


Physiological plasticity in lizard embryos exposed to high-altitude hypoxia

Gerardo A. Cordero¹  | Bea Angelica Andersson¹ | Jeremie Souchet² |
Gaëlle Micheli² | Daniel W.A. Noble³ | Eric J. Gangloff^{2,4} | Tobias Uller^{1*} |
Fabien Aubret^{2*}

¹Department of Biology, Lund University, Lund, Sweden

²Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis, Moulis, France

³Ecology & Evolution Research Centre, School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, NSW, Australia

⁴Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, Iowa, USA

Correspondence

Gerardo Antonio Cordero, Department of Biology, Lund University, Sölvegatan 37, SE-223 62 Lund, Sweden.

Email: antonio.cordero@biol.lu.se

*Tobias Uller and Fabien Aubret have shared senior authorship.

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Abstract

Coping with novel environments may be facilitated by plastic physiological responses that enable survival during environmentally sensitive life stages. We tested the capacity for embryos of the common wall lizard (*Podarcis muralis*) from low altitude to cope with low-oxygen partial pressure (hypoxia) in an alpine environment. Developing embryos subjected to hypoxic atmospheric conditions (15–16% O₂ sea-level equivalent) at 2,877 m above sea level exhibited responses common to vertebrates acclimatized to or evolutionarily adapted to high altitude: suppressed metabolism, cardiac hypertrophy, and hyperventilation. These responses might have contributed to the unaltered incubation duration and hatching success relative to the ancestral, low-altitude, condition. Even so, hypoxia constrained egg energy utilization such that larger eggs produced hatchlings with relatively low mass. These findings highlight the role of physiological plasticity in maintaining fitness-relevant phenotypes in high-altitude environments, providing impetus to further explore altitudinal limits to ecological diversification in ectothermic vertebrates.

1 | INTRODUCTION

The geographic expansion of species often involves colonization of novel environments featuring conditions rarely encountered or not experienced in the recent history of populations (Schluter, 2000; Aubert, 2013). This requires organisms to cope with conditions to which they are not expected to be adapted, which may be particularly challenging to developing offspring. Assuming the occurrence of heritable variants suited to sub-optimal conditions, natural selection should favor the persistence of individuals best able to cope with environmental stress during sensitive life stages (Schmalhausen, 1949; Waddington, 1957; Levins, 1968; West-Eberhard, 2003; Atkinson, & Thorndyke, 2001). On the other hand, plastic physiological responses might mitigate environmental stress, thereby promoting offspring survival and influencing the direction of subsequent evolution of colonizing populations (Hammond, Cardullo, & Ghalambor, 2006;

Atkinson, & Thorndyke, 2001; Ghalambor, McKay, Carroll, & Reznick, 2007; McNab, 2002).

Physiological traits are well suited to examine phenotypic shifts in response to new environments (Hammond et al., 2006; Storz, Scott, & Cheviron, 2010; Storz, 2016; Piersna, & van Gils, 2011; Rezende, Gomes, Ghalambor, Russell, & Chappell, 2005; Chown et al., 2010). Indeed, physiological processes often comprise evolvable reaction norms (Mueller, Eme, Burggren, Roghair, & Rundle, 2015), such as in responses to altered atmospheric oxygen (O₂) conditions in stressful high-altitude environments (Rezende et al., 2005; Hammond et al., 2006; Bouverot, 1985; Powell, & Hopkins, 2010; Storz et al., 2010; Beall, 2006). In addition to low temperature, the low partial pressure of O₂ (hypoxia) at high altitudes (> 2,000 m above sea level [ASL]) renders embryonic development challenging, as fewer O₂ molecules may be available to convert egg energy into tissue (Vleck, & Vleck, 1996; Noble, 1991; Wangensteen, Rahn, Burton, & Smith, 1974; Rahn, Carey,

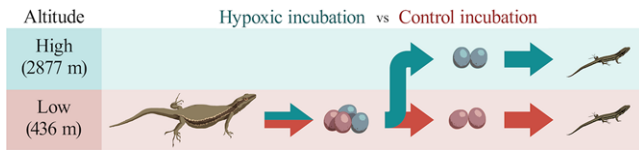


FIGURE 1 Experimental eggs were collected from gravid females sampled from low-altitude *Podarcis muralis* populations in the foothills of the Pyrenees (300–500 m above sea level). Within 48 hr of oviposition, clutches of eggs were evenly split in two batches of eggs (half-clutches). For each clutch, one half-clutch was transplanted to a high-altitude laboratory at 2,877 m where atmospheric PO_2 was 70–73 kPa (equivalent to 15–16% O_2 at sea level); while the second half-clutch underwent incubation at low altitude (436 m; control) where PO_2 (93–96 kPa; 20.8% O_2) approximated sea level conditions (101.3 kPa; 20.95% O_2). *Illustration credit:* Bea Angelica Andersson [Color figure can be viewed at wileyonlinelibrary.com]

Balmas, Bhatia, & Paganelli, 1977; Carey, Larson, Hoyt, & Bucher, 1984; Bouverot, 1985; Monge, & Leon-Velarde, 1991; Leon-Velarde, & Monge, 2004). Thus, altitudinal hypoxia is thought to impose a limit on the geographic distribution of vertebrate species (Powell, & Hopkins, 2010; Storz et al., 2010).

In birds, the suppression of embryonic metabolism is a common and effective means of reducing O_2 demand while undergoing egg incubation at high altitude (Monge, Leon-Velarde, & Gomez de la Torre, 1988; Leon-Velarde, & Monge, 2004; Lague, Chua, Farrell, Wang, & Milsom, 2016). This suppression is often associated with reduced heart rate and slower growth (Beattie, & Smith, 1975; Wangenstein et al., 1974; Leon-Velarde, & Monge, 2004). Artificial selection and comparison of high- versus low-altitude avian populations revealed that these physiological responses can evolve (Beattie, & Smith, 1975). Similarly, physiological processes that enhance O_2 transport have the potential to evolve in birds (Rahn et al., 1977; Hammond et al., 2006; Storz, 2016; Monge, & Leon-Velarde, 1991). Whether responses to cope with hypoxia in high-altitude environments are general to non-avian reptiles is unclear (McNab, 2002; Powell, & Hopkins, 2010), though laboratory manipulations at low altitude would support this assumption (Kam, 1993; Warburton, Hastings, & Wang, 1995; Andrews, 2002; Du, Thompson, & Shine, 2010; Eme, Altimiras, Hicks, & Crossley, 2011; Harrison, Shingleton, & Callier, 2015; Liang, Sun, Ma, & Du, 2015; Smith, Telemeco, Angilletta, & VandenBrooks, 2015; Cordero, Karnatz, Svendsen, & Gangloff, 2017; Crossley, Ling, Nelson, Gillium, Conner et al., 2017).

Oviparous reptiles display little or simple parental care (While, Halliwell, & Uller, 2015), and most embryonic development will occur after eggs are laid in unattended subterranean nests (Packard, & Packard, 1988; Ackerman, & Lott, 2004). As a result, embryos must adjust to fluctuations in nest temperature, moisture, and gas concentrations (e.g., acute hypoxia) (Packard, & Packard, 1988; Ackerman, & Lott, 2004; Deeming, & Thompson, 1991). Such stress-induced physiological responses might promote offspring survival when the partial pressure of O_2 is consistently low (i.e., chronic hypoxia), contributing to the invasion of alpine ecosystems in response to climate warming (Storz et al., 2010; Ortega, Mencia, & Perez-Mellado, 2016) and, perhaps,

rapid adaptive evolutionary responses to life at high altitude (Rezende et al., 2005).

We incubated eggs from low-altitude populations of the common wall lizard (*Podarcis muralis*) to test the hypothesis that embryos employ metabolic adjustments known to facilitate survival in hypoxic conditions. We predicted reduced growth via metabolic suppression, leading to decreases in hatchling size, mass, and energy content in hypoxia at 2,877 m ASL. We also tested the hypothesis that cardiovascular compensatory changes induce heart enlargement in hatchlings (Crossley, & Burggren, 2009).

2 | MATERIALS AND METHODS

2.1 | Egg collection and experimental design

Podarcis muralis is widely distributed across southern and central Europe from sea level to approximately 2,500 m ASL (Speybroeck, Beukema, Bok, & Van Der Voort, 2016). Breeding occurs during April–June with females producing up to three clutches of eggs (Speybroeck et al., 2016). In May–June 2016, we captured gravid *P. muralis* females in the foothills of the French Pyrenees (Department of Ariège; 300–500 m ASL) and transported them to the laboratory of Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis (436 m ASL; 38.898556 N, – 77.037852 E). The partial pressure of O_2 (PO_2) at this locality was 93–96 kPa, which translates to a sea level atmospheric O_2 concentration of 20.8% (Bouverot, 1985).

Females were housed individually in plastic enclosures (26 × 38 × 23 cm) with heat lamps, water, and sand boxes for nesting. Food was provided ad libitum. Sand boxes were visually inspected daily for signs of nesting, in which case eggs were immediately removed, weighed, labeled, and individually placed in plastic cups filled with moist vermiculite (1:5 water to vermiculite). Eggs from 22 clutches were then transferred to an environmental chamber set to a constant 24°C (100% air humidity).

We implemented a split-clutch design with eggs assigned to low altitude (control) and high altitude (hypoxia) treatments (Figure 1). The group assignment of the first egg in a clutch was randomly chosen with subsequent eggs alternating between treatment groups. Within 48 hr of oviposition, hypoxia eggs were transported to the Observatoire Midi-Pyrénées in Pic du Midi de Bigorre (2,877 m ASL; 38.898556 N, – 77.037852 E). Atmospheric conditions in this alpine environment were hypoxic as PO_2 was 70–73 kPa (sea level equivalent: 15–16% O_2) (Bouverot, 1985). Hypoxia eggs were otherwise incubated under the same temperature and moisture laboratory regime as in the control (normoxia) treatment in Moulis (24°C; 100% air humidity).

2.2 | Embryonic metabolism

Embryonic heart rate is a reliable indicator of metabolism and cardiovascular function in non-avian reptiles (Crossley, & Burggren, 2009). Thus, we measured heart rate weekly ($6.8 \text{ d} \pm 2 \text{ SD}$) during egg incubation in *P. muralis* using the Buddy® digital egg monitor (MK2; Avitronics, Cornwall, UK) (Aubret, Tort, & Blanvillain, 2013). Briefly, Buddy® directs infrared light onto the egg and registers the net amount of

infrared light absorbed by blood, thus blood flow changes caused by heart beats can be used to estimate heart rate (Lierz, Gooss, & Hafez, 2006). Eggs were briefly (≤ 5 min) placed in the digital egg monitor to control for potential temperature changes owing to exposure to infrared sensors (Sartori, Taylor, Abe, & Crossley, 2015). Egg temperature did not increase and ambient temperature remained stable ($\sim 24^\circ\text{C}$) during sampling.

Carbon dioxide (CO_2) production ($\dot{V}\text{CO}_2$) was measured during the last third of the egg incubation period. In reptiles, physiological compensation for reduced growth rate might be employed during this time (discussed in Spencer and Janzen (2011)). Following a stop-flow respirometry protocol (Lighton, 2008; Lighton, & Halsey, 2011), 16 eggs drawn from eight split clutches (control: $N = 8$; hypoxia: $N = 8$) were individually placed in 65-ml sealed glass jar chambers with moist vermiculite (50 ml) held at a constant 24°C , for example, Cordero et al. (2017). The egg chamber was then flushed and sealed for 180 min, incurrent air flow (200 ml/min; from chamber to respirometer) was restored and water scrubbed from air using drierite desiccant (Hammond Drierite, Xenia, Ohio, USA). Carbon dioxide was measured using the FC-10 analyzer (Sable Systems International, Las Vegas, Nevada, USA). Using Sable Systems ExpeData software, CO_2 production rate ($\dot{V}\text{CO}_2$ in ml/hr corrected for water vapor pressure of excurrent air) was calculated by integrating the change in $\dot{V}\text{CO}_2$ instantaneous level over the period the chamber was sealed (Lighton, & Halsey, 2011). Repeated respirometry measurements alternated (within 24 hr) between control and hypoxia eggs on days 38, 45, and 52 post-oviposition. Heart rate was measured immediately before each trial.

2.3 | Hatchling morphology and physiology

Field sampling and experimental protocols were approved by the Préfecture de l'Ariège (Arrêté #09-2016-01). Following guidelines for the use of live reptiles in laboratory research (HACC, 2004), hatchlings were humanely euthanized by blunt force trauma to the head (AVMA, 2013). This method has proven effective for experimental reptiles (reviewed in Nevarez, Strain, da Cunha, & Beaufre, 2014). Hatchling carcasses were weighed before preserving in 10% buffered formalin according to standard methodology for preservation of reptile specimens (McDiarmid, Foster, Guyer, Gibbons, & Chernoff, 2012).

The ventral surface of preserved hatchlings was imaged using a scanner (CanoScan LiDE 120) and snout-vent length (SVL) was measured in ImageJ 1.5 (U.S. National Institutes of Health). Hearts were then excised and dried to a constant mass by placing on absorbent paper while exposed to room temperature (25°C) air for 1 hr. Only the ventricular region of dissected hearts was examined. Carcasses were dried to a constant mass in an oven set to 60°C for 24 hr. All dry tissue measurements were recorded to the nearest 0.00001 g using an analytical balance (R200D Sartorius Research, Germany).

To estimate the energetic cost of hatchling tissue production, dry hatchling carcasses were subjected to standard calorimetric procedures (Lighton, 2008), using an oxygen bomb calorimeter (Parr 6200 Calorimeter). Energy density of hatchling tissue was initially estimated by pooling samples by clutch and treatment. The mean energy density

per treatment (control: $22.005 \text{ kJ/g} \pm 0.208 \text{ SE}$, $N = 20$; hypoxia: $21.912 \text{ kJ/g} \pm 0.158 \text{ SE}$, $N = 20$) was multiplied by dry hatchling mass to obtain per individual values of total hatchling energy content (Vitt, 1978). Note that yolk sacs were no longer herniated in hatchlings, thus energetic estimates are inclusive of any potentially internalized residual yolk mass. This potential source of variation was likely negligible because energy content values were within the expected range for *P. muralis* hatchlings that had yolk sacs removed (Ji, & Braña, 1999).

2.4 | Statistical analyses

Treatment effects on physiology and morphology were tested using mixed-effect linear and non-linear models (Zuur, Ieno, Walker, Saveliev, & Smith, 2009). Incubation duration, hatchling mass (wet and dry), SVL, dry heart mass, and hatchling energy content were analyzed as response variables in univariate models. Initial egg mass was entered as a covariate with clutch of origin as a random effect. Survival probability (i.e., hatching success) was tested using a generalized linear mixed effect model (with a Bernoulli error distribution) with initial egg mass as a covariate and clutch of origin as a random effect.

Dry heart mass models included dry hatchling mass to control for potential body mass effects. We also used Pearson's correlation tests to explore the relationship of dry heart mass versus heart rate (averaged across egg incubation) and hatchling water content. Decreased heart rate is generally associated with ventricular enlargement (heart hypertrophy) (Crossley, & Burggren, 2009), and body fluid mass should correlate with increased ventricular stroke volume caused by hypertrophy (Faber, Green, & Thornburg, 1974; Wagman, Hu, & Clark, 1999; Convertino, 1991). Models for heart rate and CO_2 production included an AR1 (autoregressive order of 1) covariance structure that accounted for temporal autocorrelation due to repeated measures. Heart rate was fitted with a third-order polynomial model according to expected heart rate trends in embryos (Burggren, & Warburton, 1994).

Mixed-effect models were evaluated with type III sums of squares analysis of variance (ANOVA), with main effects considered statistically significant if $P < 0.05$. Factors with $P < 0.10$ and their interactions were retained in final models. Models were validated by examining plots of residual distributions and residual versus fitted values (Zuur et al., 2009). The residuals for all models were normally distributed (Shapiro-Wilk's tests, $P > 0.10$), with the exception of incubation duration and initial egg mass. Thus, to meet model assumptions, analyses on incubation duration and initial egg mass were performed on \log_{10} -transformed data (Zuur et al., 2009). Note that initial egg mass for control ($0.298 \text{ g} \pm 0.010 \text{ SE}$, $N = 50$) versus hypoxia ($0.314 \text{ g} \pm 0.010 \text{ SE}$, $N = 52$) treatments differed at the onset of experimentation (ANOVA: $F_{1,78} = 4.43$, $P = 0.038$). We therefore included initial egg mass (\log_{10} -transformed) as a covariate in most analyses on hatchling traits, which also accounted for variation in maternal energetic investment. To interpret main effects in the presence of these interactions, model inputs were centered and standardized (mean of zero and unit standard deviation) and models were compared with likelihood ratio tests (Schielzeth, 2010). Analyses were conducted using the *lme4* package of the R programming language (R Development Core Team, 2017).

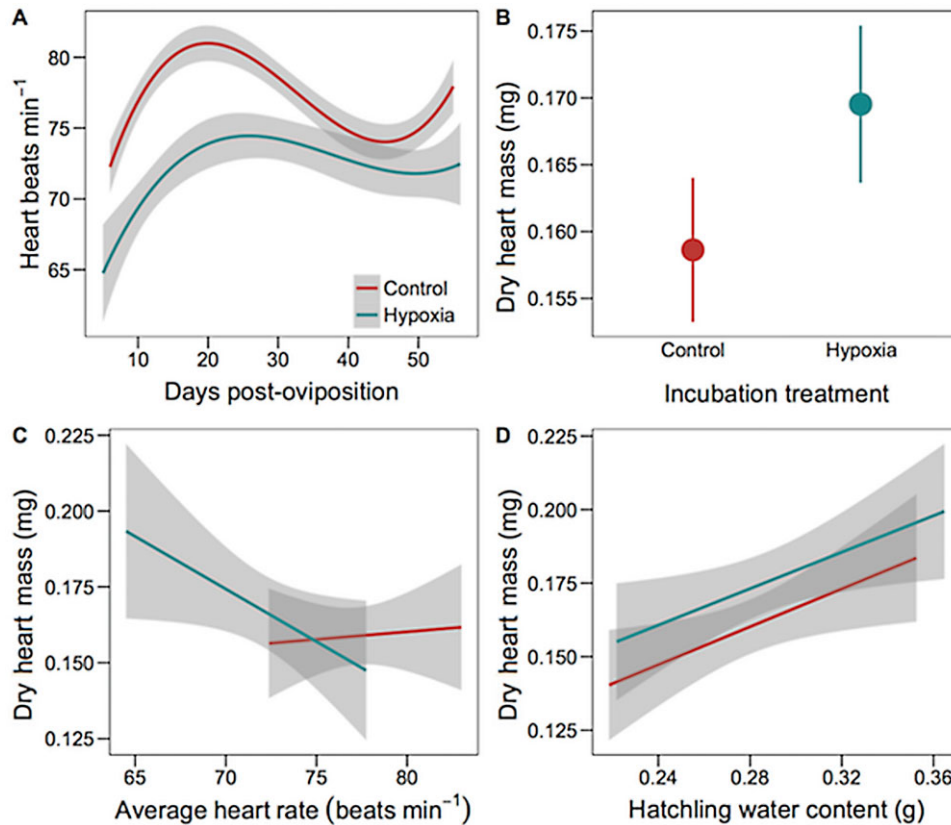


FIGURE 2 Embryonic *Podarcis muralis* from control (low altitude) and hypoxia (high altitude) groups differed in heart rate (A). Dry heart mass (covariate-adjusted means \pm SE) was slightly reduced in hypoxia hatchlings (B). Dry heart mass was negatively correlated with embryonic heart rate (averaged across egg incubation) in hypoxia but not in control hatchlings (C). There was a positive correlation of dry heart mass and hatchling water content in both groups (D); shaded regression intervals represent the standard error of the fit. [Color figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

3.1 | Embryonic metabolism

Embryos from the hypoxia group exhibited lower heart rate values (beats per minute) during egg incubation than embryos from the control group (ANOVA: $F_{1,100} = 30.8$, $P < 0.0001$; Figure 2A). Dry heart mass was lower in control hatchlings, although this did not reach statistical significance (LRT: $\chi^2 = 2.23$, $df = 1$, $P = 0.134$) (Figure 2B). Dry heart mass was negatively correlated with heart rate (averaged across egg incubation) in hatchlings from the hypoxia treatment ($r = -0.33$, $P = 0.049$), but there was no correlation in the control group ($r = 0.05$, $P = 0.753$) (Figure 2C). Dry heart mass was positively correlated with hatchling water content in hypoxia and control groups (control: $r = 0.38$, $P = 0.019$; hypoxia: $r = 0.43$, $P = 0.022$) (Figure 2D).

Embryos from hypoxia and control groups differed in CO_2 production (ANOVA; $F_{1,14} = 7.16$, $P = 0.018$). Treatment and sampling intervals interacted such that embryos of the hypoxia treatment initially displayed a lower mean for CO_2 production (days 38.5 and 45.5 post-oviposition), but exceeded that of the control group when near hatching (day 52.5, Figure 3A; treatment by day interaction: $F_{1,30} = 6.54$, $P = 0.015$). During CO_2 sampling intervals, heart rate in hypoxia embryos remained lower than in the control group ($F_{1,14} = 22.1$, $P = 0.0003$) (Figure 3B).

3.2 | Hatching success, hatchling energy content, and body size

Raw means for hatchling success, incubation duration, and morphological and physiological variables are listed in Table 1. Although hatching success was marginally affected by initial egg mass, there was a treatment by initial egg mass interaction that approached significance (LRT: $\chi^2 = 3.828$, $df = 1$, $P = 0.050$). This interaction contributed to a slight decrease in hatching success in the Hypoxia treatment that featured larger initial egg mass (Tables 1 and 2). Both hatchling dry mass (LRT: $\chi^2 = 12.7$, $df = 1$, $P = 0.0003$) and total energy content (LRT: $\chi^2 = 4.43$, $df = 1$, $P = 0.035$) were significantly affected by the interaction between incubation treatment and egg mass, such that lizards from large eggs were lighter at hatching if incubated under hypoxic conditions (Figure 4A and B and Table 2). In contrast, hypoxic conditions did not affect incubation duration, wet hatchling mass, or SVL (Table 2).

4 | DISCUSSION

Developing *P. muralis* embryos exposed to hypoxic atmospheric conditions at 2,877 m ASL exhibited physiological adjustments that mirrored typical acclimation responses in vertebrates. These included:

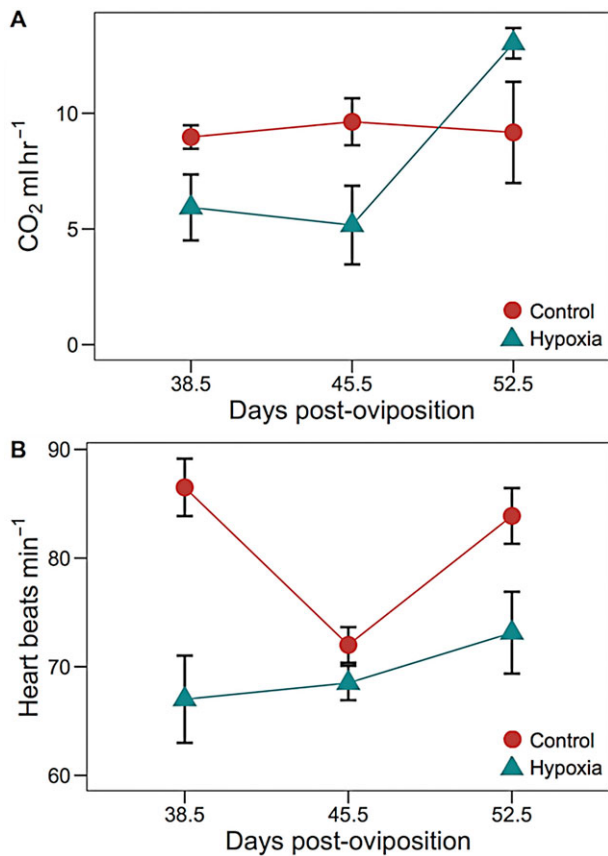


FIGURE 3 Embryonic *Podarcis muralis* from control (low altitude; $N = 8$) and hypoxia (high altitude; $N = 8$) groups differed in CO_2 production rate (A) and heart rate (B) during the last third of the egg incubation period; means \pm SE are displayed. The marked increase in $\dot{V}\text{CO}_2$ (A), relative to heart rate (B), in hypoxia embryos on day 52.5 post-oviposition is indicative of a hyperventilatory response [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Hatching Success and Raw Morphological and Physiological Means (\pm SE) for Hatching *Podarcis muralis* Incubated in Low-Altitude (Control) versus High-Altitude (Hypoxia) Environments

	Control	Hypoxia
Hatchling success	88%	77%
Initial egg mass (g)	0.294 \pm 0.007 $N = 50$	0.312 \pm 0.008 $N = 52$
Incubation duration (d)	60.045 \pm 0.315 $N = 43$	60.682 \pm 0.273 $N = 38$
Snout-vent length (cm)	2.282 \pm 0.015 $N = 43$	2.298 \pm 0.017 $N = 40$
Wet hatchling mass (g)	0.327 \pm 0.006 $N = 43$	0.334 \pm 0.007 $N = 38$
Dry hatchling mass (g)	0.053 \pm 0.001 $N = 42$	0.052 \pm 0.001 $N = 35$
Dry Heart mass (mg)	0.158 \pm 0.005 $N = 42$	0.167 \pm 0.006 $N = 35$
Total hatchling energy content (kJ)	1.149 \pm 0.027 $N = 38$	1.133 \pm 0.026 $N = 35$

TABLE 2 Estimates and 95% Profiled Confidence Intervals from Mixed Effects Models on Hatching Traits

Variable	Estimate	2.5% CI	97.5% CI
Hatching success ($N_T = 102, N_C = 23$) (log odds)			
Intercept	-4.255	-11.016	-2.049
Treatment (hypoxia)	1.519	0.008	3.496
Egg mass (log scale)	0.054	-2.213	1.786
Treatment \times initial egg mass	-1.706	-3.948	0.003
Incubation duration (log ₁₀ d) ($N_T = 81, N_C = 21$)			
Intercept (log scale)	4.096	4.083	4.11
Treatment (Hypoxia)	0.004	-0.004	0.013
Egg mass (log scale)	-0.005	-0.011	0.002
Dry mass (mg) ($N_T = 62, N_C = 21$)			
Intercept	52.992	50.154	55.749
Treatment (hypoxia)	-2.37	-4.688	-0.077
Egg mass (log scale)	5.079	3.113	7.107
Treatment \times initial egg mass	-4.886	-7.436	-2.335
Wet mass (g) ($N_T = 77, N_C = 21$)			
Intercept	0.332	0.315	0.349
Treatment (hypoxia)	0.002	-0.011	0.013
Egg mass (log scale)	0.014	0.002	0.027
Treatment \times initial egg mass	-0.013	-0.025	0
Dry heart mass (mg) ($N_T = 66, N_C = 22$)			
Intercept	0.159	0.148	0.169
Treatment (hypoxia)	0.011	-0.003	0.025
Dry mass	0.007	0.001	0.014
Snout-vent length (cm) ($N_T = 79, N_C = 21$)			
Intercept	2.29	2.261	2.32
Treatment (hypoxia)	-0.007	-0.045	0.03
Egg mass (log scale)	0.049	0.024	0.074
Energy content (kJ) ($N_T = 70, N_C = 21$)			
Intercept	1.166	1.111	1.219
Egg mass (log scale)	0.108	0.064	0.154
Treatment (hypoxia)	-0.056	-0.109	-0.004
Treatment \times initial egg mass	-0.062	-0.119	-0.004

All continuous predictor variables are centered at zero and normalized to unit standard deviation. Initial egg mass was not included in the final model on heart mass, as it was not statistically significant ($P > 0.10$). Bolded estimates are significant ($P < 0.05$), whereas italics is a $P < 0.10$. N_T , total number of observations; N_C , total number of clutches.

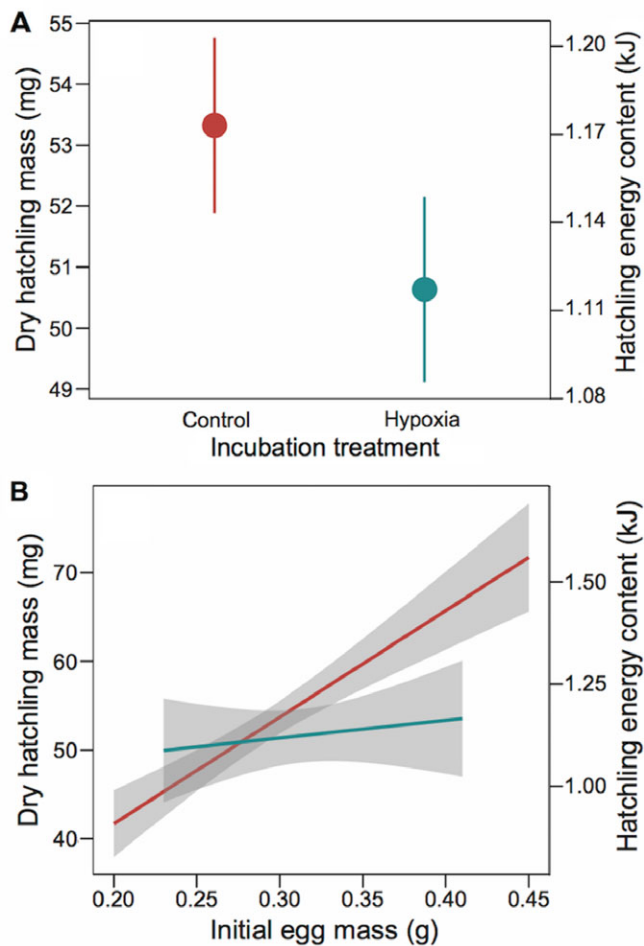


FIGURE 4 The relationships of dry hatchling mass, hatchling energetic content, and initial egg mass were altered in *Podarcis muralis* subjected to high-altitude hypoxia (A and B). Means adjusted for initial egg mass are displayed (range bars and shaded regression intervals = \pm SE) [Color figure can be viewed at wileyonlinelibrary.com]

(i) suppressed embryo metabolism, as indicated by reduced heart rate (Laughlin, 1978; Crossley, & Altimiras, 2005), (ii) elevated CO_2 production, that is, hypoxia-induced hyperventilation (Peacock, 1998; Powell, & Hopkins, 2010; Bouverot, 1985; Storz et al., 2010), and (iii) some evidence for cardiac hypertrophy, that is, heart enlargement (Du et al., 2010; Crossley, & Burggren, 2009). These physiological responses supported our predictions to some extent. However, contrary to what we expected, SVL and incubation duration were comparable to siblings that underwent egg incubation in ancestral atmospheric conditions (i.e., low-altitude environments), whereas effects of hypoxia on mass depended on the initial egg mass. Furthermore, we found that the effects of egg resources on hatchling traits were moderated by hypoxic incubation (more below).

Hypoxic environments suppressed embryonic metabolism in *P. muralis*, which is a common physiological response to hypoxia in vertebrates (Laughlin, 1978; Monge, & Leon-Velarde, 1991; Crossley, & Burggren, 2009). Hypoxia is expected to affect ATP demand and supply pathways, which ultimately decrease cellular respiration rates by downregulating ion-pumping and protein synthesis (Hochachka, Buck, Doll, & Land, 1996; Bickler, & Buck, 2007). This

common homeostatic response ensures survival without necessarily compromising embryonic development if O_2 delivery to tissues is enhanced (Crossley, & Burggren, 2009). Our results corroborate this expectation, though we did not directly measure compensatory biochemical changes in blood, for example enhanced O_2 affinity to hemoglobin (Storz et al., 2010; Storz, 2016). Still, we demonstrated that hatchling heart mass was negatively correlated with embryonic heart rate in *P. muralis* incubated under hypoxic conditions, suggesting that suppressed metabolism coincided with predicted cardiovascular alterations that enhance blood volume and circulation (discussed below).

While we provide evidence of suppressed metabolism throughout incubation, we did observe a spike in CO_2 production without a concomitant increase in heart rate when near hatching at high altitude. This might have been the result of excess arterial CO_2 that accumulated as a consequence suppressing metabolism during development in hypoxia (see Wei et al., 2007). Whether this hyperventilatory response in *P. muralis* was caused by compensatory growth in hypoxia is difficult to ascertain, as embryos might have initiated the transition from chorioallantoic gas exchange to air breathing (i.e., lung use) in preparation for hatching (Baumann, 1984; Thompson, 2007). Thus, pulmonary hyperventilation, a well-known acclimation response to high-altitude hypoxia (Bouverot, 1985; Peacock, 1998; Powell, & Hopkins, 2010; Swenson, & Bärtsch, 2014), probably reflected elevated $\dot{V}\text{CO}_2$ in pre-hatching *P. muralis*. Specifically, hypoxia will stimulate arterial chemoreceptors that promote the release of CO_2 (reviewed in Storz et al. (2010)), enabling maintenance of normal blood pH and O_2 in post-embryonic life stages at high altitude (reviewed in Bouverot (1985)).

Across many birds and reptiles, hypoxia has been shown to reduce hatchling size, mass, and in some cases, incubation duration (reviewed in Hempleman, Adamson, and Bebout (1993); Kam (1993); Leon-Velarde and Monge (2004); Du et al. (2010); Cordero et al. (2017); Crossley et al. (2017)). Altered body composition and lower growth rates are predicted from metabolic suppression because mitosis will be slowed (Douglas et al., 2005; Harrison et al., 2015), which may lead to extended incubation duration under chronic hypoxia (Sun, Wang, Pike, Liang, & Du, 2014). Indeed, *P. muralis* had lower dry mass and total energy content, suggesting that the conversion from yolk to tissue was affected by hypoxia. Even so, these changes did not elicit the expected shift in skeletal size (SVL) and incubation duration, suggesting that total cardiac output was similar to in low altitude if larger hearts afforded greater ventricular stroke in hypoxia (Burggren, & Warburton, 1994; Crossley, & Burggren, 2009; Warburton et al., 1995). In general, developing animals, including reptiles (see Crossley et al. (2017)), may match mass-specific metabolic demands to survive and grow in hypoxic incubation environments (Harrison et al., 2015).

We also showed that *P. muralis* incubated in hypoxia did have a slight increase in total hatchling water content, which may compensate for any tissue-specific changes. An increase in total body water (hypervolemia) is a common cardiovascular response associated with physiological stress and may require a larger heart to pump out more blood (i.e., greater stroke volume) (Gregg, & Wiggers, 1933;

Convertino, 1991; Wagman et al., 1999; Faber et al., 1974). Even though hypoxia induced slightly larger dry heart mass, we did not observe a strong response. To test these hypotheses, future work should address how cellular metabolism, including blood circulation and biochemistry, are affected in embryos exposed to hypoxic conditions. Note that normal growth in *P. muralis* might have simply been able to ensue because hypoxic treatments were not extreme, as suggested by montane lizards that successfully develop at < 10% O₂ (Andrews, 2002). Still, we demonstrated physiological strategies that might be employed to achieve this.

The effect of hypoxia on mass (dry) and energy content of embryos depended on the initial egg mass. *P. muralis* embryos from larger eggs under normal developmental conditions were able to assimilate more energy from yolk reserves than those from smaller eggs, but this relationship was constrained in siblings developing under hypoxic conditions. This response is consistent with lower energy utilization of embryos under hypoxia, suggesting that less energy could be converted into tissue to begin with. In fact, hatchling energy content in the hypoxia treatment was lower than expected for the given incubation temperature (Ji, & Braña, 1999). Of potential interest would be to test how *P. muralis* respond to hypoxia across diverse thermal regimes, as developmental temperatures may set the tolerance limit for low O₂ availability in lizard species of alpine ecosystems (e.g., *Sceloporus*; Smith et al., 2015).

Whether physiological adjustments that promote successful development in hypoxia-incubated embryos affect post-hatching performance and survival is as of yet unclear (but see Wearing, Conner, Nelson, Crossley, and Crossley (2017)). However, constraints on yolk utilization, and hence hatchling size, may have important fitness consequences (Sinervo, 1993; Warner, 2014). In alligators, laboratory-simulated hypoxia has been shown to cause a reduction in both embryonic and post-hatching growth rates (Owerkovicz, Eelsey, & Hicks, 2009; Crossley, & Altimiras, 2005) and may even compromise cognitive capacity in some hatchling lizards (Sun et al., 2014). As adults, reptiles are expected to cope well with high-altitude hypoxia owing to their low basal metabolic rates (McNab, 2002; Jackson, 2007), though empirical evidence to test this generalization is lacking (Powell, & Hopkins, 2010). Future work that examines the impact of hypoxia on long-term fitness traits will be important in establishing whether this is the case.

In addition to the general physiological responses that are likely to explain the ability of embryos to respond functionally to high-altitude hypoxia, low-altitude populations may also be adapted to acute hypoxia (e.g., during flooding of nests (Deeming, & Thompson, 1991; Ackerman, & Lott, 2004)) (Rezende et al., 2005). Moreover, the oviparous ancestor of modern lizards evolved in an atmosphere with 15–16% O₂ at sea level (Huey, & Ward, 2005). If so, resilience to hypoxia might be facilitated by the activation of latent physiological variation inherent to most reptiles. Although we did not sample high-altitude *P. muralis*, we succeeded in demonstrating that the capacity to cope with hypoxia already exists in low-altitude populations. This is foundational to future research aiming to directly compare the capacity of physiological responses across altitudinal gradients. To the best of our knowledge, our study is the first to examine physiologi-

cal responses to high-altitude hypoxia in situ in developing non-avian reptiles.

5 | CONCLUSIONS

Collectively, our findings support the hypothesis that plastic physiological responses to high-altitude hypoxia may facilitate the maintenance of fitness-related phenotypes in *P. muralis*. Although potential trade-offs and effects of other environmental parameters warrant further examination, we propose that cardiovascular and metabolic plasticity in embryos should facilitate altitudinal range expansion in *P. muralis*, and other non-avian reptiles, in response to climate warming.

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ORCID

Gerardo A. Cordero  <http://orcid.org/0000-0002-9137-1741>

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