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Hace constar:

Que el trabajo descrito en la presente memoria, titulado: "The function of red colouration in the spiny-footed lizard (*Acanthodactylus erythrurus*, Schinz 1833)", ha sido realizado bajo su dirección por M^a Belén Fresnillo Barba en la Unidad Docente de Ecología del Departamento de Ciencias de la Vida de la Universidad de Alcalá, dentro del Programa de Doctorado "Ecología, Conservación y Restauración de Ecosistemas", reuniendo todos los requisitos necesarios para su aprobación como Tesis Doctoral .

Alcalá de Henares, 15 de mayo de 2014

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Departamento de Ciencias de la Vida
Unidad Docente de Ecología

**The function of red colouration in the
spiny-footed lizard (*Acanthodactylus
erythrurus*, Schinz 1833)**

Memoria presentada para optar al grado de Doctora
por la Universidad de Alcalá

Programa de doctorado:
"Ecología, Conservación y Restauración de
Ecosistemas" (D330)

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*Si caminas solo,
irás más rápido;
si caminas acompañado,
irás más lejos*

Proverbio chino

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* Nota: Esta Tesis Doctoral está escrita en formato bilingüe para aspirar a la Mención Internacional.

* Note: This PhD Thesis is written in a bilingual format to aspire to the International Mention.

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RESUMEN

En muchos grupos animales, la coloración conspicua es una señal visual importante en la comunicación intra e interespecífica. Esta coloración puede tener diferentes funciones dependiendo de la parte del cuerpo donde se muestra, y también de la edad o el estado reproductivo del individuo que la muestra. La coloración conspicua es común en lacértidos, y la lagartija colirroja (*Acanthodactylus erythrurus*) en concreto, constituye un buen ejemplo para estudiar algunas de las funciones de la coloración conspicua y los posibles cambios de función a lo largo de la ontogenia, ya que las crías desarrollan una coloración roja muy llamativa en la parte ventrolateral de la cola, que se extiende en los juveniles hacia las patas traseras. Esta coloración se pierde cuando los machos alcanzan la madurez sexual, pero es retenida, e incluso incrementada, en las hembras adultas durante el celo, aunque desaparece en hembras grávidas.

En la presente tesis doctoral, abordamos varias posibles funciones de la coloración conspicua de la lagartija colirroja. Una hipótesis es la posible función de la coloración roja como un mecanismo antidepredatorio. Para ello comprobamos si la coloración roja en individuos de diferentes clases de edad estaba relacionada con comportamientos arriesgados. Grabamos el comportamiento en el campo de individuos de todas las clases de edad, y calculamos varios índices relacionados con su actividad, el uso de parches despejados lejos de los refugios y los movimientos estereotipados de patas y cola. Encontramos que los individuos más rojos (menos naranjas) pasaban más tiempo más lejos de los refugios, lo cual es consistente con una función

antidepredatoria de la coloración roja. Comprobamos también si la coloración de la cola de las crías de lagartija colirroja actuaba como un señuelo para los depredadores aviares. Para ello usamos modelos de lagartija contruidos a base de plastilina y escayola que bien tenían la cola pintada de rojo o con bandas claras y oscuras. Los modelos fueron usados tanto en un estudio en el campo como en un experimento en cautividad con un depredador común de lagartijas (*Falco tinnunculus*). Encontramos que las colas rojas incrementaban la visibilidad de los modelos en el campo, pero también que éstas eran un señuelo efectivo que desviaba los ataques aviares de las zonas más vulnerables del cuerpo hacia la cola, apoyando así la función antidepredatoria.

Otra hipótesis sobre la función de la coloración conspicua cuando aparece en juveniles (la hipótesis de reducción de agresividad) establece que la coloración conspicua sirve para reducir la agresividad de los adultos de la misma especie. Por lo tanto, testamos la influencia de la coloración de los juveniles de lagartija colirroja en la agresividad recibida por parte de los adultos mediante la grabación en vídeo de encuentros en condiciones de cautividad entre adultos de ambos sexos y juveniles que, o bien presentaban su coloración natural, o una pintura roja o blanca cubriendo sus zonas naturalmente rojas. Nuestros resultados apoyan la hipótesis de la reducción de agresividad, ya que los juveniles más rojos recibieron menos agresiones por parte de los adultos.

Cuando la coloración conspicua aparece en adultos, se suele relacionar con selección sexual. Por ello comprobamos si la coloración roja de las hembras adultas, que previamente se ha sugerido que tiene una función relacionada con la reproducción, es usada por los machos

adultos en la selección de pareja. Para ello ofrecimos pares de hembras que diferían en su edad/tamaño/madurez sexual (adultas o juveniles) y/o su color (rojo o blanco) a machos adultos y anotamos el tiempo que el macho pasaba cerca o en contacto con cada hembra. Encontramos que los machos pasaban significativamente más tiempo cerca de las hembras adultas independientemente de su coloración, y que entre las hembras adultas prefirieron las rojas, sugiriendo que la coloración roja de las hembras es un ornamento sexual.

La coloración roja podría estar informando sobre el estado reproductivo de la hembra, para comprobar esta hipótesis estudiamos la posible relación entre la coloración roja y las hormonas sexuales que regulan su ciclo reproductor, concretamente β -estradiol y progesterona. Exploramos la variación natural en los niveles hormonales y la coloración de las hembras a lo largo del ciclo reproductivo, establecimos las asociaciones existentes entre color y hormonas, y finalmente, manipulamos las concentraciones de β -estradiol y progesterona comprobando sus efectos sobre la coloración. Nuestros resultados sugieren que el color rojo de las hembras adultas indica su estado reproductivo, es decir, su fertilidad, ya que las hembras más rojas estaban más cerca de la ovulación. Además, concentraciones elevadas de β -estradiol o de β -estradiol junto con progesterona disparan la pérdida de la coloración roja inmediatamente después de la ovulación.

En conclusión, la coloración roja de la lagartija colirroja es un carácter multifuncional que cumple diferentes funciones en las diferentes clases de edad. Las señales son costosas, por lo tanto, usar la misma señal

para diferentes funciones, o reutilizar la misma señal en diferentes clases de edad debería ser ventajoso. En la lagartija colirroja, el retraimiento de la coloración roja hacia partes más ventrales a lo largo del proceso ontogenético sugiere que la presión de depredación podría estar modificando la expresión de la señal, y pone de manifiesto que los individuos de diferentes clases de edad podrían enfrentarse a fuerzas selectivas muy diferentes. Este trabajo constituye un buen ejemplo de cómo las señales son moldeadas durante el proceso evolutivo, y proporciona evidencias sobre la conservación de un carácter multifuncional a lo largo de la ontogenia de una especie, un aspecto poco estudiado con anterioridad.

ABSTRACT

In many animal taxa, conspicuous colouration is a visual signal used for communication purposes among conspecifics or heterospecifics. This kind of colouration may have different functions depending on the body part where it is present and also depending on the age or reproductive status of the individual showing it. Conspicuous colouration is common in lizards, and spiny-footed lizards (*Acanthodactylus erythrurus*) are particularly good subjects to test different hypotheses explaining the functions of conspicuous colouration and possible changes in these functions during ontogeny, as hatchlings develop conspicuous red colouration on the ventrolateral part of their tails that spreads towards the hind limbs in juveniles. This colouration is lost when males achieve sexual maturity, but is retained, and even enhanced, in adult females while they are sexually receptive, although it is lost when they become gravid.

In this PhD thesis we addressed several possible functions of the red colouration in spiny-footed lizards. One hypothesis is the possible function of red colouration as an antipredatory mechanism. We tested whether red colouration in individuals of all age classes was related to their risky behaviours, so we recorded lizard behaviour in the field and calculated several indices of lizard activity, the use of open areas far from refuges and stereotyped movements of limbs and tail. We found that redder (less orange) individuals spent more time further from refuges, a result consistent with the antipredatory function of red colouration. We also tested whether red colouration in the tail of hatchlings was a decoy

for avian predators. Using plasticine and plaster lizard models with red or striped dark and light tails, both in the field and with a common lizard predator (*Falco tinnunculus*) in captivity, we found that red tails increased model conspicuousness in the field, but also that they were an effective decoy for avian predators, diverting attacks from vulnerable body parts towards the tail, thus supporting the antidepredatory function.

Another hypothesis about the function of conspicuous colouration in juveniles (the aggression-avoidance hypothesis) states that it reduces aggression from adult conspecifics. Therefore, we tested the influence of juvenile red colouration on the aggression received from adults by conducting videotaped encounters in captivity between adults of both sexes and juveniles that showed either their natural red colouration, or a painted red or white colouration covering their natural red parts. Our results supported the hypothesis, as redder juveniles received less adult aggression.

When conspicuous colouration is present in adults, it is commonly associated with sexual selection. Therefore, we tested whether red colouration in adult females, that has already been suggested to have a mating-related function, is used by males in mate selection. We presented adult males with pairs of females differing in their age/size/sexual maturity (adult or juvenile) and/or their colour (red or white) and recorded the time the male spent near or in contact with each female. We found that males spent more time near adult females regardless of their colour, and within adult females, they preferred the red ones, suggesting that female red colouration is a sexual ornament.

Red colouration might be providing information about the female reproductive status, and to test this hypothesis we studied the possible link between colouration and female sex steroid hormones that regulate their reproductive cycle, specifically β -estradiol and progesterone. We explored natural variation of colouration and hormone levels along the reproductive cycle, determined the relationships between colouration and hormones and, finally, experimentally manipulated plasma β -estradiol and progesterone concentrations to check the effects on colouration. Our results suggest that red colour in adult females signals the reproductive status, i.e., fertility, as redder females were closer to ovulation. Moreover, high concentrations of either β -estradiol alone or β -estradiol together with progesterone trigger the loss of red colouration immediately following ovulation.

In conclusion, red colouration in spiny-footed lizards is a multifunctional signal that is used for different purposes at different age classes. Signals are costly to produce, so using the same signal for different functions or reutilizing the same signal at different age classes should have advantages. In spiny-footed lizards, the retraction of the red colouration to more ventral parts along the ontogenetic process suggests that predation pressures might be modulating the expression of the signal, and points out that individuals of different age classes may be suffering very different selective pressures. This study is also a good example of how signals can change during the evolutionary process, and provides evidences about the conservation of a multifunctional signal during the ontogeny of a species, something poorly studied.



Chapter 1

GENERAL INTRODUCTION

Colour signals in animals

Colouration is a visual signal used in many animal taxa for communication purposes (Cott 1940). Many species use colour signals for intraspecific communication (reviewed by Rowland 1979), with colour providing information to conspecifics about an individual sex (Cooper & Burns 1987; Price *et al.* 2008) or age (Kemp 2006; Hawkins, Hill & Mercadante 2012), or including more detailed information about its fertility (Pitcher, Rodd & Rowe 2007; Rowe *et al.* 2010) or sexual attractiveness (Kemp & Rutowski 2011; Olsson, Andersson & Wapstra 2011; Deere *et al.* 2012; Rutowski & Rajyaguru 2013). Colouration may also inform about the health status of the signaller (del Cerro *et al.* 2010; Barrios-Miller & Siefferman 2013). In addition, several species of very different animal groups use colour signals to advertise dominance (Senar 2006; Midamegbe *et al.* 2011), being conspicuous colours usually associated with dominant behaviours (Miles *et al.* 2007; Ardia, Broughton & Gleicher 2010).

Colouration is also commonly used for interspecific communication, for example as a species recognition signal (Losos 1985; Alatalo, Gustafsson & Lundberg 1994), and can also be related to predator-prey interactions involving crypsis, mimicry and aposematism (Ruxton, Sherratt & Speed 2004; Stevens & Ruxton 2012). It may even serve to attract predator attacks towards expendable body parts (Telemeco, Baird & Shine 2011), or in association with some behaviours,

to inform the predator that it has been spotted (Murphy 2007), so the potential prey is alert and ready to escape.

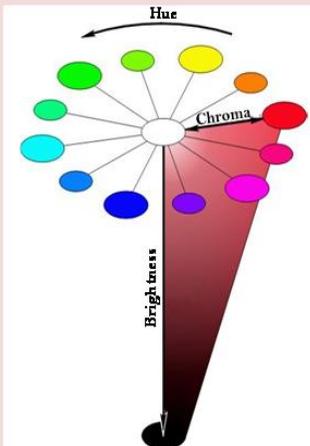
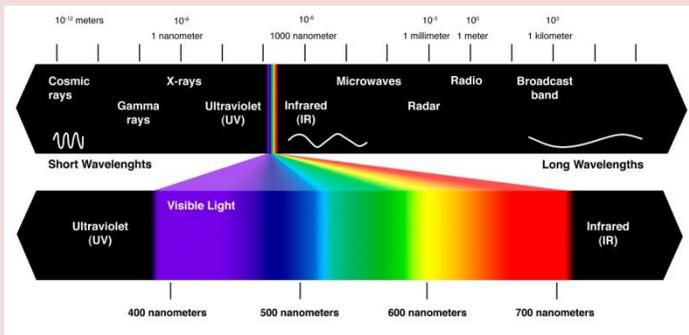
Colour is a complex signal that can be defined by three parameters: brightness, chroma and hue (Hill & McGraw 2006; Box 1.1). However, the spectral reflectance of an object is not the only factor influencing the colour signal, as signals can only be perceived if they are distinguishable from the background (Espmark, Amundsen & Rosenqvist 2000). For example, the visibility of a colour signal depends on ambient light conditions and its contrast with the background colour (Endler 1993; Vorobyev & Osorio 1998). Moreover, the spectral sensitivity of the viewer will also exert a strong influence on the perceived colour. Different species of vertebrates have different spectral ranges (Bowmaker 2008), so a colour signal can be more conspicuous to conspecifics than to other viewers if their spectral sensitivities differ (Håstad, Victorsson & Ödeen 2005; Willink, Bolaños & Pröhl 2014). Colour signals are complex, and the information given by a conspicuously coloured patch may be codified not only in the colour itself, but also in the size of the patch (Pryke *et al.* 2002).

Why do some animals change their colouration?

Colour changes are relatively common in vertebrates, although the time scale of these changes varies enormously. When colouration changes are relatively quick (from seconds to hours), they are called physiological colour changes, but when colouration changes involve

Box 1.1

Light of wavelengths between 400 and 700 nm (violet to red) is seen as colour by humans. However, the range of visible colours varies between taxa (Bowmaker 2008; Bradbury & Vehrencamp 2011). The phenomenon of colour depends on the way an animal perceives different wavelengths of light and is not only an inherent property of the object, so the viewer's physiology dictates how colours are perceived. For example, while some species (e.g. most birds) can perceive the ultraviolet (between 300 and 400 nm), others (e.g. humans) cannot (Hill & McGraw 2006).



Brightness or value, chroma or saturation, and hue are the colour parameters most commonly used in the study of colouration. They can be easily visualized in the colour wheel defined by Albert Munsell (1912). Brightness is how light/dark an object is, chroma or saturation is referred to the amount of different wavelengths included in the colour (unsaturated colours include more wavelengths than saturated ones) and hue is the wavelength of light (what we normally call colour).

longer processes (from days to weeks), they are called morphological colour changes (Bagnara & Hadley 1973).

Colour changes can be associated with ontogeny, as different life stages can be under different selective pressures (Booth 1990; Hawlena *et al.* 2006; Wilson, Heinsohn & Endler 2007; Hawlena 2009), that is, some colours may be advantageous in one life stage but disadvantageous in others. It is also common that individuals change their colouration during the breeding season (Sköld *et al.* 2008; Stelkens *et al.* 2008; Chan, Stuart-Fox & Jessop 2009) to signal fertility or genetic/phenotypic quality to potential mates. Different predation pressures may also promote changes in prey colouration to avoid predation (Hemmi *et al.* 2006; Rosenblum 2006; Farallo & Forstner 2012). Moreover, colour changes can also be triggered by abiotic factors such as temperature (Silbiger & Munguia 2008; Langkilde & Boronow 2012) or illumination (Shiraki, Kojima & Fukada 2010; Darnell 2012). In the following sections we are focusing on the three first causes of colour change mentioned above.

Colour changes during ontogeny

The same colouration can be expressed in both adults and juveniles, but changes in colouration between different life stages in the same species (ontogenetic changes) are relatively common (Booth 1990; Fig. 1.1). Different life stages can be under different selective pressures, so a colouration that may be adaptive for juveniles can be disadvantageous for adults and vice versa. Ontogenetic colour changes

can be associated with changes in size, vulnerability or habitat use (Booth 1990; Hawlena *et al.* 2006; Wilson, Heinsohn & Endler 2007; Hawlena 2009). Excluding aposematic species, ontogenetic colour changes generally imply a change from cryptic colouration in juveniles to avoid predation to conspicuous colouration in sexually mature individuals, often related to sexual selection. However, some species undergo ontogenetic colour changes in the opposite direction, with conspicuous colouration in juveniles that becomes cryptic in adults (Thresher 1978; Londei 2005; Landová *et al.* 2013; Fig. 1.1).

Conspicuous colouration in juveniles has been, in general, poorly studied, but communication between age classes seems to be an important intraspecific function of juvenile colouration. For example in birds, parental investment in offspring feeding has been shown to be modulated by colouration of chick mouth (de Ayala *et al.* 2007) or plumage (Ligon & Hill 2010). Male birds have also been suggested to defend more vigorously chicks with brighter feather colouration (Barrios-Miller & Siefferman 2013). Another suggested function for juvenile colouration in different animal taxa is the signalling of subordination or age to reduce male adult aggression (Clark & Hall 1970; Hill 1989; Ochi & Awata 2009), although the signalling function and its effects on adult aggressiveness is still under debate (Cooper & Greenberg 1992; Hawkins, Hill & Mercadante 2012). Furthermore, it is still unknown whether juvenile colouration is subject to social selective pressures similar to those promoting adult colour variation (Fargallo *et al.* 2014).

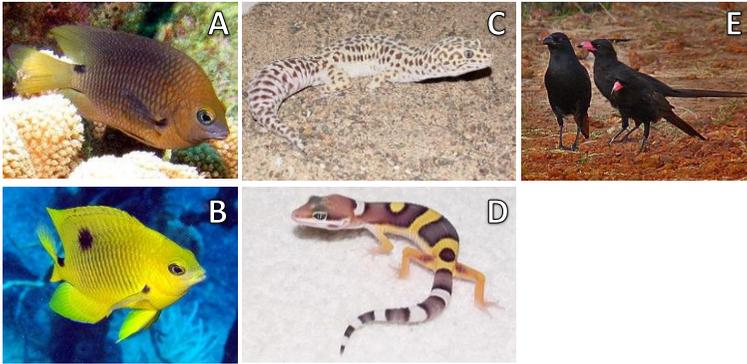


Fig. 1.1. Examples of species with conspicuously coloured juveniles and cryptic adults. (A) Adult three-spot damselfishes (*Stegastes planifrons*) have dull brown colouration that can be easily concealed within the reef, while (B) juveniles of the same species develop very conspicuous yellow colouration which does not match the background. (C) Adult leopard geckos (*Eublepharis macularius*) have brown dots over their yellowish colouration to match the ground colour, while (D) juveniles display a colourful pattern of bands and dots. (E) Similarly, juvenile piapiacs (*Ptilostomus afer*) have pink colouration in their beaks that is lost in adults.

Colour changes during the breeding season

Sexually selected characters were defined by Darwin (1871) as heritable traits associated with mating success. Several species of very different taxa develop sexually selected colourful patches which are supposed to provide information about the phenotypic and/or genetic quality of the individual carrying them (Weiss 2006; Cuervo, Palacios & Barbosa 2009; Kemp & Rutowski 2011). Sexually selected traits should be reliable signals of quality, because if low-quality individuals were able to exhibit the same signal as high-quality ones, the evolutionarily strategy of signalling would be destabilised. Although honest signalling

might require costly signals (Zahavi 1975), alternative explanations that do not require costly signalling have also been postulated (Box 1.2).

In species showing sexual dichromatism, the sex having lower parental investment (commonly the male) usually develops more conspicuous colouration (Andersson 1994; Figs. 1.2A and 1.2B). Colour patches in males developed during the breeding season commonly serve to establish dominance hierarchies to get access to mates (Pryke, Lawes & Andersson 2001; López, Martín & Cuadrado 2004), with more colourful males being also more dominant. Colour signals expressed during the breeding season can also be used to attract females (Waitt *et al.* 2003; Bajer *et al.* 2010), with more colourful males increasing mating success, and thus fitness, compared to less coloured ones. But the effects of sexual selection are not always restricted to males, because female traits may be also affected (Clutton-Brock 2009). Female conspicuous colouration, which is developed during the breeding season in some taxa (e.g. reptiles; Cooper & Greenberg 1992), has been paid little attention, as it was generally understood as a consequence of genetic correlation between male and female traits (e.g., Muma & Weatherhead 1989). However, female colouration can evolve independently of male colour by direct selection on females (Amundsen 2000). Indeed, conspicuous colouration in several species with conventional sex roles is present exclusively in females (mammals: Nunn 1999; fish: Amundsen & Forsgren 2001; birds: Heinsohn, Legge & Endler 2005; reptiles: Chan, Stuart-Fox & Jessop 2009; Figs. 1.2C and 1.2D). Female conspicuous colouration is frequently expressed seasonally during the breeding

Box 1.2

Communication often takes place between individuals that do not coincide entirely in their interests. However, honest communication frequently persists. Evolutionary biologists have tried to explain how communication can originate and be maintained among individuals with partially conflicting interests. Zahavi (1975) proposed that the mechanism maintaining signal honesty may be the payment of handicaps (costs) by the signaller. This is often referred to as the handicap principle or costly signalling. Grafen (1990) added to the handicap principle the idea that high-quality signals must be more costly for low-quality signallers (C_L) than for high-quality ones (C_H). Both types of individuals will gain the same benefits, so different costs will make the optimal signal intensity lower for low-quality individuals (S^*_L) than for high-quality ones (S^*_H).

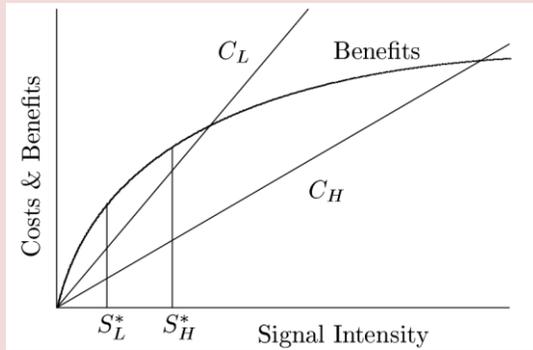


Figure 7.2a in Johnstone (1997).

Several critiques of the handicap principle have emerged (Higham 2014), leading to the idea that high costs are not necessary for honest signalling. According to these critiques, whether the signal costs are high or low for a given individual is not the important point, because what really matters is that high-quality individuals will obtain net benefits by displaying the high-quality signal, but those benefits could not be achieved by low-quality individuals. Other theories, such as the punishment of the cheaters or the presence of signals that are impossible to fake, have been also postulated as alternatives to explain the maintenance of honest signals in animal communication (Higham 2014).

period and, in these cases, it often signals some aspect of their reproductive status, either stimulating or rejecting courtship by males (Nunn 1999; Amundsen & Forsgren 2001; Chan, Stuart-Fox & Jessop 2009).

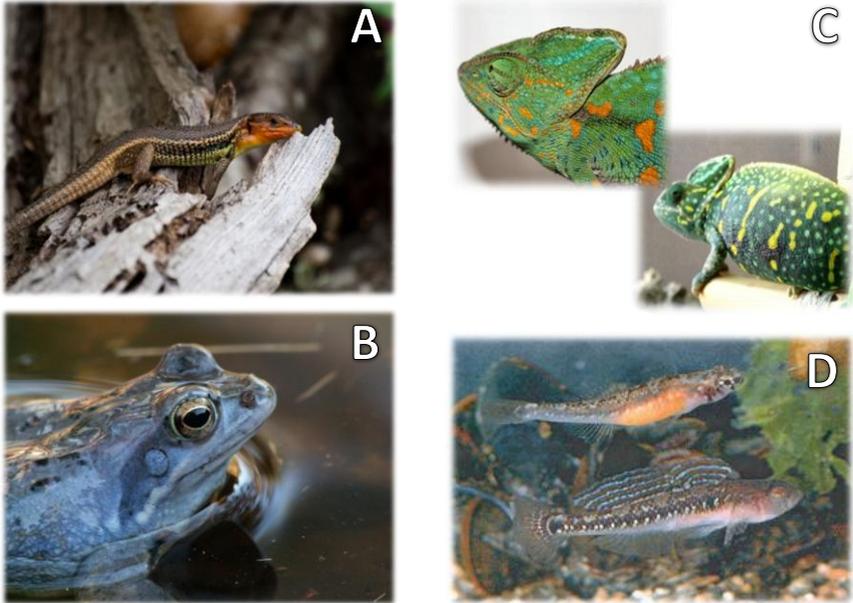


Fig. 1.2. Examples of colouration changes during the breeding season. (A) Males of the large psammodromus (*Psammodromus algirus*) and (B) the moor frog (*Rana arvalis*) display conspicuous colouration during the breeding season that disappears after reproduction. (C) Females of the common chameleon (*Chamaeleo chamaeleon*) change their body colouration from orange patches during the receptive phase to yellow and blue patches during gravidity and (D) females (upper fish) of the two-spotted goby (*Gobiusculus flavescens*) develop orange bellies during the breeding season.

Colour changes caused by predation pressures

As we mentioned above, signals are supposed to incur a cost for signallers, and one of the most important costs of conspicuous colouration may be increased conspicuousness to predators (Endler 1980; Arnold 1984; Haskell 1996). Predation is a strong selective pressure driving the evolution of prey traits, and has been shown to affect prey behaviours like refuge use or feeding rates (Sih 1992; Amo, López & Martín 2003), and also prey morphology (Losos *et al.* 2006) and colour (Garcia & Sih 2003; Hemmi *et al.* 2006). One of the most generalized colour changes caused by predator pressure involves background matching in order to increase prey camouflage and thus reduce predation risk (Garcia & Sih 2003; Hemmi *et al.* 2006; Fig. 1.3).

Background matching is not the only strategy involving colour changes to reduce predation pressure. Transformational mimics are species that mimic different models during their life stages (Mathew 1935; Zaret 1977). Sometimes, these models may be aposematic (Booth 1990), or very aggressive, with the considerable protection from predators these features might confer (Fig. 1.3). Other species rely on the conspicuous colouration of non-vital body parts to divert predator attacks to these lures, increasing the probability of escaping without serious injuries (Van Buskirk *et al.* 2004; Hawlena *et al.* 2006; Watson *et al.* 2012). This is taken to the extreme in several reptile species with conspicuous colouration in the tail (Pianka & Vitt 2006), which is autotomous and can therefore be detached from the rest of the body without compromising survival (Arnold 1988; Bateman & Fleming

2009). Conspicuous tail colouration can be conserved throughout the whole life, or can be lost during the ontogeny due to changes in habitat use or predation risk (Booth 1990; Hawlena *et al.* 2006; Hawlena 2009).

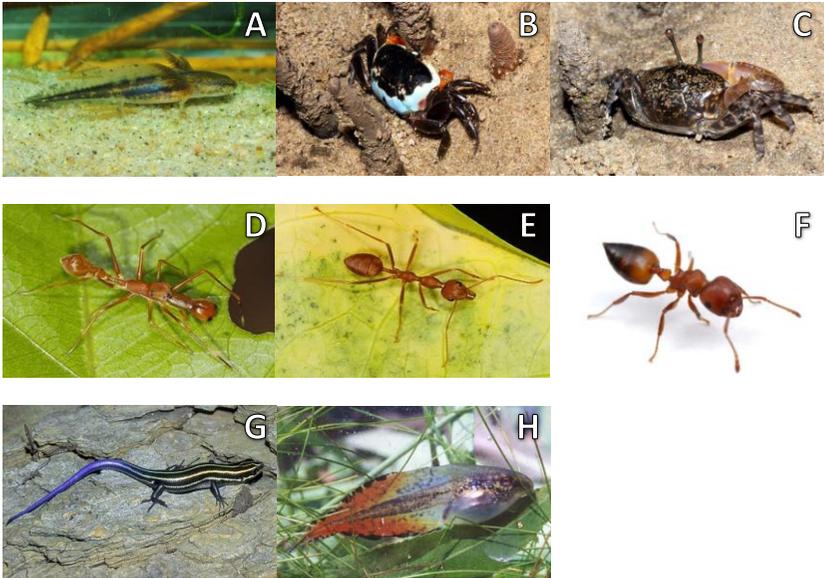


Fig. 1.3. Examples of colour changes caused by predation pressures. **Background matching:** (A) Streamside salamander (*Ambystoma barbouri*) larvae undergo colour changes to better match the background in response to predator presence, and fiddler crabs (*Uca vomeris*) change their (B) colourful social communication badges for (C) cryptic dull mottled carapaces when predation risk increases. **Transformational mimics:** Different instars of the spider (D) *Myrmarachne* sp. mimic ant species like (E) *Oecophylla smaragdina* and (F) *Crematogaster castanea* along its ontogeny. **Lures to divert predator attacks from vital body parts:** (G) Five-lined skink (*Plestiodon fasciatus*) juveniles show bright blue tails that fade away during ontogeny, and (H) tadpoles of the frog *Hyla versicolor* develop red tails that are lost during metamorphosis.

Proximal causes of colour change

The regulation of colour changes differs depending on the type of colour change studied. As mentioned above, there are two types of colour changes depending on the temporal scale in which they occur: physiological changes (temporal scale from seconds to hours) and morphological changes (temporal scale from days to weeks). Physiological colour changes usually appear in response to illumination, temperature or background colouration changes (King, Hauff & Phillips 1994), and they are subjected to neuromuscular (Messenger 2001) or, more commonly, neuroendocrine (Nery & Castrucci 1997) regulation. Morphological colour changes, however, are often triggered by environmental factors (e.g. changes in the photoperiod; Musolin & Numata 2003), which induce the production of certain hormones ultimately responsible for the colour change. For example, seasonal changes in sex steroid hormones induce breeding colouration (Jessop, Chan & Stuart-Fox 2009). In the following sections we are focusing on pigmentary colouration and the hormonal regulation of morphological colour changes.

How is colour produced?

In animals, colouration is the result of selective absorption, reflection and/or refraction of the light incident on the skin or other external structures, and can be classified as structural and pigmentary. Structural colours are those produced by the physical microstructure of

the integument, and are present in many taxa (Prum & Torres 2004; Mähthger *et al.* 2009; Grindstaff *et al.* 2012; Lo & Lee 2014). Pigmentary colours are also widespread in animals (Blount & McGraw 2008; van den Brink *et al.* 2012; Weiss, Foerster & Hudon 2012), and are the consequence of certain molecules (pigments) that absorb part of the light spectrum. Pigments are sometimes embedded in proteins of the integumentary tissue (e.g. hair or feathers), but very often are located inside skin cells called chromatophores (Bagnara & Hadley 1973). There are different types of chromatophores depending on the pigment they contain, mainly: melanophores contain melanin, and usually produce black or brown colouration (McGraw 2006a), xanthophores contain carotenoids, and usually produce yellow or orange colouration (McGraw 2006b), and erythrophores can contain both carotenoids and pteridines, and usually produce red and orange colouration (McGraw 2006c).

Pigments like carotenoids or pterins also have important physiological functions as antioxidants and elements of the immune system (Olson & Owens 1998; Oettl & Reibnegger 2002; Hörak *et al.* 2006). Therefore, the development of pigmentary colouration may involve trade-offs with other physiological processes, and only individuals in good health state could invest more pigments in producing the conspicuous colouration. Moreover, some of these pigments, like pterins, can be produced by animals endogenously from their precursors (Ziegler 1965), but others, like carotenoids, can only be obtained through the diet (Britton 1998), what implies differences in the signalling value of the colouration depending on the pigments involved.

Hormonal regulation of colouration

Physiological colour changes like skin darkening or lightening are commonly regulated by several kinds of hormones like pituitary (e.g. melanophore-stimulating hormone or MSH), adrenal (e.g. adrenocorticotrophic hormone or ACTH) and neural (e.g. catecholamines) ones (Bagnara & Hadley 1973). Morphological colour changes like the development of breeding colouration are, as mentioned above, more related with sex steroid hormones like testosterone in males, and progesterone and estradiol in females. Despite the relatively well studied effect of testosterone on male colouration during the reproductive season (Cooper, Mendonca & Vitt 1987; Fargallo *et al.* 2007; Miles *et al.* 2007; Ardia, Broughton & Gleicher 2010), there are few studies analysing the hormonal regulation of female breeding colouration (but see some exceptions in mammals: Dixson 1983, fishes: Sköld *et al.* 2008, birds: Grindstaff *et al.* 2012 and reptiles: Cooper & Greenberg 1992).

The relation between sex steroid hormones and female colouration does not seem to be simple or common for all vertebrates. In mammals, for example, estradiol seems to be closely related to an increase in female breeding colouration (Dixson 1983). In birds, most studies have focused on the role of testosterone on the intensity of female ornaments, not always finding a positive relationship (e.g. Grindstaff *et al.* 2012). In reptiles, previous research has been biased towards families of the suborder Iguania, in which conspicuous colouration is generally present during gravidity. According to these studies, female breeding colouration in lizards is triggered by

progesterone, while estradiol may only accelerate progesterone effects (Cooper & Greenberg 1992). In fishes, no significant relation between sex steroid hormones and female colouration has been found (Sköld *et al.* 2008). According to this disparity of results, more studies are necessary to understand the role that sex steroid hormones may play in the regulation of female breeding colouration in vertebrates.

Study species

The spiny-footed lizard (*Acanthodactylus erythrurus*) is a medium-sized lizard (Seva Román 1982; Carretero & Llorente 1993) that is present in southern and central Iberian Peninsula and northern Africa (Belliere 2006). In populations in central Spain, newborn lizards appear in mid-August and are active until November. At the beginning of April the following spring, these lizards become active again, and they do not reach sexual maturity until their second spring (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992). Therefore, all individuals can be divided in three rough age categories, hereafter referred to as hatchlings (from hatching until the first winter), juveniles (from first to second winter) and adults (from the second winter onwards).

Colouration in this species undergoes ontogenetic changes (Fig. 1.4). Hatchlings show a strongly contrasted dorsal pattern of dark and light bands that run along their body. This pattern starts reticulating in juveniles, and becomes dark and light patches in adults (Seva Román 1982). Hatchlings have red colouration on the ventrolateral part of their

tails which spreads towards the rear part of the hind limbs in juveniles (Seva Román 1982; Carretero & Llorente 1993). Red colouration on the tail is clearly visible from above in hatchlings and small juveniles (Fig. 1.4). Juvenile males lose the red colour when they are around one year old, while juvenile females retain it through adulthood (Cuervo and Belliure 2013; Seva Román 1982). In adults, colour differences are evident, mainly at the beginning of the breeding season (Fig. 1.4). Sexually receptive females increase the intensity of the red colouration retained from the juvenile phase until they are gravid, when they become pallid yellow, nearly white (Cuervo & Belliure 2013). Males, however, show white colouration on the rear part of their hind limbs and the ventrolateral part of their tails along the whole reproductive season (Seva Román 1982; Fig. 1.4). Adult males also develop yellow spots on their flanks, which are brighter during the breeding season (Seva Román 1982).

The function of the red colouration might change from one life stage to another. The presence of the red colouration in the autotomizable tail, where it is visible from above in early life stages, may imply an antipredatory function (Arnold 1988; Bateman & Fleming 2009). The red colour would eventually be lost during ontogeny due to changes in behaviour or habitat use (Hawlena *et al.* 2006; Hawlena 2009). Besides, a reduction in adult aggression has been suggested for other species in which juveniles show juvenile-specific or female-like colouration (Clark & Hall 1970; Werner 1978). Consequently, both functions (antipredatory and aggression avoidance) might be present in

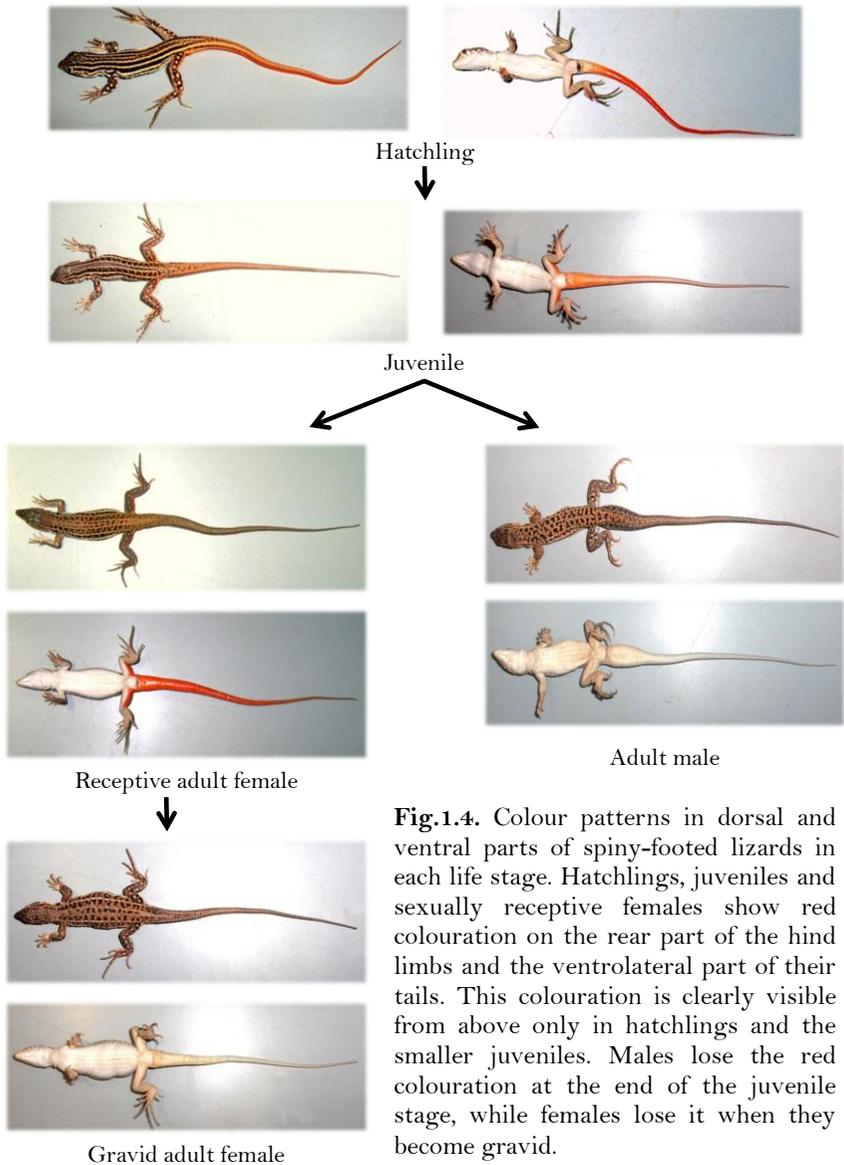


Fig.1.4. Colour patterns in dorsal and ventral parts of spiny-footed lizards in each life stage. Hatchlings, juveniles and sexually receptive females show red colouration on the rear part of the hind limbs and the ventrolateral part of their tails. This colouration is clearly visible from above only in hatchlings and the smaller juveniles. Males lose the red colouration at the end of the juvenile stage, while females lose it when they become gravid.

hatchlings and juveniles. Red colouration in adult females has been suggested to have a mating-related function (Cuervo & Belliure 2013), as females change their colouration in accordance with the different phases of the reproductive cycle, what might imply also a hormonal regulation of these colour changes (Cooper & Greenberg 1992). However, it is unknown whether this colouration is a sexual ornament (Weiss 2006), a courtship stimulus (Cooper & Crews 1988) or a sex indicator (Cooper & Burns 1987).

Whether red colouration displayed through spiny-footed lizard ontogeny has several functions or not is still unknown, although it seems plausible that the mating-related function suggested for adult females (Cuervo & Belliure 2013) is not present in hatchlings or juveniles. The presence of the same conspicuous colouration in different ontogenetic stages of a species is relatively common (e.g. Muehter, Greene & Ratcliffe 1997; Husak *et al.* 2004) but the study of this colouration in all life stages is not (but see Werner 1978). The global study of the functions and proximal causes of these traits may provide insight into the forces driving the evolution of such signals under different selective pressures. Taking into account all the information about this species, we considered the spiny-footed lizard as a good model organism to study different functions of a conspicuous colouration at different life stages, as well as some of the proximal causes (namely hormonal regulation) of morphological (seasonal) colour changes in adult females.

Objectives and hypotheses

General aim and hypotheses

The **general aim** of this PhD Thesis is to understand the functions of the red colouration that spiny-footed lizards develop on their hind limbs and tails along different life stages, and also some proximal causes (hormonal regulation) of this colouration in adult females. The presence of the same signal for different purposes in different life stages of the same species is not commonly studied, and may help understand how a signal appears and is maintained along the ontogenetic process, despite the different selective pressures probably present along this process.

The **general hypothesis** is that the red colouration on hind limbs and tails of spiny-footed lizards play different roles in different life stages and that this colouration in adult females is hormonally regulated.

Specific aims and thesis outline

This PhD thesis is structured in eight chapters, including this general introduction (Chapter 1). Chapters 2 to 6 are research chapters that correspond to scientific papers submitted to scientific journals. Chapter 7 consists of a general discussion of the main results found in the five research chapters. Finally, Chapter 8 shows the main conclusions of this PhD thesis.

The specific aims of this PhD thesis are:

Specific aim 1: To test the hypothesis that the red colouration present in spiny-footed lizards has an antipredatory function. Predictions derived from this hypothesis are addressed in Chapters 2 and 3.

Chapter 2: *Ontogenetic shifts in risky behaviours are related to body size and colouration in spiny-footed lizards.*

In this chapter, we tested prediction 1.1: lizards with redder tails will behave more riskily. Specifically, we tested whether risky behaviours were associated with the colour displayed by spiny-footed lizards at different ages/sizes.

Chapter 3: *Red tails are effective decoys for avian predators.*

In this chapter, we tested prediction 1.2: lizards with red tails will receive a larger proportion of attacks in the tail than lizards without red tails. Specifically, we compared the number of avian attacks in the tail or other body parts between lizard models with red or striped dark and light tails. We also addressed the possible increase in conspicuousness lizards might suffer derived from exhibiting red tails.

Specific aim 2: To test the hypothesis that red colouration present in hind limbs and tails of juvenile spiny-footed lizards plays a role in inter age-class communication, reducing adult aggression towards juveniles. Predictions derived from this hypothesis are addressed in Chapter 4.

Chapter 4: *Red colouration in juvenile spiny-footed lizards (Acanthodactylus erythrurus) reduces adult aggression.*

In this chapter, we tested prediction 2.1: naturally redder juveniles will receive less adult aggression; and prediction 2.2: juveniles painted red will receive less adult aggression than juveniles painted white. Specifically, we compared the number of times juveniles were bitten and attacked by adults of both sexes when they displayed their natural colouration or experimental adult-like or juvenile-like colouration.

Specific aim 3: To test the hypothesis that red colouration on the hind limbs and tails of adult female spiny-footed lizards is a sexual ornament selected by male mate choice. One prediction derived from this hypothesis is addressed in Chapter 5.

Chapter 5: *Male mate choice based on female colouration in the spiny-footed lizard.*

In this chapter, we tested prediction 3.1: adult males will spend more time with adult females painted red than with adult females painted white. We also tested whether other visual and chemical cues of female size, age and/or sexual maturity affected male mate choice.

Specific aim 5: To test the hypothesis that colour changes showed by adult female spiny-footed lizards during the breeding season are hormonally controlled. Predictions derived from this hypothesis are addressed in Chapter 6.

Chapter 6: *Hormonal control of seasonal colour change in female spiny-footed lizards (Acanthodactylus erythrurus)*.

In this chapter, we tested prediction 4.1: plasma hormone levels will be related to colouration; and prediction 4.2: changes in plasma hormone levels will affect colouration. Specifically, we studied the relation between natural plasma estradiol and progesterone levels and female colouration along the breeding season, and the effects of the experimental manipulation of these hormones plasma levels on female colouration.

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Chapter 2

**ONTOGENETIC SHIFTS IN
RISKY BEHAVIOURS ARE
RELATED TO BODY SIZE AND
COLOURATION IN SPINY-
FOOTED LIZARDS**

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Fresnillo B., Belliure J. and Cuervo J. J. Ontogenetic shifts in risky behaviours are related to body size and colouration in spiny-footed lizards (In preparation).

CAMBIOS ONTOGENÉTICOS EN LOS COMPORTAMIENTOS ARRIESGADOS ESTÁN RELACIONADOS CON EL TAMAÑO CORPORAL Y LA COLORACIÓN EN LA LAGARTIJA COLIRROJA

Resumen

Los cambios ontogenéticos en la coloración de los animales normalmente consisten en que los juveniles de coloración críptica desarrollan colores conspicuos cuando alcanzan la madurez sexual. Sin embargo, hay varias especies en las que los juveniles desarrollan colas de colores conspicuos que pasan a ser crípticas en adultos. En lacértidos se ha sugerido que la presencia de colas de colores llamativos está asociada a comportamientos arriesgados, ya que esas colas podrían ser un mecanismo antidepredatorio. El objetivo de este estudio era testar si el color de la cola estaba asociado a los comportamientos arriesgados en la lagartija colirroja (*Acanthodactylus erythrurus*), cuyos juveniles presentan colas rojas. Grabamos el comportamiento de individuos de todas las clases de edad en libertad y calculamos varios índices relacionados con los comportamientos arriesgados. Tal y como esperábamos, encontramos una relación entre el comportamiento arriesgado y la coloración de la cola, ya que los individuos más rojos (menos naranjas) permanecieron más lejos de los refugios, probablemente debido a que la percepción del riesgo de depredación disminuye en individuos con colas más rojas o a que la coloración roja esté asociada a mayores demandas energéticas que fueren a los individuos más rojos a buscar comida en áreas menos

seguras. El color no estuvo relacionado con ningún otro índice de comportamiento, pero encontramos una relación negativa entre el nivel de actividad y el tamaño corporal, lo cual sugiere un cambio hacia un comportamiento menos activo asociado al crecimiento, probablemente porque los requerimientos energéticos y de termorregulación cambian con el tamaño corporal. Cuando las temperaturas a la sombra eran bajas, los individuos permanecieron más lejos de los refugios, probablemente para alcanzar sus necesidades de termorregulación. Entre los adultos, las hembras permanecieron más lejos de los refugios que los machos, probablemente debido a su preferencia por una mayor temperatura corporal. En conclusión, la asociación entre la coloración y los comportamientos arriesgados en la lagartija colirroja es consistente con la hipótesis de que ese color es un mecanismo antidepredatorio que permite comportamientos más arriesgados cuando son necesarios.

PALABRAS CLAVE: *Acanthodactylus erythrurus*, coloración conspicua, espectrofotometría, mecanismo antidepredatorio, reptil, riesgo de depredación

ONTOGENETIC SHIFTS IN RISKY BEHAVIOURS ARE RELATED TO BODY SIZE AND COLOURATION IN SPINY-FOOTED LIZARDS

Abstract

Ontogenetic colour changes in animals generally involve cryptic juveniles developing conspicuous colouration when they achieve sexual maturity. However, there are several species in which juveniles develop conspicuously coloured tails that become cryptic in adults. In lizards, it has been suggested that colourful tails are associated with risky behaviours, as they might be an antipredatory mechanism. The aim of this study was to test whether tail colouration was related to risky behaviours in spiny-footed lizards (*Acanthodactylus erythrurus*), a species with red-tailed juveniles. Behaviour was recorded in free-ranging lizards of all age classes and several risk-related behavioural indices were calculated. As predicted, a link between risky behaviour and tail colouration was found, as redder (less orange) lizards stayed further from refuges, maybe because redder colouration was associated with lower perception of predation risk, or with higher energetic requirements that forced these lizards to look for food in less safe areas. Colouration was not related to any other behavioural index, but the level of activity was negatively related to lizard size, suggesting a change towards less active behaviour associated with growth, probably because energetic and thermoregulatory needs depends on body size. When temperatures in the shade were low, lizards stayed further from refuges,

probably to meet their thermoregulatory requirements. Among adults, females stayed further from refuges than males, possibly due to their higher preferred body temperature. In conclusion, the link between red colouration and risky behaviour in spiny-footed lizards is consistent with this colour being an antipredatory mechanism that allows lizards to behave more riskily when necessary.

KEY WORDS: *Acanthodactylus erythrurus*, antipredatory mechanism, conspicuous colouration, predation risk, reptile, spectrophotometry

Introduction

Changes in colouration between different life stages of an organism (ontogenetic changes) are relatively common in animals (Booth 1990). Very often, conspicuous colouration appears in adulthood associated to sexual selection, for example signalling dominance or attractiveness (Senar 2006; Blount & McGraw 2008). However, some ontogenetic colour changes imply a change from conspicuous colouration in juveniles to cryptic colouration in adults (Thresher 1978; Londei 2005; Landová *et al.* 2013). The presence of conspicuous colouration in juveniles may seem counterintuitive, because cryptic colouration is generally expected to avoid predation (Wilson, Heinsohn & Endler 2007), except in aposematic species (Mappes, Marples & Endler 2005). Different hypotheses have tried to explain ontogenetic colour changes from conspicuous juveniles to cryptic adults (Booth 1990). In some species, for example, juveniles mimic aposematic models, thus reducing predation (Jackson & Drummond 1974; Huey & Pianka 1977), but as juveniles grow and become larger than the model, a change to cryptic colouration may be necessary to prevent an increase in predation risk (Pough 1974). In other species, the change from gregarious juveniles to solitary adults may lead to the loss of conspicuous juvenile colouration (which might be useful in drawing group members together) when this signal is no longer needed (Rowell 1967). Moreover, palatability or vulnerability to predators may change from one life stage to another and, accordingly, conspicuous colouration may be affordable in some life stages but not in others (Booth 1990).

A dramatic example of ontogenetic colour change occurs in some lizard species characterized by conspicuous colours in juvenile tails that fade away when growing up (Hawlena *et al.* 2006; Hawlena 2009). Several hypotheses have been postulated to explain tail conspicuous colouration in juvenile lizards. First, it might inform conspecific adult males about the age class (i.e., the sexual immaturity) of the bearer, thus indicating it is not a competitor, and reducing adult male aggressiveness (Clark & Hall 1970; Werner 1978). Second, it might have a pursuit deterrent function, if associated with behaviours that make this colouration more visible, by signalling to the potential predator that it has been spotted and, therefore, that the pursuit may be costly, because the possibilities of capturing an alert prey are small (Ruxton, Sherratt & Speed 2004; Cooper 2011a). And third, it might deflect predator attacks from head and body towards expendable body parts, for example the tail in the case of species with tail autotomy (Hawlena *et al.* 2006; Telemeco, Baird & Shine 2011).

Deflecting predator attacks towards expendable body parts may be an advantage for very active individuals, as high activity rates imply high detectability, and thus high predation risk (Jackson, Ingram & Campbell 1976). Moreover, very active individuals spend more time in activities that reduce their capability of detecting a predator (Lima & Dill 1990). In most lizard species, juveniles need to move more than adults in order to thermoregulate (Martín & López 2003; Herczeg, Török & Korsós 2007) and obtain enough resources for somatic growth (Nagy 2000). The increased predation risk could not be diminished by

cryptic colouration, as crypsis requires immobility of the potential prey (Jackson, Ingram & Campbell 1976). Therefore, deflecting attacks towards an expendable body part such as the autotomous tail might be the best solution to increase survival rates of individuals with high mobility (Cooper 1998). A change towards more cryptic colouration may be advantageous when lizards achieve the minimum body size for sexual maturation and activity levels decrease (Hawlena 2009).

The spiny-footed lizard (*Acanthodactylus erythrurus*) is a good model organism to study the relation between behaviour and colour design across life stages, as young lizards develop conspicuous red colouration on the rear part of the hind limbs and the ventrolateral part of the tail that is lost in adults (Seva Román 1982). In the present study, we analysed the behaviour of free-ranging spiny-footed lizards of different ages/sizes to test whether red colouration on hind limbs and tail is associated with risky behaviours. If red colouration is an antipredatory mechanism, we predict that redder animals will behave more riskily, i.e., they will (1) move more actively, (2) move further away from refuges, and/or (3) make more tail and limb displays (as their tail and hind limbs will be more visible, the displays will be more efficient in advertising predators that the potential prey is alert and ready to escape; Cooper 2011a; Font, Carazo & Kramer 2012). The data will also allow us to test whether adults of both sexes behave differently, as we expect that adult males will be more active than adult females, at least during the mating season (Baird, Timanus & Sloan 2003).

Materials and methods

Study species

The spiny-footed lizard is medium sized (snout-vent length (SVL) and total length up to around 80 and 230 mm, respectively; Seva Román 1982; Carretero & Llorente 1993), although sizes can vary between populations. In populations in central Spain, newborn lizards appear in mid-August and are active until November. At the beginning of April the following spring, these lizards become active again, and they do not reach sexual maturity until the following spring (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992), when males are 58–65 mm SVL and females 60–66 mm SVL (Bauwens & Díaz-Uriarte 1997). Therefore, all individuals can be divided in three rough age categories, hereafter referred to as hatchlings (from hatching until the first winter), juveniles (from first to second winter) and adults (from the second winter onwards). Mean home range area is around 180 m² for juveniles, 550 m² for adult females and 630 m² for adult males (Seva Román 1982).

Colouration in this species undergoes ontogenetic changes, with dorsal pattern changing from strongly-marked dark and light bands in hatchlings to a reticulated pattern in adults (Seva Román, 1982). Hatchlings and juveniles of both sexes develop red colouration on the ventrolateral part of the tail, and juveniles also on the rear part of the hind limbs (Seva Román 1982; Carretero & Llorente 1993). The red colour is clearly visible from above in hatchlings and small juveniles

(personal observation). Juvenile males lose the red colour when they are around one year old, while juvenile females retain it through adulthood (Seva Román 1982). Adult males show white colouration on the rear part of the hind limbs and the ventrolateral part of the tail along the whole reproductive season (Seva Román 1982). In adults, sexual dichromatism is evident at the beginning of the reproductive season, when sexually receptive females increase the intensity of the red colouration, but it is less obvious later on, when females are gravid, as red colouration becomes pallid yellow, nearly white (Cuervo & Belliure 2013).

Behavioural observations

Lizard behaviour was studied from July to September 2010 and from April to August 2011 in Chapinería, south-western Madrid Region, Spain (N40°22'; W4°13'). The study area was a Mediterranean oak forest with meadow structure, where oaks (*Quercus ilex* L.) and lavender (*Lavandula stoechas* Lam.) dominated vegetation patches surrounded by open areas. Lizards were located from 10:00 to 18:30 local time, only in sunny days, and avoiding the central hours of the day when temperatures were high, as lizards reduce their activity in these conditions (Seva Román & Escarré i Esteve 1980). The activity of focal individuals was followed by one observer (always the same person, wearing clothes of similar colours) at a distance of 4-6 m, dictating behavioural observations in real time to a portable digital voice recorder. Activity recording did not begin until the animal was moving without apparent influence of the observer's presence, normally 2-3 min after

lizard location. If the focal individual showed a strong reaction to the observer, the recording was suspended. We decided that recordings lasted a maximum of 20 min, as lizard behaviour is considered to be well described within that temporal range (Hawlena *et al.* 2006). Recordings less than 10-min long might not be representative of a lizard's behaviour, so they were discarded and, therefore, all recordings included in the analyses lasted between 10 and 20 min.

For each focal individual, we recorded age class (hatchling, juvenile, or adult), a rough SVL estimate, tail status (complete or incomplete), and the exact moment when any of the following activities occurred: to move, to stop, to go into a sunny patch, to go into a shaded patch, and to display (to move stereotypically) the tail and/or the limbs. Whenever possible, the distance to the closest refuge was estimated when the animal stopped, considering a refuge any dense shrub of at least 50 cm tall or any crevice or burrow where the animal could hide. The age class was defined according to the physical characteristics of the lizards (colour design, SVL and corpulence) and considering that hatchlings become juveniles during their first winter and juveniles become adults during their second winter. The sex was determined, only in adult lizards, according to the base of the tail, much wider in males than in females (Blasco 1975). Once the focal observation had finished, we also recorded the local time, geographic coordinates (with a GPS), ground temperatures both in full sun and in deep shade (with an electronic digital thermometer to the nearest 0.1°C) and the percentage

of ground covered by shrubs (the main refuge used by this species) in a 10 m-radius circle centred at the place where the focal lizard was located.

After recording behaviour, as many lizards as possible were captured by noosing. A distance of at least 100 m was kept between every recording place and subsequent ones (this means an area of more than 31 000 m² around the recording place) when the focal individual could not be captured to minimize the risk of recording the same individual twice. If the animal was captured, the next lizard found was considered the next focal individual. Captured lizards were carried in individual cloth bags to the lab for colour measurement (see Colour Measurements below). Animals were also measured (SVL and total length with a ruler to the nearest 0.1 cm) and we could check that SVL estimates recorded in the field were fairly accurate (Pearson correlation; $r = 0.782$, $n = 82$, $P < 0.001$). For captured lizards with regenerating tails, we considered that a tail was incomplete when it was at least 1 cm shorter than the minimum tail length for that particular age class. For non-captured lizards, the tail was considered to be incomplete when a part of it was clearly missing, even though tail regeneration had started. All captured lizards were toe-clipped before their release to allow identification in case of recapture. Animals captured between April and June 2011 were kept in captivity for other studies. The rest of the animals were released in exactly the same places where they had been located on the next visit to the study area (2-7 days after capture).

All voice recordings were transferred to Excel sheets, where time devoted to each behaviour was calculated. The observation time

was calculated as the total recording time minus the time the observer was not sure of the exact position of the lizard, for example when it was on the other side of a dense shrub. We then calculated a number of behavioural indices: (1) movements per minute (MPM), as the number of times an individual started moving per minute; (2) proportion of time spent moving (PTM), as the percentage of the observation time that the focal lizard was moving; (3) mean distance to refuge (MDR), as the average distance to the closest refuge weighted by the time spent at different distances; (4) tail displays per minute (TDM), as the number of tail waving movements per minute; and (5) limb displays per minute (LDM), as the number of stereotyped movements of any limb per minute. MPM and PTM have been commonly used to describe the foraging mode of lizards (Perry *et al.* 1990; Belliure *et al.* 1996; Hawlena *et al.* 2006; Hawlena 2009), assuming that foraging is the main factor affecting activity rates, although social behaviour or thermoregulation might also influence activity levels (Cooper 2011b).

Colour measurements

Colour measurements are described in detail elsewhere (Cuervo & Belliure 2013). Briefly, we quantified the spectral properties of lizard colouration by taking reflectance readings (with a USB 2000 spectrometer and a DT-MINI-2-GS tungsten halogen light source, Ocean Optics, Dunedin, USA) in the range of 320-700 nm (Whiting *et al.* 2006). Four body regions (the rear part of both hind limbs and the ventral part of the tail around 1 cm and 2.5 cm from the cloaca) were

measured three times each. Then, reflectances at 1-nm intervals in the range studied were calculated using AVICOLOR software (Gomez 2006). From the reflectance data, three colour parameters were calculated as follows: brightness as the mean reflectance between 320 and 700 nm, red chroma as the sum of reflectances between 630 and 700 nm divided by the sum of reflectances between 320 and 700 nm, and hue as the wavelength where the maximum reflectance was recorded (Montgomerie 2006). Since the three measurements in each body part were highly repeatable (repeatability according to Lessells and Boag (1987); brightness: $0.769 \leq r \leq 0.929$; red chroma: $0.935 \leq r \leq 0.953$; hue: $0.270 \leq r \leq 0.850$, $F_{58,118} \geq 2.10$, $P < 0.001$ in the 12 tests), we calculated mean values for each body part. Likewise, as measurements in the four body regions were positively correlated (Pearson correlations, brightness: $0.415 \leq r \leq 0.834$, red chroma: $0.368 \leq r \leq 0.926$, $n = 59$, $P < 0.001$ in the 12 tests; Spearman correlations for non-normally distributed raw data, hue: $0.307 \leq r_s \leq 0.668$, $n = 59$, $P \leq 0.018$ in 5 tests, but the correlation between the right hind limb and the tail at 2.5 cm from the cloaca was marginally non-significant: $r_s = 0.233$, $n = 59$, $P = 0.075$), they were all unified in single brightness, red chroma and hue values for each individual (the mean of the four body parts), and these values were then used for further analyses (Cuervo & Belliure 2013).

Statistical analyses

A total of 95 lizards were observed and their behaviours recorded, and 82 of them were also captured. Colour measurements were

taken for all captured juveniles and females (55 lizards), but, unfortunately, colouration could only be measured in four adult males (see Table 1 for age and sex class distribution of samples). Analyses testing for sex differences were based on the 41 adults recorded, although not all of them were captured.

Body size (SVL) and age class were strongly correlated (Spearman correlation; $r_s = 0.916$, $n = 95$, $P < 0.001$), so we used SVL instead of age class in all analyses, as this variable provided more precise information. However, when SVL was retained in the final model (see last paragraph of this section), the final model was repeated replacing SVL by age class to check which age classes differed in their behaviour. We also found a strong correlation between year and day of the year (Spearman correlation; $r_s = -0.760$, $n = 95$, $P < 0.001$), so we only included day of the year in our models because we expected more

Table 1. Sample sizes of behavioural records and captures for the different age and sex classes of spiny-footed lizards in 2010 and 2011. Colour parameters were measured in all captured lizards except in 21 adult males (7 in 2010 and 14 in 2011).

Age class	Sex	2010		2011	
		Recorded	Captured	Recorded	Captured
Adults	Females	4	4	10	10
	Males	9	8	18	17
Juveniles	-	15	9	24	21
Hatchlings	-	14	12	1	1

variability in population composition and behaviours between different days of the same year than between the same days of different years. Colouration varies along life stages in this species and was therefore related to SVL, and some of the three colour parameters were also related to one another, but correlations between these four variables were not as strong as the ones mentioned above (the strongest correlation was between brightness and red chroma: Pearson correlation, $r = 0.613$, $n = 59$, $P < 0.001$), so we included the four predictors in our models. Moreover, we checked variance inflation factors (VIF) in all models and found they were ≤ 3.126 , so we assumed that collinearity was not a problem in our analyses (O'Brien 2007).

MPM, PTM and MDR were $\log_{10}(x+1)$ transformed and TD and LD fourth-root transformed before subsequent analyses to fulfil parametric assumptions, but the other variables (including mean hue) followed a normal distribution without transformation (Kolmogorov-Smirnov tests; $P \geq 0.063$). Relationships between behavioural indices and colour parameters (brightness, red chroma and hue) were tested with general linear models (GLMs). In these models, the following predictors were also included: temperatures in the sun and in the shade, as behaviour in ectotherms is strongly influenced by ambient temperatures (Belliere, Carrascal & Díaz 1996); body size (SVL) and tail status, as they may affect lizard behaviour (Martín & Avery 1998; Hawlena *et al.* 2006); day of the year as an indicator of temporal variability; and percentage of ground covered by shrubs as an indicator of refuge availability in the area. The same procedure was used to test

for differences in behavioural indices between adults of both sexes, but replacing colour parameters by sex, adding the interaction sex x tail status, and maintaining the rest of predictors.

A backward stepwise procedure was used in all GLMs, retaining only terms associated with P values below 0.10 in final models. When any of the colour variables was retained in a model, only data from the 59 lizards with colour measurements were included in the analysis, as no spectrometric information was available for the other lizards. When the three colour variables had been eliminated during the stepwise procedure, all 95 lizards were then included in the analysis. We checked whether Akaike's information criterion (Burnham & Anderson 2002) selected the same final models using either the subset of 59 individuals with colour measurements (when a colour parameter was included in the final model) or all 95 individuals (when none of the colour parameters was included in the final model), and this was indeed the case. All statistical analyses were carried out using STATISTICA 7.1 (StatSoft Inc. 2005). All tests were two-tailed and the significance level was 0.05.

Results

MPM and PTM were not significantly related to lizard colouration, but MPM was related to SVL, with small animals being more active than large ones (Table 2 and Fig. 1a). When SVL was replaced by age class in the final model, we found that hatchlings had significantly higher MPM than adults, while juveniles had intermediate

values (age class: $F_{2,92} = 7.04$, $P = 0.001$; Tukey post-hoc tests: adults-hatchlings, $P = 0.002$; adults-juveniles, $P = 0.056$; hatchlings-juveniles, $P = 0.164$; Fig. 1b). MDR was related to lizard colouration, as lizards with higher hue values (redder, less orange) stayed further away from refuges for longer periods of time (Table 2 and Fig. 2). This was the case even when SVL was forced into the model (hue: $F_{1,55} = 6.04$, $\beta \pm SE = 0.322 \pm 0.131$, $P = 0.017$). MDR was also related to temperature in the shade, the distance to the refuge being shorter when temperature was higher (Table 2). TDM and LDM were not significantly related to lizard colouration or to any other predictor (Table 2).

Figure 1. (a) Relationship between $\log_{10}(\text{movements per minute (MPM)}+1)$ and snout-vent length (SVL) and (b) mean (\pm SE) $\log_{10}(\text{MPM}+1)$ for three age classes (hatchlings, juveniles and adults) in spiny-footed lizards ($n = 95$). For the statistical tests, see Results.

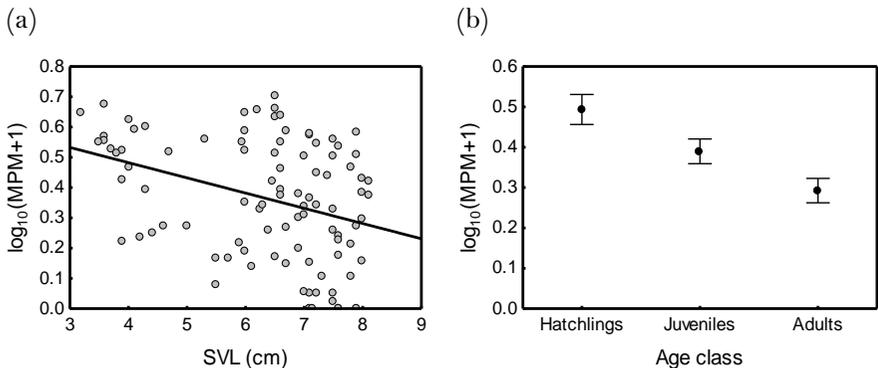
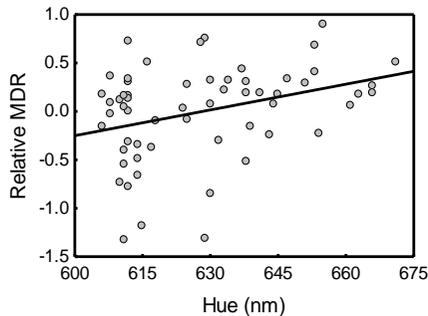


Table 2. General linear models testing the relationship between five risk-related behavioural indices (MPM: movements per minute; PTM: percentage of time spent moving; MDR: mean distance to refuge; TDM: tail displays per minute; and LDM: limb displays per minute) and colour parameters in spiny-footed lizards. Only predictors retained after a backward stepwise procedure ($P < 0.10$) are shown. Complete models included day of the year, tail status (complete or incomplete), snout-vent length (SVL), shrub cover, temperature in the sun, temperature in the shade (T shade), brightness, red chroma and hue. $N = 59$ when a colour parameter was included in the model and $N = 95$ when none of the colour parameters was included.

	Adj. R ²	Model F	Model P	Predictors	F	P	$\beta \pm SE$
MPM	0.114	13.11	< 0.001	SVL	13.11	< 0.001	-0.352 \pm 0.097
PTM	0.028	3.68	0.058	SVL	3.68	0.058	-0.195 \pm 0.102
MDR	0.137	5.60	0.006	T shade	5.43	0.023	-0.286 \pm 0.123
				Hue	7.09	0.010	0.327 \pm 0.123
TDM	-	-	-	-	-	-	-
LDM	-	-	-	-	-	-	-

Figure 2. Relationship between relative mean distance to the closest refuge (residuals after regressing mean distance to refuge (MDR) on temperature in the shade) and hue for the 59 spiny-footed lizards in which colouration was measured. Relative MDR instead of relative hue is shown so that the colour spectrum of hue (orange-red) can be readily identified.



In adults, no significant differences between sexes were found for MPM, PTM, TDM or LDM (Table 3). However, females stayed further away from refuges, i.e., took more risks, than males (Table 3). Moreover, adults with incomplete tails tended to stay closer to refuges than adults with complete tails (Table 3), a result in line with the role of the expendable tail as an antipredatory mechanism in lizards, but that will not be discussed further because the difference is not clear-cut.

Table 3. General linear models testing for differences between sexes in five risk-related behavioural indices (MPM: movements per minute; PTM: percentage of time spent moving; MDR: mean distance to refuge; LDM: limb displays per minute; and TDM: tail displays per minute) in 41 adult spiny-footed lizards. Only predictors retained after a backward stepwise procedure ($P < 0.10$) are shown. Complete models included day of the year, tail status (C: complete; I: incomplete), snout-vent length (SVL), shrub cover, temperature in the sun, temperature in the shade (T shade), sex (M: male; F: female) and the interaction tail status x sex.

	Adj. R ²	Model F	Model P	Predictors	F	P	$\beta \pm SE$
MPM	-	-	-	-	-	-	-
PTM	0.056	3.36	0.074	T shade	3.36	0.074	0.282 \pm 0.154
MDR	0.142	4.30	0.021	Sex (F) Tail (I)	4.74 4.11	0.036 0.050	0.319 \pm 0.147 -0.297 \pm 0.147
TDM	-	-	-	-	-	-	-
LDM	0.052	3.18	0.082	Day	3.18	0.082	0.274 \pm 0.154

Discussion

The aim of this study was to test the hypothesis that red tail colouration in spiny-footed lizards is an antipredatory mechanism by checking the prediction that redder lizards will behave more riskily. It has been suggested that lizards with ontogenetic changes from conspicuous to cryptic tail colouration behave more riskily before than after losing the conspicuous colouration (Hawlena *et al.* 2006; Hawlena 2009). In the present study, lizards with redder (less orange) tails behaved more riskily because they stayed further from refuges for longer periods of time, a result consistent with the hypothesis that the red colour has an antipredatory function. Conspicuously coloured lizard tails have been suggested to divert attacks from vulnerable body parts (Castilla *et al.* 1999; Watson *et al.* 2012), and, indeed, this seems to be the case in spiny-footed lizards (B. Fresnillo, J. Belliure and J.J. Cuervo, unpublished data; Chapter 3 of this thesis). The red tail would contrast more with the background than the rest of their body, thus producing a lure effect (Arnold 1984). Redder individuals might behave more riskily if redder colours were associated with a lower perception of predation risk, as the antipredatory function of red tails would compensate for the lower perception of predation risk. The lower perception of predation risk would influence the decision-making process (Lima & Dill 1990), and lizards taking more risks would have access to more resources, for example basking far from shrubs to get direct insolation or feeding not only in the safest areas, but also far from refuges. Another possible explanation is that redder colours might be associated with certain

characteristics that increase the energetic requirements of the individual and thus the home range needed to meet those requirements. For example, if juveniles of one sex, or juveniles in poor phenotypic quality (e.g. poor thermoregulatory capacity), needed to explore larger areas to find the food or the temperature required for growing, they might be forced to explore not only the safest areas, but also the less safe areas far from refuges. In this case, a hypothetical relationship between sex or phenotypic quality and colouration could readily explain the association we found between colouration and distance to the closest refuge. Clearly, more research is needed to clarify whether these speculative explanations have any basis.

Rate of movements, one of the behavioural parameters indicating lizard activity, was related to body size, but not to lizard colouration. The relationship with body size could be the consequence of the high energetic needs of smaller/younger lizards, which are immersed in intense somatic growth processes, and, indeed, hatchlings moved more often than adults (Fig. 1b). However, the increase of movements with body size could also be reflecting lizard thermoregulatory needs, as smaller lizards, which have higher heat exchanging rates (Martín & López 2003), would need to move more to maintain their preferred body temperature. Behavioural differences between individuals of different age/size have already been hypothesized (Nagy 2000) and observed (Hawlena *et al.* 2006; Herczeg, Török & Korsós 2007; Hawlena 2009) in different reptile species. As more active individuals probably suffer a higher predation risk (Jackson, Ingram & Campbell 1976), we expected

redder lizards, presumably protected to some extent from predators because of the antipredatory function of the red colour, to have higher levels of activity. Although an association between conspicuous tails and high levels of activity has often been suggested (Arnold 1984; Vitt & Cooper 1986), our results do not confirm the relationship between activity rates and colouration. It should be noted that this relationship was studied while simultaneously controlling for lizard size, something that probably has not always been done. In our sample, colouration and age/size were correlated, and this might be also the case in other species. Therefore, previous relationships found between activity rates and colouration could be simply the result of correlations between activity rates and age/size and between age/size and colouration. A possible explanation for the mismatch between colouration and level of activity in spiny-footed lizards is that the former might be more related to predation risk, while the latter, even if also related to predation risk, might be more affected by energetic or thermoregulatory needs. Lizards will be as active as they need to meet their energetic or thermoregulatory requirements, but once these requirements are met, they will not move more regardless of their colouration. Red colouration may decrease predation risk, but a better strategy is probably to behave less riskily when this is at all possible.

More tail displays were expected in redder lizards because they are commonly used by lizards as deterrent of attacks from predators (Hasson, Hibbard & Ceballos 1989), and redder colouration would probably make the movement more conspicuous, but our results do not

confirm this expectation. A problem to interpret these results is that all lizards were aware of the presence of the observer, and probably considered the observer as a predator. Therefore, once the predator (the human observer) was detected, all lizards probably displayed their tail to advertise their readiness to escape and/or to direct any potential attack to the tail, regardless of their tail colouration. It is possible that spiny-footed lizards also use tail displays when predators have not been spotted (Murphy 2007), as this might be a good strategy, mainly for lizards with conspicuous tails, when predation risk is high, so future studies should address the problem of recording lizard behaviour without interfering with this behaviour. Regarding limb displays, they were not related to colouration either, but the meaning of the foot shakes is not clear, because they could be pursuit-deterrent displays (Font, Carazo & Kramer 2012) or be involved in thermoregulation or social communication (Magnusson 1996). In our study, foot shakes of any limb were recorded, without distinguishing between fore and hind limbs. As they might have different functions, not always related to predation, an association with coloration was more difficult to find.

In this study, adult females stayed further from refuges than adult males, a difference that may have implications for predation risk, but also for thermoregulation, as shrubs provided refuge from predators but also protection from overheating (see below). Reptile females usually select higher body temperatures while gravid in order to influence the development and phenotype of the offspring (review in (Shine 2006), but females seem to prefer also higher body temperatures than males when

they are not gravid (Shine 2006). Contrary to our expectations, activity rates were similar in adults of both sexes, a similarity that has been also found in other lizard species when studied outside of the mating season (Verwajen & Van Damme 2008). During the mating season, however, males are generally more active than females (Aragón, López & Martín 2001; Wymann & Whiting 2002), a difference commonly associated with more intense defence of territories and mate search by males (Aragón, López & Martín 2001). In spiny-footed lizards, the similar activity rate of both sexes during the breeding season suggests that both sexes are equally territorial or non-territorial, something that should be studied in the future. The influence of colouration on the behavioural differences between adults of both sexes could not be addressed in this study because only four adult females with red tail were included in our sample.

Our results also suggest a relationship between lizard behaviour and thermoregulation, as lizards stayed further from refuges when ambient temperatures were lower. Lizards need to bask in order to reach their optimum temperature (Cowles & Bogert 1944), but basking implies high exposure to predators, as lizards need to be out of their refuges to thermoregulate (Amo, López & Martín 2003). Basking time is related to the thermoregulation rate of every species but also to ambient temperatures (Belluere, Carrascal & Díaz 1996), so when temperatures are low, lizards need to bask longer than when temperatures are high. In our case, when temperatures in the shade were low, lizards probably needed to stay longer in the sun to reach their optimum temperature,

and this often implied being further from refuges, as both shade and refuges were mostly provided by shrubs.

Conclusions

In this study, spiny-footed lizards with redder (less orange) tails behaved more riskily (stayed further from refuges), the expected result if red colouration in this species has an antipredatory function. The activity rate of lizards, other behaviour related to predation risk, decreased with age/size, but was not significantly related to tail colouration when simultaneously controlling for body size. In adult lizards, females behaved more riskily (stayed further from refuges) than males, possibly because females preferred higher temperatures, and these were reached basking in the sun, far from the shade of shrubs that were the main refuge.

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Chapter 3

**RED TAILS ARE EFFECTIVE
DECOYS FOR AVIAN PREDATORS**

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Fresnillo B., Belliure J. and Cuervo J. J. Red tails are effective decoys for avian predators (Submitted to *Evolutionary Ecology*).

LAS COLAS ROJAS SON UN SEÑUELO EFECTIVO PARA LOS DEPREDADORES AVIARES

Resumen

La presencia de coloración conspicua en los juveniles puede ser explicada, entre otras teorías, por la teoría del señuelo, según la cual la coloración conspicua se localiza en zonas no vitales del cuerpo para desviar los ataques dirigidos a la cabeza y el cuerpo, incrementando así las probabilidades de supervivencia. Para testar esta hipótesis elaboramos modelos de plastilina y de escayola imitando lagartijas con colas rojas o a rayas claras y oscuras, basándonos en la coloración de las crías de lagartija colirroja (*Acanthodactylus erythrurus*), que de forma natural muestran un patrón dorsal con bandas claras y oscuras y cola roja. Los modelos de lagartija fueron colocados en el campo y también presentados a cernícalos (*Falco tinnunculus*) en cautividad, un depredador aviar que se alimenta comúnmente de lagartijas. En ambos estudios se tomó nota tanto del número de modelos atacados como de la sección del cuerpo atacada (cola o resto del cuerpo), además del número de días transcurridos hasta que cada modelo fue atacado. Los resultados sugieren que los modelos de ambas coloraciones fueron reconocidos como presas y por lo tanto fueron atacados en cantidades similares, pero que en el campo, los modelos de cola roja fueron detectados, y por lo tanto atacados, antes que los de la cola rayada. A pesar de este incremento de conspicuidad, los modelos de cola roja desviaron eficazmente los ataques de las zonas más vulnerables hacia la cola,

apoyando así la teoría del señuelo. Los beneficios obtenidos al desviar los ataques a la cola han de ser mayores que los costes derivados del incremento en conspicuidad para explicar la evolución y el mantenimiento de la coloración conspicua en lacértidos.

PALABRAS CLAVE: *Acanthodactylus erythrurus*, desvío de los ataques, interacción depredador-presa, evolución de las señales.

RED TAILS ARE EFFECTIVE DECOYS FOR AVIAN PREDATORS

Abstract

A possible explanation for the presence of conspicuous colouration in juveniles is the decoy hypothesis, which states that the conspicuous colouration is present in a non-vital part of the body to divert attacks from head and trunk, thus increasing survival probability. To test this hypothesis we made plasticine and plaster lizard models with red or striped dark and light tails, based on the colour design of *Acanthodactylus erythrurus* hatchlings, which naturally show striped dark and light dorsal pattern and red tail. Lizard models were placed in the field and also presented to captive *Falco tinnunculus*, a common lizard avian predator. The number of models attacked and the body part attacked (tail or rest of the body) were recorded, as well as the number of days until a model was attacked. Our results suggest that models of both colour designs were recognized as preys and attacked at a similar rate, but, in the field, red-tail models were detected, and thus attacked, earlier than striped-tail ones. Despite this increase in conspicuousness, red-tail models effectively diverted attacks from the more vulnerable body parts to the tail, thus supporting the decoy hypothesis. Greater fitness benefits of attack diversion to the tail compared to the costs of increased conspicuousness would explain the evolution and maintenance of red tail colouration in lizards.

KEY WORDS: *Acanthodactylus erythrurus*, attack diversion, predator-prey interaction, evolution of signals

Introduction

Understanding the function of conspicuous colouration in animals is an interesting topic in evolutionary ecology, because conspicuous colouration is generally assumed to increase detection rate by predators (Arnold 1984; Endler 1980; Haskell 1996). In sexually mature animals, conspicuous colouration is often related to sexual selection (Darwin 1871; Kemp & Rutowski 2011; Stuart-Fox *et al.* 2003), a selective pressure that can lead to the expression of phenotypes that improve mating success, but at the expense of reducing survival prospects (Andersson 1994; Stuart-Fox *et al.* 2003). However, conspicuous colouration is sometimes also present in juveniles (Booth 1990) and, in these cases, sexual selection cannot be driving the evolution of the colour, so other mechanisms must be invoked. There are at least three hypotheses, different from sexual selection, to explain the existence of conspicuous colouration. First, the inter-age-class signalling hypothesis states that juvenile conspicuous colouration affects the behaviour of conspecific adults, for example reducing adult aggression (Clark & Hall 1970; VanderWerf & Freed 2003; Ochi & Awata 2009). Second, the aposematism hypothesis proposes that the conspicuous colouration gives information to predators about the toxicity or unpalatability of the prey, or at least this colouration mimics an aposematic model (Booth 1990; Kraus & Allison 2009). And third, the decoy hypothesis assumes that the conspicuous colouration is present in a non-vital part of the body to divert attacks from vital parts (Johnson,

Burt & DeWitt 2008; Watson *et al.* 2012; Kodandaramaiah, Lindenfors & Tullberg 2013).

The decoy hypothesis is more plausible in species where conspicuous colouration is present in expendable body parts. This is taken to the extreme in reptiles, where juveniles and/or adults of many lizard species show conspicuous colouration in the tail (Pianka & Vitt 2006), which is autotomous and can therefore be detached from the rest of the body without seriously compromising survival (Arnold 1988; Bateman & Fleming 2009). If conspicuous colouration is effective in diverting attacks towards non-vital body parts, showing this colouration in the autotomizable tail will make this mechanism even more effective (Pianka & Vitt 2006). Although conspicuous colouration very often increases detection rate by predators (Endler 1980; Arnold 1984; Haskell 1996), some studies on lizard tail colouration suggest that this might not be always the case (Castilla *et al.* 1999; Watson *et al.* 2012). However, even when conspicuous colouration implies costs of increased detection rates by predators, this signal may still be selected if the benefits of escaping an attack exceed the costs of increased conspicuousness (Cooper & Vitt 1991). The first studies addressing the decoy hypothesis in lizards were conducted in captivity (Clark & Hall 1970; Cooper & Vitt 1985; Vitt & Cooper 1986), because the possibilities of observing predator-prey interactions in the field are scarce. This problem is not unique for lizards, and has been partially solved in other studies by the use of plasticine (Mochida 2011; Valkonen *et al.* 2011; Watson *et al.* 2012) or plaster (Baylis, Cassey & Hauber 2012; Ruiz-Rodríguez *et al.*

2013) models, either video recording predator attacks towards prey models or inferring the attacks by the marks left on the models, despite the lack of direct observations of predator-prey interactions.

The efficiency of blue and green lizard tails in diverting attacks has already been tested (Cooper & Vitt 1985; Castilla *et al.* 1999; Watson *et al.* 2012), but there are several lizard species from different lizard families (e.g. Scincidae, Gymnophthalmidae, Lacertidae) showing red tails instead. Avian predators are known to discriminate colours (Bowmaker 2008), so different colours might cause different responses in bird predators, and the effectiveness of red tails as decoys has not been addressed yet. In the present study, our aim was to answer two specific questions: (1) do red tails increase lizard conspicuousness to avian predators? and (2) are red tails effective decoys diverting bird attacks from vital body parts? To answer these questions we used plasticine and plaster models based on the morphology of spiny-footed lizards (*Acanthodactylus erythrurus*, Schinz 1833), a species in which hatchlings have red tails and striped dark and light body. We checked whether birds attacked more and/or earlier red-tail models (striped body and red tail) than striped-tail ones (striped body and tail), and whether red-tail models received a larger proportion of attacks in the tail than striped-tail ones. Models were placed in the field and also presented to captive common kestrels (*Falco tinnunculus*, Linnaeus 1758), one of the main avian predators of *A. erythrurus*.

Materials and methods

Study species

A. erythrurus is medium-sized (snout-vent length (SVL) and total length up to around 80 and 230 mm, respectively, although size can vary among populations; Seva Román 1982; Carretero & Llorente 1993) that is present in southern and central Iberian Peninsula and northern Africa (Belluire 2006). In populations in central Spain, newborn lizards appear in mid-August and are active until November. At the beginning of April the following spring, these lizards become active again, and they do not reach sexual maturity until their second spring (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992). Therefore, all individuals can be divided in three rough age categories, hereafter referred to as hatchlings (from hatching until the first winter), juveniles (from first to second winter) and adults (from the second winter onwards).

Colouration in this species experiences ontogenetic changes. Hatchlings show a strongly contrasted dorsal pattern of dark and light bands that run along their body. This pattern starts reticulating in juveniles, and becomes dark and light patches in adults (Seva Román 1982). Hatchlings and juveniles have red colouration on the ventrolateral part of their tails (Seva Román 1982; Carretero & Llorente 1993), which is clearly visible from above in hatchlings and small juveniles (personal observation). Red colouration also appears on the rear part of the hind limbs of juveniles. Juvenile males lose the red colour

when they are around one year old, while juvenile females retain it through adulthood, specifically until they become gravid (Seva Román 1982; Cuervo & Belliure 2013).

Lizard models

We made lizard models either with plasticine or plaster, because both types of models have been widely used in studies analysing the effects of conspicuous colouration on predation (Castilla *et al.* 1999; Mochida 2011; Valkonen *et al.* 2011; Watson *et al.* 2012; Ruiz-Rodríguez *et al.* 2013) or predation pressures (Castilla & Labra 1998; Vervust, Grbac & Van Damme 2007) in different taxa. Therefore, both options seemed appropriate for the recording of avian attacks on lizard models. Plaster models were made using lizard plastic toys which we covered with a thin layer of plaster (Aquaplast Standard, Beissier S.A., Guipúzcoa, Spain). Since we used plastic toys as the base for plaster lizard models, these models were larger than real hatchlings (lizard plastic toys: mean SVL \pm SD = 56.6 \pm 7.9 mm, mean total length \pm SD = 131.8 \pm 31.4 mm, $N = 10$; hatchlings: mean SVL \pm SD = 38.3 \pm 2.6 mm, mean total length \pm SD = 107.7 \pm 10.7 mm, $N = 23$; t -Student test, $t_{31} \geq 3.32$, $P \leq 0.002$ in the two tests). However, juveniles and adults are also common preys of avian predators (Belliure 2006), so we think these models could still be easily recognized as preys, even with the mentioned difference in size in relation to real hatchlings. Plasticine models were hand made with black oil-based modelling clay (Plastilina Jovi, Barcelona, Spain) trying to mimic the size of real hatchlings (random

sample of models: mean SVL \pm SD = 38.4 ± 2.8 mm, mean total length \pm SD = 107.7 ± 7.9 mm, $N = 15$; comparison with hatchlings: t -Student test, $0.00 \leq t_{36} \leq 0.09$, $P \geq 0.928$ in the two tests).

Model colouration (in both types of models, i.e., plasticine and plaster ones) was based on real hatchlings of *A. erythrurus*. We used non-toxic paints (Satin Paint, La Pajarita, Manises, Spain) to create two experimental treatments that differed in the colour design of their tails: red-tail models (the tail was completely red in plaster models, Fig. 1A, or red with a light band on the dorsal side in plasticine models, Fig. 1C) and striped-tail models (the tail had a pattern of dark and light bands, similar to that of the body, Figs. 1B and 1D). In both colour designs, the dorsal part of the body consisted of a contrasted pattern of dark and light bands (Fig. 1). Neither the paints used in the experiment nor real lizard dorsal colouration reflect UV light (B. Fresnillo, J. Belliure & J. J. Cuervo, unpublished data; Chapter 4 of this thesis), so differences in colour discrimination between birds and humans was not probably an issue in this study. In general, plasticine models resembled more *A. erythrurus* hatchlings than plaster ones, both because of their smaller size and the light band on the tail.

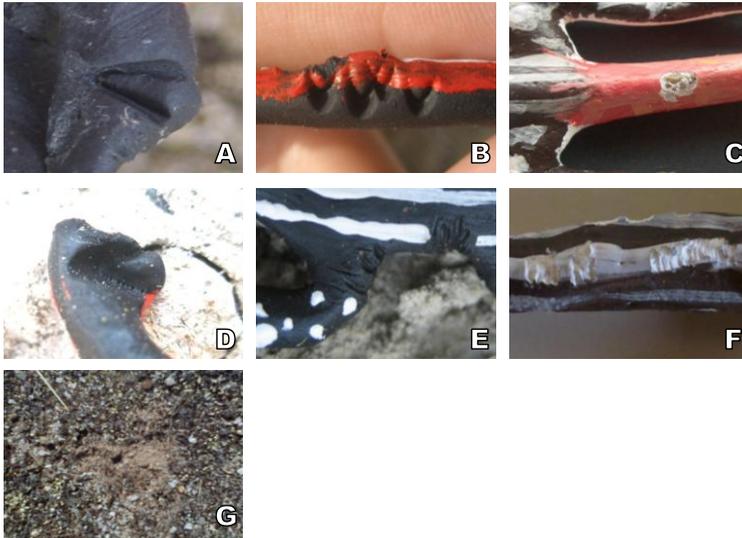
In both plaster and plasticine models, it was possible to identify the type of predator using the markings left on the model (Fig. 2). U- or V-shaped marks without teeth impressions and puncture marks were associated with birds, V-shaped marks with teeth impressions were associated with lizards, and tooth marks were associated with rodents (Fig. 2; Brodie 1993). As plaster is harder than plasticine, we are aware

that some avian attacks might not have left any mark on plaster models. Despite the possibility of detecting non-avian predation attempts, we only recorded avian attacks in this study because birds are mainly visually directed predators, while mammals and reptiles also rely on chemical cues (Greene 1988). Materials used for the construction of the models (plasticine, plaster and paint) are not odourless, and their odour might affect the behaviour of mammals and reptiles, either attracting or repelling them. Moreover, models certainly did not smell like lizards, so predators using chemical cues might not recognize models as preys. The importance of prey colouration is probably smaller for non-avian than for avian predators.

Fig. 1 Types of models used in the study: (A) red-tail plaster model, (B) striped-tail plaster model, (C) red-tail plasticine model, and (D) striped-tail plasticine model.



Fig. 2 Types of marks left in the models by predators: (A) V-shaped mark left by a bird in a plasticine model, (B) U-shaped mark left by a bird in a plasticine model, (C) puncture mark left by a bird in a plaster model, (D) V-shaped mark with teeth impressions left by a lizard in a plasticine model, (E) tooth marks left by rodents in a plasticine model, (F) tooth marks left by rodents in a plaster model, and (G) tracks left in the ground after an avian attack where the model disappeared.



Field study

We distributed lizard models in two localities in central Spain where *A. erythrurus* is common. One study area was located in Chapinería, south-western Madrid Region (N40°22'; W4°13'), a Mediterranean oak forest with meadow structure, where oaks (*Quercus ilex* L.) and lavender (*Lavandula stoechas* Lam.) dominate vegetation patches surrounded by open areas. The other study area was located in

Aranjuez, southern Madrid Region (N40°1'; W3°33'), a steppe dominated by esparto (*Stipa tenacissima* L.) where trees are scarce and the gaps between plants are mainly bare soil. The study areas are 67 km apart in a straight line, and were selected because vegetation cover differed dramatically between them (a lot fewer trees in Aranjuez), what possibly influenced prey detectability by visually directed predators (Cuadrado, Martín & López 2001; Vásquez, Ebensperger & Bozinovic 2002). Moreover, both study areas might also have different predator communities. In this way, we could have a broader view of predation pressures on *A. erythrurus* under different conditions.

The field study was carried out in two different years, 2010 and 2011, during the period with hatchling activity (August–November), so predators of this species presumably recognized lizard models as a common prey in their diet. In both localities and years, models were placed in open areas near bushes, pretending to be basking lizards. Models were placed at least 100 m apart from one another, alternating red- and striped-tail models. When both plaster and plasticine models were present in the same study area, they were intermixed and the two colour treatments were equally represented for each type of model. In 2010, we worked only in Chapinería and only with plaster models. We distributed ten plaster models (five with red tail and five with striped tail) in the area when the first hatchlings were observed. Lizard models were inspected every 4–5 days and avian attacks on two body parts, the tail and the rest of the body (i.e., head, limbs and trunk), were recorded. Lizard models were removed after an avian attack or after four visits

without avian attacks, and then new models were placed in the field (always in new locations) in order to keep the same number of red- and striped-tail models in the area. Attacks from non-avian predators (reptiles and mammals) sometimes happened and, in such cases, plaster models were repaired while plasticine models were replaced by a new model of the same characteristics. The repaired or new model was then placed at the same location but was not considered as a new model, because attacks by non-avian predators were not taken into account in this study. In 2011, we worked both in Chapinería, where plasticine and plaster models were distributed, and in Aranjuez, where only plasticine models were used. Both study areas were visited every 3 days, and 10-16 models were left in each area after every visit, always evenly distributed between red-tail and striped-tail models. The protocol for recording attacks and replacing models was the same as in 2010.

A total of 189 lizard models were placed in the field, but 85 of them (two plaster and 83 plasticine models) disappeared between visits. We considered these models as attacked (in both body parts) if there were bird tracks (Fig. 2G) on the ground where the model was (11 models), or as disappeared if there were no clear bird tracks (74 models). Lizard models considered as disappeared were excluded from statistical analyses because, in our opinion, the probability that these models were attacked by birds was low. People or cattle might have displaced some models, but most plasticine models that disappeared were very probably eaten by ants, because these insects could eat a whole plasticine model in less than 3 hours (personal observation).

Study with captive *F. tinnunculus*

F. tinnunculus is a bird of prey of the family Falconidae that is widely distributed across Eurasia and Africa (BirdLife International 2014). It commonly preys on lizards, at least in some areas (Carrillo *et al.* 1994; Vanzyl 1994; Carrillo & González-Dávila 2009), and it is a typical predator of *A. erythrurus* in Spain (Belluire 2006). The overlap in the distribution range of both species and the frequent inclusion of the lizard in the *F. tinnunculus* diet make this bird species a good model organism to study predatory interactions with our lizard models.

For this study, we used captive *F. tinnunculus* from the Wildlife Rehabilitation Centre of Santa Faz (Alicante, Spain) from August to October of three consecutive years (2011, 2012 and 2013). Only birds that were able to fly were involved in the experiment, although some of them had wing traumatism. We conducted a total of 31 trials where a *F. tinnunculus* (which was not fed 24 hours before the trial) faced a plasticine lizard model of one of the two experimental treatments: red-tail ($N = 15$) or striped-tail ($N = 16$) design. Each bird was used only once. Trials were conducted in individual cages (4.80 x 1.65 x 3.20 m) where the lizard model was placed in the centre of the concrete cage floor at the same time that the bird was placed on a perch at approximately 3 m from the lizard model and 1.3 m from the floor. Models were checked for attacks from outside the cage two or three times per day. The observer only entered the cage when an attack to the model was suspected. The trial ended when the model was attacked or after four days without attacks. If the model was attacked, the body part

attacked, i.e., with markings (tail or rest of the body), and the number of days until the model was attacked were recorded.

Statistical analyses

In the field study, Logistic Regression Models (GLZs) with binomial error distribution were used to test for the possible effects of model colour design (red or striped tail) on (1) the probability of being attacked and (2) the probability of being attacked on each body part (tail or rest of the body). For the former analysis, we used all models that were not considered as disappeared ($N = 115$), although the effect of model colour design was qualitatively identical if models disappearing without the presence of bird tracks were considered as attacked and included in the analysis ($N = 189$). For the latter analysis, we only included lizard models that had been attacked by birds on a single body part ($N = 38$), because when both parts showed markings, it was not possible to know which part was attacked first. To test for the possible effect of model colour design on the number of days until a model was attacked, a Generalized Linear Model (GLM) with Poisson error distribution was used. Only models attacked by birds ($N = 63$) were included in this analysis. All analyses in the field study simultaneously tested for differences between study areas (Chapinería and Aranjuez) and types of models (plaster and plasticine), and also checked the interactions between these two factors and model colour design. Year was excluded from our statistical analyses because it was strongly associated with the type of model in all data subsets used here (Fisher's exact test; $P < 0.001$

in the three tests), but if year was included instead of type of model, qualitatively identical results were obtained (see Results).

In the study with *F. tinnunculus*, GLZs with binomial error distribution were used to test for the possible effects of model colour design (red or striped tail) on (1) the probability of being attacked and (2) the probability of being attacked on each body part (tail or rest of the body). For the former analysis, all models were used ($N = 31$), while for the latter, only lizard models that had been attacked on a single body part were included ($N = 16$). To test for the possible effect of model colour design on the number of days until a model was attacked, a GLM with Poisson error distribution was used. Only attacked models ($N = 22$) were included in this analysis. All analyses in the study with *F. tinnunculus* simultaneously tested for differences among years (2011, 2012 and 2013) and checked the interaction between model colour design and year.

In all analyses of both studies, a backward stepwise procedure was used, retaining only terms associated with P values below 0.10 in final statistical models. If Akaike's information criterion (Burnham & Anderson 2002) was used to select final models, qualitatively identical results were obtained in all analyses (results not shown for brevity). All statistical analyses were carried out using R (R Development Core Team 2013) and lme4 package (Bates, Maechler & Bolker 2012). All tests were two-tailed and the significance level was set to 0.05.

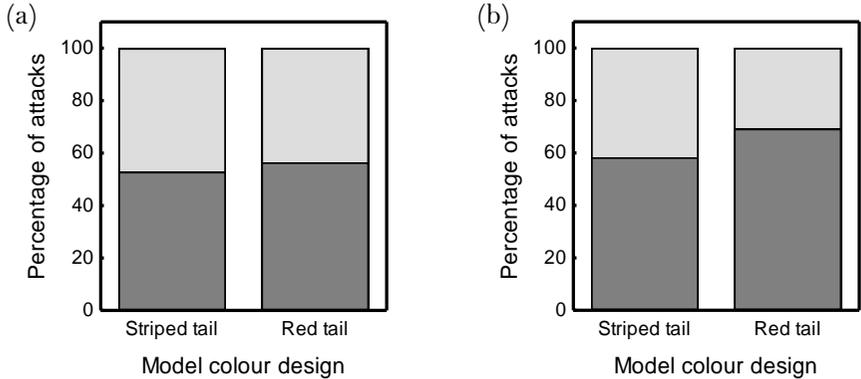
Results

Field study

Excluding models that were considered as disappeared, we recorded data for 115 models, 31 of plaster (14 with red tail and 17 with striped tail) and 84 of plasticine (48 with red tail and 36 with striped tail). A total of 63 models were attacked by birds. From the attacked models, 38 were attacked in one body part, while the rest (25 models) were attacked in both body parts (tail and rest of the body). Taking into account the total number of models placed in the field (models replacing those attacked by non-avian predators were not considered as different models), 39.16% of them were considered as disappeared, 33.33% were attacked by birds, and 27.51% were not attacked by birds.

The probability of being attacked by birds was not significantly different for red- and striped-tail lizard models (Likelihood test; $\chi^2 = 0.15$, $N = 115$, $P = 0.697$; Fig. 3a), and differences were not significant either between types of models (plaster or plasticine), or study areas (Chapinería or Aranjuez), as none of the factors was retained in the final model. However, red-tail models were attacked earlier than striped-tail ones, and plasticine models were attacked earlier than plaster ones (Table 1, Fig. 4a). The interaction between model colour design and type of model was statistically significant (Table 1), implying that the difference between colour designs depended on the type of model. We analysed separately plaster and plasticine models and found that in

Fig. 3 Percentage of models with red and striped tail that were (dark columns) and were not (light columns) attacked by birds in (a) the field study ($N = 115$) and (b) the study with *F. tinnunculus* ($N = 31$).

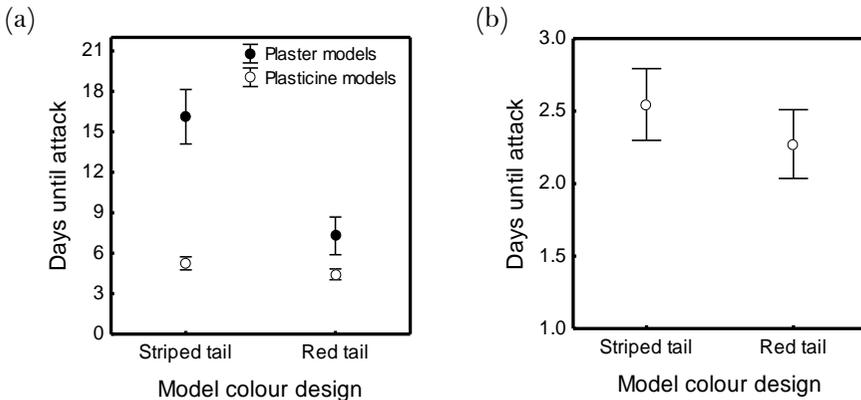


plaster models, red-tail models were attacked earlier than striped-tail ones (Likelihood test; $\chi^2 = 36.51$, $N = 19$, $P < 0.001$), but in plasticine models, the difference was not statistically significant (Likelihood test; $\chi^2 = 2.44$, $N = 44$, $P = 0.118$; Fig. 4a). When we included year instead of type of model in this analysis, we also found that red-tail models were attacked earlier than striped-tail ones (Likelihood test; $\chi^2 = 24.82$, $N = 63$, $P < 0.001$). Models were attacked earlier in 2011 than in 2010 (Likelihood test; $\chi^2 = 150.78$, $N = 63$, $P < 0.001$), and the statistically significant interaction between model colour design and year (Likelihood test; $\chi^2 = 8.81$, $N = 63$, $P = 0.003$) implied that the difference between colour designs depended on the year. We analysed separately data of both years and found that red-tail models were attacked earlier than striped-tail ones in 2010 (Likelihood test; $\chi^2 = 31.75$, $N = 16$, $P < 0.001$),

Table 1. Generalized linear model with Poisson error distribution analysing the effects of type of model (plaster or plasticine) and model colour design (red or striped tail) on the number of days until a model was attacked. The interaction between type of model and colour design is also shown. Study area and interactions including this factor were not retained in the final model after a backward stepwise procedure (see Statistical analyses for details). Only models attacked by birds were included in the analysis ($N = 63$).

	d.f.	χ^2	P
Type of model	1	124.61	< 0.001
Colour design	1	30.47	< 0.001
Type of model x Colour design	1	8.49	0.004

Fig. 4 Mean (\pm SE) number of days until a red- or a striped-tail model was attacked in (a) the field study depending on the type of model (plaster or plasticine) ($N = 38$) and (b) the study with *F. tinnunculus* ($N = 16$). Only models attacked by birds are included.



but the difference was not statistically significant in 2011 (Likelihood test; $\chi^2 = 1.88$, $N = 47$, $P = 0.170$).

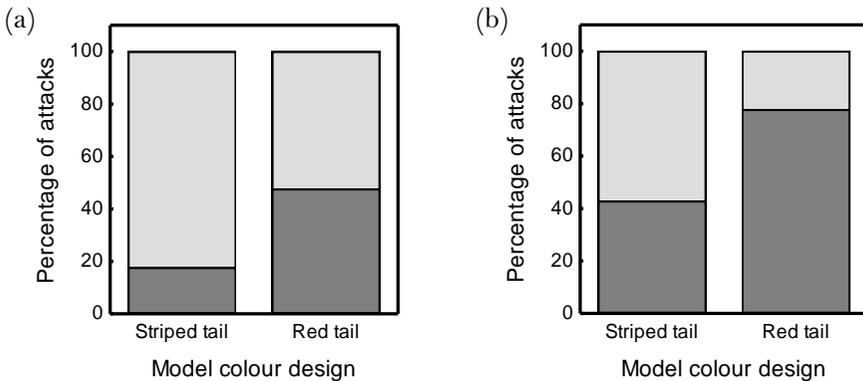
The proportion of attacks to each body part (tail or rest of the body) was different for red- and striped-tail models, as the probability of being attacked in the tail was higher for red-tail models (Likelihood test; $\chi^2 = 3.91$, $N = 38$, $P = 0.048$; Fig. 5a). Neither type of model nor study area had a significant effect on the part of the body that was attacked, as none of these factors or their interactions was retained in the final model.

Study with *F. tinnunculus*

A total of 31 models were presented to the birds, and 22 of them were attacked. From the attacked models, 16 were attacked in one body part, while the rest (6 models) were attacked in both body parts. The probability of being attacked by a captive *F. tinnunculus* was not significantly different for red- or striped-tail lizard models (Likelihood test; $\chi^2 = 0.08$, $N = 31$, $P = 0.779$; Fig. 3b), and did not differ significantly among years either, as no factor was retained in the final model. We did not find significant differences in the number of days until a model was attacked between model colour designs (Likelihood test; $\chi^2 = 0.17$, $N = 22$, $P = 0.680$; Fig. 4b), or among years, as no factor was retained in the final model. The part of the body (tail or rest of the body) that was attacked in red- and striped-tail lizard models did not

differ significantly either (Likelihood test; $\chi^2 = 2.08$, $N = 16$, $P = 0.150$; Fig. 5b).

Fig. 5 Percentage of attacks on each body section (tail: dark columns; rest of the body: light columns) for red- and striped-tail lizard models in (a) the field study ($N = 38$) and (b) the study with *F. tinunculus* ($N = 16$). Only models attacked by birds in one body part are included.



Discussion

Our first aim was to check whether red tails increase lizard conspicuousness to avian predators. The field study showed that red-tail models were attacked earlier than striped-tail ones, suggesting that the red colour indeed increased lizard model conspicuousness and, thus, detectability by avian predators. Previous studies checking the conspicuousness of green (Castilla *et al.* 1999) and blue (Watson *et al.* 2012) tails concluded that they were not significantly more conspicuous than brown or black ones respectively. Blue and green colours are

relatively common among lizards (Arnold 1984; Pianka & Vitt 2006), possibly because of their low conspicuousness at large distances in a vegetation background, although they might serve as a lure at closer distances (Arnold 1984). Red colour, however, is very conspicuous against a vegetation background (e.g. Schaefer *et al.* 2006), what may explain the increased detectability of red tail models in our study.

The field study also showed that red-tail models were not always attacked earlier than striped-tail ones, but only when certain type of model was used (plaster models) or in a particular year (2010). Unfortunately, our experimental design did not allow us to elucidate if there was a difference between types of models or between years, but both possibilities can be discussed. In the case that red tail conspicuousness differed between types of models, red-tail models were attacked earlier only in plaster models, what might be explained by their larger size or by their lack of a light band on the tail (Fig. 1), as both characteristics might make red tails particularly conspicuous in plaster models. However, in the case that red tail conspicuousness differed between years, red-tail models were attacked earlier only in 2010, what might be explained by changes in vegetation structure or predator abundance between years (e.g. lower vegetation cover or higher avian predator density in 2010) that were not investigated.

In free ranging lizards, dorsal colour design is very important in terms of camouflage (Lancaster *et al.* 2007; Calsbeek & Cox 2012), so we expected striped-tail models to match background colouration better than red-tail ones and, consequently, a higher rate of attacks in red-tail

models. However, our results did not confirm this expectation, as models of both colour designs received similar number of attacks in the field study. The fact that red-tail models were attacked earlier but not more often than striped-tail ones is counterintuitive, but the long periods between consecutive model inspections (3-5 days) might have contributed to the mismatch between two parameters (timing and number of attacks) that should be related to each other. The fact that both colour designs were attacked at a similar rate suggests, at least, that both were recognized as preys by avian predators. Similar attack rates in conspicuously and non-conspicuously coloured lizard models have also been observed in previous studies with models showing green or blue tails (Castilla *et al.* 1999; Watson *et al.* 2012). The study with *F. tinnunculus* showed similar attack rates and time until attack for lizard models of both colour designs, an expected result due to the experimental setup of this study (relatively small cages, high conspicuousness for both colour designs on the bare concrete cage floor, 24 hours fasting, etc.), but only if both colour designs were recognized as preys, as indeed seemed to be the case.

Our second aim was to check whether red tails are effective decoys diverting bird attacks from vital body parts. The field study corroborated this hypothesis, as red-tail models received a larger proportion of attacks in the tail than striped-tail models. If red tails divert attacks from vital body parts, the probability of surviving an attack will increase, particularly when the tail can be easily detached from the rest of the body without seriously compromising lizard survival

(Arnold 1988; Bateman & Fleming 2009). The benefits of increased probability of surviving a predator attack might outweigh the costs of increased conspicuousness, i.e., the costs of receiving more attacks by predators (Cooper & Vitt 1991). If this is the case, the positive net effect of red tail colouration on survival rate might have driven the evolution and maintenance of this trait in *A. erythrurus*. In the study with *F. tinunculus*, a similar pattern with a larger proportion of attacks in the tail for red-tail models was observed, but the difference between both colour designs was not statistically significant, maybe partially because of the small sample size of this study (Fig. 5b).

The present study suggests that red tails in lizards are effective decoys for avian predators, a function that has been also suggested for green and blue tails (Castilla *et al.* 1999; Watson *et al.* 2012). The tail colour developed in different lizard species does not seem to be strongly phylogenetically constrained, as the same colour can be present in species of different families (e.g. red tails are found in *Morethia ruficauda*, family Scincidae, *Vanzosaura rubricauda*, family Gymnophthalmidae, or *Acanthodactylus erythrurus*, family Lacertidae), while different colours can be present in species of the same genus (e.g. *Acanthodactylus erythrurus* and *A. schreiberi* juveniles have red tails, and *A. boskianus* and *A. beershebensis* juveniles have blue tails). Therefore, the specific tail colouration shown in every species might be more influenced by environmental variables (e.g. the contrast with the background; Håstad, Victorsson & Ödeen 2005; Rosenblum 2006), other traits of the lizard (e.g. the contrast with the rest of the body; Arnold 1984), or even the

predator's visual characteristics (e.g. different avian taxa can discriminate wavelengths at slightly different spectral ranges; Håstad, Victorsson & Ödeen 2005). In general, predation pressure seems to affect prey colouration (Rosenblum 2006), although the particular factors driving the evolution of conspicuous tail colouration in reptiles are not well known yet.

If conspicuously coloured lizard tails are an effective decoy for avian predators, we wonder why some lizard species lose this anti-predatory mechanism during the ontogenetic process (Booth 1990). *A. erythrurus* lizards, for example, lose the red colouration when they reach sexual maturity in the case of males, or when they become gravid in the case of females (Seva Román 1982; Cuervo & Belliure 2013). This ontogenetic colour change might be due to differences in behaviour between age classes, as hatchlings and juveniles usually need to be more active than adults in order to obtain enough food to meet the high energy demands of their intense somatic growth. This high activity rate would make hatchlings and juveniles more easily detected and, thus, more vulnerable to predators (Jackson, Ingram & Campbell 1976), so the development of an anti-predatory mechanism, such as a lure to divert attacks to expendable body parts, may be advantageous. However, adults would not need to be so active, and might increase their survival rates by developing more cryptic colouration (Arnold 1984; Vitt & Cooper 1986). These changes in behaviour between age classes have been observed in some lizard species that lose the conspicuous tail colouration when growing up (Hawlana 2009; Hawlana *et al.* 2006) and also seem to occur

in *A. erythrurus* (B. Fresnillo, J. Belliure, & J. J. Cuervo, unpublished data, Chapter 2 of this thesis).

Conclusions

This study suggests that lizards with conspicuous red tails are more easily detected by avian predators, but also that red tails effectively divert avian predator attacks from vital body parts to the expendable tail. If the benefits of diverting attacks to the tail exceed the costs of being conspicuous to predators, red tails will have a positive net effect on survival and, thus, on fitness, then favouring the evolution and maintenance of this conspicuous colouration. Therefore, our results support the decoy hypothesis to explain conspicuous red colouration in juvenile lizards.

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Chapter 4

**RED COLOURATION IN JUVENILE
SPINY-FOOTED LIZARDS
(*ACANTHODACTYLUS*
ERYTHRURUS) REDUCES ADULT
AGGRESSION**

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Fresnillo B., Belliure J. and Cuervo J. J. Red colouration in juvenile spiny-footed lizards (*Acanthodactylus erythrurus*) reduces aggression from adults (Submitted to Animal Behaviour).

LA COLORACIÓN ROJA EN JUVENILES DE LAGARTIJA COLIRROJA (*ACANTHODACTYLUS ERYTHRURUS*) REDUCE LAS AGRESIONES DE LOS ADULTOS

Resumen

En muchos taxones animales la coloración es una señal visual utilizada en comunicación intraespecífica, por ejemplo entre clases de edad. Se ha sugerido que la coloración de los juveniles reduce la agresividad de los adultos en algunas especies, lo que se ha denominado la hipótesis de reducción de agresividad. La lagartija colirroja (*Acanthodactylus erythrurus*) es un buen modelo para estudiar esta hipótesis, dado que los juveniles presentan una coloración roja muy llamativa en las patas traseras y en la cola que desaparece en adultos. Para comprobar la influencia de la coloración de los juveniles sobre la agresividad de los adultos, grabamos en video enfrentamientos en cautividad entre adultos de ambos sexos y juveniles que, o bien mostraban su coloración roja natural, o bien presentaban las partes naturalmente rojas cubiertas con pintura blanca o roja. Anotamos el número de veces que los juveniles eran mordidos y atacados. Los juveniles no pintados que tenían valores más altos de tono (más rojos, menos naranjas) fueron menos atacados. Además, los juveniles pintados de rojo fueron mordidos menos que los pintados de blanco. Ambos resultados apoyan la hipótesis de reducción de agresividad, ya que los juveniles más rojos recibieron menos agresiones por parte de los adultos. Concluimos que la coloración roja podría ser un indicador de edad o inmadurez sexual y que la variación en el tono del rojo (más rojo o más

naranja) podría estar relacionada con otras características como la calidad individual o el sexo del juvenil.

PALABRAS CLAVE: coloración juvenil, comunicación entre clases de edad, enfrentamientos controlados, espectrofotometría, imitación de las hembras, reducción de agresividad, señalización del estatus

RED COLOURATION IN JUVENILE SPINY-FOOTED LIZARDS (*ACANTHODACTYLUS ERYTHRURUS*) REDUCES AGGRESSION FROM ADULTS

Abstract

In many animal taxa, colouration is a visual signal used for communication among conspecifics, for example between age classes. Juvenile colouration has been hypothesised to reduce aggression from adults in some species, in what is called the aggression-avoidance hypothesis. Spiny-footed lizards (*Acanthodactylus erythrurus*) are good subjects for testing this hypothesis, as juveniles develop conspicuous red colouration on their hind limbs and tails that fades in adulthood. To test the influence of juvenile colouration on adult aggressiveness, we conducted videotaped encounters in captivity between adults of both sexes and juveniles with their naturally red colouration, or experimentally painted either red or white covering their natural red parts. Then we recorded the number of times juveniles were bitten and attacked. In unpainted juveniles, the higher the red hue (i.e., more red, less orange) was, the less they were attacked. Moreover, juveniles painted red were bitten less than those painted white. Both results support the aggression-avoidance hypothesis, as redder juveniles were subject to less adult aggression. We conclude that red colouration might indicate age or sexual immaturity, and natural variation in red hue (more red or more orange) might be related to other characteristics like individual quality or sex.

KEY WORDS: Aggression avoidance, female mimicry, inter-age class communication, juvenile colouration, spectrophotometry, staged encounter, status signalling

Introduction

Animal communication involves a wide variety of signals (Bradbury & Vehrencamp 2011). One of them is colouration, a visual signal used in many species for interspecific or intraspecific communication (Cott 1940). In interspecific communication, colour signals are common subjects of study in predator-prey interactions due to aposematism and mimicry (Stevens 2007). Colouration can also be useful to divert predator attacks towards expendable body parts (Telemeco, Baird & Shine 2011), or in association with some behaviours, inform the predator that it has been spotted (Murphy 2007). Although colour signals may play an important role in interspecific communication, much research has also focused on intraspecific functions such as signalling of sex (Cooper & Burns 1987; Price *et al.* 2008), fertility (Pitcher, Rodd & Rowe 2007; Rowe *et al.* 2010), sexual attractiveness (Kemp & Rutowski 2011; Olsson, Andersson & Wapstra 2011; Deere *et al.* 2012; Rutowski & Rajyaguru 2013), dominance (Senar 2006; Midamegbe *et al.* 2011), health status (del Cerro *et al.* 2010; Barrios-Miller & Siefferman 2013) and age (Kemp 2006; Hawkins, Hill & Mercadante 2012). Colour signals require accurate visual ability of the receptor, and, indeed, many species can detect and discriminate colours (Pichaud, Briscoe & Desplan 1999; Kelber, Vorobyev & Osorio 2003), some of them even in the ultraviolet spectrum (Jacobs 1992).

Communication between age classes is an important intraspecific function of colour signals in many taxa. For example, in birds, parental

investment in offspring feeding and defence has been shown to be modulated by chick colouration in mouth and feathers respectively (de Ayala *et al.* 2007; Barrios-Miller & Siefferman 2013). It has also been proposed that juvenile colouration reduces adult aggression towards juveniles in what has been called the aggression-avoidance hypothesis, which is supported by studies in different animal groups (Clark & Hall 1970; Hill 1989; Ochi & Awata 2009). The mechanism underlying the reduction in adult aggressive behaviour associated with juvenile-like colouration has been explained in birds by the status-signalling hypothesis (VanderWerf & Freed 2003) and the female mimicry hypothesis (Vergara & Fargallo 2007), but these mechanisms have not generally been studied in other taxa. In both hypotheses, the ultimate function of the colour signal is aggression avoidance, but the underlying mechanism is different: while female mimicry implies that adult males cannot distinguish between females and juveniles, status signalling suggests that juvenile colouration announces subordination and is not necessarily mimetic. The signalling function of juvenile colouration and its effects on adult aggressiveness are still under debate (Cooper & Greenberg 1992; Hawkins, Hill & Mercadante 2012), so more studies seem necessary to understand the factors driving the evolution of such signals.

Lizards are good subjects for testing the aggression-avoidance hypothesis, as juveniles of several species have conspicuous colouration that fades when they reach sexual maturity (Cooper & Greenberg 1992; Carpenter 1995; Hawlena *et al.* 2006). This hypothesis was first proposed

in lizards by Clark and Hall (1970), who suggested that juveniles develop such colouration to show aggressive adult males that they are not competitors, and are therefore attacked less. It has been suggested that this mechanism is beneficial to both adults and juveniles, as it reduces the possibility of the former attacking their own offspring and lessens adult aggression on the latter with the associated stress and/or risk of injury. The few previous studies analysing the role of juvenile lizard colouration in inter-age class interactions have used different methods and reached different conclusions, even within the same species (Clark & Hall 1970; Werner 1978; Cooper & Vitt 1985; Husak *et al.* 2004).

Two main types of aggression may be seen in lizards: attacking (fast direct approach), which can be considered low-intensity aggression, and biting, which can be considered high-intensity aggression. Although biting may sometimes cause lethal injuries, more often it just increases the probability of mortality, for example, by causing open wounds that are susceptible to infection, or the loss of part of the tail, thus depriving of an anti-predatory mechanism and fat stores (Bauwens 1981; Arnold 1984; Arnold 1988; Wilson 1992). The loss of the tail may be even more important for juveniles than for adults, as the former will need to invest resources in tail regeneration that will not be available for somatic growth (Bateman & Fleming 2009). Furthermore, attacks (no biting) might also reduce juvenile survival rate, for example, if subordinate individuals are forced to live in suboptimal habitats (Carothers 1981), or if escaping from attack involves loss of foraging opportunities (Cooper

2000; Cooper *et al.* 2003; Pérez-Cembranos, Pérez-Mellado & Cooper 2013). Therefore, if a signal of age or sexual immaturity can deter adult aggression, it will increase juvenile survival and thus individual fitness, and will be evolutionarily selected.

The spiny-footed lizard (*Acanthodactylus erythrurus*, Schinz 1833) is a good model organism for testing the aggression-avoidance hypothesis, as juveniles of this species have conspicuous red colouration on their hind limbs and tails that fades in adulthood. Aggression-avoidance could be an explanation for juvenile colouration in this species, because juveniles and adults coexist (Seva Román 1982), and although juveniles usually occupy more open areas than adults (Seva Román 1982), there are frequent encounters between individuals of both age classes in which juveniles flee from adults (personal observation). In the present study, we tested the aggression-avoidance hypothesis in spiny-footed lizards, specifically checking (i) whether the number of times unmanipulated juveniles were attacked and bitten by adults of both sexes was related to red colour parameters (brightness, red chroma and hue), and (ii) whether the number of times attacked and bitten by adults of both sexes differed between juveniles painted red (imitating juvenile colouration) or white (imitating adult colouration). According to the aggression-avoidance hypothesis, redder juveniles were expected to be attacked and bitten less. Aggressiveness was also expected to be higher in adult males than in adult females, as inter-age class encounters were conducted during the mating period, when male lizards are assumed to be more aggressive (Baird, Timanus & Sloan 2003).

Materials and methods

Study species

The spiny-footed lizard is medium-sized (snout-vent length (SVL) and total length up to around 80 and 230 mm respectively; Seva Román 1982; Carretero & Llorente 1993), although sizes can vary among populations. In populations in central Spain, this species reaches sexual maturity when males are 58-65 mm SVL and females 60-66 mm SVL (Bauwens & Díaz-Uriarte 1997), during their second spring. At our study site, the mating period runs from May to June, and newborn lizards appear in mid-August and are active until November. At the beginning of April the following spring, these lizards become active again, and they do not reach sexual maturity until the following spring. These observations agree with those described for other populations in central Spain (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992). Therefore, all individuals can be divided in three rough age categories, hereafter referred to as hatchlings (from hatching until the first winter), juveniles (from first to second winter) and adults (from the second winter onwards).

Colouration in this species undergoes ontogenetic changes, in which the dorsal pattern goes from strongly-marked dark and light bands in hatchlings to a reticulated pattern in adults (Seva Román 1982). Juveniles develop red colouration on the rear part of their hind limbs and the ventrolateral part of their tails (Seva Román 1982; Carretero & Llorente 1993). Juvenile males lose the red colour at the end of their

second summer, whereas juvenile females retain it through adulthood (Seva Román 1982). In the second spring, when animals of both sexes reach sexual maturity, sexual dichromatism becomes evident: while males show white colouration on the rear part of their hind limbs and the ventrolateral part of their tails, females retain red colouration in these body parts until they are gravid, when they lose their red colour and become pallid yellow, nearly white (Seva Román 1982; Cuervo & Belliure 2013).

Captures and Captivity Conditions

A total of 47 adults (21 females and 26 males) and 49 juveniles were captured using a fishing pole with a loop of dental floss at the end from April to June 2010 and 2011 in Chapinería, south-western Madrid Region, Spain (N40°22'; W4°13'). The area is a Mediterranean oak forest with meadow structure, where oaks (*Quercus ilex* L.) and lavender (*Lavandula stoechas* Lam.) dominate vegetation patches surrounded by open areas. Lizards were placed in individual cloth bags in the shade immediately following capture to prevent overheating. After a maximum of six hours from capture, they were transported at a temperature of around 22°C to the Alcalá University Animal Research Centre, Madrid, for the experimental study. Lizards were then placed in individual terrariums (42 x 26 x 19 cm) with a thin layer of sand on the bottom and available shelter. The light cycle was 12:12 h light:dark. Room temperature was 25°C and a bulb hanging over the edge of each individual terrarium provided a temperature gradient for

thermoregulation. Lizards were supplied with food (*Tenebrio molitor*) dusted with vitamins once a day and water *ad libitum*. No lizard was kept in captivity for over 79 days (mean + SD = 43.47 + 17.16 days, $N = 96$). All animals were released after the study in exactly the same places where they had been captured. All of them behaved normally and looked healthy when released.

Experiment in Captivity

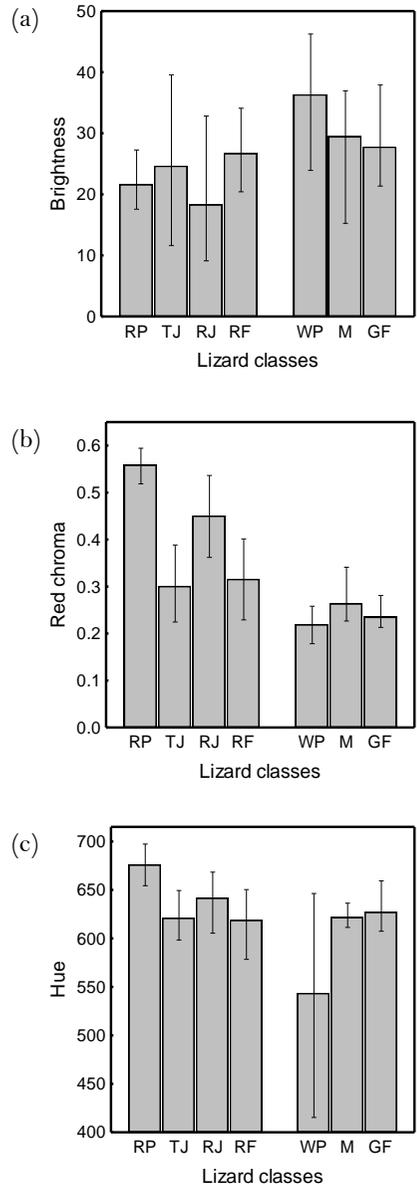
We classified lizards as juveniles or adults according to their SVL (Bauwens & Díaz-Uriarte 1997). Mean (+ SD) SVL for adults and juveniles was 74.6 (+ 3.9) mm (range = 67 - 82 mm, $N = 47$) and 48.7 (+ 4.8) mm (range = 39 - 63 mm, $N = 49$) respectively. Adults were sexed according to the base of the tail, which is much wider in males than in females (Blasco 1975), but juveniles could not be sexed because sexual dimorphism in juveniles of this species is not evident in spring. All adult females captured were considered to be sexually receptive because they all showed the typical red colouration of sexually receptive females in this species (Cuervo & Belliure 2013) and there were no copulation marks or oviductal eggs in any of them.

Lizards were kept in their individual terrariums at least three days before trials for acclimatization. We videotaped (JVC GZ-MG680 camera) 30-minute encounters between lizard pairs (one adult and one juvenile) in a neutral trial terrarium (77 x 55 x 43 cm) with a thin layer of sand on the bottom. Both lizards were placed in the trial terrarium at

the same time to avoid the effects of prior residency on the outcome of encounters (Cooper & Vitt 1987; Olsson & Shine 2000). Trials were performed from 10 to 17 h local time, within the species' period of activity (Busack 1976). A bulb hanging over the trial terrarium provided light in the middle of the terrarium. The temperature in the trial terrarium was recorded in some encounters to ensure that it was within the activity range for the species (mean + SD = $32.9 \pm 2.1^\circ\text{C}$, range = $29.7 - 35.3^\circ\text{C}$, $N = 63$; Belliure Carrascal & Díaz, 1996). No lizard was involved in more than one trial per day to prevent extenuation or excessive stress. All encounters took place during the mating period (from mid-April to the end of June) in both years.

Two kinds of interactions between an adult of either sex and a juvenile were designed based on juvenile colouration: painted (hereafter, painted trials) and unpainted (hereafter, natural-colour trials). In the painted trials, naturally red parts of juveniles were painted either white (adult-like colouration) or red (juvenile-like colouration) with nontoxic paint (Satin Paint, La Pajarita, Manises, Spain). Paint colour partially overlapped with the range of natural colouration which develops this species (Fig. 1). It was attempted to mimic the reddest colouration found in juveniles of this species with red paint and to mimic adult colouration with white paint (see Supplementary material for reflectance spectra).

Figure 1. Mean, maximum and minimum (a) brightness, (b) red chroma and (c) hue of hind legs and ventral tail in spiny-footed lizards and of the paints used for red and white experimental treatments. Red paint (RP; $N = 12$) and white paint (WP; $N = 12$) were measured on the lizard. Lizard classes: TJ = trial juveniles (all juveniles used in the natural-colour trials, $N = 78$); RF = receptive females (all females used in painted or natural-colour trials, $N = 20$); RJ = reddest juveniles (some of the reddest juveniles of this species from several populations, $N = 12$); M = males (males from the study population, $N = 5$); GF = gravid females (gravid females from the study population, $N = 20$). Classes RJ, M and GF include individuals not used in this study and are shown for comparative purposes. All colour measurements were taken on day of capture except for TJ, which were taken immediately after natural-colour trials.



A total of 216 interactions were recorded, but 13 videos were discarded due to immobility of one of the animals (as this might imply a strong reaction to manipulation or captivity) and one video was discarded due to an error in juvenile colour measurement. Therefore, 202 interactions were actually used for the study, 124 painted trials and 78 natural-colour trials. Some individuals (41 juveniles and 33 adults) were used in both painted and natural-colour trials, but each adult-juvenile pair was used only once. Mean (\pm SD) difference in SVL between adults and juveniles within pairs was 26.2 (\pm 5.3) mm (range = 14.5 - 39.0 mm, $N = 202$). For each trial, we counted (on video recordings) the number of aggressive behaviours that adults directed at juveniles, specifically attacking (fast direct approaches; low-intensity aggression) and biting (high-intensity aggression).

In the painted trials, each adult was recorded from two to five times. Each trial consisted of an interaction with a juvenile of one of the two colour treatments (white or red). All adults took part in at least one trial with each colour treatment and participated in approximately the same number of trials per colour treatment. There were 63 trials with juveniles painted red (29 trials with 16 different adult females and 34 trials with 19 different adult males) and 61 trials with juveniles painted white (28 trials with 16 different adult females and 33 trials with 19 different adult males). A total of 45 juveniles were used in this experiment, 34 of which were painted both white and red, but always presented to different adults in every trial. The first colour treatment presented to an adult was chosen at random, but ensuring a similar

number of adults in the first trial with red ($N = 19$) and white ($N = 16$) treatments.

In the natural-colour trials, we measured the reflectance of hind limbs and tails of juveniles after each interaction as a measurement of the colour shown by the juvenile (see Colour Measurements below). Each adult was recorded from one to three times. A total of 35 trials were recorded for 20 different adult females and 43 trials for 25 different adult males. We used 45 juveniles for these trials, of which 33 were used twice, but always with adults of different sex.

Colour Measurements

Colour measurements are described in detail elsewhere (Cuervo & Belliure 2013). Briefly, we quantified the spectral properties of juvenile colouration after each natural-colour trial by taking reflectance readings (with a USB 2000 spectrometer and a DT-MINI-2-GS tungsten halogen light source, Ocean Optics, Dunedin, USA) in the range of 320–700 nm (Whiting *et al.* 2006). Four body regions (the rear part of both hind limbs and the ventral part of the tail around 1 cm and 2.5 cm from the cloaca) were measured three times each. Then, reflectances at 1-nm intervals in the range studied were calculated using AVICOLOR software (Gomez 2006). From the reflectance data, three colour parameters were calculated as follows: brightness as the mean reflectance between 320 and 700 nm, red chroma as the sum of reflectances between 630 and 700 nm divided by the sum of reflectances between 320 and 700 nm, and hue as the wavelength where the

maximum reflectance was recorded (Montgomerie 2006). Since the three measurements in each body part were highly repeatable (repeatability according to Lessells and Boag (1987); brightness: $0.638 \leq r \leq 0.919$; red chroma: $0.860 \leq r \leq 0.896$; hue: $0.612 \leq r \leq 0.666$; $F_{78,158} \geq 5.70$, $P < 0.001$ in the 12 tests), we calculated the means for each body part. Likewise, as measurements in the four body regions were positively correlated (Pearson correlations; brightness: $0.535 \leq r \leq 0.878$; red chroma: $0.454 \leq r \leq 0.801$; hue: $0.235 \leq r \leq 0.547$; $N = 79$, $P \leq 0.038$ in the 18 tests), they were all unified in single brightness, red chroma and hue values for each individual (the mean of the four body parts), and these values were then used for further analyses (Cuervo & Belliure 2013).

Statistical Analyses

In natural-colour trials, the effect of juvenile colouration on adult aggressiveness (number of times attacked and bitten) was analysed with generalized linear mixed models (GLMMs) with a Poisson error distribution. The predictors included in these models were juvenile SVL, adult sex and juvenile colour parameters (brightness, red chroma and hue) measured immediately after each trial. In painted trials, differences in the number of times juveniles painted red and white were bitten and attacked were checked using similar analyses, but due to overdispersion in the number of attacks, in this particular case, a GLMM with negative binomial error distribution was used instead. Predictors in these models were juvenile SVL, experimental treatment (red or white), adult sex and

the interaction between experimental treatment and adult sex. All analyses in both types of trials included adult identity as a random factor. Number of times bitten was positively related to times attacked in both natural-colour and painted trials (natural-colour trials: $\beta + \text{SE} = 0.184 + 0.044$, $Z = 4.20$, $N = 78$, $P < 0.001$; painted trials: $\beta + \text{SE} = 0.090 + 0.023$, $Z = 3.91$, $N = 124$, $P < 0.001$), so all analyses of the number of times bitten were repeated including times attacked as another predictor. A backward stepwise procedure was used in all GLMMs, retaining only terms associated with P values below 0.10 in final models. We also checked whether Akaike's information criterion (Burnham & Anderson 2002) selected the same final models, and this was indeed the case.

We checked whether juvenile painting itself had any effect on adult behaviour by comparing the times attacked and bitten, and times bitten controlled for number of times attacked between naturally-red juveniles and those painted red. The predictors included in the GLMMs with Poisson error distribution were juvenile SVL, type of juvenile (painted red or naturally red), adult sex and the interaction between the type of juvenile and adult sex. Adult identity was also included as a random factor. The number of times juveniles painted red and unpainted (i.e., naturally red) were attacked and bitten did not differ significantly, as no predictor was retained in final models (GLMMs including type of juvenile as the only predictor; times attacked: $\beta + \text{SE} = 0.125 + 0.104$; times bitten: $\beta + \text{SE} = 0.145 + 0.305$; times bitten controlled for times attacked: $\beta + \text{SE} = -0.100 + 0.330$; $-0.30 \leq Z \leq 1.21$, $N = 141$, $P \geq 0.228$

in the three tests), suggesting that the paint itself did not significantly affect adult behaviour.

All statistical analyses were carried out using R (R Development Core Team 2013). GLMMs with Poisson error distribution were implemented using the lme4 package (Bates, Maechler & Bolker 2012), and for GLMMs with negative binomial error distribution, the glmmADMB package was used (Fournier *et al.* 2012). All statistical tests were two-tailed and the significance level was 0.05.

Ethical Note

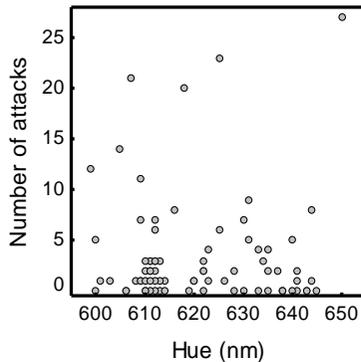
This study was conducted following the ASAB/ABS Guidelines for the Treatment of Animals in Behavioural Research and complied with the laws of Spain and the Madrid Autonomous Region. Permission to capture and keep spiny-footed lizards in captivity was given by the Madrid Autonomous Region Environment Department.

Results

Natural red colouration of juveniles was related to adult attacks: the final model explaining the times attacked only included hue, with juveniles that showed higher hue (more red, less orange) being attacked less (GLMM; $\beta + SE = -0.021 + 0.008$, $Z = -2.62$, $N = 78$, $P = 0.009$; Fig. 2). However, when times bitten, either absolute or relative to times attacked, was analysed, no predictor was retained in final models, i.e.,

times bitten was not significantly related to juvenile colouration. These results also imply that adult males and females did not differ significantly in the number of times they attacked or bit juveniles showing their natural red colouration.

Figure 2. Relationship between the number of adults attacks on juvenile spiny-footed lizards and juvenile hue (wavelength with maximum reflectance) in natural-colour trials. Reflectance was measured on the rear part of hind legs and ventral tail (see Colour Measurements).



Red Juveniles painted red or white in painted trials did not differ in the number of times attacked, as no predictor was retained in the final model (GLMM including experimental treatment as the only predictor: $\beta + SE = 0.238 + 0.253$, $Z = 0.94$, $N = 124$, $P = 0.346$). However, juveniles painted red were bitten less than those painted white, because experimental treatment was the only predictor retained in the final model and the effect was statistically significant (GLMM: $\beta + SE = -$

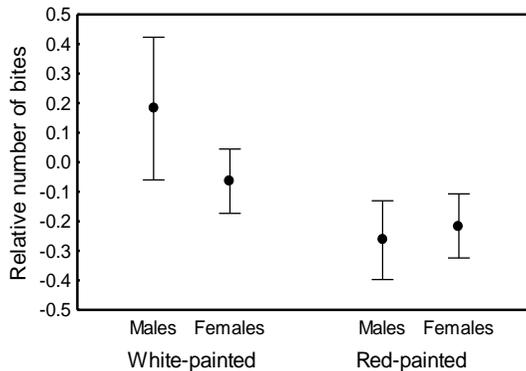
0.528 + 0.258, $Z = -2.05$, $N = 124$, $P = 0.041$). Qualitatively identical results were found when number of times attacked was included in the model (Table 1, Fig. 3). Our results also imply that there were no significant differences between adults of both sexes in the number of times painted juveniles were attacked or bitten, although a non-significant trend for adult males biting more than adult females was found when times bitten was controlled for times attacked (Table 1, Fig. 3).

As we found significant differences in the number of times juveniles painted red and white were bitten, and given that both groups showing red colouration were bitten a similar number of times (see Statistical Analyses), we explored whether juveniles showing natural red colouration were also bitten less than juveniles painted white. We repeated the same analysis used to compare juveniles painted red and white, but using the natural colour group instead of the one painted red. As expected, juveniles showing natural red colouration were bitten less than juveniles painted white (GLMM; bites: $\beta + SE = 0.690 + 0.270$, $Z = 2.58$, $P = 0.010$; bites controlled for attacks: $\beta + SE = 0.858 + 0.304$, $Z = 2.83$, $P = 0.005$).

Table 1. GLMM explaining the number of times juveniles were bitten in painted trials ($N = 124$). Juvenile SVL and interaction between experimental treatment and adult sex were removed from the final model after a backward stepwise procedure (see Statistical Analyses for details of the test).

	$\beta \pm SE$	Z	P
Intercept	-2.066 ± 0.489	-4.23	< 0.001
Number of attacks	0.114 ± 0.025	4.65	< 0.001
Experimental treatment	-0.782 ± 0.287	-2.73	0.006
Adult Sex	1.050 ± 0.560	1.88	0.061

Figure 3. Mean ($\pm SE$) relative number of times juvenile spiny-footed lizards painted red or white were bitten (controlled for number of attacks) by adults of both sexes in painted trials. Relative numbers of times bitten were residuals from a GLMM including number of attacks as the only predictor (see Statistical Analyses and Table 1).



Discussion

In this study, adult spiny-footed lizards were less aggressive towards redder juveniles in both painted and natural-colour trials: juveniles painted red were bitten less than those painted white, and of the naturally red juveniles, the redder (less orange) ones were attacked less. Therefore, our results support the aggression-avoidance hypothesis, i.e., that juvenile red colouration reduces aggression by adults. Similar results have been found in previous studies with different lizard species comparing adult aggression towards juvenile-like and adult-like juveniles (Werner 1978). Other studies, however, have not found a reduction in aggression towards conspicuously coloured juveniles (Cooper & Vitt 1985; Husak *et al.* 2004), but these studies used a different approach, so their results may not be comparable to ours.

This study enabled us to test the importance of the presence/absence of red juvenile colouration (painted trials) and of the natural variation in red juvenile colouration (natural-colour trials) to adult aggressiveness. The combination of three different results suggest that the probability of juveniles being bitten is reduced simply by displaying red colouration, regardless of small variations in the expression of the signal. First, red juveniles (both naturally red and painted red) were bitten less than those painted white. Second, naturally red juveniles and those painted red were bitten a similar number of times. And third, there was no significant relationship between natural red colour expression and times bitten. There was an escalation in

intensity of aggression (i.e., an increase in the number of times bitten) towards juveniles painted white, possibly because adults considered adult-like juveniles real adults and thus potential competitors, at least for some period of time and despite their smaller size (Rohwer 1977; Martín & Forsman 1999; Laubach *et al.* 2013). On the contrary, both naturally red juveniles and those painted red showed the typical red colouration of their age class, so adults behaved as they normally do with juveniles having subordinate behaviour that do not imply real competition for resources, i.e., without resorting to high intensity aggression (biting; Werner 1978). Thus, the presence of red colouration in juveniles reduced adult aggression, but only high-intensity aggression, as the number of attacks did not differ significantly between juveniles painted red or white.

We found a negative relationship between hue in natural-colour trials and number of attacks, i.e., redder (less orange) juveniles were attacked less by adults. A possible interpretation of this result is that small variations in the expression of the signal (the red colour), specifically in hue, could be giving information that modulates adult behaviour, reducing low-intensity aggression towards redder juveniles. But what kind of information could these small variations in hue be providing? In adult lizards, it has been suggested that colour is related to individual phenotypic condition (Cuervo & Shine 2007; Fitze *et al.* 2009; Martín & López 2009). To our knowledge, however, the link between colouration and phenotypic condition has never been studied in juvenile lizards and such studies on juveniles of other taxa are scarce

(but see de Ayala *et al.* 2007; Bailey 2011; Tringali & Bowman 2012; van den Brink *et al.* 2012). Colouration might also provide information on juvenile sex, as is the case in other lizard species (e.g. *Sceloporus undulatus*; Cox *et al.* 2005), but as we were unable to identify the sex of the juveniles involved in our study, this explanation remains purely speculative. Clearly, future studies with this species should address possible links between juvenile colouration, phenotypic condition and sex.

A problem when interpreting the relationship between juvenile colouration and adult aggression in natural-colour trials was that the relationship did not necessarily imply causality, i.e., the colour might not be the signal perceived by the adult and causing a change in its behaviour. The relationship might appear, for example, if colouration is related to another phenotypic trait that is the signal perceived by the adult. If the colour itself were not the signal affecting adult behaviour, a good candidate to mediate between colouration and adult aggression could be the pheromone composition of the juvenile scent, as chemical communication was also possible in our trials. In animals, a link between odours and visual cues has been suggested, for example to help in the recognition of conspecifics or potential mates (McLennan 2003; Tedore & Johnsen 2013). Chemical communication is very important in reptile social behaviour (Mason & Parker 2010), and can give information about an individual's age (López & Martín 2005; Martín & López 2013) or sex (Parker & Mason 2012), even after experimentally painting the animals (Cooper & Vitt 1988). Pheromones can also be used to establish

dominance hierarchies in lizards (Khannoon, El-Gendy & Hardege 2011), so the relationship we found between times attacked and hue might have been reflecting responses to the chemical composition of juvenile scent if a hue-pheromone combination was present.

Body size was expected to have an effect on adult behaviour because it is used by reptiles to assess fighting ability, larger individuals being stronger competitors than smaller ones (Andersson 1994; Olsson & Shine 2000). Body size is usually correlated, among other features, with the ability to cause injury (Arnott & Elwood 2009). In our study, juveniles of different sizes were used in the trials and juvenile SVL was therefore included in our statistical models. However, no significant effect of juvenile body size on adult aggression was found in any statistical test for either of the two types of trials (painted and natural colour trials). The reason juvenile body size had no significant effect on the number of times attacked or bitten may be the considerable difference in body size between the juvenile and the adult in each encounter, which was always more than 14 mm SVL and usually more than 20 mm SVL (see Experiment in Captivity). Juvenile fighting ability was probably always perceived by adults as modest, and small variations in juvenile size would not affect that perception.

We expected adult males to be more aggressive towards juveniles than adult females because trials were recorded during the mating season, when adult male lizards are usually more aggressive to defend territories or to gain access to females (Baird, Timanus & Sloan 2003). However, only a non-significant difference in the number of times

adults bit juveniles (controlled for the times they attacked) was found between sexes, with males tending to bite more than females, but only in painted trials. This similarity in the number of times adults of both sexes bit and attacked could be due to both sexes being territorial, but with a similar degree of territoriality, or to both sexes being non-territorial. Home ranges have been defined for age and sex classes in this species (Seva Román 1982), but the possible defence of these areas has not yet been studied. In reptiles, territorial species (in which aggressive encounters are common) have developed stereotyped aggressive displays to assess the fighting ability of the opponent, and thereby avoid escalation of aggression unless necessary (Whiting, Nagy & Bateman 2003). This kind of behaviour has not been described for spiny-footed lizards, and no such display was observed during our trials. However, fighting ability in our staged encounters could have been assessed exclusively by the size of the opponent (Olsson & Shine 2000), making any other display unnecessary. Based on the lack of references on territoriality in spiny-footed lizards and our personal field and lab observations, we think this species might be non-territorial, although more studies are necessary to clarify this point.

This study tested the hypothesis that red colouration in juvenile spiny-footed lizards was associated with a reduction in aggression by adults, and found results consistent with the hypothesis. As mentioned in the Introduction, two main mechanisms have been hypothesised to explain the reduction in aggressiveness associated with juvenile colouration, the status-signalling hypothesis (Rohwer 1977) and the

female mimicry hypothesis (Rohwer, Fretwell & Niles 1980). Our experimental design does not allow us to discriminate between these two mechanisms, but current knowledge on the ecology of the species would be more in accordance with the status-signalling hypothesis. The female mimicry hypothesis implies that juveniles enjoy reduced aggressiveness from adult males by mimicking adult females. In spiny-footed lizards, red colouration is present in juveniles and in sexually receptive females (Seva Román 1982; Cuervo & Belliure 2013), but a peculiarity of this and other lizard species is that males looking for mating opportunities try to bite females in order to hold them down and ensure copulation (e.g. Bosch & Zandee 2001). Therefore, it would be a poor strategy for juveniles to mimic receptive females, as this might increase rather than reduce aggression by adult males.

Conclusions

Our results support the aggression-avoidance hypothesis, suggesting that red colouration in juvenile spiny-footed lizards reduces aggression from adults. The mere presence of this colour signal in juveniles, regardless of small variations in its expression, informs adults of both sexes about age or sexual immaturity (and thus possibly subordination). Moreover, the hue of the red colouration might be related to characteristics other than age (e.g., phenotypic quality or sex) which could modulate adult tolerance of juveniles.

Acknowledgements

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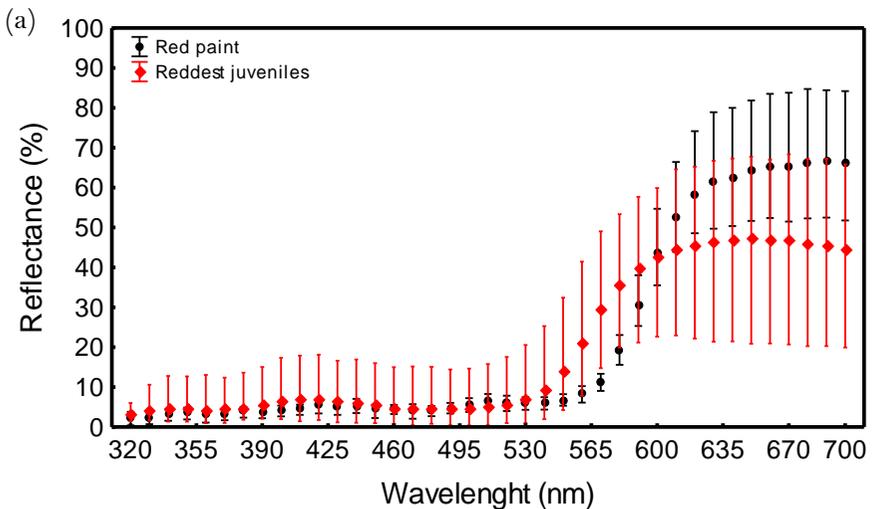
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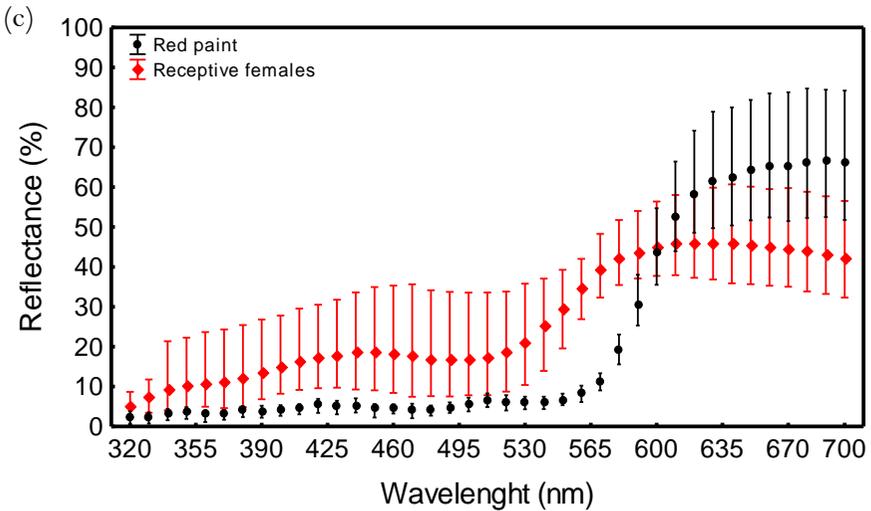
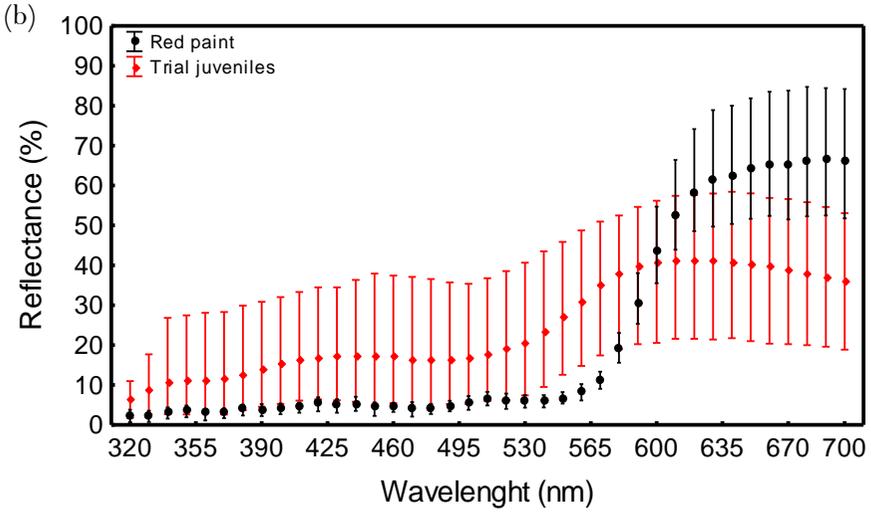
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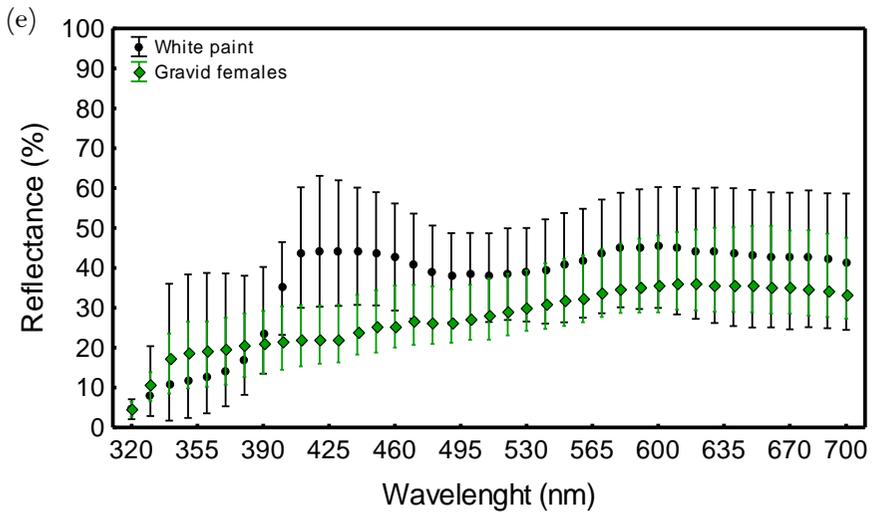
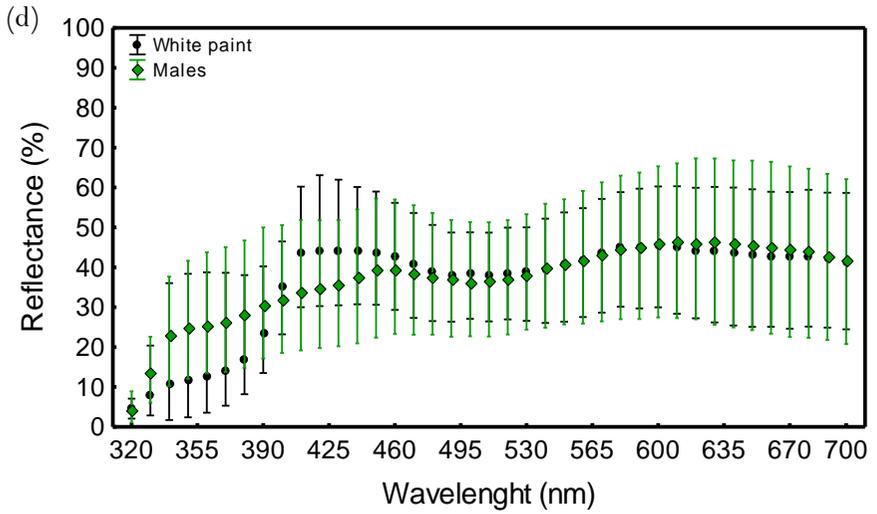
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SUPPLEMENTARY MATERIAL

Figure S1. Mean, maximum and minimum reflectance spectra of (a) red paint ($N = 12$) and natural red colouration in some of the reddest juvenile spiny-footed lizards (from several populations, $N = 12$), (b) red paint and natural red colouration in juveniles used in the natural colour trials ($N = 78$), (c) red paint and natural red colouration in receptive females used in painted or natural colour trials ($N = 20$), (d) white paint ($N = 12$) and natural white colouration in adult males from the studied population ($N = 5$), and (e) white paint and natural white colouration in gravid females from the studied population ($N = 20$). See Colour Measurements and the legend of Figure 1 for further details.









Chapter 5

**MALE MATE CHOICE BASED ON
FEMALE COLOURATION IN THE
SPINY-FOOTED LIZARD**

Este capítulo reproduce el texto del siguiente manuscrito:

This chapter reproduces the text of the following manuscript:

Belliure J., Cuervo J. J. and Fresnillo B. Male mate choice based on female coloration in the spiny-footed lizard (In preparation).

LA SELECCIÓN DE PAREJA POR PARTE DE LOS MACHOS SE BASA EN LA COLORACIÓN DE LAS HEMBRAS EN LA LAGARTIJA COLIRROJA

Resumen

La coloración conspicua está normalmente relacionada con procesos de selección sexual en vertebrados, y cuando son las hembras las que la presentan, suele influir en la selección de pareja por parte de los machos. En este estudio evaluamos el papel de la coloración de las hembras de lagartija colirroja (*Acanthodactylus erythrurus*) en la selección de pareja por parte de los machos, ya que las hembras de esta especie presentan coloración roja en las patas traseras y en la cola al inicio de la edad adulta, que se convierte en blanca más avanzada la temporada. Las preferencias de cortejo de los machos se investigaron desde un enfoque experimental en el cual les ofrecíamos pares de hembras que diferían en su edad/tamaño/madurez sexual (adultas y juveniles) y en la coloración de la cola (roja o blanca). Los machos prefirieron cortejar a las hembras adultas (usando tanto señales visuales como químicas), y cuando la coloración de las hembras adultas variaba, prefirieron a las hembras que mostraban coloración roja. Estos resultados sugieren que la coloración forma parte de la estimación que realizan los machos del valor reproductivo de la hembra. Dado que la coloración roja aparece en todos los juveniles de la especie y también en hembras adultas hasta la ovulación, sugerimos que la coloración roja al principio de la edad adulta en las hembras podría ser un indicador de fertilidad, así como de una baja probabilidad de encuentros anteriores con otros machos (es decir, de una

baja competencia espermática). Los machos obtendrían beneficios al aparearse con estas hembras debido a la mayor probabilidad de que sean fértiles y de que el macho sea el progenitor de su descendencia.

PALABRAS CLAVE: *Acanthodactylus erythrurus*, coloración de las hembras, olor de las hembras, señalización de fertilidad, selección de pareja por parte del macho, reptiles, selección sexual, lagartija colirroja

MALE MATE CHOICE BASED ON FEMALE COLOURATION IN THE SPINY FOOTED LIZARD

Abstract

Conspicuous colouration in vertebrates is frequently involved in mate selection processes, and when it is present in females, it may influence male mate decisions. In this study, we evaluated the role of female colouration on male mate choice in the spiny-footed lizard (*Acanthodactylus erythrurus*), a species in which females show red colouration in the tail and hind legs at the beginning of their adulthood, that becomes white later on in the season. Male courtship preferences were investigated through an experimental approach, where they were offered pairs of females that differed in age/size/maturity (adult and juvenile) and tail colouration (red and white). Male lizards preferred adult females for courtship (using both visual and chemical cues), and when adult female colouration could be chosen, they preferred females showing red colouration. These results suggest that red colouration in adult female spiny-footed lizards is a sexual ornament that is involved in male mate selection. Given that red colouration is present in all juveniles in this species, and that it is present in adult females till ovulation, we hypothesize that red colouration at the beginning of female adulthood might be an indicator of sexual receptiveness, but we cannot rule out the possibility that it is also indicating reduced sperm competition risk. Males would benefit from mating with these females because they would increase their probability of fertilizing the females and siring their offspring, thus increasing the reproductive success. Females might also

benefit from exhibiting the red colouration if it favoured mating with higher quality males or reproducing earlier. Our results also suggest that males can discriminate, both visually and chemically, between adult and juvenile females, preferring always the former regardless of their colour. This preference might be due to the uncertainty about the capacity of juvenile females to be fertilized, but also to the higher fecundity of larger females.

KEY WORDS: *Acanthodactylus erythrurus*, female colouration, fertility signalling, male mate choice, reptiles, sexual selection, spiny-footed lizard

Introduction

Colouration is a type of visual signal commonly used by many animal taxa, and understanding its meaning and functionality constitutes a primary focus in evolutionary and behavioural ecology. One of the functions of animal colouration most commonly addressed has been the role as sexual ornaments involved in mate choice (Andersson 1994; Espmark, Amundsen & Rosenqvist 2000; Waitt *et al.* 2003; Kemp & Rutowski 2011; Hutter, Zala & Penn 2011; Baldwin & Johnsen 2012; Richards-Zawacki, Wang & Summers 2012; Flamarique *et al.* 2013). The most frequent mate choice strategy is quality-based discrimination, in which individuals increase reproductive success by choosing a mate that would produce more and/or higher-quality offspring. In this context, colouration has been often associated with mate quality in a wide number of species, from invertebrates to mammals (Weiss 2006; Kemp and Rutowski 2011; Olsson, Andersson & Wapstra 2011; Rutowski, Nahm & Macedonia 2010).

In species with conventional sex roles, conspicuous colouration is mainly present in males, and mate selection is associated with females. This is because females typically have a larger a priori investment in reproduction, and, therefore, selection has favoured careful mate choice by females (Trivers 1972). Males with the most conspicuous colouration have been demonstrated to be high quality individuals actively selected by females in many species, and, as a consequence, male colouration may experience direct selection. Nevertheless, conspicuous colouration has

also been found in females of species with conventional sex roles (Montgomerie & Thornhill 1989; Cooper & Greenberg 1992; Watkins 1997; Nunn 1999; Cuadrado 2000; Amundsen & Forsgren 2001; Baird 2004; Heinsohn, Legge & Endler 2005; Weiss 2006; Chan, Stuart-Fox & Jessop 2009; Stuart-Fox & Goode 2014), but the significance of colour in females has been less commonly addressed relative to the attention given to males.

Although mate choice is traditionally associated with female-controlled mating systems, males should also have mating preferences whenever the reproductive benefits outweigh the costs of being choosy (Trivers 1972; Parker 1983). For example, if males contribute significant parental care, or are limited in the number and quality of females they can fertilize, selection may favour careful mate choice by males (Dewsbury 1982; Sargent, Gross & Van Den Berghe 1986; Schwagmeyer & Parker 1990; Olsson 1993). Male mating preferences have been observed in some taxa, such as birds (Jones & Hunter 1993; Amundsen 2000), fish (Amundsen & Forsgren 2001) or insects (Bonduriansky 2001), and the most commonly observed male mating preferences are those that tend to maximize a male's expected fertilization success from each mating. In spite of this, even in circumstances where males can receive direct benefits from mate choice, male preferences are unlikely to evolve without a cost (Bonduriansky 2001; Kokko & Monaghan 2001). If a male could mate without any cost at all, he should never reject a female even if she provided very low fecundity benefits, because mating with this female would not imply a trade-off with future mating. Examples of

mating costs include the so-called “opportunity costs” of mating with one female over another of potentially higher quality (Kokko & Monaghan 2001), or the physiological cost of sperm production, which may limit the number of females that a male can successfully inseminate over a set period of time (Reinhold, Kurtz & Engqvist 2002). Despite the intuitive idea that most males should practice some type of mate choice, testing of the phenomenon across taxa and mating systems has been limited compared to the voluminous literature devoted to mate selection by females.

As in the case of females, male mate choice is expected to depend on male's ability to assess female quality based on certain traits, for example female ornaments, elucidating how honest these traits are in indicating female quality (Bonduriansky 2001). Indeed, although female ornaments such as conspicuous colouration may be the result of genetic correlation with male ornaments (Muma & Weatherhead 1989), direct selection on female colouration may occur (Amundsen, Forsgren & Hansen 1997; Burns 1998; Amundsen & Forsgren 2001). One of the hypothesis for direct selection is that female colouration can be a sexual ornament signalling phenotypic and/or genotypic quality to males (Bonduriansky 2001; Weiss 2006; Weiss, Kennedy & Bernhard 2009; South & Arnqvist 2011; Weiss *et al.* 2011). Although there is growing evidence that males prefer to mate with ornamented females, it has been suggested that the production of costly ornaments may reduce female fecundity, hence favouring males with a preference for females with average ornamentation (Fitzpatrick, Berglund & Rosenqvist 1995;

Kokko 1998). Therefore, the association of female ornaments such as conspicuous colouration with male mate decisions remains poorly understood.

Species in which conspicuous colouration is restricted to females provide perfect study cases to investigate female colouration and male mate selection, as genetic correlation between sexes can be discarded and female colouration may be the result of direct selection by male choice. One species in which adult females show bright colours while adult males do not is the spiny-footed lizard (*Acanthodactylus erythrurus*, Schinz 1833). Adult females show red colouration on the ventrolateral part of the tail and the rear part of the hind legs that is absent in adult males. This red colour, particularly on legs, is conspicuous when the lizard is looked at from behind. Information on factors affecting adult female colouration in this species is scarce, but we know that red colouration is present when adult females emerge after winter hibernation, increases in intensity at the beginning of the reproductive season, possibly reaching a peak around ovulation, and fades to become pallid yellow, nearly white, when they are gravid (Cuervo & Belliure 2013). This pattern of seasonal colour change suggests that red colouration in females of this species might have a mating-related function (Cuervo & Belliure 2013), but this hypothesis has not been tested so far.

Our aim in this study was to evaluate the role of red female colouration on male mate selection in the spiny-footed lizard. The red colour does not appear *de novo* in adult females of this species, because it is also present in juveniles of both sexes, but only adult females maintain

the trait during adulthood. The influence of female colouration on male mate choice could shed some light on this sexual dichromatism. We performed an experiment in captivity to investigate the effect of female colouration on male mate preference. The mating system of the species is unknown, but promiscuity and multiple mating has been suggested for most lacertid species (Olsson & Madsen 2001; Laloi *et al.* 2004; Salvador *et al.* 2008). If male mate choice based on female colouration occurred in the spiny-footed lizard, it would be discussed in the light of fertilization and paternity probabilities.

Materials and methods

Study species

Spiny-footed lizards are medium-sized lacertids (snout-vent length (SVL) up to around 8 cm) living in open habitats of Northern Africa and the Iberian Peninsula (Seva Román 1982; Carretero & Llorente 1995; Pérez-Mellado 1998). The species reaches sexual maturity at the age of one year and a half, during their second spring (Belluire 2009). In central Spain, sexual maturity is reached when males are 5.8-6.5 cm SVL and females 6.0-6.6 cm SVL (Bauwens & Díaz-Uriarte 1997), and the mating period occurs around June (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992). Juveniles develop red colouration on the rear part of their hind limbs and the ventrolateral part of their tails (Seva Román 1982; Carretero & Llorente 1995). Juvenile males lose the red colour and show white colouration when they

are adults, but juvenile females retain the red colour on these body parts through adulthood (Seva Román 1982; Belliure 2009; Cuervo & Belliure 2013). When females are gravid, they lose their red colouration that becomes pallid yellow, nearly white (Cuervo & Belliure 2013). Average clutch size is 4.4 eggs (range 1-8; Belliure 2009), and in some populations females seem to be able to lay two clutches in the same season (Escarré & Vericad 1981; Seva Román 1982; Barbadillo, Castilla & Borreguero 1987; Castilla, Barbadillo & Bauwens 1992; Carretero & Llorente 1995).

Captures and captivity conditions

A total of 46 lizards (16 males and 30 females) were captured by noosing between May and June 2009 in Chapinería, south-western Madrid Region, central Spain (N40°22'; W4°13'). Lizards were carried to the Alcalá University Animal Research Centre, Madrid, where they were weighed (with an electronic balance to the nearest 0.1 g) and measured (with a ruler to the nearest 0.1 cm). All males were adult (mean SVL \pm SE = 7.96 \pm 0.06 cm; range = 7.60 – 8.40 cm, $N = 16$), and from the 30 females, 16 were adult (7.86 \pm 0.08 cm; range = 7.20 – 8.60 cm) and 14 juvenile (6.49 \pm 0.11 cm; range = 5.90 – 7.00 cm). According to their SVL, juvenile females could have reached sexual maturity (Bauwens & Díaz-Uriarte 1997). At the time of capture, all juvenile females showed their characteristic red colouration in legs and tail, while all adult females had already lost their red colouration and showed the nearly white colour already described for the species (Cuervo & Belliure 2013).

Lizards were placed in individual terrariums (42 x 26 x 19 cm) with a thin layer of sand on the bottom and available shelter, and maintained with a light cycle of 12:12 h light:dark. Room temperature was 25°C and a bulb hanging over the edge of each individual terrarium provided a temperature gradient for thermoregulation. Lizards were supplied with food (*Tenebrio molitor*) dusted with vitamins once a day and water ad libitum, and were kept in their individual terrariums at least three days before trials for acclimatization.

Mate choice experimental design

Male mate preference was studied using dichotomous choice trials, where focal males were given a choice between two stimuli (Ritchie 1996; Fresnillo & Belliure 2011) in a series of different experiments. Some experiments aimed to simulate real mating options that males may experience under natural conditions, but experimental manipulations allowed us to create females with characteristics that are not found in nature (for example juvenile females without red colouration, see below). As we were interested in the role of female colouration in male mate choice, the first pair of stimuli should have been a white adult female and a red adult female. However, as the two colours correspond to different phases of the reproductive cycle and seasonal colour change from red to white occurs almost simultaneously in all females (Cuervo & Belliure 2013), we were not able to find both types of adult females, but only white ones. Therefore, the only naturally red females in this study were juveniles, and the first pair of stimuli had to

be a naturally white adult female and a naturally red juvenile female. It might be argued that males cannot be interested in juvenile females unless they are sexually receptive, but, according to their body size, all females in the study, including juveniles, were potentially sexually mature (Bauwens & Díaz-Uriarte 1997). An obvious inconvenience of these pairs of females is that they do not only differ in colouration, but also in age, body size, and possibly sexual maturity (adults are older, larger, and possibly more sexually mature than juveniles).

The only way we found to compare white and red adult females was to manipulate experimentally their colour with paint. In this way, the second pair of stimuli was an adult female painted white on the rear part of the hind limbs and the ventrolateral part of the tail and an adult female painted red on the same body parts. The former simulate an adult female already gravid and the latter simulate a sexually receptive adult female. Moreover, using experimental painting, we could also disentangle the natural colour/size association (i.e., white/adult/large and red/juvenile/small) of our first pair of stimuli. Thus, the third pair of stimuli was an adult (i.e. large) female painted red on the rear part of the hind limbs and the ventrolateral part of the tail and a juvenile (i.e. small) female painted white on the same body parts. The former simulate a sexually receptive adult female and the latter has a combination of characteristics that does not exist in nature, because juvenile females are always red.

Although we were mainly interested in the function of female colouration in male mate choice, chemical cues also play an important role in intraspecific communication of lizards (Mason 1992; Cooper 1994), and pheromones are involved in eliciting male courtship in some species (López & Martín 2001), and that femoral secretions allow discrimination between adult and juvenile individuals in the spiny-footed lizard (López & Martín 2005), although female secretions have not been studied yet. Therefore, we also considered olfactory (chemical) female cues (odours) and not only visual female cues (colours) in male mate choice. Thus, the fourth pair of stimuli was odour from an adult female and odour from a juvenile female.

To summarize, the experimental design included (A) three experiments with pairs of females that differed in colouration: naturally white adult female versus naturally red juvenile female (Experiment 1), adult female painted white versus adult female painted red (Experiment 2), and adult female painted red versus juvenile female painted white (Experiment 3); and (B) one experiment with pairs of chemical stimuli: adult female odour versus juvenile female odour (Experiment 4).

Mate choice trials

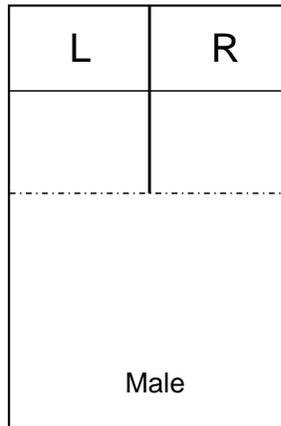
Trials were conducted in an experimental terrarium (77 x 55 x 43 cm, Figure 1). Both options for selection were offered at one end of the terrarium, one at the right side and the other at the left side (R and L areas in Figure 1), separated by an opaque cardboard wall. The focal male was introduced at the other end of the terrarium, at a distance of

approximately 50 cm from both options, which were simultaneously visible for the male from this position (Figure 1). In Experiments 1, 2 and 3, both trial females were placed into identical transparent plastic compartments (20 x 12 x 14 cm), at the R and L areas of the experimental terrarium, and an opaque cardboard wall prevented visual contact between them and defined a “selection area” of 15 cm, where the presence of the male was interpreted as a willingness to mate with that particular female (Figure 1). The transparent walls of the plastic compartments prevented any physical contact between females or between the females and the male. In Experiment 4, the transparent plastic compartments were removed and two blotting paper pieces (16 x 10 cm) containing odour (secretions) from an adult and a juvenile female were placed at both R and L areas of the experimental terrarium separated by the opaque cardboard wall. In this experiment, physical contact between the male lizard and the stimulus (the piece of paper) was possible. Males freely moved, explored, and approached both stimuli during 45 minutes, and selection was defined as the amount of time males spent in contact or close (in the selection area) to each option.

Two trials were performed per male in every experiment in order to offer each stimulus at both right and left sides of the terrarium, thus controlling any male preference for one side of the terrarium independent of the stimulus offered. Disposition of stimuli at both sides of the terrarium in the first of both trials alternated between males. Results of both trials per male and experiment were averaged for subsequent analyses. Male behaviour was videotaped (JVC GZ-MG680

camera), so there was no human disturbance during the trial. All males spent a variable time (from 2 to 10 minutes) motionless at the beginning of the trial, time that was discarded for the analyses.

Figure 1. Experimental terrarium for male mate choice trials. Focal male was offered two options, one at the right (R) and one at the left (L) side, separated by an opaque wall, that defined a “selection area” of 15 cm (dotted line).



Trials were performed from 10 to 17 h local time, within the species activity period (Busack 1976; Belliure 2009). Temperature at the laboratory during the trials was always around 25°C, but a bulb hanging over the trial terrarium provided light and heat in the middle of the terrarium, allowing a temperature of around 32°C at the trial terrarium, within the activity range for the species (Belliure, Carrascal & Díaz 1996). No lizard was involved in more than one trial per day, and each

pair of stimuli was used only once in the study. Trials were discarded if the male did not move or did not visit each of both options at least once.

Females used in Experiment 1 showed their natural colouration, but females used in Experiments 2 and 3 were experimentally painted with nontoxic paint (Satin Paint, La Pajarita, Manises, Spain) to mimic either regular white or the reddest natural colouration showed by the species. The most extreme red colouration was chosen on purpose to make the two females of each pair in Experiments 2 and 3 as different as possible in colouration. In any case, we checked that experimental white and red colouration were within the natural colour range showed by the species (B. Fresnillo, J. Belliure and J.J Cuervo unpublished data; Chapter 4 of this thesis).

Statistical Analyses

A total of 128 trials were recorded, 32 (two per male) per experiment, but 24 trials were discarded because the male did not move or did not visit each of both options at least once, or because a female escaped from its compartment during the trial. Therefore, the trials used in the study were 32 for Experiment 1, 20 for Experiment 2, 22 for Experiment 3, and 30 for Experiment 4, and after averaging results from both trials of the same male and experiment, samples sizes were 16, 10, 11 and 15, respectively. For all experiments, male choosiness was assessed as time in seconds (out of the first 30 minutes of valid time in each video) spent (a) in contact with the stimulus (the piece of paper in Experiment 4 or the transparent wall of the plastic compartment in

Experiments 1, 2 and 3), and (b) close (< 15 cm) to the stimulus. We chose two ways of assessing male choosiness because we did not know a priori how males would behave when choosing an option. Having contact with a stimulus might imply a stronger interest for that stimulus than simply staying close to it, although we assume that staying close also imply a choice.

Paired t-tests were used to analyse within-males differences in the time spent in contact or close to each stimulus of the pair in each experiment. Time was \log_{10+1} -transformed to approach normality. All statistical analyses were carried out using STATISTICA v. 8.0. (StatSoft Inc. 2007).

Results

Results from Experiment 1 showed that males spent a similar amount of time close to adult and juvenile females with natural colours ($t_{15} = 0.95$, $p = 0.359$), but preferred to spend more time in contact with the adult female ($t_{15} = 2.13$, $p = 0.0497$; Figure 2). A similar pattern was obtained when odours of adult and juvenile females were offered to males in Experiment 4: males spent a similar amount of time close to adult and juvenile female odours ($t_{14} = 1.61$, $p = 0.129$), but preferred to spend more time in contact with the adult female odour ($t_{14} = 2.41$, $p = 0.030$; Figure 3).

Figure 2. Paired results for time spent by males in contact with the transparent wall of compartments containing adult and juvenile females.

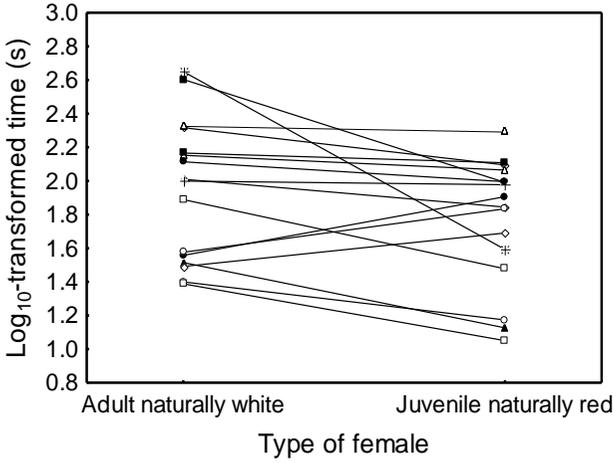
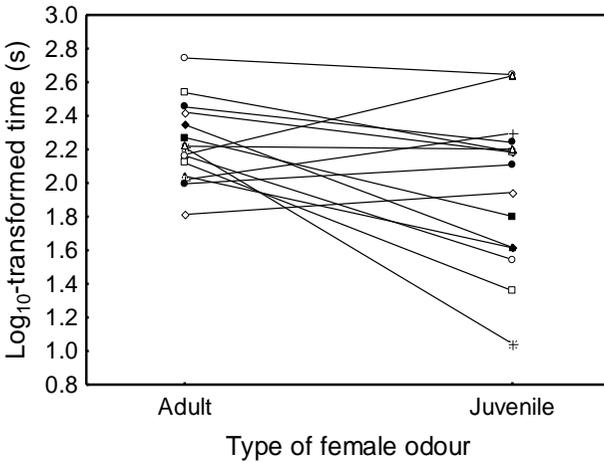


Figure 3. Paired results for time spent by males in contact with the piece of paper containing odour of adult and juvenile females.



When both adult females differed in their colouration (experimentally white or red, Experiment 2), males spent more time close to red adult females ($t_9 = -2.65$, $p = 0.027$; Figure 4), although the time in contact with both females did not differ significantly ($t_9 = -0.71$, $p = 0.498$). When colouration was experimentally reversed in adult and juvenile females, males spent more time close to red adult than to white juvenile females (Experiment 4; $t_{10} = -3.07$, $p = 0.012$; Figure 5), although the time in contact with both females did not differ significantly ($t_{10} = -0.19$, $p = 0.855$).

Figure 4. Paired results for time spent by males in the selection area of red and white painted adult females.

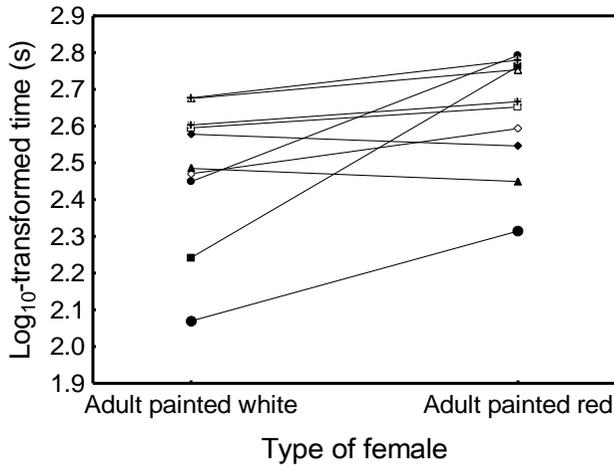
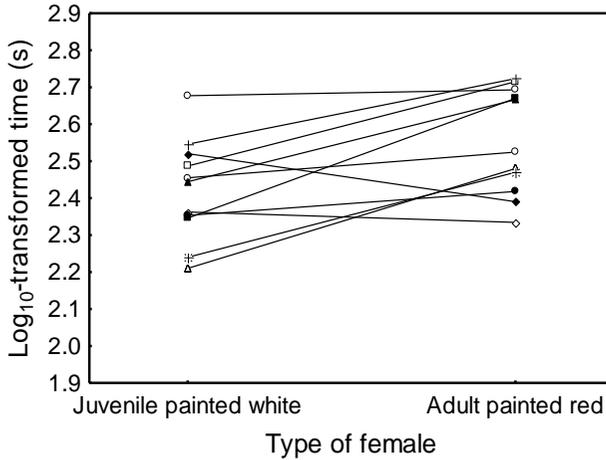


Figure 5. Paired results for time spent by males in the selection area of reversed adult and juvenile red and white painted females



Discussion

The results of this study show that male spiny-footed lizards discriminated between two different female stimuli and chose one of them. The choice was expressed by differences in the amount of time they spent in the proximity of each female stimulus. Such behaviour has been associated to courtship preferences, i.e., to male mate choice, in similar studies (Hoysak & Godin 2007; Jeswiet & Godin 2011). Our aim was to test whether red colouration in adult females was an attractive trait for males, thus providing insights into the possible function of this trait. Through a sequence of four experiments in which males were offered four different pairs of female stimuli, we found that male mate

choice exists in the spiny-footed lizard, and that at least two female traits influence male mate preferences in this species: age/size/maturity and colouration. Males of the spiny-footed lizard preferred adult females to juvenile ones, using both visual and chemical cues, and when both females were adults (i.e., both with similar age/size/maturity), they preferred red to white females. Although mate choice has been traditionally associated with female-controlled mating systems, it is becoming increasingly clear that males should also show mating preferences when the benefits of being choosy outweigh the costs (Dewsbury 1982; Hoysak & Godin 2007; Härdling, Gosden & Aguilé 2008), but also that mating-related costs are necessary for male mate choice to evolve (Bonduriansky 2001; South, Steiner & Arnqvist 2009). In the following sections we will discuss (1) the possible factors determining the existence of male mate choice in the spiny-footed lizard, (2) the role of female colouration in male assessment of female reproductive value, and (3) the possible benefits of red colouration for females.

Male mate choice and possible factors driving male mate selection

Since females are generally a limiting resource for males (Bateman 1948), it is not obvious why males should exhibit mate choice. Males in conventional mating systems are expected to mate indiscriminately to maximize the number of fertilizations they obtain and thus their reproductive success (Anderson 1994; Bonduriansky

2001). Therefore, the existence of male mate choice is better understood when mating involves costs for males, and males may discriminate between potential mates by preferring the ones that provide the largest increase in male fitness (Admunsen 2000; Bonduriansky 2001; Parker & Pizzari 2010). Recent theoretical models predict that male mate choice can be selected for when (1) there is substantial male effort in terms of searching, courtship, mating, or mate guarding (Pomiankowski 1987); (2) females are scarce due to a biased operational sex ratio (Van Den Berghe & Warner 1989); (3) individual quality varies among females (Johnstone, Reynolds & Deutsch 1996); (4) males invest in parental care (Sargent, Gross & Van Den Berghe 1986); and (5) male fertilization success varies among females (Nakatsuru & Kramer 1982; Verrell 1985; Bonduriansky 2001; Edward & Chapman 2011).

Although most of these aspects have not been studied in the spiny-footed lizard, we know that mating can be costly for male lizards. For example, spermatogenesis and maintenance of gonadal tissue is an energetically demanding process (Kenagy & Trombulak 1986; Gage 1991; Simmons *et al.* 1993). Therefore, producing ejaculates is not a cheap activity for males (Dewsbury 1982; Simmons & Kotiaho 2002), and evidences have been provided for reptiles (Olsson *et al.* 2007). Moreover, courtship is also costly, because it requires considerable time and energy (e.g., Shine & Mason 2005; Sozou & Seymour 2005) and exposes males to higher predation risk (e.g., Daly 1978; Pruden & Uetz 2004). In the case of our study species, we can assume that these male mating costs are probable. On the other hand, although the reproductive system of the

species is unknown, promiscuity and multiple mating has been found in most lacertids studied so far (Olsson & Madsen 2001; Laloï *et al.* 2004; Salvador *et al.* 2008), so the risk of sperm competition may be also present in our species. Probable male mating costs and sperm competition in the spiny-footed lizard would both promote male mate choice according to theoretical models (see above), and indeed our study corroborated the existence of male mate choice in this species. Males would benefit from female phenotypes associated with fertility and reduced sperm competition, as observed in other species (Wedell, Gage & Parker 2002; Friberg 2006; Cornwallis & Birkhead 2007).

The reproductive value of female colouration

Male spiny-footed lizards preferred adult to juvenile females regardless of female colouration, as supported by results of Experiments 1, 3, and 4. This preference might be due simply to the uncertainty about the sexual maturity of juvenile females and thus their probability of being fertilized. However, adult females are also larger than juvenile ones, so this preference agrees with the fact that female size may determine fecundity in lizards (Andrews 1985; Qualls & Shine 1995; Qualls & Andrews 1999; Bonduriansky 2001; Rhainds 2010), and thus influence male mate choice (McLain & Boromisa 1987; Verrell 1985; Olsson 1993). But when males were offered adult females showing white or red colouration, they selected red adult females, therefore suggesting that female colouration adds relevant information about sexually mature females to males, and that this information is relevant for mate selection.

As mentioned above, male spiny-footed lizards may exert mate choice in a context of ensuring paternity. Two aspects may affect the probability of paternity when males mate with adult females: (i) fertility conditions of females according to the specific moment of their reproductive cycle, and (ii) risk of sperm competition according to previous encounters of females with other males. Females are fertile around or before ovulation, because following ovulation, the eggs cannot be fertilized (Whittier & Tokarz 1992), but paternity is also influenced by sperm competition and female cryptic selection (Parker & Pizzari 2010; Wedell, Gage & Parker 2002), so males will be favoured by mates in which the probability of sperm competition is low. Therefore, male spiny-footed lizards should have the ability to assess in adult females both fertility condition and probability of previous encounters with other males. Is there any phenotypic trait in adult female spiny-footed lizards that could advertise both aspects? As it was mentioned above, red colouration of adult females does not appear *de novo* because it is also present in hatchlings and juveniles of both sexes. Adult females maintain red colouration through adulthood, when it is present at the beginning of the reproductive season and increases around ovulation (Cuervo & Belliure 2013). Thus, red colour in adult females might function as a sexual ornament signalling that they are in the fertile period, when probability of fertilization after mating is high. In contrast, when red colouration fades and becomes white, females are probably already gravid, when fertilization is no longer possible (Whittier & Tokarz 1992), and mating would imply costs of sperm depletion to males. Indeed, sperm depletion and limited ejaculatory capacity of males may

exert a relevant influence in male mate choice (Dewsbury 1982). Regarding the probability of previous encounters of the female with other males, it will be lower early than late in the season. As adult females show red colour early in the reproductive cycle and white colour late in the reproductive cycle, red colour is indicating that females are in the early phases of their cycle and thus with less probabilities of having met other males than when they show white colour. Although we think that the intensity of the red colouration is indicating mainly the fertility status of the female, we cannot rule out the possibility that it is also indicating to some degree the probability of previous encounters with other males, i.e., of sperm competition.

Another relevant aspect of ornaments that may influence mate preference is the cost of production and maintenance of the signal. It has been suggested that the production and maintenance of costly ornaments may reduce female fecundity, hence favouring males with a preference for females with average ornamentation (Fitzpatrick, Berglund & Rosenqvist 1995; Kokko 1998). The nature of red colouration in adult female spiny-footed lizards a priori excludes high costs related to the signal production, as the trait is already present in juvenile females. Costs related to maintenance or to the increment of colouration around ovulation are unknown, but probably lower than the costs of producing a new signal. In case that female fecundity is reduced to some extent, disadvantages for males would be probably outweighed by the benefits of an ornament that indicates fertility and low probability of sperm competition. This reasoning is valid at least at the beginning of female

adulthood, i.e., in the first reproductive event. Whether females change from white to red colouration during a second ovulation, either in the same or in the following year, has not been clarified for the species yet. The development of red colouration in a second ovulation during the same reproductive season would imply that the putative function of colouration indicating low sperm competition risk is not possible in this second reproductive event, and red colouration would signal female fertility exclusively.

Possible benefits of male mate selection for females

From a female's perspective, red colouration should have evolved and been maintained only if this signal provides benefits that outweigh the probable costs of the signal (Maynard Smith & Harper 2003). Would female spiny-footed lizards benefit from signalling fertility and low probability of previous encounters with males? We know that females can increase their reproductive success by producing more and/or higher-quality offspring, and signalling fertility might provide benefits in both aspects. First, an attractive trait like red colouration might make that females mate with more males, and this can increase the number of offspring, for example by a higher fertility insurance (Krokene *et al.* 1998; Byrne & Whiting 2008), but also offspring quality, as high competition among males might allow the female to mate with (and/or choose sperm from) the highest quality male. Second, showing fertility as soon as possible after hibernation implies higher probability of laying eggs early in the season, and this can again increase the number of

offspring, for example increasing the probability of a second clutch, but also offspring quality, as offspring from early clutches tend to be larger and have higher survival rates (Olsson & Shine 1997). And third, signalling fertility with certain colouration implies that a different colouration will signal that the female is not fertile, and the colouration signalling the non-fertile period may have a courtship rejection function (Cuadrado 2000, Hager 2001), preventing male aggression related to courtship and copulation.

Conclusions

The results of this study suggest that red colouration in adult female spiny-footed lizards is a sexual ornament that is involved in male mate selection. Males prefer to mate with adult females showing red instead of white colouration. Red colouration is probably indicating sexual receptiveness, but we cannot rule out the possibility that it is also indicating reduced sperm competition risk. Male preference to mate with females showing red colouration would be beneficial for males, because they would increase their probability of fertilizing the females and siring their offspring, thus increasing the reproductive success. Females might also benefit from exhibiting the red colouration if it favoured mating with higher quality males or reproducing earlier. Our results also suggest that males can discriminate, both visually and chemically, between adult and juvenile females, preferring always the former regardless of their colour. This preference might be due to the

uncertainty about the capacity of juvenile females to be fertilized, but also to the higher fecundity of larger females.

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Chapter 6

**HORMONAL CONTROL OF
SEASONAL COLOUR CHANGE IN
FEMALE SPINY-FOOTED LIZARDS
(*ACANTHODACTYLUS*
ERYTHRURUS)**

Este capítulo reproduce el texto del siguiente manuscrito:
This chapter reproduces the text of the following manuscript:

Fresnillo B., Belliure J. and Cuervo J. J. Hormonal control of seasonal colour change in female spiny-footed lizards (*Acanthodactylus erythrurus*) (Submitted to Journal of Animal Ecology).

CONTROL HORMONAL DEL CAMBIO DE COLORACIÓN ESTACIONAL EN HEMBRAS DE LA LAGARTIJA COLIRROJA (*ACANTHODACTYLUS ERYTHRURUS*)

Resumen

La coloración en las hembras se expresa generalmente de forma estacional y normalmente señala de alguna forma el estado reproductivo de la hembra, lo cual sugiere la existencia de una relación entre color y hormonas sexuales. En este estudio examinamos las relaciones entre dos hormonas sexuales (progesterona y β -estradiol) y la coloración durante el periodo reproductivo en hembras de lagartija colirroja (*Acanthodactylus erythrurus*) mediante una aproximación observacional y otra experimental. En primer lugar exploramos la variación natural tanto en las concentraciones plasmáticas de las hormonas como en la coloración de las hembras a lo largo del ciclo reproductivo. Después de identificar las asociaciones clave, manipulamos las concentraciones hormonales en condiciones de cautividad en tres grupos experimentales de hembras (tratadas con β -estradiol, tratadas con progesterona, y control) y monitorizamos los posibles efectos en la coloración. La variación temporal en los niveles hormonales durante la época reproductora sugiere la existencia de dos periodos ovulatorios para algunas hembras de esta población. Encontramos asociaciones negativas entre el β -estradiol y el brillo y entre la progesterona y el chroma del rojo durante la ovulación temprana, cuando todas las hembras adultas eran sexualmente activas, pero esas asociaciones no fueron significativas

durante la ovulación tardía, cuando tanto hembras sexualmente activas como inactivas estaban presentes en el campo. Las hembras tratadas con β -estradiol, que experimentaron un incremento tanto en β -estradiol como en progesterona, perdieron su coloración rojiza antes que las hembras de los otros dos grupos experimentales, pero el tratamiento con progesterona no tuvo efectos sobre la coloración de las hembras. Nuestros resultados sugieren que los niveles altos bien únicamente de β -estradiol, bien de β -estradiol combinado con progesterona, disparan la pérdida de la coloración rojiza en las hembras de lagartija colirroja, y que la progesterona por sí misma no afecta a la coloración. Nosotros proponemos la hipótesis de que son hormonas diferentes las que controlan los cambios de coloración de las hembras durante la época reproductora, dependiendo de la fase del ciclo reproductivo cuando esa coloración se expresa: la progesterona controlaría la coloración que se expresa tras la ovulación y el β -estradiol controlaría la coloración que se expresa antes de la ovulación.

PALABRAS CLAVE: aplicación hormonal transdermal, coloración sexual, espectrofotometría, inducción hormonal del cambio de color, radioinmunoensayo, reptil, señalización con color

HORMONAL CONTROL OF SEASONAL COLOUR CHANGE IN FEMALE SPINY-FOOTED LIZARDS (*ACANTHODACTYLUS ERYTHRURUS*)

Abstract

Female colouration is frequently expressed seasonally and often signals some aspects of female reproductive status, suggesting a link between colour and sex steroid hormones. In the present study, we examine the relationships between two steroid hormones (progesterone and β -estradiol) and reproductive colouration in female spiny-footed lizards (*Acanthodactylus erythrurus*) through observational and experimental approaches. We first explored natural variation of plasma steroid hormone levels and colouration of females during the reproductive cycle. After identification of key relationships, plasma hormone concentrations were manipulated under captive conditions by creating three experimental female groups (β -estradiol treated, progesterone treated, and control), and the possible effects on colouration were monitored. Temporal variation in hormone levels during the reproductive season suggests the presence of two ovulation periods for some females in this population. We found negative relationships between β -estradiol and brightness and between progesterone and red chroma during the early ovulation period, when all adult females were sexually active, but these relationships were no longer significant during the late ovulation period, when both sexually active and inactive females were present in the field. β -estradiol treated

females, which experienced a rise in both β -estradiol and progesterone levels, lost their red colouration earlier than females of the other two experimental groups, but progesterone treatment had no significant effect on female colouration. Our results suggest that high levels of either β -estradiol alone or β -estradiol together with progesterone trigger the loss of red colouration in female spiny-footed lizards, and that progesterone alone does not affect colouration. We hypothesize that different hormones might regulate female breeding colour change depending on the phase of the reproductive cycle when the conspicuous colouration is expressed: progesterone when colouration is expressed after ovulation and β -estradiol when it is expressed before ovulation.

KEY WORDS: colour signalling, hormone-induced colour change, radioimmunoassay, reptile, sex-specific colouration, spectrophotometry, transdermal hormone application

Introduction

Colouration is an important feature for many aspects of animal life. For example, it may influence the establishment of dominance hierarchies (Gerald 2001; Miyai *et al.* 2011; Tringali & Bowman 2012), be involved in antipredatory mechanisms such as crypsis, aposematism or mimicry (Ruxton, Sherratt & Speed 2004), or affect thermoregulation in poikilotherms (Norris 1967; Hegna *et al.* 2013). However, one of the most studied functions of animal colouration is mate selection (Kemp & Rutowski 2011; Baldwin & Johnsen 2012; Richards-Zawacki, Wang & Summers 2012; Flamarique *et al.* 2013). In species showing sexual dichromatism, the sex having lower parental investment (commonly the male) is usually more conspicuously coloured, and sexual selection has been invoked to explain this phenomenon (Andersson 1994). But female conspicuous colouration, which is relatively common in some taxa (e.g. reptiles; Cooper & Greenberg 1992), has been paid little attention, partly because it was traditionally understood as the consequence of a genetic correlation with the male trait (e.g. Muma & Weatherhead 1989). However, female conspicuous colouration can evolve independently of male colour by direct selection on females (Amundsen 2000). Indeed, conspicuous colouration is present only in females of several species that maintain conventional sex roles (e.g. mammals (Nunn 1999), fish (Amundsen & Forsgren 2001), birds (Heinsohn, Legge & Endler 2005), or reptiles (Chan, Stuart-Fox & Jessop 2009).

Female conspicuous colouration is frequently expressed seasonally and is therefore generally considered to signal some aspects of female reproductive status, for example stimulating or rejecting courtship by males (Nunn 1999; Amundsen & Forsgren 2001; Heinsohn, Legge & Endler 2005; Chan, Stuart-Fox & Jessop 2009). The relationship between female colouration and reproductive cycle has led to investigate the link between colouration and sex steroid hormones (hereafter steroid hormones) in mammals (Dixson 1983), fishes (Sköld *et al.* 2008), birds (Grindstaff *et al.* 2012) and reptiles (Cooper & Greenberg 1992). However, studies of hormonal regulation of female colour change are still scarce, in contrast to the relatively well studied effect of testosterone on colouration during the reproductive season in males (Cooper, Mendonca & Vitt 1987; Fargallo *et al.* 2007; Miles *et al.* 2007; Ardia, Broughton & Gleicher 2010). The studies analysing the hormonal regulation of female breeding colouration in birds have mainly focused on testosterone (T), but the relationships between β -estradiol (E_2) or progesterone (P_4) and female breeding colouration have also been studied in other groups and do not seem to be simple. In monkeys, for example, E_2 is closely related to female breeding colouration (Dixson 1983), while in reptiles, P_4 seems to be the hormone most commonly triggering female changes in colouration (Cooper & Greenberg 1992). In fish, however, no significant relationships between steroid hormone levels and female colouration have been found (Sköld *et al.* 2008).

Lizards are good study organisms for understanding the endocrine basis of female-specific colouration because: (1) the

conspicuous colouration of females is common in many taxa (Cooper & Greenberg 1992), (2) the hormonal regulation of their reproductive cycle is relatively well known (Norris & Lopez 2011), and (3) several authors have suggested a link between female colouration, the different reproductive stages and steroid hormones (Cooper & Greenberg 1992; Calisi & Hews 2007; Jessop, Chan & Stuart-Fox 2009). However, experiments investigating the effect of steroid hormones on female breeding colouration are scarce and strongly biased towards lizard families included in the suborder Iguania (e.g. Agamidae, Chrotaphytidae and Phrynosomatidae; Cooper & Greenberg 1992; Calisi & Hews 2007; Jessop, Chan & Stuart-Fox 2009). Consequently, research on other species becomes necessary to achieve a better understanding of the hormonal mechanisms affecting female colouration and to promote an integrative view on the possible role of steroid hormones on the regulation of female breeding colouration.

E_2 and P_4 are considered the most important hormones regulating the reproductive cycle in female lizards (Norris & Lopez 2011). Plasma E_2 concentration reaches a maximum prior to ovulation, while P_4 concentration increases during ovulation (Carnevali *et al.* 1991; Ciarcia, Paolucci & Difiore 1993; Díaz, Alonso-Gómez & Delgado 1994) and maintains high levels until oviposition. The relationship between these steroid hormones and female lizard colouration appears to be complex. P_4 has been suggested to increase conspicuous colouration in females of several species (Cooper & Ferguson 1972a; Cooper & Ferguson 1972b; Medica, Turner & Smith 1973; Cooper & Clarke 1982;

Cooper & Crews 1987). However, the effect of E_2 is not clear. In most cases, a direct effect of E_2 on female colouration has not been found (e.g. Medica, Turner & Smith 1973; Cooper & Clarke 1982), but E_2 can speed up the effect of P_4 on colouration (Cooper & Ferguson 1972a; Cooper & Ferguson 1972b).

In this study, we investigated plasma P_4 and E_2 levels in relation to colour expression in female spiny-footed lizards (*Acanthodactylus erythrurus*, Schinz 1833), a species belonging to the family Lacertidae. Adult females of this species display red colouration at the beginning of the reproductive season, but the red colour gradually changes to yellowish white when they become gravid (Cuervo & Belliure 2013). We first studied temporal changes in colouration (hereafter colour profile) and plasma hormone concentrations (hereafter hormonal profile) in the field along the breeding season, and then tested for possible relationships between colour and hormonal profiles at different stages of the reproductive cycle. Finally, we experimentally manipulated plasma steroid hormone concentrations in captivity to test their effects on colouration.

Materials and methods

Study species

The spiny-footed lizard is medium-sized (snout-vent length (SVL) and total length up to around 80 and 230 mm respectively; Seva Román 1982; Carretero & Llorente 1993), although mean size varies

among populations) that reach sexual maturity during their second spring at around 57-65 mm SVL (Busack & Jaksić 1982; Bauwens & Díaz-Uriarte 1997). The phenology of the reproductive cycle varies among populations, but in central Spain, spiny-footed lizards hibernate during November-March, emerge from hibernation in April, and begin to mate in May, with females laying eggs during June-July and most eggs hatching in late August and September (Pollo & Pérez-Mellado 1990; Castilla, Barbadillo & Bauwens 1992). In southern Spain, reproductive events occur approximately one month earlier (Busack & Jaksić 1982; Seva Román 1982; Cuervo & Belliure 2013). There is no evidence of multiple clutches in populations in southern Spain (Busack & Klosterman 1987), but a second clutch might be possible in central Spain (Castilla, Barbadillo & Bauwens 1992).

Colouration in this species varies both ontogenetically and seasonally. Dorsal pattern changes from strongly-marked dark and light bands in hatchlings to a reticulated pattern in adults (Seva Román 1982). Juveniles of both sexes develop red colouration on the rear part of their hind limbs and the ventrolateral part of their tails (Seva Román 1982; Carretero & Llorente 1993). Juvenile males lose the red colour when they are around one year old, while juvenile females retain it through adulthood (Seva Román 1982). Adult females at the beginning of the reproductive season increase the intensity of the red colouration retained from the juvenile phase until they are gravid, when the red colouration is gradually lost and becomes pallid yellow, nearly white (Cuervo & Belliure 2013). In contrast, adult males show white colouration on the

rear part of their hind limbs and the ventrolateral part of their tails along the whole reproductive season (Seva Román 1982).

Field Study

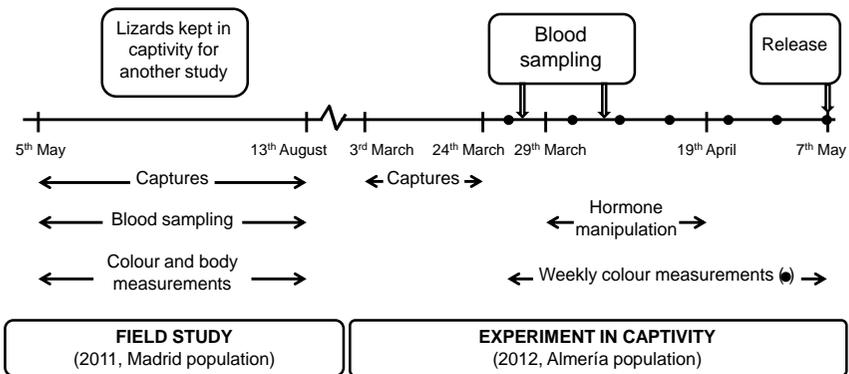
A total of 27 adult females were captured during the breeding season of 2011 in Chapinería, Madrid Region, central Spain (N40°22'; W4°13'). They were captured by noosing at a weekly basis from May to August (Fig. 1), so plasma hormone levels and colouration were sampled across the whole reproductive season. Lizards were placed in individual cloth bags in the shade immediately following capture to prevent overheating. After a maximum of six hours since their capture, they were carried to the lab for immediate blood sampling (see Blood Sampling and Hormone Assays) and colour measurements (see Colour Measurement). Animals were also measured (SVL and total length with a ruler to the nearest 0.1 cm), weighed (with an electronic balance to the nearest 0.1 g) and toe-clipped with a unique code for future individual identification. Lizards were kept in captivity for up to 79 days to be used in another study and then released at the same places where they had been captured.

Experiment in Captivity

A different spiny-footed lizard population was studied in 2012 due to the low population density found in Madrid the previous year. We captured lizards before the mating season (March, Fig. 1) from the

western part of Cabo de Gata Natural Park in Almería, southern Spain (N 36°49'; W 2°18'). A total of 45 females and 45 males were captured, taken to the lab, and measured following the same procedures used in the 2011 field study. Pairs of animals (one female and one male) were placed in outdoor terraria (80 x 60 x 43 cm) at Finca Experimental La Hoya, a facility of the Estación Experimental de Zonas Áridas (CSIC) in Almería. Pairs were kept together to allow mating when females were receptive and thus egg fertilization. Shelter was provided with a brick and two wood panels over a thick layer of sand. Animals were supplied with food (*Tenebrio molitor* or *Acheta domestica*) dusted with vitamins once a day and water *ad libitum*. All lizards were released at the same places where they had been captured after up to 64 days in captivity (Fig. 1).

Fig. 1. Chronogram of field and lab work carried out during 2011 and 2012. Study sites are specified in brackets together with the year. Blood samples in 2012 were taken on 27th March and 8th April. Colour measurement batches (●) were taken weekly from 26th March until 7th May (the other measurement batches were on 2nd, 9th, 16th, 21st and 30th April).



Females were randomly assigned to one of the following experimental groups: E group was treated with E_2 , P group was treated with P_4 , and C (control) group was manipulated but received no hormone treatment. Each experimental group consisted of 15 females, and there were no significant morphological or colorimetric differences among groups before the manipulation (one-way ANOVAs; $F_{2,42} \leq 1.93$, $P \geq 0.158$ for tests on body mass, SVL, total length, brightness, red chroma and hue).

Hormone manipulation began when all individuals had been in captivity for at least five days (Fig. 1). Circulating levels of E_2 and P_4 were manipulated through the non-invasive technique of transdermal hormone application (Meylan, Dufty & Clobert 2003). E_2 (Sigma E8875) and P_4 (Sigma P0130) were diluted in commercial sesame oil (4.5 μg of hormone per ml of oil) and 4.5 μl of the respective dilution was applied daily on the dorsal side of females of E and P groups for transdermal absorption. The same amount of sesame oil was applied daily to females of C group using the same procedure. Manipulations took place at night, when individuals were inactive. Hormone dosages were determined according to previous testosterone experiments carried out in males of this species (Belluire, Smith & Sorci 2004), because E_2 and P_4 have never been experimentally manipulated in spiny-footed lizards. In any case, hormone doses were similar to or lower than those used in other experiments with E_2 and P_4 in other lizard species (Cooper & Ferguson 1972b; Cooper & Ferguson 1973). Experimental treatments lasted until

most females had started to lose the red colouration, specifically for 22 days (from 29th March to 19th April, Fig. 1).

Female colouration was measured weekly from three days before the hormonal treatment started until the release day (seven measurement batches in total, Fig. 1). Two blood samples were taken from each female during the experiment, the first one on the day before the hormone manipulation started, and the second one 12 days later (Fig. 1). The blood sampling technique was the same used in 2011 (see Blood Sampling and Hormone Assays). Five females (four of E group and one of P group) died during the experiment, and one pair (male and female) from E group escaped during the last week of the experiment. Therefore, the number of females included in the analyses testing the effect of hormone treatment on colouration was 15 in C group, 14 in P group and 10 in E group.

Blood Sampling and Hormone Assays

Blood samples were obtained from the *sinus orbitalis* with 20- μ l capillary tubes following Woodley & Moore (1999). A maximum of 120 μ l of blood was obtained from each animal. Samples were immediately centrifuged to separate plasma from blood cells and plasma was stored at -20°C until hormone assays were performed. This sampling technique has been previously used with no apparent effects on lizard health (Whiting *et al.* 2006; Jessop, Chan & Stuart-Fox 2009).

We measured plasma steroid hormone concentrations (P_4 and E_2) in each blood sample using commercially available EIA kits (Cayman Chemical, Ann Arbor, MI, USA) and their associated protocols. Linearity was tested in plasma pools for each hormone and found to be satisfactory. All samples were tested in duplicate in each assay. In the 2011 assays, only one plate was run per hormone, and the within-assay coefficient of variation was 9.83 for P_4 and 13.31 for E_2 . In 2012, two plates were run per hormone, and between- and within-assay coefficients of variation were respectively 15.64 and 10.48 for P_4 , and 12.65 and 23.62 for E_2 .

Colour Measurements

Colour measurements are described in detail elsewhere (Cuervo & Belliure 2013). Briefly, we quantified the spectral properties of female colouration by taking reflectance readings (with a USB 2000 spectrometer and a DT-MINI-2-GS tungsten halogen light source, Ocean Optics, Dunedin, USA) in the range of 320–700 nm (Whiting *et al.* 2006). Four body regions were measured three times each, namely the rear part of both hind limbs and the ventral part of the tail around 1 cm (hereafter proximal part of the tail) and 2.5 cm (hereafter distal part of the tail) from the cloaca. We calculated reflectances at 1-nm intervals in the studied range using AVICOLOR software (Gomez 2006). From the reflectance data, three colour parameters were calculated as follows: brightness as the mean reflectance between 320 and 700 nm, red chroma (red saturation) as the sum of reflectances between 630 and 700 nm

divided by the sum of reflectances between 320 and 700 nm, and hue as the wavelength where the maximum reflectance was recorded (Montgomerie 2006). Reflectance was not measured for regenerated parts of the tail or for hind limbs with wounds or scars, because all these characteristics dramatically affected colouration. Thus, analyses for each body part included different sample sizes in some cases.

Repeatability of colour measurements (Lessells & Boag 1987) in the four body parts during the 2011 field study was high for brightness and red chroma ($0.630 \leq r \leq 0.983$, $P < 0.001$), but very low for hue in the distal part of the tail ($r = 0.170$, $P = 0.072$), and medium for hue of the other three body parts ($0.440 \leq r \leq 0.595$, $P < 0.001$). In the 2012 experimental study, repeatability was calculated using exclusively the first measurement batch (i.e., 528 measurements from 45 females), although qualitatively identical results were obtained using all seven measurement batches (i.e., 3522 measurements from 45 females; results not shown for brevity). Repeatability was medium-high for brightness ($0.381 \leq r \leq 0.728$, $P < 0.001$), high for red chroma ($0.900 \leq r \leq 0.940$, $P < 0.001$) and medium for hue ($0.307 \leq r \leq 0.529$, $P < 0.001$) in the four body parts.

Variation in colour parameters among individuals was significantly higher than measurement error in all cases of both studies, except in hue of the distal part of the tail in the field study (see above). Despite this exception, and following previous studies (Cuervo & Belliure 2013), we averaged the three measurements for each colour parameter and body part and, subsequently, calculated the mean of the

four body parts to obtain a single value of brightness, red chroma and hue for each individual. In any case, if the hue value for each female of the field study was calculated excluding the distal part of the tail (i.e., including only the hind limbs and the proximal part of the tail), qualitatively identical results were obtained (results not shown for brevity).

Statistical Analyses

Data from the 2011 field study did not need any transformation to meet parametric assumptions (normality of residuals and homocedasticity), but hormone concentrations, brightness and red chroma of the 2012 experiment in captivity were $\log_{10}(x+1)$ -transformed, while hue was x^8 -transformed. After hormone treatment, hormone concentrations did not meet parametric assumptions even after transformation, so non-parametric tests with raw data were used to check for differences in hormone concentrations after the treatment among experimental groups.

The possible relationships between female colour parameters (brightness, red chroma and hue) and natural plasma P_4 and E_2 concentrations were tested using Pearson correlations. For the 2011 field study, these relationships were analysed separately for two periods: (i) before the first egg laying was observed in captivity, i.e., before 15th June (5th May - 14th June, $n = 17$ females), when all adult females were sexually active, and (ii) after 15th June (21st June - 13th August, $n = 10$ females), when both sexually active and inactive females seemed to be

present in the field (Castilla, Barbadillo & Bauwens 1992) (see Natural Hormonal and Colour Profiles During the Reproductive Season in Results). For the 2012 experimental study, these relationships were analysed before hormone manipulation, i.e., before reproduction (late March), and included all females ($n = 45$). All these analyses were carried out with STATISTICA 7.1 (StatSoft Inc. 2005).

The possible effects of the experimental manipulation of steroid hormone levels (three experimental groups: E, P and C) on female colour parameters were tested using two-way ANOVAs with repeated measures in one factor (measurement batch). We simultaneously tested for temporal effects (seven colour measurement batches) and checked the interaction between both factors. When the interaction term was statistically significant, one-way ANOVAs were performed for each measurement batch. Post-hoc Tukey tests were used when one-way ANOVAS showed significant differences among treatments. All these analyses were performed using R software (R Development Core Team 2013) and the nlme package (Pinheiro *et al.* 2013).

All statistical tests were two-tailed and the significance level was 0.05.

Results

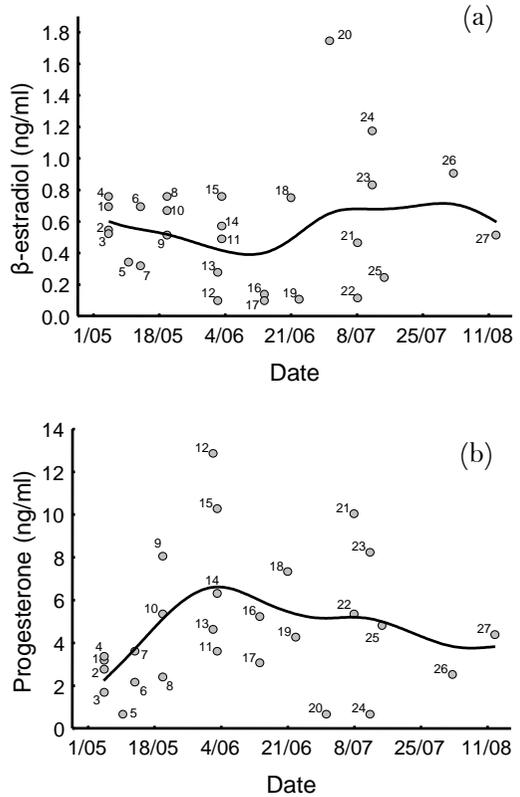
Natural hormonal and colour profiles during the reproductive season

All female spiny-footed lizards captured during the 2011 field study in Madrid were sexually mature according to their SVL (mean \pm SD = 73.2 ± 3.4 mm; range: 69 - 81 mm, $n = 27$ females). Mean (\pm SD) plasma P_4 and E_2 concentrations during the whole sampling period were 4.73 ± 3.09 ng/ml (range: 0.68 - 12.85 ng/ml, $n = 27$ females) and 0.56 ± 0.37 ng/ml (range: 0.10 - 1.75 ng/ml, $n = 27$ females) respectively.

During the first period analysed (before first egg laying), we found a significant reduction in E_2 concentration (Pearson correlation; $r = -0.525$, $n = 17$, $P = 0.030$), suggesting that the E_2 maximum concentration (which occurs shortly before ovulation in vertebrate females) happened a few days before our first captures (Fig. 2a). We also found a marginally non-significant increase in P_4 concentration during this first period (Pearson correlation; $r = 0.470$, $n = 17$, $P = 0.057$), as P_4 levels rose until the first days of June and started decreasing afterwards (Fig. 2b). During the second period analysed (after the first egg laying), non-significant relationships with date were found for any steroid hormone (Pearson correlations; $-0.176 \leq r \leq -0.015$, $n = 10$, $P \geq 0.627$ in the two tests), maybe partly due to the great variation in hormone concentrations in this second period. Both steroid hormone profiles (Fig. 2) suggest the existence of two reproductive cycles in Madrid population (hereafter early and late cycles). In the early cycle, all adult females

Fig. 2.

Plasma concentration of (a) β -estradiol and (b) progesterone in 27 adult female spiny-footed lizards in Madrid population during the reproductive season of 2011. Each point is labelled with the female identification number. Distance-weighted least-squares fit is represented as a continuous line.



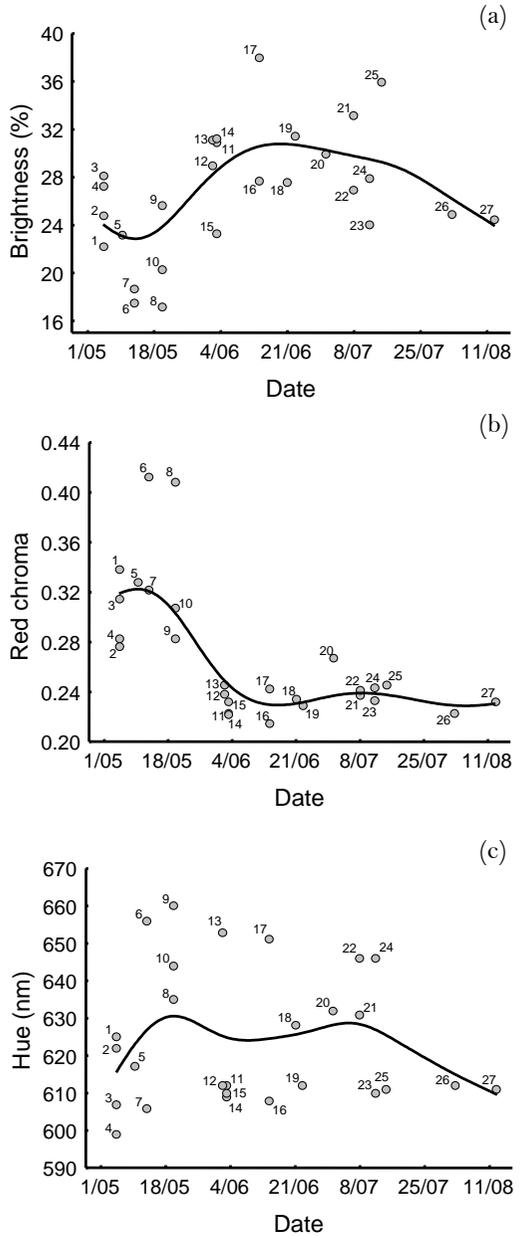
would be sexually active, because, in general, hormone levels matched the typical temporal variation in a female reproductive cycle, while the great variation in hormone concentrations in the second cycle would suggest that not all females started a second reproductive cycle. Ovulation during the early cycle probably happened in mid-May, immediately following the first E_2 peak, which might have happened a few days before the first captures (Fig. 2a). Maximum P_4 values were found at the beginning of June (Fig. 2b), presumably during gravidity of

this early cycle. These dates agree with other observations for the same year and area, as first copulations in the field were observed in mid-May and the first egg laying (a female kept in captivity for other experiments) was observed on 15th June. According to the hormonal profile, some females could have had a late ovulation at the beginning of July, when plasma E_2 concentration raised again and reached a plateau, although other females showed low E_2 levels at this time of the year (Fig. 2a). P_4 also reached a plateau the first days of July, right after E_2 did (Figs. 2a and 2b), presumably during gravidity of the females reproducing in the late cycle.

Regarding the colour profile, at the beginning of the reproductive season (mid-May), and coinciding with ovulation of the early cycle, we found maximum red chroma and minimum brightness values, while hue values were rising (Fig. 3). Females with high red saturation and low brightness were the reddest to the human eye. At the beginning of June, immediately following the P_4 peak, we found a drop in red chroma, a peak in brightness, and a small decrease in hue (Fig. 3). Females with low red saturation and high brightness were perceived by humans as pallid yellow. We did not perceive any clear increase of red colouration coinciding with the possible ovulation of the late cycle (beginning of July), but a large variation in brightness and hue was recorded during this period.

Fig. 3.

Mean (a) brightness, (b) red chroma and (c) hue in 27 adult female spiny-footed lizards in Madrid population during the reproductive season of 2011. Each point is labelled with the female identification number. Distance-weighted least-squares fit is represented as a continuous line.



Relationships between natural hormone levels and colouration

In the 2011 field study, brightness and red chroma were negatively related to plasma E_2 and P_4 concentrations respectively, but only in the period before the first egg laying (Table 1). During the second period, these relationships were in the same direction but non-statistically significant (Table 1). A non-significant trend for a positive relationship between red chroma and plasma E_2 concentration was also found during the first period (Table 1). Hue was not significantly related to plasma P_4 and E_2 concentrations in any of the two periods (Table 1).

Table 1. Pearson correlation coefficients (r) between plasma levels of sex steroid hormones (β -estradiol and progesterone) and colour parameters (brightness, red chroma and hue) in female spiny-footed lizards from Madrid population during the two periods analysed (first period: before the first egg laying observed in the season, i.e., between 5th May and 14th June 2011, $n = 17$ females; second period: after the first egg laying observed in the season, i.e., between 21st June and 13th August 2011, $n = 10$ females) and from Almería population before the mating season (late March 2012, $n = 45$ females).

		β -estradiol		Progesterone	
		r	P	r	P
First period of 2011	Brightness	-0.561	0.019	0.172	0.510
	Red chroma	0.478	0.052	-0.527	0.030
	Hue	-0.008	0.974	-0.078	0.767
Second period of 2011	Brightness	-0.259	0.471	0.097	0.789
	Red chroma	0.536	0.111	-0.368	0.296
	Hue	0.233	0.517	-0.185	0.609
Before the mating season of 2012	Brightness	-0.297	0.048	0.083	0.590
	Red chroma	0.116	0.447	0.071	0.642
	Hue	-0.080	0.600	-0.137	0.371

In the 2012 study in Almería, brightness and plasma E_2 concentration were negatively correlated before the reproductive season, i.e., before experimental hormone manipulation (Table 1). However, all other relationships between colour parameters and plasma hormone concentrations before the experiment were far from significant (Table 1).

Experimental manipulation of hormone levels

Plasma steroid hormone concentrations did not differ significantly among experimental groups before the manipulation started (one-way ANOVAs; $F_{2,42} \leq 0.10$, $P \geq 0.769$ in the two tests). After 12 days of experimental treatment, plasma E_2 concentration was, as expected, higher in E group than in the other two groups (Kruskal-Wallis test; $\chi^2 = 30.62$, d.f. = 2, $P < 0.001$, $n_E = n_P = n_C = 15$; post-hoc multiple comparisons: between E and the other two groups, $Z \geq 4.18$, $P < 0.001$ in the two tests, between C and P groups, $Z = 1.02$, $P = 0.931$). However, P_4 concentration was higher in both P and E groups than in C group (Kruskal-Wallis test; $\chi^2 = 27.72$, d.f. = 2, $P < 0.001$, $n_E = n_P = n_C = 15$; post-hoc multiple comparisons: between C and the other two groups, $Z \geq 4.33$, $P < 0.001$ in the two tests, between E and P groups, $Z = 0.31$, $P = 1.000$), even though P_4 was only applied to females in P group. Therefore, females in E group had increased levels of both E_2 and P_4 after E_2 treatment.

All colour parameters changed over time (Table 2), with a general trend towards brighter and less red (both in saturation and hue) colours in all groups (Fig. 4). Moreover, the interaction between

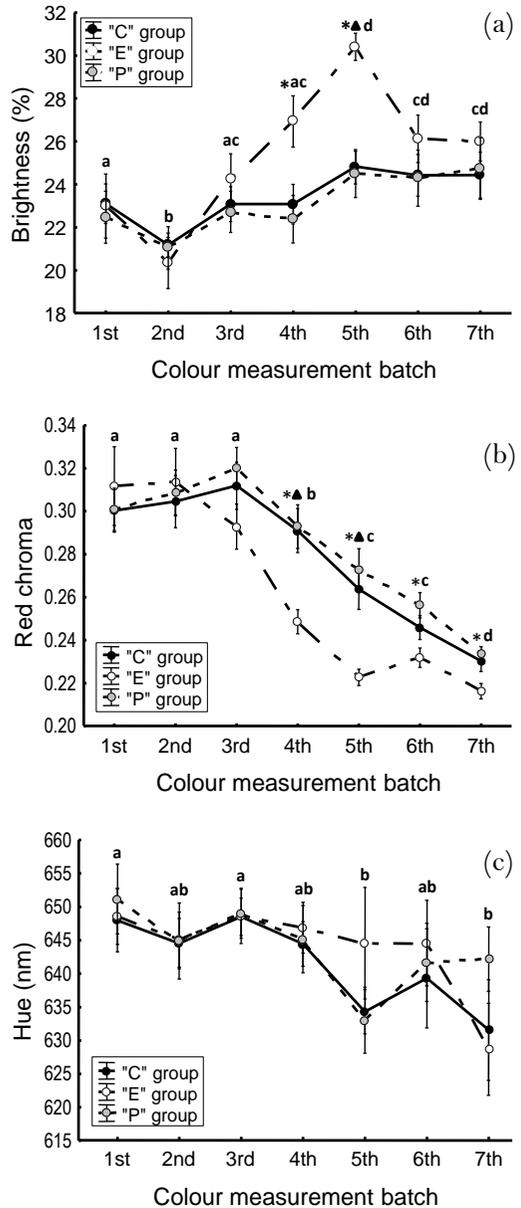
Table 2. Two-way ANOVAs with repeated measures in one factor (measurement batch) testing for experimental treatment effects (differences among three experimental groups: females treated with progesterone, females treated with β -estradiol and females not treated with hormones) and temporal effects (differences among seven colour measurement batches taken at a weekly basis from 26th March to 7th May 2012) on brightness, red chroma, and hue of 39 female spiny-footed lizards from Almería population.

		d.f.	<i>F</i>	<i>P</i>
Brightness	Treatment effect	2,36	1.78	0.183
	Temporal effect	6,216	15.80	< 0.001
	Interaction time x treatment	12,216	2.45	0.005
Red chroma	Treatment effect	2,36	2.72	0.080
	Temporal effect	6,216	83.25	< 0.001
	Interaction time x treatment	12,216	3.52	< 0.001
Hue	Treatment effect	2,36	0.02	0.858
	Temporal effect	6,216	5.57	< 0.001
	Interaction time x treatment	12,216	0.91	0.542

measurement batch and experimental treatment was statistically significant for both brightness and red chroma (Table 2). Subsequent analyses on each measurement batch revealed significant differences in brightness among experimental groups in the fourth and fifth measurement batches (one-way ANOVAs; $F_{2,36} \geq 4.65$, $P \leq 0.016$ in the two tests), and Tukey post-hoc tests showed that females of E group were brighter than females of P group in the fourth and fifth measurement batches ($P < 0.016$ in the two tests) and brighter than females of C group in the fifth measurement batch ($P < 0.001$; Fig. 4a). Regarding red chroma, we found significant differences among experimental groups in the fourth, fifth, sixth and seventh measurement

Fig. 4.

Mean (\pm SE) (a) brightness, (b) red chroma and (c) hue in three experimental groups (E: females treated with β -estradiol, $n = 10$; P: females treated with progesterone, $n = 14$; C: females not treated with hormones, $n = 15$) of female spiny-footed lizards from Almería population during seven colour measurement batches taken at a weekly basis from 26th March to 7th May 2012. Different letters indicate significant differences in colour parameters between colour measurement batches after Tukey post-hoc tests. Triangles indicate statistically significant differences between E and C groups and asterisks between E and P groups for that measurement batch after Tukey post-hoc tests



batches ($F_{2,36} \geq 4.30$, $P \leq 0.021$ in the four tests; Fig. 4b). Subsequent Tukey post-hoc tests showed that females of E group were less red saturated than females of the other two groups in the fourth and fifth measurement batches ($P \leq 0.011$ in the four tests; Fig. 4b) and less red saturated than females of P group in the sixth and seventh measurement batches ($P \leq 0.020$ in the two tests; Fig. 4b). The experimental treatment had no significant effect on hue in any measurement batch (Table 2, Fig. 4c).

Females of the three experimental groups lost their red colouration (higher brightness and lower red chroma) by the end of the experiment, but females in E group lost the red colour earlier than females in C and P groups (Figs. 4a and 4b). P_4 treatment had no significant effect on female colouration as females of P and C groups did not differ in brightness, red chroma or hue in any measurement batch (Fig. 4).

Discussion

Hormonal and colour profiles

The seasonal hormonal profile found in female spiny-footed lizards is consistent with the presence of two ovulation periods in Madrid (central Spain) population. This could be due to different timing in sexual maturation among females of diverse sizes (Bauwens & Díaz-Uriarte 1997), because females lay two clutches in the same reproductive season (Seva Román 1982; Castilla, Barbadillo & Bauwens 1992), or

both. Second clutches have been suggested for some populations of spiny-footed lizards after finding different egg sizes inside a female (Seva Román 1982) or analysing their reproductive organs (Castilla, Barbadillo & Bauwens 1992). Even when second clutches have been suggested, not all females would lay a second clutch. For example, in a population in central Spain, only five out of 24 females showed evidences of a second clutch in a year with a dry spring (Castilla, Barbadillo & Bauwens 1992). Moreover, in the same population, no female showed any evidence of a second clutch in a year with a cold and wet spring (Castilla, Barbadillo & Bauwens 1992). Therefore, there might be no second clutches in "bad" years, but some females would be able to lay a second clutch in "good" years. Late in the reproductive season of those "good" years, some females would be laying second clutches while other females would have already finished their reproduction. In our case, April and May 2011 were relatively hot months with typical rainfall in Madrid (AEMET 2011), so 2011 might be considered a "good" year and second clutches in some females could be expected. However, we cannot rule out the possibility that some small females laid first clutches late in the season. Whatever was the reason for late ovulation, in July in Madrid there would be both sexually active and inactive females. The simultaneous presence of both types of females would result in the lack of clear peaks in the hormonal profile and a large variation in plasma hormone concentrations in this period, in agreement with the results we found (Fig. 2).

Regarding female colour profiles, red colouration was more intense during the ovulation period of the early cycle, changing slowly afterwards into the typical whitish colouration of gravid females. A re-intensification of red colour during the late reproductive cycle has been reported for other populations of spiny-footed lizards where most females lay more than one clutch per year (Seva Román 1982). However, this was not evident in our study population in Madrid (Fig. 3), maybe due to the presence of both sexually active and inactive females during the late cycle. Despite no clear re-intensification of the red colour during the late cycle, the fact that early cycle ovulation and red colour intensification happened at the same time agrees with the mating-related function previously suggested for female red colouration in this species (Cuervo & Belliure 2013).

Relationships between steroid hormones and colouration

In the first period analysed in 2011 (before the first egg laying was observed), all captured females were probably sexually active, so hormone levels followed a relatively clear pattern. The negative relationships between P_4 and red chroma and between E_2 and brightness suggest that (i) females before ovulation, characterized by low levels of P_4 and high levels of E_2 (Norris & Lopez 2011), were red coloured (i.e., high red saturation and low brightness), while (ii) females during gravidity, characterized by high levels of P_4 and low levels of E_2 (Norris & Lopez 2011), were white coloured (i.e., low red saturation and high brightness). These colour changes agree with seasonal colour changes

previously observed in this species (Seva Román 1982; Cuervo & Belliure 2013). During the second period analysed in 2011 (after the first egg laying was observed), the negative relationships between P_4 and red chroma and between E_2 and brightness were not statistically significant, and the presence of sexually active and inactive females during the late cycle might help explain these negative but non-significant relationships.

Despite the negative relationship found between redness and plasma P_4 levels in the first period of the 2011 field study, our experiment in 2012 suggests that P_4 does not significantly affect female colouration in spiny-footed lizards. P_4 has been suggested to induce conspicuous colouration in females of other lizard species (Cooper & Greenberg 1992), although it has no effect on female colouration in other animal groups (e.g. Czaja *et al.* 1977). In the lizard species where P_4 induces female conspicuous colouration, this colour is displayed during vitellogenesis, at the same time as P_4 levels increase, and is retained during gravidity until egg laying (Cooper & Greenberg 1992; Jessop, Chan & Stuart-Fox 2009). In female spiny-footed lizards, however, red colouration intensifies during the early phases of vitellogenesis, when P_4 levels are still low, and disappears immediately following ovulation, when P_4 levels reach a peak (Figs. 2 and 3). We hypothesize that P_4 might be responsible for colour intensification in species where this colour is maintained during gravidity, while P_4 would not affect colouration in species where the conspicuous colouration is lost after ovulation.

Despite the negative relationships found between brightness and E_2 levels in the first period of the 2011 field study and before the reproductive season in 2012, our experiment showed that E_2 treatment was responsible for an increase in brightness and a reduction in redness. To explain these apparently contradictory results with the observational and experimental approach, we should consider that, in the natural hormone cycle of female lizards, ovulation takes place between a peak of E_2 and a peak of P_4 (Norris & Lopez 2011). The loss of red colouration (lower red saturation, higher brightness) in female spiny-footed lizards starts around ovulation, when E_2 concentration is naturally decreasing. Consequently, when the relationship between E_2 concentration and colouration was analysed during the first period of the 2011 field study (period that started slightly before ovulation and finished in late gravidity), females slightly before or around ovulation, with high E_2 levels, were darker (i.e., redder), while females sometime after ovulation (i.e., gravid), with much lower plasma E_2 concentration, were lighter (less red). Regarding the negative relationship found before the breeding season of 2012, females that had not begun any hormonal change for reproduction, with low E_2 levels, were dark (i.e., red), but females already preparing for reproduction, with higher E_2 levels, were intensifying red colouration, i.e., they were even darker. However, in the experiment, we increased plasma E_2 (and P_4) concentration in females treated with E_2 , what made them lose their red colouration earlier than females in the other experimental groups. Thus, increased E_2 levels (alone or in association with increased P_4 concentrations) trigger the loss of red colouration in females of this species. Once the mechanism to lose

the red colouration is triggered, the loss of red colouration continues even if E_2 levels decrease.

In general, E_2 has not been suggested to significantly affect female breeding colouration when this colour is maintained during gravidity (Cooper & Greenberg 1992). However, E_2 effects on female breeding colouration have been found in species of different taxa (Czaja *et al.* 1977; Dixson 1983; Calisi & Hews 2007) where females develop conspicuous colouration during vitellogenesis and lose this colour after ovulation, i.e., the pattern we found in female spiny-footed lizards. Therefore, we hypothesize that E_2 might affect colouration in species where the conspicuous colouration is lost after ovulation, but not in species where this colour is maintained during gravidity.

In our experimental study, E_2 treatment increased both E_2 and P_4 levels, possibly due to an inhibition of the aromatase, the enzyme responsible for a key step in the biosynthesis of E_2 (Simpson *et al.* 1994). Aromatase inhibition might have caused an accumulation of the non-processed P_4 , a precursor of T and E_2 (Pieau, Dorizzi & Richard-Mercier 1999), in females treated with E_2 . As a consequence, we were unable to discriminate between the effects of increased levels of the two hormones together or E_2 alone. Previous studies in which experimental hormone manipulation was assumed to affect only the treated hormone should be interpreted cautiously, because possible effects on other hormones have not been generally checked (Cooper & Ferguson 1972a; Cooper & Ferguson 1972b; Cooper & Ferguson 1973; Medica, Turner & Smith 1973; Cooper & Crews 1987).

Conclusions

The results of this study are consistent with two ovulation periods during the same reproductive season in a spiny-footed lizard population in central Spain, although they do not clarify whether this happened because some females laid second clutches, because smaller females ovulated later, or both. Re-intensification of red colouration during the late reproductive cycle was not evident in our study. In a hormone manipulation experiment, high P_4 levels did not have any significant effect on female colouration, but high levels of both E_2 and P_4 together caused the loss of red colouration. The role of E_2 in regulating the expression of red colouration in female spiny-footed lizards agrees with other vertebrate species in which conspicuous colouration is also displayed when females are sexually receptive (before ovulation). However, it disagrees with species in which conspicuous colouration is displayed when females are gravid (after ovulation), because in these cases, the key hormone regulating colouration is P_4 . Thus, different hormones might regulate female breeding colouration depending on the reproductive phase when the conspicuous colour is expressed.

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Chapter 7

GENERAL DISCUSSION

The function of red colouration in the spiny footed lizard

Spiny-footed lizards show red colouration at different stages of their development, i.e., at different ages. During the period they are hatchlings (from birth until the first winter), the red colouration is present in the tail and is clearly visible from above. At this age, red colouration is a lure that diverts attacks from visual predators (mainly birds) towards the autotomizable tail (Chapter 3), thus increasing the probability of surviving an attack. Our results also suggest that red colouration increases lizard conspicuousness in the field and thus detectability by predator. Despite this, redder lizards behave more riskily, staying further from refuges for longer periods of time (Chapter 2), what supports the antipredatory function of the red colouration. The balance between the costs of attracting more attacks and the benefits of diverting these attacks towards the tail should be positive to explain the evolution and maintenance of this trait. One might think that the conspicuous colouration should be concealed on the ventral part of the tail, and displayed only when the lizard is attacked, thus reducing detection by predators (Arnold 1984). However, the degree of vigilance this behaviour requires (the predator must be detected well in advance to display the concealed colouration) would make this strategy unaffordable for hunting lizards with reduced vigilance (Lima & Dill 1990). In this case, the continuously visible conspicuous colouration might be a better strategy in terms of costs-benefits.

Red colouration spreads towards the rear part of the hind limbs (which is clearly visible from behind) in juvenile spiny-footed lizards, i.e., after the first winter, suggesting that, at this age, it does not only act as a decoy (Arnold 1984). Indeed, our results suggest that red colouration on the tail and hind limbs of juveniles may function as an intraspecific signal of age or sexual immaturity, effectively reducing adult aggression (Chapter 4). The lack of red colouration on the hind limbs of hatchlings might be explained by (i) the small number of active adults in the field in the period with hatchlings (August-November; Seva Román 1982), (ii) the small size of hatchlings compared to adults, what would reduce their potential as competitors, and/or (iii) the fact that the mating season is over when newborn lizards emerge, so mating competition is supposed to be absent (Baird, Timanus & Sloan 2003), making the intraspecific signalling unnecessary for hatchlings. However, juveniles, which are larger than hatchlings, coexist with adults along the whole reproductive season, so a signal of age or sexual immaturity to prevent adults from treating juveniles as potential rivals is more necessary in this age class (Clark & Hall 1970).

Red colouration retracts to more ventral parts of the tail across the ontogenetic process, and completely disappears in males when they are around one year old. However, it is retained on the ventrolateral part of the tail and the rear part of the hind limbs in females through adulthood (Seva Román 1982). The fact that both males and females change their behaviour towards a less active foraging pattern as they become older and larger (Chapter 2) and that the red colouration of adult

females is not present on the dorsal part of the tail, suggest that red colouration in adult females is more related to an intraspecific than to an interspecific (antipredatory) function. In fact, a mating-related function had already been suggested for female colouration (Cuervo & Belliure 2013), and our results support this function, as we found that redder females are closer to ovulation (Chapter 6), and that males prefer to mate with females showing red rather than white colouration (Chapter 5).

Our results also suggest that the loss of red colouration in the dorsal parts of the tail along ontogeny is advantageous, because, as we mentioned above, red colouration increases the conspicuousness of the lizard. Therefore, the red colour may only be an effective antipredatory mechanism in very active lizards, which are anyway conspicuous to predators because of their continuous movement, the long time spent far from refuges and their reduced vigilance (Jackson, Ingram & Campbell 1976; Arnold 1984). Generally, hatchlings and juveniles are more vulnerable to predation because of their high activity rates (Chapter 2), their smaller size, the poor ossification of their skulls, and the unfamiliarity with their surroundings that makes finding a refuge more difficult (Arnold 1984). Thus, the presence of conspicuous tail colouration may be advantageous for small/young lizards because they would be easily detected by predators even without red tails, and the conspicuous tail can divert predator attacks to the expendable tail. However, it would not be advantageous for adults, which move less (Hawlena 2009; Chapter 2), and are less conspicuous to predators (Metcalf 1984).

Spiny-footed lizard red colouration as a multifunctional signal

A multifunctional signal has, as its own name points out, multiple functions, i.e., it can stimulate the sensory system of different types of receivers, exerting an influence on them (Bitton & Doucet 2014). It is common that a trait which is sexually selected by mate choice is also affected by intrasexual competition, acting as a signal for mates and conspecific same-sex competitors (Morris, Tudor & Dubois 2007; Hoi & Griggio 2008). Bird songs or colour patches are common multifunctional signals used in both male-male competition and mate choice (Graves 1990; Morris, Tudor & Dubois 2007; Hoi & Griggio 2008; Reichard *et al.* 2011). Sometimes, even the same signal is intended for both conspecifics and heterospecifics (Bitton & Doucet 2014).

Producing signals is neither easy nor costless. The mechanisms needed to produce signals can determine the type of signals an organism can exhibit (Endler 1993). The evolution of a signal can also be influenced by the phylogeny of the species, or by genetic or developmental linkage of the trait to be used as a signal with other traits, like the relation between body size and call acoustics in anurans (Gingras *et al.* 2013). Signallers suffer pressures to economize their signals, reducing the costs of production without compromising the communication of information, because, otherwise, their fitness would be negatively affected (Bradbury & Vehrencamp 2011). Therefore, if the same signal can fulfil several functions, it will be advantageous for the signaller in terms of costs associated with communication.

The results of this PhD thesis suggest that red colouration in spiny-footed lizards is a multifunctional signal that have different functions at different ages or stages of development. It is a trait retained and shaped throughout the ontogeny of the species, making then unnecessary the development of new signals for some intraspecific and interspecific signalling functions.

Producing a signal requires sometimes the use of scarce resources that may also be used for other purposes, posing a trade-off between costs and benefits of signalling. Colour signals, in general, are not expensive to produce, but some pigments may have other physiological functions (Olson & Owens 1998; McGraw, Adkins-Regan & Parker 2005). Red colouration in spiny footed lizards is produced by pterins (J.J. Cuervo, J. Belliure and J.J. Negro, unpublished data), which, in contrast to other pigments such as carotenoids (Britton 1998), can be synthesized *de novo* by animals (Ziegler 1965). Although the physiological functions of pterins have not been widely studied, they seem to play a role in oxidative processes (Oettl & Reibnegger 2002; McGraw 2005). Thus, retaining pterins synthesized during earlier phases of development will be less costly than synthesising these pigments *de novo* in different age classes. In the case that adult females developed again full red colouration during a second ovulation within the same year or in subsequent years, the signal should be re-synthesised, with the consequent production costs. The re-intensification of red colouration during a second ovulation period has been suggested for some populations (Seva Román 1982), although our study did not

find this pattern in a population in central Spain (Chapter 6). However, at the beginning of the 2011 mating season, we recaptured three adult females showing red colouration that were also red and adult in 2010, suggesting that a re-intensification of colouration between years is possible.

According to the functions described here for red colouration in spiny-footed lizards, we speculate that red colouration in this species might have first evolved as a signal to reduce the probability of predators attacking vital body parts (e.g. head and trunk) of very active foragers like hatchlings and juveniles, increasing the effectiveness of an already autotomizable tail (Pianka & Vitt 2006). A similar signal with the same or different colour is very common among close relatives of spiny-footed lizards (e.g. *A. schreiberi*, *A. boskianus* and *A. beershebensis*). This signal might have been associated by adults with age or sexual immaturity, i.e., with low levels of intraspecific competition for resources, reducing then adult aggression towards juveniles retaining this colouration, juveniles that could actually be their own offspring. The retention of red colouration by adult females to indicate sexual receptivity would be possibly related to the smaller costs of reusing a signal instead of producing a new one. The subsequent loss of red colouration, possibly indicating that the female is no longer sexually receptive, was then regulated by the same hormones that controlled the reproductive cycle. The loss of red colouration in males might be also associated with hormones, because high levels of testosterone reached when males start their sexual maturation have been suggested to

mediate in the expression of secondary sexual characters in other lizard species (Cox *et al.* 2005). An evolutionary path of red colouration in the opposite direction, i.e., appearing in adult females and spreading to earlier ontogenetic stages, seems less probable. If red colouration first appeared in adult females to signal their sexual receptivity, males would have associated this colour with receptive females. Thus, if a juvenile developed this colouration, it would have suffered harassment and bites from males trying to mate, and would obtain no benefits from the expression of this signal, making it difficult that the signal could be maintained.

The presence of the same colouration along ontogeny in this species does not seem to be physiologically constrained, as adult male spiny-footed lizards develop yellow spots on the flanks, suggesting that the expression of signals of other colours is possible in this species. Therefore, the reduction of costs of signalling seems a better explanation for the colouration pattern shown by this species.

Future research

Many aspects of the function of the red colouration in spiny-footed lizards still need to be clarified. Therefore, future studies should address some questions derived from this thesis:

- * The hue of juvenile red colouration might be related to individual quality or sex, so future studies should find a reliable

method to sex young juveniles and check whether red colouration differs between sexes. Moreover, the possible relationships between juvenile colouration and different proxies of phenotypic quality (e.g., health, performance, body condition, etc.) should also be studied.

* Regarding adult female colouration, it is still necessary to clarify if red colouration could be giving information about female quality or if it only indicates the reproductive state of the female. We should (i) determine the maximum expression of red colouration for each female, and (ii) study the possible relationships between the maximum red colouration and different proxies of phenotypic quality (e.g., health, performance, body size and condition, fecundity, etc.). One step further would be (iii) to study the possible relationship between the maximum expression of female red colouration and their offspring phenotypic quality.

* In case that red colouration in adult females could signal female quality, future studies should check for possible trade-offs between colouration and reproduction, as pterins used as pigments would not be available as antioxidants.

* It will also be interesting to clarify if a re-intensification of the red colouration occurs in those cases in which adult females undergo a second ovulation cycle within the same year. Likewise, it would be important to study the longevity of the

species and clarify if some females may live for more than one year as adults. If this could happen, the possible re-intensification of the red colouration in subsequent years should be investigated.

* If a re-intensification of the red colouration in adult females is confirmed, it would be interesting to know whether the re-intensification is also triggered by steroid hormones and whether the same mechanisms operate between and within years.

* Progesterone has been commonly related to the appearance of conspicuous colouration in several lizard species, but it does not seem to affect female spiny-footed lizard red colouration. Future studies should focus on the hormonal regulation of conspicuous colouration in female vertebrates taking into account the phase of the reproductive cycle when the conspicuous colouration appears.

* Loss of red colouration in females is triggered by high β -estradiol levels (alone or together with high progesterone levels), but it would be interesting to know whether hormones (e.g. high testosterone levels) are also responsible for the loss of red colouration in male spiny-footed lizards.

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Chapter 8

CONCLUSIONS

Conclusiones Generales

- ❧ La lagartija colirroja disminuye su nivel de actividad, y por lo tanto su conspicuidad, a lo largo de la ontogenia. Una elevada actividad probablemente implica una elevada presión de depredación, lo cual podría haber dirigido la evolución de la coloración roja en la cola, claramente visible desde arriba en los individuos más jóvenes, que se mantendría mientras el comportamiento activo (y por lo tanto arriesgado) fuese necesario para satisfacer las necesidades energéticas.
- ❧ Los individuos más rojos permanecen fuera de los refugios durante periodos de tiempo más prolongados que aquellos más naranjas, lo cual es consistente con una función antidepredatoria de la coloración roja.
- ❧ La cola roja de las crías provoca que los depredadores aviares localicen a los individuos más fácilmente, pero a su vez funciona como un señuelo que desvía de forma efectiva los ataques de las zonas vitales del cuerpo (cabeza y tronco) hacia la cola, que es autotomizable. Por lo tanto, la cola roja es un mecanismo antidepredatorio efectivo.
- ❧ La coloración roja de los juveniles actúa como una señal comunicativa entre clases de edad, reduciendo de manera efectiva el número de agresiones de alta intensidad (mordiscos) por parte de los

adultos de la misma especie; podría estar indicando edad o inmadurez sexual.

-  Los juveniles más rojos reciben menos ataques por parte de los adultos que los más naranjas; esta variación natural en el tono del rojo podría estar indicando la calidad del individuo o su sexo.
-  Los machos son capaces de discriminar, tanto mediante señales visuales como químicas, entre hembras juveniles y adultas, y prefieren aparearse con estas últimas independientemente de su coloración.
-  La coloración roja de las hembras adultas es un ornamento sexual, ya que los machos prefieren aparearse con hembras adultas que presentan coloración roja en lugar de coloración blanca.
-  La coloración roja de las hembras adultas está relacionada con su ciclo reproductivo, siendo las hembras más rojas las que están más cercanas al momento de la ovulación.
-  El cambio estacional en la coloración de las hembras adultas está regulado por hormonas, ya que niveles altos de estradiol (quizás solo o combinado con niveles altos de progesterona) provoca la pérdida de la coloración roja tras la ovulación.

- ❧ La coloración roja de la cola y las patas traseras de la lagartija colirroja es una señal multifuncional, cumple diferentes funciones en diferentes clases de edad, aunque podría existir más de una función en la misma clase de edad (e.g., la coloración roja en juveniles podría tener una función de reducción de agresividad y una función antidepredatoria).

General Conclusions

- ❧ Spiny-footed lizards decrease their level of activity, and thus their conspicuousness, during ontogeny. The high predation pressure that an intense activity probably implies, might have driven the evolution of red coloration on the tail, clearly visible from above early in life, which is maintained while an active (and therefore risky) behaviour is needed to meet energetic requirements.
- ❧ Lizards with redder colouration stay further from refuges for longer periods of time than those with more orange coloration, what is consistent with the antipredatory function of the red colouration.
- ❧ Red tail in hatchlings increases lizard conspicuousness to visually oriented predators, but it also serves as a lure that effectively diverts attacks from vital body parts (head and body) to the autotomizable tail. Thus, the red tail is an effective antipredatory mechanism.
- ❧ Red colouration in juveniles is an inter age-class communication signal which effectively reduces high intensity aggression (biting) from adult conspecifics and might indicate age or sexual immaturity.
- ❧ Juveniles with redder coloration are less attacked by adults than those with more orange coloration, and this natural variation in red hue might indicate individual quality or sex.

- ❧ Males can discriminate, both with visual and chemical cues, between juvenile and adult females, and prefer to mate with the latter regardless of their colouration.
- ❧ Red colouration in adult females is a sexual ornament, as males prefer to mate with adult females showing red instead of white coloration.
- ❧ Red colouration in adult females is associated with their reproductive cycle, being redder females closer to ovulation.
- ❧ Seasonal colour change in adult females is hormonally controlled, as high levels of β -estradiol (maybe alone or combined with high levels of progesterone) induce the loss of red colouration after ovulation.
- ❧ Red colouration on tails and hind limbs of spiny-footed lizards is a multifunctional signal, with different functions at different age classes, although there might be more than one function in the same age class (e.g., red colouration in juveniles might serve for both aggression-avoidance and antipredatory functions).

RELACIÓN DE IMÁGENES USADAS:

Portada y contraportada

Paisaje de Chapinería: M^a Jesús Barriero

A. erythrurus portada: Belén Fresnillo

A. erythrurus contraportada: Patricia Cruz

Capítulo 1

Página 13:

Espectro visible: <http://deserthighlandspr.com/wp-content/uploads/2013/02/Visible-spectrum.jpg>

Parámetros del color: http://www.ncsu.edu/scivis/lessons/colormodels/color_models2.html (modificado por Belén Fresnillo)

Página 16:

Fig. 1.1 A: © 2004–2014 Florent Charpin

Fig. 1.1 B: © 2004–2014 Florent Charpin

Fig. 1.1 C: © 2003–2014 Tony Gamble

Fig. 1.1 D: © 2004–2014 Tony Gamble

Fig. 1.1 E: Frank Derriks

Página 19:

Fig. 1.2 A: hhhhalberto (flickr.com)

Fig. 1.2 B: Piet Spaans (fr.wikipedia.org)

Fig. 1.2 C: José Marmaneu (Grubial - Grupo de Biología Alicantina)

Fig. 1.2 D: Elisabet Forsgren (Fig.1 from Amundsen T. & Forsgren E. (2001) PNAS 98:13155-13160)

Página 21:

Fig. 1.3 A: © 2009 Michael Graziano

Fig. 1.3 B: © 2013 Denis Riek

Fig. 1.3 C: © 2013 Denis Riek

Fig. 1.3 D: © 2006-2014 by Krushnamegh Kunte

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Fig. 1.3 G: © J. Harding

Fig. 1.3 H: RIVP (Rhode Island Vernal Pools)

Página 27:

Fig. 1.4: Belén Fresnillo

Capítulo 3

Página 97:

Fig. 1: Belén Fresnillo

Página 98:

Fig. 2: Belén Fresnillo

