especie ocupa un nicho propio. Las especies que tienen nichos parecidos en el mismo hábitat entran en competición por la comida y quizás por otros recursos. Dos especies no pueden ocupar el mismo nicho de un mismo hábitat. La comparación de especies muy cercanas viviendo en hábitats diferentes revelaría aspectos del comportamiento que son importantes para la adaptación de los individuos al ambiente. La teoría de la selección natural fue mejorada y afinada con la ayuda de la genética, la cual permitió poner en evidencia la posibilidad de mutaciones o saltos además de la selección de caracteres hereditarios.

Cuando Darwin se refería a herencia ligada al sexo o a las condiciones de vida, estaba en realidad tratando la expresión de caracteres y no su transmisión, y cuando se refería a "acción directa de las condiciones de vida" o a "uso y desuso" no estaba tratando la herencia lamarckista, sino lo que ahora se llama plasticidad fenotípica. Es la propiedad de un genotipo determinado para producir distintos fenotipos en respuesta a distintas condiciones ambientales.

La evolución de nuevos fenotipos se explicaría por ejemplo con la hipótesis de adaptación y asimilación genética de West-Eberhard (1983) o con la idea de la existencia de variación genética críptica de Caroll (2005) o con la propuesta de Kirschner y Gerhart (2005), las cuales exponen que la flexibilidad de los procesos claves conservados permitiría mantener mucha variación genética que apenas afecta al fenotipo, pero que sin dicha flexibilidad se determinaría cambios letales. Estos modelos describiendo la plasticidad fenotípica presuponen una gran cantidad de variación genética mantenida en poblaciones y que conmovería a cualquier rasgo fenotípico, sin depender de nuevas mutaciones para determinar cambios fenotípicos. Otra forma de crear novedad sería mediante la determinación por factores ambientales de fenotipos alternativos simultáneos.

La variación en los múltiples genes que intervienen en el desarrollo de cualquier rasgo permite, por asimilación o acomodación genética, la facilitación creciente de la adaptación a los cambios. Un factor ambiental puede inmediatamente afectar a muchos individuos de una población de una sola vez, mientras que una mutación afecta inicialmente sólo a un individuo.

La selección natural tendría varios efectos sobre la distribución de frecuencias de fenotipos en una población. Resultado de una mutación, de una recombinación, o de una deriva genética, la variación tiende a aumentar de una generación a la siguiente. Esta tendencia está controlada por la selección estabilizadora, la cual actúa relativamente más severamente en los extremos de una distribución de frecuencias. Cuando hay presión selectiva diferencial a lo largo de un gradiente fenotípico, la selección direccional interviene, y resultaría de una desviación de la media de distribución de frecuencias o en una distribución distorsionada sin alterar la media. También se hallaría una presión selectiva sobre los caracteres típicos de una población, es decir que la selección es más fuerte con los animales cerca de la media que con los que están en los extremos, es una selección disruptiva, pero es muy rara. En este caso, se tiende a obtener una distribución binomial y a obtener dos especies a partir de una (Fig. 1).

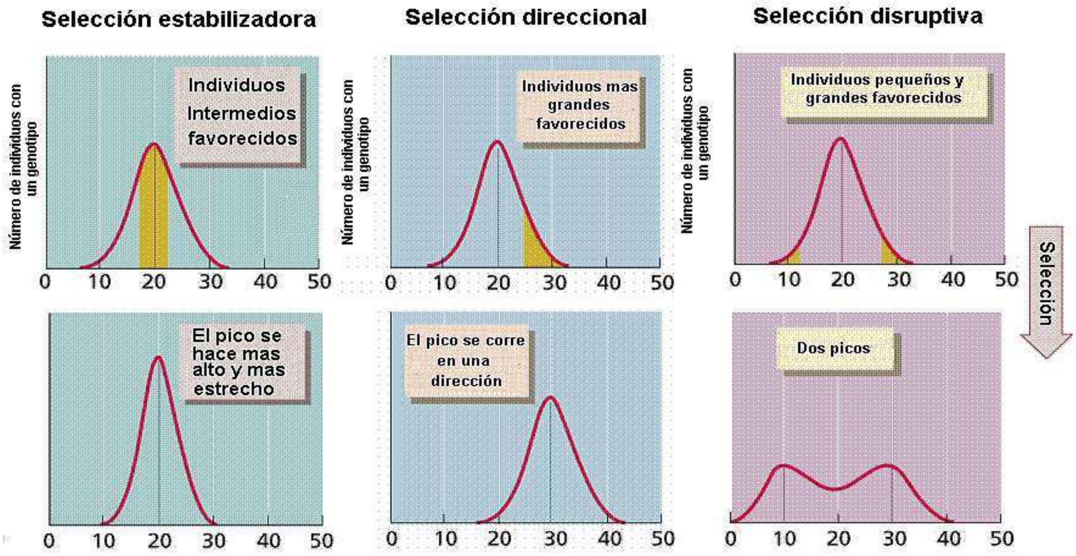
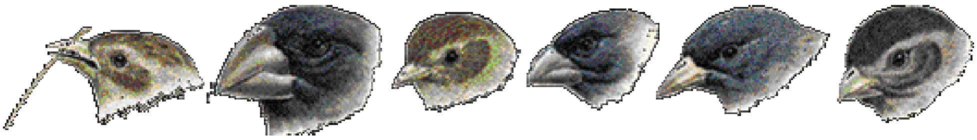


Fig. 1: *Tipos de selección natural*
- Selección direccional: se favorece un fenotipo extremo.
- Selección estabilizadora: se favorece un fenotipo intermedio.
- Selección disruptiva: se favorecen los dos fenotipos extremos, que con el tiempo se separará en dos subpoblaciones.

ESPECIACIÓN – CONCEPTO DE ESPECIE



Los seres vivos están en perpetua competición para mejorar su situación, pero también para permitir la reproducción de sus genes, su patrimonio genético. Cuando se habla de transformación dentro de una especie se trataría de microevolución, al nivel de especiación, sería la macroevolución. Ahí se encuentra todo un conflicto entre los que optan a favor de la evolución por saltos y los que opinan que la evolución es gradual. La polémica se amplía cuando la misma definición de especie es cuestionada.

> *"La colección de todos los cuerpos organizados, nacidos los unos de los otros, o con padres comunes, y los que se aparean entre ellos pueden llamarse especie."* Cuvier (1778)
>
> *"Lo que se denomina especie en zoología y botánica, es la reunión de individuos que emparentan y coinciden en muchos índices y difieren entre ellos sólo en algunas modificaciones accidentales."* Brongniart (1837)
>
> *"La especie es una serie de variedades encerradas en un círculo, y que empezando en un punto dado, se terminará en este mismo punto, después de haber seguido la circunferencia."* Brongniart (1837)

Finalmente Ernst Mayr (1942) tuvo la última palabra con la definición siguiente:

> *"Defino las especies biológicas como grupos naturales de poblaciones que se cruzan y están aisladas reproductivamente (genéticamente) de otros grupos parecidos."*

Mecanismos de aislamiento
Se definen dos tipos de aislamiento según el momento de formación de un cigoto: las barreras precigóticas por una parte, con por ejemplo un aislamiento de los hábitats (parejas potenciales habitan el mismo lugar pero no se encuentran) o un aislamiento temporal (parejas potenciales se encuentran pero escogen no aparearse), también cuando las parejas copulan pero los gametos masculinos no son transferidos o cuando los gametos masculinos son transferidos, pero no hay fertilización; y por otra parte las barreras postcigóticas cuando el cigoto se muere temprano en la embriogénesis, o produce híbridos F1 inviables, o híbridos F1 que sobreviven pero son estériles o con híbridos F2 inviables o estériles.

Mecanismos precigóticos
- Aislamiento ecológico: las poblaciones que ocupan el mismo territorio viven en hábitats distintos y no entran en contacto.
- Aislamiento estacional: la fecundación tiene lugar en momentos distintos del año o del día.
- Aislamiento etológico: la atracción sexual entre machos y hembras es débil o no existe.
- Aislamiento mecánico: impide la cópula entre los animales o la polinización en las plantas, al poseer estructuras reproductoras incompatibles.
- Aislamiento gamético: los gametos masculinos y femeninos no se atraen.

El concepto biológico de especie, desarrollado por Dobzhansky y Mayr, se refiere a conjuntos de poblaciones con capacidad de intercambio reproductivo entre sí, pero con ausencia de intercambio con otros conjuntos. Sin aislamiento genético, la mezcla de características entre variedades impediría la especiación. El aislamiento genético se debería a una separación geográfica o a cualquier impedimento para el movimiento de los individuos. Este es el modelo de la **especiación en alopatría** o alopátrida (Fig. 2).

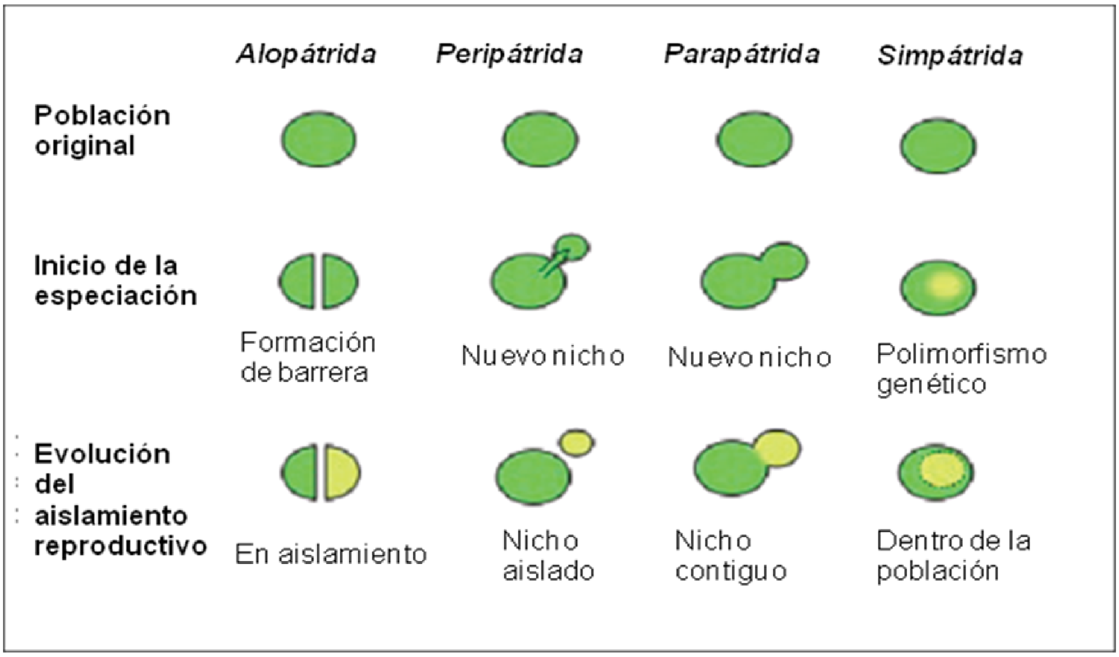


Fig. 2: Diferentes mecanismos de especiación y los resultados con una población inicial

Además de la especiación alopátrida existe la controvertida alternativa de la **especiación simpátrida**, la cual sugiere que el aislamiento reproductivo se puede producir dentro de una población sin barreras geográficas (Fig. 2). A diferencia de otros modelos, la especiación simpátrida no exige que la distancia geográfica a gran escala reduzca el flujo de genes entre las partes de una población. La explotación de un nuevo nicho podría automáticamente reducir el flujo de genes como en el caso de los insectos herbívoros que prueban una nueva planta hospedadora.

Por ejemplo, hace 200 años, los antepasados de las moscas del espino (*Rhagoletis pomonella*) ponían sus huevos solamente sobre espinos blancos; pero hoy, estas moscas depositan los huevos sobre espinos blancos (que es nativa de América) y sobre manzanos domésticos (que han sido introducidas en América por los inmigrantes). Las hembras generalmente prefieren poner sus huevos sobre el tipo de fruto donde crecieron y los machos tienden a buscar pareja sobre el tipo de fruto en el cual crecieron. De este modo, las moscas del espino blanco generalmente acaban copulando con otras moscas del espino blanco y las moscas del manzano generalmente con otras moscas del manzano. En consecuencia, la posibilidad de reproducción entre individuos de diferentes frutales se ve reducida. Pero, esta especiación simpátrida está muy debatida, desde la negación de Coyne y Orr (2004) hasta su defensa por Schilthuizen (2001).

Sin embargo, se han formulado otros modelos para explicar la aparición de especies como por ejemplo el modelo de **especiación parapátrida** (Fig. 2). Se caracteriza por la no existencia de barrera extrínseca al flujo génico con una población continua donde los individuos se reproducen aleatoriamente. La reproducción es más probable con los vecinos geográficos. Este modelo de especiación puede ocurrir por cambios cromosomales que aíslan las poblaciones o por fuerte adaptación local. Recientemente, se habló de **radiación adaptativa**. Se define por la evolución de la diversidad ecológica en un linaje con rápida multiplicación. Es la diferenciación de un ancestro en una serie de especies que habitan una variedad de ambientes y que difieren en los caracteres usados para explotar tales ambientes. La radiación adaptativa es el resultado de la selección natural divergente impuesta por el ambiente y de la competencia interespecífica

En el caso de que nuevas especies surjan por el mismo proceso mediante el cual ocurre la diferenciación de fenotipos, esto es, bajo el proceso de selección natural divergente por efectos ambientales y competencia por los recursos: se trata de **especiación ecológica**. Es un mecanismo de especiación competitiva en el que el aislamiento reproductivo es colateralmente favorecido por la selección.

No es necesariamente una selección disruptiva, ni tiene por qué producirse en simpatría. Simplemente requiere que la adaptación de variedades fenotípicas a distintas condiciones ambientales (geográficamente separadas o juntas) conlleve una divergencia genética que, irremediablemente, induzca aislamiento antes o después del apareamiento. La especiación alopátrida sólo es ecológica si la selección natural, no la sexual, determina los cambios que acaban produciendo aislamiento.

Finalmente, la **especiación adaptativa** es el resultado de la adaptación a distintos nichos ecológicos que no existían previamente, sino que son creados por la propia divergencia; mejor dicho, los nichos creados formaban parte de un nicho mayor que los englobaba. Este es el modelo de divergencia de caracteres de Darwin, remozado y basado en ecuaciones y nuevos conocimientos.

De momento se aceptaría el hecho de que la especiación sea en gran parte de tipo alopátrida ecológica. Pero nuevos ejemplos de especiación simpátrida lo ponen en duda, o por lo menos recelan de su supremacía en los fenómenos de especiación (por ej., moscas frugívoras de Norteamérica, salmónidos de Islandia, los espinosos, cíclidos de los lagos del Camerún o el Rift africano; Schiltzhuzen 2001; Dieckmann et al. 2004).

Los cambios serían determinados fundamentalmente por adaptación a las diferentes condiciones ambientales reinantes a ambos lados de la barrera, aunque se deberían también a la selección sexual por divergencia en los caracteres atractivos en poblaciones separadas.

SELECCIÓN SEXUAL - lucha por la reproducción

Noción de selección sexual

Darwin (1859) definió la selección natural como los procesos que permiten preservar los caracteres, supuestamente adaptados, útiles para la lucha por la supervivencia con el objetivo de reproducirse. Estas estructuras como el plumaje vistoso de algunos pájaros o un canto elaborado, son a menudo energéticamente costosas de desarrollar y mantener, pero también, en términos de supervivencia; sobre todo hacen parecer a los machos más débiles. En este sentido, la selección sexual tendría la capacidad de producir y mantener caracteres aparentemente mal adaptados.

A pesar de su efecto negativo sobre la viabilidad de los individuos estos caracteres han evolucionado y siguen presentes. Estas observaciones condujeron a Darwin a proponer la teoría de la selección sexual ("*The descent of man, and selection in relation to sex*" 1871). Esta teoría sugiere que estos caracteres aparentemente superfluos o costosos para la supervivencia, tendrían un beneficio a nivel de la reproducción, aumentando el éxito reproductor del individuo. La selección sexual no implicaría una lucha por la supervivencia, sino una lucha entre individuos del mismo sexo por el acceso a la reproducción con individuos del otro sexo (i.e. los individuos que tienen una ventaja sobre los otros para reproducirse, tendrán una descendencia mayor a la cual se transmite este beneficio).

La selección sexual actúa para afinar el carácter secundario sexual de los morfotipos hasta tener diferencias entre machos y hembras o entre los tipos de machos (en la mayoría de los casos). Los caracteres sexuales primarios se reflejan en la diferencia básica entre un macho y una hembra al nivel del sistema reproductivo, genitales y las glándulas asociadas. Los caracteres sexuales secundarios son los rasgos formado por la acción de la selección sexual. Estos rasgos secundarios son a menudo útiles para ayudar en la competición intrasexual para reproducirse, o para atraer a la pareja. Los colores vistosos serian señales de estrategias agresivas para los otros machos, o un plumaje elaborado sería una señal para atraer las hembras. La selección sexual seguiría los mismos tres modelos que la selección natural: dirección, estabilización y perturbación; pero la mayoría de la acción de la selección sexual promueve una selección direccional aumentando por ejemplo el tamaño del rasgo o la intensidad de la señal.

Se caracteriza por un lado la **selección intrasexual**: relacionada con la evolución y mantenimiento de fenotipos que permiten competir por el sexo opuesto (competencia entre individuos de mismo sexo), y por otro lado la **selección intersexual**, la evolución y mantenimiento de fenotipos que permiten atraer al sexo opuesto (elección de pareja).

La probabilidad de que ocurra selección sexual surge de la variación en el éxito de apareamiento entre los individuos: el éxito reproductivo de un sexo está usualmente limitado por los recursos, en tanto que el del otro sexo está limitado por las oportunidades de apareamiento. A base de las observaciones de Bateman (1948), Trivers afina la teoría de Darwin (1972). Las hembras serían el sexo limitante e invertirían más en la descendencia que los machos. Muchas hembras no son fértiles porque están criando o desarrollando crías. Entonces, los machos se encontrarían en superioridad numérica, y se podrían desarrollar unos caracteres u ornamentos con el fin de competir entre ellos o atraer las hembras. Esto explica la selección sexual como una función de la selección intersexual donde las hembras elegirían a los machos basándose en ornamentos elaborados o comportamientos por parte de los machos. Alternativamente existe la selección intrasexual donde los machos competirían para el acceso a las hembras o al territorio. La competición macho-macho puede ser muy peligrosa con combates con armas elaboradas con el objetivo de ganar acceso a las hembras para reproducirse (por ej., cuernos en ungulados).

¿Por qué existen los rasgos vistosos y por qué las hembras los prefieren?

La selección intersexual puede verse involucrada porque estos caracteres secundarios aportarían ventajas directas a las hembras. Si las preferencias de las hembras no implican costes, se favorecerán hembras cada vez más selectivas y rasgos de los machos cada vez más exagerados. El reconocimiento de especies, el cuidado parental, la defensa territorial, proveer alimento, o una mayor fertilidad por parte del macho son posibles **beneficios directos** de la selección sexual para las hembras.

Pero también se plantea la posibilidad de **beneficios indirectos**. Fisher fue el primero en exponer una hipótesis: coevolución de rasgos exagerados/preferencia. La preferencia de las hembras evoluciona inicialmente debido a que el rasgo de los machos seria favorecido por selección natural o la preferencia evoluciona por deriva génica y se espera que la descendencia lleve el carácter. Una vez que la preferencia de las hembras existe, los machos con el rasgo serán aún más exitosos (ventaja en selección natural y sexual). Habrá por lo tanto, una creciente fuerza selectiva apoyando preferencias fuertes y rasgos extremos ("*proceso runaway*"). El proceso se detendrá cuando no exista variación genética para la preferencia o exageración del carácter, o cuando los costes de viabilidad del rasgo compensen a los beneficios de la selección sexual.

La selección sexual dentro de las poblaciones llegaría indirectamente a un aislamiento sexual entre poblaciones, sin ningún enlace o relación con el ambiente u otra especie; el modelo de Runaway de Fisher en el que se observa coevolución y divergencia entre los ornamentos o señales de los machos y las preferencias de pareja de las hembras, inspiró otros modelos recientes como los de Kirkpatrick (1982) y Lande (1981); modelos similares pero con base de genética y matemática. Lande demostró que si hay variaciones genéticas al nivel del rasgo del macho y también al nivel de las preferencias de las hembras, se podría generar una covarianza genética positiva de los dos con una reproducción asortativa (Lande 1981; 1982). Está claro que si la distribución de los rasgos y preferencias no se solapan entre las poblaciones, se esperaría un aislamiento. Los modelos de Lande se interpretan como modelos de especiación. También Wilkinson y Reillo (1994) aportaron pruebas experimentales apoyando la hipótesis del Runaway con la mosca de ojos pedunculados y la correlación genética entre preferencia/ancho de los ojos.

Modelo del Handicap

Uno de los beneficios indirectos sería que la preferencia de las hembras se vea beneficiada al producir descendencia de alta calidad genética: "*modelos de buenos genes*" Zahavi (1975; 1977). Supone que algunos machos en una población poseen un rasgo que reduce su supervivencia. Estos rasgos de los machos serian indicadores de alta calidad genética ya que a pesar de tener un rasgo tan "desventajoso" son capaces de sobrevivir. Estos modelos se llaman también modelo del "*handicap*" por el hecho de que se refiere al coste de supervivencia de los rasgos de los machos.

Sin embargo, para impedir que los machos de baja calidad mintieran, se supone que el coste del rasgo desventajoso debe ser menor para los machos de alta calidad; sólo ellos pueden costearlo. El rasgo exagerado llega a ser una **señal honesta** para la calidad de los machos (prueba experimental en golondrinas Safran et al. 2005).

Hamilton y Zuk (1982) afinaron la hipótesis con predicciones intraespecíficas: (1) Las hembras basan su elección de pareja en la expresión del carácter vistoso, el que se relaciona con la expresión de buenos genes frente al parasitismo; (2) el nivel de desarrollo del rasgo debería relacionarse inversamente con la intensidad de la infección; (3) la resistencia al parasitismo debería ser heredable.

Los rasgos evolucionan debido a su utilidad como indicadores de resistencia heredable a parásitos y enfermedades. Sólo los machos resistentes con bajas cargas parasitarias, pueden producir ornamentos sexuales exagerados. Los machos con altas cargas de parásitos y de baja calidad presentan una expresión reducida de sus ornamentos sexuales. Al escoger aparearse con un macho con una condición vistosa, la hembra recibiera beneficios para su descendencia en forma de genes para la resistencia contra los parásitos.

La elección continúa de machos desventajosos eliminará a los machos de baja calidad del fondo génico. Luego de unas pocas generaciones habrá sólo machos de alta calidad genética y la selectividad de las hembras será inútil. ¿Qué mantiene la variación genética en la calidad (*fitness*) de los machos? Habría sólo machos de alta calidad en la población y cualquier variación genética remanente será no-heredable; las hembras no ganarán nada de su elección.

Finalmente, una posibilidad alternativa es que la preferencia de las hembras sea un simple efecto colateral (pleiotrópico) de evolución sensorial que ya ha evolucionado por otras razones: **sesgo sensorial** o atracción pasiva. Por ejemplo, las crestas artificiales aumentan el éxito reproductivo de algunas especies de pinzones de distintos géneros. También, en las hembras de ranas endémicas de Ecuador, *Physalaemus coloradorum* al agregar una frecuencia a los sonidos, prefirieron las llamadas experimentales a las normales de su propia especie (Kirkpatrick y Ryan 1991; Ryan y Rand 1993; Ryan 1998). Los caracteres seleccionados son simplemente más conspicuos y captarían la atención de una hembra a mayor distancia que las señales menos intensas. La evolución de los caracteres masculinos, entonces, no tendría ninguna relación con las teorías de hándicaps o con los modelos de buenos genes.

Selección sexual y especiación

¿Influye la selección sexual en la especiación? La discusión de este punto es reciente. Con las síntesis modernas, los procesos de elección de pareja para la reproducción y del reconocimiento entre especies son diferentes, sin embargo, nuevos análisis reconocen que forman parte de un continuo: las hembras podrían discriminar entre algunos machos porque son de menor calidad o menos estimulantes para las hembras, pero cuando este proceso llegue a la discriminación de grupos geográficos, después contribuiría al reconocimiento entre especies (Ryan y Rand 1993). El reconocimiento de especies predomina en los debates actuales sobre comportamientos sexuales, con la concomitante asunción de que los cambios en el sistema de reconocimiento entre especies, con la selección sexual, llevarían a una hibridación deletérea o a una confusión entre las especies.

Si hubiese suficiente variación al nivel de preferencia de pareja, los distintos fenotipos evolucionarían por diferentes caminos, creándose un aislamiento reproductivo efectivo y una especiación rápida (Fig. 3).

West-Eberhard (1983) argumentó que la evolución social incluyendo la selección intra- e intersexual, causaría especiación. Coyne y Orr (2004) subrayan que ahora parece obvio el enlace entre la selección sexual y la especiación a pesar de que siguen algunas críticas como la de Paterson (1985). La adaptación ambiental de las señales y las preferencias para el apareamiento son una potente fuente de la selección sobre la comunicación sexual y podría indirectamente provocar un aislamiento sexual.

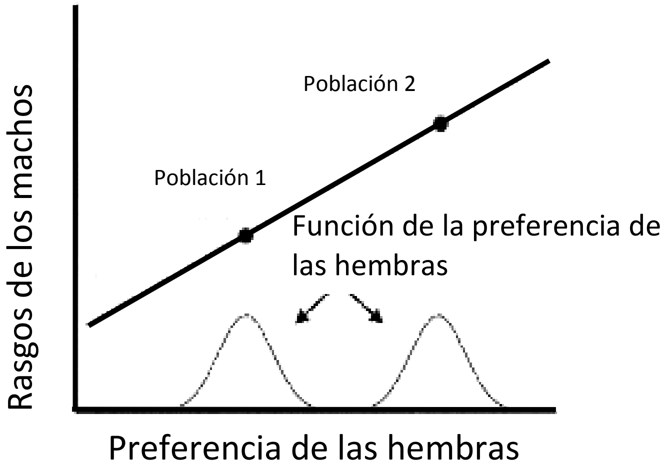


Fig. 3: Relación entre los rasgos de los machos y las preferencias de las hembras y su posible evolución hacia la especiación.

Al contrario, modelos anti-coevolución predicen a partir de fuentes alternativas de variación en los rasgos y preferencias actuando en contra de la covarianza en las poblaciones, que los rasgos y señales estarían fuera de los cambios evolutivos. Los rasgos de los machos evolucionarían sin la influencia de las hembras o preferencias de ellas, pero dentro de otro contexto, como por ejemplo los comportamientos de búsqueda de alimento (Ryan 1990; Macías García y Ramírez 2005).

Estos rasgos, llamativos en su mayoría, sirven para la comunicación entre individuos de la misma especie o no, en especial en las relaciones sociales y sexuales. Algunos de estos rasgos se calificarían de señales. Las señales intervienen directamente en la selección sexual y en la reproducción e indirectamente en la supervivencia de los individuos, lo que lleva a pensar que influyen en la eficacia biológica o más en general que intervienen en los procesos evolutivos.

COMUNICACIÓN

> *"La comunicación incluye necesariamente la interacción de dos sistemas distintos dentro de la misma especie, un emisor y un receptor, que pueden estar sometidos a muy diferentes presiones y restricciones evolutivas."* Endler (1993)

Regnier y Wilson en 1971 definieron la comunicación como la emisión por un individuo de un estímulo que provoca una reacción en otro individuo, la reacción puede ser beneficiosa para el emisor, o para el receptor o para los dos. Este estímulo es el vector de una información. La huida, o camuflaje (desde la percepción de un depredador), la detección y localización de una fuente de alimento adecuada, de la pareja sexual receptiva, del sitio de puesta o refugio adecuado, el reclutamiento de congéneres, su rango social, la apreciación de la densidad de la población para limitar la sobreexplotación del hábitat, son manifestaciones de la adaptación de la comunicación de los organismos a los ecosistemas. Están todas fundadas sobre la información, consciente o inconsciente y suponen la solicitud casi constante de un material sensorial omnipresente.

Al igual que la especiación o noción de especie, la definición de comunicación y señales no está clara (Bradbury y Vehrencamp 1998; Maynard-Smith y Harper 2003). Se encuentran varias propuestas de definiciones:

> *"La comunicación es un proceso en el cual los animales usan sus órganos sensoriales para enviar y recibir información sobre el mundo; es consecuencia de un proceso de selección natural, siendo influenciada por múltiples factores, de forma compleja y diferente en cada especie." Darwin (1872)*
>
> *"La comunicación como cualquier alteración de un individuo por mediación de una señal que altere el comportamiento de otro en un contexto adaptativo." Regnier y Wilson (1971)*
>
> *"La comunicación se puede separar entre "comunicación verdadera", "información robada" y "coacción", dependiendo de si la transferencia de información beneficia a ambos, emisor y receptor, sólo al primero, o sólo al segundo." Marler y Peters (1977)*
>
> *"La honestidad de la información transferida, que dependerá de si los intereses del emisor y receptor divergen o coinciden, generando diferentes escenarios evolutivos de favorecimiento mutuo de transferencia de la información o de "carreras de armamentos" que aseguran la honestidad de esa información." Maynard-Smith y Harper (2003)*
>
> *"La provisión de información por parte de un emisor a un receptor y el subsiguiente uso de esa información por parte de éste último para decidir su respuesta." Bradbury y Vehrencamp (1998)*

> *"Se produce comunicación cuando se puede demostrar que el comportamiento de un animal tiene un efecto en el comportamiento de otro." Dawkins (1995)*

Tocar, ver, sentir, gustar, oír, situarse en el espacio y en el tiempo, es informarse para decidir la mejor elección. La sensibilidad es una de las características fundamentales de la vida. Para sobrevivir y evolucionar, todos los seres vivos deben percibir, localizar, y a veces identificar las informaciones que vienen de los organismos de la biocenosis, pertenezcan o no a la misma especie. Todos los seres vivos, desde los más simples hasta los más complejos son capaces de recibir indicaciones del mundo alrededor y reaccionar a ellas. Las señales más comunes son las que advierten del peligro (alarma), las señales sexuales, aparéntales y las señales sociales. La información varía según su aspecto, según el medio en el que se propaga (agua, aire) y según la complejidad de los organismos.

Existen una gran variedad de señales: unimodales (llamadas sexuales por ejemplo) o multimodales (cortejo sexual) que implica estudiar todas las señales en su conjunto para explicar funciones biológicas. Todas las señales tienen un significado y son el producto en su mayoría de la selección natural y sexual. Las señales pueden ser simples y estereotipadas en los mensajes simples, pero también variables para reforzar el mensaje. Las funciones de las señales se resumirían en tres principales: (1) señalización de la identidad, (2) del estado motivacional, (3) comunicación sobre el medio.

La **señalización de la identidad** (1) se caracteriza por la capacidad de diferenciar las especies cercanas a nivel morfológico, químico, sonoro, etc. Permite también el reconocimiento entre individuos de la misma especie, para evitar por ejemplo la reproducción interespecífica o con individuos emparentados. Además las señales de identidad se utilizarían para el reconocimiento de grupo social, como en muchos insectos sociales. Uno de las funciones sería el mantenimiento de la cohesión social mediante moléculas químicas.

El **estado de motivación** (2) se define con dos puntos: el comportamiento agonístico para defender recursos y el comportamiento sexual para reproducirse. Las señales de comportamientos agonísticos, ocurren por ejemplo durante la defensa de territorio, del alimento, de la pareja sexual, o del acceso a la reproducción. Son en su mayoría visuales como posturas o huidas. Las señales sexuales se encontrarían durante las llamadas sexuales, para atraer y encontrar al sexo opuesto e informar de su estado de celo. También durante el cortejo los organismos aportan información con señales visuales, sonoras, químicas o comportamentales.

La **comunicación sobre el medio ambiente** (3) se podría explicar con dos ejemplos: los gritos de alarma en presencia de un depredador, y la información de la localización de la fuente de recursos alimenticios (baile de las abejas). En los cercopitecos verdes existen varios gritos de alarma según el tipo de peligro que se acerca. Por ejemplo un ruido específico para las serpientes, otro para los leopardos, y otro para las águilas. Esta variedad de gritos se interpretaría con la respuesta adecuada correspondiente a cada peligro. Si es la serpiente se quedan de pie y buscan al animal; si es el leopardo se suben a un árbol; y si es un águila se esconden en la vegetación.

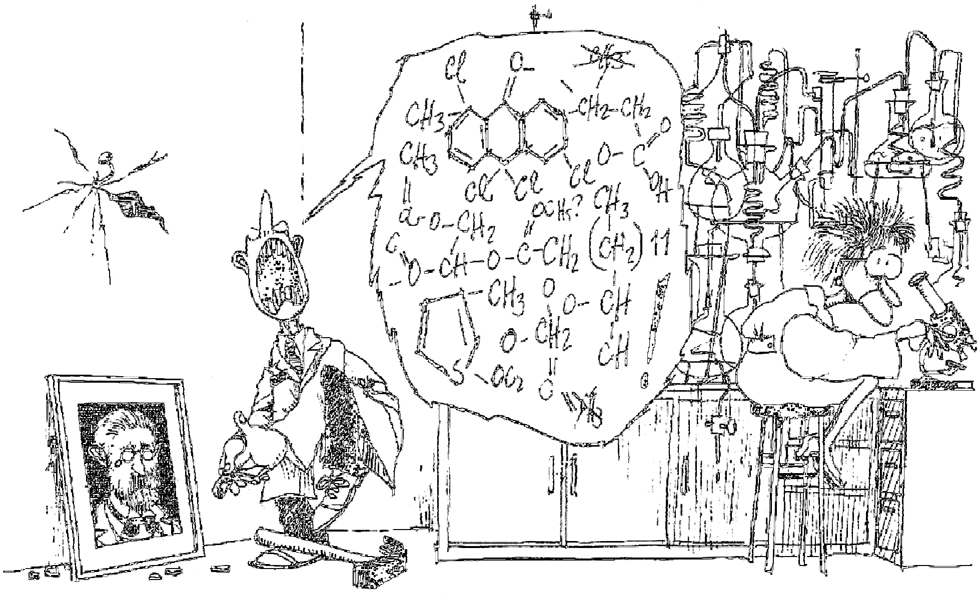
Señales Sexuales

Esta forma de comunicación sexual se encuentra en numerosos organismos desde las bacterias hasta los humanos (Bernstein C y H 1997). Los caracteres secundarios sexuales permiten una comunicación entre machos y hembras, aumentando o modulando la probabilidad de interacciones sexuales entre individuos.

Las señales favorecidas por la selección sexual tienen también un importante papel en el reconocimiento y la divergencia entre especies (Darwin 1871; Anderson 1994). Los mecanismos de reconocimiento sexual utilizan señales visuales, olfativas, auditivas y/o táctiles.

La mayor parte de los trabajos se han centrado especialmente en señales visuales y sus relaciones con los procesos evolutivos (Maynard-Smith y Harper 2003). Los otros tipos de señalización como la acústica y, sobre todo, la química han recibido, en general, menos atención (Alberts 1992; Wyatt 2003).

COMUNICACIÓN QUÍMICA



La percepción de calidades químicas ambientales o quimiorrecepción está presente en muchos organismos. El sentido de recepción de las señales químicas es uno de los sentidos más antiguos, más diversificados y complejo.

Desde hace mucho tiempo el papel de los olores en los insectos ha interesado a los investigadores. En el siglo XVIII, los coleccionistas utilizaban las hembras vírgenes de las mariposas para atraer a los machos. J.H. Fabre fue el primero que hizo experimentos de atracción entre machos y hembras de *Saturnia pyri* en 1879. Pero el científico no sabía cómo explicar lo que observaba: *"si es el olor el responsable de la atracción de los machos, ellos deberían tener nariz, pero no se observa ninguna"*. Más tarde el mismo suponía que "*el órgano (de la olfacción) está en las antenas. Es admisible, pero es complicado de entender que un palo de círculos de cornea articulado pueda ser la nariz*".

En 1919, K von Frish demostró el papel de las antenas en la percepción de los olores en las abejas. No fue hasta 1959 que A. Butenandt y Karlson lograron caracterizar la primera feromona: bombykol, extraída de las hembras de *Bombix mori* para atraer a los machos. El siguiente año, M. Barbier (1982) aislaron e identificaron la sustancia real de la glándula mandibular de la reina de las abejas que inhibe el desarrollo de los ovarios de las obreras.

A partir de ahí nació una nueva disciplina: la **Ecología Química**, ciencia de las relaciones químicas entre los seres vivos o entre el mundo mineral y el vivo. Las moléculas activas son de tipo atractivo como las feromonas, o los mensajes intraespecíficos utilizados en las relaciones sexuales, o la diferenciación de castas en insectos. Otras, como las defensas químicas de tipo repulsivo son más mensajeros interespecíficos de especies en competición (Barbier 1982). Pioneros como E. Sondheimer y J. Simeone y la utilización de la cromatografía de gases acoplada a la espectrometría de masas ayudaron a la progresión de esta nueva disciplina y a un mejor conocimiento de los mediadores químicos de los animales y plantas.

QUIMIORRECEPCIÓN

La quimiorrecepción permite al animal detectar y reconocer las señales químicas de su entorno. Depende de la conformación fisicoquímica de la energía contenida en productos químicos de peso molecular bajo que son a menudo liposolubles; son los olores. La quimiorrecepción implica que las substancias informativas volátiles (medio terrestre) o disueltas (medio acuático) no tengan valor nutritivo. Todas las substancias comprometidas en la comunicación química se calificaron de metabolitos secundarios, es decir productos derivados del metabolismo general y que no juegan ningún papel vital, pero que son propios de cada especie; y podrían expresar la diversidad del mundo vivo. De los insectos a los mamíferos todos los animales utilizan en diferentes grados señales para comunicarse entre ellos, localizar las fuentes de comida o enterarse de un peligro. La característica principal de la comunicación química es la especificidad. El animal es capaz de extraer y reconocer un número de moléculas biológicas significativas a partir de un ruido de fondo químico. Estas moléculas provocan después comportamientos específicos. Las capacidades de discriminación serian muy altas.

El termino de feromona propuesto en 1959 por Karlson, Luscher y Butenanlt, fue objeto de polémica. En las relaciones entre individuos, el sistema de comunicación se descompondría en tres elementos: la emisión de una señal química por un individuo, la recepción y el reconocimiento de esta señal por otro individuo, y la reacción del individuo receptor en función de la información traducida. Si los individuos emisor y receptor son de la misma especie, se hablaría de comunicación intraespecífica y las moléculas serian feromonas. Si los individuos son de especies diferentes, se trata entonces de comunicación interespecífica con sustancias aleloquímicas (Whittaker y Feeny 1971) (Fig. 4). La comunicación intraespecífica juega un papel importante en el comportamiento sexual y la vida social. La interespecífica interviene más en la relación con los depredadores, presas, parásitos, y hospedadores.

Ectomonas

Señales químicas emitidas por un organismo y que modifican el comportamiento o fisiología de otros organismos

Compuestos aleloquímicos	**Feromonas**
Acción interespecífica	***Acción intraespecífica***
Alomonas (Secreciones repulsivas, defensivas, veneno...)	Feromonas incitativas (Sexuales, alarmas, pistas, sociales, agregación...)
Acción benéfica para el individuo que emite la señal	*Señal química que provoca una modificación inmediata del comportamiento del individuo que la recibe. Efectos reversibles*
Kairomonas (Atracción alimenticia, alarma de peligro)	Feromonas modificantes (Castas, gregarización...)
Acción beneficiosa para el individuo que recibe la señal	*Señal química que provoca importantes modificaciones de la fisiología de los individuos que la perciben. Efectos irreversibles*

Fig.4: Clasificación de las señales químicas.

Muchas feromonas (incluyendo las feromonas sexuales de las moscas) no son un único compuesto sino una composición de varios tipos de moléculas en una proporción específica (Wyatt 2003). La omnipresencia y la variedad de feromonas se explicarían por la selección natural. El desarrollo evolutivo de las feromonas sexuales en un pez, por ejemplo, podría haber comenzado con un pez macho que detectara las hormonas sexuales de las hembras en fase de reproducción. Los machos más sensibles llegarían allí primero. Después de varias generaciones, habría una selección para que la sensibilidad de detección aumente.

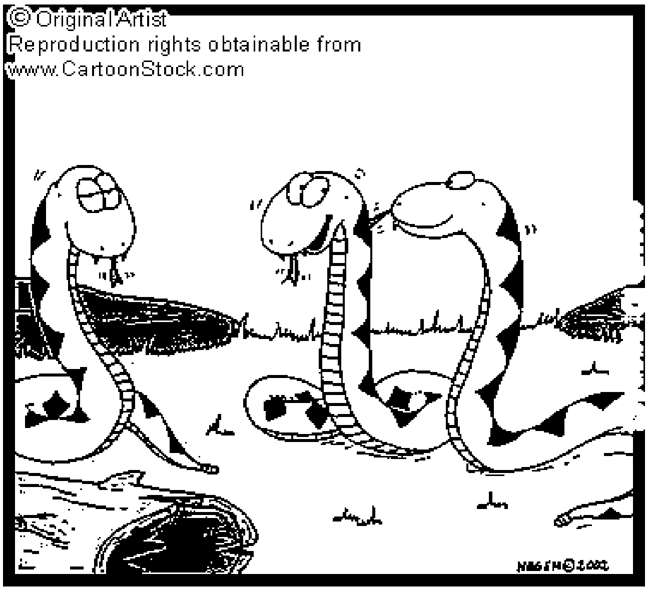
La composición y proporción relativa de los componentes de las feromonas son altamente específicas de cada especie. Existe una enorme diversificación de la composición de las feromonas en relación con las especies. Como cualquier señal, las feromonas están sometidas a las presiones selectivas naturales y sexuales (Greenfield 2002). La distinción entre las modalidades de las feromonas y otras señales ayuda a entender cómo han evolucionado. Si se añade, o se transforma o se quita sólo una molécula, se cambia totalmente la marca, lo que también permite tener una diversidad importante de este tipo de señal. Las sustancias químicas son importantes para el reconocimiento entre individuos, los índices de dominancia, el marcaje territorial (Howard y Blomquist 2005; Brennan y Kendrick 2006), y la elección de pareja (Johansson y Jones 2007). También, la composición de las feromonas y de los grupos de compuestos, estarían bajo el control de unos pocos genes (Löfstedt 1990). Estudios genéticos han demostrado que un simple "desliz" genético generaría nuevas feromonas con sólo cambiar las vías de elaboración de las enzimas que fabrican los compuestos químicos (Xue et al. 2007).

El concepto de feromona hizo frente a los períodos claves de controversia respecto a las feromonas de mamíferos, como con el ejemplo del lémur de cola anillada, que agita la cola cubierta de sustancias químicas para señalizar su estatus de dominancia. En los años 70, un grupo de investigadores que estudiaba mamíferos sostuvo que el término de feromona no debería ser usado para las señales químicas de los mamíferos, citando en particular el complejo de olores variables que los mamíferos utilizan para distinguir a los congéneres durante la elección de pareja o el altruismo. Estos olores individuales no parecían adaptarse a la definición de feromona de Karlson y Lüscher.

Las pequeñas moléculas específicas de cada especie que verdaderamente se adaptan a la definición de feromona clásica han sido identificadas en mamíferos ahora. El más espectacular fue el descubrimiento en 1996 de que la feromona sexual de las hembras de elefante asiático es una pequeña molécula (Z)-7-acetato dodecen-1-il, que también se encuentra en 140 especies de polillas como un componente de las feromonas del órgano sexual femenino (Wyatt 2003).

Otro grupo de investigadores - biólogos principalmente moleculares - aceptó la idea de que en los mamíferos se hablara de feromonas, pero que éstas son exclusivamente detectadas por un sistema sensorial especializado, el órgano vomeronasal (VNO), más bien que por el epitelio olfativo principal de la nariz. Finalmente, en 2005, una serie de estudios moleculares en ratones, utilizando las combinaciones de marcadores genéticos y de los "knockouts", confirmó que las feromonas podían estimular ambos sistemas sensoriales, que eran integrados en el cerebro (Wyatt 2003). Por tanto los individuos no necesitarían un VNO para recibir las señales de las feromonas después de todo.

QUIMIORRECEPCIÓN EN REPTILES



Stop whispering in my ear, it tickles!

La quimiorrecepción es utilizada en una multitud de contextos (Wyatt 2003). En muchos animales, las señales químicas son importantes para el reconocimiento de especies y tendrían consecuencias para la especiación, como en algunas moscas (Roberts y Uetz 2004; Symonds y Elgar 2004; Mas y Jallon 2005; revisado en Smadja y Butlin 2009). Johnston (2000, 2001) define las "señales químicas" como compuestos químicos o mezclas secretadas por un individuo que afectan a un segundo individuo de la misma especie y sugiere que este término debe ser utilizado como un nombre genérico para olores implicados en la comunicación.

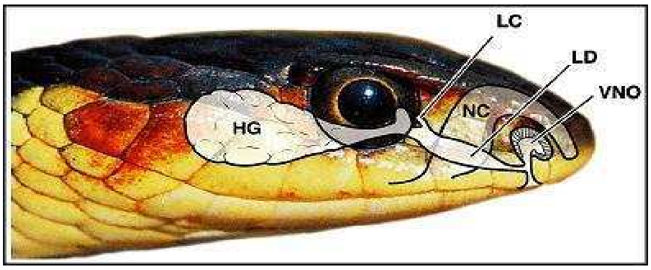
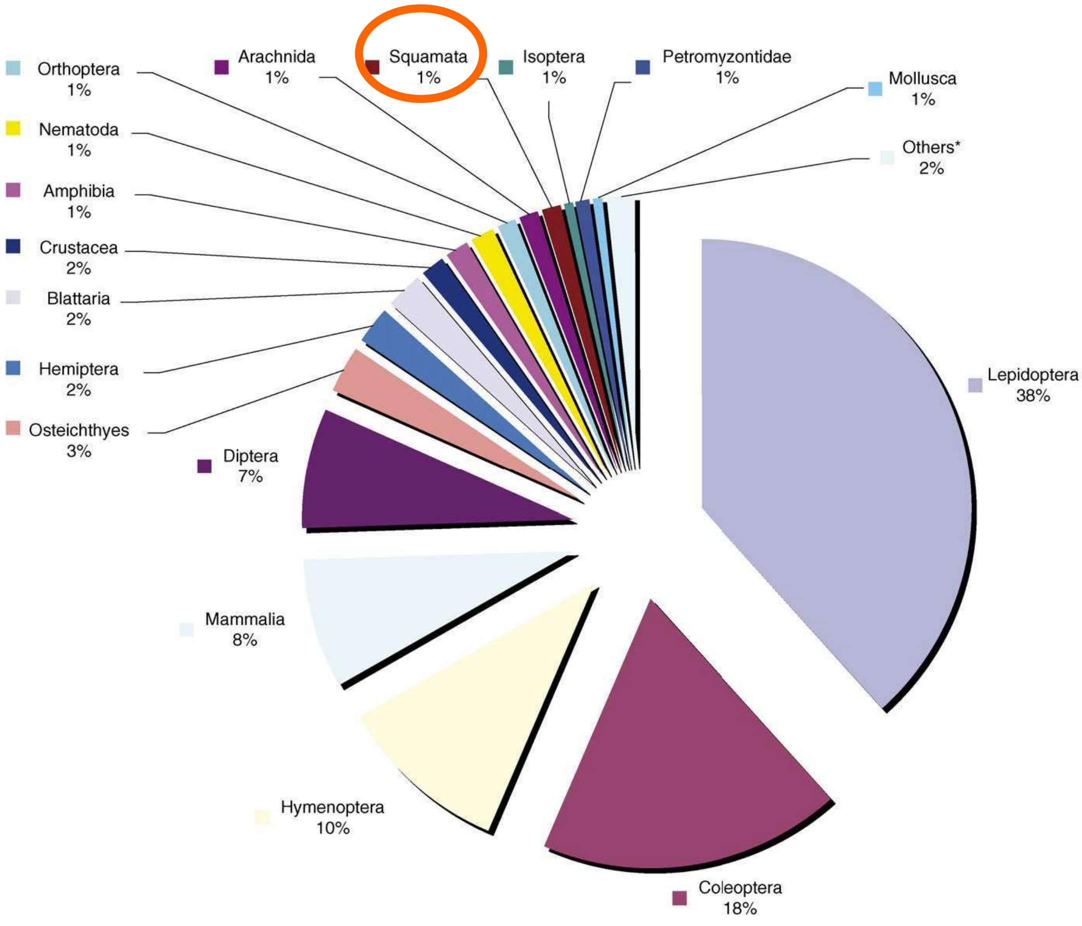


Fig. 5: Sistema vomeronasal de *una serpiente* (HG=Glándula Harderiana, LC=canal lagrimal, NC=cavidad nasal, LD=conducto lacrimal, VNO=órgano vomeronasal)

El estudio del sistema vomeronasal de los reptiles (VNO) permitió algunas de las primeras demostraciones del papel del VNO en comportamientos como el reproductivo o los relativos a la alimentación (Fig. 5). En muchas serpientes y lagartijas, los estímulos químicos son una fuente importante de información relativa al entorno, y al medio ambiente, eso tendría importantes consecuencias para el *fitness* (Halpern 1992; Mason 1992; Schwenk 1995). Ha sido demostrado en serpientes que este VNO es crítico en varios tipos de comunicación (Halpern 1987; Wysocki y Meredith 1987; Graves et al. 1991). La mayoría de las serpientes tendrían la habilidad de seguir el rastro de olores en el sustrato; estos olores poseerían una función en la localización de la pareja sexual, de la agregación durante la hibernación, del seguimiento de una presa, o al contrario, de avisar de la presencia de un depredador o conespecífico al que evitar (Ford y Low 1984; Ford 1986; Constanzo 1989; Mason 1992; Greene et al. 2001; LeMaster y Mason 2001).



TRENDS in Ecology & Evolution

Fig. 6: Diversidad Taxonómica en la investigación de las feromonas (Symonds y Elgar 2009).

Fig. 7: Ilustraciones de extracciones linguales en lagartijas (*Podarcis hispanica*) y serpientes (*Boa constrictor constrictor*) (arriba), además de poros femorales en lagartijas (*Podarcis hispanica – Uta sp*) (abajo).

Las serpientes se orientan utilizando solamente información química que está en el entorno, y esto tiene consecuencias en su éxito reproductivo (Shine et al. 2005). Las hembras de serpientes de al menos cinco familias, producen rastros de feromonas para que los machos las localicen durante la reproducción (e.g., Ford y Low 1984; LeMaster y Mason 2001; LeMaster et al. 2001). Estas feromonas depositadas por las hembras informan sobre la especie, el sexo, el atractivo sexual, el estado reproductor y sobre el camino que seguir para llegar al emisor (Ford 1982; Ford y Schofield 1984; O'Donnell et al. 2004).

La comunicación sexual ha sido, en general, menos explorada en reptiles, con respecto a otros grupos (Fig. 6). Sin embargo, los reptiles son un grupo muy diversificado, con variados sistemas de selección sexual, con complejas interacciones ecológicas y una fuerte dependencia del medio (Pleguezuelos 1997; Zug et al. 2001). Al igual que en los invertebrados, en los reptiles las señales químicas juegan un papel muy importante en los procesos evolutivos en especial en la reproducción. Es el caso de las lagartijas y las serpientes, que poseen un sistema sensorial químico muy bien desarrollado, donde los estímulos químicos serían la base de un reconocimiento intra-específico y contribuir a procesos de especiación (Shine et al. 2002; LeMaster y Mason 2003). Los compuestos químicos secretados y depositados durante la reproducción podrían ser también la base de un identificación entre especies y servir para evitar las cópulas entre individuos de distintas especies o de especies simpátridas (Cooper y Vitt 1986; Mason 1992; Shine et al. 2002) o entre individuos de la misma especie pero de poblaciones distintas (LeMaster y Mason 2003).

Muchas especies de lagartijas disponen, en posición ventral, de estructuras epidérmicas similares a poros por medio de los cuales secretan sustancias químicas (Fig. 7). Se trata de una secreción holocrina, producida por las glándulas femorales; especialmente abundantes en los machos durante el periodo reproductivo (Mason 1992; Alberts 1993). Estas substancias informarían sobre el estatus de dominancia del macho y su habilidad competitiva (Aragón et al. 2001; López y Martín 2002). También estas secreciones femorales transmitirían a las hembras información sobre la calidad del macho (López et al. 2002; Olsson et al. 2003), o servirían como una herramienta para el reconocimiento entre especies (Gómez et al. 1993; Cooper y Pérez-Mellado 2002). Por su implicación en la selección sexual, estas secreciones y su evolución llevarían a procesos de especiación.

Además, el tipo de señales utilizadas en comunicación intraespecífica estaría sometido a selección, seleccionándose aquellas señales que estimulen el sistema sensorial del receptor de manera más efectiva, dependiendo a veces del medio ambiente (Hipótesis del sesgo sensorial o "*sensory drive*": Boughman 2002). La divergencia en las señales utilizadas en selección sexual se debería a que las condiciones ambientales locales imponen selección variable sobre estas señales o sobre los sistemas sensoriales que las detectan; especies o poblaciones distintas ocupan hábitats con diferentes condiciones que favorecen el uso de uno u otro tipo de señal. Las diferentes propiedades de las señales emitidas por los individuos afectan a su conspicuidad y facilidad de detección por coespecíficos, pero otros factores como la capacidad de transmisión o persistencia en el hábitat también son importantes (Boughman 2002; Leal y Fleishman 2002; Fox y Shipman 2003). Se ha examinado esta idea respecto a señales visuales que muestran variación en diferentes hábitats (Leal y Fleishman 2004), y a como estas señales han divergido entre poblaciones alopátricas que ocupan distintos hábitats (Endler 1980) llevando a procesos de especiación (Boughman 2001). Aunque la quimiorrecepción modula una gran variedad de comportamientos reproductivos en reptiles, se desconoce prácticamente si la variabilidad interpoblacional en el uso de señales químicas y visuales implicadas en selección sexual elucidarían los procesos evolutivos de especiación.

En este contexto, en esta tesis se pretende examinar con observaciones y mediante experimentos si existen variaciones poblacionales en el tipo y características de las señales químicas utilizadas en selección sexual, debido a la distinta efectividad de cada tipo de señal en hábitats con condiciones ambientales diferentes. Así como si este uso diferencial en el tipo de señales empleado puede conducir a procesos de divergencia, aislamiento reproductivo y especiación mediados por selección sexual.

EL GENERO *PODARCIS* Y EL COMPLEJO DE ESPECIES DE *PODARCIS HISPANICA*

El género *Podarcis* (Wagler, 1830) es un grupo de lagartijas amplio y diverso que se encuentra en el área mediterránea (Arnold 1973, 1989; Arnold et al. 2007) (Fig. 8). El género *Podarcis* se localiza desde Europa central hasta al desierto del Sahara. Algunas de estas especies están en peligro de extinción (Capula et al. 2002) mientras que otras tienen una capacidad de colonización elevada, en especial con la ayuda del transporte humano (Corti et al. 1999).

Fig. 8: Distribución geográfica del género *Podarcis* en Europa

Datos taxonómicos

Desde que fue descrita por Arnold (1973), la taxonomía de *Podarcis* es muy controvertida y polémica, por su inestabilidad y variabilidad. Se han creado muchos debates alrededor de este género y dentro del género (Böhme 1997; Arnold 2000; Lanza y Boscherini 2000; Böhme 2004; Speybroek y Crochet 2007).

Entre las lagartijas de este género que ocupan la Península Ibérica, la primera distinción dentro del género fue la de *P. muralis* de Centro Europa y el norte de la Península Ibérica, después se describieron *P. bocagei* y *P. carbonelli* que se distribuyen sólo en la parte oeste de la Península Ibérica (Arnold y Burton 1978; Pérez-Mellado 1981a,b). El resto fue llamado *P. hispanica* (Steindachner, 1870). Además, una forma endémica, nominada *P. atrata* (Boscá 1916) se situó en las Islas Columbretes, Valencia (Castilla et al. 1998a,b).

Pero se admitió también que *P. hispanica* (*Ph*) tiene un polimorfismo alto y se podría distinguir del tipo del norte de África (Pérez-Mellado y Galindo-Villardón 1986). En 1987, Guillaume sugirió que se encuentran 8 formas o subespecies de *Ph*. En 2001, Sá-Sousa describió 4 formas sólo en Portugal: *P. bocagei ss, P. carbonelli,* y dos morfotipos (1 y 2) de *Ph*. Recientemente, Geniez (2001) añadió más formas o subespecies pero de momento no están confirmadas.

Actualmente, se acepta que *P. vaucheri, P. bocagei* y *P. carbonelli* sean consideradas a nivel de especies, pero al respecto de *Ph* se habla más de un grupo artificial (Montori et al. 2005). Finalmente aparte de los dos morfotipos de *Ph* se conoce un tercer morfotipo que también se encuentra bajo el nombre de *P. liolepis* que se sitúa en el noreste de la Península Ibérica y en el área mediterránea de Francia (Harris y Sá-Sousa 2002; Speybroek y Crochet 2007).

En resumen, los estudios de taxonomía clasificaron dentro del género *Podarcis* la existencia claras de especies distintas como *P. muralis*. Sin embargo, alrededor de *P. hispanica*, no está tan claro. Se acepta la posibilidad de subespecies, morfotipos, pero nada definido a nivel de especie.

Ayuda de las herramientas moleculares

Se han realizado numerosos análisis moleculares utilizando varias técnicas como las aloenzimas (Bea et al. 1986; Guillaume 1987), los patrones de filogeografía (Mayer y Tiedemann 1980; Busack 1986; Copula 1994a,b; 1997) o el ADN mitocondrial para poder identificar la posible filogenia de este género (Fu 1998; 2000; Harris et al. 1998; 2005; Arnold et al. 2007). Primero los trabajos mostraron que el mismo género se clasificaría en clados monofiléticos (Arnold 1973; 1989). Se caracterizan 4 clados geográficamente coherentes: Balcánico, Italiano, Tirreno-Balear e Ibero-Magrebí (Harris y Arnold 1999; Carretero 2008). La posición de algunas especies o subespecies es todavía objeto de debate, pero el clado Ibero-Magrebí es considerado como monofilético, a pesar de las más que probables relaciones internas (Harris et al. 2005; Poulakatis et al. 2005; Arnold et al. 2007).

Recientemente, se han estudiado las relaciones internas y el numero de linajes en este clado, encontrándose 12 linajes evolutivos distintos (Harris y Sa-Sousa 2002; Sa-Sousa y Harris 2002; Pinho et al. 2006) (Fig. 9 y 10). Únicamente *P. hispanica* es valorada como parafilética;

con dos “taxones hermanos” *P. bocagei* y *P. carbonelli* (Pinho et al. 2006); el resto de las especies definidas claramente son monofiléticas. Además nuevos análisis con marcadores nucleares, que permiten estudiar el flujo genético entre las formas de este género, han afirmado que sólo existe una barrera de flujo entre *P. bocagei* y *P. carbonelli*. Sin embargo, de momento no se sabe mucho sobre el resto de las formas que estarían en contacto; sólo que a lo mejor el flujo sigue (Pinho et al. 2007, 2008).

Una combinación de alta divergencia genética dentro del clado y su aparentemente parafilia sugiere que *P. hispanica* forma un **complejo de especies**, donde los morfotipos descritos (dos subespecies, *P. bocagei*, *P. carbonelli* y los tipos de *P. hispanica*) corresponden a los linajes definidos con los análisis de ADN mitocondrial. Se caracterizarían por un grupo de varios morfotipos, tipos, o subespecies que tendrían características distintas (a nivel de coloración, morfología, etc.) y que viven en sitios similares hasta en el mismo tipo de hábitat. Pero establecer el nivel de separación taxonómico entre ellas es más complicado; estos morfotipos están muy cerca a nivel genético (con algunas diferencias) pero quedan muchas dudas como para poder definirlas como especies propiamente.

Actualmente se observa una distribución enredada de los linajes del complejo en la Península Ibérica y el norte de África. En el noreste se encuentra el tipo 3 o linaje *P. liolepis*. En el sur y centro se localiza el morfotipo 2. El morfotipo 1 está situado en el noroeste. Pero este linaje está ahora en cuestión, con la nominación de dos morfotipos 1a y 1b. En el sur de la Península se caracterizan varios grupos como *P. hispanica s.s.*, *P. hispanica* “Sierra Nevada” y *P. vaucheri*. Esta última se encuentra también en el norte de África; además se diferenció un tipo de *P. hispanica* en Túnez. Algunos de estos grupos de *Ph* están en simpatría con otros linajes como *P. bocagei* con *Ph 1,* o *Ph 2* con *P. carbonelli*.

Los análisis moleculares reforzaron el conocimiento de las especies bien definidas del género, y la clasificación del complejo de *Podarcis* pero este aun sigue “turbio”. No se conoce si estos morfotipos cercanamente emparentados son especies distintas o más bien una sola especie ampliamente distribuida con características diferentes según el medio.

Fig. 9: Diferentes fotos del complejo de Podarcis: *P. bocagei, P. carbonelli, P. hispanica 1 & 2, P. vaucheri, P. liolepis,* y *P. hispanica ss.*

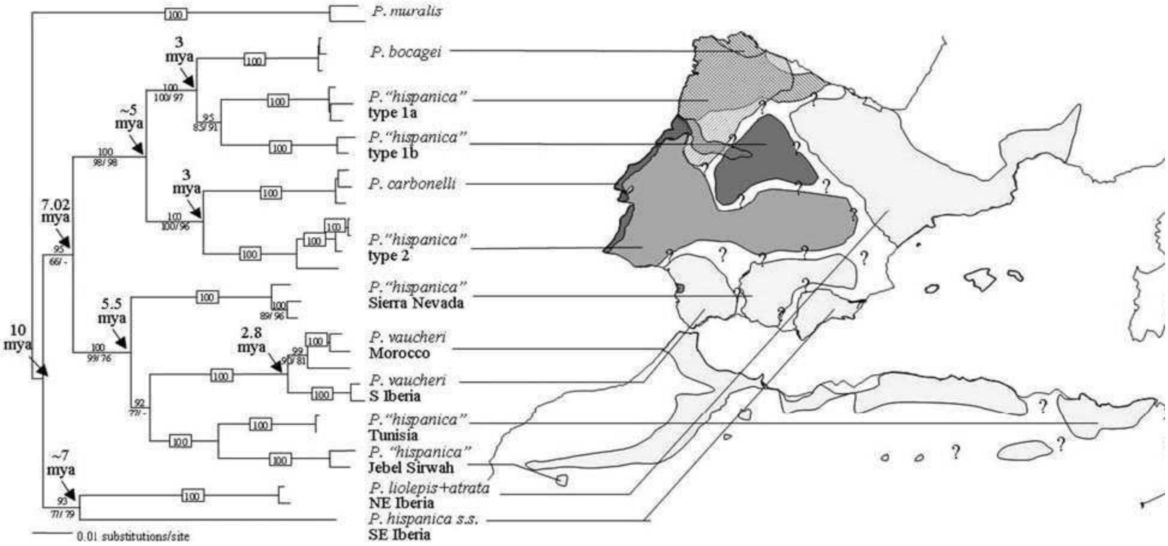


Fig. 10: Distribución estimada del género *Podarcis* en la zona Ibérica y las relaciones filogenéticas posibles entre los diferentes tipos de *Podarcis* en África del Norte y la Península Ibérica basada en análisis mitocondrial y de ADN (Pinho et al. 2006; Sá-Sousa 2000, 2001; Pinho et al. 2008).

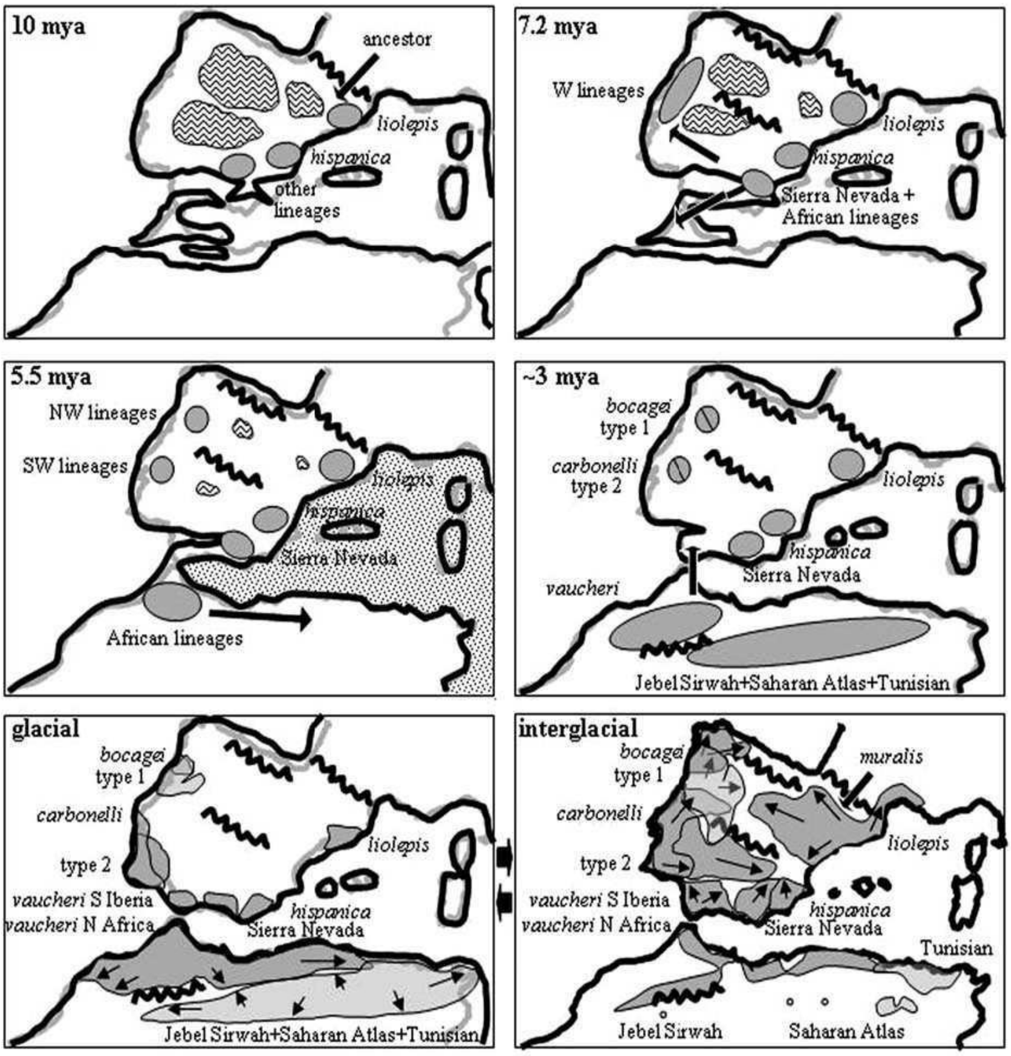


Fig. 11: Escenario paleo geográfico de la evolución de *Podarcis* en la Península Ibérica y norte de África basada en análisis de citocromo b (Pinho et al. 2006; Carretero 2008).

Escenario filo-paleo-geológico

Con todas estas características distintas de este género, cabría preguntarse sobre cómo se ha llegado a este patrón con tantas formas/subespecies/especies; ¿con qué escenario paleogeológico? Pinho en 2006 propuso uno (Fig. 11). Hace 10 millones de años (ma.), en el mioceno medio-superior, los Pirinéos estaban formándose y el clima era húmedo, subtropical; lo que permitió la dispersión y diversificación de los grupos hacia la Península Ibérica. Después, se juntó el archipiélago del Estrecho Bético (7.2 ma.). A la transformación gradual del archipiélago se añadió una crisis de aumento de la salinidad que coincidió con una sequía global del clima (Krijgsman et al. 2000). Esto pudo tener un efecto de aislamiento de los clados presentes en el oeste y este (Krijgsman et al. 2000). Además *Podarcis* es un género que depende de la humedad del entorno, entonces los cambios climáticos les debieron afectar bastante y provocar cambios importantes, influyendo sobre su diversificación (Sa-Sousa 2001b; Kaliontzopoulou et al. 2008). La evolución de la vegetación y geografía, además del clima árido del momento, hizo que los grupos de *Podarcis* se dispersaran y aislaran unos de otros (Favre et al. 2007; Pinho et al. 2006).

Cuando se formo el Estrecho de Gibraltar de la Península, se produjo el aislamiento de las *Podarcis* del norte de África que tendrían una evolución separada de las de la Península (Hsü et al. 1977; Duggen et al. 2003). Durante el plioceno (3 ma.) el clima era más frío, seco y estacional (van Dam 2006). Lo que provocó una separación de las partes oeste y este del norte (Pinho et al. 2006). También fue en este momento cuando se sugiere que ocurrió la separación del tipo de norte de África con el tipo de la península (Pinho et al. 2006). Durante el periodo de glaciación, las sierras del norte y las centrales fueron cubiertas por la tundra con las estepas y la taiga (Tarroso 2008). *Podarcis* tuvo como refugio unos pocos territorios costeros (Gomez y Lunt 2007). Sin embargo la glaciación no implicó necesariamente una separación de todos los linajes, algunos han podido compartir refugios glaciales (Gomez y Lunt 2007). En África, la glaciación correspondió a un periodo húmedo (Prentice y Jolly 2000), lo que permitió una expansión de los grupos. Finalmente, el periodo interglaciar llegó y permitió la interconexión e introgresión entre los linajes. Pudieron dispersarse por toda la península e invadir nuevos hábitats.

En resumen, se han definido varios linajes, grupos, formas, subespecies, y especies del genero *Podarcis*, distribuidos por toda la Península Ibérica y el norte de África. Pero quedan dudas sobre el establecimiento de los linajes del complejo *Podarcis hispanica*, y sobre lo que ocurre en los sitios de contacto. ¿Hay flujo de genes en estos puntos? ¿La especiación y divergencia han sido suficientemente importantes para que estos linajes se comporten como especies distintas? ¿Sigue existiendo un proceso de evolución que va en la dirección de crear un aislamiento fuerte entre los linajes? ¿O al contrario, un flujo de material genético impide este proceso de separación? ¿Estas formas distintas tienen también un comportamiento diferente? ¿La comunicación entre las formas dentro del grupo se caracteriza como interespecífica o intraespecífica? Con estas preguntas, hemos reflexionado en esta tesis sobre lo que pasaría en las zonas de contacto entre varios tipos del complejo de especies de *Podarcis hispanica* a nivel de la comunicación química: ¿Existirá un aislamiento precopulatorio entre individuos de distintos tipos dentro de este complejo que permitiría una posible especiación?

MODELO DE ESTUDIO

- *Podarcis hispanica* en la región de Madrid -

Fig. 12: Fotografías de las lagartijas *Podarcis hispanica* en varias zonas de las region de Madrid.

La lagartija Ibérica, *Podarcis hispanica,* es una pequeña lagartija común (50-70 mm) que vive en hábitats rocosos pero en distintos entornos ambientales y geográficos de la Península Ibérica. Esta lagartija ha desarrollado una habilidad a nivel del reconocimiento químico, y es capaz de discriminar químiosensorialmente entre coespecíficos y heteroespecíficos (*Psammodromus algirus*, Gómez et al. 1993 - *Podarcis carbonelli*, Cooper y Pérez-Mellado 2002; Barbosa et al. 2006) y entre sexos (Gómez et al. 1993; López y Martín 2001b; López et al. 2002). Las señales químicas de los machos son importantes en las interacciones macho-macho (López y Martín 2002a,b; Carazo et al. 2007). Además, en por lo menos una población de *Ph*, las hembras prefieren estar en áreas marcadas con olores o marcas químicas de machos que tienen altas proporciones de unos esteroides determinados en sus secreciones femorales (López y Martín 2005c; López et al. 2008). Las hembras parecen utilizar las secreciones de los machos como fuente de información decisiva en su elección de pareja. Si la preferencia de la hembra por las secreciones del macho evolucionase de forma diferente a éstas se podría dar el caso de aislamiento reproductivo.

Estudios recientes morfológicos y de biología molecular han sugerido la existencia de procesos de especiación en marcha en poblaciones de lo que tradicionalmente ha sido reconocida como una sola especie, la lagartija Ibérica (*Podarcis hispanica*). Se considera ahora como una especie parafilética que forma un "complejo de especies" con al menos cinco líneas monofiléticas (Guillaume 1987; Harris y Sá-Sousa 2001, 2002; Sá-Sousa et al. 2002; Pinho et al. 2007), que ocupan distintas condiciones ambientales, pero cuyas poblaciones están en contacto en diversas partes, especialmente en el Centro de la Península Ibérica.

De modo que esta lagartija es una buena candidata para estudiar si la selección sexual mediada por señales químicas puede explicar procesos de aislamiento reproductivo y especiación.

Se han identificado dos tipos alopátricos en el suroeste y la parte central de la Península Ibérica. *P. hispanica* de tipo 1 se encuentra en sitios elevados del Noroeste, donde las condiciones ambientales son húmedas, mientras que el tipo 2 ocupa el centro y sur de la Península con clima mediterráneo (Sa-Sousa 2000; Sa-Sousa et al. 2002). Sin embargo, estos dos tipos podrían estar en contacto geográficamente sin producirse aislamiento. Por ejemplo, en la sierra de Guadarrama (al norte de Madrid), viven poblaciones separadas pero sin aislamiento geográfico, es decir, los individuos de la población tipo 1 pueden encontrarse con los individuos de la población tipo 2 (Mellado y Olmedo 1981; García-Paris et al. 1989).

Zona de estudio

Esta zona de contacto entre morfotipos de *Podarcis hispanica* se localiza en gran parte en la comunidad autónoma de Madrid (España Central) que tiene una superficie de 8.000 Km. (Fig. 13). Sus límites describen un triángulo aproximado, en el que su base está en la linde con la provincia de Toledo, al sur, y su vértice superior en el puerto de Somosierra, al norte. La región está situada en el centro de la Meseta Central, en la parte septentrional de la Submeseta Sur, entre el Sistema Central (al norte y noroeste) y el río Tajo (al sur y sureste), limitando al norte y al oeste con Castilla y León (provincias de Segovia y Ávila) y al este y al sur con Castilla-La Mancha (provincias de Toledo, Guadalajara y Cuenca).

El relieve de la región de Madrid está definido por tres grandes unidades: la sierra y la llanura del río Tajo, separadas entre sí por el piedemonte.

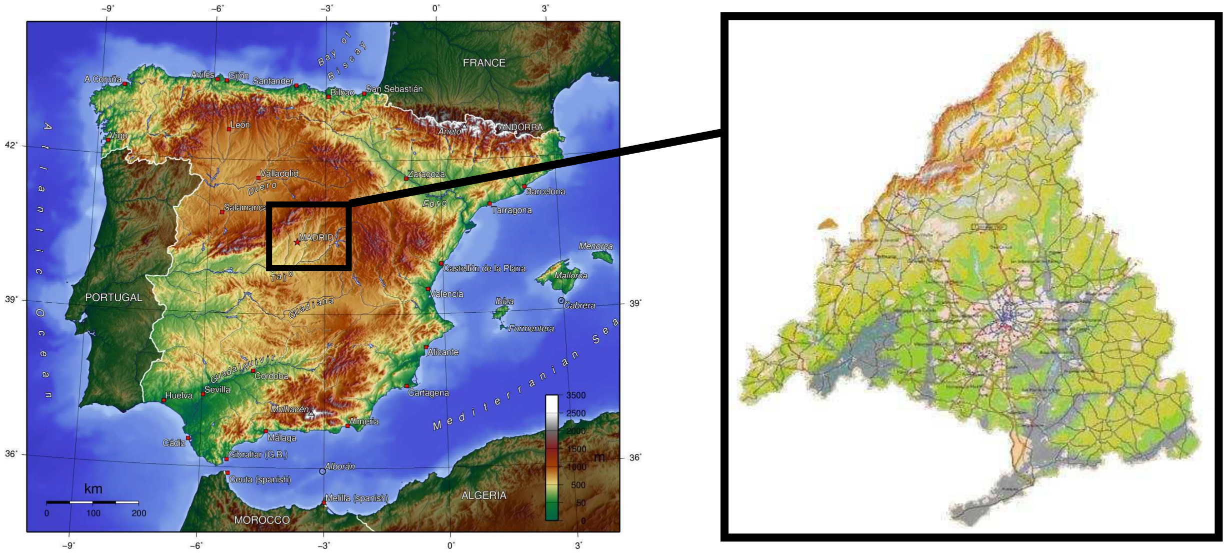


Fig. 13: Mapa de España y del centro Ibérico con la región de Madrid.

La Sierra madrileña está estructurada en falla. Las Sierras de Guadarrama, Ayllón (la parte más occidental de ésta, conocida como Sierra de Somosierra) y Gredos (la parte más oriental de ésta) conforman un paisaje típico de montaña, con altitudes máximas de 2.428 m (Peñalara, el pico más alto de la región), 2.129 m (Peña Cebollera o Pico de las Tres Provincias) y 1.770 m (Alto del Mirlo), respectivamente. Otros picos importantes son La Maliciosa (2.227 m) y Siete Picos (2.138 m), ambos en la Sierra de Guadarrama. En lo que respecta a su litología, el granito y el gneiss son las rocas dominantes en la dos primeras sierras, mientras que la pizarra y las cuarcitas lo son en la de Ayllón (este macizo presenta los materiales rocosos más antiguos de la zona de Madrid, formados hace 450 millones de años). Las montañas de Madrid que tienen más de 2.200 m de altitud pertenecen a la Sierra de Guadarrama. Estos picos, ordenados según altura, son los siguientes: Peñalara, 2.428 m, Risco de los Claveles, 2.387 m, Cabezas de Hierro, 2.383 m, Risco de los Pájaros, 2.334 m, Dos Hermanas, 2.285 m, Cerro de Valdemartín, 2.280 m, Bola del Mundo, 2.265 m, La Maliciosa, 2.227 m, y El Nevero, 2.209 m.

Las campiñas, páramos y vegas configuran geomorfológicamente la segunda unidad de relieve, articulada alrededor de la cuenca del río Tajo. Aquí se encuentran las mínimas altitudes de la comunidad autónoma: 430 m en el cauce del río Alberche y 467 m en

Fuentidueña de Tajo. Esta unidad presenta una composición del terreno menos uniforme que la de la Sierra. Las calizas, arcillas, yesos y margas son abundantes en los páramos, mientras que las arenas, margas arenosas, margas yesíferas y arcillas dan forma a las campiñas. Las vegas, por último, quedan perfiladas por las arenas, gravas y limos.

A modo de transición entre la Sierra y las llanuras arenosas del río Tajo, aparece la llamada Rampa de la Sierra o piedemonte, que se extiende desde la confluencia de los ríos Jarama y Lozoya, al norte de la provincia, hasta el suroeste de la comunidad, formando una franja paralela a la Sierra. No se trata exactamente de una unidad de relieve, aunque sí cabe definirla así desde un punto de vista geomorfológico. Se compone fundamentalmente de arenas, arcillas, margas y otros materiales detríticos.

Entre la máxima y mínima altitud de la región (Peñalara y Villa del Prado), se origina un desnivel de unos 2.000 m, que se salvan a lo largo de poco más de 100 Km. (la altura media de la provincia es de 650 m, aproximadamente). Este complejo relieve convierte a Madrid en una zona de contrastes medioambientales con una variedad de hábitats para colonizar y adaptarse. En ella se puede encontrar la mayor parte de los pisos bioclimáticos de la Península Ibérica (crioromediterráneo, oromediterráneo, supramediterráneo y mesomediterráneo), además de una rica variedad de ecosistemas.

A pesar de su reducida superficie (8000 Km.), la Comunidad de Madrid presenta dos climas diferenciados, consecuencia de su ubicación entre el Sistema Central y el valle del Tajo. Las zonas más altas de las Sierras de Guadarrama y Ayllón tienen clima de montaña, con temperaturas frías o muy frías en invierno y suaves en verano. Aquí las precipitaciones son abundantes: pueden superar los 1500 mm al año y son en forma de nieve durante el invierno y parte de la primavera.

El resto del territorio madrileño posee un clima mediterráneo continental, de carácter atenuado en el piedemonte y extremado en la llanura mesetaria, en la que se sitúa la capital. En estas zonas los inviernos son frescos, con temperaturas inferiores a los 8ºC, heladas nocturnas muy frecuentes y nevadas ocasionales (tres o cuatro al año). Por el contrario, los veranos son calurosos, con temperaturas medias superiores a los 24ºC en julio y agosto y con máximas que muchas veces superan los 35ºC. La oscilación diaria es de aproximadamente de

10ºC. Las precipitaciones no suelen superar los 700 mm al año y se concentran especialmente en la primavera, seguida del otoño.

***Background Podarcis hispanica*, el área de Madrid y la comunicación química**

Recientemente se ha demostrado, mediante análisis químicos, que los machos de dos poblaciones de la Sierra de Guadarrama, situados en condiciones ambientales diferentes (altitud y clima), poseen variaciones en la composición y proporción de las secreciones femorales (Martín y López 2006a). Los machos de la población situada más al norte (i.e. en el Valle de la Fuenfría) se parecerían a los del tipo 1. Los individuos viven en un microclima más húmedo, tienen en las secreciones femorales más ésteres céreos y una abundancia mayor de ácidos grasos de cadena larga que los de la población del monte de Golondrina (un poco más al sur, cerca del pueblo de Cercedilla); los cuales se asemejan a los del tipo 2 y están en un medio más seco (Martín y López 2006a). Estas diferencias permitirían una mayor persistencia y eficiencia de las señales químicas en el ambiente húmedo. Este análisis caracterizó la presencia y abundancia de algunos compuestos como el colesterol, el dehidrocolesterol (=provitamina D_3), el ácido hexadecanoico, hexadecanol, y octadecanol en las dos poblaciones y como estos compuestos se distribuyen en diferentes proporciones. Además, los machos de cada tipo (poblaciones de Fuenfría y Golondrina) se discriminaron entre ellos por medio de las señales químicas (Martín y López 2006a). Los machos tuvieron un mayor “interés” por los olores de individuos de su población que por los de la otra. Esto significaría que los machos además de discriminar tendrían tendencia a sociabilizarse con individuos de su misma población.

Sin embargo, esto no está tan claro en el caso de las hembras. Unos experimentos de reconocimiento quimiosensorial demostraron que las hembras tuvieron el mismo interés químico por olores procedentes de los machos de su población que por los de la otra población. Esto no implica que ellas no discriminen los dos tipos de secreciones, tampoco que no las diferencien, pero la variación química interpoblacional podría no tener importancia para ellas a la hora de preferir el olor de un macho.

El grado de diferencia morfológica de *P. hispanica* ha sido mucho tiempo polémico, pero ahora se aceptaría la alta variabilidad de formas en la Península (Barbadillo et al. 1999). La lagartijas del género *Podarcis* son muy variables en tamaño, formas, y colores, y la variación ocurre no solamente entre especies reconocidas, sino también entre poblaciones e individuos (Arnold y Ovenden 2002) (Fig. 10). Sabiendo ésto, entender el camino de la historia evolutiva a partir del efecto de adaptaciones locales o de la plasticidad fenotípica (o encontrando un fenotipo cohesivo) parece muy complicado. La distinción entre los morfotipos de *Podarcis* no puede ser concedida ni tampoco necesariamente atribuida a la evolución de la separación. Madrid (Central España) está entre dos áreas de distribución descritas por Pinho et al. (2006), donde se encuentra por lo menos dos de los morfotipos (1 y 2). Se consideraría que Madrid o el centro de España es una zona de contacto de varias formas de *Podarcis* y en especial de *Ph*. En efecto, en esta zona de contacto se localizo en el campo más de dos tipos del complejo de especies de *Podarcis hispanica*; se observaron varias poblaciones que viven en distintos hábitats con diferencias a nivel morfológico, de coloración o de señales químicas (Fig. 12).

Poblaciones estudiadas

Durante el desarrollo de la tesis, se ha capturado machos y hembras de *Podarcis hispanica* en varias localidades del área de Madrid (Fig. 14). Tres de estos sitios se encuentran en la zona montañosa del centro de la península (Fuenfría, Golondrina y Pedrezuela) y otros dos se sitúan en la parte más baja, en el Sur de la comunidad (Belmonte del Tajo y Aranjuez). Estas poblaciones fueron elegidas porque muestran características distintas a primera vista con respecto a la morfología y coloración.

Fig. 14: Localización de las poblaciones estudiadas de *Podarcis hispanica* en la zona de contacto (Madrid).

En el norte, hemos capturado lagartijas de una población que ocupa un hábitat con rocas y paredes de granito al borde de un bosque de pinos de la reserva natural del Valle de la Fuenfría (40° 47’ N, 4° 03’ W; 1750 m altitud). Además, capturamos individuos de unas poblaciones que se encuentran en rocas dentro de un bosque de robles de un monte llamado “La Golondrina”. Debajo de este monte está el pueblo de Cercedilla (40° 44’ N, 4° 02’ W;

1250 m altitud), como a unos 10 Km. La tercera población del norte se localiza en viejos muros que separan parcelas de cultivo o casas viejas en ruinas en el pueblo de Pedrezuela (40° 44' N, 3° 36' W; 800 m altitud).

En el sur, hemos capturado individuos cerca de construcciones humanas, como en un jardín público en el pueblo de Belmonte del Tajo (40° 8' N, 3° 20' W; 735 m altitud) y en rocas de caliza y yeso en zonas sin vegetación arbórea, cerca de campos de cultivos de olivos alrededor de Aranjuez (40° 2’ N, 3° 37’ W; 494 m altitud).

Para la última parte de este trabajo hemos estudiado también las lagartijas de las Islas Columbretes *Podarcis atrata*, cerca de la costa valenciana (39º 55' N, 0º 40' E; 65m altitud), Esta lagartija endémica forma parte del complejo de especies de *Podarcis hispanica* pero se distingue de *P. hispanica* continental a nivel genético (Castilla 1998a,b).

En esta tesis se propone estudiar y conocer si existe reconocimiento intraespecífico a nivel químico entre las poblaciones consideradas de *Podarcis hispanica,* y, si existen, cuáles son los mecanismos de aislamiento reproductivo que podrían dar lugar a un proceso de especiación dentro de este complejo de especies en un área de contacto: la zona de Madrid.

PROCEDIMIENTO

- Parte 1 - Morfología y señales químicas de las lagartijas

En un primer paso, se estudiaron las diferentes poblaciones a nivel morfológico y químico (Capítulo 1). Pesamos a los individuos, y medimos el tamaño corporal y la condición corporal, además del tamaño de la cabeza (largo, ancho y alto). La condición corporal se utilizó como un índice de las reservas de grasa y una estimación de la condición física o estado nutricional de los individuos (Bonnet y Naulleau 1994). Finalmente, anotamos el número de poros femorales de cada pata posterior de cada individuo y contamos el número de los pequeños pero distintivos ocelos azules laterales. En general estas manchas se encuentran en la parte lateral de los machos y son muy vistosas. Los puntos azules (ocelos) tendrían un papel en el reconocimiento entre sexos y en las relaciones entre machos (López et al. 2004). Así que medir estos dos caracteres (poros y ocelos) nos daría una idea del desarrollo de las señales visuales y químicas de los individuos de *P. hispanica* en diferentes poblaciones.

Después, extrajimos las secreciones de las glándulas femorales de los machos, presionando alrededor de los poros femorales, que fueron inmediatamente guardadas en viales de cristal con tapas de teflón. Los viales se conservaron a -20°C hasta su análisis. Las secreciones fueron mezcladas con 250 µl de n-hexano y analizadas con un cromatógrafo de gases acoplado a un espectrómetro de masas (Finnigan-ThermoQuest Trace 2000 GC-MS) (Capítulo 1).

Con estos datos de morfología y química, intentamos ver si los individuos de diferentes poblaciones muestran variaciones en morfología o en la composición y proporción de compuestos químicos en las secreciones femorales de los machos, según su localización geográfica o hábitat (Capítulo 1).

- Parte 2 - Comunicación química y comportamiento interpoblacional

En un segundo paso, estudiamos el comportamiento pre-reproductivo y copulatorio de las lagartijas de varias poblaciones de *P. hispanica* y su reconocimiento interpoblacional a base de señales químicas (Capítulos 2 y 3). Para eso diseñamos experimentos de reconocimiento quimiosensorial basados en las tasas de protusiones linguales frente a diferentes olores, realizamos pruebas de elección de olores por parte de las hembras y finalmente hicimos cruces reproductivos inter e intrapoblacionales.

a. Reconocimiento químico

Se ha demostrado que las lagartijas reaccionan frente a una variedad de estímulos químicos, aumentando o variando su número de protusiones linguales (Cooper y Burghardt 1990). La tasa de protusiones linguales ("*tongue-flicks*"; TFs) se utiliza como un índice cuantitativo para demostrar la detección de señales químicas (Cooper y Pérez-Mellado 2002).

Para examinar las diferentes respuestas o detección por parte de los machos y hembras, les presentamos olores procedentes de varios individuos de diferentes poblaciones y sexos. Comparamos el número de protusiones linguales según el tipo de olor presentado como estímulo impregnado en un bastoncillo de algodón (Cooper y Burghardt 1990). Probamos olores de las secreciones femorales de los machos y de la parte cloacal de las hembras, pues estas áreas son frecuentemente investigadas mediante protusiones linguales durante la reproducción o en los encuentros sociales (López y Martín 2001a,b, 2002a; López et al. 2002).

También los individuos fueron confrontados con compuestos químicos (estándares comerciales) que se encuentran de forma natural en las secreciones femorales, como algunos esteroides o alcoholes (Capítulos 4 y 5), para ver cómo reaccionan, si muestran un interés y si estos comportamientos difieren según las poblaciones o sexos estudiados.

b. Preferencia de olores por parte de las hembras

Las hembras se sometieron a pruebas en las que se les ofrecían sustratos marcados con olores de machos, de su misma población o de otra, en un mismo terrario (Capítulos 2 y 3). Si las hembras se situaban en un lado con un olor determinado se consideraba que habían elegido temporalmente estar en ese lado con este olor (Martín y López 2000, 2006c; Olsson et al. 2003). Para eso, contamos el número de veces que cada hembra fue observada en el lado de cada olor y analizamos las diferencias de respuesta según los estímulos y según las poblaciones estudiadas. También se analizó la elección o preferencia de olores de otra forma.

Supusimos que las hembras estarían interesadas en un compuesto químico especial de las secreciones femorales de los machos. Así que en vez de comparar los olores entre poblaciones, miramos en cada par de olores presentado cuales eran las proporciones del compuesto en cuestión (Capítulo 3).

c. Copulas inter e intrapoblacionales

Hicimos encuentros entre machos y hembras de la misma o distinta población para examinar el éxito de cópulas intra e interpoblación (Capítulo 3). Cada macho se cruzó y pudo copular con hembras de su población y de otra. Apuntamos si había o no cópulas, la duración de la cópula, el tiempo de latencia, el número de intentos, el comportamiento de la hembra, etc. Para obtener una idea de la barrera pre-reproductiva entre las poblaciones de esta zona de contacto.

- Parte 3 - Reconocimiento de compuestos de las señales químicas

En un tercer paso, intentamos definir la capacidad de reconocimiento de algunos compuestos químicos por parte de las lagartijas (Capítulos 4 y 5). Las secreciones femorales de los machos de *P. hispanica* están compuestas de varios tipos de moléculas químicas, pero en especial de esteroides, ácidos carboxílicos y alcoholes (Martín y López 2006a). Partiendo de la hipótesis que las secreciones femorales de los machos difieren en composición y proporción de los compuestos entre las poblaciones estudiadas (Capítulo 1), y que los compuestos químicos podrían informar sobre la salud, estatus, etc. de los emisores (López y Martín 2007), comparemos la respuesta de los individuos frente a compuestos químicos tales como los esteroides y los alcoholes. Al igual que anteriormente, hicimos experimentos de reconocimiento quimiosensorial y comparamos el número de veces que las lagartijas sacaban la lengua en dirección al estímulo químico entre las diferentes poblaciones estudiadas. Primero analizamos si las lagartijas de todas las poblaciones tienen el mismo interés por lo esteroides (Capítulo 4). Se ha visto en otras especies de lagartijas que algunos esteroides como el colesterol o el colesta-5,7-dien-3-ol podrían ser una señal honesta (Martín y López 2006a, 2007). Además suponemos que estos compuestos varían en las secreciones según la población (Capítulo 1). Así que queríamos ver si las lagartijas de distintas poblaciones también reaccionaban o tenían una atracción diferente por algunos esteroides. Finalmente, nos interesamos por los alcoholes y su posible interés químico por parte de las lagartijas.

Preguntándonos, si los individuos de *P. hispanica* además de discriminar los esteroides, reconocían los alcoholes (presentes en las secreciones) y tenían un cierto interés por estos compuestos (Capítulo 5).

- Parte 4 - Relación entre el sistema inmune y las señales químicas

En la cuarta parte, nos cuestionamos por la relación entre la calidad del sistema inmune y las señales químicas (Capítulo 6). Las señales químicas informarían de manera honesta sobre el estado de salud de los individuos, y las hembras utilizarían estas señales químicas como índice de calidad de los machos. Entonces, activamos el sistema inmune de los machos y observamos los que ocurría en las señales químicas Para eso, utilizamos dos grupos de machos de *P. hispanica*: uno control y un experimental. Los experimentales fueron tratados con una inyección de paredes de bacterias (*E. coli)* para inducir la respuesta inmune activando los anticuerpos de las lagartijas, pero sin producir efectos patógenos. De la misma manera que en los otros capítulos, comparamos la composición y proporción de los compuestos químicos de las secreciones femorales antes y después del tratamiento y entre los machos controles y experimentales. Los compuestos que varían con la activación del sistema inmune indicarían a las hembras el estado de salud de los machos.

- Parte 5 - Comunicación química entre dos especies muy cercanas

Finalmente en la quinta parte, estudiamos la respuesta de las lagartijas Ibéricas con otra especie, pero del mismo complejo de especies: *P. atrata* (Capítulos 7 y 8). Al mismo tiempo de observar la comunicación química entre estas dos especies, se estudiaron las consecuencias (i.e. riesgo de hibridación) de una posible introducción de la lagartija peninsular en las Islas Columbretes donde vive *P. atrata*. Por una parte, comparamos las secreciones femorales a nivel de la composición y de la proporción de compuestos (Capítulo 8). También examinamos el reconocimiento quimiosensorial entre los individuos endémicos de las Islas y los continentales para ver cómo responden frente a olores de individuos de su población, en comparación a los olores de la otra “especie” (Capítulo 7). Según si los individuos son capaces de reconocerse quimiosensorialmente como especies o poblaciones muy distintas, se podría estimar cuál es el riesgo de hibridación resultante de la introducción de una especie invasora en las islas, aunque el riesgo de hibridación no es el único que ocurriría después de una invasión.

OBJETIVOS

- Describir y comparar la morfología y la composición de las señales químicas de los individuos de lagartija Ibérica (*Podarcis hispanica*) de las distintas poblaciones estudiadas en la región de Madrid; discutiendo la posible relación de la variación encontrada con sus hábitats y localizaciones geográficas (Capítulo 1).

- Examinar la capacidad de las lagartijas para reconocer químicamente los olores de otras lagartijas de las otras poblaciones estudiadas. Analizar si existe discriminación y un mayor interés por los olores de individuos de su propia población, y si las hembras prefieren sustratos marcados con olores de machos de su población, o si, por el contrario, no existe una diferencia interpoblacional a nivel de comunicación química (Capítulos 2 y 3).

- Las secreciones femorales están compuestas de esteroides, ácidos carboxílicos y alcoholes entre otros compuestos. Se propone examinar las variaciones interpoblacionales en el reconocimiento quimiosensorial de unos esteroides de las secreciones de los machos que parecen importantes en los procesos de selección sexual de *P. hispanica*, y estimar la importancia relativa de estos esteroides según las poblaciones (Capítulo 4). Además de los esteroides, comprobar si las lagartijas discriminan y muestran un interés significativo por los alcoholes presentes en las secreciones, y si existen variaciones entre las poblaciones (Capítulo 5).

- Según el principio del Hándicap, las señales honestas, deben ser costosas. El coste puede venir de la existencia de un compromiso entre la expresión de las señales sexuales y el mantenimiento del sistema inmunitario. Por eso se pretende estudiar la posible relación entre el sistema inmune y las señales químicas de los machos. Para ello se van a examinar los cambios en la composición de las secreciones femorales de los machos después de un tratamiento experimental con LPS para activar su sistema inmune sin producir efectos patógenos (Capítulo 6).

- Se va a examinar la composición de las señales químicas y el reconocimiento quimiosensorial entre dos “especies” del género *Podarcis* que son genéticamente próximas: una (*P. atrata*) es endémica de las Islas Columbretes y la otra (*P. hispanica*) de la Península Ibérica (Capítulos 7 y 8). Se quiere comprobar si las diferencias encontradas entre estas especies en base a análisis genéticos, se reflejan también a nivel de sus señales químicas (secreciones femorales de los machos), y si ésto afecta a la comunicación química y al reconocimiento interspecífico.

CAPÍTULO 1

Las variaciones morfológicas y de señales químicas entre poblaciones de la lagartija Ibérica, *Podarcis hispanica,* reflejarían un proceso de especiación críptica dentro de este complejo de especies

La lagartija Ibérica, *Podarcis hispanica,* parece formar un complejo de especies con varios tipos monofiléticos. Las comparaciones genéticas y morfológicas entre tipos sugieren la existencia de procesos de aislamiento reproductivo en marcha. En esta lagartija, las feromonas sexuales son importantes para la elección de pareja y también para el reconocimiento entre especies, de modo que diferencias en las secreciones químicas de lagartijas de poblaciones diferentes dentro del mismo complejo de especies podrían llevar a un aislamiento reproductivo. En el centro de España, se encuentran por lo menos dos tipos de esta lagartija en áreas sin barreras geográficas aparentes. En este trabajo, estudiamos la morfología y las características químicas de las secreciones femorales de cinco poblaciones de *P. hispanica* de esta zona de contacto. Planteamos la hipótesis de que las diferencias interpoblacionales en la composición y proporciones de compuestos químicos de las secreciones femorales podrían tener implicaciones en los procesos evolutivos de estas lagartijas. Los resultados mostraron diferencias morfológicas y químicas entre las poblaciones. Con respecto a la morfología, observamos dos morfotipos: las lagartijas de las poblaciones del Norte son más robustas, pesadas, grandes, y tienen más poros femorales pero menos ocelos laterales azules que los individuos de las poblaciones del Sur. Además, existen diferencias en la composición química de las secreciones femorales de los machos entre poblaciones. La mayor parte de esta variación se explicaría por adaptación local al hábitat ocupado por cada población, con el objetivo de maximizar la eficacia de las marcas químicas en el substrato, y/o podría ser el resultado de dietas diferentes según el sitio. Finalmente, encontramos una correlación entre la clasificación Norte vs. Sur y la variación de algunos compuestos químicos, en especial el colesta-5,7-dien-3-ol. Los machos de las poblaciones del Norte tuvieron una proporción más alta de este esteroide en las secreciones que los del Sur. Todos estos resultados sugieren que en estas poblaciones distintas podrían haber evolucionado diferentes tipos de señales (Norte vs. Sur), lo que ayudaría a entender los procesos evolutivos en esta zona de contacto del complejo de especies *P. hispanica*, y las posibles causas que explicarían los mecanismos de especiación críptica en marcha en estas lagartijas.

Interpopulational differences in morphology and chemical signals of Iberian wall lizards, *Podarcis hispanica,* could reflect cryptic speciation processes within this species complex

Marianne Gabirot, Pilar López, José Martín

Abstract The Iberian wall lizard, *Podarcis hispanica*, seems to form a “species complex” with several monophyletic types. Genetic and morphological comparisons between types suggest the existence of ongoing reproductive isolation processes. In this lizard, sex pheromones are important for mate choice and species recognition and, thus, differences in chemical secretions between lizard populations within the species complex could lead to reproductive isolation. In Central Spain, at least two types of this lizard could be found living in areas without geographic isolation. In this paper, we studied morphology of lizards and chemical characteristics of femoral secretions of five populations of *P. hispanica* from this contact zone. We hypothesized that interpopulational differences in composition and proportion of chemical compounds in femoral gland secretions may have implications for the evolutive processes of these lizards. The results showed morphological and chemical differences between populations. With respect to morphology, we observed two morphotypes of lizards: lizards from Northern populations were more robust, heavier and larger, and had more femoral pores, but less blue ocelli, than lizards from the Southern populations. Moreover, there were differences in chemical composition of femoral secretions between populations. Most of this variation could be explained by local adaptation to the habitat occupied by each population, in order to maximize efficiency of substrate scent marks, and/or could result from different diets in different areas. Finally, we found a correlation between the classification North vs. South and variations of some chemical components, especially for cholesta-5,7-dien-3-ol. Males from the North had higher proportions of this steroid in secretions than Southern males. All these results suggest that different types of signals might have evolved in these populations (North vs. South), which could help understanding the evolutive processes in this contact zone of the *P. hispanica* species complex, and the possible causes that could explain ongoing cryptic speciation processes in these lizards.

Résumé Le lézard ibérique *Podarcis hispania* semble former un complexe d’espèces avec plusieurs types monophylétiques. Les comparaisons génétiques et morphologiques entre les types ont suggéré l’existence de processus d’isolation reproductif en évolution. Chez ce lézard les phéromones sexuelles sont importantes pour le choix de partenaire reproductif et pour la reconnaissance inter espèces ; par conséquent des différences dans les secrétions chimiques entre les populations de lézards dans un même complexe d’espèces pourraient provoquer une isolation sexuelle. Au centre de l’Espagne, se trouvent au moins deux morphotypes, sans barrière géographique observable. Dans ce travail, nous avons étudié la morphologie et les caractéristiques chimiques des sécrétions des glandes fémorales de cinq populations du lézard *Podarcis hispanica* de cette zone de contact. Nous supposons que les différences de composition et proportion des substances chimiques entre les populations pourraient avoir des implications dans les processus évolutifs de ces animaux. Les résultats ont montré des variations au niveau morphologique et chimique entre les populations. Pour ce qui se rapporte à la morphologie, nous avons observé deux types de lézards : ceux qui viennent des populations du Nord de la zone étudiée, qui sont plus robustes, gros et larges, avec un nombre de pores fémoraux plus important mais moins de points bleus latéraux que les animaux des populations du Sud de Madrid. De plus, nous avons noté des différences au niveau de la

composition des substances fémorales entre les populations. La plupart des variations est dû aux adaptations locales des habitats occupés par les populations, dans le but de maximiser l'efficacité des marques chimiques Sur les substrats, ou bien elles pourraient aussi venir des différentes sources alimentaires des distinctes zones. Finalement, nous avons trouvé, une corrélation entre la classification Nord vs. Sud et la variation de certains composés chimiques, spécifiquement le cholesta-5,7-dien-3-ol. Les mâles du Nord ont une proportion plus importante de ce stéroïde dans ces secrétions que les mâles du Sud. Tous ces résultats suggèrent que différents types de signaux pourraient avoir évolué chez ces populations (Nord vs. Sud), et ceci pourrait aider à comprendre les processus évolutifs dans cette zone de contact du complexe d'espèces *P. hispanica*, et les possibles causes qui expliqueraient ces cette spéciation cryptique en marchent chez ce lézard.

Manuscrito en preparación

INTRODUCTION

Male mating ornaments and female preferences when choosing male partners could evolve in different ways in different populations and this could facilitate speciation mechanisms (Boughman 2001; Panhuis et al. 2001). Reproductive isolation might occur between individuals that display distinct sexual signals, and once that interpopulation recognition and isolation are effective, they may result in ongoing processes of speciation (e.g., Shaw and Parsons 2002; Shine et al. 2002). Among the different types of signals, specific chemical compounds released by one individual could or could not affect a second individual, depending very often of whether both individuals are or are not conspecifics. Therefore, these chemical signals could be a very good way to inform about the species identity of individuals (Johnston 2000, 2001). Chemical signals are important for mate and species recognition and may result in premating reproductive isolation and speciation in many animals (Smadja and Butlin 2009). Especially in lizards, intraspecific communication is often based on chemical substances deposited passively on the substrate as lizards move through their home ranges. These chemicals are often produced by femoral glands, which are epidermal structures on the ventral Surface of the thigh connected to glands that produce copious amounts of holocrine secretion, especially in males and during the mating season (reviewed in Mason 1992; Alberts 1993). Behavioral experiments with several Lacertid lizard species demonstrated that lizards (both sexes) explore the substrate scent-marks by tongue flicking. Also when lizards meet a congener, such as during social encounters, lizards investigated each other by tongue-flicking the cloacal and femoral regions. Femoral secretions of males could inform during intrasexual communication about the social status or competitive ability to other males (Aragón et al. 2001; López and Martín 2002; Martín et al. 2007). Moreover, females could use some chemical cues from femoral glands of males as signals of a male’s individual quality in order to chose their mate partner (Martín and López 2000, 2006; López et al. 2002; Olsson et al. 2003). Thus, because femoral gland secretions are involved in sexual selection, it is likely that they may also lead to speciation processes. For example, chemical cues prevented heterospecific mating between closely related species of skinks of the genus *Eumeces* (Cooper and Vitt 1987).

Podarcis hispanica, a small lacertid lizard common in Iberian Peninsula, has well developed chemical recognition abilities, and is able to discriminate between conspecifics and heterospecifics (Cooper and Pérez-Mellado 2002; Barbosa et al. 2006), and between sexes by chemical cues alone (Gómez et al. 1993; López and Martín 2001; López et al. 2002). Chemical cues of males are important in male-male interactions (López and Martín, 2002; Carazo et al. 2007). Moreover, in at least one population, females prefer to stay on areas scent marked by males with high proportions of cholesta-5,7-dien-3-ol (=provitamin D_3) in their femoral secretions, which may signal a better immune response of those males (López and Martín 2005; López et al. 2009). Therefore, it seems that female mate choice decisions are partially based on characteristics of chemical signals of males.

Previous studies suggested the existence of ongoing speciation processes between populations of *P. hispanica* within taxa previously considered to be conspecifics Molecular works have provided relationships and genetic distances between populations. Genetics and morphological comparisons suggest that the Iberian wall lizard, *P. hispanica*, is paraphyletic, and forms a species complex with at least five monophyletic lineages (Guillaume 1987; Harris and Sá-Sousa 2001, 2002; Sá-Sousa et al. 2002; Pinho et al. 2007). This common lizard is widespread at rocky habitats inside many different environments of the Iberian Peninsula. In the western and central parts of the Iberian Peninsula two allopatric types have been identified. In Northwestern Iberia, *P. hispanica* type 1 occurs, mainly in highlands and where Atlantic humid environmental conditions prevail, while *P. hispanica* type 2 occurs in Central and Southern Iberia, where Mediterranean dry conditions are typical (Sá-Sousa 2000; Sá-Sousa et al. 2002). Although their populations are mainly allopatric, both types have been reported from the Madrid province (Central Spain). In this area, two populations live close together without geographical isolation, and individuals of both types may find each other easily (Mellado and Olmedo 1981; García-Paris et al. 1989).

Recently, analyses showed that closed populations of *P. hispanica* from the Guadarrama Mountains (Central Spain), inhabiting different altitudes and environmental conditions, differ in the chemical composition of femoral gland secretions of males (Martín and López 2006a) and have also morphological and coloration differences (Gabirot et al. unpublished data). Males of the population inhabiting more humid microclimates have secretions with higher proportion of compounds (e.g., long chain alcohols) that may favor persistency and efficiency of chemical signals in humid environments.

Moreover, different rates of chemosensory exploration between these closed populations showed that males can discriminate by chemical cues alone between males of their own and the other population (Martín and López 2006a, b). In contrast, females detect scent of males, but do not seem able to discriminate between scents of males of the two populations (Martín and López 2006a, b). Similarly, males of *P. bocagei* and *P. hispanica* from the North of Portugal are able to discriminate chemically between conspecifics and heterospecifics, but females are not (Barbosa et al. 2006). These results suggest that despite of clear differences in morphology and chemical signals between populations of *P. hispanica* lizards, there is not clear interpopulational discrimination at the level of chemical recognition and premating reproductive isolation. These results support that reproductive isolation and speciation between these populations of *P. hispanica* is not clear, or that speciation is still incomplete.

Podarcis lizards are highly variable in size, shape, escalation and color patterns, not only between currently recognized species (Arnold and Ovenden 2002), but also between populations and individuals. Given this, disentangling the track of evolutionary history from the effects of local adaptation or phenotypic plasticity (or finding a cohesive phenotype) can be a difficult task. Morphological distinction between *Podarcis* forms can neither be taken for neither granted nor necessarily attributed to separate evolution. Madrid is an area where at least two types (1 and 2) of *P. hispanica* could be found. This area could be considered as a contact zone, where perhaps more than two types could be found. Several populations very distinct at different levels, such as morphology or chemical signals, seem to live in this zone. Observations in the field around Madrid showed that there are more than two different populations of *P. hispanica* with clear variations in coloration and morphology (Gabirot et al. unpublished data).

In this paper, we captured lizards from five populations of this contact area, occupying different geography localizations. We studied three populations from the Northern area of Madrid, one from the North and two from the Northwest, one from the mountains and two from the mountain basin at low altitude. In the South, we studied two isolated populations located at similar low altitude. All these populations showed morphological and coloration differences (Gabirot et al. unpublished data). We first compared the morphological characteristics of these populations, and then analyzed interpopulational differences in composition and proportions of chemical compounds in femoral gland secretions of males. We hypothesized that interpopulational differences in chemical signals of male lizards *P. hispanica*, probably resulting from local adaptation to the habitat occupied for each population, could be leading to different evolutions of chemical signals and, thus, it is possible that in the future, these differences lead to reproductive isolation processes between populations within this species complex.

MATERIALS AND METHODS

Study populations

During February-March 2008, we captured by noosing male and female *P. hispanica* lizards at five localities within the Madrid Province (Central Spain) (Fig. 1). Three of these were localized in the mountain Northern area (‘Fuenfría’, ‘Golondrina’ and ‘Pedrezuela’), and the two other were situated in the plain Southern area (‘Belmonte’ and ‘Aranjuez’). We chose these populations because they show distinct morphological and coloration characteristics (Gabirot et al., unpubl. data). In the North, we captured lizards from a population occupying different granite rock-cliffs at the edge of a pine forest in the upper part of ‘Fuenfría’ Valley (40°47’ N, 4°03’ W; 1750 m altitude) (21 males and 26 females), on granite rocky outcrops in a large oak forest (‘Golondrina’) near Cercedilla village (40°44’ N, 4°02’ W; 1250 m altitude) (29 males and 27 females), and from old stone walls near to cultivate fields in the ‘Pedrezuela’ village (40°44' N, 3°36' W; 800 m altitude) (19 males and 16 females). In the South, we captured 22 males and 17 females on human constructions in a public garden in the ‘Belmonte del Tajo’ village (40°8' N, 3°20' W; 735 m altitude) and other lizards on chalk and gypsum rocks in deforested bushy hills near ‘Aranjuez’ (40°2’ N, 3°37’ W; 494 m altitude) (21 males; and 32 females).

Fig.1: Localization of the five populations of *Podarcis hispanica* studied in the center of Spain.

All lizards were individually housed at “El Ventorrillo” Field Station (Cercedilla, Madrid) about 5 Km from the capture sites of the Northern populations, in indoor 60 x 40 cm PVC terraria containing sand substratum and rocks for cover. Cages were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle, and were screened from each other using cardboard. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and water was provided *ad libitum*. Lizards were returned to their exact capture sites with good health condition at the end of experiments. Captures and observations were performed under license from the Environmental Agency of Madrid Government ("Consejería del Medio Ambiente de la Comunidad de Madrid", Spain).

Morphological characteristics

For each population, we made the following morphological measures of each individual: body mass (or weight) (with a digital balance to the nearest 0.01 g) and body size (snout-to-vent length, SVL with a ruler to the nearest 1 mm). We calculated the 'body condition' as the body mass divided by the SVL. Also, we used the residuals of the regression of weight on SVL, both ln corrected (r=0.86; $F_{1,228}$=677.77; P<0.0001) as a 'body condition index' (BCI), which may represent an index of the relative amount of fat stored, and hence an estimation of individual physical condition or nutritional status (Bonnet and Naulleau 1994).

We also made morphological measurements of the head using digital calipers (to the nearest 0.05 mm). Head length was the distance between the tip of the snout and the posterior side of the parietal scales. Head width was the greatest distance between the external sides of the parietal scales. Head depth was the greatest distance from the highest portion of the head to the bottom of the lower jaw. We transformed the head size measurements by using the residuals of the regression of head size on SVL, both ln corrected (regression: head length: r=0.79, $F_{1,228}$=394.79, P<0.0001; head width: r=0.71, $F_{1,228}$=239.23, P<0.0001; head depth: r=0.67, $F_{1,228}$=185.86, P<0.0001).

We also counted under a magnifying glass the number of femoral pores on the right and left hindlimbs of lizards and calculated an average number for both sides. Finally, we noted the number of small but distinctive and conspicuous blue ocelli that runs along each of the body sides on the outer margin of the belly of males, and calculated an average number for both sides. These ocelli seem to have a role in sex recognition and intrasexual social relationships between males (López et al. 2002).

We used GLM models to analyze differences in morphological variables between populations and between sexes, including the interaction between sexes and populations in the models.

Analyses of femoral gland secretions

Immediately after capture in the field, we extracted femoral gland secretion of males by gently pressing with forceps around the femoral pores, and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at –20 °C until analyses. We also used the same procedure on each sampling occasion but without collecting secretion, to obtain blank control vials that were treated in the same manner to compare with the lizard samples. Before the analyses we added 250 µl of n-hexane (Sigma, capillary GC grade) to each vial, We analyzed lipophilic compounds in samples by using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl / 95 % dimethylsiloxane) column (Thermo Fisher, Trace TR-5, 30 m length x 0.25 mm ID, 0.25-mm film thickness) and 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 at 30 cm/sec, with injector temperature at 250 °C. The oven temperature program was as follows: 50 °C isothermal for 5 min, then increased to 270 °C at a rate of 10 °C/min, isothermal for 1 min, then increased to 315 °C at rate of 15 °C/min, and finally isothermal (315 °C) for 10 min. Ionization by electron impact (70 eV) was carried out at 250°C. Mass spectral fragments below m/z=39 were not recorded. Impurities identified in the solvent and/or the control vial samples are not reported. Initial tentative identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards from Sigma-Aldrich Chemical Co. For unidentified or unconfirmed compounds we report here their characteristic ions, which we used together with retention times and characteristic m/z ratios to confirm whether these compounds were present in a given individual.

For the statistical analyses of femoral secretions, the relative amount of each component was determined as the percent of the total ion current (TIC). The relative areas of the peaks were transformed following Aitchison’s formula: $Zij=\ln(Yij/g(Yj))$, where Zij is the standardized peak area i for individual j, Yij is the peak area i for individual j, and $g(Yj)$ is the geometric mean of all peaks for individual *j* (Aitchison 1986; Dietemann et al. 2003). The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. The transformed areas were used as variables in a principal component analysis. The eight principal components (PC) extracted (all eigenvalues >1; which explained 73.30 % of variance) were used as independent variables in a multivariate analysis of variance (MANOVA) to determine whether the five populations of male *P. hispanica* differed in the relative abundances of compounds.

In addition, we used these eight extracted PCs as covariates in a subsequent discriminant analysis. We used this test to verify whether chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard.

RESULTS

Interpopulational differences in morphology

Weight, SVL, body condition and BCI were significantly different between sexes (MANOVA: Wilks' $\lambda=0.52$, $F_{2,219}=99.80$, $P<0.0001$) and between populations (Wilks' $\lambda=0.52$, $F_{8,438}=20.80$, $P<0.0001$), and the interaction between population and sexes was also significant (Wilks' $\lambda=0.83$, $F_{8,438}=5.40$, $P<0.0001$). Males were significantly greater than females in all variables (ANOVAs: weight, $F_{1,228}=87.46$, $P<0.0001$; SVL, $F_{1,228}=17.10$, $P<0.0001$; body condition, $F_{1,228}=17.15$, $P=0.00005$; BCI, $F_{1,228}=123.90$, $P<0.0001$).

For males, we found significant differences between populations in weight ($F_{4,107}=35.89$, $P<0.0001$), SVL ($F_{4,107}=38.80$, $P<0.0001$) and body condition ($F_{4,107}=38.75$, $P<0.0001$, but not for the BCI ($F_{4,107}=1.53$, $P=0.19$) (Table 1). Males from Fuenfría and Golondrina populations were heavier and larger than individuals from Aranjuez and Belmonte (Tukey's tests, $P<0.001$ for all comparisons). Males from Pedrezuela were intermediate in size between all other populations, with a weight similar to males from Aranjuez ($P=0.17$) but with a SVL similar to that of Golondrina males ($P=0.11$) (Table 1).

In females, there were significant differences between populations in weight ($F_{4,113}=3.20$, $P=0.015$), SVL ($F_{4,113}=11.43$, $P<0.0001$), body condition ($F_{4,113}=11.43$, $P<0.0001$) and BCI ($F_{4,113}=12.94$, $P<0.0001$) (Table 1). Females from Fuenfría and Golondrina were significantly greater than individuals from Belmonte and Aranjuez (Tukey's tests, $P<0.05$ for all). Golondrina and Fuenfría females were similar in size between them ($P>0.50$ for all), as were similar Aranjuez and Belmonte females ($P>0.50$ for all).

Pedrezuela females were half way between Fuenfría and Aranjuez females, with a size, weight and body condition closed to females from Golondrina and Belmonte ($P>0.10$ for all).

For the weight measures, only one significant difference was found; Belmonte females were significant less heavy than Fuenfría females (Tukey's test, $P=0.006$); the rest of comparisons were not significant ($P>0.10$). Finally for the BCI, females from Aranjuez had positive values, differing significantly from all other populations that had negative BCI values ($P<0.006$ for all).

Respect to head size (length, width, depth), we find overall significant differences between sexes (MANOVA, Wilks' $\lambda=0.24$, $F_{3,217}=227.75$, $P<0.0001$) and between populations (Wilks' $\lambda=0.85$, $F_{12,554}=2.96$, $P=0.0004$), also the interaction was significant (Wilks' $\lambda=0.88$, $F_{12,574}=2.28$, $P=0.007$) (Table 1). Separated analyses for each sex showed that males' head size differed significantly between populations (MANOVA, Wilks' $\lambda=0.77$, $F_{12,278}=2.32$, $P=0.007$), also females differed significantly in head size (MANOVA, Wilks' $\lambda=0.72$, $F_{12,291}=3.17$, $P=0.0002$). However, differences between populations of males were only significant for head depth (ANOVA, $F_{4,107}=5.68$, $P=0.0003$), but not for head length ($F_{4,107}=0.93$, $P=0.44$) and width ($F_{4,107}=2.29$, $P=0.064$) (Table 1). Males from Fuenfría had significantly deeper heads than males from Belmonte (Tukey's test, $P=0.001$) or Pedrezuela ($P=0.043$) but did not significantly differ from males from Aranjuez ($P=0.99$) and Golondrina ($P=0.32$).

Morphological measures	Populations				
	Aranjuez	Belmonte	Golondrina	Fuenfría	Pedrezuela
Females					
Weight (g)	2.88 ± 0.13	2.46 ± 0.17	2.93 ± 0.14	3.26 ± 0.14	2.78 ± 0.18
SVL (cm)	5.0 ± 0.1	5.0 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.2 ± 0.1
Body condition (g)	2.76 ± 0.15	2.74 ± 0.15	3.53 ± 0.12	3.89 ± 0.14	3.05 ± 0.14
BCI	0.03 ± 0.02	-0.07 ± 0.02	-0.14 ± 0.02	-0.13 ± 0.02	-0.08 ± 0.02
Head length (mm)	11.03 ± 0.12	11.13 ± 0.17	11.83 ± 0.14	12.00 ± 0.13	11.51 ± 0.17
Head width (mm)	6.56 ± 0.07	6.50 ± 0.10	6.74 ± 0.08	6.91 ± 0.08	6.59 ± 0.11
Head depth (mm)	4.82 ± 0.06	4.79 ± 0.08	4.98 ± 0.07	5.09 ± 0.07	4.75 ± 0.09
Femoral pores	13.7 ± 0.2	16.4 ± 0.3	15.6 ± 0.2	16.0 ± 0.2	15.3 ± 0.3
Males					
Weight (g)	3.35 ± 0.17	3.06 ± 0.17	4.66 ± 0.17	5.46 ± 0.15	3.84 ± 0.18
SVL (cm)	5.1 ± 0.1	5.0 ± 0.1	5.8 ± 0.1	6.2 ± 0.1	5.5 ± 0.1
Body condition (g)	2.97 ± 0.15	2.76 ± 0.12	4.18 ± 0.14	4.85 ± 0.13	3.64 ± 0.17
BCI	0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.04 ± 0.02
Head length (mm)	12.67 ± 0.20	12.41 ± 0.19	14.23 ± 0.20	14.87 ± 0.17	13.77 ± 0.21
Head width (mm)	7.26 ± 0.13	7.47 ± 0.13	8.17 ± 0.13	8.52 ± 0.11	7.97 ± 0.14
Head depth (mm)	5.32 ± 0.10	5.60 ± 0.10	6.06 ± 0.10	6.11 ± 0.08	5.91 ± 0.10
Femoral pores	16.2 ± 0.3	17.5 ± 0.3	18.3 ± 0.3	17.7 ± 0.2	17.2 ± 0.3
Blue ocelli	4.5 ± 0.5	4.9 ± 0.5	1.9 ± 0.5	1.7 ± 0.4	5.9 ± 0.5

Table 1: Morphological characteristics (mean ± SE) of *P. hispanica* lizards (males and females) from five distinct populations of the Madrid area (Aranjuez, Belmonte, Golondrina, Fuenfría and Pedrezuela).

With respect to the number of femoral pores, males had significantly more pores than females (two-way ANOVA, $F_{1,220}$=131.80, P<0.0001) (Table 1), the population had a significant effect on the number of femoral pores without sex consideration ($F_{4,220}$=23.05, P<0.0001), and the interaction between populations and sexes was significant ($F_{4,220}$=2.84, P=0.025). Lizards from Aranjuez had significantly less femoral pores than lizards from Golondrina (Tukey's tests, P=0.0001 for males and females), Fuenfría (P=0.001 for males, and P=0.0001 for females), or Belmonte (P=0.017 for males, and P=0.0001 for females). Pedrezuela males had a similar number of femoral pores than Aranjuez males (P=0.11), but Pedrezuela females had significantly more pores than Aranjuez females (P=0.02). Both males and females, from Golondrina, Fuenfría and Belmonte had a similar number of femoral pores (P>0.50 for all).

With respect to the number of blue ocelli of males, there were significant differences between populations (ANOVA, $F_{4,107}$=15.13, P<0.0001) (Table 1). Aranjuez, Belmonte and Pedrezuela males had significantly more blue ocelli than males from Fuenfría and Golondrina (Tukey's tests, P>0.05 for all comparisons); while there were not significant differences between males from Belmonte, Aranjuez and Pedrezuela (P>0.50 for all), nor between Fuenfría and Golondrina (P =0.99).

Interpopulational differences in chemical composition of femoral secretions

We found 53 lipophilic compounds in femoral gland secretions of male *P. hispanica* (Table 2). The lipophilic fraction of femoral gland secretions of males is a mixture of steroids (83.69 % of TIC), and carboxylic acids ranged between n-C_{14} and n-C_{22} and their esters (10.30 %), but we found also five alcohols between n-C_{16} and n-C_{24} (3.53 %), a furanone (1.18 %), four waxy esters (1.10 %), squalene (0.60 %), and two terpenoids (0.28 %) (Table 2). On average, the five most abundant chemicals were cholesterol (63.24 % of TIC), followed by cholesta-5,7-dien-3-ol (5.16 %), hexadecanoic acid (3.73 %), campesterol (3.66 %), octadecenoic acid (2.46 %) and octadecanoic acid (1.77 %) (Table 2).

There were 34 chemical compounds presented in all populations, but we encountered differences between populations in the presence/absence of 19 compounds in femoral secretions. With respect to steroids, we observed nine differences in presence/absence of chemicals between populations (Table 2).

Compounds	RT (min)	Fuenfría	Pedrezuela	Golondrina	Belmonte	Aranjuez
Steroids:						
Un.steroid.(145,213,248,353,368,387)	29.92	0.01 ± 0.01	-	0.17 ± 0.05	1.49 ± 0.56	-
Cholesta-2-4-diene	30.58	0.68 ± 0.11	2.66 ± 0.44	0.44 ± 0.08	2.59 ± 0.46	0.96 ± 0.35
Cholesta-3.5-diene	30.81	0.42 ± 0.10	0.23 ± 0.04	0.30 ± 0.07	0.13 ± 0.03	0.25 ± 0.07
Un.steroid (155,197,251,350,365)	30.96	1.32 ± 0.16	1.00 ± 0.14	0.55 ± 0	0.45 ± 0.06	0.45 ± 0.17
Cholesta-5,7,9(11)-trien-3-ol	31.06	1.62 ± 0.18	1.07 ± 0.24	0.94 ± 0.11	0.65 ± 0.11	0.29 ± 0.07
Un.steroid (207,251,350,365)	31.13	0.40 ± 0.08	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.07	0.08 ± 0.04
Un.steroid (143,195,207,351,366)	31.20	0.19 ± 0.02	0.08 ± 0.01	0.15 ± 0.04	0.18 ± 0.05	0.22 ± 0.06
Un. steroid (141,156,209,350,365)	31.37	0.37 ± 0.05	0.03 ± 0.01	0.30 ± 0.06	2.47 ± 0.42	-
Un. steroid (155,197,251,365,379)	31.64	0.06 ± 0.01	0.21 ± 0.02	0.08 ± 0.02	0.43 ± 0.07	0.45 ± 0.18
Un.steroid (195,209,251,365,379)	31.84	-	0.07 ± 0.01	0.27 ± 0.07	0.51 ± 0.08	0.32 ± 0.12
Cholesterol	32.43	59.74 ± 2.79	62.33 ± 1.68	66.61 ± 2.00	53.03 ± 2.51	74.51 ± 2.04
Cholestanol	32.47	1.40 ± 0.14	0.53 ± 0.08	0.90 ± 0.11	0.60 ± 0.06	0.55 ± 0.12
Cholesta-5.7-dien-3-ol.	32.65	13.41 ± 1.85	2.68 ± 0.54	8.02 ± 1.33	1.16 ± 0.19	0.54 ± 0.17
Un.steroid (105,213,255,353,368,386,415)	32.75	0.02 ± 0.01	0.03 ± 0.02	0.35 ± 0.11	0.09 ± 0.03	0.39 ± 0.16
Ergosterol	33.00	-	0.05 ± 0.02	-	0.17 ± 0.11	-
Campesterol	33.17	1.61 ± 0.22	3.76 ± 0.28	3.27 ± 0.36	5.46 ± 0.28	4.22 ± 0.57
Cholest-4-en-3-one	33.41	0.17 ± 0.03	0.53 ± 0.17	0.19 ± 0.05	0.20 ± 0.02	0.92 ± 0.38
Ergosta-5,8-dien-3-ol	33.50	2.43 ± 0.30	1.58 ± 0.22	2.38 ± 0.37	1.31 ± 0.24	0.56 ± 0.14
Cholesta-4,6-dien-3-one	33.69	0.24 ± 0.06	0.53 ± 0.08	0.29 ± 0.06	0.40 ± 0.06	-
Sitosterol	33.92	0.65 ± 0.10	0.74 ± 0.16	0.94 ± 0.15	1.18 ± 0.11	1.13 ± 0.23
Ergostanol	34.02	0.07 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	0.11 ± 0.02	0.33 ± 0.11
Stigmasterol	34.13	0.31 ± 0.06	0.27 ± 0.13	0.28 ± 0.04	1.22 ± 0.22	0.44 ± 0.26
Un.steroid (221,253,281,355,380,430)	34.30	2.23 ± 0.32	0.70 ± 0.18	1.01 ± 0.16	-	-
Cholest-5-en-3-one	34.38	-	-	-	1.33 ± 0.24	0.91 ± 0.28
Ergosta-5.22-dien-3-ol	34.47	-	0.13 ± 0.07	0.12 ± 0.03	0.15 ± 0.04	-
Un.steroid (214,267,395)	35.30	0.12 ± 0.04	0.21 ± 0.11	-	0.56 ± 0.44	0.22 ± 0.09
Carboxylic acids and their esters:						
Tetradecanoic acid	20.64	0.16 ± 0.04	0.38 ± 0.13	0.22 ± 0.06	0.24 ± 0.05	0.85 ± 0.55
Pentadecanoic acid	21.68	0.13 ± 0.02	0.15 ± 0.12	0.10 ± 0.03	0.18 ± 0.05	0.41 ± 0.19
Hexadecanoic acid. methyl ester	22.33	-	0.05 ± 0.02	-	0.09 ± 0.02	0.25 ± 0.08
Hexadecenoic acid	22.54	0.16 ± 0.02	0.40 ± 0.20	0.25 ± 0.07	0.57 ± 0.33	0.28 ± 0.09
Hexadecanoic acid	22.76	3.68 ± 0.32	4.36 ± 0.65	3.11 ± 0.35	5.98 ± 0.51	1.54 ± 0.23
Hexadecanoic acid, ethyl ester	22.98	-	0.37 ± 0.11	-	0.19 ± 0.06	0.40 ± 0.17
9,12-octadecadienoic acid	24.35	0.10 ± 0.01	0.11 ± 0.02	0.12 ± 0.02	0.27 ± 0.08	0.06 ± 0.02
Octadecenoic acid	24.43	1.99 ± 0.18	1.76 ± 0.20	2.76 ± 0.57	4.82 ± 1.41	1.01 ± 0.21
Octadecanoic acid	24.60	1.39 ± 0.12	2.52 ± 0.34	1.41 ± 0.13	2.55 ± 0.23	0.99 ± 0.18
Octadecanoic acid, ethyl ester	24.82	-	0.51 ± 0.23	-	0.14 ± 0.04	0.55 ± 0.23
Eicosanoic Acid	26.31	0.46 ± 0.09	0.63 ± 0.15	0.76 ± 0.11	0.59 ± 0.17	0.64 ± 0.18
Docosanoic acid	28.00	-	0.01 ± 0.01	-	0.01 ± 0.01	-
Docosanoic acid, ethyl ester	28.21	-	0.45 ± 0.12	-	0.21 ± 0.05	0.23 ± 0.12
Alcohols:						
Hexadecanol	21.02	0.23 ± 0.05	-	0.19 ± 0.07	0.16 ± 0.04	0.16 ± 0.05
Octadecanol	23.87	0.26 ± 0.05	0.69 ± 0.16	0.19 ± 0.06	0.29 ± 0.08	-
Eicosanol	25.67	0.17 ± 0.03	0.55 ± 0.13	0.28 ± 0.08	0.21 ± 0.05	0.81 ± 0.28
Docosanol	27.33	0.23 ± 0.05	0.52 ± 0.15	0.23 ± 0.04	0.23 ± 0.04	0.73 ± 0.26
Tetracosanol	29.80	0.03 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.07 ± 0.02	0.01 ± 0.01
Waxy esters:						
Unidentified waxy ester 1	29.45	0.28 ± 0.08	0.98 ± 0.20	-	1.37 ± 0.47	0.75 ± 0.23
Unidentified waxy ester 2	35.57	0.58 ± 0.10	2.61 ± 0.60	0.42 ± 0.08	2.84 ± 0.45	0.69 ± 0.26
Unidentified waxy ester 3	38.06	0.23 ± 0.06	0.29 ± 0.06	0.20 ± 0.05	0.09 ± 0.03	0.37 ± 0.23
Unidentified waxy ester 4	38.27	0.63 ± 0.11	1.78 ± 0.26	0.47 ± 0.10	2.26 ± 0.30	0.82 ± 0.16
Others:						
Tetradecanone	22.11	0.20 ± 0.05	0.27 ± 0.11	0.13 ± 0.03	0.15 ± 0.03	0.18 ± 0.06
Unidentified Furanone	24.19	0.12 ± 0.02	0.10 ± 0.03	0.06 ± 0.01	-	-
Squalene	30.07	0.93 ± 0.26	0.70 ± 0.10	0.66 ± 0.20	0.35 ± 0.04	0.40 ± 0.19
Unidentified terpenoid 1	30.83	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.02	0.03 ± 0.01	0.13 ± 0.07
Unidentified terpenoid 2	31.94	0.48 ± 0.09	-	0.48 ± 0.12	0.05 ± 0.01	-

Table 2: Lipophilic compounds found in femoral gland secretions of male lizards, *P. hispanica* from five distinct populations of the Madrid area. The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (± 1SE). Characteristics (*m/z*) are reported for some unidentified compounds.

An unidentified steroid (with retention time, *RT*, of 29.92) was situated in Fuenfría, Golondrina and Belmonte populations but not in Aranjuez and Pedrezuela. Cholesta-4,6-dien-3-one and an unidentified steroid (*RT*=31.37) were found in all populations except Aranjuez. While another unidentified steroid (*RT*=31.84) was presented in all populations but in Fuenfría. The unidentified steroid (*RT*=35.30) was seen in all populations except in Golondrina. Ergosterol was observed only in Pedrezuela and Belmonte populations, and ergosta-5,22-dien-3-ol was only found in Pedrezuela, Golondrina and Belmonte. The unidentified steroid (*RT*=34.30) was only in the three Northern populations, while cholest-5-en-3-one was only situated in the two Southern populations.

With respect to the carboxylic acids and their esters, we found five differences in presence/absence between populations. The ethyl esters of hexadecanoic, octadecanoic and docosanoic acids were only seen in Belmonte, Aranjuez and Pedrezuela populations. Docosanoic acid was present only in Pedrezuela and Belmonte.

Finally for the rest of compounds, we had several differences, such as for hexadecanol, absent in Pedrezuela, or for octadecanol that was not found in Aranjuez. The unidentified waxy ester (*RT*=35.57) was presented in all populations but in Golondrina, while the furanone was only observed in the three Northern populations, and the unidentified terpenoid (*RT*=31.94) was only encountered in Fuenfría, Golondrina and Belmonte.

Moreover, multivariate analyses on the eights PCs for the relative proportions of all compounds showed that there were significant differences between populations (MANOVA, Wilks' $\lambda<0.0001$, $F_{32,355}=453.39$, $P<0.0001$) (Table 3).

According to the correlations of compounds with the PCs, the PC-1 scores were related positively to proportion of tetradecanone, hexadecanoic acid, eicosanol, docosanol, cholesta-3,5-diene, cholesterol, cholestanol, campesterol, cholest-4-en-3-one, sitosterol and an unidentified steroid (*RT*=31.20) and negatively with the proportions of ergosterol. There were significant differences in PC-1 score values between populations (ANOVA, $F_{4,103}=120.49$, $P<0.0001$) (Table 3). All comparisons between populations were significant (Tukey's tests: $P<0.001$) except between Fuenfría and Golondrina ($P=0.11$). Males from Aranjuez had the highest positive values for this PC-1, whereas Belmonte had the lowest scores.

The PC-2 scores were negatively correlated with proportions of cholest-4-en-3-one, hexadecanoic acid methyl ester, and the ethyl esters of hexadecanoic, octadecanoic and docosanoic acids, whereas they were, related positively with the proportions of octadecanol, an unidentified furanone, cholesta-4,6-dien-3-one and two unidentified steroids (*RT*=31.37, *RT*=34.30). PC-2 scores varied significantly with the population of origin (ANOVA, $F_{4,103}$=1072.12, $P<0.0001$) (Table 3). We observed a gradient of PC-2 scores between Golondrina and Aranjuez populations. Golondrina, Fuenfría and Pedrezuela had positive values, while Belmonte and Aranjuez had negative values. Moreover, Golondrina had significantly higher PC-2 scores than all other populations (Tukey's tests: $P<0.0002$). Then, Fuenfría showed significantly higher values than Pedrezuela, Belmonte and Aranjuez ($P<0.0002$), PC-2 scores of Pedrezuela population were significantly higher than those of Belmonte and Aranjuez ($P<0.0002$); and those of Belmonte were significantly higher than those of Aranjuez ($P<0.0002$). Lizards from Aranjuez had secretions with the lowest PC-2 scores of all populations.

The PC-3 scores were related negatively with proportions of the unidentified terpenoid 1 and the steroid (*RT*=31.84) and positively with proportions of the waxy ester 1 and the steroid (*RT*=35.30). There were significant differences between populations in PC-3 scores (ANOVA, $F_{4,103}$=205.99, $P<0.0001$) (Table 3). We observed a decreasing gradient of PC-3 scores from Fuenfría, then Pedrezuela, Belmonte, Aranjuez and Golondrina. Lizards from Fuenfría, Pedrezuela and Belmonte had positive PC-3 values, while values were negative for Aranjuez and Golondrina populations. All comparisons between populations were significant (Tukey's tests: $P<0.0002$) except between Belmonte and Pedrezuela (P=0.99).

The PC-4 scores were related positively to proportions of hexadecanol, the unidentified terpenoid 2 and the steroid (*RT*=29.92), and significant differences were found between populations (ANOVA, $F_{4,103}$=235.98, $P<0.0001$) (Table 3). We found a gradient of populations from higher to lower: PC-4 scores from Belmonte, then Fuenfría, Golondrina, Aranjuez and until Pedrezuela. Males from Belmonte, Fuenfría and Golondrina had positive PC-4 values, while Aranjuez and Pedrezuela had negative values. All post-hoc comparisons between populations were significant (Tukey's tests: $P<0.003$); only Fuenfría and Golondrina did not differ (P=0.70).

The PC-5 scores were correlated negatively with proportions of the waxy esters 2 and 4, and there were significant differences between populations (ANOVA, $F_{4,103}$=2.98, P=0.02) (Table 3). Only one significant difference was found: between Fuenfría and Belmonte (Tukey's test: P=0.03); but the rest of comparisons between populations were not significant (P>0.10 for all).

The PC-6 scores were related positively with proportions of hexadecanoic acid and octadecadienoic acid, and there were significant differences between populations (ANOVA, $F_{4,103}$=2.62, P=0.03) (Table 3). But, all Tukey's comparisons between populations were not significant (P>0.20).

Moreover, the PC-7 (i.e. tetracosanol) and PC-8 scores (unidentified steroid, RT=31.64) were not affected by the population of origin of lizards ($F_{4,103}$=0.58, P=0.67 for PC-7; $F_{4,103}$=0.61, P=0.65 for PC-8).

When we compared in particular the proportion of one compound, the cholesta-5,7-dien-3-ol, we found a significant effect of the population of origin on the proportions of this steroid (ANOVA: $F_{4,103}$=76.25, P<0.0001). There was a decreasing gradient of proportions, where Fuenfría was the population with highest levels, then Golondrina, followed by Pedrezuela, Aranjuez, and Belmonte with the lowest proportions. All comparisons between populations were significant (Tukey's test: P<0.0001).

In addition, the discriminant analysis showed that PCs scores of chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard (Wilks' λ=0.0001, $F_{32,355}$=453.39, P<0.0001) (Fig. 2).

Then, we calculated the squared Mahalanobis distances of individuals with all other individuals, and compared them between populations. These distances were affected by the population of origin of males ($F_{16,412}$=2537.26, P<0.0001).

Separated analyses showed that males from Fuenfría did not have the same relative distance to all populations ($F_{4,92}$=2353.98, P<0.0001) (Fig. 3); the distance increased with the geographical distance to populations: from lowest values for Golondrina, then greater distances with Pedrezuela, Belmonte and the greatest distance with Aranjuez. All comparisons of these distance's scores between populations were significant (Tukey's tests: P<0.0002 for all). We observed a similar result for Golondrina males; there were significant different distances depending of the population of origin ($F_{4,92}$=3348.76, P<0.0001) (Fig. 3) and this distance increased as a gradient, from Fuenfría, then Pedrezuela, Belmonte and finally with the greatest distances to

Aranjuez. All comparisons between populations were significant (Tukey's tests: $P<0.0002$).

Conversely, for the Southern populations, Aranjuez and Belmonte, we observed a similar effect, but opposite. Distances increased as populations are more located to the North ($F_{4,76}=3052.32$, $P<0.0001$ for Aranjuez; $F_{4,84}=2082.10$, $P<0.0001$ for Belmonte) (Fig. 3). The distance between Belmonte or Aranjuez was lower with their respective closer population (Aranjuez or Belmonte), but then increased as a gradient from Pedrezuela, then Fuenfría and Golondrina. All comparisons between populations of these distances were significant (Tukey's tests: $P<0.0002$).

Finally, for Pedrezuela lizards, we also observed a variation with the population of origin ($F_{4,68}=410.11$, $P<0.0001$) (Fig. 3), where the distance between the secretions from Pedrezuela and Belmonte was lower but significant, then there was an increasing gradient from Aranjuez to Fuenfría and Golondrina; all comparisons between populations were significant (Tukey's tests: $P<0.0002$), except between Fuenfría and Golondrina ($P=0.10$) or between Fuenfría and Aranjuez ($P=0.57$).

PCs	eigenvalue	Fuenfría	Pedrezuela	Golondrina	Belmonte	Aranjuez
PC-1	18.87	0.59 ± 0.08^{b}	-0.59 ± 0.10^{c}	0.29 ± 0.08^{b}	-1.47 ± 0.09^{d}	1.08 ± 0.09^{a}
PC-2	9.24	0.77 ± 0.03^{b}	0.32 ± 0.03^{c}	0.97 ± 0.03^{a}	-0.64 ± 0.03^{d}	-1.67 ± 0.03^{e}
PC-3	4.60	1.34 ± 0.06^{a}	0.15 ± 0.08^{b}	-1.42 ± 0.06^{d}	0.20 ± 0.0^{b}	-0.27 ± 0.07^{c}
PC-4	3.21	0.49 ± 0.06^{b}	-1.98 ± 0.07^{d}	0.37 ± 0.06^{b}	0.84 ± 0.06^{a}	-0.19 ± 0.07^{c}
PC-5	1.99	-0.37 ± 0.19^{b}	-0.10 ± 0.22^{ab}	0.23 ± 0.19^{ab}	0.47 ± 0.20^{b}	-0.26 ± 0.21^{ab}
PC-6	1.68	0.31 ± 0.19	-0.50 ± 0.22	0.18 ± 0.19	-0.27 ± 0.20	0.15 ± 0.21
PC-7	1.23	0.06 ± 0.20	0.22 ± 0.09	0.02 ± 0.20	-0.24 ± 0.21	-0.04 ± 0.22
PC-8	1.16	0.17 ± 0.20	0.9 ± 0.23	-0.15 ± 0.20	0.09 ± 0.21	-0.21 ± 0.22

Table 3: Principal components (PCs) scores (mean ± SE) from a PCA for relative proportions of compounds in femoral secretions of *P. hispanica* from five distinct populations in the Madrid area. Same small letters on means indicated lack of significant post-hoc differences (Tukey's tests) between populations within each PC.

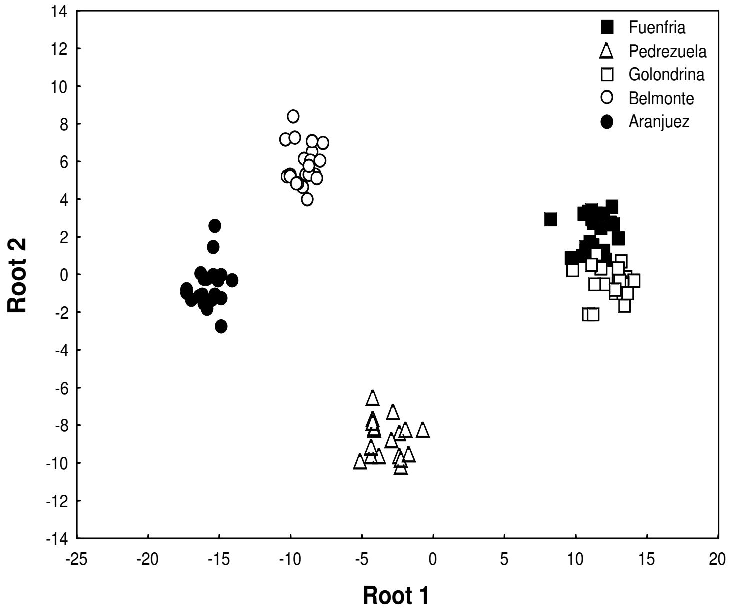


Fig.2: Separation of the principal component of chemical secretions in a discriminant analysis based on population of origin.

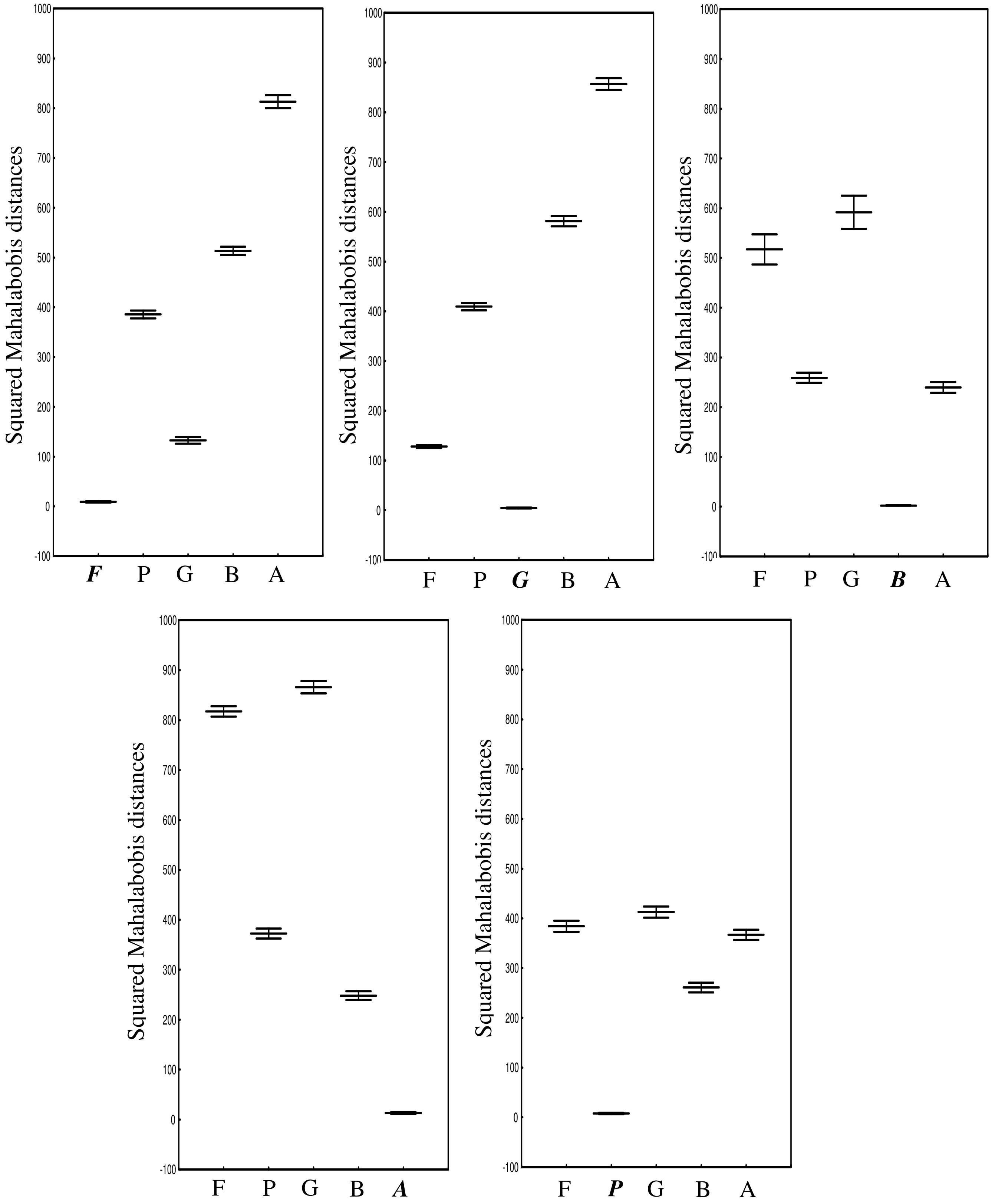


Fig. 3: Squared Mahalabobis distances (mean ± SE), from discrimimant analysis of chemical components' PCs scores, of different populations *P. hispanica* males (Fuenfria "F", Pedrezuela "P", Golondrina "G", Belmonte "B" and Aranjuez "A").

DISCUSSION

Our results first showed that individuals of *P. hispanica* from different populations differed in some morphological aspects. We could differentiate between individuals from the South and North of this contact zone. Lizards from Fuenfría, Golondrina and Pedrezuela populations (i.e., Northern part) were characterized by being larger, heavier, and more robust, and with greater heads; than individuals from Aranjuez and Belmonte (i.e., Southern part). We used here the body condition index (BCI), which is the relative variation of weight corrected by the SVL. Positive values of BCI could be explained by observed weight being superior to predicted weight, while negative values have predicted weights that were greater than observed weight. Thus, females from Aranjuez with negatives values of BCI were heavier with respect to their size than females from other populations. Finally, we observed a gradient in size, weight and head size between the two more geographically distant, extreme populations (Aranjuez and Fuenfría), with greater values in the North that decreased as populations were located more to the South

These differences in morphology between populations could be explained by the different habitats where these populations live. Firstly, Northern and Southern populations are localized in two geographically contrasting sites; mountains (cold, humidity and high altitude) vs. lowland (hot, dry and low altitude). Variations of body size of many animals, and in particular of vertebrates, are often related to climatic factors. Many species show trends in body size that conform to Bergmann's rule, individuals from colder environments being larger than those from warmer areas (e.g., Yom-Tov and Nix 1986). Moreover, animals in cold sites could have color adaptations in order to maximize capture of sun or heat, and more tests could be interesting in order to see whether, in addition to body size, the climate of a habitat could affect coloration of lizards to allow a better adaptation to abiotic local conditions.

Similarly, respect to chemical signals, there are differences between Northern and Southern populations of *P. hispanica*. First, males from Northern populations (Golondrina and Fuenfría) had more femoral pores than males from Southern populations (Aranjuez and Belmonte). Only lizards from the Northern Pedrezuela population had a number of pores that was lower than in the other Northern populations but that was close to those of Southern populations. In addition, the analyses of chemical composition of femoral secretions showed that similarly to other lizard species, femoral gland secretions of *P. hispanica* have carboxylic acids and steroids as predominant components (reviewed in Weldon et al. 2008). Among steroids, cholesterol was the main steroid, which was also found in abundance in secretions of most lizard species (Weldon et al. 2008). More interestingly, chemicals found in femoral gland secretions of male *P. hispanica* varied in composition and proportions between populations. The analyses pointed out that each population had a singular chemical composition and proportion. Each population could be characterized separately in base to chemical composition of femoral secretions. These differences could be due to local adaptation to the habitats where each population lives. Also, these lipidic secretions might be related to alimentation (Symond 2009). Thus, this multiple chemical variability could be the consequence of distinct diets, or more exactly of different available food sources. Molecules from trophic sources of each population would be present in cellules, and could finally be secreted by femoral glands. Diet could be very variable between populations, between seasons, and between individuals (due to different prey items or ability to find food).

Furthermore, for some compounds in femoral secretions, we observed a correlation with the geographical classification of lizard populations (North vs. South). Steroids such as some unidentified steroids and cholesta-4,6-dien-3-one, or the alcohol octadecanol were more abundant in secretions from Northern populations than in Southern ones. Finally, relative proportions of several ethyl esters of carboxylic acids, and cholest-4-en-3-one were more abundant in Southern than in Northern males. These differences in chemical compounds might be related to different microclimatic conditions in the geographical area occupied for each population of lizards (Alberts 1992; Escobar et al. 2001, 2003). Thus, Northern lizards occupy areas characterized by high levels of humidity and low temperature; whereas Southern males occupy more dry and hot areas (Gabirot et al. unpublished data). If the persistence of scent marks in the habitat was a requisite for their efficiency, then there might be selection for the presence of different chemicals in femoral secretions used to mark substrates (see Alberts 1992).

Moreover, chemicals in femoral secretions of Aranjuez male lizards were characterized by higher proportions of cholesterol than in other populations. Cholesterol is a steroid of animal origin that was found in abundance in femoral and precloacal gland secretions of most lizard species (Alberts et al. 1992; Escobar et al. 2001; Lopez and 2005). The abundance and ubiquity of cholesterol in secretions was thought to be useful to constitute an unreactive apolar matrix that delivers the compounds that are the true semiochemicals (Escobar et al. 2003). Thus, cholesterol could confer a better stabilizer for secretions, especially in habitats with high evaporation rates. The hot conditions in the South of the study area could difficult the persistence of substrate scent marks for longer, while the presence of high proportions of cholesterol could allow a better persistence of these secretions.

Therefore, it is likely that selection for a better efficiency of chemical signals used in intraspecific communication had led to differences in composition of femoral secretions of the different populations of male Iberian wall lizards inhabiting different environments; with less volatile and stable molecules being found in habitats where evaporation rate conditions are higher. The question that arises is whether these differences in chemicals affect recognition systems and whether this may have consequences for speciation.

Femoral gland secretions of males and especially some compounds could advertise residence in a home range, and/or convey information about social status and competitive ability (Aragón et al. 2001; López and Martín 2002), or even transmit information about a male's quality, which females may use to select mates (Martín and López 2000; López et al. 2002; Olsson et al. 2003). Previous studies showed that *P. hispanica* have a efficient chemosensory discrimination of chemical compounds found in femoral secretions, and that females could made their partner scent choice using these chemical compounds, and especially cholesta-5,7-dien-3-ol, which may signal a better cell-mediated immune response (López and Martín 2005; López et al. 2009). The steroid cholesta-5,7-dien-3-ol is a precursor for vitamin D_3, and is often found in the skin, where it will transform into vitamin after exposition to sun UV-B irradiation (Fraser 1995; Holick et al. 1995; Carman et al. 2000). Vitamin D_3 is essential in calcium metabolism of lizards and other vertebrates (Allen et al. 1994; Laing and Fraser 1999), thus, this compound could inform conspecifics, especially females, on the health status of males. Interestingly our results showed that variation of cholesta-5,7-dien-3-ol between populations was notable, and also responded to the classification North vs. South. Lizards from Southern populations had lower proportions of cholesta-5,7-dien-3-ol than those from Northern populations. Thus, this could be interpreted as that lizards from the North secrete more cholesta-5,7-dien-3-ol, probably because their intraspecific communication relies more on this compound. While Southern lizards might not use this chemical signal so much, but rely more on another type of signals. The comparison of the potential importance of visual signals (i.e., number of blue ocelli) might support this. The number of blue ocelli in males differed between populations; individual from the North have less blue ocelli than Southern lizards. Many animals display color in fleshy structures which may be accurate indicators of quality due to their potentially rapid response to changes in condition (Lozano 1994; Faivre et al, 2003). In many lizards, males show a conspicuous row of small distinctive blue ocelli that runs along their body side on the outer margin of the belly. These ocelli could be a visual signal used in intrasexual selection processes (López et al. 2002, 2004). As it occurs in other lacertid lizards, blue ocelli may be a reliable signal of sex, body size/age and/or body condition (López et al. 2004).

Considering the differences in number of femoral pores and blue ocelli between Northern and Southern populations in this contact area, we could suggest that these populations might be adapted to different habitats and in consequence might be using different type of signals in communication. These elements lead us to hypothesize that populations from the mountains of North of Madrid, with cold and humid habitat, might use chemical communication more intensively than lizards from Southern populations. In contrast, in the populations from South of Madrid, where the habitat is drier and hotter, other type of communication, such as the visual one, could prevail.

These characteristics of Northern population are closed to those described for the morphotype 1 of *P. hispanica* by Sá-Sousa (2000), while individuals from the South look more like the description of the morphotype 2 (Pérez-Mellado and Galindo 1986; Guillaume 1987; Sá-Sousa 2000; Sá-Sousa et al. 2002). We observed a particular result for lizards from Pedrezuela population; these lizards live in the North but they have chemical and morphological differences with respect to other Northern lizards such as, for example, those from Fuenfría. This population could be adapted to this place and could have evolved differently than the other Northern populations. Experiments of chemical recognition and genetic tests could clarify whether lizards from Pedrezuela are or are not closer to Fuenfría lizards or whether this population of lizards is totally different. All these populations studied living in distinct areas could have evolved in different way and used different communication signals or different cues in mate choice. These chemical differences between North and South or between populations could be related with similar genetic variations, but further molecular comparisons are necessary to conclude this. Thus, these results suggest the existence of ongoing reproductive isolation and speciation between types in the *P. hispanica* complex, which merits further studies.

In summary, in this paper, we compared five different populations within the species complex of *P. hispanica* in a contact zone where we can found at least two previously described morphotypes. Results showed that morphologically and chemically, we could differentiate at least between two classes or types of lizards: lizards from the North and from the South of the region. The Northern individuals were more robust, heavier and larger with more femoral pores, but less blue ocelli, than Southern lizards. Moreover, chemical femoral secretions varied between populations. Most of this variation could be due to local adaptation to different habitats and could be consequence of different alimentations in different areas. Finally, we noted a correlation between the classification North vs. South and some chemical components, especially for cholesta-5,7-dien-3-ol. Males from the Northern populations have higher proportions of this steroid in secretions than Southern males. Because this compound could be an honest signal of male quality in some lizards, we could hypothesize that the importance of this signal could differ between Northern and Southern populations. Finally, all these results point out that these populations could have evolved different types of signals. These results give more cues on the possibility that in this contact zone there could be an ongoing speciation process between *P. hispanica* populations.

Acknowledgments We thank "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an “El Ventorrillo” CSIC grant to MG. The experiments enforced all the present Spanish laws and were performed under license from the Environmental Organisms of Madrid Community where they were carried out.

References

Aitchison, J. 1986. The statistical analysis of compositional data: Monographs in statistics and applied probability. London: Chapman and Hall.

Alberts, A.C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. Am. Nat. **139**: 62–89.

Alberts, A.C. 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behav. Evol. **41**: 255–260.

Alberts, A.C., Pratt, N.C., and Phillips, J.A. 1992. Seasonal productivity of lizard femoral glands: relationships to social dominance and androgen levels. Physiol. Behav. **51**: 729–733.

Allen, M.E., Bush, M., Oftedal, O.T., Roscoe, R.,Walsh, T., and Holick, M.F. 1994. Update on vitamin D and ultraviolet light in basking lizards.

Proc. Am. Assoc. Zoo Vet. **25**: 314–316.

Aragón, P., López, P., and Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Arnold, E.N., and Ovenden, D.W. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Harper Collins, London.

Barbosa, D., Font, E., Desfilis, E., and Carretero, M.A. 2006. Chemically mediated species recognition in closely related Podarcis wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Bonnet, X., and Naulleau, G. 1994. Utilisation d'un indice de condition corporelle (BCI) pour l'étude de la reproduction chez les serpents. C. R. Acad. Sci. **317**: 34–41.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature **411**(6840): 900–901.

Carazo, P., Font, E., and Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895–902.

Carman, E.N., Ferguson, G.W., Gehrmann, W.H., Chen, T., and Holick, M.F. 2000. Photobiosynthetic opportunity and ability for UVB generated vitamin D synthesis in freeliving house geckos (*Hemidactylus turcicus*) and Texas spiny lizards (*Sceloporus olivaceous*). Copeia **2000**: 245–250.

Cooper, W.E., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., and Vitt, L.J. 1987. Ethological isolation, sexual behavior and pheromones in the fasciatus species group of the lizard genus *Eumeces*. Ethology **75**: 328–336.

Dietemann, V., Peeters, C., Liebig, J., Thivet, V., and Hölldobler, B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc. Natl. Acad. Sci. USA **100**: 10341–10346.

Escobar, C.M., Escobar, C.A., Labra, A., and Niemeyer, H.M., 2003. Chemical composition of precloacal secretions of two *Liolaemus fabiani* populations: are they different? J. Chem. Ecol. **29**: 629–638.

Escobar, C.A., Labra, A., and Niemeyer, H.M. 2001. Chemical composition of precloacal secretions of Liolaemus lizards. J. Chem. Ecol. **27**: 1677–1690.

Favre, E., François, L., Fluteau, F., Cheddadi, R., Thévenod, L., and Suc, J.P. 2007. Messinian vegetation maps of the Mediterranean region using models and interpolated pollen data. Geobios **40**: 433–43.

Fraser, D.R. 1995. Vitamin D. Lancet **345**: 104–107.

García-Paris, M., Martín, C., and Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain-Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Gómez, A., Font, E., and Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica*. In: Lacertids of the Mediterranean Region (E.D. Valakos, W. Böhme, V. Pérez-Mellado and P. Maragoú, eds.) Athens, Greece: Helenic Zoological Society. pp. 213–230.

Guillaume, C.P. 1987.) Les petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: Univ. Sci. Techn. Langedoc.

Harris, D.J., and Sá-Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75–81.

Holick, M.F., Tian, X.Q., and Allen, M. 1995. Evolutionary importance for the membrane enhancement of the production of vitamin D_3 in the skin of poikilothermic animals. Proc. Natl. Acad. Sci. USA **92**: 3124–3126.

Johnston, R.E. 2000. Chemical communication and pheromones: The types of chemical signals and the role of the vomeronasal system. In: The Neurobiology of Taste and Smell, 2nd edition. Ed. by T.E. Finger, W.L. Silver & D. Restrepo. Wiley, NY, pp. 101–127.

Johnston, R.E. 2001. Neural mechanisms of communication: from pheromones to mosaic signals. In: Chemical Signals in Vertebrates, Vol. 9. Ed. By A. Marchlewska-Koj, J. J. Lepri & D. Muller-Schwarze. Plenum, N.Y.

Laing, C. J., and Fraser, D. R. 1999. The vitamin D system in iguanian lizards. Comp. Biochem. Physiol. **123**: 373–379.

López, P., Gabirot, M., and Martín, J. 2009. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturw. **96**: 65–69.

López P., and Martín, J. 2005. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–465.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

López, P., Martín, J., and Cuadrado, M. 2004. The role of lateral blue spots in intrasexual relationships between male Iberian rock-lizards, *Lacerta monticola*. Ethology **110**: 543–561.

Lozano, G.A. 1994. Carotenoids, parasites, and sexual selection. Oikos **70**: 309–311.

Martín, J., and López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B **267**: 1265–1269.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., Moreira, P.L., and López, P. 2007. Status-signaling chemical badges in male Iberian rock lizards. Funct. Ecol. **21**: 568–576.

Mason, R.T. 1992. Reptilian pheromones. In: Gans C, Crews D, eds. Biology of the Reptilia. Vol. 18. Chicago: University of Chicago Press, pp. 114–228.

Mellado, J., and Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, Act. Verteb. **8**: 299–300.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B, Suppl. **270**: 254–S256.

Panhuis, T.M., Butlin, R., Zuk, R.M., and Tregenza, T. 2001. Sexual selection and speciation. Trends Ecol. Evol. **16**: 364–371.

Pérez-Mellado, V., and Galindo-Villardón, M.P. 1986. Sistemática de *Podarcis* (Sauria Lacertidae) ibéricas y Norteafricanas mediante técnicas multidimensionales. Ediciones de la Universidad de Salamanca, Salamanca.

Pinho, C., Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Sá-Sousa, P. 2000. A predictive distribution model for the Iberian wall lizard (*Podarcis hispanicus*) in Portugal. Herpetol. J. **10**: 1–11.

Sá-Sousa, P., Vicente, L., and Crespo, E.G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. Amphib-Rept. **23**: 55–69.

Shaw, K.L., and Parsons, Y.M. 2002. Divergence of mate recognition behavior and its consequences for genetic architectures of speciation. Am. Nat. **159**: S61–S75.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Smadja, C., and Butlin, R.K. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity **102**: 77–97.

Weldon, P.J., Flachsbarth, B., and Schulz, S. 2008. Natural products from the integument of nonavian reptiles. Nat. Prod. Rep. **25**: 738–756.

Yom-Tov, Y., and Nix, H. 1986. Climatological correlates for body size of five species of Australian mammals. Biol. J. Linn. Soc. **29**: 245–262.

CAPÍTULO 2

Las variaciones interpoblacionales en el reconocimiento químico y en la elección de olores de machos por parte de las hembras de la lagartija Ibérica *Podarcis hispanica* podrían reflejar procesos de especiación críptica.

Comparaciones genéticas y morfológicas entre tipos sugieren la existencia un complejo de especies de la lagartija Ibérica (*Podarcis hispanica*), con varios tipos monofiléticos, en procesos activos de aislamiento reproductivo. En estas lagartijas, las feromonas sexuales son importantes en la elección de pareja y el reconocimiento interespecífico y, por lo tanto, las diferencias entre tipos en las secreciones químicas podrían llevar a un aislamiento reproductivo. En el centro de la Península Ibérica, se encuentran varias poblaciones con características diferenciadas de morfología, coloración y señales químicas, que ocupan áreas cercanas sin aislamiento geográfico. En esta zona de contacto, estudiamos la comunicación interpoblacional mediante señales químicas entre cinco poblaciones diferentes de *P. hispanica.* Los resultados no indicaron una discriminación quimiosensorial clara de los olores de individuos de la propia población, sino sólo una preferencia por el área de origen (norte vs. sur). Los individuos discriminaron y mostraron más interés por olores de lagartijas de varias poblaciones su propia área frente a las de la otra área. Sin embargo, es interesante que sólo los machos de las poblaciones más alejadas geográficamente distinguieran y señalaron más interés por olores de hembras de su propia población que por los de las otras poblaciones. Además, hicimos pruebas de elección en las que las hembras de tres poblaciones podían elegir entre sustratos con marcas olorosas de machos de distintas poblaciones. Las hembras de las poblaciones más alejadas geográficamente pasaron más tiempo en zonas con marcas olorosas de machos de su propia población, mientras que las hembras de la población intermedia no prefirieron marcas olorosas de machos de su población. La habilidad de los machos de algunas poblaciones para discriminar entre señales químicas de hembras de distintas poblaciones y el reconocimiento del área de origen por los dos sexos indicaría la existencia de aislamiento reproductivo parcial entre algunas de estas poblaciones de esta zona de contacto. Estos resultados apuntarían a que el aislamiento reproductivo entre poblaciones dentro del complejo de *P. hispanica* no está tan claro, pero que, al menos entre las poblaciones más distantes geográficamente, existiría un proceso de especiación críptica mediado por aislamiento reproductivo basado en feromonas.

Interpopulational variation in chemosensory recognition and female choice of males' scent in the Iberian wall lizard *Podarcis hispanica* may reflect cryptic speciation processes

Marianne Gabirot, Pilar López, José Martín

Abstract Genetic and morphological comparisons between types suggest the existence of a "species complex" with several monophyletic types in the Iberian wall lizard (*Podarcis hispanica*), with a ongoing reproductive isolation processes. In these lizards, sex pheromones are important for mate choice and species recognition and, thus, differences between lizards' types in chemical secretions could lead to reproductive isolation. In Central Spain, there are several distinct populations with distinct morphology, coloration and chemical signals characteristics that occupy nearby areas without geographic isolation. In this contact zone, we studied interpopulational communication by chemical cues between five distinct populations of *P. hispanica*. Our results did not indicate a clear chemosensory discrimination of scents of individuals from the own population, but only an origin area preference (north vs. south). Individuals discriminated and showed more interest for scents of several populations of lizards from their own area than from the other area. However, interestingly, only males from geographically farther populations distinguished and had more interest for scent of females from their own populations than from other populations. In addition, we made choice trials where females of three populations could choose between scents marked substrates of males from different populations. Females from the two more geographically distant populations spent more time on scent marks of males from their own population; while females from the intermediate population did not prefer scent marks of males from their population. The ability of males from some populations to discriminate between chemical signals of female populations and the area of origin recognition by both sexes may indicate the existence of partial reproductive isolation between some of these populations in this contact zone. All these results support that reproductive isolation between distinct populations in the *P. hispanica* complex is not clear, but that, at least between the more geographically distant populations there could exist an ongoing cryptic speciation process mediated by pheromone reproductive isolation.

Résumé Des comparaisons génétiques et morphologiques ont suggérées l'existence un complexe d'espèces du lézard *Podarcis hispanica,* qui comprend plusieurs types monophylétiques, avec un processus d'isolation reproductive en marche mais le comportement sexuel est pour le moment inconnu. Ces lézards utilisent les phéromones sexuelles pour faire leurs choix de partenaire ou pour la distinction entre espèces, donc des différences au niveau des secrétions chimiques de cet animal pourraient conduire à un isolement reproductif. Dans la partie centrale de la péninsule ibérique, au moins deux morphotypes de ce complexe ont été identifiés sans aucune isolation géographique notable. En conséquence, dans cette zone de contact, nous avons étudié la communication interpopulationale à base de signaux chimiques entre différentes populations caractérisées par une morphologie et signaux chimiques distincts. De plus, les femelles de chaque population sont mises en présence de plusieurs odeurs de mâles (de sa population ou non) et pourront faire le choix d'être en présence ou non de ces secrétions. Nos résultats ont montré l'existence d'une discrimination significative utilisant les signaux chimiques de la part des femelles et des mâles, mais pas de préférence claire pour les odeurs de leur propres populations, seulement une préférence pour les secrétions de la zone. Les lézards du nord et sud de la zone

de contact ont été plus intéressés par les secrétions des populations de leur zone que de l'autre (Sud vs. Nord). Seulement, les mâles des populations situées aux extrêmes de cette zone, reconnaissaient et avaient un intérêt élevé pour les odeurs des femelles de leur propre population. De plus, les femelles de ces populations extrêmes ont passé plus de temps en présence des secrétions des mâles de leur population plutôt que des autres populations ; tandis que la population intermédiaire n'a pas de préférence pour un type de sécrétion. En conclusion, la discrimination par les mâles entre les différentes odeurs de femelles, de différentes populations et la reconnaissance de la zone d'origine des secrétions par les deux sexes indiqueraient la possibilité de processus de spéciation entre les populations de cette zone de contact. Tous ces résultats supportent une fois de plus que l'isolation reproductive entre population du complexe *Podarcis hispanica* n'est pas claire, mais lorsque l'on étudie les populations les plus éloignées un processus de spéciation cryptique est en marche avec une barrière reproductive à base de phéromones.

Enviado a *Behavioral Ecology and Sociobiology*

INTRODUCTION

Sexual selection is one of the mechanisms that may facilitate speciation due to divergence in male mating signals and female preferences (Boughman 2001; Panhuis et al. 2001), and, thereafter, several species recognition mechanisms may prevent interspecific matings (e.g., Shaw and Parsons 2002; Shine et al. 2002). In many animals, chemical signals are important for mate and species recognition and may result in premating reproductive isolation and speciation (reviewed in Smadja and Butlin 2009). In reptiles, chemical signals released at the time of reproduction may provide the basis for species recognition and avoidance of interspecific mating among closely related sympatric species (Cooper and Vitt 1986; Mason 1992; Shine et al. 2002) or between different populations of the same species (LeMaster and Mason 2003).

Chemical compounds from femoral gland secretions in many lizards are used in intraspecific communication (Mason 1992; Aragón et al. 2001). The femoral pores are epidermal structures on the ventral surface of the thigh connected to glands that produce copious amounts of holocrine secretion, especially in males and during the mating season (reviewed in Mason 1992; Alberts 1993; Martín and López in press). Behavioral experiments of several Lacertid lizard species showed that both male and female lizards responded by tongue-flicking to substrate scent marks, but also, during social encounters, lizards investigated each other by tongue-flicking the cloacal and femoral regions. Furthermore, these experiments showed that femoral gland secretions of males could advertise residence in a home range, and/or convey information about social status and competitive ability (Aragón et al. 2001; López and Martín 2002), or even transmit information about a male's quality, which females may use to select mates (Martín and López 2000; 2006a,b; López et al. 2002; Olsson et al. 2003; reviewed in Martín and López in press). Thus, because femoral gland secretions are involved in sexual selection they may lead to speciation processes. For example, chemical cues prevented heterospecific matings between closely related species of skinks of the genus *Eumeces* (Cooper and Vitt 1987).

The Iberian wall lizard, *Podarcis hispanica* is a small (50-70 mm adult snout-to-vent length, SVL) lacertid lizard common and widespread at rocky habitats inside many different environments of the Iberian Peninsula. This lizard has well developed chemical recognition abilities, and is able to discriminate between conspecifics and heterospecifics (Cooper and Pérez-Mellado 2002; Barbosa et al.

2006) and between sexes by chemical cues alone (Gomez et al. 1993; López and Martín 2001; López et al. 2002). Chemical cues of males are important in male-male interactions (López and Martín 2002; Carazo et al. 2007). Moreover, in at least one population, females prefer to stay on areas scent marked by males with high proportions of cholesta-5,7-dien-3-ol (=provitamin D_3) in their femoral secretions, which may signal a better immune response of those males (López and Martín 2005; López et al. 2008). Therefore, it seems that female mate choice decisions may be, at least partially, based on characteristics of chemical signals of males.

Between populations of several taxa of this species complex (*Podarcis hispanica*), molecular studies showed the possibility of relationships and genetic distances, which suggest the existence of ongoing speciation processes within taxa previously considered to be conspecific. Moreover, molecular and morphological studies suggest that the Iberian wall lizard is paraphyletic, and forms a species complex with at least five monophyletic lineages (Guillaume 1987; Harris and Sá-Sousa 2001, 2002; Sá-Sousa et al. 2002; Pinho et al. 2007; Carretero 2008). For example, two allopatric types have been identified in the Iberian Peninsula: in Northwestern Iberia, *P. hispanica* type 1 occurs, mainly in highlands and where Atlantic humid environmental conditions prevail, while *P. hispanica* type 2 occurs in Central and Southern Iberia, where Mediterranean dry conditions are typical (Sá-Sousa 2000; Sá-Sousa et al. 2002).

Although populations of the two types of *P. hispanica* are mainly allopatric, both types have been reported from the Madrid region (Central Spain). In this area, the two populations live close together without geographical isolation, and individuals of both types may find each other easily (Mellado and Olmedo 1981; García-Paris et al. 1989). Nearby populations of *P. hispanica* from the Guadarrama Mountains (Madrid, Central Spain), inhabiting different altitudes and environmental conditions, differ in the chemical composition of femoral gland secretions of males (Martín and López 2006a) and have also morphologic and coloration differences (Gabirot et al. unpublished data). Males of the population inhabiting more humid microclimates have secretions with higher proportion of compounds (e.g., long chain alcohols and waxy esters) that may favor persistency and efficiency of chemical signals in humid environments. Moreover, different rates of chemosensory exploration show that males can discriminate by chemical cues alone between males of their own and the other population (Martín and López

2006a,b). In contrast, females detect scent of males, but do not seem able to discriminate between scents of males of the two populations (Martín and López 2006a,b). Similarly, males of *P. bocagei* and *P. hispanica* from the North of Portugal are able to discriminate chemically between conspecifics and heterospecifics, but females are not (Barbosa et al. 2006). These results suggest that despite of clear differences in morphology and chemical signals between these two closed populations of *P. hispanica* lizards, there is not clear interpopulational discrimination at the level of chemical recognition and pre-mating reproductive isolation. Therefore, reproductive isolation and speciation between these two populations of *P. hispanica* complex is not clear.

Podarcis lizards are highly variable in size, shape, escalation and color pattern, not only between currently recognized species (Arnold and Ovenden 2002), but also between populations and individuals. Given this, disentangling the track of evolutionary history from the effects of local adaptation or phenotypic plasticity (or finding a cohesive phenotype) can be a difficult task. Morphological distinction between *Podarcis* forms can neither be taken for granted nor necessarily attributed to separate evolution.

In this paper, we captured *P. hispanica* lizards from five distinct populations (respect to morphology, chemical signals and coloration) from different geographical areas of the Madrid region in Central Spain. This is an area where at least two types (1 and 2) of *P. hispanica* can be found. This area could be a contact zone, where perhaps more than two types could be found. In fact, observations and captures around Madrid showed that there are more than two distinct populations of *P. hispanica* with variation in coloration, morphology and chemical signals (Gabirot et al. unpublished data; see chapter 1). Thus, we studied three populations from the northern area of Madrid, one from the north and two from the northwest, one from the mountains and two from the mountain basin at low altitude. In the south, we captured two isolated populations at similar low altitude. We studied chemosensory recognition and discrimination between these populations from the contact area in order to better understand the interpopulation chemical communication and the possible existence of cryptic speciation process. We hypothesized that, in addition to visual differences, individuals could recognize scents of lizards of their own population by chemical cues alone. Moreover, individuals from close populations could have more “chemical interest” between them than for individuals from farther (geographically) populations. Differences in chemosensory recognition may reflect the existence of cryptic on-going speciation processes within these populations. Finally to help understanding the role of chemical communication between populations in potential pre-mating isolation processes, we made a scents choice experiment, where females of each population were presented and could choose between males’ scent marked substrates from different populations. We hypothesized that interpopulational differences in chemical signals of male lizards’ *P. hispanica*, and/or in female mate preferences, could be leading to reproductive isolation processes between populations of this lizard species.

MATERIALS AND METHODS

Study populations

Fig.1: Localization of the five populations studied of *Podarcis hispanica* in the Madrid province in the center of Spain.

During February-March 2008, we captured by noosing male and female *P. hispanica* lizards at five localities within the Madrid Province (Central Spain) (Fig. 1). We chose these populations because they showed clearly distinct morphological and chemical characteristics (cf. Chapter 1). These populations are separated one from each other by distances between 10 and 100 km, but there are not current geographical barriers that could isolate them. We selected three populations (Aranjuez, Fuenfría and Pedrezuela), that are the populations more geographically distant from each other (around 100km between them), and, thus, with less possibility of contact. Then we selected another two populations (Belmonte and Golondrina) geographically located in the middle or between the more extreme populations. ‘Fuenfría’, ‘Golondrina’ and ‘Pedrezuela’ were located in the mountain northern area, and (‘Belmonte’ and ‘Aranjuez’ were situated in the plain southern area (Fig. 1). In the north, we captured lizards from a population occupying different granite rock-cliffs at the edge of a pine forest in the upper part of ‘Fuenfría’ Valley (40°47’ N, 4°03’ W; 1750 m altitude) (24 males and 27 females), on granite rocky outcrops in a large oak forest (‘Golondrina’) near Cercedilla village (40°44’ N, 4°02’ W; 1250 m altitude) (18 males and 24 females), and from old stone walls near cultivated fields in the ‘Pedrezuela’ village (40°44' N, 3°36' W; 800 m altitude) (14 individuals of each sex) (Fig. 1). In the south, we captured lizards on walls of human buildings in a public garden in the ‘Belmonte del Tajo’ village (40°8' N, 3°20' W; 735 m altitude) (10 males and 16 females) and on chalk and gypsum rocks in deforested bushy hills near ‘Aranjuez’ (40°2’ N, 3°37’ W; 494 m altitude) (8 males and 12 females) (Fig. 1).

All lizards were individually housed at “El Ventorrillo” Field Station (Cercedilla, Madrid) about 5 Km from the capture sites of the northern populations, in indoor 60x40 cm PVC terraria containing sand substratum and rocks for cover. Cages were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle, and were screened from each other using cardboard. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and water was provided *ad libitum*. Lizards were returned to their exact capture sites with good health condition at the end of experiments. Captures and observations were performed under license from the Environmental Agency of Madrid Government (“Consejería del Medio Ambiente de la Comunidad de Madrid”, Spain).

Chemosensory recognition between populations

Lizards have been shown to react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt 1990). Tongue-flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues (e.g., Cooper and Pérez-Mellado 2002). Thus, to test for differential responses to scents we made comparisons of TF rate by lizards (males and females) in response to chemical stimuli arising from cotton applicators impregnated with scents of male and female *P. hispanica* of the five different populations (Aranjuez, Golondrina, Fuenfría, Pedrezuela and Belmonte) or with deionized water (odorless control) (Cooper and Burghardt 1990). Water was used to gauge baseline TF’s rates in the experimental situation. We obtained lizard scents from the femoral pores of males or from the cloacal area of females because these are the bodily areas most frequently and intensely investigated by tongue-flicking during social encounters (López and Martín 2001, 2002; López et al. 2002).

Therefore, after first dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water, we rolled the tip over those bodily areas (of one population and sex per applicator). We used a new applicator in each trial.

First, males were exposed to scents from males and then to scents from females of each population tested. Finally we studied the reaction of females to scent of males of each population. Every lizard was exposed to each stimulus and order of presentation was counterbalanced. One trial was conducted per day for each animal. Trials were conducted in outdoor conditions during April, which coincided with the mating season of lizards in their original natural populations (P. López and J. Martín unpublished data), and between 11:00-13:00 h (GMT) when lizards were fully active.

To begin a trial, the experimenter slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. Lizards usually did not flee from the swab, but explore it repeatedly by tongue-flicking or ignore it after the firsts TFs. The numbers of TFs directed at the swab were recorded for 60 s beginning with the first TF. To examine differences in number of TFs directed at the swab among treatments, we used repeated measures two-way ANOVAs examining the effects of scent stimuli (within factor: Fuenfría *vs.* Golondrina *vs.* Aranjuez *vs.* Pedrezuela *vs.* Belmonte *vs.* water) and population of origin of the responding lizard (between factor). We included the interaction in the model to analyze whether responses to the different scents differed as a function of the population of the responding lizard. Because the interactions were significant (see results), we further used separated repeated measures one-way ANOVAs to test the effect of scent stimuli within each population of responding lizards. Analyses were made separately for responding males and females. Data were log-transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honestly significant difference (HSD) tests (Sokal and Rohlf 1995).

Females' choice of substrates scent-marked by males

In this experiment, we used only lizards from three populations, two from the northern mountains (Fuenfría and Golondrina) and one from the southern plains (Aranjuez). We placed in the males' terraria several absorbent paper strips (35x10 cm) fixed to the floor, and left them there for three weeks to allow males to scent-mark the substrates. The experiments of females' choice of scent-marks were performed at the end of April, coinciding with the mating season of this lizard. Females' cages had two basking platforms (two identical flat tiles) placed symmetrically at each end of the cage, and rocks for cover in the center. At the beginning of experiments (09:00 h, GTM; when females where still inactive) we fixed, wearing fresh gloves, on one tile one paper strip from one individual male, and on the other tile a paper from another individual male. Different papers from each male were used in different choice test with different females. Each female was presented with two scents types per trial, one from a male from her own population, which serves as a control and other from a male from each of the three populations in three different trials. Thus, for example, females from Aranjuez population were tested in three different days in these trials: "Aranjuez vs. Fuenfría", "Aranjuez vs. Golondrina" and "Aranjuez vs. Aranjuez". The individual males tested and the positions of papers were randomly determined. Each female was tested twice in each of the three trials, once a day, with papers from two different pairs of males. Each trial lasted 2 h (from 11:00 h GMT, shortly after females appeared from refuges and until 13:00 h GMT when females hid again). Females were monitored each 10 min from a hidden point. If a female was located on a tile with paper strip, she was designated as haven chosen temporarily that particular paper, whereas, if she was located outside of the tiles, she was designated as having made no choice (Martín and López 2000, 2006c; Olsson et al. 2003). From these observations, we calculated the time spent by females in each of these three areas. At the end of the trials the papers were removed and the cage was thoroughly rinsed with clean water.

For each trial, females were confronted to scent of males from their own population (control side) against another male scent (experimental side). We used this design because the time spent by females in one side depended on the time spent on the other side. Thus, we compared the time spent by females in the experimental side between treatments (other population 1 vs. other population 2 vs. own population). We used two-way repeated measures ANOVA models to test for differences between the two days of the trial and between the three treatments, both as within-subject factors (Sokal and Rohlf 1995). Tests were conducted separately for responding females from each population.

RESULTS

Chemical recognition between populations

Responses of males to scent of males

All male *P. hispanica* directed TFs to the swab in all conditions. There were significant differences between scent treatments in numbers of TFs directed by males (two-way repeated measures ANOVA: $F_{5,380}=125.17$, $P<0.0001$; Fig. 2), and between the populations of origin of the responding males ($F_{4,76}=3.40$, $P=0.001$), which responded in a different way to the different stimuli tested (interaction: $F_{20,380}=12.68$, $P<0.0001$) (Fig. 1). Therefore, we analyzed separately the responses of males of each population, showing that the number of TFs differed significantly between the scents presented in all cases (one-way repeated measures ANOVAs: $32.00<F<54.32$; $P<0.0001$ for all; see below) (Fig. 2). In all populations, males discriminated between scents of any male and water (Tukey's tests: $P<0.005$ in all cases).

Aranjuez's males directed a significantly higher number of TFs to scent of males of their own population than to scent of males from the three northern populations (one-way repeated measures ANOVA: $F_{5,85}=36.46$, $P<0.0001$; Tukey's tests: $P=0.00014$ to Fuenfría, $P=0.00012$ to Golondrina, and $P=0.00012$ to Pedrezuela), which did not differ between them ($P>0.50$ for all) (Fig. 2a). In contrast, Aranjuez's males did not differ significantly in their responses to scent of males from their own population and males from the other southern population (Belmonte; $P=0.09$), but responses to Belmonte's males were significantly higher than to males from Golondrina and Pedrezuela populations ($P<0.0001$). Responses to Belmonte's and Fuenfría's males were not significantly different ($P=0.15$) (Fig. 2a).

Similarly, Belmonte's males directed significantly more TFs in direction to scent of males from their own population than to scent of males from the three northern populations (one-way repeated measures ANOVA: $F_{5,40}=35.98$, $P<0.0001$; Tukey's tests, Fuenfría: $P=0.0002$; Golondrina: $P=0.002$; Pedrezuela: $P=0.02$), which did not differ significantly between them ($P>0.50$ for all) (Fig. 2b). Belmonte's males directed a non significant different number of TFs to scent of males of their own population and to males from Aranjuez ($P=0.18$). Responses to Aranjuez's males did not differ significantly of responses to males from the three northern populations ($P>0.10$ in all cases)

The number of TFs directed by Fuenfría's males were significantly higher in response to scent of males of their own population than to scent of males from any other populations (one-way repeated measures ANOVA: $F_{5,115}=54.42$, $P<0.0001$; Tukey's tests: $P<0.0002$ for all), and there were no significant differences between the number of TFs directed to scent of males from these other populations ($P>0.07$ for all) (Fig. 2c).

Golondrina's males directed a significantly higher number of TFs in response to scent of males of their own population than to males from Aranjuez, Belmonte and Pedrezuela (one-way repeated measures ANOVA: $F_{5,80}=32.00$, $P<0.0001$; Tukey's tests: $P<0.0001$ for

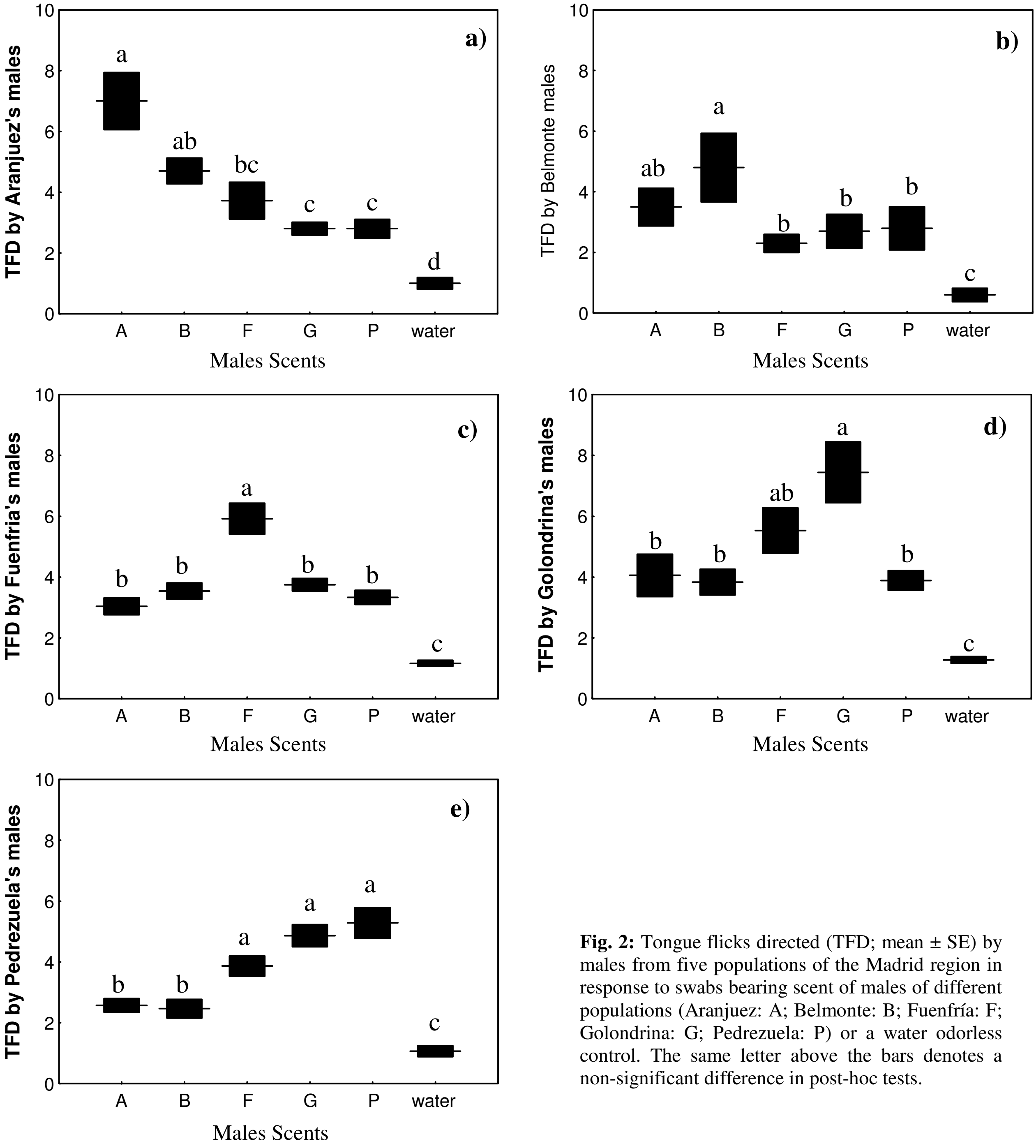


Fig. 2: Tongue flicks directed (TFD; mean ± SE) by males from five populations of the Madrid region in response to swabs bearing scent of males of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a non-significant difference in post-hoc tests.

all)(Fig. 2d). The number of TFs in response to scent of males of their own population and Fuenfría males were no significant different (P=0.12), and the latter was not significant different from the rest of populations (P>0.50 for all).

Males from Pedrezuela directed significantly more TFs in response to males of their own population than to males of the two southern populations (one-way repeated measures ANOVA: $F_{5,60}$=39.19, P<0.0001; Tukey's tests: Aranjuez: P=0.0001; Belmonte: P=0.0001), which were not significantly different between them (P=0.99) (Fig. 2e). However, responses to males of their own

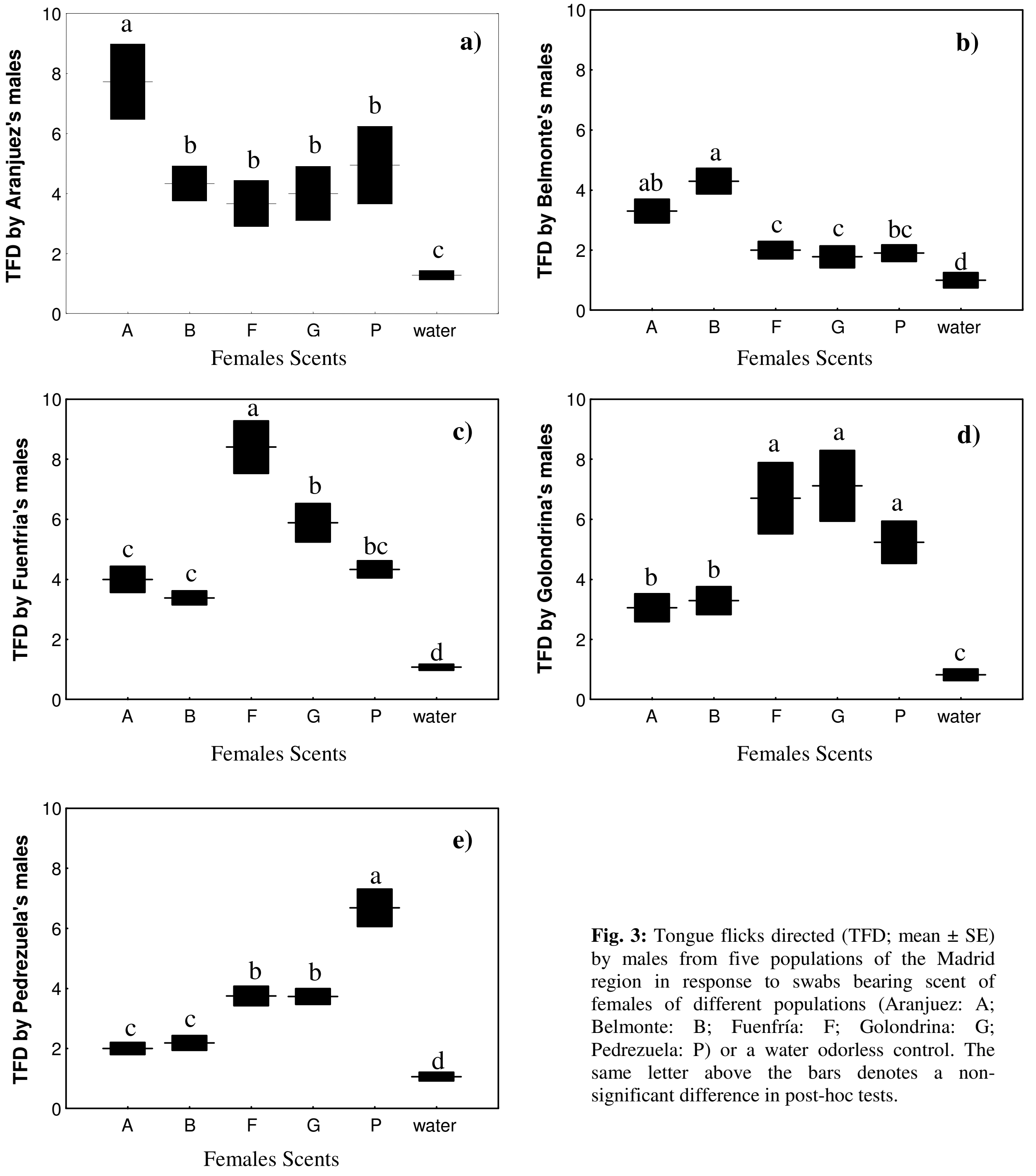


Fig. 3: Tongue flicks directed (TFD; mean ± SE) by males from five populations of the Madrid region in response to swabs bearing scent of females of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a non-significant difference in post-hoc tests.

population did not significantly differ from responses to males of the other two northern populations (P>0.50 for all). The TFs directed by Pedrezuela males were higher in response to scent of males from northern than southern populations (P<0.0001 for all).

Responses of males to scent of females

All male *P. hispanica* directed TFs to the *swab in all conditions. There were* significant differences between treatments in numbers of TFs directed by males (two-way repeated measures ANOVA:

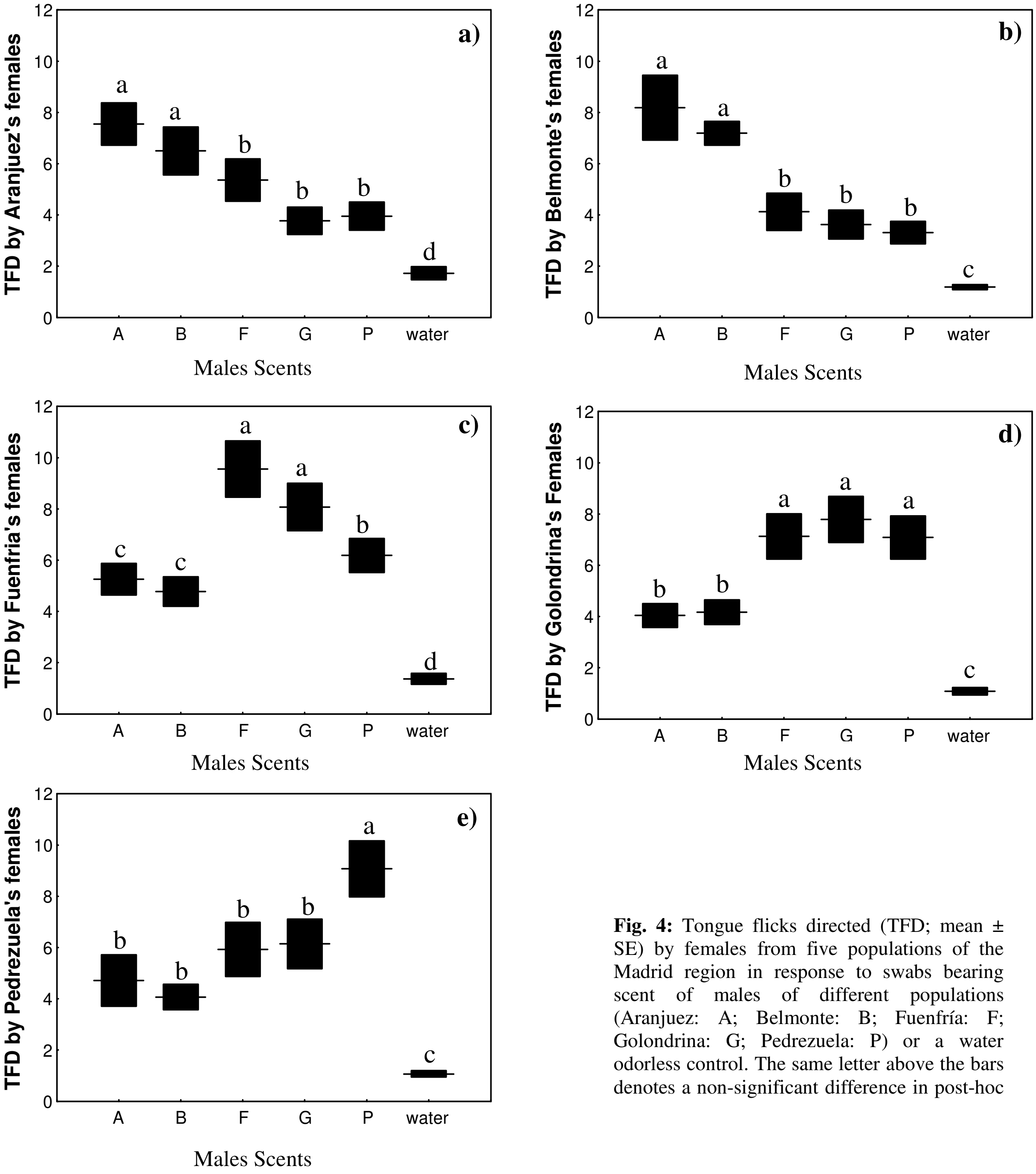


Fig. 4: Tongue flicks directed (TFD; mean ± SE) by females from five populations of the Madrid region in response to swabs bearing scent of males of different populations (Aranjuez: A; Belmonte: B; Fuenfría: F; Golondrina: G; Pedrezuela: P) or a water odorless control. The same letter above the bars denotes a non-significant difference in post-hoc

$F_{5,400}$=98.74, $P<0.0001$; Fig. 3), there were significant differences between the populations of origin of the responding males ($F_{4,80}$=5.65, P=0.0004), which responded in a different way to the stimuli tested (interaction: $F_{20,400}$=13.85, $P<0.0001$) (Fig. 3). Therefore, we analyzed separately each male population, showing that the number of TFs differed between treatments in all populations (one-way repeated measures ANOVAs: 21.89<F<4.94; $P<0.0001$ for all; see below) (Fig. 3). In all cases, males discriminated between scents of any female and water (Tukey's tests: $P<0.005$ in all cases).

Aranjuez's males directed a significantly higher number of TFs to scent of females of their own population than to scent of females from all other populations (one-way repeated measures ANOVA: $F_{5,85}$=24.39, $P<0.0001$; Tukey's tests: $P<0.0001$ for all), which did not vary significantly between them ($P>0.50$ for all) (Fig. 3a).

Belmonte's males directed significantly more TFs in response to scent of females from their own population than to females from the northern populations (one-way repeated measures ANOVA: $F_{5,40}$=21.89, $P<0.0001$; Tukey's tests: Fuenfría: P=0.0001; Golondrina: P=0.0001; Pedrezuela: P=0.0007); which did not significantly differ between them ($P>0.50$) (Fig. 3b). However, Belmonte males showed a non significant different number of TFs in response to scent of females of their own population than to Aranjuez females (P=0.54).

The number of TFs by Fuenfría males were significantly higher in response to scent of females of their own population than to females from any other population (one-way repeated measures ANOVA: $F_{5,125}$=74.84, $P<0.0001$; Tukey's tests: $P<0.0002$ for all) (Fig. 3c). There were non significant differences between the number of TFs directed to the two southern populations (P=0.94) nor between Golondrina and Pedrezuela populations (P=0.21). However, the scent of females from the southern populations elicited significantly less TFs than females from the northern populations ($P<0.001$ for all) but those from Pedrezuela, which did not differ significantly from the southern populations ($P>0.18$).

Golondrina's males directed a significantly higher number of TFs in response to scent of females from the northern populations, including their own population, than to females from the southern populations (one-way repeated measures ANOVA: $F_{5,80}$=28.00, $P<0.0001$; Tukey's tests: $P<0.0001$ for all) (Fig. 3d). The number of TFs directed by Golondrina males in response to the two southern populations was no significantly different

(P>0.50 for all), and similarly, there were non significant differences between responses to the scent of females of the three northern populations (P>0.50 for all).

Males from Pedrezuela directed significantly more TFs in response to scent of females of their own population than to scent of females from any other population (one-way repeated measures ANOVA: $F_{5,70}$=49.48, P<0.0001; Tukey's tests: P<0.0001 for all) (Fig. 3e). However, responses to scent of females from the two other northern populations were significantly higher than to females from the two southern populations (P>0.001 for all). Responses to scent of females from the two southern populations did not differ significantly between them (P=0.98), and similarly there were not significant differences between the two other northern populations (P=0.99).

Responses of females to scent of males

All female *P. hispanica* directed TFs to the swab in all conditions. There were significant differences between treatments in numbers of TFs directed by females (two-way repeated measures ANOVA: $F_{5,490}$=129.43, P<0.0001; Fig. 4), there were non significant differences between the overall responses of the populations of origin of the responding females ($F_{4,98}$=0.82, P=0.51), but females responded in a different way to the stimuli tested depending on their population of origin (interaction: $F_{20,490}$=11.87, P<0.0001). Therefore, we analyzed for each population separately, showing that the number of TFs differed between treatments in all populations (one-way repeated measures ANOVAs tests: 27.30<F<49.21; P<0.0001 for all; see below) (Fig. 4). Females discriminated between scents of any male and water (Tukey's tests: P<0.005 in all cases) (Fig. 4).

Aranjuez's females directed a significantly higher number of TFs in response to scent of males of their own population than to males from the three northern populations (one-way repeated measures ANOVA: $F_{5,105}$=27.30, P<0.0001; Tukey's tests: P=0.014 to Fuenfría, P=0.0001 to Golondrina and P=0.0001 to Pedrezuela), which did not differ significantly between them (P>0.50 for all) (Fig. 4a). Furthermore, Aranjuez's females did not significantly differ in their responses to scent of males of their own population or males from the other southern population (Belmonte, P=0.50). Aranjuez females directed significantly more TFs in response to scent of males from Belmonte than to those from Golondrina (P=0.004) and Pedrezuela populations (P=0.01), but responses to

Belmonte and Fuenfría scents did not differ (*P*=0.61) (Fig. 4a).

Belmonte's females directed significantly more TFs in response to scent of males of their own population than to males from the three northern populations (one way repeated measures ANOVA: $F_{5,75}=33.67$, $P<0.0001$; Tukey's tests: Fuenfría: $P=0.0002$; Golondrina: $P=0.0001$; Pedrezuela: $P=0.0001$) (Fig.4b), which did not differ significantly between them ($P>0.50$ for all). Belmonte females directed a non significant different number of TFs to scent of males from their own population than to males from the other southern population (Aranjuez, $P=0.18$).

The number of TFs directed by Fuenfría females were significantly higher in response to scent of males of their own population than males from the two southern populations (one-way repeated measures ANOVA: $F_{5,130}=46.84$, $P<0.0001$; Tukey's tests: $P<0.0001$ for all) and from one of the northern populations (Pedrezuela, $P=0.005$), which did not significantly differ between them ($P>0.50$ for all) (Fig. 4c). Responses to scent of males of their own population and males from Golondrina were non significant different ($P=0.79$), nor were different the responses to males from Golondrina and Pedrezuela ($P=0.21$). Responses to scent of males from Golondrina were significantly higher than to males from the three southern populations (Fig. 4c).

Golondrina's females directed a significantly higher number of TFs in response to scent of males from the northern populations (Fuenfría, Pedrezuela and their own population) than to scent of males from the southern populations (Aranjuez and Belmonte) (one-way repeated measures ANOVA: $F_{5,115}=49.21$, $P<0.001$; Tukey's tests: $P<0.0001$ for all) (Fig. 4d). Responses to males from the northern populations, including their own, did not differ significantly between them ($P>0.50$ for all), nor did differ responses to males from the southern populations ($P>0.50$ for all) (Fig. 4d).

Females from Pedrezuela directed significantly more TFs in response to scent of males from their own population than to males from all the other southern and northern populations (one-way repeated measures ANOVA: $F_{5,65}=33.12$, $P<0.0001$; Tukey's tests: Aranjuez: $P=0.0001$; Belmonte: $P=0.0001$; Fuenfría: $P=0.01$; Golondrina: $P=0.0001$) (Fig. 4e). There were non significant differences between responses to males of these populations ($P>0.50$ for all).

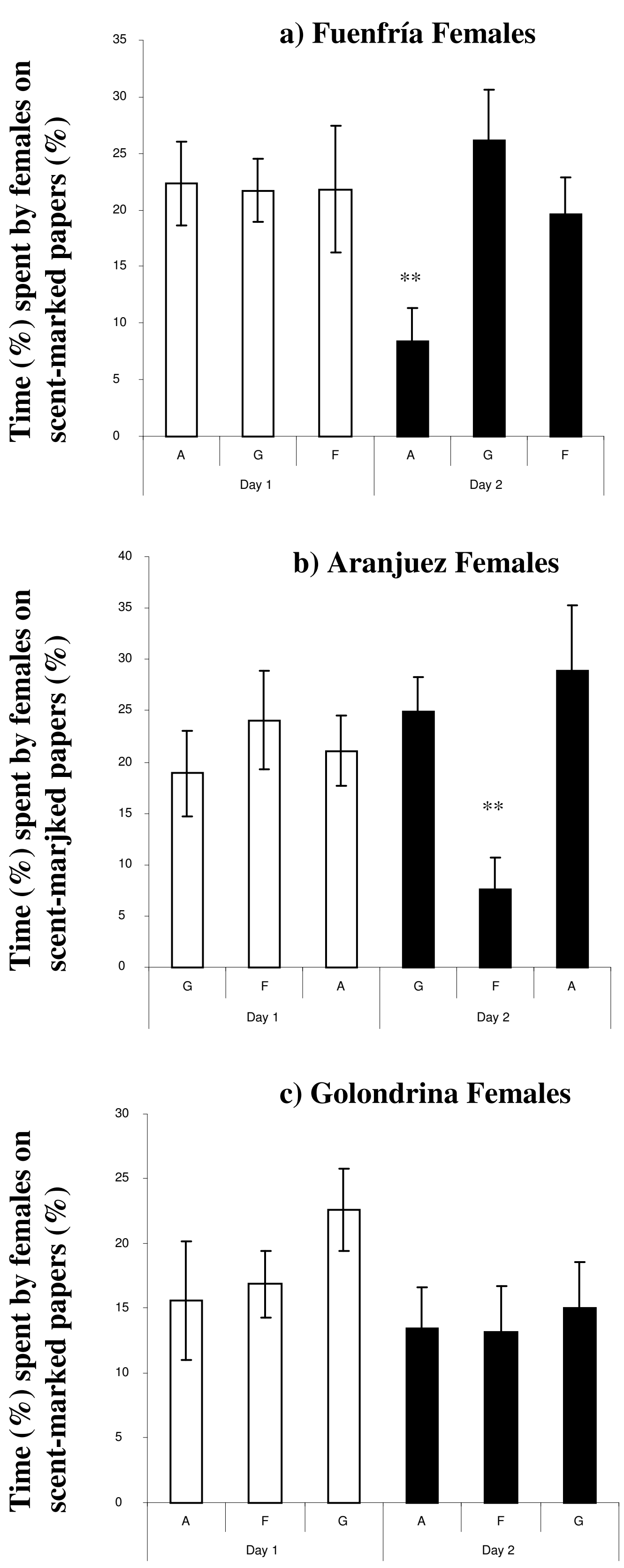


Fig. 5: Time (%) spent by females on papers scent marked by males of three different populations (Aranjuez: A; Fuenfría: F; Golondrina: G). Females were tested in two different days within each treatment.

Females' choice of substrates scent-marked by males

Fuenfría females

There were no overall significant differences between times spent by Fuenfría females (log-transformed number of observations) on paper strips scent marked by experimental males between the two days of the experiment (repeated measures two-way ANOVA: $F_{1,19}$=1.37 P=0.25) nor between males' populations ($F_{2,38}$=2.24 P=0.12) but the interaction between day and males' population was significant ($F_{2,38}$=4.14 P=0.02) (Fig. 5a). Thus, we conducted separated analyses for each day. For the first day, we did not find any difference between the males' populations tested (repeated measures one-way ANOVA: $F_{2,38}$=0.005 P=0.99). However, during the second day, Fuenfría females spent significantly less time (repeated measures one-way ANOVA: $F_{2,38}$=8.75 P=0.0007) on papers scent marked by males from Aranjuez than on papers scent marked by males from Fuenfría (Tukey's test: P=0.03) or Golondrina (P=0.0006).

Aranjuez females

There were not significant differences on the time spent by Aranjuez females on paper strips of males between days (repeated measures two-way ANOVA: $F_{1,19}$=0.07 P=0.79) nor between males' populations ($F_{2,38}$=2.48 P=0.09) but the interaction between day and males' populations was significant ($F_{2,38}$=5.02 P=0.01) (Fig. 5b). Separated analyses showed that, for the first day, there were not any significant difference between the males' populations tested (repeated measures one-way ANOVA: $F_{2,38}$=0.64 P=0.52). However, during the second day, Aranjuez females spent significantly less time (repeated measures one-way ANOVA: $F_{2,38}$=6.95 P=0.002) on papers scent marked by males from Fuenfría than on papers scent marked by Aranjuez (Tukey's test: P=0.003) or Golondrina males (P=0.018).

Golondrina females

Time spent by Golondrina females on papers scent marked by males did not significantly differ between days (repeated measures two-way ANOVA: $F_{1,19}$=2.07 P=0.16), nor between males' populations ($F_{2,36}$=1.06 P=0.35), and the interaction between day and males' populations was not significant ($F_{2,36}$=0.34 P=0.70) (Fig. 5c). Thus, females from Golondrina spent the same amount on time on papers scent marked by males from any population in both days of the experiment.

DISCUSSION

Our results first showed that individuals of *P. hispanica* from each population of the Madrid region could clearly detect scents of lizards from any population in comparison to an odorless control (i.e., water). However, lizards displayed different tongue flick (TF) rates depending of the population of origin of the lizard's scent presented. Both, females and males differed in their responses to scents from lizards from the different populations. Males showed more "interest" (i.e., a higher TF rate) for scents from males from their own area (i.e., north vs. south); males from the northern populations made more TFs in response to scent of northern populations males than to scent of southern males. Conversely, southern males made more TFs in response to scents from southern males than to scent from northern males. Only males from Fuenfría population showed a clearly higher response to scents from males of their own population. For the rest of populations, there were not higher responses to scent of males from their own population, but only an "area preference" (north vs. south).

Moreover, males also discriminated between scents from females from the different areas. Males from northern populations showed more interest for scents from northern females than for scent from southern females; similarly, the converse occurred in southern males. However, we observed one interesting difference: males from the more geographically distant populations (i.e., Aranjuez, Fuenfría and Pedrezuela) showed a clear discrimination and interest (i.e., higher TF rates) for scents of females from their own population against scent of females from any other population. Then, there was a further secondary intermediate interest for scent of females from their area (north or south), and finally a lower interest for females from the other area. In contrast, for the populations geographically less distant (i.e., Belmonte and Golondrina) we did not observe a discrimination and a higher interest for females from their own population, but only a discrimination of females from their area (north vs. south).

In addition, for the males' scents recognition by females, we found similar results. There was recognition of the area of origin of the male (south vs. north). Females from northern populations made more TFs in response to scent of northern males than to southern males, and *viceversa*, but there were not differences between populations within each area. We found only a clear higher interest of Pedrezuela females to scents of males from their own population against the other populations.

Therefore, these results showed that male and female *P. hispanica* lizards can recognize and discriminate between scents of individuals from the different areas (north vs. south), and that only male from the more distant extreme populations show different responses to females scents from their own population than from any other populations. Moreover, other study shows that the chemicals in femoral secretions of males also varied between these populations (Gabirot et al. unpublished data; see Chapter 1). We found a correlation between the classification north vs. south and the abundance of certain chemical compounds in femoral secretions, especially for the abundance of the steroid cholesta-5,7-dien-3-ol (Gabirot et al. unpublished; see chapter 1). Males from the northern populations have higher proportions of this steroid in secretions than southern males. Thus, it is likely that individuals could discriminate these differences in chemical composition between populations and could recognize the area of origin of lizards based on scent alone.

Nevertheless, the interpopulational differences in chemosensory recognition and chemical interest might not necessarily imply that females would not copulate with males from any other population but with those from their own, or at least that females would not choose to stay on scent marked substrates by males of other populations. The experiment of males' scent choice by females showed that females could vary their election depending of scents type and depending of the day of experiment. In the first day, when females had not been previously in contact with males' scent, females did not differ in the amount of time spent on scent marked areas between the different scents. Females did not show a preference for being on the side with scent marks of males from their own population. However, in the second day, responses changed. Females from the extreme populations (Aranjuez and Fuenfría) decreased the percent of time spent on the side with scent marks of males from their respective farther populations; Fuenfría females showed the same interest for scent marks from Fuenfría and Golondrina males, but a lower interest for Aranjuez males' scents. Conversely, Aranjuez females spent more time on scent marks of Golondrina and Aranjuez males than on scent marks of Fuenfría's males. However, females from the geographically intermediate population of Golondrina did not show a choice for any of the scent marks of males.

These scent choice experiments demonstrated that females seemed to prefer areas scent marked by males based on the criterion of the population of origin of the male; but only females from the more geographically distant populations (Aranjuez and Fuenfría) and during the second day. If a female established in a male territory, independently of the male population, she would have the same probability of mating with males from their own or from other populations. Thus, the existence of matings between Fuenfría and Golondrina lizards could be possible, but also matings between Aranjuez and Golondrina lizards; while mating between lizards from the distant populations of Aranjuez and Fuenfría might be more difficult if females avoid scent marks of males of the other population.

Differences in scent choice were also dependent on the day of the trial. The first day, females did not prefer spending more time on one type of scent, but the second day females from extreme populations changed their behavior and showed scent preferences. In our experiment, the first day was the first one that females had been exposed to scent marks of males within that mating season. It is likely that detection of a new male odor induced females to be interested on and to spent similar amounts of time on any scent mark, independently of the origin of that male. Females might, thus, acquire enough information on the characteristics of those male that allows them to make later a possible scent choice. Thus, during the second day, females might recognize the different types of scent presented and make their choice of a given scent. Scent choice could be more restrictive after having been exposed to scent of different males, that when first encountering a scent mark of a male within that mating season.

The lack of premating reproductive isolation for the Golondrina population might be firstly explained by an incapacity of female *P. hispanica* from Golondrina to discriminate between populations of males (however TF experiments showed that there is at least a discrimination of the area of origin), or perhaps simply by a lack of female mate choice criteria. Females might select to establish in areas scent marked by any male (Fuenfría or Aranjuez), and could accept mating with any male with enough genetic relatedness. However, female *P. hispanica* could select scents of individual males using the proportion of chemicals in femoral gland secretions (presented in all male populations), as cholesta-5,7-dien-3-ol (=provitamin D_3). Previous studies showed that female *P. hispanica* from Golondrina population can actually detect changes in concentration of this lipid and preferred scents with higher proportions of this compound (López and Martín 2005; Martín and López 2006d). The chemical criteria of individual preference by females would explain the occurrence of gene flow and hybridization between related, but even between clearly distinct species, within the Genus *Podarcis* observed in the laboratory (e.g., *P. bocagei* x *P. carbonelli*, Galán 2002) and based on genetic analyses (Capula 2002; Pinho et al. 2007).

Our results indicate that the more far or isolated the populations are the more effective the chemical recognition is. Individuals from geographically extreme populations discriminate scent from their populations and have more interest for scents of their own population. Moreover, females showed a scent preference for scents of their own populations. At least, between Aranjuez and Fuenfría populations, chemical signals of males varied significantly (Gabirot et al. unpublished; Chapter 1) and these differences could be enough large as to allow lizards to discriminate and use them for population recognition in sexual selection behaviors. This is one more cue indicating that there exists a cryptic speciation process in the Madrid region, with the presence of several populations with different degrees of speciation. This would explain the results of the current experiments of tongue flick discrimination and scent preferences. These data convey that at least there are two different types of populations in the southern and northern areas of the Madrid region, where individuals can recognize between them differently and show a scent preference for lizards of their own area.

In summary, in the Madrid region we can observe several distinct populations of *P. hispani*ca with a clear chemical discrimination between themselves. Individuals, both males and females, from southern or northern populations have more interest for scents from their own area than for scents from the other area. Moreover, males from the more distant populations (Aranjuez, Fuenfría and Pedrezuela) can discriminate and have more interest for scent of females from their own population than from other populations, but always maintaining the primary differentiation between southern and northern populations. The clear ability of males to discriminate between female types would, thus, indicate that there is a cryptic speciation process, probably mediated by the role of chemical signals in sexual interactions. In addition, females also seem to discriminate male chemicals between areas (north vs. south), but not between populations. Moreover, extreme populations (Aranjuez and Fuenfría) show a preference for scents of males from their own population; while for the Golondrina population (intermediate population), there is no preference for scents from their area, and, thus, the probability of successful mating may not depend on the population of origin of a male. All these results support that reproductive isolation and speciation between distinct populations in the *P. hispanica* complex is not clear, but that between the more extreme populations there could be a more advanced reproductive isolation and speciation process, which merits further studies.

Acknowledgments We thank "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an “El Ventorrillo” CSIC grant to MG. The experiments enforced all the present Spanish laws and were performed under license from the Environmental Organisms of Madrid Community where they were carried out.

References

Alberts, A.C. 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behav. Evol. **41**: 255–260.

Aragón, P., López, P., and Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Arnold, E.N., and Ovenden, D.W. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe.Harper Collins, London.

Barbosa, D., Font, E., Desfilis, E., and Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature **411**: 900–901.

Capula, M. 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). Amphib-Rept. **23**: 313–321.

Carazo, P., Font, E., and Desfilis; E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895–902.

Carretero, M.A. 2008. An integrated assessment of a group with complex systematics: the Iberomaghrebian lizard genus *Podarcis* (Squamata, Lacertidae). Integrative Zool. **4**: 247–266.

Cooper, W.E., and Burghardt, G.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J. Chem. Ecol. **16**: 45–65.

Cooper, W.E., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Cooper, W.E., and Vitt, L.J. 1987. Ethological isolation, sexual behavior and pheromones in the fasciatus species group of the lizard genus *Eumeces*. Ethology **75**: 328–336.

Galán, P. 2002. Hibridación en laboratorio de *Podarcis bocagei* y *Podarcis carbonelli*. Bol. Asoc. Herpetol. Esp. **13**: 28–31.

García-Paris, M., Martín, C., and Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain-Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Gómez, A., Font, E., and Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica.* In: Lacertids of the Mediterranean Region (E.D. Valakos, W. Böhme, V. Pérez-Mellado and P. Maragoú, eds.) Athens, Greece: Helenic Zoological Society. pp. 213–230.

Guillaume, C.P. 1987. Les petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: Univ. Sci. Techn. Langedoc.

Halpern, M. 1992. Nasal chemical senses in reptiles: structure and function. In: Gans, C., Crews, D. (Eds.), Hormones, Brain and Behavior. Biology of the Reptilia. Physiology E, vol. 18. University of Chicago Press, Chicago, pp. 423–523.

Harris, D.J., and Sá-Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75–81.

LeMaster, M.P., and Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red-sided garter snakes, *Thamnophis sirtalis parietalis*. J. Chem. Ecol. **29**: 1027–1043.

López, P., Gabirot, M., and Martín, J. 2008. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturw. **96**: 65–69.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–465.

López P., and Martín, J. 2005. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

Martín, J., and López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B **267**: 1265–1269.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., and López, P. 2006c. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

Martín, J., and López, P. 2006d. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc. R. Soc. Lond. B **273**: 2619–2624.

Martín, J., and López, P. (In press). Pheromones and reproduction in Reptiles. In: Norris, D.O., and Lopez, K.H. (Eds.) Hormones and Reproduction in Vertebrates. Reptiles. Academic Press, San Diego, California.

Mason, R.T. 1992. Reptilian pheromones. In: Gans, C., Crews, D. (Eds.), Biology of the Reptilia, vol 18. University of Chicago Press, Chicago. pp. 114–228.

Mellado, J., and Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, Act. Verteb. **8**: 299–300.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B, Suppl. **270**: S254–S256.

Panhuis, T.M., Butlin, R., Zuk, R.M., and Tregenza, T. 2001. Sexual selection and speciation. Trends Ecol. Evol. **16**: 364–371.

Pinho, C., Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Sá-Sousa, P. 2000. A predictive distribution model for the Iberian wall lizard (*Podarcis hispanicus*) in Portugal. Herpetol. J. **10**: 1-11.

Sá-Sousa, P., Vicente, L., and Crespo, E.G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. Amphib-Rept. **23**: 55–69.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Smadja, C., and Butlin, R.K. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity **102**: 77–97.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. New York: Freeman.

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

Shaw, K.L., and Parsons, Y.M. 2002. Divergence of mate recognition behavior and its consequences for genetic architectures of speciation. Am. Nat. **159**: S61–S75.

CAPÍTULO 3

La elección de pareja por las hembras basada en feromonas que indican la calidad del macho podría impedir un aislamiento reproductivo efectivo dentro de un complejo de especies de lagartijas

Las feromonas sexuales tienen un papel importante en la elección de pareja y el reconocimiento entre especies, especialmente en la lagartija Ibérica *Podarcis hispanica*. Esta lagartija forma en realidad un complejo de especies con varios tipos monofiléticos, que varían en morfología y genética. Los tipos dentro de este complejo estarían en pleno proceso de especiación, pero se desconoce el comportamiento reproductivo real entre tipos. Así, las diferencias entre tipos a nivel de las señales químicas llevarían a un aislamiento reproductivo. En el centro de la Península Ibérica, al menos dos de estos morfotipos viven sin una barrera geográfica. En este trabajo hemos analizado si existe un aislamiento reproductivo real entre dos poblaciones de *P. hispanica* a partir de comparaciones morfológicas, químicas y comportamentales. Estas dos poblaciones de la Sierra de Guadarrama, ocupan distintos medios, y difieren en características morfológicas y de las señales químicas. Además los machos pueden discriminar químicamente entre individuos de las dos poblaciones, pero no las hembras. Así, hemos estudiado en el laboratorio si las hembras de estas poblaciones podrían reconocer y elegir sustratos marcados con olores por machos de su población en contra de sustratos marcados por machos de la otra población, o si las hembras elegirían marcas químicas de los machos basándose en la "calidad" del olor de los machos, independientemente de la población de origen. Finalmente, con el fin de ver si el aislamiento reproductivo (pre-copulatorio) es efectivo o no, provocamos encuentros intersexuales entre machos y hembras de la misma o diferente población. Los resultados mostraron que, a pesar de las claras diferencias a nivel morfológico y químico entre poblaciones, no hubo una clara discriminación quimiosensorial interpoblacional. Las hembras no prefirieron las marcas olorosas de los machos de su población. Sin embargo, las hembras parecían basar su elección de pareja en criterios individuales de calidad, que serían señalados de manera similar por feromonas que se encuentran en los machos de las dos poblaciones. Finalmente, los encuentros sexuales confirmaron que la población de origen de machos y hembras no afectó a la probabilidad o duración de las cópulas. Sugerimos que los diferentes hábitats donde se localizan estas poblaciones podrían favorecer la selección de diferentes morfologías y señales químicas en las secreciones, pero que la elección de pareja por parte de las hembras basada en la calidad individual de los machos podría estar evitando un aislamiento reproductivo pre-cópula efectivo entre estas dos poblaciones.

Female mate choice based on pheromones signaling male quality could preclude an effective reproductive isolation within a lizard species complex

Marianne Gabirot, Pilar López, José Martín

Abstract Sex pheromones are important for mate choice and species recognition and especially for the Iberian wall lizard, *Podarcis hispanica*. This species forms a "species complex" with several monophyletic types, differing in morphology and genetic. There could be an ongoing reproductive isolation processes, but the actual reproductive behavior between types is not known. Thus, differences between lizards' types in chemical secretions could lead to reproductive isolation. In Central Spain, there are at least two morphotypes that could be found without geographic isolation. We analyzed whether there is actual pre-mating reproductive isolation between two populations of *P. hispanica* using morphological, chemical and behavioral data. These populations from the Guadarrama Mts., inhabiting different environments, differ in their morphological and/or chemical signals characteristics. Moreover, males can discriminate individuals by chemical cues, from their population or from the other population, but females seem not able to discriminate. Thus, we examined in the laboratory whether females recognized and chose to establish on areas scent marked by males of their own population against areas marked by males of the other population, or whether females selected scent marked areas based on "quality" characteristic of males' scent, independently of the population of origin of that male. Finally, to test for an effective premating reproductive isolation between populations, we staged intersexual encounters between males and females of the same or different population. The results showed that despite of clear differences in morphology and chemical signals between populations, there was not clear interpopulational chemosensory discrimination. Females did not show preferences for the scent-marks of males from their own population. However, females seemed to base their mate selection on criteria of individual male quality that was similarly signaled by pheromones shared by males of both populations. Finally, in staged encounters, the population of origin of males and females did not affect the probability or the duration of copulations. We suggest that the different environments occupied by each population might be selecting for different morphologies and chemicals in secretions, but that pheromone mediated female mate choice based on cues of individual quality could be precluding an effective pre-mating reproductive isolation between populations.

Résumé Les phéromones sexuelles jouent un rôle important au niveau des comportements reproductifs et de la reconnaissance entre espèces, notamment chez le lézard *Podarcis hispanica*. Cette espèce ou plutôt complexe d'espèces se caractérise par l'existence de plusieurs morphotypes variant au niveau morphologique et génétique; ce complexe serait en processus de spéciation. Si ces morphotypes diffèrent au niveau des signaux chimiques, et que la communication n'est plus effective, la reproduction serait interrompue. Dans la partie centrale de la péninsule ibérique, deux des morphotypes de *Podarcis hispanica* cohabitent sans barrière géographique. Nous avons analysé l'actuelle barrière reproductive entre deux populations de cette espèce à partir de comparaisons morphologiques, chimiques et comportementales. Ces deux populations possèdent une composition chimique des secrétions fémorales différentes. De plus, les mâles montrent une discrimination entre les odeurs de leur population vis-à-vis de l'autre ; mais pas au niveau des femelles. En conséquence, nous avons testé si les femelles de ces deux populations pouvaient reconnaître et choisir de s'établir dans une zone avec des odeurs de mâles de leur population ou de l'autre ; ou bien si les femelles choisissent les marques chimiques des mâles a partir de caractéristique individuelle i.e. indice

de qualité des mâles, indépendamment de population d'origine. Finalement, nous procéderont à des croisements reproductifs entre ces deux populations afin de tester s'il y a ou non un isolement reproductif effectif. Malgré les nettes différences au niveau morphologiques et chimiques entre ces populations, les résultats ont montré qu'il n'y a pas une claire discrimination quimiosensorielle interpopulationnelle. Les femelles n'ont pas eu de préférence pour les odeurs de mâles de leur population. Cependant, elles pourraient faire leur choix de partenaire selon un critère individuel synonyme de qualité, signalé par des indices chimiques chez les deux populations. Les croisements sexuels confirment le fait que ces deux populations ne montrent pas de barrière reproductive pour le moment. La probabilité et le temps de copulation ne varient pas selon le type de croisement : inter et intra population. Donc, les habitats où vivent ces populations auraient permis la sélection de ces différentes morphologies et signaux chimiques, mais pour le moment le choix de partenaire des femelles selon la qualité individuelle des mâles pourrait stopper une possible isolation pré-reproductive entre ces deux populations.

INTRODUCTION

Speciation is considered to result from the evolution of reproductive isolating mechanisms that prevent gene exchange between newly arising taxa (Coyne and Orr 2004). Reproductive isolation often evolves as a consequence of divergent natural selection on traits between different habitats (Schluter 2001; Rundle and Nosil 2005), which may be subsequently amplified by sexual selection (Panhuis et al. 2001). The progressive accumulation of adaptations to different environments may alter the secondary sexual traits used in mate recognition, and/or the mating preferences, which can lead to reproductive isolation between populations (Coyne and Orr 2004). Alternatively, natural selection may promote the evolution of different traits in different environments, leading to genetic differences between populations, but if sexual traits and the criteria used in mate recognition and mate choice do not diverge, sexual selection may preclude an effective premating reproductive isolation.

Interspecific recognition mechanisms use behavior, visual, olfactory, auditory and tactile cues. Chemical signals are important for species recognition and may result in speciation in many animals, such as in some flies (Mas and Jallon 2005), beetles (Symonds and Elgar 2004) or spiders (Roberts and Uetz 2004). Lizards and snakes have a well developed chemosensory system (Mason 1992) and chemical stimulus can be the basis of intraspecific recognition and speciation in many species, such as in sympatric species of sea snakes (Shine et al. 2002), in different populations of red-garter snakes (LeMaster and Mason 2003), or in closely related species or populations of lizards (Cooper and Vitt 1986; Barbosa et al. 2005, 2006; Martín and López 2006a, b).

In many lizards, chemical cues of males, such as those secreted by the femoral glands, are used in intraspecific communication especially during the reproductive season (Mason 1992; Alberts 1993). Chemical cues of males may inform other males on a male's status and competitive ability (Aragon et al. 2001; Carazo et al. 2007; Martin et al. 2007a), and also transmit to females information on male's quality that females may use to select potential mates (Olsson et al. 2003; Martín and López 2006c). Thus, because chemical signals (pheromones) of male lizards are involved in female mate choice and male-male competition, they may also be relevant in the context of interspecific recognition and reproductive isolation (Cooper and Vitt 1986; Cooper and Perez-Mellado 2002; Barbosa et al. 2005, 2006; Martín and López 2006a, b), provided that females from different populations based their mate preferences on those male pheromones that differ between populations.

Molecular studies have provided relationships and genetic distances between populations of several taxa, which suggest the existence of ongoing speciation processes within taxa previously considered to be conspecific. For example, molecular and morphological studies suggest that the Iberian wall lizard, *Podarcis hispanica*, is paraphyletic, and forms a species complex with at least five monophyletic lineages (Guillaume 1987; Harris and Sá-Sousa 2001, 2002; Sá-Sousa et al. 2002; Pinho et al. 2007). This small common lizard widespread at rocky habitats inside many different environments of the Iberian Peninsula, has well developed chemical recognition abilities, and is able to discriminate between conspecifics and heterospecifics (Cooper and Pérez-Mellado 2002; Barbosa et al. 2006), and between sexes by chemical cues alone (Gomez et al. 1993; López and Martín 2001; López et al. 2002). Chemical cues of males are important in male-male interactions (López and Martín, 2002; Carazo et al. 2007). Moreover, in at least one population, females prefer to stay on areas scent marked by males with high proportions of cholesta-5,7-dien-3-ol (=provitamin D_3) in their femoral secretions, which may signal a better immune response of those males (López and Martín 2005; López et al. 2009). Therefore, it seems that female mate choice decisions are, at least partially, based on characteristics of chemical signals of males. We hypothesized that interpopulational differences in chemical signals of male lizards *P. hispanica*, and/or in female mate preferences, could be leading to reproductive isolation processes between populations of this lizard species.

Recently, analyses showed that two closed populations of *P. hispanica* from the Guadarrama Mountains (Central Spain), inhabiting different altitudes and environmental conditions, differ in the chemical composition of femoral gland secretions of males (Martín and López 2006a). Males of the population inhabiting more humid microclimates have secretions with higher proportion of compounds (e.g., long chain alcohols and waxy esters) that may favor persistency and efficiency of chemical signals in humid environments. Moreover, different rates of chemosensory exploration show that males can discriminate by chemical cues alone between males of their own and the other population (Martín and López 2006a,b). In contrast, females detected scent of males, but did not seem able to discriminate between scents of males of the two populations (Martín and López 2006a,b).

Similarly, males of *P. bocagei* and *P. hispanica* from the North of Portugal are able to discriminate chemically between conspecifics and heterospecifics, but females are not (Barbosa et al. 2006). These results suggest that, even if there are differences in chemicals in male femoral secretions, females might be basing their mate recognition, and mate preferences, on chemical cues that are shared by males from different populations. However, the failure to detect chemical discrimination by females in these tests does not discard that female still discriminated and preferred to establish in areas scent marked by males of their own population, thus increasing their opportunities to mate with these males. Moreover, females may reject or allow copulations from males from different populations based on chemical cues, or other type of additional cues. The lack of successful copulations between males and females from different populations is a requisite for an effective pre-mating reproductive isolation.

In this paper, we first explored whether males of two populations of *P. hispanica* lizards from the Guadarrama Mts., inhabiting different environments, differ in their morphological and/or chemical signals characteristics. Then we examined in the laboratory whether females recognized and chose to establish on areas scent marked by males of their own population against areas scent marked by males of the other population, or whether females selected scent marked areas based on some characteristic of males' scent, which could predict the "quality" of a particular individual male, independently of the population of origin of that male. Finally, to test for an effective premating reproductive isolation between populations, we staged intersexual encounters between males and females of the same or different population and analyzed their mating behavior and whether copulations occur. We hypothesized that females mating preferences for certain features of a mating signal that characterized males of their population could promote a rapid divergence between populations. However, if females based their mate choice on characteristics shared from males from the two populations, this lack of discrimination might be precluding an effective reproductive isolation, even if other selective pressures were promoting genetic divergence between populations.

MATERIAL AND METHODS

Study animals

We captured by noosing during March, before the start of their mating season, adult male and female *P. hispanica* lizards at two close localities in the Guadarrama Mts. (Madrid Province, Spain). For all experiments, we used 43 females and 25 males from rock-cliffs at the edge of a pine forest (‘Fuenfría’, 40°47’ N, 4°03’ W, 1750 m altitude), and we caught 45 females and 25 males from rocky outcrops inside a large oak forest (‘Golondrina’, 40°44’ N, 4°02’ W, 1250 m altitude). These two areas are only 6 km distant and they are not isolated geographically, although the intermediate area is not occupied by stable populations of *P. hispanica* (Martín and López 2006a,b). These two sites differ in the altitudinal range, which results in different microclimates, but both sites have similar rocky microhabitats that are adequate for *P. hispanica* (unpublished data). Within each population, we captured lizards in different places over large areas (10 km²) to ensure that individuals had not had previous interactions, which may affect their responses (López and Martín 2002).

Lizards were individually housed at “El Ventorrillo” Field Station, about 5 km from the capture sites, in outdoor 80x50cm PVC terraria containing sand substratum, rock for cover and water *ad libitum*. They were fed every day mealworm larvae (*Tenebrio molitor*). The photoperiod and ambient temperature were those of the surrounding region. Lizards were held in captivity at least one week before testing to allow acclimation to laboratory conditions. Cages of males and females were in different places to avoid contact between them before the experiments. All animals were healthy, did not show adverse behavioral or physiological changes during the tests, and were returned to their capture sites at the end of trials. The capture and experiments were performed under license from the “Comunidad de Madrid” Environmental Agency.

Morphological characteristics of males

We measured males’ body weight with a digital balance to the nearest 0.01 g, and the snout-to-vent length (SVL) with a ruler to the nearest 1 mm. We calculated individual values of body condition as the residuals from the regression equation of ln mass (g) on ln SVL (mm), which may represent an index of the relative amount of fat stored, and hence an estimation of individual physical condition or nutritional status (Bonnet and Naulleau 1994). We also made morphological measurements of the head of males using digital calipers (to the nearest 0.05 mm). Head length was the distance between the tip of the snout and the posterior side of the parietal scales. Head width was the greatest distance between the external sides of the parietal scales. Head depth was the greatest distance from the highest portion of the head to the bottom of the lower jaw.

We also counted under a magnifying glass the number of femoral pores on the right and left hind limbs of males and calculated an average number for both sides. Finally, we noted the number of small but distinctive and conspicuous blue ocelli that runs along each of the body sides on the outer margin of the belly, and calculated an average number for both sides. These ocelli seem to have a role in sex recognition and intrasexual social relationships between males (López et al. 2002).

Analysis of femoral gland secretions of males

The femoral gland secretions of males were extracted by gently pressing with forceps around the femoral pores immediately after capture. We collected secretion in glass vials with Tetlon-lined stoppers. Vials were stored at -20 °C until analyses. We used the same procedure without collecting secretion in order to have blank control vials. Before the analyses, we added 250 µl of n-hexane to each vial. Samples were analyzed in a Finnigan-ThermoQuest Trace 2000 gas chromatograph-mass spectrometer (GC-MS) equipped with a 30 m Supelco, Equity-5 column, and temperature programmed from 50-280 °C at 5 °Cmin^{-1} and kept at 280 °C for 30 min (see Martín and López 2006a for details of analyses). Identification of compounds was done by comparison of mass spectra in the NIST/EPA/NIH 1998 library, and later confirmed with authentic standards. The relative amount of each compound was determined as the percentage of the total ion current (TIC). The relative areas of the peaks were transformed following Aitchison's formula: $Zij=\ln(Yij/g(Yj))$, where Zij is the standardized peak area i for the individual j, Yij is the peak area i for individual j and $g(Yj)$ is the geometric mean of all peaks for individual j (Aitchison 1986; Dietemann et al. 2003).

Female choice of males' scents

We placed in males' terraria several absorbent paper strips (35x10 cm) fixed to the floor, and left them there for three weeks to obtain the scents from males. Mate choice experiments were performed at the end of April, coinciding with the mating season of this lizard species. Females' cages had two basking platforms (two identical flat tiles) placed symmetrically at each end of the cage, and rocks for cover in the center. At the beginning of experiments (09.00 h, GTM; when females where still inactive) we fixed, wearing fresh gloves, on one tile one paper strip from one male of one population, and on the other tile a paper from a male of the other population. Different papers from each male were used in four choice tests against the papers of other four males from the other population. The males tested and the positions of papers were randomly determined. Each female was tested twice, once a day, with papers from two different pairs of males (own vs. other population). Each trial lasted 5 h (from 11.00 h GMT, shortly after females appeared from refuges and until 16.00 h GMT when females hid again). Females were monitored each 15 min from a hidden point. If a female was located on a tile with paper strip, she was designated as haven chosen temporarily that particular paper, whereas, if she located out side of the tiles, she was designated as having made no choice (Martín and López 2000, 2006c; Olsson et al. 2003). At the end of the trials the papers were removed and the cage was thoroughly rinsed with clean water.

We counted the number of times that each female was observed on each particular stimulus paper in each trial, and used a repeated measures three-way ANOVA to test for differences between the two days of the trial and between types of males (male from her own population vs. male from other population), both as within-subject factors. The population of origin of the female (‘Fuenfría’ vs. ‘Golondrina’) was included as a between subject factor to test whether the responses varied between populations (Sokal and Rohlf 1995). Also, because a previous study suggested that, at least, females from ‘Golondrina’ may prefer scents of males with relatively higher amounts of cholesta-5,7-dien-3-ol in secretions (López and Martín 2005), we made a similar three-way ANOVA but classifying the two males within each pair according to the relative abundance of this lipid in their secretions (higher vs. lower) independently of the population of origin.

In addition, individual males were also classified according to the attractiveness of their scent; the paper on which a female spent greater than 50 % of her time (excluding time in the no choice) was designed as the preferred paper in the trial (Martín and López 2000). Each individual male was assigned an attractiveness index, calculated as the proportion of females that preferred a paper with his scent. Then, we used the morphological variables or the transformed areas of chemical compounds in femoral secretions of males (see above) as independent variables in forward stepwise general regression models (GRM) with attractiveness indexes of males as the dependent variable.

Mating behavior

We staged encounters between male and female lizards from the same or from different populations to study whether successful copulations occurred and the differences in copulatory behavior depending on the population of origin of the male and the female. Each male encountered two females, once per day over two days. Half of males were presented first with a female from their own population and the day after with a female from the other population, and the converse for the other half of males. Each female was used only once with a single male. The individual males and females used in each encounter were chosen at random. In each trial, we gently took a female from her cage and placed her in a male's cage. From a blind we observed whether or not a copulation occurred, and recorded the duration of the copulation (i.e., since the first cloacal contact with hemipenis intrusion until the moment that cloacae of individuals were physically separated). The female was removed from the male's cage immediately after the copulation finished or after 30 min since the start of the trial, if copulation did not occur.

We used General Linear Mixed Models (GLMM) in the statistic software of SAS (SAS, 1989-96 Institute Inc., Cary, NC, USA) to test the dependent variable with normal distribution (i.e., duration of copulation; Kolmogorov-Smirnoff's test: $P>0.10$) and Generalized Linear Mixed Models (GLIMMIX) when the dependent variable was binomial (i.e., whether or not a copulation occurred). In this analysis, the male individual was used twice so, we defined male identity as a random factor. We also included in the models the population of the male and the female and the order of presentation as categorical predictors.

RESULTS

Morphology of male lizards

Males from the two populations differed significantly respect to their morphological characteristics (weight, SVL, condition, head size, number of femoral pores and number of lateral blue ocelli) (MANOVA: Wilk's $\lambda=0.16$, $F_{8,11}=6.9$, $P=0.002$; Table 1). Males from Fuenfría were significantly larger and heavier, had significantly greater heads, and had significantly higher body condition than males from Golondrina (Table 1). However, males of the two populations did not differ significantly in the number of femoral pores or blue ocelli (Table 1).

Chemicals in femoral gland secretions of males

The lipophilic fraction of femoral secretions of male lizards' *P. hispanica* consisted in several fatty acids, alcohols, waxy esters, squalene and steroids (Table 1). The most abundant compounds were cholesterol and cholesta-5,7-dien-3-ol. The other compounds were found in minor quantities. There were significant overall differences between populations in the relative proportion of compounds in femoral secretions of males (MANOVA: Wilks' λ=0.046, $F_{8,1}$=28.54, P<0.0001; Table 1). Univariate protected ANOVAs showed that males from Fuenfría had significantly lower proportions of low molecular weight (C_{12}-C_{18}) fatty acids, cholesterol and campesterol, and significantly greater proportions of alcohols and cholesta-4,6-dien-3-one than males from Golondrina (Table 1). Males from Fuenfría also tended to have greater proportions of fatty acids of high molecular weight (C_{20}-C_{24}) and of waxy esters than males from Golondrina, but although not significantly,. For the other compounds there were no significant differences between populations.

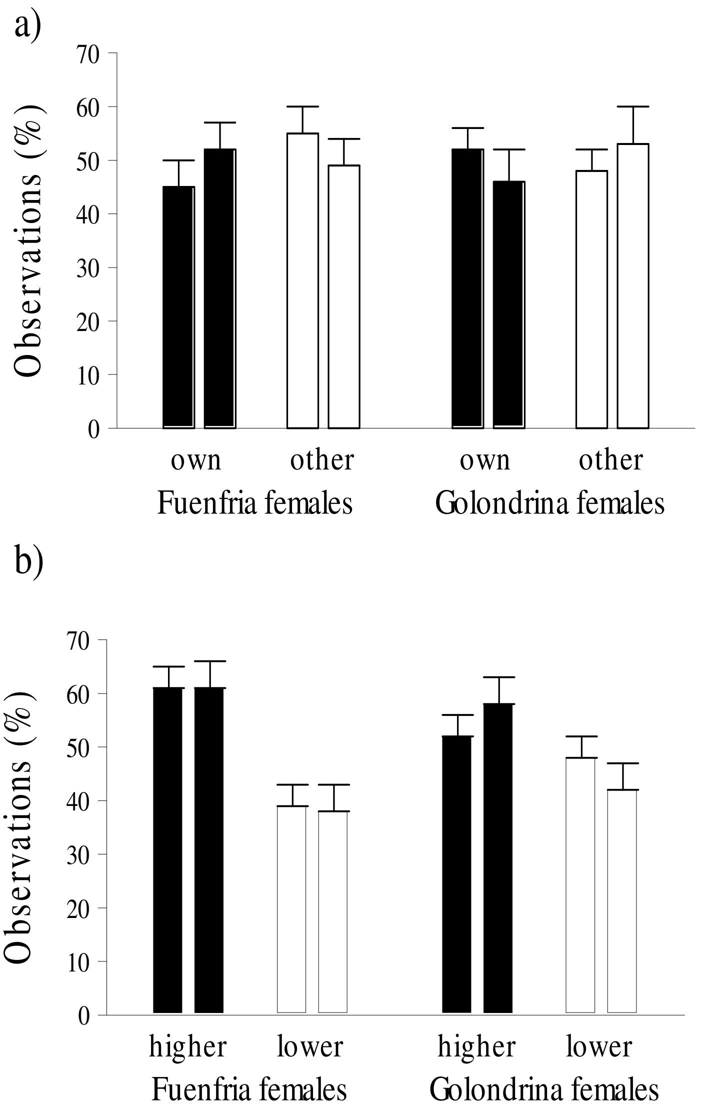


Fig. 1: Percent number of times (mean+SE), during each of two scent's choice trials, that female *P. hispanica* lizards from two populations (Fuenfria or Golondrina) were observed on paper strips scent marked by (a) males from the same or from the other population, or (b) by the male within each pair with relatively higher or lower proportions of cholesta-5,7-dien-3-ol in its femoral secretions, independently of the population of origin

Choice of males' scent by females

There were no significant differences between time spent by females (log-transformed number of observations) on paper strips scent marked by a male of their own population or by a male from the other population (repeated measures three-way ANOVA: $F_{1,38}$=0.08, P=0.77), the population of origin of the female had not significant effect on these responses ($F_{1,38}$=0.08, P=0.77), and the interaction between population of male and female population was not significant ($F_{1,38}$=0.07, P=0.79) (Fig. 1a). However, the overall number of observations of females varied significantly between the two days of the trial ($F_{1,38}$=5.09, P=0.03). And the interaction between day and female population was significant ($F_{1,38}$=33.91, P<0.0001), but this variation between days did not affect the female choice of paper strips (interaction of male population x day: $F_{1,38}$=0.01, P=0.94; three way interaction: $F_{1,38}$=1.64, P=0.21).

Analyzing the attractiveness of males, considering their morphology, we found that none of the morphological variables (weigh, size, condition, head size, etc) had any significant relation with the attractiveness indexes of their scent (i.e., no variable entered the GRM model with significance). A similar lack of relationships was found when we analyzed separately each population. However, when we analyzed the possible relationships between male attractiveness and the major chemical compounds in femoral secretions, we found a significant positive relationship between the attractiveness index of a male and the relative proportions of cholesta-5,7-dien-3-ol in his secretions (GRM: R^2=0.42, $F_{1,17}$=12.55, P=0.0025; Fig. 2). A relationship that was similar when we calculated the attractiveness indexes of males from the responses of females of each of the two populations separately (GRM, Fuenfria females: R^2=0.27, $F_{1,16}$=5.92, P=0.027; Golondrina females: R^2=0.33, $F_{1,17}$=8.23, P=0.011).

Moreover, in the trials of choice of males' scent by females, when we classified the two males within each pair according to the relative abundance of cholesta-5,7-dien-3-ol in their secretions (higher vs. lower) independently of the population of origin of the male, females spent significantly more time on paper strips scent-marked by the male, within each pair, with the higher proportion of cholesta-5,7-dien-3-ol (repeated measures three-way ANOVA: $F_{1,38}$=14.58, P=0.0005; Fig. 1b). This effect was similar independently of the population of origin of the female ($F_{1,38}$=0.09, P=0.77), and there were no significant differences between days ($F_{1,38}$=2.17, P=0.15), although the interaction between female population and day of the trial was significant ($F_{1,38}$=35.40, P<0.0001). All the other interactions were non significant (P>0.16 in all cases).

	Fuenfría	Golondrina	$F_{1,18}$	P
Morphology:				
Weight (g)	5.1 ± 0.2	4.1 ± 0.2	16.55	**0.0007**
SVL (mm)	63 ± 1	57 ± 1	13.36	**0.0018**
Condition (weight/SVL)	0.89 ± 0.01	0.80 ± 0.01	12.22	**0.002**
Head length (mm)	14.90 ± 0.18	13.18 ± 0.15	8.30	**0.009**
Head width (mm)	9.29 ± 0.25	8.12 ± 0.12	4.15	0.05
Head depth (mm)	6.28 ± 0.10	5.66 ± 0.06	7.09	**0.01**
Femoral pores	18.4 ± 0.4	18.7 ± 0.5	0.22	0.64
Blue ocelli	3 ± 1	4 ± 1	0.19	0.66
Chemicals:				
Fatty acids (C_{12}-C_{15})	1.58 ± 0.22	2.91 ± 0.41	12.23	**0.0026**
Fatty acids (C_{16}-C_{18})	7.44 ± 1.87	8.50 ± 0.97	5.43	**0.03**
Fatty acids (C_{20}-C_{24})	1.49 ± 0.38	0.73 ± 0.09	3.70	0.07
Alcohols	2.25 ± 0.36	0.87 ± 0.05	14.60	**0.0012**
Waxy esters	2.01 ± 0.67	0.62 ± 0.28	3.62	0.07
Squalene	0.45 ± 0.12	0.42 ± 0.04	3.02	0.10
Cholesterol	52.14 ± 2.24	63.88 ± 1.22	6.55	**0.02**
Cholesta-5,7-dien-3-ol	19.34 ± 0.97	13.12 ± 1.14	0.01	0.91
Ergosta-5,8-dien-3-ol	3.37 ± 0.14	2.43 ± 0.08	0.09	0.77
4,4-Dimethyl-cholesta-5,7-dien-3-ol	2.04 ± 0.17	1.01 ± 0.20	3.19	0.09
Campesterol	0.33 ± 0.07	0.48 ± 0.06	4.53	**0.04**
Cholesta-4,6-dien-3-one	0.83 ± 0.15	0.16 ± 0.05	12.18	**0.003**
Cholestanol	0.46 ± 0.31	0.61 ± 0.25	1.54	0.23
Minor steroids	6.21 ± 0.64	4.24 ± 0.38	0.01	0.93

Table 1: Morphological measurements (mean ± SE) and lipophilic chemical compounds (mean+SE of TIC area) found in femoral gland secretions of male *Podarcis hispanica* from Fuenfría and Golondrina populations

* Results (*F*, *P*) from protected one-way ANOVAs on transformed data (see methods) are shown. The bold values indicate that *P* is significant at the 0.05 level

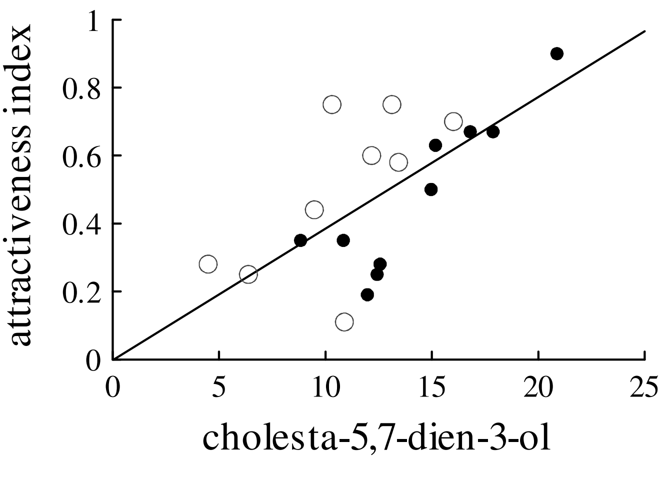


Fig. 2: Relationship between relative proportions of cholesta-5,7-dien-3-ol in femoral gland secretions and the attractiveness index scores of male lizards *P. hispanica* from two populations, Fuenfria (o) or Golondrina (•)

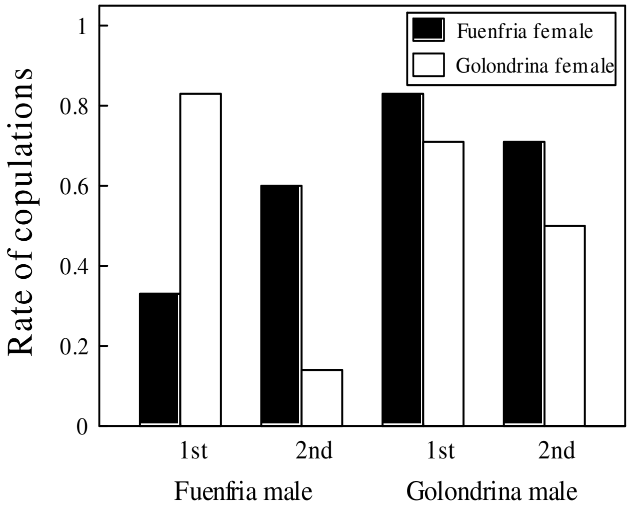


Fig. 3: Rate of successful copulations between male lizards *P. hispanica* from two populations (Fuenfria or Golondrina) with females from their own or from the other population, in two successive copulation trials of the same male with different individual females (see methods)

Mating behavior

The overall frequency of successful copulations in this experiment was 0.58 (29 copulations from 50 staged encounters) (Fig. 3). The probability of occurrence of a copulation was not significantly dependent of the population of the male (GLIMMIX: $F_{1,19}=1.25$, $P=0.27$) nor of the population of the female ($F_{1,19}=0.21$, $P=0.65$), and the interaction was not significant ($F_{1,19}=0.09$, $P=0.76$). The probability of copulation was significantly higher in the first encounter than in the second one (order effect: $F_{1,19}=9.06$, $P=0.007$), but this effect was independent of the population of the male or of the female ($P>0.20$ for all interactions of order with all other effects) (Fig. 3).

The duration of successful copulations did not differ depending of the population of the male (GLMM, $F_{1,26}=3.60$, $P=0.06$) or the population of the female ($F_{1,26}=1.84$, $P=0.18$) and the interaction between male and female populations was not significant ($F_{1,26}=2.71$, $P=0.11$). Moreover, the duration of copulation was not significant affected by the order of female presentation ($F_{1,26}=0.39$, $P=0.54$). All other interactions were not significant ($P>0.40$ in all cases).

DISCUSSION

Our results showed that males from these two closed populations of *P. hispanica* differ in some important aspects. Morphologically, males of Fuenfría are more robust and bigger, and have large heads than males of Golondrina. Also, although the femoral secretions of males are composed by similar chemical compounds, the relative proportions of these chemicals are different (see also Martín and López 2006a). These observed differences in morphology and femoral secretions could be related with small genetic differences between populations.

These variations described here might have arisen due to the different environments in the geographical area occupied by each population. Although lizards occupy similar rocky microhabitats in the two populations, there are notorious differences in elevation, temperature and humidity (Gabirot et al. unpublished data). Lizards from Fuenfría occupy areas characterized by high levels of humidity and cold temperatures; whereas males from Golondrina occupy drier and warmer areas than males from Fuenfría. Thus, for example, differences in body size may be simply due to different growth rates or other life history parameters of lizards promoted by altitude-related differences in thermal opportunities, food availability, predation risk, etc (e.g., Sears 2005; Iraeta et al. 2006), but also natural selection may have favored individuals with morphological characters more adapted to each environment. Similarly, differences in proportion of chemicals in femoral secretions might be related to different microclimatic conditions, reflecting selection for the persistency and efficiency of chemical signals in different environments; less volatile compounds and with a higher chemical stability being favored in lizards inhabiting more humid climatic conditions (Alberts 1992; Martín and López 2006a). The question that arises is whether these differences are so great as to promote reproductive isolation.

In fact, differences in chemical compounds seem relevant for population recognition based on previous experiments that showed that male *P. hispanica* can discriminate between scents from males from their own or from other population (Martín and López 2006a, b), which can have consequences for rival recognition in intrasexual contests and from scent marked territories (López and Martín 2002; Carazo et al. 2007).

In contrast to males, females do not seem to discriminate between male populations based on chemical cues, although females clearly detect scent of males of the two populations from a baseline odor (Martin and Lopez 2006a,b). Moreover, the results of the current experiment further showed that females do not prefer, nor reject, areas scent marked by males based on the criterion of the population of origin of the male. Therefore, if a female established in a male territory, independently of the male population, she would have the same probability of mating with males from their own or from the other population. Furthermore, the results of staged encounters showed that males and females are equally likely to copulate, and copulation duration is similar, independently of their population of origin. Therefore, our results indicate that there is a lack of pre-mating and mating reproductive isolation between these two populations, in instead of differences in morphology and chemical sexual signals described here. Although it remains to be analyzed whether interpopulation mating results in the same reproductive success and whether “hybrids” have similar fitness, our data suggest that reproductive isolation between these two populations is at least incomplete.

This lack of reproductive isolation might be firstly explained by an incapacity of female *P. hispanica* to discriminate between populations of males, or perhaps simply by a lack of female mate choice criteria. Females might select to establish in areas scent marked by any male, and accept mating with any male with enough genetic relatedness. This would explain the occurrence of gene flow and hybridization between related, but even between clearly distinct species, within the Genus *Podarcis* observed in the laboratory (e.g., *P. bocagei* x *P. carbonelli*, Galán 2002) and based on genetic analyses (Capula 2002; Pinho et al. 2007).

However, our study showed that female *P. hispanica* select scent of individual males using the proportion of chemicals in femoral gland secretions. Females prefer males with relatively higher amounts of cholesta-5,7-dien-3-ol (=provitamin D_3) in femoral secretions. Previous studies showed that female *P. hispanica* can actually detect changes in concentration of this lipid (Martín and López 2006d). This mate choice criterion may be explained by the positive relationship between the amount of cholesta-5,7-dien-3-ol in secretions and the quality of the immune response of a male observed in this species (López and Martín 2005; López et al. 2009) and in other lacertid lizard species (Martín and López 2006c). Cholesta-5,7-dien-3-ol is a precursor for vitamin D_3 and, in humans and other mammals, there is considerable scientific evidence that the active form of vitamin D is a potent immune system modulator with a variety of effects on immune system function that may enhance immunity (Griffin et al. 2003; Hayes et al. 2003). In lizards, experimental dietary supplementations and challenge of the immune system suggest that there may be a trade-off between physiological regulation of the immune system and the allocation of essential nutrients (provitamins) to sexual ornaments (Martin and Lopez 2006e; Martín et al. 2007b; López et al. 2009), which may allow to honestly signal male quality via chemical cues.

Interestingly, average amounts of cholesta-5,7-dien-3-ol in males' secretions did not vary between our study populations, although interindividual variability is high in both populations. Therefore, despite of females used this signal to select a male, the population of origin would not affect to the mate selection, but rather the individual characteristics of each male. This mate selection based on specific characteristics of chemical signals of males that not differ between populations would lead to a lack of effective reproductive isolation. In fact, our results confirmed that successful copulation with similar characteristics can occur between males and females of both populations.

In summary, in this study, we have found that despite of clear differences in morphology and chemical signals between two populations of *P. hispanica* lizards, there is not clear interpopulational discrimination at the level of chemical recognition and pre-mating reproductive isolation. While an anterior study showed that males can discriminate between these populations by chemical cues (Martín and López 2006a, b), females do not seem to discriminate between male chemicals, nor showed preferences for the scent of males from their own population, and probabilities of successful mating do not depend on the population of origin. However, females seem to base their mate selection on criteria of individual male quality that is signaled by pheromones shared by males of both populations. All these results support that reproductive isolation and speciation between distinct types in the *P. hispanica* complex is not clear and merits further studies.

Acknowledgments We thank "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an “El Ventorrillo” CSIC grant to MG. The experiments enforced all the present Spanish laws and were performed under license from the Environmental Organisms of Madrid Community where they were carried out.

References

Aitchison, J. 1986. The statistical analysis of compositional data: Monographs in statistics and applied probability. London: Chapman and Hall.

Alberts, A.C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. Am. Nat. **139**: 62–89.

Alberts, A.C. 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behav. Evol. **41**: 255–260.

Aragón, P., López, P., and Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Barbosa, D., Desfilis, E., Carretero, M.A., and Font E. 2005. Chemical stimuli mediate species recognition in *Podarcis* wall lizards. Amphib-Rept. **26**: 257–263.

Barbosa, D., Font, E., Desfilis, E., and Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Bonnet, X., and Naulleau, G. 1994. A body condition index (BCI) in snakes to study reproduction. Comp. Rend. S. Acad. Sci. – Sér. III – Sci. Vie **317**: 34–41.

Capula, M. 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). Amphib-Rept. **23**: 313–321.

Carazo, P., Font, E., and Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895–902.

Cooper, W.E., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Coyne, J.A., and Orr, H.A. 2004. Speciation. Sunderland: Sinauer Associates.

Dietemann, V., Peeters, C., Liebig, J., Thivet, V., and Hölldobler, B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc. Natl. Acad. Sci. USA **100**: 10341–10346.

Galán, P. 2002. Hibridación en laboratorio de *Podarcis bocagei* y *Podarcis carbonelli*. Bol. Asoc. Herpetol. Esp. **13**: 28–31.

Gómez, A., Font, E., and Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica*. In: Valakos ED, Böhme W, Pérez-Mellado V, Maragoú P, eds. Lacertids of the Mediterranean Region. Athens, Greece: Helenic Zoological Society, pp. 213–230.

Griffin, M.D., Xing, N., and Kumar, R. 2003. Vitamin D and its analogs as regulators of immune activation and antigen presentation. Annu. Rev. Nutrition **23**: 117–145.

Guillaume, C.P. 1987. Les petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: Univ. Sci. Techn. Langedoc.

Harris, D.J., and Sá-Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75–81.

Hayes, C.E., Nashold, F.E., Spach, K.M., and Pedersen, L.B. 2003. The immunological functions of the vitamin D endocrine system. Cell. Mol. Biol. **49**: 277–300.

Iraeta, P., Monasterio, C., Salvador, A., and Díaz, J.A. 2006. Mediterranean hatchling lizards grow faster at higher altitude: a reciprocal transplant experiment. Funct. Ecol. **20**: 865–872.

LeMaster, M.P., and Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red-sided garter snakes, *Thamnophis sirtalis parietalis*. J. Chem. Ecol. **29**: 1027–1043.

López, P., Gabirot, M., and Martín, J. 2009. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturw. **96**: 65–69.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–465.

López, P., and Martín, J. 2005. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P, Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr Behav **28**: 154–163.

Martín, J., and López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B **267**: 1265–1269.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., and López, P. 2006c Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

Martín, J., and López, P. 2006d. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herpetol. **60**: 556–561.

Martín, J., and López, P. 2006e. Vitamin D supplementation increases the attractiveness of males' scent for female

Iberian rock lizards. Proc. R. Soc. Lond. B. **273**: 2619–2624.

Martín, J., López, P., Gabirot, M., and Pilz, K.M. 2007b. Effects of testosterone supplementation on chemical signals of male Iberian wall lizards: consequences for female mate choice. Behav. Ecol. Sociobiol. **61**: 1275–1285.

Martín, J., Moreira, P.L., and López, P. 2007a. Status-signaling chemical badges in male Iberian rock lizards. Funct. Ecol. **21**: 568–576.

Mas, F., and Jallon, J.M. 2005. Sexual isolation and cuticular hydrocarbon differences between *Drosophila santomea* and *Drosophila yakuba*. J. Chem. Ecol. **31**: 2747–2752.

Mason, R.T. 1992. Reptilian pheromones. In: Gans C, Crews D, eds. Biology of the Reptilia. Vol. 18. Chicago: University of Chicago Press,. pp. 114-228.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B, Suppl. **270**: S254–S256.

Panhuis, T.M., Butlin, R., Zuk, R.M., and Tregenza, T. 2001. Sexual selection and speciation. Trends Ecol. Evol. **16**: 364–371.

Pinho, C., Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Roberts, J.A., and Uetz, G.W. 2004. Chemical signaling in a wolf spider: a test of ethospecies discrimination. J. Chem. Ecol. **30**: 1271–1284.

Rundle, H.D., and Nosil, P. 2005. Ecological speciation. Ecol. Lett. **8**: 336–352.

Sá-Sousa, P., Vicente, L., and Crespo, E.G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. Amphib-Rept. **23**: 55–69.

Schluter, D. 2001. Ecology and the origin of species. Trends Ecol. Evol. **16**: 372–380.

Sears, M.W. 2005. Geographic variation in the life history of the sagebrush lizard: the role of thermal constraints on activity. Oecologia **143**: 25–36.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. New York: Freeman.

Symonds, M.R.E., and Elgar, M.A. 2004. The mode of pheromone evolution: evidence from bark beetles. Proc. R. Soc. Lond. B **271**: 839–846.

CAPÍTULO 4

Las variaciones interpoblacionales en las respuestas quimiosensoriales a esteroides de las secreciones femorales de los machos de *Podarcis hispanica* reflejarían las diferencias en las señales químicas entre poblaciones

Las secreciones femorales de los machos de la lagartija Ibérica *Podarcis hispanica* se componen de proteínas y lípidos (principalmente esteroides y ácidos grasos); pero se piensa que los lípidos son los principales compuestos con un papel en la comunicación intraespecífica. Entre los esteroides, el más abundante es el colesterol, que en otras especies de lagartijas podría señalar el tamaño corporal y la dominancia. Otro esteroide importante en las secreciones femorales es el colesta-5,7-dien-3-ol, un precursor de la vitamina D_3. Las hembras de *P. hispanica* elegirían su pareja usando este esteroide, que indicaría la calidad del sistema inmune. La lagartija Ibérica parece formar un “complejo de especies”, con diferentes morfotipos que frecuentemente no están aislados geográficamente. Estudios previos han mostrado que diferentes poblaciones de este complejo de especies, que viven en una zona de contacto en Madrid (centro de España), difieren en la composición y proporciones de compuestos químicos en las secreciones, especialmente con respecto a estos esteroides. En este trabajo, comparamos las respuestas quimiosensoriales de cinco poblaciones diferenciadas de *P. hispanica* a dos esteroides (colesterol y colesta-5,7-dien-3-ol). Los animales discriminaron y tuvieron un interés significativamente mayor (i.e., altas tasas de protusiones linguales) por estos esteroides, pero hubo diferencias entre poblaciones. Las lagartijas de poblaciones con temperaturas frías y humedad relativa elevada (i.e., Norte de Madrid) mostraron respuestas elevadas a estos esteroides, mientras que lo contrario ocurrió en lagartijas de poblaciones que ocupan hábitats secos y cálidos (i.e., Sur de Madrid). Interesantemente, la magnitud de las respuestas quimiosensoriales al colesta-5,7-dien-3-ol en cada población refleja la abundancia de este compuesto en las secreciones de los machos de cada población. Estos resultados sugieren que la importancia del colesta-5,7-dien-3-ol en las secreciones de los machos es menor para las lagartijas de las poblaciones del sur, las cuales podrían usar más bien otro tipo de compuestos químicos u otro tipo de señales (i.e., visuales) para la comunicación. Por lo tanto, las poblaciones que viven en áreas distintas podrían haber evolucionado de manera distinta y usar diferentes señales comunicativas en la selección sexual, lo cual apoyaría la existencia de procesos en marcha de aislamiento reproductivo y especiación entre estas poblaciones.

Interpopulational variations in chemosensory responses to selected steroids from femoral secretions of male lizards *Podarcis hispanica* may reflect population differences in chemical signals

Marianne Gabirot, Pilar López, José Martín

Abstract Femoral secretions of male Iberian wall lizards, *Podarcis hispanica*, are composed of both proteins and lipids (mainly steroids and fatty acids), but the latter are thought to be the main compounds involved in intraspecific communication. Among steroids the most abundant is cholesterol, which in other lizard species may signal body size and dominance. Other major steroid in femoral secretions is cholesta-5,7-dien-3-ol, a precursor for vitamin D_3. Female *P. hispanica* could make their partner scent choice using this steroid, which may signal a better immune response. The Iberian wall lizard seems to form a "species complex" with different morphotypes that are often not geographically isolated. Previous experiments showed that different populations of this species complex, living in a contact zone in Madrid (Central Spain), differed in composition and proportions of chemical compounds in secretions, especially with respect to these steroids. Here, we compared the chemosensory responses of *P. hispanica* lizards from five distinct populations to two steroids (cholesterol and cholesta-5,7-dien-3-ol). Lizards discriminated and had a significant high interest (i.e., high tongue-flick rates) for these steroids, but showed interpopulational differences. Animals from populations with cold temperatures and high relative humidity (i.e., northern Madrid), showed higher responses to these steroids, whereas the converse occurred for lizards from populations occupying dry and hot habitats (i.e., southern Madrid). Interestingly, the magnitude of the chemosensory responses to cholesta-5,7-dien-3-ol in each population mirrored the abundance of this compound in secretions of males of each population. These results suggest that the importance of cholesta-5,7-dien-3-ol in males' secretions is lower for lizards from the southern populations, which could rather use other type of chemicals or other type of signal (i.e., visual) for communication. Therefore, populations living in distinct areas could have evolved in different way and use different communication signals in sexual selection, which may support the existence of ongoing reproductive isolation and speciation processes between these populations.

Résumé Les secrétions fémorales du lézard *Podarcis hispanica* comprennent des protéines et des lipides, en majorité ces composés jouent un rôle dans la communication. Parmi les stéroïdes, le cholestérol est une des majeurs molécules chimiques des secrétions des glandes fémorales et pré cloacales chez de nombreuses espèces de lézards. Il aurait comme fonction la signalisation du statut de dominance aux congénères. Un autre stéroïde important est le cholesta-5,7-dien-3-ol qui est un précurseur de la vitamine D_3. Les signaux chimiques et spécialement le cholesta-5,7-dien-3-ol seraient utilisés par les femelles de *Podarcis hispanica* pour choisir leur partenaire reproductif; il serait un signal de qualité informant sur le système immunitaire des mâles. Ce lézard se définit comme un complexe d'espèces qui regroupe plusieurs morphotypes. Dans le centre de la péninsule, deux des morphotypes vivent sans barrière géographique apparente. De plus des études antérieures ont montré que dans cette zone de contact, plusieurs populations peuvent être observées et se différencient à plusieurs niveaux : morphologie, signaux chimiques, ainsi qu'une reconnaissance entre individus a pu être mise en évidence. Les individus se situant au nord de cette zone dans un habitat plutôt montagneux (froid et humide) ont une concentration et proportions de ces stéroïdes (cholestérol et cholesta-5,7-dien-3-ol) plus élevée que les mâles des populations du sud. En

conséquence, nous avons comparé la réaction face à ces stéroïdes (i.e., discrimination) des individus de ces populations (sud et nord). Les résultats ont montré que les lézards ont un certain intérêt pour ces composés, mais différent selon la population d'origine. Plus la population se trouve au nord de cette région avec un habitat froid, humide et d'altitude élevé, plus les lézards ont un intérêt pour ces stéroïdes ; et *vice versa* pour ceux du sud (i.e., températures élevées et peu d'humidité). Ces individus du sud reconnaissent ces composés mais montrent moins d'intérêt pour eux. Ces molécules chimiques pourraient être importante dans leur communication mais moins qu'elles ne le sont pour les lézards du nord ; ceux du sud utiliseraient un autre type de molécules des secrétions ou bien même un autre type de signal. Ces populations qui vivent dans des zones bien distinctes avec des conditions climatiques différentes pourraient avoir évolué différemment et développé différents signaux pour communiquer ou pour se reproduire ; tous ces résultats supportent une fois de plus l'existence d'un processus de spéciation en marche dans cette zone de contact.

Enviado a *Journal of Chemical Ecology*

INTRODUCTION

Chemical signals released during the reproduction are important for mate and species recognition and may provide the basis for premating reproductive isolation and speciation in many animals (Mason 1992). If some variations in chemical signals occur, these variations could avoid interspecific mating among closely related sympatric species (Cooper and Vitt 1987; Shine et al. 2002) or between different populations of the same species (LeMaster and Mason 2003). In many lizards, intraspecific communication is based on chemical cues from femoral gland secretions (Mason 1992; Aragón et al. 2001). Especially secretions of chemical signals often come from femoral pores, which are epidermal structures on the ventral surface of the thigh connected to glands that produce copious amounts of holocrine secretion, particularly in males and during the mating season (reviewed in Mason 1992; Alberts 1993). Because of the ventral location of the femoral pores, the secretions seem to be passively deposited on the substrate as lizards move through their home ranges. Behavioral experiments with several Lacertid lizard species showed that both male and female lizards responded by tongue-flicking to substrate scent marks, but also, during social encounters, lizards investigated each other by tongue-flicking the cloacal and femoral regions. Femoral gland secretions of males could advertise residence in a home range, and/or convey information about social status and competitive ability (Aragón et al. 2001; López and Martín 2002; Martín and López 2007; Martín et al. 2007), or even transmit information about a male's quality, which females may use to select mates (Martín and López 2000, 2006a; López et al. 2002; Olsson et al. 2003). Thus because femoral gland secretions are involved in sexual selection they may also lead to speciation processes.

The Iberian wall lizard, *Podarcis hispanica*, is a small diurnal lacertid lizard, living in rocky habitats and in artificial walls of the Iberian Peninsula. This lizard use multiple types of signals in social behavior (i.e., carotenoid dependent coloration and chemical secretions from femoral glands) and can discriminate between sexes by either visual or chemical cues alone (López and Martín 2001; Cooper and Pérez-Mellado 2002; López et al. 2002). Manipulations of visual and chemical traits showed that, at close range, chemical cues seem more important than color patterns in sex recognition and individual rival recognition, but, at long range, male bright coloration alone allows sex identification and elicits aggressive responses of males (López and Martín 2001, 2002; López et al. 2002). Femoral secretions are composed of both proteins and lipids, but the latter are thought to be the main compounds involved in communication (Mason 1992). Among steroids, cholesterol is a steroid of animal origin that was found in abundance in femoral and precloacal gland secretions of most lizard species (Alberts et al. 1992; Escobar et al. 2001; Lopez and Martin 2005a,b,c). The abundance and ubiquity of cholesterol in secretions was thought to be useful to constitute an unreactive apolar matrix that delivers the compounds that are the true semiochemicals (Escobar et al. 2003). Nevertheless, the total amounts or relative proportions of cholesterol in femoral secretions might be depended on size, age or sex steroid levels, because lipid metabolism, femoral gland production and percentage of lipid in secretions depend on androgen levels in lizards (Alberts et al. 1992; Sheridan 1994). Thus, cholesterol might also have a communicative function, for example as a potential signal of dominance as occurs in *Lacerta monticola* lizards where cholesterol is the predominant component too (Martin and Lopez 2007). Moreover, other major steroid found in secretions is cholesta-5,7-dien-3-ol, a precursor for vitamin D_3, which is often found in the skin, where it will transform into vitamin D after exposition to sun UV-B irradiation (Fraser 1995; Holick et al. 1995; Carman et al. 2000). Vitamin D_3 is essential in calcium metabolism of lizards and other vertebrates (Allen et al. 1994; Laing and Fraser 1999). However, very often, the synthesis of vitamin D_3 in the skin is not sufficient to meet physiological requirements, and lizards require dietary intake of vitamin D (Allen et al. 1994; Ferguson et al. 2005). In the lizard *P hispanica*, individuals can discriminate cholesta-5,7-dien-3-ol by chemosensory cues alone and females could made their partner scent choice using the chemical signals of males, especially this steroid, which may signal a better cell-mediated immune response (López and Martín 2005, López et al. 2009).

Podarcis lizards are highly variable in size, shape, escalation and color pattern, not only between currently recognized species (Arnold and Ovenden 2002), but also between populations and individuals. Given this, disentangling the track of evolutionary history from the effects of local adaptation or phenotypic plasticity (or finding a cohesive phenotype) can be a difficult task. Morphological distinction between *Podarcis* forms can neither be taken for granted nor necessarily attributed to separate evolution. Recently molecular and morphological studies suggest that the Iberian wall lizard, is paraphyletic, and forms a species complex with at least five monophyletic lineages, and suggest the existence of ongoing speciation processes within taxa previously considered to be conspecific (Guillaume 1987; Harris and Sá-Sousa 2001, 2002; Sá-Sousa et al. 2002; Pinho et al. 2007). Although these populations of the *P. hispanica* species complex are mainly allopatric, both types have been reported from the Madrid region (Central Spain). In this area, two populations live close together without geographical isolation, and individuals of both types may find each other easily (Mellado and Olmedo 1981; García-Paris et al. 1989). Thus, Madrid is a contact zone where at least two types (1 and 2) of *P. hispanica* could be found. Moreover, more than two populations with distinct morphology, color and chemical signals were observed in this region (Gabirot et al. unpublished; see chapter 1).

In this paper, we studied five distinct populations of *P. hispanica* in the contact zone of Madrid. We tested three populations from the north of Madrid and two southern populations. All these populations showed morphological and chemical differences (Gabirot et al. unpub. data; see Chapter 1). Males from the northern populations have in their femoral secretions a higher proportion of steroids, and in particular of cholesta-5,7-dien-3-ol, than males from the southern populations (Gabirot et al. unpubl. data;; see Chapter 1). We aimed to study whether there were interpopulational differences in chemosensory recognition of two of these major steroids (cholesterol and cholesta-5,7-dien-3-ol), by *P. hispanica* lizards. We presented steroid compounds and compared the tongue flick responses of individuals of both sexes from distinct populations in order to understand the implication of chemical signals in the evolutive processes of the *P. hispanica* species complex. We hypothesized that interpopulational differences in chemical signals of male lizards *P. hispanica*, and in responses to these chemical, could be leading to reproductive isolation processes between populations of this lizard species.

MATERIALS AND METHODS

Study populations

During the spring 2008, we captured by noosing male and female *P. hispanica* lizards at five localities within the Madrid region (Central Spain). Three of these were localized in the mountain northern area (‘Fuenfría’, ‘Golondrina’ and ‘Pedrezuela’), and the two other were situated in the plain southern area (‘Belmonte’ and ‘Aranjuez’). We chose these populations because they show distinct morphological and coloration characteristics (Gabirot et al. unpubl. data;

see Chapter 1). In the north, we captured 24 males and 27 females from a population occupying different granite rock-cliffs at the edge of a pine forest in the upper part of ‘Fuenfría’ Valley (40°47’ N, 4°03’ W; 1750 m altitude), 18 males and 24 females on granite rocky outcrops in a large oak forest (‘Golondrina’) near Cercedilla village (40°44’ N, 4°02’ W; 1250 m altitude), and 28 lizards (14 of each sex) from old stone walls near to cultivated fields in the ‘Pedrezuela’ village (40°44' N, 3°36' W; 800 m altitude). In the south, we captured lizards (10 males and 16 females) on human constructions in a public garden in the ‘Belmonte del Tajo’ village (40°8' N, 3°20' W; 735 m altitude) and 8 males and 12 females on chalk and gypsum rocks in deforested bushy hills near ‘Aranjuez’ (40°2’ N, 3°37’ W; 494 m altitude).

All lizards were individually housed at “El Ventorrillo” Field Station (Cercedilla, Madrid) about 5 Km from the capture sites of the northern populations, in indoor 60x40 cm PVC terraria containing sand substratum and rocks for cover. Cages were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle, and were screened from each other using cardboard. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and water was provided *ad libitum*. Lizards were returned to their exact capture sites with good health condition at the end of experiments. Captures and observations were performed under license from the Environmental Agency of Madrid Government (“Consejería del Medio Ambiente de la Comunidad de Madrid”, Spain).

Chemical compound recognition by lizards

To measure the discrimination of chemical cues, we used a bioassay based on tongue-flick (TF) rates, where lizards respond by distinct TF rates to different chemical stimuli (Cooper and Burghardt 1990; Cooper 1994).

In this experiment, we compared TF rate by lizards (males and females) in response to stimuli arising from cotton applicators bearing scents of (1) cholesterol, (2) cholesta-5,7-dien-3-ol, and (3) dichloromethane (DCM). (1) cholesterol is the most abundant steroid in femoral secretions; (2) cholesta-5,7-dien-3-ol is found in femoral gland secretions, and it seems to have a role in scent marking and intraspecific communication (López and Martín 2005b; López et al. 2009); (3) DCM was used as a control to gauge baseline TF rates under the experimental conditions.

We prepared chemical stimuli the day of the tests by dissolving each compound (authentic standards, GC grade, Sigma-Aldrich, St. Louis, MO) in DCM (30 mg ml^{-1}) in glass vials with Teflon-lined stoppers. We shook the solution for 1 min using a vortex, and kept the vials in a refrigerator. Trials were made between 11:00 and 13:00 h (GMT). Immediately before the trials, we prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) for 3 s in vials containing DCM alone, or DCM with other chemicals dissolved in it. In each experiment, every lizard was presented with each chemical stimulus in a randomized order. Swabs with all stimuli were visually similar for humans. A new swab was used in each trial. To begin a trial, the same experimenter slowly approached the home terrarium of each lizard and slowly moved the cotton swab to a position 1 cm anterior to the lizard's snout. The experimenter was blind to the chemical presented. The numbers of TFs directed to the swab were recorded for 60 s, beginning with the first TF.

To examine differences in number of directed TFs between conditions, we used three-way repeated measures ANOVA with chemical stimuli as a within factor, and population and sex as categorical between variables. Data were log-transformed to ensure normality and homogeneity of variances. Pairwise comparisons used Tukey's honestly significant difference tests (Sokal and Rohlf 1995).

We also analyzed whether the numbers of TFs directed to each chemical compound stimulus by each population were related to the relative proportions of those compounds in femoral secretions of males. Males from these populations have different proportion of cholesterol and cholesta-5,7-dien-3-ol in secretions (Gabirot et al. unpubl. data; see. chapter 1); males from the northern populations having secretions with higher proportions of cholesta-5,7-dien-3-ol. To test for this possible relation between the number of TFs to these compounds and the relative proportions of these steroids in male secretions, we used non-parametric spearman's rank-order correlations (Siegel and Castellan 1988).

RESULTS

All individual *P. hispanica* directed TFs to the swab in all conditions. There were significant differences in number of TFs directed by lizards to the different stimuli presented (three-way repeated measures ANOVA, stimuli: $F_{2,372}=428.51$, $P<0.0001$), males and females made a similar overall number of TFs (sex: $F_{1,86}=0.003$ $P=0.96$), and the interaction between sex and stimuli was not significant ($F_{2,372}$=0.84, P=0.42) (Fig. 1). Both male and female lizards from all population made a significant higher number of TFs in response to both steroids (i.e., cholesterol or cholesta-5,7-dien-3-ol) than in response to DCM (i.e., control) (Tukey's tests: P<0.0001 for all), and significantly more TFs in response to cholesta-5,7-dien-3-ol than to cholesterol (P=0.0001).

However, the population of origin affected significantly the overall numbers of TFs ($F_{4,186}$=6.26 P=0.0001), and the interaction between chemical stimuli and population was also significant ($F_{8,372}$=2.17, P=0.03). The interaction between sex and population was not significant ($F_{4,186}$=0.91, P=0.46). Thus, there was a gradient in the magnitude of the responses, where the numbers of TFs increased when the population was situated more to the north. Lizards from Aranjuez made less TFs in response to both steroids than lizards from Fuenfría or Pedrezuela (Tukey's tests: P<0.05). However, the rest of post-hoc comparisons were not significant.

Finally, we calculated the relation between the average proportions of steroids in femoral secretions of males in each population (cf. Chapter 1) and the average number of TFs made by lizards from each population in direction to these compounds. For cholesterol, we did not observe any significant relationship (Spearman's correlation: r_s=-0.30, t=-0.54, N=5; P=0.62). While for cholesta-5,7-dien-3-ol, in those populations where the relative proportions of this steroid in femoral secretion were higher, the numbers of TFs directed to swabs bearing this compound were significantly higher (Spearman´s correlation: r_s=0.90, t=3.57, N=5; P=0.03). Lizards with higher proportions of this steroid in their femoral secretion (i.e., Fuenfría population) made higher number of TFs in response to this compound than lizards with lower proportions (i.e., Aranjuez).

DISCUSSION

Lizards from all the studied populations discriminated and showed a significant high interest (i.e., high TF rates) for the two steroids tested (cholesterol and cholesta-5,7-dien-3-ol) in comparison to a control. However, interestingly, lizards from different populations did not display the same interest for these steroids. Lizards from the southern population (i.e., Aranjuez) made less tongue flicks in response to cholesterol and cholesta-5,7-dien-3-ol than lizards from the northern populations (i.e., Pedrezuela and Fuenfría). The Belmonte and Golondrina populations showed an intermediate response between those of southern and northern populations. We might interpret this result as an outcome of an interpopulational gradient of interest for these steroids related to the geographical localization (or climatic characteristics) of each population. Populations that live in habitats with cold temperatures and high relative humidity (i.e., northern Madrid) show a higher interest for steroids; while the converse occurs in population with a dry and hot habitat (i.e., southern Madrid), where the interest for steroids decreases.

Moreover, other study showed that male *P. hispanica* lizards from the north and south of the Madrid region have different chemical signals (Gabirot et al. unpublished; see Chapter 1). Femoral secretions of males differ in proportions and composition; males from northern populations (Fuenfría, Golondrina and Pedrezuela) have higher relative proportions of cholesta-5,7-dien-3-ol in femoral secretions than individuals from southern populations (Aranjuez and Belmonte). Fuenfría was the population with the highest proportion of this compound and Aranjuez the one with the lowest proportion. In previous studies, cholesta-5,7-dien-3-ol has been show to have a role in mate choice by Fuenfría and Golondrina females (Gabirot et al. unpublished data; see Chapter 3). Females selected establishing in a territory scent marked by individual males with relatively higher amounts of cholesta-5,7-dien-3-ol in their femoral gland secretions, independently of the male population (Golondrina vs. Fuenfría). This mate choice criterion may be explained by the

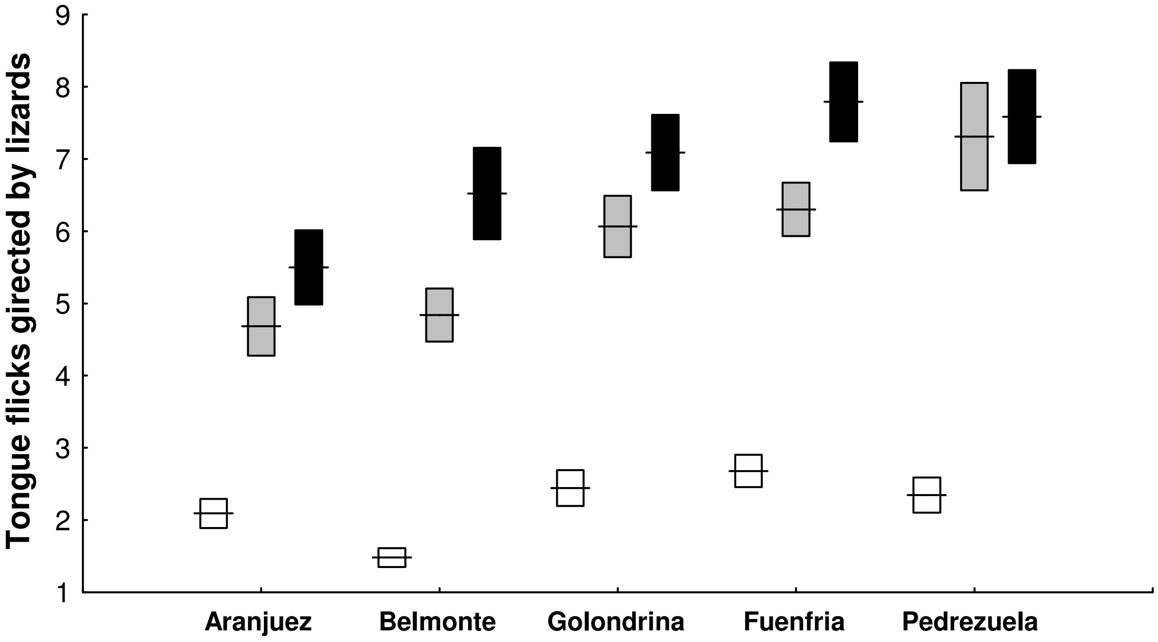


Fig 1: Tongue flicks directed (mean ± SE) by *P. hispanica* lizards from five populations in response to different chemical compounds presented on cotton swabs: cholesterol (in grey), cholesta-5,7-dien-3-ol (in black) and control DCM (in white).

positive relationship between the amount of cholesta-5,7-dien-3-ol in secretions and the quality of the immune response of a male observed in this lizard (López and Martín 2005c; López et al. 2009) and in other Lacertid lizard species (Martín and López 2006a). Other experiment showed that female *P. hispanica* can actually detect changes in concentration of this lipid in femoral secretions (Martín and López 2006b). Thus, it is likely that in northern populations, where the relative proportions of cholesta-5,7-dien-3-ol are higher, individuals showed a higher interest for this compound, and could use it as a honest signal in sexual selection behaviors. While in southern populations, where this steroid appears in smaller proportions in femoral secretions, individuals showed a lower interest for it. Therefore, in the southern populations this steroid might not have the same importance and role in reproductive processes than in the northern populations. If individuals had an interest for this steroid because it may bear information, there could be a trend to increase its presence in chemical signals. Conversely, if this compound had not or lose the informative signal, individuals could not have reasons to secrete this costly compound in higher proportions. For Aranjuez lizards, cholesta-5,7-dien-3-ol is found in low proportions in secretions. Individuals recognize it, and have some interest for it. Thus, this chemical could still be important but not so much as for Fuenfría lizards. Aranjuez lizards could rather use other type of compounds in secretions or other type of signal (e.g., visual) for communication.

These chemical differences (in behavior and composition) could be due to an adaptation to different habitats, with temperature, humidity and trophic variations. The environment can have an important role in how pheromones are used and conveyed. The humidity and temperature are important for the persistency and efficiency of chemical secretions on substrates (Alberts 1992). The loss of chemical communication in some lineages (e.g., fireflies; Branham and Wenzel 2003) might be a result of environmental selection against pheromone use (Symonds and Elgar 2009). Thus, in a habitat where the temperature is high, the secretions will not have the same efficiency, and perhaps importance, than in cold mountain populations where the secretions will stay for longer time and might have an important function in communication. Northern populations living in a habitat more humid and cold, and with higher proportions of some steroids in secretions, could have evolved a higher use of chemical signals than in southern populations where the habitat is more dry and hot. In contrast, in the populations from south of Madrid, other type of communication, such as the visual one, could prevail (Gabirot et al. unpubl. data). Considering the chemosensory behavior differences between northern and southern populations in the region of Madrid, we could support that these populations could be adapted to different environments and in consequence use different types of sexual signals in communication. All these results support the existence of ongoing reproductive isolation and cryptic speciation between types in the *P. hispanica* complex, which merits further studies.

Acknowledgments We thank "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an “El Ventorrillo” CSIC grant to MG. The experiments enforced all the present Spanish laws and were performed under license from the Environmental Organisms of Madrid Community where they were carried out.

References

Alberts, A.C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. Am. Nat. **139**: S62–S89.

Alberts, A.C. 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behav. Evol. **41**: 255–260.

Allen, M.E., Bush, M., Oftedal, O.T., Roscoe, R., Walsh, T., and Holick, M.F. 1994. Update on vitamin D and ultraviolet light in basking lizards. Proc. Am. Assoc. Zoo Vet. **25**: 314–316.

Aragón, P., López, P., and Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Arnold, E.N., and Ovenden, D.W. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Harper Collins, London.

Branham, M.A., and Wenzel, J.W. 2003. The origin of photic behavior and the evolution of sexual communication in fireflies (Coleoptera: Lampyridae). Cladistics **19**: 1–22.

Carman, E.N., Ferguson, G.W., Gehrmann, W.H., Chen, T.C. and Holick, M.F. 2000. Photobiosynthetic opportunity and ability for UVB generated vitamin D synthesis in freeliving house geckos (*Hemidactylus turcicus*) and Texas spiny lizards (*Sceloporus olivaceous*). Copeia **2000**: 245–250.

Cooper, W.E. 1994. Chemical discrimination by tongue-flicking in lizards: A review with hypotheses on its origin and its ecological and phylogenetic relationships. J. Chem. Ecol. **20**: 439–487.

Cooper, W.E., and Burghardt, G.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J. Chem. Ecol. **16**: 45–65.

Cooper, W.E., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Cooper, W.E., and Vitt, L.J. 1987. Ethological isolation, sexual behavior and pheromones in the fasciatus species group of the lizard genus *Eumeces*. Ethology **75**: 328–336.

Escobar, C.M., Escobar, C.A., Labra, A., and Niemeyer, H.M. 2003. Chemical composition of precloacal secretions of two *Liolaemus fabiani* populations: are they different? J. Chem. Ecol. **29**: 629–638.

Escobar, C.A., Labra, A., and Niemeyer, H.M. 2001. Chemical composition of precloacal secretions of Liolaemus lizards. J. Chem. Ecol. **27**: 1677–1690.

Ferguson, G.W., Gehrmann, W.H., Karsten, K.B., Landwer, A.J., Carman, E.N., Chen, T.C. and Holick, M.F. 2005. Ultraviolet exposure and vitamin D synthesis in a sun-dwelling and a shade-dwelling species of *Anolis*: are there adaptations for lower ultraviolet B and dietary vitamin D3 availability in the shade? Physiol. Biochem. Zool. **78**: 193–200.

Fraser, D.R. 1995. Vitamin D. Lancet **345**: 104–107.

García-Paris, M., Martín, C., and Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain-Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Guillaume, C.P. 1987. Les petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: Univ. Sci. Techn. Langedoc.

Harris, D.J., and Sá-Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75–81.

Holick, M.F., Tian, X.Q., and Allen, M. 1995. Evolutionary importance for the membrane enhancement of the production of vitamin D3 in the skin of poikilothermic animals. Proc. Natl Acad. Sci. USA **92**: 3124-3126.

Laing, C.J., and Fraser, D.R. 1999. The vitamin D system in iguanian lizards. Comp. Biochem. Physiol. B **123**: 373–379.

LeMaster, M.P., and Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red-sided garter snakes, *Thamnophis sirtalis parietalis*. J. Chem. Ecol. **29**: 1027–1043.

López, P., Gabirot, M., and Martín, J. 2009. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturw. **96**: 65–69.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461-465.

López, P., and Martín, J. 2005a. Chemical compounds from femoral gland secretions of male Iberian rock lizards, *Lacerta monticola cyreni*. Z. Naturforsch. C **60**: 632–636.

López, P., and Martín, J. 2005b. Age related differences in lipophilic compounds found in femoral gland secretions of male spiny-footed lizards, *Acanthodactylus erythrurus*. Z. Naturforsch. C **60**: 915–920.

López, P., and Martín, J. 2005c. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

Martín, J., and López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B **267**: 1265–1269.

Martín, J., and López, P. 2006a. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

Martín, J., and López, P. 2006b. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herpetol. **60**: 556–561.

Martin, J., and Lopez, P. 2007. Scent may signal fighting ability in male Iberian rock lizards. Biol. Lett. **3**: 125–127.

Martín, J., Moreira, P.L., and López, P. 2007. Status-signaling chemical badges in male Iberian rock lizards. Funct. Ecol. **21**: 568–576.

Mason, R.T. 1992. Reptilian pheromones. In: Gans C, Crews D, eds. Biology of the Reptilia. Vol. 18. Chicago: University of Chicago Press, pp. 114–228.

Mellado, J., and Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, Act. Verteb. **8**: 299–300.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B, Suppl. **270**: S254–S256.

Pinho, C., Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Sá-Sousa, P., Vicente, L., and Crespo, E.G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. Amphib-Rept. **23**: 55–69.

Sheridan, M.A. 1994. Regulation of lipid-metabolism in poikilothermic vertebrates. Comp. Biochem. Physiol. B **107**: 495–508.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Siegel, S., and Castellan, N. J. 1988. Nonparametric statistics for the behavioral sciences, 2nd edn. NewYork: McGraw-Hill.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. New York: Freeman.

Symond, M.R.E., and Elgar, M.A. 2009. The evolution of pheromone diversity. Trends Ecol. Evol. **23**: 220–228.

CAPÍTULO 5

Reconocimiento químosensorial de alcoholes presentes en las secreciones femorales de los machos de la lagartija Ibérica *Podarcis hispanica*

En algunos lacértidos, las marcas químicas de los machos pueden informar del estatus y habilidad competitiva, o incluso trasmitir información sobre las características de un macho que serviría a las hembras para seleccionar parejas. Las secreciones femorales de los machos de lagartija Ibérica, *Podarcis hispanica,* se componen de esteroides, ácidos carboxílicos, ésteres céreos, alcoholes y otros compuestos. Los esteroides son los compuestos más abundantes, pero otros compuestos importantes en las secreciones son los alcoholes que parecen funcionar como señales en otras especies. En este trabajo, exploramos la importancia de los alcoholes en la comunicación de *P. hispanica*. Diseñamos un experimento de reconocimiento quimiosensorial mediante protusiones linguales para saber si los individuos identifican y muestran interés por explorar varios alcoholes (hexadecanol, octadecanol, docosanol y tetracosanol) que se encuentran de forma natural en las secreciones femorales de los machos. Los resultados mostraron que las lagartijas discriminaban y tuvieron intereses diferentes por los alcoholes examinados. Las lagartijas manifestaron un interés mayor por el tetracosanol, lo que podría sugerir que este compuesto funciona como señal en las secreciones de los machos. Además, las diferencias en las respuestas entre dos poblaciones distintas de *P. hispanica* indicarían que estos alcoholes tendrían también un papel en el reconocimiento interpoblacional. Estudios previos han mostrado respuestas quimiosensoriales y el posible papel señalizador de los esteroides en las secreciones de los machos, la existencia de respuestas adicionales a los alcoholes proporcionaría más oportunidades para señalizar otras características de los machos, o reforzaría la fiabilidad de las características señalizadas por los esteroides. Finalmente, este trabajo resalta el importante papel de la comunicación química en la biología de las lagartijas y la alta especificidad y complejidad de este sentido de comunicación.

Chemosensory recognition of alcohols found in femoral gland secretions of male Iberian wall lizards *Podarcis hispanica*

Marianne Gabirot, Pilar López, José Martín

Abstract In some Lacertid lizards, scent marks of males can convey information about social status and competitive ability, or even transmit information about a male's characteristics, which females may use to select mates. The femoral secretion of male Iberian wall lizards, *Podarcis hispanica*, is a mix of steroids, carboxylic acids, waxy esters, alcohols and other compounds. Steroids are the more abundant compounds, but other main compounds in secretions are alcohols, which seem to have a signaling function in other lizard species. In this paper, we explored the importance of alcohols in communication of *P. hispanica* lizards. We designed an experiment of chemosensory recognition by tongue flicks in order to know whether individuals could identify and show an exploratory interest for several alcohols (hexadecanol, octadecanol, docosanol and tetracosanol) that are naturally found in males' femoral secretions. Results showed that lizards can discriminate and show different interests for tested alcohols. Lizards showed a higher interest for tetracosanol, which could suggest that this compound might have a signaling role in secretions of males. Moreover, some differences in responses between two distinct populations of *P. hispanica* could indicate that these alcohols might also have a role in interpopulational recognition. Previous studies have showed chemosensory responses to, and the possible signaling role of, steroids from male's secretions. The existence of additional responses to alcohols might provide more opportunities to signal other characteristics of males, or reinforce the reliability of traits signaled by steroids. Finally, this work enlightens the important role of chemical communication in lizard's biology and the very specificity and complexity of this communication sense.

Résumé Les marques chimiques laissées par les lézards pourraient avertir sur le territoire ou sur informer sur le statut social ou l'habilité compétitive, ou encore transmettraient des renseignements sur les caractéristiques individuels des mâles, caractéristiques à partir desquelles les femelles choisiraient leur partenaire sexuel. Les secrétions fémorales des mâles de *Podarcis hispanica* sont un mélange de stéroïdes, acides carboxyles d'esters acéreux, d'alcools et autres composés. La majeur proportion des secrétions est faite de stéroïdes, mais les alcools occupent aussi une partie importante et ont une fonction de signal chez d'autres lézards. Par conséquent, dans ce travail nous avons testé l'importance des alcools dans la communication du lézard *Podarcis hispanica*. Pour cela, nous avons procédé à des expériences de reconnaissance chimique par extraction linguale dans le but de savoir si ces animaux peuvent distinguer et avoir un intérêt pour ces différents alcools : hexadecanol, octadecanol, docosanol et tetracosanol. Les résultats ont montré un nombre de tirage de langue significatif en direction des substances testées. De plus, les lézards ont eu un intérêt élevé pour le tetracosanol. Cette molécule pourrait informer sur le statut des mâles. La comparaison des réactions face aux alcools gras entre deux populations de *Podarcis hispanica* laisse penser que les alcools pourraient jouer un rôle dans la reconnaissance interpopulationnale. Ces composés chimiques présents dans les secrétions fémorales des mâles seraient une possibilité de plus pour signaler aux congénères. Finalement ce travail apporte plus de preuves sur le rôle de la communication chimique ainsi que sa spécificité et complexité.

In Press in *Journal of Herpetology*

INTRODUCTION

The chemosensory system and chemical communication are well developed in lizards and snakes (Mason 1992). In many lizards, chemical secretions from femoral glands of males play a prominent role in social relationships of many species (reviewed in Mason 1992; Alberts 1993; Martín and López in press). Behavioral experiments showed that scent marks of males could advertise residence in a home range, and/or convey information about social status and competitive ability (Lopez and Martin 2002; Carazo et al. 2007; Martin et al. 2007), or even transmit information about a male's characteristics, which females may use to select mates (Martin and Lopez 2000, 2006d; Olsson et al. 2003; Lopez and Martin 2005a).

However, in spite of the potential importance of chemical signals in lizard social biology, only a few studies have described the chemical composition of gland secretions in a few species of lizards (reviewed in Weldon et al. 2008). Secretions are mainly composed of both proteins and lipids, which are thought to be the main compounds involved in communication (Mason 1992; Martín and López in press). Moreover, although some studies examined the responses to chemicals used for prey identification in foraging contexts (Cooper and Pérez-Mellado 2001; Cooper et al. 2002a,b), the chemosensory behavioral responses of lizards to specific chemicals from femoral secretions have been little studied (Martín and López 2006c,d, 2008). However, variations in the chemosensory responses to different chemicals would be an important first indication of the potential role of some compounds as chemical signals.

The small lacertid Iberian wall lizard, *Podarcis hispanica*, is a common lizard living in rocky habitats and on artificial walls of the Iberian Peninsula. This lizard uses multiple types of signals in social behavior (i.e., coloration and chemical secretions from femoral glands) and can discriminate between sexes by either visual or chemical cues alone (López and Martín 2001; Cooper and Pérez-Mellado 2002; López et al. 2002), but chemical signals seem more important than color patterns in sex recognition and individual rival recognition (López and Martín 2001, 2002; López et al. 2002; Carazo et al. 2008).

The lipophilic composition of femoral secretions from male *P. hispanica* is a mix of steroids, carboxylic acids, alcohols and other minor compounds (Martin and Lopez 2006a). Among steroids, cholesterol is the most abundant component, which is also found in abundance in femoral gland secretions of most lizard species (Weldon et al. 2008). The other major steroid in secretions is cholesta-5,7-dien-3-ol, which might function as an honest sexual signal in mate choice processes (López and Martín 2005a; Martin and Lopez 2006d; López et al. 2009). Previous studies showed that female *P. hispanica* can discriminate these two steroids and are able to assess changes in concentration of cholesta-5,7-dien-3-ol (Martín and López 2006c). However, although most previous studies have examined the role of steroids as potential chemical signals, there are also other main compounds in secretions such as alcohols. One study in the lizard *Lacerta m. monticola* showed that two alcohols (hexadecanol and octadecanol) may be a reliable status badge (i.e., signaling aggressive behavior) (Martin et al. 2007). These alcohols are also found in secretions of *P. hispanica,* so it is likely that alcohols could have a similar role in sexual selection or intraspecific communication of this lizard.

In this paper, we explored the importance of alcohols that are naturally found in femoral secretions of male Iberian wall lizards, *P. hispanica* in their intraspecific communication. For this, we designed an experiment of chemosensory recognition, by measuring tongue-flick rates, to test whether lizards recognized and showed different chemosensory responses to several alcohols also found in males' secretions.

In addition, chemical signals are important for species recognition and may result in speciation in many animals (reviewed in Smadja and Butlin 2009). Chemical stimuli can be the basis of interspecific recognition and speciation in snakes (Shine et al. 2002; LeMaster and Mason 2003) and lizards (Cooper and Vitt 1986; Barbosa et al. 2006; Martín and López 2006a,b). Iberian wall lizards, *P. hispanica,* seem to form a "species complex" with several morphotypes that might be cryptic species (Harris and Sa-Sousa 2002; Pinho et al. 2007). Although populations of the different types of *P. hispanica* are mainly allopatric, several non-isolated distinct populations are found in the Madrid region (Central Spain) (Mellado and Olmedo 1981; García-Paris et al. 1989; Gabirot et al. unpublished data). Here, we used two nearby but distinct populations of *P. hispanica* from the Guadarrama Mountains, inhabiting different altitudes and environmental conditions, which differ in morphology and in the chemical composition of femoral gland secretions of males (Martín and López 2006a). We tested whether individuals from these different populations showed different chemosensory responses to chemical stimuli (i.e., alcohols) as an indication of the potential role of these alcohols as signals used in interpopulational or interspecific recognition.

MATERIALS AND METHODS

Study animals

We used *P. hispanica* lizards from two distinct populations from the north of the Madrid province (Central Spain); we captured 20 males and 19 females from a population occupying different granite rock-cliffs at the edge of a pine forest in the upper part of 'Fuenfría' Valley (40°47' N, 4°03' W; 1750 m altitude), and 18 males and 31 females were caught on granite rocky outcrops in a large oak forest ('Golondrina') near Cercedilla village (40°44' N, 4°02' W; 1250 m altitude). These two populations are not isolated geographically, but show clear differences in morphology, coloration and chemical signals (Martín and López, 2006a).

All lizards were individually housed at "El Ventorrillo" Field Station (Cercedilla, Madrid), in indoor 60x40 cm PVC terraria containing sand substratum and rocks for cover. Cages were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle, and were screened from each other using cardboard. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and water was provided *ad libitum*. Lizards were held in captivity at least one week before testing to allow acclimation to laboratory conditions. Lizards from different populations were housed in different rooms. All lizards were healthy during the trials. They did not show behavioral or physiological changes due to possible stress of experiments, and all maintained or increased their original body mass. Lizards were returned to their exact capture sites at the end of experiments.

Chemosensory recognition trials

Several studies have shown that lizards respond to different chemical stimuli by changing the rate of tongue extrusions (Cooper and Burghardt 1990; Cooper 1994). Therefore, tongue-flick (TF) rates can be used as a bioassay for measuring discrimination of chemical cues. In this experiment, we compared TF rate by lizards (males and females) in response to stimuli arising from cotton applicators bearing scents of (1) cholesterol, (2) hexadecanol, (3) octadecanol (4) docosanol, (5) tetracosanol and (6) dichloromethane (DCM). The rationale for testing these chemicals was that: (1)

cholesterol is the most abundant chemical in femoral secretions. We previously knew that *P. hispanica* lizards show high chemosensory responses and discriminate cholesterol from other chemicals (Martín and López 2006c), so we used cholesterol as a control indicating a high chemosensory response; (2) hexadecanol and (3) octadecanol are present in the femoral secretions and could be putative chemical signals signaling male dominance status, as it occurs in other lacertid lizard species (Martin et al. 2007); the rest of alcohols, (4) docosanol and (5) tetracosanol, are also found in the femoral secretions of males but their function is unknown. Finally, (6) DCM was used as a control to gauge baseline TF rates under the experimental conditions because DCM was used to dissolve other chemicals used in the tests.

We prepared chemical stimuli the day of the tests by dissolving each compound (authentic standards, GC grade, Sigma-Aldrich, St. Louis, MO) in DCM (30 mg.ml^{-1}) in glass vials with Teflon-lined stoppers. We shook the solution for 1 min using a vortex, and kept the vials in a refrigerator. Trials were made between 11:00 and 13:00 h (GMT). Immediately before the trials, we prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) for 3 s in vials containing DCM alone, or DCM with other chemicals dissolved in it. After impregnating the swab, the DCM quickly evaporated in a few seconds leaving the chemical dissolved in it deposited on the swab. The control solution of DCM alone evaporated entirely without leaving any solid residual. Swabs with all stimuli were visually similar for humans. In each experiment, every lizard was presented with each chemical stimulus in a randomized order. A new swab was used in each trial. To begin a trial, the same experimenter (MG) slowly approached the home terrarium of each male and slowly moved the cotton swab to a position 1 cm anterior to the lizard's snout. The experimenter was blind to the chemical presented. The numbers of TFs directed to the swab were recorded for 60 s, beginning with the first TF.

To examine differences in number of directed TFs by individuals (males and females) among stimuli presented (chemical compounds), we used a three-way repeated measures ANOVA with chemical stimuli as a within factor, and with the sex (male vs. female) and population of origin (Golondrina vs. Fuenfría) of the responding lizard as between factors. Further, because responses of males and females were different (see results), we performed two-way repeated measures ANOVAs separately for males and females, with chemical stimuli as a within factor, and population of origin as a between factor. Data were log-transformed to ensure normality and homogeneity of variances. Pairwise comparisons used Tukey's honestly significant difference tests (Sokal and Rohlf 1995).

RESULTS

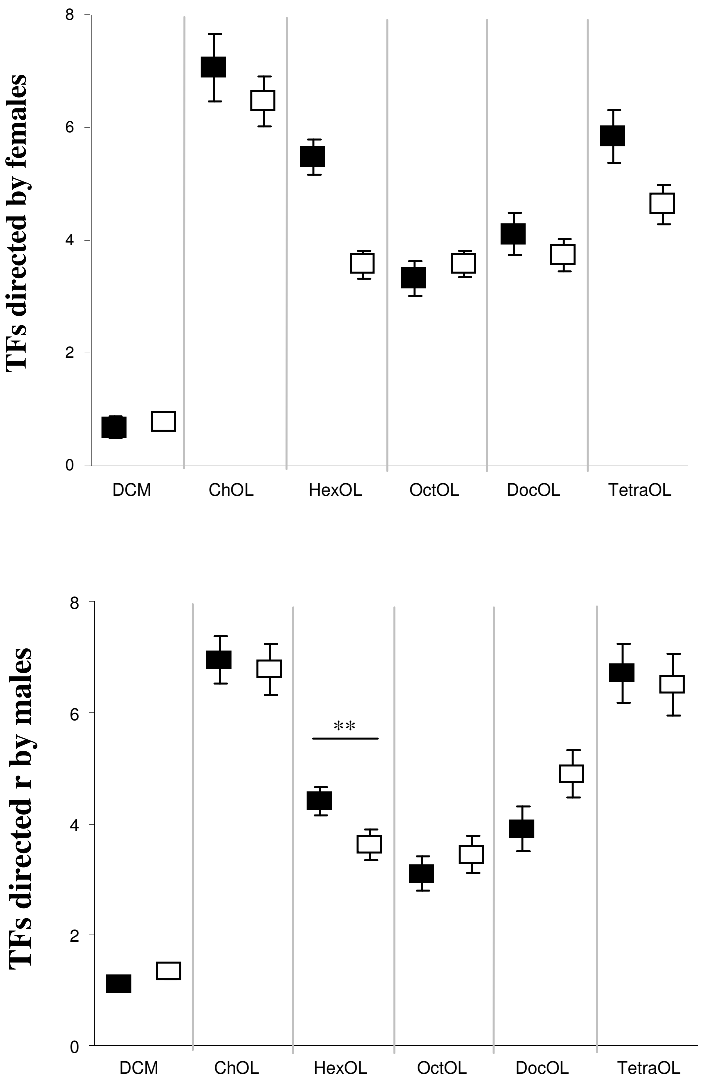


Fig. 1: Mean (± SE) tongue flick rates directed by (up) female and (down) male *P hispanica* from two populations (Fuenfría in black and Golondrina in white) in response to several chemicals: cholesterol (ChOL), Hexadecanol (HexOL), Octodecanol (OctOL), Tetracosanol (TetraOL), Docosanol (DocOL) and Dichloromethane (DCM).

The sex and the population of origin of lizards had not significant effects on the overall number of TFs (three-way repeated measures ANOVA: sex: $F_{1,84}$=2.57, P=0.11; population: $F_{1,84}$=0.95, P=0.33); and the interaction between sex and population was not significant ($F_{1,84}$=2.57, P=0.11). However, lizards' TF rates differed significantly between chemical stimuli (scent: $F_{5,420}$=285.13, P<0.0001), and the magnitude of the responses to the different stimuli varied significantly between males and females (scent x sex: $F_{5,420}$=7.00, P<0.0001), as well as between populations (scent x population: $F_{5,420}$=5.41, P<0.0001), and the interaction between sex and population was not significant ($F_{5,420}$=0.38, P=0.86) (Fig. 1). Thus, we made two separated analyses for males and females (see below). In all cases, the TFs responses of lizards in response to any chemical compound were significantly higher than in response to control DCM (Tukey's test: P<0.05 for all).

Responses by females

We found significant differences in TF rates of females between chemical stimuli (two-way repeated measures ANOVA: scent: $F_{5,240}$=175.64, P<0.0001) (Fig. 1a), females from different populations made non significant different overall numbers of TFs (population: $F_{1,48}$=2.78, P=0.10), but the interaction between scent stimuli and population was significant ($F_{5,240}$=3.63, P=0.003), suggesting that females from different populations responded differentially to the different chemical stimuli..

Females from Fuenfría made significantly more TFs in response to cholesterol, tetracosanol and hexadecanol than in response to octadecanol and docosanol (Tukey's tests: P<0.001 for all). There were no significant differences between responses to cholesterol, tetracosanol and hexadecanol (P>0.50 for all) nor between octadecanol and docosanol (P=0.99). Females from Golondrina showed a significantly higher number of TFs in response to cholesterol than to all the other chemical compounds (P<0.001 for all), and TF rates in response to any alcohol (tetracosanol, docosanol, hexadecanol and octadecanol) were not significantly different (P>0.08 for all).

Responses by males

We found significant differences between scent stimuli in TFs directed by males (two-way repeated measures ANOVA $F_{5,180}$=123.90, P<0.0001) (Fig. 1b); the population of origin had not significant effects on overall TFs elicited by males ($F_{1,36}$=0.31, P=0.58), but the interaction between scent stimuli and the population was significant ($F_{5,180}$=2.27, P=0.049). However, only for hexadecanol, we observed significant differences between populations; males from Fuenfría showed a significantly higher number of TFs in response to this compound than males from Golondrina (Tukey's test: P<0.0001).

Males from Fuenfría made a significantly higher number of TFs in response to cholesterol and tetracosanol than to hexadecanol, docosanol and octadecanol (Tukey's test: P<0.03 for all). There were no significant differences between cholesterol and tetracosanol (P=0.99), nor between hexadecanol and docosanol (P=0.95), nor between docosanol and octadecanol (P=0.67). Finally, males from Fuenfría elicited significantly higher TF rates in response to hexadecanol than to octadecanol (P=0.02).

For males from Golondrina, there were similar results. Individuals elicited significantly more TFs in response to cholesterol and tetracosanol than to hexadecanol, docosanol and octadecanol (P<0.03 for all); only the TFs number in response to tetracosanol and hexadecanol were similar (P=0.11). There were no significant differences between cholesterol and tetracosanol (P=0.99), nor between hexdecanol and docosanol (P=0.31), nor between hexadecanol and octadecanol

(*P*=0.99), but there were significant differences in the responses to docosanol and octadecanol (*P*=0.048).

DISCUSSION

This study first showed that *P. hispanica* lizards can discriminate between a control (i.e., DCM) and some chemical compounds tested (cholesterol and several alcohols) dissolved in this control solution. Further, we knew that cholesterol elicited higher chemosensory responses by these lizards (Martín and López 2006c). So, the comparison of the responses to the several alcohols tested and cholesterol could inform on the relative importance of the observed responses. We found that lizards had similar high responses to some alcohols (hexadecanol and tetracosanol) than to cholesterol. These results strongly suggest that *P. hispanica* lizards could detect and identify alcohols present in femoral secretions of males in the same way as they can discriminate steroids. Males and females differed in their responses to alcohols, which may suggest that the same chemicals in femoral secretions may convey different messages or be differentially important for males and females, as it has been similarly shown in other lacertid lizards (Martin and Lopez 2008).

We observed the highest number of tongue flicks (TF) in response to tetracosanol or 'lignoceric alcohol'. This compound is a fatty alcohol with a hydrocarbon chain of 24 carbons. This chemical is also present in very large amounts in secretions of other lacertid lizard *Acanthodactylus erythrurus* (Lopez and Martin 2005b). The high chemosensory responses of lizards to this alcohol might be related to its metabolic functions. Some studies suggest that fatty alcohols may decrease cholesterol levels in preventing low-density lipoprotein oxidation (Shimura et al. 1987; Fraga et al. 1997). Thus, higher levels of tetracosanol in secretions might be an indication of health or condition. But, at this moment there is no knowledge about the role and effects of this compound in reptiles, nor on its potential role as a chemical signal, and further experiments are needed.

Also, lizards showed relatively high chemosensory responses to hexadecanol, but it seems that it was no clearly discriminated from the similar octadecanol and docosanol. The small chemical differences between these alcohols (i.e., hydrocarbon chains with 16, 18 or 20 carbons respectively) might explain that lizards did not discriminate them, or alternatively that differences in the responses are too small as to yield significant results in our experiment.

Perhaps, not just one alcohol could have a signaling function, but the mix of several alcohols and the different relative proportions of each one could be the true signal (see Johnston 2001). Hexadecanol, but also octadecanol, could have a function signaling male dominance, as it occurs in the lizard *L. m. monticola* (Martín et al. 2007). Hexadecanol elicits male aggressive behavior in this lizard. The immune response of male *L m. monticola* was correlated with the abundances of hexadecanol (and octadecanol) in the femoral secretions, suggesting that both a male's dominance status and the quality of chemical signals are linked with the quality of the immune system (Martin et al. 2007).

Interestingly, only for hexadecanol we found a population difference in chemosensory responses. Male *P. hispanica* from Fuenfría showed responses to hexadecanol higher than those of males from Golondrina. These two populations have distinct characteristics at several levels such as morphology and chemical signals (Martin and Lopez 2006a). However, lizards from these populations have similar proportions of hexadecanol in femoral secretions (a relative proportion of 0.03%, Martin and Lopez 2006a). In spite of this similarity, the relative importance of specific chemicals in communication might differ between populations. Fuenfría individuals could have evolved distinct responses or obtain different information from this compound than Golondrina lizards. Here the interpopulational variations in the responses to hexadecanol could be one more cue indicating that these populations are different.

In summary, *P. hispanica* lizards show differential chemosensory responses and can discriminate several alcohols found in femoral secretions of males. The presence of some of these alcohols in secretions might have relation with health or social status of individuals. Alcohols could signal this information alone or be associated with other chemical compounds in femoral secretions, such as steroids, that also seem to have a signaling function (López and Martín 2005a). Thus, alcohols presented in males' secretions may provide more opportunities to signal a male quality via different circumstances, but also may reinforce the reliability of the signal when several types of chemicals may be perceived simultaneously. Finally, these alcohols could also have a role in interpopulational chemosensory recognition. However, more experiments are clearly needed to know the potential signaling role of alcohols in femoral secretions of lizards.

Acknowledgements

We thank "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an "El Ventorrillo" CSIC grant to MG. The experiments enforced all the present Spanish laws and were performed under license from the Environmental Organisms of Madrid Community where they were carried out.

References

Alberts, A.C. 1993. Chemical and behaviour studies of femoral gland secretions in iguanid lizard. Brain Behavior Evolution **41**: 255–260.

Barbosa, D., Font, E., Desfilis, E., and Carretero, M.A. 2006. Chemically mediated species recognition in closely related Podarcis wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Carazo, P., Font, E., and Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Animal Behaviour **74**: 895–902.

Carazo, P., Font, E., and Desfilis, E. 2008. Beyond 'nasty neighbours' and 'dear enemies'? Individual recognition by scent marks in a lizard (*Podarcis hispanica*). Animal Behaviour **76**: 1953–1963.

Cooper, W.E. 1994. Chemical discrimination by tongue-flicking in lizards: A review with hypotheses on its origin and its ecological and phylogenetic relationships. J. Chem. Ecol. **20**: 439–487.

Cooper, W.E., and Burghardt, G.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J. Chem. Ecol. **16**: 45–65.

Cooper, W.E., and Pérez-Mellado, V. 2001. Chemosensory responses to sugar and fat by the omnivorous lizard *Gallotia caesaris* with behavioral evidence suggesting a role for gustation. Physiology and Behavior **73**: 509–516.

Cooper, W.E., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Cooper, W. E., Perez-Mellado, V., and Vitt, L. J. 2002a. Responses to major categories of food chemicals by the lizard *Podarcis lilfordi*. Journal of Chemical Ecology **28**: 709–720.

Cooper, W. E., Perez-Mellado, V., and Vitt, L. J. 2002b. Lingual and biting responses to selected lipids by the lizard *Podarcis lilfordi*. Physiology and Behavior **75**: 237–241.

Fraga, V., Menendez, R., Amor, A.M., Gonzalez, R.M., Jimenez S., and Mas R. 1997. Effect of policosanol on in vivo and in vitro rat liver microsomal lipid peroxidation. Arch. Med. Res. **28**: 355–360.

García-Paris, M., Martín, C., and Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain-Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phylog. Evol. **23**: 75–81.

Johnston, R.E. 2001. Neural mechanisms of communication: from pheromones to mosaic signals. In: Chemical Signals in Vertebrates, Vol. 9. Ed. by A. Marchlewska-Koj, J. J. Lepri & D. Muller-Schwarze. Plenum, N.Y.

LeMaster, M.P., and Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red-sided garter snakes, *Thamnophis sirtalis parietalis*. J. Chem. Ecol. **29**: 1027–1043.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–465.

Lopez, P., and Martín, J. 2005a. Chemical compounds from femoral gland secretions of male Iberian rock lizards, *Lacerta monticola cyreni*. Z. Naturforsch. C **60**: 632–636.

Lopez, P., and Martín, J. 2005b. Age related differences in lipophilic compounds found in femoral gland secretions of male spiny-footed lizards, *Acanthodactylus erythrurus*. Z. Naturforsch. C **60**: 915–920.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

López, P., Gabirot, M., and Martín, J. 2009. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturwissenschaften **96**: 65–69.

Martín, J., and López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B **267**: 1265–1269.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., and López, P. 2006c. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herpetol. **60**: 556–561.

Martín, J., and López, P. 2006c. 2006d. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Functional Ecology **20**: 1087–1096.

Martín, J., and López, P. 2006c. 2008. Intersexual differences in chemosensory responses to selected lipids reveal different messages conveyed by femoral secretions of male Iberian rock lizards. Amphibia-Reptilia **29**: 572–578.

Martín, J., and López, P. 2006c. In press. Pheromones and reproduction in Reptiles. *In* D. O. Norris and K. H. Lopez (eds.). Hormones and Reproduction in Vertebrates. Reptiles. Academic Press, San Diego, California.

Martín, J., Moreira, P. L., and López, P. 2007. Status-signalling chemical badges in male Iberian rock lizards. Functional Ecology **21**: 568–576.

Mason, R.T. 1992. Reptilian pheromones. In: Gans C, Crews D, eds. Biology of the Reptilia. Vol. 18. Chicago: University of Chicago Press, pp. 114–228.

Mellado, J., and Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, Act. Verteb. **8**: 299–300.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B, Suppl. **270**: S254–S256.

Pinho, C., Harris, D. J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biological Journal of the Linnean Society **91**: 121–133.

Shimura, S., Hasegawa, T., Saburo, T., and Suzuki, T. 1987. Studies on the effect of octacosanol on motor endurance in mice. Nutr. Rep. Int. **36**:10–29.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Smadja, C., and Butlin, R.K. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heridity **102**: 77–97.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. New York, Freeman.

Symond, M.R.E., and Elgar, M.A. 2009. The evolution of pheromone diversity. Trends Ecol. Evol. **23**: 220–228.

CAPÍTULO 6

La activación del sistema inmune afecta a los ornamentos sexuales químicos de los machos de la lagartija Ibérica

Numerosos animales utilizan las señales químicas en la selección sexual, pero no está claro cómo estas señales sexuales han podido evolucionar para informar honestamente sobre la condición de un macho. Es posible que exista un compromiso entre mantener el sistema inmune y la elaboración de los ornamentos sexuales. Hemos estimulado el sistema inmune de lagartijas Ibéricas *Podarcis hispanica* con un antígeno bacteriano (LPS), sin efectos patógenos, para explorar como la activación del sistema inmune afectaría a las señales químicas de los machos. La activación inmune resultó en una disminución de la proporción de uno de los compuestos principales de las secreciones femorales de los machos (colesta-5,7-dien-3-ol = pro-vitamina D_3), un compuesto que se sabe es seleccionado en el olor de los machos por las hembras, y cuya forma activa (vitamina D_3) tiene una multitud de efectos importantes sobre las funciones del sistema inmune. Este resultado sugiere la existencia de un compromiso potencial entre la regulación fisiológica del sistema inmune y la asignación de nutrientes esenciales (vitaminas) para elaborar los ornamentos sexuales químicos de los machos de lagartija.

Immune activation affects chemical sexual ornaments of male Iberian wall lizards

Pilar López, Marianne Gabirot, José Martín

Abstract Many animals use chemical signals in sexual selection, but is not clear how these sexual traits might have evolved to signal honestly male condition. It is possible that there is a trade-off between maintaining the immune system and the elaboration of ornaments. We experimentally challenged the immune system of male Iberian wall lizards, *Podarcis hispanica*, with a bacterial antigen (LPS), without pathogenic effects, to explore whether the immune activation affected chemical ornaments. Immune activation resulted in decreased proportions of a major chemical in femoral secretions (cholesta-5,7-dien-3-ol = provitamin D_3) known to be selected in scent of males by females, and which active form (vitamin D) has a variety of important effects on immune system function. This result suggests the existence of potential a trade-off between physiological regulation of the immune system and the allocation of essential nutrients (vitamins) to sexual chemical ornaments in male lizards.

Résumé De nombreux animaux utilisent des signaux chimiques lors de la sélection sexuelle, mais l'évolution de ces traits en signaux honnêtes de la condition des mâles n'est pas encore claire. Il est possible qu'il existe un compromis entre le maintien du système immunitaire et l'élaboration des ornementations. Nous avons mis à l'épreuve le système immunitaire des lézards *Podarcis hispanica* grâce à l'antigène bactérien (LPS), sans effet pathogène, pour explorer comment l'activation du système immunitaire peut affecter les signaux chimiques des mâles. Lors de cette activation, les proportions d'un des majeurs composés des secrétions chimiques des mâles (cholesta-5,7-dien-3-ol = provitamine D_3), connu comme indice pour les femelles pour choisir leur partenaire sexuel et dont la forme active (vitamine D) a de nombreux effets importants sur le système immunitaire. Ce résultat suggère l'existence d'un réel compromis entre la régulation physiologique du système immunitaire et l'attribution des nutriments essentiels (vitamines) aux ornements mâles utilisés lors de la sélection sexuelle.

Naturwissenschaften 2009, **96**(1):65-69.

INTRODUCTION

Many animals use chemical signals in mate choice (Johnasson and Jones 2007) and some studies have suggested that only healthier males may afford to produce attractive 'chemical ornaments' (Penn and Potts 1998; Rantala et al. 2002, 2003; Zala et al. 2004; López and Martín 2005), but is not clear how these chemical signals have evolved to be used as sexual signals. A requisite for sexual signals to be evolutionarily stable and useful for sexual selection is that signals are costly to the signaler, and that the cost is correlated with the signaler's quality (Zahavi 1975; Pomiankowski 1988; Grafen 1990). One of the possible costs is the energetic expenditure of producing and maintaining components of the immune system, which may have a major effect on condition, thus creating a trade-off because resources are used up by both the condition dependent sexual advertisement and the immune system (Wedekind and Folstad 1994; Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). Therefore, only individuals in good condition could mount a strong immune defense and produce an extravagant sexual ornament (Westneat and Birkhead 1998).

Chemical signals play a prominent role in social relationships of many lizards (Mason 1992). Chemical secretions from the femoral glands of males may reliably convey information about a male's quality or genetic compatibility, which females may use to choose prospective mates (Olsson et al. 2003; Martín and López 2006a,b). The Iberian wall lizard, *Podarcis hispanica*, is a small (50-70 mm adult snout-to-vent length) diurnal lacertid lizard, common in rocky habitats of the Iberian Peninsula. Females prefer scent marks of males with high proportions of cholesta-5,7-dien-3-ol in their femoral secretions, which may signal a better cell-mediated immune response (López and Martín 2005). Also, increased testosterone levels decrease proportions of cholesta-5,7-dien-3-ol in secretions of males (Martín et al. 2007).

We explored here whether costs of immunity affected the expression of chemical ornaments (i.e., chemicals in femoral secretions) of male lizards, *P. hispanica*, which would allow honest signaling of male condition. Previous observational studies have found relationships between health state and elaboration of sexual signals in this and other related lizards (e.g., López and Martín 2005; Martín et al. 2008), but simple correlations may confound costs of immunity *per se* because of pathogen effects on host health. However, to look for immunity costs, we can expose individuals to an antigen that is identified by the immune system as a pathogen and that elicits an immune response without being pathogenic (e.g., Bonneaud et al. 2003; Staszewski and Boulinier 2004; Uller et al. 2006). Thus, we experimentally challenged the immune system of male lizards *P. hispanica* with a bacterial antigen (LPS), without pathogenic effects, and examined whether their chemical signals were affected by the activation of the immune system. We predicted that if these chemical sexual traits honestly reflected current health, and if a trade-off between immune function and elaboration of chemical ornaments existed, activation of the immune system should reduce the “intensity” of chemical displays.

MATERIAL AND METHODS

Study animals

We captured by noosing 20 adult male lizards *P. hispanica* of similar body sizes, at the beginning of April 2006, just before the start of their mating season, near Cercedilla (Madrid province, Spain). Lizards were individually housed at "El Ventorrillo" Field Station, 5 km from the capture site, in outdoor 80 x 50 cm PVC terraria with sand substratum, rocks for cover, and water *ad libitum*. We fed lizards every day with three mealworm larvae and ensured that all lizards ate all larvae to standardize feeding regimes of all individuals. Similar captivity conditions ensured that all lizards had similar body condition and similar access to food, thus ensuring that differences in the quality of the individuals or of their home ranges did not affect or compensate for the effects of the activation of the immune system. All animals were returned with good condition to their capture sites at the end of trials.

Activation of the immune system

To activate the immune systems of male lizards we used lipopolysaccharide (LPS) from the cell wall of the bacteria, *E. coli* (serotype 0111-B4; Sigma-Aldrich). LPS induces an inflammatory response by non-specifically activating B and T lymphocytes, and producing specific anti-LPS antibodies, but has no pathogenic effects (Janeway et al. 2001), although LPS also induces immunopathological effects such as fever (Deen and Hutchison 2001). Ten male lizards (‘LPS-males’) were injected intraperitoneally with LPS (2.5 μg per g of body weight) diluted in 0.05 ml of PBS (phosphate buffered saline). The serotype was similar, and the concentration similar or lower than those previously used to stimulate the immune system in other lizards (Deen and Hutchison 2001; Uller et al. 2006). Ten control males (‘C-males’) were injected with the same volume (0.05 ml) of PBS alone. Lizards were injected twice: in April, ten days after capture, and in May, three weeks after the first injection. Lizards were assigned at random to the treatments, and were handled “blind” by the same investigator using the same protocol for injections. All lizards were kept under similar captivity conditions.

Chemical signals

We examined whether the experimental immune challenge affected the proportion of cholesterol and cholesta-5,7-dien-3-ol in femoral secretions of males because previous experiments suggested that these steroids may be important as secondary sexual signals (López and Martín 2005). We extracted samples of femoral secretion immediately after capture, and again one month after males were first injected with LPS or PBS alone. Samples were analyzed by gas chromatography–mass spectrometry (GC-MS) (Finnigan-ThermoQuest Trace 2000) equipped with a Supelco, Equity-5 column (for details of chemical analyses see Martín and López 2006c). We identified chemicals by comparison of mass spectra in the NIST/EPA/NIH 1998 library, and later confirmed them with authentic standards (from Sigma-Aldrich Chemical Co.).

We calculated the percent of the total ion current (TIC) to determine the relative amount of each component. These measurements were carried out blind. We transformed areas of cholesterol and cholesta-5,7-dien-3-ol before and after the experimental treatment following Aitchison’s (1986) formula, and used them as dependent variables in two-way repeated measures ANOVAs (Sokal and Rohlf 1995) testing the effects of temporal changes after the experimental manipulation (within factor; ‘time’), treatment group (between factor, C-males *vs.* LPS-males) and the interaction between time and treatment. This interaction effect tested for actual changes due to the treatment after considering natural variation between individuals, and temporal changes within individuals due, for example, to captivity conditions or natural seasonal changes. Pairwise comparisons used Tukey’s honestly significant difference (HSD) tests (Sokal and Rohlf 1995).

RESULTS

There were not significant differences in overall levels of cholesta-5,7-dien-3-ol in femoral gland secretions between treatments (repeated measures two-way ANOVA: treatment effect, $F_{1,18}$=0.16, P=0.70), but there were significant differences between the beginning and the end of the experiment (time effect, $F_{1,18}$=19.60, P=0.0003), and the interaction was significant ($F_{1,18}$=4.99, P=0.038). Thus, at the beginning of the experiment there were not significant differences between C- and LPS-males in proportions of cholesta-5,7-dien-3-ol (Tukey's test: P=0.50), but, after the immune activation, LPS-males decreased the proportion of cholesta-5,7-dien-3-ol (P=0.001), whereas C-males maintained it (P=0.43) and, at the end of the experiment, C-males had significantly higher proportions of cholesta-5,7-dien-3-ol in their femoral secretions than LPS-males (P=0.03) (Fig. 1a).

Overall levels of cholesterol in femoral secretions did not significantly differ between treatments (repeated measures two-way ANOVA: $F_{1,18}$=0.30, P=0.59), but were significantly higher at the end of the experiment ($F_{1,18}$=17.60, P=0.0005), and the interaction was not significant ($F_{1,18}$=0.04, P=0.84). Thus, both groups of males increased proportions of cholesterol during the experiment, but there were not differences between treatments at the beginning or at the end of the experiment (Fig. 1b).

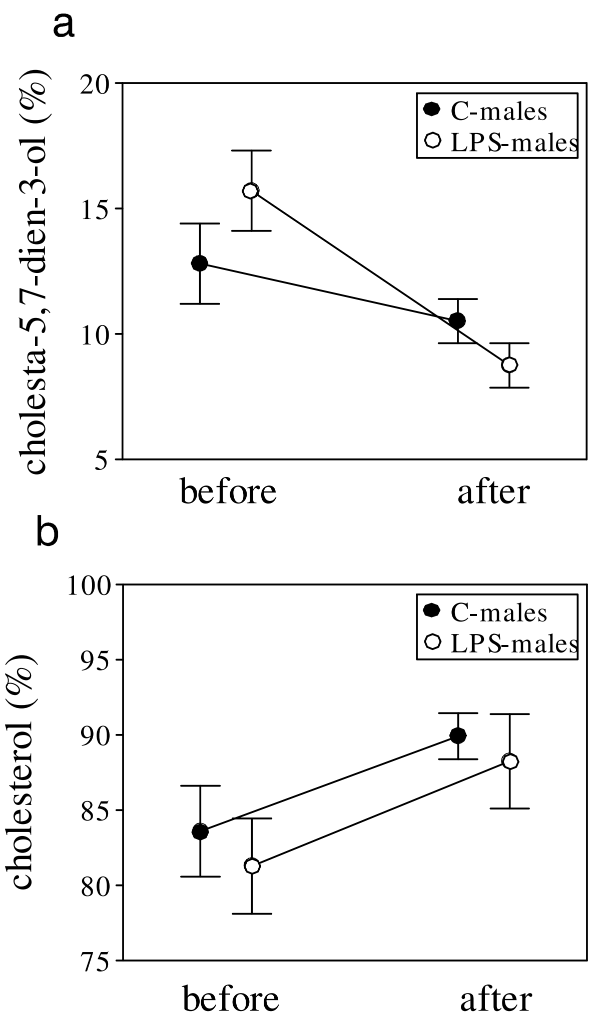


Fig. 1: Mean (± SE) relative proportions of **a** cholesta-5,7-dien-3-ol and **b** cholesterol in femoral gland secretions of male lizards *P. hispanica*, before and one month after the injection of control PBS (C-males) or LPS (LPS-males).

DISCUSSION

Our experiment showed that activation of the immune system resulted in decreased proportions of cholesta-5,7-dien-3-ol in femoral secretions of male lizards *P. hispanica*. This suggests that there is a potential trade off between the immune system and the elaboration of chemical sexual signals. Males that increased the costs of the immune response might not be able to allocate enough cholesta-5,7-

dien-3-ol to femoral secretions without detrimental effects for metabolic functions. This result may explain that proportions of this steroid in secretions are related to health state in this and other lacertid lizards (López and Martín 2005; Martín and López 2006a). Also, the immunosuppressive effects of supplemented testosterone can decrease proportions of cholesta-5,7-dien-3-ol in secretions of this lizard (Martín et al. 2007). Because female *P. hispanica* detect changes in concentration of this lipid (Martín and López 2006d) and prefer scent marks of males with higher proportions of it (López and Martín 2005), it is likely that proportions of cholesta-5,7-dien-3-ol may reliably signal health of a male to females. In contrast, proportions of cholesterol were not affected by the immune challenge, which may suggest that this major steroid constitutes an unreactive apolar matrix that aids in the delivery of other truly semiochemicals.

Cholesta-5,7-dien-3-ol is a precursor for vitamin D_3 and, in humans and other mammals, there is considerable scientific evidence that the active form of vitamin D is a potent immune system modulator with a variety of effects on immune system function that may enhance innate immunity and inhibit the development of autoimmunity (Griffin et al. 2003; Hayes et al. 2003). Most cells of the immune system, including T cells and antigen-presenting cells, such as dendritic cells and macrophages have vitamin D receptors (Lin and White 2004). Recent evidence demonstrates that vitamin D_3 promotes creation of antimicrobial peptides within keratinocytes, which enable these cells to recognize and respond to microbes and to protect wounds against infection (Schauber et al. 2007). Given that other better known physiological aspects of reptiles are similar to those of mammals (Janeway et al. 2001; Hill et al. 2008), similar functions of vitamin D are very likely to occur in lizards too. Therefore, activation of the immune system might require lizards to allocate larger amounts of systemic cholesta-5,7-dien-3-ol (provitamin D) to the immune function. Consequently, less cholesta-5,7-dien-3-ol would be available to be allocated to femoral secretions.

Our results with chemical signals are similar to those relative to carotenoid-base visual signals in birds and fish, where there is a well documented trade-off between elaboration of visual traits and immunity (Blount et al. 2003; Faivre et al. 2003; Grether et al. 2004; McGraw and Ardia 2004). This is explained because carotenoids are important in the up-regulation of various aspects of the immune functioning and cellular protection against oxidation by free radicals (e.g., Chew 1993; von Schantz et al. 1999; McGraw 2005). These data together with our results suggest a 'scenario' for the evolution of chemical signals as honest signals of male quality that is extremely similar to visual signals based on carotenoids or other pigments with immunological and antioxidant functions. Therefore, our study supports that the trade-off between physiological functions, such as regulation of the immune system, and the allocation of essential nutrients to sexual ornaments (e.g., carotenoids in visual signals and vitamins in chemical signals) may be the common basis of honest sexual displays in many animals, and that only the sensory mechanism used to detect these "essential chemicals" would actually differentiate visual from chemical signals.

Acknowledgements We thank three anonymous reviewers for helpful comments and "El Ventorrillo" MNCN Field Station for use of their facilities. Financial support was provided by the projects MEC-CGL2005-00391/BOS and MCI-CGL2008-02119/BOS, and by an "El Ventorrillo" CSIC grant to MG. The experiments enforced all Spanish laws and were performed under license from the Environmental Organisms of Madrid Community.

References

Aitchison, J. 1986. The statistical analysis of compositional data. Chapman and Hall, London.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R., and Surai, P.F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science **300**: 125–127.

Bonneaud, C., Mazuc, J., González, G., Haussy, C., Faivre, B., and Sorci, G. 2003. Assessing the cost of mounting an immune response. Am. Nat. **161**: 367–379.

Chew, B. P. 1993. Role of carotenoids in the immune response. J. Dairy Sci. **76**: 2804–2811.

Deen, C.M., and Hutchison, V.H. 2001. Effects of lipopolysaccharide and acclimation temperature on induced behavioral fever in juvenile *Iguana iguana*. J. Therm. Biol. **26**: 55–63.

Faivre, B., Grégoire, A., Préault, M., Cézilly, F., and G. Sorci. 2003. Immune activation rapidly mirrored in a carotenoid-based secondary sexual trait. Science **300**: 103.

Grafen, A. 1990. Biological signals as handicaps. J. Theor. Biol. **144**: 517–546.

Grether, G.F., Kasahara, S., Kolluru, G.R., and Cooper, E.L. 2004. Sex-specific effects of carotenoid intake on the immunological response to allografts in guppies (*Poecilia reticulata*). Proc. R. Soc. Lond. B **271**: 45–49.

Griffin, MD, Xing, N, and Kumar, R. 2003. Vitamin D and its analogs as regulators of immune activation and antigen presentation. Ann. Rev. Nutrition **23**: 117–145.

Hayes, C.E., Nashold, F.E., Spach, K.M., and Pedersen, L.B. 2003. The immunological functions of the vitamin D endocrine system. Cell. Mol. Biol. **49**: 277–300.

Hill, R.W., Wyse, G.A., and Anderson, M. 2008. Animal Physiology, 2nd edn. Sinauer Associates, Sunderland, MA.

Janeway, C.A., Travers, P., Walport, M., and Shlomchik, M. 2001. Immunobiology. The Immune System in Health and Disease, 5th edn. Garland Publishing, New York.

Johansson, B.G., and Jones, T.M. 2007. The role of chemical communication in mate choice. Biol. Rev. **82**: 265–289.

Lin, R., and White, J.H. 2004. The pleiotropic actions of vitamin D. Bioessays **26**: 21–28.

Lochmiller, R.L., and Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos **88**: 87–98.

López, P., and Martín, J. 2005. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P., and Martín, J. 2006a. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

López, P., and Martín, J. 2006b. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc. R. Soc. Lond. B **273**: 2619–2624.

Martín, J., Amo, L., and López, P. 2008. Parasites and health affect multiple sexual signals in male common wall lizards, *Podarcis muralis*. Naturwiss. **95**: 293–300.

Martín, J., and López, P. 2006c. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006d. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herp. **60**: 556–561.

Martín, J., López, P., Gabirot, M., and Pilz, K.M. 2007. Effects of testosterone supplementation on chemical signals of male Iberian wall lizards: consequences for female mate choice. Behav. Ecol. Sociobiol. **61**: 1275–1285.

Mason, R.T. 1992. Reptilian pheromones In: Gans C, Crews D (eds) Biology of the Reptilia, vol 18. University of Chicago Press, Chicago, pp 114–228.

McGraw, K.J. 2005. The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? Anim. Behav. **69**: 757–764.

McGraw, K.J., and Ardia, D.R. 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am. Nat. **162**: 704–712.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B (Suppl) **270**: 254–256.

Penn, D., and Potts, W.K. 1998. Chemical signals and parasite mediated sexual selection. Trends Ecol. Evol. **13**: 391–396.

Pomiankowski, A.N. 1988. The evolution of female mate preferences for male genetic quality. Oxford Surv. Evol. Biol. **5**: 136–184.

Rantala, M.J., Jokinen, I., Kortet, R., Vainikka, A., and Suhonen, J. 2002. Do pheromones reveal male immunocompetence? Proc. R. Soc. Lond. B **269**: 1681–1685.

Rantala, M.J., Kortet, R., Kotiaho, J.S., Vainikka A., and Suhonen, J. 2003. Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. Funct. Ecol. **17**: 534–540.

Schauber, J., Dorschner, R.A., Coda, A.B., Büchau, A.S., Liu, P.T., Kiken, D., Helfrich, Y.R., Kang, S., Elalieh, H.Z., Steinmeyer, A., Zügel, U., Bikle, D.D., Modlin, R.L., and Gallo, R.L. 2007. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D–dependent mechanism. J. Clinic. Inv. **117**: 803–811.

Sheldon, B.C., and Verhulst, S. 1996. Ecological immunology: costly parasite defence and trade-offs in evolutionary ecology. Trends Ecol. Evol. **11**: 317–321.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. Freeman, New York.

Staszewski, V., and Boulinier, T. 2004. Vaccination: a way to address questions in behavioral and population ecology? Trends Parasitol. **20**: 17–22.

Uller, T., Isaksson, C., and Olsson, M. 2006. Immune challenge reduces reproductive output and growth in a lizard. Funct. Ecol. **20**: 873–879.

von Schantz, T.V., Bensch, S., Grahn, M., Hasselquist, D., and Wittzell, H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc. R. Soc. Lond. B **266**: 1–12.

Wedekind, C., and Folstad, I. 1994. Adaptive or nonadaptive immunosuppression by sex hormones? Am. Nat. **143**: 936–938.

Westneat, D.F., and Birkhead, T.R. 1998. Alternative hypothesis linking the immune system and mate choice for good genes. Proc. R. Soc. Lond. B **265**: 1065–1073.

Zahavi, A. 1975. Mate selection-A selection for a handicap. J. Theor. Biol. **53**: 205–214.

Zala, S.M., Potts, W.K., and Penn, D.J. 2004. Scent-marking displays provide honest signals of health and infection. Behav. Ecol. **15**: 338–344.

CAPÍTULO 7

El reconocimiento químico entre especies podría reducir la frecuencia de hibridación entre las lagartijas nativas y las introducidas

La introducción de especies en las islas debido a la actividad humana puede tener consecuencias muy importantes sobre las poblaciones endémicas insulares. La hibridación entre *Podarcis atrata* (Boscá 1916), lagartija endémica de las Islas Columbretes (Mediterráneo, España) y la lagartija Ibérica, *Podarcis hispanica* (Steindachner 1870), de la Península Ibérica, podría producirse potencialmente ya que algunos haplotipos continentales han sido detectados ya en las islas, porque estas dos especies son muy cercanas a nivel genético, y debido a la cada vez mayor frecuencia de visitas humanas a las islas. Sin embargo, las decisiones relativas a la reproducción de muchas lagartijas están frecuentemente mediadas por mecanismos de reconocimiento entre especies basados en señales químicas. Bajo esta asunción, incluso si algunas *P. hispanica* continentales fueran introducidas en las islas, el reconocimiento químico entre especies podría hacer que fuera poca frecuente la hibridación con *P. atrata* insulares. Hemos examinado si existe reconocimiento químico interespecífico entre *P. atrata* insulares y *P. hispanica* continentales. Los resultados han mostrado que las lagartijas de ambos sexos respondieron con más intensidad (es decir, tuvieron una tasa mayor de protusiones linguales) al olor de individuos coespecíficos que al de heterospecíficos. El reconocimiento químico de coespecíficos por la lagartija insular *P. atrata* reduciría la posibilidad de hibridación con *P. hispanica* introducidas desde el continente, con lo que se protegerían las características genéticas de la especie insular.

Chemosensory species recognition may reduce the frequency of hybridization between native and introduced lizards

Marianne Gabirot, Aurora M. Castilla, Pilar López and José Martín

Abstract The introduction in islands of alien species mediated by anthropogenic activity can cause catastrophic consequences on small populations of island endemics. Hybridization between the endangered and endemic insular lizard *Podarcis atrata* (Boscá 1916) from the Columbretes islands (Mediterranean, Spain) and the common mainland lizard *Podarcis hispanica* (Steindachner 1870) could potentially occur because mainland haplotypes have already been detected in the island, because of the close genetic relatedness between these two species, and because of the increasing frequency of visitors to these islands. However, reproductive decisions of lizards are often mediated by species recognition mechanisms based on chemical cues. Under that assumption, even if some mainland *P. hispanica* lizards were introduced in the islands, interspecific recognition might make rare an eventual hybridization with insular *P. atrata*. We examined interspecific chemical recognition between the insular *P. atrata* and the mainland *P. hispanica*. Our results showed that lizards of both sexes responded more strongly (i.e., directed a significant higher number of tongue-flicks) to scents of conspecific individuals than to scents of heterospecifics. Chemical recognition of conspecifics by endemic island *P. atrata* lizards may reduce the occurrence of hybridization with introduced mainland *P. hispanica* and protect the insular gene pool.

Résumé L'introduction d'espèces étrangères dans les îles par l'activité humaine aurait des conséquences catastrophiques sur les populations insulaires endémiques. L'hybridation entre *Podarcis atrata* (Boscá 1916), lézard endémique des îles Columbretes (Mediterranean, Spain) et le lézard commun de la péninsule ibérique *Podarcis hispanica* (Steindachner 1870) pourrait potentiellement se produire du fait que (1) certains haplotypes continentaux ont déjà été détectés sur l'île, (2) que ces deux espèces sont relativement proche génétiquement, et (3) que la fréquence des visites humaines a augmenté sur l'île. De plus, les lézards utiliseraient en grande partie les signaux chimiques lors de la reconnaissance entre espèces et/ou lors des décisions de reproduction. A partir de cette hypothèse, même si quelques *P. hispanica* lézards continentaux sont introduits dans les îles, la reconnaissance interspécifique raréfierait la possibilité d'hybridation. Nous avons examiné la reconnaissance chimique interspécifique entre l'insulaire *P. atrata* et le continental *P. hispanica*. Les résultats ont montré que les lézards des deux sexes montrent plus d'intérêt (i.e., un nombre plus grand de tirage de langue) en direction d'odeurs d'individus de sa propre espèce que de l'autre espèce. Cette différence interspécifique au niveau de la reconnaissance chimique contribuerait à la protection du lot génétique insulaire face au risque de l'hybridation avec les congénères communs continentaux.

Canadian Journal of Zoology, 2010, **88** (1): 73-80.

INTRODUCTION

Small isolated islands are ideal scenarios where evolutionary processes may lead to genetic divergence of local populations of animals and plants and the appearance of endemic new species (Thorpe and Malhotra 1996; Coyne and Orr 2004). However, the introduction in islands of alien species mediated by anthropogenic activity can cause catastrophic consequences for the often endangered small populations of endemics (Fritts and Rodda 1998). Island endemics are often closely related to common mainland species that can be easily introduced and hybridize with the island species, thus reversing the island speciation processes (Seehausen et al. 2008). Many endangered species are considered to be seriously threatened by crossbreeding with closely related species as a result of introduction by human mediated activities (Gunnel et al. 2008; Oliveira et al. 2008; reviewed in Rhymer and Simberloff 1996; Allendorf et al. 2001).

The endemic Columbretes wall lizard, *Podarcis atrata* (Boscá 1916), is an endangered lizard from the Columbretes islands (Mediterranean, Spain) (Castilla et al. 1998a,b). This is the European lizard with the smallest (19 Ha) distribution area, and has a small population size (total of ca. 12000 individuals) (Castilla and Bauwens 1991). *P. atrata* is closely related to the mainland Iberian wall lizard, *Podarcis hispanica* (Steindachner 1870) (Pinho et al. 2006, 2007), which is very common and widely distributed in the Iberian Peninsula (Barbadillo et al. 1999).

The Columbretes archipelago is a Natural Park that receives fishermen, visitors and divers from the Spanish mainland and other European countries. In addition, the number of boat arrivals has considerably increased during recent years (Columbretes Natural Park, own data). The light house, the housing facilities for game keepers and researchers, and the paths connecting to the disembarkation sites are all being newly rebuild. These activities bear a high risk of accidental transportation of lizards from the mainland to the islands. In fact, haplotypes from mainland lizards have been already found in these islands (Castilla et al. 1998b). Even if it is unclear whether those haplotypes were retained or due to recent introgression, if they were due to recent introductions from mainland lizards, then, they may represent a threat (e.g., hybridization, competition) and a consequent loss of genetic isolation for insular lizards (Castilla et al. 1998b).

The occurrence of gene flow and hybridization between related species of lizards, and even between clearly distinct species of the Genus *Podarcis* has been observed in the laboratory (e.g., *P. bocagei* x *P. carbonelli*, Galán 2002) and inferred from genetic analyses of field populations (*P. sicula* x *P. tiliguerta*, Capula 2002; *P. hispanica* of various genetically distinct types, Pinho et al. 2007).

Human mediated secondary contact, hybridization and interbreeding between different taxa are challenges of increasing relevancy in conservation biology (Rhymer and Simberloff 1996; Allendorf et al. 2001). Some studies have reported complete replacement of haplotypes in natural populations due to hybridization (Gyllesten and Wilson 1987; Dowling and Hoeh 1991; Bernatched et al. 1995). Hybridization between species commonly results in outbreeding depression (Wang et al. 2007) and is involved in the risk of extinction of many species. For this reason, hybridization is of major concern for the appropriate implementation of species conservation (Sutherland et al. 2006; Gunnel et al. 2008; Oliveira et al. 2008; Vincenzi et al. 2008). Some management actions include the removal of non-native species to eliminate or prevent hybridization (Copp et al. 2008).

Interestingly, however, there are some cases where related species can live together while showing a low percentage of hybrid genotypes, suggesting that there is a mechanism for species recognition that acts as a barrier to hybridization (Shine et al. 2002; Kingston and Gwilliam 2007). Even if individuals of different populations or species share the same habitats, successful reproduction might not always occur between them. For example, two subspecies of the mouse *Peromyscus maniculatus* do not hybridize even though their ranges overlap (Campbell 1995). Therefore, some species might have mechanisms to avoid hybridization, and behavioral studies may contribute to clarify the actual relevance of the conservation problem of hybridization (Caro 1998).

It is well known that sexual selection is an important mechanism that may facilitate reproductive isolation (Boughman 2001; Panhuis et al. 2001). Understanding the pattern of mating and the cues that individuals use to select mates can help o identify the potential for hybridization between species (Grant and Grant 1997). Species recognition may prevent interspecific mating between sympatric related taxa (Cooper and Vitt 1986; Shine et al. 2002). Thus, if reproductive isolation is mediated by species recognition, the risk of interspecific hybridization should be low, even if heterospecifics are common.

In many species of lizards, including the Iberian wall lizard *P. hispanica*, the chemical senses play important roles in intraspecific communication, mate choice and species recognition (Cooper and Vitt 1986; Barbosa et al. 2005, 2006; López and Martín 2005; Martín and López 2006a, b; see review in Mason 1992). Chemosensory recognition is well developed in *P. hispanica,* and chemicals in femoral gland secretions of males vary between populations (Martín and López 2006a,b). Also, sex identification of females is based mainly on chemical cues (Gómez et al. 1993; López and Martín 2001; López et al. 2002), and female pheromones are necessary to elicit male courtship (López and Martín 2001; Cooper and Pérez-Mellado 2002) as it occurs in other lizards (Cooper et al. 1986). Chemical cues of males are also important in male-male interactions (López and Martín 2002; Carazo et al. 2007) and it seems that female mate choice decisions are, at least partially, based on characteristics of chemical signals of males (López and Martín 2005; Martín and López 2006c; Martín et al. 2007).

In this paper, we examined whether interspecific chemical recognition exists between the insular *P. atrata* and the mainland *P. hispanica*, which could prevent mating and hybridization between these two species. We expected that if chemical recognition existed, chemosensory responses to scent of conspecific lizards should be different than those observed to scent of heterospecifics. The existence of interspecific recognition may have important consequences for conservation strategies of the threatened *P. atrata*, because we hypothesized that if *P. atrata* could maintain their reproductive isolation by recognition of conspecifics over heterospecifics, then the threat of hybridization with introduced lizards may be reduced.

MATERIAL AND METHODS

Study area and species

The Columbretes wall lizard *P. atrata*, is a small (mean adult snout-vent length of 50-70 mm) lizard endemic from the Columbretes archipelago (39º55' N, 0º40' E, Mediterranean, Castellón, Spain), that inhabits three small islands characterized by high aridity and vegetation dominated by perennial shrubs, herbs and grasses (Castilla and Bauwens 1991, 2000; Castilla 2000). We collected 15 males and 15 females in the main island Columbrete Grande (13 ha) during the reproductive season in April 2008.

The Iberian wall lizard, *P. hispanica*, is a small lizard (mean adult snout-vent length of 49-61 mm) with a broad distribution in Spain and the south east coast of France (Barbadillo et al. 1999). We collected lizards in three different populations of Central Spain (Madrid province) during the reproductive season in March 2008. In the first area (Fuenfría, 40° 47' N, 4° 03' W) we captured 20 males and 21 females (Ph1), in the second (Golondrina, 40° 44' N, 4° 02'W) 17 males and 34 females (Ph2), and in the third (Aranjuez, 40° 2' N, 3° 37' W) we captured 12 males and 11 females (Ph3). The three zones are characterized by having a large cover of rocks, inside pine or oak forest or Mediterranean shrubland.

We chose these populations for logistical reasons, but also because it is very difficult to predict the mainland origin of the putatively colonizing population. Apart from accidental introductions from coastal zones, we should also consider intentional introductions from very distant areas. A recent example, the lizard *Podarcis pytiusensis* from the Balearic islands (Mediterranean) has been introduced in islands of the Basque Country (Atlantic, North Spain) (Sanz-Azkue et al. 2005). In other studies that examined hybridization in islands due to human activities, recent artificial introductions have been assumed from different localities and not from those closest to the island (Gantenbein and Largiadèr 2002).

We captured lizards with a noose and immediately transported them to the Field Station "El Ventorrillo" (Cercedilla, Madrid). Lizards were kept individually in indoor 60 x 40 cm PVC terraria containing sand substratum and rocks for cover. Terraria with lizards of different populations and sexes were housed separately to avoid visual or chemical contact among them outside experimentation. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) dusted with multivitamin powder for reptiles, and fruit and tomatoes (for *P. atrata* only). Water was provided *ad libitum*. All terraria were heated with 40 W spotlights during 6 h/day, and overhead lighted (36 W full-spectrum daylight tubes) on a 10:14 light/dark cycle.

Lizards were held in captivity at least one week before conducting the tests to allow their acclimation to the laboratory conditions. All lizards were healthy during the trials, they did not show behavioral or physiological changes due to possible stress of experiments, and all maintained or increased their original body mass. All lizards were returned to their exact capture sites at the end of experiments (middle of May 2008).

Scent recognition experiments

Lizards have been shown to react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper and Burghardt 1990). Tongue-flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues and of discrimination of conspecifics and heterospecific lizards (e.g., Cooper and Pérez-Mellado 2002; Barbosa et al. 2006). To test for differential responses to scents we made comparisons of TF rate by lizards (males and females) in response to chemical stimuli arising from cotton applicators impregnated with scent of male and female *P. hispanica* of the three mainland populations, or of island male and female *P. atrata*, or with deionized water (odorless control) (Cooper and Burghardt 1990). Water was used to gauge baseline TF's rates in the experimental situation. We do not use here a pungency control, because in many previous studies, including those with wall lizards (e.g., Cooper and Pérez-Mellado 2002), it has already been well established that these lizards can discriminate scent of conspecifics lizards from biologically irrelevant scents.

We tested lizard scents from the femoral pores of males or from the cloacal area of females because these are the bodily areas most frequently and intensely investigated by tongue-flicking during social encounters (López and Martín 2001, 2002; López et al. 2002). Therefore, after first dipping the cotton tip (1 cm) of a wooden applicator attached to a long stick (50 cm) in deionized water, we rolled the tip over those bodily areas (of one population and sex per applicator). Individuals that donor the scent were the same individuals used as responding individuals in other trials in different days. A new swab from a different donor individual was used in each trial.

Individual *P. atrata* were confronted randomly to scents of males and females of their own species and of three mainland populations of *P. hispanica* (Ph1, Ph2, and Ph3). Thus, we analyzed the responses of island *P. atrata* lizards to a simulated introduced *P. hispanica* lizard from the mainland, in comparison with their responses to conspecifics. Also, we randomly presented to individuals of *P. hispanica* scents from conspecific males and females of their own populations, and scent from male and female *P. atrata*. Thus, we compared the responses of a simulated introduced mainland *P.*

hispanica lizard when finding island *P. atrata* lizards, in comparison with their responses to conspecifics of their own mainland population. We were interested on those social interactions that may lead to reproductive interactions, and possible hybridization. These include male-male recognition, which is needed to prevent intrasexual competition, and male-female recognition, which is required for matings. Female-female recognition is not important in these lizards, because there is not competition between females for accessing to mates. Therefore, we did not consider female-female chemical recognition because it is irrelevant for potential hybridization.

Every lizard was exposed to each stimulus and order of presentation was counterbalanced. One scent presentation was conducted per day for each animal. Trials were conducted during April, which coincided with the mating season of lizards in their original natural populations (Castilla and Bauwens 1991, 2000; P. López and J. Martín unpublished data), and between 1100-1300 h (GMT) when lizards were fully active. Because temperature strongly influences tongue-flick rates of lizards (Van Damme et al. 1990), lizards were maintained at their preferred body temperatures (i.e., *P. hispanica* = 35.5 °C, *P: atrata* = 35.7 °C; see Bauwens et al. 1995) inside each terrarium at least 1 h before and during the experiments. To begin a trial, the experimenter slowly approached the terrarium and slowly moved the cotton swab to a position 1 cm anterior to the lizards' snout. Lizards usually did not flee from the swab, but explored it repeatedly by tongue-flicking or ignored it after the first TFs. The numbers of TFs directed at the swab were recorded for 60 s beginning with the first TF. Only data from individuals that respond to the swab without fleeing were included in the analyses.

Statistical analyses were made separately for responding males and females, and for responses to male and female scent. To examine differences in number of TFs directed at the swab among treatments, we used repeated measures one- or two-way ANOVAs examining the effects of the different scent stimuli presented to the same individual (within factor) and, in the case of *P. hispanica* responses, the population of the responding lizard (between factor; Ph1 vs. Ph2 vs. Ph3). We included the interaction in the models to analyze whether responses to the different scents differed as a function of the population of the responding lizard. Data were log-transformed to ensure normality. Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honestly significant difference (HSD) tests (Sokal and Rohlf 1995). All analyses were performed using STATISTICA (StatSoft, Inc. 2001), data analysis software system, version 6. www.statsoft.com).

RESULTS

We found significant differences in the number of TFs directed by lizards to the different scent stimuli presented (one-way repeated measures ANOVAs, $P<0.0001$ in all cases) (Fig. 1, 2). In all cases, male and female lizards from different populations directed significantly more TFs to scents of other lizards than to water (Tukey's tests, $P=0.00013$ in all cases), indicating detection of lizard scents in all cases.

Responses of island lizards

Insular male *P. atrata* directed significantly more TFs to scent of conspecific males than to scent of males from any of the three mainland *P. hispanica* populations (one-way repeated measures ANOVA, $F_{4,56}=82.25$, $P<0.0001$; Tukey's tests, $P=0.00013$ in all cases). Responses of male *P. atrata* to all the different stimuli from male *P. hispanica* were not significantly different ($P>0.97$ in all cases) (Fig. 1a).

Similar results were obtained when males *P. atrata* were confronted with female *P. hispanica* ($F_{4,56}=72.67$, $P<0.0001$; Tukey´s tests, $P=0.00025$ in all cases). Responses of male *P. atrata* to all the different stimuli from female *P. hispanica* were not significantly different ($P>0.30$ in all cases) (Fig. 1a).

Female *P. atrata* directed significantly more TFs to scent of conspecific males than to scent from male *P. hispanica* from any of the three mainland populations ($F_{4,56}=74.16$, $P<0.0001$; Tukey's tests, $P=0.00013$ in all cases) (Fig. 1b). Responses to all the different stimuli from male *P. hispanica* were not significantly different ($P>0.95$ in all cases).

Responses of mainland lizards

Male *P. hispanica* of the three mainland populations directed significantly fewer TFs to scents of insular male *P. atrata* than to scents of conspecific males from their own population (two-way repeated measures ANOVA, $F_{2,92}=319.02$, $P<0.0001$; Tukey's tests, $P<0.001$ in all cases) (Fig. 2a). The population of origin of responding male *P. hispanica* affected significantly to their responses to conspecific males from their own population ($F_{2,46}$=10.87, P=0.00014). This was explained because males from Fuenfría directed more TFs than males from Aranjuez to scents of males from their own populations (Tukey's tests, Ph1 vs. Ph2, P=0.21; Ph1 vs. Ph3, P=.0.92; Ph2 vs. Ph3, P=0.02), but male *P. hispanica* from all populations directed a similar lower number of TFs to male *P. atrata* (P>0.97 in all comparisons between populations) than to all conspecific males.

Mainland male *P. hispanica* directed significantly fewer TFs to scents of insular female *P. atrata* than to scents of conspecific females from their own population ($F_{2,84}$=198.28, P<0.0001; Tukey's tests, P=0.0001 in all cases) (Fig. 2a). The population of origin of responding male *P. hispanica* affected significantly to their responses to conspecific females from their own population ($F_{2,42}$=3.48, P=0.04). This was explained because males from Aranjuez directed more TFs than males from Fuenfría to scents of females from their own populations (Tukey's tests, Ph1 vs. Ph2, P=0.68; Ph2 vs. Ph3, P=0.035; Ph1 vs. Ph3, P=0.20), but male *P. hispanica* from all populations directed a similar lower number of TFs to female *P. atrata* (P>0.95 in all comparisons between populations) than to conspecific females.

Mainland female *P. hispanica* directed significantly fewer TFs to scents of insular male *P. atrata* than to scents of conspecific males from their own population ($F_{2,122}$=405.99, P<0.0001; Tukey's tests, P<0.0001 in all cases) (Fig. 2b), independently of the population of origin ($F_{2,61}$=0.68, P=0.51).

DISCUSSION

We initiated this study with the supposition that hybridization between the endangered island *P. atrata* and the common mainland *P. hispanica* could occur because 1) mainland *P. hispanica* haplotypes have been already detected in the Columbretes island (Castilla et al. 1998b), 2) there are close genetic relatedness between both species (Pinho et al. 2006, 2007), 3) gene flow and hybridization occur between *Podarcis* species (Galán 2002; Capula 2002; Pinho et al. 2007), and 4) there is a strong risk of *P. hispanica* incursions from mainland sites during frequent boat contact (Columbretes Natural Park, own data). However there is no evidence that actual hybridization between the two lizard species currently occurs.

The results of our study suggest that a breakdown in mechanisms allowing species discrimination via chemical cues would be necessary to allow reproductive interactions between *P. hispanica* and *P. atrata*. Interspecific reproductive interactions might occasionally occur between other wall lizard species as a consequence of weak species recognition mechanisms (Cooper and Pérez-Mellado 2002; Barbosa et al. 2005, 2006; Martín and López 2006a,b).

In our experiment, the different tongue-flick rates of lizards in response to different scents indicated that *P. hispanica* and *P. atrata* detected and discriminated between scents based on chemical cues alone. In all cases the rate of TF directed to scent of lizards was higher than to the control baseline odor

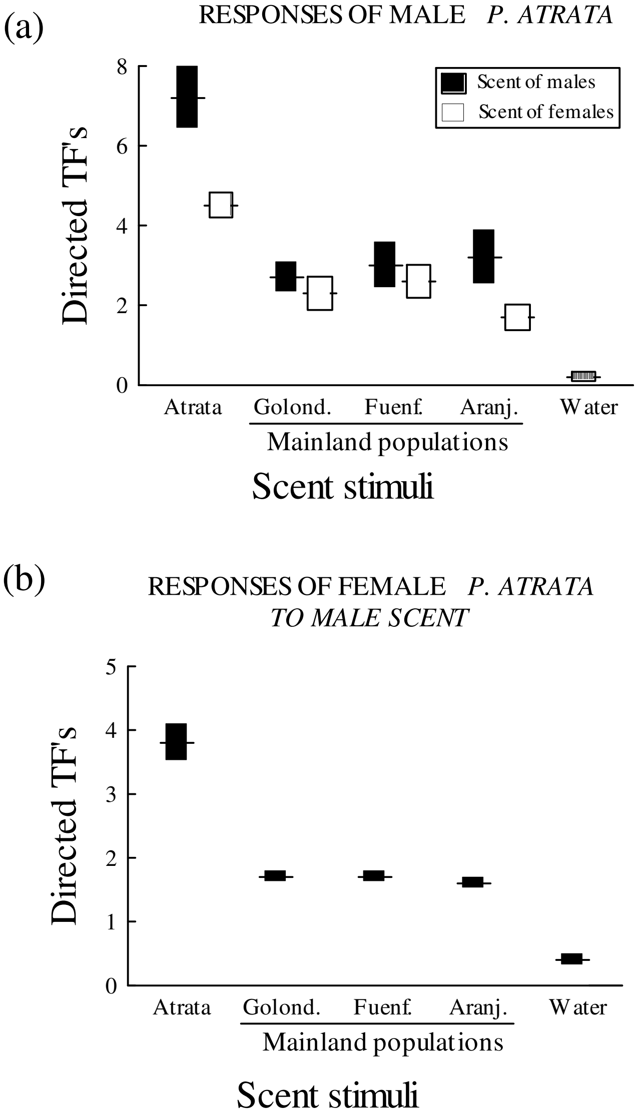


Fig. 1: Mean (± 1 SE) number of tongue-flicks directed to the swab by island (a) male or (b) female *Podarcis atrata* in response to control deionized water, or scents from males (black boxes) and females (open boxes) from three mainland populations of *Podarcis hispanica* (Golondrina, Fuenfría, Aranjuez)

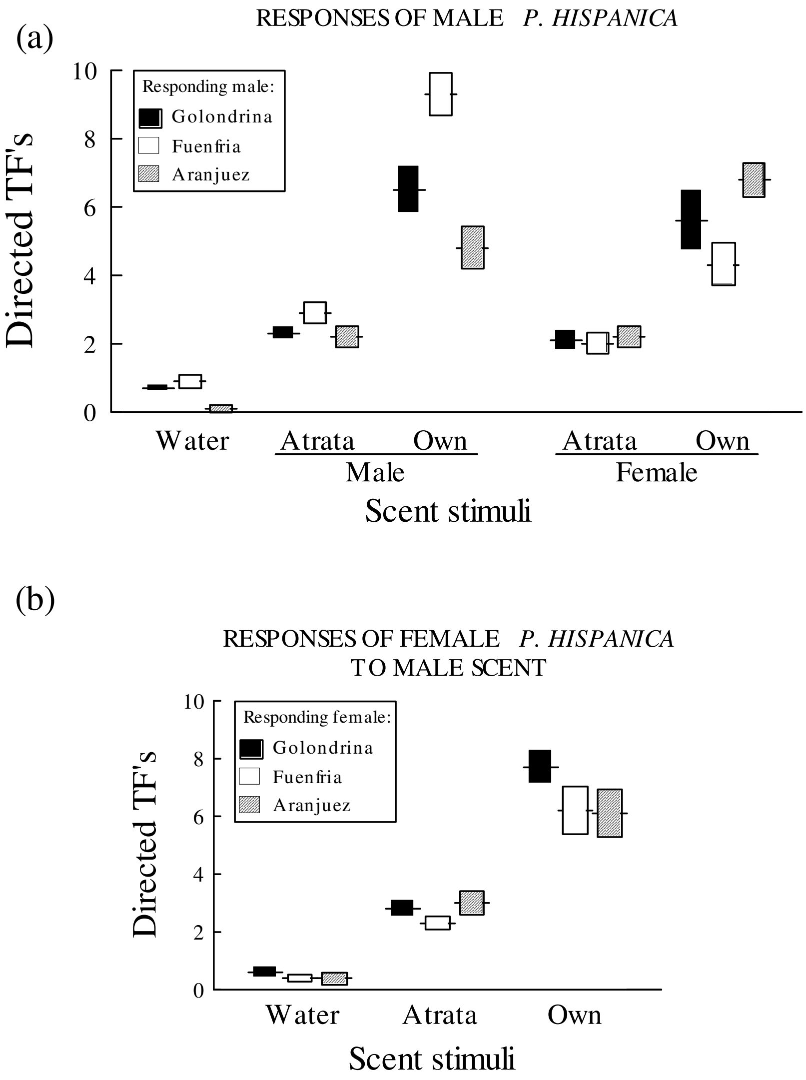


Fig. 2: Mean (± 1 SE) number of tongue-flicks directed to the swab by (a) male or (b) female *Podarcis hispanica* from three mainland populations (Golondrina: black boxes, Fuenfría: open boxes, Aranjuez: striped boxes), in response to control deionized water, or scents from island male or female *Podarcis atrata* and from male or female *Podarcis hispanica* from their respective own mainland populations

(water), indicating that detection of the lizards scent occurred in all cases.

Our results clearly demonstrated that lizards of both species and sexes responded more strongly to scents of individuals of their own species than to scents of the other species. This suggests that lizards of both sexes and species have more "interest" for exploring the scent of conspecific lizards than the scent from the other species. This indicates the occurrence of a clear interspecific chemosensory discrimination between these two species.

These results suggest that chemicals of *P. hispanica* and *P. atrata* used in intraspecific communication might be different in composition and/or proportions, which might be related to genetic differences between the two species. Preliminary chemical analyses appear to support such prediction (Gabirot et al. unpubl, data; see Chapter 8). For example, cholesta-5,7-dien-3-ol is a major steroid found in femoral secretions of mainland male *P. hispanica* (López and Martín 2005; Martín and López 2006a) but it is very scarce in secretions of male *P. atrata* (Gabirot et al. unpubl, data; see Chapter 8). That compound is apparently selected by female *P. hispanica* when selecting scent-marks of conspecific males (López and Martín 2005; Martín and López 2006c). Similarly, in a species complex of rodents, it has been shown that a greater genetic relatedness between two individuals is associated with more similarity in the qualities of their individual scents (Heth et al. 2001).

Our results on chemosensory abilities of *P. atrata* suggest a similar role of chemical cues in social behavior and reproduction in this species too. Therefore, reproductive decisions may be linked to a previous chemosensory recognition of chemicals that identify each species. Therefore, our results could be a preliminary cue of the high improbability of reproduction between *P. hispanica* and *P. atrata*. If individuals do not recognize or discriminate between species, interspecific matings could occur. However, further experiments of mate choice and staged encounters should be done to ensure that premating reproductive isolation may actually prevent hybridization between island and mainland lizards.

Implications for conservation

Considering the results of our study, the risk of hybridization between both lizard species may not be as high as expected (due to the high degree of connectivity with the mainland) because of the interspecific differences in chemical recognition. Therefore, the maintenance of the insular genetic pool appears to be protected. However, even if we suggest that introduced lizard may not hybridize with endemic island lizards based on chemical discrimination, we recommend governmental managers to interpret our results with caution, since we have only examined one of the potential effects of species introductions. We should be aware that many other dangers are associated to species introductions, such as competition for food, habitat and nesting sites, or transmission of parasites or new diseases (Lockwood et al. 2007).

Behavioral studies of species interactions can contribute to a greater understanding of conservation problems such as the actual risk of hybridization (Caro 1998). Thus, even in small islands with a high degree of connectivity with the mainland, the risk of hybridization should not be considered high if behavioral experiments indicate that reproductive isolation is guaranteed. There are many other endemic lizards inhabiting other small islands in the Mediterranean where the risk of hybridization with common species is a conservation issue. Further research involving visual and chemical reproductive interactions between species would provide valuable insight into the mechanisms governing possible hybridization and species recognition in mate choice (e.g., Grant and Grant 1997; Kingston and Gwilliam 2007).

In any case, irrespective of our results, efforts should be directed to prevent the introduction of mainland lizards in the islands. The introduction of alien competitors or predators can create novel ecological contexts in which the adaptive antipredatory responses of native prey may not be successful and this can lead to extinctions of island species (Shea and Chesson 2002; Courchamp et al. 2003; Blackburn et al. 2004).

Acknowledgments We thank two anonymous reviewers for helpful comments, ‘El Ventorrillo’ MNCN Field Station for using their facilities, and the Generalitat Valenciana (GV) and the Secretaría General de Pesca Marítima (MAPA) for permission to work in the islands and to collect the lizards. We also thank to the game keepers of the Natural Park of Columbretes for their help in capturing lizards, to Pep (Cruz Roja del Mar) for facilitating a cool room to keep the lizards before transportation, and to the boats CAT-CAT and Clavel I for transportation. Financial support was provided by an ‘El Ventorrillo’ CSIC grant to MG, by a contract “Ramón and Cajal” (CSIC, MEC) to AMC, and by the projects MEC-C GL2005-00391/BOS and MCI-CGL2008-02119/BOS. Captures and experiments were performed under license from the Environmental Agencies of the Madrid Government (“Consejería del Medio

Ambiente de la Comunidad de Madrid", Spain) and by the GV.

References

Allendorf, F.W., Leary, R.F., Spruell, P., and Wenburg, J.K. 2001. The problems with hybrids: setting conservation guidelines. Trends Ecol. Evol. **16**: 613–622.

Barbadillo, L.J., Lacomba, J.I., Pérez-Mellado, V., Sancho, V., and López-Jurado, L.F. 1999. Anfibios y Reptiles de la Península Ibérica, Baleares y Canarias. Geoplaneta, Barcelona.

Barbosa, D., Desfilis, E., Carretero, M.A., and Font, E. 2005. Chemical stimuli mediate species recognition in *Podarcis* wall lizards. Amphib.-Rept. **26**: 257–263.

Barbosa, D., Font, E., Desfilis, E., and Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Bauwens, D., Garland, T. Castilla, A.M., and Van Damme, R. 1995. Evolution of sprint speed in lacertid lizards: morphological, physiological, and behavioral covariation. Evolution **49**: 848–863

Bernatched, L., Glémet, H., Wilson, C.C., and Danzmann, R.G. 1995. Introgression and fixation of Artic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. **52**: 179–185.

Blackburn, T.M., Cassey, P., Duncan, R.P., Evans, K.L., and Gaston, K.J. 2004. Avian extinctions and mammalian introductions on oceanic islands. Science **305**: 1955–1958.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature **411**: 944–947.

Campbell, N.A. 1995. Biologie. Edition du Renouveau Pédagogique Inc., Quebec.

Capula, M. 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). Amphib.-Rept. **23**: 313–321.

Carazo, P., Font, E., and Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895–902.

Caro, T. 1998. Behavioral ecology and conservation biology. Oxford University Press, New York.

Castilla, A.M. 2000. Among-islet variation in color, morphological and scalation characters in *Podarcis atrata* from the Columbretes Archipelago, Mediterranean Sea. J. Herpetol. **34**: 142–146.

Castilla, A.M., and Bauwens, D. 1991. Observations on the natural history, present status, and conservation of the insular lizard *Podarcis hispanica atrata*. Biol. Conserv. **58**: 69–84.

Castilla, A.M., and Bauwens, D. 2000. Reproductive characteristics of the lacertid lizard *Podarcis atrata*. Copeia **2000**: 748–756.

Castilla, A.M., Fernández-Pedrosa, V., Harris, J.D., González, A., Latorre, A., and Moya, A., 1998a. Mitochondrial DNA divergence suggests that *Podarcis hispanica atrata* (Squamata: Lacertidae) from the Columbretes islands merits specific distinction. Copeia **1998**: 1037–1040.

Castilla, A.M., Fernández-Pedrosa, V., Backeljau, T., González, A., Latorre, A., and Moya, A. 1998b. Conservation genetics of insular *Podarcis* lizards using partial cytochrome b sequences. Mol. Ecol. **7**: 1407–1411.

Cooper, W.E., Jr., and Burghardt, G.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J. Chem. Ecol. **16**: 45–65.

Cooper, W.E., Jr., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. **292**: 523–527.

Cooper, W.E., Jr., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Cooper, W. E., Jr., Garstka, W.R., and Vitt, L.J., 1986. Female sex pheromone in the lizard *Eumeces laticeps*. Herpetologica **42**: 361–366.

Copp, G.H., Warrington, S., and Wesley, K.J. 2008. Management of an ornamental pond as a conservation site for a threatened native fish species, crucian carp *Carassius carassius*. Hydrobiologia **59**: 149–155.

Courchamp, F., Chapuis, J.L., and Pascal, M. 2003. Mammal invaders on islands: impact, control and control impact. Biol. Rev. **78**: 347–384.

Coyne, J.A., and Orr, H.A. 2004. Speciation. Sinauer Associates, Sunderland.

Dowling, T.E.,, and Hoeh, W.R. 1991. The extent of introgression outside the contact zone between *Notropis cornutus* and *Notropis chysocephalus* (Teleostei: Cyprinidae). Evolution **45**: 944–956.

Fritts, T.H., and Rodda, G.H. 1998. The role of introduced species in the degradation of island ecosystems: A case study of Guam. Annu. Rev. Ecol. Syst. **9**: 113–140.

Galán, P., 2002. Hibridación en laboratorio de *Podarcis bocagei y Podarcis carbonelli*. Bol. Asoc. Herpetol. Esp. **13**: 28–31.

Gantenbein, B., and Largiadèr, C.R. 2002. *Mesobuthus gibbosus* (Scorpiones: *Buthidae*) on the island of Rhodes- hybridization between Ulysses' stowaways and native scorpions? Mol. Ecol. **11**: 925–938.

Gómez, A., Font, E., and Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica*. In Lacertids of the Mediterranean region. Edited by E.D.Valakos, W. Böhme, V. Pérez-Mellado, and P. Maragoú. Helenic Zoological Society, Athens, Greece. pp. 213–230.

Grant, P.R., and Grant, B.R. 1997. Hybridization, sexual imprinting and mate choice. Am. Nat. **149**: 1–28.

Gunnell, K., Tada, M.K., Hawthorne, F.A., Keeley, E.R., and Ptacek, M.B. 2008. Geographic patterns of introgressive hybridization between native Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and introduced rainbow trout (*O. mykiss*) in the South Fork of the Snake River watershed, Idaho. Conserv. Genet. **9**: 49–64.

Gyllesten, U., and Wilson , A.C. 1987. Interspecific mitochondrial DNA transfer and the colonization of Scandinavia by mice. Genet. Res. **49**: 25–29.

Heth, G., Todrank, J., Busquet, N., and Baudoin, C. 2001. Odour-genes covariance and differential investigation of individual odours in the *Mus* species complex. Biol. J. Linn. Soc. **73**: 213–220.

Kingston, J.J., and Gwilliam, J. 2007. Hybridization between two sympatrically breeding species of fur seal at Iles Crozet revealed by genetic analysis. Conserv. Genet. **8**: 1133–1145.

Lockwood, J.L., Hoopes, M.F., and Marchetti, M.P. 2007. Invasion ecology. Blackwell, Oxford.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., and Martín, J. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–465.

López, P., and Martín, J. 2005. Female Iberian wall lizards prefer male scents that signal a better cell-mediated immune response. Biol. Lett. **1**: 404–406.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154-163.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., and López, P. 2006c. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herpetol. **60**: 556–561.

Martín, J., López, P., Gabirot, M., and Pilz, K.M. 2007. Effects of testosterone supplementation on chemical signals of male Iberian wall lizards: consequences for female mate choice. Behav. Ecol. Sociobiol. **61**: 1275–1285.

Mason, R.T. 1992. Reptilian pheromones. *In* Biology of the Reptilia, vol 18. *Edited by* C. Gans and D. Crews. University of Chicago Press, Chicago. pp. 114–228.

Oliveira, R., Godinho, R., Randi, E., Ferrand, N., and Alves, P.C. 2008. Molecular analysis of hybridisation between wild and domestic cats (*Felis silvestris*) in Portugal: implications for conservation. Conserv. Genet. **9**: 1–11.

Panhuis, T.M., Butlin, R., Zuk, R.M., and Tregenza, T. 2001. Sexual selection and speciation. Trends Ecol. Evol. **16**: 364–371.

Pinho, C., Ferrand, N., and Harris, J. 2006. Reexamination of the Iberian and North African *Podarcis* (Squamata: Lacertidae) phylogeny based on increased mitochondrial DNA sequencing. Mol. Phylogenet. Evol. **38**: 266–273.

Pinho, C.. Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Rhymer, J.M., and Simberloff, D. 1996. Extinction by hybridization and introgression. Annu. Rev. Ecol. Syst. **27**: 83–109.

Sanz-Azkue, I., Gosá, A., and García-Etxebarria, K. 2005. Origin and progress of the Ibiza wall lizard (*Podarcis pityusensis*) introductions on the Cantabrian coast. Munibe **56**: 159–166.

Seehausen, O., Takimoto, G., Roy, D., and Jokela, J. 2008. Speciation reversal and biodiversity dynamics with hybridization in changing environments. Mol. Ecol. **17**: 30–44.

Shea, K., and Chesson, P. 2002. Community ecology theory as a framework for biological invasions. Trends Ecol. Evol. **17**: 170–176.

Shine, R., Reed, R.N., Shetty, S., LeMaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Sokal, R.R., and Rohlf, F.J. 1995. Biometry. Freeman, New York.

Sutherland, W.J., Armstrong-Brown, S., Armsworth, P.R., Brereton, T., Brickland, J., Campbell, C.D., Chamberlain, D.E., Cooke, A.I., Dulvy, N.K., Dusic, N.R., Fitton, M., Freckleton, R.P., Godfray, C.J., Grout, N., Harvey, H.J., Hedley, C., Hopkins, J.J. Kift, N.B. Kirby, J., Kunin, W.E. MacDonald, D.W., Marker, B., Naura, M., Neale, A.R., Oliver, T., Osborn, D., Pullin, A.S., Shardlow, M.E.A., Showler, D.A., Smith, P.L., Smithers, R.J., Solandt, J.L., Spencer, J., Spray, C.J., Thomas, C.D., Thompson, J., Webb, S.E., Yalden, D.W., and Watkinson, A.R. 2006. The identification of 100 ecological questions of high policy relevance in the UK. J. Appl. Ecol. **43**: 617–627.

Thorpe, R.S., and Malhotra, A. 1996. Molecular and morphological evolution within islands. Philos. Trans. R. Soc. Lond. B Biol. Sci. **351**: 815–822.

Van Damme, R., Bauwens, D., Vanderstighelen, D., and Verheyen, R.F. 1990. Responses of the lizard *Lacerta vivipara* to predator chemical cues: the effect of temperature. Anim. Behav. **40**: 298–305

Vincenzi, S., Crivelli, A.J., Jesensek, D., Rubin, J.F., Poizat, G., and De Leo, G.A., 2008. Potential factors controlling the population viability of newly introduced endangered marble trout populations. Biol. Conserv. **141**: 198–210.

Wang, I.A., Gilk, S.E., Smoker, W.W., and Gharrett, A.J. 2007. Outbreeding effect on embryo development in hybrids of allopatric pink salmon (*Oncorhynchus gorbuscha*) populations, a potential consequence of stock translocation Aquaculture, **272**: S152–S160.

CAPÍTULO 8

Diferencias en las señales químicas explicarían el reconocimiento interespecífico entre la lagartija insular endémica *Podarcis atrata* y la lagartija continental *P. hispanica*

Las señales químicas podrían ser la base del reconocimiento interespecifico y de especiación en muchos animales. La lagartija de las Islas Columbretes, *Podarcis atrata* es muy cercana a nivel genético a la Lagartija Ibérica continental *P. hispanica* (i.e., están incluidas las dos en el complejo de especies de *Podarcis hispanica*), pero un estudio anterior de reconocimiento quimiosensorial sugirió que sería necesario un fallo del mecanismo de discriminación química interespecífico para que ocurrieran interacciones reproductivas e hibridación entre estas dos especies. Estos resultados indicaban que las sustancias químicas utilizadas en la comunicación intraespecífica podrían diferir en composición y/o proporciones entre estas dos especies. En este trabajo, utilizamos cromatografía de gases acoplada a espectrometría de masas (GC-MS) para estudiar la composición de la fracción lipofílica de las secreciones de las glándulas femorales de los machos de *P. atrata* y *P. hispanica*. Los análisis han mostrado que los químicos encontrados en las secreciones femorales varían en composición y proporciones entre las especies y las poblaciones. Siete esteroides, como el colest-3-ene, y dos ésteres céreos sin identificar, son exclusivos de las lagartijas de las islas. Al contrario, otros nueve esteroides y otros seis compuestos fueron observados únicamente en las poblaciones continentales de *P. hispanica*. Hubo también diferencias en las proporciones de los compuestos compartidos entre las especies. Además, todas estas diferencias fueron más elevadas entre estas dos especies que entre las poblaciones mismas de *P. hispanica*. Las diferencias químicas podrían ser consecuencia de diferencias genéticas, pero también podrían ser explicadas por una adaptación a distintos hábitats, con condiciones climáticas o recursos alimenticios diferentes. Los compuestos que son específicos de cada especie, o las diferencias entre los patrones de las moléculas, permitirían el reconocimiento entre especies. Por lo tanto estos resultados de la composición química de las secreciones y estudios previos de reconocimientos químicos refuerzan el hecho de que las diferencias genéticas entre *P. atrata* y *P. hispanica* podría resultar en un aislamiento reproductivo efectivo entre estos taxa.

Differences in chemical signals could explain species recognition between an endemic island lizard, *Podarcis atrata*, and related mainland lizards, *P. hispanica*

Marianne Gabirot, Aurora M. Castilla, Pilar López, José Martín

Abstract Chemical signals can be the basis of interspecific recognition and speciation in many animals. The Columbretes Islands wall lizard, *Podarcis atrata* is very close genetically to the mainland Iberian wall lizard *P. hispanica*, but a previous study of chemosensory recognition suggested that a breakdown in mechanisms allowing species discrimination via chemical cues would be necessary to allow reproductive interactions and hybridization between these two species. These results suggested that chemicals used in intraspecific communication might differ in composition and/or proportions between these two species. In this paper, we used gas chromatography-mass spectrometry (GC-MS) to characterize the chemical composition of the lipophilic fraction from femoral gland secretions of male *P. atrata* and *P. hispanica*. The analysis showed that chemicals found in femoral secretions varied in composition and proportions between species and between populations. Seven steroids, such as cholest-3-ene, and two unidentified waxy esters, were exclusive of *P. atrata* lizards from the islands. In contrast, nine different steroids and other six compounds were only found in mainland *P. hispanica*. There were also differences in proportions of shared compounds between species. Moreover, all these differences were higher between these two species than between any population of *P. hispanica.* Chemical differences might be consequence of genetic differences, but they could also be explained by adaptation to different habitats with different climatic conditions or diet resources. Compounds that are specific of each species, or differences in the pattern of compounds, could explain species recognition. Therefore, these results of chemical composition and previous studies of chemosensory recognition reinforce the fact that the genetic differences between *P. hispanica* and *P. atrata* may result in an effective reproductive isolation between these two taxa.

Résumé Les signaux chimiques pourraient être à la base de la reconnaissance inter-spécifique et de la spéciation chez de nombreux animaux. Le lézard des îles Columbretes, *Podarcis atrata* est très proche génétiquement du lézard ibérique *P. hispanica*, mais une étude chimiosensorielle antérieure suggère que l'existence d'une cassure dans le mécanisme de discrimination entre espèces via signaux chimiques serait nécessaire pour empêcher les interactions reproductives et l'hybridation entre ces deux espèces. Les résultats ont suggéré que les composés chimiques utilisés lors de la communication intra-spécifique pourraient différer en composition et/ou en proportions entre ces deux espèces. Dans ce travail, nous avons utilisé la chromatographie sur gaz et spectrométrie de masse (GC-MS) pour caractériser la composition chimique de la fraction lipophilique des sécrétions des glandes fémorales des mâles de *P. atrata* et *P. hispanica*. L'analyse montra que les composés chimiques trouvés dans les secrétions fémorales variaient en composition et proportions entre les espèces et populations. Sept stéroïdes, comme le cholesta-3-ene, et deux non-identifiés esters acéreux, sont exclusifs du lézard insulaire, *P. atrata*. Au contraire, neuf différents stéroïdes et six autres composés sont trouvés seulement chez le lézard continental *P. hispanica*. De plus, il y avait des variations dans les proportions des composés partagés entre les espèces. Aussi, toutes ces différences étaient plus importantes entre ces deux espèces, qu'entre les populations de *P. hispanica*. Les différences chimiques seraient la conséquence de variations

génétiques, mais aussi pourraient être expliquées par l'adaptation à différents habitats avec des conditions climatiques ou ressources alimentaires différentes. Les composés qui sont spécifiques de chaque espèce, ou les différences du patron chimique, pourraient expliquer la reconnaissance entre les espèces. Finalement, ces résultats de la composition chimique et l'antérieure étude de reconnaissance chimique renforcent le fait que les différences génétiques entre *P. hispanica* et *P. atrata* pourraient conclure à une isolation de reproduction effective entre ces deux taxa.

In Press in *Biochemical Systematics and Ecology*

INTRODUCTION

Signals favored by sexual selection may play an important role in species recognition, reproductive isolation and speciation (Andersson 1994; Boughman 2001; Panhuis et al. 2001). Species recognition mechanisms based on behavior, visual, olfactory, auditory and tactile cues may prevent interspecific mating between sympatric related taxa (Cooper and Vitt 1986; Shine et al. 2002). Lizards and snakes have a well developed chemosensory system (Mason 1992) and chemical stimulus can be the basis of interspecific recognition and speciation in reptiles and many other animals (reviewed in Smadja and Butlin 2009), such as in sympatric sea snakes species (Shine et al. 2002), or in different populations of the same species of red-garter snakes (LeMaster and Mason 2003) and in closely related species or populations of lizards (Cooper and Vitt 1986; Barbosa et al. 2005, 2006; Martin and Lopez 2006a,b).

Many lizards have epidermal structures on the ventral surface of the thigh (femoral pores) connected to holocrine femoral glands that secrete chemical cues. These secretions are especially abundant in males during the reproductive season (Mason 1992; Alberts 1993), and chemicals secreted are thought to be important compounds involved in communication and sexual selection (Mason 1992; Martin and Lopez 2006c). Femoral gland secretions of males could advertise residence in a home range and/or inform other males on a male's status and competitive ability (Aragón et al. 2001 2006; Carazo et al. 2007; Martín et al. 2007). They may also transmit information about a male's quality, which females may use to select potential mates (Olsson et al. 2003; Martin and Lopez 2006c). Finally, femoral secretions can also be used in the context of interspecific recognition (Cooper and Vitt 1986; Cooper and Perez-Mellado 2002; Gabirot et al. in press), and potentially lead to reproductive isolation and speciation processes. For example, in *Podarcis* lizards, chemosensory recognition is well-developed. These lizards can discriminate between conspecifics and more genetically distant heterospecifics (*P. hispanica* vs. *P. bocagei* or *P. carbonelli* vs. *P. bocagei*), and between sexes by chemical cues alone (López and Martín 2001; Cooper and Pérez-Mellado 2002; López et al. 2002; Barbosa et al. 2005, 2006).

Podarcis lizards are highly variable in size, shape, escalation and color pattern, not only between currently recognized species (Arnold and Ovenden 2002), but also between populations and individuals. Recently molecular and morphological studies suggest that the Iberian wall lizard, *P. hispanica*, is paraphyletic, and forms a “species complex” with at least five monophyletic lineages. This suggests the existence of ongoing speciation processes within taxa previously considered to be conspecific (Harris and Sá-Sousa 2001, 2002; Pinho et al. 2007). The genetic divergence’s analyses in this “species complex” help to distinguish the island lizard *P. atrata* from the mainland *P. hispanica* (Castilla et al. 1998b).

The Columbretes wall lizard, *P. atrata*, is an endangered and endemic lacertid lizard from the Columbretes archipelago (Mediterranean, Spain) (Castilla et al. 1998a,b). This is the European lizard with the smallest (19 Ha) distribution area, and has a small population size (total of ca.12000 individuals) (Castilla and Bauwens 1991). In contrast, in the Iberian Peninsula, the Iberian wall lizard, *P. hispanica,* is common and widely distributed (Barbadillo et al., 1999). *P. atrata* is genetically distinct (Castilla et al. 1998b), but much more closely related to some populations of *P. hispanica* than previously thought (Harris and Sa-Sousa 2002) and the risk of possible hybridization of the endemic island *P. atrata* with introduced mainland *P. hispanica* has been suggested (Castilla et al. 1998a; Gabirot et al. in press; see Chapter 7).

These two lizard species that are genetically very closed could, however, diverge in chemical signals or in communication, thus allowing species recognition and avoiding hybridization. In a previous experiment, differential tongue-flick rates of lizards in response to different scents indicated that *P. hispanica* and *P. atrata* can discriminate between species based on chemical cues alone (Gabirot et al. in press; see Chapter 7). Lizards of both species and sexes have more “interest” for exploring the scent of conspecific lizards than the scent from lizards of the other species (Gabirot et al. in press; see Chapter 7). This indicates the occurrence of a clear interspecific chemosensory discrimination and suggests that chemicals of *P. hispanica* and *P. atrata* used in intraspecific communication might be different in composition and/or proportions. Other studies have shown that chemical secretions of lizards of different taxa differ in composition and proportion of compounds, even when the species are sympatric and closely related, which would allow species recognition (Martín and López 2006a). In this paper, we aimed to explore whether there were differences between femoral secretions of male *P. atrata* from Columbretes islands and male *P. hispanica* from different mainland populations. For this, we used gas chromatography-mass spectrometry (GC-

MS) to analyze and compare the chemical composition of the lipophilic fraction of femoral gland secretions of males of both species.

METHODS

Study area and species

The Columbretes wall lizard *P. atrata*, is a small lizard (mean adult snout-vent length of 50-70 mm) endemic from the Columbretes archipelago (39°55’ N, 0°40’ E, Mediterranean Sea, Castellón, Spain), which inhabits three small islands characterized by high aridity and vegetation dominated by perennial shrubs, herbs and grasses (Castilla and Bauwens 1991, 2000; Castilla 2000). We collected by noosing 15 adult males in the main island, Columbrete Grande (13 ha), during the reproductive season in April 2008.

The Iberian wall lizard, *P. hispanica*, is a small lizard (mean adult snout-vent length of 49-61 mm) with a widespread distribution in Spain and the south east coast of France (Barbadillo et al. 1999). We captured by noosing adult male *P. hispanica* lizards at five localities within the Madrid Province (Central Spain) during the reproductive season in May-June 2008 (Fig. 1). Three of these were located in the mountain northern area (‘Fuenfría’, ‘Cercedilla’ and ‘Pedrezuela’), and the other two were located in the plain southern area (‘Belmonte’ and ‘Aranjuez’). In the north, we captured lizards from a population occupying granite rock-cliffs at the edge of a pine forest in the upper part of ‘Fuenfría’ Valley (‘Ph1’) (40°47’ N, 4°03’W; 1750 m altitude; *n* = 21 males), and from old stone walls near to cultivated fields in the ‘Pedrezuela’ village (‘Ph2’) (40°44’ N, 3°36’ W; 800 m altitude; *n* = 19 males). Finally, we caught lizards on granite rocky outcrops in a large oak forest (‘Golondrina’, ‘Ph3’) near Cercedilla village (40°44’ N, 4°02’ W; 1250 m altitude; *n*=29 males). In the south, we captured lizards on human constructions in a public garden in the ‘Belmonte del Tajo’ village (‘Ph4’) (40°8’ N, 3°20’ W; 735 m altitude; *n* = 22 males), and on chalk and gypsum rocks in deforested bushy hills

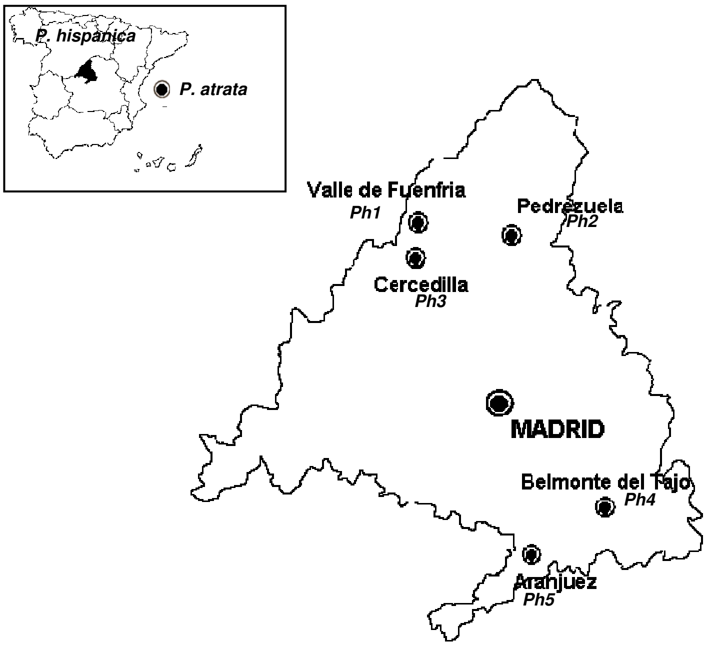


Fig.1: Localization of the populations studied of *Podarcis hispanica* and of *Podarcis atrata*

near 'Aranjuez' ('Ph5') (40°2' N, 3°37' W; 494 m altitude; $n = 21$ males).

Analyses of femoral gland secretions

Immediately after capture in the field, we extracted femoral gland secretion of males by gently pressing with forceps around the femoral pores, and collected secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at −20 °C until analyses. We also used the same procedure on each sampling occasion but without collecting secretion, to obtain blank control vials that were treated in the same manner to compare with the lizard samples. Before the analyses we added 250 µl of n-hexane (Sigma, capillary GC grade) to each vial, We analyzed lipophilic compounds in samples by using a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5 % diphenyl/95 % dimethylsiloxane) column (Thermo Fisher, Trace TR-5, 30 m length x 0.25 mm ID, 0.25-mm film thickness) and 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 at 30 cm/sec, with injector temperature at 250 °C. The oven temperature program was as follows: 50 °C isothermal for 5 min, then increased to 270 °C at a rate of 10 °C/min, isothermal for 1 min, then increased to 315 °C at a rate of 15 °C/min, and finally isothermal (315 °C) for 10 min. Ionization by electron impact (70 eV) was carried out at 250°C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the solvent and/or the control vial samples are not reported. Initial identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards from Sigma-Aldrich Chemical Co. For unidentified or unconfirmed compounds we report here their characteristic ions, which we used together with retention times and characteristic *m/z* ratios to confirm whether these compounds were present in a given individual.

For the statistical analyses of secretions, the relative amount of each component was determined as the percent of the total ion current (TIC). Then, relative areas of the peaks were transformed following Aitchison's formula: [$Z_{ij,} = \ln(Y_{ij}/g(Y_j))$], where *Zij* is the standardized peak area i for individual *j*, *Yij* is the peak area *i* for individual *j*, and g(*Yj*) is the geometric mean of all peaks for individual *j* (Aitchison 1986; Dietemann et al. 2003). The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. The transformed areas were used as variables in a principal component analysis. The eight principal components (PC) extracted (with eigenvalues > 1; which explained 82.26% of variance) were used as independent variables in a multivariate analysis of variance (MANOVA) to determine whether the five populations of male *P. hispanica* (mainland) and *P. atrata* (island) differed in the relative proportion of compounds. In addition, we used these extracted principal components (all 8 PCs) as covariates in a subsequent discriminant analysis. We used this test to verify whether chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard.

In a further similar analysis, we selected the 28 compounds that were presented in all populations, re-standardized their TIC areas to 100% and used a MANOVA with transformed areas to test for differences between populations in the relative proportions of shared compounds.

RESULTS

We found 47 lipophilic compounds in femoral glands secretions of male *P. atrata* (see Table 1). These secretions were a mixture of steroids (78.47% of TIC), carboxylic acids ranged between n-C_{14} and n-C_{20} and their esters (9.89%), six waxy esters (8.30%), four alcohols between n-C_{16} and n-C_{22} (2.75%), squalene (0.35%), a ketone (0.13%), and an unidentified terpenoid (0.11%). On average, the five most abundant chemicals were cholesterol (63.38% of TIC), an unidentified waxy ester (3.61%), octadecanoic acid (3.19%), hexadecanoic acid (2.98%), and campesterol (2.66%) (see Table 1).

The femoral gland secretions of male *P. hispanica* (all five populations pooled) were composed by 53 lipophilic compounds, which were a mixture of steroids (83.64% of TIC), and carboxylic acids ranged between n-C_{14} and n-C_{22} and their esters (10.30%), but we found also five alcohols between n-C_{16} and n-C_{24} (1.26%), four waxy esters (3.53%), squalene (0.60%), two terpenoids (0.40%), a ketone (0.18%), and a furanone (0.09%) (see Table 1). On average, the five most abundant chemicals were cholesterol (63.24% of TIC), cholesta-5,7-dien-3-ol (5.16%), hexadecanoic acid (3.73%), campesterol (3.66%) and octadecenoic acid (2.46%) (Table 1). However, when considered separately, there were some clear differences between populations of *P. hispanica* (see below).

There were differences between *P. hispanica* and *P. atrata* in the presence/absence of several compounds in femoral secretions. Male *P. atrata* from Columbretes islands had nine chemical compounds (seven steroids and two waxy esters) that were not found in mainland *P. hispanica* males (see Table 1). On the other hand, secretions from mainland *P. hispanica* (all populations pooled) had 15 compounds (nine steroids, three carboxylic acids, one alcohol, one furanone and one terpenoid) that were not found in island *P. atrata* lizards (see Table 1).

Also, proportion of the main classes of chemical compounds in secretions varied between species. In comparison with *P. hispanica, P. atrata* had significantly higher overall proportions of alcohols (ANOVA: $F_{1,119}$=44.22 P<0.0001) and waxy esters ($F_{1,119}$=38.53, P<0.0001), and significantly lower proportions of low molecular weight (C_{14-18}) fatty acids ($F_{1,119}$=28.39, P<0.0001), high molecular weight (C_{20}-C_{22}) fatty acids ($F_{1,119}$=6.75, P=0.01), and steroids ($F_{1,119}$=23.07, P<0.0001).

Multivariate analyses on the 8 PCs for all the 62 compounds found in femoral secretions showed that there were significant differences in the relative proportions of some compounds between species and/or populations (MANOVA, *Wilk's* λ=0.0001, $F_{40,473}$=904.56, P<0.0001). Moreover, the discriminant and the canonical analyses showed that PCs scores of chemical compounds in femoral secretions of male *P. atrata* clearly allowed differentiating them from males from any of the five mainland populations of *P. hispanica* (Fig. 2). This analysis classified correctly 100 % of individual *P. atrata* as belonging to this species. Moreover, the squared Mahalanobis distances between groups showed that all *P. hispanica* individuals had significantly shorter distances (based on chemical components) to individuals from any *P. hispanica* population than to *P. atrata* individuals ($F_{5,535}$=632.92, P<0.0001) (Fig. 3).

According to the correlations of relative proportions of all compounds found in femoral secretions with the PCs, the PC-1 scores were related negatively to the proportion of tetracosanol, cholesta-2,4-diene, ergosta-5,8-dien-3-ol, cholesta-4,6-dien-3-one and stigmasterol, and were related positively with the proportions of the unidentified waxy esters 1 and 3, cholest-3-ene and six unidentified steroids (2, 3, 11, 12, 13 and 15). PC-1 scores varied significantly between populations (ANOVA, $F_{5,115}$=2092.48, P>0.0001) (see Table 2). Island *P. atrata* had positive and significant different PC-1 scores than all *P. hispanica* populations, which had negative values (Tukey's tests: P<0.0002 for all). PC1 scores of Ph1 and Ph3 were not significantly different (P>0.60), but both differed significantly from values of Ph4

Compounds Characteristic ions observed (m/z)	RT (min)	*Podarcis hispanica*					*P. atrata*
		Ph1	Ph2	Ph3	Ph4	Ph5	
Steroids:							
Un.ster.(18,145,213,248,353,368,387)	29.92	0.01 ± 0.01	-	0.17 ± 0.05	1.49 ± 0.56	-	-
Cholesta-2-4-diene	30.58	0.68 ± 0.11	2.66 ± 0.44	0.44 ± 0.08	2.59 ± 0.46	0.96 ± 0.35	-
Un.steroid (141,156,209,281,350,365)	30.64	-	-	-	-	-	0.44 ± 0.06
Un.steroid (143,253,350,367)	30.74	-	-	-	-	-	0.09 ± 0.01
Cholesta-3.5-diene	30.81	0.42 ± 0.10	0.23 ± 0.04	0.30 ± 0.07	0.13 ± 0.03	0.25 ± 0.07	0.05 ± 0.01
Un.steroid (155,197,251,350,365)	30.96	1.32 ± 0.16	1.00 ± 0.14	0.55 ± 0	0.45 ± 0.06	0.45 ± 0.17	0.79 ± 0.10
Cholesta-5,7,9(11)-trien-3-ol	31.06	1.62 ± 0.18	1.07 ± 0.24	0.94 ± 0.11	0.65 ± 0.11	0.29 ± 0.07	0.94 ± 0.18
Un.steroid (207,251,350,365)	31.13	0.40 ± 0.08	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.07	0.08 ± 0.04	0.18 ± 0.04
Un.steroid (143,195,207,351,366)	31.20	0.19 ± 0.02	0.08 ± 0.01	0.15 ± 0.04	0.18 ± 0.05	0.22 ± 0.06	0.35 ± 0.17
Un. steroid (141,156,209,350,365)	31.37	0.37 ± 0.05	0.03 ± 0.01	0.30 ± 0.06	2.47 ± 0.42	-	0.52 ± 0.12
Un. Steroid (155,197,251,365,379)	31.64	0.06 ± 0.01	0.21 ± 0.02	0.08 ± 0.02	0.43 ± 0.07	0.45 ± 0.18	0.31 ± 0.08
Un.steroid (195,209,251,365,379)	31.84	-	0.07 ± 0.01	0.27 ± 0.07	0.51 ± 0.08	0.32 ± 0.12	0.34 ± 0.22
Cholesterol	32.43	59.74 ± 2.79	62.33 ± 1.68	66.61 ± 2.00	53.03 ± 2.51	74.51 ± 2.04	63.38 ± 2.26
Cholestan-3β-ol	32.47	1.40 ± 0.14	0.53 ± 0.08	0.90 ± 0.11	0.60 ± 0.06	0.55 ± 0.12	1.33 ± 0.18
Cholesta-5,7-dien-3β-ol	32.65	13.41 ± 1.85	2.68 ± 0.54	8.02 ± 1.33	1.16 ± 0.19	0.54 ± 0.17	0.62 ± 0.30
4-Methyl-cholestan-3β-ol	32.73	-	-	-	-	-	0.12 ± 0.03
Un.steroid (105,213,255,353,368,386,415)	32.75	0.02 ± 0.01	0.03 ± 0.02	0.35 ± 0.11	0.09 ± 0.03	0.39 ± 0.16	-
Ergosterol	33.00	-	0.05 ± 0.02	-	0.17 ± 0.11	-	0.09 ± 0.02
Campesterol	33.17	1.61 ± 0.22	3.76 ± 0.28	3.27 ± 0.36	5.46 ± 0.28	4.22 ± 0.57	2.66 ± 0.37
Cholest-4-en-3-one	33.41	0.17 ± 0.03	0.53 ± 0.17	0.19 ± 0.05	0.20 ± 0.02	0.92 ± 0.38	1.33 ± 0.42
Un.steroid (215,374,384,400,416)	33.45	-	-	-	-	-	2.02 ± 0.33
Ergosta-5,8-dien-3-ol	33.50	2.43 ± 0.30	1.58 ± 0.22	2.38 ± 0.37	1.31 ± 0.24	0.56 ± 0.14	-
Cholesta-4,6-dien-3-one	33.69	0.24 ± 0.06	0.53 ± 0.08	0.29 ± 0.06	0.40 ± 0.06	-	
Un.steroid (267,339,366,383)	33.72	-	-	-	-	-	0.48 ± 0.09
Sitosterol	33.92	0.65 ± 0.10	0.74 ± 0.16	0.94 ± 0.15	1.18 ± 0.11	1.13 ± 0.23	1.25 ± 0.17
Ergostanol	34.02	0.07 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	0.11 ± 0.02	0.33 ± 0.11	0.13 ± 0.03
Stigmasterol	34.13	0.31 ± 0.06	0.27 ± 0.13	0.28 ± 0.04	1.22 ± 0.22	0.44 ± 0.26	-
Un.steroid (267, 366,381)	34.17	-	-	-	-	-	0.37 ± 0.04
Un.steroid (221,253,281,355,380,430)	34.30	2.23 ± 0.32	0.70 ± 0.18	1.01 ± 0.16	-	-	-
Un.steroid (143,253,354,380,395,413)	34.31	-	-	-	-	-	0.30 ± 0.07
Cholest-5-en-3-one	34.38	-	-	-	1.33 ± 0.24	0.91 ± 0.28	-
Ergosta-5.22-dien-3-ol	34.47	-	0.13 ± 0.07	0.12 ± 0.03	0.15 ± 0.04	-	0.36 ± 0.08
Un.steroid (214,267,395)	35.30	0.12 ± 0.04	0.21 ± 0.11	-	0.56 ± 0.44	0.22 ± 0.09	-
Carboxylic acids and their esters:							
Tetradecanoic acid	20.64	0.16 ± 0.04	0.38 ± 0.13	0.22 ± 0.06	0.24 ± 0.05	0.85 ± 0.55	0.21 ± 0.05
Pentadecanoic acid	21.68	0.13 ± 0.02	0.15 ± 0.12	0.10 ± 0.03	0.18 ± 0.05	0.41 ± 0.19	0.25 ± 0.08
Hexadecanoic acid. methyl ester	22.33	-	0.05 ± 0.02	-	0.09 ± 0.02	0.25 ± 0.08	0.11 ± 0.04
Hexadecenoic acid	22.54	0.16 ± 0.02	0.40 ± 0.20	0.25 ± 0.07	0.57 ± 0.33	0.28 ± 0.09	0.17 ± 0.05
Hexadecanoic acid	22.76	3.68 ± 0.32	4.36 ± 0.65	3.11 ± 0.35	5.98 ± 0.51	1.54 ± 0.23	2.98 ± 0.20
Hexadecanoic acid, ethyl ester	22.98	-	0.37 ± 0.11	-	0.19 ± 0.06	0.40 ± 0.17	-
9,12-Octadecadienoic acid	24.35	0.10 ± 0.01	0.11 ± 0.02	0.12 ± 0.02	0.27 ± 0.08	0.06 ± 0.02	0.18 ± 0.04
Octadecenoic acid	24.43	1.99 ± 0.18	1.76 ± 0.20	2.76 ± 0.57	4.82 ± 1.41	1.01 ± 0.21	2.24 ± 0.21
Octadecanoic acid	24.60	1.39 ± 0.12	2.52 ± 0.34	1.41 ± 0.13	2.55 ± 0.23	0.99 ± 0.18	3.19 ± 0.22
Octadecanoic acid, ethyl ester	24.82	-	0.51 ± 0.23	-	0.14 ± 0.04	0.55 ± 0.23	-
Eicosanoic acid	26.31	0.46 ± 0.09	0.63 ± 0.15	0.76 ± 0.11	0.59 ± 0.17	0.64 ± 0.18	0.53 ± 0.21
Docosanoic acid	28.00	-	0.01 ± 0.01	-	0.01 ± 0.01	-	0.03 ± 0.01
Docosanoic acid, ethyl ester	28.21	-	0.45 ± 0.12	-	0.21 ± 0.05	0.23 ± 0.12	-
Alcohols:							
Hexadecanol	21.02	0.23 ± 0.05	-	0.19 ± 0.07	0.16 ± 0.04	0.16 ± 0.05	0.26 ± 0.06
Octadecanol	23.87	0.26 ± 0.05	0.69 ± 0.16	0.19 ± 0.06	0.29 ± 0.08	-	1.58 ± 0.45
Eicosanol	25.67	0.17 ± 0.03	0.55 ± 0.13	0.28 ± 0.08	0.21 ± 0.05	0.81 ± 0.28	0.59 ± 0.10
Docosanol	27.33	0.23 ± 0.05	0.52 ± 0.15	0.23 ± 0.04	0.23 ± 0.04	0.73 ± 0.26	0.32 ± 0.10
Tetracosanol	29.80	0.03 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.07 ± 0.02	0.01 ± 0.01	-
Waxy esters:							
Un.waxy ester of hexadecanoic acid 1	28.47	-	-	-	-	-	0.08 ± 0.04
Un. waxy ester of octadecanoic acid 2	29.45	0.28 ± 0.08	0.98 ± 0.20	-	1.37 ± 0.47	0.75 ± 0.23	0.74 ± 0.16
Un.waxy ester of hexadecanoic acid 3	35.00	-	-	-	-	-	0.03 ± 0.01
Un. waxy ester of hexadecanoic acid 4	35.57	0.58 ± 0.10	2.61 ± 0.60	0.42 ± 0.08	2.84 ± 0.45	0.69 ± 0.26	3.61 ± 0.69
Un. waxy ester of octadecnoic acid 5	38.06	0.23 ± 0.06	0.29 ± 0.06	0.20 ± 0.05	0.09 ± 0.03	0.37 ± 0.23	1.47 ± 0.26
Un. waxy ester of octadecanoic acid 6	38.27	0.63 ± 0.11	1.78 ± 0.26	0.47 ± 0.10	2.26 ± 0.30	0.82 ± 0.16	2.37 ± 0.29
Others:							
Tetradecanone	22.11	0.20 ± 0.05	0.27 ± 0.11	0.13 ± 0.03	0.15 ± 0.03	0.18 ± 0.06	0.13 ± 0.03
Unidentified furanone	24.19	0.12 ± 0.02	0.10 ± 0.03	0.06 ± 0.01	-	-	-
Squalene	30.07	0.93 ± 0.26	0.70 ± 0.10	0.66 ± 0.20	0.35 ± 0.04	0.40 ± 0.19	0.35 ± 0.08
Unidentified terpenoid 1	30.83	0.09 ± 0.03	0.07 ± 0.03	0.08 ± 0.02	0.03 ± 0.01	0.13 ± 0.07	-
Unidentified terpenoid 2	31.94	0.48 ± 0.09	-	0.48 ± 0.12	0.05 ± 0.01	-	0.11 ± 0.05

Table 1: Lipophilic compounds found in femoral gland secretions of male lizards, *Podarcis hispanica* from five distinct populations of the mainland Iberian Peninsula, and *P. atrata* from Columbretes Islands. The relative amount of each component was determined as the percent of the total ion current (TIC) and reported as the average (± SE). Characteristics (m/z) are reported for some unidentified compounds.

and Ph5 ($P<0.0002$); Ph4 and Ph5 were significantly different ($P=0.00012$).

The PC-2 scores correlated positively with the proportion of tetracosanol, cholesterol, cholestanol, cholesta-3,5-diene and campesterol, and negatively with the proportions of ergosterol. There were significant differences between populations ($F_{5,115}=144.64$; $P<0.0001$) (Table 2). Males from Ph1, Ph2 and Ph5 had positive values whereas Ph2, Ph4 and *P. atrata* had negative values. PC-2 scores of Ph1 and Ph5 ($P=0.11$) and those of *P. atrata* and Ph2 ($P=0.31$) were not significantly different, whereas all the rest of comparisons were significantly different ($P<0.00011$ for all).

PC-3 scores described negatively the relative proportions of hexadecanoic acid ethyl and methyl esters, octadecanoic acid ethyl ester, docosanoic acid ethyl ester, and cholest-5-en-3-one and positively the proportions of octadecanol,

PCs	*Eigenvalue*	***Podarcis hispanica***					***P. atrata***
		Ph 1	***Ph 2***	***Ph 3***	***Ph 4***	***Ph 5***	
PC-1	16.59	-0.33 ± 0.02^{b}	-0.36 ± 0.02^{b}	-0.31 ± 0.02^{b}	-0.59 ± 0.02^{a}	-0.06 ± 0.02^{c}	2.81 ± 0.02^{d}
PC-2	15.33	0.79 ± 0.07^{a}	-0.71 ± 0.08^{b}	0.4 ± 0.07^{c}	-1.48 ± 0.08^{b}	1.09 ± 0.08^{a}	-0.43 ± 0.10^{c}
PC-3	7.67	0.96 ± 0.03^{a}	-0.05 ± 0.04^{a}	0.97 ± 0.03^{c}	-0.18 ± 0.04^{d}	-1.79 ± 0.04^{e}	0.20 ± 0.05^{b}
PC-4	3.88	1.37 ± 0.06^{a}	0.28 ± 0.07^{b}	-1.57 ± 0.06^{d}	0.15 ± 0.06^{bc}	-0.20 ± 0.07^{c}	0.03 ± 0.08^{bc}
PC-5	2.84	-0.12 ± 0.04^{c}	2.06 ± 0.05^{a}	0.10 ± 0.04^{b}	-1.19 ± 0.05^{d}	-0.47 ± 0.05^{e}	-0.08 ± 0.06^{bc}
PC-6	1.83	0.30 ± 0.20	-0.46 ± 0.23	0.17 ± 0.20	-0.19 ± 0.21	0.04 ± 0.22	0.002 ± 0.27
PC-7	1.67	-0.35 ± 0.20	-0.01 ± 0.23	0.27 ± 0.20	0.35 ± 0.20	-0.21 ± 0.22	-0.09 ± 0.27
PC-8	1.16	-0.11 ± 0.20	0.02 ± 0.23	0.11 ± 0.20	0.21 ± 0.21	0.31 ± 0.22	0.08 ± 0.27

Table 2: Principal components (PCs) scores (mean ± SE) from a PCA for relative proportions of compounds in femoral secretions of *P. hispanica* populations (Ph1: Aranjuez, Ph2: Belmonte, Ph3: Golondrina, Ph4: Fuenfría and Ph5: Pedrezuela) and *P. atrata*. Same small letters on means indicated lack of significant posthoc differences (Tukey's tests) between populations within each PC.

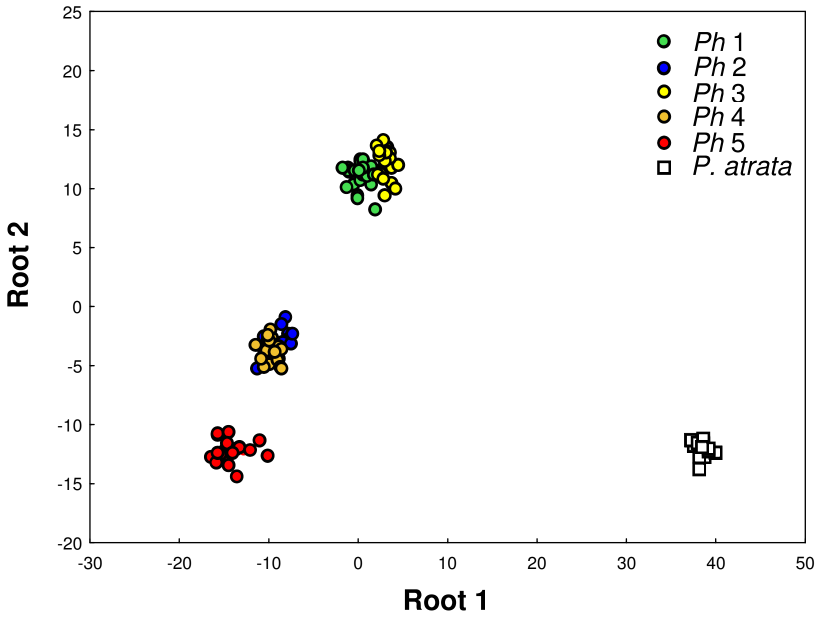


Fig.2: Separation of the principal components describing chemical femoral secretions in a discriminant analysis based on population of origin (Ph1: Aranjuez, Ph2: Belmonte, Ph3: Golondrina, Ph4: Fuenfria and Ph5: Pedrezuela).

the unidentified steroid 7, and the unidentified terpenoid 2. PC-3 values differed significantly between populations ($F_{5,115}$=610.65, P<0.0001) (Table 2). Males from Ph1 and Ph2 had similar positive values (P=0.99), while other populations had negative values. All other comparisons of PC-3 scores between populations were significant (P<0.0002 for all).

The PC-4 scores were negatively related to the proportions of the unidentified steroid 16 and positively with the proportions of the unidentified steroid 9. PC-4 scores differed significantly between populations ($F_{5,115}$=205.39, P=0.0006) (Table 2). Males from Ph1 had positive PC-4 scores, while the rest of populations had PC-4 scores around zero and negative. The comparisons between populations were all significant (P<0.0002), except between Ph2 and Ph4 (P=0.84) or between *P. atrata* and Ph2

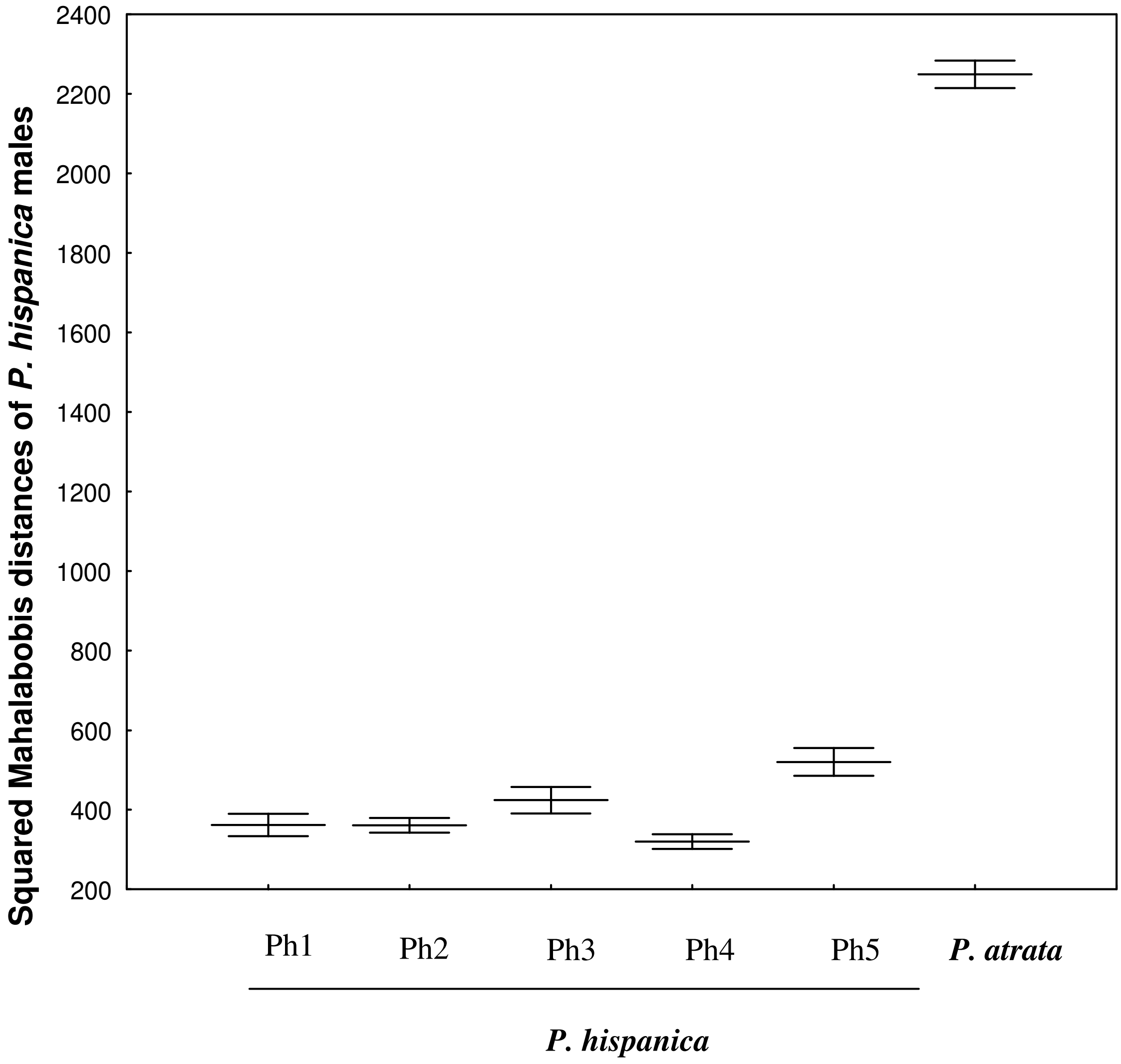


Fig. 3: Squared Mahalabobis distances (mean ± SE), obtained from a discrimimant analysis of chemical components' PCs scores, of individual *P. hispanica* to individuals from all *P. hispanica* populations (Ph1: Aranjuez, Ph2: Belmonte, Ph3: Golondrina, Ph4: Fuenfria and Ph5: Pedrezuela) or to *P. atrata* individuals.

(*P*=0.31), Ph5 (*P*=0.34) or Ph4 (*P*=0.89).

Finally, the proportion of hexadecanol in secretions (i.e., PC-5 scores) differed with the population of origin ($F_{5,115}$=386.05, *P*<0.0001) (Table 2). Males from Ph2 showed positive PC-5 scores whereas all other populations had negative values. All comparisons of PC-5 scores between populations were significant (*P*<0.0001), excepted between *P. atrata* and Ph1 (*P*=0.99) and Ph2 (*P*=0.18).

There were not significant differences between populations for PC-6 ($F_{5,115}$=1.59, *P*=0.16), PC-7 ($F_{5,115}$=1.75, *P*=0.12) or PC-8 ($F_{5,115}$=0.76, *P*=0.57) (Table 2).

In an additional analysis, when we selected only the 28 compounds shared (i.e. presented) between all populations, the relative proportion of these shared chemicals also varied significantly with the population of origin (MANOVA, *Wilks' λ*=0.03, $F_{125,452}$=8.29, *P*<0.0001).

DISCUSSION

The analyses of chemical composition showed that similarly to other lacertid lizards, femoral gland secretions of *P. hispanica* and *P. atrata* have steroids and carboxylic acids as predominant components (reviewed in Weldon et al., 2008). Among steroids, in both species, cholesterol was the main steroid, which was also presented in abundance in secretions of many other lizard species (Weldon et al., 2008). More interestingly, chemicals found in femoral gland secretions of males differed in composition and proportion between the two species, and secondarily between populations of *P. hispanica*. The chemical differences between island *P. atrata* and mainland *P. hispanica* were higher than between continental populations of *P. hispanica* alone. For some chemicals, there were clear differences in presence/absence between species. Some steroids and waxy esters were presented only in secretions of *P. atrata*, whereas other chemicals such as tetracosanol or cholesta-2,4-diene, ergosta-5,8-dien-3-ol or stigmasterol, were found only in secretions of *P. hispanica*. Also, the proportions of the main classes of compound varied between species. For example lizards from the islands had secretions with higher proportions of alcohols and waxy esters. In addition, the proportions of shared chemicals also varied between *P. atrata* and *P. hispanica*.

These chemical differences between species could result from the different habitats where each lizard species lives. A first possible explanation is that these differences in lipidic secretions might be the consequence of different diets (Symonds and Elgar, 2009), or different available food sources in the island and in the mainland. Interestingly, for example, the few existing studies on the diet of *P. atrata* indicates that this species includes in its diet marine isopods and poisonous scorpions, while this has never been reported for any mainland populations of *P. hispanica* (Castilla et al., 2008c,d; Castilla and Herrell, 2009). Although, to confirm this relationship we would need more studies of diet, and also experiments examining whether different diets lead to differences in femoral secretions.

Alternatively, or in addition, these variations in chemical composition and proportions in secretions might be correlated to the different microclimatic conditions of different geographical areas. Presumably, the variation of femoral chemical composition also would reflect the environment where each lizard species lives (Alberts, 1992; Escobar et al., 2001, 2003). Therefore, it is likely that selection for a better efficiency of chemical signals used in intraspecific communication had led to differences in composition of femoral secretions of lizards inhabiting different environments (see Alberts, 1992). The abiotic conditions in a small island and in the continent may be very different (e.g., altitude, temperature, wind, relative humidity) and this could have influenced the characteristics of chemical secretions of lizards. A study showed that femoral secretions of two populations of *P. hispanica* inhabiting areas with different microclimatic conditions differ in the abundance of more stable waxy esters and long chain fatty acids, which are more abundant in lizards from areas with higher humidity levels (Martin and Lopez, 2006a). These less volatile compounds would confer more stability to secretions under conditions where high levels of humidity increase evaporation (Alberts, 1992). Similarly, our results indicate that waxy esters, which would confer more stability to secretions in the humid conditions of the island environment, are more abundant in *P. atrata.*

Finally, differences in chemicals in femoral secretions may simply result from genetic differences between populations and species. The Iberian wall lizard, *P. hispanica* is paraphyletic with respect to *P. bocagei*, *P. carbonelli*, and *P. atrata* and appears to form a species complex (Harris and Sa-Sousa, 2002; Pinho et al., 2007). Genetic divergence is high between the population of *P. atrata* from the Columbretes islands and a population of *P. hispanica* from the continent, which justified that it was described as a different species (Castilla et al., 1998a,b; Harris and Sa-Sousa, 2001, 2002). Chemical differences in femoral secretions may result of colonization and adaptation to different habitats; or just from random genetic divergence due to the geographical isolation, such that each species could have evolved in different ways and produce different chemical signals. Similarly, in some rodents, genetic differences within a species complex seem to explain differences in scent characteristics (Heth and Todrank, 2000; Heth et al., 2001; Talley et al., 2001).

The question that arises is whether these differences in chemical signals affect recognition systems and whether this may have had consequences for speciation. A previous experiment of chemosensory recognition by tongue-flick tests between *P. hispanica* and *P. atrata* lizards (Gabirot et al., 2010) and the results of the current study suggest that these differences in chemical signals could allow species recognition. This chemosensory species discrimination could have been an important requisite to preclude reproductive interactions between *P. hispanica* and *P. atrata*. This is something that has been already observed within other wall lizard species (Cooper and Pérez-Mellado, 2002; Barbosa et al., 2005, 2006; Martín and López, 2006a,b). Compounds that are specific for femoral secretions of each species could allow species recognition. Furthermore, chemical recognition between species could not be only based on differences in one or a few compounds alone, but also on the pattern of compounds found in each species. In vertebrates, pheromones are often a mixture of several chemical compounds with different properties (Müller-Schwarze, 2006). These multicomponent pheromones may have different functions, but may also act together providing specific “odor profiles”, also named “gestalts”, “patterns”, or “mosaics” (Johnston, 2005). Not only the mixture of chemicals, but also the relative proportions or concentrations of these chemicals, are often needed to be biologically active as a pheromone. Thus, a vertebrate pheromone may be defined as a group of active compounds in a secretion that supply information to, or change behavior in, another conspecific (Müller-Schwarze, 2006), and that could also allow species recognition.

In summary, our study showed that chemical signals (femoral secretions) of *P. hispanica* and *P. atrata* lizards were different in composition and proportions of chemical compounds. Moreover, this difference is higher between these two species than between any distinct population of *P. hispanica*. All these chemical differences could be the consequence of random genetic variation, but could also be due to adaptation to different habitats, with differences in climatic conditions or diet resources. This chemical variation could be used by individuals to recognize lizards from their own species. Therefore, these results of chemical composition and previous studies of chemosensory recognition reinforce the fact that the genetic differences between *P. hispanica* and *P. atrata* may reflect reproductive isolation between these two taxa. Our results could be a preliminary cue of the high improbability of reproduction between *P. hispanica* and *P. atrata*. If individuals do recognize and discriminate between species, which may be based on differences in chemical compounds in femoral secretions, interspecific mating could not occur. However, further experiments of mate choice and staged encounters should be done to ensure that premating reproductive isolation may actually prevent hybridization between island and mainland lizards.

Acknowledgments We thank 'El Ventorrillo' MNCN Field Station for using their facilities, the Generalitat Valenciana (GV) and the Secretaría General de Pesca Marítima (MAPA) for permission to work in the islands and to collect the lizards. We also thank to the game keepers of the Natural Park of Columbretes for their help in capturing lizards, to Pep (Cruz Roja del Mar) for facilitating a cool room to keep the lizards before transportation, and to the boats CAT-CAT and Clavel I for transportation. Financial support was provided by an 'El Ventorrillo' CSIC grant to MG, by a contract "Ramón and Cajal" (CSIC, MEC) to AMC, and by the MEC projects CGL2005-00391/BOS and MCI-CGL2008-02119/BOS. Captures and experiments were performed under license from the Environmental Agencies of the Madrid Government ("Consejería del Medio Ambiente de la Comunidad de Madrid", Spain) and by the GV.

References

Aitchison, J. 1986. The Statistical Analysis of Compositional Data: Monographs in Statistics and Applied Probability. Chapman and Hall, London.

Alberts, A.C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. Am. Nat. **139**: 62–89.

Alberts, A.C. 1993. Chemical and behavioral studies of femoral gland secretions in iguanid lizards. Brain Behav. Evol. **41**: 255–260.

Andersson, M. 1994. Sexual Selection. Princeton University Press. Princeton, New Jersey.

Aragón, P., López, P., and Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Aragon, P., Meylan, S., and Clobert, J. 2006. Dispersal status-dependent response to the social environment in the Common Lizard, *Lacerta vivipara*. Funct. Ecol. **20**: 900–907.

Arnold, E.N., and Ovenden, D.W. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Harper Collins, London.

Barbadillo, L.J., Lacomba, J.I., Pérez-Mellado, V., Sancho, V., and López-Jurado, L.F. 1999. Anfibios y Reptiles de la Península Ibérica, Baleares y Canarias. Geoplaneta, Barcelona.

Barbosa, D., Desfilis, E., Carretero, M.A., and Font, E. 2005. Chemical stimuli mediate species recognition in *Podarcis* wall lizards. Amphib.-Rept. **26** : 257–263.

Barbosa, D., Font, E., Desfilis, and E., Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature **411**: 944–947.

Carazo, P, Font, E., and Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895-902.

Castilla, A.M. 2000. Among-islet variation in color, morphological and scalation characters in *Podarcis atrata* from the Columbretes Archipelago, Mediterranean Sea. J. Herpetol. **34**: 142–146.

Castilla, A.M., and Bauwens, D. 1991. Observations on the natural history, present status, and conservation of the insular lizard *Podarcis hispanica atrata*. Biol. Cons. **58**: 69–84.

Castilla, A.M., and Bauwens, D. 2000. Reproductive characteristics of the lacertid lizard *Podarcis atrata*. Copeia **2000**: 748–756.

Castilla, A.M., and Herrel, A. 2009. The scorpion *Buthus occitanus* as a profitable prey for the endemic lizard *Podarcis atrata* in the volcanic Columbretes islands (Mediterranean, Spain). J. Arid Environm. **73**: 378–380.

Castilla, A.M., Fernández-Pedrosa, V., Backeljau, T., González, A., Latorre, A., and Moya, A. 1998a. Conservation genetics of insular *Podarcis* lizards using partial cytochrome b sequences. Mol. Ecol. **7**: 1407–1411.

Castilla, A.M., Fernández-Pedrosa, V., Harris, J.D., González, A., Latorre, A., and Moya, A. 1998b. Mitochondrial DNA divergence suggests that *Podarcis hispanica atrata* (Squamata: Lacertidae) from the Columbretes islands merits specific distinction. Copeia **1998**: 1037–1040.

Castilla, A.M., Herrel, A., and Gosá, A. 2008c. Mainland versus island differences in behaviour of *Podarcis* lizards confronted with dangerous prey: the scorpion *Buthus occitanus*. J. Nat. Hist. **42**: 2331–2342.

Castilla, A.M., Vanhooydonck, B., and Catenazzi, A. 2008d. Feeding behavior of the Columbretes lizard *Podarcis atrata*, in relation to the marine species, *Ligia italica (Isopoda, Crustaceae)*. Belgium J. Zool. **138**: 146–148.

Cooper, W.E., Jr., and Pérez-Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. A **292**: 523–527.

Cooper, W.E., Jr., and Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Dietemann, V., Peeters, C., Liebig, J., Thivet, V., and Hölldobler, B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc. Natl. Acad. Sci. USA **100**: 10341–10346.

Escobar, C.A., Labra, A., and Niemeyer, H.M. 2001. Chemical composition of precloacal secretions of *Liolaemus* lizards. J. Chem. Ecol. **27**: 1677–1690.

Escobar, C.M., Escobar, C.A., Labra, A., and Niemeyer, H.M. 2003. Chemical composition of precloacal secretions of two *Liolaemus fabiani* populations: are they different? J. Chem. Ecol. **29**: 629–638.

Gabirot, M., Castilla, A.M., López, P., and Martín, J. 2010. Chemosensory species recognition may reduce the frequency of hybridization between native and introduced lizards. Can. J. Zool.

Harris, D.J., and Sá-Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., and Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phyl. Evol. **23**: 75–81.

Heth, G., and Todrank, J. 2000. Individual odour similarities across species parallel phylogenetic relationships in the *S. erenbergi* superspecies of mole rats. Anim. Behav. **60**: 789–795.

Heth, G., Todrank, J., Busquet, N., and Baudoin, C. 2001. Odour-genes covariance and differential investigation of individual odours in the *Mus* species complex. Biol. J. Linn. Soc. **73**: 213–220.

Johnston, R.E. 2005. Communication by mosaic signals: individual recognition and underlying neural mechanisms. In: Mason, R.T., LeMaster M. P., Müller-Schwarze, D. (Eds.), Chemical Signals in Vertebrates, Vol. 10. Springer, New York, pp. 269-282.

LeMaster, M.P., and Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red-sided garter snakes, *Thamnophis sirtalis parietalis*. J. Chem. Ecol. **29**: 1027–1043.

López, P., and Martín, J. 2001. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards, *Podarcis hispanica*. Ethology **107**: 901–912.

López, P., Martín, J., and Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

Martín, J., and López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., and López, P. 2006b. Pre-mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., and López, P. 2006c Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

Martín, J., Moreira, P.L., and López, P. 2007. Status-signaling chemical badges in male Iberian rock lizards. Funct. Ecol. **21**: 568–576.

Mason, R.T. 1992. Reptilian pheromones. In: Gans, C., Crews, D. (Eds.), Biology of the Reptilia, Vol. 18. University of Chicago Press, Chicago, pp. 114–228.

Müller-Schwarze D. 2006. Chemical ecology of Vertebrates. Cambridge University Press, Cambridge.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., and Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. B **270**: 254–S256.

Panhuis, T.M., Butlin, R., Zuk, R.M., and Tregenza, T. 2001. Sexual selection and speciation. Trends Ecol. Evol. **16**: 364–371.

Pinho, C., Harris, D.J., and Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex: the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Lin. Soc. **91**: 121–133.

Shine, R., Reed, R.N., Shetty, S., LeMaster, M., and Mason, R.T. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution **56**: 1655–1662.

Smadja, C., and Butlin, R.K. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity **102**: 77–97.

Symonds, M.R.E., and Elgar, M.A. 2009. The evolution of pheromone diversity. Trends Ecol. Evol. **23**: 220–228.

Talley, H.M., Laukaitis, C.M., and Karn, R.C. 2001. Female preference for male saliva: implications for sexual isolation of *Mus musculus* subspecies. Evolution **55**: 631–634.

Weldon, P.J., Flachsbarth, B., and Schulz, S. 2008. Natural products from the integument of nonavian reptiles. Nat. Prod. Rep. **25**: 738–756.

RESULTADOS y DISCUSION integrados

- Parte 1 - Morfología y señales químicas de las lagartijas

La lagartija Ibérica *Podarcis hispanica* pertenece a un taxón muy complejo y sobretodo "inestable". Esta especie se compone de individuos con morfología similar en general pero con unas variaciones intrapoblacionales sustanciales. En la región de Madrid por su localización, su historia paleo-geográfica y su dispersión, se encuentran diferentes poblaciones con una variedad de morfologías y coloraciones; algunas de estas poblaciones viven en localidades muy cercanas sin aislamiento geográfico efectivo (Gabirot et al. datos no publicados). Durante esta tesis, hemos estudiado individuos (hembras y machos) de varias poblaciones en la región de Madrid. Las poblaciones están separadas entre sí por entre 10 y 100 Km. de distancia: las más alejadas son las de Fuenfría, Pedrezuela y Aranjuez. Los individuos de Fuenfría se encuentran en el noroeste, en la parte de la Sierra de Guadarrama, Pedrezuela en el Norte debajo del Puerto de Somosierra, y Aranjuez en el extremo Sur, cercana a Toledo. A estas tres poblaciones añadimos otras dos intermedias: Golondrina, justo al Sur de Fuenfría, y Belmonte al este y cerca de Aranjuez. Las poblaciones las hemos clasificado como del Norte (Fuenfría, Golondrina y Pedrezuela), o del Sur de Madrid (Aranjuez y Belmonte).

Comparación morfológica (Capítulo 1)

Además del dimorfismo sexual, en estas poblaciones de la zona de Madrid existen otras diferencias a nivel morfológico: los individuos del Sur y los del Norte son muy distintos. Las poblaciones de Fuenfría y Golondrina, más la de Pedrezuela (i.e. del Norte) se caracterizan por tener individuos más robustos, con cuerpos y cabezas más grandes, y con un peso mayor. Al contrario los individuos del Sur (Aranjuez y Belmonte) se caracterizan por tener individuos más estilizados, con cuerpos y cabezas más pequeños, y menos pesados. Se observó un gradiente entre los dos extremos geográficos (de Fuenfría a Aranjuez). Un gradiente de tamaño, peso, y tamaño de la cabeza, que va de los más grandes en el Norte hasta los más pequeños en el Sur.

Los individuos del Norte tienen un número mayor de poros femorales que los individuos del Sur. Al contrario, los individuos del Norte tienen menos ocelos azules laterales que los del Sur. Estos ocelos azules podrían ser una señal visual utilizada en los procesos de comunicación y de selección intrasexual (López et al. 2004). Muchos animales tienen colores llamativos como indicadores potenciales de calidad individual porque esta coloración tiene una respuesta rápida a los cambios de condiciones (Lozano 1994; Favre et al. 2003). En algunas lagartijas, en los géneros *Lacerta* y *Podarcis* por ejemplo, el macho muestra unos ocelos pequeños laterales situados justo después de las patas delanteras (Barbadillo et al. 1999). En *Lacerta monticola*, el número de estos ocelos está relacionado con el tamaño corporal, la edad, la condición corporal y la dominancia (machos más viejos) (López et al. 2004). Existe dimorfismo sexual en este carácter y por eso tendrían un papel importante durante la selección sexual (López et al. 2004).

Relación de la morfología y el hábitat

Estas diferencias de morfología entre las poblaciones se explicarían porque las poblaciones ocupan distintos hábitats, localizaciones geográficas y/o condiciones climatológicas diferentes. La variación del tamaño corporal de los individuos de muchos animales estaría relacionada con el factor climático. Muchas especies tienden a tener un tamaño de cuerpo que sigue la regla de Bergman: los individuos en condiciones frías tienen un cuerpo mayor que los de zonas más cálidas (Yom-To y Nix 1986). Entonces, en la población de Fuenfría, la selección natural presionaría en dirección a alcanzar tamaños corporales mayores que permitirían una mejor eficacia frente a las condiciones abióticas del hábitat. Además los animales en condiciones ambientales frías tendrían una coloración adaptada para poder captar los rayos de sol o el calor con más eficacia. También la iluminación típica de cada hábitat sería importante en relación a las señales visuales y su evolución (Endler y Thery 1996). Como complemento se examinaría la coloración de los individuos de cada población, para ver si también a este nivel visual existen variaciones y si están de acuerdo con lo esperado por la adaptación al clima local. Así, en un hábitat más cálido, como es el caso de Aranjuez, un tamaño más pequeño y una coloración más reflectante (o menos absorbente) serían más eficaces para la termorregulación y también para disminuir el riesgo de depredación en este tipo de hábitat.

Variación de las señales químicas (Capítulo 1)

Las secreciones femorales de los machos de cada población se componen en su mayoría de esteroides, ácidos carboxílicos, alcoholes y otros compuestos como el escualeno (Tabla 1; Capítulo 1). El compuesto más abundante es el colesterol, seguido por el colesta-5,7-dien-3-

ol, y después por el ácido hexadecanoico, el campesterol y el ácido octadecenoico. Esto está de acuerdo con lo que se ha encontrado en otras especies de lagartijas (Weldon et al. 2008). Existen diferencias entre las poblaciones estudiadas en la presencia/ausencia de algunos compuestos químicos en las secreciones femorales de los machos. Por ejemplo, las lagartijas del Norte no tienen colest-5-en-3-one, ni tampoco los etil-ésteres de ácidos carboxílicos que sí aparecen en las del Sur; o también las secreciones de los machos de Aranjuez no contienen octadecanol, al contrario que todas las otras poblaciones.

La composición de las señales químicas varían según las poblaciones estudiadas; cada población tiene su propia composición, y también proporción, de compuestos químicos en las secreciones femorales. Esta alta variación podría ser una adaptación local al hábitat donde vive cada población. Estas secreciones son dependientes de la alimentación y una variación en los nutrientes provocaría una síntesis diferente al nivel de las glándulas femorales como por ejemplo ocurre en las moscas *Drosophila serrata* las cuales modifican los compuestos químicos cuticulares cuando se les modifica la dieta (Symond et al. 2009; Rouault et al. 2004). Entonces, en cada hábitat se encontrarían nutrientes diferentes; las fuentes de alimentación varían además con las zonas geográficas pero también con la estación, con el clima, con el tipo de hábitat. etc. Las secreciones serían un reflejo instantáneo de lo que pasa en el organismo: el individuo consume un tipo de nutriente, entra en su organismo y en sus células, y finalmente sería secretado por las glándulas femorales. Así que una parte de esta variación química entre las poblaciones vendría de las posibles diferencias en la dieta. Pero para comprobar esto, se requieren más estudios de comparación del hábitat y de la dieta

Además, para algunos compuestos de las secreciones femorales, se encontró una relación con la clasificación Norte vs. Sur. Algunos esteroides como el colesta-5,7-dien-3-ol, el ergosta-5,8-dien-3-ol, el tetracosanol y el escualeno se hallan en mayor proporción en las secreciones de los machos de las poblaciones del Norte que en las del Sur. Al contrario, el colesterol está en mayor proporción en las del Sur. Estas variaciones entre las zonas Norte y Sur podrían estar relacionadas con las diferencias de microclimas entre estas áreas geográficas (Alberts 1992; Escobar et al. 2001,2003). Las lagartijas del Norte ocupan hábitats con un nivel de humedad alto y temperaturas bajas; al contrario las del Sur se localizan en sitios muy secos y calurosos. Como la persistencia de las marcas químicas es necesaria para su eficacia en el hábitat, en cada zona se produciría una selección de los compuestos que permiten una mayor duración de las señales químicas, y por eso podríamos observar diferencias según el hábitat (Norte vs. Sur) con condiciones abióticas distintas.

La selección de compuestos tendente a una mejor eficacia de las señales químicas utilizadas en la comunicación intraespecífica llegaría a producir diferencias en la composición de las secreciones femorales entre las distintas poblaciones de los machos de lagartija Ibérica que viven en medios ambientales diferentes; con moléculas menos volátiles y estables en los hábitats donde la evaporación es elevada.

Algunos de los compuestos de las secreciones tienen un papel en la comunicación entre los individuos y especialmente pueden informar sobre el estado de salud de los machos, lo que serviría a las hembras para elegir su pareja potencial de reproducción. Estudios anteriores han sugerido que en esta especie el colesta-5,7-dien-3-ol sería una señal para las hembras que emplearían en su elección de pareja; las hembras lo reconocen y distinguen mediante su sistema quimiosensorial dentro de las marcas químicas de los machos. El colesta-5,7-dien-3-ol es un precursor de la vitamina $D_{3,}$ con importantes funciones metabólicas (absorción del calcio y regulación del sistema inmune) (Griffin et al. 2003; Hayes et al. 2003). Es interesante que este compuesto aparezca en mayor proporción en las lagartijas de las poblaciones del Norte que en las del Sur. Eso podría interpretarse como que los individuos del Norte secretan más colesta-5,7-dien-3-ol porque la comunicación intraespecífica depende más de este compuesto. Al contrario los animales del Sur no lo utilizarían tanto, o bien podrían depender más de otros tipos de señales. Esto último, lo sugiere la comparación de la importancia de las señales visuales (i.e., ocelos azules) y químicas (número de poros femorales) entre las poblaciones. Los machos del Sur tienen un mayor número de ocelos azules, pero menos poros femorales, que los del Norte. Así, estas diferencias entre las poblaciones apoyarían que los individuos de cada población se han podido adaptar a las condiciones climáticas geográficas de cada sitio, lo que resultaría en un uso diferencial del tipo de señales usadas para comunicarse. Estos elementos nos permiten pensar que los individuos de la Sierra del noroeste de Madrid en un hábitat con humedad y temperaturas más bajas utilizarían e invertirían más en las señales químicas que los individuos del Sur; y al revés, los individuos del Sur en un área más seca y calurosa podrían utilizar más otro tipo de comunicación como la visual.

En resumen, las características del hábitat donde se encuentran las poblaciones jugarían un papel importante en la evolución de las variaciones de morfología y señales químicas encontradas en las poblaciones de la zona de Madrid; las condiciones ambientales de cada sitio afectarían a cada población y conducirían a distintos procesos evolutivos. Las poblaciones estudiadas en la región de Madrid son diferentes a varios niveles: morfológico y señales químicas. Todas estas diferencias caracterizadas entre las poblaciones también se observarían a nivel genético, pero se necesitan más análisis moleculares para confirmarlo. Esta adaptación a un hábitat diferente también afectaría a la comunicación intraespecífica y a las señales involucradas. Estas poblaciones que viven en áreas con condiciones climáticas y geográficas distintas desarrollarían tácticas diferentes o comunicación a base de señales de tipos diferentes, cada una adaptada a su medio ambiente. Estas variaciones en las señales podrían tener consecuencias a nivel de la comunicación inter- e intrasexual, afectando a la capacidad de reproducción entre poblaciones, como ocurre con los peces del género *Pseudotropheus* de los lagos Malawi o Victoria (Danley et al. 2001).

- Parte 2 - Comunicación química y comportamiento interpoblacional

Reconocimiento químico interpoblacional (Capítulo 2)

Los resultados de los experimentos de reconocimiento químiosensorial mostraron claramente que cada población de la región de Madrid estudiada distinguiría los olores de lagartijas de un control no oloroso (agua), pero además, los individuos respondieron de forma distinta según el origen poblacional del olor presentado. Los machos tienen un interés mayor (i.e., un mayor número de protusiones linguales) por los olores de machos de su propia área (Norte vs. Sur). Los machos de las poblaciones del Norte (Fuenfría, Golondrina y Pedrezuela) sacaron más la lengua en respuesta a olores de machos del Norte, y lo contrario para las lagartijas del Sur (Belmonte y Aranjuez). Sin embargo, únicamente hemos encontrado un interés mayor por olores de machos de su propia población, en los machos de Fuenfría, pero en el resto sólo se observó un reconocimiento y mayor interés por olores de machos de su área, pero sin distinguir los machos de su propia población de las otras de su área.

También, los machos discriminaron entre olores de hembras según su población de origen. De la misma manera encontramos una preferencia de área: es decir que los machos de las zonas del Norte hicieron más protusiones linguales en respuesta a olores de hembras de las poblaciones del Norte, y lo contrario para los machos del Sur. Sin embrago, encontramos una diferencia para las poblaciones más extremas (i.e., Aranjuez, Fuenfría y Pedrezuela); los machos de estas poblaciones tuvieron una discriminación y un interés mayor por los olores de hembras de su propia población que por los olores de las hembras de otras poblaciones; manteniendo el interés relativo por hembras de su zona. Por ejemplo, los individuos de Aranjuez tuvieron respuestas más elevadas a olores de hembras de su población, después por las hembras de Belmonte, y finalmente por las hembras de las poblaciones del Norte sin diferenciar entre ellas. Para estas poblaciones extremas (a nivel geográfico) se observó primero una preferencia por olores de hembras de su población, después una preferencia por zona y finalmente un menor interés por los olores de hembras de la otra zona. Por el contrario, los machos de las poblaciones menos aisladas a nivel geográfico (i.e. Belmonte y Golondrina), no demostraron estas preferencias por olores de hembras de su propia población, sino sólo un interés mayor por olores de hembras de su zona.

Las hembras reaccionaron de manera similar a los machos en cuanto al reconocimientos de olores de machos: las hembras mostraron un reconocimiento y preferencia de olores según el origen de la población únicamente según su zona (Norte vs. Sur). Las hembras del Norte sacaron más veces la lengua en respuesta a olores de machos de su propia zona que a los de la otra, sin diferenciar entre el origen de las poblaciones dentro de cada zona. Sólo las hembras de Pedrezuela mostraron respuestas elevadas a los olores de machos de su población frente a olores de machos de otras poblaciones de su zona.

Por lo tanto, las lagartijas de las cinco poblaciones discriminaron y reconocieron los olores presentados según el área de origen de las secreciones. Sólo los machos de las poblaciones extremas mostraron una discriminación interpoblacional de olores de hembras; en el resto se encontró sólo un reconocimiento químico y preferencia por olores según la zona (Norte vs. Sur). Pero, el hecho de que las poblaciones e individuos no mostraran una preferencia por su población, o al contrario que haya una preferencia de zona, no significa que no puedan ocurrir cópulas entre individuos de distintas poblaciones o zonas. Las hembras podrían elegir territorios marcados con olores de machos de su propia población, o de su zona, o, por el contrario, no tener ninguna preferencia a la hora de elegir entre dos territorios con olores de machos de poblaciones o zonas distintas.

Elección de olores de machos por parte de las hembras (Capítulos 2 y 3)

El experimento de elección de olores de machos por parte de las hembras informó que la elección varía según el origen poblacional del macho y además, varía según el día del experimento. Este experimento se hizo sólo con tres de las cinco poblaciones: Fuenfría y Aranjuez (las dos extremas) y Golondrina (intermedia y cercana a la del Norte).

En el primer día, las hembras no habían tenido contacto con ningún olor de macho, ni visto ningún macho en esta temporada reproductiva, y no mostraron una preferencia por un tipo de olor; pasaron el mismo tiempo en lados del terrario con olores de machos de distintas poblaciones. Pero en el segundo día de las pruebas, las hembras tuvieron una preferencia por un olor, o bien un rechazo por el otro tipo de olor. Las hembras de las poblaciones extremas (Aranjuez y Fuenfría) disminuyeron el tiempo pasado en las zonas con olores de machos de la población más lejana a la suya. Las hembras de Aranjuez estuvieron menos tiempo en las zonas con olores de machos de la población de Fuenfría, pero mantuvieran el mismo porcentaje de tiempo pasado en los olores de machos de Aranjuez y Golondrina; y lo contrario para las hembras de Fuenfría. Sin embargo, las hembras de Golondrina no mostraron cambios en el porcentaje de tiempo pasado según el origen de los olores, ni según el día de la prueba. Por lo tanto, este experimento de elección de olores de machos por parte de las hembras sugiere que las hembras podrían tener preferencias por algunos olores según el origen de la población, pero eso sólo a partir del segundo día del experimento, tras haber tenido contacto previo con olores de machos.

Si las hembras se establecen en un territorio, independientemente del origen poblacional del macho que lo ha marcado, tendrían la misma posibilidad de reproducirse con machos de su población que con los de las otras. El primer día del experimento, las hembras no conocerían los diferentes olores, pero el segundo día ya tendrían más experiencia. Cuanto más se presenta un olor a un individuo más lo conoce, obtiene más información, y más lo reconoce. Así por ejemplo, el segundo día tras reconocer el olor de la población de Fuenfría, las hembras de Aranjuez pudieron no necesitar, o querer, más información acerca de estos machos y pasar menos tiempo en presencia de estos olores, mientras que para los olores de su población o de Golondrina su interés seguiría siendo alto. Lo mismo pasaría en las hembras de Fuenfría, cuyo interés por las secreciones de los machos de Aranjuez disminuiría con las sucesivas presentaciones.

Cópulas inter e intrapoblacionales (Capítulo 3)

Finalmente, los encuentros reproductivos y los experimentos de elección de olores indicaron que los machos y hembras de las dos poblaciones cercanas del Norte, Fuenfría y Golondrina, tienen comportamientos sexuales similares con individuos de su misma población o de la otra. Las hembras de Fuenfría y Golondrina no mostraron una preferencia por olores de machos de su misma o de la otra población. Además, en los encuentros intersexuales el número de cópulas y la duración de las cópulas fueron similares, independientemente de las poblaciones de origen de la pareja. Por lo tanto, a pesar de que entre estas dos poblaciones existen diferencias a nivel morfológico y químico, cuando se trata de reproducirse, los individuos de distintas poblaciones copularon entre ellos. Falta por saber si los huevos procedentes de cópulas intrapoblacionales son más fértiles, y/o si las crías son más viables, que los resultantes de cópulas interpoblacionales. Pero, al menos a nivel precopulatorio, no hubo diferencias, ni barreras de aislamiento entre poblaciones cercanas como Fuenfría y Golondrina.

Esta falta de aislamiento reproductivo a nivel precopulatorio se explicaría por una incapacidad de las hembras de Golondrina para discriminar entre los olores de machos de poblaciones distintas, pero de la misma zona. Los experimentos de reconocimiento quimiosensorial han señalado que en esta población los individuos podían discriminar los olores de individuos de su población de los olores de individuos del Sur, pero que entre olores de poblaciones del Norte la discriminación no está tan clara. Un estudio anterior comparando las poblaciones de Fuenfría y Golondrina sugiero que sólo los machos, pero no las hembras, podían discriminar entre olores de diferentes poblaciones (Golondrina vs. Fuenfría). Aquí encontramos los mismos resultados. Los machos sí que muestran un reconocimiento poblacional, pero las hembras sólo uno de zona. Quizás las hembras no diferenciaron entre olores de machos de poblaciones de la misma zona. Las hembras se establecerían en cualquier área marcada por cualquier macho (de su zona) y aceptarían reproducirse con todos los machos que tienen una relativa similitud genética, morfológica, de coloración, etc. Esta incapacidad podría explicar el hecho de que haya un flujo de genes y hibridación posible entre taxones del género *Podarcis*, incluso entre especies bien distintas (por ej., observaciones en laboratorio, *P. bocagei* x *P. carbonelli*, Galán 2002). Otra explicación alternativa, sería que las hembras si diferencian los olores de los machos, pero no eligen según la población de origen sino según otro criterio. Las hembras seleccionarían el olor de un macho basándose en un criterio de calidad individual (indicado por compuestos de las secreciones femorales) y no según el origen poblacional.

Los resultados han demostrado que las poblaciones más lejanas geográficamente fueron las más diferenciadas, y eso a varios niveles: morfológico, químico, y de comportamiento precopulatorio en el reconocimiento químico y elección de olores. Lo que apoya la idea de que las poblaciones en la región de Madrid están en proceso de adaptación a diferentes hábitats y a largo plazo podrían llegar a un proceso de especiación. Las distintas poblaciones podrían estar en diferentes etapas de este proceso. Sólo los machos mostraron una preferencia poblacional a nivel químico; las hembras sólo una preferencia del área de origen. Además los experimentos de elección de olores y cruces reproductivos mostraron que las hembras siguen sin diferenciar, o seleccionar, entre los tipos de machos. Pero los resultados indican que entre el Norte y el Sur de Madrid las poblaciones e individuos se reconocen, se diferencian y prefieren olores de su zona, basándose sólo en señales químicas. Pero cuando las poblaciones son de la misma zona, la comunicación se hace casi como si fueran de la misma población.

De acuerdo con la historia genética y los escenarios paleó-geológicos, la zona de Madrid tendría los dos morfotipos definidos 1 y 2. Las poblaciones estudiadas aquí mostraron que entre las del Sur y las del Norte se podía observar una discriminación química. Cabría preguntarse sobre el origen de las poblaciones estudiadas. Si las del Sur y del Norte tienen el mismo origen o si vienen de orígenes distintos. La primera posibilidad sería que ambas zonas y todas las poblaciones tengan el mismo origen, por ejemplo, con una población ancestral del Sur que se fue dispersando hacia el Norte hasta llegar a la sierra madrileña. Durante la dispersión, las poblaciones van adaptándose a cada hábitat que se encuentran. La adaptación y evolución sería gradual en la zona de Madrid. La otra hipótesis sería que las poblaciones tienen un origen distinto. Las del Norte vendrían de una población ancestral del morfotipo 1 y las del Sur de Madrid del morfotipo 2; estaría ocurriendo una doble dispersión hacia el centro de Madrid. Un análisis genético podría solucionar esta duda, pero los resultados de comportamiento y reconocimiento químico nos permiten pensar que sería más correcta la segunda hipótesis de la doble dispersión. Los individuos del Norte serían cercanos al morfotipo 1 y los del Sur al morfotipo 2, mientras que el centro de la zona madrileña sería una zona de contacto con posibilidad de híbridos entre los dos tipos.

Las observaciones de comunicación entre poblaciones cercanas como Fuenfría y Golondrina sugieren que el proceso de especiación no ha terminado, sino al contrario que está sólo empezando en esta zona. La comunicación química precopulatoria de las poblaciones de los extremos parece indicar que estas poblaciones son más específicas y están más aisladas.

- Parte 3 - Reconocimiento de compuestos de las señales

Interés diferente por los esteroides (Capítulo 4)

Sin embargo, durante los cruces interpoblacionales entre Fuenfría y Golondrina, las hembras parece que seleccionaron los olores de los machos según la proporción de un compuesto de la secreciones femorales. Las secreciones femorales de las lagartijas se componen de proteínas y lípidos, y estos últimos están involucrados en los mecanismos de comunicación (Mason 1992). En las lagartijas las marcas químicas de las secreciones de los machos podrían advertir sobre el territorio, el estatus social y la habilidad competitiva (Aragón et al. 2001; López y Martín 2002a; López et al. 2003; Martín et al. 2007), o informar sobre características de los machos que las hembras puedan utilizar para elegir una pareja (Martín y López 2000, 2006c; López et al. 2002, 2003; Olsson et al. 2003; López y Martín 2005c).

Dentro de los esteroides, el colesterol es un compuesto de origen animal que se encuentra en abundancia en las secreciones de los machos de muchas lagartijas (Alberts et al. 1992; Escobar et al. 2001; López y Martín 2005a,b,c). Estudios anteriores han mostrado que los esteroides serían importantes en la selección sexual de las lagartijas y en particular de las *Podarcis*. El reconocimiento de esteroides y en especial del colesta-5,7-dien-3-ol revela que todas las poblaciones estudiadas aquí pueden discriminar este compuesto. Además las poblaciones indicaron un interés distinto por este esteroide según el origen de los individuos. Las lagartijas de poblaciones con temperaturas frías y humedad relativa elevada (i.e., Norte de Madrid) tuvieron respuestas elevadas a este compuesto, mientras que lo contrario ocurrió en lagartijas de poblaciones que ocupan hábitats secos y cálidos (i.e., Sur de Madrid). Resultó interesante que la intensidad de protusiones linguales en respuesta al colesta-5,7-dien-3-ol se relacionaba con la abundancia de este compuesto en las secreciones de los machos de cada población. Esto sugiere una vez más que este esteroide es importante en la comunicación de las lagartijas en especial en las de la zona Norte, pero la importancia parece menor para las lagartijas del Sur, las cuales utilizarían otro tipo de señal (e.g., visual).

Finalmente, los experimentos de cruces entre las poblaciones de Fuenfría y Golondrina han sugerido que las hembras podrían utilizar como índice de calidad de un macho la proporción de un compuesto en sus secreciones femorales como una señal para decidir si reproducirse con el individuo en cuestión. Las hembras de ambas poblaciones prefieren las secreciones de machos con altas proporciones de colesta-5,7-dien-3-ol (= provitamina D_3). Este criterio de elección de pareja podría explicarse por la relación entre el colesta-5,7-dien-3-ol en las secreciones y la calidad de la respuesta del sistema inmune de los machos que existe en esta y otras especies de lagartijas (López y Martín 2005c; Martín y López 2006c; López et al. 2009). Por lo tanto podría existir un compromiso entre destinar este compuesto al metabolismo general o a las secreciones femorales. En estas poblaciones cercanas del Norte, las hembras no tienen un interes mayor por los machos de su propia poblacion, sino que más bien eligen marcas quimicas según un criterio comun a las dos poblaciones, la abundancia de colesta-5,7-dien-3-ol.

Reconocimiento de los alcoholes (Capítulo 5)

Los resultados indicaron qué *P. hispanica* puede discriminar quimiosensorialmente esteroides como el colesta-5,7-dien-3-ol; las hembras pueden elegir su pareja en base a la proporción de este compuesto en las secreciones de los machos. Pero, a pesar que los esteroides son más abundantes en las secreciones femorales, las secreciones se componen de otro tipo de moléculas. Quizás los esteroides no sean los únicos compuestos con un papel en la comunicación y reconocimiento químico o en la selección sexual. Los alcoholes, también presentes en las secreciones, podrían también ser importantes en la comunicación química entre individuos.

En un primer paso, se probó si los individuos de *P. hispanica* eran capaces de detectar, o no, algunos de los alcoholes que se encuentran en las secreciones femorales de los machos. Los individuos discriminaron los alcoholes testados (hexadecanol, octadecanol, docosanol y tetracosanol) del control (i.e., agua), y algunos alcoholes, como el hexadecanol y el tetracosanol, despertaron un interés mayor a los individuos. Por lo tanto, de la misma manera que las lagartijas pueden reconocer los esteroides en las secreciones femorales, también lo harían con algunos alcoholes.

Las lagartijas sacaron la lengua con mucho más interés frente al tetracosanol o lignoceril alcohol (i.e. alcohol graso con 24 carbonos). Este compuesto podría ser importante en la señalización química de la condición de un macho, ya que estudios previos han sugerido que el tetracosanol disminuiría el nivel de colesterol afectando a la oxidación LDL en mamíferos (Hagiwara 1987, Shimura et al. 1987; Hagiwara 1987). Pero de momento no se conoce el papel y los efectos de este compuesto químico en los reptiles.

Además, las lagartijas detectaron y tuvieron un cierto interés por los alcoholes hexadecanol, octadecanol y docosanol, pero no hubo una clara discriminación entre ellos. De modo que considerarían estos alcoholes como muy parecidos o idénticos. Esto se explicaría porque estos compuestos tienen una conformación química y una masa muy similar (cadenas de hidrocarbonos con 16 a 20 carbonos) además sus proporciones en las secreciones femorales de los machos también son muy parecidas; lo que proporcionaría una distinción más complicada aun entre estos alcoholes. También es posible que no solamente fuera un alcohol en concreto el que interesara a las lagartijas como señal, sino un complejo o una mezcla de varios alcoholes en proporciones y concentraciones variables. El hexadecanol además del octadecanol tendrían una función en la señalización de la dominancia en la lagartija *Lacerta. m. montícola* (Martín et al. 2007); el hexadecanol sería una señal del estatus de dominancia de los machos: sólo los machos con comportamiento más agresivo tienen un nivel alto de este alcohol.

En nuestro estudio, únicamente se encontraron diferencias de interés quimiosensorial entre las poblaciones por el hexadecanol: los machos de Fuenfría mostraron más interés por el hexadecanol que los de Golondrina. Esta diferencia podría venir del hecho que en sus secreciones también existieran diferencias en este compuesto, sin embargo las proporciones en las secreciones femorales son muy parecidas. Por tanto es posible que estas dos poblaciones utilicen de manera distinta este compuesto y su información. Este resultado es un dato más para clasificar como diferentes las poblaciones dentro de la zona de Madrid.

En resumen, la lagartija *P. hispanica* muestra un interés por los alcoholes presentados en este trabajo. Tal vez estos compuestos son una señal química y transmiten información a los individuos, pero de momento no se puede concluir si tienen un papel de comunicación sobre el estado fisiológico o de salud del individuo, o sobre la especie, etc. Además, son necesarias más pruebas con el hexadecanol y el octadecanol para comprobar si existe también en esta especie una relación de estos compuestos con el estatus de dominancia de los machos. Los alcoholes serían una oportunidad más para informar sobre la identidad y calidad de un macho, pero también podrían reforzar el mensaje de otras señales químicas.

- Parte 4 - Relación entre el sistema inmune y las señales químicas (Capítulo 6)

Numerosos animales utilizan señales químicas en la selección sexual. Pero existen dudas sobre cómo han evolucionado estos caracteres sexuales como señales honestas de la condición de los individuos. Los ornamentos y señales costosos podrían sufrir un compromiso entre mantener un buen sistema inmune y la elaboración de estos caracteres sexuales secundarios. En este capítulo hemos examinado experimentalmente la relación entre el sistema inmune y las señales químicas de los machos de una población de *P. hispanica*, activando su sistema inmune con una inyección de un antígeno bacteriano (LPS, lipopolisacáridos de la pared celular de *E. coli*), pero sin efectos patógenos sobre los individuos.

Los resultados indicaron que la activación del sistema inmune modificaba la proporción de algunos compuestos en las secreciones femorales de los machos. Estos cambios sugieren la posibilidad de un compromiso entre la activación del sistema inmune y la elaboración de las señales químicas de esta especie.

En las secreciones femorales de los machos, las proporciones de colesta-5,7-dien-3-ol disminuyeron después de la activación inmunológica. Esto se explicaría si el aumento de los costes del sistema inmune debido a su activación, llevaran a una imposibilidad de los individuos para destinar suficiente colesta-5,7-dien-3-ol a las secreciones, sin modificar negativamente las funciones metabólicas en las que participa este esteroide. Este trabajo confirma que las secreciones femorales y la proporción de este compuesto estarían relacionados con el estado de salud en esta y otras especies de lagartijas (López y Martín 2005c; Martín y López 2006c). Otro estudio mostró que un suplemento experimental de testosterona en la misma especie implicó una disminución de este esteroide en las secreciones femorales de los machos (Martín et al. 2007). Además las hembras son capaces de detectar cambios en la proporción de este compuesto en las secreciones de los machos (Martín y López 2006d,e), y las hembras prefieren olores de machos con altas proporciones de este compuesto (López y Martín 2005c). Las proporciones de colesta-5,7-dien-3-ol en las señales químicas de los machos se relacionarían con el estado de salud de los machos, lo que podría ser detectado por las hembras. Al contrario, el colesterol no se vio afectado por la activación inmunológica, lo que apoya la idea de que este compuesto (el esteroide más abundante en las secreciones) seria únicamente una matriz apolar no reactiva que ayudaría a mantener la eficacia de las señales de los otros compuestos de las secreciones (Escobar et al. 2003).

De acuerdo con otros estudios, las señales químicas de *P. hispanica* proporcionarían información a los demás individuos, sobre el estado de salud de los machos, existiendo un compromiso con el papel de estos compuestos químicos, en especial los de las vitaminas, en las cadenas metabólicas. De modo que las moléculas químicas secretadas por las glándulas femorales serian una señal honesta.

- Parte 5 - Comunicación química con *P. atrata* (Capítulos 7 y 8)

En las Islas Columbretes, cerca de Valencia, vive la lagartija endémica *Podarcis atrata*, la cual es una especie muy cercana genéticamente a *Podarcis hispanica*. Ambas pertenecen al mismo género y al mismo complejo de especies, y se han diferenciado hace sólo unos miles de años según el estudio filogenético de este género (Pinho et al. 2007). Las visitas de humanos a estas islas han aumentado considerablemente en los últimos años, y varios individuos de *P. hispanica* continentales han sido encontrados en las islas. Por tanto, está aumentando el riesgo de que los individuos introducidos de *P. hispanica* pudieran hibridar con la especie endémica de las islas, *P. atrata*. Por eso hemos intentado estudiar los procesos de comunicación y especialmente el reconocimiento quimiosensorial entre especies para saber si entre estas dos especies cercanas existe o no una barrera reproductiva interespecífica que impidiera la mezcla de genes, además de contemplar la comunicación química entre especies del mismo complejo de especies.

Los resultados de los experimentos de reconocimiento quimiosensorial revelaron que *P. hispanica* y *P. atrata* detectaron y discriminaron entre los olores de cada especie en base a señales químicas, y también diferencian los olores de machos y hembras del control (Capítulo 7). Los individuos de los dos sexos mostraron más interés por el olor de su propia especie que por el de la otra, lo que indicaría una clara discriminación química entre estas especies. Esto sugiere que los compuestos de las secreciones químicas de estas dos especies serian diferentes en composición y proporciones.

El análisis de las secreciones femorales de los machos de *P. atrata* reveló que las sustancias químicas mayoritarias son los esteroides, ácidos carboxílicos, alcoholes, ésteres céreos, escualeno, y algunos otros compuestos como terpenoides (Capítulo 8). Los cinco compuestos más abundantes en las secreciones son el colesterol, un éster céreo sin identificar, el ácido octadecanoico, el ácido hexadecanoico, el campesterol, y el ácido octadecenoico. Las señales químicas de las lagartijas endémicas y continentales difieren en la proporciones de los compuestos químicas, pero, sobre todo, varían en composición (i.e., presencia/ausencia): siete esteroides y dos ésteres céreos han sido encontrados en las secreciones de *P. atrata* pero no en *P. hispanica*; y al revés, nueve esteroides, tres ácidos carboxílicos, un alcohol, una furanona y un terpenoide están presentes únicamente en las *P. hispanica* continentales. Estas diferencias a nivel de las señales químicas entre estas dos especies jugarían un papel importante a la hora de reconocerse como especies distintas. Estas variaciones podrían ser debidas a una adaptación diferencial entre el hábitat de las islas y el continental, pero también serían el reflejo de la variación ya descrita a nivel genético entre estas especies.

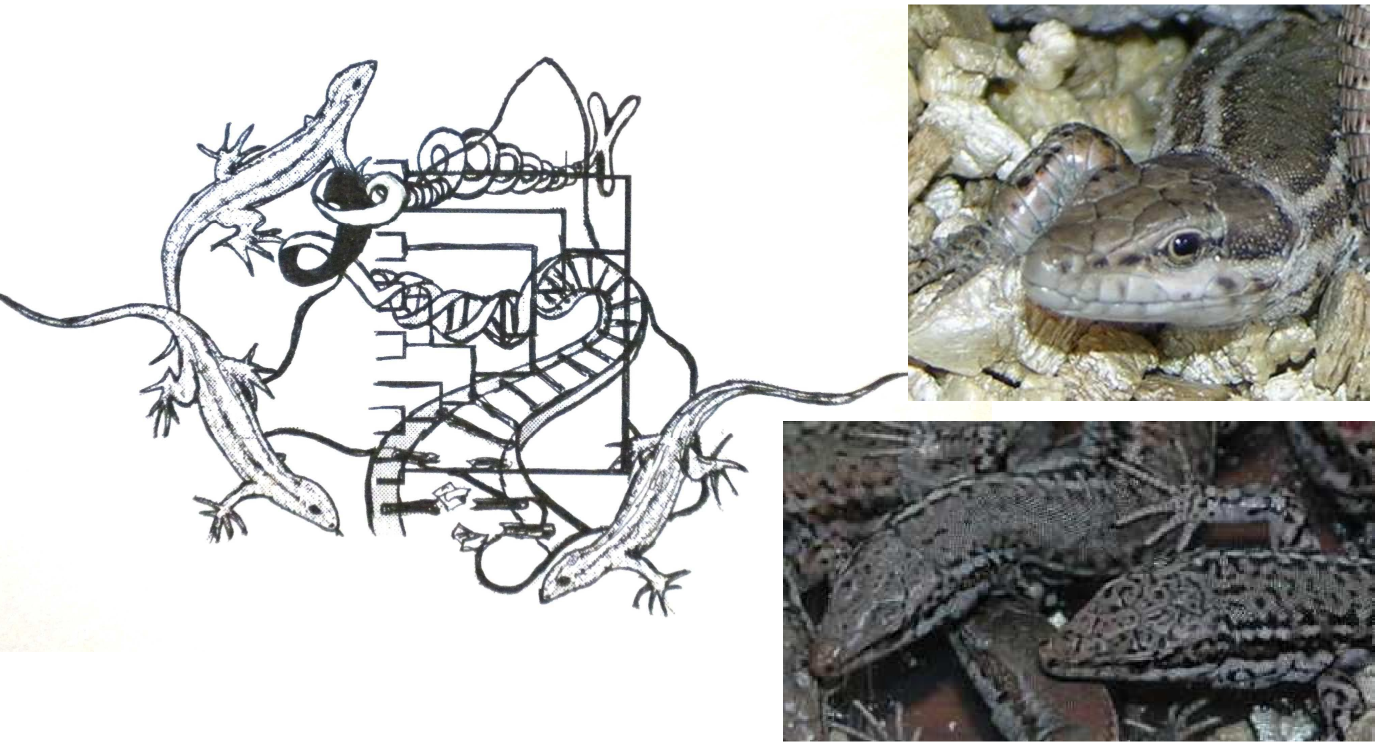
Anteriormente, hemos comparado las variaciones químicas entre poblaciones de la misma especie, poblaciones distintas a varios niveles, y encontramos diferencias significativas en la proporción y composición de las secreciones femorales de los machos, sin embargo estas diferencias se acentúan cuando analizamos dentro de este complejo, especies definidas ya como tales y no poblaciones o morfotipos.

Podarcis atrata también desarrolló un sistema de reconocimiento químico en los procesos de comunicación y de selección sexual. Los resultados de estos capítulos (7 y 8) serian unos primeros índices de la imposibilidad de reproducción entre estas dos especies insular y continental. Si los individuos insulares no reconocieran a los individuos continentales como de una especie distinta y tuvieran el mismo interés a nivel químico, entonces las cópulas entre ellos serian posibles. Pero, aunque hacen falta otros experimentos de cruces reproductivos interespecíficos, parece que ese no es el caso. A pesar de la presencia escasa de lagartijas continentales en las Islas Columbretes, de momento no se han podido observar individuos híbridos, ni tampoco se han observado comportamientos de acercamiento o comunicación entre especies.

Finalmente aunque no se crucen, ni vayan a mezclar su patrimonio genético con la especie endémica de las Islas Columbretes, la introducción de la especie continental sigue siendo peligrosa, pues puede tener consecuencias indirectas sobre la población endémica. La especie introducida podría encontrar un hábitat favorable en las islas y podría convertirse en un competidor por los recursos alimenticios o del hábitat con la especie endémica.

El riesgo de hibridación entre lagartijas de especies cercanas del continente e insulares podría no ser alto gracias a un reconocimiento químico entre especies, explicado por las diferencias en las señales químicas entre especies. Aunque existiera una conexión entre el continente y las islas, el riesgo de hibridación estaría atenuado por el posible aislamiento reproductivo y efectivo entre estas especies. El patrimonio genético de la especie insular (y endémica) está todavía protegido por esta barrera interespecífica. Pero se necesitan más experimentos de comportamiento reproductivo con cruces para apoyar estos resultados de reconocimiento químico entre especies.

CONCLUSIONES



- Las poblaciones de *Podarcis hispanica* estudiadas en la Comunidad de Madrid manifiestan diferencias morfológicas pero también a nivel de las señales químicas. Los individuos de las poblaciones del Norte son más robustos y grandes, mientras que las lagartijas del Sur son más pequeñas. Las secreciones femorales de los machos se componen en su mayoría de esteroides, alcoholes y ácidos carboxílicos. La composición y proporción de sustancias químicas varían entre poblaciones, en especial respecto al esteroide colesta-5,7-dien-3-ol. Las lagartijas que viven en las zonas serranas del norte, con humedad alta y temperaturas bajas tienen proporciones más altas de este esteroide que los machos del sur que viven en áreas más áridas y con temperaturas altas. Las características del hábitat donde se encuentra cada población conducirían a diferentes adaptaciones locales, lo que podría tener efectos en los procesos de especiación.

- La capacidad de las lagartijas para discriminar y reconocer los olores de lagartijas depende del área de origen. La distinción entre estas poblaciones de la zona de contacto de Madrid es efectiva entre las zonas del Norte y del Sur. Unicamente los machos de las poblaciones extremas muestran una discriminación interpoblacional de los olores de hembras; en el resto sólo existe un reconocimiento químico y una preferencia por olores según la zona (Norte vs. Sur). Estas preferencias por parte de las hembras podrían llevar a un aislamiento reproductivo entre poblaciones de las dos zonas.

- Pero cuando se refiere a las poblaciones más cercanas o intermedias, la discriminación y preferencias no tienen lugar. Los experimentos de cruces entre poblaciones cercanas indican que las hembras no tienen un interés mayor por los machos de su propia poblacion, sino que parecen utilizar como índice de calidad de un macho la proporción de colesta-5,7-dien-3-ol en sus secreciones femorales, independientemente de su población de origen. Los encuentros intra e interpoblacionales indicaron que los machos y hembras de dos poblaciones cercanas del Norte tienen comportamientos sexuales similares con individuos de su misma población o de la otra. De modo que el aislamiento reproductivo entre estas poblaciones cercanas no es enteramente efectivo.

- Las lagartijas de las poblaciones del Norte utilizarían el esteroide colesta-5,7-dien-3-ol, para obtener información sobre los machos, mientras que las del Sur no muestran un interés tan notable por este compuesto. Las diferencias de interés en cada población se relacionan con la abundancia de este compuesto en las secreciones de los machos de cada sitio. La comparación del número de ocelos azules laterales y poros femorales, más el análisis de la comunicación química sugieren que estas poblaciones del Norte y Sur se han adaptado a diferentes hábitats, y han evolucionado para utilizar diferentes tipos de señales: las del Norte tendrían una comunicación química más desarrollada que las del Sur, que utilizarían más otro tipo de señales como las visuales.

- Las lagartijas son capaces de detectar algunos de los alcoholes presentes en las secreciones femorales de los machos. Los individuos discriminan los alcoholes testados (hexadecanol, octadecanol, docosanol y tetracosanol) del control y algunos alcoholes, como el tetracosanol, despiertan un interés mayor. Además, los animales detectan y muestran un cierto interés por los otros alcoholes pero sin discriminar entre ellos. Es posible que no sea sólo un alcohol en concreto el que interese a las lagartijas como señal, sino una mezcla de varios alcoholes en proporciones variables. Únicamente se encuentran diferencias de interés quimiosensorial entre las poblaciones por el hexadecanol: los machos de Fuenfría muestran más interés por el hexadecanol que los de Golondrina. Los alcoholes podrían ser una oportunidad más para informar sobre la identidad y calidad de un macho, pero también podrían reforzar el mensaje de otras señales químicas.

- La activación del sistema inmune mediante la inyección de bacterias (LPS) modifica la composición química de las secreciones femorales de los machos. Las proporciones de colesta-5,7-dien-3-ol disminuyen después de la activación del sistema inmune. Esto se podría interpretar como un compromiso entre el mantenimiento del sistema inmune y la asignación de compuestos químicos, en especial el colesta-5,7-dien-3-ol, a las secreciones femorales. Este compromiso permitiría mantener la honestidad de las señales químicas.

- Finalmente, dos especies próximas (*P. atrata* y *P. hispanica*) del complejo de especies de *Podarcis hispanica*, que viven en sitios distintos y son genéticamente diferentes, son también distintas a nivel de las señales químicas. Las lagartijas insulares *P. atrata* y las continentales *P. hispanica* tienen una composición y proporción química de las secreciones femorales diferentes. Siendo además estas diferencias entre especies más importantes que las que hay entre las distintas poblaciones de *P. hispanica*. Estas varaciones químicas explicarían el reconocimiento interespecífico por parte de los individuos de ambas especies, lo que impediría los cruces reproductivos entre estas especies cercanas y permitiría la protección de la identidad genética de la especie endémica insular.

REFERENCIAS

Alberts, A.C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. Am. Nat. **139**: s62–s89.

Alberts, A.C. 1993. Chemical and behaviour studies of femoral gland secretions in iguanid lizard. Brain. Behav. Evol. **41**: 255–260.

Alberts, A.C., Pratt, N.C., y Phillips, J.A. 1992. Seasonal productivity of lizard femoral glands: relationships to social dominance and androgen levels. Physiol. Behav. **51:** 729–733.

Andersson, M. 1994. S*exual selection*. Princeton University Press. Princeton, New Jersey.

Aragón, P., López, P., y Martín, J. 2001. Chemosensory discrimination of familiar and unfamiliar conspecifics by lizards: implications of field spatial relationships between males. Behav. Ecol. Sociobiol. **50**: 128–133.

Arnold, E.N. 1973. Relationships of the Palearctic lizards assigned to the genera Lacerta, Algyroides and Psammodromus (Reptilia: Lacertidae). Bull. British Mus. (Nat. Hist.). Zool. **25**: 289–366.

Arnold, E.N. 1989. Towards a phylogeny and biogeography of the Lacertidae: relationships within an Old–World family of lizards derived from morphology. Bull. British Mus. (Nat. Hist.). Zool. **55**: 209–257.

Arnold, E.N. 2000. Using fossils and phylogenies to understand evolution of reptile communities on islands. Bönn. Zool. Monogr. **46**: 309–323.

Arnold, E.N., Arribas, O.J., y Carranza, S. 2007. Systematics of the Palaearctic and Oriental lizard tribe Lacertini (Squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. Zootaxa **1430**: 1–8.

Arnold, E.N., y Burton, J.A. 1978. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Collins, London.

Arnold, E.N., y Ovenden, D.W. 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Harper Collins, London.

Ayala, F. 2007. Darwin y el Diseño Inteligente. Alianza Editorial, S.A. Madrid.

Baird, T. A., Fox, S. E., y McCoy, J. K. 1997. Population differences in the roles of size and coloration in intra– and intersexual selection in the collared lizard, *Crotaphytus collaris*: influence of habitat and social organization. Behav. Ecol. **8**: 506–517.

Barbadillo, L.J., Lacomba, J.I., Pérez–Mellado, V., Sancho, V., y López–Jurado, L.F. 1999. Anfibios y Reptiles de la Península Ibérica, Baleares y Canarias. Geoplaneta, Barcelona.

Barbier, M. 1982. Les pheromones, aspect biochimiques et biologiques. Masson, Paris.

Barbosa, D., Font, E., Desfilis, E., y Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. J. Chem. Ecol. **32**: 1587–1598.

Bateman, A.J. 1948. Intra–sexual selection in *Drosophila*. Heredity **2**: 349–368.

Bea, A., Gosa, A., Guillaume, C.P., y Geniez, P. 1986. *Podarcis hispanica sebastiani* (Klemmer, 1964), nomen novum pro *Podarcis hispanica hispanica* (Steindachner, 1870) del Monte Urgull e Isla de Santa Clara (San Sebastian, España). Rev. Esp.Herpetol. **1**: 188–205.

Bernstein, C., y Bernstein, H. 1997. Sexual communication. J. Theor. Biol. **188**(1): 68–79.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R., y Surai, P.F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science **300**: 125–127.

Böhme, W. 1997. A note on the gender of the genus Podarcis (Sauria: Lacertidae). Bonn. Zool. Beitr. **47**: 187–8.

Böhme, W. 2004. Do endings of adjective flectible species names affect stability? A final note on the gender of *Podarcis* Wagler, 1830 (Reptilia, Lacertidae). Bonn. Zool. Beitr. **53**: 291–3.

Bonnet, X., y Naulleau, G. 1994. Utilisation d'un indice de condition corporelle (BCI) pour l'étude de la reproduction chez les serpents. C. R. Acad. Sci. **317**: 34–41.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature **411**(6840): 900–1.

Boughman, J.W. 2002. How sensory drive can promote speciation? Trends Ecol. Evol. **17**: 571–577.

Bradbury, J. W., y Vehrencamp, S. L. 1998. Principles of animal communication. Sunderland, MA, Sinauer.

Brennan, P.A., y Kendrick, K.M. 2006. Mammalian social odours: attraction and individual recognition. Philos. Trans. R. Soc. Lond. B Biol. Sci. **361**: 2061–2078.

Brongniart, A. 1837. bull. Soc. Géol. Fr. 1 t.8.

Busack, S.D. 1986. Biogeographic analysis of the herpetofauna separated by the formation of the Strait of Gilbraltar Natl. Geograph. Res. **2**: 17–36.

Capula, M. 1994a. Genetic variation and differentiation in the lizards, *Podarcis wagleriana* (Reptilia: Lacertidae). Biol. J. Linn. Soc. **52**: 177–196.

Capula, M. 1994b. Evolutionary relationships of *Podarcis* lizards from Sicily and the Maltese Islands. J. Zool. Syst. Evol. Res. **32**: 180–192.

Capula, M. 1997. Genetic divergence and phylogenetic inferences in the genus *Podarcis* (Reptilia: Lacertidae) In: Roèk Z, Hart S, eds. Herpetology 97. Abstracts III World Congress of Herpetology, Prague, Czech Republic.

Capula, M. 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). Amphib. Rept. **23**: 313–321.

Carazo, P., Font, E., y Desfilis, E. 2007. Chemosensory assessment of rival competitive ability and scent mark function in a lizard (*Podarcis hispanica*). Anim. Behav. **74**: 895–902.

Caroll, S.B. 2005. Endless forms most beautiful. The new science of Evo Devo. Norton, New York.

Carretero, M.A. 2008. An integrated assessment of a group with complex systematics: the Iberomaghrebian lizard genus *Podarcis* (Squamata, Lacertidae). Integr. Zool. **4**: 247–266.

Castilla, A.M., Fernández–Pedrosa, V., Backeljau, T., González, A., Latorre, A., y Moya, A. 1998b. Conservation genetics of insular *Podarcis* lizards using partial cytochrome b sequences. Mol. Ecol. **7**: 1407–1411.

Castilla, A.M., Fernández–Pedrosa, V., Harris, J.D., González, A., Latorre, A., y Moya, A. 1998a. Mitochondrial DNA divergence suggests that *Podarcis hispanica atrata* (Squamata: Lacertidae) from the Columbretes islands merits specific distinction. Copeia **1998**: 1037–1040.

Comte, F. 2009. Dieux et Darwin, débat sur les orignes de l'homme. Ed. JC Lattès, Paris.

Constanzo, J.P. 1989. Conspecific scent trailing by garter snakes (*Thamnophis sirtalis*) during autumn: further evidence for use of pheromones in den location. J. Chem. Ecol. **15**: 2531–2538.

Cooper, W.E., Jr., y Burghardt, G.M. 1990. A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J. Chem. Ecol. **16**: 45–65.

Cooper, W.E., y Greenberg, N. 1992. Reptilian coloration and behavior. Biology of the Reptilia, Vol 18 (eds. C. Gans and D. Crews), pp. 298–422. University of Chicago Press, Chicago.

Cooper, W.E., Jr., y Pérez–Mellado, V. 2002. Pheromonal discrimination of sex, reproductive condition, and species by the lacertid lizard *Podarcis hispanica*. J. Exp. Zool. **292**: 523–527.

Cooper, W.E., Jr., y Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). Behaviour **97**: 1–9.

Corti, C., Masseti, M., Delfino, M., y Pérez–Mellado, V. 1999. Man and herpetofauna of the Mediterranean islands. Rev. Esp. Herpetol. **13**: 83–100.

Coyne, J.A. y Orr, H.A. 2004. Speciation. Sinauer, Sunderland M.A.

Cuvier, G. 1798. Tableau élémentaire de l'histoire naturelle des animaux, in–8, pp. 3.

Danley, P.D., y Kocher, T.D. 2001. Speciation in rapidly diverging systems: lessons from Lake Malawi. Mol. Ecol. **10:**1075–1086.

Darwin, C. 1872. The expression of emotions in man and animals. Philosophical Library, New York.

Darwin, C. 1859. On the origin of species by means of Natural Selection, or the preservation of favoured races in the struggle for life. John Murray, London.

Darwin, C. 1871. The descent of man and selection in relation to sex. John Murray, London.

Dawkins. R. 1995. River out of Eden. BasicBooks, Weidenfeld and Nicolson. Harper Collins.

Dieckmann, U., Doebeli, M., Metz, J.A.J., y Tautz, D. 2004. Adaptive Speciation. Cambridge University Press, Cambridge.

Dobzhansky, T. 1937. Genetic and the origin of species. Columbia University Press, New York.

Duggen, S., Hoernie, K, van den Bogaard, P., Rupke, L., y Morgan, J.P. 2003. Deep roots of the Messinian salinity crisis. Nature **422**: 602–5.

Endler, J.A. 1980. Natural selection on color patterns in *Poecilia reticulata*. Evolution **34**: 76–91.

Endler, J.A. 1993. The color of light in forests and its implications, Ecol. Monogr. **63**: 1–27.

Endler, J.A., y Thery, M. 1996. Interacting effects of lek placement, display behavior, ambient light and color patterns in three neotropical forest–dwelling birds. Am. Nat. **148**: 421–452.

Escobar, C.M., Escobar, C.A., Labra, A., y Niemeyer, H.M. 2003. Chemical composition of precloacal secretions of two *Liolaemus fabiani* populations: are they different? J. Chem. Ecol. **29**: 629–638.

Escobar, C.A., Labra, A., y Niemeyer, H.M. 2001. Chemical composition of precloacal secretions of Liolaemus lizards. J. Chem. Ecol. **27**: 1677–1690.

Fabre, J.H. 1879. Souvenirs entomologiques.

Favre, E., François, L., Fluteau, F., Cheddadi, R., Thévenod, L., y Suc, J.P. 2007. Messinian vegetation maps of the Mediterranean region using models and interpolated pollen data. Geobios **40**: 433–43.

Faivre, B., Gregoire, A., Preault, M., Cezilly, F., y Sorci, G. 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science **300**: 29–31.

Fisher, R.A. 1930. The genetical theory of atual selection. Oxford University Press, Oxford.

Ford, N. B. 1982. Species specificity of sex pheromone trails of sympatric and allopatric garter snakes (*Thamnophis*). Copeia **1982:** 10–13.

Ford, N. B. 1986. The role of pheromone trails in the sociobiology of snakes. In: Chemical Signals in Vertebrates IV. Ecology, Evolution and Comparative Biology. Ed. by D. Duvall, D. Muller–Schwarze & R. M. Silverstein, pp. 261–278, New York: Plenum.

Ford, N.B., y Low, J.R. Jr. 1984. Sex pheromone source location by garter snakes. J. Chem. Ecol. **10**: 1193–1199.

Ford, N.B., y Schofield, C.W. 1984. Species specificity of pheromone trails in the plain garter snakes, *Thamnophis radix*. Herpetologica **40**: 51–55.

Fox, S.F., y Shipman, P.A. 2003. Social behavior at high and low elevations: Environmental release and phylogenetic effects in Liolaemus, pp. 310–355, in Lizard Social Behavior. S. F. Fox, J. K. McCoy, and T. A. Baird (eds). John Hopkins University Press, New York.

Fu, J. 1998. Toward the phylogeny of the Family Lacertidae. Implications from Mitochondrial DNA 12S and 16S gene sequences (Reptilia: Squamata). Mol. Phylog. Evol. **9**: 118–30.

Fu, J. 2000. Toward the phylogeny of the family Lacertidae–Why 4708 base pairs of mtDNA sequences cannot draw the picture. Biol. J. Linn. Soc. **71**: 203–17.

Galán, P. 2002. Hibridación en laboratorio de *Podarcis bocagei* y *Podarcis carbonelli*. Bol. Asoc. Herpetol. Esp. **13**: 28–31.

García–Paris, M., Martín, C., y Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain–Madrid: Ministerio de Agricultura, Pesca y Alimentación.

Geniez, P. 2001. Variation géographique des lézards du genre *Podarcis* (Reptilia, Sauria, Lacertidae) dans la péninsule Ibérique, l'Afrique du Nord et le sud de la France. In Mémoire présenté pour l'obtention du diplôme de l'École Pratique des Hautes Etudes Montpellier.

Gómez, A., Font, E., y Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica*. In: Lacertids of the Mediterranean Region. Ed. Valakos, W. Böhme, V. Pérez–Mellado & P. Maragoú, Helenic Zoological Society, pp.213–230, Greece, Athens.

Gómez, A., y Lunt, D.H. 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N, eds. Phylogeography in Southern European Refugia: Evolutionary Perspectives on the Origin and Conservation of European Biodiversity, pp. 155–88, Springer, Dordrecht.

Graves, B. M., Halpern, M., y Friesen, J. L. 1991. Snake aggregation pheromones: source and chemosensory mediation in western ribbon snakes (*Thamnophis proximus*). J. Comp. Psychol. **105**: 140–144.

Greene, M.J., Stark, S.L., y Mason, T. 2001. Pheromone Trailing Behavior of the Brown Tree Snake, *Boiga irregularis*. J. Chem. Ecol. **27**: 2193–2201.

Greenfield, M.D. 2002. Signalers and Receivers, Oxford University Press, Oxford.

Griffin, M.D., Xing, N., y Kumar, R. 2003. Vitamin D and its analogs as regulators of immune activation and antigen presentation. Annu. Rev. Nutrition **23**: 117–145.

Guillaume, C.P. 1987. Les Petits Lacertidés du Bassin Mediterranéen Occidental (Genera *Podarcis* et *Archeolacerta* essentiellement). PhD Thesis. Montpellier, France: Univ. Sci. Techn. Langedoc.

Hagiwara, Y. 1987 Antilipaemic agents containing hexacosanol used to treat hypertension, arteriosclerosis, diabetes mellitus, heart disease and obesity. JP A 62 099323.

Halpern, M. 1987. The organization and function of the vomeronasal system. Annu. Rev. Neurosci. **10**: 325–362.

Halpern, M. 1992. Nasal chemical senses in reptiles: structure and function. In: Gans C, Crews D, editors. Biology of the Reptilia, Vol. 18, Brain, hormones, and behavior. University of Chicago Press, p 423–523, Chicago.

Hamilton, W.D., y Zuk, M. 1982. Heritable true finess and right birds: a role for parasites? Science **218**: 384–387.

Harris, D.J., y Arnold, E.N., 1999. Relationships and evolution of wall lizards *Podarcis* (Reptilia: Lacertidae) based on mitochondrial DNA sequences. Copeia **1999**: 719–754.

Harris, D.J., Arnold, E.N., y Thomas, R.H. 1998. Relationships of the lacertid lizards (Reptilia: Lacertidae) estimated from mitochondrial DNA sequences and morphology. Proc. R. Soc. Lond. B.–Biol. Sci. **265**: 1939–1948.

Harris, D.J, Pinho, C., Carretero, M.A., Corti, C., y Böhme, W. 2005. Determination of genetic diversity within the insular lizard *Podarcis tiliguerta* using mtDNA sequence data, with a reassessment of the phylogeny of *Podarcis*. Amphib.–Rept. **26**: 401–407.

Harris, D.J., y Sá–Sousa, P. 2001. Species distinction and relationships of the western Iberian *Podarcis* lizards (Reptilia, Lacertidae) based on morphology and mitochondrial DNA sequences. Herpetol. J. **11**: 129–136.

Harris, D.J., y Sá–Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? Mol. Phylog. Evol. **23**: 75–81.

Hayes, C.E., Nashold, F.E., Spach, K.M., y Pedersen, L.B. 2003. The immunological functions of the vitamin D endocrine system. Cell. Mol. Biol. **49**: 277–300.

Howard, R.W., y Blomquist, G.J. 2005. Ecological, behavioural, and biochemical aspects of insect hydrocarbons. Annu. Rev. Entomol. **50**: 371–393.

Hsü, K.J., Montadert, L., Bernoulli, D., Bianca, C.M., Erickson, A., Garrison, R.E., Kidd, R.B., Méliéres, F., Müller, C., y Wright, R. 1977. History of the Mediterranean salinity crisis. Nature **267**: 399–403.

Johansson, B.G., y Jones, T.M. 2007. The role of chemical communication inmate choice. Biol. Rev. Camb. Philos. Soc. **8:**, 265–289.

Johnston, R.E. 2000. Chemical communication and pheromones: The types of chemical signals and the role of the vomeronasal system. In: The Neurobiology of Taste and Smell, 2nd edition. Ed. by T.E. Finger, W.L. Silver & D. Restrepo, pp. 101–127, Wiley, New York.

Johnston, R.E. 2001. Neural mechanisms of communication: from pheromones to mosaic signals. In: Chemical Signals in Vertebrates, Vol. 9. Ed. By A. Marchlewska–Koj, J. J. Lepri & D. Muller–Schwarze, Plenum, New York.

Kaliontzopoulou, A., Brito, J., Carretero, M.A., Larbes, S., y Harris, D.J. 2008. Modelling the partially unknown distribution of wall lizards *Podarcis* in North Africa: ecological affinities, potential areas of occurrence and methodological constraints. Can. J. Zool. **86**: 992–1101.

Karlson, P., y Butenandt, A. 1959. Pheromones (ectohormones) in insects. Annu. Rev. Entomol. **4:** 39–58.

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution **36**: 1–12.

Kirkpatrick, M., y Ryan, M.J. 1991. The paradox of the lek and the evolution of mating preferences Nature **350**: 33–38.

Kirschner, M.W., y Gerhart, J.C. 2005. The plausibility of life. Resolving Darwin's dilemma. Yale University Press, New Haven.

Krijgsman, W., Garcés, M., Agustí, J., Raffi, I., Taberner, C., y Zachariasse, W.J. 2000. The "Tortonian salinity crisis" of the Eastern Betics (Spain). Earth Planet. Sci. Lett. **181**: 497–511.

Kwiatkowski, M.A., y Sullivan, B.K. 2002. Geographic variation in sexual selection variation among populations of an iguanid lizard, *Sauromalus obesus* (Zater). Evolution **56**: 2039–2051.

Lamark, J.B. 1809. Philosophie Zoologique.

Lande, R. 1981. Models of speciation by sexual selection on polygenic traits. Proc. Natl. Acad. Sci. USA. **78**: 3721–3725.

Lande, R. 1982. Rapid origin of sexual isolation and character divergence in a cline. Evolution **36**: 213–223.

Lanza, B., y Boscherini, S. 2000. The gender of the genera *Podarcis* Wagler 1830 (Lacertidae), *Pelamis* Daudin 1803 (Hydrophiidae) and *Uropeltis* Cuvier 1829 (Uropeltidae). Tropic. Zool. **13**: 327–9.

Leal, M., y Fleishman, L.J. 2002. Evidence for habitat partitioning based on adaptation to environmental light in a pair of sympatric lizard species. Proc. R. Soc. Lond. B **269**: 351–359.

Leal, M., y Fleishman, L.J. 2004. Differences in visual signal design and detectability between allopatric populations of *Anolis* Lizards. Am. Nat. **163**: 26–39.

LeMaster, M.P., y Mason, R.T. 2001. Evidence for a female sex pheromone mediating male trailing behavior in the red–sided garter snake, *Thamnophis sirtalis parietalis*. Chemoecol. **11**: 149–152.

LeMaster, M.P., y Mason, R.T. 2003. Pheromonally mediated sexual isolation among denning populations of red–sided garter snakes, *Thamnophis sirtalis parietalis.* J. Chem. Ecol**. 29**: 1027–1043.

LeMaster, M.P., Moore, I.T., y Mason, R.T. 2001. Conspecific trailing behavior of red–sided garter snakes, *Thamnophis sirtalis parietalis*, in the natural environment. Anim. Behav. **61**: 827–833.

Löfstedt, C. 1990. Population variation and genetic control of pheromone communication systems in moths. Entomol. Exp. Appl. **54**: 199–218.

López, P., Aragon, P., y Martín, J. 2003. Responses of female lizards, *Lacerta monticola*, to males' chemical cues reflect their mating preference for older males. Behav. Ecol. Sociobiol. **55**: 73–79.

López, P., Gabirot, M., y Martín, J. 2009. Immune activation affects chemical sexual ornaments of male Iberian wall lizards. Naturw. **96**: 65–69.

López, P., y Martín, J. 2001a. Fighting rules and rival recognition reduce costs of aggression in male lizards, *Podarcis hispanica*. Behavioral Ecology and Sociobiology **49**: 111–116.

López, P., y Martín, J. 2001b. Pheromonal recognition of females takes precedence over the chromatic cue in male Iberian wall lizards *Podarcis hispanica*. Ethology **107:** 901–912.

López, P., y Martín, J. 2002a. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. Behav. Ecol. Sociobiol. **51**: 461–463.

López, P., y Martín, J. 2002b. Effects of female presence on intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 491–496.

Lopez, P., y Martin, J. 2005a. Chemical compounds from femoral gland secretions of male Iberian rock lizards, *Lacerta monticola cyreni*. Z. Naturforsch. C **60**: 632–636.

Lopez, P., y Martın, J. 2005b. Age related differences in lipophilic compounds found in femoral gland secretions of male spiny–footed lizards, *Acanthodactylus erythrurus*. Z. Naturforsch. C **60**: 915–920.

Lopez, P., y Martın, J. 2005c. Female Iberian wall lizards prefer male scents that signal a better cell–mediated immune response. Biol. Lett. **1**: 404–406.

López, P., y Martín, J. 2007. Scent may signal fighting ability in male Iberian rock lizards. Biol. Lett. **3**: 125–127.

López, P., Martín, J., y Cuadrado, M. 2004. The role of lateral blue spots in intrasexual relationships between male Iberian rock–lizards, *Lacerta monticola*. Ethology **110**: 543–561.

López, P., Martín, J., y Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. Aggr. Behav. **28**: 154–163.

Lozano, G.A. 1994. Carotenoids, parasites, and sexual selection. Oikos **70**: 309–311.

Macedonia, J.M., James, S., Wittle, L.W., y Clark, D.L. 2000. Skin pigments and coloration in the Jamaican radiation of Anolis lizards. J. Herpetol .**34**: 99–109.

Macías Garcia, C., y Ramirez, E. 2005. Evidence that sensory traps can evolve into honest signals. Nature **434**: 501–505.

Marler, P., y Peters, S. 1977. Selective vocal learning in a sparrow. Science **198**: 519–521.

Martín, J., y López, P. 2000. Chemoreception, symmetry and mate choice in lizards. Proc. R. Soc. Lond. B. **267**: 1265–1269.

Martín, J., y López, P. 2006a. Interpopulational differences in chemical composition and chemosensory recognition of femoral gland secretions of male lizards *Podarcis hispanica*: implications for sexual isolation in a species complex. Chemoecol. **16**: 31–38.

Martín, J., y López, P. 2006b. Pre–mating mechanisms favoring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. Evol. Ecol. Res. **8**: 643–658.

Martín, J., y López, P. 2006c. Links between male quality, male chemical signals, and female mate choice in Iberian rock lizards. Funct. Ecol. **20**: 1087–1096.

Martín, J., y López, P. 2006d. Chemosensory responses by female Iberian wall lizards, *Podarcis hispanica*, to selected lipids found in femoral gland secretions of males. J. Herpetol. **60**: 556–561.

Martín, J., y López, P. 2006e. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. Proc. R. Soc. Lond. B **273**: 2619–2624.

Martín, J., Moreira, P.L., y López, P. 2007. Status–signaling chemical badges in male Iberian rock lizards. Funct. Ecol. **21**: 568–576.

Mas, F., y Jallon, J.M. 2005. Sexual isolation and cuticular hydrocarbon differences between *Drosophila santomea* and *Drosophila yakuba*. J. Chem. Ecol. **31**: 2747–2752.

Mason, R.T. 1992. Reptilian pheromones. In: Gans, C. et Crews, D. (eds.), Biology of the reptilia. Vol. 18. Univ. Chicago Press, Chicago, pp. 114–228.

Mayer, W., y Tiedemann, F. 1980. Elektrophoretische Untersuchungen an europäischen Arten der Gattungen *Lacerta* and *Podarcis*. I. Die *Podarcis*. Formen der griechischen Inseln Milos und Skiros. Z. Zool. Syst. Evol. **18**: 147–52.

Maynard Smith, J., y Harper, D. 2003. Animal Signals. Oxford University Press, Oxford.

Mayr, E. 1942. Systematic and the origin of species. Columbia University press, New York.

McGraw, K.J. 2005. The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? Anim. Behav. **69**: 757–764.

Mellado, J., y Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, Acta Vertebrata **8**: 299–300.

Montori, A., Llorente, G.A., Alonso–Zarazaga, M.A. *et al.* 2005. Conclusiones de nomenclatura taxonomía para las especies de anfibios y reptiles de España. Asociación Herpetológica Española, Barcelona.

O'Donnell, R.P., Ford, N.B., Shine, R., y Mason, R.T. 2004. Male red–sided garter snakes, *Thamnophis sirtalis parietalis*, determine female mating status from pheromone trails. Anim. Behav. **68**: 677–683.

Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B., y Wittsell, H. 2003. Major histocompatibility complex and mate choice in sand lizards. Proc. R. Soc. Lond. B (Suppl.) **270**: S254–S256.

Paterson, H.E.H. 1985. The recognition concept of species. In E.S. Vrba. Ed. Species and Speciation, pp21–29. Transvaal Museum, Pretoria.

Pérez–Mellado, V. 1981a. La lagartija de Bocage *Podarcis bocagei* (Seoane, 1884): Primeros datos sobre su distribución, colorido y ecología. Amphib.–Rept. **3/4**: 253–268.

Pérez–Mellado, V. 1981b. Nuevos datos sobre la sistemática y distribución de *Podarcis bocagei* (Seoane, 1884) (Sauria, Lacertidae) en la Península Iberica. Amphib.–Rept. **2**: 259–265.

Pérez–Mellado, V., y Galindo–Villardón, M.P. 1986. Sistemática de *Podarcis* (Sauria Lacertidae) ibéricas y norteafricanas mediante técnicas multidimensionales. Ed. de la Universidad de Salamanca, Salamanca.

Pinho, C., Ferrand, N., y Harris, D.J. 2006. Reexamination of the Iberian and North African *Podarcis* (Squamata, Lacertidae) phylogeny based on increased mitochondrial DNA sequencing. Mol. Phylog. Evol. **38**: 266–273.

Pinho, C., Harris, D.J., y Ferrand, N. 2007. Comparing patterns of nuclear and mitochondrial divergence in a cryptic species complex, the case of Iberian and North African wall lizards (*Podarcis*, Lacertidae). Biol. J. Linn. Soc. **91**: 121–133.

Pinho, C., Harris, D.J., y Ferrand, N. 2008. Non–equilibrium estimates of gene flow inferred from nuclear genealogies suggest that Iberian and North African wall lizards (*Podarcis* spp.) are an assemblage of incipient species. BMC Evol. Biol. **8**: 63.

Pleguezuelos, J.M. 1997. *Coluber hippocrepis* Linnaeus, 1758. In: Fauna Ibérica, vol. 10: 347–358. Ramos, M.A. et al., Eds, Museo Nacional de Ciencias Naturales, C.S.I.C. Madrid.

Poulakakis, N., Lymberakis, P., Valakos, E.D., Zouros, E., y Mylonas, M. 2005. Phylogenetic relationships and biogeography of *Podarcis* species from the Balkan Peninsula, by bayesian and maximum likelihood analyses of mitochondrial DNA sequences. Mol. Phylog. Evol. **37**: 845–857.

Prentice, I.C., y Jolly, D. 2000. Mid–Holocene and glacial–maximum vegetation geography of the northern continents and Africa. J. Biogeogr. **27**: 507–519.

Regnier, F.E., y Wilson, E.O. 1971. Chemical communication and "propaganda" in slave–maker ants. Science **172**: 267–269.

Roberts, J.A., y Uetz, G.W. 2004. Chemical signalling in a wolf spider: a test of ethospecies discrimination. J. Chem. Ecol. **30**: 1271–1284.

Rouault, J.D., Marican, C., Wicker–Thomas C., y Jallon J.M. 2004. Relations between cuticular hydrocarbons (HC) polymorphism, resistance against desiccation and breeding temperature; a model for HC evolution in *D. melanogaster* and *D. simulans.* Genetica **120**: 195–212.

Ryan, M.J. 1990. Sexual selection, sensory systems, and sensory exploitation. Oxford Surv. Evol. Biol. **7**: 156–195.

Ryan, M.J. 1998. Sexual selection receiver biases and the evolution of sex differences. Science **281**: 1999–2003.

Ryan, M.J., y Rand, A.S. 1993. Sexual selection and signal evolution– the ghost of biases past. Philos. Trans. R. Soc. Lond. B. **340**:187–195.

Safran, R.J., Neuman, C. R., McGraw, K. J., y Lovette I. J. 2005. Dynamic paternity allocation as a function of male plumage color in barn swallows. Science 309: 2210–2212.

Sá–Sousa, P. 2000. A predictive distribution model for the Iberian Wall Lizard (*Podarcis hispanicus*) in Portugal. Herpetol. J. **10**: 1–11.

Sá–Sousa, P. 2001a. A Controversa Sistemática das Lagartixas do género *Podarcis* Wagler 1830 (Sauria: Lacertidae) em Portugal (PhD thesis). University of Lisboa, Lisbon.

Sá–Sousa, P. 2001b. Comparative chorology between *Podarcis bocagei* and *P. carbonelli* (Sauria, Lacertidae) in Portugal. Rev. Esp. Herpetol. **15**: 85–97.

Sá–Sousa, P., y Harris, D.J. 2002. *Podarcis carbonelli* Pérez– Mellado, 1981 is a distinct species. Amphib.–Rept. **23**: 459–68.

Sá–Sousa, P., Vicente, L., y Crespo, E.G. 2002. Morphological variability of *Podarcis hispanica* (Sauria, Lacertidae) in Portugal. Amphib. Rept. **23**: 55–69.

Schilthuizen, M. 2001. Frogs, Flies and Dandelions. Oxford University Press, Oxford.

Schwenk, K. 1995. Of tongue and noses: chemoreception in lizards and snakes. Trends Ecol. Evol. **10**:7–12.

Shimura, S., Hasegawa, T., Takano, S., y Suzuki, T. 1987. Studies on the effect of octacosanol on motor endurance in mice. Nutr. Rep. Int. **36**:10–29.

Shine, R. 2005. Life–history evolution in reptiles. Annu. Rev. Ecol. Evol. Syst. **36**: 23–46.

Shine, R., Reed, R.N., Shetty, S., Lemaster, M., y Mason, R.T 2002. Reproductive isolationg mechanisms between two sympatric sibling species of sea snakes. Evolution*,* **56**: 1655–1662.

Smadja, C., y Butlin, R.K. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity **102**: 77–97.

Sokal, R.R., y Rohlf, F.J. 1995. Biometry. Freeman, New York.

Speybroek, J., y Crochet, P.A. 2007. Species list of the European herpetofauna–a tentative update. Podarcis **8**: 8–34.

Symonds, M.R.E., y Elgar, M.A. 2004. Species overlap, speciation and the evolution of aggregation pheromones in bark beetles. Ecol. Lett. **7**: 202–212.

Symonds, M.R.E., y Elgar, M.A. 2009. The evolution of pheromone diversity. Trends Ecol. Evol. **23**: 220–228.

Tarroso, P. 2008. Late–Quaternary Landscape Dynamics in the Iberian Peninsula and Balearic Islands (MSc thesis). University of Oporto, Operto.

Trivers, R.L. 1972. Parental investment and sexual selection. In B. Campbell, ed. Sexual Selection and the Descent of Man (1871–1971), pp136–179. Heinemann, London.

van Dam, J.A. 2006. Geographic and temporal patterns in the late Neogene (12–3 Ma) aridification of Europe: the use of small mammals as paleoprecipitation proxies. Palaeogeogr. Palaeoclimat.Palaeoecol. **238**: 190–218.

Von Frisch, K. 1919. Über den Geruchsinn der Bienen und seine blütenbiologische Bedeutung. Zool. Jb. Abt. Allg. Zool. Physiol. **37**: 1–238.

Weldon, P.J., Flachsbarth, B., y Schulz, S. 2008. Natural products from the integument of nonavian reptiles. Nat. Prod. Rep. **25**: 738–756.

West–Ebergard, M.J. 1983. Sexual selection, social competition and speciation. Q. Rev. Biol. **58**: 155–183.

Whittaker, R. H., y Feeny, P. P. 1971. Allelochemics: Chemical interactions between species. Science **171**:757–770.

Wilkinson, G.S., y Reillo, P.R. 1994. Female choice response to artificial selection on an exagerated male trait in a stalk–eyed fly. Proc. R. Soc. Lond. B. **255**: 1–6.

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

Wysocki, C.J., y Meredith, M., 1987. The vomeronasal system. In: Finger, T.E., Silver, W.L. (Eds.), Neurobiology of Taste and Smell, pp. 125–150, Wiley, New York.

Xue, B., Rooney A.P., Kajikawa, M., Okada, N., y Roelofs, W.L. 2007. Novel sex pheromone desaturases in the genomes of corn borers generated through gene duplication and retroposon fusion. Proc. Natl. Acad. Sci. USA **104**: 4467–4472.

Yom–Tov, Y., y Nix, H. 1986. Climatological correlates for body size of five species of Australian mammals. Biol. J. Linn. Soc. **29**: 245–262.

Zahavi, A. 1975. Mate selecton–a selection for a hándicap. J. Theor. Biol. **53**: 205–214.

Zahavi, A. 1977. The cost of honesty (further remarks on the handicap principle). J. Theor. Biol. **67**: 603–605.

Zug, G.R., Vitt, L.J., y Caldwell, J.P. 2001. Herpetology: An Introductory Biology of Amphibians and Reptiles. 2nd ed. Academic Press, San Diego CA.