

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

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19 Running head: Incubation requirements and range limits

20 **Abstract** Studies on range limits clarify the factors involved in the extent of species
21 occurrence and shed light on the limits to adaptation. Alpine lizards have to adapt to a steep
22 gradient if they are to persist as elevation decreases and temperature increases. We studied
23 elevational variation in the thermal dependence of fitness-related traits (incubation time,
24 hatching rate, and survivorship, size, and condition of hatchlings) to assess the role of
25 incubation requirements in distribution range limits of the alpine endemism *Iberolacerta*
26 *cyreni*. We captured gravid females from two core (summit) and two marginal (low-
27 elevation edge) populations, we incubated their eggs at three temperatures (22, 26 and
28 30°C), and we monitored phenotypic effects. Viability of eggs and hatchlings decreased,
29 independently of elevation, as incubation temperature increased. Hatching success and
30 embryo survivorship were lower for clutches from low-elevation areas than for those from
31 mountain summits, showing that lizards face difficulties to thrive at the low-elevation edge
32 of their range. Such difficulties were partly counterbalanced by faster postnatal growth at
33 lower elevations, leading to increased adult size and higher fecundity. High incubation
34 temperature had detrimental effects also at low-elevation areas, and no elevational variation
35 in the thermal dependence of hatchling traits was detected. We suggest that temperature
36 effects on egg development and lack of selective pressures strong enough to foster local
37 adaptation at marginal areas, combined with extended egg retention, may contribute to shape
38 range limits of these alpine oviparous reptiles.

39 **Keywords** Growth rates · Incubation requirements · Lacertidae · Local adaptation ·
40 *Iberolacerta* · Temperature effects · Thermoregulation

41 **Introduction**

42

43 Geographic ranges, conceived as the spatial projection of the realized ecological niche
44 (Hutchinson, 1957), reflect the ability of species to thrive under a given set of
45 environmental conditions (Kearney & Porter, 2004). As a consequence, the study of their
46 limits may help to disentangle the factors involved in the extent of species occurrence,
47 and it provides a useful framework for analyzing the limits to adaptation at the edge of
48 the range (Holt & Keitt, 2005). In fact, the concept of limiting factors (temperature, pH,
49 salinity, etc.), or physical factors that restrict the occurrence of organisms to particular
50 habitats, can be traced back to the early days of ecology, with an emphasis on interactions
51 between physiology and the physical environment (Huey, 1991). Nevertheless, the task is
52 challenging, not only due to the complexity of the physiological, ecological, and
53 evolutionary interactions involved, but also because these interactions may be subject to
54 unknown rates of adjustment under the current scenario of global change (Quintero &
55 Wiens, 2013).

56 Empirical studies comparing core and marginal populations within the distribution
57 range provide an adequate framework to tackle relevant questions about range limits
58 (Díaz, Pérez-Tris, Bauwens, Pérez-Aranda, Carbonell, Santos & Tellería, 2007; Kawecki,
59 2008). When species occur along ecological gradients, studying adaptation at range
60 margins can provide insight into the plasticity of their responses to environmental
61 variation (Byars, Papst & Hoffmann, 2007; Vergeer & Kuni, 2013). Such plasticity may
62 determine if populations are able to thrive under the new conditions at the edge of their
63 range, or if, on the contrary, they encounter a barrier for their survival (Angert &
64 Schemske, 2005).

65 Steep environmental gradients, such as those found in mountains, serve as

66 excellent testing grounds for adaptation to novel environments. Moreover, alpine species
67 show clearly restricted distribution patterns, which could reflect their specialisation to
68 cold habitats and their inability to cope with changes along the mountain slope (Merrill,
69 Gutiérrez, Lewis, Gutiérrez, Díez & Wilson, 2008). Therefore, examining their responses
70 to factors that are strongly correlated with elevation can help to identify environmental
71 thresholds for their occurrence. Among these factors, temperature turns out to be
72 particularly important (Körner, 2007), not only because its major effects on organisms are
73 pervasive, but also because it can reveal the potential responses of species to global
74 warming. If species are not able to adjust their phenotypes to temperature changes either
75 by developmental plasticity or genetic differentiation, they may experience range
76 contractions or even extinctions (Williams, Shoo, Isaac, Hoffmann & Langham, 2008).

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

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

111

112 **Methods**

113

114 *Study species and study areas*

115 Rock lizards *Iberolacerta cyreni* (adult snout vent length [SVL] of 73-80 mm) are

116 heliothermic, actively foraging, diurnal lacertid lizards, which are endemic to the Iberian
117 Sistema Central. The species presents a restricted distribution range since it is only found
118 above 1,600 m, preferably in rocky outcrops and mixed-shrub formations (Monasterio,
119 Salvador & Díaz, 2010a). The Sistema Central presents contrasting seasonal conditions,
120 with cold wet winters and short dry summers. Its mountain bases (1,200-1,700 m a.s.l.)
121 are covered with deciduous Pyrenean oak (*Quercus pyrenaica*) forests, which in some
122 areas (particularly in the Sierra de Guadarrama) are progressively substituted by Scots
123 pine (*Pinus sylvestris*) forests at higher altitudes. These forested areas, that can spread
124 from 1,500 to 2,100 m a.s.l., gradually become less dense until vegetation is dominated
125 by a mosaic of dense mixed-shrub formations (of perennial *Juniperus communis* and
126 *Cytisus oromediterraneus*) interspersed with small meadows of *Festuca* and other
127 grasses. These alpine areas above the tree line (1,700-2,300 m a.s.l.) are also
128 characterized by extensive patches of large granite rocks and scree interspersed among
129 shrub formations.

130 Within the Sistema Central, *I. cyreni* is found in several mountain ranges
131 (Guadarrama, Gredos, Béjar, La Serrota, and La Paramera), but most of its distribution
132 range corresponds to the ranges of Guadarrama and Gredos. For that reason, we chose
133 two study areas in each of these two mountain ranges (Fig. 1), one at high elevation
134 (2100-2300 m a.s.l.: Bola del Mundo in Guadarrama and Circo in Gredos) and another
135 one at low elevation (1800-1850 m a.s.l.: Puerto de la Morcuera in Guadarrama and
136 Plataforma in Gredos). This design included an explicit biogeographic scenario given that
137 high elevation sites correspond to populations that are in the centre of their distribution
138 range (core areas, at the mountain summits), whereas low elevation sites correspond to
139 populations located at its lower limit. This is supported by previous analyses showing that
140 altitude is the independent variable that most accurately predicts variations in the

141 abundance of *I. cyreni*. Thus, we selected our low-altitude study sites on the basis of a
142 previously published model predicting that lizard populations should disappear below
143 1,650 m a.s.l. (Monasterio et al., 2010a); between this elevation and the 1,800 m a.s.l.
144 altitude of our study sites, the species is too scarce to allow capture of the minimum
145 number of females required to attain a reliable sample size. It is also important to note
146 that the two sampled areas in each mountain range are connected by intermediate
147 populations, so that distributional range is not confounded with meta-population structure
148 (as if each mountain was a meta-population); instead, each mountain has its own core and
149 edge area, and our design provides appropriate replicates of edge effects along altitudinal
150 gradients.

151

152 ***Collection and husbandry of adults, eggs and juveniles***

153 Between 7/06/2012 (first captures at the Guadarrama low-elevation site) and 21/06/2012
154 (last captures at the Gredos high-elevation site), we noosed 48 gravid females (SVL: 72.7
155 ± 0.7 mm; body mass: 8.4 ± 0.2 g) that were transported to our laboratory in the
156 Department of Zoology (Universidad Complutense de Madrid) on the same day of
157 capture. Mean laying dates ranged between 27/06/2012 (Guadarrama, low-elevation) and
158 8/07/2012 (Gredos, high-elevation). Thus, we are confident that our results should not be
159 confounded by among-population differences in time of capture of gravid females or time
160 in captivity before oviposition. It should also be noted that although gravid females
161 readily lay eggs in captivity, their reproductive output still depends on the environmental
162 conditions they experienced in the wild, specially in the case of species with long-lasting
163 intrauterine embryogenesis such as *I. cyreni* (Braña, Bea & Arrayago, 1991). This is
164 because in lizards the hormonal and physiological coupling among clutch size, egg size,
165 and clutch mass occurs during early vitellogenesis (Sinervo & Licht, 1991).

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

172 Lizards were housed in white opaque wall terraria ($40 \times 60 \times 30$ cm; 2 females
173 per cage) which were covered by a green net (0.5-cm mesh) that prevented escape. The
174 mesh provided a shrubby-like shelter while still allowing daylight to enter the cages.
175 Terraria were filled with moistened earth averaging 10 cm in depth, covered by a leaf
176 litter layer. A 60 W lamp suspended over one end of the cage created a photothermal
177 gradient (ca. 25-50°C) allowing thermoregulation within the preferred temperature range
178 (Bauwens, Garland, Castilla & Van Damme, 1995). UV light was also provided. An
179 earthenware tile (ca. 10×15 cm) and a thin section of fallen wood provided additional
180 shade and shelter. Lizards were daily fed crickets (*Acheta domesticus*) and mealworms
181 (*Tenebrio mollitor*), dusted with a commercial vitamin and calcium supplement delivered
182 according to manufacturer's recommendations. Food and water were provided *ad libitum*.

183 Gravid females were monitored daily to detect changes in body shape or body
184 mass that might indicate egg-laying. When it was clear that a female had laid, eggs were
185 searched and located, removed from the cage, counted, weighed and individually placed
186 in 150 ml plastic cups filled with ca. 35 g of moistened vermiculite (10 g vermiculite per
187 8 g water, equivalent to -200 kPa). Eggs were completely covered by the vermiculite,
188 and the jars were closed with a tightly fitting screw top to minimize evaporative water
189 loss. Eggs of the same clutch were distributed evenly over three incubators (MMM
190 FriocellTM, Germany) set at constant temperatures of 22, 26 and 30 °C. These values were

191 chosen based on a previous study (with eggs incubated at 24, 28 and 32 °C) in which high
192 temperatures (32 °C) negatively affected the reproductive success of these lizards
193 (Monasterio et al., 2011). We thus lowered incubation temperatures to decrease negative
194 effects while still being able to detect thermal effects on incubation. Searches for new
195 hatchlings were conducted daily towards the end of the incubation period. Hatchlings
196 were weighed, measured (snout-vent length, SVL), and individually marked by toe
197 clipping. The incubation period (in days) was calculated as the time elapsed between egg
198 deposition and hatching. After females had laid their eggs, they were released at their site
199 of capture.

200 Immediately after hatching, newborns were placed in individual plastic terraria
201 (265 mm length x 162 mm width x 150 mm height) that offered rock and sand substrates.
202 A 60W lamp 25 cm above the rock acted as a heat source allowing lizards to bask and
203 UV light was also provided. As per adults, food and water were provided *ad libitum*. To
204 monitor growth of juveniles, we measured (SVL to the nearest mm) and weighed (to the
205 nearest mg) all lizards after 21 days of captivity, when the experiment was completed.
206 Growth rates of juveniles were estimated both for size (SVL) and body mass using the
207 formula:

208 $\text{Growth rate (in days}^{-1}\text{)} = \text{Ln (measurement at an age of 21 days / measurement at hatching)} / 21.$

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

212

213 ***Characterisation of field thermal environments***

214 To characterize the thermal environments available at each study site, we registered soil
215 temperatures using Thermochron Ibutton temperature loggers that were placed at 5 cm

216 depth in soil under bare ground. We used four sampling points at each study site
217 separated by at least 100 m. Temperature was recorded every hour from 1st July to 13th
218 September 2012 (75 days x 24 h x 4 loggers x 4 sites). It is important to note that we do
219 not intend to provide an accurate description of temperatures available at nesting sites,
220 because very few clutches have been found in the field, usually under rocks, and as a
221 consequence nesting sites remain basically unknown. Instead, our aim was to provide a
222 coarse characterisation of thermal variability between and within sites, and to compare
223 the effects of several sources of spatio-temporal variation on the thermal environment.

224

225 *Statistical analyses*

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

235 We checked the assumptions of parametric tests (normality and homogeneity of
236 variances) before using GLM's. In addition, the mixed GLM, used for testing the
237 significance of clutch (random factor) and temperature, altitude, and area (fixed factors)
238 on hatchling phenotypes, requires leaving the random effects independent of the fixed
239 effects (Searle, Casella & McCulloch, 1992) by constructing appropriate error terms.
240 Statistica 7.0 uses Satterthwaite's method of denominator synthesis to find the linear

241 combinations of sources of random variation that serve as error terms for each effect.
242 Thus, the degrees of freedom for the denominator mean square can be fractional rather
243 than integer values, meaning fractions of sources of variation were used in synthesizing
244 error terms for significance testing. We estimated body condition at hatching and at an
245 age of 21 days using GLM's (ANCOVA's) with body mass as the dependent variable and
246 SVL as the covariate. Data are reported as mean \pm 1 SE.

247

248 **Results**

249

250 *Body size and reproductive investment of females*

251 Females differed in size between areas (Gredos vs. Guadarrama) and altitudes (mean
252 SVL \pm 1 SE and sample size: Gredos, core areas: 73.1 ± 0.7 mm, $N = 31$; Gredos, low-
253 elevation edge of the range: 75.5 ± 1.0 mm, $N = 15$; Guadarrama, core areas: 69.7 ± 0.8
254 mm, $N = 45$; Guadarrama, low-elevation edge of the range: 71.6 ± 0.9 mm, $N = 57$).

255 Females from Gredos were larger than those from Guadarrama, and lower elevation
256 females were larger than higher elevation ones (two-way ANOVA: area $F_{1, 144} = 13.13$, P
257 < 0.001 ; altitude: $F_{1, 144} = 4.54$, $P = 0.034$; area x altitude interaction: $F_{1, 144} = 0.04$, $P =$
258 0.844).

259 Lower elevation females laid larger and heavier clutches than higher elevation
260 ones, whereas mean egg mass was larger for females from Guadarrama than for those
261 from Gredos (Table 1); clutch mass was more dependent on clutch size ($\beta = 0.981$) than
262 on mean egg mass ($\beta = 0.552$). However, altitudinal differences disappeared after
263 controlling for female size (SVL) both for clutch size (ANCOVA; altitude: $F_{1, 42} = 2.77$, P
264 $= 0.103$; SVL: $F_{1, 42} = 18.06$, $P < 0.001$) and clutch mass (altitude: $F_{1, 42} = 1.76$, $P =$
265 0.192 ; SVL: $F_{1, 42} = 22.98$, $P < 0.001$), showing that the greater reproductive investment

266 of low elevation females was mainly a consequence of their larger body size. Egg mass,
267 however, was higher in Guadarrama (mean egg mass of 0.51 ± 0.1 g, vs. 0.47 ± 0.1 g for
268 Gredos; see Table 1) even after controlling for the effects of SVL (area: $F_{1,42} = 5.66$, $P =$
269 0.022 ; SVL: $F_{1,42} = 0.40$, $P = 0.531$).

270

271 ***Hatching success***

272 Overall, females laid 47 clutches, with a mean clutch size of 6.34 ± 0.21 eggs. We
273 incubated a total of 298 eggs of which we obtained 245 hatchlings (Table 2), although
274 one of the hatchlings was found dead ($N = 244$ for subsequent analyses). A log-linear
275 model with hatching success as the response variable, and area, altitude and temperature
276 as the design factors provided a very high goodness-of-fit to the observed data (χ^2 for the
277 overall model = 6.06, $df = 18$, $P = 0.996$), and included both an interaction between area
278 and hatching success (partial association: $\chi^2 = 16.22$, $df = 1$, $P < 0.001$) and, more
279 importantly, an interaction between elevation and hatching success (partial association: χ^2
280 = 11.76, $df = 1$, $P < 0.001$). The first interaction indicates that hatching success was
281 higher for eggs from Gredos than for those from Guadarrama, whereas the second
282 interaction shows that hatching success increased with elevation (Table 2). Temperature
283 did not influence hatching success (partial association: $\chi^2 = 0.04$, $df = 2$, $P = 0.981$).
284 Interestingly, the interaction between area, altitude, and hatching success was close to
285 zero (partial association: $P = 0.983$). We can therefore conclude that hatching success
286 decreases near the lower limit of the altitudinal range, independently of the mountain belt
287 considered and incubation temperature experienced.

288

289 ***Incubation time***

290 Incubation time decreased dramatically with increasing temperature (28.5 ± 0.2 , $36.3 \pm$

291 0.3, and 51.0 ± 0.3 days for 30, 26, and 22°C, respectively), and it was somewhat longer
292 for eggs from Guadarrama than for those from Gredos (38.2 ± 0.8 , and 39.3 ± 1.0 days ,
293 respectively), but it did not differ significantly between high and low elevation sites
294 (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
295 after controlling for the effects of egg mass ($F_{1, 71.31} = 1.90$, $P = 0.172$). Familial (i.e.
296 clutch) effects were significant ($F_{42, 191} = 3.10$, $P < 0.001$).

298

299 *Size, body condition and growth rates*

300 A mixed GLM with incubation temperature, area (Gredos vs. Guadarrama) and altitude
301 as fixed factors, clutch as the random factor, and egg mass at oviposition as the covariate,
302 showed that temperature had a strong effect on SVL at hatching, with higher
303 temperatures producing smaller hatchlings (Table 3; temperature: $F_{2, 189} = 37.90$, $P <$
304 0.001 ; in this and the following analyses, non-significant results are omitted). Familial
305 (i.e. clutch) effects were significant ($P \leq 0.012$) for all dependent variables except for
306 growth rates (see below). Hatchling mass also decreased at higher temperature
307 (temperature: $F_{2, 189} = 11.53$, $P < 0.001$; egg mass: $F_{1, 189} = 9.08$, $P = 0.002$). Because
308 SVL and body mass of hatchlings were positively correlated ($r = 0.691$, $P < 0.001$), and
309 they both decreased at higher temperature, body condition at hatching showed no
310 significant between-groups differences (ANCOVA with body mass as the dependent
311 variable and SVL at hatching and egg mass at oviposition as the covariates).

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

316 among temperature treatments, being higher for juveniles that came from warmer
317 incubation treatments (temperature: $F_{2, 156} = 42.63$, $P < 0.001$).

318 After three weeks of postnatal growth, the larger growth rates of juveniles coming
319 from eggs incubated at warmer temperatures (especially at 30 °C) allowed them to
320 overpass the size of their siblings incubated at 22 °C (Table 3). Thus, at an age of 21 days
321 they had compensated their initial disadvantage in both SVL (temperature: $F_{2, 156} = 35.42$,
322 $P < 0.001$) and body mass (temperature: $F_{2, 156} = 27.88$, $P < 0.001$), and they had reached
323 the highest body condition (Table 3) of the three temperature treatments (ANCOVA with
324 body mass as the dependent variable and SVL at hatching and egg mass at oviposition as
325 the covariates; temperature: $F_{2, 155} = 3.24$, $P = 0.042$). No significant between-group
326 differences in body size, mass or condition at the age of three weeks were found for area,
327 elevation, or any of the interaction effects examined, although lower elevation juveniles
328 were slightly larger and heavier than higher elevation ones.

329

330 *Embryo survival*

331 Of the 245 neonates that hatched successfully, 19 were not viable, including the one that
332 died shortly after hatching plus other 18 individuals that showed hampered mobility,
333 could not feed successfully, and died before the end of the experiment. These symptoms
334 were identical to those previously observed for this species when incubated at high
335 temperatures (32 °C; (Monasterio et al., 2011)). A log-linear model with survivorship of
336 embryos (from hatching to an age of 21 days) as the response variable, and area, altitude
337 and temperature as the design factors (χ^2 for the overall model = 14.33, $df = 20$, $P =$
338 0.814), included only an interaction between area and survival (partial association: $\chi^2 =$
339 21.29, $df = 1$, $P < 0.001$; Table 2), although the interaction between temperature and
340 survival was close to significance (partial association: $\chi^2 = 5.34$, $df = 2$, $P < 0.069$), with

341 survivorship decreasing as incubation temperature increased (survival rates of 0.97, 0.93,
342 and 0.86 for juveniles incubated at 22, 26, and 30 °C, respectively).

343 Differences among study sites in overall embryo survival (from eggs to a
344 postnatal age of 21 day) were highly significant (Table 2: $\chi^2 = 44.24$, $df = 3$, $P < 0.001$).
345 In all populations except Morcuera (i.e. Guadarrama 1800 m, where survivorship was
346 lowest), embryo survival was higher for eggs incubated at 22 °C than for those incubated
347 at 30 °C (Table 2). The difference between survival rates of embryos incubated at 22 and
348 30 °C was higher for the coolest site (Guadarrama, 2200 m) than for the other three
349 locations with higher and more similar soil temperatures ($F_{1,2} = 21.88$, $P = 0.043$). After
350 controlling for the effects of such difference, survival was significantly higher at 22 than
351 at 30 °C ($F_{1,2} = 40.80$, $P = 0.024$). In other words, embryo survival was lowest at the
352 worst quality site (Guadarrama 1800 m), and it was lower at 30 than at 22 °C, especially
353 at the coolest site (Guadarrama 2200 m).

354

355 *Variation of soil temperatures in the field*

356 A main effects ANOVA with soil temperature as the dependent variable and time of day,
357 month, area and elevation as categorical predictors explained 71.1% of the variance in
358 soil temperatures ($F_{27,28772} = 2,618.11$, $P < 0.001$), showing significant differences
359 between times of day (Fig. 3), months, elevations, and areas (contributions to R^2 of 0.575,
360 0.060, 0.042, and 0.033, respectively). Low temperatures at the high-elevation site in
361 Guadarrama produced a significant interaction between elevation and area that increased
362 overall R^2 to 0.748, indicating that altitudinal differences in the thermal environment
363 were more pronounced for Guadarrama than for Gredos. Differences among loggers were
364 also large, as shown by the high R^2 value (0.871) of the full factorial model with logger,
365 time of day, and month as categorical predictors. Moreover, ranges of daily temperatures

366 suggest a wide range of thermal opportunities at each of the four study sites (Guadarrama,
367 low-elevation, mean range of daily temperatures ± 1 SE: 22.8 ± 0.5 °C; Guadarrama,
368 high-elevation: 19.8 ± 0.5 °C; Gredos, low-elevation: 20.4 ± 0.4 °C; Gredos, high-
369 elevation: 22.9 ± 0.5 °C; $N = 75$ days in all cases). Because mean ranges were highest at
370 Guadarrama low-elevation and Gredos high-elevation, neither the main effects of site nor
371 altitude were significant in a two-way ANOVA, whereas their interaction was highly
372 significant (area: $F_{1,296} = 0.57$, $P = 0.452$; altitude: $F_{1,296} = 0.40$, $P = 0.530$; interaction:
373 $F_{1,296} = 32.07$, $P < 0.001$).

374

375 **Discussion**

376

377 Our results describe a scenario in which eggs and hatchlings from mothers captured at
378 low elevation areas showed reduced survivorship, which suggests that reproduction is
379 impaired at the lower limit of the elevational distribution range. Such impairment might
380 be partly counterbalanced by faster postnatal growth at lower elevations, leading to
381 increased female size and higher fecundity. However, interactions between the effects of
382 temperature and elevation were not significant either for incubation time, hatchling size,
383 hatchling mass, body condition, or growth rates, indicating a lack of elevational variation
384 in the thermal dependence of the phenotypic traits studied. Moreover, high temperatures
385 (above 30 °C), which were unfavourable for the success of high-elevation eggs, at least in
386 Guadarrama (Monasterio et al., 2011) and results of this study), were also detrimental for
387 low-elevation eggs. Remarkably, the great variety of soil temperatures available at all
388 sites examined suggests a wide range of thermal opportunities that is likely to keep the
389 intensity of selective pressures at the edge of the range far from being able to foster local
390 adaptation.

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

407 Although strictly speaking, and given our limited sample size (only two high and
408 two low-elevation sites), we can not rule out the importance of local site effects *per se*,
409 our data suggest that high temperatures are detrimental for eggs and hatchlings both at
410 core and low elevation areas, because the survival of propagules to an age of 3 weeks was
411 lowest at 30 °C in the four populations examined. Although the difference in survival
412 rates between propagules incubated at 22 and 30 °C was maximal at the highest and
413 coolest site (Guadarrama, 2200 m) and minimal at the warmest site (Guadarrama, 1800
414 m), this was not due to a higher hatching/survival rate of low-elevation eggs incubated at
415 higher temperature, but to a poorer survivorship of those incubated at low temperature. In

416 fact, survival rate was lowest (< 0.5) at the Guadarrama low-elevation site independently
417 of incubation temperature, whereas eggs from the Gredos high-elevation site hatched and
418 developed quite successfully at all incubation temperatures (rates within the range 0.91-
419 0.97). In a previous study of clutches from females captured at middle elevations (1850-
420 1950 m), Monasterio et al. (2011) found that increased incubation temperatures had a
421 negative effect on juvenile survival (38% viable juveniles at 32°C), and our results
422 confirm that this species is unable to cope with progressively warmer incubation
423 conditions ($> 30-32$ °C) within the thermal range encompassed in these studies (22-32°C).
424 We can therefore conclude that high incubation temperatures are detrimental for *I. cyreni*
425 also at the lower limit of its elevational range.

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

439 A possible explanation for the observed lack of variation is that females may be
440 able to compensate for clinal variation in thermal conditions along the elevational

441 gradient through nest-site selection (Doody, 2009; Huang & Pike, 2011). Accordingly,
442 adaptation to warmer temperatures in *I. cyreni* may be precluded by the great variety of
443 soil temperatures available at low-elevation sites, that should lower the intensity of
444 selective pressures near the edge of the range. This hypothesis is consistent with the fact
445 that, even with a low sample size of only four loggers per site, variation among loggers
446 was large, particularly at warmer times of day. This suggests that maternal manipulation
447 of hatchling phenotypes via nest site selection (Shine & Harlow, 1996) may indeed
448 hamper the evolution of physiological adaptations, providing another example of how
449 behavioral adjustments can buffer environmental variation and thereby reduce selective
450 pressures for evolutionary change (Huey, Hertz & Sinervo, 2003).

451 On the other hand, juveniles that came from eggs incubated at higher temperature
452 were able to counterbalance their smaller hatchling size by growing faster than those
453 incubated at cooler temperature (Table 3). Also, and more importantly, juveniles from
454 lower elevation areas grew faster than those from core populations. These results suggest
455 that in marginal populations selection could favour the genotypes that encode for faster
456 growth, as a way of compensating for their lower hatching success. Differences in
457 postnatal growth rates could explain, at least in part, why adult females were larger in
458 populations located close to their distributional limit. Lizards inhabiting lower elevations
459 could benefit from longer activity periods. This, combined with the potential for faster
460 growth rates evidenced by our common garden experiment, may promote larger sizes at
461 maturity (Du, Waner, Langkilde, Robbins & Shine, 2012). Moreover, their larger body
462 size should allow lower-elevation females to lay more eggs (and therefore heavier
463 clutches), which is another way to compensate the reduced hatching success of low-
464 elevation embryos (Du, Ji & Shine, 2005). In fact, the faster postnatal growth rates of
465 lower elevation hatchlings might aid to counterbalance the negative effects of high

466 incubation temperature on hatchling size near the lower limit of the distribution range of
467 this alpine species. Interestingly, a related pattern of elevational covariation was found in
468 Guadarrama for the lacertid *Psammodromus algirus*, in which the genotypes that encode
469 for fast growth occur in lowland habitats with low food availability, where lizards attain
470 small adult sizes and lay smaller clutches of larger eggs (Iraeta, Salvador & Díaz, 2013).
471 However, for *P. algirus* the fast-growing environment is the high elevation one (Iraeta et
472 al., 2006), located 300-400 m below the lower limit of *I. cyreni*'s elevational range. This
473 pattern of countergradient variation, which is found in *P. algirus* but not in *I. cyreni*,
474 supports the idea that temperate lacertids may be more able to adapt to cooler climates
475 than to warmer ones.

476 Reproductive traits were also distinctly different in the two mountain ranges
477 examined. The soil temperatures registered by loggers indicate that the thermal gradient
478 is steeper at Guadarrama, where lizards from the low-elevation site, besides showing the
479 worst performance levels of the four populations examined, are scarcer (Monasterio et al.,
480 2010a) and thermoregulate less effectively (Monasterio, Salvador, Iraeta & Díaz, 2009)
481 than at higher elevation. Given its harsher and more continental climate, Guadarrama
482 may have poorer conditions than Gredos in terms of productivity and associated prey
483 availability, which are important determinants of life-history variation in lacertid lizards
484 (Iraeta et al., 2006; Lorenzon, Clobert & Massot, 2001). According to our results,
485 hatching success was lower in Guadarrama, and body size of females was larger in
486 Gredos. However, and despite their smaller body size, females from Guadarrama laid
487 larger eggs than those from Gredos, even at the cost of a slight increase in the duration of
488 incubation, maybe to counterbalance their reduced hatching success. Previous studies
489 indicate that in lizards the survival advantage of large offspring is greater under
490 conditions of food shortage (Ferguson & Fox, 1984) and/or in drought years (Iraeta et al.,

491 2006; Sinervo, Doughty, Huey & Zamudio, 1992).

492 The reasons why reproductive success was so low for eggs from the Guadarrama
493 low elevation site, independently of incubation temperature, remain unclear. However, it
494 can be argued that reproductive failure may be caused by environmental constraints on
495 thermoregulatory abilities of gravid females (Schwarzkopf & Shine, 1991; Shine &
496 Harlow, 1993). It is well known that uterine retention is selectively advantageous in
497 montane reptiles because it allows developing eggs to be kept warm by their mother's
498 thermoregulatory behaviour, and thus to avoid detrimentally low nest temperatures that
499 hamper both hatching success and hatchling viability (Shine, 2002). In a comparative
500 study of egg retention in lacertid lizards, Braña et al. (1991) showed that the related
501 species *Iberolacerta monticola*, together with oviparous populations of *Zootoca vivipara*,
502 are the species with more advanced intrauterine embryogenesis, and also the ones that
503 reach highest elevations in northern Spain; however, there is no evidence of intraspecific
504 variability (Braña et al., 1991), and our results confirmed that incubation times were
505 almost identical at high and low elevation sites. On the other hand, it is also known that
506 early events of reptilian embryogenesis are involved in tissue differentiation and
507 organogenesis to ensure the production of viable phenotypes, whereas late development
508 is characterized by physiological changes and growth in size (Deeming & Ferguson,
509 1991). As a consequence, the hatching success and viability of *I. cyreni*'s eggs and
510 hatchlings may be more dependent on the thermoregulatory effectiveness of gravid
511 females than on the thermal properties of nesting sites (see Shine & Harlow, 1993, for an
512 experimental demonstration of the effects of maternal thermoregulation on offspring
513 viability in a viviparous scincid). Interestingly, previous studies have shown that *I. cyreni*
514 from Guadarrama thermoregulates less efficiently, probably due to a scarcity of
515 appropriate refuges such as large rocks, at lower elevation (Monasterio et al., 2010a;

516 Monasterio et al., 2009). In fact, forest belts may prevent the expansion of alpine lizards
517 towards lower elevations (Huang, Porter, Tu & Chiou, 2014) as a result of their low
518 thermal quality and reduced availability of refuges (Monasterio et al., 2009). Moreover,
519 the high success of eggs and hatchlings from the Gredos high elevation site,
520 independently of incubation temperature, is consistent with careful thermoregulation by
521 gravid females during the first weeks of embryonic development, that would be favoured
522 by high diurnal operative temperatures readily available at alpine environments (Shine,
523 Elphick & Barrott, 2003) and high cover of large rocks providing good-quality refuges
524 (Monasterio et al., 2010a).

525 In summary, our study demonstrates that at the lower limit of the distribution
526 range of *I. cyreni*, reproductive output of adult females is lower than at core, high-
527 elevation areas, and that no specific adaptations to higher temperatures have evolved in
528 the former ones, perhaps due to the availability of nesting sites with suitable thermal
529 conditions as elevation decreases along mountain slopes. Another non alternative
530 explanation for the lack of variation in thermal reaction norms could be gene swamping
531 caused by asymmetrical gene flow from core areas with cold-adapted genotypes to lower
532 and warmer areas (Sexton, McIntyre, Angert & Rice, 2009). This seems plausible for *I.*
533 *cyreni* because core and marginal populations occur continuously, separated by a
534 relatively short distance, along a steep environmental gradient. We could therefore
535 hypothesize that migration among highly connected populations across mountain slopes
536 could cause enough gene flow to prevent the fixation of genetic novelties that could be
537 favoured in warmer incubation environments. Thus, if the traits we studied are
538 genetically based, range limits could appear abruptly as a result of the inability of low-
539 elevation lizards to adapt to higher temperatures. Although the role of gene swamping in
540 shaping range limits requires careful study given the complexity of the interactions

541 involved, our results suggest it would be worthwhile testing this hypothesis, which has
542 recently fostered the debate on the evolution of range limits (Moeller, Geber & Tiffin,
543 2011; Paul, Seth & Angert, 2011).

544

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553

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694 Table 1. Mean values (\pm 1SE) for clutch size, mean egg mass, and clutch mass of
 695 clutches from two areas or mountain ranges (Guadarrama and Gredos) and elevations
 696 (low and high). Results of two-way ANOVA's are also shown.
 697

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

698

699

700 Table 2. Number of eggs from each of the four study sites that were incubated at each
 701 experimental temperature, number of eggs that succeeded to hatch (with hatching rate in
 702 parentheses), survivorship of hatchlings to an age of 21 days (with survival rate in
 703 parentheses), and overall embryo survival (survivorship from eggs to a post-natal age of
 704 21 days). Note that a total number of 12 hatchlings were separated from the rest of their
 705 cohort, due to logistical reasons, during the three-weeks period of postnatal growth. As a
 706 consequence, they were excluded from the rates shown in the last two columns.

707

Incubation temperature	Guadarrama 1800 m				Guadarrama 2200 m			
	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

Incubation temperature	Gredos 1800 m				Gredos 2100 m			
	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

708

709

710 Table 3. Mean values (\pm 1SE) for body size (SVL), mass, and condition (body mass
711 adjusted for SVL and egg mass at oviposition), both at hatching and at an age of 21 days,
712 classified according to elevation (low vs. high), incubation temperature, and area (i.e.
713 mountain range: Guadarrama vs. Gredos).
714

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

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716

717 **Figure legends**

718

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

722 **Figure 2.** Size and mass specific growth rates of low elevation (black circles) and high
723 elevation (white circles) juveniles hatched from eggs incubated at different temperatures.

724 **Figure 3.** Daily variation of mean (\pm 95 % CI) soil temperatures available during the
725 incubation period of *I. cyreni* in the four study sites.