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Embryonic and juvenile snakes (*Natrix maura*, Linnaeus 1758) compensate for high elevation hypoxia via shifts in cardiovascular physiology and metabolism

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Abstract

The colonization of novel environments requires a favorable response to conditions never, or rarely, encountered in recent evolutionary history. For example, populations colonizing upslope habitats must cope with lower atmospheric pressure at elevation, and thus reduced oxygen availability. The embryo stage in oviparous organisms is particularly susceptible, given its lack of mobility and limited gas exchange via diffusion through the eggshell and membranes. Especially little is known about responses of Lepidosaurian reptiles to reduced oxygen availability. To test the role of physiological plasticity during early development in response to high elevation hypoxia, we performed a transplant experiment with the viperine snake (*Natrix maura*, Linnaeus 1758). We maintained gravid females originating from low elevation populations (432 m above sea level [ASL]—normoxia) at both the elevation of origin and high elevation (2877 m ASL—extreme high elevation hypoxia; approximately 72% oxygen availability relative to sea level), then incubated egg clutches at both low and high elevation. Regardless of maternal exposure to hypoxia during gestation, embryos incubated at extreme high elevation exhibited altered developmental trajectories of cardiovascular function and metabolism across the incubation period, including a reduction in late-development egg mass. This physiological response may have contributed to the maintenance of similar incubation duration, hatching success, and hatchling body size compared to embryos incubated at low elevation. Nevertheless, after being maintained in hypoxia, juveniles exhibit reduced carbon dioxide production relative to oxygen consumption, suggesting altered energy pathways compared to juveniles maintained in normoxia. These findings highlight the role of physiological plasticity in maintaining rates of survival and fitness-relevant phenotypes in novel environments.

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KEYWORDS

cardiovascular physiology, developmental plasticity, embryonic metabolism, high elevation hypoxia, juvenile metabolism, maternal effect, respirometry

1 | INTRODUCTION

Environments experienced during early developmental stages can have profound consequences for juvenile and adult phenotypes (Bodensteiner, Agudelo-Cantero et al., 2021; Lindström, 1999; Mousseau & Dingle, 1991; Mousseau & Fox, 1998; Abdel-Tawwab et al., 2019; O'Dea et al., 2019). Thus, the physiological and behavioral responses of early life history stages to environmental variation have important fitness consequences for individuals and can determine the fate of populations (Liu et al., 2022; Sun et al., 2021). Embryos respond to variation in abiotic nest conditions, such as temperature (Booth, 2018; Noble et al., 2018; Refsnider et al., 2019; While et al., 2018) and water availability (Packard et al., 1980; Gutzke & Packard, 1987; Du & Shine, 2008; Bodensteiner et al., 2015). In oviparous reptiles, most embryonic development occurs after the eggs are laid in unattended subterranean nests (Ackerman & Lott, 2004; Packard & Packard, 1988). The egg environment limits embryonic motility and therefore the range of behavioral responses available to embryos (Cordero et al., 2018; Telemeco et al., 2016, but see Du & Shine, 2022), thereby prioritizing physiological responses to adverse conditions (Galli et al., 2023; Hall & Sun, 2021). In subterranean nests, additional challenges are low oxygen levels and fluctuations in gas concentrations (Ackerman & Lott, 2004; Deeming & Thompson, 1991; Packard & Packard, 1988; Seymour & Ackerman, 1980; Stahlschmidt & DeNardo, 2009). As examples, oxygen concentration in both turtle and crocodile nests decreases throughout the incubation period (Ackerman, 1977; Lutz & Dunbar-Cooper, 1984; Prange & Ackerman, 1974; Whitehead, 1987). Gas exchange is diffusion-limited (Vitt & Caldwell, 2013) for these two groups of Archelosaurian reptiles, with gases passing via diffusion through pores in the calcareous eggshell and through spaces between the fibers of the egg membranes. However, no such pores have been observed in the shell of Lepidosaurian eggs, and so gases presumably diffuse between the fibers of the single shell membrane (Packard et al., 1977). The structural difference between eggs of these two reptilian clades likely has important implications for their ability to compensate for reduced oxygen availability through increased gas exchange capacity, warranting studies in a variety of taxa to uncover broader patterns across oviparous reptiles (Vitt & Caldwell, 2013). A limit on gas diffusion capacity is especially relevant at the end of incubation when the oxygen demand of the embryos increases (Cordero et al., 2017b; Dmi'el, 1970; Sartori et al., 2017; Tate et al., 2015), potentially leading to a mismatch between oxygen demand and supply (Gangloff & Telemeco, 2018; Hall & Warner, 2020).

Exposure to hypoxia during development may induce plastic changes in cardiovascular, muscular, or mitochondrial function to

increase performance capacity under these oxygen-limited conditions (Eme et al., 2013; Galli et al., 2016, 2023; Sun et al., 2015). Such stress-induced physiological responses might promote offspring survival in response to consistently low O₂ partial pressure. In other cases, however, exposure to hypoxia during the embryo stage can negatively affect development via reduction in metabolism, in embryonic mass, and in hatching success (Cordero et al., 2017b; Crossley & Altimiras, 2005; Williamson et al., 2017a). The acclimation of nonavian reptiles to hypoxia during embryonic development varies depending on the taxonomic group (i.e., Testudines, Squamata, Crocodylia; Porteus et al., 2011) and most of these responses are related to the hypoxia induced by the burial of eggs in subterranean nests (Cordero et al., 2017b; Crossley & Altimiras, 2005; Stahlschmidt & DeNardo, 2008, 2009; Kam, 1993; lungman & Piña, 2013; Wearing et al., 2017; Williamson et al., 2017b). The effects of high elevation hypoxia during this stage, especially on squamate reptiles, have only received recent interest due to expected upward range expansion as climates warm (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020; Souchet et al., 2020, 2021). In general, lizard embryos respond to the reduced partial pressure of oxygen at high elevations by a reduction of metabolic rate without impacting fitness-relevant aspects of the hatchling phenotype (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020). In snakes, the embryonic response to reduced oxygen depends on incubation temperature (Souchet et al., 2021). At a cool incubation temperature (24°C), the metabolic rates of embryos and the phenotypes of juveniles at hatchling are not modified (Souchet et al., 2021). But when incubated in high elevation hypoxia and at warmer temperatures, physiology and development are impacted (increased heart rate and reduced hatchling mass at 28°C; decreased heart rate, reduced hatchling size and mass at 32°C) and juvenile snakes exhibit reduced swimming performance (Souchet et al., 2020, 2021). These combined effects on early-life phenotypes may bear implications for upslope range expansion.

In mountainous regions, the impacts of climate change are particularly pronounced (Chen et al., 2011; Dirnböck et al., 2011; Nogués-Bravo et al., 2008). Formerly inhospitable habitats at high elevations have warmed and become thermally suitable for some low-altitude species (Marshall et al., 2020; Parmesan, 2006; Sinervo et al., 2010, 2018; Pauchard et al., 2016). Nevertheless, the colonization of high elevation environments depends on a species' ability to cope with low atmospheric pressure and thus reduced oxygen availability (Bouverot, 1985; Powell & Hopkins, 2010; Storz, 2021). Therefore, through physiological plasticity, adaptive evolutionary responses, or adaptive plasticity (Rezende et al., 2005), oviparous species may be able to colonize alpine ecosystems in response to climate warming (Ortega et al., 2016; Storz et al., 2010;

Storz, 2021). In this context, the viperine snake (*Natrix maura*, Linnaeus, 1758), is a useful model species to study the potential impacts of high elevation hypoxia during embryonic development. Indeed, this species has been exposed to fluctuating temperatures throughout its evolutionary history, repeatedly migrating along the elevational gradient to colonize mountainous environments in conjunction with historical warming and cooling cycles (Gómez & Lunt, 2007). In contemporary times, this species has been recorded up to 1000 m above sea level [ASL] in the western Pyrenees Mountains and 1700 m ASL in the east (Martinez-Rica & Reiné-Viñales, 1988; Pottier, 2016; Santos, 2015).

The potential responses in cardiovascular and respiratory systems specifically are essential in understanding the limits of physiological plasticity in facilitating colonization of novel habitats, such as at high elevation (Tian et al., 2017). In high elevations the atmospheric pressure decreases, with associated reduction in the partial pressure of gases, including oxygen, carbon dioxide, and water vapor (Millet & Debevec, 2020; Richalet, 2020). The reduction in oxygen partial pressure at this high elevation provides a useful experimental environment to test the physiological and developmental responses to reduced oxygen availability (Cordero et al., 2017a; Kouyoumdjian et al., 2019; Souchet et al., 2020, 2021). To assess the potential impact of high elevation hypoxia on the cardiovascular-respiratory system of snake embryos and the effect of maternal developmental adjustments to low oxygen levels, we exposed wild-caught gravid females and eggs of the viperine snake to three early-life developmental treatments: gestation and incubation in high elevation hypoxia (~72% oxygen availability relative to sea level), gestation in normoxia and incubation in high elevation hypoxia, and gestation and incubation in normoxia (we here use “gestation” to refer to the period of within-egg development before oviposition). We tested the impacts of these treatments on key physiological markers related to development and growth: embryonic heart rate, oxygen consumption rate, and carbon dioxide production rate. Further, we measured body size and body mass of juveniles at hatching and at ages 14 and 28 days, which have important ties to fitness in early life (Kissner & Weatherhead, 2005; Gangloff & Sparkman, & Bronikowski, 2018; Mack et al., 2017; Manjarrez & San-Roman-Apolonio, 2015). We predicted that high-elevation hypoxia will reduce both heart rate and metabolic rate in embryos and juveniles, resulting in reduced body size and energy stores. If maternal developmental adjustments to high elevation hypoxia can potentially prepare the embryo and reduce the impact of this low level of oxygen availability during incubation, we expected that these negative consequences will be mitigated in embryos from mothers exposed to hypoxia preoviposition.

2 | MATERIALS AND METHODS

2.1 | Female capture and housing

Gravid female *Natrix maura* ($N = 22$) were captured by hand along the banks of the Lez River (Department of Ariège, France) in June 2018.

Capture sites spanned from 422 to 745 m ASL. Immediately after capture, we measured snout-vent length (SVL) using a measuring tape (to the nearest 0.1 cm; mean $SVL \pm SD$: 57.8 ± 8.9 cm). Fifteen gravid females were maintained at low elevation at the Station d'Ecologie Théorique et Expérimentale du CNRS in Moulis (SETE-CNRS; 42.958394 N, 1.086440 E; low elevation at 436 m ASL; native elevation; normoxia; 95% sea-level equivalent O_2 availability; $PO_2 \sim 20.1$ kPa). The seven other gravid females were maintained in extreme high elevation at the Observatoire Midi-Pyrénées at Pic du Midi de Bigorre (42.936389° N, 0.142472° E; extreme high elevation at 2877 m ASL, above current range limits; high elevation hypoxia; 72% sea-level equivalent O_2 availability; $PO_2 \sim 15.3$ kPa). Gravid females were identified by palpation for detection of eggs. We were unable to identify the precise stage of development at capture, though females spent the majority of the gravid period in captivity (mean $\pm SD$: 42.5 ± 9.3 days). Females in the hypoxia treatment were transplanted to extreme high elevation within 7 days of capture (~92% of the captive gravid period spent at high elevation), resulting in approximately 40% of the total development period occurring during gestation in the experimental treatment (Andrews & Mathies, 2000; Shine, 1983). All females were fed and returned to their exact site of capture within 2 weeks after egg-laying.

2.2 | Embryo incubation and measurements

Eggs ($N = 257$) were obtained between July 3, 2018 and July 28, 2018 (mean clutch size $\pm SD = 11.7 \pm 5.0$ eggs). After accounting for female size, there was no difference in clutch size among females from the different treatments (linear model with female SVL as covariate; $p = 0.18$). A total of 28 eggs were infertile or died within the first 7 days postlaying, leaving 229 eggs from 22 females for experiments (Figure 1). Eggs were individually marked for identification with a pencil and allocated to three treatments. A total of 97 eggs laid by seven females at extreme high elevation were maintained at extreme high elevation (EHE), 65 eggs laid by eight females at low elevation were maintained at low elevation (LE), and 67 eggs laid by seven females at low elevation were transplanted to extreme high elevation (LE-EHE; Figure 1). All eggs were placed by clutch in a plastic container ($10 \times 15 \times 6$ cm) on a 2 cm layer of wet vermiculite and incubated at 28°C (ExoTerra Model PT-2445, Rolf C. Hagen Inc.). Water bowls placed within each incubator, directly under the incubator's fan, ensured high levels of humidity throughout incubation, indicated by condensation on the incubator walls (Souchet et al., 2020). During incubation, we weighed each egg using a digital scale (MYCO MZ-100, OnBalance; to the nearest 0.01 g) within 12 h of oviposition, and then every 7 days until hatching (Figure 1). Embryo heart rates were also measured at 28°C using a Buddy digital egg monitor (MK2, Avitronics). Each egg was gently placed onto the sensor pad for heart rate reading (a stable reading was obtained after approximately 30 s) under the standardized protocol described for eggs (Aubret et al., 2016; Cordero et al., 2017a;

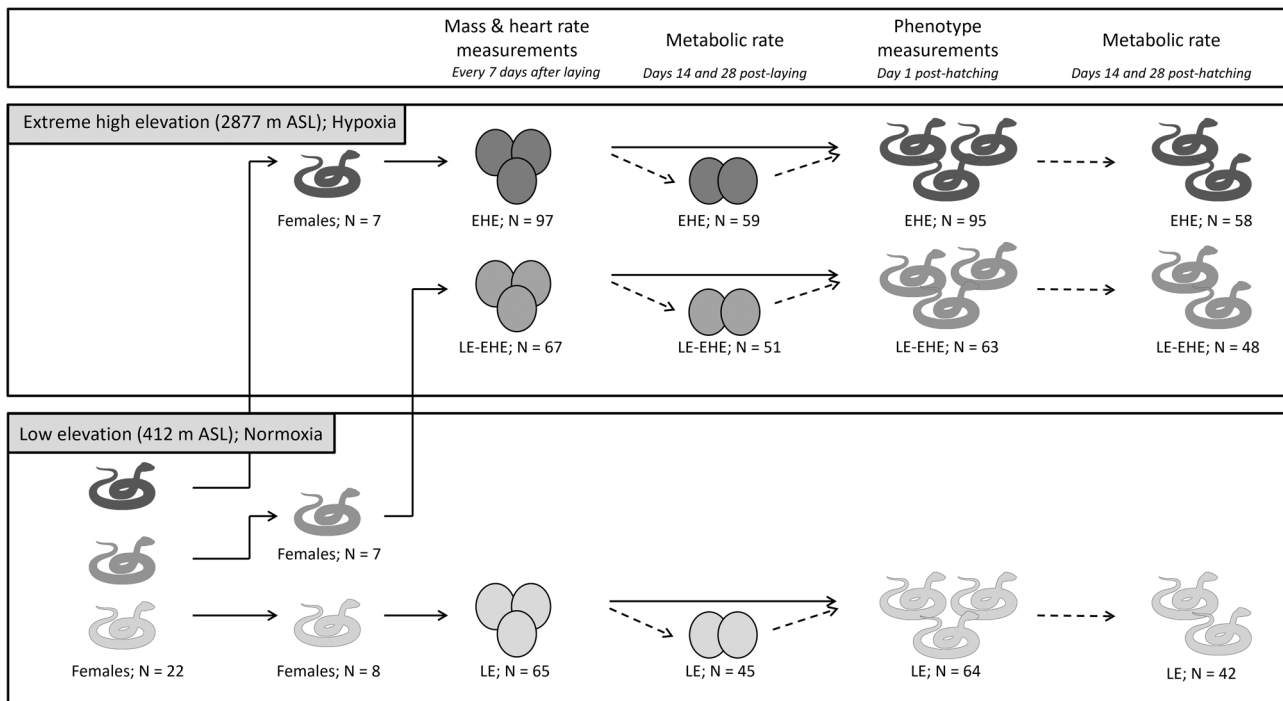


FIGURE 1 Experimental design. Gravid *Natrix maura* females were captured at low elevation from populations in the foothills of the Pyrénées (422 to 745 m ASL). A third of them were transplanted and maintained for gestation at the extreme high elevation laboratory at 2877 m ASL, while two thirds of them were maintained for gestation at the low elevation laboratory at 436 m ASL. Within 48 h of oviposition, the complete clutch from half of the females gestating at low elevation were transported for incubation at the extreme high-elevation laboratory, while the complete clutches from the other females were incubated at the low-elevation laboratory. Clutches from females gestating at the extreme high elevation laboratory were maintained at this elevation. Eggs mass and embryo heart rate were measured throughout incubation. At hatching, a number of morphological traits were measured in juveniles. A subset of eggs and juveniles in each treatment were tested for $\dot{V}O_2$ consumption and $\dot{V}CO_2$ production in the environment where eggs were incubated. ASL, above sea level.

Souchet et al., 2020, 2021), first at 7 days postoviposition and then every 7 days until hatching (Figure 1). At 14 and 28 days postoviposition, we measured gas exchange at 28°C in a subset of 155 embryos (Figure 1). Each egg was individually placed in a 250 mL custom-made plastic metabolic chamber, further sealed with parafilm, fitted with valves to control in- and out-flow, then placed in an incubator (ExoTerra Model PT-2445, Rolf C. Hagen Inc.). We used closed-system respirometry (Foxbox-C Field O_2 and CO_2 Analysis System, Sable Systems, Inc.) to measure gas exchange [oxygen consumption rate ($\dot{V}O_2$) and carbon dioxide production rate ($\dot{V}CO_2$), corrected for barometric pressure]. We first flushed the chamber for 10 min at a flow rate of 400 mL min^{-1} using the internal Foxbox pump, then closed valves to seal the chamber for 60 min. We then opened the valves to re-establish air flow for 7 min to ensure values equilibrated, dried air of water vapor through a plastic column of recharged Drierite, and measured O_2 and CO_2 . We then immediately repeated this procedure to procure two measurements for each individual at each temperature, both of which were included in data analysis (see below). Raw data were analyzed with ExpeData software (v.1.7.30, Sable Systems, Inc.) to calculate $\dot{V}O_2$ and $\dot{V}CO_2$ (in $\mu\text{L/h}$) by integrating the change in instantaneous gas concentrations over the period the chamber was sealed

(Lighton, 2018). We calculated the respiratory quotient (RQ) as the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$.

2.3 | Juvenile housing and measurements

A total of 226 embryos (of 229) from 22 females successfully hatched between August 18 and September 21, 2018 (overall hatching success = 98.7%). Another 4 neonates died shortly after hatching, leaving 222 hatchlings for morphological measurements. Hatchlings were sexed via hemipene eversion, individually marked for identification with a medical cauterizer (low temperature power handle Model HIT0 with 0.05 mm tip Model H100, Bovie®; Winne et al., 2006) within 24 h of emergence (Figure 1), and raised together by hatching date in plastic containers ($15 \times 10 \times 5 \text{ cm}$) in an incubation chamber (ExoTerra Model PT-2445, Rolf C. Hagen Inc.) at a constant temperature of 20°C (as in Souchet et al., 2020, 2021). At hatching, we weighed the yolk leftover in the eggshell (residual egg yolk) and juveniles using a digital scale (MYCO MZ-100, OnBalance; to the nearest 0.01 g) and we measured juvenile SVL using a measuring tape (to the nearest 0.1 cm). At 14 and 28 days posthatching, all juveniles were measured again for SVL and body mass and a subset of 148 juveniles (from eggs previously tested; Figure 1) were measured for

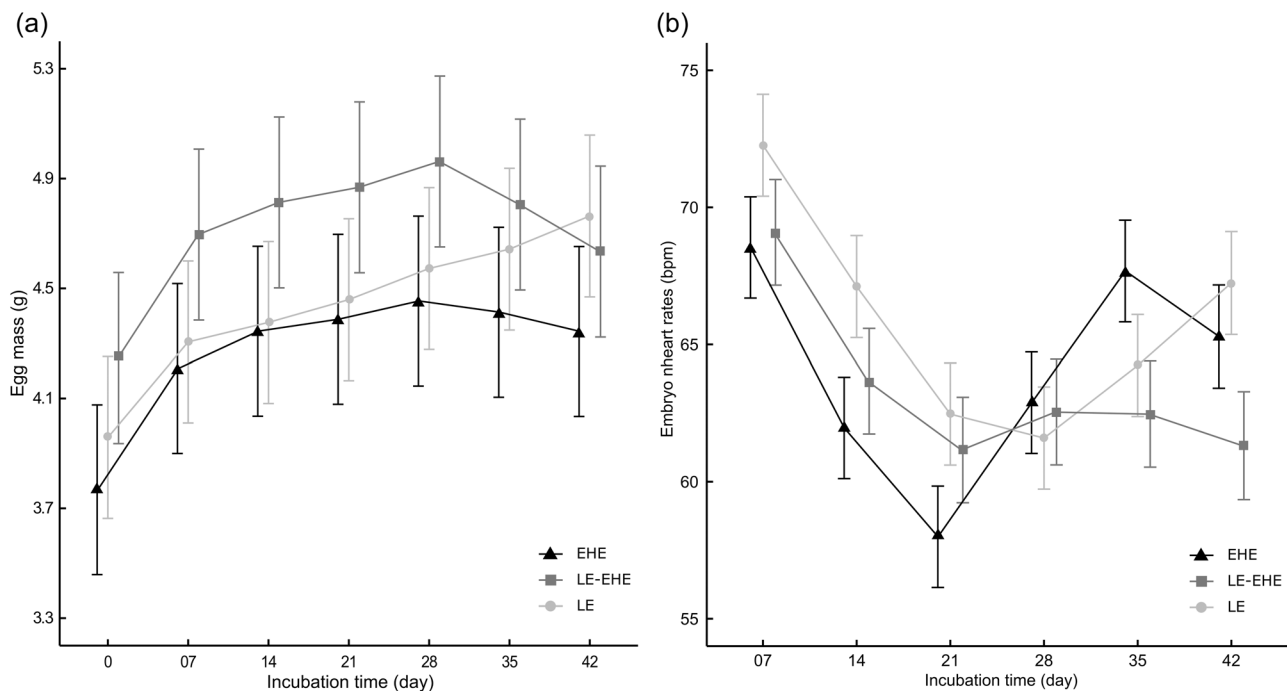


FIGURE 2 Egg mass (a) and heart rate (b) through incubation at 28°C in embryos of the snake *Natrix maura* (Figure 1). The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; $N = 97$; triangle), gestation at low elevation and incubation at extreme high elevation (LE-EHE; $N = 67$; square) and gestation at low elevation and incubation at low elevation (LE; $N = 65$; circle). Least-squares means \pm SE estimated by linear mixed models are plotted.

resting metabolic rate at 20°C under the same protocol as eggs, with juvenile snakes placed in the metabolic chambers and left undisturbed for 60 min before measurements. We also calculated body condition as the residuals of the \log_{10} -mass on \log_{10} -SVL linear regression at hatching day, 14 days, and 28 days posthatching. Juveniles were fasted until the completion of experiments, at which time they were fed and released 1 week after the last day of tests, 35 days posthatching, at the maternal capture site.

2.4 | Data analysis

We first assessed the effect of treatment and time on egg mass and embryo heart rate. We used a linear mixed effect model, including the main effect of treatment (LE, LE-EHE, and EHE), time (days postlaying, treated as categorical to account for expected nonlinearity), and their interaction to assess differences in trajectories in the groups across development. For the egg mass, the model includes the initial egg mass at oviposition as a covariate. Then we assessed the influence of treatment on measures of offspring phenotype using linear mixed effects models, including the main effect of treatment (LE, LE-EHE, and EHE), incubation duration, residual egg yolk, body mass, body size (SVL) and body condition. We used generalized linear mixed-effect models (binomial outcome) of the same structure to assess the effect of treatment on hatching success and sex ratio. Finally, we assessed the influence of treatment and time on gas exchange [oxygen consumption rate ($\dot{V}O_2$), carbon dioxide production rate ($\dot{V}CO_2$) and RQ] of embryos

and juveniles. We again used linear mixed effect models including the main effect of treatment (LE, LE-EHE, and EHE), time (days postlaying or posthatching), and the interaction of treatment and time. We also included the covariate of heart rate for measures in embryos to assess whether variation in gas exchange was linked to changes in heart rate and the covariate of body mass for measures in juveniles) to account for the expected effect of body size on metabolic rate. To account for the nonindependence of siblings we included the clutch of origin as a random effect in all models. In models for which we measured individuals repeatedly (egg mass, embryo heart rates, and gas exchange), we also included individual as a random effect. We used type III sums of squares to assess the significance of main effects, incorporating a Kenward-Roger denominator degree of freedom approximation (Kenward & Roger, 1997). We also conducted a pairwise comparison of least-squares means, adjusting p values for multiple comparisons with the Tukey method. All analyses were conducted in the programming language R 3.6.1 (R Development Core Team, 2017) with the lme4 package (Bates et al., 2014) and emmeans package (Lenth et al., 2018). Data figures were made with the ggplot2 package (Wickham, 2016).

3 | RESULTS

3.1 | Egg mass and embryonic heart rates

Egg mass trajectories differed among the three treatment groups (Figure 2 and Table 1; Table S1). For all treatments, the egg mass

increased between oviposition and day 28 of incubation. After 28 days, the mass of eggs in the LE group continued to increase to the end of incubation, while the mass of eggs in both groups incubated in hypoxia decreased (Figure 2a), resulting in eggs at low elevation having 7.3% greater mass. Embryo heart rate trajectories differed between the three treatments (Figure 2b and Table 1; Table S1). For all treatments, heart rates decreased similarly between 7 and 21 days postoviposition and remained stable between 21 and 28 days. After 28 days, embryos in the LE and EHE treatments increased their heart rates while embryos in the LE-EHE treatment remained stable (Figure 2b), resulting in LE-EHE embryos with heart rates reduced by 4.3% compared to the other two treatment groups.

3.2 | Hatching success and morphological measurements

Hatching success (overall 98.7%) and hatchling sex ratio (overall 51.3% female) did not differ between embryos incubated in the three treatments (Table 2). The different treatments did not influence incubation duration or residual egg yolk mass (Table 2), nor did the treatments alter the body size (SVL), body mass, or body condition at the three time points these traits were measured (hatching day, 14 days, and 28 days posthatching; Table 2). For each trait measured, the post hoc comparison of least-squares means from the models did not indicate any significant pairwise difference between treatments.

3.3 | Embryo and juvenile metabolic rate

Embryonic oxygen consumption rate ($\dot{V}O_2$) significantly increased between 14 and 28 days postoviposition in all treatments (Figure 3a and Table 3; Table S2). Nevertheless, embryos in the LE treatment consumed 27.4% more oxygen at 14 days postoviposition than both other groups, but at 28 days postoviposition oxygen consumption did not differ between treatments (Table S2). The carbon dioxide produced by embryos ($\dot{V}CO_2$; Figure 3a and Table 3) generally followed the same trend. The exception is that at 28 days postoviposition, embryos in the LE treatment produced 16.3% more carbon dioxide than the other groups (Table S2). Embryonic RQ was significantly affected by day of incubation and its interaction with the

treatment (Figure 4a and Table 3). At 14 days of incubation, the post hoc comparison of least-squares means from the model (Figure 4a; Table S2) indicates that embryonic RQ was similar between all treatments. The increase in $\dot{V}CO_2$ in the LE treatment at 28 days resulted in differences between groups at 28 days postoviposition, whereby the LE treatment exhibited higher RQ values than the other two treatment groups (mean LSM RQ values for LE: 0.77; LE-EHE: 0.69; EHE: 0.69; Figure 4a; Table S2).

Oxygen consumption rate ($\dot{V}O_2$) of juvenile snakes was similar among all treatments and did not change across posthatching measurements (Figure 3b; Table S2), but was dependent on juvenile body mass (Table 3). Carbon dioxide production ($\dot{V}CO_2$) was similar among juveniles in the EHE and LE-EHE treatments, significantly increasing between 14 and 28 days posthatching (Figure 3b and Table 3). The rate of carbon dioxide production by juveniles in the LE treatment was higher than other treatments by 27.6% at day 14 and 13.0% at day 28 and did not change between 14 and 28 days posthatching (Table S2). Juvenile RQ was significantly affected by the treatments, the time since hatching, and their interaction (Figure 4b and Table 3). At 14 days posthatching, the post hoc comparison of least-squares means from the model (Figure 4b; Table S2) indicates that juvenile RQ was similar between both EHE and LE-EHE treatments (LSM RQ values of 0.56 and 0.57, respectively), but higher in juveniles in the LE treatment (LSM RQ of 0.78). At 28 days posthatching, the RQ of juveniles in the LE and LE-EHE treatments was maintained, while the RQ of the EHE treatment slightly but significantly increased, compared to the RQ at 14 days posthatching (LSM RQ values for LE: 0.76; EHE: 0.61; LE-EHE: 0.59; Figure 4b; Table S2).

4 | DISCUSSION

Our study examined how low oxygen partial pressure in extreme high elevation (i.e., high elevation hypoxia) experienced during gestation and incubation affects the cardiovascular physiology of viperine snakes in early life-history stages, and the potential long-term fitness consequences. We also explored if gravid females maintained in extreme high elevation can buffer the potential negative effects of high elevation hypoxia for their offspring. Exposure of embryos to hypoxia during postoviposition

TABLE 1 Results of linear mixed effect models testing for the effect of incubation treatment, time of incubation (days postlaying), and their interaction on the egg mass and on embryonic heart rates during embryonic development of the snake *Natrix maura* (Figures 1 and 2a,b).

| | Egg mass | Embryo heart rates |
|-------------------------|--|---|
| Day | $F_{6,1346.36} = 241.93$; $p < 0.001^{***}$ | $F_{5,1095.92} = 38.47$; $p < 0.001^{***}$ |
| Treatment | $F_{2,18.73} = 0.29$; $p = 0.755$ | $F_{2,18.88} = 0.57$; $p = 0.578$ |
| Treatment × Day | $F_{12,1346.35} = 14.18$; $p < 0.001^{***}$ | $F_{10,1096.02} = 6.13$; $p < 0.001^{***}$ |
| Egg mass at oviposition | $F_{1,170.36} = 672.11$; $p < 0.001^{***}$ | – |

Note: The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; $N = 97$), gestation at low elevation and incubation at extreme high elevation (LE-EHE; $N = 67$) and gestation at low elevation and incubation at low elevation (LE; $N = 65$). Significant factors shown in bold with three ($p < 0.001$) asterisks.

TABLE 2 Results of linear mixed effect models testing for the effect of treatment at hatching, 14 days, and 28 days posthatching on the phenotype of juvenile snakes *Natrix maura* (Figure 1).

| | LE | LE-EHE | EHE | F (dfn, dfd) | p-value |
|--|----------------|---------------|----------------|-----------------|-----------|
| Incubation duration (day) | 52.72 ± 3.11 | 52.08 ± 3.97 | 50.40 ± 2.75 | 2.01 (2, 35.49) | p = 0.149 |
| Hatching success (% of success) | 98.96 | 96.92 | 100.00 | 0.58 (2, 18.20) | p = 0.571 |
| Sex ratio (% of females) | 51.58 | 52.38 | 50.00 | 0.04 (2, 15.66) | p = 0.965 |
| Body mass (g) at hatching | 2.72 ± 0.48 | 2.82 ± 0.60 | 2.71 ± 0.34 | 0.42 (2, 33.67) | p = 0.663 |
| Body size (cm) at hatching | 15.35 ± 0.69 | 15.06 ± 1.10 | 15.16 ± 0.85 | 1.27 (2, 29.42) | p = 0.295 |
| Body condition at hatching | -0.016 ± 0.051 | 0.018 ± 0.042 | -0.001 ± 0.032 | 0.99 (2, 24.75) | p = 0.386 |
| Residual egg yolk (g) | 0.88 ± 0.50 | 0.84 ± 0.47 | 0.60 ± 0.44 | 2.39 (2, 20.21) | p = 0.117 |
| Body mass (g) at 14 days posthatching | 2.53 ± 0.46 | 2.64 ± 0.54 | 2.52 ± 0.32 | 0.31 (2, 33.74) | p = 0.739 |
| Body size (cm) at 14 days posthatching | 15.70 ± 0.74 | 15.41 ± 1.08 | 15.55 ± 0.85 | 1.15 (2, 29.53) | p = 0.331 |
| Body condition at 14 days posthatching | -0.016 ± 0.043 | 0.022 ± 0.034 | -0.004 ± 0.033 | 2.63 (2, 22.80) | p = 0.094 |
| Body mass (g) at 28 days posthatching | 2.29 ± 0.42 | 2.37 ± 0.50 | 2.27 ± 0.28 | 0.28 (2, 32.55) | p = 0.756 |
| Body size (cm) at 28 days posthatching | 15.87 ± 0.79 | 15.51 ± 1.13 | 15.76 ± 0.87 | 1.06 (2, 29.95) | p = 0.378 |
| Body condition at 28 days posthatching | -0.016 ± 0.43 | 0.025 ± 0.039 | -0.006 ± 0.032 | 3.02 (2, 22.06) | p = 0.069 |

Note: The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; N = 95), gestation at low elevation and incubation at extreme high elevation (LE-EHE; N = 63) and gestation at low elevation and incubation at low elevation (LE; N = 64). Least-squares means ± SE or percentages are given.

Abbreviations: EHE, gestation at extreme high elevation and incubation at extreme high elevation; LE, gestation at low elevation and incubation at low elevation; LE-EHE, gestation at low elevation and incubation at extreme high elevation.

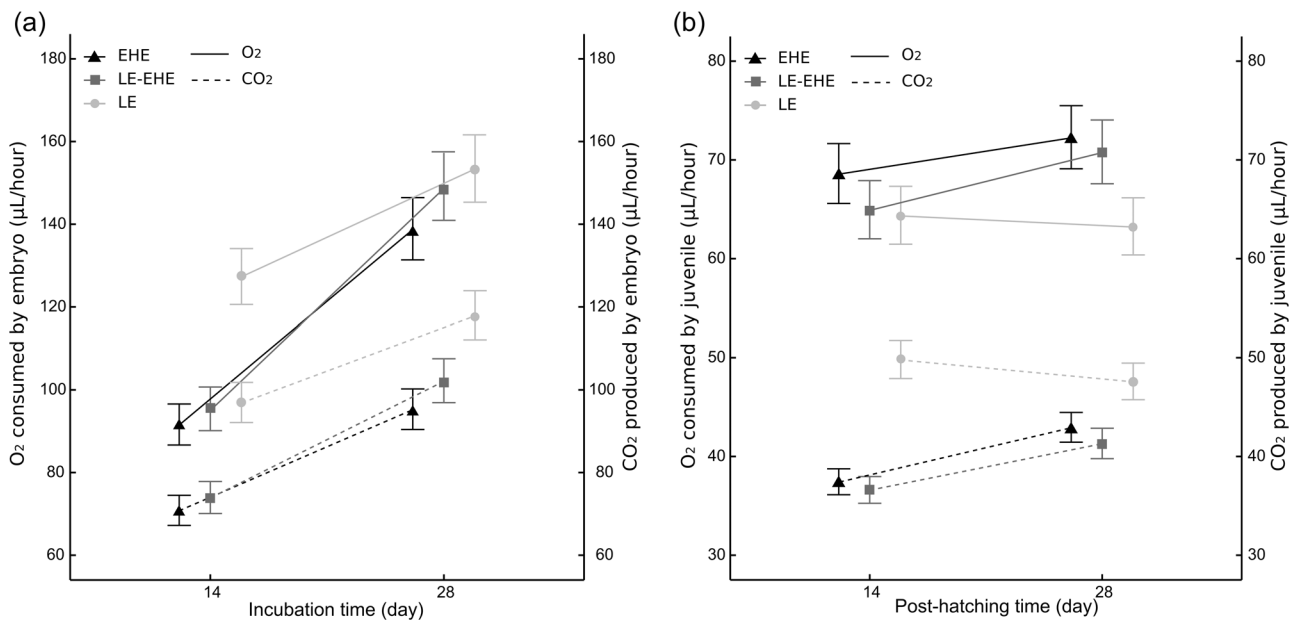


FIGURE 3 Rate of oxygen consumption and rate of carbon dioxide production by embryos (a) and juveniles (b) at 14 and 28 days postoviposition in embryos and posthatching in juveniles of the snake *Natrix maura*. The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; triangle; a: N = 59; and b: N = 58), gestation at low elevation and incubation at extreme high elevation (LE-EHE; square; a: N = 51; and b: N = 48) and gestation at low elevation and incubation at low elevation (LE; circle; a: N = 45; and b: N = 42). Least-squares means ± SE estimated by linear mixed models are plotted.

TABLE 3 Results of linear mixed effect models testing for the effect of incubation treatment, the age at measurement (day postoviposition or day posthatching), and their interaction on the rate of oxygen consumption ($\dot{V}O_2$; Figure 3a), the rate of carbon dioxide production ($\dot{V}CO_2$; Figure 3b), and the respiratory quotient (RQ; Figure 4a,b) in embryo and juvenile snakes (*Natrix maura*; Figure 1).

| | Embryo $\dot{V}O_2$ | Embryo $\dot{V}CO_2$ | Juvenile $\dot{V}O_2$ | Juvenile $\dot{V}CO_2$ | Embryo RQ | Juvenile RQ |
|------------------------|---|---|---|--|--|---|
| Day | $F_{1,149.09} = 283.32$ $p < 0.001^{***}$ | $F_{1,149.07} = 188.92$ $p < 0.001^{***}$ | $F_{1,380.11} = 0.74$ $p = 0.772$ | $F_{1,364.51} = 4.54$ $p = 0.034^*$ | $F_{1,148.60} = 37.92$ $p < 0.001^{***}$ | $F_{1,436.89} = 4.40$ $p = 0.037^*$ |
| Treatment | $F_{2,18.80} = 4.24$ $p = 0.030^*$ | $F_{2,18.81} = 8.10$ $p = 0.002^{**}$ | $F_{2,17.76} = 1.43$ $p = 0.267$ | $F_{2,16.80} = 21.22$ $p < 0.001^{***}$ | $F_{2,18.16} = 2.26$ $p = 0.133$ | $F_{2,17.51} = 76.23$ $p < 0.001^{***}$ |
| Day \times treatment | $F_{2,146.70} = 10.84$ $p < 0.001^{***}$ | $F_{2,146.69} = 1.51$ $p = 0.223$ | $F_{2,427.62} = 2.13$ $p = 0.120$ | $F_{2,427.50} = 12.36$ $p < 0.001^{***}$ | $F_{2,146.12} = 9.31$ $p < 0.001^{***}$ | $F_{2,428.69} = 7.04$ $p < 0.001^{***}$ |
| Heart rate | $F_{1,259.88} = 0.08$ $p = 0.772$ | $F_{1,259.42} = 0.03$ $p = 0.858$ | - | - | $F_{1,229.28} = 1.03$ $p = 0.312$ | - |
| Body mass | - | - | $F_{1,55.40} = 62.50$ $p < 0.001^{***}$ | $F_{1,34.05} = 80.84$ $p < 0.001^{***}$ | - | $F_{1,44.76} = 0.20$ $p = 0.655$ |

Note: The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; embryo: $N = 59$; and juvenile: $N = 58$), gestation at low elevation and incubation at extreme high elevation (LE-EHE; embryo: $N = 51$; and juvenile: $N = 48$) and gestation at low elevation and incubation at low elevation (LE; embryo: $N = 45$; and juvenile: $N = 42$). Significant factors shown in bold with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

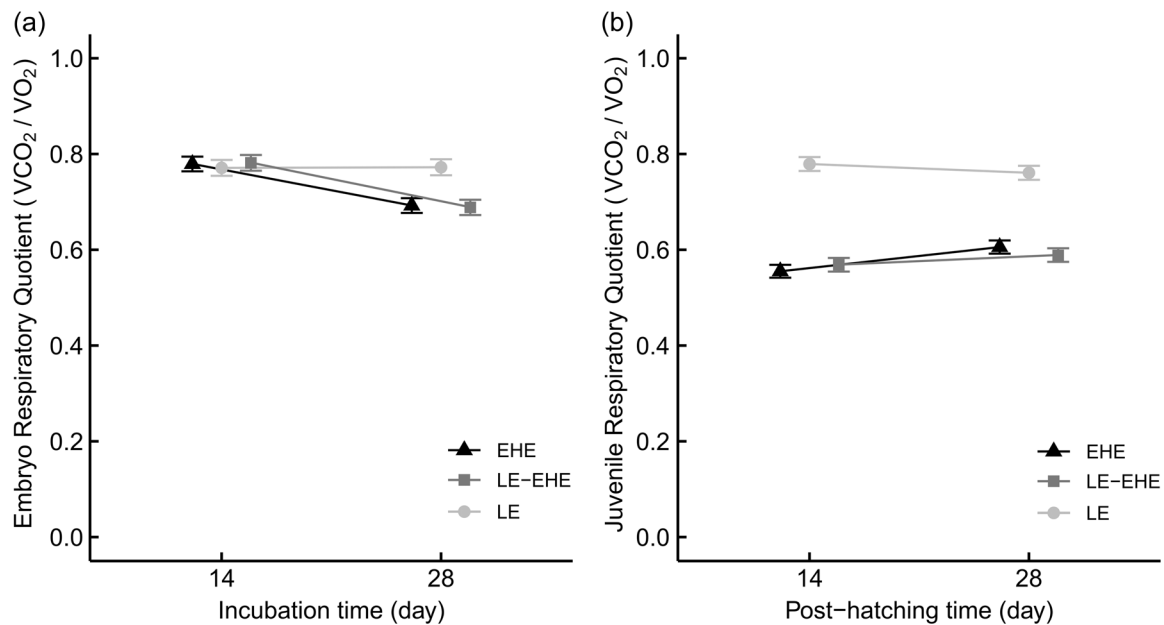


FIGURE 4 Embryo respiratory quotient (a) and juvenile respiratory quotient (b) in each treatment at 14 and 28 days postoviposition in embryos and posthatching in juveniles of the snake *Natrix maura*. The three treatments are gestation at extreme high elevation and incubation at extreme high elevation (EHE; triangle; a: $N = 59$; and b: $N = 58$), gestation at low elevation and incubation at extreme high elevation (LE-EHE; square; a: $N = 51$; and b: $N = 48$) and gestation at low elevation and incubation at low elevation (LE; circle; a: $N = 45$; and b: $N = 42$). Least-squares means \pm SE estimated by linear mixed models are plotted.

development but not gestation (LE-EHE treatment group) lead to a modest decrease in embryonic heart rate relative to the other treatment groups at the end of incubation. While a decrease in egg mass is normal late in the incubation period, hypoxia experienced at extreme high elevation during all of development led to a greater decrease in egg mass relative to the other groups. Importantly, however, this reduction in late-stage egg mass did not then result in smaller hatchlings. Regardless of the elevation experienced during preoviposition gestation, incubation at

extreme high elevation did not affect size and mass at hatching or in early life. Nevertheless, high elevation hypoxia induced a decrease in RQ (increase in O_2 consumption relative to CO_2 production), suggesting either a change in ventilation patterns or a shift in the energy substrate used by embryos. These physiological responses might have buffered impacts of reduced oxygen availability on growth and development, thus allowing embryos to maintain similar incubation durations, hatching success, and morphological phenotype of juveniles relative to those developing

at low elevation. Nevertheless, after being maintained in high elevation hypoxia, juveniles demonstrated shifts in metabolic pathways compared to juveniles maintained in normoxia. Thus, physiological responses allow hatchlings and very young snakes to maintain important fitness-relevant traits, but the longer-term effects of such metabolic shifts are unknown.

4.1 | Maternal effects and hatchling phenotype

The preoviposition developmental window is especially critical in that organogenesis, including differentiation of nervous tissue and cardiovascular organs, occurs while embryos are still being carried by the mother (Ackerman & Lott, 2004; Packard & Packard, 1988). Even so, the potential for maternal effects to impact offspring phenotype is generally reduced in oviparous (compared to viviparous) species (MacLeod et al., 2021). In our experiment, we detected no evidence of maternal ability to “prime” embryos for postoviposition life in a reduced oxygen environment: preoviposition maternal exposure to hypoxia resulted in little effect on embryos or hatchlings. Furthermore, we found no evidence that hypoxia altered maternal energetic resources provided to each embryo (i.e., residual egg yolk), though it is possible that moms altered the specific content (e.g., hormones, macronutrient ratios) of the yolk under the different experimental conditions (Carter et al., 2018; Hayward & Wingfield, 2004; Price, 2017). Nevertheless, hypoxia is expected to affect ATP demand and supply pathways, which can ultimately decrease cellular respiration rates by downregulating ion pumping and protein synthesis (Bickler & Buck, 2007; Hochachka et al., 1996). This common homeostatic response ensures survival without necessarily compromising embryonic development if O₂ 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 O₂ affinity to hemoglobin or hemoglobin concentration (Gangloff et al., 2019; Lu et al., 2015; Storz et al., 2010; Storz, 2016). In accordance with expectations based on previous work in nonsnake reptiles (Cordero et al., 2017a; Crossley et al., 2017; Du et al., 2010; Galli et al., 2023; Kam, 1993), physiological adjustments resulted in no effect of hypoxia on incubation duration, nor was phenotype at hatching or phenotype after being maintained 4 weeks at their elevation of incubation affected (Table 2). The consistency of embryo and hatchling phenotype across treatment groups suggests that physiological mechanisms may compensate for suboptimal environmental conditions (here, hypoxia). The lack of difference in the two high-elevation incubation treatments (LE-EHE and EHE) further points to the response of the embryo itself (rather than maternal effects) as the origin of these compensatory pathways, though further experiments are needed to elucidate more precisely the nature of these responses.

4.2 | Embryonic and juvenile metabolism

Developing viperine snake embryos exposed to high elevation hypoxia exhibited typical vertebrate physiological adjustments. First, suppressed embryonic metabolism is indicated by the reduction of the heart rate throughout incubation (Cordero et al., 2017a; Crossley & Burggren, 2009; Kouyoumdjian et al., 2019; Laughlin, 1978). Second, the decreasing RQ at the end of incubation indicates an augmentation of O₂ consumption relative to CO₂ production. This suggests a response of hypoventilation across the embryonic membranes, which is counter to the response of embryonic alligators (Crossley et al., 2017). After hatching, the RQ of juveniles was lower for both groups incubated and maintained at extreme high elevation. This result suggests that high elevation hypoxia induces a lasting shift in energy substrate, with reduced RQ indicating a reliance on stored lipids or potentially a shift in ventilation patterns relative to cellular metabolism (Bouverot, 1985; Cordero et al., 2017a; Peacock, 1998; Powell & Hopkins, 2010; Storz et al., 2010). Other aspects of metabolic plasticity not observed during this experiment during development may have contributed to the ability of juveniles in hypoxia to maintain or improve their metabolism compared to juveniles in normoxic conditions. It is possible that development in high elevation hypoxia improves the oxygen-carrying capacity of the blood via increased hemoglobin concentrations and higher hematocrit values as found in adults of a variety of lizard species (González-Morales et al., 2015; Lu et al., 2015; Megía-Palma et al., 2020; Newlin & Ballinger, 1976; Vinegar & Hillyard, 1972; Weathers & White, 1972). While a response of increasing density of red blood cells may maintain the performance of juveniles in high elevation hypoxia, this phenotypic plasticity could carry metabolic costs in the form of increased blood viscosity and the energy cost of blood circulation (Dunlap, 2006; Hedrick et al., 1986). Because of the pleiotropic effects of shifts in physiological pathways, potential ramifications of these responses, both positive and negative, may only be evident in later developmental stages.

Importantly, we note that we conducted our experiment in an ecological context, exposing early-life snakes to a feasible level of moderate hypoxia which could be experienced in the near future if upward range expansion continues. Embryos in our high elevation hypoxia treatment developed in reduced atmospheric partial pressure that results in approximately 72% oxygen availability relative to sea level. This experimental design is different from other studies which exposed developing embryos to pharmacological levels of hypoxia, often below 50% relative availability. For example, crocodylian embryos exposed to greater hypoxia reduced heart rates and oxygen arterial pressure and increased heart size relative to controls, resulting in reduced mass and growth rates (Crossley & Altimiras, 2005; Owerkovicz et al., 2009). Similarly, turtle embryos exposed to high levels of chronic hypoxia reduced growth rates and body size (Wearing et al., 2015). We did not observe such negative consequences on fitness-relevant traits under a more moderate level of hypoxia exposure, similar to results in other studies conducted in

the context of potential upslope expansion (Cordero et al., 2017a, 2017b; Kouyoumdjian et al., 2019; Li et al., 2020; Souchet et al., 2020, 2021). Further experiments testing the limits of physiological and developmental plasticity in this ecological context, combined with more detailed studies of *in vivo* physiological systems, are necessary to form a more complete picture of how developing vertebrates respond to abiotic challenges.

4.3 | General conclusion

Collectively, our findings support the hypothesis that plastic physiological responses to high elevation hypoxia may facilitate the maintenance of early-life fitness-related phenotypes in *Natrix maura*. At the same time, we found no evidence that maternal preoviposition exposure to low-oxygen environments affected development or juvenile phenotype, suggesting a limit in the ability of mothers to “prime” their offspring for such novel conditions. However, our experimental design limited the range of responses available to females, specifically in nest site selection. Future work should be directed to quantifying the potential of maternal behavioral choices in affecting offspring development and survival, as has been identified in a variety of reptilian taxa (Bodensteiner et al., 2023; Burger & Zappalorti, 1986; Escalona et al., 2009; Peet-Paré & Blouin-Demers, 2012; Pike et al., 2010; Refsnider et al., 2010). Even though early-life traits are maintained, population establishment will depend on the long-term costs associated with life in reduced oxygen availability and the consequences of reduced performance (Bodensteiner, Gangloff et al., 2021; Galli et al., 2023; Gangloff et al., 2019). Furthermore, it would be fruitful to quantify expression of important genes relevant to differentiation and growth in response to hypoxia, as has recently been done for lizard embryos exposed to thermal stress (Sanger et al., 2021). Uncovering the mechanistic bases for developmental plasticity in response to environmental challenges is essential in predicting how embryos would respond to simultaneous threats (e.g., hypoxia and high temperature). If developing embryos are robust to extreme variation in oxygen availability, as for example in the present experiment, then this early life-history stage should not put a brake on the gradual upslope migration of this species. We propose that metabolic plasticity in embryos and early life should facilitate elevational range expansion in *Natrix maura*, in response to continued climate warming.

AUTHOR CONTRIBUTIONS

Jérémie Souchet, Fabien Aubret, and Eric J. Gangloff: contributed to experimental design and logistics. **Jérémie Souchet and Alicia Josserand:** conducted experiments. **Jérémie Souchet and Eric J. Gangloff:** conducted statistical analyses. **Jérémie Souchet, Alicia Josserand, Fabien Aubret, and Eric J. Gangloff:** drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

Data are available in the publically-availability repository Mendeley here: <https://data.mendeley.com/datasets/csxmt9tbcd/1>.

ETHICS STATEMENT

All experimental protocols (including animal collection, housing, experimentation and release) were approved by the DREAL Midi-Pyrénées (Direction Régionale de l'Environnement, de l'Aménagement et du Logement) and by the Préfecture of Ariège, Aude, Haute-Garonne, Hautes-Pyrénées and Pyrénées Orientales districts (Arrêté Préfectoral No. 2017-s-02 du 30 mars 2017) and ethical committee (Ministère de l'enseignement supérieur, de la recherche et de l'innovation: APAFIS#16359-201808011445465 v4). All experiments were carried out in accordance with the approved guidelines. Animal caretakers and handlers were trained to use wildlife in scientific purposes (Decree No. 2013-118 du 01 février 2013 and approval of the Ministry of Agriculture under No. I-75-MNHN-F1-15 du 17 juin 2015).

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