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

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## 1 INTRODUCTION

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

1  
2  
3 24 populations at high-elevation to the double constraints of low oxygen availability and sub-  
4  
5 25 optimal temperatures.  
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8 26  
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10 27 Ectotherm physiology and behavioural processes are strongly dependent on environmental  
11  
12 28 temperatures (Huey and Stevenson, 1979; Angilletta et al., 2002; Gillooly et al., 2002;  
13  
14 29 Deutsch et al., 2008; Huey et al., 2012), and therefore they are often utilized as a model in  
15  
16 30 climate change related experiments and studies (Pen et al., 2010; Sinervo et al., 2010;  
17  
18 31 Bestion et al., 2015a; Dahlhoff et al., 2019). Our current study focuses on embryo  
19  
20 32 development and hatching success, because the production of viable and fit offspring is a  
21  
22 33 required condition for successful dispersal and population establishment in novel  
23  
24 34 environments (Baguette et al., 2012). Further, incubation temperature is the main driver of  
25  
26 35 embryonic development and hatchling phenotype in ectotherms such as reptiles (Deeming &  
27  
28 36 Ferguson, 1991; Deeming, 2004; Booth, 2006; Goodman, 2008; Warner, 2014; Refsnider et  
29  
30 37 al., 2019). The influence of incubation temperatures (notably above the optimal range;  
31  
32 38 Andrews and Schwarzkopf, 2012) on hatchling phenotype is especially well known in  
33  
34 39 reptiles, affecting development, sex determination, incubation duration, body size, growth  
35  
36 40 rate, locomotor performance, cognitive abilities, and post-natal behaviour (Shine, 2004;  
37  
38 41 Deutsch et al., 2008; Daufresne et al., 2009; Gardner et al., 2011; Sheridan & Bickford, 2011;  
39  
40 42 Bestion et al., 2015b; Cunningham et al., 2017; Noble et al., 2018; Pellerin et al., 2019;  
41  
42 43 Refsnider et al., 2019). Additionally, the effects of low oxygen availability on physiology have  
43  
44 44 attracted recent attention, including in the context of altitudinal range shifts driven by  
45  
46 45 climate change (Powell and Hopkins, 2010; Storz et al., 2010; Jacobsen, 2020). For instance,  
47  
48 46 it was shown that common wall lizards (*Podarcis muralis*, Laurenti, 1768) transplanted to  
49  
50 47 high elevation enhanced oxygen-carrying capacity by increasing hematocrit and blood  
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3 48 hemoglobin concentration, though transplanted lizards still suffered a reduction in running  
4  
5 49 endurance (Gangloff et al., 2019). Further, reptile embryos exposed to hypoxia increased  
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8 50 heart rates in some studies (Du et al., 2010a; Souchet et al., 2020), while in other cases  
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10 51 hypoxia leads to decreased heart rates and cardiac hypertrophy (Cordero et al., 2017a;  
11  
12 52 Kouyoumdjian et al., 2019). In viperine snakes, exposure to hypoxia during incubation  
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14  
15 53 resulted in hatchlings that were smaller in body size and slower swimmers (a proxy for  
16  
17 54 predator avoidance and food acquisition in snakes; Jayne & Bennett, 1990; Kingsolver et al.,  
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19  
20 55 2001) compared to their siblings incubated at lower elevation (Souchet et al., 2020).  
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23 56

24  
25 57 Recent work suggests that the interaction of high temperature and oxygen limitation will  
26  
27 58 alter embryo development (Jackson, 2007; Flewelling & Parker, 2015; Smith et al., 2015;  
28  
29 59 Gangloff & Telemeco, 2018; Hall & Warner, 2020; Li et al., 2020). Here we experimentally  
30  
31 60 tested the effect of high temperature (*i.e.*, current populations caught in the climatic debt),  
32  
33 61 low oxygen availability (*i.e.*, populations having shifted their range in altitude in the near  
34  
35 62 future), and the combined effect of high temperature and low oxygen (*i.e.*, extreme high  
36  
37 63 elevation in the year 2070) on the development, hatching success and hatchling phenotype  
38  
39 64 in a temperate snake species (viperine snake, *Natrix maura*, Linnaeus 1758). This is a first  
40  
41 65 step toward assessing the colonization potential to high elevation in a potentially upward-  
42  
43 66 migrating species. We used a split-clutch design and incubated eggs in four ecologically  
44  
45 67 relevant treatments: 1) oxygen availability at native elevation (normoxia; 436m above sea  
46  
47 68 level [ASL]) and 32°C incubation temperature (*i.e.*, populations lagging behind climate  
48  
49 69 change); 2) low oxygen availability (2877 m ASL), 24°C incubation (*i.e.*, range shifted in  
50  
51 70 altitude); 3) low oxygen availability, 32°C incubation (*i.e.*, high altitude in the year 2070), and  
52  
53 71 4) a normal oxygen availability, 24°C incubation control treatment (*i.e.*, recent past  
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3 72 conditions). We monitored embryo heart rates (a proxy for metabolism and cardiovascular  
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5 73 function; Crossley & Burggren, 2009) and egg mass throughout the incubation and measured  
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8 74 fitness-relevant aspects of hatchling phenotypes (body size and swimming performance) at  
9  
10 75 hatching. This factorial design allowed us to tease apart the individual and combined effects  
11  
12 76 of incubation temperature and oxygen levels on embryo development and hatchling  
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14  
15 77 phenotypes. Based on our previous work (Souchet et al., 2020), we expected that the  
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17  
18 78 extreme high elevation will decrease egg mass and induce higher heart rates throughout  
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20 79 incubation. Moreover, we predicted incubation duration will be shorter and the hatchlings  
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22 80 will be smaller in high-elevation hypoxia. Further, we predicted that the combined  
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24  
25 81 constraints imposed by higher metabolic rates induced by warmer incubation temperature  
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28 82 (Huey, 1982; Angilletta, 2009; Dillon et al., 2010) and oxygen limitation on juveniles will  
29  
30 83 result in a reduced performance capacity. Specifically, we predicted that embryos incubated  
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32 84 at extreme high elevation will produce slower-swimming juveniles and that embryos  
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34  
35 85 developing under conditions of both high temperature and high-elevation hypoxia will be  
36  
37  
38 86 the slowest. Finally, we further partitioned treatment groups to test whether the effects of  
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40 87 embryonic environment would be ontogenetically stable even after hatchlings were  
41  
42 88 transplanted to the alternative elevation.  
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45 89

## 90 **MATERIALS AND METHODS**

### 91 ***Experimental design***

92 We captured 17 gravid females viperine snake along the banks of the Lez River (Department  
93 of Ariège, France), between May and July 2017. This aquatic species (Vacher and Geniez,  
94 2010) has been recorded up to 1000 m above sea level [ASL] in France (Aubret et al., 2015;  
95 Pottier, 2016) and 1500 m ASL in Spain (Martinez-Rica & Reiné-Viñales, 1988; Santos, 2015).

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2  
3 96 The viperine snake has been exposed to fluctuating temperatures and has migrated along  
4  
5 97 the elevational gradient throughout its evolutionary history, colonizing mountainous  
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8 98 environments repeatedly in conjunction with historical warming and cooling cycles (Gómez  
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10 99 & Lunt, 2007). Capture sites spanned from 412m to 715m ASL. Each female was maintained  
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12  
13 100 in the Station d'Ecologie Théorique et Expérimentale du Centre National de la Recherche  
14  
15 101 Scientifique (SETE-CNRS; 42.958394 N, 1.086440 E) and laid a single clutch for a total of 205  
16  
17 102 eggs between 21 June 2017 and 22 July 2017 (mean clutch size  $\pm$  SD =  $11.9 \pm 4.9$  eggs). Three  
18  
19 103 eggs were infertile, leaving 202 eggs for the experiment. All females were returned to their  
20  
21  
22 104 exact site of capture within two weeks of egg-laying.  
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26  
27 106 We first investigated how temperature (cool temperature at constant 24°C; and hot  
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29 107 temperature at constant 32°C) and oxygen availability interact to influence embryonic  
30  
31  
32 108 development. Oxygen treatments were normoxia at the SETE-CNRS (low elevation at 436 m  
33  
34 109 ASL, 95% sea-level equivalent O<sub>2</sub> availability, PO<sub>2</sub> ~20.1 kPa) and high-elevation hypoxia at  
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36  
37 110 the Observatory Midi-Pyrénées of the Pic du Midi de Bigorre (42.936389 N, 0.142472 E,  
38  
39 111 above current range limits at 2877 m ASL, 72% sea-level equivalent O<sub>2</sub> availability, PO<sub>2</sub> ~15.3  
40  
41  
42 112 kPa). This difference in elevation results in a decrease in atmospheric pressure, with  
43  
44 113 associated reduction in the partial pressure of gases, including oxygen, carbon dioxide, and  
45  
46 114 water vapor (Millet & Debevec, 2020; Richalet, 2020). Most relevant to our hypotheses is the  
47  
48 115 25% reduction in oxygen availability at the Pic du Midi de Bigorre lab in comparison to sea  
49  
50 116 level (Bouverot, 2012). Eggs were weighed using a digital scale (to the nearest 0.01 g) within  
51  
52  
53 117 12 hours of oviposition and individually marked for identification with a pencil. We used a  
54  
55 118 split-clutch design and allocated eggs to four incubation treatments within 24 hours of  
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57 119 oviposition (Figure 1): Low Elevation and Cool temperature (LEC; normoxia at constant 24°C),  
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2  
3 120 Low Elevation and Hot temperature (LEH; normoxia at constant 32°C), Extreme High  
4  
5 121 Elevation and Cool temperature (EHEC; hypoxia at constant 24°C) and Extreme High  
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7  
8 122 Elevation and Hot temperature (EHEH; hypoxia at constant 32°C). Because egg mass  
9  
10 123 influences both embryo metabolism and hatching phenotype (Nelson et al., 2004; Aubret,  
11  
12 124 2013), and egg mass varied among clutches (Kruskal-Wallis test:  $H= 148.42$ ,  $Df= 15$ ,  $P <$   
13  
14 125  $0.001$ ), eggs were ranked within each clutch from lightest to heaviest and alternately  
15  
16 126 assigned to treatments in order to ensure no difference in egg mass between treatments  
17  
18 127 (Kruskal-Wallis test:  $H=0.151$ ,  $Df= 3$ ,  $P = 0.985$ ). LEC, LEH, EHEC and EHEH treatment quarter-  
19  
20 128 clutches were placed in a plastic container (20 cm x 15 cm x 5 cm) on a 2 cm layer of wet  
21  
22 129 vermiculite (1:5 water to vermiculite by volume) and incubated in four identical incubation  
23  
24 130 chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc., USA). Water bowls placed within  
25  
26 131 each incubator, directly under the incubator's fan, ensured high levels of humidity  
27  
28 132 throughout incubation (indicated by condensation on the incubator walls).  
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37 134 Out of 202 eggs, 177 embryos from 16 females successfully hatched (87.6% hatching success  
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39 135 rate) while 25 died at various stages during incubation. Another 17 neonates died shortly  
40  
41 136 after hatching (between 24 hours to two weeks). We measured morphology (Test2, Figure1;  
42  
43 137 see below) first on all 177 hatchlings at their incubation location (low or extreme high  
44  
45 138 elevation). Our experimental design allowed us to measure the effects of temperature and  
46  
47 139 hypoxia during incubation on juvenile development and performance. It also allowed us to  
48  
49 140 measure the short-term effects on juvenile development and performance in acute high-  
50  
51 141 elevation hypoxia after translocation to extreme high elevation. In order to assess these  
52  
53 142 questions, at nine days post-hatching (after all yolk was assimilated; Ji et al., 1999) we  
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55 143 measured morphology and swimming performance (Test 3, Figure1; see below) first on all  
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3 144 160 hatchlings at their incubation elevation (low or extreme high elevation). After this first  
4  
5 145 measurement, half of the hatchlings in the LEC and LEH treatments were transferred to  
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7  
8 146 extreme high elevation while half of the hatchlings from the EHEC and EHEH treatments  
9  
10 147 were brought down to the low elevation site. All juveniles were then tested for swimming  
11  
12  
13 148 performance and morphology at 11 days, 25 days, and at 40 days post-hatching (respectively  
14  
15 149 one day, two weeks and one month of acclimation for transferred juveniles; Test 4, Figure 1).  
16  
17  
18 150 Once tests were completed, young snakes were fed with small dead minnows (0.5 g to 1 g)  
19  
20 151 and released between 42 and 45 days post-hatching at the maternal capture site.  
21  
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23 152

### 24 25 153 ***Egg mass and heart rate measurements***

26  
27 154 We weighed each egg using a digital scale (to the nearest 0.01 g) within 12 hours of  
28  
29 155 oviposition, and then every 7 days until hatching (Figure 1; Test 1). Embryo heart rates were  
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31  
32 156 first measured at 7 days of incubation and then every 7 days until hatching (Figure 1; Test 1)  
33  
34  
35 157 at the same temperature as incubation. To measure embryo heart rates, we used the Buddy  
36  
37 158 digital egg monitor (MK2, Avitronics, Cornwall, UK) under the standardized protocol  
38  
39 159 described for eggs (Aubret, 2013; Cordero et al., 2017a; Souchet et al., 2020). Each egg was  
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41  
42 160 gently placed onto the sensor pad for heart rate reading (a stable reading was obtained after  
43  
44  
45 161 approximately 30 seconds) and then returned to its clutch. All eggs were only briefly ( $\leq$   
46  
47 162 1min) placed in the digital egg monitor to mitigate potential temperature changes owing to  
48  
49 163 exposure to infrared sensors (Sartori et al., 2015; Hulbert et al., 2017). Heart rates can be  
50  
51  
52 164 influenced by a variety of factors (Clark et al., 2006; Du et al., 2010b) and are linked to  
53  
54 165 metabolic rate in some circumstances (Kouyoumdjian et al., 2019), though this relationship  
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56  
57 166 may become less clear especially late in development (Sartori et al., 2017). We also  
58  
59 167 calculated the total number of heart beats (THB) of embryos throughout embryonic  
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3 168 development using the formula  $THB = \text{average heart rate} \times \text{total minutes of developmental}$   
4  
5 169 duration (Du et al., 2009, 2011).  
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10 171 ***Hatchling measurements***

11  
12 172 Hatching occurred between 8 August 2017 and 29 September 2017 (Figure 1; Test 2) and  
13  
14 173 hatchlings were individually marked for identification with a medical cauterizer (Model HIT0,  
15  
16 174 Bovie, USA) on the ventral scales (Winne et al., 2006) within 24 hours of emergence.  
17  
18 175 Hatchlings were weighed using a digital scale (to the nearest 0.01 g), measured for snout-  
19  
20 176 vent length (SVL) and total body length (TL) using a measuring tape (to the nearest 0.1 cm),  
21  
22 177 and sexed via hemipene eversion. Since sex is genetically determined in snakes, we did not  
23  
24 178 expect an effect of treatment on sex determination, but tested for differential effects  
25  
26 179 between the sexes in developing embryos which could result in skewed hatchling sex ratios.  
27  
28 180 We calculated body condition as the residual of the  $\log_{10}$ -mass on  $\log_{10}$ -SVL linear regression  
29  
30 181 at hatching day. Finally, we weighed the yolk leftover in the eggshell (residual egg yolk) using  
31  
32 182 a digital scale (to the nearest 0.01 g). Juveniles were housed together by hatching date in  
33  
34 183 plastic containers (15 cm x 10 cm x 5 cm) with a water dish, shelter and paper towel as a  
35  
36 184 substrate in incubation chambers (ExoTerra Model PT-2445, Rolf C. Hagen Inc. Canada) set at  
37  
38 185 constant 20°C. Our experience with viperine snake shows that cooler temperatures (below  
39  
40 186 thermal optimum for performance or preferred temperatures) results in higher juvenile  
41  
42 187 survivorship (93% survival at one month in this species; J.S. and F.A. unpubl. data). Juveniles  
43  
44 188 were measured again at 9 days, 11 days, 25 days and 40 days post-hatching for SVL, TL and  
45  
46 189 body mass prior to performance testing.  
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59 191 ***Swimming performance***  
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3 192 For this test, we were interested in measuring the maximal swimming speed to evaluate the  
4  
5 193 potential limitation of hypoxia on this ecologically-relevant performance. To estimate the  
6  
7  
8 194 swimming speed we used a procedure that has been validated for snakes (Shine & Shetty,  
9  
10 195 2001; Aubret, 2004; Aubret et al., 2005), modified effectively for juveniles (Souchet et al.,  
11  
12 196 2020). A high-definition wide-angle digital camera (25 fps, Sony Model HDR-XR160E, Sony  
13  
14 197 Corporation) was fitted above a linear swimming track (100 cm x 20 cm x 20 cm) and used to  
15  
16 198 record swimming trials. The tank was filled to a depth of 5 cm with water maintained at 25°C  
17  
18 199 using aquarium heaters. A standard testing temperature of water at 25°C was used because  
19  
20 200 it approximates the optimal temperature for swimming speed of the viperine snake (Hailey  
21  
22 201 & Davies, 1986; Aubret et al., 2015). At 9 days, 11 days, 25 days and 40 days post-hatching,  
23  
24 202 each snake was acclimated to 25°C for 30 minutes and swam 10 consecutive lengths. Raw  
25  
26 203 data were extracted from video files by measuring swimming speed ( $\text{cm}\cdot\text{s}^{-1}$ ) for each length  
27  
28 204 (ten per individual and day of measurement) with the software Tracker (Brown, 2019). The  
29  
30 205 fastest performance from all trials was utilized for swimming analysis. Analyzing the average  
31  
32 206 swimming speed of the 10 trials gives the same qualitative results; however, since our focus  
33  
34 207 is performance capacity, we include results for maximum swimming speed here.  
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#### 44 209 ***Data analysis***

45  
46 210 We first assessed the influence of the temperature and elevation of incubation, and time of  
47  
48 211 development on egg mass and embryo heart rate (Test 1). We used linear mixed-effect  
49  
50 212 models, including as main effects the temperature of incubation (cool: 24°C; warm: 32°C),  
51  
52 213 the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation  
53  
54 214 hypoxia), the age at measurement (0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days post-laying)  
55  
56 215 treated as a categorical effect to account for the expected nonlinear response over time  
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58  
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2  
3 216 (Burggren & Warburton, 1994; Cordero et al., 2017a; Sartori et al., 2017), and all three- and  
4  
5  
6 217 two-way interactions. We then assessed the influence of temperature and elevation of  
7  
8 218 incubation on eight measures of hatchling phenotype at hatching (Test 2): survival to  
9  
10 219 hatching, sex, incubation time, total number of heartbeats (THB), body mass, body size (SVL),  
11  
12  
13 220 body condition, and residual egg yolk. We used linear mixed-effect models, including in all  
14  
15 221 models the same main effects of the temperature of incubation (cool: 24°C; warm: 32°C),  
16  
17  
18 222 the elevation of incubation (low elevation: normoxia; extreme high elevation: high-elevation  
19  
20 223 hypoxia), and interactions as above. Finally, we assessed the influence of the temperature  
21  
22  
23 224 and elevation of incubation on swimming performance of juveniles (Test 3 and Test 4). We  
24  
25 225 used linear mixed-effect models, including as the main effects the temperature of incubation  
26  
27  
28 226 (cool: 24°C; warm: 32°C), the elevation of incubation (low elevation: normoxia; extreme high  
29  
30 227 elevation: high-elevation hypoxia), the age at measurement (9, 11, 25 and 40 days post-  
31  
32 228 hatching), the location of test (low elevation or extreme high elevation), and all four-, three-  
33  
34  
35 229 and two-way interactions. We also include as covariates the total body length (TL) and the  
36  
37 230 sex of juveniles.

39  
40 231  
41  
42 232 To account for the non-independence of siblings we included the clutch of origin as a  
43  
44  
45 233 random effect (intercept) in all models. In models for which we measured individuals  
46  
47 234 repeatedly (egg mass, embryo heart rates, and swimming performance), we also included  
48  
49  
50 235 individual as a random effect (intercept), nested within clutch. We used type III sums of  
51  
52 236 squares to assess the significance of main effects, incorporating a Kenward-Roger  
53  
54 237 denominator degree of freedom approximation (Kenward & Roger, 1997). We also  
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56  
57 238 conducted a pairwise comparison of least-squares means and adjusted p-values for multiple  
58  
59 239 comparisons with the Tukey method. All analyses were conducted with the lme4 package  
60

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2  
3 240 (Bates et al., 2014) and the emmeans package (Lenth, 2016) and figures were made with the  
4  
5 241 ggplot2 package (Wickham, 2016) in the programming language R 3.6.1 (R Development  
6  
7  
8 242 Core Team, 2019).  
9

10 243

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12  
13 244 **RESULTS**14  
15 245 ***Test 1: Egg mass and embryonic heart rates***

16  
17 246 The main effects of elevation, temperature, and time of measurement (days post-laying) and  
18  
19 247 their interaction significantly altered egg mass trajectories (Table 1, Figure 2A). Eggs  
20  
21 248 incubated at 24°C (*i.e.*, LEC and EHEC) gained mass for 35 days post-oviposition before  
22  
23 249 decreasing (Table 1, Figure 2A), while the mass of eggs incubated at 32°C (*i.e.*, LEH and  
24  
25 250 EHEH) decreased throughout the incubation (Table 1, Figure 2A). The post-hoc comparison  
26  
27 251 of least-squares means from the model (Table S1) indicates that eggs masses were similar in  
28  
29 252 eggs incubated at the same incubation temperature whatever the oxygen availability.  
30  
31 253 Nevertheless, at the last day of measurement (28 days post-hatching for LEH and EHEH; 63  
32  
33 254 days post-hatching for LEC and EHEC) the egg mass of both treatments in extreme high  
34  
35 255 elevation were significantly less than those of the low elevation treatments (Figure 2A; Table  
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37 256 S1). Eggs incubated at 24°C (*i.e.*, at LEC and EHEC) maintained higher mass (mean difference  
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39 257  $\pm$  SE:  $0.36 \pm 0.08$  g) across the incubation period compared to eggs incubated at 32°C (*i.e.*, at  
40  
41 258 LEH and EHEH).  
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51 259  
52 260 Heart rate trajectories were also significantly altered by elevation and temperature, time of  
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54 261 measurement (days post-laying), and by the interaction between temperature and both  
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56 262 elevation and time of measurement (Table 1, Figure 2B). Heart rates of embryos incubated  
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58 263 at 32°C (*i.e.*, at LEH and EHEH) increased rapidly during the first 7 days of incubation before  
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3 264 decreasing for the remainder of the incubation (Table 1, Figure 2B), while embryos  
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5 265 incubated at 24°C (*i.e.*, at LEC and EHEC) maintained stable heart rates throughout  
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8 266 incubation (Table 1, Figure 2B). Post-hoc comparison of least-squares means (Table S1)  
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10 267 indicates that embryos from LEH treatment maintained higher heart rates (mean difference  
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12  
13 268  $\pm$  SD:  $7.25 \pm 1.17$  bpm) across the incubation period compared to EHEH. Further, eggs in the  
14  
15 269 EHEH treatment exhibited much higher heart rates (mean difference  $\pm$  SE:  $33.39 \pm 1.00$  bpm)  
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18 270 compared to both embryo groups incubated at 24°C (*i.e.*, at LEC and EHEC).  
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### 22 272 ***Test 2: Hatching success and morphological measurements***

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25 273 Hatching success of embryos was dependent on incubation temperature and the interaction  
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28 274 with the elevation (LEC = 90.2%, LEH = 91.8%, EHEC = 94.1% and EHEH = 74.5%; Table 2).  
29  
30 275 Post-hoc comparison of least-squares means (Table S2) indicates that hatching success  
31  
32 276 differed between eggs in the EHEC and EHEH treatments. We observed that of the 25 dead  
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34  
35 277 embryos, half of them are from EHEH. Moreover, in this treatment, 92% of the death  
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37  
38 278 appeared in the last stage of development. Elevation and temperature levels did not affect  
39  
40 279 the hatchling sex ratio (LEC = 56.5%; LEH = 57.8%; EHEC = 47.9%; and EHEH = 42.1% females;  
41  
42 280 Table 2). Incubation duration differed between embryos incubated in the four treatments as  
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44  
45 281 a function of temperature and its interaction with the elevation (Table 2, Figure 3A). All  
46  
47 282 treatment groups are significantly different from each other (Table S2). Snakes in the LEC  
48  
49  
50 283 treatment incubated  $2.25 \pm 0.28$  days longer than EHEC, EHEC treatment incubated  $29.01 \pm$   
51  
52 284  $0.29$  days longer than EHEH, and EHEH treatment incubated  $2.66 \pm 0.30$  days longer than  
53  
54 285 LEH. Only the temperature of incubation affected THB of embryos (Table 2, Figure 3B). THB  
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56  
57 286 did not differ in embryos from the same incubation temperature (Table S2) and THB were  
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59 287 greater in the cool incubation temperature treatments (*i.e.*, LEC and EHEC) compared to  
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3 288 warm (*i.e.* LEH and EHEH). Moreover, the residual egg yolk was also significantly affected by  
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6 289 the temperature of incubation (Table 2, Figure 3F). Comparison of least-squares means from  
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8 290 the model (Table S2) indicates that residual egg yolk was similar for the treatments within an  
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10 291 incubation temperature (*i.e.*, LEC vs EHEC and LEH vs EHEH), but that snakes in the LEC and  
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12 292 EHEC treatments retained an average of 0.29 g (33.6%) more residual egg yolk compared to  
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15 293 the LEH and EHEH treatments.

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20 295 Elevation and temperature and their interaction influenced body mass and body size (SVL) at  
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22 296 hatching (Tables 2 & S2, Figures 3C & 3D). Elevation and temperature influenced hatchlings'  
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24 297 body condition (Tables 2 & S2, Figure 3E). In all cases, the two cool treatments (*i.e.*, at LEC  
25  
26 298 and EHEC) did not significantly differ from each other. For the body mass at one day post-  
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28 299 hatching, LEH treatment did not differ from either LEC or EHEC treatments. However, the  
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30 300 EHEH treatment was  $0.39 \pm 0.12$  g (13.2%) lighter compared to the three other treatments.  
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32  
33 301 For body size at one day post-hatching, cool-temperature treatments were  $0.72 \pm 0.17$  cm  
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35 302 (5.1%) longer than snakes in the LEH treatment, which in turn were  $0.49 \pm 0.18$  cm (4.7%)  
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37 303 longer than snakes in the EHEH treatment. Finally, the different treatments also influenced  
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39 304 the body condition at one day post-hatching (Tables 2 & S2, Figure 3E), with snakes in the  
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41 305 LEH treatment having a 35.4% higher body condition compared to the three other  
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43 306 treatments.

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### 50 308 ***Tests 3 & 4: Swimming performance***

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52 309 Globally, maximum swimming speed (Table 3) was influenced by the effect of incubation  
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54 310 temperature (24°C and 32°C), the time of measurement (9, 11, 25, and 40 days post-  
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56 311 hatching), and their interaction with test location (low elevation and extreme high

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3 312 elevation). Moreover, size positively influenced swimming speed within each treatment  
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6 313 group, with longer snakes swimming faster (slope estimate  $\pm$  SE:  $2.71 \pm 0.31$ ; Table 3, Figure  
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8 314 4).

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13 316 At nine days post-hatching, for the first swimming performance measurement (Test 3)  
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15 317 conducted at the elevation of incubation, the post-hoc comparison of least-squares means  
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17 318 (Table S3) indicates that maximum swimming speed was similar for both treatments at the  
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19 319 cool incubation temperature (*i.e.*, LEC vs EHEC; Figure 5A). Juveniles from LEH treatments  
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21 320 swam significantly faster (by 22.0%) than LEC and EHEC (Figure 5A; Table S3). Finally, the  
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23 321 juveniles from the EHEH treatment swam significantly faster (by 10.4%) compared to the LEC  
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25 322 treatment (Figure 5A; Table S3). After translocation to the opposite oxygen level treatment,  
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27 323 maximum swimming speed was only significantly altered in the EHEH treatment at 25 days  
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29 324 post hatching (Figure 5B; Table S3). That is, individuals translocated to low elevation (EHEH-  
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31 325 LE) swam faster (by 18.7%) compared to siblings retained at extreme high elevation (EHEH-  
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33 326 EHE). These results remained qualitatively unchanged when measuring swimming speed  
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35 327 expressed as body length per second (analysis not shown).  
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45 329 The proportion of residual variance attributed to clutch was up to 67% (for egg mass) and  
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47 330 the inclusion of this random effect significantly improved model fit for most traits measured  
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49 331 (Table S4). Siblings most strongly covaried for traits related to offspring size (egg mass and  
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51 332 body mass at hatching) as well as developmental duration (incubation duration and total  
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53 333 heart beats). Only heart rate and sex ratio were not influenced by significant maternal  
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55 334 effects.  
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**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 (Figure 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 & Ferguson, 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 reduced heart rate (Table 1, Figure 2; Laughlin, 1978; Monge & Leon-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.

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3 360 We observed the strongest effects on development in embryos incubated at extreme high  
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5 361 elevation and at high temperature, suggesting that these factors interact to limit the  
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8 362 functional capacity of ectotherms. Gas exchange in embryos is diffusion-limited, likely  
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10 363 constraining their ability to compensate for reduced oxygen availability through increased  
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12 364 oxygen transport capacity (Vitt & Caldwell, 2013). These effects are then exacerbated by the  
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15 365 increased demand induced by high temperatures. Under conditions of high temperature and  
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17 366 low oxygen availability, we expect reductions in maximal performance, limitations on  
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19 367 physiological processes generally, and potentially reductions of critical thermal limits  
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21 368 (Gangloff & Telemeco, 2018). For example, recent work demonstrated that lizard embryos  
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23 369 suffer a mismatch between oxygen supply and demand at high temperatures, which may  
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25 370 serve as the proximal cause of death (Hall & Warner, 2020). Our results demonstrate for the  
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27 371 first time these effects in snake embryos, in concordance with previous work studying  
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29 372 embryonic development *in ovo* under varying temperatures and levels of oxygen availability  
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31 373 in other reptile taxa (Birds: Vimmerstedt et al., 2019; Crocodiles: lungman and Piña, 2013;  
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33 374 Lizards: Flewelling and Parker, 2015; Smith et al., 2015; Li et al., 2020; Turtles: Liang et al.,  
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35 375 2015). For example, embryos of the lizard *Podarcis muralis* increase incubation times in  
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37 376 conditions of hypoxia when incubated at 28°C, but not at 24°C (Cordero et al., 2017a;  
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39 377 Kouyoumdjian et al., 2019). In this study, we found that snake embryos incubated in warm  
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41 378 temperature and in hypoxia were less likely to survive to hatching, especially because the  
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43 379 last-stage embryos have higher oxygen demand (Dmi'el, 1970; Sartori et al., 2017), and,  
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45 380 when they did survive, were smaller than snakes in other treatment groups (Table 2, Figure  
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47 381 3D). In accordance with previous work (Shine, 2004; Daufresne et al., 2009; Du et al., 2009;  
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49 382 Gardner et al., 2011; Sheridan and Bickford, 2011; Noble et al., 2018; Refsnider et al., 2019),  
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51 383 our results show that snakes incubated at warm temperatures were smaller and shorter  
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3 384 than their counterparts, and hatched after fewer total heartbeats, regardless of oxygen  
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5 385 availability (Table 2, Figure 3C & 3D). Moreover, there was less residual egg yolk in both  
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8 386 warm treatments (*i.e.*, LEH and EHEH) compared to cool treatments (*i.e.*, LEC and EHEC) and  
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10 387 yet these animals were also smaller, suggesting higher basal metabolic demands associated  
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12 388 with high-temperature incubation may reduce growth efficiency (conversion of yolk to body  
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14 389 mass). Hatchlings incubated at cool temperatures in hypoxia did not exhibit reduced body  
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18 390 size or mass (Table 2; Figure 3C & 3D). This result demonstrates that reduced metabolic rates  
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20 391 and increased ability to assimilate energy stores associated with cool temperatures mitigate  
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22 392 the negative impacts of reduced oxygen availability (Jackson, 2007; Gangloff & Telemeco,  
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25 393 2018).

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29 395 Swimming speed is an ecologically relevant trait important to predator avoidance and food  
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31 396 acquisition in snakes (Jayne & Bennett, 1990; Kingsolver et al., 2001), that typically  
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34 397 correlates (positively) with body length (Shine and Shetty, 2001; Aubret et al., 2015).  
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36 398 Although this trend was found within each treatment group (Figure 4), it was not observed  
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39 399 across treatments: snakes incubated under both hypoxia and high temperatures  
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41 400 demonstrated the fastest swimming speeds compared to all other treatment groups, despite  
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44 401 exhibiting the smallest body size on average (Table 3, Figure 4). Previous studies in other  
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46 402 ectothermic species demonstrate that cool incubation temperatures produce faster  
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49 403 swimmers (Shine, 1999; Angilletta & Dunham, 2003; Watkins & Vraspir, 2006; Gahm et al.,  
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51 404 2020). At nine days post hatching, juveniles in this experiment did not follow this trend:  
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54 405 juveniles from warm treatments (*i.e.*, LEH and EHEH) were faster swimmers than their  
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57 406 siblings from cool treatments (*i.e.*, LEC and EHEC) in both absolute and relative swimming  
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59 407 speed. Most surprisingly, juveniles from the EHEH treatment were also faster than juveniles  
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3 408 from LEH despite smaller body size and conditions of oxygen limitation (Figure 5A). One  
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5 409 potential explanation for this finding is that warm incubation temperature and oxygen  
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7 410 limitation may reduce the optimal temperature for performance (Gangloff & Telemeco,  
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9 411 2018). We suggest that juveniles from EHEH potentially reduced their optimal temperature  
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11 412 for performance, thus swimming faster than the other groups at the test temperature of  
12  
13 413 25°C. Alternatively, exposure to hypoxia during development may have induced plastic  
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15 414 changes in cardiovascular, muscular, or mitochondrial function to increase performance  
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17 415 capacity (Eme et al., 2013; Sun et al., 2015; Galli et al., 2016). Further experiments directed  
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19 416 towards quantifying the effects of incubation temperature on the entire thermal  
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21 417 performance curve are necessary to fully characterize how incubation temperature  
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23 418 influences both physiology and performance across a range of temperatures (Taylor et al.,  
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25 419 2020).

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35 421 After relocation to low elevation, juveniles from the EHEH treatment swam faster than  
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37 422 siblings remaining at extreme high elevation, which maintained swimming speeds similar to  
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39 423 other treatment groups measured at both extreme high and low elevation (Figure 5B). In  
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41 424 birds and mammals the acclimation to high-elevation hypoxia can include an alteration of  
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43 425 cardio-respiratory pathways, a modification of blood composition, and increased muscle  
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45 426 performance (Monge & Leon-Velarde, 1991b; Beall et al., 2002; Storz et al., 2004; Lague et  
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47 427 al., 2016). Similar effects have been demonstrated in other reptiles (Iungman & Piña, 2013;  
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49 428 González-Morales et al., 2015; Lu et al., 2015; Wearing et al., 2015; Jochmans-Lemoine &  
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51 429 Joseph, 2018; Gangloff et al., 2019). These modifications may allow the maintenance of  
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53 430 locomotor performance such as swimming. Furthermore, these physiological and anatomical  
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55 431 changes due to development in chronic hypoxia serve to improve performance under  
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3 432 normoxic conditions, similar to athletes training at high altitudes for competition at sea level  
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6 433 (*e.g.*, Khodaei et al., 2016). Repeated measurements throughout ontogeny are necessary to  
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8 434 quantify the time frame over which such compensatory mechanisms remain relevant  
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10 435 (Mitchell et al., 2018). Finally, although we cannot speculate on the adaptive value of such  
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12 436 behavior at this stage, this response to a double constraint (high incubation temperature  
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14 437 and low oxygen level) may be yet another case of informed dispersal in reptiles (as in  
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16 438 *Zootoca vivipara*, Lichtenstein, 1823 and *Natrix maura*; Clobert et al., 2009; Bestion et al.,  
17  
18 439 2015a; Aubret et al., 2016): environmental clues may convey important information about  
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20 440 the quality of the natal environment and foster dispersal behaviour and/or dispersal  
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22 441 enhancing traits (*i.e.*, high locomotor performance). Importantly, the high level of observed  
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24 442 maternal effects (Table S4) indicates the necessity of a split-clutch design in any experiment  
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26 443 measuring similar traits in Squamate reptiles. Future work directed towards partitioning this  
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28 444 estimate into narrow-sense heritability and maternal effects will be important to predict the  
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30 445 evolutionary response to novel conditions within populations, especially at the colonization  
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32 446 front.  
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#### 42 448 **General conclusion**

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44 449 Our results suggest that even though body size, development, and physiology are altered,  
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46 450 and hatching success is lowered, the majority of embryos developing in high-elevation  
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48 451 hypoxia produced viable young snakes. Furthermore, these snakes were able to equal or  
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50 452 exceed the swimming performance of snakes incubated under native conditions. We stress  
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52 453 that the results of this experiment represent an extreme case of abiotic limitation (exposing  
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54 454 developing embryos from low elevation to a 32°C incubation temperature and 72% sea-level  
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56 455 equivalent O<sub>2</sub> availability). Such approaches are important to identify patterns among  
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3 456 mechanisms, pathways, and constraints, allowing subsequent experimental designs to be  
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6 457 refined accordingly. In some species of Squamates, embryos transplanted to extreme high  
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8 458 elevation do not suffer reductions in survival and maintain fitness-relevant traits to hatching  
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10 459 (Du et al., 2010a; Cordero et al., 2017a; Kouyoumdjian et al., 2019; Li et al., 2020). In this  
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12  
13 460 context, the plastic physiological responses might mitigate environmental stress, thereby  
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15 461 promoting offspring survival and influencing the direction of subsequent evolution of  
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18 462 colonizing populations (Atkinson & Thorndyke, 2001; McNab, 2002; West-Eberhard, 2003;  
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20 463 Hammond et al., 2006; Ghalambor et al., 2007). Our results tentatively suggest that the  
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22  
23 464 embryonic stage will not be the limiting factor in the potential for viperine snake to utilize  
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25 465 expansion to high-elevation habitat as a strategy to track favorable environmental  
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28 466 temperatures. However, the success this colonization could be threatened if temperatures  
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30 467 continue to rise, especially at high elevation.  
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25  
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27  
28 480 (APAFIS#16359-201808011445465 v4). All experiments were carried out in accordance with  
29  
30 481 the approved guidelines. Animal caretakers and handlers were trained to use wildlife in  
31  
32 482 scientific purposes (Decree No. 2013-118 du 01 février 2013 and approval of the Ministry of  
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34 483 Agriculture under No. I-75-MNHN-F1-15 du 17 juin 2015).  
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For Peer Review

**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 the snake *Natrix maura* (Test 1 Figure 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.

	<b>Egg mass</b>	<b>Embryo heart rates</b>
<b>Temperature</b>	$F_{1,426.8} = 2602.33$ ; <b>P &lt; 0.001***</b>	$F_{1,1142.3} = 635.04$ ; <b>P &lt; 0.001***</b>
<b>Elevation</b>	$F_{1,283.7} = 8.02$ ; <b>P = 0.003 **</b>	$F_{1,1144.2} = 13.22$ ; <b>P &lt; 0.001***</b>
<b>Day</b>	$F_{9,1130.0} = 30.92$ ; <b>P &lt; 0.001***</b>	$F_{9,1179.6} = 11.82$ ; <b>P &lt; 0.001***</b>
<b>Temperature x Elevation</b>	$F_{1,426.1} = 22.57$ ; P = 0.500	$F_{1,1143.0} = 9.61$ ; <b>P = 0.002 **</b>
<b>Temperature x Day</b>	$F_{4,1138.8} = 97.60$ ; <b>P &lt; 0.001***</b>	$F_{4,1194.3} = 7.21$ ; <b>P = 0.007 **</b>
<b>Elevation x Day</b>	$F_{9,1139.8} = 5.66$ ; <b>P &lt; 0.001***</b>	$F_{9,1191.2} = 2.38$ ; P = 0.123
<b>Temperature x Elevation x Day</b>	$F_{4,1138.5} = 5.75$ ; <b>P &lt; 0.001***</b>	$F_{4,1191.8} = 0.24$ ; P = 0.626

**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 the snake *Natrix maura* (Test 2, Figure 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  $\pm$  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
	<i>LSM</i> $\pm$ SE	<i>LSM</i> $\pm$ SE	<i>LSM</i> $\pm$ SE	<i>LSM</i> $\pm$ SE	<i>F</i> ( <i>dfn</i> , <i>dfd</i> ) <i>P</i> -value	<i>F</i> ( <i>dfn</i> , <i>dfd</i> ) <i>P</i> -value	<i>F</i> ( <i>dfn</i> , <i>dfd</i> ) <i>P</i> -value
Hatching success	-	-	-	-	4.04 (1, 184.4) <b>P = 0.046 *</b>	2.43 (1, 185.2) P = 0.120	5.72 (1, 185.2) <b>P = 0.018 *</b>
Sex	-	-	-	-	0.09 (1, 163.7) P = 0.764	2.56 (1, 165.0) P = 0.112	0.22 (1, 166.9) P = 0.643
Incubation duration (days)	65.59 $\pm$ 0.42	36.59 $\pm$ 0.44	67.85 $\pm$ 0.43	33.93 $\pm$ 0.43	24415.32 (1, 158.4) <b>P &lt; 0.001***</b>	0.99 (1, 158.8) P = 0.321	145.83 (1, 159.1) <b>P &lt; 0.001***</b>
Total number of embryo heart beats	7650391 $\pm$ 105481	6954688 $\pm$ 111257	7689242 $\pm$ 105997	7036319 $\pm$ 106591	108.49 (1, 158.8) <b>P &lt; 0.001***</b>	0.86 (1, 159.4) P = 0.355	0.11 (1, 159.9) P = 0.744
Body mass (g) at hatching	2.87 $\pm$ 0.12	2.56 $\pm$ 0.12	3.00 $\pm$ 0.12	2.97 $\pm$ 0.12	11.87 (1, 158.7) <b>P &lt; 0.001***</b>	24.39 (1, 158.7) <b>P &lt; 0.001***</b>	7.20 (1, 158.9) <b>P = 0.008 **</b>
Body length (cm) at hatching	15.30 $\pm$ 0.20	14.09 $\pm$ 0.21	15.37 $\pm$ 0.20	14.58 $\pm$ 0.20	67.12 (1, 158.8) <b>P &lt; 0.001***</b>	5.27 (1, 159.3) <b>P = 0.023 *</b>	2.83 (1, 159.8) P = 0.094
Body condition at hatching	-0.029 $\pm$ 0.010	-0.007 $\pm$ 0.010	-0.015 $\pm$ 0.010	0.031 $\pm$ 0.010	30.95 (1, 158.8) <b>P &lt; 0.001***</b>	18.47 (1, 159.3) <b>P &lt; 0.001***</b>	3.69 (1, 159.9) P = 0.056
Residual egg yolk (g)	0.655 $\pm$ 0.055	0.431 $\pm$ 0.061	0.815 $\pm$ 0.056	0.456 $\pm$ 0.56	34.61 (1, 60.8) <b>P &lt; 0.001***</b>	3.46 (1, 162.0) P = 0.065	1.81 (1, 163.4) P = 0.180

**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 the snake *Natrix maura* (Tests 3 & 4, Figure 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.

	<b>F (dfn, dfd)</b>	<b>P-value</b>
<b>Sex</b>	0.70 (1,145.5)	P = 0.401
<b>Total body length (cm)</b>	73.81 (1,169.2)	<b>P &lt; 0.001 ***</b>
<b>Temperature</b>	49.80 (1,156.4)	<b>P &lt; 0.001 ***</b>
<b>Elevation</b>	0.45 (1,165.5)	P = 0.501
<b>Day</b>	12.62 (3,500.6)	<b>P &lt; 0.001 ***</b>
<b>Location of test</b>	0.02 (1,442.8)	P = 0.964
<b>Temperature x Elevation</b>	0.002 (1,163.5)	P = 0.962
<b>Temperature x Day</b>	4.84 (3,506.1)	<b>P = 0.003 **</b>
<b>Temperature x Location of test</b>	0.01 (1,442.8)	P = 0.909
<b>Elevation x Day</b>	1.14 (3,487.8)	P = 0.331
<b>Elevation x Location of test</b>	9.80 (1,440.7)	<b>P = 0.002 **</b>
<b>Day x Location of test</b>	13.85 (2,460.9)	<b>P &lt; 0.001 ***</b>
<b>Temperature x Elevation x Day effect</b>	0.18 (3,487.1)	P = 0.913
<b>Temperature x Elevation x Location of test</b>	0.04 (1,446.9)	P = 0.839
<b>Temperature x Day x Location of test</b>	3.25 (2,457.5)	<b>P = 0.040 *</b>
<b>Elevation x Day x Location of test</b>	0.04 (2,456.9)	P = 0.961
<b>Temperature x Elevation x Day x Location of test</b>	0.79 (2,456.6)	P = 0.456

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3 Experimental design. Eggs were collected from gravid females (represented by different colors) sampled from low-elevation population of the snake *Natrix maura* in the foothills of the Pyrenees, France (412 m to 715 m ASL). Within 24hr 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 ASL, 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 ASL, 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 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 color represents incubation temperature treatment (cool or warm) and snake pattern represents incubation elevation treatment (low or extreme high elevation).

20 **Figure 2**

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Egg mass (A) and embryo heart rate (B) measured at the same temperature as the incubation temperature through incubation duration in the snake *Natrix 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

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3 25 (LEH; N = 49; red circle). Least-squares means  $\pm$  SE estimated by linear mixed-effect models  
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6 26 are plotted.

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10 28 **Figure 3**

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13 29 Hatching phenotypes in juveniles of the snake *Natrix maura*: incubation duration (A), total  
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15 30 number of embryo heartbeats (B), body mass (C), body size (D), body condition (E), and  
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18 31 residual egg yolk (F) for each incubation treatment: low elevation at 24°C (LEC; N = 46; blue  
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20 32 circle), extreme high elevation at 24°C (EHEC; N = 48; blue triangle), low elevation at 32°C  
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23 33 (LEH; N = 45; red circle), and extreme high elevation at 32°C (EHEH; N = 38; red  
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25 34 triangle). Least-squares means  $\pm$  SE estimated by linear mixed-effect models are plotted.  
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28 35 Significant differences between least-squares means are shown with one ( $P < 0.05$ ) or three  
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30 36 ( $P < 0.001$ ) asterisks.

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35 38 **Figure 4**

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37 39 Maximum swimming speed as a function of body length in juveniles of the snake *Natrix*  
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40 40 *maura* incubated in four treatments: low elevation at 24°C (LEC; N = 41; blue circle), extreme  
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42 41 high elevation at 24°C (EHEC; N = 41; blue triangle), low elevation at 32°C (LEH; N = 41; red  
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44 42 circle), and extreme high elevation at 32°C (EHEH; N = 37; red triangle). Raw data for each  
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46 43 individual are plotted with regression lines and 95% CI.

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52 45 **Figure 5**

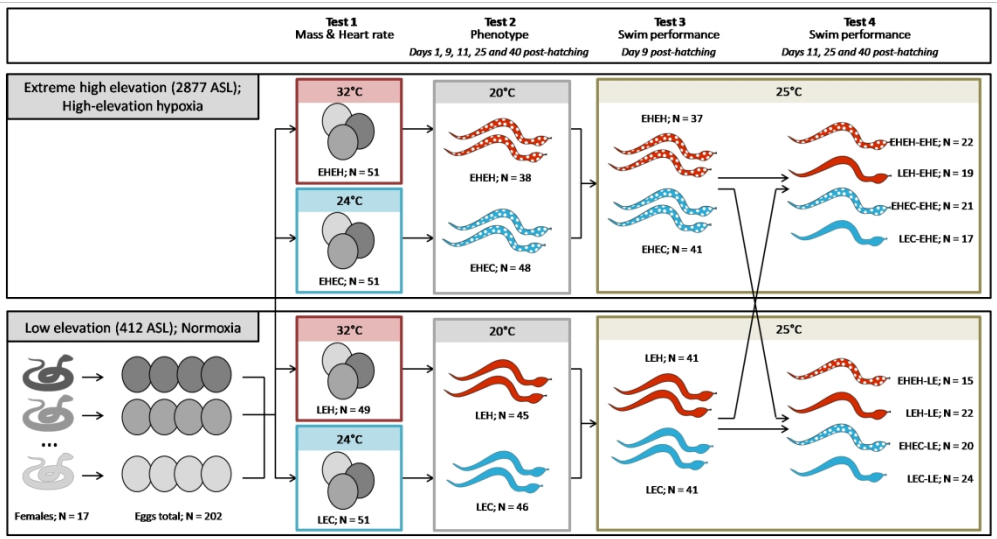
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54 46 Maximum swimming speed in juveniles of the snake *Natrix maura* for each incubation  
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57 47 treatment. First at 9 days post-hatching (A) at elevation of incubation (low elevation or  
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59 48 extreme high elevation) for all juveniles of the four incubation treatments: low elevation at  
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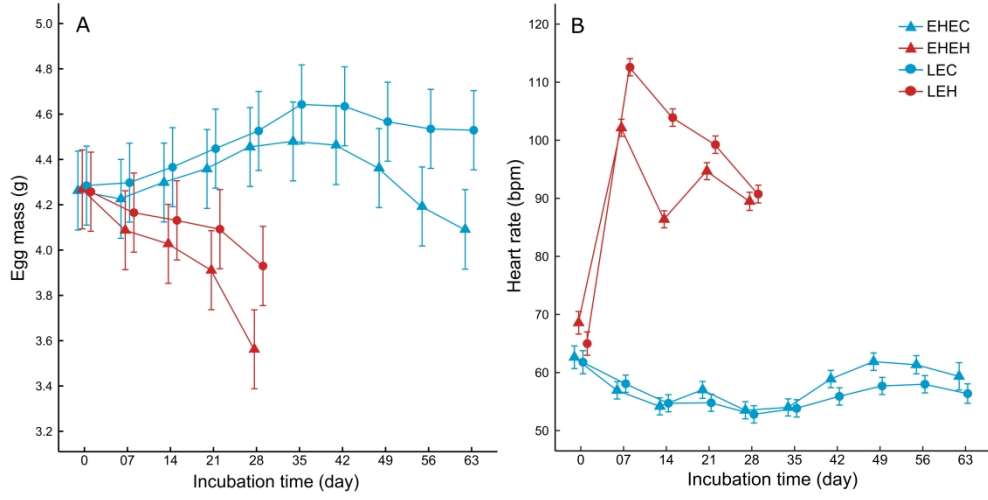
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7 51 37; red triangle). Second, at 11 days post-hatching (B) at the same elevation as incubation  
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9 52 for half of the juveniles: low elevation at 24°C (LEC-LE; N = 24; blue circle), extreme high  
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11 53 elevation at 24°C (EHEC-EHE; N = 21; blue triangle), low elevation at 32°C (LEH-LE; N = 22; red  
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13 54 circle), and extreme high elevation at 32°C (EHEH-EHE; N = 22; red triangle). Also at 11 days  
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15 55 post-hatching (B) at opposite elevation as incubation for the other half of the juveniles: low  
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17 56 elevation at 24°C (LEC-EHE; N = 17; blue triangle), extreme high elevation at 24°C (EHEC-LE;  
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19 57 N = 20; blue circle), low elevation at 32°C (LEH-EHE; N = 19; red triangle), and extreme high  
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21 58 elevation at 32°C (EHEH-LE; N = 15; red circle). Least-squares means  $\pm$  SE estimated by linear  
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23 59 mixed-effect models are plotted. Significant differences between least-squares means are  
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25 60 shown with one ( $P < 0.05$ ) or three ( $P < 0.001$ ) asterisks.  
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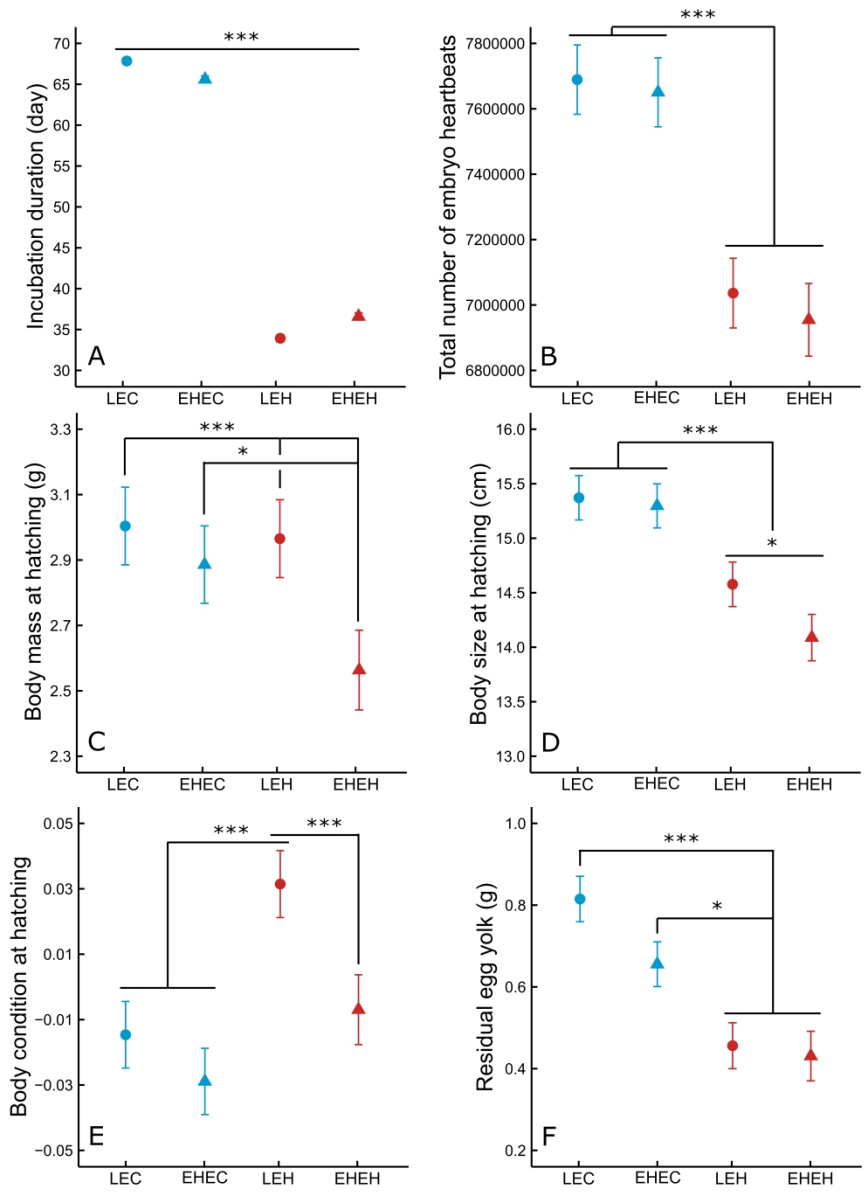


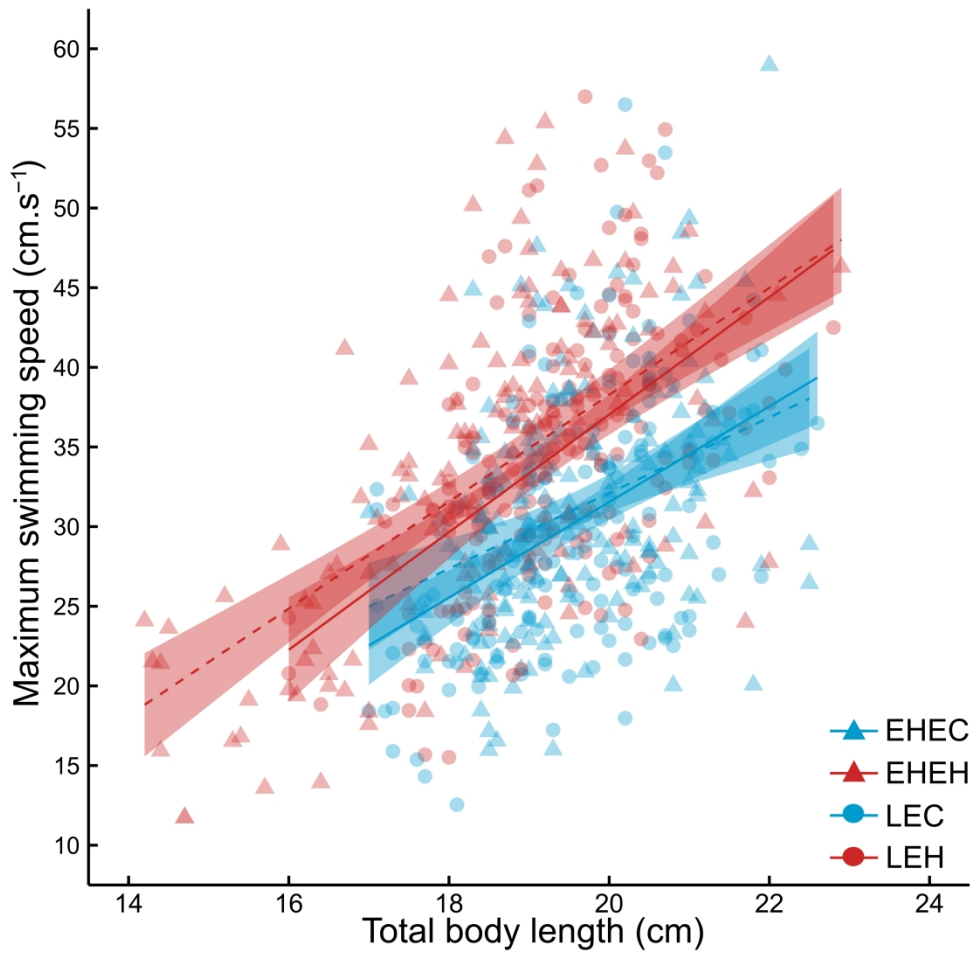
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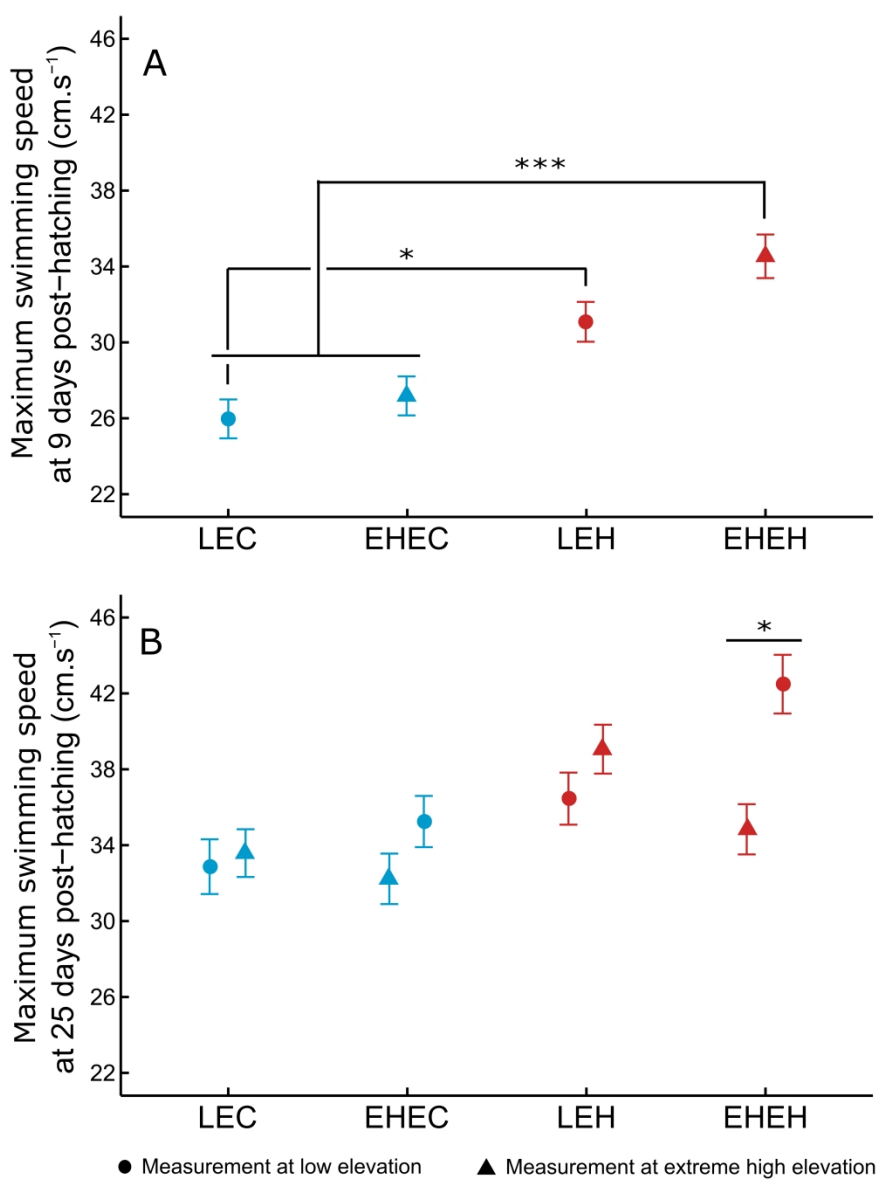


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**Table S1:** Results of the pairwise comparison of least-squares means and adjusted p-values for multiple comparisons with the Tukey method from the linear mixed-effect model testing for the effect of incubation temperature, incubation elevation, age at measurement (day post hatching), and their interaction on embryo traits in the snake *Natrix maura*. 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 differences between least-squares means shown in bold with one (P < 0.05) two (P < 0.01) or three (P < 0.001) asterisks.

	Egg mass			Embryo heart rate		
	Estimate	SE	P-value	Estimate	SE	P-value
EHEC D0 - EHEH D0	-0.005	0.090	1.000	-5.941	2.650	0.971
EHEC D0 - LEC D0	-0.022	0.090	1.000	0.851	2.676	1.000
EHEC D0 - LEH D0	0.005	0.091	1.000	-2.348	2.703	1.000
EHEH D0 - LEC D0	-0.016	0.090	1.000	6.792	2.675	0.868
EHEH D0 - LEH D0	0.010	0.091	1.000	3.593	2.702	1.000
LEC D0 - LEH D0	0.027	0.091	1.000	-3.199	2.726	1.000
EHEC D0 - EHEC D7	0.037	0.040	1.000	5.708	2.352	0.920
EHEH D0 - EHEH D7	0.180	0.040	<b>0.005 **</b>	-33.564	2.343	<b>&lt; 0.001 ***</b>
LEC D0 - LEC D7	-0.013	0.041	1.000	3.706	2.372	1.000
LEH D0 - LEH D7	0.092	0.041	0.971	-47.589	2.411	<b>&lt; 0.001 ***</b>
EHEC D7 - EHEH D7	0.138	0.090	1.000	-45.213	1.976	<b>&lt; 0.001 ***</b>
EHEC D7 - LEC D7	-0.072	0.091	1.000	-1.150	1.976	1.000
EHEC D7 - LEH D7	0.061	0.091	1.000	-55.645	1.986	<b>&lt; 0.001 ***</b>
EHEH D7 - LEC D7	-0.210	0.090	0.949	44.063	1.965	<b>&lt; 0.001 ***</b>
EHEH D7 - LEH D7	-0.078	0.091	1.000	-10.432	1.975	<b>&lt; 0.001 ***</b>
LEC D7 - LEH D7	0.132	0.091	1.000	-54.494	1.975	<b>&lt; 0.001 ***</b>
EHEC D7 - EHEC D14	-0.072	0.040	0.999	2.747	1.975	1.000
EHEH D7 - EHEH D14	0.060	0.041	1.000	15.755	1.964	<b>&lt; 0.001 ***</b>
LEC D7 - LEC D14	-0.068	0.041	1.000	3.367	1.964	1.000
LEH D7 - LEH D14	0.034	0.041	1.000	8.671	1.995	<b>0.009 **</b>
EHEC D14 - EHEH D14	0.271	0.090	0.528	-32.205	1.965	<b>&lt; 0.001 ***</b>
EHEC D14 - LEC D14	-0.068	0.091	1.000	-0.530	1.966	1.000
EHEC D14 - LEH D14	0.167	0.091	0.999	-49.721	1.986	<b>&lt; 0.001 ***</b>
EHEH D14 - LEC D14	-0.338	0.091	0.095	31.675	1.965	<b>&lt; 0.001 ***</b>
EHEH D14 - LEH D14	-0.103	0.091	1.000	-17.516	1.986	<b>&lt; 0.001 ***</b>

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3	LEC D14 - LEH D14	0.235	0.091	0.847	-49.191	1.986	< 0.001 ***
4	EHEC D14 - EHEC D21	-0.060	0.041	1.000	-2.838	1.955	1.000
5	EHEH D14 - EHEH D21	0.117	0.041	0.634	-8.294	1.955	<b>0.014</b> *
6	LEC D14 - LEC D21	-0.082	0.041	0.996	-0.082	1.964	1.000
7	LEH D14 - LEH D21	0.039	0.042	1.000	4.660	2.006	0.953
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9							
10	EHEC D21 - EHEH D21	0.447	0.090	<b>0.001</b> ***	-37.661	1.946	< <b>0.001</b> ***
11	EHEC D21 - LEC D21	-0.090	0.091	1.000	2.226	1.956	1.000
12	EHEC D21 - LEH D21	0.266	0.091	0.597	-42.224	1.976	< <b>0.001</b> ***
13	EHEH D21 - LEC D21	-0.537	0.091	< <b>0.001</b> ***	39.887	1.956	< <b>0.001</b> ***
14	EHEH D21 - LEH D21	-0.181	0.091	0.995	-4.563	1.977	0.957
15	LEC D21 - LEH D21	0.356	0.091	0.059	-44.450	1.986	< <b>0.001</b> ***
16							
17							
18	EHEC D21 - EHEC D28	-0.097	0.041	0.937	3.511	1.955	0.999
19	EHEH D21 - EHEH D28	0.349	0.041	< <b>0.001</b> ***	5.196	2.024	0.852
20	LEC D21 - LEC D28	-0.078	0.041	0.998	1.992	1.975	1.000
21	LEH D21 - LEH D28	0.163	0.042	<b>0.047</b> *	8.500	2.028	<b>0.017</b> *
22							
23	EHEC D28 - EHEH D28	0.893	0.090	< <b>0.001</b> ***	-35.976	2.034	< <b>0.001</b> ***
24	EHEC D28 - LEC D28	-0.071	0.091	1.000	0.707	1.977	1.000
25	EHEC D28 - LEH D28	0.525	0.091	< <b>0.001</b> ***	-37.235	2.009	< <b>0.001</b> ***
26	EHEH D28 - LEC D28	-0.964	0.091	< <b>0.001</b> ***	36.683	2.045	< <b>0.001</b> ***
27	EHEH D28 - LEH D28	-0.368	0.091	<b>0.037</b> *	-1.259	2.076	1.000
28	LEC D28 - LEH D28	0.596	0.091	< <b>0.001</b> ***	-37.942	2.019	< <b>0.001</b> ***
29							
30	EHEC D28 - EHEC D35	-0.025	0.041	1.000	-0.490	1.964	1.000
31	LEC D28 - LEC D35	-0.117	0.041	0.651	36.683	2.045	< <b>0.001</b> ***
32	EHEC D35 - LEC D35	-0.163	0.091	0.999	-1.259	2.076	1.000
33	EHEC D35 - EHEC D42	0.016	0.041	1.000	-37.942	2.019	< <b>0.001</b> ***
34	LEC D35 - LEC D42	0.008	0.041	1.000	-4.892	1.975	0.898
35	EHEC D42 - LEC D42	-0.171	0.091	0.998	2.991	1.986	1.000
36	EHEC D42 - EHEC D49	0.101	0.041	0.903	-2.988	1.996	1.000
37	LEC D42 - LEC D49	0.068	0.041	1.000	-1.792	1.975	1.000
38	EHEC D49 - LEC D49	-0.205	0.091	0.966	4.187	1.987	0.989
39	EHEC D49 - EHEC D56	0.170	0.041	<b>0.022</b> *	0.534	2.054	1.000
40	LEC D49 - LEC D56	0.032	0.041	1.000	-0.299	1.975	1.000
41	EHEC D56 - LEC D56	-0.342	0.091	0.086	3.355	2.046	1.000
42	EHEC D56 - EHEC D63	0.101	0.046	0.977	1.994	2.741	1.000
43	LEC D56 - LEC D63	0.006	0.043	1.000	1.614	2.130	1.000
44	EHEC D63 - LEC D63	-0.438	0.094	<b>0.003</b> **	2.974	2.805	1.000
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**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 the snake *Natrix maura*. The four incubation treatments are low elevation at 24°C (LEC), low elevation at 32°C (LEH), extreme high elevation at 24°C (EHEC), and extreme high elevation at 32°C (EHEH). Significant differences between least-squares means shown in bold with one ( $P < 0.05$ ) or three ( $P < 0.001$ ) asterisks.

	Hatching success	Sex	Incubation duration (day)	Total number of heart beats	Body mass at hatching (g)	Body size at hatching (cm)	Body condition at hatching	Residual egg yolk (g)
<b>EHEC - EHEH</b>	0.194 ± 0.062 <b>P = 0.011 *</b>	0.058 ± 0.011 P = 0.951	29.01 ± 0.29 <b>P &lt; 0.001 ***</b>	695703 ± 94019 <b>P &lt; 0.001 ***</b>	0.323 ± 0.076 <b>P = 0.011 *</b>	1.21 ± 0.18 <b>P &lt; 0.001 ***</b>	-0.022 ± 0.009 P = 0.064	0.225 ± 0.072 <b>P = 0.011 *</b>
<b>EHEC - LEC</b>	0.037 ± 0.062 P = 0.935	-0.086 ± 0.010 P = 0.840	-2.25 ± 0.28 <b>P &lt; 0.001 ***</b>	-38851 ± 88811 P = 0.972	-0.118 ± 0.072 P = 0.359	-0.07 ± 0.17 P = 0.971	-0.015 ± 0.008 P = 0.311	-0.160 ± 0.068 P = 0.091
<b>EHEC - LEH</b>	0.020 ± 0.063 P = 0.989	-0.099 ± 0.010 P = 0.780	31.66 ± 0.28 <b>P &lt; 0.001 ***</b>	614072 ± 88776 <b>P &lt; 0.001 ***</b>	-0.079 ± 0.072 P = 0.687	0.72 ± 0.17 <b>P &lt; 0.001 ***</b>	-0.061 ± 0.008 <b>P &lt; 0.001 ***</b>	0.199 ± 0.068 <b>P = 0.020 *</b>
<b>EHEH - LEC</b>	-0.157 ± 0.062 P = 0.058	-0.144 ± 0.011 P = 0.559	-31.26 ± 0.29 <b>P &lt; 0.001 ***</b>	-734554 ± 94583 <b>P &lt; 0.001 ***</b>	-0.441 ± 0.077 <b>P &lt; 0.001 ***</b>	-1.28 ± 0.18 <b>P &lt; 0.001 ***</b>	0.008 ± 0.009 P = 0.828	-0.384 ± 0.072 <b>P &lt; 0.001 ***</b>
<b>EHEH - LEH</b>	-0.174 ± 0.063 <b>P = 0.030 *</b>	-0.157 ± 0.011 P = 0.493	2.66 ± 0.30 <b>P &lt; 0.001 ***</b>	-81631 ± 95322 P = 0.827	-0.402 ± 0.077 <b>P &lt; 0.001 ***</b>	-0.49 ± 0.18 <b>P = 0.036 *</b>	-0.038 ± 0.009 <b>P &lt; 0.001 ***</b>	-0.025 ± 0.073 P = 0.985
<b>LEC - LEH</b>	-0.017 ± 0.063 P = 0.993	-0.013 ± 0.011 P = 0.999	33.91 ± 0.28 <b>P &lt; 0.001 ***</b>	652923 ± 89836 <b>P &lt; 0.001 ***</b>	0.039 ± 0.073 P = 0.951	0.79 ± 0.17 <b>P &lt; 0.001 ***</b>	0.046 ± 0.008 <b>P &lt; 0.001 ***</b>	0.359 ± 0.069 <b>P &lt; 0.001 ***</b>



**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 the snake *Natrix maura*. 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) or three (P < 0.001) asterisks.

	<i>Estimate</i>	<i>SE</i>	<i>P-value</i>
<b>EHEC D9 - EHEH D9</b>	-7.358	1.386	<b>&lt; 0.001 ***</b>
<b>EHEC D9 - LEC D9</b>	1.211	1.290	1.000
<b>EHEC D9 - LEH D9</b>	-3.905	1.305	0.423
<b>EHEH D9 - LEC D9</b>	8.568	1.396	<b>&lt; 0.001 ***</b>
<b>EHEH D9 - LEH D9</b>	3.453	1.339	0.752
<b>LEC D9 - LEH D9</b>	-5.115	1.308	<b>0.035 *</b>
<b>EHEC D9 - EHEC-EHE D11</b>	3.416	1.330	0.761
<b>EHEC D9 - EHEC-LE D11</b>	1.872	1.356	1.000
<b>EHEH D9 - EHEH-EHE D11</b>	3.237	1.320	0.836
<b>EHEH D9 - EHEH-LE D11</b>	4.531	1.541	0.463
<b>LEC D9 - LEC-LE D11</b>	2.853	1.262	0.925
<b>LEC D9 - LEC-EHE D11</b>	-0.586	1.450	1.000
<b>LEH D9 - LEH-LE D11</b>	1.436	1.307	1.000
<b>LEH D9 - LEH-EHE D11</b>	-2.419	1.397	0.998
<b>EHEC-EHE D11 - EHEC-LE D11</b>	-1.543	1.710	1.000
<b>EHEH-EHE D11 - EHEC-LE D11</b>	5.993	1.785	0.187
<b>LEC-EHE D11 - LEC-LE D11</b>	3.439	1.733	0.985
<b>LEH-EHE D11 - LEH-LE D11</b>	3.855	1.718	0.932
<b>EHEC-EHE D11 - EHEC-EHE D25</b>	-8.464	1.450	<b>&lt; 0.001 ***</b>
<b>EHEC-LE D11 - EHEC-LE D25</b>	-9.940	1.489	<b>&lt; 0.001 ***</b>
<b>EHEH-EHE D11 - EHEH-EHE D25</b>	-3.538	1.420	0.811
<b>EHEH-LE D11 - EHEH-LE D25</b>	-12.488	1.733	<b>&lt; 0.001 ***</b>
<b>LEC-EHE D11 - LEC-EHE D25</b>	-6.313	1.610	<b>0.034 *</b>
<b>LEC-LE D11 - LEC-LE D25</b>	-10.467	1.357	<b>&lt; 0.001 ***</b>
<b>LEH-EHE D11 - LEH-EHE D25</b>	-2.962	1.532	0.990

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3	<b>LEH-LE D11 - LEH-LE D25</b>	-9.403	1.443	<b>&lt; 0.001 ***</b>
4	<b>EHEC-EHE D25 - EHEC-LE D25</b>	-3.020	1.709	0.998
5	<b>EHEH-EHE D25 - EHEH-LE D25</b>	-7.656	1.831	<b>0.013 *</b>
6	<b>LEC-EHE D25 - LEC-LE D25</b>	-0.714	1.734	1.000
7	<b>LEH-EHE D25 - LEH-LE D25</b>	-2.586	1.714	1.000
8				
9	<b>EHEC-EHE D25 - EHEC-EHE D40</b>	-2.137	1.448	1.000
10	<b>EHEC-LE D25 - EHEC-LE D40</b>	-0.724	1.485	1.000
11	<b>EHEH-EHE D25 - EHEH-EHE D40</b>	-2.375	1.417	0.999
12	<b>EHEH-LE D25 - EHEH-LE D40</b>	4.279	1.719	0.813
13	<b>LEC-EHE D25 - LEC-EHE D40</b>	-2.154	1.609	1.000
14	<b>LEC-LE D25 - LEC-LE D40</b>	0.607	1.355	1.000
15	<b>LEH-EHE D25 - LEH-EHE D40</b>	-2.342	1.525	1.000
16	<b>LEH-LE D25 - LEH-LE D40</b>	4.080	1.419	0.517
17				
18	<b>EHEC-EHE D40 - EHEC-LE D40</b>	-1.607	1.709	1.000
19	<b>EHEH-EHE D40 - EHEH-LE D40</b>	-1.002	1.835	1.000
20	<b>LEC-EHE D40 - LEC-LE D40</b>	2.046	1.735	1.000
21	<b>LEH-EHE D40 - LEH-LE D40</b>	3.836	1.714	0.934
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**Table S4:** Residual variance estimates from linear and generalized linear mixed models. Traits were measured in embryos and in juveniles of the snake *Natrix maura*. The significance of model fit improved by inclusion of clutch as a random effect is indicated by one (P < 0.05) two (P < 0.01) or three (P < 0.001) asterisks. See text for model details.

	Residual	Individual	Clutch				
	variance	variance	variance	Proportion of residual variance	$\chi^2$	Df	P-value
<b>Egg mass</b>	0.041	0.165	0.418	0.67	197.48	1	< 0.001 ***
<b>Embryo heart rate</b>	94.550	0.000	3.765	0.038	13.50	1	0.999
<b>Hatching success</b>	0.252	-	0.000	~ 0	4.23	1	<b>0.040</b> *
<b>Sex</b>	0.097	-	0.007	0.067	2.27e-13	1	> 0.99
<b>Incubation duration</b>	1.754	-	2.252	0.56	104.20	1	< 0.001 ***
<b>Total number of heart beats</b>	1.815 x10 <sup>11</sup>	-	1.137 x10 <sup>11</sup>	0.39	51.59	1	< 0.001 ***
<b>Residual egg yolk</b>	0.107	-	0.011	0.093	5.95	1	<b>0.015</b> *
<b>Body mass at hatching</b>	0.119	-	0.182	0.60	120.86	1	< 0.001 ***
<b>Body size at hatching</b>	0.646	-	0.426	0.40	53.71	1	< 0.001 ***
<b>Body condition at hatching</b>	0.002	-	0.001	0.33	59.78	1	< 0.001 ***
<b>Swimming speed</b>	22.010	11.697	3.186	0.086	9.72	1	<b>0.002</b> **