

High temperatures limit developmental resilience to high-elevation hypoxia in the snake *Natrix maura* (Squamata: Colubridae)

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Received 16 July 2020; revised 5 October 2020; accepted for publication 5 October 2020

Climate change is generating range shifts in many organisms, notably along the altitudinal gradient. However, moving up in altitude exposes organisms to lower oxygen availability, which may negatively affect development and fitness, especially at high temperatures. To test this possibility in a potentially upward-colonizing species, we artificially incubated developing embryos of the viperine snake *Natrix maura* Linnaeus 1758, using a split-clutch design, in conditions of extreme high elevation or low elevation at two ecologically-relevant incubation temperatures (24 and 32 °C). Embryos at low and extreme high elevations incubated at cool temperatures did not differ in development time, hatchling phenotype or locomotor performance. However, at the warmer incubation temperature and at extreme high elevation, hatching success was reduced. Further, embryonic heart rates were lower, incubation duration longer and juveniles born smaller. Nonetheless, snakes in this treatment were faster swimmers than siblings in other treatment groups, suggesting a developmental trade-off between size and performance. Constraints on development may be offset by the maintenance of important performance metrics, thus suggesting that early life-history stages will not prevent the successful colonization of high-elevation habitat even under the dual limitations of reduced oxygen and increased temperature.

ADDITIONAL KEYWORDS: climate change – developmental plasticity – embryonic development – embryonic metabolism – heart rate – high-elevation hypoxia – incubation temperature – swimming performance.

INTRODUCTION

The effects of global warming on biodiversity are a growing concern at all levels of ecosystem functioning

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(Parmesan, 2006; Scheffers *et al.*, 2016; Pecl *et al.*, 2017). Range shifts toward higher latitudes and elevations are now commonly observed as organisms and populations alter their geographic distributions to track their thermal requirements (Sinervo *et al.*, 2018). The rapid contemporary pace of global warming has resulted in a process coined “thermophilization”, where community compositions shift in favour of warm-affinity species (Devictor *et al.*, 2012; De Frenne *et al.*, 2013; Stuart-Smith *et al.*, 2015; Fadrique *et al.*, 2018). Many organisms, however, are unable to track the temperature changes due to habitat fragmentation, slow dispersal and long life spans (Sinervo *et al.*, 2010; Bertrand *et al.*, 2016; Lenoir *et al.*, 2020), leading to climatic debts in community responses to macroclimate warming (Devictor *et al.*, 2012; Alexander *et al.*, 2018; Zellweger *et al.*, 2020). Consequently, many organisms’ and populations’ ecological requirements are rapidly becoming mismatched with their thermal environment, potentially leading to local extirpation (Whitfield *et al.*, 2007; Sinervo *et al.*, 2010; Bestion *et al.*, 2015b), unless successful range shifts occur towards more suitable thermal latitudes or altitudes (Lenoir *et al.*, 2020). Although altitudinal range shifts have been reported in a wide range of organisms (Walther *et al.*, 2002; Parmesan & Yohe, 2003; Bässler *et al.*, 2013; Pauchard *et al.*, 2016; Freeman *et al.*, 2018), moving up in elevation exposes organisms to lower oxygen availability, potentially impacting reproduction, dispersal and overall range shift dynamics (Powell and Hopkins, 2010; Storz *et al.*, 2010; Jacobsen, 2020). Moreover, under the IPCC’s current projection (Mokhov & Eliseev, 2012), temperatures are expected to keep rising, even at high elevation (Jacobsen, 2020). This may eventually expose newly established populations at high-elevation to the double constraints of low oxygen availability and sub-optimal temperatures.

Ectotherm physiology and behavioural processes are strongly dependent on environmental temperatures (Huey and Stevenson, 1979; Angilletta *et al.*, 2002; Gillooly *et al.*, 2002; Deutsch *et al.*, 2008; Huey *et al.*, 2012), and therefore they are often utilized as a model in climate change related experiments and studies (Pen *et al.*, 2010; Sinervo *et al.*, 2010; Bestion *et al.*, 2015a; Dahlhoff *et al.*, 2019). Our current study focuses on embryo development and hatching success, because the production of viable and fit offspring is a required condition for successful dispersal and population establishment in novel environments (Baguette *et al.*, 2012). Further, incubation temperature is the main driver of embryonic development and hatchling phenotype in ectotherms such as reptiles (Deeming & Thompson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014; Refsnider *et al.*, 2019). The influence of incubation temperatures [notably above the optimal range (Andrews and Schwarzkopf,

2012)] on hatchling phenotype is especially well known in reptiles, affecting development, sex determination, incubation duration, body size, growth rate, locomotor performance, cognitive abilities and post-natal behaviour (Shine, 2004; Deutsch *et al.*, 2008; Daufresne *et al.*, 2009; Gardner *et al.*, 2011; Sheridan & Bickford, 2011; Bestion *et al.*, 2015b; Cunningham *et al.*, 2017; Noble *et al.*, 2018; Pellerin *et al.*, 2019; Refsnider *et al.*, 2019). Additionally, the effects of low oxygen availability on physiology have attracted recent attention, including in the context of altitudinal range shifts driven by climate change (Powell and Hopkins, 2010; Storz *et al.*, 2010; Jacobsen, 2020). For instance, it was shown that common wall lizards (*Podarcis muralis* Laurenti, 1768) transplanted to extreme high elevation areas enhanced oxygen-carrying capacity by increasing hematocrit and blood hemoglobin concentration, though transplanted lizards still suffered a reduction in running endurance (Gangloff *et al.*, 2019). Further, reptile embryos exposed to hypoxia increased heart rates in some studies (Du *et al.*, 2010a; Souchet *et al.*, 2020), while in other cases hypoxia led to decreased heart rates and cardiac hypertrophy (Cordero *et al.*, 2017a; Kouyoumdjian *et al.*, 2019). In viperine snakes, exposure to hypoxia during incubation resulted in hatchlings that were smaller in body size and slower swimmers [a proxy for predator avoidance and food acquisition in snakes (Jayne & Bennett, 1990; Kingsolver *et al.*, 2001)] compared to their siblings incubated at lower elevation (Souchet *et al.*, 2020).

Recent work suggests that the interaction of high temperature and oxygen limitation will alter embryo development (Jackson, 2007; Flewelling & Parker, 2015; Smith *et al.*, 2015; Gangloff & Telemeco, 2018; Hall & Warner, 2020; Li *et al.*, 2020). Here we experimentally tested the effect of high temperature (i.e. current populations caught in the climatic debt), low oxygen availability (i.e. populations having shifted their range in altitude in the near future), and the combined effect of high temperature and low oxygen (i.e. extreme high elevation in the year 2070) on the development, hatching success and hatchling phenotype in a temperate snake species, the viperine snake *Natrix maura* Linnaeus, 1758. This is a first step toward assessing the colonization potential to high elevation in a potentially upward-migrating species. We used a split-clutch design and incubated eggs under four ecologically relevant treatments: (1) oxygen availability at native elevation (normoxia; 436 m a.s.l.) and 32 °C incubation temperature (i.e. populations lagging behind climate change); (2) low oxygen availability (2877 m a.s.l.), 24 °C incubation (i.e. range shifted in altitude); (3) low oxygen availability, 32 °C incubation (i.e. high altitude in the year 2070); and (4) a normal oxygen availability, 24 °C incubation control treatment (i.e.

recent past conditions). We monitored embryo heart rates [a proxy for metabolism and cardiovascular function (Crossley & Burggren, 2009)] and egg mass throughout the incubation and measured fitness-relevant aspects of hatchling phenotypes (body size and swimming performance) at hatching. This factorial design allowed us to tease apart the individual and combined effects of incubation temperature and oxygen levels on embryo development and hatchling phenotypes. Based on our previous work (Souchet *et al.*, 2020), we expected that the extreme high elevation would decrease egg mass and induce higher heart rates throughout incubation. Moreover, we predicted incubation duration would be shorter and the hatchlings would be smaller in high-elevation hypoxia. Further, we predicted that the combined constraints imposed by higher metabolic rates induced by warmer incubation temperature (Huey, 1982; Angilletta, 2009; Dillon *et al.*, 2010) and oxygen limitation on juveniles would result in a reduced performance capacity. Specifically, we predicted that embryos incubated at extreme high elevation would produce slower-swimming juveniles and that embryos developing under conditions of both high temperature and high-elevation hypoxia would be the slowest. Finally, we further partitioned treatment groups to test whether the effects of embryonic environment would be ontogenetically stable even after hatchlings were transplanted to the alternative elevation.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

We captured 17 gravid female viperine snakes along the banks of the Lez River (Department of Ariège, France), between May and July 2017. This aquatic species (Vacher and Geniez, 2010) has been recorded up to 1000 m a.s.l. in France (Aubret *et al.*, 2015; Pottier, 2016) and 1500 m a.s.l. in Spain (Martinez-Rica & Reiné-Viñales, 1988; Santos, 2015). The viperine snake has been exposed to fluctuating temperatures and has migrated along the elevational gradient throughout its evolutionary history, colonizing mountainous environments repeatedly in conjunction with historical warming and cooling cycles (Gómez & Lunt, 2007). Capture sites spanned from 412 to 715 m a.s.l. Each female was maintained at the Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique (SETE-CNRS; 42.958 394 °N, 1.086 440 °E) and laid a single clutch for a total of 205 eggs between 21 June 2017 and 22 July 2017 (mean clutch size \pm SD = 11.9 \pm 4.9 eggs). Three eggs were infertile, leaving 202 eggs for the experiment. All

females were returned to their exact site of capture within 2 weeks of egg laying.

We first investigated how temperature (cool temperature at constant 24 °C; and hot temperature at constant 32 °C) and oxygen availability interact to influence embryonic development. Oxygen treatments were normoxia at the SETE-CNRS (low elevation at 436 m a.s.l., 95% sea-level equivalent O₂ availability, PO₂ ~20.1 kPa) and high-elevation hypoxia at the Observatory Midi-Pyrénées of the Pic du Midi de Bigorre (42.936 389 °N, 0.142 472 °E, above current range limits at 2877 m a.s.l., 72% sea-level equivalent O₂ availability, PO₂ ~15.3 kPa). This difference in elevation results in a decrease in atmospheric pressure, with associated reduction in the partial pressure of gases, including oxygen, carbon dioxide and water vapour (Millet & Debevec, 2020; Richalet, 2020). Most relevant to our hypotheses is the 25% reduction in oxygen availability at the Pic du Midi de Bigorre laboratory in comparison to sea level (Bouvetot, 2012). Eggs were weighed using a digital scale (to the nearest 0.01 g) within 12 h of oviposition and individually marked for identification with a pencil. We used a split-clutch design and allocated eggs to four incubation treatments within 24 h of oviposition (Fig. 1): (1) Low Elevation and Cool temperature (LEC; normoxia at constant 24 °C); (2) Low Elevation and Hot temperature (LEH; normoxia at constant 32 °C); (3) Extreme High Elevation and Cool temperature (EHEC; hypoxia at constant 24 °C); and (4) Extreme High Elevation and Hot temperature (EHEH; hypoxia at constant 32 °C). Because egg mass influences both embryo metabolism and hatching phenotype (Nelson *et al.*, 2004; Aubret, 2013), and egg mass varied among clutches (Kruskal-Wallis test: $H = 148.42$, d.f. = 15, $P < 0.001$), eggs were ranked within each clutch from lightest to heaviest and alternately assigned to treatments in order to ensure no difference in egg mass between treatments (Kruskal-Wallis test: $H = 0.151$, d.f. = 3, $P = 0.985$). LEC, LEH, EHEC and EHEH treatment quarter-clutches were placed in a plastic container (20 × 15 × 5 cm) on a 2 cm layer of wet vermiculite (1:5 water to vermiculite by volume) and incubated in four identical incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc., USA). 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).

Out of 202 eggs, 177 embryos from 16 females successfully hatched (87.6% hatching success rate) while 25 died at various stages during incubation. Another 17 neonates died shortly after hatching (between 24 h to 2 weeks). We measured morphology (Test 2, Fig. 1; see below) first on all 177 hatchlings at their incubation location (low or extreme high elevation). Our experimental design allowed us to

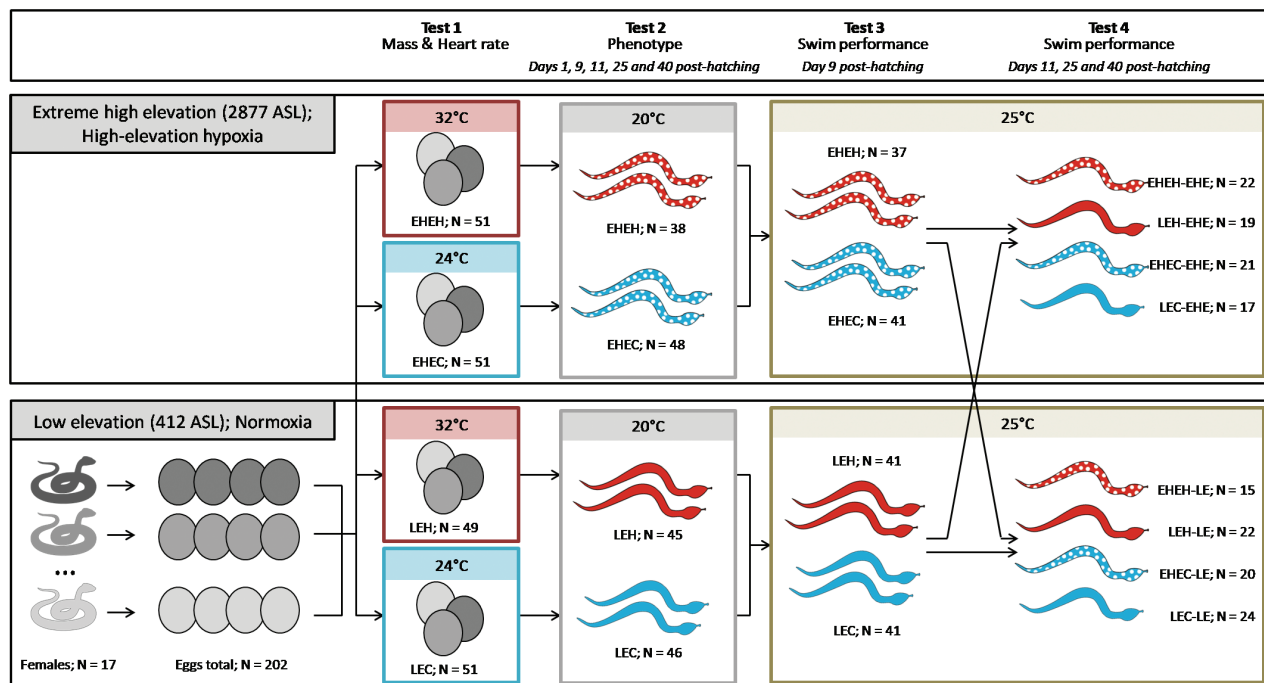


Figure 1. Experimental design. Eggs were collected from gravid females (represented by different colours) sampled from low-elevation populations of *N. maura* in the foothills of the Pyrenees, France (412 m to 715 m a.s.l.). Within 24 h of oviposition clutches were evenly split into four groups of eggs with similar average egg mass. For each clutch, two quarter-clutches were transplanted to the extreme high elevation site (2877 m a.s.l., Observatoire Midi-Pyrénées du Pic du Midi de Bigorre), with one quarter-clutch incubated at 24 °C and the other at 32 °C. The two remaining quarter-clutches were incubated at 24 °C and at 32 °C at the low-elevation site (436 m a.s.l., Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche Scientifique). Egg mass and embryo heart rate were measured throughout incubation (Test 1). At hatching a number of phenotypic traits were measured in juveniles (Test 2). All hatchlings were first tested for swimming performance in the environment that their eggs were incubated (Test 3). Each treatment was then again split in half with half of each treatment group translocated to the alternative environment for additional swimming measures (Test 4). Snake colour represents incubation temperature treatment (blue = cool or red = warm) and snake pattern represents incubation elevation treatment (solid = low or spotty = extreme high elevation).

measure the effects of temperature and hypoxia during incubation on juvenile development and performance. It also allowed us to measure the short-term effects on juvenile development and performance in acute high-elevation hypoxia after translocation to extreme high elevation. In order to assess these questions, at 9 days post-hatching [after all yolk was assimilated (Ji *et al.*, 1999)] we measured morphology and swimming performance (Test 3, Fig. 1; see below) first on all 160 hatchlings at their incubation elevation (low or extreme high elevation). After this first measurement, half of the hatchlings in the LEC and LEH treatments were transferred to extreme high elevation while half of the hatchlings from the EHEC and EHEH treatments were brought down to the low elevation site. All juveniles were then tested for swimming performance and morphology at 11 days, 25 days and at 40 days post-hatching (respectively 1 day, 2 weeks and 1 month of acclimation for transferred juveniles; Test 4, Fig. 1). Once tests were completed, young

snakes were fed with small dead minnows (0.5 to 1 g) and released between 42 and 45 days post-hatching at the maternal capture site.

EGG MASS AND HEART RATE MEASUREMENTS

We weighed each egg using a digital scale (to the nearest 0.01 g) within 12 h of oviposition, and then every 7 days until hatching (Fig. 1, Test 1). Embryo heart rates were first measured at 7 days of incubation and then every 7 days until hatching (Fig. 1, Test 1) at the same temperature as incubation. To measure embryo heart rates, we used the Buddy digital egg monitor (MK2, Avitronics, Cornwall, UK) under the standardized protocol described for eggs (Aubret, 2013; Cordero *et al.*, 2017a; Souchet *et al.*, 2020). Each egg was gently placed onto the sensor pad for heart rate reading (a stable reading was obtained after approximately 30 s) and then returned to its clutch. All eggs were only briefly (≤ 1 min) placed in the

digital egg monitor to mitigate potential temperature changes owing to exposure to infrared sensors (Sartori *et al.*, 2015; Hulbert *et al.*, 2017). Heart rates can be influenced by a variety of factors (Clark *et al.*, 2006; Du *et al.*, 2010b) and are linked to metabolic rate in some circumstances (Kouyoumdjian *et al.*, 2019), though this relationship may become less clear especially late in development (Sartori *et al.*, 2017). We also calculated the total number of heart beats (THB) of embryos throughout embryonic development using the formula $\text{THB} = \text{average heart rate} \times \text{total minutes of developmental duration}$ (Du *et al.*, 2009, 2011).

HATCHLING MEASUREMENTS

Hatching occurred between 8 August 2017 and 29 September 2017 (Fig. 1, Test 2) and hatchlings were individually marked for identification with a medical cauterizer (Model HIT0, Bovie, USA) on the ventral scales (Winne *et al.*, 2006) within 24 h of emergence. Hatchlings were weighed using a digital scale (to the nearest 0.01 g), measured for snout-vent length (SVL) and total body length (TL) using a measuring tape (to the nearest 0.1 cm) and sexed via hemipene eversion. Since sex is genetically determined in snakes, we did not expect an effect of treatment on sex determination, but tested for differential effects between the sexes in developing embryos which could result in skewed hatchling sex ratios. We calculated body condition as the residual of the \log_{10} -mass on \log_{10} -SVL linear regression at hatching day. Finally, we weighed the yolk leftover in the eggshell (residual egg yolk) using a digital scale (to the nearest 0.01 g). Juveniles were housed together by hatching date in plastic containers (15 × 10 × 5 cm) with a water dish, shelter and paper towel as a substrate in incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc., Canada) set at a constant 20 °C. Our experience with viperine snakes shows that cooler temperatures (below thermal optimum for performance or preferred temperatures) results in higher juvenile survivorship (93% survival at 1 month in this species; J. Souchet and F. Aubret, unpubl. data). Juveniles were measured again at 9 days, 11 days, 25 days and 40 days post-hatching for SVL, TL and body mass prior to performance testing.

SWIMMING PERFORMANCE

For this test, we were interested in measuring the maximal swimming speed to evaluate the potential limitation of hypoxia on this ecologically-relevant performance. To estimate the swimming speed we used a procedure that has been validated for snakes (Shine & Shetty, 2001; Aubret, 2004; Aubret *et al.*, 2005), modified effectively for juveniles (Souchet *et al.*, 2020). A high-definition wide-angle digital camera (25

frame per second, Sony Model HDR-XR160E, Sony Corporation) was fitted above a linear swimming track (100 × 20 × 20 cm) and used to record swimming trials. The tank was filled to a depth of 5 cm with water maintained at 25 °C using aquarium heaters. A standard testing temperature of water at 25 °C was used because it approximates the optimal temperature for swimming speed of the viperine snake (Hailey & Davies, 1986; Aubret *et al.*, 2015). At 9 days, 11 days, 25 days and 40 days post-hatching, each snake was acclimated to 25 °C for 30 min and swam ten consecutive lengths. Raw data were extracted from video files by measuring swimming speed ($\text{cm}\cdot\text{s}^{-1}$) for each length (ten per individual and day of measurement) with the software Tracker (Brown, 2019). The fastest performance from all trials was utilized for swimming analysis. Analyzing the average swimming speed of the ten trials gives the same qualitative results; however, since our focus is performance capacity, we include results for maximum swimming speed here.

DATA ANALYSIS

We first assessed the influence of the temperature and elevation of incubation, and time of development on egg mass and embryo heart rate (Test 1). We used linear mixed-effect models, including as main effects the temperature of incubation (cool: 24 °C; warm: 32 °C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), the age at measurement (0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post-laying) treated as a categorical effect to account for the expected nonlinear response over time (Burggren & Warburton, 1994; Cordero *et al.*, 2017a; Sartori *et al.*, 2017), and all three- and two-way interactions. We then assessed the influence of temperature and elevation of incubation on eight measures of hatchling phenotype at hatching (Test 2): survival to hatching, sex, incubation time, total number of heartbeats (THB), body mass, body size (SVL), body condition and residual egg yolk. We used linear mixed-effect models, including in all models the same main effects of the temperature of incubation (cool: 24 °C; warm: 32 °C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), and interactions as above. Finally, we assessed the influence of the temperature and elevation of incubation on swimming performance of juveniles (Test 3 and Test 4). We used linear mixed-effect models, including as the main effects the temperature of incubation (cool: 24 °C; warm: 32 °C), the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation hypoxia), the age at measurement (9, 11, 25 and 40 days post-hatching), the location of test (low elevation or extreme high elevation), and all four-, three- and two-way

interactions. We also included as covariates the total body length (TL) and the sex of juveniles.

To account for the non-independence of siblings we included the clutch of origin as a random effect (intercept) in all models. In models for which we measured individuals repeatedly (egg mass, embryo heart rates and swimming performance), we also included individual as a random effect (intercept), nested within clutch. 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 and adjusted *P*-values for multiple comparisons with the Tukey method. All analyses were conducted with the lme4 package (Bates *et al.*, 2014) and the emmeans package (Lenth, 2016) and figures were made with the ggplot2 package (Wickham, 2016) in the programming language R v.3.6.1 (R Development Core Team, 2019).

RESULTS

TEST 1: EGG MASS AND EMBRYONIC HEART RATES

The main effects of elevation, temperature and time of measurement (days post-laying) and their interaction significantly altered egg mass trajectories (Table 1; Fig. 2A). Eggs incubated at 24 °C (i.e. LEC and EHEC) gained mass for 35 days post-oviposition before decreasing (Table 1; Fig. 2A), while the mass of eggs incubated at 32 °C (i.e. LEH and EHEH) decreased throughout the incubation (Table 1; Fig. 2A). The post hoc comparison of least-squares means from the model (Supporting Information, Table S1) indicates that egg masses were similar in eggs incubated at the same incubation temperature whatever the oxygen availability. Nevertheless, at the last day of measurement (28 days post-hatching for LEH and

EHEH; 63 days post-hatching for LEC and EHEC) the egg mass of both treatments at extreme high elevation were significantly less than those of the low elevation treatments (Fig. 2A; Supporting Information, Table S1). Eggs incubated at 24 °C (i.e. at LEC and EHEC) maintained higher mass (mean difference ± SE: 0.36 ± 0.08 g) across the incubation period compared to eggs incubated at 32 °C (i.e. at LEH and EHEH).

Heart rate trajectories were also significantly altered by elevation and temperature, time of measurement (days post-laying) and by the interaction between temperature and both elevation and time of measurement (Table 1; Fig. 2B). Heart rates of embryos incubated at 32 °C (i.e. at LEH and EHEH) increased rapidly during the first 7 days of incubation before decreasing for the remainder of the incubation (Table 1; Fig. 2B), while embryos incubated at 24 °C (i.e. at LEC and EHEC) maintained stable heart rates throughout incubation (Table 1; Fig. 2B). Post hoc comparison of least-squares means (Supporting Information, Table S1) indicates that embryos from LEH treatment maintained higher heart rates [mean difference ± SD: 7.25 ± 1.17 beats per minute (bpm)] across the incubation period compared to EHEH. Further, eggs in the EHEH treatment exhibited much higher heart rates (mean difference ± SE: 33.39 ± 1.00 bpm) compared to both embryo groups incubated at 24 °C (i.e. at LEC and EHEC).

TEST 2: HATCHING SUCCESS AND MORPHOLOGICAL MEASUREMENTS

Hatching success of embryos was dependent on incubation temperature and the interaction with the elevation (LEC = 90.2%, LEH = 91.8%, EHEC = 94.1% and EHEH = 74.5%; Table 2). Post hoc comparison of least-squares means (Supporting Information, Table S2) indicates that hatching success differed between eggs in the EHEC and EHEH treatments. We observed

Table 1. Results of linear mixed-effect model testing for the effects of incubation temperature, incubation elevation, age at measurement (days post-oviposition), and their interaction on embryo developmental parameters in *N. maura* (Test 1, Fig. 2). The four incubation treatments are extreme high elevation at 24 °C (EHEC; *N* = 51), extreme high elevation at 32 °C (EHEH; *N* = 51), low elevation at 24 °C (LEC; *N* = 51), and low elevation at 32 °C (LEH; *N* = 49). Significant factors shown in bold with two (*P* < 0.01) or three (*P* < 0.001) asterisks

	Egg mass	Embryo heart rates
Temperature	$F_{1426.8} = 2602.33; P < \mathbf{0.001}^{***}$	$F_{11142.3} = 635.04; P < \mathbf{0.001}^{***}$
Elevation	$F_{1283.7} = 8.02; P = \mathbf{0.003}^{**}$	$F_{11144.2} = 13.22; P < \mathbf{0.001}^{***}$
Day	$F_{91130.0} = 30.92; P < \mathbf{0.001}^{***}$	$F_{91179.6} = 11.82; P < \mathbf{0.001}^{***}$
Temperature × elevation	$F_{1426.1} = 22.57; P = 0.500$	$F_{11143.0} = 9.61; P = \mathbf{0.002}^{**}$
Temperature × day	$F_{41138.8} = 97.60; P < \mathbf{0.001}^{***}$	$F_{41194.3} = 7.21; P = \mathbf{0.007}^{**}$
Elevation × day	$F_{91139.8} = 5.66; P < \mathbf{0.001}^{***}$	$F_{91191.2} = 2.38; P = 0.123$
Temperature × elevation × day	$F_{41138.5} = 5.75; P < \mathbf{0.001}^{***}$	$F_{41191.8} = 0.24; P = 0.626$

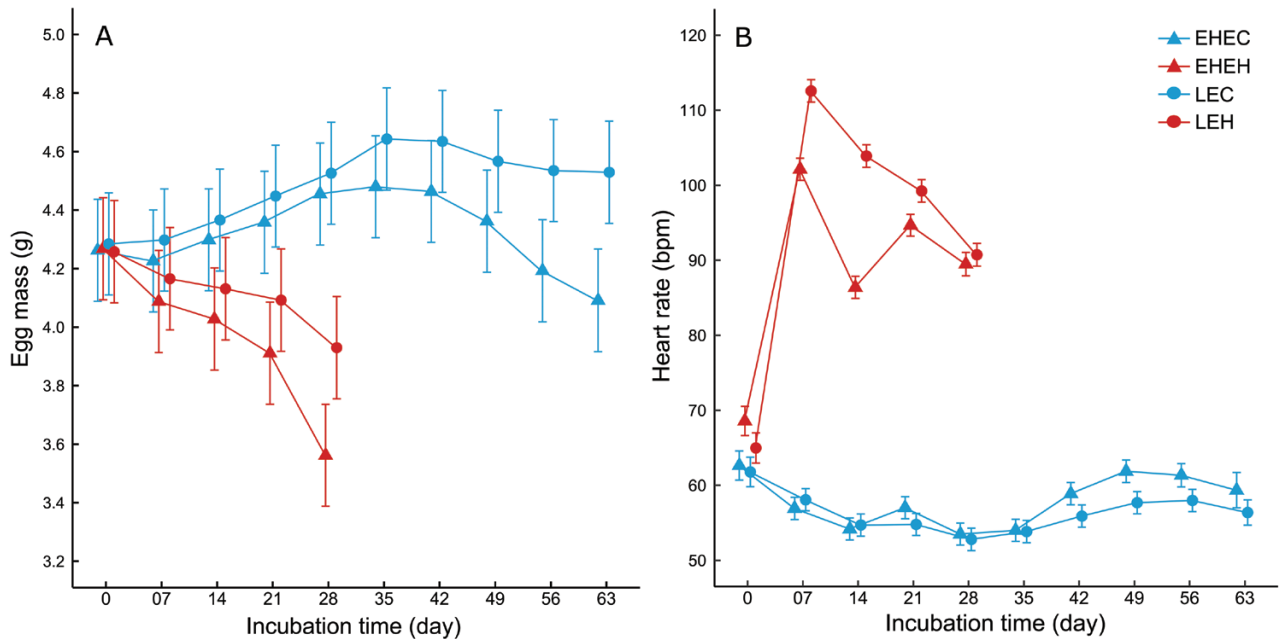


Figure 2. Egg mass (A) and embryo heart rate (B) measured at the same temperature as the incubation temperature through incubation duration in *N. maura* at extreme high elevation at 24 °C (EHEC; $N = 51$; blue triangle), extreme high elevation at 32 °C (EHEH; $N = 51$; red triangle), low elevation at 24 °C (LEC; $N = 51$; blue circle) and low elevation at 32 °C (LEH; $N = 49$; red circle). Least-squares means \pm SE estimated by linear mixed-effect models are plotted.

than of the 25 dead embryos, half of them were from EHEH. Moreover, in this treatment, 92% of the deaths appeared in the last stage of development. Elevation and temperature levels did not affect the hatchling sex ratio (LEC = 56.5%, LEH = 57.8%, EHEC = 47.9% and EHEH = 42.1% females; Table 2). Incubation duration differed between embryos incubated in the four treatments as a function of temperature and its interaction with the elevation (Table 2; Fig. 3A). All treatment groups were significantly different from each other (Supporting Information, Table S2). Snakes in the LEC treatment incubated 2.25 ± 0.28 days longer than EHEC, EHEC treatment incubated 29.01 ± 0.29 days longer than EHEH, and EHEH treatment incubated 2.66 ± 0.30 days longer than LEH. Only the temperature of incubation affected THB of embryos (Table 2; Fig. 3B). THB did not differ in embryos from the same incubation temperature (Supporting Information, Table S2) and THB were greater in the cool incubation temperature treatments (i.e. LEC and EHEC) compared to warm (i.e. LEH and EHEH). Moreover, the residual egg yolk was also significantly affected by the temperature of incubation (Table 2; Fig. 3F). Comparison of least-squares means from the model (Supporting Information, Table S2) indicates that residual egg yolk was similar for the treatments within an incubation temperature (i.e. LEC vs. EHEC and LEH vs. EHEH), but that snakes in the LEC and EHEC treatments retained an average

of 0.29 g (33.6%) more residual egg yolk compared to the LEH and EHEH treatments.

Elevation and temperature and their interaction influenced body mass and body size (SVL) at hatching (Table 2; Supporting Information, Table S2; Fig. 3C, D). Elevation and temperature influenced hatchlings' body condition (Table 2; Supporting Information, Table S2; Fig. 3E). In all cases, the two cool treatments (i.e. at LEC and EHEC) did not significantly differ from each other. For body mass at 1 day post-hatching, LEH treatment did not differ from either LEC or EHEC treatments. However, the EHEH treatment was 0.39 ± 0.12 g (13.2%) lighter compared to the three other treatments. For body size at 1 day post-hatching, cool-temperature treatments (EHEC and LEC) were 0.72 ± 0.17 cm (5.1%) longer than snakes in the LEH treatment, which in turn were 0.49 ± 0.18 cm (4.7%) longer than snakes in the EHEH treatment. Finally, the different treatments also influenced the body condition at 1 day post-hatching (Table 2; Supporting Information, Table S2; Fig. 3E), with snakes in the LEH treatment having a 35.4% higher body condition compared to the three other treatments.

TESTS 3 & 4: SWIMMING PERFORMANCE

Globally, maximum swimming speed (Table 3) was influenced by the effect of incubation temperature (24 and 32 °C), the time of measurement (9, 11, 25 and

Table 2. Results of linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, and their interaction on the juvenile traits at hatching in *N. maura* (Test 2, Fig. 3). The four incubation treatments are low elevation at 24 °C (LEC; *N* = 46), low elevation at 32 °C (LEH; *N* = 45), extreme high elevation at 24 °C (EHEC; *N* = 48), and extreme high elevation at 32 °C (EHEH; *N* = 38). Least-squares means (LSE) ± SE are given. Significant factors shown in bold with one (*P* < 0.05), two (*P* < 0.01) or three (*P* < 0.001) asterisks

	EHEC	EHEH	LEC	LEH	Temperature effect	Elevation effect	Temperature x elevation effect
	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE	F (dfn, dfd) <i>P</i> -value	F (dfn, dfd) <i>P</i> -value	F (dfn, dfd) <i>P</i> -value
Hatching success	-	-	-	-	4.04 (1, 184.4) <i>P</i> = 0.046*	2.43 (1, 185.2) <i>P</i> = 0.120	5.72 (1, 185.2) <i>P</i> = 0.018*
Sex	-	-	-	-	0.09 (1, 163.7) <i>P</i> = 0.764	2.56 (1, 165.0) <i>P</i> = 0.112	0.22 (1, 166.9) <i>P</i> = 0.643
Incubation duration (days)	65.59 ± 0.42	36.59 ± 0.44	67.85 ± 0.43	33.93 ± 0.43	24 415.32 (1, 158.4) <i>P</i> < 0.001***	0.99 (1, 158.8) <i>P</i> = 0.321	145.83 (1, 159.1) <i>P</i> < 0.001***
Total number of embryo heart beats	7 650 391 ± 105 481	6 954 688 ± 111 257	7 689 242 ± 105 997	7 036 319 ± 106 591	108.49 (1, 158.8) <i>P</i> < 0.001***	0.86 (1, 159.4) <i>P</i> = 0.355	0.11 (1, 159.9) <i>P</i> = 0.744
Body mass (g) at hatching	2.87 ± 0.12	2.56 ± 0.12	3.00 ± 0.12	2.97 ± 0.12	11.87 (1, 158.7) <i>P</i> < 0.001***	24.39 (1, 158.7) <i>P</i> < 0.001***	7.20 (1, 158.9) <i>P</i> = 0.008**
Body length (cm) at hatching	15.30 ± 0.20	14.09 ± 0.21	15.37 ± 0.20	14.58 ± 0.20	67.12 (1, 158.8) <i>P</i> < 0.001***	5.27 (1, 159.3) <i>P</i> = 0.023*	2.83 (1, 159.8) <i>P</i> = 0.094
Body condition at hatching	-0.029 ± 0.010	-0.007 ± 0.010	-0.015 ± 0.010	0.031 ± 0.010	30.95 (1, 158.8) <i>P</i> < 0.001***	18.47 (1, 159.3) <i>P</i> < 0.001***	3.69 (1, 159.9) <i>P</i> = 0.056
Residual egg yolk (g)	0.655 ± 0.055	0.431 ± 0.061	0.815 ± 0.056	0.456 ± 0.56	34.61 (1, 60.8) <i>P</i> < 0.001***	3.46 (1, 162.0) <i>P</i> = 0.065	1.81 (1, 163.4) <i>P</i> = 0.180

Abbreviations: dfd, degrees of freedom denominator; dfn, degrees of freedom numerator; F, distribution.

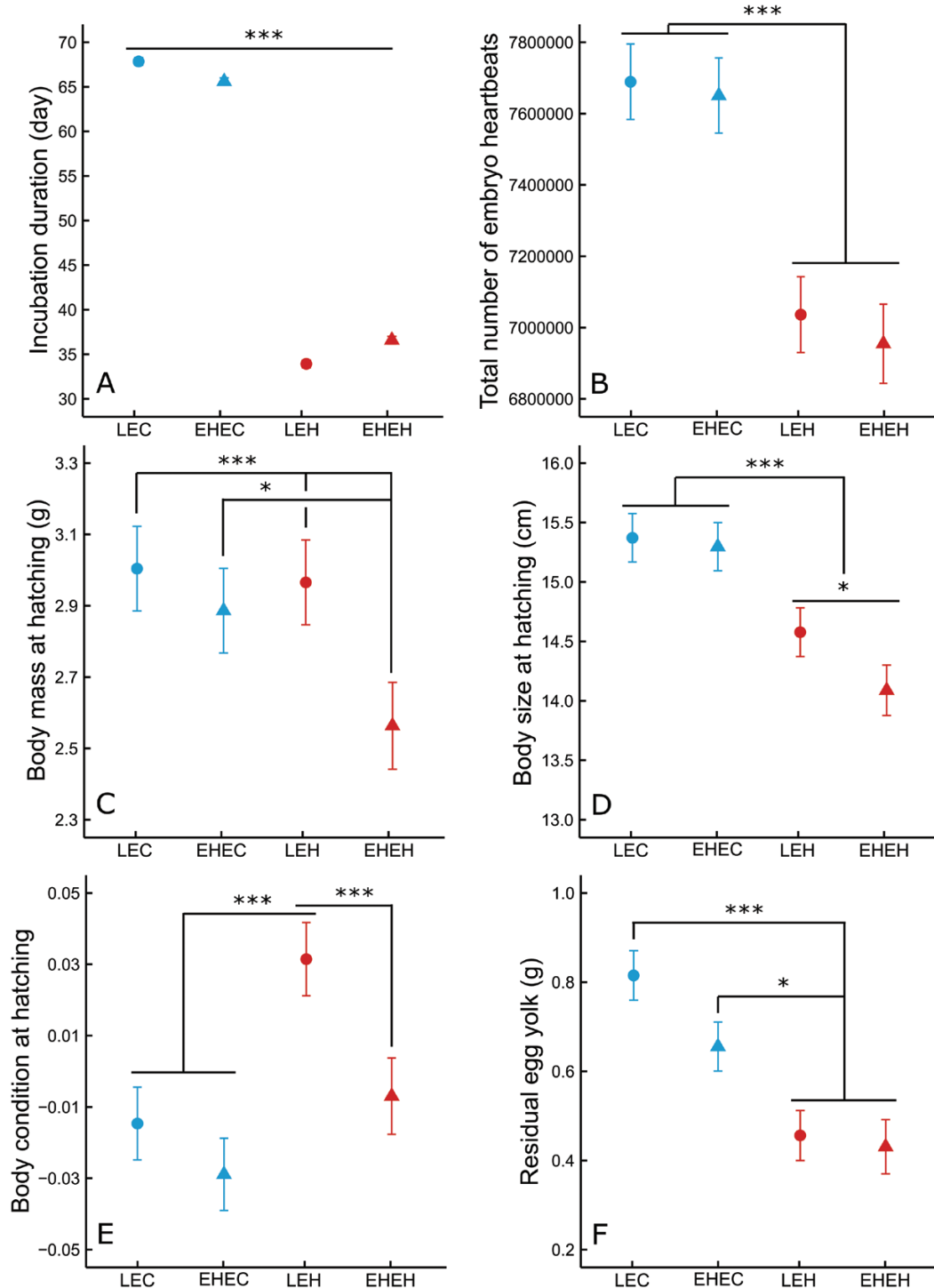


Figure 3. Hatching phenotypes in juveniles of *N. maura*: incubation duration (A), total number of embryo heartbeats (B), body mass (C), body size (D), body condition (E) and residual egg yolk (F) for each incubation treatment: low elevation at 24 °C (LEC; $N = 46$; blue circle), extreme high elevation at 24 °C (EHEC; $N = 48$; blue triangle), low elevation at 32 °C (LEH; $N = 45$; red circle) and extreme high elevation at 32 °C (EHEH; $N = 38$; red triangle). Least-squares means \pm SE estimated by linear mixed-effect models are plotted. Significant differences between least-squares means are shown with one ($P < 0.05$) or three ($P < 0.001$) asterisks.

Table 3. Results of linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (days post-hatching), test elevation, and their interaction on the maximum swimming performance in juveniles in *N. maura* (Tests 3 & 4, Figs 4, 5). Sex and total body length (TL) were included as covariates. The four incubation treatments are extreme high elevation at 24 °C (EHEC; $N = 41$), extreme high elevation at 32 °C (EHEH; $N = 37$), low elevation at 24 °C (LEC; $N = 41$), and low elevation at 32 °C (LEH; $N = 41$). Significant factors shown in bold with one ($P < 0.05$), two ($P < 0.01$) or three ($P < 0.001$) asterisks

	F (dfn, dfd)	P-value
Sex	0.70 (1145.5)	$P = 0.401$
Total body length (cm)	73.81 (1169.2)	$P < 0.001$***
Temperature	49.80 (1156.4)	$P < 0.001$***
Elevation	0.45 (1165.5)	$P = 0.501$
Day	12.62 (3500.6)	$P < 0.001$***
Location of test	0.02 (1442.8)	$P = 0.964$
Temperature × elevation	0.002 (1163.5)	$P = 0.962$
Temperature × day	4.84 (3506.1)	$P = 0.003$**
Temperature × location of test	0.01 (1442.8)	$P = 0.909$
Elevation × day	1.14 (3487.8)	$P = 0.331$
Elevation × location of test	9.80 (1440.7)	$P = 0.002$**
Day × location of test	13.85 (2460.9)	$P < 0.001$***
Temperature × elevation × day effect	0.18 (3487.1)	$P = 0.913$
Temperature × elevation × location of test	0.04 (1446.9)	$P = 0.839$
Temperature × day × location of test	3.25 (2457.5)	$P = 0.040$*
Elevation × day × location of test	0.04 (2456.9)	$P = 0.961$
Temperature × elevation × day × location of test	0.79 (2456.6)	$P = 0.456$

40 days post-hatching) and their interaction with test location (low elevation and extreme high elevation). Moreover, size positively influenced swimming speed within each treatment group, with longer snakes swimming faster (slope estimate \pm SE: 2.71 ± 0.31 ; Table 3; Fig. 4).

At 9 days post-hatching, for the first swimming performance measurement (Test 3) conducted at the elevation of incubation, the post hoc comparison of least-squares means (Supporting Information, Table S3) indicates that maximum swimming speed was similar for both treatments at the cool incubation temperature (i.e. LEC vs. EHEC; Fig. 5A). Juveniles from LEH treatments swam significantly faster (by 22.0%) than LEC and EHEC (Fig. 5A; Supporting Information, Table S3). Finally, the juveniles from the EHEH treatment swam significantly faster (by 10.4%) compared to the LEC treatment (Fig. 5A; Supporting Information, Table S3). After translocation to the opposite oxygen level treatment, maximum swimming speed was only significantly altered in the EHEH treatment at 25 days post hatching (Fig. 5B; Supporting Information, Table S3). That is, individuals translocated to low elevation (EHEH-LE) swam faster (by 18.7%) compared to siblings retained at extreme high elevation (EHEH-EHE). These results remained qualitatively unchanged when measuring swimming speed expressed as body length per second (analysis not shown).

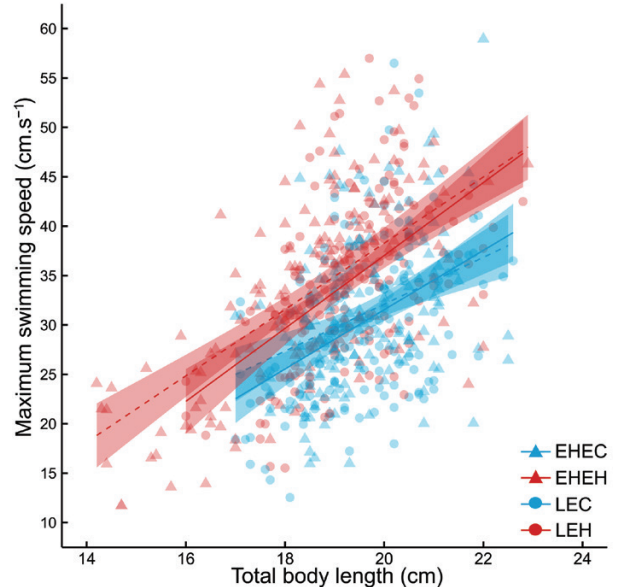


Figure 4. Maximum swimming speed as a function of body length in juveniles of *N. maura* incubated in four treatments: low elevation at 24 °C (LEC; $N = 41$; blue circle), extreme high elevation at 24 °C (EHEC; $N = 41$; blue triangle), low elevation at 32 °C (LEH; $N = 41$; red circle) and extreme high elevation at 32 °C (EHEH; $N = 37$; red triangle). Raw data for each individual are plotted with regression lines and 95% CI.

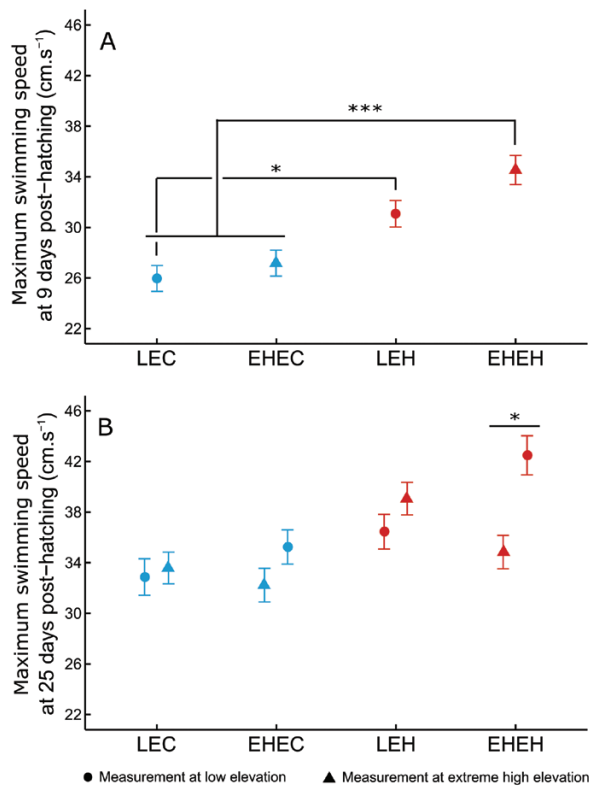


Figure 5. Maximum swimming speed in juveniles of *N. maura* for each incubation treatment. First, at 9 days post-hatching (A) at elevation of incubation (low elevation or extreme high elevation) for all juveniles of the four incubation treatments: low elevation at 24 °C (LEC; $N = 41$; blue circle), extreme high elevation at 24 °C (EHEC; $N = 41$; blue triangle), low elevation at 32 °C (LEH; $N = 41$; red circle) and extreme high elevation at 32 °C (EHEH; $N = 37$; red triangle). Second, at 25 days post-hatching (B) at the same elevation as incubation for half of the juveniles: low elevation at 24 °C (LEC-LE; $N = 24$; blue circle), extreme high elevation at 24 °C (EHEC-EHE; $N = 21$; blue triangle), low elevation at 32 °C (LEH-LE; $N = 22$; red circle) and extreme high elevation at 32 °C (EHEH-EHE; $N = 22$; red triangle). Also at 25 days post-hatching (B) at opposite elevation as incubation for the other half of the juveniles: low elevation at 24 °C (LEC-EHE; $N = 17$; blue triangle), extreme high elevation at 24 °C (EHEC-LE; $N = 20$; blue circle), low elevation at 32 °C (LEH-EHE; $N = 19$; red triangle) and extreme high elevation at 32 °C (EHEH-LE; $N = 15$; red circle). Least-squares means \pm SE estimated by linear mixed-effect models are plotted. Significant differences between least-squares means are shown with one ($P < 0.05$) or three ($P < 0.001$) asterisks.

The proportion of residual variance attributed to clutch was up to 67% (for egg mass) and the inclusion of this random effect significantly improved model fit for most traits measured (Supporting Information, Table S4).

Siblings most strongly covaried for traits related to offspring size (egg mass and body mass at hatching) as well as developmental duration (incubation duration and total heart beats). Only heart rate and sex ratio were not influenced by significant maternal effects.

DISCUSSION

Our study demonstrates the impact of high-elevation hypoxia coupled with temperature regime on development, physiology and early-life performance in an oviparous ectotherm. Irrespective of oxygen availability during incubation, eggs incubated at cool temperature (i.e. LEC and EHEC) maintained higher mass and much lower heart rates throughout incubation compared to siblings incubated at a warmer temperature (Fig. 2; Table 1). The longer incubation duration combined with reduced heart rate at the cool incubation temperature suggests a lowered metabolic rate (Table 1), as expected (Deeming & Thompson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014). At warm incubation temperatures (i.e. LEH and EHEH), viperine snake embryos in extreme high-elevation hypoxia exhibited typical physiological adjustments to hypoxia found in other taxa, including increased heart rate (Table 1; Fig. 2B; Laughlin, 1978; Monge & León-Velarde, 1991; Crossley & Altimiras, 2005; Crossley & Burggren, 2009; Du *et al.*, 2011; Cordero *et al.*, 2017a, b; Kouyoumdjian *et al.*, 2019). Importantly, this trend was not exhibited in snakes incubated at extreme high elevation and low temperatures and furthermore is counter to that we demonstrated in our previous study conducted at an intermediate incubation temperature of 28 °C (Souchet *et al.*, 2020), suggesting that this is an effect of combined increased metabolism and reduced oxygen availability. Reduced heart rates were observed only in embryos incubated at the warmer temperature and extreme high elevation. The interaction of temperature and oxygen availability also influenced other important fitness-related parameters, including offspring development times, hatching success, body size at birth and swimming performance. Notably, the potential negative consequences of reduced oxygen availability were exacerbated by high incubation temperatures.

We observed the strongest effects on development in embryos incubated at extreme high elevation and at high temperature, suggesting that these factors interact to limit the functional capacity of ectotherms. Gas exchange in embryos is diffusion-limited, likely constraining their ability to compensate for reduced oxygen availability through increased oxygen transport capacity (Vitt & Caldwell, 2013). These effects are then exacerbated by the increased demand induced by high

temperatures. Under conditions of high temperature and low oxygen availability, we expect reductions in maximal performance, limitations on physiological processes generally and potentially reductions of critical thermal limits (Gangloff & Telemeco, 2018). For example, recent work demonstrated that lizard embryos suffer a mismatch between oxygen supply and demand at high temperatures, which may serve as the proximal cause of death (Hall & Warner, 2020). Our results demonstrate, for the first time, these effects in snake embryos, in concordance with previous work studying embryonic development *in ovo* under varying temperatures and levels of oxygen availability in other reptile taxa [birds (Vimmerstedt *et al.*, 2019); crocodiles (Iungman and Piña, 2013); lizards (Flewelling and Parker, 2015; Smith *et al.*, 2015; Li *et al.*, 2020); turtles (Liang *et al.*, 2015)]. For example, embryos of the lizard *Podarcis muralis* increase incubation times in conditions of hypoxia when incubated at 28 °C, but not at 24 °C (Cordero *et al.*, 2017a; Kouyoumdjian *et al.*, 2019). In this study, we found that snake embryos incubated at warm temperature and in hypoxia were less likely to survive to hatching, especially because the last-stage embryos have higher oxygen demand (Dmi'el, 1970; Sartori *et al.*, 2017), and, when they did survive, were smaller than snakes in other treatment groups (Table 2; Fig. 3D). In accordance with previous work (Shine, 2004; Daufresne *et al.*, 2009; Du *et al.*, 2009; Gardner *et al.*, 2011; Sheridan and Bickford, 2011; Noble *et al.*, 2018; Refsnider *et al.*, 2019), our results show that snakes incubated at warm temperatures were smaller and shorter than their counterparts, and hatched after fewer total heartbeats, regardless of oxygen availability (Table 2; Fig. 3C, D). Moreover, there was less residual egg yolk in both warm treatments (i.e. LEH and EHEH) compared to cool treatments (i.e. LEC and EHEC) and yet these animals were also smaller, suggesting higher basal metabolic demands associated with high-temperature incubation may reduce growth efficiency (conversion of yolk to body mass). Hatchlings incubated at cool temperatures in hypoxia did not exhibit reduced body size or mass (Table 2; Fig. 3C, D). This result demonstrates that reduced metabolic rates and increased ability to assimilate energy stores associated with cool temperatures mitigate the negative impacts of reduced oxygen availability (Jackson, 2007; Gangloff & Telemeco, 2018).

Swimming speed is an ecologically relevant trait important to predator avoidance and food acquisition in snakes (Jayne & Bennett, 1990; Kingsolver *et al.*, 2001), that typically correlates (positively) with body length (Shine and Shetty, 2001; Aubret *et al.*, 2015). Although this trend was found within each treatment group (Fig. 4), it was not observed across treatments: snakes incubated under both hypoxia and high

temperatures demonstrated the fastest swimming speeds compared to all other treatment groups, despite exhibiting the smallest body size on average (Table 3; Fig. 4). Previous studies in other ectothermic species demonstrate that cool incubation temperatures produce faster swimmers (Shine, 1999; Angilletta & Dunham, 2003; Watkins & Vraspir, 2006; Gahm *et al.*, 2020). At 9 days post hatching, juveniles in this experiment did not follow this trend: juveniles from warm treatments (i.e. LEH and EHEH) were faster swimmers than their siblings from cool treatments (i.e. LEC and EHEC) in both absolute and relative swimming speed. Most surprisingly, juveniles from the EHEH treatment were also faster than juveniles from LEH despite smaller body size and conditions of oxygen limitation (Fig. 5A). One potential explanation for this finding is that warm incubation temperature and oxygen limitation may reduce the optimal temperature for performance (Gangloff & Telemeco, 2018). We suggest that juveniles from the EHEH treatment potentially reduced their optimal temperature for performance, thus swimming faster than the other groups at the test temperature of 25 °C. Alternatively, exposure to hypoxia during development may have induced plastic changes in cardiovascular, muscular or mitochondrial function to increase performance capacity (Eme *et al.*, 2013; Sun *et al.*, 2015; Galli *et al.*, 2016). Further experiments directed towards quantifying the effects of incubation temperature on the entire thermal performance curve are necessary to fully characterize how incubation temperature influences both physiology and performance across a range of temperatures (Taylor *et al.*, 2020).

After relocation to low elevation, juveniles from the EHEH treatment swam faster than siblings remaining at extreme high elevation, which maintained swimming speeds similar to other treatment groups measured at both extreme high and low elevation (Fig. 5B). In birds and mammals, the acclimation to high-elevation hypoxia can include an alteration of cardio-respiratory pathways, a modification of blood composition and increased muscle performance (Monge & León-Velarde, 1991; Beall *et al.*, 2002; Storz *et al.*, 2004; Lague *et al.*, 2016). Similar effects have been demonstrated in other reptiles (Iungman & Piña, 2013; González-Morales *et al.*, 2015; Lu *et al.*, 2015; Wearing *et al.*, 2015; Jochmans-Lemoine & Joseph, 2018; Gangloff *et al.*, 2019). These modifications may allow the maintenance of locomotor performance such as swimming. Furthermore, these physiological and anatomical changes due to development in chronic hypoxia serve to improve performance under normoxic conditions, similar to athletes training at high altitudes for competition at sea level (e.g. Khodae *et al.*, 2016). Repeated measurements throughout ontogeny are necessary to quantify the time frame over which

such compensatory mechanisms remain relevant (Mitchell *et al.*, 2018). Finally, although we cannot speculate on the adaptive value of such behaviour at this stage, this response to a double constraint (high incubation temperature and low oxygen level) may be yet another case of informed dispersal in reptiles [as in *Zootoca vivipara* Lichtenstein, 1823 and *N. maura* (Clobert *et al.*, 2009; Bestion *et al.*, 2015a; Aubret *et al.*, 2016)]: environmental clues may convey important information about the quality of the natal environment and foster dispersal behaviour and/or dispersal enhancing traits (i.e. high locomotor performance). Importantly, the high level of observed maternal effects (Supporting Information, Table S4) indicates the necessity of a split-clutch design in any experiment measuring similar traits in squamate reptiles. Future work directed towards partitioning this estimate into narrow-sense heritability and maternal effects will be important to predict the evolutionary response to novel conditions within populations, especially at the colonization front.

CONCLUSION

Our results suggest that even though body size, development and physiology are altered, and hatching success is lowered, the majority of embryos developing in high-elevation hypoxia produced viable young snakes. Furthermore, these snakes were able to equal or exceed the swimming performance of snakes incubated under native conditions. We stress that the results of this experiment represent an extreme case of abiotic limitation (exposing developing embryos from low elevation to a 32 °C incubation temperature and 72% sea-level equivalent O₂ availability). Such approaches are important to identify patterns among mechanisms, pathways and constraints, allowing subsequent experimental designs to be refined accordingly. In some species of squamates, embryos transplanted to extreme high elevation do not suffer reductions in survival and maintain fitness-relevant traits at hatching (Du *et al.*, 2010a; Cordero *et al.*, 2017a; Kouyoumdjian *et al.*, 2019; Li *et al.*, 2020). In this context, the plastic physiological responses might mitigate environmental stress, thereby promoting offspring survival and influence the direction of subsequent evolution of colonizing populations (Atkinson & Thorndyke, 2001; McNab, 2002; West-Eberhard, 2003; Hammond *et al.*, 2006; Ghalambor *et al.*, 2007). Our results tentatively suggest that the embryonic stage will not be the limiting factor in the potential for viperine snake to utilize expansion to high-elevation habitat as a strategy to track favourable environmental temperatures. However, the success

this colonization could be threatened if temperatures continue to rise, especially at high elevation.

ACKNOWLEDGEMENTS

We are grateful to the staff of Observatoire Midi-Pyrénées for logistical support in Pic du Midi de Bigorre as well as Isabel Verdaguer, Joaquim Soler and Zuleica Alonso for their help in the laboratory. We thank three anonymous reviewers for their helpful comments. This work was supported by the French Laboratory of Excellence project “TULIP” (ANR-10-LABX-41; ANR-11-IDEX-0002-02), INTERREG POCTEFA ECTOPYR (No. EFA031/15), and the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 752299. 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 Préfectures of Ariège, Aude, Haute-Garonne, Hautes-Pyrénées and Pyrénées Orientales (Arrêté Préfectoral No. 2017-s-02 du 30 mars 2017) and ethical committee (APAFIS#16359-201808011445465 v4). All experiments were carried out in accordance with the approved guidelines. Animal caretakers and handlers were trained to use wildlife for 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). Author contributions: J.S., H.P. and F.A. were responsible for experimental design and logistics; J.S., C.B., E.D., H.L.C. and M.P. conducted the experiments; J.S. and E.J.G. carried out the statistical analyses; J.S., E.J.G. and F.A. drafted the manuscript; all authors contributed critically to the drafts and gave final approval for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Results of the pairwise comparison of least-squares means and adjusted *P*-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (days post hatching), and their interaction on embryo traits in *N. maura*.

Table S2. Results of the pairwise comparison of least-squares means and adjusted *P*-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, and their interaction on the juvenile traits in *N. maura*.

Table S3. Results of the pairwise comparison of least-squares means and adjusted *P*-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (days post-hatching), test location, and their interaction on the maximum swimming performance in juveniles of *N. maura*.

Table S4. Residual variance estimates from linear and generalized linear mixed models.