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Living at the edge: lower success of eggs and hatchlings at lower elevation may shape range limits in an alpine lizard

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Studies on range limits clarify the factors involved in the extent of species occurrence and shed light on the limits to adaptation. We studied the effects of elevational variation on the thermal dependence of fitness-related traits (incubation time, hatching rate, and survivorship, size, and condition of hatchlings) to assess the role of incubation requirements in distribution range limits of the alpine endemic *Iberolacerta cyreni*. We captured gravid females from two core (summit) and two marginal (low-elevation edge) populations, we incubated their eggs at three temperatures (22, 26, and 30 °C), and we monitored phenotypic effects. Viability of eggs and hatchlings decreased, independently of elevation, as incubation temperature increased. Hatching success and embryo survivorship were lower for clutches from low-elevation areas than for those from mountain summits, showing that lizards face difficulties thriving at the low-elevation edge of their range. Such difficulties were partly counterbalanced by faster postnatal growth at lower elevations, leading to increased adult size and higher fecundity. High incubation temperature had detrimental effects also at low-elevation areas, and no elevational variation in the thermal dependence of hatchling traits was detected. We suggest that temperature effects on egg development and the lack of selective pressures strong enough to foster local adaptation at marginal areas, combined with extended egg retention, may contribute to shape the range limits of these alpine oviparous reptiles. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **00**, 000–000.

KEYWORDS: alpine lizard – elevation – growth rate – hatching success – incubation temperature – local adaptation – range limits.

INTRODUCTION

Geographic ranges, conceived as the spatial projection of the realized ecological niche (Hutchinson, 1957), reflect the ability of species to thrive under a given set of environmental conditions (Kearney & Porter, 2004). As a consequence, the study of their limits may help to disentangle the factors involved in the extent of species occurrence and it provides a useful framework for analysing the limits to adaptation at the edge of the range (Holt & Keitt, 2005). In fact, the concept of limiting factors (temperature, pH, salinity, etc.) or physical factors that restrict the occurrence of organisms to particular habitats, can be traced back to the early days of ecology, with an emphasis on the interactions between physiology and the physical environment (Huey, 1991). Nevertheless, the task is challenging, not only because of the complexity of the physiological, ecological, and evolutionary interactions involved, but also because these

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interactions may be subject to unknown rates of adjustment under the current scenario of global change (Quintero & Wiens, 2013).

Empirical studies comparing core and marginal populations within the distribution range provide an adequate framework with which to tackle relevant questions about range limits (Díaz *et al.*, 2007; Kawecki, 2008). When species occur along ecological gradients, studying adaptation at range margins can provide insight into the plasticity of their responses to environmental variation (Byars, Papst & Hoffmann, 2007; Vergeer & Kuni, 2013). Such plasticity may determine if populations are able to thrive under the new conditions at the edge of their range, or if, on the contrary, they encounter a barrier for their survival (Angert & Schemske, 2005).

Steep environmental gradients, such as those found in mountains, serve as excellent testing grounds for adaptation to novel environments. Moreover, alpine species show clearly restricted distribution patterns, which could reflect their specialization to cold habitats and their inability to cope with changes along the mountain slope (Merrill *et al.*, 2008). Therefore, examining their responses to factors that are strongly correlated with elevation can help to identify environmental thresholds for their occurrence. Among these factors, temperature turns out to be particularly important (Körner, 2007), not only because its major effects on organisms are pervasive but also because it can reveal the potential responses of species to global warming. If species are not able to adjust their phenotypes to temperature changes either by developmental plasticity or by genetic differentiation, they may experience range contractions or even extinctions (Williams *et al.*, 2008).

Alpine lizards are models particularly well suited to test for adaptation to new thermal conditions because, on the one hand, they are strongly dependent on temperature but, on the other, they face steep changes in the thermal environment, which may require behavioural, ecological, and/or physiological adjustments (Navas, 2003; Espinoza, Wiens & Tracy, 2004). Embryonic stages of oviparous species may be particularly responsive to thermal variation because eggs are unable to thermoregulate behaviourally. Therefore, lizards are expected to fine-tune the thermal dependence of life-history traits along the mountain slope in order to adjust hatching dates and to enhance offspring survival (Braña & Ji, 2000; Shine & Olsson, 2003). In fact, recent studies suggest a link between thermal incubation requirements and elevational range limits (Monasterio *et al.*, 2011, 2013). However, there is little evidence of geographical variation in the life histories of alpine or high-elevation lizards, although such information is needed to provide a better understanding of the physiological and/or ecological mechanisms shaping their distribution range limits.

Our goal here was to study elevational variation of reproductive traits (female body size, clutch size, mean egg mass, clutch mass, incubation time, hatchling size, and postnatal growth rates of juveniles) in the Iberian rock lizard, *Iberolacerta cyreni,* and to discuss how our findings might help in understanding its alpine confinement. For that purpose, we compared two populations (one at a core, high-elevation site, and the other at a marginal, low-elevation site) in each of the two mountain ranges that encompass most of its world distribution. We have previously shown that increased incubation temperature (32 °C) has detrimental effects on the reproduction of this species by reducing both hatching success and the number of viable hatchlings (Monasterio *et al.*, 2011). Moreover, we found that areas at the lower limit of the distribution range may represent a barrier to the expansion of *I. cyreni* as a result of the higher soil temperatures in such areas (Monasterio *et al.*, 2011). However, there are no data on how populations of this species adjust, or do not adjust, their reproductive strategy to thermal changes along mountain slopes. Thus therefore, we addressed the following questions: (1) Are high incubation temperatures equally detrimental for populations near mountain summits and for populations close to the lower edge of the elevational range? Or are there any elevational differences, either in prenatal or postnatal developmental stages, in the thermal responses of eggs or juveniles?; and (2) Can these results shed light on the inability of *I. cyreni* to adapt to novel environmental conditions along the elevational gradient, and thereby explain its disappearance at the lower edge of its distribution range?

MATERIAL AND METHODS

STUDY SPECIES AND STUDY AREAS

Rock lizards [*I. cyreni*; adult snout vent length (SVL) of 73–80 mm] are heliothermic, actively foraging, diurnal lacertid lizards, which are endemic to the Iberian Sistema Central. The species presents a restricted distribution range because it is only found above 1600 m, preferably in rocky outcrops and mixed-shrub formations (Monasterio, Salvador & Díaz, 2010a). The Sistema Central presents contrasting seasonal conditions, with cold, wet winters and short, dry summers. Its mountain bases (1200–1700 m a.s.l.) are covered with deciduous Pyrenean oak (*Quercus pyrenaica)* forests, which in some areas (particularly in the Sierra de Guadarrama) are progressively substituted by Scots pine (*Pinus sylvestris*) forests at higher altitudes. These forested areas, which can spread from 1500 to 2100 m a.s.l., gradually become less dense until vegetation is dominated by a mosaic of dense mixed-shrub formations (of perennial *Juniperus communis* and *Cytisus oromediterraneus*) interspersed with small meadows of *Festuca* and other grasses. These alpine areas above the tree line (1700–2300 m a.s.l.) are also characterized by extensive patches of large granite rocks and scree interspersed among shrub formations.

Within the Sistema Central, *I. cyreni* is found in several mountain ranges (Guadarrama, Gredos, Béjar, La Serrota, and La Paramera), but most of its distribution range corresponds to the ranges of Guadarrama and Gredos. For that reason, we chose two study areas in each of these two mountain ranges (Fig. 1): the first at high elevation (2100–2300 m a.s.l.: Bola del Mundo in Guadarrama and Circo in Gredos) and the second at low elevation (1800–1850 m a.s.l.: Puerto de la Morcuera in Guadarrama and Plataforma in Gredos). This design included an explicit biogeograpical scenario given that high-elevation sites correspond to populations that are in the centre of their distribution range (core areas, at the mountain summits), whereas low-elevation sites correspond to populations located at the lower limit of their distribution range. This is supported by previous analyses showing that altitude is the independent variable that most accurately predicts variations in the abundance of *I. cyreni*. Thus, we selected our low-altitude study sites on the basis of a previously published model predicting that lizard populations should disappear below 1650 m a.s.l. (Monasterio *et al.*, 2010a); between this elevation and the 1800-m a.s.l. altitude of our study sites, the species is too scarce to allow capture of the minimum number of females required to attain a reliable sample size. It is also important to note that the two sampled areas in each mountain range are connected by intermediate populations, so that the distribution range is not confounded with meta-population structure (as if each mountain was a meta-population); instead, each mountain has its own core and edge area, and our design provides appropriate replicates of edge effects along altitudinal gradients.

COLLECTION AND HUSBANDRY OF ADULTS, EGGS, AND JUVENILES

Between 7 June 2012 (the date of first captures at the Guadarrama low-elevation site) and 21 June 2012 (the date of last captures at the Gredos high-elevation site), we noosed 48 gravid females (with SVL of 72.7 ± 0.7 mm and body mass of 8.4 ± 0.2 g) that were transported to our laboratory in the Department of Zoology (Universidad Complutense de Madrid) on the same day of capture. Mean laying dates ranged between 27 June 2012 (Guadarrama; low-elevation site) and 8 July 2012 (Gredos; high-elevation site).

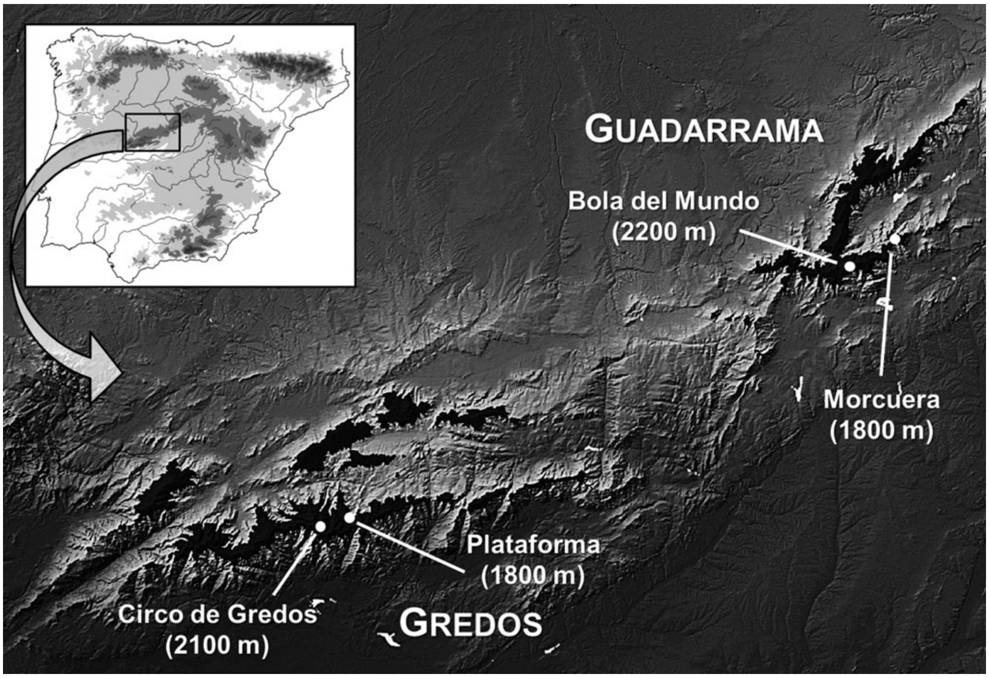

Figure 1. Distribution map of *Iberolacerta cyreni* in the Iberian Peninsula (shaded areas in the enlarged panel of the Sistema Central mountain range) showing the location of the study sites.

Thus, we are confident that our results should not be confounded by among-population differences in the time of capture of gravid females or time in captivity before oviposition. It should also be noted that although gravid females readily lay eggs in captivity, their reproductive output still depends on the environmental conditions they experienced in the wild, especially in the case of species with long-lasting intrauterine embryogenesis, such as *I. cyreni* (Braña, Bea & Arrayago, 1991). This is because in lizards the hormonal and physiological coupling among clutch size, egg size, and clutch mass occur during early vitellogenesis (Sinervo & Licht, 1991).

For the analysis of the relationships between female body size, geographical area (Gredos vs. Guadarrama) and altitude, we increased sample size to $N = 148$ by adding records from previous studies (Monasterio *et al.*, 2010a, 2011) from several other localities in both mountain belts that could again be classified as high-altitude sites (core alpine areas located at mountain summits) vs. marginal sites (lower altitude areas beyond which *I. cyreni* is absent).

Lizards were housed in white opaque wall terraria (40 cm × 60 cm × 30 cm; two females per cage) which were covered with a green net (0.5-cm mesh) that prevented escape. The mesh provided a shrubby-like shelter whilst still allowing daylight to enter the cages. Terraria were filled with moistened earth averaging 10 cm in depth, covered by a leaf litter layer. A 60-W lamp suspended over one end of the cage created a photothermal gradient (*c.* 25–50 °C), allowing thermoregulation within the preferred temperature range (Bauwens *et al.*, 1995). Ultraviolet (UV) light was also provided. An earthenware tile (*c.* 10 cm × 15 cm) and a thin section of fallen wood provided additional shade and shelter. Lizards were fed, daily, with crickets (*Acheta domesticus*) and mealworms (*Tenebrio mollitor*), dusted with a commercial vitamin and calcium supplement delivered according to the manufacturer's recommendations. Food and water were provided *ad libitum*.

Gravid females were monitored daily to detect changes in body shape or body mass that might indicate egg-laying. When it was clear that a female had laid, the eggs were searched for and located, removed from the cage, counted, weighed, and placed individually in separate, 150-mL plastic cups filled with *c.* 35 g of moistened vermiculite (10 g of vermiculite per 8 g of water, equivalent to −200 kPa). The eggs were completely covered by the vermiculite, and the jars were closed with a tightly fitting screw top to minimize evaporative water loss. Eggs of the same clutch were distributed evenly over three incubators (MMM Friocell, Germany) set at constant temperatures of 22, 26, and 30 °C. These values were chosen based on the results of a previous study (with eggs incubated at 24, 28, and 32 °C) in which high temperatures (32 °C) negatively affected the reproductive success of these lizards (Monasterio *et al.*, 2011). We thus lowered the incubation temperatures to decrease the negative effects but still allow the thermal effects on incubation to be detected. Searches for new hatchlings were conducted daily towards the end of the incubation period. Hatchlings were weighed, measured (SVL), and individually marked by toe clipping. The incubation period (in days) was calculated as the time elapsed between egg deposition and hatching. After females had laid their eggs, they were released at their site of capture.

Immediately after hatching, newborns were placed in individual plastic terraria (265 mm length × 162 mm width × 150 mm height) that offered rock and sand substrates. A 60-W lamp, 25 cm above the rock, acted as a heat source allowing lizards to bask, and UV light was also provided. As per adults, food and water were provided *ad libitum*. To monitor growth of juveniles, we measured (SVL to the nearest mm) and weighed (to the nearest mg) all lizards after 21 days of captivity, when the experiment was complete. The growth rates of juveniles were estimated both for size (SVL) and for body mass using the formula:

Growth rate (in day^{-1}) = Ln (measurement at an age of 21 days/measurement at hatching)/21.

These estimates reflect the proportionate increase in size or mass on a per-day basis (Sinervo, 1990). At the end of the experiment, juveniles were released at their mother's site of capture.

CHARACTERIZATION OF FIELD THERMAL ENVIRONMENTS

To characterize the thermal environments available at each study site, we registered soil temperatures using Thermochron Ibutton temperature loggers that were placed at 5 cm depth in soil under bare ground. We used four sampling points at each study site, separated by at least 100 m. Temperature was recorded every hour from 1 July to 13 September 2012 (75 days × 24 h × four loggers × four sites). It is important to note that we do not intend to provide an accurate description of temperatures available at nesting sites because very few clutches have been found in the field, usually under rocks, and as a consequence nesting sites remain basically unknown. Instead, our aim was to provide a coarse characterization of thermal variability between and within sites, and to compare the effects of several sources of spatio-temporal variation on the thermal environment.

STATISTICAL ANALYSES

We analyzed our data using the general linear models (GLM) and log-linear analysis modules of the statistical package Statistica 7.0. For log-linear analyses (used for testing the significance of two- and higher-order interactions among hatching success or embryo survival as the response variable, and area, altitude, and temperature as the design factors), we provide maximum likelihood ratio chi-square tests for the overall fit of the model and for the partial association of each effect included in the model; the partial association evaluates the significance of the respective effect by comparing the model that includes all effects (i.e. interactions) of the same order with the model without the respective effect.

We checked the assumptions of parametric tests (normality and homogeneity of variances) before using GLMs. In addition, the mixed GLM, used for testing the significance of clutch (random factor) and temperature, altitude, and area (fixed factors) on hatchling phenotypes, requires the random effects to be independent of the fixed effects (Searle, Casella & McCulloch, 1992) by constructing appropriate error terms. Statistica 7.0 uses Satterthwaite's method of denominator synthesis to find the linear combinations of sources of random variation that serve as error terms for each effect. Thus, the degrees of freedom for the denominator mean square can be fractional rather than integer values, meaning that fractions of sources of variation were used in synthesizing error terms for significance testing. We estimated body condition at hatching and at an age of 21 days using GLMs [analysis of covariance (ANCOVA)], with body mass as the dependent variable and SVL as the covariate. Data are reported as mean ± 1 SE.

RESULTS

BODY SIZE AND REPRODUCTIVE INVESTMENT OF FEMALES

Females differed in size between areas (Gredos vs. Guadarrama) and altitudes (mean SVL ± 1 SE and sample size: Gredos, core areas: 73.1 ± 0.7 mm, $N = 31$; Gredos, low-elevation edge of the range: 75.5 ± 1.0 mm, $N = 15$; Guadarrama, core areas: 69.7 ± 0.8 mm, $N = 45$; and Guadarrama, low-elevation edge of the range: 71.6 ± 0.9 mm, $N = 57$). Females from Gredos were larger than those from Guadarrama, and females from lower elevations were larger than females from higher elevations (two-way ANOVA: area $F_{1,144} = 13.13$, $P < 0.001$; altitude: $F_{1,144} = 4.54$, $P = 0.034$; area × altitude interaction: $F_{1,144} = 0.04$, $P = 0.844$).

Females from low elevations laid larger and heavier clutches than females from high elevations, whereas mean egg mass was larger for females from Guadarrama than for those from Gredos (Table 1); clutch mass was more dependent on clutch size ($\beta = 0.981$) than on mean egg mass ($\beta = 0.552$). However, altitudinal differences disappeared after controlling for female size (SVL), both for clutch size (ANCOVA; altitude: $F_{1,42} = 2.77$, $P = 0.103$; SVL: $F_{1,42} = 18.06$, $P < 0.001$) and for clutch mass (altitude: $F_{1,42} = 1.76$, $P = 0.192$; SVL: $F_{1,42} = 22.98$, $P < 0.001$), showing that the greater reproductive investment of females from low-elevation areas was mainly a consequence of their larger body size. Egg mass, however, was higher in Guadarrama (mean egg mass of 0.51 ± 0.1 g, vs. 0.47 ± 0.1 g for Gredos; see Table 1), even after controlling for the effects of SVL (area: $F_{1,42} = 5.66$, $P = 0.022$; SVL: $F_{1,42} = 0.40$, $P = 0.531$).

HATCHING SUCCESS

Overall, females laid 47 clutches, with a mean clutch size of 6.34 ± 0.21 eggs. We incubated a total of 298 eggs, from which we obtained 245 hatchlings (Table 2), although one of the hatchlings was found dead ($N = 244$ for subsequent analyses). A log-linear model with hatching success as the response variable, and area, altitude, and temperature as the design factors, provided a very high goodness-of-fit to the observed data (χ^2 for the overall model = 6.06, d.f. = 18, $P = 0.996$), and included both an interaction between area and hatching success (partial association: $\chi^2 = 16.22$, d.f. = 1, $P < 0.001$) and, more importantly, an interaction between elevation and hatching success (partial association: $\chi^2 = 11.76$, d.f. = 1, $P < 0.001$). The first interaction indicates that hatching success was higher for eggs from Gredos than for those from Guadarrama, whereas the second interaction shows that hatching success increased with elevation (Table 2). Temperature did not influence hatching success (partial association: $\chi^2 = 0.04$, d.f. = 2, $P = 0.981$). Interestingly, the interaction between area, altitude, and hatching success was close to zero (partial association: $P = 0.983$). We can therefore conclude that hatching success decreases near the lower limit of the altitudinal range, independently of the mountain belt considered and incubation temperature experienced.

INCUBATION TIME

Incubation time decreased dramatically with increasing temperature (28.5 ± 0.2, 36.3 ± 0.3, and 51.0 ± 0.3 days for 30, 26, and 22 °C, respectively), and it was somewhat longer for eggs from Guadarrama than for those from Gredos (38.2 ± 0.8 days

Table 1. Clutch size, mean egg mass, and clutch mass of clutches from two areas or mountain ranges (Guadarrama and Gredos) and elevations (low and high)

	Guadarrama		Gredos		Two-way ANOVA					
					Elevation		Area		Elevation × area	
	Low ($N = 10$)	High ($N = 11$)	Low ($N = 14$)	High ($N = 12$)	$F_{1,43}$	P	$F_{1,43}$	P	$F_{1,43}$	P
Clutch size	6.5 ± 0.3	5.5 ± 0.3	7.1 ± 0.5	6.0 ± 0.2	7.11	0.011	1.95	0.170	0.06	0.812
Mean egg mass (g)	0.49 ± 0.02	0.53 ± 0.02	0.48 ± 0.01	0.46 ± 0.01	0.43	0.515	5.45	0.024	3.19	0.081
Clutch mass (g)	3.18 ± 0.20	2.93 ± 0.15	3.42 ± 0.23	2.76 ± 0.11	5.85	0.020	0.04	0.838	1.16	0.287

Values are given as mean ± 1 standard error (SE). The results of two-way analysis of variance (ANOVA) are also shown.

Table 2. Number of eggs from each of the four study sites that were incubated at each experimental temperature, number of eggs that successfully hatched (with hatching rate in parentheses), survivorship of hatchlings to an age of 21 days (with survival rates in parentheses), and overall embryo survival (survivorship from eggs to a postnatal age of 21 days)

	Guadarrama 1800 m				Guadarrama 2200 m			
Incubation temperature	Eggs incubated	Eggs hatched	Hatchling survivorship	Embryo survival rate	Eggs incubated	Eggs hatched	Hatchling survivorship	Embryo survival rate
22 °C	22	12 (0.55)	10 of 11 (0.91)	0.48	21	18 (0.86)	16 of 17 (0.94)	0.80
26 °C	22	15 (0.68)	10 of 15 (0.67)	0.45	21	16 (0.76)	14 of 15 (0.93)	0.70
30 °C	21	13 (0.62)	10 of 13 (0.77)	0.48	19	17 (0.89)	11 of 17 (0.65)	0.58
Total	65	40 (0.62)	30 of 39 (0.77)	0.47	61	51 (0.84)	41 of 49 (0.84)	0.69

	Gredos 1800 m				Gredos 2100 m			
Incubation temperature	Eggs incubated	Eggs hatched	Hatchling survivorship	Embryo survival rate	Eggs incubated	Eggs hatched	Hatchling survivorship	Embryo survival rate
22 °C	32	28 (0.88)	24 of 24 (1.00)	0.86	24	23 (0.96)	19 of 19 (1.00)	0.95
26 °C	35	30 (0.86)	30 of 30 (1.00)	0.86	24	23 (0.96)	22 of 22 (1.00)	0.96
30 °C	33	27 (0.82)	26 of 27 (0.96)	0.79	24	23 (0.96)	22 of 23 (0.96)	0.92
Total	100	85 (0.85)	80 of 81 (0.99)	0.83	72	69 (0.96)	63 of 64 (0.98)	0.94

Note that a total number of 12 hatchlings were separated from the rest of their cohort, for logistical reasons, during the 3-week period of postnatal growth. As a consequence, they were excluded from the rates shown in Hatchling survivorship and Embryo survival rate.

and 39.3 ± 1.0 days, respectively), but it did not differ significantly between high- and low-elevation sites (temperature: $F_{2,191} = 2{,}048.33$, $P < 0.001$; area: $F_{1,46.88} = 6.03$, $P = 0.018$; altitude: $F_{1,12.32} = 0.0002$, $P = 0.989$). However, the difference between areas became non-significant after controlling for the effects of egg mass ($F_{1,71.31} = 1.90$, $P = 0.172$). Familial (i.e. clutch) effects were significant ($F_{42,191} = 3.10$, $P < 0.001$).

Size, body condition, and growth rates

A mixed GLM, with incubation temperature, area (Gredos vs. Guadarrama), and altitude as fixed factors, clutch as the random factor, and egg mass at oviposition as the covariate, showed that temperature had a strong effect on SVL at hatching, with higher temperatures producing smaller hatchlings (Table 3; $F_{2,189} = 37.90$, $P < 0.001$; in this and the following analyses, non-significant results are omitted). Familial (i.e. clutch) effects were significant ($P \leq 0.012$) for all dependent variables except for growth rates (see below). Hatchling mass also decreased at higher temperature ($F_{2,189} = 11.53$, $P < 0.001$; egg mass: $F_{1,189} = 9.08$, $P = 0.002$). Because SVL and body mass of hatchlings were positively correlated ($r = 0.691$, $P < 0.001$), and they both decreased at higher temperature, body condition at

Table 3. Body size [snout vent length (SVL)], mass, and condition (body mass adjusted for SVL and egg mass at oviposition), both at hatching and at an age of 21 days, classified according to elevation (low vs. high), incubation temperature, and area (i.e. mountain range: Guadarrama vs. Gredos)

	Low elevation			High elevation		
	22 °C	26 °C	30 °C	22 °C	26 °C	30 °C
SVL at hatching (mm)						
Guadarrama	30.41 ± 0.33	29.70 ± 0.30	29.15 ± 0.32	31 ± 0.27	30.93 ± 0.29	29.18 ± 0.28
Gredos	30.80 ± 0.21	30.83 ± 0.21	30.00 ± 0.22	30.5 ± 0.24	30.02 ± 0.24	29.48 ± 0.24
Mass at hatching (g)						
Guadarrama	0.53 ± 0.02	0.55 ± 0.02	0.52 ± 0.02	0.56 ± 0.01	0.56 ± 0.02	0.51 ± 0.1
Gredos	0.55 ± 0.01	0.56 ± 0.01	0.54 ± 0.02	0.52 ± 0.01	0.53 ± 0.01	0.50 ± 0.01
Body condition at hatching (g)						
Guadarrama	0.53 ± 0.02	0.55 ± 0.01	0.54 ± 0.02	0.53 ± 0.01	0.53 ± 0.01	0.52 ± 0.01
Gredos	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.52 ± 0.01	0.54 ± 0.01	0.53 ± 0.01
SVL at 21 days (mm)						
Guadarrama	32.80 ± 0.50	35.30 ± 0.50	34.40 ± 0.50	33.10 ± 0.39	34.54 ± 0.43	34.23 ± 0.47
Gredos	33.42 ± 0.32	35.47 ± 0.28	35.38 ± 0.32	32.75 ± 0.36	34.14 ± 0.34	34.57 ± 0.32
Mass at 21 days (g)						
Guadarrama	0.71 ± 0.04	0.87 ± 0.04	0.85 ± 0.04	0.74 ± 0.03	0.81 ± 0.03	0.83 ± 0.03
Gredos	0.76 ± 0.02	0.87 ± 0.02	0.87 ± 0.02	0.68 ± 0.03	0.80 ± 0.03	0.81 ± 0.02
Body condition at 21 days (g)						
Guadarrama	0.77 ± 0.02	0.81 ± 0.02	0.85 ± 0.02	0.79 ± 0.02	0.77 ± 0.02	0.80 ± 0.02
Gredos	0.80 ± 0.02	0.80 ± 0.01	0.82 ± 0.01	0.77 ± 0.02	0.82 ± 0.02	0.81 ± 0.02

Values are given as mean ± 1 standard error (SE).

hatching showed no significant between-group differences (ANCOVA with body mass as the dependent variable and SVL at hatching and egg mass at oviposition as the covariates).

Relative growth rates in SVL differed significantly between temperatures and elevations, being higher for lizards that came from clutches obtained at lower elevation and for those incubated at warmer temperatures (Fig. 2; temperature: $F_{2,156} = 88.55$, $P < 0.001$; altitude: $F_{1,65.93} = 4.75$, $P = 0.033$). Relative growth rates in body mass differed only among temperature treatments, being higher for juveniles treated at warmer incubation temperatures (temperature: $F_{2,156} = 42.63$, $P < 0.001$).

After 3 weeks of postnatal growth, the faster growth rates of juveniles from eggs incubated at warmer temperatures (especially at 30 °C) allowed them to surpass the size of their siblings incubated at 22 °C (Table 3). Thus, at an age of 21 days they had compensated for their initial disadvantage in both SVL (temperature: $F_{2,156} = 35.42$, $P < 0.001$) and body mass (temperature: $F_{2,156} = 27.88$, $P < 0.001$), and they had reached the highest body condition (Table 3) of the three temperature treatments (ANCOVA with body mass as the dependent variable and SVL at hatching and egg mass at oviposition as the covariates; temperature: $F_{2,155} = 3.24$, $P = 0.042$). No significant between-group differences in body size, mass, or condition at the age of 3 weeks were found for area, elevation, or any of the interaction effects examined, although juveniles from low-elevation sites were slightly larger and heavier than those from high-elevation sites.

EMBRYO SURVIVAL

Of the 245 neonates that hatched successfully, 19 were not viable, including the one that died shortly after hatching plus another 18 individuals that showed hampered mobility, could not feed successfully, and died before the end of the experiment. These symptoms were identical to those previously observed for this species when incubated at high temperatures (32 °C; Monasterio *et al.*, 2011). A log-linear model with survivorship of embryos (from hatching to an age of 21 days) as the response variable, and area, altitude and temperature as the design factors (χ^2 for the overall model = 14.33, d.f. = 20, $P = 0.814$), included only an interaction between area and survival (partial association: $\chi^2 = 21.29$, d.f. = 1, $P < 0.001$; Table 2), although the interaction between temperature and survival was close to significance (partial association: $\chi^2 = 5.34$, d.f. = 2, $P < 0.069$), with survivorship decreasing as incubation temperature increased (survival rates of

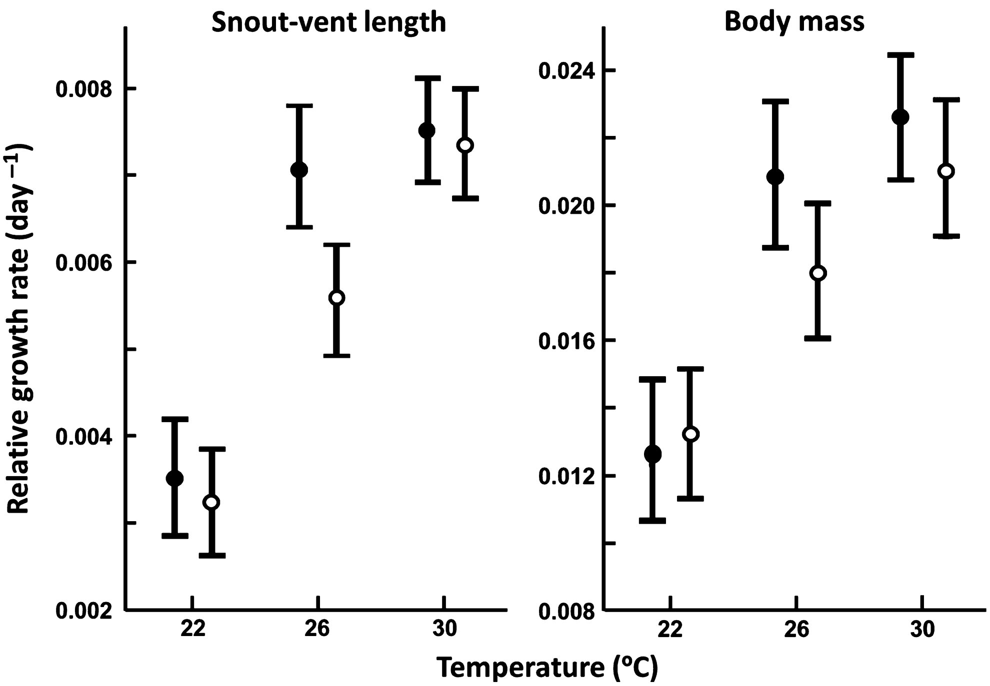

Figure 2. Size- (snout-vent length) and body mass-specific growth rates of juveniles, from low-elevation (black circles) and high-elevation (white circles) sites, hatched from eggs incubated at different temperatures.

0.97, 0.93, and 0.86 for juveniles incubated at 22, 26, and 30 °C, respectively).

Differences among study sites in overall embryo survival (from eggs to a postnatal age of 21 days) were highly significant (Table 2: $\chi^2 = 44.24$, d.f. = 3, $P < 0.001$). In all populations except Morcuera (i.e. Guadarrama, 1800 m, where survivorship was lowest), embryo survival was higher for eggs incubated at 22 °C than for those incubated at 30 °C (Table 2). The difference between survival rates of embryos incubated at 22 and 30 °C was higher for embryos from the coolest site (Guadarrama, 2200 m) than for embryos from the other three locations with higher and more similar soil temperatures ($F_{1,2} = 21.88$, $P = 0.043$). After controlling for the effects of this difference, survival was significantly higher at 22 °C than at 30 °C ($F_{1,2} = 40.80$, $P = 0.024$). In other words, embryo survival was lowest at the worst quality (i.e. warmest) site (Guadarrama, 1800 m), and it was lower at 30 °C than at 22 °C, especially at the coolest site (Guadarrama, 2200 m).

VARIATION OF SOIL TEMPERATURES IN THE FIELD

A main-effects ANOVA, with soil temperature as the dependent variable, and time of day, month, area, and elevation as categorical predictors, explained 71.1% of the variance in soil temperatures ($F_{27,28,772} = 2618.11$, $P < 0.001$), showing significant differences between times of day (Fig. 3), months, elevations, and areas (contributions to R^2 of 0.575, 0.060, 0.042, and 0.033, respectively). Low temperatures at the high-elevation site in Guadarrama produced a significant interaction between elevation and area that increased overall R^2 to 0.748, indicating that altitudinal differences in the thermal environment were more pronounced for Guadarrama than for Gredos. Differences among loggers were also large, as shown by the high R^2 value (0.871) of the full factorial model, with logger, time of day, and month as categorical predictors. Moreover, the ranges of daily temperatures suggest a wide range of thermal opportunities at each of the four study sites (Guadarrama, low-elevation site, mean range of daily temperatures ± 1 SE: 22.8 ± 0.5 °C; Guadarrama, high-elevation site: 19.8 ± 0.5 °C; Gredos, low-elevation site: 20.4 ± 0.4 °C; and Gredos, high-elevation site: 22.9 ± 0.5 °C; $N = 75$ days in all cases). Because mean ranges were highest at Guadarrama low-elevation and Gredos high-elevation sites, neither the main effects of site nor altitude were significant in a two-way ANOVA, whereas their interaction was highly significant (area: $F_{1,296} = 0.57$, $P = 0.452$; altitude: $F_{1,296} = 0.40$, $P = 0.530$; and interaction: $F_{1,296} = 32.07$, $P < 0.001$).

DISCUSSION

Our results describe a scenario in which eggs and hatchlings from mothers captured at low-elevation

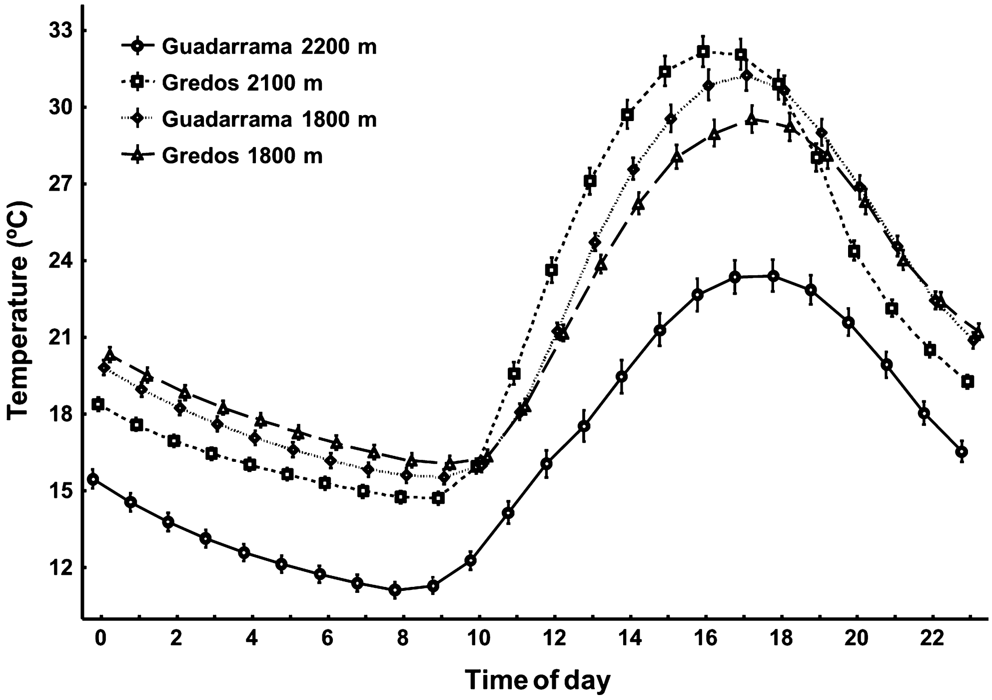

Figure 3. Daily variation of mean (± 95% confidence interval) soil temperatures available during the incubation period of *Iberolacerta cyreni* at the four study sites.

areas showed reduced survivorship, which suggests that reproduction is impaired at the lower limit of the elevational distribution range. Such impairment might be partly counterbalanced by faster postnatal growth at lower elevations, leading to increased female size and higher fecundity. However, interactions between the effects of temperature and elevation were not significant for incubation time, hatchling size, hatchling mass, body condition, or growth rates, indicating a lack of elevational variation in the thermal dependence of the phenotypic traits studied. Moreover, high temperatures (above 30 °C), which were unfavourable for the success of high-elevation eggs, at least in Guadarrama (Monasterio *et al.*, 2011; and the results of this study), were also detrimental for eggs from low-elevation sites. Remarkably, there is a great variety of soil temperature available at all sites examined suggesting a wide range of thermal opportunities even at marginal areas. Therefore, it is likely that the intensity of selective pressures may not be fostering local adaptation at the edge of the range.

It should be emphasized that our study was set to describe potential abiotic limits on the distribution range of a lizard species confined to alpine habitats. Previous work, comparing *I. cyreni* with another lower-elevation lacertid species (*Psammodromus algirus*), showed that thermal requirements of embryonic development, rather than competition, is a major ecophysiological factor determining the elevational range limits of these oviparous lizards (Monasterio *et al.*, 2011). In fact, *P. algirus* is found at elevations below 1600 m, whereas *I. cyreni* is found at elevations above 1600 m, with no overlap between them. Also, variations in the abundance of *I. cyreni* are negatively correlated with altitude (Monasterio *et al.*, 2010a). Moreover, we found no experimental evidence of competition between *I. cyreni* and wall lizards (*Podarcis muralis*, the only species whose distribution range overlaps with that of *I. cyreni*), although intraspecific interactions seemed to explain the behaviour of adult rock lizards (Monasterio, Salvador & Díaz, 2010b). All of this evidence supports the view that abiotic factors are crucial in shedding light on the alpine confinement of these lizards, and that elevational variations in reproductive success and in the thermal sensitivity of eggs and embryos may be important to shape distribution range limits.

Although strictly speaking, and given our limited sample size (only two high-elevation sites and two low-elevation sites), we cannot rule out the importance of local site effects per se, our data suggest that high temperatures are detrimental for eggs and hatchlings, both at core and low-elevation areas, because the survival of propagules to an age of 3 weeks was lowest at 30 °C in the four populations examined. Although the difference in survival rates between propagules incubated at 22 and 30 °C was maximal at the highest and coolest site (Guadarrama, 2200 m) and minimal at the warmest site (Guadarrama, 1800 m), this was not because of a higher hatching/survival rate of eggs from low-elevation sites incubated at higher temperature but rather because of a poorer survivorship of those incubated at low temperature. In fact, survival rate was lowest (< 0.5) at the Guadarrama low-elevation site, independently of incubation temperature, whereas eggs from the Gredos high-elevation site hatched and developed quite successfully at all incubation temperatures (rates within the range 0.91–0.97). In a previous study of clutches from females captured at middle elevations (1850–1950 m), Monasterio *et al.* (2011) found that increased incubation temperatures had a negative effect on juvenile survival (38% of juveniles were viable at 32 °C), and our results confirm that this species is unable to cope with progressively warmer incubation conditions (> 30–32 °C) within the thermal range encompassed in these studies (22–32 °C). We can therefore conclude that high incubation temperatures are detrimental for *I. cyreni* also at the lower limit of its elevational range.

Despite the fact that both offspring survival and (as a consequence) reproductive success of adults were lower at the edge of the distribution range, the hatchlings from low elevation areas that managed to hatch did not show any compensatory adaptive modifications, such as larger body size or better body condition. Regardless of site of origin, higher temperatures produced smaller (SVL) and lighter (body mass) hatchlings. In fact, a relevant new finding of this study is the lack of elevational variation in the thermal dependence of traits, such as incubation time or hatchling size or condition (Table 3). This lack of variation differs from previous research on other lacertid species, whose incubation times have been found to vary along altitudinal or latitudinal gradients in order to fine-tune reproductive success and phenology to environmental variation (Iraeta *et al.*, 2006; Oufiero & Angilletta, 2006; Díaz *et al.*, 2012). However, it should be noted that the thermal differences in these studies might be larger than those reported here.

A possible explanation for the observed lack of variation is that females may be able to compensate for clinal variation in thermal conditions along the elevational gradient through nest-site selection (Doody, 2009; Huang & Pike, 2011). Accordingly, adaptation to warmer temperatures in *I. cyreni* may be precluded by the great variety of soil temperatures available at low-elevation sites, which should lower the intensity of selective pressures near the edge of the range. This hypothesis is consistent with the fact that, even with a low sample size of only four loggers per site, variation among loggers was large, particularly at warmer times of day. This suggests that maternal manipulation of hatchling phenotypes via nest site selection (Shine & Harlow, 1996) may indeed hamper the evolution of physiological adaptations, providing another example of how behavioural adjustments can buffer environmental variation and thereby reduce selective pressures for evolutionary change (Huey, Hertz & Sinervo, 2003).

On the other hand, juveniles that came from eggs incubated at higher temperature were able to counterbalance their smaller hatchling size by growing faster than those incubated at cooler temperature (Table 3). Also, and more importantly, juveniles from lower elevation areas grew faster than those from core populations. These results suggest that in marginal populations, selection could favour the genotypes that encode for faster growth, as a way of compensating for their lower hatching success. Differences in postnatal growth rates could explain, at least in part, why adult females were larger in populations located close to their distributional limit. Lizards inhabiting lower elevations could benefit from longer activity periods. This, combined with the potential for faster growth rates evidenced by our common garden experiment, may promote larger sizes at maturity (Du *et al.*, 2012). Moreover, their larger body size should allow females from areas of low elevation to lay more eggs (and therefore heavier clutches), which is another way to compensate for the reduced hatching success of embryos from areas of low elevation (Du, Ji & Shine, 2005). In fact, the faster postnatal growth rates of hatchlings from areas of low elevation might aid in counterbalancing the negative effects of high incubation temperature on hatchling size near the lower limit of the distribution range of this alpine species. Interestingly, a related pattern of elevational covariation was found in Guadarrama for the lacertid *P. algirus*, in which the genotypes that encode fast growth occur in lowland habitats with low food availability, where lizards attain small adult sizes and lay smaller clutches of larger eggs (Iraeta, Salvador & Díaz, 2013). However, for *P. algirus*, the fast-growing environment is the high-elevation one (Iraeta *et al.*, 2006), located 300–400 m below the lower limit of *I. cyreni*'s elevational range. This pattern of countergradient variation, which is found in *P. algirus* but not in *I. cyreni*, supports the idea that temperate lacertids may be more able to adapt to cooler climates than to warmer ones.

Reproductive traits were also distinctly different in the two mountain ranges examined. The soil temperatures registered by loggers indicate that the thermal gradient is steeper at Guadarrama, where lizards from the low-elevation site, besides showing the worst performance levels of the four populations examined, are scarcer (Monasterio *et al.*, 2010a), and thermoregulate less effectively (Monasterio *et al.*, 2009), than at higher elevation. Given its harsher and more continental climate, Guadarrama may have poorer conditions than Gredos in terms of productivity and associated prey availability, which are important determinants of life-history variation in lacertid lizards (Lorenzon, Clobert & Massot, 2001; Iraeta *et al.*, 2006). According to our results, hatching success was lower in Guadarrama, and body size of females was larger in Gredos. However, and despite their smaller body size, females from Guadarrama laid larger eggs than those from Gredos, even at the cost of a slight increase in the duration of incubation, perhaps to counterbalance their reduced hatching success. Previous studies indicate that in lizards the survival advantage of large offspring is greater under conditions of food shortage (Ferguson & Fox, 1984) and/or in drought years (Sinervo *et al.*, 1992; Iraeta *et al.*, 2006).

The reasons why reproductive success was so low for eggs from the Guadarrama low-elevation site, independently of incubation temperature, remain unclear. However, it can be argued that reproductive failure may be caused by environmental constraints on the thermoregulatory abilities of gravid females (Schwarzkopf & Shine, 1991; Shine & Harlow, 1993). It is well known that uterine retention is selectively advantageous in montane reptiles because it allows developing eggs to be kept warm by their mother's thermoregulatory behaviour, thus avoiding detrimentally low nest temperatures that hamper both hatching success and hatchling viability (Shine, 2002). In a comparative study of egg retention in lacertid lizards, Braña *et al.* (1991) showed that the related species *Iberolacerta monticola*, together with oviparous populations of *Zootoca vivipara*, are the species with more advanced intrauterine embryogenesis, and also those that reach the highest elevations in northern Spain; however, there is no evidence of intraspecific variability (Braña *et al.*, 1991), and our results confirmed that incubation times were almost identical at high- and low-elevation sites. On the other hand, it is also known that early events of reptilian embryogenesis are involved in tissue differentiation and organogenesis to ensure the production of viable phenotypes, whereas late development is characterized by physiological changes and growth in size (Deeming & Ferguson, 1991). As a consequence, the hatching success and viability of *I. cyreni*'s eggs and hatchlings may be more dependent on the themoregulatory effectiveness of gravid females than on the thermal properties of nesting sites (see Shine & Harlow, 1993, for an experimental demonstration of the effects of maternal thermoregulation on offspring viability in a viviparous scincid). Interestingly, previous studies have shown that *I. cyreni* from Guadarrama thermoregulates less efficiently, probably because of a scarcity of appropriate refuges, such as large rocks, at lower elevation (Monasterio *et al.*, 2009, 2010a). In fact, forest belts may prevent the expansion of alpine lizards towards lower elevations (Huang *et al.*, 2014) as a result of their low thermal quality and reduced availability of refuges (Monasterio *et al.*, 2009). Moreover, the high success of eggs and hatchlings from the Gredos high-elevation site, independently of incubation temperature, is consistent with careful thermoregulation by gravid females during the first weeks of embryionic development, which would be favoured by high diurnal operative temperatures readily available at alpine environments (Shine, Elphick & Barrott, 2003) and high cover of large rocks providing good-quality refuges (Monasterio *et al.*, 2010a).

In summary, our study demonstrates that at the lower limit of the distribution range of *I. cyreni*, reproductive output of adult females is lower than at core, high-elevation areas, and that no specific adaptations to higher temperatures have evolved in the populations at the limits, perhaps because of the availability of nesting sites with suitable thermal conditions as elevation decreases along mountain slopes. Another non-alternative explanation for the lack of variation in thermal reaction norms could be gene swamping caused by asymmetrical gene flow from core areas with cold-adapted genotypes to lower and warmer areas (Sexton *et al.*, 2009). This seems plausible for *I. cyreni* because core and marginal populations occur continuously, separated by a relatively short distance, along a steep environmental gradient. We could therefore hypothesize that migration among highly connected populations across mountain slopes could cause enough gene flow to prevent genetic novelties, favoured in warmer incubation environments, to become fixed. Thus, if the traits we studied are genetically based, range limits could appear abruptly as a result of the inability of lizards from areas of low elevation to adapt to higher temperatures. Although the role of gene swamping in shaping range limits requires careful study, given the complexity of the interactions involved, our results suggest that it would be worthwhile to test this hypothesis, which has recently fostered the debate on the evolution of range limits (Moeller, Geber & Tiffin, 2011; Paul, Seth & Angert, 2011).

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