Differences Physiological preferred temperatures and evaporative water loss rates in two sympatric lacertid species

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Abstract
Sister species living in sympatry offer the opportunity to study the degree of divergence in their ecological, physiological and life-history traits. It has been hypothesized that closely related species with overlapping distribution should differ in their niche to reduce competition for resources. Furthermore, the investigation of sympatric species may shed light on how they may coexist without outcompeting each other. In the present study, we assess the degree of physiological divergence in two sympatric lacertid lizards, Podarcis bocagei and Podarcis guadarramae lusitanicus. These species share a Pliocene ancestry and overlap at a both geographical and ecological scale. We assessed their thermal preferences and water loss rates, two physiological traits considered stable across congeneric species. We found that the two species differ in both traits, with P. bocagei selecting higher temperature than P. g. lusitanicus and losing more water than the latter at and above its preferred temperature. The results also showed that for both species body size has a relevant impact on thermal and hydric traits, with bigger individuals losing proportionally less water and selecting higher temperatures. These results, combined with previous evidence, suggest that physiological mechanisms, ecological preferences and morphology probably allow these two species to overlap in their distribution while selecting different microhabitats and thus decreasing possible competition between them.

Keywords: Lacertidae, Podarcis, Sister species, Thermal ecology, Water balance, Preferred temperature

1. Introduction
Closely related species with sympatric distribution offer the opportunity to study the extent of divergence in their ecology, physiology and life-history traits (Connell, 1983). It has been hypothesized that closely related species belonging to the same ecological guild and living in sympatry should differ to reduce competition (Schulter, 1994). Alternatively, specific combinations, or assemblages of species are expected to exclude one another (Diamond, 1975).
Patterns of divergence in sympatric species are common and encompass a large variety of taxa. For example, interspecific competition and competitive exclusion appear to be relevant in shaping ant communities (Andersen and Patel, 1994), and niche partitioning might be a powerful force segregating fish species in terms of ecological and morphological space (Wikramanayake, 1990). In amphibians, the degree of character displacement can be associated with the intensity of competition between tadpoles of closely related species sharing the same ponds (Pfennig and Murphy, 2002). Depending on the case, either genetic or non-genetic changes can lead to reduced competition through niche shift associated with phenotypic plasticity or character displacement (Pfennig and Murphy, 2002; Price et al., 2003).

Lizards represent an excellent model to study adaptation, competition, niche shift and habitat segregation. For example, Anolis lizards from the Caribbean islands have been intensively investigated in this regard on each island, either one or more Anolis species are present, with different species assemblages on each island (Roughgarden, 1995). If several species live on the same island, they segregate into different ecomorphs that evolved several times independently (Losos, 1990). It has also been shown that sympatric lizards differ in scale morphology, likely the result of adaptation to different climatic niches (Wegener et al., 2014). Thus, in Anolis lizards, interspecific competition has driven the evolution of morphology, behavior, and physiology, shaping the species composition on each island to reduce niche overlap among species (Losos, 1994).

Lizards are also excellent model organisms to investigate thermal adaptation, with seminal studies dating back to the first decades of the previous century (Bogert, 1949). An overwhelming amount of evidence has been collected regarding the thermal ecology of many species, as well as the relationship between environmental temperature, body temperature and fitness (Cossins and Bowler, 1987; Angilletta, 2009). Ectotherms rely on external sources of heat to raise their body temperature and many of them actively thermoregulate (Angilletta, 2009). One of the most important themes in thermal biology is related to the concept of the preferred temperature (Tpref) that represents a range of temperatures, usually narrow, within which most physiological functions are performed nearly at their maximum (Huey and Stevenson, 1979). For lizards, it has been hypothesized that Tpref should be either an evolutionary labile or static trait, depending on the view and the history of the considered group. The labile hypothesis focuses more on local adaptation, while the static one stresses the evolutionary rigidity of Tpref, considering it an umbrella parameter for many physiological functions (Hertz et al., 1983). On the other hand, thermal ecology is strongly intertwined with water balance. For instance, it has been shown that dehydrated lizards tend to prefer lower body temperature (Crowley, 1987). However, for a long time most studies on lizards focused solely on the thermal aspect of their ecology.

The current state of knowledge on water balance, evaporative water loss (EWL) rates and the effect of temperature on the hydric status of lizards is poor, particularly if compared with the amount of studies focused
solely on lizards’ thermal biology. For example, in desert-dwelling lizards, differences in EWL were shown to be attributable to habitat selection (Dmi’el, 2001), while in Anolis lizards it has been found that species living in drier and warmer climates tend to have fewer dorsal scales, probably to reduce cutaneous water loss (Wegener et al., 2014). However, there is surprisingly little evidence regarding physiological differences among sympatric lizards, especially from temperate climates. García-Muñoz and Carretero (2013), for example, showed that Algyroides marchi and Podarcis hispanica select similar temperatures but are characterized by different water loss rates, possibly associated with different microhabitat selection. To our knowledge, EWL has never been studied at various temperatures for sympatric sister taxa of lizards.

Podarcis wall lizards have been used as model species to investigate many aspects of reptile biology, from evolution (Harris and Arnold, 1999; Herrel et al., 2008) to morphology and life-history traits (Bauwens and Diaz-Uriarte, 1997; Kaliontzopoulou et al., 2012). Conversely, the thermal and hydric ecology of Podarcis species has been investigated in some species but little is known on the relation between thermal ecology and water balance, with studies usually focusing on the thermal preferences of a single species (Ji and Braña, 1999). Regarding EWL rates, information is even more scarce, with data available only for a few species, showing that, in general, either congeneric or heterogeneric species living in sympatry differ in their relative EWL (García-Muñoz and Carretero, 2013; Osojnik et al., 2013; Carneiro et al., 2015). Podarcis species of the Iberomaghrebian clade are an intriguing case to study ecophysiology, due to their high level of genetic diversity coupled with a complex pattern of evolutionary and biogeographical history (Carretero, 2008; Kaliontzopoulou et al., 2011).

In the present study, we examined these two ecophysiological traits, Tpref and EWL, using two sister taxa of lacertid lizards, Podarcis bocagei and P. guadarramae lusitanicus (sensu Geniez et al., 2014). These two species were once included into the Podarcis hispanica species complex, from which several lineages have been elevated to species or subspecies level (Harris and Sá-Sousa, 2002). Most of the forms in the complex show an allopatric or parapatric distribution, while P. bocagei and P. g. lusitanicus are largely sympatric in their distribution range in north-western Iberian Peninsula (Kaliontzopoulou et al., 2011; Geniez et al., 2014). These two species are characterized by one of the closest genetic distances among the recognized species pairs within the complex (Kaliontzopoulou et al., 2011). In contrast with this phylogenetic proximity, recent studies highlighted how these two closely related species differ substantially in morphological traits and microhabitat use (Kaliontzopoulou et al., 2012; Gomes et al., 2016). In particular, P. bocagei is more robust and seems associated with a more ground-dwelling life style in comparison with P. g. lusitanicus, which has a more compressed head, associated with a more saxicolous life-style. Furthermore, the former is restricted to Atlantic climate, while the latter occurs both in Atlantic and Mediterranean areas (Loureiro et al., 2008). This mixture of phylogenetic affinity, coupled with overlap in their distribution and divergence in microhabitat use and
morphology, makes this two-species system a good candidate to investigate the existence of a divergence in physiological traits directly linked to thermal and hydric biology.

In this context, we expected that (i) if the labile view of Tpref is found in the study system, the two species should differ in thermal preferences due to current or past adaptive processes, even though they are sister taxa; (ii) if instead one of the two species selects for higher temperatures, it should also lose more water, assuming a trade-off between thermal preference and the rate of evaporative water loss; (iii) considering species distribution, climate affinity, and microhabitat use, P. g. lusitanicus would be more resistant to water loss than P. bocagei.

2. Methods
2.1. Individual sampling and housing
Lizards were collected by noosing near Moledo, a coastal locality in northern Portugal (41°50 N, 8°52 W) where the two species live in syntopy. To avoid the effects of reproduction, body condition and ontogeny on Tpref and EWL, only males with intact tails were captured (Carretero et al., 2005). Samplings took place during August and September 2015. To avoid recaptures across sampling sessions, we applied photo-identification using I3S Classic (Van Tienhoven et al., 2007; Sacchi et al., 2016; download at http://www.reijns.com/i3s/). Lizards were brought to the lab where their snout-vent length (SVL) and head height (HH) were measured to the nearest 0.01 mm using a digital calliper and their body mass was measured to the nearest 0.0001 g using a precision balance (Sartorius M-Pact AX224; Sartorius AG, Goettingen, Germany). All measurements were taken by the same person (MS). Lizards were housed in individual terraria (40 cm × 25 cm × 25 cm) for two days before experimentation. During this period, lizards had ad libitum access to water and shelter, but no food was given to prevent digestion from affecting Tpref and EWL (Van Damme et al., 1991). During captivity, lizards were exposed to a natural light cycle regime with daytime air temperature set at 27 °C, and nighttime air temperature set at 20 °C. By the end of the experiment, all lizards were released at the point of capture.

2.2. Preferred temperature
We individually exposed 37 P. bocagei and 32 P. g. lusitanicus to thermal gradients (± 20–50 °C) by putting them in 100 cm × 30 cm × 40 cm acrylic terraria provided with a 150 W infrared lamp at one end. A thin layer of sand (~5 mm) acted as a substrate. Room temperature was kept at 20 °C by an air conditioning system to guarantee the establishment of the gradient inside the terraria. Individuals were granted 24 h of habituation to experimental conditions before the experiment started. Then, every hour, for nine repetitions (10:30-18:30), we measured cloacal temperature with a contact thermometer (Hibok 18, precision: 0.1 °C) fitted with a k-type thermocouple probe. We limited each contact with lizards to 10–15 s to prevent them from changing body
temperature during the readings. This procedure might cause some stress to the lizards, but we believe it represented the best option, considering that the use of infrared thermometers or trailing thermocouples attached to the cloaca is not feasible on small species (Trullas et al., 2007; Carretero, 2012). To prevent the lizards from losing water during the experiment, possibly altering the pattern of Tpref (Crowley, 1987), a cup of water was provided in the middle of each gradient. By the end of the experiment, lizards were returned to their original terraria. We calculated the mean temperature (± standard deviation), the temperature distribution and the 50% central range as a proxy for thermal preference (Hertz et al., 1993). A two sample t-test was used to compare thermal preferences of the two species, while F-statistics from linear models were used to assess the effect of SVL and body mass on thermal preferences

2.3. Evaporative water loss rate
The same individuals tested for Tpref were tested for EWL rates after one day of recovery with water available. Three temperatures were set for the experiment: 27, 32 and 37 °C. These temperatures were chosen after a small sample of P. bocagei (10 adult males, different from the ones tested here) preferred temperatures of approximately 32 °C (using the same procedure as described in Section 2.2). We thus used this value as the reference temperature and then added and subtracted 5 °C. Notice that lizards were kept at 27 °C during captivity. This temperature thus should provoke less pronounced hydric stress, while 32 and 37 °C were expected to elicit increasingly higher water loss. We randomized the order at which every individual was tested with respect to temperature treatment and allowed a one-day recovery after each test. With this design, the lizards’ body masses did not statistically change from the beginning to the end of the experiment (F2,204 = 0.216, P > 0.80).

Before the EWL test, lizards were weighed to the nearest 0.0001 g and then put into individual PVC cylindrical boxes (10 cm × 9 cm) with ventilation holes on the top and bottom sides. Groups of 6 individual boxes were placed into a sealed incubator (Binder KB 53, precision at 25 and 40 °C: ± 0.2 and 0.3 °C, respectively) with 125 g of silica gel. This amount of silica gel provided stable and dry conditions (< 15% RH) inside the incubator throughout the experiment. Every hour, for 9 hours, we removed and weighed each individual with the same balance.

Faeces account for a small, but possibly relevant portion of lizards’ body mass. Following earlier study methodologies (Carneiro et al., 2015), we did not remove faeces when we found them in the plastic boxes. Even though some lizard species are able to survive upon losing 30% of their body mass in water (Hertz, 1980), our aim was to test for differences between species and not for overall resistance to water loss. We thus ceased
the experiment if an individual exceeded 5% of body mass loss (Table S1 in the supplementary online Appendix).

2.4. EWL data analysis
Given the unbalanced and time-dependent structure of the data and the possible subject-specific effects, we fitted a linear mixed-effects model (Pinheiro and Bates, 2000). Water loss was the dependent variable and three variables and their interactions were the predictors. Considering that the species differed in body mass (see Section 3.1), and were thus likely to lose water in function of their body mass, we used percentage of water loss instead of total water loss. These two variables are highly correlated (Pearson’s correlation coefficient: rp = 0.93) and by using percentage we could directly test for species divergence in water loss. The set of explanatory variables was temperature × species × time (where temperature was either 27, 32 or 37 °C; this set was meant to mimic a realistic thermal scenario that lizards could face in nature). Considering that water loss could vary in a non-linear way through time, we also fit the same model with a quadratic term on the time factor. Body length and body mass were added as covariates to account for potential effects of body size on EWL. Individuals were treated as random effects. The starting model was reduced following Zuur et al. (2009) and normality of the model's residuals was checked graphically (Pinheiro and Bates, 2000).

The statistical analysis was performed in R version 3.3.2 (R Core Team, 2016) using the nlme package (Pinheiro et al., 2017) for the linear mixed models. Figures were produced using the package ggplot2 (Wickham, 2009).

3. Results
3.1. Species morphology
The two species did not differ significantly in SVL (P. bocagei: 54.51 ± 5.35 mm, P. g. lusitanicus: 53.44 ± 4.52 mm; two sample t-test = 1.01, P = 0.31, df = 67), but they were statistically different in their HH (P. bocagei: 5.89 ± 0.59 mm, P. g. lusitanicus: 5.30 ± 0.59 mm; two sample t-test = 4.42, P = 0.0001, df = 67) and body mass (P. bocagei: 3.75 ± 0.78 g, P. g. lusitanicus: 3.29 ± 0.73 g; two sample t-test = 2.55, P = 0.013, df = 66.6). This translates in adult males of P. bocagei being more robust, heavier and with higher heads than P. g. lusitanicus, while the overall length of the body is similar for the two species (Fig. 1).
3.2. Preferred temperature

The two species selected statistically different temperatures (two sample t-test = 2.92, P < 0.01, df = 604). P. bocagei preferred warmer temperatures in comparison with P. g. lusitanicus (31.1 ± 0.13 °C and 30.6 ± 0.11 °C, respectively). Mean difference between the two species was 0.50 °C and it was also reflected in the temperature distributions (Fig. 2a and Fig. S1 in the supplementary online Appendix). Such a small absolute difference is associated with a small effect size (Cohen’s d = 0.23; Cohen, 1992). Tpref does not appear to show a clear time-trend pattern (Fig. S2). Following Hertz et al. (1993), the ranges of the preferred temperature (50% central distribution of the data) were very similar between species: 29.8—32.6 °C for P. bocagei and 29.4—32.0 °C for P. g. lusitanicus. For both species, Tpref was positively correlated with SVL and body mass, with bigger and heavier individuals selecting higher temperatures (SVL: F1,591 = 18.97, P < 0.0001; Body mass: F1,591 = 40.58, P < 0.0001; Fig. 2b).
Fig. 2. Preferred temperature of Podarcis bocagei and P. g. lusitanicus. Top: boxplot of the temperature distributions for both species. See the text for mean and 50% values. Bottom: linear regression of temperature on the body length. Shaded areas represent the 95% confidence interval.

3.3. Evaporative water loss rate

The quadratic model described the data better than the linear one (Log-Likelihood-χ² (LR-χ²) = 162, d.f. = 22/14, P < 0.0001). This suggests that the EWL trend was non-linear with respect to time (Fig. 3A). After reduction, the best model included the two covariates (SVL and body mass) and the three interaction terms species × temperature, time × temperature and time × species.

Fig. 3
Fig. 3. (A) Evaporative water loss rate (EWL) as percentage over time, for both species at all temperature treatments. Dots represent measurement data with standard errors, lines represent the fit of the best linear mixed model (see the text for parameterization). (B) EWL as a function of body length for both species. (C) EWL at the three temperature treatments for both species. (D) EWL as a function of time separated for each temperature treatment.

Water loss was negatively correlated with SVL for both species ($\chi^2 = 27.57$, d.f. = 22/17, $P < 0.0001$; Fig. 3B). Similarly, increasing body mass led to reducing proportionally the loss of water ($\chi^2 = 22.22$, d.f. = 22/17, $P < 0.0001$). Thus, P. bocagei appeared to lose more water (in terms of absolute values) if compared with P. g. lusitanicus for small individuals, while larger individuals lost proportionally less water.

The significance of the interaction term species × temperature ($\chi^2 = 17.71$, d.f. = 22/16, $P < 0.0001$) reflects the fact that, depending on the treatment, the two species lost different amounts of water. In particular, by the end of the experiment, lizards diverged clearly at both 32 and 37 °C, with P. bocagei losing more water than P. g. lusitanicus (Fig. 3C). The pattern is even clearer if loss in terms of absolute values is considered (Fig. S3). Table 1 shows average amounts of water loss for each temperature treatment, in terms of both absolute values and percentages. The interaction term time × temperature reflects the fact that different temperatures imposed
increasing rates of water loss (Fig. 3D). Finally, the interaction term time × species indicates that the species differed in the overall loss of water across time (Fig. 3A), regardless of the treatment.

4. Discussion

The results of this study suggest that P. bocagei and P. g. lusitanicus differ in thermal and hydric physiology. Regarding the thermal preferences, we detected a significant difference, with P. bocagei selecting higher temperatures than P. g. lusitanicus. In general, higher body temperatures are beneficial to lizards (Angilletta et al., 2010), allowing them to increase their fitness in terms of gametes production (Licht, 1971), digestion efficiency (Harlow et al., 1976), predation avoidance (Christian and Tracy, 1981) and locomotion (Bauwens et al., 1995), among others. Thermal preference has been assumed to be either an evolutionarily stable trait, or a labile one, depending on the evidence at hand (Hertz et al., 1983). For example, limited difference has been seen in the thermal preferences of 13 lacertid species (Bauwens et al., 1995). On the other hand, other groups of lizards showed variation in thermal preferences depending on climate (Rodríguez-Serrano et al., 2009) and habitat (Hertz et al., 2013).

The results of our thermal experiment show a relatively small divergence in thermal preference of P. bocagei and P. g. lusitanicus, considering that they are sister taxa with a relatively recent evolutionary divergence (Pinho et al., 2008; Kaliontzopoulou et al., 2011) and with large geographic overlap (Geniez et al., 2014). In the present study, the absolute difference of Tpref between the species was indeed relatively small in terms of absolute values. It is thus likely that from a thermal ecology perspective, the investigated species are an example of evolutionary rigidity for this trait (Hertz et al., 1983). Nonetheless, the differences we detected could still bear an ecological meaning, considering that niche divergence can occur quickly in nature (Pearman et al., 2008), that thermal preference is possibly associated with genetic variation (Good, 1993), and that selective pressure to diverge is expected in closely related species living in sympatry (Schulter, 1994). For instance, we found that P. bocagei selected higher temperatures than P. g. lusitanicus. However, the latter is commonly associated with drier and warmer micro-climates, and this pattern seems to hold also at the micro-habitat selection level (Gomes et al., 2016). Further, the geographic distribution of P. bocagei is restricted to Atlantic areas (cooler and wetter) while P. g. lusitanicus occurs in both Atlantic and Mediterranean areas (warmer and drier; Loureiro et al., 2008). Thus, the biological consequences of the divergence in Tpref should be investigated under field conditions and using multiple populations, to understand how thermal preferences relate with other ecological factors and species distribution.
The results of the EWL experiment are clear and could offer an ecological and a physiological framework to understand the direction of the divergence in Tpref. For both species, bigger individuals lost less water than smaller ones, suggesting that surface-to-volume ratio is an important factor that contributes to EWL rate even for small body size lizard species (Hertz, 1980; Neilson, 2002). In absolute values, P. g. lusitanicus lost less water than P. bocagei at every temperature, and the pattern is similar if percentages are considered: increasingly higher temperatures amplify the difference between the two species. In terms of proximate causes, within-genus divergence in EWL rate can be attributed to peripheral vasomotor changes, involving vasodilation and vasoconstriction of the skin blood vessels (Eynan and Dmi’el, 1993).

It is extremely interesting to note that P. g. lusitanicus lost less water in proportion to P. bocagei, despite being more flattened and lighter, thus having a higher surface-to-volume ratio than the latter (Fig. 1). These observations, coupled with the fact that the experimental setting prevented individuals from behavioral adjustment, support the hypothesis that the difference in EWL rate between these species can be attributed to physiological mechanisms, allowing P. g. lusitanicus to lose less water than P. bocagei, despite its higher surface/volume ratio.

Anatomical and physiological differences could not only explain the divergence between species, but also account for the habituation curve in response to temperature. The curvilinear time-trend in EWL appears to be determined by a rapid decline in hour-by-hour loss of water (Fig. 3A). This pattern likely reflects a stress-induced physiological response that ultimately allowed lizards to reduce their water loss through time by habituation to the experimental temperature (Eynan and Dmi’el, 1993). Alternatively, by losing water through time, lizards had progressively less water to lose in relation to their body mass. However, lizards can be quite resistant to water loss. Some tropical species can tolerate up to 30% of body mass loss (Hertz, 1980) and some desert-dwelling species can resist up to 50% of loss in body mass (Munsey, 1972). In the present study, we limited maximum water loss at 5% of body mass. Podarcis species live in temperate, Mediterranean, and continental climate areas (Sindaco and Jeremčenko, 2008), and the upper limit of 37 °C (in terms of perceived temperature) that we set was meant to mimic a realistic thermal scenario that these species could face when active during the hottest months of the year. Moreover, our experiment was designed to mimic a diel cycle during which individuals had little access to water, with the aim of testing physiological differences between sister species living in the same area. It is thus possible that Podarcis species could tolerate a much higher amount of water loss (Munsey, 1972; Hertz, 1980).

Finally, the results of this study highlight that lizards lose water in function of temperature and that such change is not linear, as reflected by the divergence in the slopes of water loss across time. Therefore, studies testing EWL in lizards should carefully select the temperature at which such assessment takes place. A very low
temperature, like 27 °C used here (or lower), could fail to detect patterns of interest between species. For example, Carneiro et al. (2015) did not detect differences between males of two sympatric species of Podarcis lizards that were tested for EWL at ∼24 °C. This temperature is very unlikely to be biologically meaningful for these species, as the reported Tpref for P. muralis is 31.76 °C, and for P. liolepis it is 31.97 °C, as assessed in the same paper of Carneiro et al. (2015). We thus recommend assessing thermal preference before testing for water loss and then using Tpref as a reference temperature for EWL assessment. This protocol offers a meaningful estimate of the amount of water that a lizard is losing at its preferred temperature and allows for a realistic comparison between species. Alternatively, if the main aim is to understand how EWL changes with temperature, it might be useful to measure EWL at various temperatures. Such a protocol would resemble the common one used to estimate performance curves (Angilletta, 2006).

Concluding, our experiment highlighted that sister taxa species of lizards living in sympatry might differ in both thermal and hydric physiology. In the present case, P. g. lusitanicus selected lower temperatures than P. bocagei, and lost less water than the latter, especially at its preferred and higher temperatures. Previous findings suggest that, within a lizard genus, physiological and anatomical differences, in terms of peripheral blood flow and skin properties, could offer a proximate explanation for the observed results. In terms of causal factors, evolutionary divergence in sympatric sister taxa in terms of micro-habitat and micro-climate selection could have boosted such physiological differences.

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