Adaptive color variation along an elevational gradient.
The case of the Mediterranean lizard Psammodromus algirus.

Variación adaptativa de la coloración en un gradiente altitudinal. El caso del lacértido mediterráneo Psammodromus algirus.

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Memoria presentada por la Licenciada Senda Reguera Panizo para optar al Grado de Doctora en Biología por la Universidad de Granada.

Tesis realizada bajo la dirección del Dr. Gregorio Moreno Rueda

V°B° director

Fdo: Dr. Gregorio Moreno Rueda

La doctoranda

Fdo: Lda. Senda Reguera Panizo

Granada, 2014

El Dr. **Gregorio Moreno Rueda**, Profesor Ayudante Doctor de la Universidad de Granada

CERTIFICAN:

Que los trabajos de la investigación desarrollada en la Memoria de Tesis Doctoral: "**Adaptive color variation along an elevational gradient. The case of the Mediterranean lizard *Psammodromus algirus***", son aptos para ser presentados por la Lda. Senda Reguera Panizo ante el Tribunal que en su día se designe, para optar al Grado de Doctora por la Universidad de Granada.

Y para que así conste, en el cumplimiento de las disposiciones vigentes, extiendo el presente certificado

En Granada a 27 de noviembre de 2014

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Que el trabajo ha sido realizado por la doctorando bajo la dirección del director de la tesis y hasta donde nuestro conociemiento alcanza, en la realización del trabajo, **se han respetado los derechos de otros autores a ser citados cuando se ha utilizado sus resultados o publicaciones.**

En Granada a 27 de noviembre de 2014

V°B° director

Fdo: Dr. Gregorio Moreno Rueda

La doctoranda

Fdo: Lda. Senda Reguera Panizo

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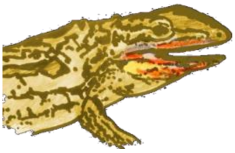

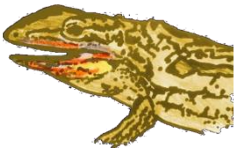

A Granada

(y a todo, todas, y todos lo que ella implica)

El camino sigue y sigue desde la puerta.
El camino ha ido muy lejos, y si es posible he de seguir recorriéndolo
con pie fatigado hasta llegar a un camino más ancho donde se
encuentran senderos y cursos.
¿Y de ahí adónde iré? No podría decirlo.

J.R.R Tolkien. El Señor de los Anillos: La Comunidad del Anillo

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Chapter 1
PRESENTATION AND SYNOPSIS

Presentación

En boca de Miguel Delibes: "El planeta en el que habitamos es como un gran motor. Los organismos que lo habitan son las piezas. El tener todas las piezas sin que el mecanismo funcione no sirve de nada. Pero por otro lado, los mecanismos del motor no se pondrán en marcha si falta alguna de esas piezas". Es imprescindible, por lo tanto, no solo conservar las piezas, si no también conocer estos mecanismos que hacen que el motor siga funcionando. En un marco de cambio global como en el que nos encontramos, los mecanismos de funcionamiento del "motor" se están modificando. Y a las "piezas" de este planeta no les queda más remedio que adaptarse o desaparecer. Para conocer la posible respuesta de los organismos al cambio global, es de vital importancia conocer los mecanismos evolutivos que han llevado a los organismos a ser tal y como son hoy en día, así como predecir la respuesta de estos organismos ante el posible cambio en su distribución y entender el reto que supone a las especies enfrentarse a condiciones ambientales cambiantes.

Conocer los mecanismos evolutivos no siempre es fácil. Pero podemos valernos de herramientas para simular estos mecanismos, por ejemplo mediante la experimentación. También podemos buscar situaciones que simulen estos mecanismos, por ejemplo el seguimiento de una isla recién formada a lo largo de un periodo prolongado de tiempo. En este aspecto, los gradientes geográficos de factores ambientales nos proporcionan un ámbito de estudio muy interesante. A escala planetaria el gradiente latitudinal nos proporciona cambios graduales de condiciones climáticas y de los ecosistemas asociados a ellas. A escala más local, los gradientes altitudinales también nos proporciona un escenario parecido, pero mucho más abarcable a nivel logístico. Una vez nos planteamos cómo estudiar los mecanismos, hay que plantearse qué "piezas" nos pueden proporcionar más información. Para ello el organismo o los organismos que estudiemos deben cumplir ciertas características ligadas al mecanismo que queramos estudiar.

En esta tesis se pretende estudiar las posibles respuestas y/o adaptaciones de una especie modelo en un gradiente altitudinal. De este modo, se pretende predecir respuestas del tipo fisiológico o comportamental, como consecuencia del posible ascenso distribucional en altitud de la especie objeto de estudio. Para ello se ha escogido como modelo una especie de lacértido, la lagartija colilarga (*Psammodromus algirus*, Linneo 1758), y se ha centrado la atención en las poblaciones localizadas en el mayor gradiente altitudinal de la Península Ibérica, situado en el macizo montañoso de Sierra Nevada. La intención de esta tesis es ver las adaptaciones de este lacértido al gradiente ambiental que proporciona el gradiente altitudinal de 2200 m (rango en el que habita la lagartija) que encontramos en la ladera sur de esta sierra. Concretamente, desde el estudio de la variación en la coloración de esta lagartija para poder entender qué fuerzas evolutivas han podido seleccionar la coloración que presenta actualmente el animal, y profundizar también en los cambios fisiológicos ligados al estrés oxidativo en esta especie a lo largo del gradiente. La coloración en un animal se encuentra estrechamente ligada a los niveles de estrés oxidativo, bien porque dicha coloración puede proteger frente a los daños producidos por agentes oxidantes, como la radiación ultravioleta, o bien porque los propios pigmentos que producen la coloración actúan como antioxidantes o están en las rutas metabólicas de producción de antioxidantes dentro del organismo. En el sistema de estudio escogido, cabe esperar que los individuos localizados a diferentes altitudes se encuentren adaptados a las condiciones ambientales locales quedando así reflejado en su fenotipo. Y frente a un hipotético caso de cambio global, en el que los individuos de las poblaciones de zonas bajas asciendan en altitud, podrán sobrevivir o no en función de su capacidad de adaptación.

Abstract

Understanding the evolutionary processes from the pass, and under organisms that have evolved to be as we currently know, is the way to predict how they will respond to environmental changes in the future. In the global climate change framework, the species (especially those which are ectotherms and depend tightly on environmental temperatures) are shifting their distribution in latitude and elevation, finding new environmental conditions to cope with in this process. Those geographical gradients that show at the same time environmental gradients, and the organisms that have been selected to inhabit in these gradients, are useful tools to study the processes of adaptation. In this sense, elevational gradients are very useful to perform this kind of research.

This thesis aims to understand lacertid adaptations within an elevational gradient, using as a model the lizard Large Psammodromus (*Psammodromus algirus*) in the southern slope of Sierra Nevada Mountain Range (Granada, Spain), along a 2200 m elevational gradient (300-2500 m asl [above sea level]). Being the hypotheses as follow:

1) Dorsal coloration may be darker in highland individuals, to improve thermoregulation and warming (temperatures are colder), and to protect them against damages caused from ultraviolet radiation (more intense).

2) Given that ultraviolet radiation is an important oxidant agent, and that its intensity increases in elevation, it would be expected that oxidative stress (oxidative imbalance) will be greater in highland individuals.

3) On the contrary, it is possible those lizards from highlands had evolved under these conditions and that they have characteristics that protect them against ultraviolet damage (for example, darker skin coloration).

4) Furthermore, dorsal coloration may be as similar as possible to the background where they inhabit, being cryptic colorations selected to reduce predations risk.

5) Ventral coloration and color badges associated to this area (yellowish pigmented patch in the gular regions, and orange pigmented patch in the commissures, in this species) may be colorful and more conspicuous in

places where the availability of resources is higher. Moreover, they may be more contrasted in older lizards (assuming badges are status signals), and they may be more conspicuous in males than females (assuming badges are sexual characters involved in mate choice).

For these purposes, individuals of *P. algirus* were sampled in six different localities along an elevation gradient, during the activity period of the lizards for a period of four years (2010-2013). About 450 adult lizards were caught (males and females) and their dorsal and ventral coloration was studied, obtaining color parameters (lightness, chroma and hue) using a spectrophotometer. Oxidative stress levels were also quantified in terms of oxidative damage (in lipids and proteins) and antioxidant capacity biomarkers (aiming to detect ultraviolet radiation damage at that level).

As it was expected, results showed that *P. algirus* individuals are darker at highlands (above 2000 m asl). Being lightness positively correlated with temperatures (lighter lizards are in warmer places), and negatively correlated with ultraviolet radiation (smaller lightness values in stronger ultraviolet radiation places). Furthermore, dorsal skin coloration in terms of hue was significantly related to substrate color, suggesting a positive selection for cryptic coloration as an antipredator strategy.

Contrary to the expectations, oxidative damage was lower in highland lizards. This finding suggests that lizards from highlands are better adapted to harmful effects of ultraviolet radiation. However, the experiment run in laboratory conditions, where lizards were exposed to artificial ultraviolet light, showed that ultraviolet radiation seems to have no effects on the oxidative stress levels of this lizard. Furthermore, it was observed that lizards from lowlands had higher oxidative damage levels in lipids and proteins, which suggests that lowland environments are more oxidizing than the highland environment. Probably the higher temperature conditions and the presence of more contaminants make lowland environments more oxidizing for the lizards than highlands, regardless the higher solar radiation conditions.

In relation with ventral color badges, *P. algirus* showed a multiple signal system where diverse color badges convey the same quality, while different qualitites can be signaled by the same color signal. Accordingly, males showed orange commissures as a mean of signaling their sex, body size and age. Females (as males) showed yellowish pigmentation in throat and chest. These yellowish patches were more saturated and larger in older and larger lizards, being probably a status signal of the bearer. In addition, colourful individuals show more often signals of predator attacks. Probably, not because of the conspicuousness, but regard to these individuals are more active and spend more time exposed to predators. Finally, it was observed that lizards invest more resources in coloration in the highlands (greater proportion of individuals with color badges).

These findings suggest *P. algirus* is a well-adapted lizard to the environmental conditions found in an elevational gradient. This suggests that the adaptations that have taken place allow this lizard to inhabit in high-elevation areas, where almost any other Mediterranean lizard cannot do it. Dark dorsal colorations may be positive in terms of thermoregulation and protection against ultraviolet radiation damage. This matches with the finding that there are no differences in terms of thermal preferences and of ultraviolet radiation effects on oxidative stress levels between lizards from lowlands and highlands. However, it is suggested that lizards from lowlands cope with more oxidizing conditions that highland lizards.

Additionally, the fact that lizards from highlands are more colorful in terms of sexual badges, suggest that they are able to invest more resources in colorations. This might be due to their more resources, as a result of their less oxidative stress levels and their possibility to invest pigments in colorations instead of antioxidant molecules. This might be also due to visual signals dominating over the chemical ones, which might have been selected in a more colder and humid environment.

In summary, it seems that the lizard *P. algirus* has undergone a suitable evolution along the elevation gradient, especially at highlands. This will result in a positive factor in a future scenario where lowlands populations would shift their distribution in elevation (or latitude) as consequence of global climate change.

Resumen

Conocer los procesos evolutivos que han ocurrido en el pasado, y que han dado lugar a los organismos tal y como los conocemos en el presente, es una manera de predecir como responderán a los cambios ambientales en el futuro. En el marco de cambio climático global las especies (sobre todo de ectotermos que dependen más estrechamente de la temperatura) ascienden en latitud y altitud, encontrándose en el proceso con nuevas condiciones ambientales que afrontar. Los estudios en ambientes geográficos que presentan a su vez gradientes ambientales, así como los organismos que habitan en estos gradientes, nos proporcionan una amplia información de los mecanismos evolutivos y las adaptaciones que han ocurrido a lo largo de la evolución de dichos organismos. En este sentido, los gradientes altitudinales funcionan como una buena herramienta para el estudio de la ecología evolutiva.

Con el trabajo contenido en esta tesis se pretende conocer las adaptaciones de los lacértidos en los gradientes altitudinales, utilizando como modelo las poblaciones de lagartija colilarga (*Psammodromus algirus*) en la ladera sur de Sierra Nevada (Granada, España), a lo largo de un gradiente de 2200 m de altitud (300-2500 m snm). Siendo las hipótesis de trabajo las siguientes:

1) La coloración dorsal será más oscura a mayor altitud, para favorecer la termorregulación (temperaturas más bajas) y para proteger de la radiación ultravioleta (más intensa).

2) Puesto que la radiación ultravioleta es conocida como un importante agente oxidante, esperamos que las lagartijas a mayor altitud estén más afectadas por la radiación ultravioleta y tengan mayor estrés oxidativo que las de altitudes bajas.

3) En caso contrario, es posible que o bien se han seleccionado rasgos que protegen frente al daño oxidativo de la radiación (coloraciones más oscuras), o bien que la radiación ultravioleta no es un agente oxidante para este animal.

4) Además el color dorsal será similar al color del sustrato, seleccionándose coloraciones crípticas para disminuir el riesgo de ataques por depredadores aéreos.

5) La coloración ventral y las señales de coloración visuales asociadas a esta zona del cuerpo (en el caso de la especie de estudio: mancha amarilla en el pecho y comisura naranjas en el lateral de la boca) será más vistosa en ambientes donde la disponibilidad de recursos sea mayor. Además será más intensa en lagartijas de más edad (asumiendo mayor estatus social), y serán más conspicuas en machos que en hembras (asumiendo que son señales sexuales utilizadas en la selección de pareja).

Para testar estas hipótesis se muestrearon 6 poblaciones a lo largo de este gradiente, durante cuatro años (2010-2013). Se capturaron cerca de 450 lagartijas adultas (machos y hembras) y se estudió su coloración (mediante los parámetros de brillo, saturación y matiz) dorsal y ventral mediante la utilización de un espectrofotómetro. También se estudiaron los niveles de estrés oxidativo *in situ* y en un experimento en el laboratorio (para conocer el daño por radiación ultravioleta). El estrés se midió a través de biomarcadores del daño oxidativo en lípidos y proteínas y de la capacidad antioxidante de los animales.

Los resultados obtenidos mostraron que, efectivamente, los individuos de *P. algirus* son más oscuros dorsalmente a mayor altitud (por encima de los 2000 m). Habiendo además una correlación positiva con las temperaturas medias ambientales (piel más clara en zonas de temperaturas más altas), y negativa con la radiación ultravioleta de cada área (piel más oscura en zonas con mayor intensidad de radiación). Además, existió una relación significativa entre el matiz de color de la piel dorsal de los animales y el color del sustrato en el que habitan, sugiriendo una selección de coloraciones crípticas.

Respecto a los niveles de estrés oxidativo, en contra de nuestras predicciones, los animales de mayor altitud presentaron menor daño oxidativo (no hubo diferencias en la capacidad antioxidante). Esto podría deberse a que la coloración más oscura se ha seleccionado en beneficio para proteger frente a los daños negativos causados por la radiación ultravioleta. Sin embargo, en el experimento realizado en condiciones de laboratorio en el que se expuso a un grupo de lagartijas a radiación ultravioleta, y a un grupo control a un espectro de luz visible, no se encontraron diferencias en los niveles de estrés oxidativo entre las poblaciones de baja, media, o elevada altitud. No obstante, si se observó que las lagartijas de altitudes elevadas presentaban menor daño oxidativo. Probablemente esto se deba a que las condiciones de las zonas de menor altitud son más oxidantes, por ejemplo porque la temperatura ambiental es mayor, o por la presencia de sustancias contaminantes procedentes de las actividades humanas. Además, aunque no se detectaron daños de la radiación ultravioleta a nivel de daño oxidativo, no se puede descartar la exisencia de daños a otros niveles, o a largo plazo.

En cuanto a la coloración ventral, *P. algirus* muestra un sistema de señales múltiples en el que varias señales informan sobre una misma característica del individuo, pero al mismo tiempo, varias características pueden ser señaladas por el mismo rasgo de color. Por ejemplo, los machos presentaron comisuras naranjas como indicativo del sexo, del tamaño y de la edad del portador. La mancha amarilla en el pecho o la garganta estuvo presente en machos y hembras. Esta mancha amarilla fue más saturada y de mayor tamaño en animales de mayor longitud y de más edad, pudiendo ser una señal de estatus social del portador. Además los individuos más vistosos presentan señales de haber sufrido intentos de depredación con más frecuencia, aunque es probablemente se deba a que son más activos, y no tanto a que son más conspicuos para los depredadores. Por último, se observó que las lagartijas invirtieron más en señales de coloración a mayor altitud (mayor proporción de individuos con manchas pigmentarias).

Estos resultados sugieren que *P. algirus* es un lacértido bien adaptado a las condiciones que encuentra en un gradiente altitudinal, y que se han seleccionado rasgos que permiten su presencia en altitudes elevadas, en condiciones en las que no se encuentran casi ningún otro lacértido mediterráneo. La coloración dorsal más oscura podría estar beneficiando en términos de termorregulación y de protección frente a radiaciones más intensas, ya que no se encuentra que las lagartijas de zonas elevadas estén perjudicadas por la menor temperatura o la mayor radiación ultravioleta. No obstante, parece ser que las lagartijas de altitudes más bajas están sometidas a condiciones más oxidantes que las de zonas altas. Por otro lado, parece ser que en los ambientes más fríos y húmedos que nos encontramos en las altitudes elevadas han seleccionado señales visuales más intensas, probablemente en detrimento de otras señales como las químicas.

En cualquier caso, parece ser que *P. algirus* está bien adaptada a los ambientes que encuentra a lo largo del gradiente altitudinal, y esto la beneficiará en un escenario futuro si las poblaciones de menor altitud tuvieran que ascender en altitud o latitud debido al cambio climático.

Chapter 2
GENERAL INTRODUCTION

Why to study the coloration of animals? How coloration evolves and which evolutionary forces influence it.

Organisms' adaptations and the evolutionary mechanism can be studied from diverse approaches: distribution, behavior, reproduction investment, social organization, inter- and intra-specific interactions, physiology, etc. Accordingly, coloration is a trait that can merge all these approaches into one, since coloration is largely involved in many aspects of the biology of the organism; from behavior to physiology, including reproduction or predation. These characteristics are under selection, and if the environment changes it could also be expected a change of the organism's characteristics. Furthermore, if the change in environmental condition is gradually, we should find gradual changes in the organism's characteristics according to these gradients.

Why to study the coloration of animals? How coloration evolves and which evolutionary forces influence it.

Coloration along with body-shape is the first trait that conspecific and interspecific individuals see from another individual, although probably odor is the first signal that they detect. Communication based on visual signals depends on different conditions: distance between sender and receiver, the habitat, the climate conditions (Bradbury and Vehrencamp 2011). The high color variation that exists in the biosphere is not only restricted to animals, and their function is almost equal in some aspects in all taxa, being communication or predation avoidance probably the most important. The diversity of colorations and color patterns not only exists between taxa, but also in the same taxon we can find variation between populations, or between individuals within the same population, too. This huge diversity makes coloration to be a relevant and interesting character in many areas of study including physiology, evolutionary ecology, or genetics (Grill and Rush 2000; Hill and McGraw 2006b).

In the animal kingdom, coloration participates in numerous fundamental components of the organism biology including thermoregulation, predation avoidance, and communication (Grill and Rush 2000; Endler 1990; Hill and McGraw 2006b; Bradbury and Vehrencamp 2011). Consequently, many evolutionary forces influence this character. With regard to the communication, for an efficient information flow between intraspecifics to take place, color patterns should be conspicuous and attractive, so that detection by intraspecifics is easier. However, when the color function in the opposite, that means to hide information, *e.g.* about the presence of the individual to minimize detection by predators, color patterns should be inconspicuous or cryptic (Endler 1990; Caro 2005). At this level, sexual and natural selection conflict each other, since sexual selection favors more conspicuous male displays (Andersson 1994), whereas natural selection favors less conspicuous displays (Caro 2005). In lizards, for example, inconspicuous colorations are preferred for dorsal body side, whereas attractive signals are selected for ventral and lateral sides (Pérez i de Lanuza 2012; Norris and Lowe 1964). In this way, lizards achieve to be cryptic for predators while being conspicuous for conspecifics.

How does coloration get involved in the biology of animals and which evolutionary forces cast it?

The main goal in the biology of the organisms is to reproduce and bequeath their genetic information to the next generation. However, before doing that, individuals must overcome diverse obstacles along their lives. Therefore, in order to pass along their genes to the next generation, individuals must survive, find a partner, and sometimes take care of their offspring. Adaptations to get all these aims are under selection, and coloration largely partakes.

Antipredator strategies are essential for the survival of the individuals. To be depredated has obvious costs that individuals can try to minimize by cryptic or antipredator coloration (Husak et al. 2006; Caro 2005). Cryptic colorations usually appear in the most exposed parts of the body, such as dorsal side or limbs, and the colors match well with environmental background (Endler 1990). The degree of crypsis or conspicuousness of an animal is obtained by measuring the similarity between its color pattern parameters with those of the background (Endler 1990). In order to maximize crypsis, the difference with background should be minimized (Endler 1990). An alternative strategy is the presence of disruptive color patterns such as stripes or patches along the body (Pleguezuelos et al. 2010), or hence aposematic colorations (*e.g.* Niskanen and Mappes 2005). Anyway, selection by predation is an attractive hypothesis to explain the variation in color background and color patterns in many species within the animal kingdom (Slagsvold et al. 1995; Crisp et al 1979; Stevens and Merilaita 2009; Lai et al. 2008; Rosenblum et al. 2004; Hoekstra 2006; Brown and Thorpe 1991). However, when we think that predation pressure is modeling the coloration patterns it is not always possible to be demonstrated (Stuart-Fox et al. 2004). Otherwise, conspicuous coloration can be selected if the strategy is diverting predator's attention to a specific part of the body, as happens in several lizard species. Lizards have tail autotomy and regenerate it after autotomy, a widespread trait as an adaptation for escape from predators (Fox et al. 1994). Linked to that, some species have evolved conspicuous colors in tails to distract the predators' attention, and improve the tail autotomy strategy, especially during immature states (Hawlena 2009).

Another important goal for organisms to survive is to keep the body in good physiological conditions in order to optimize their physiological functions. Body temperature is an important trait to achieve this objective, but not every taxon is capable to produce and keep thermal energy as endotherms do. Ectotherms are those animals that need of environmental thermic energy to rise and maintain their body temperature in optimal values. For obtaining or keeping this optimal body temperature, different strategies have evolved in ectotherms such as basking behaviors (Crisp et al. 1979) or larger body size (*e.g.* Bergmann's Rule; Bergman 1847; Ashton 2002; Ashton and Feldman 2003). Regarding coloration, in this group of animals is especially important the color, and particularly dark (melanin-based) colors of the skin (Gunn 1998; Karl et al. 2009; Clusella-Trullas et al. 2007). The "thermal melanism hypothesis" proposes that darker (low skin reflectance) individuals should heat faster and reach higher equilibrium temperatures than lighter (higher reflectance) ones, assuming a similar body size (Gates 1980). Based on this idea, Clusella-Trullas et al. (2007) assessed the following four predictions that may be derived from the hypothesis: i) melanistic diurnal species inhabit cooler areas than lighter colored species, ii) melanism results in greater fitness in cold climates, iii) there is a trade-off between melanism and body size and, iv) color, behavior, and physiology are coadapted. Different studies point out the relation between melanism and environmental temperatures (*eg. Rana temporaria*; Alho et al. 2010), and the benefits of dark tegument in environments with inefficient thermal conditions. Environments with these conditions are located for example in northern latitudes, or at higher elevations, as well as closed and forested habitats in contrast to open and desert ones. Reptiles, because of their ectotherm condition, rely upon external heat sources to increase body temperature (Vitt and Caldwell 2009) and are good models to study the thermal melanism hypothesis (*e.g.* Clusella-Trullas et al. 2008; Clusella-Trullas et al. 2009; Janse van Rensburg et al. 2009). It should be expected that melanism in reptiles provides thermally adaptive benefits (*eg. Vipera berus*; Forsman 1995; Tanaka 2005). In colder habitats, ectotherms may expend more time basking (Carrascal et al. 1992), implying more time exposed to predators. Darker coloration allows in several examples to heat up faster, reducing the basking time necessary for thermoregulation, leaving time for other duties, such as foraging, and moreover, reducing the predation susceptibility (Gvoždík 2002). Occasionally, benefits of being darker arise from a combination of traits, such as darker teguments and bigger body size (to keep thermal energy) or thermoregulatory behaviors (*e.g.* basking) (Tanaka 2009; Tanaka 2005; Jambrich and Jandzik 2012; Gvoždík 1999; Gabirot et al. 2013).

An important handicap for ectotherms, and especially for those that are heliotherms, is that they spend a long time basking under solar radiation. This means that they are long exposed to damages induced by ultraviolet radiation (UVR) (Iwai et al. 1999; Blaustein et al. 1998; Chang and Zheng 2003; Dahms et al. 2011; Liu et al. 2006). In this regard, especially melanin-based coloration protect organisms against UVR-damage (Brenner and Hearing 2007; Ortonne 2002), thanks to this compounds' capacity to absorb and dissipate UVR-energy (Kollias et al. 1991).

How, who and what to communicate? The visual signals evolution.

Once the individual has covered survival and metabolic requirements, the next important need is to find a partner to breed and leave offspring. At this level of the life history of the organisms, intra-specific communication plays an important role in the fitness of the individuals, not only during mating but also during territory defense and dominance hierarchies. Consequently, intra-specific communication works during intra- and inter-sexual interactions.

The channels of communication can be diverse and use really different mechanisms. The most important thing is that the signal being emitted may stand out over the environment and other signals (Endler 1990; Endler 1993). Channels or mechanisms of communication can be auditive, tactile, chemical, and visual, or a combination of several, depending on the taxon and also on the environment (Bradbury and Vehrencamp 2011). Occasionally, depending on the environmental conditions, one or other channel is more effective and is evolutionarily selected. For example, chemical signals are usually selected in animals that inhabit dry and hot environments (Abert 1992). This is because of the composition of the compounds used in chemical signalization, a combination of holocrine "waxy" secretion produced by glands. The secretions are composed of lipid and protein substances, *e.g.* steroids, fatty acids, alcohols, within others, in different proportions (Martín and López 2014). The secretory glands can be located in different parts of the animal, being precloacal or preanal a very frequent position. In reptiles are important the femoral pores, present both in females and males, but it is in males where gain prominence, especially during the mating season (Martín and López 2014). The condition-dependence of chemical signals is closely related to health condition and good quality of the individual that is producing them (Martín and López 2014). In this sense, the mechanism of evolution of chemosignals is rather similar to the visual signals. Both are expensive to produce and honestly inform the receivers about the good qualities of the sender (Zahavi 1975). Chemosignals closely depend on environmental conditions to spread (Abert 1992; Martín et al. 2013; Endler and Basolo 1998); because of their chemical composition, lipophilic compound, do not spread well in wet atmospheres (Abert 1992; Endler and Basolo 1998). Accordingly, it should be expected that other mechanisms of communication, such as behavior or visual signals may work better and may be selected in wet and cold environments.

The intra-specific communication based on visual signals works when these signals (usually color ornamentations) convey information about the senders' characteristics and qualities to the receivers (Dale 2006; Griffith et al. 2006; Hill and McGraw 2006b; Senar 2006). Since different ornaments can reveal different qualities or life strategies i) the same individual can show multiple color signals (multicomponent signals; (Martín and López 2009; Langkilde and Boronow 2010; Cuervo and Belliure 2013); ii) the same population can show individuals with different color morphotypes (chromatic polymorphism; Sinervo and Lively 1996; Bleay, Comendant and Sinervo 2007; Pérez i de Lanuza et al. 2012; Blouin-Demers et al. 2013; San-José et al. 2014); and/or iii) the same species can show sexual dichromatism, being males usually the ones displaying color ornamentations (Díaz 1993), although also females can show color ornamentations in some species (Calisi et al. 2008; Cuervo and Belliure 2013). Furthermore, sexual dichromatism can be in the ultraviolet (UV) spectral range (Pérez i de Lanuza and Font 2007; Font, Pérez i de Lanuza, and Sampedro 2009; Pérez i de Lanuza 2012; Pérez i de Lanuza and Font 2014a). Color signals usually are developed during breeding season and inform to other males (intra-sexual communication) about its dominance and strength, or inform to females (inter-sexual communication) about their quality (Martín et al. 2008; Cote et al. 2010; Martin et al. 2013; Molnár et al. 2013); information flux can be bidirectional from females to males too, although in fewer cases (Cuervo and Belliure 2013; Stuart-Fox and Goode 2014). For an efficient communication in courtship or territoriality, the difference between the animal and environmental background should be maximized (Endler 1990).

In lizards (but also in other taxa), males' dominance and social status can be reflected through different color badges (Díaz 1993; Olsson, 1994a; Sinervo and Lively, 1996; Bastiaans et al. 2013; Hamilton et al., 2013). Frequently, these males are more aggressive, have stronger bite force, which is reflected in larger and more robust heads (Martín and López 2009; Kaliontzopoulou et al. 2012). They also own and guard larger territories, which usually results in mating with more females (Salvador and Veiga 2001). Dominance traits are conveyed by color ornamentations, both pigment-based and structural-based colorations (Sinervo and Lively 1996; Díaz 1993; Bastiaans et al. 2013), as it is illustrated in the following examples. Dominant *Lacerta schreiberi* males have throat colorations that are brighter and more saturated in UV and blue wavelengths, and they have darker and greener coloration in dorsal body surface (Martín and López 2009). Likewise, males of *Psammodromus algirus* with larger and more orange head colorations dominate over dull males and keep larger territories and mate with more females (Díaz 1993). Frequently, aggressive and dominant behaviors are related to higher levels of testosterone in blood, and it has been observed that testosterone can especially induce the orange or yellowish ventral patches development (Sinervo et al. 2000; Salvador et al. 1997; Olsson et al. 2007). These badges not only convey the male's fighting capacity, but also avoid future fights between unequal males, since weak males can recognize strong males from the distance and avoid hand to hand combats (Olsson 1994a).

Under inter-sexual communication, females act as selective agents choosing for those males with better characteristics. Females select "attractive" males as mates because they should produce better quality offspring that will be reproduce more and eventually transmit better their genes along generations. Females benefit from mating with high-quality males by having genetically superior offspring (with higher return of fitness; Calsbeek and Sinervo 2002; Olsson et al. 2003; but see Noble et al. 2013), and also indirectly by reproducing in high-quality territories (with more probabilities of survival for the offspring; Calsbeek and Sinervo 2002). In this sense, coloration traits again work as signals that inform females about the males' qualities. Continuing with this idea, males with better body condition usually display more colorful and attractive color patches (Folstad and Karter 1992; Guindre-Parker and Love 2014). For example, in the sand lizard (*Lacerta agilis*), males with better body condition show larger and more saturated green pigmentary patches around the throat and chest (Olsson 1994b). In Pyrenean populations, they also reflect more saturated patches in the UV spectral range (Pérez i de Lanuza and Font 2007). In the same way, males of *Podarcis muralis* and *Timon lepida* display more UV saturated patches when their body condition is better (Pérez i de Lanuza et al. 2014; Font et al. 2009). A good body condition can reflect a good health status of the bearer, but also other traits, such as physiological status represented by a good immune response or good oxidative balance. Both physiological conditions are closely related to coloration traits and usually those males with good health status can display more attractive color ornamentations. Females can also show breeding coloration, although it is less frequent, indicating quality or receptiveness to males (Cuervo and Belliure 2013; Stuart-Fox and Goode 2014; Calisi et al. 2008; Comendant et al. 2003). In some cases, coloration indicates the female's receptiveness, whereas in other species it conveys the gravid status of the female, avoiding in this way undesirable meetings with males.

To be sexy has its costs. "Honest signals" and the "Handicap Principle".

The proper functioning of "honest signals" states that displaying this signal must inflict a cost for the bearer ("the Handicap Principle"; Zahavi, 1975; Zahavi and Zahavi 1997). Costs can be given at different levels: predation, parasitism, physiological imbalance (*e.g.* immune response or oxidative stress). Although not impossible, faking these kinds of signals is difficult, because their production has costs for the bearer at different levels. As a consequence, cheater males will be eliminated from the population because if they are not strong or dominant enough (as their ornaments signaled), their fitness will be reduced as a consequence of increased parasitism, oxidative stress or fights with dominant males. A good example of this type of signals is the species studied in this thesis, *Psammodromus algirus*. Salvador et al. (1996) demonstrated that although subordinate individuals of *P. algirus* are able to produce breeding coloration with the correct testosterone stimuli, they do not do due to the cost of fighting with larger males and the costs of increased parasitism in testosterone-treated individuals.

Individuals with more attractive colorations are not only more visible for conspecifics, but also for predators. On the other hand, brighter colorations may attract predators, increasing predation risk (Zuk and Kolluru 1998). Usually antipredator strategies are selected to cope with this handicap, such as changing escape behavior and activity (Cabido et al. 2009; Martín and López 1999a; Martín et al. 2009), developing color badges in less visible parts of the body as ventral side (Pérez i de Lanuza 2012), or developing color signals in the UV spectral range, which predators such as mammals cannot perceive (Font et al. 2009; Pérez i de Lanuza and Font 2014b; but see Modarressie et al. 2013)). However, most of the time, the more conspicuous is an individual, the more susceptible of suffering a predator attack is (*e.g.* Martín and López 1999b; Martín and López 2001; Husak et al. 2006; but see Olsson 1993; Stuart et al. 2012).

Parasite-derived costs of coloration have also been largely studied. Hamilton and Zuk were the pioneers in this area (Hamilton and Zuk 1982), but many works have proved their hypothesis (Møller et al. 1999). The fact is that higher levels of testosterone, which are frequently necessary to develop color ornamentations, may provoke a weakening of the immune system (Salvador et al. 1996; Fargallo et al. 2007; Folstad and Karter 1992), a situation that is exploited by parasites to infect the bearer's body (Salvador et al. 1996; Salvador and Veiga 2000; Calisi et al. 2008). In this case, what the color ornamentation is signaling to receivers is the bearer's capacity to cope with this parasitic infection, something females select as a good quality for their offspring. On the contrary, those that cannot cope with parasites' effects can suffer a deterioration of their sexual signals. For example, the presence of parasites can reduce the size of the melanin-color patches, as well as carotenoid-based colorations (Fitze and Richner 2002); or reduce color brightness in lizards (Calisi et al. 2008).

However, the immune system can be weakened by other procedures, apart from higher testosterone levels in the organism. Pigments used to produce color badges are also directly or indirectly involved in immune response, which results in the production of pigment-based colorations (carotenoids and melanins) weakening the immune response (Alonso-Álvarez et al. 2004b; Guindre-Parker et al. 2013) or destabilizing the oxidative balance in the organism (Olsson et al. 2012; Galván and Alonso-Álvarez 2008). On the one hand, melanins are involved in the pathway of synthesis of gluthatione (Ortonne 2002; Hill and McGraw 2006a), an important substance directly related to keep the oxidative balance. Notwithstanding, also corticosterone are involved in melanogenesis pathway, being in part a depressant of melanin synthesis (Roulin et al. 2008). On the other hand, carotenoids play a quite similar role as melanins. However, animals are unable to synthesize them, and they have to obtain carotenoids in the food, and then use the compounds in different physiological functions of the organism (*e.g.* as antioxidants, immune response, or color ornaments). This multiple function is what provokes the trade-off between carotenoid-based coloration production and physiological functions of the organism (Fitze et al. 2009; San-José et al. 2013; Alonso-Álvarez et al. 2008). Furthermore, carotenoid-based ornaments can reflect the presence of these compounds in the individual's diet due to several reasons: the availability of these compounds in the environment is limited, the foraging ability of the bearer to get the necessary resources (pigments) to produce them, and the capacity to distribute the resources between the diverse functions in the organism. Nevertheless, more studies about the hypothesis of the trade-off between the availability of pigment resources in diet and their re-allocation in pigmentary patches are necessary, since some attempts to demonstrate this relation have failed (Isaksson and Andersson 2008; Fitze et al. 2009) suggesting, however, that pigments ingested in diet have other effects in physiological functions.

Definitely, coloration is costly in terms of resources, and only individuals with enough resources to spare in badges would be capable to produce them. In this case, resources refer to the availability of enough pigments in order to use them in physiological functions, coloration and homeostasis. It can also refer to the resources required to cope with predators (*e.g.* shelter availability) or parasites (*e.g.* good body condition derived from a good feeding, to cope with parasites effects). Cheaters are not possible because the cost associated to these signals is too high to "low-quality" individuals (Olson and Owens 1998).

In view of these aspects about coloration, when we talk about the evolution of coloration and color signals we must consider not only which evolutionary forces are affecting them, but also which mechanisms of production are working on them. Moreover, owing to the cost of producing coloration, we can predict that color ornaments may be brighter and more attractive in: those environments with a higher availability of resources, and also in those where mating opportunities are scarce (such as higher density populations, or males population ratio biased) forcing individuals to do their best for mating chance. An example of this was found in *Zootoca vivipara*, whose change in red coloration depends on the oxidative stress balance of the lizard. However, the situation is different if the lizard is in a cute or in long-term stress situation. When lizards experience low food availability, they invest more resources in red coloration, as a "terminal investment" in a probable last reproduction event (Cote et al. 2010). Geographic variation of resources may lead to differential selection in color ornamentations and differences in morphotypes and strategies (Endler 1995; Dunn et al. 2008; Chui and Doucet 2009). Moreover, not only foraging resources vary geographically, and other selective pressure can differentially work on coloration expression (Comendant et al. 2003; Forsman and Shine 2006; San-José et al. 2012).

Why to study oxidative stress?

"All aspects of aerobic life involve free radicals and antioxidants: you cannot escape them, nor should you wish to" (Halliwell 2007)

Why to study oxidative stress?

“All aspects of aerobic life involve free radicals and antioxidants: you cannot escape them, nor should you wish to” (Halliwell 2007). The study of the oxidative stress in organisms is important and useful because of the important overlaps between ecology of stress and many aspects of the organisms (von Schantz et al. 1999; Costantini 2008; Monaghan et al. 2009; Dowling and Simmons 2009; Costantini et al. 2010; Metcalfe and Alonso-Álvarez 2010; Costantini 2014). Oxidative stress is involved in such important aspects as reproduction (Bertrand et al. 2006; Kim et al. 2010a; Olsson et al. 2009), or aging and lifespan (Harman 2006; Pérez-Campo et al. 1998; Robert et al. 2007; Dowling and Simmons 2009; Selman et al. 2012; Finkel and Holbrook 2000). For this reason, oxidative stress is considered one of the most important ecological and evolutionary forces (von Schantz et al. 1999; Costantini 2008; Monaghan et al. 2009; Dowling and Simmons 2009; Costantini et al. 2010; Metcalfe and Alonso-Álvarez 2010; Costantini 2014). Consequently, the more we know about oxidative axis running, the more we know about evolutionary ecology of the organisms (Boonstra 2013). Oxidative balance inside of the organisms tightly depend on the environmental conditions, therefore, it should be expected that in changing environments, physiological and consequently oxidative stress conditions may change too (Costantini and Dell’Omo 2006).

As explained above, oxidative balance is also tightly related to coloration. On the one hand, pigments usually used in sexual colorations are also involved in synthesis pathways of antioxidants (Alonso-Álvarez et al. 2004; Galván and Alonso-Álvarez 2008). However, concerning to this thesis, it is also important the melanin-based coloration and the protection they play against oxidative damage produced by UVR from the Sun. Negative effects of UVR on oxidative balance, as well as the protection-mechanisms carried out by melanins in this sense are developed in subsequent sections of this thesis.

What is oxidative stress?

There are lots of reactive species in the organism, especially of oxygen and nitrogen (but also aldehydes, quinones, or epoxies), which are continuously produced by metabolism and also induced by the environment (Jones 2008; Finkel and Holbrook 2000). Frequently reactive species are employed in important cellular events such as gene modulation, local and systemic defense response, or apoptosis and necrosis, among others (Halliwell 2007; Scandalios 2002; Figure 2.1). However, these reactive oxygen and nitrogen reactive species (RONS) also mediate damage in molecule, cell and tissue structures (Finkel and Holbrook 2000). Fortunately, antioxidant substances exist and combat RONS. Therefore, antioxidants can be defined as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell 2007; Halliwell and Gutteridge 1995). In other words, antioxidant defenses control levels of RONS rather than eliminate them (Halliwell 2007) and in this way they maintain an equilibrium between RONS production and RONS-mediated damage. However, when the balance between antioxidants and pro-oxidants (*e.g.* RONS) breaks, an oxidative imbalance happens and oxidative stress occurs in the organism (Helmut Sies 1991; Halliwell and Gutteridge 1995; Halliwell 2007; Scandalios 2002; Figure 2.1). Usually this imbalance leads to potential damages. Accordingly, (Halliwell and Whiteman 2004) defined oxidative damage as “the biomolecular damage caused by attacks of reactive species upon the constituents of living organisms”.

Fortunately, organisms are protected against oxidative damage by antioxidant defenses and damage-repair mechanisms, which work to maintain RONS levels at equilibrium and minimize RONS damages in the organism (Sies 1991; Finkel and Holbrook 2000; Blokhina et al. 2003). Changes in RONS levels lead to the activation or silencing of genes encoding transcription factors that subsequently can derive in antioxidant enzymes production (Scandalios 2002). However, the protection against oxidative damage has a cost. Production and maintenance of antioxidants implies energy and resources consumption and, therefore, it is costly (Halliwell 2007). This explains why the study of oxidative stress is so interesting. The existence of a trade-off between allocate resources to keep oxidative balance or for other metabolic functions (*e.g.* immune response or mating coloration), is central to our understanding of life-history evolution (Monaghan et al. 2009; Dowling and Simmons 2009).

Oxidative stress is a host of facts that happen in the organism; therefore, we can approach the study of oxidative stress from different perspectives. In other words, we can consider different biomarkers to quantify the magnitude of this oxidative imbalance, that is, the oxidative stress. In particular: i) we can quantify RONS concentration, ii) we can evaluate oxidative damage in cell structures, lipids, proteins, and/or DNA, iii) we can measure antioxidant capacity through enzymatic and non-enzymatic antioxidants' activity, hence iv) we can measure repairing mechanisms. The best option would be the combination of different biomarkers in order to have a wider approach of the oxidative stress levels in the organism.

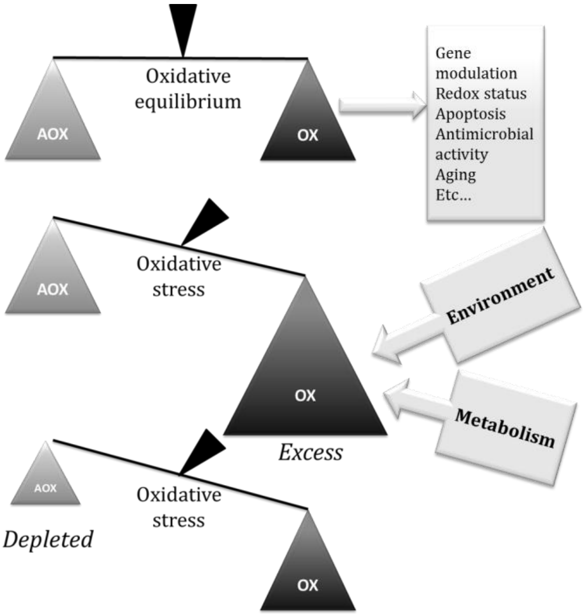

Figure 2.1. Oxidative stress results from imbalance between oxidants (OX) meanly reactive oxygen species and antioxidants (AOX). Under normal circumstances, cells are able to balance the production of oxidants and antioxidants (such as catalase and superoxide dismutase), resulting in redox equilibrium. Even OX are used in physiological functions of the organism (Gene modulation, antimicrobial activity, etc). OX production can be triggered by extern (environment) and endogenous (metabolism) factors. Oxidative stress occurs when cells are subjected to excess levels of ROS, or as a result of depletion in antioxidant defenses (*adapted from Scandalios 2002*).

How oxidative stress is triggered and what are the consequences

These various oxidative molecular species can be generated from several different sources, both endogenously and exogenously. On the one hand, the own metabolism of organisms produces this species endogenously, *e.g.* in mitochondrias during aerobic metabolism, peroxisomes, etc… (Finkel and Holbrook 2000). Consequences of high metabolism rates have been described by the "free-radical theory of aging" (Harman 2006; Pérez-Campo et al. 1998; Robert et al. 2007; Dowling and Simmons 2009; Selman et al. 2012; Finkel and Holbrook 2000). Accordingly, organisms with higher growth rates (therefore, with higher metabolism rates) shows less antioxidant capacity and higher oxidative damage (Monaghan et al. 2009; Kim et al. 2010a; 2010b).

On the other hand, several environmental factors can exogenously trigger RONS production, *e.g.* environmental toxins, temperature, oxygen concentration, ultraviolet radiation, ionizing radiation, etc (Finkel and Holbrook 2000). Environmental components, such as contaminants, produce oxidative stress at different levels (increment of oxidative damages in lipids and activity of antioxidant enzymes) in diverse animal groups (Berglund et al. 2007; Regoli 2000; Prevodnik et al. 2007). In particular, iron and cadmium, boost lipid peroxidation and catalase, gluthatione-S-peroxidases and gluthatione reductase activity in the pied flycatcher (*Ficedula hypoleuca*; Berglund et al., 2007). Similarly, pesticides and trace elements (*e.g.* heavy metals) increase antioxidant enzymes activity in the East Pacific green turtle (*Chelonia mydas;* Labrada-Martagón *et al.*, 2011). Moreover, environmental variables also produce oxidative imbalance. It is known that tolerance to sub-zero temperatures and its subsequent anoxia increase antioxidant enzymes during freezing in reptiles in order to deal with oxygen free radical overgeneration during thawing or reoxygenation (*e.g.* in the garter snake *Thamnophis sirtalis* (Hermes-Lima and Storey 1993a;1993b); and the European common lizard *Lacerta vivipara*; (Voituron et al. 2006)). In a similar way, animals that are under hypoxia conditions during diving (*e.g.* the freshwater turtle *Trachemys scripta*) have tolerance mechanisms to deal with oxidative stress during the anoxic-aerobic transition of recovery, what increases the antioxidant activity during tissues reoxygenation (Storey 1996; Hermes-Lima and Zenteno-Savín 2002). Furthermore, high temperatures, together with UVR, provoke oxidative damages in symbiotic algae and consequently bleaches in corals (Lesser 1997; Lesser 1996). Finally, ultraviolet radiation by photooxidation is an important stressor that could produce several sublethal effects - even mortality -on living organisms (Belden et al. 2000; Dahms et al. 2011).

Ultraviolet radiation effects on organisms and especially on oxidative stress levels

Visible and UV wavelength from the light spectrum are considered biologically active. Light can biochemically excite biomolecules to a higher energy state. However, given it is an unstable state, they return almost immediately to a lower state. During this transition, energy is released and may be transmitted to other molecules via electron transfer (Porter 1967). These reactions cascade is known as photooxidation and is an important cause of oxidative imbalance in the organism (Chuang and Chen 2013; Dahms and Lee 2010). For instance, aqueous mediums (*e.g.* cellular cytoplasm) in the presence of UVR result in the production of hydrogen peroxide and other reactive oxygen species (ROS). ROS are formed by the univalent reduction of molecular O_2 due to spin restrictions on its valence electrons, yielding superoxide radicals (O^{2-}). Further reduction of superoxide leads to the generation of hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•). Furthermore, the transfer of absorbed energy from activated photosensitizers to ground state O_2 (photodynamic action) produces highly reactive singlet oxygen (O•) (Halliwell and Gutteridge 1999). In summary, UVR initiates a series of redox reactions resulting in the release of free reactive oxygen and nitrogen species, which triggers an oxidative imbalance in cells and tissues (Dahms and Lee 2010). For this reason, UVR is an important environmental oxidative stressor, and therefore, it can produce damages from tissue (Chang and Zheng 2003; Liu et al. 2006) to DNA (Tadokoro et al. 2003; Ravanat et al. 2001), including at molecule and cell level (Halliwell and Gutteridge 1999). This consequently can end in sub-lethal or lethal effects in critical situations (Dahms and Lee 2010; Belden et al. 2000; Dahms et al. 2011). Frequent damages provoked by UVR are derived from the oxidative imbalance induced by radiation. For instances, it has been proved that UVR can provoke immune suppression in mice (Iwai et al. 1999), or damages at skin level (Chang and Zheng 2003; Liu et al. 2006), through the oxidative stress pathway. Actually, harmful UVR-effects have been detected in almost every animal kingdom, from zooplankton (Zellmer 1995) to mammals (Matts and Fink 2010), including corals (Lesser 1997; Lesser 1996), earthworms (Chuang and Chen 2013), fishes (Zamzow 2004; Charron et al. 2007), amphibians (Marquis et al. 2008; Blaustein et al. 1998; Belden et al. 2000), reptiles (Chang and Zheng 2003; Liu et al. 2006), and damages in human skin (Matts and Fink 2010). Although the magnitude of the UVR-damages depends on the organism, finally all of these damages lead to fitness loss (Hylander et al. 2014; Alonso-Álvarez et al. 2004).

Organisms have several mechanisms to cope with UVR-damage (and consequently the oxidative stress derived from photooxidation). The strategies can be divided in behavioral responses, physic barriers, and/or physiological mechanisms. The main behavioral strategy is to avoid the stressor. For instance, avoiding UVR by sheltering or through crepuscular or nocturnal activity (Zellmer 1995; Hansson and Hylander 2009; Holtby and Bothwell 2008). Among physic barriers, skin is the first and most important, since it covers the whole organism body surface. It does not only protect against solar radiation and its UV-component, but also against contaminants, parasites and pathogens. The characteristics of the skin *e.g.* thickness, color, and/or structure, are key to the protection (Porter 1967). In reptiles, almost all the UV-wavelengths penetrating the atmosphere would also penetrate their body wall (Porter 1967). Although wavelength range is reduced when we take into account the presence of the peritoneum. In standard conditions, the skin with its superficial layer of keratinous scales absorbs around 70% of the total radiation incident on the animal, including UVR (Porter 1967). Even though reptiles lack of fur or feathers, important structures of protection against UV-effects, keratinous scales together with the skin also can stop part of the UVR. For example, in the Cope's rat snake (*Elaphe taeniura*) it is known that only 5.1% of UVR-B crosses the keratinous layer to the epidermal stratum germinativum (Chang and Zheng 2003). Structural barriers, such as skin, usually are reinforced by the presence of sun-screen molecules. Either because these molecules absorb UVR or because they combat oxidant species, together they help to cope with oxidative stress. Photoprotective compounds have been found in several animal groups from invertebrates to vertebrates (Hansson and Hylander 2009; Cope et al. 2001; Hofer and Mokri 2000). Serving in this role are pigments such as melanins and carotenoids (Zellmer 1995; Hessen 1996; Gunn 1998), as well as non-pigments such as mycosporine-like amino acids, exclusive from some invertebrates and algae (Hansson and Hylander 2009). Structure and function vary among compounds (Hansson and Hylander 2009).

Melanins are able to absorb UV-wavelength and dissipate this energy as heat, but free radicals can be produced (photohomolysis) in this process (Hansson and Hylander 2009; Ortonne 2002). Melanins can also scavenge ROS, so they can protect pigmentary cells against oxidative stress (Rozanowska et al. 1999). However, efficiency in ROS scavenging is not too effective and depends on the radical lifetime and how close melanins and ROS are (Rozanowska et al. 1999). Carotenoids are also pigments that can inhibit photo-sensitive oxidation and be an efficient quenching of ROS (Edge et al. 1997; El-Agamey et al. 2004). However, unlike melanins, which are synthetized by the organism, they must be ingested in the diet. Quenching of singlet oxygen mainly leads to energy dissipation as heat, whereas the reaction of carotenoids (or any antioxidant) with free radicals will lead to electron transfer or possibly to additional reactions (El-Agamey et al. 2004). Antioxidant molecules are not only associated to physics barriers; they are also present in other tissues and especially in blood from where antioxidant species are distributed to the rest of the organism.

Physiological antioxidant mechanisms are dominated by antioxidant enzymes, but also by non-enzymatic antioxidants, such as pigments (as discussed previously), vitamin E, or gluthatione. The total antioxidant capacity can be approach by the trolox-equivalent antioxidant capacity (TEAC), a method that includes both enzymatic and no-enzymatic antioxidant cell mechanisms. But also antioxidant enzymes activity can be individually quantified, and the most used in these kinds of researches are: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione transferase (GST), DT-diaphorase (DTD), and peroxidase.

Phenotypic variation in geographical gradients: an especial one, the elevational gradients

The ecological environment is not equal throughout the planet. Because of the form of the planet and its position around the Sun, ecosystems and their environmental conditions change not only geographically along the latitude and longitude, but also along the time (seasonal and along years variation). We can easily observe gradual variation (although not always is so gradual) in environmental factors. Temperature is probably the most important, since several other environmental variables depend on it. Furthermore, it has gained importance during the last decades and nowadays, since it is one of the main environmental factors affected by global climate change (Graae et al. 2012). We find a warm-cold temperature gradient from the ecuador to the poles. In the same way, solar radiation, and its more harmful component, the UVR, increases toward the ecuador. However, due to the ozone layer deplete it is expected an increase towards the southern latitudes (Madronich et al. 1998; Newman and McKenzie 2011). According to this latitudinal gradient, or in other differential environments, the phenotypic adaptations that we observe in organisms can be explained as result of selection by the breadth of climatic conditions they experience along the diverse geographical patterns (Stevens 1992).

Thus, geographical diversity in environmental conditions offers us diverse examples of variation in the characteristics of the organisms; however, this variation can be observed at different levels of organism biology. In a first approach, we observe an enrichment of the species diversity towards the equator (Stevens 1989), associated to the use of their niche and diverse environmental variables. Furthermore, the genetic diversity varies geographically due to the genetic population dynamic (Nevo 1978). And finally, this is reflected at the phenotypic level, related to morphology and physiology. Bergmann's rule is well known, as an explanation of body size increase in animals with latitude (or towards colder environments) to optimize the body thermodynamic (Bergman 1847). Moreover, variations in tegument coloration have been reported in relation to diverse selective forces. Under temperature selection, the "thermal melanism hypothesis" states that individuals or species with darker teguments may be found in colder or low thermal-quality environments (Clusella-Trullas et al. 2007; Janse van Rensburg, Mouton and van Niekerk 2009; Clusella-Trullas et al. 2009). According to that, we can find darker individuals in the same species in northern latitudes (Alho et al. 2010). UVR also selects for darker colorations (rise in melanins) that protect against its harmful effects (Jablonski and Chaplin 2010; Brenner and Hearing 2007); however, in this case the cline that we observe is darker skin in individuals towards the equator, *e.g.* in humans (Parra 2007). Together with abiotic variables, biotic variables also vary geographically and act as selective agents. An important effect results from predation pressure that selects for cryptic coloration, depending on ground (or environmental) color (Thorpe and Brown 1989; Husak et al. 2006; Jambrich and Jandzik 2012; Vidal et al. 2007); or differential parasites incidence, as it has been seen in birds and the darken of feathers in localities where parasitism is higher (Roulin et al. 2009)

Studies in latitudinal gradients can help to understand selection on characters and organism adaptation to diverse environmental conditions. Furthermore, studies along climatic gradients can help to assess the effects of temperature on ecological processes in the current global warming scenario (Graae et al. 2012). These geographical gradients allow us to compare organism responses to different conditions, as well as to understand the past and present, and predict future responses to these changing environments. Nevertheless, there can arise some kind of logistical problems by using latitudinal gradients: geographical ranges are usually large, which implies more time, expensive budgets, and more staff to carry out any research within them. A good alternative for these kinds of studies are elevational gradients.

Why are elevational gradients interesting?

Elevational gradients, as latitudinal gradient, offer a very interesting opportunity for carrying out research and studies in evolutionary ecology (Stevens 1992; Körner 2007; Keller et al. 2013). Not only environmental variables change with elevation, but also the vegetation physiognomy, geological resources, and human uses of the habitat. Many of these variables vary in a similar way than in latitudinal gradients (Stevens 1992; Körner 2007; Graae et al. 2012), which makes possible in some cases to compare the studies in one and another geographical gradient. This similarity allows to carry out similar studies and extrapolate results obtained in elevational gradients to latitudinal conditions, but with the advantage of shorter distance. Consequently, budgets and logistic requirements are in general smaller (Körner 2007). However, we must be cautious since not all environmental variables change in the same direction and intensity in both geographical gradients. For example, UVR increases with elevation (Blumthaler et al. 1997; Sola et al. 2008) but decreases with latitude (Madronich et al. 1998; Newman and McKenzie 2011). In case of ectotherms, temperature and UVR are the most important environmental variables that may affect their biology. The first one, because, as ectotherms, they depend on environmental temperature to keep their bodies warm and carry out their vital functions in a correct way (Vitt and Caldwell 2009). The second variable is important because many of the ectotherms species are heliotherms and keeping their optimal body temperature depends on basking under solar radiation to obtain the necessary thermal energy (Vitt and Caldwell 2009). Moreover, at higher elevations, where temperature is colder, they spend more time basking (Carrascal et al. 1992), and consequently, more time exposed to UVR. Both environmental variables have been considered as important selective pressures, and as discussed in previous sections, coloration is a good trait candidate for being under selection in elevational gradients. Although not only temperature and UVR can affect coloration, but also predation pressure, resources availability, or different sexual selection pressure that usually change with elevation too (Badyaev 1997; Fox et al. 1994; Cooper 2010).

According to these qualitative changes in the environment along the elevational gradient, organisms might also change in their characteristics (Badyaev 1997; Blackburn and Ruggiero 2001; Castella et al. 2013; Fu et al. 2004; Leaché et al. 2010; Sinsch and Lehr 2010; Welter-Schultes 2000; Gabirot et al. 2013). Frequently, phenotypic variations in altitude match with those observed in latitude (Ashton et al. 2000; Ashton and Feldman 2003; Ashton 2002; Blackburn and Ruggiero 2001), as well as some ecological patterns such as biodiversity variation (Stevens 1992; Stevens 1989). According to the "thermal melanism hypothesis" (Clusella-Trullas et al. 2007), we found that many species get darker with altitude, as a consequence of selection by colder temperatures (Leaché et al. 2010; Sinsch and Lehr 2010). Although the photoprotective effect of dark coloration has been less studied in relation to elevational gradients, it is also known that melanin-based coloration protects against harmful effects of UVR from the Sun (Brenner and Hearing 2007; Jablonski and Chaplin 2010; Porter and Norris 1969). Therefore, it is likely that darker colorations at high elevations may be selected. Furthermore, other colorations such as carotenoid-based red coloration can also protect against UVR at high elevations and may be selected too (Cooper 2010). In this regard, it has been suggested that high-elevation populations may have a relatively increased tolerance to high levels of UVR, as for example, the common frog *Rana temporaria*, which shows a higher UVR-tolerance in highland populations than in lowlands (Marquis et al. 2008; Marquis and Miaud 2008).

In the global warming changing scenario our planet is experiencing, there are many different taxa that are changing their distributions (Hickling et al. 2006). Frequently they ascend in latitude (Perry et al. 2005; Hickling et al. 2006; Moreno-Rueda et al. 2012), but in a similar way, there are many others that shift their elevational limits (Wilson et al. 2005; Lenoir et al. 2005). We already know that there are numerous examples of species adapted to high elevations conditions or along the elevation gradient. However, what happens with those species that inhabit in lowlands, but whose distribution could change in the future to higher altitudes (or latitudes)? Are these species adapted to suffer new environmental conditions they might have to cope with? At this point, it is where studies in elevational gradients (or in a similar way, those in latitudinal gradients) can help us to understand how species are adapted to different environmental conditions and whether they are ready to cope with the new conditions they will find in the future (Benito et al. 2011). For example, species that ascend in elevation looking for more adequate temperature conditions (because warming of lowlands), would they be able to cope with the higher UVR existing at highlands compared to lowlands? In previous sections, it was discussed the harmful effects provoked by UVR in organisms, as well as the tight relations to the oxidative stress pathway. Organisms whose distributions shift in elevations may cope with this problem as many other taxa have done already.

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Chapter 3
OBJETIVES AND THESIS STRUCTURE

Objetives

In the global climate change framework, a major concerning is to understand the evolutionary processes from the pass, under which the organisms have evolved to be as we currently know, is the way to predict how they will respond to environmental changes in the future.

Those geographical gradients that show at the same time environmental condition gradients, and the organisms that have been selected to inhabit in these gradients, are useful tools to study the processes of adaptation. In this sense, elevational gradients are very useful to perform this kind of research. The species found along wide geographic or environmental ranges over these changing territories provide promising information about the evolutionary mechanisms that molded their current specific traits. Among all the characters that defines one species, one population, or even one individual, those specific traits that are constrained by several evolutionary pressures at the same time might be the ones that provide with more information to the adaptive mechanisms *per se.*

This thesis aims to provide some knowledge in regard to the adaptive mechanism of the large Psammodromus, *Psammodromus algirus* Linneo 1758, along an altitudinal range of 2200 m asl (from 300 m to 2500 m). This elevational gradient also entails a variation in the environmental conditions. For this purpose we studied the coloration of this lizard within the elevational gradient framework. And also the change in oxidative stress, an important biomarker of the physiological condition of organisms, tightly related to coloration in diverse aspects.

The specific objectives of this thesis can be summarized as following:

1. To study the variation in dorsal coloration of *P. algirus* along an elevational gradient (Chapter 5). Three non-exclusive hypotheses might be proposed to explain this variation:

1.a Darker coloration is favorably selected because it facilitates the thermal gain in less thermic efficient environments (“thermal melanism hypothesis”).

1.b Darker coloration is favorably selected because it is protective against ultraviolet radiation coming from the sun (more intensive in higher elevations).
1.c Cryptic colorations to background specific patterns at each population altitude are favorably selected to increase lizard inconspicuousness to aerial predators.

2. To quantify variation of oxidative stress (*e.g.* oxidative damage and antiosidant capacity) in *P. algirus* along the elevational gradient *in situ* (Chapter 6), in regard to the second hypothesis derived from the first objective. This aim is based on the understanding of ultraviolet radiation as one of the most harmful oxidative agents in nature.

3. To verify the possible harmful effects of the ultraviolet radiation on a generalist species as *P.algirus* along an elevational gradient, continuing with the idea of ultraviolet radiation as an important oxidative agent for the lizard. The aim is based on the following premises: i) ultraviolet radiation is more intense at highlands. ii) lizards from higher elevations are adapted under this higher radiation conditions. For this purpose oxidative damage, antioxidant capacity and immune response were quantified after lizards' exposition under ultraviolet radiation bulb in a laboratory experiment. Lizards were from different populations from different elevations along a 2200 m elevational gradient (Chapter 7).

4. To quantify the grade of crypsis between dorsal coloration of the lizar *P. algirus* and the surrounders where it inhabits (Cahpter 8), in order to verify the third hypothesis derived from the first objective. It was carried on studying the color of the lizards and the elements of the environment, and quantifying the grade of similitude between the colorations in regard to study the grade of crypsis.

5. To study the variation of ventral and lateral coloration of the lizar *P. algirus* along an elevational gradient (Chapter 9). Assuming that the resources availability fluctuates along this gradient (*e.g.* food, activity period, mates), we expected that the resource allocation in visual signals (partially related to sexual and dominancy behaviors) might be higher in those environments with higher resource availability.

Objetivos

En un marco de cambio global como en el que se encuentra nuestro planeta, es de vital importancia conocer cuáles han sido los rasgos de las especies que se han seleccionado en el pasado. Esto es fundamental para poder conocer cómo funcionan estas especies en el presente, y poder predecir de algún modo cómo reaccionarán en el futuro.

Para ello, los espacios geográficos en los que las condiciones ambientales (tanto bióticas como abióticas) cambian de una manera más o menos gradual son de gran ayuda para entender cómo han sido seleccionados estos rasgos adaptativos. Además, aquellas especies que se distribuyen de una manera más o menos generalista a lo largo de estos gradientes geográficos y ambientales aportan una rica información acerca de los mecanismos que han influido en la selección de los rasgos que las caracterizan. De todo el conjunto de rasgos que caracterizan a una especie, población o incluso a cada uno de los individuos que la componen, aquellos que se ven influenciados por varias fuerzas evolutivas de manera simultánea, son quizás los que más información aportan a nivel de conocimiento de los mecanismos de adaptación.

El objetivo principal de esta tesis es conocer las adaptaciones de la lagartija colilarga, *Psammodromus algirus* Linneo 1758 a lo largo de un gradiente altitudinal de 2200 m (desde los 300 m snm hasta los 2500 m snm; que implica un gradiente ambiental). Para ello nos hemos basado en uno de los rasgos más estudiados en ecología evolutiva: la coloración. Además, hemos prestado especial atención a la variación en el estrés oxidativo, uno de los factores fisiológicos que más puede verse relacionado con diversos aspectos de la coloración.

Siguiendo el orden de los capítulos, los objetivos concretos fueron los siguientes:

1. Estudiar la variación en la coloración dorsal de *P. algirus* a lo largo un gradiente altitudinal (Chapter 5). Planteando tres posibles hipótesis no excluyentes para explicar dicha variación.

1.a Selección de coloraciones más oscuras para favorecer la termorregulación en ambientes menos eficientes en cuanto a energía térmica ("thermal melanism hypothesis").

1.b Selección de coloraciones más oscuras para favorecer la protección frente a los daños de la radiación ultravioleta procedente del Sol, en ambientes con mayor intensidad de dicha radiación (a mayores altitudes).

1.c Selección de coloraciones crípticas con respecto al color del sustrato, o el ambiente en el que se desenvuelven las lagartijas, para conseguir así pasar más desapercibida para sus depredadores aéreos.

2. Comprobar si existe una variación en los niveles de estrés oxidativo (*e.g.* daño oxidativo y capacidad antioxidante) *in situ* a lo largo del gradiente altitudinal en la lagartija colilarga (Chapters 6). Este objetivo surge base a la hipótesis de la selección de coloraciones más oscuras en lugares con mayor radiación ultravioleta, y asumiendo a la radiación ultravioleta como uno de los agentes oxidantes más dañinos a nivel de variables ambientales.

3. Comprobar el posible efecto perjudicial de la radiación ultravioleta sobre una especie generalista como *P. algirus* en un gradiente altitudinal, continuando con la idea de la radiación ultravioleta como posible agente oxidante para esta lagartija. El objetivo se basa en las siguientes premisas: 1) La radiación ultravioleta es más intensa a mayor elevación. 2) Las lagartijas de zonas alpinas están localmente adaptadas para resistir mejor este tipo de radiación. Para ello, cuantificamos el daño oxidativo, la capacidad antioxidante y la respuesta inmune, tras someter a radiación ultravioleta a un grupo de

machos adultos de lagartija colilarga procedentes de poblaciones situadas a diferentes altitudes a lo largo de un gradiente de 2200 m (Chapter 7).

4. Evaluar el grado de cripsis de la coloración dorsal de *P. algirus* respecto al medio en el que se desenvuelve (Chapter 8), para comprobar la tercera hipótesis planteada en el primer objetivo. Se llevó a cabo mediante el estudio de la coloración dorsal de las lagartijas, y el color de diferente elementos del medio en el que estas se desenvuelven, y evaluando la similitud de estas coloraciones para establecer el grado de cripsis.

5. Estudiar la variación en la coloración ventral y lateral de la lagartija colilarga a lo largo de un gradiente altitudinal (Chapter 9). Esperamos que la inversión en estas señales visuales (asociadas en parte a comportamientos de dominancia y apareamiento) sea mayor en aquellos ambientes en los que la disponibilidad de recursos sea mayor. Nos basamos en la asunción de que la disponibilidad de recursos para las lagartijas tales como el alimento, el periodo de actividad y la disponibilidad de pareja, no es la misma a lo largo del gradiente.

Chapter 4
GENERAL METHODOLOGY

Description of the species: Why Psammodromus algirus?

Taxonomic framework

Kingdom: Animalia
Phyllum: Chordata
Class: Reptilia
Order: Squamata
Family: Lacertidae
Subfamily: Gallotiinae
Genus: *Psammodromus*
Species: *P. algirus*
(Linnaeus, 1758)

Conservation status is considered as the Least Concern in both the World Category (IUCN; (Mateo-Miras et al. 2009) and the Spanish Category (Carretero et al. 2002).

Psammodromus algirus is a medium-large size lacertid lizard. Adult individuals in our study area show snout-vent lengths (SVL) from 53 to 95 mm, being 66.8±0.32 mm on average (mean±se). In this population, no sexual dimorphism in body size was detected (ANOVA; $F_{(1,491)} = 1.22$, $p = 0.27$).

This lizard is abundant in a wide variety of Mediterranean habitats, from open sandy areas to forests, although shrubby Mediterranean landscapes are its preferred. It occurs in most of the Iberian Peninsula (except the Euro Siberian fringe), furthermore in northern Tunisia, northern Algeria, and northern and central Morocco, Conigli islet near Lampedusa island (Italy), and the South-East coast of France (Figure 4.2). However, probably the most important characteristic of the *P. algirus* distribution is that we can find this lizard along a wide elevation range -from sea level to 2800 m- precisely the range found in our study area, in Sierra Nevada Mountain (O. Jiménez-Robles, personal communication). The elevational limit depends on the latitude, being lower in the North and higher in South, as it is expected. The wide elevational range of distribution along with the wide range of environmental conditions that the lizard has to deal with makes *P. algirus* an interesting species to perform studies on evolutionary ecology.

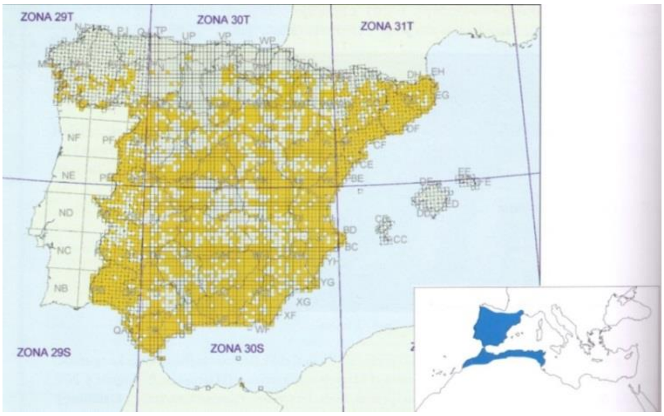


Figure 4.2 Iberian (big map) and world (small) distribution maps of *Psammodromus algirus*. Antropic introduced populations from the Balearic Islands are not included. (*Map from Carretero et al. 2002*).

Mating season for *P. algirus* takes place during spring and the first months of summer; although this period is changing depending on the region (Salvador 2011). Females lay clutches from the middle of May (also depending on the region); they lay between 3 and 13 eggs per clutch, depending on the locality and the size of the female (Salvador 2011). On average, eggs hatch 90 days after females lay them. Hatchlings begin to appear in the field since the beginning of August. Hatchlings feed and grow before winter, and reach maturity during the next mating season; although probably they don't reproduce until their second mating season (*pers. observ.*). In addition to that, during the mating season, males from some populations develop orange and yellow colorations (Díaz 1993, Carretero 2002).

Coloration in this species, especially ventral coloration, is tightly related to breeding and can vary geographically. In this regard, there are two well differenced phylogenetic lineages. Dorsal ground-coloration is more similar among populations, although some patterns can be more frequent in some populations than in others, and usually they do not show sexual dimorphism. Dorsally, the *P. algirus* shows from pale to dark brown coloration, with two dorsolateral yellow lines and a third mid-vertebral line in eastern populations (Salvador 2011). However, in terms of ventral coloration, it is shown a pale gray to white coloration. Moreover, some individuals (both males and females) can show yellowish patches (Figure 4.3) that can extend from throat to chest and vary in extension and intensity (Carretero 2002; Salvador and Veiga 2001). In western populations, males develop orange pigmentation around the head, but only during breeding season (Verdú-Ricoy et al. 2010; Díaz 1993). In the rest of the populations, males only develop few orange (or sometimes yellow) sub-labial scales around the commissures of the mouth (Figure 4.3), frequently visible only when the lizard open the mouth (Carretero 2002; Salvador and Veiga 2001). In the flank area, the lizards can show a different number of blue eyespots (ocelli; Figure 4.3). Usually, this number and size depend on the age, sex and population (Carretero 2002). Finally, it is known that this species counts with UV-reflecting patches in the flanks that are not visible to the human eye (Pérez i de Lanuza and Font 2010).

Figure 4.3. Pictures illustrating the color and location of the different color patches of a male of the *Psammodromus algirus* lizard from the Sierra Nevada Mountain. Ocelli are signaled by blue, commissure by orange and yellow gular patch by yellow arrows.

Genetic and geographical differentiation

During the last decade, the genetic differentiation of the *P. algirus* has been object of discussion. This started when (Busack and Lawson 2006) concluded that the origin of *P. algirus* was in the African continent and suggested a subsequent colonization of the Iberian Peninsula during Miocene-Pliocene (2.98-3.23 My ago). Furthermore, they split the Iberian population into two different species; on the one hand, the *P. manuelae* with a north-western distribution, and on the other hand, the *P. jeanneae* with a south-eastern distribution (Busack, Salvador, and Lawson 2006). At the same time, in an independent study, (Carranza et al. 2006) demonstrated the existence of two clades: one in the eastern Ibearian Peninsula, and other in the western Peninsula and North Africa (Figure 4.4a). In addition to that, they suggested that the western clade invaded North Africa after the opening of the Strait of Gibraltar. Recently, (Verdú-Ricoy et al. 2010) denied the existence of two species. Instead of that, they proposed two mitochondrial lineages; an eastern lineage confined to Iberia, and a western lineage present in both Iberia and North Africa (Figure 4.4b). Lizards studied in this thesis belonged to the eastern populations.

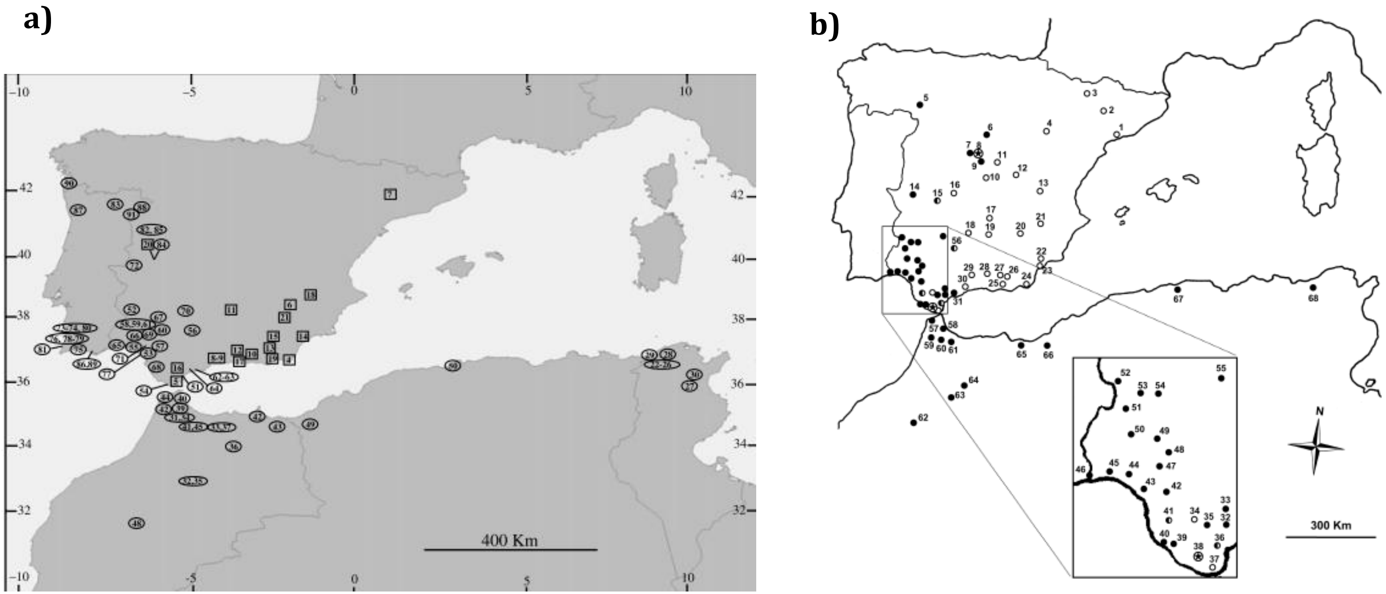


Figure 4.4. Distribution map of the two different lineages of *Psammodromus algirus*. a) From (Carranza et al. 2006): ovals mark haplotypes belonging to the "western clade" and rectangles correspond to the "eastern clade". b) From (Verdú-Ricoy et al. 2010): solid symbols represent the western lineage, open symbols represent the eastern lineage and half-solid symbols indicate the localities representing both lineages (type localities for P. manuelae [8] and P. jeanneae [38] highlighted with asterisk; (Busack, Salvador, and Lawson 2006)).

The study area: Why the Sierra Nevada Mountain system?

The field work was performed in the southern side of the Sierra Nevada Mountain system (30S 443587 4085814), in the south-east of the Iberian Peninsula (Province of Granada). The Sierra Nevada Mountain system counts with the highest elevation range in the Iberian Peninsula, from 200 m to 3481 m asl (Mulhacén Peak). For the purpose of sampling the lizards, locations were six points along the elevational gradient, separated each other by approximately 500 m in elevation (Figure 4.5). Particularly, the locations were found at 300 m, 700 m, 1200 m, 1700 m, 2200 m, and 2500 m asl. Locations were selected carefully, according to similar variables in terms of slope and vegetation structure, considering that vegetation type changes with elevation.

Vegetation structure was measured in each location. Transects of 50 meters were performed randomly, and every meter it was recorded the presence or absence of vegetation, and the height and the kind of plants. Plant height was classified as: < 25 cm, 25 - 50 cm, and > 50 cm, because those plant-height values determine major habitat differences for the shrub-linked lizard *P. algirus*. This process was performed five times in each location. Differences in vegetation cover and structure unavoidably appeared in this altitudinal gradient, as expected. However, two important components of the landscape, vegetation cover (Figure 4.6a) and vegetation height (Figure 4.6b), showed no clear pattern in altitude. These results suggest that all of our sampling locations comprised the shrubby vegetation physiognomy where *P. algirus* lives (Salvador 2011).

The study area covers the low and high Mediterranean habitats, where precipitation and temperatures are subject to the Mediterranean climate. In the Sierra Nevada Mountain system, the annual temperature varies from 17.6 to 3.5 °C, depending on the elevation (261 to 3471 m asl respectively) (Junta de Andalucía 2001; Figure 4.5). In particular, in the study area, air temperature ranges from 25.0±5.09 °C to 17.2±4.87 °C, depending on the elevation gradient (300 to 2500 m asl) (Zamora-Camacho et al. 2013). According to this, environmental temperature differs 8 °C on average between the lowest and the highest sampling location, during the activity season of *P. algirus* (March to September). Environmental temperature was recorded during the first year of this study, 1m above the ground, under a shade, using a Hibok 14 thermometer (accuracy 0.1 °C) (more details in Zamora-Camacho et al., 2013). The precipitation in Sierra Nevada Mountain is similar to the Mediterranean climate regimen. Above 2000 m asl, precipitation falls usually in form of snow during the cold season, and the snow covers the ground for around 6 months of the year (November-May); below 2000 m asl, precipitations fall during spring and autumn, keeping the environment relatively humid during these months. However, summer is hot and dry. Relative irradiance increases with elevation on average 6-8 %Km^{-1} for UVR-A and 7-11 %Km^{-1} for UVR-B in the northern slope of the mountain range (Sola et al. 2008). In particular, in the study area, UVR-B ranges from 1.28±0.03 to 4.8±0.37 μWcm^{-2}nm^{-1} between the lowest and the highest sampling locations. UVR was recorded using a BIC compact four-channel spectroradiometer (Ocean Optics model USB2000+UV-VIS). Radiation was measured twice a day at the beginning of August, every minute during a 10 min period. We used these data to predict radiation along the day, by using a polynomial model. Values of UVR-B were obtained from the 305 nm wavelength channel to pick lizards' activity period (11:00–18:00 h).

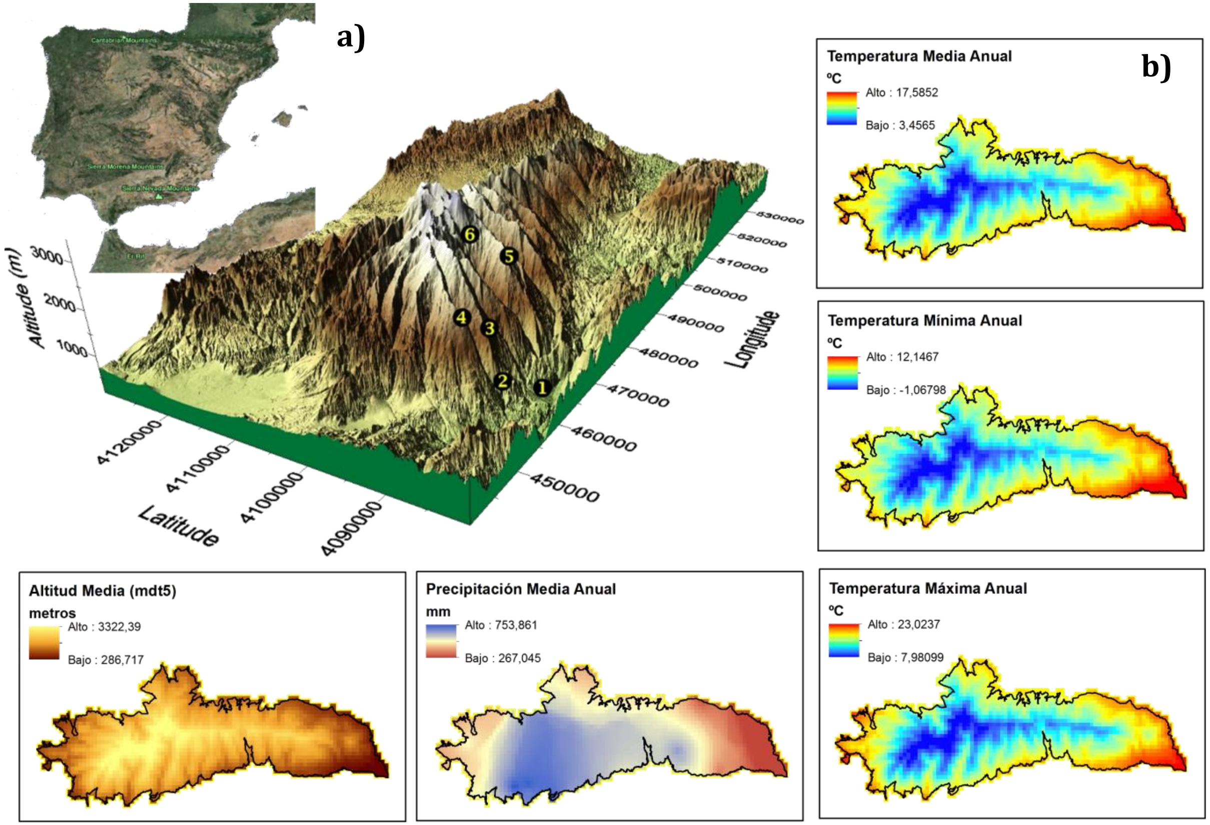


Figure 4.5. a) Three-dimensional map of the Sierra Nevada Mountain system, showing the location of the six sampling localities; (1) 300 m asl, (2) 700 m asl, (3) 1200 m asl, (4) 1700 m asl, (5) 2200 m asl, and (6) 2500 m asl. The location of the Sierra Nevada Mountain range in the Iberian Peninsula is also indicated in the upper map. b) The other maps show information of elevation, temperature, and precipitation in the geographical area of the Sierra Nevada Mountain system.

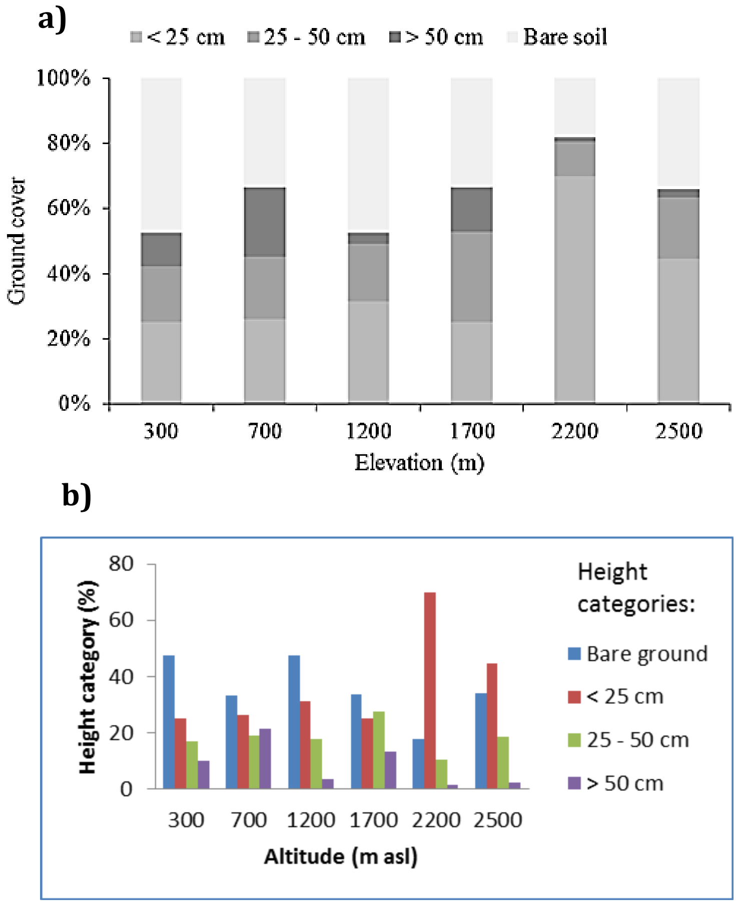


Figure 4.6 Proportion of bare soil and 3 vegetation-height categories in each elevation (top). Percentage of ground surface covered for each vegetation-height category in altitude (Bottom) (n = 253 measures in every altitude).

Detailed description of each sampling location.

300-location is at 300 m asl (30 S 464355 4082005; Figure 4.5 [1]), on the riverside of the river Guadalfeo, close to the village of Orgiva. The climate here is characterized by hot summers and generally frost-free winters. Precipitation falls during spring and autumn. Vegetation is composed by fruit trees plantations, *Populus alba*, and allocthonous *Eucalyptus sp*. Moreover, natural shrubby areas are dominated by *Rosmarinus officinalis* and *Retama sphaerocarpa*, with *Genista sp*., *Artemisia sp*. and *Thymus sp* (Figure 4.7 [1]). The geology is represent by Neogene-Quarternary sediments, especially important Unconsolidated, colluvial and alluvial gravel, sand, and minor silt in relationship with the river Guadalfeo (REDIAM 2010)

<u>700-location</u> is at 700 m asl (30 S 461402 4085716; Figure 4.5 [2]). It is next to a ravine caused by a spring stream, close to the village of Cañar. The climate is similar to the 300-location. Although trees are not abundant, there are some extensive orchards of *Olea europaea* and solitary *Prunus dulcis* and *Ficus carica*. Shrubby vegetation is dominated by *Cystus sp*., *Retama sphaerocarpa*, *Rosmarinus officinalis* and *Genista sp*., and a few *Foeniculum vulgaris*, *Capparis spinosa* and *Ruta graveolens* (Figure 4.7 [2]). The geology is made by the Neogene-Quarternary basin made up of sediments as marls, sands and gravels (REDIAM 2010).

<u>1200-location</u> is at 1200 m asl (30 S 462894 4087558; Figure 4.5 [3]), close to a spring stream and some irrigation canals. The climate consists of hot summers with infrequent frosts in winter and a few precipitations during spring and autumn. Vegetation is dominated by *Castanea sativa* and some dispersed *Morus nigra* and *Ficus carica* trees. Moreover, shrub formations dominated the landscape, consisting mainly of *Genista sp*. and *Cystus sp* (Figure 4.7 [3]). In relationship with geology, it is represented by the Alpujarride complex. This included in the base colored metapelitic formation made up of schists, graphitic micaschists, and quartzschists. Along the complex it is organized metaquartzites with calcschists in the uppermost part. Finally in the overlying carbonate rock formation

is made up of calcareous and dolomitic marbles both with the characteristic white-grey colors (REDIAM 2010).

<u>1700-location</u> is at 1700 m asl (30 S 461418 4089413; Figure 4.5 [4]), where climate is characterized by warm summers and frequent frosts with snowfall in winter. Landscape is dominated by an oak forest of *Quercus rotundifolia* and *Q. pyrenaica*. Where the forest is absent, shrubby areas are formed of *Genista sp*. and *Cystus sp* (Figure 4.7 [4]). To respect to the geology, it is found the Nevado-Filabride complex distingued two main tectonic groups of rocks, the lowed made up of graphite bearing schists (with dark colors and chipping forms) and quartzities, and the upper comprising a great variety of rocks including schists, marbles, gneiss and serpentinites (REDIAM 2010).

<u>2200-location</u> is at 2200 m asl (30 S 471812 4091221; Figure 4.5 [5]) around a little pond, which forms spring thaw. The climate is characterized by warm summers and very frequent freezes with abundant snowfalls in winter, covering the ground with a snow layer of variable thickness. Landscape is occupied in part by dense plantations of *Pinus sylvestris* with some forest clearings, but dominated by alpine meadows; the dominant shrub is *Genista florida* (Figure 4.7 [5]). It is an example of Nevado-Filabride complex in geology meaning, as well as spot 1700asml (REDIAM 2010).

<u>2500-location</u> is at 2500 m asl (30 S 471830 4096768; Figure 4.5 [6]) between some streams from spring thaw and the river Mulhacén. The climate is similar to that in the 2200-location, but harsher in

winter. There are not trees here, being above the timberline. Landscape is dominated by Creeper *Juniperus communis* and *Genista florida* as well as sub-alpine meadows (Figure 4.7 [6]). In geology terms, as well as spot 1700 and 2000asml, it is represent the the Nevado-Filabride complex, specially distinguished with quartzities as upper rocks with more resistance to erosion (REDIAM 2010).

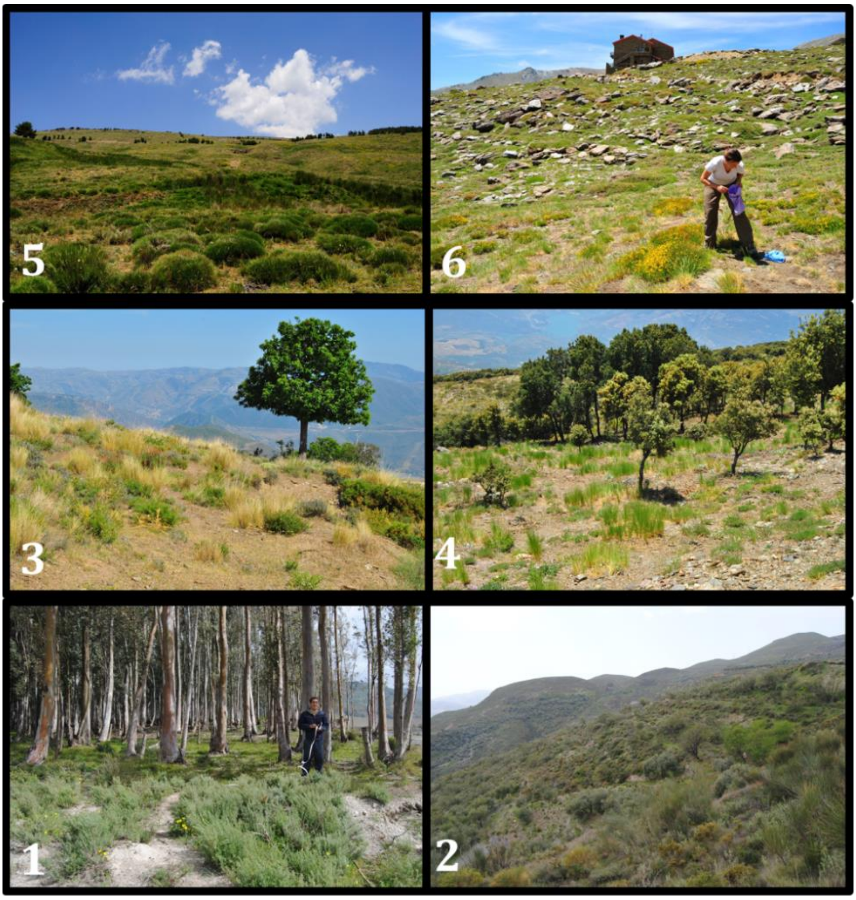


Figure 4.6. Pictures of each location at six different elevations as an example of the common vegetation in the areas selected for sampling the lizards. Locations go from left-bottom to right-top: [1] 300 m asl, [2] 700 m asl, [3] 1200 m asl, [4] 1700 m asl, [5] 2200 m asl, and [6] 2500 m asl.

General procedures with the lizards

During the period between 2010 and 2013, 492 lizards were captured by hand (255 females and 237 males) during their activity season (Since March until October). The number of individuals by sex and for each location was as follow: in the six sampling locations ($N_{(\text{elevation})}$ = ♀♀/♂♂) N_{300} = 48/60; N_{700} = 38/22; N_{1200} = 32/19; N_{1700} = 38/35; N_{2200} = 51/42; N_{2500} = 48/59. Lizards were captured under official permission of the Regional Government of Andalusia (Junta de Andalucía) and the Sierra Nevada National Park (references GMN/GyB/JMIF and ENSN/JSG/JEGT/MCF). All lizards were released at the capture site after the study, and no lizard was injured as a consequence of this study. When it was necessary, lizards were transported to a lab facility in the Department of Zoology at the University of Granada, inside cotton bags allowing transpiration. Once in the laboratory, lizards were kept in plastic terraria (20×13×9 cm) with a heat cable in one end and food (larvae of *Tenebrio molitor*) and water in form of vitamined aqua-gel *ad libitum*. Heat resource was on during three hours a day simulating the peak of temperature that take place in the field (from 12h to 15h).

General processing of the lizards consisted of measuring the length of the body size from snout to vent (SVL), the tail when it was complete or regenerated (also the status of the tail was noted as complete, incomplete, or regenerated), and the front and hind limb using a metal ruler to the nearest 1 mm. Head width, length, and height were also measured to the nearest 0.01 mm using a digital caliper. Biometric variables were used in statistical analyses, but they were removed from the final models when it was oppotune. In addition to that, lizards were weighed with an electronic balance (Model Radwag WTB200) to the nearest of 0.01 g. Sex was determined mainly on the basis of femoral pore development (more conspicuous in males; Iraeta et al. 2011). Age class was also established following an objective criterion to consider sub-adult and adult age categories. Knowing the exact moment of maturity of the individuals needs of death and dissection of the individuals to evaluate the gonadal development, therefore, maturity traits this species show in field were used to infer their age class (adult or juvenile). In the case of males, adults show an orange commissure and conspicuous femoral pores, so we considered the SVL of the smallest male with this trait as the limit between sub-adult and adult. In the case of females, gravid condition is a sign of maturity, so we considered the SVL of the smallest gravid female as the limit between sub-adult and adult. In addition to that, since body size varies along elevation, we decided to apply these criteria differently to each sampling locality (Table 4.1).

Table 4.1. Body length (SVL, snout-vent length in mm) limit between sub-adult and adult males and females for each sampling-locality at six different elevations (meters about sea level).

Elevation	SVL Males	SVL Females
300	55	53
700	55	54
1200	55	56
1700	55	59
2200	62	65
2500	63	67

Analyzing coloration

Studies of color have traditionally relied on human observation; however, related problems arise through this approach. Firstly, human eye can only perceive wavelength in the visible spectrum (400-700 nm). Moreover, human observations on color may not be only biased by the observer's perception, but also by the color space that surrounds the observed surface. For this reasons, the development of new methodologies based on spectral measurements under standardized conditions incorporates some advantages for analyzing color elements (Montgomerie 2006; Grill and Rush 2000; Endler 1990). Advantages can be summarized in: i) Spectral data are less susceptible to researcher bias. ii) A single spectrum contains an enormous quantity of information about the color characteristics of a sample. iii) Resolution and range of spectrophotometers can far exceed the capabilities of human vision (Grill and Rush 2000).

Changes in color are reflected in the spectrum shape as curve slopes, amplitude, position, and number of peaks. The large amount of data that a spectrum provides can be handled in different ways. Two of the most used techniques for analyzing spectrum data are the one based on the principal component analysis (or PCA), and the other one based on a segment classification (SC) analysis (Montgomerie 2006; Grill and Rush 2000; Endler 1990). In any case, both techniques finally describe the three main components of color (Montgomerie 2006): brightness, chroma, and hue. *Brightness* or lightness is a measure for the total amount of light coming from an unit of area of a surface at a particular angle (Montgomerie 2006). The values range from 0 to 100, being 0 "black" color or the absence of reflectance, and 100 "white" color or total reflectance of the spectrum (Figure 4.8b). *Chroma* or saturation is the radiance in a specific part of the spectrum in relation to the radiance from the whole visible spectrum (Montgomerie 2006). It is measured as the distance from the centre (0) of the color space to its circumference (100) where pure spectral colors are represented (Figure 4.8b). *Hue* is the technical term for what we call "color" in everyday speech

(Montgomerie 2006). Hue indicates which wavelengths contribute most to the total radiance and can be represented as an angle (0 to 360°, being 0° for red, 90° for yellow, 180° for green, and 270° for blue; Figure 4.8b). Although PCA analysis is slightly better since it provides better estimates of color components -specially hue- SC provides better estimates of color components in colors that falls into the yellow/green category (Grill and Rush 2000).

In this thesis, the coloration characteristics were measured using a spectrophotometer (Minolta CM-2600d/2500d) that measures reflectance of surfaces in the L*a*b* color space (Figure 4.8a) of the Commission Internationale d'Eclairage (CIE), which describes all the colors visible for most of the diurnal terrestrial vertebrates (Montgomerie 2006). For this purpose, the equipment emits a beam and measures the reflected light on the surface whose color we want to know (*e.g.* lizards' skin). This is repeated three times and the spectrophotometer offers automatically the average measurement. The area of the surface measured is a circle with a diameter of 3 mm.

The L*a*b* color (Figure 4.8a) space is based on the SC technique, being L* the total brightness (calculated as the area under the reflectance curve, equation (1)) and a* and b*, the brightness in each of the four equal wavelength intervals of the spectral range (calculated as the "regional brightness", equations (2) and (3)): *Blue* (-b = 400-474 nm); *Green* (-a = 475-549 nm); *Yellow* (+b = 450-624 nm); *Red* (+a = 625-700 nm). The relative brightness in each region is obtained as follows:

$B_{-b} = Q_{-b}/Q_{Total}$; $B_{-a} = Q_{-a}/Q_{Total}$; $B_{+b} = Q_{+b}/Q_{Total}$; $B_{+a} = Q_{+a}/Q_{Total}$.

$$^{(1)}\ L^* = \sum Q(\lambda,x) = Q_{Total}$$

$$^{(2)}\ a^* = B_{+a}-B_{-a}$$

$$^{(3)}\ b^* = B_{+b}-B_{-b}$$

Where Q is the total quantal capture for wavelength λ at a focal distance of x. Next, chroma and hue are estimated from the "regional brightness" as follows (equations (4) and (5)):

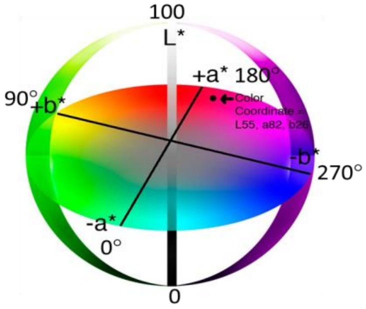
$$^{(4)}\ C^* = [(a^*)^2+(b^*)^2]^{1/2}$$

$$^{(5)}\ H^* = \arcsin((b^*)/C^*) = \arccos((a^*)/C^*)$$

Additional details and explanations can be found in Endler(1990) and Grill and Rush (2000).

Color measures of the lizards were obtained from dorsal and ventral side: two points for the dorsal surface, one at pileus and other at the middle back (avoiding yellow lines), and two points for the ventral surface, one at the gular region and one on the right side of the chest. Since pileus and back, and throat and chest were pairwise correlated (Table 4.2), back and throat data were only included in the subsequent statistical analyses.

a) L*a*b* color space



b) LCH color space

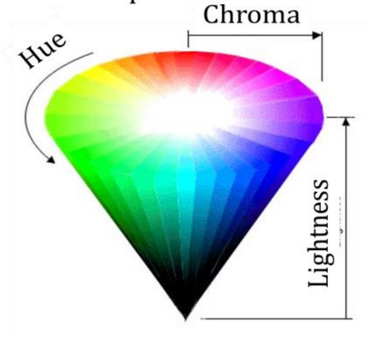


Figure 4.8. a) Representation of the L*a*b* color space from the CIE, and b) representation of the LCH color space being L = lightness, C = chroma, and H = hue.

Table 4.2. Pearson's correlations matrix for the three color components lightness (L), chroma (C), and hue angle (H) of *Psammodromus algirus*; dorsal-color variables measured in pileus, and back and ventral-color variables measured in throat and chest. Pearson correlations were ran due to the normality of the data, with elevation as covariate

	Pileus L*	**Pileus C***	**Pileus H***
Back L*	0.65¤¤	0.47¤¤	0.34¤¤
	366	*366*	*366*
Back C*	0.44¤¤	0.58¤¤	0.04
	366	*366*	*366*
Back H*	0.17¤¤	-0.01	0.65¤¤
	369	*369*	*369*
	Throat L	**Throat C**	**Throat H**
Chest L	0.64***	-0.41***	0.26***
	366	*366*	*366*
Chest C	-0.53***	0.81***	-0.15*
	366	*366*	*366*
Chest H	0.20***	-0.12*	0.67***
	369	*369*	*369*

Significant values ($p<0.05$) indicated as bold. The Pearson correlation parameter appears on the upper side and the sample size appears on the bottom side.

Analyzing oxidative stress

Oxidative stress processes combine various components such as free radical production, antioxidant defenses, oxidative damage, and repair mechanisms (Monaghan et al. 2009; Scandalios 2002). For this reason, several biomarkers can be used to quantify oxidative stress levels. However, because there is not one single "standard" biomarker for oxidative stress, it would be convenient to use more than one in the same study (Halliwell and Gutteridge 1999). For this thesis, oxidative damage (in lipids and proteins) and antioxidant capacity were quantified (through total antioxidant capacity and the activity of diverse specific antioxidant enzymes).

Oxidative damage

Different methods were used to quantify the oxidative damage. In a first study (chapter 6), attention was focused on lipid-peroxidation levels, determined according to (Buege and Aust 1978). This method is based on malondialdehyde (MDA) levels generated as a result of lipid peroxides degradation. In the presence of thiobarbituric acid, MDA reacts producing a colored thiobarbituric acid-reacting substance (TBAR). The color change is measured at 535 nm and the amount of TBAR in the sample means the level of lipid peroxidation expressed as MDA per g of tissue. In a second work (chapter 7), oxidative damage produced in the cells was measured in more detail, and apart from free malondialdehyde (FrMDA), lipid and protein peroxidation biomarkers were evaluated: protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH), and protein hydroperoxides (PrOOH) (Grintzalis et al. 2013).

Antioxidant capacity

In order to assess the antioxidant capacity, trolox-equivalent antioxidant capacity (TEAC) was measured as the antioxidant mechanism (chapter 7), as well as the activity of diverse antioxidant enzymes (chapters 6 and 7): superoxide dismutase (SOD), catalase (CAT), glutathione reductase

(GR), glutathione peroxidase (GPX), glutathione S-transferase (GST), and DT-diaphorase (DTD) (Schlesier et al. 2002).

For this purpose, firstly, tissue samples were homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, and 0.1% triton X-100 (v/v), pH 7.8) at a ratio of 1:9 (w/v). Homogenates were centrifuged at 30000 g for 30 min in a Centrikon H-401 centrifuge. After centrifugation, the supernatant was collected and frozen at -80 °C until being analyzed. All enzymatic assays were performed at 25±0.5 °C using a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). The enzymatic reactions were started adding the extracted tissue, except for SOD, where xanthine oxidase was used.

The specific methodology for the assay quantification and the conditions of these biomarkers are thoroughly explained in the pertinent chapters.

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Chapter 5
Dorsal coloration of *P. algirus*

Senda Reguera, Francisco J Zamora-Camacho, and Gregorio Moreno-Rueda (2014) **The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high elevations.** *Biological Journal of the Linnean Society.* 112(1): 132-141. SCI: 2.535

Abstract

Elevational gradients offer a good opportunity to study organisms’ adaptations to clinal environmental variables. In elevation, the most influential variables on organisms are temperature and ultraviolet solar radiation, the first decreasing whereas the second increasing with elevation. Both variables affect ectotherms' biology, as ectotherms depend on environmental temperature for thermoregulation, frequently being heliotherms. Here, we studied dorsal coloration in the lizard *Psammodromus algirus* (Linnaeus, 1758) along a wide elevational gradient (2200 m) in Sierra Nevada (SE Spain). We hypothesize that lizards’ skin will be darker in elevation, that is, in environments with colder temperatures and higher UVR intensity. Results show that individual dorsal colorations turned darker at high elevation. We propose two non-mutually exclusive explanations for this result: i) darker dorsal surface would favour faster warming in high elevations, where temperature is colder, and ii) darker dorsal surface would protect against UVR, stronger in high elevations. We found significant relationships between both temperature and ultraviolet radiation and population dorsal darkness, giving mixed support for the two explanations. Moreover, dorsal hue was positively correlated with substrate hue, suggesting that hue evolved to maximize crypsis. Our study, therefore, suggests that geographic variation in dorsal coloration in this lizard is adaptive, and darkness coloration might have evolved in response to adverse conditions (low temperature and high ultraviolet radiation) of high elevations.

Keywords: darkened coloration - body color - elevational gradient - thermal melanism - ultraviolet radiation - *Psammodromus algirus*.

Introduction

The environment gradually varies with elevation (Körner 2007) and accordingly, organisms distributed in a wide elevational range may be locally adapted, generating a cline for several traits with elevation (Welter-Schultes 2000; Blackburn and Ruggiero 2001; Ashton and Feldman 2003). In fact, animals may show adaptive variation in coloration in relation to elevation. For example, darker individuals are expected at higher elevations, where temperatures are colder, pattern which may be explained by the "thermal melanism hypothesis" (Clusella-Trullas et al. 2007). This hypothesis states that, compared to light individuals, darker ones have an advantage under low-temperature conditions, because they heat up faster at a given level of solar radiation (Watt 1969; Clusella-Trullas et al. 2007; Clusella-Trullas et al. 2008). Heating up faster is important for ectotherms, especially for reptiles, which largely depend on solar radiation for thermoregulation (Vitt and Caldwell 2009). Populations distributed along an elevational range face temperature gradients, which provide a good opportunity to study the evolution of body coloration and its relationship with thermoregulation (Clusella-Trullas et al. 2007).

Another hypothesis, non-mutually exclusive with the previous one, that could explain why organisms darken along elevation is based on the increasing ultraviolet radiation (UVR) intensity with elevation ("protection against UV damage hypothesis"; Porter and Norris 1969). Most ectotherm organisms (specially, heliotherms) rely on solar radiation to thermoregulate, but this radiation may have harmful effects at high elevation, where it is stronger. Moreover, given that thermal energy availability is lower at higher elevations, alpine reptiles, which furthermore spend more time sunbathing (Carrascal et al. 1992; Martín and López 1999; Gvoždík 2002), would be more time exposed to the harmful effects of solar radiation. High levels of UVR produces damage on DNA (Ravanat et al. 2001), increases eggs and embryos mortality, and reduces larvae growth and development in amphibians (Belden et al. 2000; Marquis et al. 2008; Lizana and Pedraza 2008), and causes tissues-damages and cellular oxidative stress (Chang and Zheng 2003). Consequently, organisms show several strategies to protect themselves from harmful effects of UVR, being sun-screen factors one of the most important. Sun-screen factors, such as carotenoid and melanin pigmentation (Zellmer 1995; Hessen 1996; Gunn 1998), protect against UVR absorbing it (Hofer and Mokri 2000). Accordingly, given that UVR increases with elevation because the atmosphere is thinner (Blumthaler et al. 1997), we expect individuals to darken at higher elevations.

Ectotherms tend to be darker in colder latitudes (Vidal, et al. 2007; Alho et al. 2010). Regarding elevation, melanic morphs of arthropods are more frequent at high elevations (Zellmer 1995; Hessen 1996; Rajpurohit et al. 2008; more examples in Clusella-Trullas et al. 2007). In reptiles, darker *Cordylus spp.* occur more frequently in colder and foggy areas, where melanism is interpreted as an advantage for heating up faster (Janse van Rensburg et al. 2009). Moreover, melanic *Cordylus spp.* heat up faster, as a consequence of their low reflectance (Clusella-Trullas et al. 2009). *Sceloporus occidentalis* lizards from populations sited at high elevations show darker colorations (Leaché et al. 2010), and *Podarcis hispanica* lizards inhabiting colder environments are darker than conspecifics in warmer sites (Gabirot et al. 2013).

On the other hand, geographic variation of darkness in lizard coloration may be due to non-physiological selective pressures. For example, pressure of predation selects for cryptic coloration, which may be darker depending on substrate color (Thorpe and Brown 1989; Husak et al. 2006; Jambrich and Jandzik 2012). In this sense, clinal variation in substrate coloration might explain different coloration in animals.

Here we studied the shifts in the dorsal coloration of a lizard, the large Psammodromus (*Psammodromus algirus* Linnaeus, 1758), along a 2200 m elevational gradient in south-eastern Spain, as possible adaptation in a clinal-variation of environmental conditions. This species inhabits a wide gradient of elevations and environmental conditions (Salvador 2011). As an ectotherm organism, *P. algirus* closely depends on environmental temperatures to thermoregulate (Diaz et al. 2006). These circumstances make *P. algirus* a suitable organism for this study. *Psammodromus algirus* is distributed between 200-2600 m asl (metres above sea level) in Sierra Nevada Mountain (SE Spain). In this mountain, environmental temperatures strongly decrease with elevation (Zamora-Camacho et al. 2013), while UVR increases (Sola et al. 2008). We hypothesize that, in this elevation gradient, lizards' dorsal coloration darkens with elevation, under selective pressures such as colder environmental temperatures (because darker surfaces heat up faster) and/or higher UVR levels (because darker surfaces best protect against the harmful effects of UVR). Alternatively, changes in dorsal coloration may be a consequence of variation in substrate color as a consequence of predation pressure, because higher similarity of dorsal coloration to the surroundings implies less detectability by predators.

Therefore, the main goal of this study is to test the hypothesis that dorsal coloration varies with elevation in *P. algirus*. Here, according to aforementioned, we predict darker individuals at highlands. In a second step, we tentatively test the different hypotheses explaining the variation in dorsal coloration with elevation. We test these hypotheses by correlating the average dorsal coloration of lizards in our six population of study, with average temperature, UVR, and substrate color in the study zones.

Material and methods

General methods

Psammodromus algirus is a lacertid lizard 60-80 mm snout-vent length (SVL), abundant in shrubby Mediterranean habitats. It extends from south-eastern France to Morocco, including most of the Iberian Peninsula, along a wide elevation range (0-2600 m asl), where it is exposed to a wide range of environmental conditions (Salvador 2011). Dorsal coloration spans from pale to dark brown, with two dorsolateral yellow lines and a third mid-vertebral line in eastern populations (Salvador 2011). Ventral ground-coloration goes from white to grey and frequently with yellow pigmentation in the throat region that varies in extension and intensity (Carretero 2002). In western populations, males show orange pigmentation around the gular region during the breeding season (Salvador 2011), but not in eastern populations, where males only show an orange spot in the commissures (Carretero 2002). Dorsal pattern does not have sexual dimorphism.

Sampling was performed on Sierra Nevada (SE Spain 30S 443587W 4085815N), between 300-2500 m asl. We established six locations for sampling separated by approximately 500 m in elevation (300, 700, 1200, 1700, 2200, 2500 m asl; Figure 4.5), chosen according to similarity in slope and vegetation structure (more details in Chapter 4 General methodology). In the Sierra Nevada, mean annual temperature goes from 17.6 to 3.5 °C, according to elevation (261 to 3471 m asl respectively; Figure 4.5), and in the study-area, air environmental temperature during the activity period (March to September) of *P. algirus* differs 8 °C on average (mean±sd) between the lowest (300 m asl; 25.0±5.09 °C) and the highest populations (2500 m asl; 17.2±4.87 °C; Zamora-Camacho et al. 2013). Relative irradiance increases with elevation on average 6-8 $\%Km^{-1}$ for UV-A radiation and 7-11$\%Km^{-1}$ for UV-B (Sola et al. 2008). In addition, UVR (300 nm wave length) values on average went from 1.28±0.03 $\mu Wcm^{-2}nm^{-1}$ in lowlands to 4.80±0.37 $\mu Wcm^{-2}nm^{-1}$ in highlands (see measurement methodology below).

During 2010-2013 we captured by hand 492 lizards (255 females and 237 males) during their activity season (March to October) in the six populations ($N_{(elevation)}$ = ♀♀/♂♂; N_{300} = 48/60; N_{700} = 38/22; N_{1200} = 32/19; N_{1700} = 38/35; N_{2200} = 51/42; N_{2500} = 48/59). Individuals were transported to a lab facility where color characters were measured with a spectrophotometer (Minolta CM-2600d/2500d). Color measures had the three chromatic values of the L*C*H* color space, based on the L*a*b* color-space of the *Commission Internationale d'Eclairage* (CIE) (Figure 4.7), which describes all the colors visible for most of the diurnal terrestrial vertebrates (Montgomerie 2006). The coordinate L* represents *Lightness*, from black (L* = 0) to white (L* = 100). We also considered *chroma* (color saturation), $C^* = [(a^*)^2+(b^*)^2]^{1/2}$ (0 in the centre of the color-space [unsaturated] and increases according to the distance from the centre); and hue angle, H* = arc-tangent(b*, a*) in radians (after transforming radians into grades, it defines coloration as 0° for red, 90° for yellow, 180° for green, and 270° for blue). Measures were obtained from two points (3mm in diameter) of the dorsal surface, one at pileus and other at middle back (avoiding yellow lines).

Furthermore, snout-vent length (SVL) was measured with a metal ruler (accuracy 1 mm), and lizards were weighed with an electronic balance (Model Radwag WTB200, accuracy 0.01 g). We characterized sex attending mainly to femoral pores development (more conspicuous in males). To avoid the effect of ontogenetic shift in dorsal coloration (*manova*-test with color traits as dependent variable and age category as factor; $F_{(1,\ 387)} = 4.19$, $p < 0.001$), we only considered adult individuals in the analyses. We considered as adult those males with body size larger than the SVL of the smallest male with orange commissure and those females with body size larger than the SVL of the smallest gravid female. Both of them are objective characters that reveal sexual maturity. These criterions were applied separately for each population, as body size (and thus minimal adult body size) varied with elevation (see Table 4.1).

Lizards were captured under permission (references GMN/GyB/JMIF and ENSN/JSG/JEGT/MCF) of the Junta de Andalucía and Parque Nacional de Sierra Nevada (Spanish Government) and were released at the capture site within a week. No lizard was damaged as a consequence of this study.

Statistical analyses

Variation in dorsal color with elevation

In order to achieve the main goal of our study, we tested for differences in dorsal color of lizards among elevations. In this analysis, individual lizards were used as statistic units blocked by locations (= elevation). We used lineal models (Quinn and Keough 2002) ran with R version 2.15.2 (R Core Team 2012). Initially, six variables were considered for describing dorsal coloration: Pileus L*, Pileus C*, Pileus H*, Back L*, Back C*, Back H*. We generated a matrix of Pearson's partial correlations (controlling for elevation) to examine the relationships among the color variables. The matrix of correlations showed that pileus and back coloration were highly correlated (all $p < 0.05$; Table 4.2), so we decided to include only back data in further analyses. Finally, we ran mixed effect linear models (LMM) for each color components (Zuur et al. 2009): lightness, chroma, and hue. We introduced in these analyses elevation (corresponding to the six populations sampled) and sex (male and female) as fixed factors, and body size as co-variable. Body size was estimated with a principal component analysis (PCA) in order to combine mass and SVL, as both were highly correlated ($r = 0.9$, $p < 0.001$, $N = 356$). In addition, we introduced month (from March to August) and year (from 2010 to 2013) as random factors, as we wanted to correct the possible variance they could be introducing in the model, but we were not interested in their effect on our dependent variables (color components). We checked all interactions among independent variables, but only significant interactions remained in final models. For each model, we tested normality and homoscedasticity in residuals. Differences in coloration between pairs of populations were tested with Tukey's "Honest Significant Difference" post hoc test (Tukey HSD).

Relationship between population-level dorsal color and environmental variables

In order to achieve the secondary goal of our study (a tentative test of three hypotheses explaining elevational variation in dorsal color in this lizard), we tested the relationship among lizards' dorsal average coloration (at the level of location) with the characteristics of their location: environmental temperature, UVB-radiation, vegetation cover, and substrate coloration. We measured these environmental variables in every population: i) We recorded environmental temperature one metre above the ground, under a shade, using a thermometer Hibok 14 (accuracy 0.1 °C) (more details in Zamora-Camacho et al. 2013). ii) We measured UVB-radiation ($\mu Wcm^{-2}nm^{-1}$) using a BIC compact 4-channel radiometer (Biospherical Inc, CA, USA). Radiation was measured twice a day at the beginning of August, every minute during a ten minute period. We used these data to predict radiation along the day, by using a polynomial model. Values of UVB-radiation were obtained by the 305 nm wave length channel to pick lizards' activity period (11:00-18:00 h). iii) We measured vegetation cover at the middle of the growing season (spring). To measure the vegetation structure in each sampling station, we randomly set 5 transects 50-meter-long and recorded the presence or absence of vegetation (from grass-size to bush-size; see Chapter 4 General Methodology) every metre. iv) We also measured ground coloration taking pictures of the substrate where lizards were captured and processing these pictures with Adobe Photoshop CS5 software (Pereira and Amat 2010). After standardising the pictures, we measured the average coloration in the L*a*b* color space in 25 points (5×5 pixels area), and estimated L*C*H* as described above (More details in Chapter 8).

Finally, using populations as statistical units, we correlated (Spearman correlations) mean values of lizards' dorsal coloration for each elevation with mean environmental temperature, mean UVB-radiation, percentage of vegetation cover, and mean values of substrate color.

Results

Variation in dorsal color with elevation

We found significant differences in all three dorsal color components of *P. algirus* (lightness, chroma and hue angle) with elevation (Table 5.1, Figure 5.1a). Individuals from the two highest populations were darker (smaller values of lightness) than those in lower populations (Figure 5.1b). Dorsal coloration was less vivid (smaller values of chroma) in the two highest populations (Figure 5.1c). Individuals turned redder (smaller values of hue angle) in 1700 and especially in 2200 and 2500 m asl than in 300-1200 m asl. Post hoc Tukey HSD tests revealed significant differences between both 2200 and 2500 populations and the rest of populations for the three color components measured (Table A1). Sexual differences were found only for chroma (Table 5.1). Males had more vivid dorsal coloration (mean±se; ♀ = 12.26±0.18, ♂ = 13.03±0.21). Finally, body size was an important predictor for lightness, even after controlling for elevation (Table 5.1). Larger individuals had higher values of lightness ($\beta = 2.38$). Nevertheless, we found a significant interaction between elevation and body size for dorsal lightness, given that the positive correlation between dorsal lightness and body size was found only in the populations at 300, 700 and 1700 m asl (Figure 5.2).

Relationship between population-level dorsal color and environmental variables

Temperature was negatively correlated with elevation, whereas UVR was positively correlated (Table 5.2; see mean population values of variables in Table A2). There was no significant correlation between elevation and vegetation cover (Table 5.2). Regarding substrate coloration, hue angle and lightness significantly covaried with elevation, substrate being darker as elevation increased (Table 5.2). Mean dorsal lightness of lizards decreased with elevation, but not significantly (Table 5.2). Moreover, lightness significantly increased with mean values of environmental temperature and decreased with UVR and vegetation cover (Table 5.2). Dorsal lightness did not covary with substrate lightness (Table 5.2). Mean values of dorsal chroma decreased significantly with elevation. Moreover, chroma increased with temperature and decreased with UVR (Table 5.2). Dorsal hue behaved similarly, decreasing significantly with elevation, increasing with temperature and decreasing with UVR (Table 5.2). Dorsal hue also was positively correlated with substrate hue (Table 5.2).

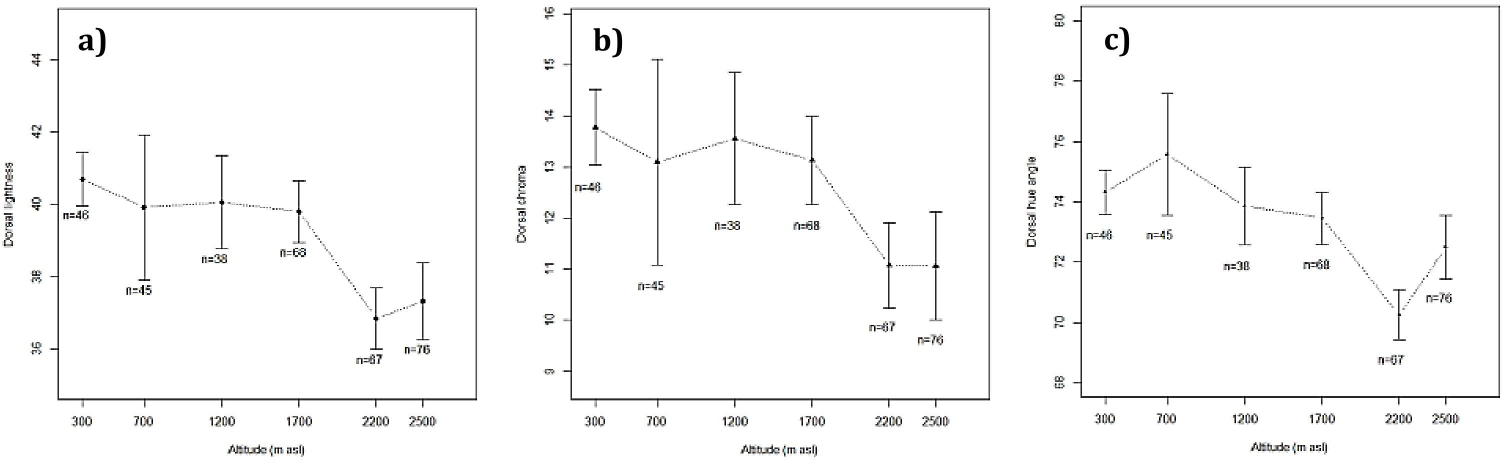

Figure 5.1. Mean values and standard error bars of dorsal coloration components (lightness [a], chroma [b], and hue angle [c]) of *Psammodromus algirus* in six populations (Elevation; m asl) along an elevation gradient in Sierra Nevada (SE Spain). Sample size (n) is under the mean value.

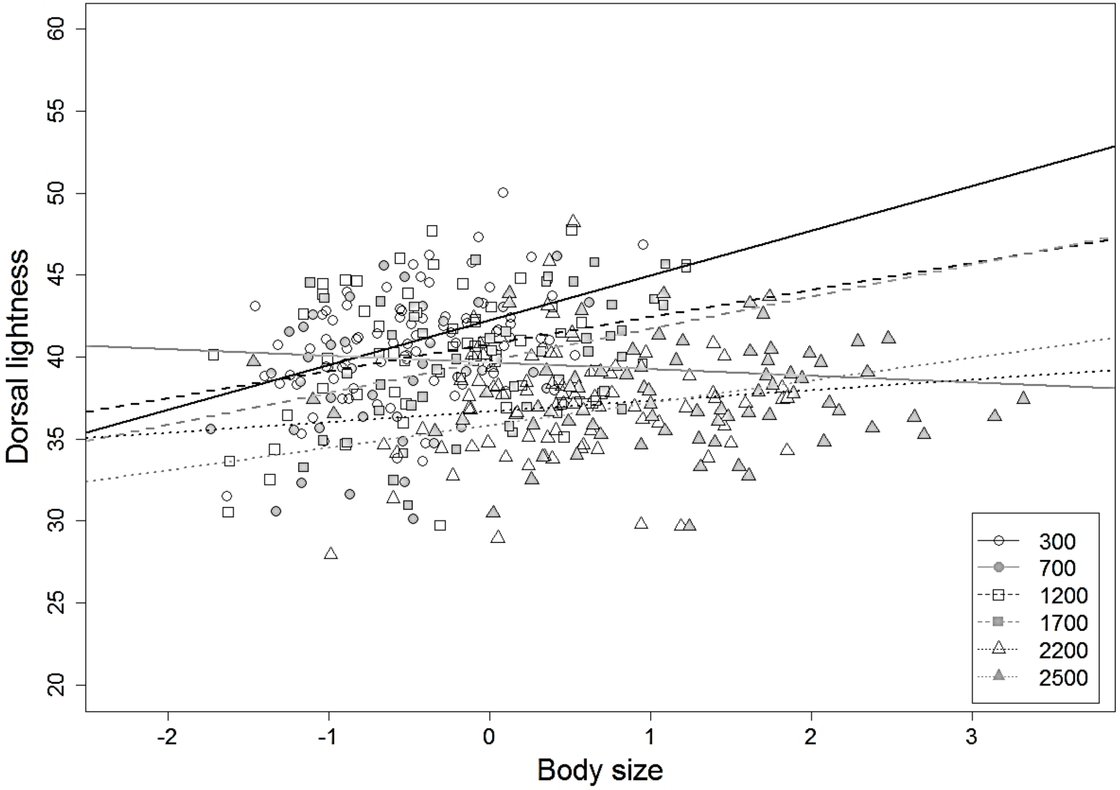

Figure 5.2. Dorsal lightness variation with body size (PCA factor of mass and snout-vent length) for each six population (i.e.. Elevation in m asl). Regression lines are represented for each elevation with lightness as dependent and body size as independent variables.

Table 5.1. Results of mixed effects linear models (anova test; F-values) for each dorsal color component. Color components were included as dependent variables (lightness, chroma, hue angle), elevation and sex as fixed factors, and body size as covariate. Linear model was corrected for random factors including year and month.

	DF	F-value	p-value
Back lightness			
Elevation	5, 341	16.52	<0.01
Body size	1, 341	8.90	<0.01
Sex	1, 341	2.80	0.10
Elevation×Size	5, 341	3.38	0.01
Back chroma			
Elevation	5, 345	19.06	<0.01
Body size	1, 345	0.64	0.42
Sex	1, 345	11.46	<0.01
Back hue angle			
Elevation	5, 345	8.77	<0.01
Body size	1, 345	0.01	0.91
Sex	1, 345	2.65	0.10

Only significant interactions are included.

Discussion

Psammodromus algirus showed dorsal color variation in the elevational gradient examined in this study. Dorsal surface was darker at the highest elevations (over two thousand metres above sea level) and had less vivid and redder coloration. In addition, for a given elevation, larger individuals showed lighter coloration, especially at low elevations. There were some differences between sexes, males having more vivid coloration.

Darker dorsal coloration at higher elevation has been observed in other animals (see Introduction). The "thermal melanism hypothesis" states that darker coloration provides benefits in terms of body warming (Clusella-Trullas et al. 2007). For instance, in *Chamaeleo spp*. and *Sceloporus spp*., when temperature is low, pigments are dispersed through all the cytoplasm, darkening the skin and improving thermoregulation (Walton and Bennett 1993; Sherbrooke et al. 1994). Consequently, darker individuals have thermal advantages under cold conditions, implying shorter periods of thermoregulation and increasing time available for other activities, which increases success in feeding opportunities, mates, defending territories and in escaping from predators (Clusella-Trullas et al. 2007). This advantage would make darker individuals in cold environments (*e.g.* in alpine habitats) to be larger and to show better body condition than lighter ones of the same population (Luiselli 1995). Our results are consistent with this hypothesis, as populations where individuals showed darker skin (lower lightness values) were located at elevations with the lowest temperatures. However, studies in a variety of lizard species on the relationship between coloration and warming rates have found mixed results, some failing to find a relationship (*e.g.* Herczeg et al. 2007), while other found that melanic lizards heated up faster than lighter lizards (*e.g.* Clusella-Trullas et al. 2009). "Thermal melanism hypothesis" also states that darker skins are in detrimental of body size (Clussella-et al. 2007). That means that smaller individuals might be darker, prediction that matches with our findings, in almost all elevations, larger lizards are lighter.

An alternative, non-exclusive, hypothesis is that dorsal darkness results from the selective pressure of UVR. UVR is more severe at higher elevations (Sola et al. 2008) and may produce damages at different levels (see Introduction). In fact, higher reflectance of the skin is common in desert lizards (Porter and Norris 1969). Accordingly, correlation between average skin lightness at the level of population and UVR intensity was significantly negative, darker individuals (low values of lightness) were found where UVR was highest, at high elevations. The negative correlation between plant cover and dorsal coloration that we found may help to discern between the "thermal melanism hypothesis" and the "protection against UVR hypothesis". The "thermal melanism hypothesis" would predict a positive correlation between covering and dorsal coloration, as in zones with higher plant cover, lizards face a cooler environment (Iraeta et al. 2010), and would need heating up faster, therefore darker lizards having an advantage. On the other hand, the "protection against UVR hypothesis" would predict a negative correlation between plant cover and dorsal coloration, given that in more open zones lizards would be more exposed to radiation and darker lizards would have an advantage. Therefore, we suggest that the negative correlation between plant cover and dorsal lightness (lizards are darker in those places with higher plant cover) tentatively gives support to the "thermal melanism hypothesis" in detriment of the "protection agains UVR damage hypothesis".

Other possible explanations for the elevational variation in dorsal darkness may be ruled out. First, in other lizards, predation selects for darker colorations that are more similar to substrate color (Brown and Thorpe 1991; Vidal et al. 2007). Although substrate darkened with elevation, dorsal lightness of lizards' skin and lightness of substrate color were not correlated, thus this explanation was not supported. Second, melanin has been related to improved immunocompetence and higher resistance against parasites and pathogens in different taxa (Wilson et al. 2001; Burtt and Ichida 2004; Moreno and Moller 2006). We have no data on how disease pressure varies with elevation in our study system, but preliminary analyses of immune response, measuring swelling foot pad after phytohaemagglutinin injection (standard method to estimate cellular immune response, Smits et al. 1999) suggest that immune response does not vary with elevation ($F_{(5,96)} = 1.064$, $p = 0.39$; unpubl. data). And third, dark coloration may be a by-product of increased aggressiveness due to high population density, as frequently occurs in island lizard populations (Raia et al. 2010). Nevertheless, we can discard this possibility in our study population, as the highest densities were found in populations at mid elevations (1200 and 1700 m asl; Zamora-Camacho et al. 2013), and there were no differences in coloration between mid and low elevations (where the least dense populations occur).

We observed slight sexual dimorphism in dorsal coloration. Males had slightly more vivid color than females. These results could be explained by the relationship of melanins and carotenoids with status and aggressiveness (Badyaev and Hill 2000). On the other hand, note that males are more active than females and they are more exposed to sunlight (Díaz 1993). Therefore, other possibility is that males accumulate more pigments (more saturated coloration) in order to protect them from radiation, or in order to heat up faster than females, and therefore to initiate activity earlier.

Chromatic components of the color (chroma and hue) also varied with elevation. We tested whether differences in substrate color acted as selective pressures favouring the noted variation (Macedonia et al. 2003). Substrate coloration significantly varied with elevation, and lizard hue was positively correlated with substrate hue among populations. This finding strongly suggests that predation selects for lizards with dorsal color similar to substrate color. Therefore, it seems that predation selects for dorsal hue, while temperature and/or UVR selects for dorsal lightness in our study population.

In conclusion, this study shows that dorsal coloration shifts in an elevational gradient in the lizard *P. algirus*, lizards from the highland populations being darker than lizards from mid- and low-land populations. We proposed three possible but non-exclusive hypotheses for this adaptation: the "thermal melanism hypothesis" (Clusella-Trullas et al. 2007), the "protection against UVR hypothesis" (Porter and Norris 1969) and the "cryptic-coloration hypothesis". Our findings give more support to the "protection against UVR hypothesis", although the "thermal melanism hypothesis" cannot be convincingly ruled out. On the other hand, the "cryptic-coloration hypothesis" did not explain why lizards are darker at high elevations, but this hypothesis explained variation in dorsal hue with elevation. Therefore, we suggest that *P. algirus* is adapted to wide ranges of factors, adaptively darkening dorsal color at high elevation in a Mediterranean mountain.

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Table 5.2. Spearman's correlation matrix for mean values of components of lizards' dorsal color (lightness [L*], chroma [C*] and hue angle [H*]), environmental temparature (Te, in °C) during the activity season, ultraviolet B radiation (UVB, in µW cm-2 nm-1), percentage of vegetation cover (%VC) and substrate coloration for each six elevations (corresponding to the studied populations).

	Elevation	**Back L***	**Back C***	**Back H***	**Te**	**UV**	**%VC**	**Substrate L***	**Substrate C***
Back L*	-0.77								
	0.07								
Back C*	-0.94	0.89							
	<0.01	0.02							
Back H*	-0.94	0.83	0.89						
	<0.01	0.04	0.02						
Te	-0.94	0.83	0.89	1.00					
	<0.01	0.04	0.02	<0.01					
UV	0.94	-0.83	-0.89	-1.00	-1.00				
	<0.01	0.04	0.02	<0.01	<0.01				
%VC	0.43	-0.83	-0.60	-0.60	-0.60	0.60			
	0.40	0.04	0.21	0.21	0.21	0.21			
Substrate L*	-0.83	0.54	0.77	0.77	0.77	-0.77	-0.14		
	0.04	0.27	0.07	0.07	0.07	0.07	0.79		
Substrate C*	0.77	-0.20	-0.60	-0.60	-0.60	0.60	-0.14	-0.71	
	0.07	0.70	0.21	0.21	0.21	0.21	0.79	0.11	
Substrate H*	-0.94	0.60	0.83	0.89	0.89	-0.89	-0.20	0.94	-0.83
	<0.01	0.21	0.04	0.02	0.02	0.02	0.70	<0.01	0.04

Sample size was six populations. P-values are in cursive, below the correlation coefficients. Significant values in **bold**.

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Chapter 6
Oxidative stress variation in P. algirus

Senda Reguera, Francisco J Zamora-Camacho, Cristina E. Trenzado, Ana Sanz, Gregorio Moreno-Rueda. **Oxidative stress decreases with elevation in the lizard *Psammodromus algirus*.** *Comparative Biochemistry and Physiology*. *Part A*, Molecular and integrative physiology. 172: 52-56. SCI: 2.371

Abstract

Oxidative stress is considered one of the main ecological and evolutionary forces. Several environmental stressors vary geographically and thus organisms inhabiting different sites face different oxidant environments. Nevertheless, there is scarce information about how oxidative damage and antioxidant defences vary geographically in animals. Here we study how oxidative stress varies from lowlands (300-700 m asl) to highlands (2200-2500 m asl) in the lizard *Psammodromus algirus*. To accomplish this, antioxidant enzymatic activity (catalase, superoxide dismutase, gluthatione peroxidase, gluthatione reductase, gluthatione transferase, DT-diaphorase) and lipid peroxidation were assayed in tissue samples from the lizards' tail. Lipid peroxidation was higher in individuals from lowlands than from highlands, indicating higher oxidative stress in lowland lizards. These results suggest that environmental conditions are less oxidant at high elevations with respect to low ones. Therefore, our study shows that oxidative stress varies geographically, which should have important consequences for our understanding of geographic variation in physiology and life-history of organisms.

Key words: antioxidant enzymes - antioxidant protection - elevation gradient - lipid peroxidation – lizards - ultraviolet radiation.

Introduction

Oxidative stress, the unbalance between the production of pro-oxidant substances and antioxidant defences (Halliwell 2007), is considered one of the most important ecological and evolutionary forces (von Schantz et al. 1999; Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009; Costantini et al. 2010; Metcalfe and Alonso-Álvarez 2010). Aerobic metabolism implies the production of pro-oxidant substances (reactive oxygen/nitrogen species, RONS; Finkel and Holbrook 2000), which may react with molecular components of the cell such as lipids, proteins and nucleid acids, producing damages in cells' machinery (Halliwell and Gutteridge 1995; Sies 1997; Halliwell 2007). Organisms are protected against oxidative damage by enzymatic and non-enzymatic antioxidant defences, which work to maintain RONS levels at equilibrium and minimize RONS damages in the organism (Sies 1997; Finkel and Holbrook 2000; Blokhina et al. 2003). Production and maintenance of antioxidants implies energy and resources consumption and therefore it is costly (Halliwell 2007). When this balance is lost and antioxidant mechanisms cannot face RONS, oxidative stress occurs in cells (Sies 1997; Jones 2008).

In the wild, oxidative stress is induced by a wide range of environmental factors including changes in oxygen availability (Storey 1996; Hermes-Lima and Zenteno-Savín 2002; Buttemer et al. 2010), high or low temperatures (Hermes-Lima and Storey 1993; Voituron et al. 2006), contaminants (Regoli 2000; Prevodnik et al. 2007; Labrada-Martagón et al. 2011), and ultraviolet radiation (UVR) (Dahms et al. 2011). These environmental factors vary geographically, and thus levels of oxidative stress and antioxidant defences should vary along a cline of these environmental factors. Nevertheless, there is a lack of studies examining geographic variation in oxidative stress in animals (Prevodnik et al. 2007; Costantini et al. 2010).

Sunlight radiation, whose UV-B wavelength component is the most harmful (Dahms et al. 2011), is an important causes of oxidative stress (Chang and Zheng 2003; Chuang and Chen 2013). UVR initiates a series of redox reactions ending in free radical formation and leading to oxidative stress in cells (Dahms and Lee 2010). Moreover, reduction of O_2 by photolytic reactions results in negative effects on oxidative balance, increasing oxygen radicals and producing lipid peroxidation as well as changes in antioxidant enzyme activities (Dahms and Lee 2010). Damages produced by UVR negatively affect organisms' fitness, by reducing sperm motility, hatching success and growth rates, as well as by increasing embryo malformation and mortality (*e.g.* Blaustein et al. 1998; Pahkala et al. 2002; Marquis et al. 2008; Hylander et al. 2014). As a consequence, UVR is an important abiotic selective agent shaping physiology and life histories of organisms (Merilä et al. 2000). Because UVR increases with elevation (Blumthaler et al.1997; Sola et al. 2008), we expect that organisms from high elevations will be more prone to suffer oxidative stress than organisms from low elevations.

However, along an elevation gradient there are other environmental factors that gradually vary, such as temperature and oxygen partial pressure (PO_2; Körner 2007; Graae et al. 2012). Both temperature and PO_2 are involved in biochemical reactions and both decrease along elevation gradient. These environmental factors may have antagonistic effects with solar radiation regarding to oxidative stress generation. Oxygen plays an important role in oxidative metabolism and oxidative damages can be reduced in presence of low PO_2 (Buttemer et al. 2010). Low temperatures slow down biochemical reactions in ectotherms (but do the reverse in endotherms), which lead to low oxidants production and, for instance, low oxidative damage in cold environments (Jena et al. 2013). Moreover, in cold environments many animals hibernate, a period in which animals decrease metabolism to the minimum. Considering that PO_2 and temperature decrease with elevation, organisms from highlands might show less oxidative stress levels than organisms from lowlands.

In this work, we studied how oxidative stress damage and antioxidant enzymatic activity vary in the lizard *Psammodromus algirus* along an elevation gradient of 2200 m. *P. algirus* is an abundant lizard in Mediterranean landscapes of south-western Europe and north-western Africa, inhabiting along a wide elevation gradient (0-2700 metres above sea level; m asl), therefore, facing a wide range of environmental conditions of temperature, PO_2 and UVR (Salvador 2011).

Oxidative stress processes combine various components such as free radical production, antioxidant defences, oxidative damage, and repair mechanisms (Monaghan et al. 2009). In this study, two of these components were assayed, lipid peroxidation (as an indicator of oxidative stress; Del Rio et al. 2005; Monaghan et al. 2009; Hõrak and Cohen 2010), and activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione transferase (GST), DT-diaphorase (DTD) (as indicators of antioxidant defences), to guess possible variation in oxidative stress of lizards along a strong environmental gradient.

Material and methods

Study area and field procedures

Samplings were performed in Sierra Nevada Mountain (SE Spain), during the second half of July, in 2011. Sampling was concentrated in a short time period in order to avoid possible biases due to seasonal changes in oxidative stress (Hermes-Lima et al. 2012). Two sample sites were located at lowlands (36° 53'N, 3° 24'W, 300 m asl and 36° 55'N , 3° 26'W, 700 m asl) and two at highlands (36° 58'N, 3° 19'W, 2200 m asl and 37° 01'N, 3° 19'W, 2500 m asl) (Figure 4.5). In Sierra Nevada, mean annual temperature goes from 3.5 to 17.6 °C (in highest and lowest elevation respectively; REDIAM; http://www.juntadeandalucía.es/medioambiente/site/rediam), and in the study area, environmental temperature during *P. algirus* activity season (March to October) differs 8 °C on average between the lowest (300 m asl; mean±sd = 25.0±5.09 °C) and the highest sampling plot (2500 m asl; 17.2±4.87 °C; Zamora-Camacho et al. 2013). Relative irradiance also varies with elevation, increasing on average 6-8 $\%Km^{-1}$ for UV-A and 7-11$\%Km^{-1}$ for UV-B radiations (Sola et al. 2008). In the study area, UV-B radiation went from 1.28±0.03 to 4.8±0.37 $\mu Wcm^{-2}nm^{-1}$ between the lowest and the highest sampling plot (measured with the 305 nm wavelength channel of a BIC compact 4-channel radiometer, Biospherical Inc, CA, USA).

We captured 19 individuals (9 from highlands and 10 from lowlands) to measure oxidative stress under natural conditions at different elevations. Only adult males were used in this study, in order to avoid confounding effects due to age or sex (Olsson et al. 2012). Specimens were captured by hand. We measured snout-vent length (SVL) with a metal ruler (accuracy 1 mm) and weighted with an electronic balance (Model Radwag WTB200, accuracy 0.01 g). To avoid killing specimens, tissue samples were taken from the tail because lizards can regenerate it. Taking a tissue sample from tail has small or null impact on lizard survival (Niewiarowski et al. 1997). We took tail sample *in situ*, preserving samples in liquid nitrogen until carried to the laboratory facilities, where were keep in a freezer at -80 °C until analyses.

Biochemical analyses

Tissue samples were homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA and 0.1% triton X-100 (v/v), pH 7.8) at a ratio of 1:9 (w/v). Homogenates were centrifuged at 30000g for 30 min in a Centrikon H-401 centrifuge. After centrifugation, the supernatant was collected and frozen at -80°C until analysed. All enzymatic assays were carried out at 25±0.5 °C using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). The enzymatic reactions were started by the addition of the tissue extract, except for SOD, where xanthine oxidase was used. The specific assay conditions were as follows.

Catalase (EC 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 concentration at 240 nm according to Aebi (1984). The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2.

Superoxide dismutase (EC 1.15.1.1) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c and 0.024 IU ml^{-1} xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome C reduction rate measured at 550 nm (McCord and Fridovich 1969).

Glutathione peroxidase (EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). A freshly prepared glutathione reductase solution (2.4 U ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.0) was added to a 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH and 0.15 mM cumene hydroperoxide. After the addition of 1 mM GSH (reduced glutathione), the NADPH-consumption rate was monitored at 340 nm.

Glutathione reductase (EC 1.6.4.2) activity was assayed as described by Carlberg and Mannervik (1975) with some modifications, by measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.15 mM GSSG.

Glutathione S-transpherase (EC 2.5.1.18) activity was determined by the method of Habig et al. (1974) adapted to microplate. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH and 1.23 mM solution of 1-chloro-2,4-dinitrobenzene in ethanol were prepared just before the assay. GST activity was monitored at 340 nm by the formation of glutathione-CDNB-conjugate.

DT-diaphorase (NAD(P)H: Quinone oxidoreductase; EC 1.6.99.2) activity was measured according to Sturve et al. (2005) and adapted by Sanz et al. (2010). The reaction mixture contained 50 mM Tris-HCl (pH 7.3), 50 µM DCPIP (2,6-dichlorophenol indophenol) and 0.5 mM NADH. Control reaction was measured with the addition of distilled water instead of sample extract. DTD activity was defined as the difference between sample and control DCPIP reduction.

Except for SOD, for which the arbitrary units have already been mentioned, for other enzymatic activities, one unit of activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the above assay conditions. To estimate the enzymatic specific activity, soluble protein of the extracts was determined by bicinchoninic acid protein assay reagent (Thermo Scientific, Pierce Biotechnology, Rockford, USA) using bovine serum albumin as the standard.

Lipid-peroxidation levels were determined according to Buege and Aust (1978), based on malondialdehyde levels generated as product of lipid peroxides degradation. In the presence of thiobarbituric acid, MDA reacts producing colored thiobarbituric acid-reacting substances (TBARS) that was measured at 535 nm expressed as MDA per g tissue. Despite this method for lipid peroxidation assay is not so accurate as HPLC analysis (Halliwell 2007), evaluation of MDA levels as TBARS determination has been widely considered as a suitable indicator of tissue oxidation in different species of insects, fish or birds (Sanz et al. 2010, 2013; Moreno-Rueda et al. 2012). Although TABARS reacts with other aldehydes, most of the chromogen formed can be ascribed to the complex MDA-TBARS even when little MDA is present, because lipid peroxides break down to release MDA during the test conditions (Gutteridge and Quinlan 1983).

For all the reagents variables, two measurements were taken from each aliquot, and the average was used in statistical analyses. Except for SOD and CAT, repeatabilities (estimated according to Nakagawa and Schielzeth 2010) of biochemical measurements were high (Table 6.1).

All reagents, including substrates, coenzymes, and purified enzymes, were obtained from Roche (Mannheim, Germany), Sigma Chemical Co. (USA) or Merck (Darmstadt, Germany).

Table 6.1. Repeatability of biochemical analyses. The value of every variable was estimated twice, from different aliquots.

Biomarkers	**F$_{(18,19)}$**	***P*-value**	**Repeatability**
MDA	155.52	<0.001	0.987
SOD	6.91	<0.001	0.747
CAT	4.14	<0.001	0.611
GPX	60.94	<0.001	0.968
GR	45.09	<0.001	0.957
GST	37.80	<0.001	0.948
DTD	164.31	<0.001	0.988

MDA= Malondialdehyde, CAT= catalase, SOD= superoxide dismutase, GPX= gluthatione peroxidase, GR= gluthatione reductase, GST= gluthatione transferase, DTD= DT-diaphorase.

Statistical analyses

We performed a Mann-Whitney U-test to examine differences in oxidative stress biomarkers (lipid peroxidation [MDA] and antioxidant enzymatic activity [SOD, CAT, GPX, GR, GST, DTD]), between lizards from low and high elevation. Statistical power (1-β) was estimated for these comparisons. Because SVL and mass were highly correlated (r = 0.93, p < 0.01), both variables were combined in a factor (called "size") by using a Principal Components Analysis. In addition, we obtained Body Condition Index (BCI), calculated from residuals of regression of log body mass on log SVL. Finally, we explored relationships among oxidative stress biomarkers themselves and with body size and BCI of lizards with Spearman's correlations. All analyses were performed by using software R 2.15.2 (R Core Team 2012).

Results

Lizards from highlands were significantly larger than lizards from lowlands (Table 6.2). However, BCI was similar between both elevations (Table 6.2). All enzymes but GST had lower activity in highland than in lowland lizards, although any difference was significant (Table 6.2). By contrast, lipid peroxidation was significantly lower in highland than in lowland lizards (Table

6.2). Therefore, lowland lizards showed higher levels of oxidative stress than highland lizards.

Lipid peroxidation (MDA concentration) was negatively correlated with body size (Table 6.3). However, considering each elevation separately, MDA and body size were not significantly correlated (highlands, $r_s = 0.04$, $p = 0.93$; lowlands, $r_s = -0.43$, $p = 0.34$). By contrast, the activity of most of enzymes was not significantly correlated with size, excepting CAT and GR, which was negatively correlated (Table 6.3). Moreover, lipid peroxidation was negatively correlated with activity of several of the antioxidant enzymes, although the correlation was only significant with GST activity (Table 6.3). BCI did not correlate with any oxidative stress biomarker but GR (Table 6.3).

Table 6.2. Body size (factor made up of snout-vent length and mass variables), BCI (residuals of regression of log body mass on log snout-vent length), lipid peroxidation levels (MDA) and antioxidant enzymatic activity for lowland and highland lizards. Table shows mean values and standard error (±se), Mann-Whitney U test results, signification value (*P-value*), and statistical power (1-β) for biochemical variables.

	Highlands Mean±se	**Lowlands Mean±se**	**U**	***P-value***	**1-β**
Size	1.04±0.26	-0.96±0.22	53	<0.01	0.90
BCI	0.03±0.02	-0.01±0.02	38	0.27	0.05
MDA	140.96±50.74	423.04±83.23	13	0.02	0.62
SOD	42.27±2.45	42.79±3.71	51	0.66	0.05
CAT	5.33±0.47	6.35±0.63	31	0.27	0.21
GPX	3.53±0.53	4.25±0.66	28	0.48	0.10
GR	9.92±0.58	12.44±1.12	23	0.07	0.37
GST	35.48±2.94	28.54±2.46	63	0.15	0.35
DTD	71.17±7.26	72.19±9.98	48	0.84	0.05

MDA= Malondialdehyde (nmol/g tissue), CAT= catalase (U/mg protein), SOD= superoxide dismutase (U/mg protein), GPX= gluthatione peroxidase (U/mg protein), GR= gluthatione reductase (mU/mg protein), GST= gluthatione transferase (mU/mg protein), DTD= DT-diaphorase (mU/mg protein). Significant differences between elevationes are in **bold**. Sample size in the experiment was 19 (9 lizards from highlands and 10 lizards from lowlands) and samples were taken *in situ* to measure basal state of the parameters.

Table 6.3. Spearman’s correlation matrix among body size (factor of mass and snout-vent length), BCI (residuals of regression of log body mass on log snout-vent length), lipid peroxidation (MDA), and antioxidant enzymatic activity. Table contains rho-values for pairwise Spearman’s correlations and sample size (in italic).

	Size	**BCI**	**MDA**	**SOD**	**CAT**	**GPX**	**GR**	**GST**
MDA	-0.58*	0.05						
	15	15						
SOD	0.01	-0.31	-0.39					
	15	15	18					
CAT	-0.54*	-0.47	0.03	0.66**				
	15	15	18	19				
GPX	-0.45	-0.15	-0.05	0.03	0.15			
	15	13	16	17	17			
GR	-0.61*	-0.71*	0.21	0.43	0.68**	0.18		
	15	15	18	19	19	17		
GST	-0.03	-0.08	-0.52*	0.62	0.44	0.17	0.23	
	15	15	18	19	19	17	19	
DTD	-0.29	-0.37	-0.03	0.52*	0.54*	0.3	0.52*	0.56*
	15	15	18	19	19	17	19	19

MDA= Malondialdehyde (nmol/g tissue), CAT= catalase (U/mg protein), SOD= superoxide dismutase (U/mg protein), GPX= gluthatione peroxidase (U/mg protein), GR= gluthatione reductase (mU/mg protein), GST= gluthatione transferase (mU/mg protein), DTD= DT-diaphorase (mU/mg protein). Significant differences between elevationes are in **bold**. Sample size in the experiment was 19 (9 lizards from highlands and 10 lizards from lowlands) and samples were taken *in situ* to measure basal state of the parameters..

Discussion

Our results show that lipid peroxidation (as biomarker of oxidative stress damage) decreases with elevation in the lizard *P. algirus*. The question arises why oxidative stress level is lower at high elevation. Sunlight (including UV-B and UV-A radiation) is an important stressor for organisms (Ortonne 2002; Chang and Zheng 2003; Dahms et al. 2011). At high elevations, where UVR is more intense than at low elevations, we could expect that individuals of *P. algirus* would present higher levels of oxidative stress. The effect of UVR is exacerbated because, as an ectothermic organism, *P. algirus* expends long periods of time sunbathing to get suitable body temperatures (Díaz 1997), which makes it more susceptible to solar radiation damages. However, our results contrast with this expectation: lizards showed lower lipid peroxidation levels at high elevations.

One possible explanation for this result is that the environment is less pro-oxidant at high elevations. At low elevations, PO_2 and environmental temperature are higher, which increases metabolic rates and therefore free radical production. In reptiles, volume of consumed O_2 is usually proportional to environmental PO_2 (Snyder and Weathers 1977), and metabolism increases with elevated temperature (Shine 2005). Indeed, that oxidative damage increases with temperature in ectotherms has already proved (Jena et al. 2013). In our study system, lizards from lowlands have a body temperature 1.5 °C on average higher than lizards from highlands (Zamora-Camacho et al. 2013). Higher metabolism and free radical production in lowland lizards would lead to higher oxidative stress levels ("the rate-of-living hypothesis"; Sohal and Weindruch, 1996; Speakman 2005).

In addition, lizards from low elevations were significantly smaller. Metabolic rates and, in consequence, RONS production rates, is higher in relatively smaller individuals (Speakman 2005), which would lead to higher levels of oxidative damage. Because body size increased with elevation, and MDA decreased with body size, is difficult to disentangle whether oxidative damage changed with elevation *per se*, or such a variation is a consequence of variation in body size. To analyse this issue, we tested separately in both elevations the relationship between body size and oxidative stress. When each elevation is considered separately, no relationship between body size and oxidative stress emerges, which suggests that variation in oxidative stress with elevation is not due to variation in body size.

Furthermore, lizards from highlands show a longer hibernation period, thus undergoing a more restricted period of feeding (Zamora-Camacho et al. 2013). According to the "oxidative damage attenuation hypothesis" (Noguera et al. 2011), short periods of restricted feeding could help to prevent oxidant production, which could contribute to explain why lizards at highlands have low tissue oxidation levels. Additionally, individuals of *P. algirus* from highlands are darker than individuals from mid and lowlands (Reguera et al. 2014; Chapter 5). Higher concentration of melanins in highland lizards might be protecting from UVR (Ortonne 2002), contributing to reduce oxidative stress.

Finally, besides higher temperatures, the lowest sampling plot is characterised by the presence of fruit trees and olive crops. These kinds of habitats usually have higher presence of contaminants and heavy metals which are known as important stressor agents (Amaral et al. 2012). More contaminants in lowlands could contribute to the fact that lizards in lowlands showed higher oxidative stress levels.

We can discard that the differences in oxidative stress between lizards from highland and lowland are due to higher enzymatic antioxidant defences in highlands than in lowlands, given that we did not find significant differences in the activity of antioxidant enzymes with elevation. Consequently, differences in oxidative stress between highland and lowland lizards cannot be explained as a consequence of differences in enzymatic antioxidant capacity, although we cannot rule out the effect of non-enzymatic antioxidant defences.

Our findings contrast with studies in humans, which find that oxidative stress increases with elevation (review in Askew 2002). One of the main variables affecting oxidative stress with elevation is temperature, but temperature differentially affects metabolism (and thus RONS production) in endotherms and in ectotherms. Endotherms increase metabolism as temperature decreases (Beamonte-Barrientos and Verhulst 2013), which might explain higher oxidative stress in humans living at higher elevations. However, ectotherms reduce metabolism as temperature decreases, and consequently oxidant molecules production (Jena et al. 2013).

In conclusion, although one may naively think that alpine habitats, being more extreme, generate more oxidative stress in lizards, our findings show that *P. algirus* lizards from high elevation have less oxidative stress levels than those from low elevation. Therefore, our findings suggest that highland environments are less stressful than lowlands. Differences in stressful conditions probably have importantly influenced the evolution of life-history in animals (Pérez-Campo et al. 1998; Alonso-Álvarez et al. 2007; Buttemer et al. 2010). Consequently, our results may explain why highland ectotherms (exposed to less oxidative conditions) show higher longevity (*e.g.* Zhang and Lu 2012) and increased growth rate (*e.g.* Iraeta et al. 2006). Our study, therefore, highlights the ecological importance of geographic variation in pro-oxidant stressors in order to cast animal evolution.

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Chapter 7
Ultraviolet radiation effects on *P. algirus*

Senda Reguera, Francisco J Zamora-Camacho, Elena Melero, Sergio García-Mesa, Cristina E Trenzado, Marco J Cabrerizo, Ana Sanz, Gregorio Moreno-Rueda. **Ultraviolet radiation does not increase oxidative stress in the lizard *Psammodromus algirus* along an elevational gradient**. (In second revision *Comparative Biochemistry and Physiology. Part A, Molecular and integrative physiology*)

Abstract

Lizards, as ectotherms, spend much time basking for thermoregulating exposed to solar radiation. Consequently, they are subjected to ultraviolet radiation (UVR), which is the most harmful component of solar radiation spectrum. UVR can provoke several types of damage, from the molecular to tissue level, and can even cause death. Photooxidation triggered by UVR produces reactive oxidative species (ROS). When antioxidant machinery cannot combat the ROS concentration, oxidative stress occurs in the organisms. Given that UVR increases with elevation, we hypothesised that lizards from high elevations should be better adapted against UVR than lizards from lower elevations. In this work, we test this hypothesis in *Psammodromus algirus* along an elevation gradient (three elevational belts, from 300 to 2500 m asl). We ran an experiment in which lizards from each elevation belt were exposed to 5-hour doses of UVR (UV-light bulb, experimental group) or photosynthetically active radiation (white-light bulb, control group) and, 24 h after the exposure, we took tissue samples from the tail. We measured oxidative damage (lipid and protein peroxidation) and antioxidant capacity as oxidative-stress biomarkers. We found no differences in oxidative stress between treatments. However, consistent with a previous work, less oxidative damage appeared in lizards from the highlands. We conclude that UVR is not a stressor agent for *P. algirus*; however, our findings suggest that the lowland environment is more oxidative for lizards. Therefore, *P. algirus* is well adapted to inhabit a large elevation range, and this would presumably favor the lizard in case it ascends in response to global climate change.

Key words: antioxidant capacity, elevation gradient, lipid hydroperoxides, lizards, oxidative damage, oxidative stress, protein hydroperoxides, ultraviolet radiation.

Introduction

Oxidative stress, recognized as a strong evolutionary and ecological force (Boonstra 2013), occurs when organisms present an imbalance between pro-oxidant and anti-oxidant molecules. Pro-oxidant molecules include free-radical oxygen species (ROS) and other reactive species (*e.g.* aldehydes or quinones; Jones 2008). These oxidative species trigger a cascade-like process, producing lipid hydroperoxides (LOOH), protein hydroperoxides (PrOOH), and finally damage to biological membranes and other lipid- and protein-containing structures (Halliwell 2007), as well as DNA (Grintzalis et al. 2013). Consequently, ROS can inflict harm at the molecular, cellular, and tissue levels (Halliwell and Gutteridge 2007), with serious fitness consequences for individuals (*e.g.* Alonso-Álvarez et al. 2004). The antioxidant machinery (enzymatic and non-enzymatic) in organisms keeps the oxidative species concentration in equilibrium. However, when antioxidant machinery cannot regulate the concentration of pro-oxidant substances, oxidative-stress levels rise (Halliwell and Gutteridge 1995; Sies 1997; Halliwell 2007).

Pro-oxidant molecules can be generated endogenously (*e.g.* mitochondria, peroxisomes, etc.) and/or exogenously (by environmental factors *i.e.*, ultraviolet radiation [UVR], toxins, ionizing radiation, etc.; Finkel and Holbrook 2000). Endogenously, mitochondria are the most important producers of oxidative species during aerobic metabolism (Finkel and Holbrook 2000). Regarding environmental factors, UVR is an important stressor that induces oxidative imbalance by photooxidation (Chuang and Chen 2013). UVR initiates a series of redox reactions that result in free oxygen radicals that trigger oxidative imbalances in cells (Dahms and Lee 2010). In this way, UVR damages cell molecules (Tadokoro et al. 2003). In addition, UVR can weaken the immune response (IR) (Garssen and van Loveren 2001; El-Ghorr and Norval 1999). Eventually, all this damage can end up in a fitness reduction (Hylander et al.2014), with sub-lethal and lethal effects in severe situations (Dahms and Lee 2010; Belden et al.2000). These harmful effects provoked by UVR have been detected in almost every animal group, from zooplankton (Rautio and Tartarotti 2010) to humans (Matts and Fink 2010), including algae (Harrison and Smith 2009), cnidaria (Tarrant et al. 2014), earthworms (Chuang and Chen 2013), fish (Zamzow 2004; Charron et al. 2007), amphibians (Blaustein et al. 1998), and reptiles (Chang and Zheng 2003).

The net effect of UVR is exacerbated in reptiles because, as ectotherms, they spend long periods of time basking for thermoregulate exposed to sunlight (Díaz 1997), making them more susceptible to solar-radiation damage. Unlike mammals or birds, reptilian skin lacks photoprotective fur or feathers, and therefore they should be more vulnerable to UVR damage (Chang and Zheng 2003). Despite this lack, reptiles have developed strategies to cope with UVR such as the use of photo-protective substances (Cope et al. 2001) or pigments such as melanins (Ortonne 2002) in the skin.

UVR is an environmental factor with pronounced spatial variation (Blumthaler et al. 1997). Given that UVR intensifies with elevation because the atmosphere layer is thinner (Körner 2007), individuals from higher elevations are more exposed to UV rays. Therefore, we initially expected that oxidative stress should be higher in high elevation lizards as a consequence of increased UVR, and we tested this hypothesis in the lizard *Psammodromus algirus* (Linnaeus 1758) along an elevational gradient of 2200 m in Sierra Nevada Mountain (SE Spain). However, against our prediction, high elevation individuals showed less oxidative damage (in terms of lipid peroxidation) than did lowland ones under natural conditions (Reguera et al. 2014b; Chapter 6). One explanation for this finding is that high elevation lizards may be locally adapted, being better protected against UVR, as happens in the high elevation populations of other species (Marquis and Miaud 2008; Marquis et al. 2008).

In the present study, we tested whether *P. algirus* is locally adapted to cope with UVR along the elevational gradient. For this, we performed a laboratory experiment in order to reduce the range of environment stressors to one: UVR. Lizards from low, mid, and high elevations were exposed to two different radiation treatments, UVR (experimental group) and photosynthetically active radiation (PAR) (control group). We predicted that (1) if UVR is an oxidative agent for this lizard, oxidative damages will be higher and the immune response lower in lizards exposed to UVR than in control lizards; and (2) if there is local adaptation against UVR along the elevational gradient, the effect of UVR will be higher in low elevation lizards.

Because there is no one single “standard” biomarker for oxidative stress (Halliwell and Gutteridge 2007), we measured oxidative damage and total antioxidant capacity (including the activity of diverse antioxidant enzymes) capacity. For this, we examined the trolox-equivalent antioxidant capacity (TEAC) and antioxidant enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST). In addition, we quantified markers of oxidative damage in tissues, specifically the main lipid and protein peroxidation products: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH; representing mainly lipid and other hydrophobic hydroperoxides), and protein hydroperoxides (PrOOH) (Grintzalis et al. 2013). At the same time, in order to evaluate possible UVR effects on the immune system, we measured skin inflammatory response to a mytogen (PHA: phytohaemagglutinin), and peroxidase activity, related to oxidative respiration of neutrophils (Mohanty and Sahoo 2010).

Material and methods

Sampling

Psammodromus algirus is a lacertid lizard widely distributed in the Iberian Peninsula and the north of the Maghreb, occurring from sea level to 2800 m asl (metres above sea level). Adult males were caught by hand at Sierra Nevada Mountain (SE Spain), from May to July of 2013. Sampling was performed in six populations located along a 2200 m elevation gradient from 300 m asl to 2500 m asl (Figure 4.5). Localities were grouped into a low (300-700 m asl), medium (1200-1700 m asl), and high (2200-2500 m asl) elevation belt for the statistical analyses. This grouping was justified by the similarity between areas in different population traits, such as activity patterns and morphology (Zamora-Camacho et al., 2013; Reguera et al., 2014a; Chapter 5). Solar irradiation varies with elevation, increasing on average 6-8 %Km^{-1} for UV-A radiation and 7-11 %Km^{-1} for UV-B (Sola et al. 2008). In the study area, noon irradiance of UV-B radiation ranged from 1.28±0.03 to 4.80±0.37 μWcm^{-2} on average between the lowest and the highest sampling plot for the experimental period (Reguera et al. 2014a,b; Chapter 4 General Methodology).

We caught 63 lizards (22 from low, 19 from medium, and 22 from high elevation) and, in the lab, kept them singly in cages (20×13×9 cm), with a heat cable at one end of the cage, switched on three hours a day (11 h-14 h), to allow thermoregulation. Lizards were fed mealworms (larvae of *Tenebrio molitor*) and given *ad libitum* access to water. Before the experimentation, the individuals were acclimated for four days (three days in the cages and one day in the experimental terrarium) in order to equal the conditions. After the experiments, lizards were released in the localities they came from, and no lizard died as a consequence of the treatments. All lizards were weighed to the nearest 0.01 g (balance model Radwag WTB200) and measured to the nearest 1.0 mm with a metal ruler. To avoid killing specimens, tissue samples were taken from the tail because it can regenerate. Taking a tissue sample from tail has a minor or null impact on lizard survival (Niewiarowski et al. 1997). Only adult individuals were included in the study to avoid confounding ontogenetic factors, given that oxidative damage and antioxidant defences are higher in juveniles than in adults (Furtado-Filho et al. 2007; Hermes-Lima et al. 2012). Moreover, only males were included in the study, because of sexual differences in oxidative stress levels (Olsson et al. 2012), and because most of the females were pregnant during the experimental period.

Experimental procedure

The experimental approach consisted of exposing individuals to different portions of solar radiation spectrum: (i) 30 individuals (from the six different localities) received photosynthetically active radiation provided by white light bulbs (control group), and (ii) 33 individuals (from the six different localities) received UVR+PAR (experimental group) provided by UV-light bulbs (Repti

Glo 10.0 compact). Lizards in the experimental group were exposed to irradiances of 1.94, 2.83 and 1217.6 μWcm^{-2} for UV-B, UV-A and PAR, respectively, which was inside the range found under natural conditions for this lizard (Reguera et al., 2014a,b; Chapter 4 General methodology). Lizards in the control group received no UVR. The spectral output of the bulbs was checked with a spectroradiometer (Ocean Optics model USB2000+UV-VIS).

Bulbs were placed 30 cm over the centre of a 100×30×50 cm terrarium with pine cork as substrate and *ad libitum* access to water. Lights were switched on for 5 h, during which time each lizard was subdued because there had no shelters. The lizards' body temperature was monitored during the experiment, recorded hourly with a 1mm diameter thermocouple (TP (K) INCONEL 600), 8 mm inside the cloaca, assembled to a Hibok 14 thermometer (accuracy 0.01 °C). Maximum body temperature (T_{max}) was included in the analyses, given that oxidative stress increases with body temperature in ectotherms (Jena et al. 2013). Tissue samples were taken 24 h after the experiment was finished, when the antioxidant response and oxidative damage peak (Chang and Zheng 2003). Samples were immediately frozen in liquid nitrogen and kept at -80 °C until biochemical analyses.

Oxidative stress evaluation

Determining antioxidant capacity

To assess the antioxidant capacity, we measured trolox-equivalent antioxidant capacity (TEAC) as the antioxidant mechanism, as well as a set of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione S-transferase (GST) (Schlesier et al. 2002). Tissue samples were homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, and 0.1 % triton X-100 (v/v), pH 7.8) at a ratio of 1:9 (w/v). Homogenates were centrifuged at 30000 *g* for 30 min in a Centrikon H-401 centrifuge. After centrifugation, the supernatant was collected and frozen at -80 °C until being analysed. All enzymatic assays were performed at 25±0.5 °C using a Power Wavex microplate scanning spectrophotometer (Bio-Tek

Instruments, USA) in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany). The enzymatic reactions were started by the addition of the extracted tissue, except for SOD, where xanthine oxidase was used. The specific assay conditions were as follows:

Total antioxidant capacity assayed as TEAC was measured according to (Erel 2004). ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) in acidic medium is oxidized by hydrogen peroxide, turning an emerald-green color. The reduction of this compound in the presence of antioxidants results in a loss of color measured at 595 nm, proportional to the total antioxidant capacity of the extract. Antioxidant activity refers to the equivalent of a water-soluble analogue of vitamin E (Trolox) used as standard. Results were expressed in terms of μmol of Trolox equivalent antioxidant capacity per litre of tissue extract (μM).

SOD (EC 1.15.1.1) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c, and 0.024 IU ml^{-1} xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to bring about a 50% inhibition of the ferricytochrome c reduction rate, measured at 550 nm (McCord and Fridovich 1969).

CAT (EC 1.11.1.6) activity was determined spectrophotochemically by measuring the decrease in the H_2O_2 concentration at 240 nm (Aebi 1984). The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and 10.6 mM H_2O_2 (freshly prepared).

GPX (EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). A freshly prepared glutathione reductase solution (2.4 U ml−1 in 0.1 M potassium phosphate buffer, pH 7.0) was added to 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH, and 0.15 mM cumene hydroperoxide (CumOOH). After the addition of 1 mM GSH (reduced glutathione), the

NADPH-consumption rate was monitored spectrophotochemically at 340 nm.

GST (EC 2.5.1.18) activity was determined by the method of Habig et al. (1974), adapted to a microplate. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH, and 1.23 mM solution of 1-chloro-2,4-dinitrobenzene in ethanol, which was prepared just before the assay. GST activity was monitored spectrophotochemically at 340 nm by the formation of glutathione-CDNB-conjugate.

Except for SOD (the arbitrary units of which have been mentioned above), for every other enzymatic activity, one unit of activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the aforementioned assay conditions. To estimate the enzymatic specific activity, the soluble protein of the extracts was determined by bicinchoninic acid protein assay reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as the standard.

Determination of oxidative damage

To quantify the oxidative damage, we evaluated lipid and protein peroxidation biomarkers: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH), and protein hydroperoxides (PrOOH) (Grintzalis et al. 2013).

At the first step, each sample was divided in two homogenates of 0.5 ml, which were mixed with 1 ml chlorophorm:methanol (2:1) followed by vigorous vortexing; then, 150 μl TCA (trichloroacetic acid) 100 % was added to the resulting mixture and briefly vortexed. After 20 min of incubation in an ice-water bath, the samples were centrifuged at 20000 *g* for 10 min. As a result of this process, three layers were formed: an upper aqueous layer, an intermediate protein disk, and a bottom chloroform layer. The aqueous layers of the two replicates of every sample were combined and stored at -20 °C to use later for the FrMDA determination. The combination of the two chloroform bottoms was concentrated by a continuous flow of nitrogen and stored at -20 °C, for the LOOH assay. One of the resulting disks of protein was solubilized in

1500 µl NaOH 0.1mM and incubated at 60 °C for 30 min to hydrolyze PrMDA from the protein-MDA complex. This was immediately cooled in an ice-water bath and centrifuged at 20000 *g* for 5 min, when the supernatant was collected and stored at -20 °C and then used for PrMDA measurement. The other resulting disk of protein was solubilised in 1800 µl NaOH 0.1M and neutralized with 200 µl HCl 1M, stored directly at -20 °C, and afterwards used for PrOOH measurement.

For the LOOH and PrOOH determination, we followed the same procedure. Firstly, the lipid pellet was dissolved in 2000 µl absolute methanol for LOOH assay. In both, the sample was incubated for 30 min with or without the presence of Fe^{+2}. The final concentration of reagents for sample +Fe was 92.5% methanol, 100 µM XO (xylenol orange), 12.5 mM H_2SO_4, and 200 µM Fe^{2+}, while the final concentration of reagents for sample -Fe was 92.5 % methanol, 100 µM XO, 12.7 mM H_2SO_4. The absorbance was measured at 560 nm, and the net absorbance difference was calculated as (Sample+Fe) - (Blank reagent+Fe), from which the absorbance difference (Sample-Fe) - (Blank reagent-Fe) was subtracted. For LOOH, the net absorbance was converted to CumOOH concentration equivalents using a standard curve (0-20 µM), or converted to H_2O_2 concentration equivalents using a standard curve (0-40 µM).The results were expressed as µmol equivalents CumOOH/mg protein of original LOOH or PrOOH homogenate, respectively.

For PrMDA and FrMDA, we followed the same procedure. Samples were incubated, with a final reagent concentration of 7 % TCA, 0.21 % TBA (thiobarbituric acid), 1mM BHA (butylhydroxyanisole) for sample reaction or 7 % TCA, 1mM BHA for sample blank, for 20 min at 100 °C. Then, samples were cooled in an ice-water bath and centrifuged at 10000 *g* for 3 min. The absorbance of supernatant was measured at 535 nm. The net absorbance (sample reaction – sample blank) was converted to MDA (malondialdehyde) concentrations from the corresponding standard curve, prepared in NaOH 0.1M solution (0-15 µM MDA) for PrMDA determination or prepared in extraction solution (100 mM phosphate buffer, 0.5mM BHA, 33 % methanol, 13.3 % TCA) (0-40µM) for FrMDA assay. The results were expressed as µmol

MDA/mg protein of PrMDA fraction (PrMDA) or μmol MDA/mg protein of original homogenate (FrMDA).

For all biochemical variables, two measurements were taken from each aliquot, and the average was used in the statistical analyses. All biochemical reagents, including substrates, coenzymes, and purified enzymes, were obtained from Roche (Mannheim, Germany), Sigma Chemical Co. (USA) or Merck (Darmstadt, Germany).

Immune response evaluation

Skin inflammatory response

To assess the T-cell-mediated immune response, we used the phytohaemagglutinin (PHA) injection test. This response is a measure of T-cell proliferation, and is assessed by subcutaneously injecting PHA and measuring the swelling that occurs after 24 h (Smits et al. 1999). Thickness of the left-hind-limb sole was measured (at a central point) using a pressure-sensitive micrometer (Mitutoyo), with an accuracy of 0.01 mm. The sole was then injected at its central point with 0.05 mg of PHA (Sigma, L- 8754) in 0.01 ml of phosphate buffered saline (PBS). Twenty-four hours later we again measured the thickness of the sole at the central inoculation site. Reaction to PHA was expressed as the difference between thickness 24 h after inoculation minus thickness just before inoculation (Belliure et al. 2004).

Peroxidase activity

The enzyme peroxidase is related to neutrophil activity during pathogen infections, and therefore its activity indirectly indicates the neutrophil concentration. Peroxidase activity was determined as described by Mohanty and Sahoo (2010). After several tests, we considered the mixture of 10 μl of sample with 90 μl of Hank's solution without Ca^{2+} or Mg^{2+} to which 35 μl of TMB (J644, AMRESCO, USA) were added. The reaction was stopped with 35 μl of sulphuric acid 1M after 2 min of incubation. The samples were read at 450 nm. The results were expressed as optical density (OD) at 450 nm.

Statistical analyses

All analyses were performed using the software R 2.15.2 (R Development Core Team, 2012). Initially, we tested possible differences in T_{max} between the control and experimental group with an ANOVA. Oxidative damage and antioxidant capacity were evaluated using lineal models (Quinn and Keough 2002).The final models included measurements of oxidative stress and the immune response as dependent variables, and treatment (as factor), population of origin (elevation, as factor), and T_{max} (which differed between treatments [see Results], as the covariate). No interaction proved significant, and thus no interaction is shown. Body mass was not included in the analyses because it had no significant effect in any case, and showed collinearity with elevation. Some variables were log-transformed to achieve normality and homoscedasticity of the residuals.

Results

Body temperature was significantly higher in control individuals than in individuals exposed to UVR ($F_{(1,59)} = 5.98$, $p = 0.02$; mean T_{max}±se: control group: 29.45±0.37 °C; experimental group: 27.94±0.47 °C). For this reason, T_{max} was included as a covariate in subsequent analyses, although models without T_{max} as a covariate gave qualitatively the same results;See in Appendix A Table A3). We found no effect of treatment on any of oxidative biomarkers (oxidative damage or antioxidant capacity; Table 7.1; Figure 7.1 and Figure 7.2). However, biomarkers of oxidative damage, such as FrMDA, PrMDA, and PrOOH significantly decreased with elevation (Table 7.1, Figure 7.1). Even though T_{max} was significantly higher in control individuals, T_{max} had no significant effect on oxidative biomarkers (Table 7.1). Finally, immune parameters (skin inflammatory response and peroxidase activity) were not correlated with any oxidative parameters (Appendix A Table A4) and neither varied between treatments or elevations (Table 7.1; Figure 7.3).

Table 7.1. F-values and degree of freedom (in brackets) for a lineal model, including oxidative damage, antioxidant capacity, and immune response variables as dependent variables, and treatment, elevation, and maximum body temperature (Tmax) as independent predictors.

	Treatment	**Elevation**	**Tmax**
Antioxidant capacity			
TEAC	(1,54) 0.42	(2,54) 1.01	(1,54) 0.27
SOD	(1,54) 1.97	(2,54) 0.10	(1,54) 0.04
CAT	(1,51) 0.77	(2,51) 2.44	(1,51) 0.11
log(GPX)	(1,50) 0.80	(2,50) 0.11	(1,50) 2.05
GST	(1,51) 0.62	(2,51) 1.01	(1,51) 0.06
Oxidative damage			
FrMDA	(1,56) 0.31	(2,56) 3.90*	(1,56) 0.57
log(PrMDA+1)	(1,55) 0.46	(2,55) 3.08*	(1,55) 0.08
log(LOOH)	(1,55) 0.01	(2,55) 0.43	(1,55) 0.53
PrOOH	(1,56) 0.31	(2,56) 4.79**	(1,56) 1.40
Immune response			
IR	(1,56) 0.66	(2,56) 0.03	(1,56) 1.69
Peroxidase	(1,51) 0.29	(2,51) 0.88	(1,51) 3.13

TEAC = trolox equivalent antioxidant capacity (µM eq Trolox), SOD= superoxide dismutase (U/mg protein), CAT= catalase (U/mg protein), GPX= glutathione peroxidase (mU/mg protein), GST= glutathione (mU/mg protein) transferase, FrMDA= free malondialdehyde (µmol/mg protein), PrMDA= malondialdehyde associated (µmol/mg protein) to protein , LOOH= lipid hydroperoxides (µmol/mg protein), PrOOH= Protein hydroperoxides (µmol/mg protein), IR=Inflammatory immune response (mm), Peroxidase (OD. at 450nm). All higher-order interactions involving covariates proved non-significant and were removed from the models. The reduced models are presented here. Results were considered significant when *p*-value <0.05. * *p*<0.05, ***p*<0.01, ****p*<0.001.

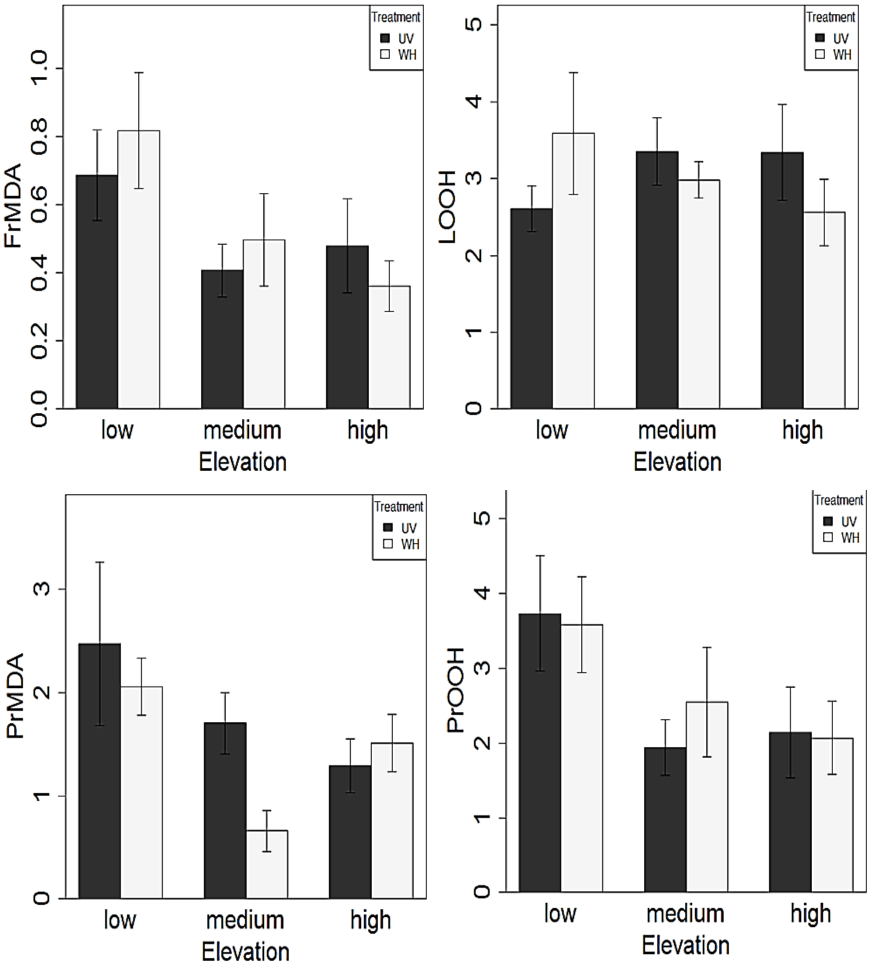

Figure 7.1. Mean values with standard error (error bars) of different biomarkers of oxidative damage by treatment (Black: UV= ultraviolet light bulb, experimental group; Grey: WH= white light bulb, control group), and by elevation (low= 300-700 m asl; medium= 1200-1700 m asl; high= 2200-2500 m asl). FrMDA= free malondialdehyde, PrMDA= malondialdehyde associated to protein, LOOH= lipid hydroperoxides, PrOOH= Protein hydroperoxides (all measured in μM/mg protein).

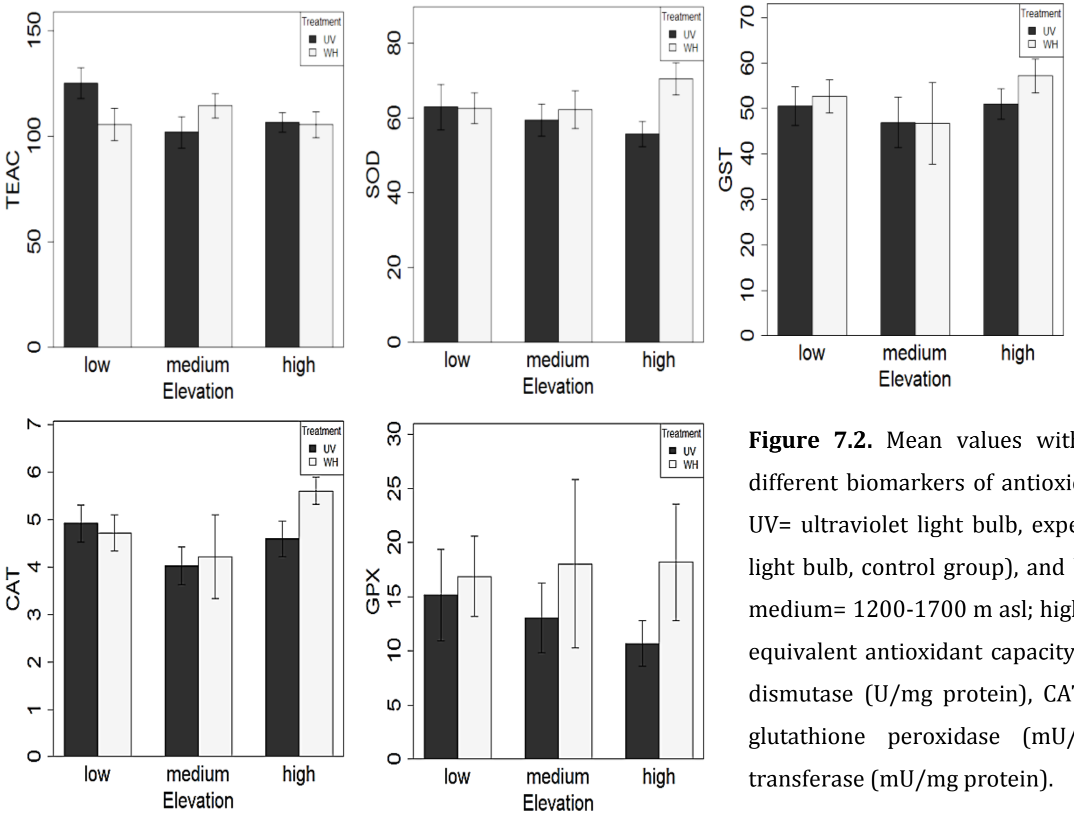

Figure 7.2. Mean values with standard error (error bars) of different biomarkers of antioxidant capacity by treatment (Black: UV= ultraviolet light bulb, experimental group; Grey: WH= white light bulb, control group), and by elevation (low= 300-700 m asl; medium= 1200-1700 m asl; high= 2200-2500 m asl). TEAC= trolox equivalent antioxidant capacity (µM eq Trolox), SOD= superoxide dismutase (U/mg protein), CAT= catalase (U/mg protein), GPX= glutathione peroxidase (mU/mg protein), GST= glutathione transferase (mU/mg protein).

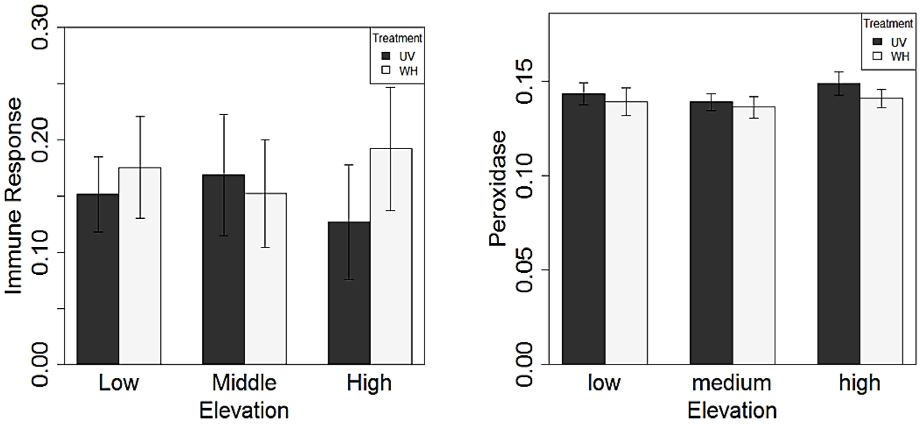

Figure 7.3. Mean values with standard error (error bars) of parameters of immune response by treatment (Black: UV= ultraviolet light bulb, experimental group; Grey: WH= white light bulb, control group), and by elevation (low= 300-700 m asl; medium= 1200-1700 m asl; high= 2200-2500 m asl). Skin inflammatory response was measured as swelling (mm) of the front limb sole after 24h of an injection of PHA (Phytohaemagglutinin). Peroxidase activity was measured in OD at 450 nm.

Discussion

Then main goal of this study was to evaluate the short-term UVR impacts on oxidative stress responses in a lizard along an elevational gradient. We found no effects of UVR on any of the oxidative biomarkers or immune parameters in the lizard *P. algirus* independently of lizard origin (low, mid, or high elevation). The mean UVR values used in the experiment were within the natural variation range of UVR measured in the field (Regueraet al. 2014a,b: Chapter4 General methodology). Therefore, our radiation values that simulated those to which lizards are exposed under natural conditions. Exposure time to UVR (5 h) was also similar to the exposure time under natural conditions, considering that *P. algirus* basks during 47.3 % of its activity time (Belliure et al. 1996). Nevertheless, in the wild, lizards do not receive all the UVR present in the environment (Ferguson et al. 2014), given that lizards also shelter to avoid overheating (Carrascal and Díaz 1989). Note that to expose animals to UVR levels higher than those present in natural conditions would prompt non-comparable responses with the situation that they undergo in nature. The question is whether natural-occurring UVR is harmful for animals and –in this study– whether high elevation lizards are less damaged by UVR than lowland lizards. In contrast to our findings, the snake *Elaphe taeniura* shows oxidative stress in response to 2 h of exposure to UVR (Chang and Zheng 2003). In that study, Chang and Zheng used UVR within the levels that some lizards are exposed in the nature (Ferguson et al. 2005, 2014).

In our study, the timing of tissue sampling was also appropriate, as oxidative damage (*e.g.* MDA and tissues damages) and antioxidant activity (*e.g.* SOD and CAT) peak at about 24 h after the exposure to UVR (Chang and Zheng 2003). Consequently, time of exposure and radiation received were sufficient for experimental lizards to develop an oxidative response, especially considering that relatively low levels of radiation and exposure were sufficient to generate oxidative stress in the snake *E. taeniura* (Chang and Zheng 2003). The fact that we did not detect oxidative stress in this lizard suggests that it is well-adapted against UVR. This finding is not completely surprising, given that *P. algirus* is basically heliothermic (Díaz and Cabezas-Díaz 2004). Therefore, it is heavily exposed to solar radiation, and has probably evolved adequate defence mechanisms to cope with harmful UVR (*e.g.* accumulation of melanin in the skin).

Although UVR usually causes several types of damage at different levels in organisms (Blaustein et al. 1998; Garssen and van Loveren 2001; Chang and Zheng 2003), this is not the first study that has failed to detect harmful effects of UVR. For example, UVR was found not to affect egg survival or larvae growth rates of the fish *Coregonus sp*. (Häkkinen et al. 2002). Moreover, the lizard *Anolis carolinensis* showed no UV-effects on immune response, probably due to photoprotective substances in the skin that absorb UVR (Cope et al. 2001), although UVR reduces the immune response in humans, rodents, and birds (El-Ghorr and Norval 1999; Garssen and van Loveren 2001). Photoprotective substances have been found in several groups from zooplankton (Hansson and Hylander 2009) to algae (Llewellyn and Airs

2010), amphibians (Hofer and Mokri 2000), and fish (Fabacher and Little 1998). UVR even induces melanization in lighter species (Häkkinen et al. 2002). Accordingly, we found that lizards living in high elevations are darker than low elevations ones (Reguera et al. 2014a), indicating higher melanin concentrations (Montgomerie 2006).

On the other hand, we found that oxidative damage (on lipids: FrMDA, PrMDA and proteins: PrOOH) decreased with elevation. This finding tallies with our previous work (with a different set of individuals), in which we found more oxidative damage in low than in high elevation lizards (Reguera et al. 2014). Therefore, the fact that *P. algirus* did not express oxidative damage inflicted by UVR, which is one of the main oxidative agents at high elevations, suggests that this lizard is well-adapted to UVR (Reguera et al., 2014a; Chapter 6). However, the fact that low elevation lizards showed more oxidative damage that did high elevation ones, may indicate a more oxidative environment at lower elevations. Besides UVR, other environmental agents may influence physiological conditions (Dahms et al. 2011). For example, temperature or oxygen pressure, which are also major oxidative agents (Costantini 2014), decrease with elevation, allowing a less oxidant environment in the high elevations. Moreover, the effects of a single stressor usually differ when considered in combination with other stressors, due to the antagonistic or synergistic nature of the interactions (Folt et al. 1999).

In this sense, within a context of global change, it is crucial to understand how species adapt to varying conditions at both global and local scales. Some studies indicate that many species are shifting their distribution pattern to higher elevations in response to climate change (Wilson et al. 2005; Benito et al. 2011; Chen et al. 2011). Therefore, it is important to know whether species or populations from lowlands ascending in elevation would have the ability to face highland environmental conditions. In this sense, the importance of temperature has been repeatedly considered, while the importance of UVR has been generally neglected. In mountains, a successful distribution shift upwards will depend on the capacity of species to inhabit environments with stronger UVR, among other biotic and abiotic factors. *P.*

might be expected to raise its elevational distribution in response to climate change in Sierra Nevada (a mountain with a maximum elevation of 3479 m asl), reflected by the observation of dense populations located around 2600 m asl and single individuals over 2700 m asl this elevation (Salvador 2011; Zamora-Camacho et al. 2013; O. Jiménez-Robles, pers. observ.).

In summary, our results confirm that *P. algirus* is a generalist organism in terms of environmental conditions. On the one hand, UVR seems not to restrict its distribution and, on the other hand, *P. algirus* inhabits a wide range of environmental temperatures (mean±se; 25.02±1.26 °C to 16.49±1.61 °C from low to high elevations, Zamora-Camacho et al. 2013). This suggests that, in a climate-warming scenario, *P. algirus* could inhabit in higher elevations, if the habitat (shrubs cover vegetation) and prey availability are adequate. Nevertheless, although UVR levels do not constitute a stressor agent for *P. algirus* in terms of oxidative stress or immune response, other stressors may be acting in low elevations, resulting in more oxidative damages in low than in high elevation individuals.

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Chapter 8
Variable crypsis in *P. algirus*

Laureano G González-Granda; Senda Reguera; Francisco J Zamora-Camacho; Elena Melero and Gregorio Moreno-Rueda. **Variable crypsis in the lizard *Psammodromus algirus* along an elevational gradient.** (In second revision *Evolutionary Ecology*).

Abstract

Predation usually selects for crypsis. Crypsis is the matching between animal coloration and the background where they are usually found, in order not to be detected by visual predators. Therefore, if background coloration varies along a gradient, animal coloration should vary parallel to background color in that gradient. We test this hypothesis in the lizard *Psammodromus algirus* along an elevational gradient. Moreover, given that this lizard is found mainly on bare soil, we predicted that its dorsal coloration should covary with bare soil coloration, but not with coloration of other elements of background rarely used by this lizard, such as rocks and vegetation. Our findings generally supported our predictions: lizard dorsal coloration covaried with coloration of bare soil along the elevational gradient, but not with coloration of rocky substrate or vegetation. Nevertheless, the maximal crypsis was with rocky substrate, followed by bare soil. Moreover, we found that the degree of crypsis decreased with elevation. This finding was expected, because as elevation increases, natural selection favors darker lizards, for improved thermoregulation and/or protection against ultraviolet radiation. Thus, selection for darker coloration conflicted with selection for crypsis, leading to reduced crypsis with elevation. In conclusion, our findings reveal that there is local adaptation for crypsis in this lizard along an elevational gradient, but this local adaptation is hindered at higher elevations as a consequence of natural selection for darker dorsal coloration.

Keywords: Crypsis - Local adaptation - Elevational gradient – Lizards coloration – Predation pressure

Introduction

Predation is one of the main selective forces in nature (Caro 2005). Predators not only select for traits directly related to predation avoidance –such as escape capacity (Vervust et al. 2007), odour (Reneerkens et al. 2005), or growth rate (Cheng and Martin 2012)–, but also affect the evolution of morphology (Irschick and Jay 2007), physiology (Rantala et al. 2011), personality (Kortet et al. 2010), communication (Zuk and Kolluru 1998), and life history of prey (Walsh and Reznick 2009).

The primary way to avoid predation is to escape from predator detection. That is, predation frequently selects for crypsis (Slagsvold et al. 1995; Dale and Slagsvold 1996; Götmark 1997; Merilaita et al. 2001; Huhta et al. 2003; Review in Caro 2005). Crypsis implies that prey present coloration similar to that of the background where they usually are found, in order to not be detected by visual predators (Stevens and Merilaita 2009). In this way, animal coloration is selected (by predation) to match with background coloration. The smaller the differences are between animal and background coloration, the more cryptic the animal is in relation to that background (Endler 1990), and the less probable will be the detection of the animal by predators. However, if background coloration varies geographically, natural selection favours a matching geographic variation in animal coloration (Rosenblum et al. 2004; Hoekstra 2006). Indeed, geographic covariation between background and animal coloration has long been reported (Vidal et al. 2007; Williams 2007; Sumner 1929; Dice and Blossom 1937; Nachman 2005). These types of parallel gradients (background and animal coloration) can provide insights into how species adapt to varying conditions, and has been considered as evidence of natural selection favouring crypsis. However, other selective agents may lead to gradients in coloration, which may also lead to spurious covariation between animal and background coloration. For example, in a previous study (Reguera et al. 2014; Chapter 5), we found that dorsal coloration of the lizard *Psammodromus algirus* is darker in highland populations in Sierra Nevada mountain (SE Spain), and substrate color is also darker with elevation. This information might appear to be evidence of natural selection on dorsal coloration varying with elevation in order to guarantee crypsis, generating the observed pattern in this lizard. However, substrate and lizard dorsal darkness were not significantly correlated, and other hypotheses –the thermal melanism hypothesis (Clusella-Trullas et al. 2007) and the protection against ultraviolet radiation (UVR) damage hypothesis (Porter 1967)– better explain the pattern of darkness found (Reguera et al. 2014; Chapter 5).

However, in the study cited, we found a correlation between substrate hue and dorsal hue in the lizard *P. algirus* along an elevational gradient, suggesting that crypsis may also be in play (Reguera et al. 2014; Chapter 5). Nonetheless, this was suggested only by a single correlation. Here, we formally test the hypothesis that dorsal color in this lizard is under predation selection by taking advantage of a number of natural "controls": We take into account that this lizard moves mainly on bare soil (for basking, foraging, territorial defence, or searching for mates or oviposition sites), being rarely found on rocks or on vegetation (Salvador 2011; personal observations). Accordingly, if dorsal coloration in this lizard has evolved as a consequence of selection by visual predators, we predict (1) that dorsal coloration in this lizard will be more similar to bare-soil coloration than to rocky-substrate or vegetation coloration, and (2) that along the elevational gradient, dorsal coloration will covary mainly with bare-soil coloration, covariation with rocky-substrate and vegetation colorations being smaller or absent. In this way, we gain reliability on our conclusions regarding the selective agent of dorsal coloration, not being confused by spurious correlations.

Moreover, covariation between background and animal coloration may not be perfect, as other selective pressures may influence the coloration gradient. For instance, in the lizard *P. algirus*, low temperatures and high UVR at higher elevations select for darker dorsal coloration (Reguera et al. 2014; Chapter 5). In this way, these selective pressures would conflict with the selective pressure caused by predators, making less cryptic lizards in highlands than in lowlands. Consequently, we also predict (3) that crypsis will decrease as elevation increases.

Materials and Methods

Study species

Psammodromus algirus is a lacertid lizard of 53-95 mm snout-vent length (SVL) in our study area, which is abundant in shrubby Mediterranean habitats (Díaz and Carrascal 1991). It extends from south-eastern France to Morocco, including most of the Iberian Peninsula, along a wide elevation range (0-2600 m asl; Salvador 2011). Dorsal coloration of this lizard goes from pale to dark brown, with three yellow stripes in eastern populations (Verdú-Ricoy et al. 2010). As an ectotherm, *Psammodromus algirus* body temperature depends on environmental temperature and radiation, frequently using a heliothermic strategy for thermoregulation (Díaz and Cabezas-Díaz 2004; Díaz 1997; Carretero and Llorente 1995). Generally, it basks at ground level near shrubs where it shelters when it detects a predator (Martín and López 1995; Martín and López 2000; Díaz 1992). *Psammodromus algirus* is an opportunistic predator of arthropods that usually follows an active foraging strategy, sometimes using a “sit and wait” strategy (Salvador 2011). Therefore, this lizard is exposed to predators when basking and foraging, as well as when guarding its territory, searching for mates or for oviposition sites. Its main predators include several visual predators such as birds (Table 1 in Salvador 2011).

Study area

The study area was located in the southern face of Sierra Nevada (SE Spain, 2°56'1‟2 - 3°38'02‟2 W and 36°56'10‟0 - 37°12'58‟9 N). Annual precipitation varies from 267 to 753 mm (Junta de Andalucía 2001). Mean annual temperatures range from 17.6 to 3.5°C from the lowest to the highest point of the mountain (Junta de Andalucía 2001; for more details on temperature in our study site, see Chapter 4 General methodology). Relative irradiance varies with elevation 6-8 % km^{-1} for UV-A radiation and 7-11% km^{-1} for UV-B (Sola et al. 2008; See also Chapter 4 General metodology).

Sampling

Sampling was carried out within the typical habitat of *P. algirus*: areas with abundant herbs and shrubs ranging from 40 to 100 cm high and relatively high vegetation cover (Díaz and Carrascal 1991). Care was taken into selecting plots with similar habitat structure despite that plant communities varied along the elevational gradient. Vegetation cover and vegetation height did not statistically differ with elevation (vegetation cover: $\chi^2_5 = 0.09$, $p = 1.00$; frequency of plants of 25-50 cm: $\chi^2_5 = 0.16$, $p = 1.00$; Figure 4.4). Six sampling plots were established along the elevational gradient, separated by approximately 500 m of elevation: 300, 700, 1200, 1700, 2200, and 2500 m asl (Figure 4.5). Sampling was concentrated in periods of lizard activity, during the activity season (March to September; Zamora-Camacho et al. 2013). A total of 568 lizards were captured from 2010 to 2013. Individuals were captured by hand and transported to the laboratory in cotton bags, where they were immediately measured. Lizards were released at the capture site without having been injured as a consequence of this study. We considered only adult individuals in the analyses. Adults were recognized as those reaching a minimum SVL for each sex and elevation, that of the smallest male with an orange commissure for males and that of the smallest gravid female for females (Table 4.1). Both of these characters objectively reveal maturity.

Color measurement

We measured dorsal color in each individual with a spectrophotometer (Minolta CM-2600d). Measurements were taken from the middle of the dorsum, avoiding the yellow stripes. Color measurements consisted of the three chromatic values of the L*a*b* color space of the Commission Internationale d'Éclairage (CIE) (Figure 4.8). This color space objectively describes all the colors visible for most diurnal terrestrial vertebrates (Montgomerie 2006). The spectrophotometer, placed over the lizard skin, shoots three beams of light through a hole of 3 mm in diameter. As a result, it takes three measurements of reflectance and automatically gives the average value of each color component. L*a*b* color space is a 3-dimensional rectangular color space. The coordinate L* represents the lightness of the color, from black (L* = 0) to white (L* = 100). Coordinates a* and b* represent the position of the color in the green-red gradient (a*) and in the blue-yellow gradient (b*), respectively (Endler 1990). From L*a*b* values, we calculated chroma (or saturation, the radiance in a specific part of the spectrum in relation to the radiance from the whole visible spectrum) as $C^* = [(a^*)^2+(b^*)^2]^{1/2}$ (measured as the percentage distance from the centre (0) of the color space to its circumference (100) where its represented pure spectral colors); and hue angle (the color in the vernacular sense) as $H^* = \tan^{-1}(b^*/a^*)$ (starting in the positive a* axis and expressed in degrees). As a result, we established the L*C*H* color space (Figure 4.8), which offers the advantage of expressing pure coloration in a single value (H*), allowing a more intuitive and comprehensive analysis (Endler 1990).

Also, we estimated color of different background elements where the lizards where found following Stevens et al. (2007) and Pereira and Amat (2010). For this, from May to July 2013, we photographed each place where a lizard was observed basking. Photographs were taken with a Canon 1000D digital-reflex camera with an 18-55 mm lens (although all photographs were taken with the lens set to 24 mm). The aperture was set to F22 to ensure the image would be focused within a wide range of depths, providing a clear image despite the irregularities of the ground. White balance was calibrated by photographing a white paper sheet in the sun and setting its color as pure white before each sampling session. A tripod was used to ensure the distance between the camera and ground was perpendicular and constant at 60 cm. Only shutter-aperture time was adjusted for each photograph so that all colors would be represented. Additionally, a standard color chart was placed on the ground so that it would appear in each photograph to allow subsequent standardization of color measurements (Stevens et al. 2007). Also, an ultraviolet filter was used for noise reduction, considering that lizards' dorsal coloration does not include prominent reflection for these wavelengths (Pérez i de Lanuza 2012). Eventually, 136 photographs were taken over the six sampling plots.

We used Adobe Photoshop CS5 software to quantify coloration in all the photographs. During image processing and prior to measurement, the white balance was calibrated again and, thus, standardized in all photographs by sampling the grey scale of the color chart present in each image with the white balance calibration tool (curve layer). Then, in each photograph, each category of elements of the environment, when present, was sampled in 20 haphazardly established points, employing the Eyedropper Point Sample tool. Four distinct categories of elements of the environment were established for measurement: bare soil (where the lizards usually live), rocks, fresh vegetation, and dry vegetation. Not all categories were present in all the photos, which affected sample size in the analyses. Point width was set to 5×5 pixels, each measurement corresponding to the mean coloration of 25 pixels. We processed the photographs directly in L*a*b* CIE color space to be comparable with lizard-coloration measurements made with the spectrophotometer. The coloration differences between lizards and environmental elements were calculated as $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$ (Endler 1990). This method to estimate degree of crypsis is the best option when accurate information on the visual system of predators is not available (Endler 1990). In our study, given that visual predators of this lizard are a set of different predators (see Salvador 2011), for which the relative importance along the elevational gradient is unknown, the use of visual models was precluded.

Statistical analyses

We tested the relationships among the different variables of color (lightness "L*", chroma "C*" and hue angle "H*") of the background elements (bare soil, rock, fresh vegetation, and dry vegetation) with Pearson's correlations. Then, we ran an ANOVA in order to test a possible variation of the background coloration along the elevational gradient, with each color component as a dependent variable and elevation (each of the six sampling plots) as a factor. To test the relationship between background coloration and lizards' dorsal coloration, we calculated the mean of each variable for each elevation plot. Then, we used a Spearman's correlation (Siegel and Castellan 1988). In this analysis, we used the six plots as statistical units to test the prediction that dorsal coloration will covary with bare soil coloration along the elevational gradient, but not with the other background elements.

In addition, we estimated the degree of crypsis as the difference between each of the lizards' dorsal coloration and the mean coloration of each element of the background for each elevation. This difference (ΔE) was calculated as $\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$ (Endler 1990). The smaller the difference is, the higher the crypsis is. We examined which background element was most similar to lizard dorsum by using a Repeated Measurements ANOVA (RM-ANOVA). Then, we tested whether or not ΔE depended on elevation with an ANOVA, using differences in color as a dependent variable and elevation as a factor. In the same way, we also examined how the variance (estimated as one standard deviation) in difference between dorsal coloration and background elements varied with elevation by using a Spearman's correlation. All analyses were performed with R 2.15.2 (R Core Team 2012).

Results

Correlations among background color variables were relatively low (Table A5). Only the three color components (L*C*H*) of bare soil significantly varied with elevation (Table 8.1). Lightness ($F_{(5,90)} = 7.45$, $p < 0.001$) and hue angle ($F_{(5,90)} = 6.68$, $p < 0.001$) increased with elevation, while chroma ($F_{(5,90)} = 2.73$, $p < 0.05$) decreased (Figure 8.1). The color of the remaining elements of the background did not vary with elevation (Table 8.1). Meanwhile, the three components of lizard dorsal coloration significantly decreased with elevation (Figure 5.1; see also Chapter 5).

The lizard's dorsal average hue per elevation was significantly correlated with average bare soil hue ($r_s = 0.89$, $p < 0.05$, N = 6 populations), as well as with fresh vegetation hue ($r_s = 0.83$, $p < 0.05$; Figure 8.2). No other correlations between lizard dorsal coloration and background-element coloration were found (Table 8.2). However, hue values for lizard dorsum and bare soil proved similar, while values of hue for fresh vegetation and lizard dorsum were very different (Figure 8.2), implying that the correlation between fresh vegetation and lizard dorsum hue was spurious.

Table 8.1. Variation of each components of the L*C*H* CIE color space (dependent variable), for each background element variable, with elevation (fixed factor with six levels: 300, 1200, 1700, 2200, and 2500 m asl). Test performed with an ANOVA.

Color component	df	F	P-value	
	Bare soil			
Lightness	5, 90	7.45	<0.001	***
Chroma	5, 90	2.73	0.024	*
Hue angle	5, 90	6.68	<0.001	***
	Rocky substrate			
Lightness	5, 50	1.89	0.112	
Chroma	5, 50	1.18	0.332	
Hue angle	5, 50	0.38	0.860	
	Fresh vegetation			
Lightness	5, 89	1.15	0.341	
Chroma	5, 89	0.34	0.888	
Hue angle	5, 89	1.03	0.407	
	Dry vegetation			
Lightness	5, 106	0.84	0.521	
Chroma	5, 106	1.54	0.182	
Hue angle	5, 106	0.66	0.653	

Significance is indicated as follows: *= P<0.05; **= P<0.01; ***= P<0.001.

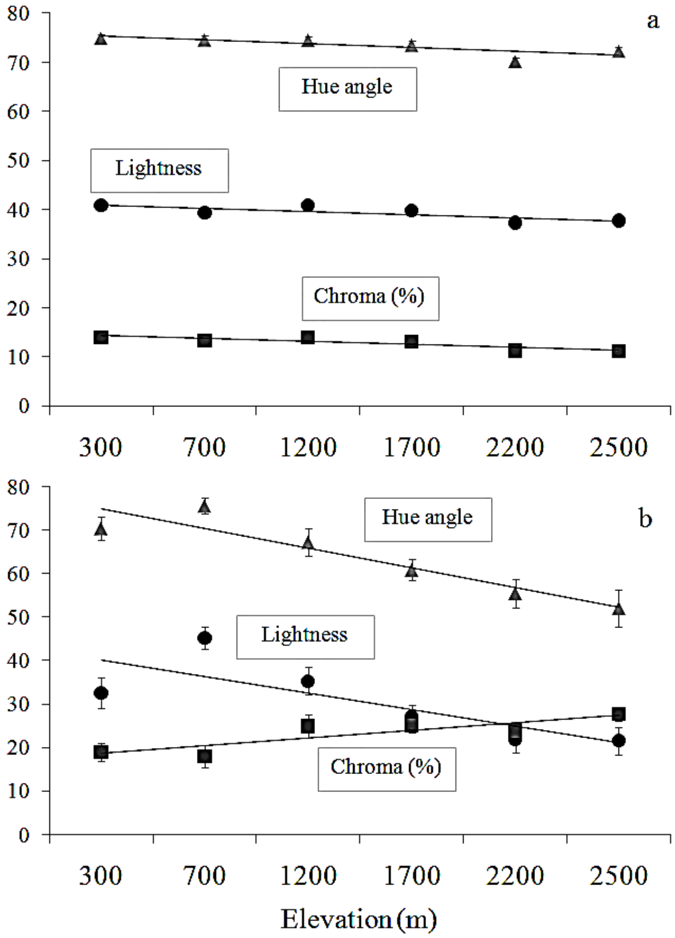

Figure 8.1 Means (points) with standard errors (bars) of lightness (black circles), chroma (grey squares), and hue angle (grey triangles) of the bare soil (a) and lizard dorsum (b) (See also Figure 5.1) along the elevational gradient. Lightness ranges from black ($L^* = 0$) to white ($L^* = 100$); chroma represents color saturation (percentage); hue angle indicates the coordinate of color (º), in strict sense. Lines indicate the regression fitting.

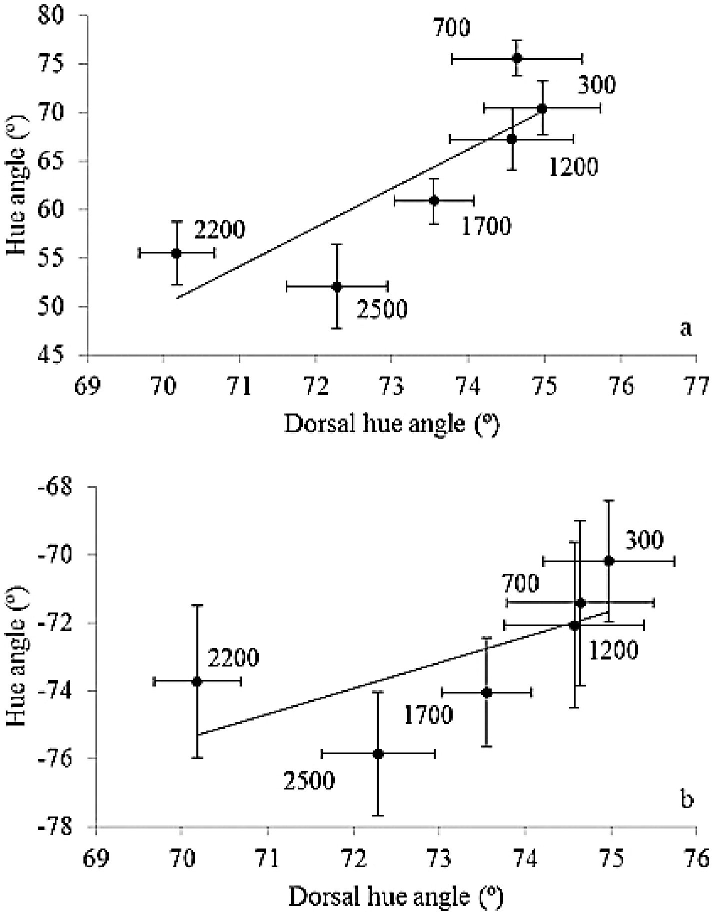

Figure 8.2 Relationship between lizards' dorsal hue with (a) bare soil hue and (b) with fresh vegetation hue, along the elevational gradient. There are represented mean values (points) and standard error bars of hue angle of both lizard dorsum and environmental elements (bare soil or fresh vegetation) for each plot (300, 1200, 1700, 2200, and 2500 m. asl).

Table 8.2 Spearman's correlation coefficients (rs) between mean lizards' dorsal L*C*H* CIE color space with background color space, for each component of the color space, along the elevation gradient (N = 6). *= p < 0.05.

Color component	Bare soil	Rock	Fresh vegetation	Dry vegetation
Lightness	0.54	0.6	-0.37	0.09
Chroma	-0.6	-0.09	-0.66	0.26
Hue angle	0.89 *	-0.14	0.83 *	0.37

Significance is indicated as follows: *= P<0.05; **= P<0.01; ***= P<0.001.

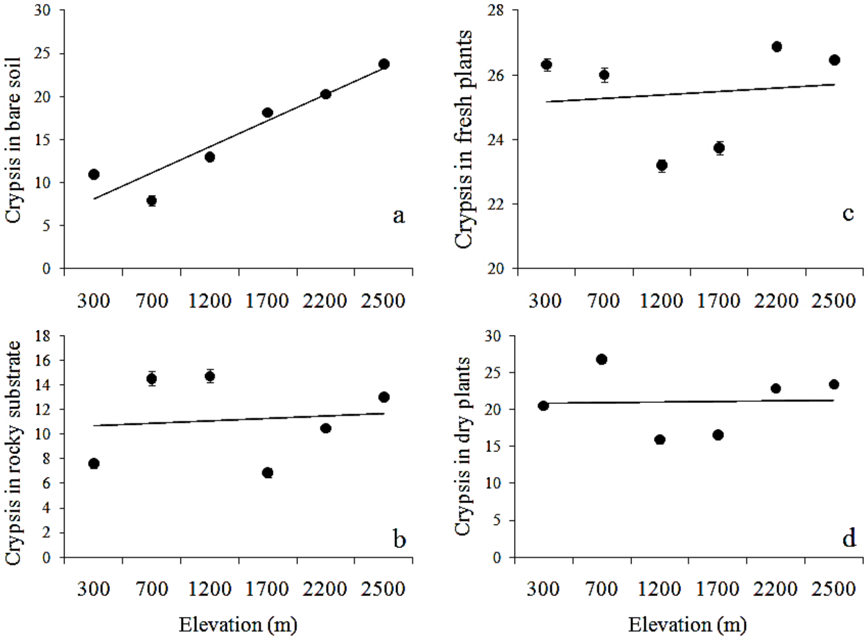

Figure 8.3 Means (points) and standard errors (bars) of difference in overall coloration (ΔE; i.e.. degree of crypsis) between dorsal coloration of lizards and each background element (a: bare soil; b: rocky substrate; c: fresh vegetation; d: dry vegetation), along the elevational gradient. Larger differences indicate less degree of crypsis. The lines indicate the regression fitting between elevation and difference in coloration

The difference between lizard dorsal color and background elements (ΔE) significantly varied among background elements (RM-ANOVA, $F_{(3, 1104)}$ = 958.18, $p < 0.001$; Figure 8.3). The difference was minimal for rocky substrate, followed by the difference with bare soil (Figure 8.3). Population significantly explained variance ΔE for every type of background element: bare soil ($F_{(5,366)} = 342.10$, $p < 0.001$), rocky substrate ($F_{(5,366)} = 75.66$, $p < 0.001$), fresh vegetation ($F_{(5,366)} = 67.79$, $p < 0.001$), and dry vegetation ($F_{(5,366)} = 31.31$, $p < 0.001$). However, although there was variation among populations for the four elements, a clear trend with elevation was observed only for the difference between dorsum and bare-soil color ($r_s = 0.94$, $p < 0.01$; Figure 8.3).

We also examined how the variance in the color difference between the lizard dorsum and the four background elements varied with elevation. We found that variance for all color differences decreased with elevation (Figure 8.4), although the correlation with elevation was significant only for the variance in the coloration difference between the lizard's dorsum and both bare soil and rocky substrate (r_s = -0.84 and -0.94, respectively, $p < 0.05$ in both cases).

Discussion

In reptiles, ventral and lateral body zones are less visible for predators, and are more variable in coloration involved in social exhibitions (Pérez i de Lanuza 2012). By contrast, dorsal coloration is less variable and frequently matches background coloration (Norris and Lowe 1964). Reptiles, being ectotherms, spend long periods of time basking (warming) and are therefore exposed to predators (*e.g.* Díaz 1997). Consequently, lizards are a good model for studying the evolution of crypsis. Moreover, elevational gradients provide a huge variation in environmental conditions (and thus selective pressures) in a relatively small geographic region, logistically facilitating the study of local adaptation (Keller et al. 2013). Our study system thus allowed us to examine how crypsis varied along an elevational gradient in a lizard, as a consequence of selective pressures on dorsal coloration varying with elevation (see below).

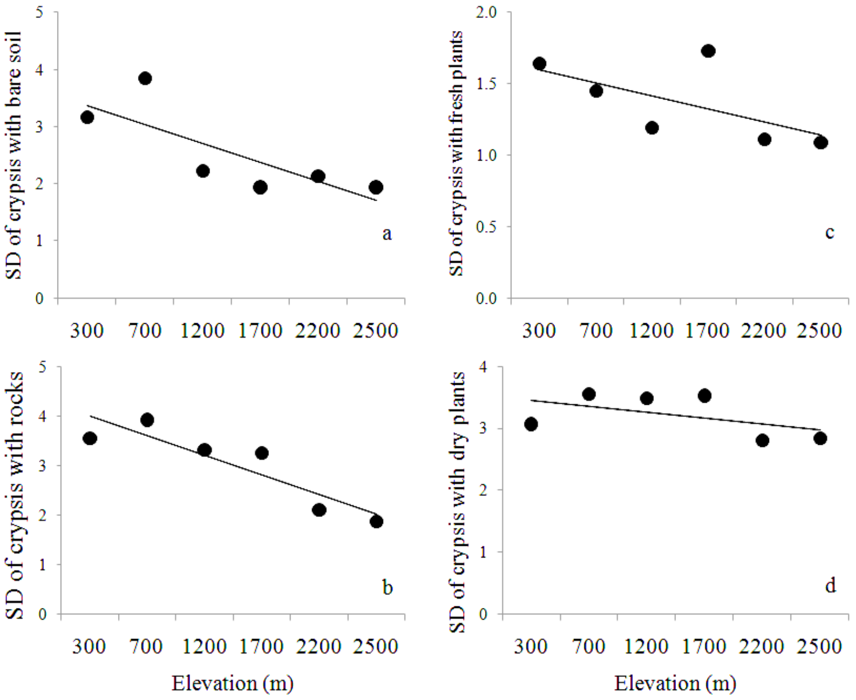

Figure 8.4 Relationship between standard deviation of the coloration difference (ΔE; *i.e.* degree of crypsis) between dorsal coloration of lizards with background elements (a: bare soil; b: rocky substrate; c: fresh vegetation; d: dry vegetation), along the elevational gradient. The lines indicate the regression fitting between elevation and the populations SD in crypsis.

Our findings show that dorsal coloration in the lizard *P. algirus* is more similar to rocky substrate than to bare soil. That is, lizards are the most cryptic when against a rocky background. We have no explanation for this finding, as this lizard is usually found on bare soil (Salvador 2011), and consequently is not a rock-dwelling lizard. One possibility is that lizards bask on rocks more than previously thought. Rocky surfaces heat up faster in the morning and preserve heat longer during the late hours of the day. Moreover, when basking, lizards present low body temperature, which reduced escape capacity (Van Damme et al. 1989; Huey et al. 1990; Bonino et al. 2011). Under these circumstances, being cryptic with rocky substrates would be more important than when the lizard is fully active on bare soil, where its body temperature allows high escape performance. Nevertheless, throughout populations, dorsal coloration did not covary with rock coloration.

Nonetheless, the crypsis hypothesis for the dorsal coloration of *P. algirus* was supported by other lines of evidence. Despite the result for rocky substrate, dorsal coloration resembled that of bare soil far more than that of vegetation. Moreover, along the elevational gradient, dorsal hue covaried with bare-soil hue, but not with vegetation or rock hue. Therefore, our prediction 1 was not fully supported, but prediction 2 was; covariation between bare soil hue and dorsal hue strongly suggests that lizards are locally adapted to background coloration in order to minimize predation risk.

On the other hand, we found that the degree of crypsis (similarity in overall coloration between lizard's dorsum and bare soil) decreased with elevation, supporting our prediction 3. We hypothesised that, given that selective pressures favouring darker dorsa emerge with elevation –for protection against UVR and/or increased thermal efficiency (Reguera et al. 2014; Chapter 5)– a conflict with selection for crypsis would appear, the result being decreased crypsis with elevation. This hypothesis was fulfilled. Local adaptation may be hindered by a number of factors (Kawecki and Ebert 2004), and our findings suggest that local adaptation in crypsis is hindered with elevation by the increase in importance of other environmental variables favouring darker dorsum in lizards.

If other factors reduce the degree of crypsis with elevation, this may result in greater predation and a lower adaptive peak of the alpine populations in comparison with mid- and low-land populations, or the adaptive peak may be maintained with highland lizards adopting alternative anti-predatory strategies (Martín et al. 2009; Cabido et al. 2009). For example, highland lizards could be faster in order to escape from predators, but we found no elevational differences in escape capacity in our study system (Francisco J. Zamora-Camacho et al. 2014). How other anti-predatory strategies (fleeing distance or time shelter) vary with elevation has not yet been studied.

Asymmetric gene flow between highlands and lowlands also could contribute to the elevational pattern in the degree of crypsis found. Storfer et al. (1999) found that salamanders *Ambystoma barbouri* in temporary ponds were selected for darker coloration, perhaps for protection against UVR. Meanwhile, in streams with predatory fishes, selection for crypsis favoured lighter salamanders. As a consequence of gene flow, the degree of crypsis decreased with proximity to temporary ponds. However, in our study, variance in crypsis decreased with elevation, which implies that phenotypic variability also decreases. Reduced phenotypic variance matches with a strong natural selection for dark dorsum in higher elevations, which would reduce variance, but not with gene flow from lower elevations, which should increase phenotypic variance (Kawecki and Ebert 2004).

There is another non-mutually exclusive explanation for the reduction of the degree of crypsis with elevation, which is that predation pressure also decreases with elevation, as a consequence of lower abundance of predators (Fox et al. 1994), thus relaxing selection for crypsis at higher elevations (Endler 1995). At this point, we do not know how predator abundance varies with elevation in our study system, although, even if predator density decreases with elevation, this does not necessarily imply lower risk of predation at higher elevations. Instead, in cold-climate zones, lizards should spend more time basking, and thus be more exposed to predators, than in hotter-climate zones (Díaz 1997). Therefore, selection for crypsis is often higher in cold-climate lizards, as has been reported elsewhere (*e.g.*, Thorpe and Brown 1989; Brown and Thorpe 1991).

In conclusion, our findings show an elevational gradient in *P. algirus* dorsal coloration, which matches with a gradient in background coloration of bare soil (where the lizard is usually found). Moreover, the degree of crypsis decreases with elevation, probably as a consequence of contrasting selective pressures (thermoregulation, UV-protection). These findings suggest the existence of local adaptation along the elevational system in the degree of crypsis according to background coloration and the existence of other selective forces.

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Chapter 9
Multiple color signals in *P. algirus*

Senda Reguera, Francisco J Zamora-Camacho, Mar Comas and Gregorio Moreno-Rueda. **Multiple color signals in the lizard *Psammodromus algirus*: different badges convey different signaller's qualities and vary with elevation**. (Submitted to *Animal Behaviour*)

Abstract

Coloration is one of the main communication tools used by animals. Signals based on coloration can indicate different qualities of the bearer. For this reason, they are used in contexts of mate choice and agonistic encounters. Nevertheless, coloration signals are under several selective pressures, which may vary geographically, causing geographic variation in signals used by animals to communicate. The lizard *Psammodromus algirus* from eastern populations shows different color patches on the ventral and lateral side: orange or yellow in the commissures, yellow in the gular region (throat), and blue blotches on the flanks. In this work, we quantitatively study the ventral coloration of this lizard. We relate these color patches to information on different characteristics of the individuals (body condition, age, body size, or gender). The entire study was performed within an elevation gradient of 2 200 m asl, allowing us to analyse the geographic variation in coloration. Color patches were more frequent and colorful at high elevations, except the ocelli, which reached a maximal number in low elevation populations. Sexual dichromatism, however, decreased with elevation. Predation risk seemed higher in colorful lizards. On the other hand, our results show that the color patches of this lizard work as multicomponent signals that indicate different aspects of the bearer. Throat chroma and hue, number of ocelli, and the color of the patch in the commissure (orange for males, yellow for females), indicate gender. However, age was indicated by throat lightness and chroma, as well as the presence of a yellow patch on the throat and a colored patch (orange or yellow) in the commissure. Therefore, different color patches indicate the same quality of the bearer, but at the same time, each color trait indicates different qualities of the bearer, working as a multicomponent signal. Consequently, the multiple patches in *P. algirus* are the result of redundant signals and multiple messages working simultaneously in a set of multicomponent signals.

keywords: elevational gradient; multicomponent signals; lizard coloration; sexual selection; sexual signals; status signals; *Psammodromus algirus*; pigmentation; visual communication.

Introduction

Many aspects of communication in the animal kingdom are based on color signals (Searcy and Nowicki 2005; Bradbury and Vehrencamp 2011), which has attracted the interest of evolutionary and behavioural ecologists (Hill and McGraw 2006). Understanding the evolution of coloration patches in animals is challenging because selection often arises from the interaction of several evolutionary pressures. While natural selection favours cryptic coloration to avoid predator attacks (Caro 2005), sexual selection selects mainly for conspicuous colorations (Andersson 1994).

In lizards, the dorsal body side is usually visible to air predators whereas ventral side remains hidden. In this way, lizards usually show cryptic coloration on the dorsum (Norris and Lowe 1964), whereas ventral coloration plays an important role in intra-specific communication and is usually colorful and conspicuous (*e.g.* Cooper and Burns 1987; Pérez i de Lanuza and Font 2007; Calisi et al. 2008). Furthermore, lizards frequently show sexual dichromatism and color polymorphism (Sinervo and Lively 1996; Blouin-Demers et al. 2013; Pérez i de Lanuza et al. 2013). The lateral side involves an intermediate situation, being visible to con-specific individuals but also to ground predators such as snakes (Pérez i de Lanuza and Font 2010).

Intra-specific communication works during inter- and intra-sexual interactions and coloration indicates several qualities of the sender to the receiver (Hill and McGraw 2006). Usually, attractive coloration appears during the breeding season. Males use color ornamentation to inform females about their quality at different levels (*e.g.* Martín et al. 2008; Cote et al. 2010; Molnár et al. 2013; Martin et al. 2013; Pérez i de Lanuza et al. 2014). Females benefit from mating with high-quality males by having genetically superior offspring (with higher return of fitness; Olsson et al. 2003; but see Noble et al. 2013), and also indirectly by reproducing in high-quality territories (with more probabilities of survival for their offspring; Calsbeek and Sinervo 2002). Females may also show breeding coloration, indicating quality or receptiveness to males (Cuervo and Belliure 2013; Stuart-Fox and Goode 2014).

On the other hand, males usually display color ornamentation during agonistic encounters with other males. Frequently, color badges work as signals of fighting ability, aggressiveness, or social status (Bastiaans et al. 2013; Díaz 1993; Olsson 1994a; Sinervo and Lively 1996), which finally translates into males with larger territories and more mating females (Salvador and Veiga 2001). Especially, orange or yellowish ventral patches are related to testosterone levels and aggressive behaviour (Olsson et al. 2007; Salvador et al. 1997; Sinervo et al. 2000). By signalling fighting ability, contesting lizards may reduce the number of costly interactions between individuals of the same sex during territory or female defence (Olsson 1994a).

Color badges serve to influence receiver behaviour (Maynard-Smith and Harper 2003). Badges convey information that receivers use to behave adaptively (avoiding fighting with a superior competitor or allowing mating with a high-quality male). However, color ornaments usually imply a cost for the bearer in order to honestly signal a certain quality (Zahavi and Zahavi 1997). On the one hand, coloration is usually costly in terms of resources. Resources are limited and those invested in conspicuous coloration are not available for *e.g.* increasing fat reserves or mounting an immune response. Therefore, only individuals with enough resources to spare in badges are able to produce them. Cheaters are not possible because the cost associated with these signals is too high for low-quality individuals (Grafen 1990). For example, the use of pigments in carotenoid-based colorations has negative effects on antioxidant mechanisms (Olsson et al. 2012) and the immune response (Alonso-Álvarez et al. 2004). Indeed, in the lizard *Psammodromus algirus*, males with larger patches of carotenoid-based breeding coloration suffer a higher parasite load and reduced survivorship (Salvador and Veiga 2000; Salvador et al. 1996). Consequently, coloration works as a “honest signal” and the intensity of pigmentation is subsequently correlated with indexes of vigour (Olson and Owens 1998). On the other hand, brighter coloration can attract predators, increasing predation risk (Zuk and Kolluru 1998). The more conspicuous the individual, the more susceptible is to predator attack (*e.g.* Martín and López 1999, 2001; Husak et al. 2006; but see Olsson 1993; Stuart et al. 2012).

Therefore, a badge may signal one or more qualities (*e.g.* gender, health, body condition, fighting ability), which may be directed to one or more types of receivers (potential mates or competitors; Brooks and Couldridge 1999; Guindre-Parker et al. 2013; Martín and López 2009). At the same time, different badges may signal the same quality or different qualities. When different badges signal the same quality of the bearer, they are redundant, and contribute to reinforce the message the sender seeks to transmit. Alternatively, different badges may signal different qualities, giving receivers a more complete picture of the different qualities of the bearer (Johnstone 1996; Rowe 1999; Candolin 2003; Hebets and Papaj 2004).

Finally, resources invested in secondary sexual characters depend on the resource availability for the individual or the individual capacity to take them. Because resources are not distributed homogeneously over space, there is geographical variation in their availability, and as a consequence, variation in signal expression (Endler 1995; Dunn et al. 2008; Chui and Doucet 2009). Moreover, not only resources vary geographically, but selective pressures can work differently depending on the location, which can translate into geographic differences in color expression (Comendant et al. 2003; Forsman and Shine 2006; San-José et al. 2012; Reguera et al. 2014a [Chapter 5]). For example, visual signals are more selected than chemical signals in wet and cold environments, because the lipidic composition of chemical signals does not work well under such conditions (Abert 1992).

In the present study, we analyse the causes of variation in several color badges that potentially work as signals in the lizard *Psammodromus algirus*. This Mediterranean lizard shows different colored patches in the ventro-lateral sides of the body: (1) Orange pigmentation around the head in breeding males (in western populations, Díaz 1993; Verdú-Ricoy et al*.* 2010) or only orange patches in the commissures, most of the time hidden except when lizards open the mouth (in south-eastern populations; Carretero 2002; Verdú-Ricoy et al*.* 2010). (2) A yellowish patch in gular region (throat and/or chest; Salvador and Veiga 2001; Carretero 2002). When the yellowish patch is not present, the ventral side is white to pale grey. (3) Blue round eyespots (ocelli) on the flanks of the body (Carretero 2002; Salvador and Veiga 2001). (4) Moreover, it has ultraviolet reflecting patches on the flanks (Pérez i de Lanuza and Font 2010). Dorsal coloration goes from pale to dark brown on the dorsum, and varies with elevation (Reguera et al. 2014a; Chapter 5). Usually they show two yellow lines along the dorsum, and even a third along the vertebral line in the western populations (Salvador 2011).

The goal of this study was to perform a quantitative analysis of the ventro-lateral coloration patches, and to relate them to different qualities of the lizard *P. algirus*, in order to disentangle the function of each color badge. We quantified 8 traits of this lizard's coloration: lightness, chroma, and hue of the throat; presence and size of the yellow patch in the throat; presence and color, as well as size, of the patch in the commissures; and total number of blue ocelli. For every color trait quantified, we addressed the following issues: (1) The existence of sexual dichromatism. (2) Seasonal variation in the expression of the quantified cues. Traits used in mating are predicted to be expressed mainly during the breeding season, while signals of status may be expressed throughout the year. (3) Whether color badges are indicative of body condition. (4) Whether color badges are indicators of age. (5) The risk of predation associated with coloration, because conspicuous badges are predicted to increase predation risk, and thus signals of predation (incomplete or regenerated tails; Arnold 1988) would be expected more often in lizards with brighter color characters. (6) Geographical variation in color badges. Previous studies have shown that the expression of sexual signals varies with elevation in birds (Badyaev 1997c), and predation pressure, population density or prey availability varies with elevation (Fox et al. 1994; Zamora-Camacho et al. 2013), resulting in different selective pressures on coloration signals. Hence, elevation gradients offer an informative framework for studying evolutionary ecology (Keller et al. 2013), in our case to study how color-based signals vary geographically. (7) Finally, whether color badges are interrelated.

Material and methods

The species

Large Psammodromus (*P. algirus* Linnaeus, 1758) is a medium-large size lacertid lizard (60-80 mm snout-vent length [SVL]), found in characteristic habitats of the Mediterranean region from North Africa and across Iberian Peninsula as far as southern France (Salvador 2011). It inhabits mainly shrubby Mediterranean landscapes (Díaz and Carrascal 1991). Moreover, the species occurs in an ample elevation gradient from 0 to more than 2600 m asl, enduring a wide range of environmental conditions (Salvador 2011).

Study area

The field work was performed in Sierra Nevada mountain system (SE Spain). There, elevation gradient goes from 200 to 3478 m asl and *P. algirus* occurs from lowlands to 2800 m asl (Fernández-Cardenete et al. 2000; O. Jiménez Robles, pers. com.). We selected 6 sampling points along the elevation gradient at 300, 700, 1200, 1700, 2200, and 2500 m asl (Figure 4.5), as similar in vegetation structure as possible (more details Chapter 4 General methodology).

General procedures

Lizards were captured by hand and brought to the lab in cotton bags. We caught 366 adult lizards during their activity season in Sierra Nevada, SE Iberian Peninsula (from March to September; Zamora-Camacho et al. 2013) from 2011 to 2013. Sex was determined mainly on the basis of femoral pore development (more conspicuous in males; Iraeta et al. 2011). We included only adults because we were interested in inter- and intra-sexual coloration signals. We considered females to be adult when they were larger than the smallest gravid female for a given population, and males as adult when they were larger than the smallest male with a colored commissure in the population given. Minimal SVL of adults varied between 53 and 67 mm depending on the population (Table 4.1). We measured lizards' SVL with a metal ruler (accuracy

1 mm). In addition, we weighed lizards with a digital balance (Model Radwag WTB200, accuracy 0.01 g). Lizards were captured with permission of the Andalusian government and National Park of Sierra Nevada (Spanish Government, references GMN/GyB/JMIF and ENSN/JSG/JEGT/MCF). We released lizards at the capture site within 1 week and no lizard was injured as consequence of this study.

Measurement of throat

We measured coloration of throat (gular region) and chest (on the underside of the right forelimb region) with a spectrophotometer (Minolta CM-2600d). The spectrophotometer, placed over the lizard skin, projected three beams of light through a hole of 3 mm diameter. As a result, it takes three measures of reflectance and automatically gives the average value of each color component. Data are measured in the L*a*b* color-space of the Commission Internationale d'Eclairage (CIE; 1976; Figure 4.8), which describes all the colors visible for most of the diurnal terrestrial vertebrates (Montgomerie 2006). L*a*b* color space is a 3-dimensional rectangular color space. L* axis represents lightness (0 is black, 100 is white); a* axis represents red-green gradient (positive values are red, negative values are green); b* axis represents blue-yellow gradient (positive values are yellow, negative values are blue). From L*a*b* values we determined the chroma (saturation is the radiance in a specific part of the spectrum in relation to the radiance from the whole visible spectrum) as $C^* = [(a^*)^2+(b^*)^2]^{1/2}$ (measured as the percentage distance from the centre [0] of the color space to its circumference [100] where pure spectral colors are represented); and hue angle (the “color” in common parlance) as $H^* = \tan^{-1}(b^*/a^*)$ (Endler 1990). Because throat and chest color parameters (lightness, chroma, and hue) were highly correlated each other (Table 4.2), we used only values of the throat.

Commissures, yellowish throat-chest patch, and ocelli

During 2012 and 2013, we noted the presence or absence of colored commissures (CO) for each lizard and in case of presence, we noted the color (yellow or orange; Figure 9.1b). We also noted the presence or absence of a yellowish patch (YP) in the throat and/or chest area (Figure 9.1a). Finally we counted the total number of ocelli in both flanks of the lizards' body. We quantified the area of CO and YP when they appeared in photographs. Photos were taken from ventral side of the body, and right side of the head (with the mouth opened). We used a Canon Power Shot SX200 IS digital camera and graph paper for size reference. Then, using the software Image J (version 1.47; Rasband, 2008) we quantified the area of CO and YP. First, we scaled the photo using the graph paper (in mm) and the "Set scale" tool, and cropped a delineated area of the colored patch. Then, we adjusted the area using the "Color threshold" tool, and measured the area of each colored patches with the "Analyse particles" tool (Figure 9.1). The software measured all the patches delineated and gave us the total selected area

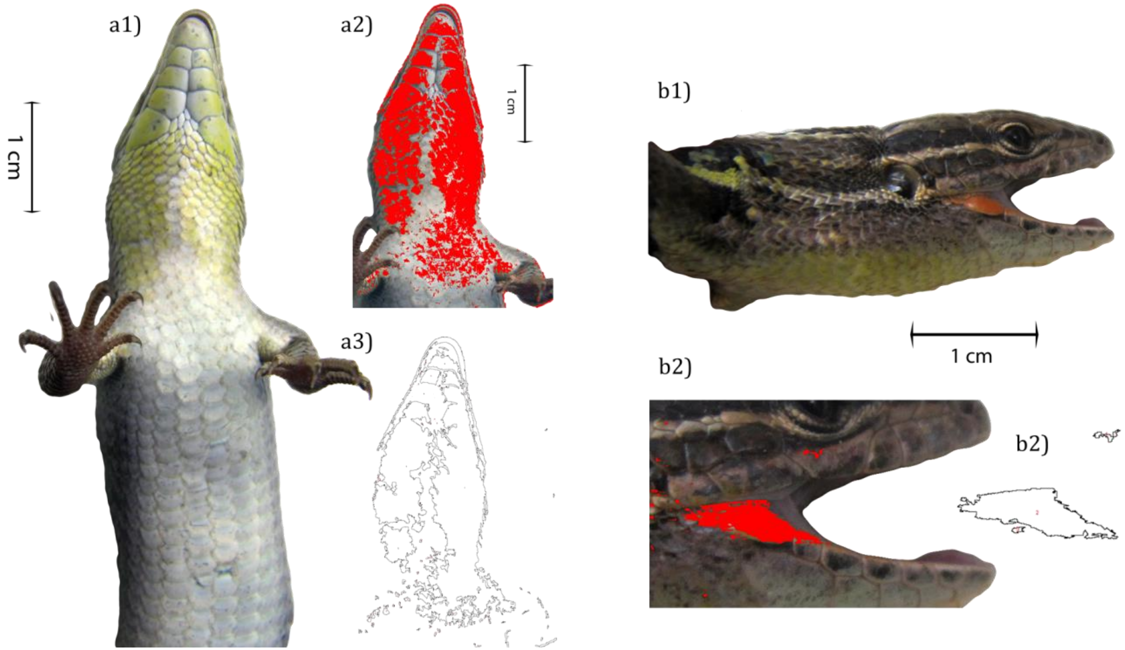

Figure 9.1. Representation of procedures for quantifying the yellow patch (a) and the commissure (b) area, using the software Image J (version 1.47). Firstly, we performed ventral (a1) and lateral (b1) photographs of the lizard. Secondly, we selected the coloured area with the "Colour threshold" tool (a2, b2). Lastly, we measured the area with the "Analyse particles" tool (a3 and b3).

Estimation of age with skeletochronology

The age of a subsample of lizards (N = 118) was determined by using skeletochronology analysis (Castanet and Smirina 1990). One toe of each lizard was clipped and preserved in ethanol 70 %. The wound provoked by the cut was properly disinfected. The toes were decalcified in 3 % nitric acid for 3 h and 30 min. Cross-sections (10 µm) were prepared using a freezing microtome (CM1850 Leica) at the Centre of Scientific Instrumentation of the University of Granada. Cross-sections were stained with Harris hematoxylin during 20 minutes and dehydrated with an alcohols chain and then were fixed with DPX (mounting medium for histology) and mounted on slides. Thereafter, were examined for the presence of LAGs using a light microscope (Leitz Dialux20) at magnifications of 400×. We took several photographs (with a camera ProgresC3) of several representative cross-sections for each individual, discarding those in which cuts were unsuitable for observing LAGs. We selected diaphysis sections in which the size of the medullar cavity was at its minimum and that of the periosteal bone at its maximum. Inferring age from the number of LAGs in cross-sections requires knowing the number of period of arrested growth in a year of life, and therefore we studied sections in juveniles, for which we knew the age to be < 1 year. The number of LAGs detected in the periosteal bone was independently counted three times by the same person but on different occasions, always blindly regarding the specimen identification.

Statistical analyses

Given that all variables of size of surface area allometrically correlated with SVL (CO area: $r = 0.43$, $p < 0.001$, N = 114, YP area: $r = 0.48$, $p < 0.001$, N = 73), we corrected these variables for SVL, and the variables included in the analyses were the residual values from the regression of each variable on SVL, all variables log-transformed. Similarly, the body condition index (BCI) was estimated using residuals from regression of mass on SVL, both variables log-transformed (Green 2001).

We ran Lineal Models (LM, Quinn and Keough, 2002) with all dependent variables having a normal distribution (in some cases, after log-transformation). These variables were: color parameters (lightness, chroma, and hue), YP area, CO area, and number of blue ocelli. By contrast, we ran a Generalized Lineal Models (GLM, Quinn and Keough 2002) with dependent variables having a distribution that differed from normal. These were: YP presence, with binomial distribution (two levels: absence or presence of YP), and CO color, with multinomial distribution (three levels: absence of color, yellow, or orange). In GLMs, we used logit as the function link.

For the goals of our study, in the first set of analyses, we used as explaining variables in the LMs and GLMs the next independent variables: (1) sex (categorical with two levels, males or females), to test possible sexual dimorphism in color characters; (2) month (categorical with five levels: April to August, both inclusive), to test variation over the activity season; (3) BCI, to estimate the relationship between condition and coloration; (4) elevation (categorical with six levels; see locations in Figure 4.5), to test for geographic variations in social coloration; and (5) tail condition (categorical with three levels: complete, regenerated, or incomplete); All the above variables were introduced into a model for each dependent variable in order to test hypotheses 1, 2, 3, 5, and 6 from the Introduction.

In a second set of analyses, we used the subsample for which age was estimated, and we added the variable (6) age, in order to test whether some color patches were indicative of age. Similarly, we examined the relationship between dependent variables and SVL, which is a proxy for age, given that lizards present indeterminate growth. This analysis was performed separately for each elevation plot, given that SVL strongly covaries with elevation in our study system (Reguera et al. 2014a; Chapter 5), and including the two variables simultaneously in the same model may provoke multicollinearity.

Finally, we examined the covariation among all dependent variables to test possible inter-relationships among different color patches. Relationships of variables with color parameters of throat (lightness, chroma, and hue) were performed with LMs or GLMs, as needed. The relationship between YP and CO presence was analysed with contingency tables.

In all cases, interactions between predictive variables were tested, but removed from the final model when they were not significant. Homoscedasticity and normality of residuals were checked for all final models. All analyses were performed with R 2.15.2 (R Core Team 2012).

Results

Sexual dichromatism

We found *P. algirus* lizards were sexually dichromatic for throat chroma and hue, commissure (CO) color category, and number of ocelli (Table 9.1; Figures 9.2-9.4). Male throats were more yellow (mean±se; Males hue = 91.03±0.75; Females hue = 88.87±0.73) and saturated (Males chroma = 15.16±0.56; Females chroma = 14.63±.041) than female throats in almost every population (Figure 9.2), and showed colored CO more often than in females (39 % of males vs. 8 % of females; Figure 9.4). Orange CO were exclusive to males (only one female had orange CO), whereas yellow CO were more frequent in females (Figure 9.4). In this respect, there was a significant interaction elevation×sex (Table 9.1), indicating that the degree of sexual dichromatism for CO varied with elevation (Figure 9.4). The presence of the yellow patch (YP) of the gular region was not sexually dimorphic, but there was a significant interaction elevation×sex, too (Table 9.1, Figure 9.2). Finally, males presented more ocelli than females did (Males = 7.70±0.16; Females = 3.11±0.12; Table 9.1). In short, male lizards presented yellower throats and more ocelli than females did, as well as orange commissures vs. yellow in females.

Seasonal variation in colored patches

During the breeding season (mainly May and June), throat color was darker, more saturated, and less yellow (Table 9.1, Figure 9.2B), and the presence of CO was more frequent (Table 9.1, Figure 9.4C). Moreover, although YP was present in the same frequency throughout the activity season (Table 9.1), the YP area was larger during May and June (Table 9.1; Figure 9.5). Therefore, throat patch color and size, as well as CO presence, changed in the breeding season. The absence of interactions season×sex and season×elevation suggests that changes in color patches were similar in both sexes and along the elevational gradient. In summary, throat size and coloration, and presence of a patch in the commissure showed seasonal changes.

Colored patches as indicators of phenotypic quality

Throat coloration was not significantly related to BCI (Table 1). Only the size of YP tended to increase with BCI (Table 9.1; estimate = 1.70, p = 0.059). Regarding age, old individuals showed lower lightness (estimate = -1.73) and higher chroma (estimate = 0.22) than did young lizards, but no relationship was found between age with throat hue and number of ocelli (Table 9.2). Similarly, throat chroma increased with SVL in all populations, while lightness tended to diminish with SVL; throat hue and number of ocelli showed no relationship with body length, supporting the previous finding for age (Table 9.3). Moreover, lizards with YP and CO (yellow or orange) had higher SVL in all populations (Table 9.4). Thus, we failed to find any relationships among coloration patches and condition, but throat coloration and the presence of a yellow patch on the throat, and orange or yellow commissures (but not the number of ocelli) were positively correlated with age, and therefore with body size and survival capacity (indicate that those individuals have lived during more time).

Colored patches and predation

Regarding predation risk, individuals with darker throats or those that showed YP or colored CO more often had incomplete or regenerated tails (Table 9.1; Figure 9.6). No other relation was found between tail breakage and coloration.

Geographic variation in colored patches

Coloration traits varied geographically with elevation. Lizards had darker, more saturated and less yellow (trending to orange) throat at higher elevations (Table 9.1, Figure 9.2A). In addition, lizards with color patches (YP and CO) were more frequent in higher populations (Table 9.1, Figures 9.3 and 9.4), although the elevational variation for CO was sex dependent (Table 9.1). By contrast, the total number of ocelli was higher at lowlands (Table 9.1; Figure 9.7).

Relationships among colored patches

Finally, we found that lizards with darker and more saturated throats more often had color patches such as YP and CO (Table 9.5). Furthermore, saturation of throat color was positively related to the area of these color patches (Table 9.5). In addition, lizards which had colored CO, were prone to have YP (χ^2 = 25.02, $p < 0.01$). However, the number of ocelli and throat hue were unrelated with the remaining color traits.

Table 9.1 (part 1). Summary for the lineal models testing the effect of elevation, sex, BCI (Body Condition Index), month, and tail condition (complete, regenerated, or incomplete) on throat colouration parameters ("L" lightness, "C" chroma, and "H" hue angle), number of ocelli, colour of the commissure (CO; absent, yellow, or orange), commissure area (CO area) controlled for SVL (snout-vent length), yellow chest patch (YP; present, or absent), and yellow chest patch area (YP area; in case it was present). Elevation, sex, month, and tail are factors, and BCI is a continuous variable. Some variables were log-transformed to improve parametric conditions of the residuals from the final model.

	Throat L		**Throat C**		**Throat H**		**Ocelli**	
	Df	**F-value**	**Df**	**F-value**	**Df**	**F-value**	**Df**	**F-value**
Elevation	5,442	30.35***	5,442	9.40***	5,442	9.40***	5,347	4.50***
Sex	1,442	0.63	1,442	7.77**	1,442	7.77**	1,347	119.93***
BCI	1,442	0.27	1,442	0.52	1,442	0.52	1,347	1.75
Month	4,442	5.47***	4,442	9.94***	4,442	9.94***	5,347	0.73
Tail	2,442	5.63**	2,442	0.60	2,442	0.60	2,347	1.45
Elevation*Sex								

All higher order interactions involving covariates that were not significant were removed from the final model. Results were considered significant when the *p*-value <0.05. § *p*=0.05 * *p*<0.05 ***p*<0.01 ****p*<0.001

Table 9.1 (part 2). Summary for the lineal models testing the effect of elevation, sex, BCI (Body Condition Index), month, and tail condition (complete, regenerated, or incomplete) on throat colouration parameters ("L" lightness, "C" chroma, and "H" hue angle), number of ocelli, colour of the commissure (CO; absent, yellow, or orange), commissure area (CO area) controlled for SVL (snout-vent length), yellow chest patch (YP; present, or absent), and yellow chest patch area (YP area; in case it was present). Elevation, sex, month, and tail are factors, and BCI is a continuous variable. Some variables were log-transformed to improve parametric conditions of the residuals from the final model.

	CO		**log(CO area)**		**YP**		**YP area**	
	Df	**χ2**	**Df**	**F-value**	**Df**	**χ2**	**Df**	**F-value**
Elevation	10	39.60 ***	5,99	1.29	5	15.40**	5,60	1.01
Sex	2	26.05 ***	1,99	1.59	1	1.39	1,60	3.12
BCI	2	0.73	1,99	0.70	1	1.98	1,60	3.72 §
Month	8	30.30 ***	4,99	1.71	4	6.20	2,60	5.16 **
Tail	4	19.57 **	2,99	0.30	2	5.72^	2,60	0.24
Elevation*Sex	10	23.58**			5	15.64**		

All higher order interactions involving covariates that were not significant were removed from the final model. Results were considered significant when the *p*-value <0.05. § *p*=0.05 * *p*<0.05 ***p*<0.01 ****p*<0.001

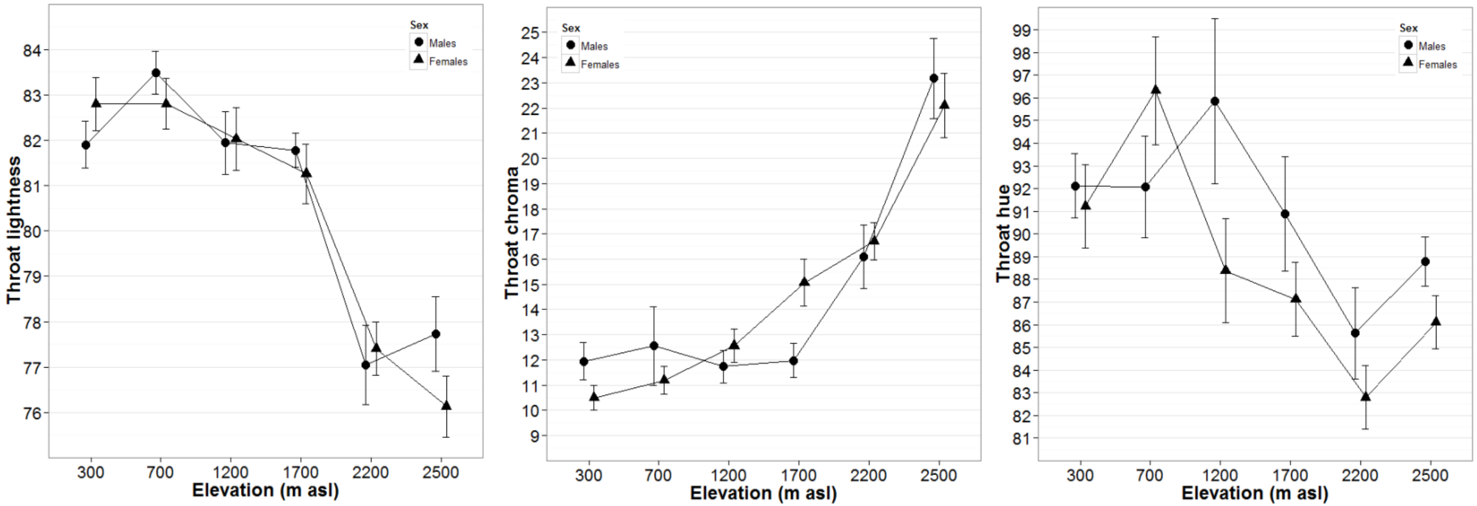

Figure 9.2A. Mean values (dot) and standard error (whiskers) of throat colour parameters (lightness, chroma, and hue) for each population (elevation from 300 to 2 500 m asl), for males (circles) and females (triangles) of the lizard *Psammodromus algirus*.

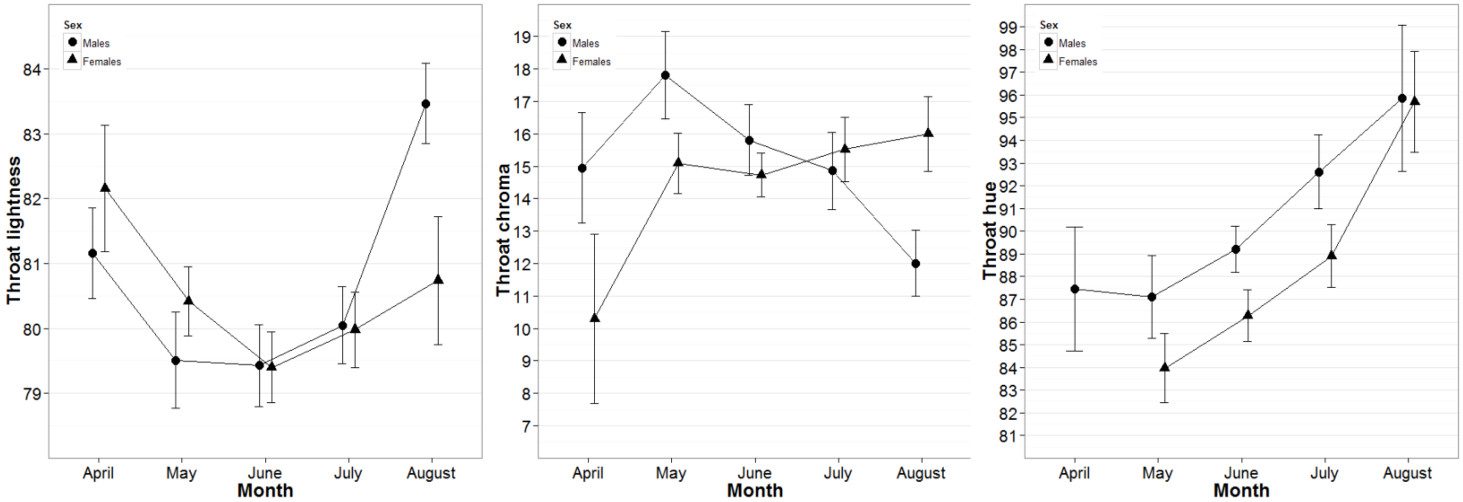

Figure 9.2B. Mean values (dot) and standard error (whiskers) of throat colour parameters (lightness, chroma, and hue) for each month along the activity season, for males (circles) and females (triangles) of the lizard *Psammodromus algirus*.

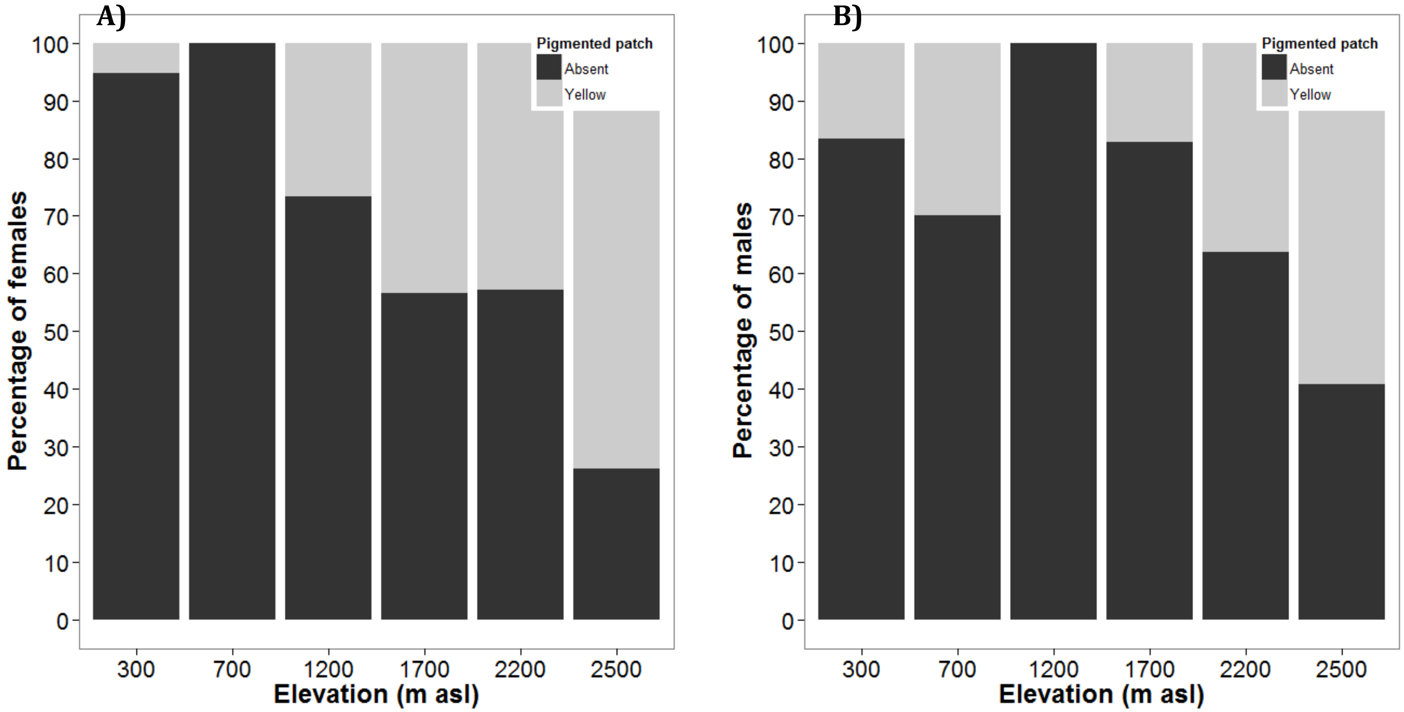

Figure 9.3. Frequency of the pigmented patch in throat in females (A) and males (B) of the lizard *Psammodromus algirus* for each population (elevation from 300 to 2 500 m asl). The variable has 2 levels: absent (in black) or present (Yellow, in light grey).

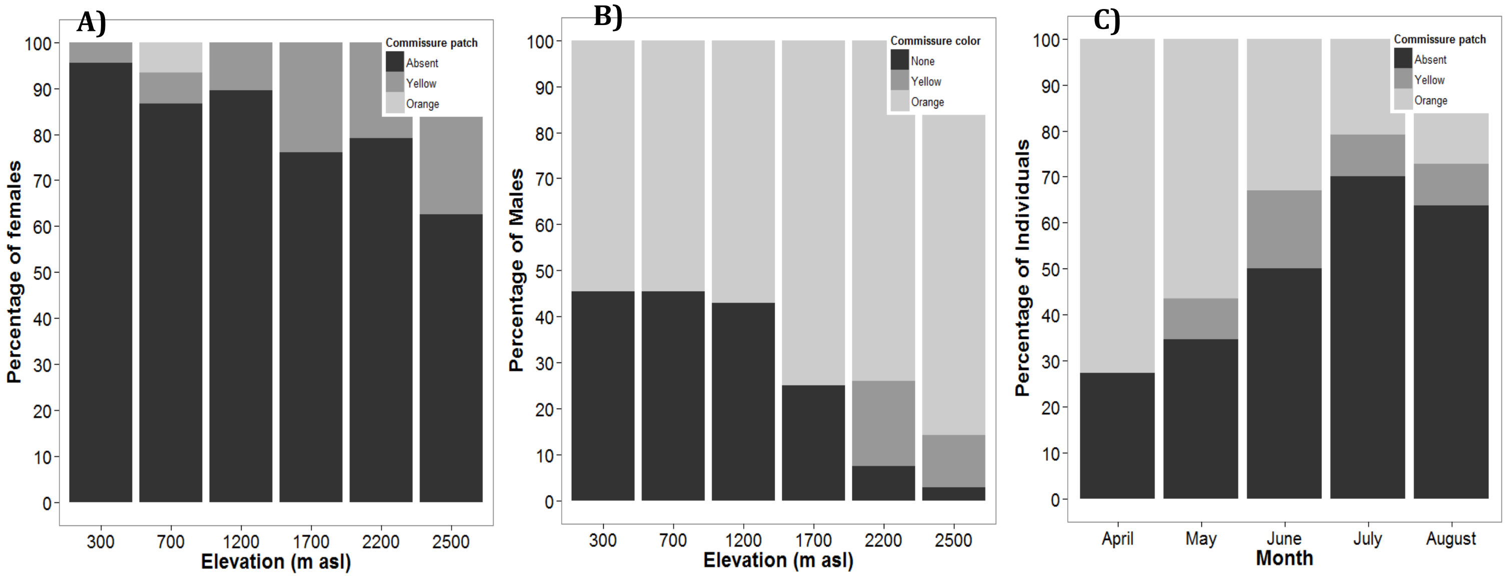

Figure 9.4. Frequency of presence of pigmented patch in the commissures in females (A) and males (B) of the lizard *Psammodromus algirus* for each population (elevation from 300 to 2 500 m asl). Figure 5C: Frequency of pigmented patch in the commissure over months of the *P. algirus* activity season. The variable has 3 levels: absent (black), yellow (dark grey), and orange (light grey).

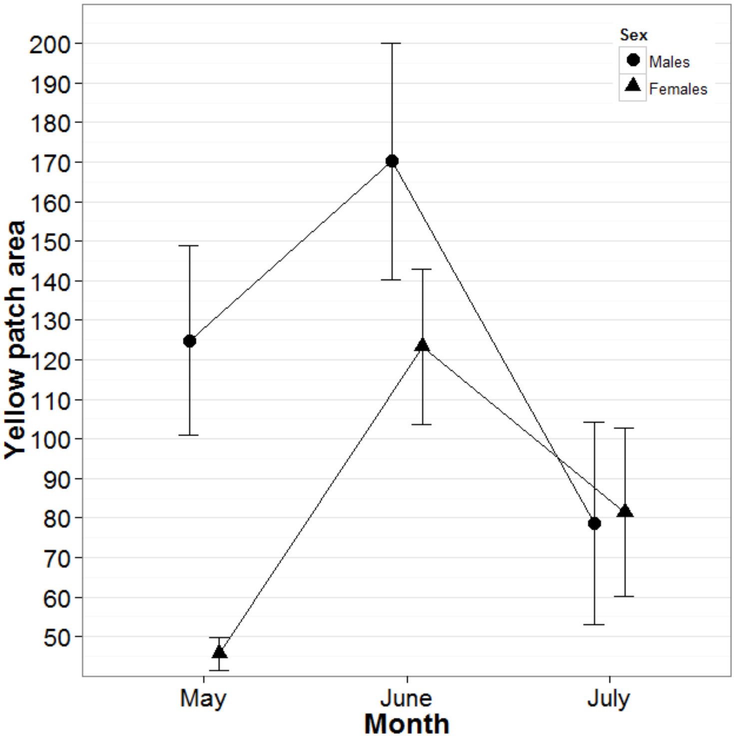

Figure 9.5. Mean values (dot) and standard error (whiskers) of throat yellowish patch area when present for each month over the activity season (individuals with yellowish patches were found only during May, June, and July), for males (circles) and females (triangles) of the lizard *Psammodromus algirus*.

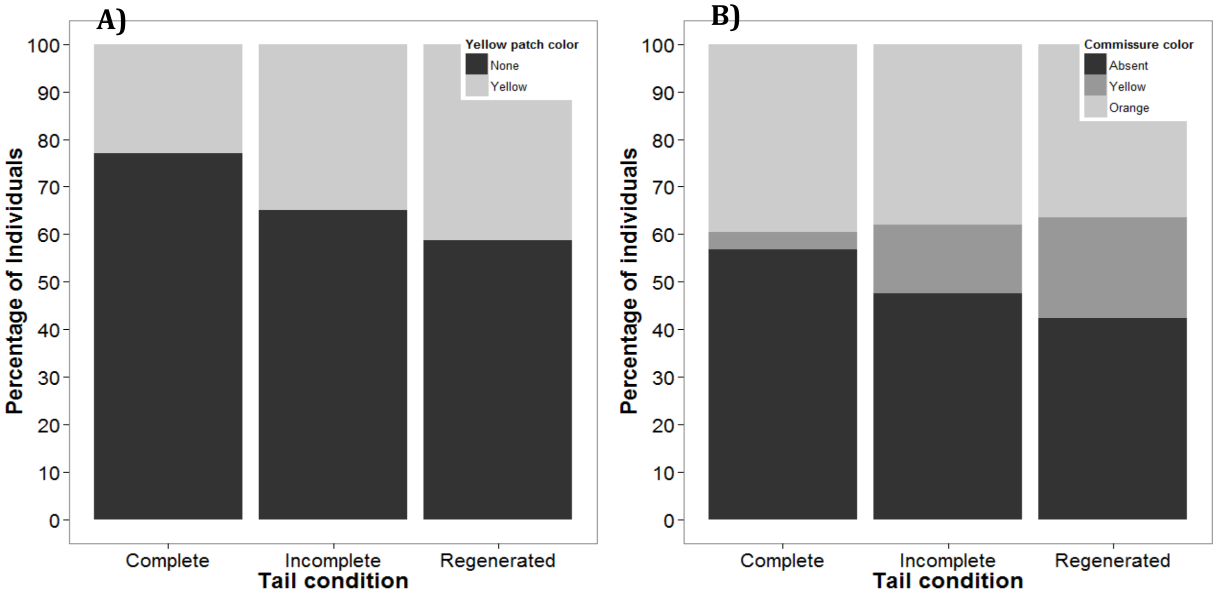

Figure 9.6. Frequency of pigmented patches in the lizard *Psammodromus algirus* in relation to their tail condition. (A) Pigmented patch in the throat, 2 levels: absent (in black) or present (Yellow, in light grey). (B) Pigmented patch in the commissures 3 levels: absent (black), yellow (dark grey), and orange (light grey)

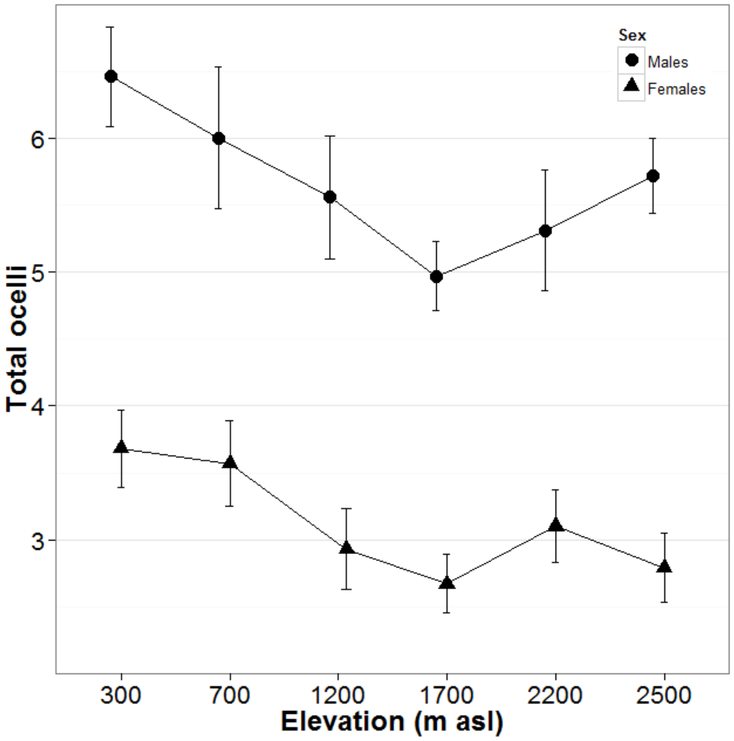

Figure 9.7. Mean values (dot) and standard error (whiskers) of number of ocelli for each population (elevation from 300 to 2 500 m asl), for males (circles) and females (triangles) of the lizard *Psammodromus algirus*.

Table 9.2. Summary for the lineal models testing the effect of elevation, sex, BCI (Body Condition Index), month, tail condition (complete, regenerated, or incomplete), and age (LAGs; lines of arrested growth) on throat colouration parameters ("L" lightness, "C" chroma, and "H" hue angle), and number of ocelli. Elevation, sex, month and tail are factors, and BCI and LAGs are continuous variables. The lineal model is very similar to the presented in Table 1, but ran with a subset of data for which we had age (LAGs) information.

	Throat L		**Log (Throat C)**		**Throat H**		**Ocelli**	
	Df	**F-value**	**Df**	**F-value**	**Df**	**F-value**	**Df**	**F-value**
Elevation	5,92	6.63 ***	5,92	5.97 ***	5,92	2.26	5,86	2.93 *
Sex	1,92	0.59	1,92	0.11	1,92	6.41 *	1,86	37.97***
BCI	1,92	1.20	1,92	0.28	1,92	0.22	1,86	0.20
Month	4,92	2.11	4,92	1.38	4,92	3.22 *	4,86	1.34
Tail	2,92	0.63	2,92	1.07	2,92	0.33	2,86	0.37
Age	1,92	16.39 ***	1,92	38.94 ***	1,92	0.34	1,86	0.77

All higher-order interactions involving covariates that were not significant were removed from the final model. Results were considered significant when the p-value <0.05. § p=0.05 * p<0.05 **p<0.01 ***p<0.001

Table 9.3. Summary for the simple regressions of SVL (snout-vent length) on ventral colour parameters ("L" lightness, "C" chroma, and "H" hue) and total number of ocelli of the lizard *Psammodromus algirus* for each population (elevation from 300 to 2 500 m asl).

	Throat L		**Throat C**		**Throat H**		**Ocelli**	
	β	**t-value**	**β**	**t-value**	**β**	**t-value**	**β**	**t-value**
300	-0.07	-1.03	0.32	3.93 ***	-0.21	-1.00	0.08	1.52
700	0.16	0.96	0.27	2.46 *	-0.49	-1.58	-0.01	-0.22
1200	-0.09	-1.10	0.29	4.17 ***	-0.37	-1.10	-0.01	-0.20
1700	-0.23	-3.91 ***	0.34	3.71 ***	-0.27	-1.09	-0.01	-0.19
2200	-0.36	-5.14 ***	0.51	5.67 ***	-0.22	-1.23	0.10	1.87
2500	-0.39	-6.36 ***	0.92	8.90***	-0.61	0.00***	-0.05	-1.57

Results were considered significant when *p*-value <0.05. § p=0.05 * p<0.05 **p<0.01 ***p<0.001

Table 9.4. Summary of the mean ± standard error of SVL for each category of yellow chest patch (absent or present) and commissure colour (absent, yellow, orange) and for each population (elevation from 300 to 2 500 m asl) and statistical test for differences ($\chi 2$-test) between categories.

	Chest yellow patch			**Commissures colour**			
	Absent	**Present**		**Absent**	**Yellow**	**Orange**	
	mean±se	**mean±se**	**$\chi 2$**	**mean±se**	**mean±se**	**mean±se**	**$\chi 2$**
Total	63.67±0.45	72.81±0.64	104.00 ***	63.72±0.55	73.85±1.18	68.45±0.54	72.66 ***
300	61.16±0.62	67.17±1.74	0.41 *	60.46±0.73	6.0	64.72±1.11	11.36 **
700	60.87±1.05	67±1.53	0.44	60.16±1.11	57.0	67.43±0.9	15.16 ***
1200	59.85±1.06	68.5±0.96	0.40 *	60.11±1.05	69.5±0.5	65.5±1.64	11.45 **
1700	64.81±0.8	69.53±1.09	0.25 **	64.48±0.99	70±2.11	66.29±0.97	6.97 *
2200	68.26±0.73	73.28±1.12	0.28 **	68.67±0.87	73.4±1.78	69.45±1.1	6.83 *
2500	69.68±1.67	76.12±0.9	0.18 **	72.39±1.83	78.46±1.43	72.77±0.95	10.81 **

Results were considered significant when the p-value <0.05. § $p=0.05$ * $p<0.05$ ** $p<0.01$ *** $p<0.001$

Table 9.5. Summary of the relationship among ventral colour parameters (“L” lightness, “C” chroma, and “H” hue) and the rest of the colour characters; yellow chest patch (YP; present, or absent), yellowish patch area (when it appeared), commissure (CO; absent, yellow, or orange), commissure area (when coloured) and number of ocelli. Statistical relationship between variables was controlled for elevation, included as factor in the models.

	YP		**YP area**			**CO colour**		**CO area**			**Ocelli**		
	Df	**χ2**	**Df**	**Estimate**	**t-value**	**Df**	**χ2**	**Df**	**Estimate**	**t-value**	**Df**	**Estimate**	**t-value**
Throat L	1	2.15	69	0.02	0.84	2	12.96**	92	-0.01	-0.54	387	0.04	1.48
Throat C	1	87.02**	69	0.03	3.51***	2	33.22**	92	0.02	3.058**	387	0.02	1.02
Throat H	1	0.71	69	0.00	0.15	2	4.16	92	0.00	0.26	387	0.01	1.10

Results were considered significant when the p-value <0.05. § p=0.05 * p<0.05 **p<0.01 ***p<0.001

Discussion

Redundant signals vs. Multiple signals

This study shows that different color signals in the lizard *P. algirus* indicate different aspects of this lizard. Although the dimensions of each signal were generally correlated (except number of ocelli and throat hue), which would support the hypothesis that they are redundant, signals were correlated differentially with various traits of the lizard, such as sex or age, suggesting that they also function as multiple signals. For example, throat chroma and hue, number of ocelli, and the color of the patch in the commissure, indicate gender in this lizard. However, age was indicated by throat lightness and chroma (but not hue), as well as the presence of the yellow patch on the throat and the colored patch (orange or yellow) in the commissure. Note, for example, that the presence of color patches in throat and commissure indicates maturation, but the color of these patches indicates the gender of the bearer. Therefore, we found that (1) different traits convey the same messages about some quality of the bearer (redundancy), (2) different qualities are indicated by different color traits (multiple messages), and (3) different qualities are indicated by different aspects of the same signal (multicomponent signals); that is, our findings imply that multiple patches in *P. algirus* are redundant signals and codify multiple messages because they work as multicomponent signals.

Whether such signals are addressed to potential mates or potential competitors remains to be properly determined. However, the fact that general throat coloration and size, as well as the presence of colored commissures are more frequent in certain months, when breeding occurs, suggests that these traits are involved in mating. Throat chroma and hue, as well as commissure color are in fact sexually dichromatic. However, the number of ocelli was sexually dimorphic, but did not change with season. Nevertheless, we measured only the number of ocelli, and thus we cannot rule out that other traits of these patches changed seasonally, such as, for example, color traits (lightness, chroma, hue), size of the ocelli, or even the UV reflectance, which is usually associated with ocelli in other species (*e.g.* Pérez i de Lanuza et al., 2014).

Throat patch

In *Psammodromus algirus*, previous studies have shown that the throat pigmented patch is sexually dimorphic in populations of central and western Spain, where breeding males show orange heads and throat, while females have yellow throats (Salvador 2011; Verdú-Ricoy et al. 2010). However, in eastern populations as that studied here, it had been concluded that there is no sexual dichromatism for this patch (Carretero 2002). Our findings using a spectrophotometer indicate that there is a subtle dichromatism in eastern populations, males being yellower and more saturated than females. A yellow patch on the throat seems to be present all the year. However, yellow patches were larger and more saturated during May and June, which matches with the breeding season in all elevations (Zamora-Camacho et al. 2013). More saturated throat coloration indicates higher pigment concentration (Montgomerie 2006), or a different structure of iridophores (San-José et al. 2013). In any case, carotenoid-based signals indicate individual quality in lizards (Fitze et al. 2009). Given that May and June is when spermatogenesis in males and vitellogenesis in females happens (Díaz et al. 1994), throat patches seem to be related to mating, presumably as a sign that the bearer is sexually receptive.

Moreover, throat patch may indicate several qualities of the bearer, such as body condition or age, working as a multicomponent signal (Grether et al. 2004). Lizards in better condition tended to show larger throat patches, although coloration of the patch was not related to body condition. Many studies have posited that color badges are indicators of good quality of the individuals (Pérez i de Lanuza et al. 2014; Langkilde and Boronow 2010; Salica and Halloy 2009a), but the size of the pigmented patches is a signal of the capacity of the bearer to invest resources in the signal (Alonso-Álvarez et al. 2004; Alonso-Álvarez et al. 2004).

Colorful pigmentary patches frequently indicates social status in lizards (Langkilde and Boronow 2010; Olsson 1994b). Coloration of the throat badge, but not its size, varied with age —and body size, which depends of age— of the lizards. Note that throat coloration indicates age or body size, while the size of the pigmentary patch seems to indicate body condition (previous paragraph). Social status in lizards is usually size dependent, and larger individuals are dominant over smaller ones (Díaz 1993; Martín and Forsman 1999). Therefore, badge color indicates size, and thus potential status. To evaluate the status of competitors by color badges may be easier than to evaluate their fighting capacity by examining their body size (Senar 2006). Younger lizards are selected to not indicate undue status, in which case they would be punished by older lizards, this explaining why younger lizards show duller throat coloration (Salvador et al. 1997; Martín and Forsman 1999).

In *Psammodromus algirus*, males with larger pigmentary badges on their throats have advantages during mating and agonistic encounters, this translating into higher reproductive success (Salvador and Veiga 2001; Salvador and Veiga 2008), but reduced long-term survivorship (Díaz 1993). Lizards with larger pigmentations have more testosterone (Olsson et al. 2012a; Salvador et al. 1996), but higher testosterone levels have negative effects on growth rates (Salvador and Veiga 2000), oxidative stress (Alonso-Álvarez et al. 2008), the immune system (Braude et al. 1998), and susceptibility to ectoparasites (Salvador et al. 1996). Moreover, colored individuals are more conspicuous to predators (Cabido et al. 2009; Forsman and Shine 2006; Husak et al. 2006; Olsson 1993; Stuart-Fox and Ord 2004). In fact, we found evidence of a cost for bearers of this signal: lizards with colorful throats showed evidence of higher predation risk (higher frequency of lost or regenerated tails). This finding may be a consequence of lizards with colorful throats being more easily detected by predators (Martín and López 1999). However, ventral coloration is almost invisible for aerial predators and conspicuousness seems not to be the real reason of more frequent predation (Olsson 1993). Besides having colorful ornaments, breeding and higher-status lizards are more active (Civantos 2002; Díaz 1993; Rose 1981), meaning that they are exposed to predators for more time, regardless of the ventral coloration. Because predators of *P. algirus* are mainly aerial, this latter explanation is more plausible. Therefore, higher predator attacks in more colored lizards seem to be the consequence of more active, and not of more conspicuous, individuals.

It should be noted that the difference in coloration between males and females was very subtle. Females in our populations showed yellow throats similar to those of males. Pigmentation patches in females are more frequent in nature than apparently (Calisi et al. 2008; Cuervo and Belliure 2013; Forsman and Shine 2006; Hager 2001; Salica and Halloy 2009a; Salica and Halloy 2009b). Females have the necessary genetic information to produce the same signals as males, but usually do not have the necessary hormonal stimulation (Olsson et al. 2012b). Yellow throat patches may be indicative of the receptiveness of the female, inducing male-male competition (Salvador and Veiga 2001), and of the high quality of the female as a mother (Svensson et al. 2006). In other species, females use a coloration similar to that of the male to avoid courtship (Hager 2001; López and Martín 2001). Moreover, female lizards use coloration as status badges involved in territorial displays (Hager 2001; Václav et al. 2007).

Commissure patch

The color of the commissure was sexually dichromatic, males presenting orange commissures, while females presented a yellow commissure or absence of commissure patch; therefore commissure color is indicative of gender in this species (also see Carretero 2002). In populations of the central and western Iberian Peninsula, males present orange pigmentation on the head during the breeding season (Díaz 1993; Verdú-Ricoy *et al.* 2010; Salvador 2011). However, we found only three adult males with orange pigmentation beyond the commissures, from the highest population (2500 m asl). This suggests that centre and western populations evolved their orange coloration as an extension of the color of the commissure –the evolution of the orange head was later (Verdú-Ricoy et al. 2010). Male lizards in our population might have the necessary biochemical machinery to produce a more widespread orange pigmentation, but they lack the necessary resources (*e.g.* pigments) or hormone concentration to produce it (Olsson 2012a). Furthermore, it is probable that the warmer and dryer environmental conditions in the south-western areas of the Iberian Peninsula have selected for chemical instead of visual signals (Abert 1992).

In addition, males with orange commissures were more often found at the beginning of the activity season than later, suggesting that the orange pigmentation disappeared after the breeding season as occurs with orange heads in central and western populations (Díaz 1993). Alternatively, this pattern may be a consequence of an increased number of yearlings reaching adulthood as the activity season advances. As it occurred with throat patch, larger and older individuals showed larger commissures. This trait is displayed only to receivers when the sender opens its mouth. Therefore, the orange commissure seems to be a cue to facilitate the estimation of the sender's mouth size. Colorful pigmentary patches indicating high social status are often hidden, and receivers view them only during gaping displays (Lappin et al. 2006).

Our study provides evidence that the commissure is costly to maintain. The presence of the commissure patch was related to increased risk of predation (broken tails). Clearly, the commissure patch does not increase detectability by predators, given that it is hidden and shown only when the lizard opens the mouth. We suggest that, as discussed above for the throat patch, the relationship between the presence of the commissure patch and predation risk may be related to high-status lizards being more active and thus more exposed to predators (Díaz 1993).

Number of ocelli

Psammodromus algirus also was sexually dimorphic for the number of ocelli, these being more numerous in males, as happens in many other species such as Timon lepida (Font et al. 2009). The potential function of ocelli in communication is unknown. The number of ocelli was not related to any quality measured in this study. We could not measure coloration or size of the ocelli, nor UV reflectance, which is usually associated with these type of blue patches (Pérez i de Lanuza and Font 2010).

Elevational variation in coloration

Previous studies showed that coloration of badges in *Psammodromus algirus* has geographic variation in the east-west gradient (Carretero 2002; Verdú-Ricoy et al. 2010; Salvador 2011). Here, we show that throat coloration varies with elevation, populations at the highest elevations showing darker and more saturated throats, the exact hue being also more orange (lower hue values). In addition, the probability of a patch appearing in the commissures increased with elevation. However, the number of ocelli was maximal in the lowest elevations. Social coloration is under different but not exclusive selective pressures, but these pressures do not have the same affect or the same intensity over entire geographic gradients. In this section, we discuss different non-mutually exclusive explanations for the observed pattern with elevation.

(1) The observed pattern may be a consequence of sexual selection varying with elevation. In birds, investment in sexual signals decreases with elevation (Badyaev 1997c; Snell-Rood and Badyaev 2008), which is consistent with our findings for number of ocelli, but inconsistent with our findings for throat and commissure-badge coloration. Badyaev (1997) and Snell-Rood and Badyaev (2008) argued that the intensity of sexual selection in birds decreases with elevation as a consequence of increased selection for monogamy and biparental care of nestlings (Badyaev 1997b; Badyaev 1997a; Badyaev and Ghalambor 2001), which is clearly not applicable to lizards that lack parental care of neonates. However, in our study, sexual dichromatism was more

evident at lower populations, females being more similar to males at higher elevations. In other words, males at low elevations invest more resources in coloration and pigmentary patches than do females, which is consistent with findings in birds (Badyaev 1997c). On the other hand, geographic variation in female preferences has been described in lizards (Kwiatkowski and Sullivan 2002). Variation in female *P. algirus* preferences for different color traits with elevation could explain the findings in our study, especially why the number of ocelli is maximal at low elevation, while the other color patches show the reverse pattern –*i.e.*, mate choice could be more based on ocelli at low elevations and on carotenoid-based coloration at high elevations. Female preferences according to elevation, however, remain to be studied.

(2) As happens with dorsal coloration, lizards turned darker ventrally, which could be consequence of a general increment of melanin concentration in the skin (Reguera et al. 2014a; Chapter 5). Coloration in lizards is based on the resulting reflectance from three skin layers: an outermost layer of xantophores and erythrophores with carotenoids, an intermediate layer of iridophores, and a final layer of melanophores, which contain melanin (Grether, Kolluru, and Nersissian 2004). Therefore, an increase in whole-body melanin with elevation could result in the pattern found for throat lightness. Similarly, Leaché et al. (2010) found that ventral blue coloration turned darker with elevation in *Sceloporus occidentalis*, a trend they related to improved thermoregulatory capacity in darker lizards.

However, the fact that lizards had more saturated coloration in throats and more frequent pigmentary patches at high elevations cannot be explained by higher melanin concentration in the skin. By contrast, it could mean that, at high elevations, lizards have more resources, or in contrast, have a lower predation risk, oxidative stress, or parasite load, possibilities which we discuss below.

(3) Predation pressure tends to decrease as elevation rises (Fox et al*.* 1994, and references therein), and consequently more conspicuous lizards could be selected at high elevations (Endler 1980; also see Cabido et al*.* 2009). For example, the lizard *Crotaphytus collaris* presents more conspicuous

colorations and greater dichromatism in populations with fewer predators (Macedonia et al. 2002). This possibility matches with the fact that our study species show reduced dorsal crypsis as elevation increases (Chapter 8). Nevertheless, as discussed above, the relationship between coloration and predation seems to be more related to activity than to increased detectability by predators for more colored lizards. Therefore, this scenario remains to be properly elucidated.

(4) The availability of carotenoids as pigments could increase with elevation for different reasons. For example, the availability of carotenoids in the diet might augment with elevation in our study area, but this point is unknown. Nonetheless, in our study system, prey availability increases with elevation (unpublished data). Given that carotenoids are incorporated from diet, carotenoid-based signals are prone to vary geographically according to carotenoid availability (review in Dale 2006). Moreover, the environment is less oxidant at high than at low elevations (Reguera et al. 2014b; Chapter 6); therefore high-elevation lizards could afford to use carotenoids in pigmentation and not as antioxidants (Alonso-Álvarez et al. 2008), this leading to more saturated ventral coloration. For example, great tits (*Parus major*) breeding in urban habitats are exposed to higher oxidative stress than rural birds, and also have significantly paler-yellow plumage (Isaksson et al., 2005). Carotenoids are also used in the immune system (*e.g.* Blount et al. 2003; Faivre 2003; Pérez-Rodríguez et al. 2008). Therefore, if pathogen pressure decreases with rising elevation, this would allow increased investment in carotenoid-based signals. In our study system, ectoparasite prevalence decreases with rising elevation (unpublished data).

(5) Finally, another possibility is that visual signals works better in high than in low elevations. Luminosity is higher in high elevations, favouring visual signals (Endler 1990). Besides, high elevation environmental conditions (wetter and colder) are more adequate for visual instead of chemical signals, given that chemical signals propagate better in dry and hot environments (Abert 1992).

Conclusions

In conclusion, our study provides a quantitative analysis of a comprehensive suite of social coloration signals in a well-studied dichromatic species. Different traits conveyed the same qualities of the bearer, but at the same time, different color traits indicated different qualities, implying that color patches in *P. algirus* were, at the same time, redundant and codify multiple messages. Different elements of the same color patch indicated different qualities of the bearer, thus color traits acted as multicomponent signals in this lizard. Throat chroma and hue, number of ocelli, and the color of the patch in the commissure, indicate gender in this lizard. Age, and consequently body size and survival capacity, was indicated by throat lightness and chroma (but not hue), as well as the presence of yellow patch in the throat and colored patch (orange as well as yellow) in the commissure. We also found evidence that colorful individuals were more exposed to predation, but this seemed not to be a consequence of the color *per se*. Most color traits were sexually dichromatic and/or appeared especially during breeding season, suggesting that they were involved in mating. Finally, coloration varied with elevation, pigmentary patches being usually more frequent and saturated at high than at low elevations, while the number of ocelli tended to decrease with rising elevation.

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Chapter 10
GENERAL DISCUSSION

General discussion

The results of this thesis have confirmed *Psammodromus algirus* as a lizard capable to adapt to different environmental conditions, even though it has been mainly consider a Mediterranean lizard. The fact that lizards from high elevations are darker suggests that *P. algirus* is locally adapted following the "thermal melanism hypothesis" (Clusella-Trullas et al. 2007), i.e. darker skin being beneficial in terms of body warming in colder environments. Nevertheless, it cannot be rejected the idea that darker teguments (rich in melanins) may protect the lizard against UVR negative effects (Brenner and Hearing 2007; Ortonne 2002).

Darker dorsal skin in lizards from higher elevations matches pretty well the predictions assessed by the "thermal melanism hypothesis" (Clusella-Trullas, van Wyk, and Spotila 2007). Individuals of *P. algirus* are darker in colder locations, but also a trade-off between body size and darker coloration apparently exists, since those lizards that are smaller (are less efficient keeping warm; Bergman 1847) are also darker. Moreover, lizards' skin is darker in those locations where cover vegetation is denser, supporting that darker color inproves thermorregulation in those places with less efficient thermal conditions. In regard to protection against UVR harmful effects, lizards are darker where UVR is more intense.

In the attempt to study the harmful effects of UVR (Belden et al. 2000; Chang and Zheng 2003; Iwai et al. 1999) and expecting that lizards from highlands might be more affected by UVR, we studied the oxidative stress levels in the lizard *P. algirus*. However, against our expectations, lizards from highlands showed less oxidative damage than those from lowlands (Marquis and Miaud 2008). That could means that darker skin coloration, or whatever other sun-screen substance, is protecting them from UVR harmful damages (Brenner and Hearing 2007; Ortonne 2002; Cope et al. 2001). So the next logic steps should be test if UVR is as important as other oxidative agents for this lizard as in other organisms (Hessen 1996; Dahms, Dobretsov, and Lee 2011; Chang and Zheng 2003). We found that individuals of *P. algirus* were not affected by UVR at the oxidative stress level regardless of the location they came from. However, lizards from locations at lowlands showed more oxidative damage. This fact may mean that populations at lower elevations are subjected to more oxidative environmental conditions than those from highlands. The climate at lowlands, with higher temperatures, and the presence of more contaminants derived from the human activity are most probably the main reason for these differences (Lesser 1997; Amaral et al. 2012; Costantini 2014). Higher temperatures can result in higher metabolic rates and higher oxidant reactive species production in the organisms (Jena et al. 2013), triggering higher oxidative damage. In addition, the presence of contaminants also triggers oxidative reactive species in organisms (Costantini 2014). Relationship between darker skin and oxidative stress were not significant in our study. Thus, darker skin in these lizards might be protective at other physiological levels, acting in long term at either the tissue or other systemic levels.

On the other hand, selection for cryptic coloration is apparently affecting dorsal coloration, too. Thermal benefits and UVR-protection apparently are more important as selective pressures at higher elevations, since lizards from highlands are less cryptic with background color than lowland individuals. Nevertheless, lizards' dorsal coloration covaries with background coloration in terms of color hue, which shows that predation pressure has an important role in dorsal color evolution (Slagsvold et al. 1995; Rosenblum et al. 2004; Caro 2005).

In regard to color badges and intra-specific communication, *P. algirus* shows pigmented patches that work as visual signals (Hill and McGraw 2006). It is noteworthy that bearers have diverse signals that convey the same message, but also diverse messages can be signaled by the same badge, acting as a multicomponent signal system (Martín and López 2009). Whereas throat chroma and hue, number of ocelli and the presence of orange pigmentation in the commissures of the mouth may be a sexual signal, it is very likely to find the presence of yellowish pigmentation in the gular region in the same degree both in males and in females. Furthermore, throat chroma and lightness, as well as the presence and saturation of the yellowish pigmentation are signals of the social status (older age and bigger size) of the sender (Díaz 1993; Olsson 1994; Salvador et al. 1997).

Nonetheless, the presence of these color badges has a "price". Colorful individuals present injured tails more often. Probably, this is not due to their conspicuousness to predators, since signals are ventrally located, but to the fact that they spend more time exposed to the predator's attacks because usually these colorful individuals are more active (Díaz 1993). Otherwise, damaged tails can also be a consequence of fights between individuals and during copulation. However, it is more likely that the more active individuals are involved more often in this type of situations.

The use of visual signals is geographically different. The resources availability or the environmental conditions provoke a different evolution of the visual signals in geographical gradients. In the elevation gradient studied here, color badges are more frequent and more intense in those lizards from highlands. This pattern may be a consequence of foraging resources are better at higher elevations, and also because colder and more humid conditions may lead to the selection of visual signals over the chemical ones (Abert 1992). The fact that lizards from higher elevations show lower oxidative stress levels might allow these lizards to invest physiological resources in coloration instead of maintaining the homeostasis of oxidative and immune systems (Alonso-Álvarez et al. 2008).

The results of this thesis evidence that in a geographical gradient, as an elevational range, evolutionary forces work in different ways. In terms of thermodynamics, the thermal energy and the increase of the thermoregulation efficiency by lizards is really important in cold places. Simultaneously, reproductive investment and consequently territoriality and mating investment are acting as selective pressures. Therefore, those individuals which had more access to certain resources, or which are more efficient to use them might be favorably selected.

This Mediterranean lizard has demonstrated that is well adapted along an elevational gradient, showing adaptive colorations to the environmental conditions at each specific altitude. Therefore it is expected that in a global warming change scenario, whether populations would raise their distribution to higher elevations, they might be able to cope with the environmental conditions.

Discussion references

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Chapter 11
CONCLUSIONS

Conclusions

1. Lizards from highlands (population above 2000 m asl elevation) are darker than those from mid- and low-lands. Following the predictions states by the "thermal melanims hypothesis": Lizards are darker in colder locations, smaller individuals are darker in almost all elevations. Furthermore, individuals are darker where vegetation cover is more dense, thus facing a colder environment. In addition, darker lizards have been selected in those places where ultraviolet radiation is more intense.

2. Oxidative stress level in *P. algirus* varies along elevation. In contrast it was expected, lizards from highlands show less oxidative damage. That evidence the existence of adaptations in highland lizards against oxidative stress damage provoked by ultraviolet radiation, but also and not exclusive that environments at low elevations are more oxidizing.

3. Ultraviolet radiation is not an important oxidant agent for *P. algirus*, at least at the level of oxidative damage.

4. Lizards' dorsal surface is similar to the color of the ground where they inhabit. Natural selection have selected for coloration as similar as possible to the ground, in order to reduce detectability by aerial predators. However, the degree of crypsis decreases with elevation, probably because in highlands other selective pressures select for darker colorations than ground in favor of thermoregulation efficiency and/or protection against ultraviolet radiation. That points to the existence of local adaptation along the elevational system in the degree of crypsis according to background coloration and the existence of other selective forces.

5. Ventral and lateral lizards' body sides frequently show colored patches. These pigmentary areas work as visual signals used by the individuals in intra- and inter-sexual communication. Different traits conveyed the same

bearer's quality, but at the same time, different bearer's qualities were indicated by the same color trait, implying that color patches in *P. algirus* were redundant signals and codify multiple messages.
5a. Most of color traits were sexually dimorphic and/or appeared especially during breeding season, suggesting that they were involved in mating.
5b. Throat chroma and hue, number of ocelli, and the color of the patch in the commissure, indicate gender in this lizard.
5c. Age was indicated by throat lightness and chrome (but not hue), as well as the presence of yellow patch in the throat and/or chest and orange (in males) and/or yellow (both sexes) patch in the commissure.

7. Colorful individuals are more exposed to predation, but not as consequence of their conspicuousness, but because they are more active and expend more time exposed to predators' attacks.

8. Lizards from highlands invest more resources in color badges than lowland lizards. Highlands provide more resources in terms of diet or less stressful environments. Also different evolutionary forces are selecting for different communication signals, being visual ones more effective in highland environments.

9. Finally, in a global warming scenario where lots of species are shifting their distribution to higher elevations, *Psammodromus algirus* appears to be well adapted to high-elevation environmental conditions, which may benefit it in a hypothetic shift of lowlands populations.

Conclusiones

1. Las lagartijas son más oscuras en las localidades a mayor altitud (por encima de los 2000 m snm). La coloración oscura concuerda con las predicciones propuestas por la "hipótesis del melanismo térmico": los individuos son más oscuros en ambientes más fríos, y en la mayoría de las localidades, los individuos más pequeños son más oscuros. Siguiendo con las prediccionres de la hipótesis de melanismo térmico, las lagartijas son dorsalmente más oscuras en lugares con mayor desidad de vegetación, relacionado esto generalmente con ambientes menos eficientes a nivel térmico. Finalmente, se han seleccionado lagartijas de piel más oscura en las localidades donde la radiación solar es más intensa, apoyando la hipótesis de la protección frente a la radiación ultravioleta.

2. Los niveles de estrés oxidativo en *P. algirus* varían a lo largo del gradiente altitudinal. En contra de lo que se esperaba, las lagartijas de altitudes mayores presentan menor daño oxidativo respecto a las de localidades bajas. Esto pone de manifiesto la existencia de adaptaciones para protegerse del daño causado por la radiación ultravioleta en las lagartijas procedentes de localidades elevadas, pero también y no de forma excluyente, que los ambientes a menor altitud son más oxidantes para estas lagartijas.

3. La radiación ultravioleta no parace ser un agente oxidante tan importante para *P. algirus*, al menos al nivel de daño oxidativo.

4. El color dorsal de las lagartijas es similar al del sustrato en el que habitan. La selección natural ha seleccionado coloraciones similares al sustrato, reduciendo así la detectabilidad por parte de los depredadores aéreos. Sin embargo, el grado de cripsis disminuye con la altitud, probablemente porque a mayor altitud actúan otras presiones de selección seleccionando coloraciones más oscuras que el suelo favoreciendo la termorregulación y/o la protección frente a los daños producidos por la

radiación ultravioleta. Esto apunta a que existen adaptaciones locales, y que la presiones de selección no son la mismas a lo largo del gradiente altitudinal.

5. Las zonas ventral y lateral del cuerpo de las lagartijas muestran manchas de pigmentación que destacan sobre el resto del cuerpo. Estas manchas de coloración funcionan como señales visuales utilizadas por los individuos en la comunicación intra- e inter-sexual. Diferentes señales informan sobre diferentes cualidades del portador de la señal. Al mismo tiempo una misma señal puede estar informando sobre diferentes cualidades, o una misma cualidad pude ser señalizada por diferentes señales. Esto implica que las señales de coloración en *Psammodromus algirus* son redundantes y codifican múltiples mensajes.

5a. La mayoría de las señales de coloración son sexualmente dimórficas y/o aparece durante la época de reproducción, sugiriendo una función durante la selección de pareja.

5b. La tonalidad del color de la garganta y el pecho, así como el número de ocelos, y el color de la mancha en las comisuras de la boca, indican el sexo del portador en esta especie.

5c. La edad es indicada por el brillo y la saturación del color de la garganta (pero no por la tonalidad), así como la presencia de pigmentación amarilla en el área de la garganta y/o el pecho, y de pigmentación naranja (en machos) y/o amarilla (en ambos sexos) en las comisuras.

7. Los individuos más coloridos están más expuestos a los ataques por depredadores, pero no por causa de su conspicuidad, si no porque son individuos más activos y por lo tanto pasan más tiempo disponibles para los depredadores.

8. Las lagartijas procedentes de mayor altitud invierten más en señales sexuales. Puede ser bien porque el hábitat a mayor altitud proporcionan más recursos a nivel de alimentación, o bien porque es un ambiente menos estresante en términos de estrés oxidativo, y les permite invertir los

pigmentos en coloración en vez de en el sistema antioxidante. Además podrían estar ocurriendo una selección de señales visuales en detrimento de otro tipo de señales, debido a las condiciones ambientales del ambiente, frío y humedad.

9. Finalmente, en un marco de cambio climático global en el que muchas de las especies están ascendiendo en altitud, *Psammodromus algirus* parece estar bien adaptada a las condiciones que encontraría en las localidades a mayor altitud, lo cual la beneficiaría en el hipotético caso de que las poblaciones de zonas bajas asciendan en altitud.

SUPPLEMENTARY MATERIAL

Appendix A: suplemmentary results

Table A1 (part 1) Post-hoc analyses for the color variation in altitude

Back lightness	**diff**	**lwr**	**upr**	**p-adj**
300-1200	-1.91	0.02	1.96	1.00
300-1700	-0.62	1.00	2.63	0.49
300-2200	1.93	3.55	5.18	0.00
300-2500	1.53	3.11	4.69	0.00
300-700	-0.25	1.55	3.36	0.14
700-2200	0.16	2.00	3.84	0.02
700-2500	-0.24	1.56	3.35	0.13
1200-1700	-0.99	0.98	2.95	0.71
1200-2200	1.56	3.53	5.50	0.00
1200-2500	1.16	3.09	5.01	0.00
1200-700	-0.59	1.53	3.65	0.31
1700-2200	0.88	2.55	4.22	0.00
1700-2500	0.49	2.11	3.72	0.00
1700-700	-1.29	0.55	2.40	0.96
2500-2200	-1.17	0.44	2.06	0.97

Table A1 (part 2) Post-hoc analyses for the color variation in altitude

Back chroma	**diff**	**lwr**	**upr**	**p-adj**
300-1200	0.06	-1.36	1.49	1.00
300-1700	0.83	-0.37	2.03	0.35
300-2200	2.73	1.53	3.93	0.00
300-2500	2.85	1.69	4.02	0.00
300-700	0.70	-0.64	2.03	0.67
700-1700	0.13	-1.22	1.49	1.00
700-2200	2.04	0.68	3.40	0.00
700-2500	2.16	0.83	3.49	0.00
1200-1700	0.77	-0.68	2.22	0.66
1200-2200	2.67	1.22	4.12	0.00
1200-2500	2.79	1.37	4.21	0.00
1200-700	0.63	-0.93	2.20	0.86
1700-2200	1.90	0.67	3.13	0.00
1700-2500	2.02	0.83	3.22	0.00
2200-2500	0.12	-1.08	1.32	1.00

Table A1(part 3) Post-hoc analyses for the color variation in altitude

Back hue	**diff**	**lwr**	**upr**	**p-adj**
300-1200	0.40	-2.66	3.46	1.00
300-1700	1.42	-1.15	4.00	0.61
300-2200	4.79	2.21	7.36	0.00
300-2500	2.69	0.18	5.19	0.03
300-700	0.33	-2.53	3.19	1.00
700-1200	0.07	-3.28	3.43	1.00
700-1700	1.09	-1.82	4.01	0.89
700-2200	4.46	1.54	7.37	0.00
700-2500	2.36	-0.49	5.21	0.17
1200-1700	1.02	-2.09	4.13	0.94
1200-2200	4.38	1.27	7.50	0.00
1200-2500	2.28	-0.77	5.34	0.27
1700-2200	3.36	0.73	6.00	0.00
1700-2500	1.26	-1.30	3.83	0.72
2500-2200	2.10	-0.47	4.67	0.18

Table A2 (part 1) Mean values and standard error (below the mean value) of lizards' dorsal color components (lightness [L*], chroma [C*] and hue angle [H*]), environmental temperature (Te, in ºC) during the activity season, ultraviolet B radiation (UVB, in µW cm-2 nm-1), percentage of vegetation cover (%VC) and components of ground coloration for each six altitudes (corresponding to the studied populations). Sample size in italic.

Elevation	**300**	**700**	**1200**	**1700**	**2200**	**2500**
Back L*	40.87	39.32	40.85	39.87	37.34	37.76
	0.38	0.54	0.62	0.43	0.4	0.34
	75	*47*	*38*	*68*	*67*	*77*
Back C*	13.99	13.29	13.92	13.16	11.24	11.13
	0.33	0.37	0.45	0.34	0.26	0.22
	75	*47*	*38*	*68*	*67*	*76*
Back H*	74.97	74.64	74.57	73.55	70.2	72.28
	0.76	0.85	0.81	0.52	0.51	0.66
	75	*47*	*38*	*68*	*67*	*76*
Te	25.02	23	20.85	19.09	16.49	17.05
	1.26	1.03	1.29	1.35	1.61	1.73
	13	*13*	*12*	*12*	*11*	*8*

Table A2 (part 2) Mean values and standard error (below the mean value) of lizards' dorsal color components (lightness [L*], chroma [C*] and hue angle [H*]), environmental temperature (Te, in ºC) during the activity season, ultraviolet B radiation (UVB, in µW cm-2 nm-1), percentage of vegetation cover (%VC) and components of ground coloration for each six altitudes (corresponding to the studied populations). Sample size in italic.

Elevation	**300**	**700**	**1200**	**1700**	**2200**	**2500**
UV	1.28	4	4.19	4.41	5.15	4.8
	0.03	0.14	0.13	0.15	0.31	0.37
	37	*37*	*37*	*37*	*37*	*37*
%VC	52.57	66.67	53.73	66.4	82.03	65.88
Substrate L*	32.62	45.21	35.32	27.11	21.79	21.6
	3.5	2.57	3.1	2.76	2.92	3.15
	10	*11*	*16*	*22*	*23*	*14*
Substrate C*	18.97	17.96	24.87	25.04	23.94	27.69
	2.08	2.54	2.74	1.64	1.48	1.66
	10	*11*	*16*	*22*	*23*	*14*
Substrate H*	70.41	75.55	67.22	60.87	55.52	52.08
	2.76	1.83	3.16	2.38	3.29	4.31
	10	*11*	*16*	*22*	*23*	*14*

Table A3. F-values and degree of freedom (in brackets) for a lineal model including oxidative damage, antioxidant capacity elements, and immune response as dependent variables, and treatment, and elevation as independent predictors.

	Treatment	**Elevation**
	Antioxidant capacity	
TEAC	(1,53) 0.09	(2,53) 0.96
SOD	(1,53) 2.55	(2,53) 0.11
CAT	(1,50) 1.33	(2,50) 2.11
log(GPX)	(1,50) 2.16	(2,50) 0.09
GST	(1,50) 1.05	(2,50) 1.06
	Oxidative damage	
FrMDA	(1,55) 0.31	(2,55) 3.35*
log(PrMDA+1)	(1,55) 1.60	(2,55) 3.74*
log(LOOH)	(1,54) 0.03	(2,54) 0.76
PrOOH	(1,55) 0.34	(2,55) 3.05§
	Immune response	
Peroxidase	(1,50) 0.05	(2,50) 0.10
IR	(1,53) 0.44	(2,53) 0.05

Table A2 Mean values and standard error (below the mean value) of lizards' dorsal color components (lightness [L*], chroma [C*] and hue angle [H*]), environmental temperature (Te, in ºC) during the activity season, ultraviolet B radiation (UVB, in µWcm^{-2}nm^{-1}), percentage of vegetation cover (%VC) and components of ground coloration for each six altitudes (corresponding to the studied populations). Sample size in italic. All higher order interactions involving covariates were not significant and were removed from the model. The reduced model is presented here. Results were considered significative when *p*-value <0.05. §*p*=0.05 * *p*<0.05 ***p*<0.01 ****p*<0.001

Table A4. Pearson's correlation coefficients among immune response (peroxidase activity, and immune response to PHA) and antioxidant capacity (TEAC, SOD, CAT, GPX, and GST) and oxidative damage (FrMDA, PrMDA, LOOH, and PrOOH) in the lizard *Psammodromus algirus* in the Sierra Nevada (SE Spain). Sample size for each correlation is in italic. Peroxidase activity is related to lymphocyte activity (immune cells) and immune response was measured as the swelling of the fore limb sole after the injection of PHA antigen (protein from cell wall). Any of the immune parameters were significantly correlated with any of the oxidative stress parameters.

	Peroxidase		**IR**	
TEAC	-0.32	*54*	-0.14	*55*
SOD	0.11	*54*	0.32	*55*
CAT	0.07	*55*	0.13	*52*
GPX	0.10	*55*	0.01	*52*
GST	0.02	*55*	0.22	*52*
FrMDA	0.18	*55*	0.14	*57*
PrMDA	0.06	*55*	0.01	*57*
LOOH	-0.21	*54*	-0.01	*57*
PrOOH	0.10	*55*	0.18	*57*

TEAC= trolox equivalent antioxidant capacity (μM eq Trolox), SOD= superoxide dismutase (U/mg protein), CAT= catalase (U/mg protein), GPX= gluthatione peroxidase (mU/mg protein), GST= gluthatione transferase (mU/mg protein), FrMDA= free malondialdehyde (μM/mg protein), PrMDA= malondialdehyde associated to protein (μM/mg protein), LOOH= lipid hydroperoxides (μM/mg protein), PrOOH= Protein hydroperoxides (μM/mg protein).

Table A5.– Sample sizes (n) and Pearson's correlation coefficients (r) among background elements and each other for each component of the L*C*H* CIE color space. Test significance is indicated as follows: *= P<0.05; **= P<0.01; ***= P<0.001.

	Bare soil		Rock		Fresh vegetation		Dry vegetation
	n						
Bare soil	96						
Rock	36		56				
Fresh vegetation	72		37		95		
Dry vegetation	83		43		82		112
	Lightness						
Bare soil	1.00						
Rock	0.30		1.00				
Fresh vegetation	-0.01		0.19		1.00		
Dry vegetation	0.23	*	0.19		0.36	***	1.00
	Chroma						
Bare soil	1.00						
Rock	0.40	*	1.00				
Fresh vegetation	0.13		-0.17		1.00		
Dry vegetation	0.46	***	0.50	***	0.26	*	1.00
	Hue angle						
Bare soil	1.00						
Rock	0.50	***	1.00		-0.17		
Fresh vegetation	0.24	*	-0.17		1.00		
Dry vegetation	0.29	*	0.07		0.06		1.00

Appendix B: Published manuscripts

LINNEAN SOCIETY BIOLOGICAL Journal

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The lizard *Psammodromus algirus* (Squamata: Lacertidae) is darker at high altitudes

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Altitudinal gradients offer a good opportunity to study organisms' adaptations to clinal environmental variables. Regarding altitude, the most influential variables on organisms are temperature and ultraviolet (UV) solar radiation, the first decreasing and the second increasing with altitude. Both variables affect ectotherms' biology, as ectotherms depend on environmental temperature for thermoregulation, frequently being heliotherms. Here, we studied dorsal coloration in the lizard *Psammodromus algirus* (Linnaeus, 1758) along a wide altitudinal gradient (2200 m) in Sierra Nevada (south-east Spain). We hypothesize that the skin will be darker with altitude, i.e. in environments with lower temperatures and higher UV radiation intensity. Results show that individual dorsal colorations became darker at high altitude. We propose two non-mutually exclusive explanations for this result: (1) darker dorsal surface would favour faster warming at high altitudes, where temperature is lower, and (2) darker dorsal surface would protect against UV radiation, stronger at high altitudes. We found significant relationships between both temperature and UV radiation and population dorsal darkness, giving mixed support for the two explanations. Moreover, dorsal hue was positively correlated with substrate hue, suggesting that hue evolved to maximize crypsis. Our study therefore suggests that geographical variation in dorsal coloration in this lizard is adaptive, and darkness coloration might have evolved in response to adverse conditions (low temperature and high UV radiation) at high altitudes. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **112**, 132–141.

ADDITIONAL KEYWORDS: altitudinal gradient – body colour – darkened coloration – thermal melanism – ultraviolet radiation.

INTRODUCTION

The environment gradually varies with altitude (Körner, 2007) and, accordingly, organisms distributed in a wide altitudinal range may be locally adapted, generating clines for several traits with altitude (Welter-Schultes, 2000; Blackburn & Ruggiero, 2001; Ashton & Feldman, 2003). In fact, animals may show adaptive variation in coloration in relation to elevation. For example, darker individuals are expected at higher elevations, where temperatures are lower, a pattern that may be explained by the 'thermal melanism hypothesis' (Clusella-Trullas, van Wyk & Spotila, 2007). This hypothesis states that, compared with light individuals, darker individuals have an advantage under low-temperature conditions, because they heat up faster at a given level of solar radiation (Watt, 1969; Clusella-Trullas *et al*., 2007, 2008). Heating up faster is important for ectotherms, especially for reptiles, which depend largely on solar radiation for thermoregulation (Vitt & Caldwell, 2009). Populations distributed along an altitudinal range face temperature gradients, which provide a good opportunity to study the evolution of body coloration and its relationship to thermoregulation (Clusella-Trullas *et al*., 2007).

A second hypothesis, non-mutually exclusive with the first, that could explain why organisms darken with altitude is based on the increasing UV radiation intensity with elevation ('protection against UV damage hypothesis', Porter & Norris, 1969). Most ectothermic organisms (in particular, heliotherms) rely on solar radiation to thermoregulate, but this radiation may have harmful effects at high altitude, where it is stronger. Moreover, given that thermal

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Oxidative stress decreases with elevation in the lizard *Psammodromus algirus*

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Ultraviolet radiation

ABSTRACT

Oxidative stress is considered one of the main ecological and evolutionary forces. Several environmental stressors vary geographically and thus organisms inhabiting different sites face different oxidant environments. Nevertheless, there is scarce information about how oxidative damage and antioxidant defences vary geographically in animals. Here we study how oxidative stress varies from lowlands (300–700 m asl) to highlands (2200–2500 m asl) in the lizard *Psammodromus algirus*. To accomplish this, antioxidant enzymatic activity (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, DT-diaphorase) and lipid peroxidation were assayed in tissue samples from the lizards' tail. Lipid peroxidation was higher in individuals from lowlands than from highlands, indicating higher oxidative stress in lowland lizards. These results suggest that environmental conditions are less oxidant at high elevations with respect to low ones. Therefore, our study shows that oxidative stress varies geographically, which should have important consequences for our understanding of geographic variation in physiology and life-history of organisms.

1. Introduction

Oxidative stress, the unbalance between the production of pro-oxidant substances and antioxidant defences (Halliwell, 2007), is considered one of the most important ecological and evolutionary forces (von Schantz et al., 1999; Costantini, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009; Costantini et al., 2010; Metcalfe and Alonso-Alvarez, 2010). Aerobic metabolism implies the production of pro-oxidant substances (reactive oxygen/nitrogen species, RONS; Finkel and Holbrook, 2000), which may react with molecular components of the cell such as lipids, proteins and nucleid acids, producing damages in cells' machinery (Halliwell and Gutteridge, 1995; Sies, 1997; Halliwell, 2007). Organisms are protected against oxidative damage by enzymatic and non-enzymatic antioxidant defences, which work to maintain RONS levels at equilibrium and minimize RONS damages in the organism (Sies, 1997; Finkel and Holbrook, 2000; Blokhina et al., 2003). Production and maintenance of antioxidants implies energy and resources consumption and therefore it is costly (Halliwell, 2007). When this balance is lost and antioxidant mechanisms cannot face RONS, oxidative stress occurs in cells (Sies, 1997; Jones, 2008).

In the wild, oxidative stress is induced by a wide range of environmental factors including changes in oxygen availability (Storey, 1996; Hermes-Lima and Zenteno-Savín, 2002; Buttemer et al., 2010), high or low temperatures (Hermes-Lima and Storey, 1993; Voituron et al., 2006), contaminants (Regoli, 2000; Prevodnik et al., 2007; Labrada-Martagón et al., 2011), and ultraviolet (UV) radiation (Dahms et al., 2011). These environmental factors vary geographically, and thus levels of oxidative stress and antioxidant defences should vary along a cline of these environmental factors. Nevertheless, there is a lack of studies examining geographic variation in oxidative stress in animals (Prevodnik et al., 2007; Costantini et al., 2010).

Sunlight radiation, whose UV-B wavelength component is the most harmful (Dahms et al., 2011), is an important causes of oxidative stress (Chang and Zheng, 2003; Chuang and Chen, 2013). UV-radiation initiates a series of redox reactions ending in free radical formation and leading to oxidative stress in cells (Dahms and Lee, 2010). Moreover, reduction of O_2 by photolytic reactions results in negative effects on oxidative balance, increasing oxygen radicals and producing lipid peroxidation as well as changes in antioxidant enzyme activities (Dahms and Lee, 2010). Damages produced by UV-radiation negatively affect organisms' fitness, by reducing sperm motility, hatching success and growth rates, as well as by increasing embryo malformation and

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Abbreviations: BCI, Body Condition Index; CAT, catalase; DTD, DT-diaphorase; MDA, malondialdehyde; m asl, metres above sea level; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione transferase; DCPIP, 2,6-dichlorophenol indophenol; RONS, reactive oxygen/nitrogen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reacting substance; UV-radiation, Ultraviolet radiation.

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