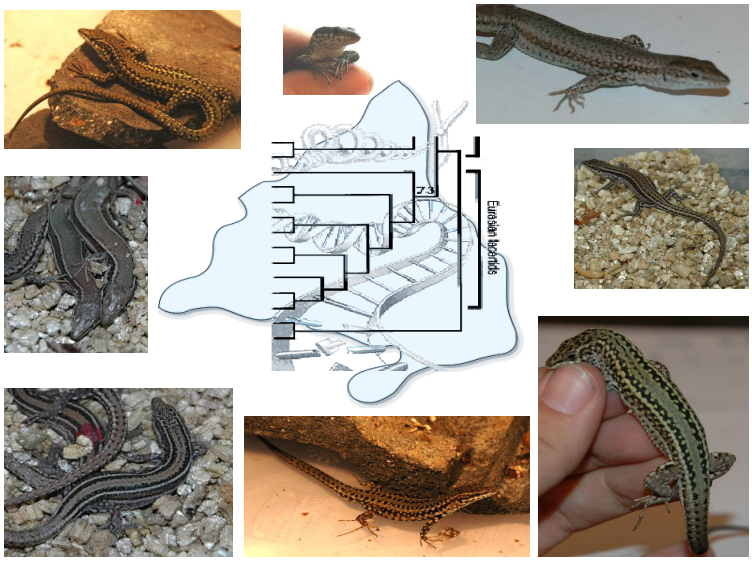
Morphological, chemical and molecular variability between populations of *Podarcis hispanica* lizards in the area of Madrid: consequences on reproductive isolation and speciation within a species complex



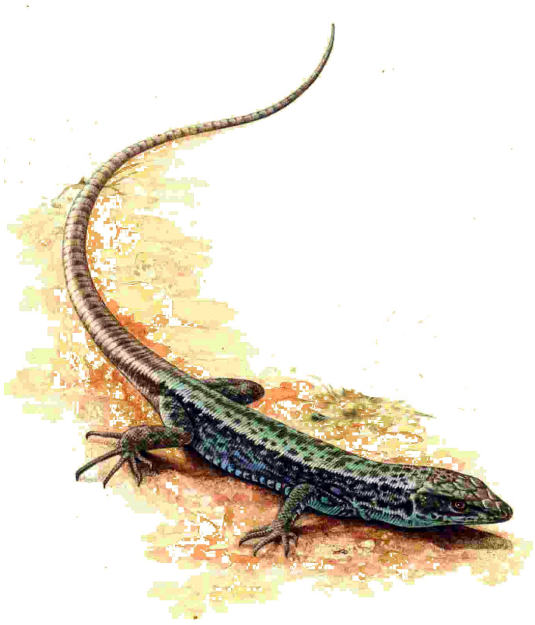
Marianne Gabirot

- Diploma de Estudios Avanzados –
- Doctorado de Comportamiento Animal y Humano –
- 2008 -

Director de investigación: JOSÉ MARTIN RUEDA
Tutor: FERNANDO COLMENARES GIL

Departamento de Psicología
Facultad de Psicología
Universidad Complutense de Madrid

Museo Nacional de Ciencias Naturales
Consejo Superior de Investigaciones



PLAN

AGRADECIMIENTOS

La parte menos científica y más personal a escribir, por lo tanto más arriesgada, si me olvido a alguien.

Primero, quisiera agradecer a José Martín y Pilar López por haberme dado esta oportunidad de trabajar y aprender de/con ellos. Por haberme hecho un hueco en su grupo de trabajo, y por tener pasencia a la hora de escribir y corregir los manuscritos.

Segundo, quisiera agradecer a mi súper ayudante de experimento, Nino. Es mi "niñera privada" de lagartijas y el "M. Giver" en la estación del Ventorrillo.

For the molecular part, I would like to thank to Staffan Bensch and lab, and especially Anna to make me see that lab experiences are not so complicate and could be funny.

Ensuite, je voudrais remercier à tous ceux qui m'ont permis de réaliser et analyser ces expériences: Isabel, Juanto, Montse, Conchi, Elisa, Oscar, Camila, Gema … et je suis sur que j'en oublie ! Plus qu'une aide professionnelle, les gars heureusement que chacun à sa manière étaient là pour me motiver, m'aider, me guider ou rassurer et bien sur me divertir ! MERCI.

Mon dernier remerciement va pour NAVACERRADA CLUB, qui m'a permis de sortir de mon isolement printanier et de me sociabiliser, car comme beaucoup d'entre nous le savent : après une bonne journée de travail avec les animaux, une bonne bière, des tapas et des taureaux sont les bien venus !

INTRODUCCION

La selección natural explicaría la evolución de caracteres supuestamente adaptados (Darwin, 1859). Darwin definió la selección natural como los procedimientos que permiten preservar los caracteres útiles para la lucha por la supervivencia con el objetivo de reproducirse. Sin embargo otros muchos caracteres parecen mal adaptados y aún así están conservados por la selección. Los caracteres sexuales de los machos, como el plumaje vistoso de algunos pájaros o un canto elaborado, podrían ser costoso en términos de supervivencia y energéticamente pero sobre todo hacen parecer a los machos más débiles (Fig. 1). Sin embargo, 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 teoria 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.

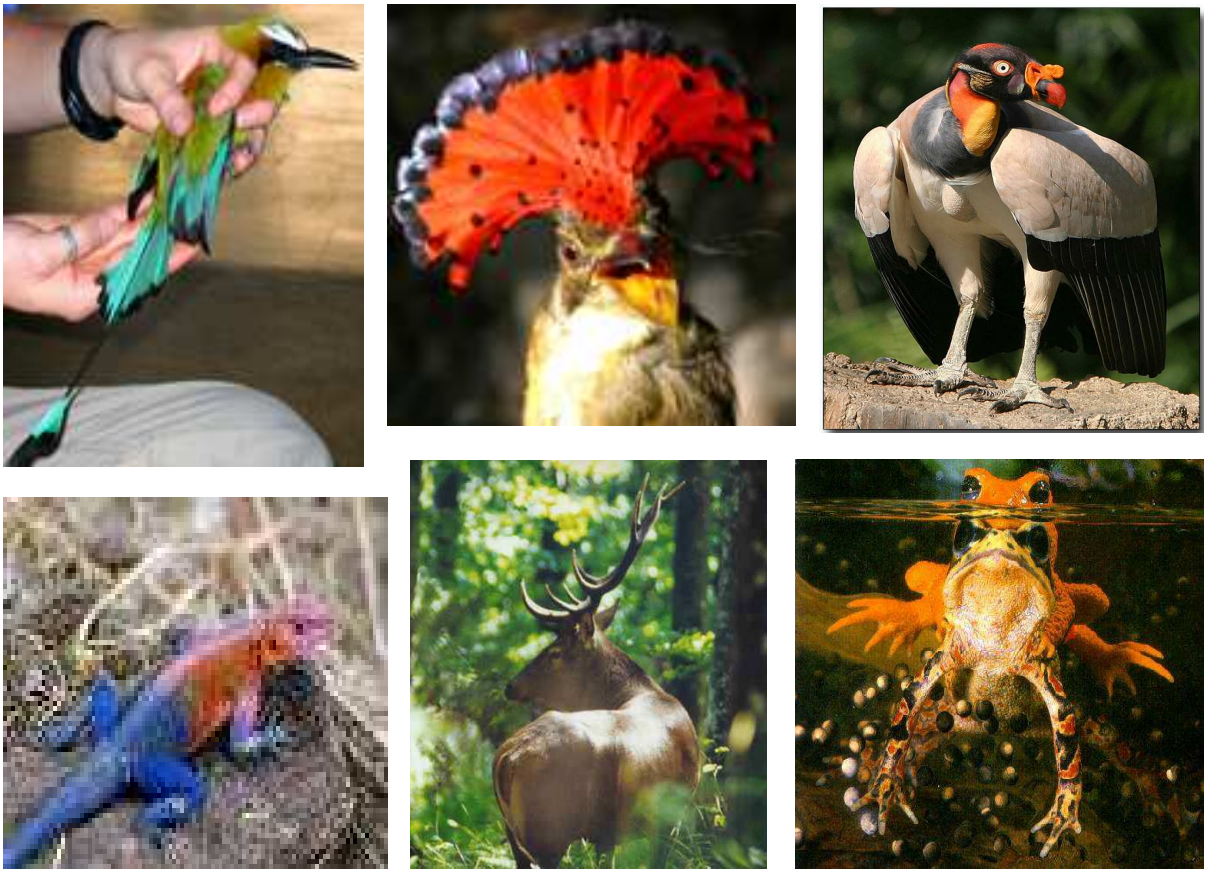


Fig. 1: Ejemplos de caracteres sexuales segundarios en aves, reptiles, mamíferos y anfibios.

Los caracteres secundarios sexuales permiten una comunicación entre machos y hembras, para facilitar el intercambio de información entre individuos. La comunicación sexual se hace gracias a señales utilizadas para aumentar, o modular, la probabilidad de interacciones sexuales entre individuos. Esta forma de comunicación sexual se encuentra en numerosos organismos desde las bacterias hasta los humanos (Bernstein C & H, 1997).

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

La quimiorrecepción es utilizada en multitud de contextos (Wyatt, 2003). En muchos animales, las señales químicas son importantes para el reconocimiento de especies y para la especiación, como en algunas moscas (Mas & Jallon, 2005). Al igual que en los invertebrados, en los reptiles las señales químicas juegan un papel muy importante. Es el caso de las lagartijas y las serpientes, que poseen un sistema sensorial químico muy bien desarrollado, donde los estímulos podrían ser la base de un reconocimiento intra-específico y contribuir a procesos de especiacion (Shine *et al*., 2002; LeMaster & Mason, 2003).
Muchas especies de lagartijas disponen, en posición ventral, de estructuras epidérmicas similares a poros por medio de los cuales secretan sustancias químicas. Se trata de secreciones holocrinas, producidas por glándulas femorales, que es especialmente abundante en los machos durante el periodo reproductivo (Fig.2) (Mason, 1992; Alberts, 1993). Se sabe que estas secreciones podrían informar sobre el estatus del macho y su habilidad competitiva (Aragon *et al*., 2001; López & Martín, 2002). También se ha visto que estas secreciones femorales podrían transmitir a las hembras información sobre la calidad del macho (Lopez *et al*., 2002; Olsson *et al*., 2003), o utilizarlo como una herramienta para el reconocimiento entre especies (Gomez *et al*., 1993; Cooper & Perez-Mellado, 2002). Por su implicación en la selección sexual, estas secreciones y su evolución podrían llevar a procesos de especiación.

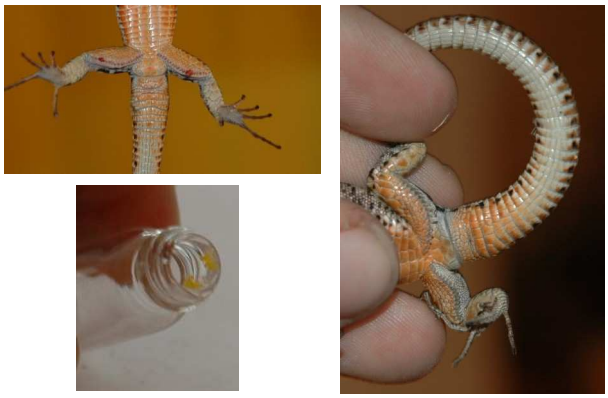


Fig. 2: Fotos de los pores femorales de la lagartija *P. hispanica* machos (arriba y a la derecha) & las secreciones que se recogen en un tubo especial para el cromatogafo con gas (abajo a la izquierda).

Además, el tipo de señales utilizadas en comunicación intraespecífica puede estar sometido a selección, seleccionándose aquellas señales que estimulen el sistema sensorial del receptor de manera más efectiva, lo que puede depender del medio ambiente (Hipótesis del "Sensory drive": Boughman, 2002). Así, la diversidad de señales observada puede evolucionar porque especies o poblaciones distintas ocupan hábitats con diferentes condiciones que favorecen el uso de uno u otro tipo de señal. Y la divergencia en las señales utilizadas en selección sexual puede deberse a que las condiciones ambientales locales imponen selección variable sobre estas señales o sobre los sistemas sensoriales que las detectan. Las diferentes propiedades de las señales emitidas por los machos afectan a su conspicuidad y facilidad de detección por conespecíficos, pero otros factores como la capacidad de transmisión o persistencia en el hábitat también son importantes (Boughman, 2002; Leal & Fleishman, 2002; Fox & Shipman, 2003). Se ha examinado esta idea respecto a señales visuales que muestran variación en diferentes hábitats (Leal & 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 si la variabilidad interpoblacional en el uso de señales químicas y visuales implicadas en selección sexual puede explicar procesos evolutivos de especiación.

En este contexto, en este trabajo se pretende examinar observacional y experimentalmente 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 señales 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.

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 & Sà-Sousa, 2001), que ocupan distintas condiciones ambientales, pero cuyas poblaciones están en contacto en diversas partes, especialmente en el Centro de la Península Ibérica (Fig. 3).

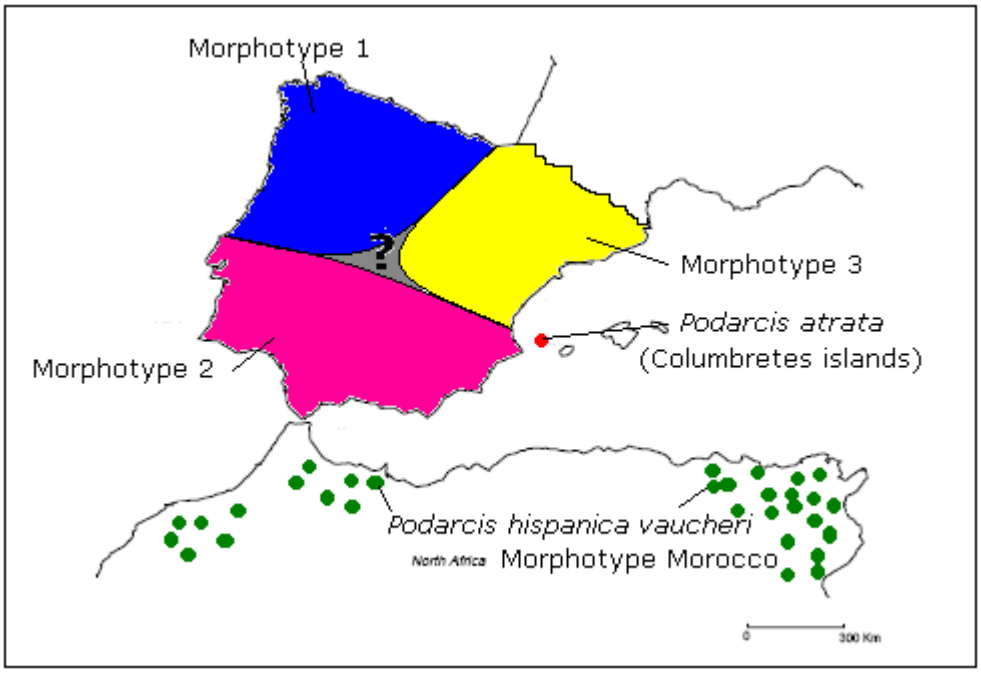


Fig. 3: Distribución de los diferentes morfotipos de *P. hispanica* en la zona ibérica & Maruecos (imagen simplificada a partir Pinho *et al*., 2008)

Esta especie ha desarrollado un sistema químico que le permite diferenciar entre especies y sexos (*Psammodromus algirus*, Gomez *et al*., 1993 - *Podarcis carbonnelli*, Cooper & Perez-Mellado, 2002). Las hembras utilizan 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.
De modo que esta "especie" 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 cuyo clima es 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 1 pueden encontrarse con los individuos de la población 2 (Mellado & Olmedo, 1981; Garcia-Paris *et al*., 1989).

Recientemente se ha demostrado, mediante análisis químico, que los machos de dos poblaciones de la Sierra de Guadarrama, situados en condiciones ambientales diferentes, poseen diferencias en la composición y proporción de secreciones (Martín & López, 2006). Los machos de tipo 1 que se encuentran en un microclima mas húmedo, tienen más ésteres céreos y una abundancia mayor de ácidos grasos de cadena larga que los de tipo 2 que viven en un medio más seco (Martín & López, 2005). Este análisis muestra 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 cómo estos compuestos se encuentran en diferentes proporciones. Los machos de cada tipo pueden discriminarse entre ellos únicamente por medio de las señales químicas (Martín & López, 2005). Sin embargo esto no está tan claro en el caso de las hembras. Diferentes experimentos de reconocimiento quimiosensorial han demostrado que las hembras no discriminan entre los dos tipos de secreciones. Sin embargo eso no significa que las hembras no muestren preferencia por un tipo de macho.

Es en este contexto en el cual este trabajo pretende estudiar y conocer el reconocimiento intra-especifico a nivel quimico y cuáles son los mecanismos de aislamiento reproductivo que podrían dar lugar a un proceso de especiación dentro este complejo de especies en la zona de Madrid.
Despues de caracterizar estas dos poblaciones del norte de Madrid a nivel quimico y morpfologico, examinaremos en laboratorio si las hembras reconocen y elijen estar en un área con olores de machos de la propia poblacion frente a otra con olor de machos de la otra pobalcion, o si las hembras seleccionan el área marcada por olores de machos segun caracteristicas individuales de los machos, independientemente de la población de origen.
Para seguir investigando el posible aislamento reproductivo entre estas dos poblaciones, haremos cruces inter y intra poblacional entre hembras y machos.
Si las hembras prefirieran reproducirse con machos de su poblacion, identificados por señales características de su poblacion, esto produciría una rapida divergencia entre las poblaciones. Sin embargo, si las hembras elejieran segun caracteristicas independemiente del origen de los machos, esto podría impedir un aislamiento reproductivo efectivo.

Ademas, en la segunda parte compararemos estas dos poblaciones con una otra poblacion de *P. hispanica* procedente de Aranjuez en el sur de Madrid (Fig. 4). Esta otra poblacion se encuentra en un habitat diferente de las del norte, con un suelo de yesos, una humedad màs baja y tempeartura màs alta. Notamos tambien que a nivel morfologico no se parece a las del norte de Madrid. Asi que caracterizaremos esta poblacion del sur al nivel morfologico, genetico y la compararemos con los rasgos de las poblaciones del norte. Observaremos tambien si existe reconocimiento quimiosensorial entre estas tres poblaciones. A partir de estos datos podremos estimar si existe un posible aislamento reproductivo entre las poblaciones más alejadas del complejo de especies de *P. hispanica* en la zona de Madrid.

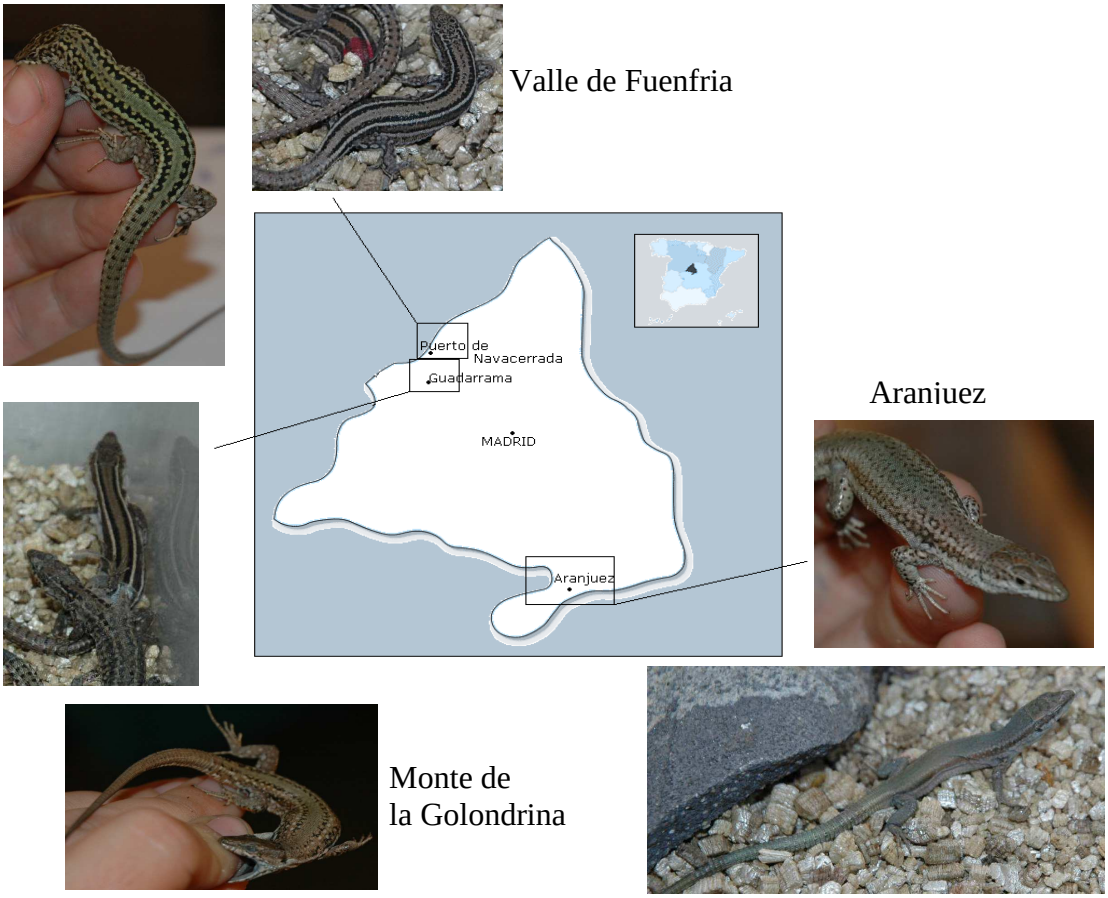
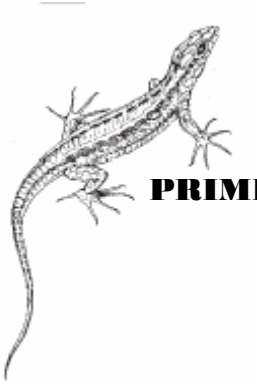


Fig. 4: zonas de capturas de los tres poblaciones de *P. hispanica* utilizadas en este trabajo: en el valle de Fuenfria (cerca del Puerto de Navacerrada), en el monte de la Golondrina (cerca de Cercedilla) & en los alrededores de Aranjuez.



PRIMERA PARTE

La elección de pareja de las hembras basada en compuestos químicos que indican calidad del macho podria impedir un aislamiento reproductiva efectivo dentro el complejo de especies de P. hispanica.

Marianne Gabirot, Pilar Lopez & José Martin

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

Marianne Gabirot, Pilar López, and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC,

José Gutiérrez Abascal 2, 28006 Madrid, Spain

Short title: Mate choice by lizards and speciation

Address correspondence to J. Martín: E-mail: Jose.Martin@mncn.csic.es.

Fax: 34-91-5 64 50 78

Fax: 34-91-5 64 50 78

No. of words in the text: 6244

Speciation is considered to result from the evolution of reproductive isolating mechanisms that prevent gene exchange between newly arising taxa (Coyne & Orr, 2004). Reproductive isolation often evolves as a consequence of divergent natural selection on traits between different habitats (Schluter, 2001; Rundle & 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 & 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 & Jallon, 2005), beetles (Symonds & Elgar, 2004) or spiders (Roberts & 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 & Mason, 2003), or in closely related species or populations of lizards (Cooper & Vitt, 1986; Barbosa *et al.* 2005, 2006; Martín & López, 2006a,b).

In many lizards, chemical cues of males, suchs 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 & López, 2006c). Thus, because chemical signals 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 & Vitt, 1986; Cooper & Perez-Mellado, 2002; Barbosa *et al*. 2005, 2006; Martín & López, 2006a,b), provided that females from different populations based their mate preferences on those male chemicals 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 & Sá-Sousa, 2001, 2002; Pinho *et al*. 2007). This 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 & Pérez-Mellado, 2002; Barbosa *et al*., 2006), and between sexes by chemical cues alone (Gomez *et al*., 1993; López & Martín, 2001; López *et al*., 2002). Chemical cues of males are important in male-male interactions (López & 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 & Martín, 2005). Therefore, it seems that female mate choice decisions are, at least partialy, 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, chemical analyses showed that two closed populations of *P. hispanica* from the Guadarrama Mountains (Central Spain), inhabiting different environmental conditions, differ in the chemical composition of femoral gland secretions of males (Martín & 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 & 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 & 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 fail to detect chemical discrimination by females in these tests does not discard that female still discriminated and preferred to stablish in areas scent marked by males of their own population, thus increasing their opportunities to mate with these males. Moreover, females may reject copulations from males from different populations based on chemical cues, or other type of additional cues. The lack of successful copulations bewteen 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, 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 her own population against areas 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 female mating preferences for certain features of a mating signal that characterized males of her own 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.

MATERIALS AND METHODS

Study animals

We captured by noosing during March, before the start of their mating season, adult *P. hispanica* lizards at two close localities in the Guadarrama Mts. (Madrid Province, Spain). In the upper part of the 'Fuenfria' Valley (40°47'N, 4°03'W, 1750 m altitude), where lizards occupy different rock-cliffs at the edge of the pine forest, we captured 10 males and 20 females, and in a large oak forest near "Cercedilla" village (40°44'N, 4°02'W, 1250 m altitude), we captured on rocky outcrops 10 males and 20 females. These two areas are only 6 km distant and they are not isolated geographically, although the intemediate area is not occupied by stable populations of *P. hispanica* (Martín & 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 & 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 licence 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 & Naulleau, 1994). We also made morphological measurements of the head of males using digital callipers (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. To estimate relative size of the head, we removed the influence of body size on head measurements by regressing each against SVL (all variables ln-transformed) and used the residuals in subsequent analyses.

We also counted under a magnifying glass the number of femoral pores on the right and left hindlimbs of males and calculated an average number for both sides. Finally, we noted the number of small but distinctive and conspicuous blue spots that runs along each of the body sides on the outer margin of the belly, and calculated an average number for both sides. These spots 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 & 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 standardised 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 & 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 (‘Fuenfria’ 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 & 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 attractive 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 & 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 occur and the differences in copulatory behavior of males depending on the population origin of 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 contrary 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 choosen 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 occured, 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; K-S P>0.1) 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 two times so, we defined it like 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 showed significant differences when comparing their morphological characteristics (weight, SVL, condition, head size, number of femoral pores and number of lateral blue oceli) (MANOVA, $F_{10,9}$=5.80 p=0.007) (Table 1). Males from Fuenfria were significantly larger and heavier, and had significantly higher body condition than males from Golondrina. Also, Fuenfria males had significantly greater heads than Golondrina males (Table 1).

The head measures (length, width and depth) were different between these two populations of males (one-way ANOVA: $F_{1,18}$=8.34, p=0.009). The head of Fuenfria males were more length ($F_{1,18}$=8.30, p=0.009), more width ($F_{1,18}$=4.15, p=0.05) and more depth ($F_{1,18}$=7.09, p=0.01) than the head of Golondrina males.

However, Fuenfria and Golondrina males did not differ significantly in the number of femoral pores or blue oceli (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, Wilk's $\lambda = 0.046$, $F_{8,11}$=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, although non significantly, to have greater proportions of fatty acids with high molecular weight (C_{20}-C_{24}) and of waxy esters than males from Golondrina. For the other compounds there were no significant differences between populations.

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 her 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 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 betwwen the two days of the trial ($F_{1,38} = 5.09$, $p = 0.03$). And the interaction bewteen 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 analized 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 attractivenex 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)

Mating behavior

The overall frequency of successful copulations in this experiment was 0.58 (29 copulations from 50 staged encounters) (Fig. 3a). 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 > 0.2 for all interactions of order with all other effects) (Fig. 3a).

The duration of successful copulations did not differ between male populations (GLMM, $F_{1,26}$=3.60 p=0.06) or between female populations ($F_{1,26}$=1.84 p=0.18) and the interaction between male and female populations was not significant ($F_{1,26}$=2.71 p=0.11) (Fig. 3b). 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 interaction were not significant (p>0.40 in all cases) (Fig. 3b).

DISCUSSION

Our results showed that males from these two populations of *P. hispanica* differ in some aspects. Morpologically, males of Fuenfria are more robust and bigger than males of Golondrina. Also, femoral secretions of males are composed by similar chemical compounds but relative proportions of these chemicals are different (see also Martín & López, 2006a). Moreover, the genetic study showed that these two populations are genetically different. Therefore, these variations in morphology and femoral secretions could be related with genetic differences between populations.

These differences 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 (unpublished data). Lizards from Fuenfria occupy areas characterized by high levels of humidity and cold, whereas males from Golondrina occupy more dry and hot areas. 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 favoured individuals with morphological characters more adapted to each environment. Similarly, differences in proportion of chemical compounds 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 favoured in lizards inhabiting more humid climatic conditions (Alberts, 1992; Martín & López, 2006a). The genetic data suggest that selection on these parameters have resulted in genetic differences between populations, and the question that arises is whether differences are so great as to promote reproductive isolation. In fact,

differences in chemical compounds seem relevant for population recognition, because previous experiments showed that male *P. hispanica* can discriminate between scents from males from their own or from the other population (Martín & López, 2006a,b), which can have consequences for rival recognition in intrasexual contests.

In contrast to males, females did not seem to discriminate between male populations basing on chemical cues, although females clearly detected scent of males of the two populations from a baseline odour (Martin & Lopez, 2006a,b). Moreover, the results of this paper further showed that females did 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 origin population. Therefore, our results indicate that there is a lack of pre-mating and mating reproductive isolation between these two populations in instead of significant differences in genetic, morphological and chemical sexual signals. Although it remains to be analyzed whether interpopulation mating result 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 and accept mating by 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).

Our study showed that female *P. hispanica* selected scent of 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 & López, 2006d). This 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 & Martín, 2005) and in other lacertid lizard species (Martín & 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 exist a trade-off between physiological regulation of the immune system and the allocation of essential nutrients (provitamins) to sexual ornaments (Martin & Lopez, 2006e; Martín *et al*. 2007b; López *et al*., unpublished data), 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, if females used this signal to select a male, the population of origin would not affect to the mate election, 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 seen that instead of clear differences in morphology and chemical signals between two populations of *P. hispanica* lizards, which probably resulted

from small genetic differences, there are not clear interpopulational discrimination at the level of chemical recognition and pre-mating reproductive isolation. Females do not seem to discriminate between male chemicals, nor show preferences for the scent of males from their own population, but seem to base their selection on criteria of individual male quality that are shared by males of both populations. Also probabilities of successful matings do not depend on the origin population. All these results support that reproductive isolation and speciation between 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 project MEC-CGL2005-00391/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. 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.

Aragón, P., López, P. & 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., & Font, E. 2005. Chemical stimuli mediate species recognition in *Podarcis* wall lizards. *Amphib-Reptil*. **26**: 257-263.

Barbosa, D., Font, E., Desfilis, E. & Carretero, M.A. 2006. Chemically mediated species recognition in closely related *Podarcis* wall lizards. *J. Chem. Ecol*. **32**: 1587-98.

Bonnet, X. & Naulleau, G. 1994. A body condition index (BCI) in snakes to study reproduction. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie*, **317**: 34-41.

Capula, M. 2002. Genetic evidence of natural hybridization between *Podarcis sicula* and *Podarcis tiliguerta* (Reptilia: Lacertidae). *Amphibia-Reptilia*, **23**(3): 313-321.

Carazo, P., Font, E. & 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. & 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. & Vitt, L.J. 1986. Interspecific odour discrimination among syntopic congeners in Scincid lizards (genus *Eumeces*). *Behaviour* **97**: 1–9.

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

Dietemann, V., Peeters, C., Liebig, J., Thivet, V. & Hölldobler, B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. *Proc. Natl. Acad. Sci. USA* **100**: 10341-46.

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

Gómez, A., Font, E. & 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

Griffin, M.D., Xing, N. & Kumar, R. 2003. Vitamin D and its analogs as regulators of immune activation and antigen presentation. *Ann. 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. & 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. & 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. & Pedersen, L.B. 2003. The immunological functions of the vitamin D endocrine system. *Cell Mol. Biol.* **49**: 277-300.

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

LeMaster, M.P. & Mason, R.T. 2003. Pheromonally Mediated Sexual Isolation Among Denning Populations of Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis. Journal of Chemical Ecology* **29**(4): 1027-1043.

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

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

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

Martín, J. & 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. *Chemoecology* **16**: 31-38.

Martín, J. & López, P. 2006b. Pre-mating mechanisms favouring or precluding speciation in a species complex: chemical recognition and sexual selection between types in the lizard *Podarcis hispanica*. *Evolutionary Ecology Research* **8**: 643-658.

Martín, J. & 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. & 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. 2006e. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. *Proc. R. Soc. B Biol. Sci.* **273**: 2619-2624.
Martín, J., Moreira, P.L. & López, P. 2007a. Status-signalling chemical badges in male Iberian rock lizards. *Funct. Ecol.* **21**: 568-576.
Martín, J., López, P., Gabirot, M. & 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.
Mas, F. & Jallon, J.M. 2005. Sexual Isolation and Cuticular Hydrocarbon Differences between *Drosophila santomea* and *Drosophila yakuba*. *Journal of Chemical Ecology* **31**(11): 2747-2752.
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,
Olsson, M., Madsen, T., Nordby, J., Wapstra, E., Ujvari, B. & 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. & Tregenza, T. 2001. Sexual selection and speciation. *Trends Ecol. Evol.* **16**: 364–371.
Pinho, C., Harris, D.J. & Ferrnad, 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. & Uetz, G.W. 2004. Chemical signaling in a wolf spider: a test of ethospecies discrimination. *Journal of Chemical Ecology*. **30**(6): 1271-1284.
Rundle, H.D, & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8**: 336–352.
Sa-Sousa, P., Vicente, L. & Crespo, E. G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. *Amphib-Reptil*. **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. & Mason, R.T. 2002. Reproductive isolationg mechanisms between two sympatric sibling species of sea snakes. *Evolution,* **56**(8): 1655-1662.
Sokal, R.R. & Rohlf, F.J. 1995. *Biometry*. Freeman, New York.
Symonds M.R.E. & Elgar M.A. 2004. The mode of pheromone evolution: evidence from bark beetles. *Proc. R. Soc. Lond. B.* **271**(1541): 839-846.

Table 1: Morphological measurements (mean ± SE) amd 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.

	Fuenfria	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 oceli	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

FIGURE LEGENDS:

Fig. 1. Percent number of times (mean+SE), during each of two scent'schoice 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 popualtion, 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.

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).

Fig. 1

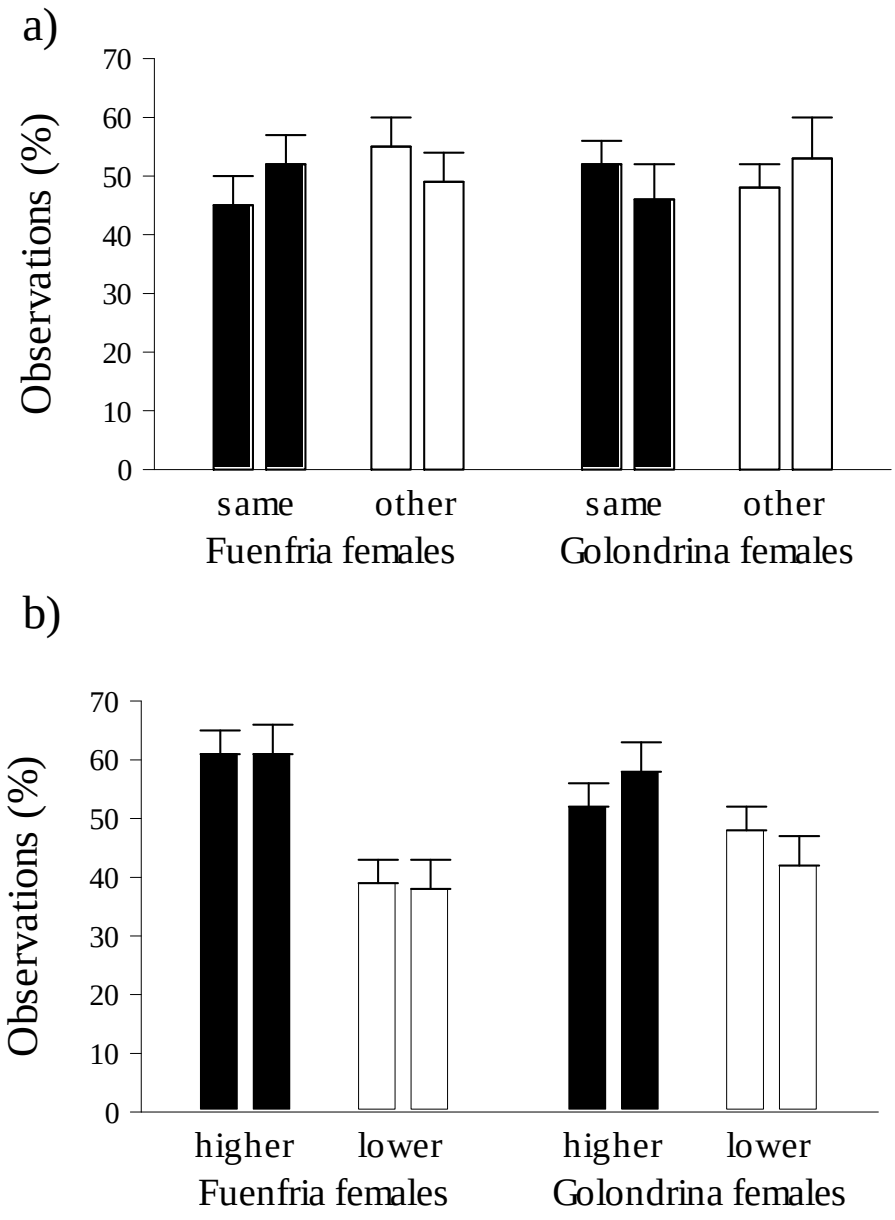

a)
Observations (%)
70
60
50
40
30
20
10
0
same
other
same
other
Fuenfria females
Golondrina females
b)
Observations (%)
70
60
50
40
30
20
10
0
higher
lower
higher
lower
Fuenfria females
Golondrina females

Fig. 2

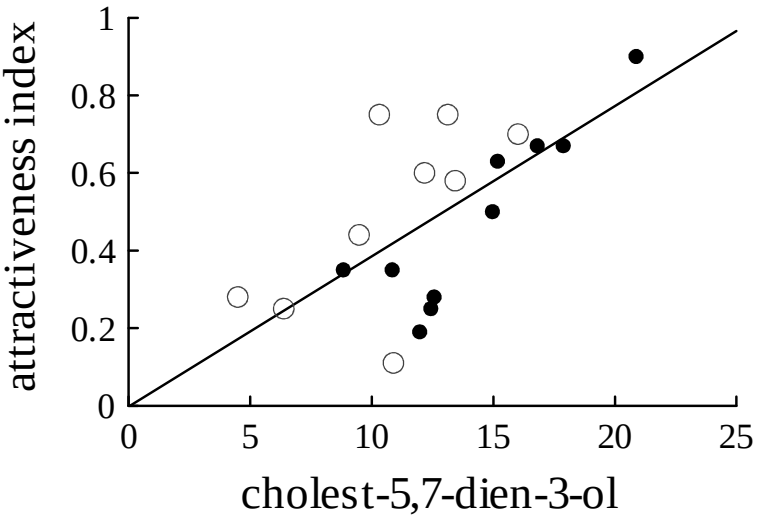
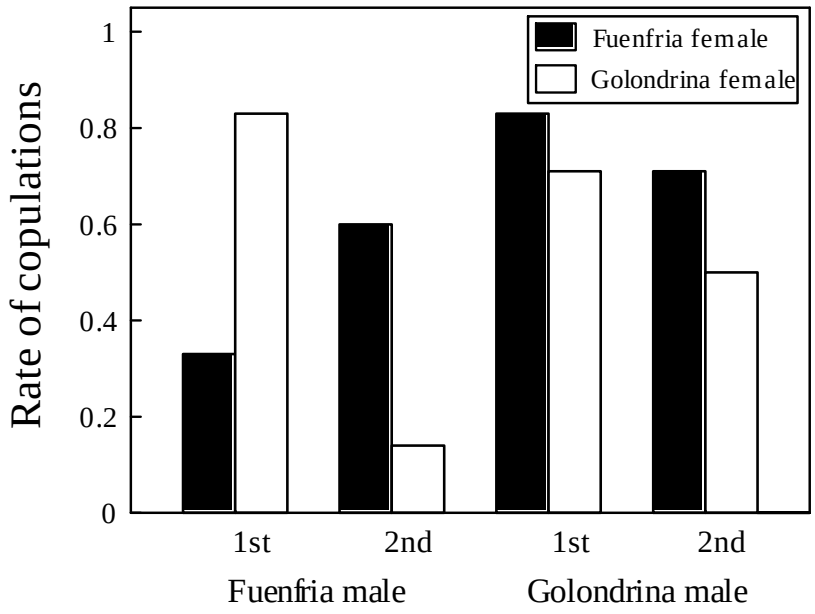

attractiveness index
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
cholest-5,7-dien-3-ol

Fig. 3

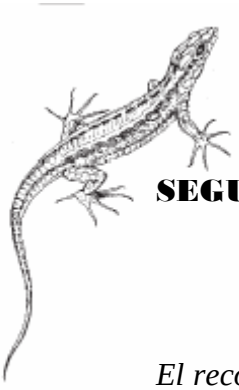

Rate of copulations
1
0.8
0.6
0.4
0.2
0
Fuenfria female
Golondrina female
1st
2nd
1st
2nd
Fuenfria male
Golondrina male

El primer trabajo mostró que a pesar de las claras diferencias morfológicas y de señales químicas entre las dos poblaciones de *P. hispanica* del norte de Madrid, y de las posibles pequeñas diferencias genéticas, no existe una clara discriminación interpoblacional entre individuos ni tampoco un aislamiento reproductivo pre-cópula efectivo. Las hembras consideran químicamente a todos los machos por igual, independientemente que sean de su población o no. Sobretodo, su elección de pareja mediante señales químicas se basaría en un compuesto presente en las secreciones femorales de los machos de las dos poblaciones, el Colesta-5,7-dien-3β-ol. Este compuesto es un precursor de la vitamina D_3, con importantes funciones metabólicas (absorción del calcio y regulación del sistema inmune). Por lo que puede existir un compromiso entre destinar este compuesto al metabolismo general o a las secreciones femorales. La secrección de este compuesto sería especialmente costoso para los machos de baja calidad y por tanto podría ser un buen índice de calidad individual de los machos para las hembras a la hora de elegir parejas.

Pero en este estudio, hemos observado dos poblaciones muy cercanas. Sabemos que son diferentes visual y químicamente pero no conocemos sus diferencias genéticas relativas. Los análisis de las variaciones genéticas y flujo genético entre estas dos poblaciones podrían explicar en parte los resultados etológicos obtenidos.

Además, ¿que pasaría si cojieramos una población más lejana geográficamente y con individuos de morfología aun más diferente? ¿Las diferencias de las señales químicas, y visuales podrían ser mayores y, por tanto, el aislamiento reproductivo sería más efectivo?

En el próximo trabajo, hemos cogido otra población de *Podarcis hispanica* del sur de la comunidad de Madrid (Aranjuez), donde el suelo está caracterizado por yeso y caliza, además de estar situada a una altitud menor que las poblaciones de la sierra (Golondrina y Fuenfria). Esta nueva población también se diferencia al nivel de coloración y del tamaño corporal. Las *P. hispanica* de la zona de Aranjuez son más pequeñas y de coloración más clara.
Compararemos los rasgos morfológicos de las tres poblaciones de *P. hispanica*. También se analizaran las diferencias genéticas entre estas poblaciones. Finalmente probaremos si existe reconocimiento quimiosensorial entre ellas para tener un primer índice de aislamiento reproductivo entre poblaciones más diferentes y alejadas.



SEGUNDA PARTE

El reconocimiento basado en señales quimicos entre individuos de P. hispanica de poblaciones del sur y del norte de Madrid, muestra un posible aislamento reproductivo.

Marianne Gabirot, Pilar Lopez, Staffan Bensch & José Martin

Morphological and genetic variability between populations of *Podarcis hispanica* lizards in the Madrid region: effects on intraspecific recognition in this species complex

Marianne Gabirot, Pilar López, Staffan Bensch and José Martín

Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC,

José Gutiérrez Abascal 2, 28006 Madrid, Spain

Short title: Interpopulational variations in lizards and speciation

Address correspondence to Marianne Gabirot: mcnmg805@mncn.csic.es

Tel.: 34-91-5 64 50 78 Ext: 1221

Fax: 34-91-5 64 50 78

No. of words in the text: 10334

1. INTRODUCTION

The concept that species are basic units of evolution, each with its own unique genetic makeup, is widely accepted amongst evolutionary biologist (Carson, 1957; Paterson, 1993; Drew, 2004; Balakrishnan, 2005). The accurate identification and description of biological species is vital and morphological taxonomists must continue to use diagnostic systems that elucidate, or are in agreement with genetic boundaries (Balakrishnan, 2005). Although molecular methods, such as DNA analyses, may assist in species resolution (Tautz *et al.*, 2003), their values needs to be assessed in light of what constitutes a species (Fittzhugh, 2006). Combining different sources of information is essential for a complete understanding of the process of differentiation between species.

Wall lizards species, *Podarcis* spp., are the predominant lizard group in southern Europe. Their taxonomy is complex and unstable, primarily because species are morphologically similar but exhibit substantial levels of intraspecific variation (Arnold & Burton, 1978). Recently, molecular studies have suggested 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 & Sá-Sousa 2001, 2002). In the western and central parts of the Iberian Peninsula two allopatric types have been described. 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 Region (Central Spain). In this area, distinct populations live close together without geographical isolation, and individuals of both types may find each other easily (Mellado & Olmedo 1981; García-Paris *et al*., 1989). We can observe that there exist a variety

of morphologies in this relatively small geographical area. For example, in the northern populations, where the habitat is compose by mountains and highlands, individuals are larger and with darker colour than in the southern populations with gypsum type habitat and plain relief (personal observ.). These distinct lizard populations contacting at this particular area provide an excellent system to study whether morphological differences between populations are due to actual genetic differences, and to search for interpopulation recognition mechanisms that might lead to reproductive isolation and speciation processes within this species complex.

Chemosensory recognition is well-developed in *P. hispanica* lizards. They can discriminate between conspecifics and more genetically distant heterospecifics (*P. bocagei*), and between sexes by chemical cues alone (Gómez *et al*., 1993; López & Martín, 2001; Cooper & Pérez Mellado, 2002; López *et al*., 2002). Moreover, the aggressive response of male Iberian wall lizards to intruding individuals depends at close range on pheromonally mediated sex recognition; males impregnated experimentally with scent of males were attacked by other males, but males impregnated with scent of females did not elicit aggressive responses (López *et al*., 2002), and females bearing male scents were attacked (López & Martín, 2001).

Recently, a chemical analysis has demonstrated that males of two closed populations of *P. hispanica* from the Guadarrama Mountains (Central Spain), inhabiting different environmental conditions, differ in the chemical composition of femoral gland secretions of males (Martín & López, 2006a). Males of the population inhabiting more humid microclimates have secretions with more 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 & López, 2006a,b). In contrast, females detected but did not seem able to discriminate between scents of males of the two populations (Martín & López, 2006a,b). A similar study shows that 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 similar chemical cues shared by males from different populations. Moreover, other experiment showed that males and females from these two populations can copulate successfully with individuals from the other population (Gabirot *et al.* unpublished data). Despite these two populations differ in morphological and chemical characteristics, it seems that premating reproductive isolation does not entirely occur. Perhaps the magnitude of these differences between these two populations are not enough large as to lead to an effective reproductive isolation process.

In this paper, we aimed to study distinct populations of *P. hispanica* lizards inhabiting different areas of Madrid Region, but where the phenotypic differences between lizard populations are more marked, and the habitats are different. For this, we captured individuals in two populations in the north Mountains and one population in the south of the Madrid community. First, we characterized and compared these populations by morphological (body size, colouration) and molecular methods (DNA microsatellites). Moreover, we further analyzed whether there were chemosensory recognitions between these three populations of Iberian wall lizards from the Madrid region.

2. METHODS

POPULATIONS SAMPLING

During March 2007, we captured by noosing males and females *P. hipanica* at three close localities in the Madrid Province (Spain). We captured 20 males and 21 females were captured from a population occupying different granite rock-cliffs at the edge of a pine forest in

the upper part of ‘Valle de la Fuenfría’ (40° 47’ N, 4° 03’W; 1750 m altitude). 19 males and 36 females were captured on rocky outcrops in a large oak forest near ‘Cercedilla’ village (40° 44’ N, 4° 02’W; 1250 m altitude). Finally, we caught 8 males and 12 females in the south of Madrid, near to Aranjuez (40°2’N, 3°37’O; 494 m altitude) from populations encountered in different rock in hills where the ground is constitute by chalk and gypsum.

Within each population, we captured lizards in different places over large areas (10 km^2) to ensure that individuals had not seen in previous contact, which might affect their responses (López & Martín, 2002). All population types were recognized based on morphology and colouration. Fuenfria lizards have flattened head and body, reticulated or stripped dark dorsal patterns and whitish pearly coloured belly, whereas Golodrina lizards have head and body moderately robust, light brown patterns and orange belly and Aranjuez individuals have low head and body robust, and they are more light-coloured with a pattern more yellow-green than mountain’s populations (for details see Pérez-Mellado & Galindo, 1986; Guillaume, 1987; Sá-Sousa, 2000; Sá-Sousa *et al*., 2002).

In the northern mountain area, the two study populations are 6 Km apart, occupying different altitudinal ranges (Fuenfria: between 1700-1800 m; Golondrina: below 1500 m) with different microclimates but with similar microhabitat structural characteristics (P. López & J. Martín, unpublished data). Between these two populations, there is an altitudinal area (aprox. between 1500-1700 m) where no stable population of *P. hispanica* is found. However, suitable habitat (i.e., rocky outcrops) is available and populations of other similar lacertid lizard species are present (Martín-Vallejo *et al*., 1995), but also some, presumably wandering, *P. hispanica* individuals are occasionally found. For example, we have found isolated individuals mathcing the morphology of lizards from the lower population (Golondrina) as close as 2 Km from the higher population of Fuenfría (P. López & J. Martín, unpublished data). These observations strongly suggest that encounters between individuals of both populations should not be rare.

However, the distance between the north and south populations is of about 100 km., so the probability of encounters between individuals from the mountains and from the south is very low.

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 terrarium 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. To avoid that lizards had contact with the scent and visual stimuli before they were tested, terrarium with lizards of different populations were housed separately. All lizards were healthy during the trials. They did not show behavioural 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. The captures and experiments 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 morphological measures of each individual: weight, size between nose and cloaca (snout-to-vent length, SLV), the body condition, head size, number of femoral pores in each side, number of blue oceli in each side. We used GLM models to analyze differences in body mass between populations and sexes, including the interaction between sexes and populations. We corrected body mass by SVL of lizards, thus, we refer through the text indistinctly to body mass and body condition.

In this paper we used the body condition for the different analyse making the regression of weight on SLV, both ln corrected (Regression: r = 0.60; $F_{1,113} = 64.76$; $p<0.0001$). The body condition is the body mass corrected by the SVL in this regression, but we also used the residuals of this regression and named body condition index (BCI). Similarly, we transformed the head size measurements corrected by the SLV. In the analysis, we used the residual of the regression head size data on SLV.

MEASUREMENTS OF COLORATION

Male and female *P. hispanica* have similar dorsal coloration (a brownish-olive background, presumably mimetic with the habitat) but differ somewhat in dorsal blackish patterns, which were the same during all the seasons. However, ventral coloration is sexually dichromatic, being bright orange to yellow to human observers, with maximum reflectance values between 600 and 700 nm (see results), in adult males of these populations during the mating season whereas it is white in females and juvenile males. Ventral coloration of males seems to be dependent of androgens (i.e. more developed during the mating season) and could be considered as a secondary sexual signal. Intraspecific sex determination at long distance is based on the presence/absence of this coloration (López & Martín, 2001; López *et al.*, 2002). As in other lizards, yellow, orange and red colorations are probably produced by carotenoid and pteridine pigments (Cooper & Greenberg, 1992; Macedonia *et al.*, 2000).

We measured reflectance of coloration from 300 to 750 nm using an Ocean Optics USB2000 spectroradiometer with a DT-1000-MINI Deuterium–Halogen light source (Ocean Optics, Inc., Dunedin, FL). To exclude ambient light and standardise measuring distance, a cylindrical metallic tube was mounted on the bifurcated fibre optic probe (Montgomerie, 2006). The probe was held at a 90° degree angle to the skin, and reflectance was measured, always by the same person (MG). We measured coloration on four standardized spots in the ventral part: on the middle of the throat (between the last chin shields and the collar; ‘throat’), between the

two forelimbs (at the middle of the second row of ventral scales from the collar; ‘breast’), close to the end of the vent (at the middle of the fourth row of ventral scales from the cloaca; ‘belly’) and on the beginning of the ‘tail’; We also measured coloration on two standardized spots in the dorsal part: one between the two forelimbs and one between the hind legs. Reflectance (R) was calculated relative to a white standard (WS-1-SS) with the OOIBase32 software (Ocean Optics, Inc.). Mean reflectance was summarized over 6 nm steps (‘binned’; Grill & Rush, 2000) before statistical analysis.

We mathematically summarized the spectra using principal component analysis (PCA) (Cuthill *et al.*, 1999; Grill & Rush, 2000). This method makes no assumptions about how reflectance variation is perceived or which aspects of the spectrum might be important (Cuthill *et al.*, 1999). In PCA of spectral data, PC1 represents variation in intensity of coloration or brightness, and subsequent PCs represent combinations of hue and chroma (Cuthill *et al.*, 1999; Grill & Rush, 2000). Principal component analysis was performed including all spectra for all males togetherbut separately for the ventral and dorsal colorations. Then, we used GLMs to examine the variation in PC scores between populations and between body positions (within effect; ‘body’: throat vs. breast vs. belly vs. tail).

In addition, we used the segment classification (SC) method (Endler, 1990; Grill & Rush, 2000) to calculate the relative contribution to total spectra of four different ‘colour classes’: UV (300-400 nm), blue-green (400-500 nm), yellow (500-600 nm) and orange-red (600-750 nm). We first calculated the area under the entire reflectance curve (i.e. the sum of the reflectances at all measured wavelengths) by using integral calculations. Then, we similarly calculated the area within each of the above four colour classes, and finally calculated the relative contribution of each colour class to the total reflectance area. Then, we used GLMs to examine the variation in contribution of each colour class between populations and between body positions (within effect; ‘body’).

POPULATIONS GENETIC STRUCTURING

We tested 27 lacertid microsatellites isolated in *Podarcis muralis* (Nembrini & Oppliger, 2003), *Lacerta vivipara* (Boudjemadi *et al.*, 1999), *Podarcis bocagei* (Pinho *et al.*, 2004), *Podarcis erhardii* (Poulakakis *et al.*, 2005a) for cross-species amplification in *P. hispanica*. Tail samples were collected and preserved in ethanol. DNA was extracted with the DNeasy Tissue extraction kit (QIAGEN) for *P. hispanica*. Initially, the primers were tested in seven *P. hispanica* individuals from the Madrid area. The PCR (polymerase chain reaction) mix contained 4 pmol of each primer, 15 nmol $MgCl_2$, 1.25 nmol dNTP, 0.5 U Ampli-taq polymerase and 10 ng template in a 10 μl reaction. PCRs were done in a GeneAmp PCR system 9700 (Applied Biosystems) and the conditions were as follows: 94°C for 2 minutes, then 35 cycles at 94°C for 30 s/T_a for 30 s/72°C for 30 s followed by 72°C for 10 minutes, where T_a is the locus specific annealing temperature. The fluorescent-labelled PCR products were separated and alleles were detected in an ABI PRISM 3730 capillary sequencer (Applied Biosystems).

Of the 27 primer pairs tested in the 7 test individuals of *P. hispanica* we deemed 14 to be potentially useful since they had polymorphic products. 4 loci were not further tested and 1 discarded due to unspecific amplification for *P. hispanica* (Runemark *et al*., 2008). The remaining loci were either monomorphic, did not amplify or had unspecific products (Runemark *et al*., 2008). The 9 apparently polymorphic and easily scored loci in *P. hispanica* were further tested in all individuals from Madrid area: in 19 individuals from the Aranjuez population, 20 from Fuenfria population and 20 from Golondrina population.

Standard population genetic analyses were performed to check all samples for evidence of linkage between loci and departure from random (outcrossing) genotypic expectations. Tests for significant genetic heterogeneity between samples were performed using Exact Test of allele and genotype frequencies, and via the departure of measures of genetic differenciation (FST) from zero and population structure, using the software computer programs GENEPOP

(Raymond & Rousset, 1995), FSTAT (Goudet, 1995), ARLEQUIN 2.0 (Schneider *et al*., 1999) and STRUCTURE 2.1 (Pritchard *et al*., 2000).

SCENTS RECOGNITION EXPERIMENT

Lizards have been shown to react to a variety of chemical stimuli with increased and differential rates of tongue extrusions (Cooper & Burghardt, 1990). Tongue-flick (TF) rate can, therefore, be used as a quantitative bioassay of detection of chemical cues (e.g., Cooper & 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 or female *P. hispanica* of different populations (Aranjuez, Golondrina and Fuenfria), or of male or female *P. muralis* (used as a control of a related but well distinct lizard species), or with deionized water (odorless control) (Cooper & Burghardt, 1990). Water was used to gauge baseline TF's rates in the experimental situation. 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 & 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 de-ionized water, we rolled the tip over those bodily areas (of one population and sex per applicator). We used a new applicator in each trial.

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 population (P. López & J. Martín unpublished data), and between 1100-1300 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. Latency to the first TF was computed as the period elapsed between presentations of the cotton swab to the first TF directed at the swab. 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: Fuenfria *vs*. Golondrina *vs*. Aranjuez *vs*.water *vs*. *P. muralis*) and population of the responding lizard (between factors). 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. 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 & Rohlf, 1995).

3. RESULTS

MORPHOLOGICAL CHARACTERISTICS

Weight, SVL, body condition and BCI were significantly different between sexes (ANOVA: $F_{3,327}$= 6.43, p= 0.0003) and between populations (ANOVA: $F_{6,327}$=9.32 p<0.0001), and the interaction between population and sexes was not significant (MANOVA: $F_{6,327}$=0.75 p=0.60). Males were significantly greater than females (ANOVAs: weight, $F_{1, 109}$= 17.53 p<0.001; SVL, $F_{1,109}$=13.99 p=0.0003; Body condition, $F_{1,109}$=13.98 p=0.0003; BCI, $F_{1,109}$=6.94 p=0.009).

Males from Fuenfria were significantly larger and heavier than males from Golondrina and Aranjuez. Also, males from Fuenfria had significantly higher body condition than Golondrina and Aranjuez males (Table 1).

For females, we only found significant differences between populations with respect to SVL and body condition (ANOVAs: SVL, $F_{2,65}$=25,49 p<0.001; body condition, $F_{2,65}$=25.48 p<0.01). Females from fuenfria were larger than Golodrina's females (Tukey's test: p=0.0007) and than Aranjuez's females (p=0.00011), and Golondrin's females were larger than Aranjuez females (p=0.00018).

Respect to the head size (length, width, depth), we made a model with these three variables and populations and sexes as categorical groups. Males had significantly greater heads than females (MANOVA, $F_{2,218}$=3.7 p=0.02) but we did not find significant difference between populations ($F_{4,218}$=1.00 p=0.40), and the interaction was not significant ($F_{4,218}$=0.71 p=0.57; Table 1).

With respect to the number of femoral pores, males had significant more pores than females (two-way ANOVA, $F_{1,109}$=45.98 p<0.001), moreover the population had a significant effect on the number of femoral pores without sex consideration ($F_{2,109}$=6.00 p=0.003), and the interaction between populations and sexes was not significant ($F_{2,109}$=1.04 p=0.35). Lizards from Aranjuez had significantly less femoral pores than lizards from Golondrina (Tukey's test: p=0.017) or Fuenfria (Tukey's test: p=0.0012; Table 1).

With respect to the blue oceli, present only in males, there was significant difference in the number of blue oceli between populations (one-way ANOVA: $F_{2,44}$=4.07 p=0.02). Males from Aranjuez population had a higher number of blue oceli than males from Fuenfria (Tukey's test: p =0.03). Males from Golondrina did not differ significantly in the number of blue oceli with respect to males from Fuenfria (Tukey's test: p =0.11) and Aranjuez (Tukey's test: p =0.57) (Table 1).

MEASUREMENTS OF COLORATION

Ventral coloration

The PCA on reflectance data of all spectra of ventral coloration produced three principal components (PCs) that together accounted for 95.08% of the variation in the original spectra. The first PC (PC-1) accounted for 82.72% of variation (eigenvalue = 57.77). The coefficients relating PC-1 to the original reflectance data were all negative and of similar magnitude (Fig. 1a), so PC-1 represented achromatic brightness variation in the original spectra. The second PC (PC-2) accounted for a further 7.49% of the variation (eigenvalue = 4.87) in the original spectra. The coefficients relating PC-2 to the original reflectance values below 540 nm were all positive, while above 540 nm they were negative (Fig. 1a). PC-2 thus represented variation in the relative amount of short- to long-wavelength reflectance. The third PC (PC-3) accounted for 4.87% of the variation (eigenvalue = 3.17), and the pattern of coefficients suggested it represented variation in the relative amounts of medium (410-600 nm) wavelengths in the negative side to both short (300-410 nm) and long (600-740 nm) wavelengths in the positive side (Fig. 1a).

Overall characteristics of ventral coloration defined by all PCs differed significantly between positions in the ventral area (MANOVA: $F_{6,344}=3.09$ $p<0.0001$) and between populations ($F_{4,344}=11.62$ $p<0.0001$) and the interaction was significant ($F_{12,344}=2.44$ $p=0.004$) (Table 2). Characteristics of ventral coloration defined by PC-1 (i.e, brightness) differed significantly between populations (two-way ANOVA: $F_{2,172}=45.43$ $p<0.0001$) and between body positions ($F_{3,172}=24.48$ $p<0.0001$) but the interaction was not significant ($F_{6,172}=1.29$ $p=0.26$). Brightness was significantly higher at the throat than at the breast (Tukey's test: $p=0.0002$) and higher than at the belly and at the tail ($p<0.0001$ in both cases). Brightness at the breast was significantly higher than at the tail ($p=0.002$) (Table 3). Moreover the model showed that the PC1 value of Fuenfria were higher than Golondrina (Tukey's test: $p=0.0003$) and than Aranjuez values in each body area ($p=0.0001$). And Aranjuez PC1-scores were lower than Golondrina scores ($p=0.02$) (Table 3; Fig. 2a).

Characteristics of ventral coloration defined by PC-2 (i.e. relative amount of short- to long-wavelengths) differed significantly between body positions (two-way ANOVA: $F_{3,172}$=20.29 p<0.0001), and between populations ($F_{2,172}$=6.04 p=0.003), but the interaction was significant ($F_{6,172}$=2.57 p=0.02) (Table 2). Values of PC-2 scores were positive and significantly higher at the throat than at other positions (Tukey's tests: p<0.0001 in all cases), while PC-2 score values were negative and similar at the breast, belly and tail (p>0.5 in all cases). Values of PC-2-scores were significantly lower in the Golondrina population than in Fuenfria (p=0.01) and but not differ to Aranjuez populations (p=0.72). While Aranjuez and Fuenfria PC-2 values did not differ (p=0.10) (Table 3; Fig. 2a).

Characteristics of ventral coloration defined by PC-3 (i.e. relative amounts of medium to both short and long wavelengths) differed significantly between body positions (two-way ANOVA: $F_{3,172}$=9.75 p<0.0001) and between populations ($F_{2,172}$=37.43 p<0.0001), and the interaction was not significant ($F_{6,172}$=1.86 p=0.08) (Table 2). PC-3 values were positive significantly higher at the throat than at other body positions (Tukey's test: breast; p=0.0003; belly: p=0.00001; tail: p=0.0001), while PC-3 scores were negative and similar at the breast, belly and tail (p>0.5 in all cases). Moreover the model showed that the PC3 values of Fuenfria were higher than Golondrina (Tukey's test: p=0.0005) and than Aranjuez values (p=0.0001) (Tables 2, 3). But there was no difference significant between Golondrina and Aranjuez PC3 values (p=0.19) (Table 3; Fig. 2a).

The analyse of colours classes in GLM showed that there were significant effects of the area and the lizard population on the relative contribution of UV, Blue-Green, Yellow and Orange-Red categories to ventral coloration, and the interactions were not significant (Table 4). In more detail, we observed a lack of significant differences between populations in the

proportion of UV coloration in the throat, belly and tail (Table 5; Fig. 3), whereas in the breast males from Aranjuez had significantly higher proportions of UV coloration than males from the other two populations (Table 5; Fig. 3).

We observed that the intensity of colour classes changed between body areas (Tables 4; Fig. 3). The UV values decreased from the throat to the tail, whereas the Yellow values increased. The Blue-Green values for the Aranjuez population were higher in the anterior part (Throat, Breast and Belly) than in the posterior. We observed a similar, but less notable, decrease for the other two populations. Finally, the orange-red coloration was more intense in the posterior part of the body part than in the anterior part for all populations (Fig. 3).

In all body areas, Aranjuez individuals showed significant higher values of Blue-Green coloration than Fuenfria and Golndrina individuals (Tuckey's tests: $p<0.05$ in all cases). Moreover, Golondrina and Aranjuez males differed in blue-green coloration in all body areas ($p<0.05$), but in the throat and tail ($p>0.05$). Blue-green coloration of Fuenfria and Golondrina were different in throat and breast ($p<0.05$). Only for the breast, these three populations showed significant difference between themselves ($p<0.05$) (Table 5).

For the Yellow coloration, the differences between population are less notable (Tables 4,5; Fig. 3). The only significant difference was between Fuenfria and Golondrina populations in throat, breast and tail (Tukey's tests: $p<0.05$ for all) (Table 5).

Finally, for the orange-red coloration, Fuenfria males showed significantly higher values than Aranjuez and Golondrina males (Tukey's tests: $p<0.05$). But there were no significant difference in this coloration type between Aranjuez and Golondrina populations in all the body areas ($p>0.05$ for all). Only for the belly, orange-red values of Golondrina males were not different from Fuenfria males' values, but differed with those of Aranjuez (Table 5; Fig. 3).

Dorsal coloration

The PCA for the spectra of dorsal coloration produced three principal components that together accounted for 95.12% of the variation in the original spectra. The first PC (PC-1) accounted for 77.96% of variation (eigenvalue = 50.67). The coefficients relating PC-1 to the original reflectance data were all positive and near 0.8 and of similar magnitude (Fig. 1b), in the same way that for the ventral coloration, we could think that PC-1 represented achromatic brightness variation in the original spectra. The second PC (PC-2) accounted for a further 10.59% of the variation (eigenvalue = 6.88) in the original spectra. The values to PC-2 below 600 nm were all negative, while above 600 nm they were positive (Fig. 1b). This represented variation suggested it represented variation in the relative amounts of short-medium (350-600 nm) wavelengths in the negative side to long (600-700 nm) wavelengths in the positive side. in the relative amount of short- to long-wavelength reflectance. The third PC (PC-3) accounted for 6.57% of the variation (eigenvalue = 4.27), and the coefficients relating PC-3 to the original reflectance values between 400 nm and 600 nm were all negative, while below 400 nm and above 600 nm, they were all positive (Fig. 1b). PC-3 thus could represent variation in the relative amount of short- to long-wavelength reflectance of dorsal coloration.

There were not overall significant differences in the characteristics of dorsal coloration between body positions (MANOVA: $F_{1,86}$=0.14 p=0.70), but there were significant differences between populations ($F_{2,86}$=12,51 p<0.0001). And the interaction was not significant ($F_{2,86}$=0.91 p=0.40) (Table 4).

Univariate test showed that differences between populations were only significant for PC-1 (i.e. brightness) ($F_{2,86}$=27.12 p<0.0001) and PC3 values (i.e. relative amounts of medium to both short and long wavelengths) between the population origin of individuals ($F_{2,86}$=4.54 p=0.01 for PC3) (Table 2; Fig.2b). Lizards from Aranjuez had significantly brighter (PC-1) dorsal colorations than lizards from Golondrina (Tukey's test: p=0.0009) and Fuenfria (p=0.0001), and lizards from Golondrina had significantly brighter dorsal coloration than

lizards from Fuenfria (p=0.00023). Respect to the PC3 values, there were not significant differences between Aranjuez and Fuenfria (p=0.69), but lizards from both populations had significantly higher values than lizards from Golondrina (p=0.02 for Aranjuez; p=0.04 for Fuenfria).

When we analysed the contribution of the different colour classes to dorsal coloration, we did not find significant differences between body areas, but there were significant differences between populations for all colour classes but for the yellow one (Table 5; Fig. 4), and the interactions were not significant. Considering only the effect of population on the coloration classes, we observed that the values of UV from Fuenfria males were higher than those from Aranjuez males (Tukey's test, p=0.046), whereas the values from Golondrina males were intermediate and did not differ between the other two populations (p>0.05). For the Blue-Green colour class, Fuenfria males had lower values than Aranjuez and Golondrina males (Table 5; Fig. 4). While for the Orange-Red colour classes, Fuenfria males had higher values than Aranjuez or Golondrina males (p<0.05). Finally, for the Yellow coloration, there was not significant difference between population (Table 5; Fig. 4).

GENETIC DIVERSITY

Each pair of loci was tested for linkage disequilibrium and genotypic independance was confirmed. Expected and observed heterozygotie values for each locus and each population are reported in Tables. Hardy-weinberg was tested at each locus for each population. We can note significant heterozygote deficiencies for the locus Lv472, Pb47, Pb73 of the Aranjuez population, for Lv472 and Lv4x of Fuenfria population and for Pb10 of Golondrina population (p<0.0006). It may be asumed that the deficit of heterozygotes most likely results from of a presence of null alleles.

GENETIC STRUCTURE

Population differention of *Podarcis hispanica* poppulations is presented by pairwaise FST coefficient (Table 6). The comparison of structure population between the populations Fuenfria or Golondrina with the Aranjuez population show a FST superior of 0.10. According to the Wright (1978) range, we can consider that populations of Fuenfria and Golondrina have a moderate to hight genetic difference with the Aranjuez population. Moreover, the genetic difference between Fuenfria and Golondrina population could be considered like low (Table 6). The gene flow between populations is shown by the estimates of the number of migrants per generation (Nm where N is the total effective number of lizards and m is the migrate rate). We note that the estimates of the number of migrants per generation is highter between Fuenfria and Golondina (~6) than Golondrina or Fuenfria population with Aranjuez population (~2).

Analysing the microsatellites data with Structure software, we could be seen on the graphics the population alleles' structure of *Podarcis hispanica* with both hypothesis of two and three different subpopulations. We can observe that the hypothsis of two subpoplations was the most probably. With the hypothesis of 2 subpopulations, Aranjuez in one side and Fuenfria and Golondrina populations, in other side, have a significant different population structure. We can note too a low different structure between Fuenfria and Golondrina populations (Fig. 5).

Moreover, we used the method of detecting the true value of number of populations with structure software datas (Evanno *et al*., 2005) (Fig. 6). The distribution of ln(k) (values out from structure software, where k is the possible number of subpopulation) showed that the true value could be 2 populations. This means that Golondrina and Fuenfria form one population but different to Aranjuez population.

CHEMOSENSORY RECOGNITION EXPERIMENT

Responses of females to scent of males

All female lizards responded to swabs by tongue flicking. There were signifcant differences among male scent stimuli (repeated measures two-way ANOVA, $F_{4,128}$=130.70, P<0.0001) and between populations of the responding females ($F_{2,32}$=31.07, p<0.0001), but the interaction was significant ($F_{8,128}$=17.23, p<0.0001) (Fig. 7).

All TF directed to scents of male lizards were significantly higher than TF directed to water (Tukey's tests, p<0.05 for all cases). Females from Aranjuez population made more TF directed to scents of males from their own population than to scent of males from Fuenfria and Golondrina populations and than to male *P. muralis* (p<0.05 in all cases). Moreover, directed TF rates by females from Aranjuez to males from Golondrina and Fuenfria and male *P. muralis* did not significantly differ between them (p>0.05 in all cases) (Fig. 7).

In contrast, females from Golondrina or Fuenfria populations also directed significantly more TF to scent of males from their own populations than to males from Aranjuez or male *P. muralis* (p>0.05 in all cases). However, although the number of TFs directed to males from Aranjuez and male *P. muralis* differed significantly (p<0.05), TF rates to males from Fuenfria and Golondrina populations were not signifcantly different (p>0.05 in both cases).

Responses of males to scent of males

All male lizards responded to swabs by tongue flicking. There were signifcant differences among males scent stimuli (repeated measures two-way ANOVA, $F_{4,180}$=210.88, P<0.0001) and between populations of the responding males ($F_{2,45}$=17.84, p<0.0001), but the interaction was significant ($F_{8,180}$=16.28, p<0.0001) (Fig. 8a).

All TFs directed to scents of male lizards were significantly higher than TFs directed to water (Tukey's tests, p<0.05 for all cases). Moreover, TFs directed to scent of male *P. muralis* were significantly higher than to water (p<0.05) but significantly lower than to scents of males from Aranjuez, Fuenfria and Golondrina populations (p<0.05 in all cases). Males from

Aranjuez made directed significantly more TFs to scents of males from their own population than to males from Golondrina and Fuenfria populations ($p<0.05$), and the number of TFs directed to males from Golondrina and Fuenfria did not signifcantly differ ($p=0.37$) (Fig. 8a).

Males from Fuenfria population directed a significantly higher number of TF to scents of males from their own population than to males from Golondrina and Aranjuez populations ($p<0.05$ in both cases). Moreover, Fuenfria males directed significantly more TFs to scent of males from Golondrina than to males from Aranjuez ($p<0.05$) (Fig. 8a).

TFs directed from Golondrina's males to scents of males from Aranjuez were significantly lower than to males from their own population ($p<0.05$), but there were no signifcant differences between TFs directed to males from Fuenfria and Golondrina, nor between males from Fuenfria and Aranjuez populations ($p>0.05$ in both cases) (Fig. 8a).

Responses of males to scent of females

There were signifcant differences among responses of males to the different female scent stimuli (repeated measures two-way ANOVA, $F_{4,176}=185.9$, $P<0.0001$) and between populations of the responding males tested ($F_{2,44}=28.17$, $p<0.0001$), but the interaction was significant ($F_{8,176}=17.35$, $p<0.0001$). Males directed significantly more TFs to scent of females from any population than to water or scent from female *P. muralis* (Tukey's tests, $p<0.05$ for all cases), but TF directed to female *P. muralis* were significantly higher than to water ($p<0.05$) (Fig. 8b).

Males from Aranjuez directed signifcantly more TFs to scent of females from their own population than to scent of females from Golondrina and Fuenfria populations ($p<0.05$ in both cases), and the number of TFs directed to females from Golondrina and Fuenfria were not significantly different ($p=0.37$) (Fig. 8b).

Males from Fuenfria directed a significantly lower number of TFs to scent of Aranjuez females than to females from their own population ($p=0.0013$), but TF directed to females from Golondrina, Fuenfria and Aranjuez populations were not signifiantly different ($p>0.05$) (Fig. 8b).

Males from Golondrina directed a significantly higher number of TFs to females from their own population than to females from Aranjuez and Fuenfria populations ($p<0.05$ in both cases), but there were no signifcant difference between TFs directed to females from Aranjuez and Fuenfria ($p=0.29$) (Fig. 8b).

4. DISCUSION

Our results showed that individuals from these three populations of *P. hispanica* differ in some aspects. Morphologically, there are difference between these populations, especially between Fuenfria and Aranjuez populations. Fuenfria and Golondrina could be characterised by the northern populations and Aranjuez is the southern population. And more specifically, Fuenfria is northern than Golondrina population. The individual from north populations are more hight, more robust than individual from the south population. Moreover the head of individual from north population is larger, heavier greater than individual from Aranjuez population. In addition, we noted that the number of femoral pores was not the same between these three populations. The significative difference was seen between the more distant populations (Fuenfria & Aranjuez). Fuenfria males have more number of pores than Aranjuez individuals.

Moreover, we observed that the number of oceli in males did differ between populations. Individual from the north of Madrid have less number of oceli than southern individuals. These oceli could be a visual signal used in intrasexual selection processes (López

et al., 2004). Many animals display colour 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 spots that runs along their body side on the outer margin of the belly. For example, blue spots are present in several specie of the genus *Lacerta* and *Podarcis* (Barbadillo *et al.*, 1999). Such colour spots might play an important role during sexual selection because this is a sexually dimorphic character (López *et al*., 2004). Blue spots may be a reliable signal of sex, body size/age and/or body condition, dominant/older male signal, like in the specie, *Lacerta monticola* (López *et al.*, 2004).

The analyses of coloration also showed different patterns for these populations. We observed ventral and dorsal colour differences between populations. The brightness of ventral coloration in the south population is higher than in the northern populations. Moreover, the major ventral colour differences were noted for the blue-green and orange-red ranges. The populations from south of Madrid have ventral coloration characterized by higher proportions of blue-green and lower proportions of orange-red colour than northern populations. Being the differences greater between the most geographically distant populations (Fuenfria and Aranjuez). We observed a similar result in the dorsal coloration. Populations from Fuenfria have coloration with higher proportions of orange-red, whereas Aranjuez and Golondrina populations have higher proportions of blue-green dorsal coloration. For the yellow ventral and dorsal colours and UV range, there were no differences between these populations.

These differences in coloration and morphology between populations could be explained by the different habitat where these populations live. Firstly, northern and southern populations are localized in two contrasting geography sites; mountains (cold, humidity and high altitude) vs. lowland (hot, dry and low altitude). Variation of body size of many animals, and in particular in mammals, is 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 (Yom-Tov & Nix, 1986). Moreover, animal in cold sites could have colour or adaptation in order to capture sun or heat more efficiently For visual signals, relevant ecological variables include the ambient light spectrum in which a signal is viewed and features of the visual background from which the signal must be discriminated. Studies of guppies (Endler, 1991) and birds (Endler & Thery, 1996) have been important in demonstrating the influence of habitat light and contrast on colour signal evolution. Moreover, Endler's (1978, 1980, 1991) work on guppies has shown how differences in spectral sensitivity between a signalling species and its predators can select for colour patterns that minimize predator detection while remaining conspicuous to the species in which the signal has evolved.

In the southern areas, the light is more present over the years, and the temperature is higher than in the mountain sites. Perhaps individuals from mountain are darker than individuals from the south. Perhaps for this reason individuals from mountain need to be darker to maximize light and heat absorption. Whereas in the south, individuals could not need to absorb all available light, but on the contrary would reflect light and head. Thus, colouration of lizards in the south of Madrid could have evolved to more vivid colouration.

In addition, Aranjuez individuals look more mimetic with their habitat. Closely related *Heliconius* species generally differ in mimetic colour pattern, as though adaptive radiation has occurred (Turner, 1976; McMillan *et al*., 1997). The sister species *H. melpomene* and *H. cydno* are sympatric throughout Central America and the Andean foothills, where they differ in mimicry and habitat use (Jijjins *et al*., 2001). Lizards from Aranjuez populations live in areas with gypsum and sandy soil and they have a yellow-green vivid dorsal coloration that could be and adaptation to this soil/habitat.

Considering the differences in number of femoral pores between mountain and south populations in the region of Madrid, we can support one more time that these populations could

be adapted to different habitats and in consequences use different type of communication signals. Fuenfria population, or in general, populations from high mountain, with habitat more humid and cold, could have more evolved the use of chemical cues than Aranjuez population where the habitat is more dry and hot. These elements conduct us to thinking that perhaps the populations from mountains of north of Madrid could use the chemical communication more intensively than Aranjuez population. In contrast, in the populations from south of Madrid could prevail other type of communication such as the visual one. The humidity and temperature are important for the persistency and efficiency of chemical secretions on substrates (Alberts, 1992). So, in a habitat where the temperature is high and humidity is low, the secretions will not have the same efficiency, and perhaps importance, than in mountain population where the secretions will stay for longer time and might have an important function in communication.

In addition to these morphological results, the genetic analyse showed a possible boundaries between these populations. In fact, the microsatellites analyses showed that there was genetic variability between these populations. The difference between both mountain populations is very small, and we can not consider these populations like genetically different. But south and north populations are significantly different. Microsatellites measures demonstrated that the genetic structure of these two populations is different.

Furthermore, the chemical recognition between these populations demonstrated that individuals of each population had more interest for scents from their own individual population than scents of other populations. Populations from the north (Golondrina and Fuenfria) made more tongue flicks to scents from northern population than to scents from the southern population (Aranjuez) and we observed this in both sexes. Lizards could consider

individuals from the other population like belonging to a different species. Perhaps, this difference in recognition could induce to a higher selectivity for intrapopulation mate choice and lead to a reproductive isolation. But with the current results we can not yet conclude that reproductive isolation exists. All these results could make think that populations from north and from south of Madrid are two types very different and that they can be in a speciation process. But to make a better conclusion we need more precopulatory experiment in order to know the real or hypothetic boundary between these populations.

Research on reproductive isolation in African cichlid fishes has focused olfactory cues in mate recognition by females of a Lake Malawi cichlid species. Female *Pseudotropheus emmiltos* were given a choice of spawning next to a conspecific male or a male of the closely-related sympatric *Pseudotropheus ainzilberi*. However, Jordan *et al*. (2003) found that females of three Lake Malawi cichlids associated more with conspecific males behind solid partitions which prevented olfactory contact, even under monochromatic light. This suggests that shape, pattern and behaviour may have been more important than colour in species discrimination in this group. This suggests that divergence of olfactory signals may have been an important influence on the explosive radiation of the East African species flock.

In summary, in this study, we have seen that furthermore of clear differences in morphology and genetic between three populations of *P. hispanica* lizards, but more especially between the mountain and south populations in Madrid area, there are clear interpopulational discrimination at the level of chemical recognition. Males and females discriminate between the scents from their own population and other population. The different morphology, colour and genetic results could be explained by an adaptation to different habitat. These populations living in distinct areas could have evolved in different way and used different communication signals or different cues in mate choice. All these results support the existence of ongoing

reproductive isolation and 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 project MEC-CGL2005-00391/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**: 62-89.

Arnold, E.N. & Burton, J.A. 1978. A field guide to the reptiles and amphibians of Britain and Europe. London: Collins.

Balakrishnan, R. 2005. Species concepts, species boundaries and species identification: a view from the tropics. *Systematic Biology* **54**: 689-693.

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

Barbadillo, L. J., Lacomba, L. J., Perez-Mellado, V., Sancho, V. & Lopez-Jurado, L. F. 1999. Anfibios y reptiles de la Peninsula Ibe´rica, Baleares, y Canarias. GeoPlaneta, Barcelona.

Boudjemadi, K., Martin, O., Simon, J.C. & Estoup, A. 1999. Development and cross-species comparison of microsatellite markers in two lizard species, *Lacerta vivipara* and *Podarcis muralis. Molecular Ecology*, **8**: 518-520.

Carson, H.L. 1957. The species as a field for gene recombination. In: Mayr E, ed. The species problem. Washington, DC: *American Association for the Advancement of Science*. **50**: 23-38.

Cooper, W.E. & 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. & Greenberg, N. 1992. Hormones, Brain, and Behavior. Ed: Chicago University Press. pp: 299-400.

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

Cuthill, I.C., Bennett, A.T.D., Partridge, J.C. & Maier, E.J. 1999. Plumage reflectance and the objective assessment of avian sexual dichromatism. *American Naturalist* **153**: 183-200.

Drew, R.A.I. 2004. Biogeography and speciation in the Dacini (Diptera: Tephritidae: Dacinae). *Bishop Museum Bulletin in Entomology* **12**: 165-178.

Endler, J.A. 1978. A predator’s view of animal colour patterns. *Evolutionary Biology*. **11**: 319-364.

Endler, J.A. 1980. Natural Selection on Color Patterns in Poecilia reticulata. *Evolution* **34**(1): 76-91.

Endler, J.A. 1990. On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society* **41**: 315-352.
Endler, J.A. 1991. Variation in the appearance of guppy color patterns to guppies and their predators under different visual conditions, *Vision Res*. **31**: 587-608.
Endler, J.A. 1993. The color of light in forests and its implications, *Ecol. Monogr*. **63**: 1-27.
Endler, J.A. & Théry, 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.
Evanno, G., Regnaut, S. & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**(8): 2611-2620.
Faivre, B., Gregoire, A., Preault, M., Cezilly, F. & Sorci, G. 2003. Immune activation rapidly mirrored in a secondary sexual trait. *Science* **300**: 29-31.
Fitzhugh, K. 2006. DNA Barcoding: an instance of technology driven science? *Bioscience* **56**: 463-463.
García-Paris, M., Martín, C. & Dorda, J. 1989. Los Anfibios y Reptiles de Madrid. Spain-Madrid: Ministerio de Agricultura, Pesca y Alimentación.
Gómez, A., Font, E. & Desfilis, E. 1993. Chemoreception in the Lacertidae: exploration and conspecific discrimination in the Spanish wall lizard, *Podarcis hispanica*. Pp. 213–230 in Valakos ED, Böhme W, Pérez-Mellado V, Maragoú P (eds) Lacertids of the Mediterranean Region. Greece-Athens: Heleni Zoological Society.
Goudet, J. 2001. FSTAT, A Program to Estimate and Test Gene Diversities and Fixation Indices. Version 2.9.3. Available from http://www.unil.ch/popgen/softwares/fstat.htm.
Grill, C.P. & Rush, V.N. 2000. Analysing spectral data: comparison and application of two techniques. *Biological Journal of the Linnean Society* **69**: 121-138.
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. & 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. & Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (Podarcis): Is *Podarcis hispanica* a species complex? *Mol. Phylog. Evol*. **23**: 75-81.
Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. 2001. Reproductive isolation caused by colour patternmimicry *Nature* **411**: 302-305.
Jordan, R., Kellogg, K., Juanes, F. & Stauffer, J.J. 2003. Evaluation of Female Mate Choice Cues in a Group of Lake Malawi Mbuna (Cichlidae). *Copeia* **1**: 181-186.
López, P. & 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. 2002. Chemical rival recognition decreases aggression levels in male Iberian wall lizards, *Podarcis hispanica*. *Behav. Ecol. Sociobiol*. **51**: 461-465.
López, P., Martín, J. & Cuadrado, M. 2002. Pheromone mediated intrasexual aggression in male lizards, *Podarcis hispanicus*. *Aggr. Behav*. **28**: 154-163.
Lopez, P., Martin, J. & 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**(2): 309-311.
Macedonia, J.M., James, S., Wittle, L.W. & Clark, D.L. 2000. Skin Pigments and Coloration in the Jamaican Radiation of Anolis Lizards. *Journal of Herpetology* **34**(1): 99-109.
Martín, J. & 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. *Chemoecology* **16**: 31-38.

Martín, J. & 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*. *Evolutionary Ecology Research* **8**: 643-658.
Martin-Vallejo, J., García-Fernández, J., Pérez-Mellado, V. & Vicente- Villardón, J.L. 1995. Habitat selection and thermal ecology of the sympatric lizards *Podarcis muralis* and *Podarcis hispanica* in a mountain region of central Spain. *Herpetol J* **5**: 181-188.
McMillan,W. O., Jiggins, C. D. & Mallet, J. 1997. What initiates speciation in passion vine butterflies? *Proc. Natl Acad. Sci. USA* **94**: 8628-8633.
Mellado, J. & Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, *Acta Vertebrata* **8**: 299–300.
Montgomerie, R. 2006. Analyzing colors. *Bird Coloration, Vol 1. Mechanisms and Measurements* (eds. G.E. Hill &. K.J. McGraw), pp 90-147. Harvard University Press, Cambridge, Massachusetts.
Nembrini, M. & Oppliger, A. 2003. Characterization of microsatellite loci in the wall lizard *Podarcis muralis* (Sauria: Lacertidae). *Molecular Ecology Notes*, **3**: 123-124.
Paterson, H.E.H. 1993. The term 'isolating mechanisms' as a canalizer of evolutionary thought. In: McEvey SF, ed. Evolution and the recognition concept of species. London: *The John Hopkins Press Ltd*, 1-10.
Pérez-Mellado, V. & Galindo, M.P. 1986. Sistemática de *Podarcis* (Sauria, Lacertidae) Ibéricas y Norteafricanas mediante técnicas multidimensionales. Spain-Salamanca: Univ. Salamanca.
Peters, A., Delhey, K., Denk, A.G. & Kempenaers, B. 2004. Trade-offs between immune investment and sexual signaling in male mallards. *Am Nat* **164**: 51–59.
Pinho, C., Sequeira, F., Godinho, R., Harris, D.J. & Ferrand, N. 2004. Isolation and characterization of nine microsatellite loci in *Podarcis bocagei* (Squamata: Lacertidae). *Molecular Ecology Notes*, **4**: 286-288.
Poulakakis, N., Goulielmos, G., Antoniou, A., Zouros, E. & Mylonas, M. 2005. Isolation and characterization of polymorphic microsatellite markers in the wall lizard *Podarcis erhardii* (Squamata : Lacertidae). *Molecular Ecology Notes*, **5:** 549-551.
Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **155**: 945-959.
Raymond, M. & Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenism. *The journal of heredity* **86**: 248.
Runemark, A., Gabirot, M., Bensch, S., Svensson, E.I., Martín, J., Pafilis, P., Valakos E.D. & Hansson B. 2008. Identification of polymorphic microsatellite loci in *Podarcis gaigeae* and *P. hispanica* and assessment of their utility in three other *Podarcis* species (Squamata: Lacertidae). *Molecular Ecology Note*. **In press**.
Sa´-Sousa, P. 2000. A predictive distribution model for the Iberian wall lizard (*Podarcis hispanicus*) in Portugal. Herpetol. J. 10: 1-11.
Sa-Sousa, P., Vicente, L. & Crespo, E. G. 2002. Morphological variability of *Podarcis hispanica* (Sauria: Lacertidae) in Portugal. *Amphib-Reptil*. **23**: 55-69.
Schneider, S., Roessli, D. & Excoffier, L. 2000. Arlequin: a software for population genetics data analysis. Version 2.0. Availabel from: http://lgb.unige.ch/arlequin/software/.
Sokal, R.R. & Rohlf, F.J. 1995. Biometry 3rd ed. USA-New York:WH Freeman.
Tautz, D., Arctander, P., Minelli, A., Thomas, R. & Vogler, P. 2003. A plea for DNA taxanomy. *Trends in Ecology and Evolution* **18**: 70-74.
Yom-Tov, Y. & Nix, H. 1986. Climatological correlates for body size of five species of Australian mammals. *Biological Journal of the Linnean Society* **29**(4): 245-262.
Wright, S. 1978. Evolution and genetics of Regulation. Vol. 4. Variability within and among populations. Univ. of Chicago Press, Chicago.

TABLES

Table 1: Morphological measurements (mean ± SE) of individual *Podarcis hispanica* from Fuenfría, Golondrina and Aranjuez populations. Results (F, p) from protected one-way ANOVAs on transformed data (see methods) are shown.

	Golondrina	*Fuenfria*	*Aranjuez*	*F*	*P*
MALES					
Weight (g)	4.15 ± 0.14	5.27 ± 0.20	3.59 ± 0.25	16.47	< 0.01*
SVL (mm)	58 ± 1	61 ± 1	52 ± 1	19.29	< 0.01*
Body condition	1.38 ± 0.03	1.53 ± 0.04	1.09 ± 0.05	19.28	< 0.01*
BCI	0.02 ± 0.02	0.11 ± 0.02	0.16 ± 0.39	7.78	0.001*
Head length (mm)	13.7 ± 0.1	14.4 ± 0.2	12.4 ± 0.2	0.46	0.63
Head width (mm)	8.2 ± 0.1	8.5 ± 0.1	7.6 ± 0.2	1.49	0.23
Head depth (mm)	5.8 ± 0.1	6.1 ± 0.1	5.4 ± 0.1	0.67	0.518
Femoral pores	19 ± 1	18 ± 1	17 ± 1	3.29	0.046*
Blue spots	3.5 ± 0.6	1.8 ± 0.5	4.6 ± 0.8	4.07	0.02*
FEMALES					
Weight (g)	4.41 ± 1.41	3.66 ± 0.13	2.71 ± 0.11	2.06	0.13
SVL (mm)	55 ± 1	58 ± 1	50 ± 1	25.49	< 0.01*
Body condition	1.23 ± 0.03	1.40 ± 0.03	0.99 ± 0.04	25.48	< 0.01*
BCI	-0.05 ± 0.08	-0.11 ± 0.01	-0.01 ± 0.03	0.35	0.70
Head length (mm)	11.2 ± 0.1	11.8 ± 0.1	10.9 ± 0.1	16.09	< 0.01*
Head width (mm)	6.8 ± 0.1	7.0 ± 0.1	6.5 ± 0.1	4.42	0.016*
Head depth (mm)	4.6 ± 0.1	4.8 ± 0.1	4.7 ± 0.1	7.70	< 0.01*
Femoral pores	16 ± 1	17 ± 1	15 ± 1	3.32	0.04*

Table 2: Results of GLM models for the variations of colour characteristics defined by PCs from a PCA on reflectance values, at four ventral body positions ('ventral area') and populations origin ('population') and at two dorsal positions ('dorsal area').

		PC-1		PC-2		PC-3	
Effect	df	*F*	*P*	*F*	*P*	*F*	*P*
Ventral Area	3	24.48	<0.0001	20.29	<0.0001	9.75	<0.0001
Error	172						
Population	2	45.43	<0.0001	6.04	0.0029	37.43	<0.0001
Error	172						
Ventral area x Population	6	1.29	0.26	2.57	0.02	1.86	0.089
Error	172						
Dorsal Area	1	0.19	0.66	1.93	0.16	2.81	0.09
Error	86						
Population	2	27.12	<0.0001	2.73	0.07	4.54	0.01
Error	86						
Dorsal area x Population	2	1.81	0.16	0.159	0.85	0.23	0.79
Error	86						

Table 3: Mean ± SE and comparision of PCs coloration (F, p by one-way ANOVA) between the three populations (aranjuez, Fuenfria & Golondrina).

			ARANJUEZ	*FUENFRIA*	*GOLONDRINA*	*F*	*P*
VENTRAL coloration	**PC1**	**Throat**	-1.97 ± 0.55	-0.04 ± 0.17	-0.85 ± 0.20	11.57	<0.0001
		Breast	-1.05 ± 0.36	0.53 ± 0.10	-0.30 ± 0.17	16.72	<0.0001
		Belly	-0.55 ± 0.15	0.78 ± 0.11	0.14 ± 0.19	13.96	<0.0001
		Tail	0.02 ± 0.25	0.80 ± 0.10	0.29 ± 0.15	6.38	0.003
	PC2	**Throat**	1.36 ± 0.40	0.58 ± 0.16	0.79 ± 0.21	2.22	0.12
		Breast	0.45 ± 0.33	-0.04 ± 0.17	-0.55 ± 0.18	4.53	0.01
		Belly	-0.42 ± 0.20	-0.01 ± 0.19	-0.81 ± 0.26	3.36	0.04
		Tail	-0.52 ± 0.20	0.08 ± 0.13	-0.50 ± 0.19	4.04	0.02
	PC3	**Throat**	0.21 ± 0.21	1.18 ± 0.19	-0.015 ± 0.15	13.31	<0.0001
		Breast	-0.89 ± 0.36	0.57 ± 0.17	-0.54 ± 0.23	10.92	0.0001
		Belly	-1.24 ± 0.43	0.33 ± 0.17	-0.21 ± 0.21	8.63	0.0007
		Tail	-0.49 ± 0.16	0.19 ± 0.13	-0.69 ± 0.14	11.32	0.0001
DORSAL coloration	**PC1**		1.08 ± 0.26	-0.59 ± 0.08	0.18 ± 0.15	26.90	<0.0001
	PC2		0.27 ± 0.27	0.15 ± 0.08	-0.29 ± 0.21	2.76	0.068
	PC3		0.39 ± 0.14	0.16 ± 0.12	-0.36 ± 0.21	4.50	0.013

Table 4: Results of GLM models for the variations of colour characteristics defined by the relative contribution of four colour classes to the total reflectance spectra at three ventral body positions ('Ventral area') and population origin ('Populations') and the same with dorsal position ('Dorsal area').

	UV (300-400 nm)		Blue-Green (400-500 nm)		Yellow (500-600 nm)		Orange-Red (600-750 nm)	
Effect	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Ventral area $F_{3,172}$	45.06	<0.0001	9.49	<0.0001	11.67	<0.0001	11.74	<0.0001
Population $F_{2,172}$	8.46	<0.0001	36.24	<0.0001	15.38	<0.0001	50.14	<0.0001
Ventral area x Population	0.55	0.76	1.45	0.19	0.49	0.81	1.02	0.41
Dorsal area $F_{1,86}$	0.041	0.84	1.22	0.27	0.506	0.479	2.03	0.157
Population $F_{2,86}$	3.81	0.026	9.11	0.0002	2.85	0.06	13.36	<0.0001
Dorsal area x Population	0.96	0.38	2.69	0.07	0.006	0.99	0.66	0.52

Table 5: Mean ± SE and comparison of coloration classes (F, p by one-way ANOVA) between the three populations (Aranjuez, Fuenfria & Golondrina)

			ARANJUEZ	*FUENFRIA*	*GOLONDRINA*	*F*	*P*
VENTRAL coloration	**UV**	**Throat**	0.15 ± 0.008	0.126 ± 0.007	0.13 ± 0.006	2.10	0.134
		Breast	0.11 ± 0.009	0.059 ± 0.009	0.07 ± 0.007	5.86	0.0056
		Belly	0.07 ± 0.009	0.015 ± 0.02	0.04 ± 0.007	2.09	0.13
		Tail	0.04 ± 0.009	0.02 ± 0.008	0.03 ± 0.007	1.12	0.33
	Blue-Green	**Throat**	0.22 ± 0.006	0.17 ± 0.005	0.20 ± 0.005	22.73	<0.0001
		Breast	0.22 ± 0.007	0.13 ± 0.010	0.17 ± 0.007	15.61	<0.0001
		Belly	0.20 ± 0.009	0.13 ± 0.013	0.145 ± 0.009	7.12	0.002
		Tail	0.18 ± 0.006	0.13 ± 0.007	0.16 ± 0.011	4.80	0.013
	Yellow	**Throat**	0.245 ± 0.004	0.22 ± 0.005	0.26 ± 0.005	13.07	<0.0001
		Breast	0.27 ± 0.011	0.25 ± 0.006	0.29 ± 0.007	8.96	0.00055
		Belly	0.29 ± 0.011	0.27 ± 0.017	0.29 ± 0.005	0.83	0.44
		Tail	0.28 ± 0.009	0.26 ± 0.006	0.29 ± 0.005	7.05	0.002
	Orange-Red	**Throat**	0.27 ± 0.007	0.34 ± 0.006	0.29 ± 0.005	32.29	<0.0001
		Breast	0.28 ± 0.006	0.40 ± 0.014	0.33 ± 0.007	21.15	<0.0001
		Belly	0.30 ± 0.008	0.39 ± 0.017	0.36 ± 0.012	6.73	0.003
		Tail	0.32 ± 0.009	0.39 ± 0.009	0.34 ± 0.011	11.11	0.0001
DORSAL coloration	**UV**		0.06 ± 0.008	-0.09 ± 0.05	0.017 ± 0.017	3.84	0.025
	Blue-Green		0.17 ± 0.005	0.13 ± 0.01	0.18 ± 0.009	8.58	0.0004
	Yellow		0.30 ± 0.01	0.30 ± 0.01	0.33 ± 0.008	2.93	0.058
	Orange-Red		0.35 ± 0.006	0.44 ± 0.01	0.34 ± 0.015	15.95	<0.0001

Table 6: Population Structure between populations (Aranjuez, Fuenfria & Golondrina), F_{ST} (at the right of the diagonal) and Nm (at the left of the diagonal) by GENEPOP and FSTAT soft wares.

Populations	Aranjuez	Fuenfria	Golondrina
Aranjuez	-	0.1027[1]	0.1582[1]
Fuenfria	2.18[2]	-	0.0392[1]
Golondrina	1.33[2]	6.12[2]	-

[1] $F_{ST}=(H_T-H_S)/H_T$
[2] using the formula, $Nm=(1-F_{ST}) / 4xF_{ST}$, derived by Wright (1969).

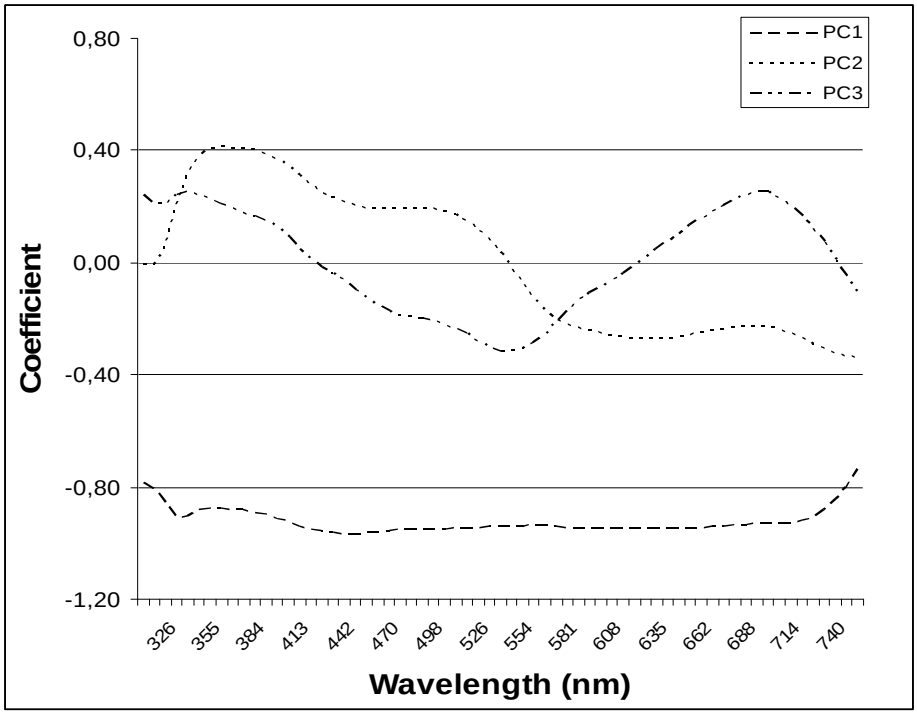
According to Wright (1978):

- $0 < F_{ST} < 0.05$: low genetic difference
- $0.05 < F_{ST} < 0.15$: moderate genetic difference
- $0.15 < F_{ST} < 0.25$: high genetic difference
- $0.25 < F_{ST}$: very high genetic difference

FIGURES

Fig. 1: Coefficients of the first three principal components from a PCA on all the reflectance spectra that characterize ventral coloration (a) and dorsal (b) of male lizards *P. hispanica*

(a)



(b)

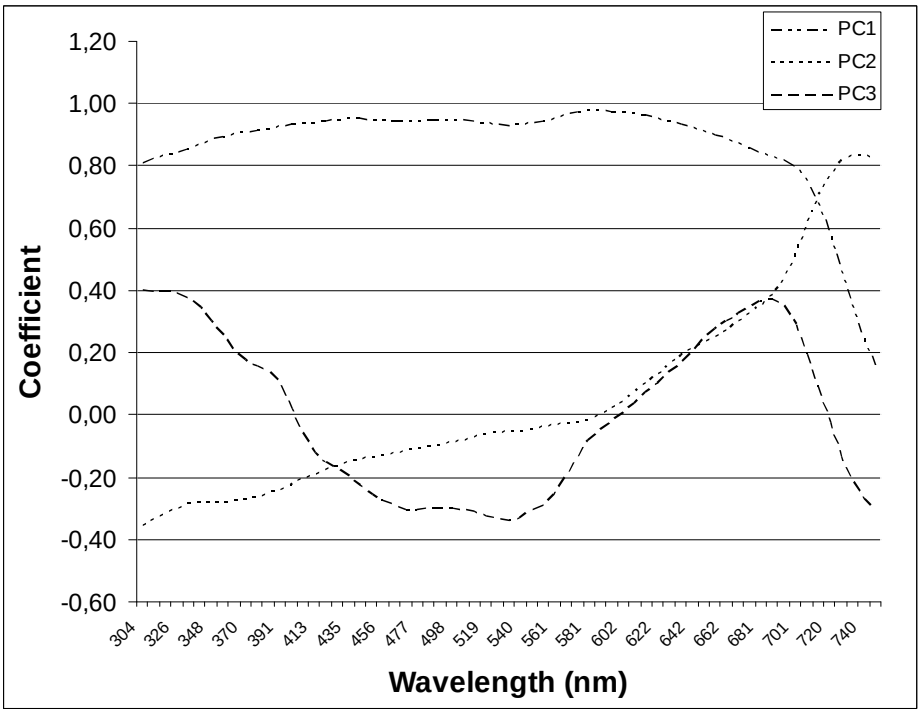
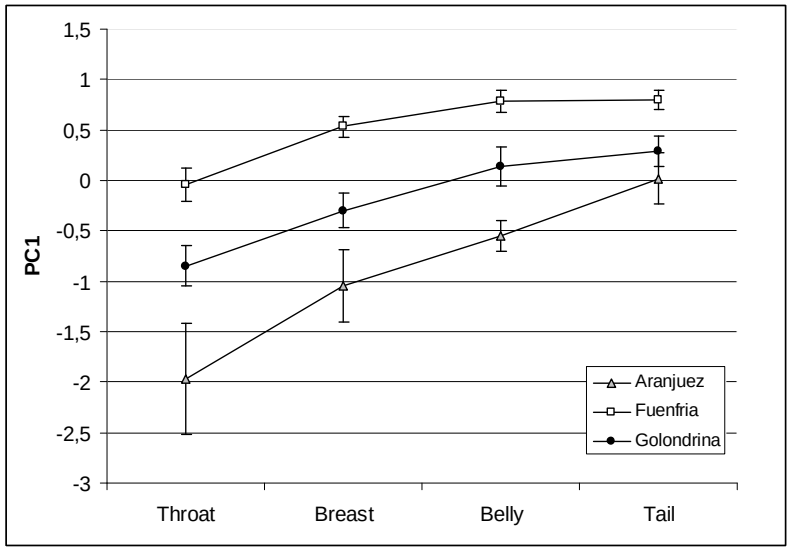
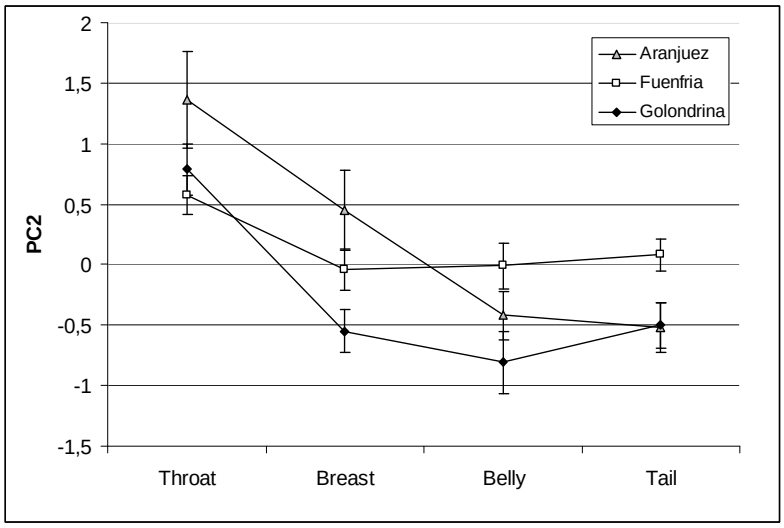
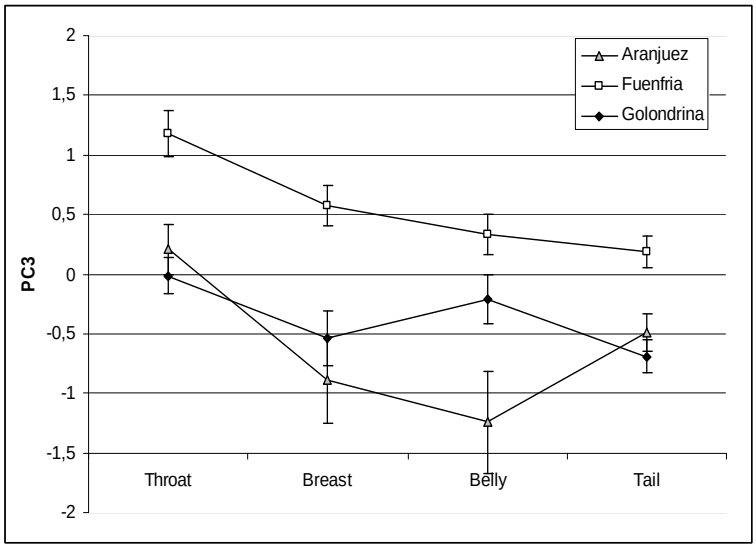


Fig. 2: PCs components of ventral (a) and dorsal (b) colour of *Podarcis hispanica* from three populations (Aranjuez, Fuenfria & Golondrina); measures captured in four body area: Throat, Breast, Belly and Tail.

a)






Aranjuez
Fuenfria
Golondrina
PC3
2
1,5
1
0,5
0
-0,5
-1
-1,5
-2
Throat
Breast
Belly
Tail

b)

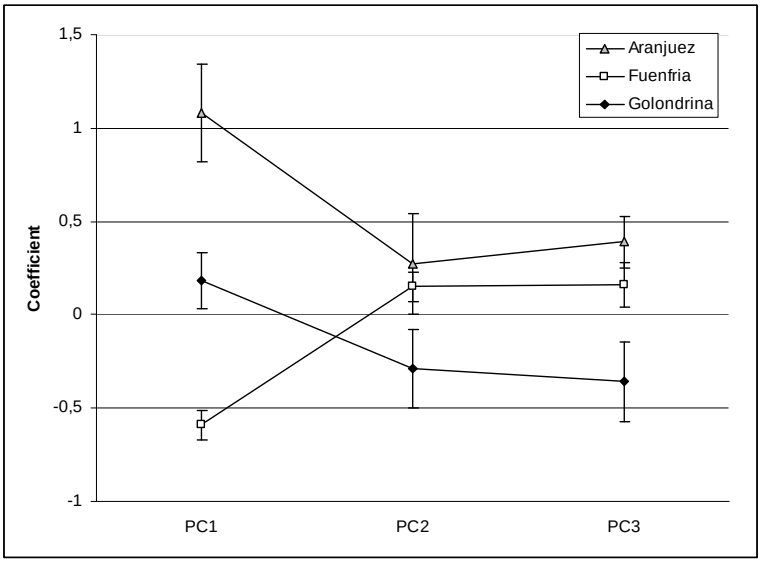

Aranjuez
Fuenfria
Golondrina
Coefficient
1,5
1
0,5
0
-0,5
-1
PC1
PC2
PC3

Fig. 3: Variation in relative contribution (mean ± SE) of four different 'colour classes' to total reflectance spectra of ventral coloration in four body site (Throat, Breast, Belly & Tail) of male lizards *P. hispanica*, from three populations (Blue: Aranjuez Population – Red: Fuenfria Population – Green: Golondrina Population).

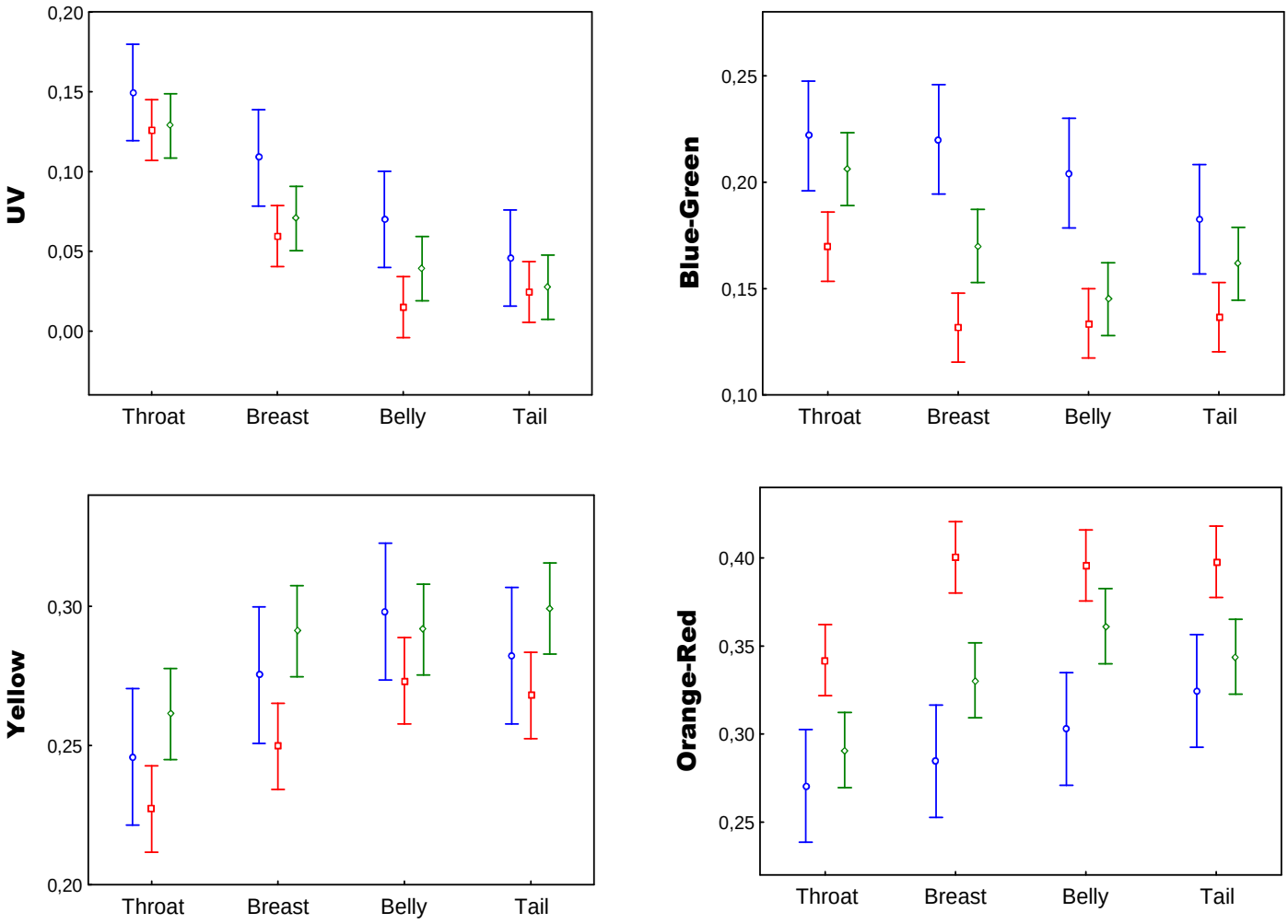


Fig. 4: Variation in relative contribution (mean ± SE) of four different 'colour classes' to total reflectance spectra of dorsal coloration of male lizards *P. hispanica*, from three populations (Aranjuez, Fuenfria & Golondrina Population).

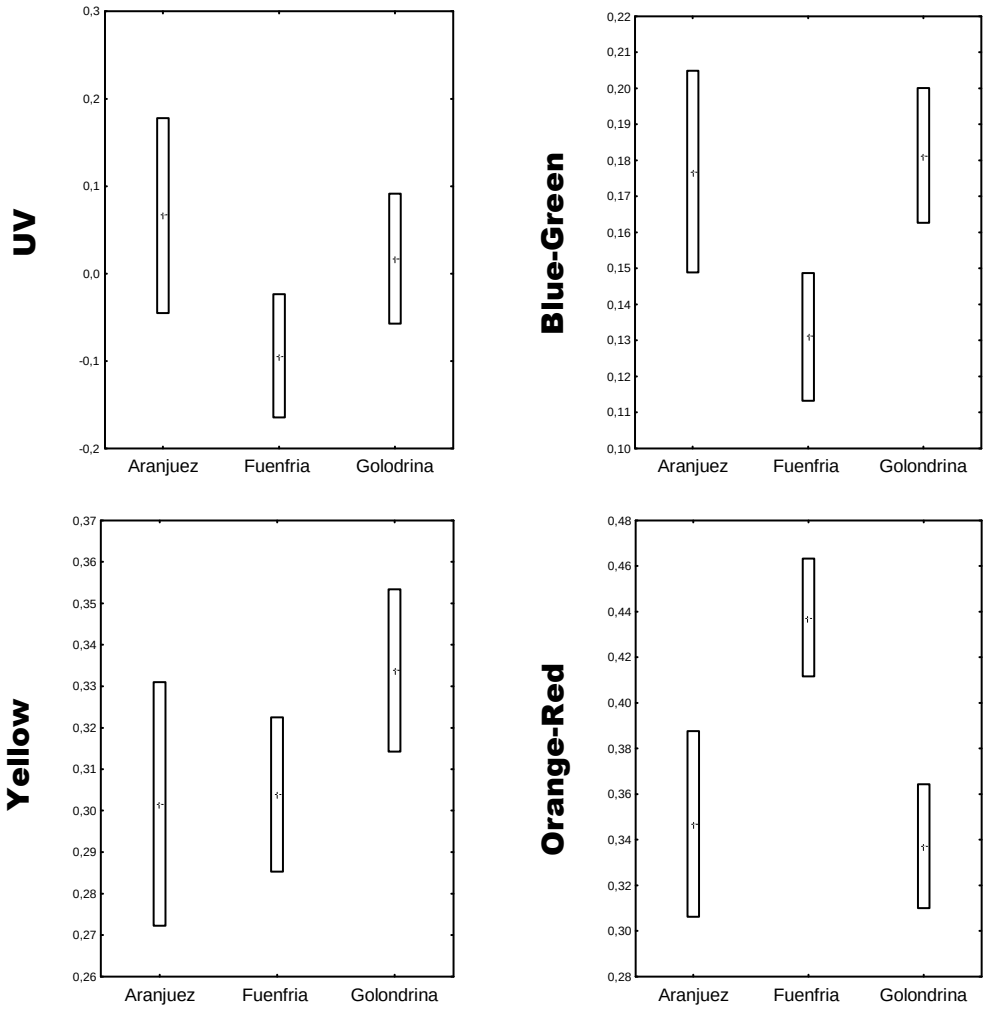
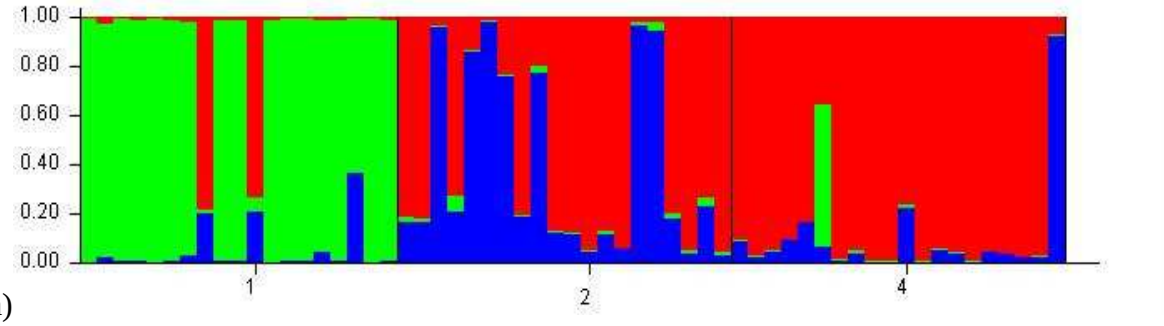
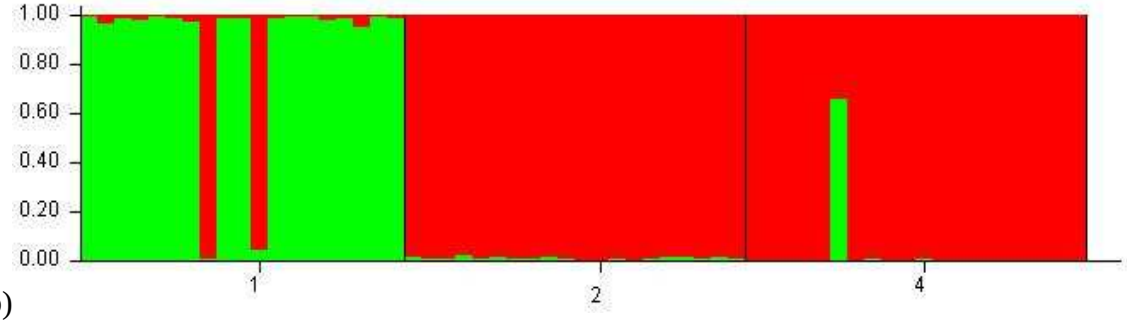


Fig. 5: Genetic Structure of *Podarcis hispanica* populations (Aranjuez: 1; Fuenfria: 2; Golondrina: 4) considering the hypothesis of 3 subpopulations (a) and 2 subpopulations (b) by STRUCTURE software.



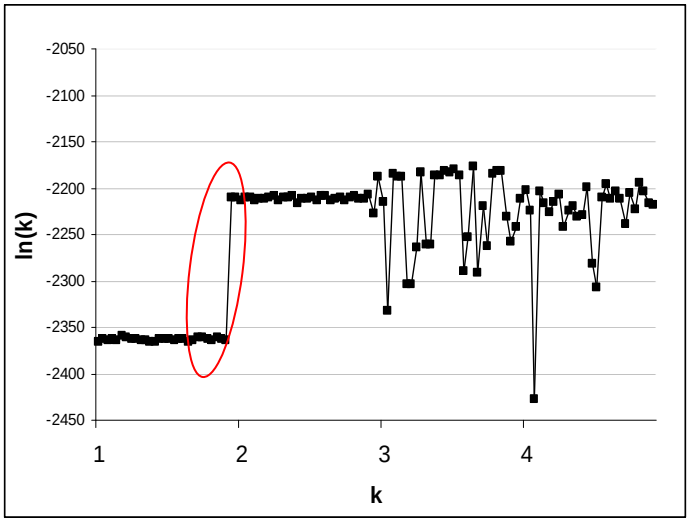
a)



b)

Fig. 6: Description of the steps for the graphical method allowing detection of the true number of populations (k) (Evanno *et al*., 2005). (a) Ln(k) data over 20 runs each k value. (b) Mean of ln(k) over each k value.

(a)



(b)

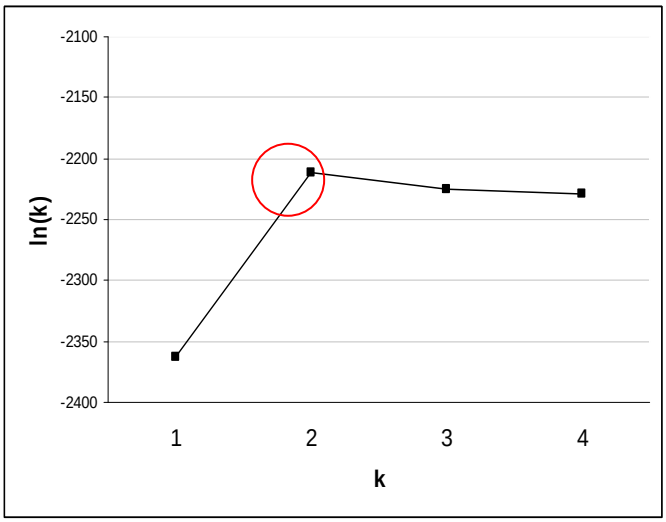


Fig. 7: Mean (+SE) of the number of tongue-flicks directed to the swab by females *P. hispanica* of three males populations (Aranjuez (gris), Fuenfria (black) & Golondrina (white)) in response to control deionized water, or scents from femoral gland secretions of male *P. hispanica* of their own or of different types, or of males *P. muralis*, presented for 60 s on cotton-tipped applicators

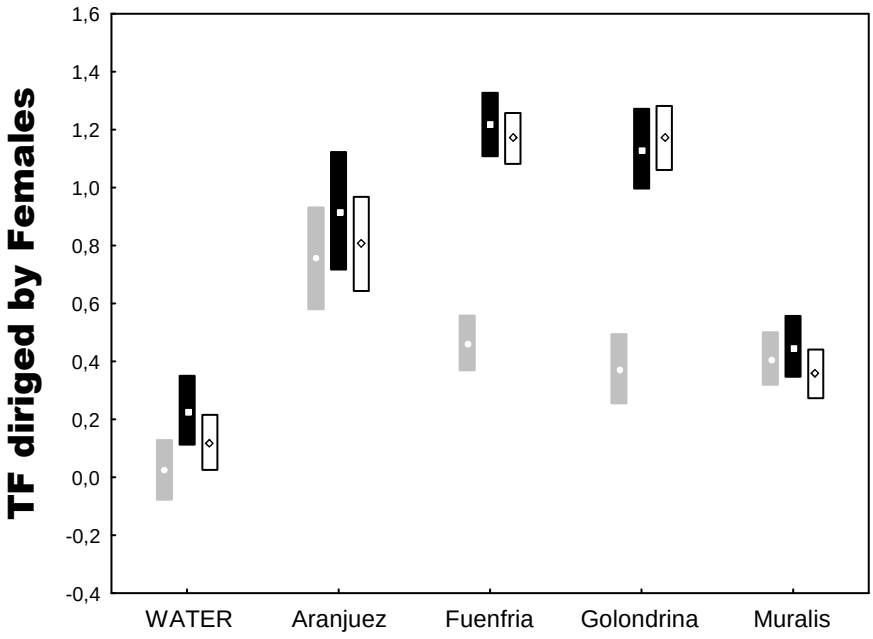
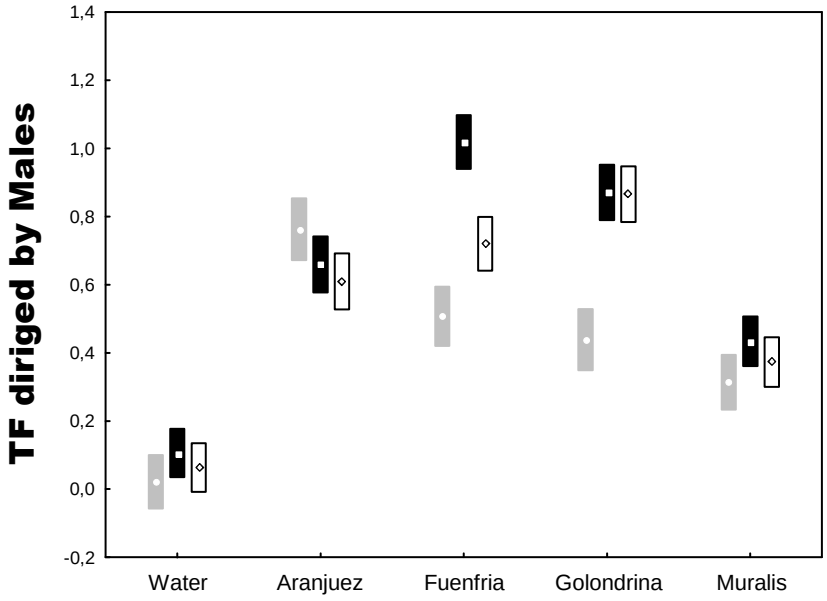
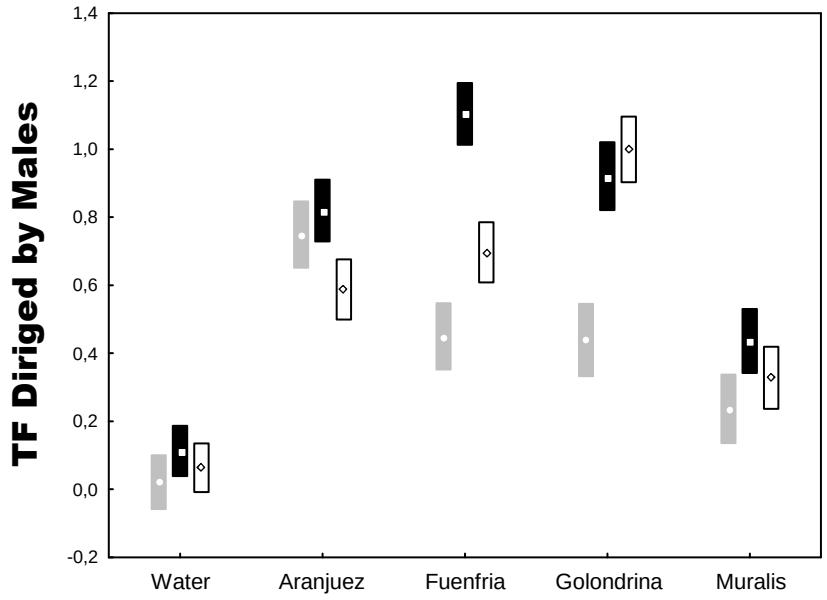


Fig. 8: Mean (+SE) of the number of tongue-flicks directed to the swab by males *P. hispanica* of three populations (Aranjuez (gris), Fuenfria (black) & Golondrina (white)) in response to control deionized water, or males scents from femoral gland secretions *P. hispanica* of their own (a) or males scents of different types (a), or males scents of *P. muralis* (a), and in response control deionized water, or females scents of *P. hispanica* of their own (b) or females scents of different types (b), or females scents of *P. muralis* (b), to presented for 60 s on cotton-tipped applicators



(a)



(b)

CONCLUSIONES

El complejo de especies de *P. hispanica* se compone de varios morfotipos que han sido estudiados y definidos a nivel molecular (Harris & Sa-Sousa, 2002; Pinho *et al*., 2006). Los tres morfotipos ibéricos principales se distribuyen de tal manera que en el centro de la Península se podría encontrar por lo menos dos de estos tipos (conocidos como 1 y 2). En esta zona se podrían observar fenómenos de aislamiento reproductivo o al contrario híbridos de estos dos tipos. Según el nivel de especiación entre estos dos tipos se observarían diferentes comportamientos reproductivos.

En la región de Madrid, se encuentra varias poblaciones de esta especie. Estas poblaciones pueden varían entre ellas a diferentes niveles: morfológico, químico y genético. En este trabajo, hemos estudiado individuos con características muy diferentes. Los individuos de la Sierra de Guadarrama (Fuenfria y Golondrina) son más grandes y robustos que los del sur de la región (Aranjuez). Además hemos notado que las poblaciones del norte estaban caracterizadas por un mayor número de poros femorales que los de Aranjuez. Estos datos morfológicos mostraron una gran diferencia entre estas poblaciones. Además, los análisis de microsatélites ayudaron a entender la variabilidad genética entre ellas. Las poblaciones del norte y la del sur tendrían una estructura genética diferente y el flujo genético entre ellas parece ser muy bajo. A estos datos moleculares, se añade un primer experimento de reconocimiento químico que apoya el hecho que estas poblaciones sean diferentes. Las poblaciones del norte y la del sur no mostraron el mismo interés a la hora de explorar quimiosensorialmente los estímulos olorosos de otras lagartijas. Los individuos de cada población mostraron un interés mayor por los individuos de su propia población que por los de la otra.

Estos datos morfológicos, moleculares y de reconocimiento químico, podrían indicarnos que entre estas poblaciones del sur y del norte de Madrid hay un inicio de aislamiento y quizás exista una barrera reproductiva. Pero, de momento no sé puede afirmar nada concluyente, se necesitan más análisis y experimentos de elección de olores de los machos por parte de las hembras, o de cruzamientos entre individuos de poblaciones distintas, para saber si existe aislamiento reproductivo efectivo.

Además en este trabajo hemos estudiado más a fondo lo que podría pasar entre poblaciones más cercanas. Antes hemos tratado dos poblaciones, norte y sur, que se encuentran separadas por al menos 100 km. de distancia entre ellas. Por eso, en el primer trabajo, hemos estudiado dos poblaciones de la Sierra de Gudarrama distanciados por unos 10 kilómetros y que se encuentran en contacto geográfico. Estas dos poblaciones (Fuenfria y Golondrina) son diferentes al nivel morfológico: los individuos de Fuenfria son más grandes y robustos que los de Golondrina. Además en la composición de las secreciones femorales existen diferencias importantes entre estas poblaciones. Al nivel de discriminación o reconocimiento químico, los machos diferenciaban entre la población de origen de la hembras y demostraban un interés mayor por las de su población que por las de la otra. Por el contrario, las hembras no parecían mostrar un interés mayor por el olor de los machos de su propia población.

Este trabajo mostró que a pesar de las diferencias morfológicas y químicas, las dos poblaciones se podrían reproducir sin diferenciar entre una pareja de su población o de la otra. Las hembras consideran “químicamente” a todos los machos por igual, independientemente de

que sean de su población o no. Sobretodo, la elección de pareja de las hembras mediante señales químicas se basaría en un compuesto presente en las secreciones femorales de los machos de las dos poblaciones, el Colesta-5,7-dien-3β-ol. Este compuesto es un precursor de la vitamina D3, con importantes funciones metabólicas (absorción del calcio y regulación del sistema inmune). Por lo que puede existir un compromiso entre destinar este compuesto al metabolismo general o a las secreciones femorales. La secreción de este compuesto sería especialmente costosa para los machos de baja calidad y por tanto podría ser un buen índice de calidad individual. Por lo tanto, las diferencias fenotípicas entre estas poblaciones cercanas no implican un aislamiento reproductivo efectivo.

Finalmente, podríamos interrogarnos sobre la historia de este complejo de especies y como una especie llegó a diferenciarse en varios morfotipos con una posible especiación efectiva. El escenario biogeográfico de la colonización de *P. hispanica* puede implicar al inicio un morfotipo ancestral que se propagó por toda la Península Ibérica. Pero al establecerse en diferentes habitats, los individuos han podido adaptarse por selección natural. Un hábitat con temperaturas bajas, altura y humedad alta no requiere las mismas adaptaciones que un hábitat más caluroso y menos húmedo. Las diferencias morfológicas y químicas entre los individuos de la Sierra de Madrid, con un hábitat mas frió y mas húmedo, y los individuos de Aranjuez, donde el hábitat es más cálido y menos húmedo, podrían ser consecuencia de las diferentes adaptaciones de cada población a diferentes habitats. Los individuos de las poblaciones de la Sierra son más grandes y más oscuros, y con pigmentación ventral más naranja, por lo que podría mostrar una buena adaptación al clima frió y de altura para poder tener suficiente energía frente al frió y para captar suficiente calor para ser activos. Al contrario, los individuos del sur de color más claro y más pequeños, podrían mostrar también una adaptación al hábitat pero de tipo más seco y caluroso. Además, se podría especular que el color de los individuos de las poblaciones del sur podría resultar de un proceso de mimetismo con el suelo de su hábitat, inducido por los depredadores.

Más localmente, entre las poblaciones de la Sierra que ocupan habitats similares pero de altitud, humedad y temperatura diferentes, muestran una variabilidad en la composición química de las secreciones femorale80s. Estas diferencias químicas podrían también ser debidas a la adaptación a una zona con condiciones diferentes. Además, si consideremos las diferencias en el número de poros femorales entre las poblaciones de la sierra y las del sur, podríamos explicar una vez más que estas poblaciones están adaptadas a diferentes habitats y que en consecuencias podrían utilizar diferentes señales de comunicación. Las poblaciones de la Sierra habrían desarrollado caracteres químicos de manera más importante que las poblaciones del sur. Al contrario, los individuos del sur utilizarían más otro tipo de comunicación, como la visual. La temperatura y humedad son esenciales para la persistencia y eficiencia de las secreciones químicas sobre el substrato. Entonces, en un hábitat donde la temperatura es alta y la humedad es baja, las secreciones no tendrían la misma eficiencia que en las poblaciones de la Sierra, donde las secreciones podrían permanecer más tiempo en el substrato y en consecuencia tener más importancia en la comunicación.

Los resultados han sugerido que la separación entre las poblaciones de *P. hispanica* en la zona de Madrid no está tan clara como parece. Entre las poblaciones del norte se podría pensar que por parte de los machos se está iniciando un proceso de especiación, pero que de momento la elección de pareja de las hembras basada en índices individuales de calidad comunes a ambas poblaciones está evitando un aislamiento reproductivo efectivo. Pero si las diferencias entre las poblaciones e individuos son más notables o importantes se podría llegar a un aislamiento reproductivo más efectivo como parece ocurrir entre las poblaciones del sur y las del norte. Un caso similar al observado en las especies de cíclidos del lago Malawi (Jordan

et al., 2003). En estos peces, las hembras tendrían preferencia por emparejarse con conespecificos con los que han tenido reconocimientos y contactos químicos previamente. Pero en el caso del complejo de especies de *P. hispanica* falta realizar varios experimentos de elección de pareja y cruzamientos entre poblaciones para comprender lo que está pasando. Además un estudio preciso del hábitat ayudaría a entender cómo las diferencias de hábitat pueden explicar la diferenciación entre las poblaciones.

BIBLIOGRAFIA

Alberts, A.C. 1993. Chemical and behaviour studies of femoral gland secretions in iguanid lizard. *Brain. Behav. Evol*. **41** : 255-260.

Andersson, M. 1994. S*exual selection*. Princeton University Press. Princeton, New Jersey.

Aragón, P., López, P. & 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.

Bernstein, C. & Bernstein H. 1997. Sexual communication. *Journal of Theorical Biology* **188**(1): 68-79.

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 in ecology & Evolution* **17**(12): 571-577.

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

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 en relation to sex. John Murray, London.

Endler, J.A. 1980. Natural Selection on Color Patterns in Poecilia reticulata. *Evolution* **34**(1): 76-91.

Fox, S. F. & 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.

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

Gómez, A., Font, E. & 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. & 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. & Sá-Sousa, P. 2002. Molecular phylogenetics of Iberian wall lizards (*Podarcis*): Is *Podarcis hispanica* a species complex? *Mol. Phylog. Evol.* **23**: 75-81.

Jordan, R., Kellogg, K., Juanes, F. & Stauffer, J.J. 2003. Evaluation of Female Mate Choice Cues in a Group of Lake Malawi Mbuna (Cichlidae). *Copeia* **1**:181-186.

Leal, M. & Fleishman, L. J. 2002. Evidence for habitat partitioning based on adaptation to environmental light in a pair of sympatric lizard species. *Proceedings of the Royal Society of London B* **269**:351–359.

Leal, M. & 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. & Mason, R.T. 2003. Pheromonally Mediated Sexual Isolation Among Denning Populations of Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis. Journal of Chemical Ecology* **29**(4): 1027-1043.

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

Martín, J. & López, P. 2005. Wall Lizards Modulate Refuge Use through Continuous Assessment of Predation Risk Level. *Ethology* **111**(2): 207-219.

Martín, J. & López, P. 2006. 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. *Chemoecology* **16**:31-38.

Mas, F. & Jallon, J.M. 2005. Sexual Isolation and Cuticular Hydrocarbon Differences between *Drosophila santomea* and *Drosophila yakuba*. *Journal of Chemical Ecology* **31**(11): 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.

Mellado, J. & Olmedo, G. 1981. Sobre las poblaciones de *Podarcis* en el Macizo de Guadarrama. Doñana, *Acta Vertebrata* **8**: 299–300.

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

Pinho, C., Ferrand, N. & Harris, D.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. & 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. *Evolutionary Biology* **8**: 63.

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

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

Shine, R., Reed, R.N., Shetty, S., Lemaster, M. & Mason, R.T 2002. Reproductive isolationg mechanisms between two sympatric sibling species of sea snakes. *Evolution,* **56**(8): 1655–1662.

Wyatt, T. D. 2003. Pheromones and Animal Behaviour. Eds. Cambridge University Press.