Transplanting gravid lizards to high elevation alters maternal and embryonic oxygen physiology, but not reproductive success or hatchling phenotype

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LK, EJG, JS, GAC, and FA contributed to experimental design and logistics. LK, EJG, JS, and FA conducted experiments. LK, EJG, and AD performed lab assays. GAC performed embryo staging. LK and EJG conducted statistical analyses and drafted the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Summary Statement

Reproducing females and developing embryos of the lizard species *Podarcis muralis* appear to be resilient, at least in the short term, to reduced oxygen availability at high elevations.

Abstract

Increased global temperatures have opened previously inhospitable habitats, such as at higher elevations. However, the reduction of oxygen partial pressure with increase in elevation represents an important physiological constraint that may limit colonization of such habitats, even if the thermal niche is appropriate. To test the mechanisms underlying the response to ecologically-relevant levels of hypoxia, we performed a translocation experiment with the common wall lizard (*Podarcis muralis*), a widespread European lizard amenable to establishing populations outside its natural range. We investigated the impacts of hypoxia on the oxygen physiology and reproductive output of gravid common wall lizards and the subsequent development and morphology of their offspring. Lowland females transplanted to high elevations increased their haematocrit and haemoglobin concentration within days and maintained routine metabolism compared to lizards kept at native elevations. However, transplanted lizards suffered from increased reactive oxygen metabolite production near the oviposition date, suggesting a cost of reproduction at high elevation. Transplanted females and females native to different elevations did not differ in reproductive output (clutch size, egg mass, relative clutch mass, or embryonic stage at oviposition) or in post-oviposition body condition. Developing embryos reduced heart rates and prolonged incubation times at high elevations within the native range and at extreme high elevations beyond the current range, but this reduced oxygen availability did not affect metabolic rate, hatching success, or hatchling size. These results suggest that this opportunistic colonizer is capable of successfully responding to novel environmental constraints in these important life-history stages.

Keywords: climate change; development; embryo; hypoxia; metabolic rate; physiological plasticity; *Podarcis muralis*; reactive oxygen metabolites; reproductive output

Introduction

In mountainous regions, the impacts of climate change are particularly pronounced owing to shifts in the altitudinal climate envelope (Chen *et al.*, 2011; Dirnböck *et al.*, 2011; Nogués-Bravo *et al.,* 2008; Walther *et al.*, 2002). Such shifts may invoke biological changes through the processes of adaptation, plasticity, and migration; or may result in extirpation (Parmesan, 2006; Sinervo *et al.*, 2010). Just as low-lying valleys provided refuge for a number of organisms during periods of glaciation, it is possible that high-elevation areas will play a similar role in times of global warming (Hewitt, 1999; Tzedakis, 2004). Indeed, the migration of some populations to higher elevations is currently observed in mountainous landscapes (Bani *et al.,* 2019; Bassler *et al.*, 2013; Chen *et al.*, 2011; Freeman *et al.*, 2018; Hickling *et al.*, 2006; Pottier, 2012; Pauchard *et al.*, 2015) and the preservation of such habitats has been proposed specifically as a refuge for lizards (Sinervo *et al.*, 2018). As a result, it is important to identify factors that both permit and limit colonization of higher-elevation habitats. Terrestrial ectothermic vertebrates, such as non-avian reptiles, are sensitive to variation in environmental temperatures and exhibit substantial metabolic plasticity (Huey, 1982). As such, they provide excellent models to understand the impacts of climate change across geographic scales (Diele-Viegas and Rocha, 2018; Huey *el al.*, 2018; Le Galliard *et al.* 2012; Walther *et al.*, 2002). However, their flexible physiologies and complex interactions among pathways in responding to many environmental factors make broad predictions difficult as the effects of changing habitats may vary dramatically across taxa (Levy *et al.*, 2017; Moore *et al.*, 2018; Pontes-da-Silva *et al.*, 2018; Sinervo *et al.*, 2010; Weatherhead *et al.*, 2012).

One potential constraint to upslope migration is the reduced oxygen partial pressure at high elevations, which causes high-altitude hypoxia (Bouverot, 1985; Powell and Hopkins, 2010). This phenomenon imposes important physiological challenges on a variety of organisms (reviewed in Storz *et al.*, 2010). Importantly, oxygen availability interacts with temperature in a context-dependent manner to influence thermal performance curves and thermal limits in terrestrial ectotherms (Gangloff and Telemeco 2018; Jackson, 2007). Acute and chronic effects of hypoxia have been fairly well studied in some mammals (humans in particular), birds, and some reptiles (Monge and Leon-Velarde, 1991; Weathers and McGrath, 1972) and the responses can be diverse (Storz *et al.*, 2010). Acute effects usually include hyperventilation and tachycardia, for example in humans brought to high altitude (Saito *et al.*, 1988). Chronic effects may involve changes to the cardiorespiratory systems, such as increased size of the lungs, changes to cardiac morphology, and increased blood pressure (Cunningham *et al.*, 1974; He *et al.*, 2013; Hillyard 1980; Powell and Hopkins, 2010). In lizards specifically, chronic hypoxia may also result in hematological and muscle composition changes (Gonzales-Morales *et al.*, 2015; Lu *et al.*, 2015; Weathers and McGrath, 1972). Such physiological shifts in oxygen-carrying capacity, however, may be insufficient to maintain performance traits or compensate for reduced oxygen in the long term. Hypoxia exposure during early development, such as embryos *in ovo*, can also dramatically affect physiology and development. Most common responses include mechanisms to facilitate oxygen diffusion and transport, such as increased vascularization of chorioallontoric membranes, increased haematocrit, and cardiac hypertrophy (Corona and Warburton, 2000; Crossley and Altimiras, 2005; Jochmans-Lemoine and Joseph, 2018; Kam, 1993; Nechaeva, 2011; Warburton *et al.*, 1995). Under certain conditions, oxygen restrictions can also result in depressed metabolism, decreased growth, and reduced survivorship to hatching (Iungman and Piña, 2013; Warburton *et al.*, 1995). These early developmental effects can persist into later life phenotypes (Sun *et al.*, 2014; Wearing *et al.*, 2017).

Previous work demonstrates the impacts of hypoxia on adult male and embryo common wall lizards (*P. muralis*) transplanted to extreme high elevation beyond the current range. Transplanted adult males increased haematocrit and haemoglobin within three weeks, but were unable to sustain this response for longer. Despite these changes in oxygen-carrying parameters, lizards transplanted to high elevation suffered reduced running performance and a reduction in body condition compared to lizards kept at lowland native elevation (Gangloff *et al.*, 2019). Embryos of this species developing in high-altitude hypoxia, however, demonstrate a potential to sustain growth and development at least until hatching. High-altitude hypoxia affected embryo physiology, resulting in reduced metabolism, cardiac hypertrophy, and hyperventilation. These physiological and morphological adjustments seemed to buffer embryos from negative impacts of hypoxia, resulting similar hatching success and hatchling body size compared to embryos developing at native lowland elevation (Cordero *et al.*, 2017a). While studies such as these provide important data on the effects of high-altitude hypoxia across life stages, relatively little is known about the effects of high altitude hypoxia exposure on maternal energetics and reproductive allocation in natural populations. If reduced oxygen availability imposes a physiological constraint, we might expect that gestating females will be less able to provide adequate resources or environments for developing embryos. This is particularly important in the context of responses to climate change, given the potential for trans-generational plasticity (e.g., maternal effects) to buffer developing offspring from the impacts of novel environmental conditions (Sinervo *et al.*, 2018; Warner, 2014). These effects have the potential to promote offspring success in future environments, such as when maternal exposure to warmer temperatures increases offspring survival in future warm environments (Shama *et al.*, 2014; Sun *et al.*, 2018).

With this study we quantify physiology and reproduction at different levels of oxygen availability on native and transplanted common wall lizards (*P. muralis*). Such data are needed to assess the potential importance of the effects of high-altitude hypoxia on reproduction and development, which in turn will determine the colonization potential of this species with a wide and expanding range (Pottier 2012; Speybroeck *et al.*, 2016). Our goals are to quantify how reproducing females physiologically respond to changes in oxygen availability, how these responses can influence reproductive output and capacity, how reduced oxygen availability affects embryo development, and how such responses might differ between lizards native to different elevations. We first measured aspects of maternal physiology and reproduction from replicate low- and high-elevation populations at native elevations and after translocation to high elevations. Then, we measured embryo development and physiology at native, high (within the natural range), and extreme high (beyond current range limit) elevations, to test five primary hypotheses:

i. Energetic limitations imposed by hypoxia will force a trade-off resulting in reduced relative reproductive investment. Because reduced oxygen availability affects the shape of performance curves (Gangloff and Telemeco, 2018), we expect that short-term (i.e., weeks) exposure to hypoxic conditions might reduce efficiency of physiological functions involved in energy processing for both gravid females and developing embryos. If lizards are unable to compensate, we expect that a maternal allocation trade-off will reduce egg mass of lizards transplanted to high elevation, resulting in a smaller relative clutch mass (RCM), a metric of reproductive investment. Because clutch size is likely determined before our experimental manipulations and females are unlikely to resorb eggs (Blackburn, 1998), we do not expect to see an effect of transplant on clutch size. If female lizards at high elevation are able to maintain the same level of reproductive investment (egg size and RCM), we expect to observe prolonged egg retention and thus laying of eggs at a later developmental stage, as a consequence of reduced oxygen availability on gravid mothers (see Mathies and Andrews, 1995). Alternatively, if oxygen limitation imposes a constraint on reproduction, mothers may sustain investment into offspring at a physiological cost to themselves, for instance resulting in reduced post-oviposition body condition.

ii. Hypoxia and reproductive stage will interact to affect reactive oxygen metabolite (ROM) production. Reactive oxygen metabolites are produced as by-products of oxidative metabolism and can damage a variety of subcellular molecules. ROMs have been implicated in senescence and as a by-product of reproduction in reptiles (Costantini, 2016; Dowling and Simmons, 2009; Robert *et al.*, 2007; Stahlschmidt *et al.*, 2013, Stier *et al.*, 2017; Webb *et al.*, 2018). ROM production generally increases as a result of cellular hypoxia (reviewed in Guzy and Schumacker 2006; Harrison *et al.*, 2015; Solaini *et al.*, 2010). Exploring the interaction of reproduction and hypoxia on ROM production is essential to both understand short-term physiological changes and potential long-term consequences of reproduction at altitude. We expect that the reactive oxygen metabolite profile of lizards will shift across reproduction and that transplanted lizards will suffer from relatively increased levels compared to lizards kept at native elevation.

iii. Developing embryos will exhibit changes in physiology in response to hypoxia to meet metabolic demands important to development and differentiation. Reptilian embryos *in ovo* often experience naturally-occurring hypoxia, such as due to flooding events or limited gas exchange in subterranean nests (reviewed in Packard and Packard 1988; Ackerman and Lott 2004; Booth, 1998). Given the resilience to these conditions exhibited by a variety of taxa, we predict that embryos will adjust physiology and development, such as through changes in heart rate and incubation time. With these responses, embryos will sustain differentiation, growth, and organogenesis, critical processes that must be fueled by aerobic metabolism and are likely buffered from moderate hypoxia (Crossley *et al.*, 2017; Iungman, and Piña 2013; Warburton et al. 1995). Physiological or morphological responses will facilitate the maintenance of oxygen consumption ($\dot{V}O_2$, an integrated index of general metabolism), though late-period increases in demand might present challenges at high elevation. Further, we predict these adjustments will allow embryos to maintain important fitness-related traits such as size and mass at birth; if energetic constraints are present this will be demonstrated by reduced body size of embryos developing at extreme high elevation.

iv. Effects of hypoxia on post-oviposition development will be reduced in embryos that were exposed to hypoxia during gestation. Exposure to hypoxia earlier in development will provide both more time for embryos to respond and a potential window for anticipatory maternal effects. We expect that the change in heart rate and incubation time predicted above will be less pronounced in embryos from females that were transplanted to high altitudes compared with embryos translocated after oviposition. Furthermore, maternal effects in response to hypoxia may provide a mechanism to prime embryos for subsequent development in low-oxygen environments, thus reducing the potentially negative impacts on development in embryos transplanted to high or extreme high elevation.

v. Lizards from high-elevation populations will demonstrate local adaptation in traits related to oxygen capacity. We predict that the responses to hypoxia of transplanted females will differ from that of lizards native to high elevation, while embryos from high-elevation populations will show reduced effects on development at extreme high elevations compared to embryos from low-elevation populations. As has been found in previous studies of transplanted lizards (He *et al.*, 2013; Gangloff *et al.*, 2019; Weathers and McGrath, 1972) we predict physiological plasticity in parameters related to oxygen-carrying capacity in blood chemistry (haematocrit and haemoglobin concentration) in reproductive females from low elevations transplanted to high elevations. Importantly, we do not expect the physiological profile of lowland lizards transplanted to high elevation to match those of lizards from high-elevation populations, as often the short-term plastic response differs from that of locally-adapted populations (He *et al.*, 2013; Jochmans-Lemoine *et al.*, 2015; Reyes *et al.*, 2018; Storz *et al.*, 2010; Velotta *et al.*, 2018). We predict that such shifts in oxygen capacity will allow lizards to maintain resting metabolic rates but perhaps bear other physiological or reproductive consequences given the increased energetic demands of reproduction (Angilletta and Sears, 2000; Foucart et *al.*, 2014). Furthermore, we predict that embryos from high-elevation populations will respond less dramatically to development at extreme high elevations compared to lizard embryos native to low elevations.

Taken together, these observations allow us to evaluate how hypoxia may constrain physiology in reproducing and developing lizards and how these responses may differ between populations native to different elevations. Given the dependence of successful colonization on recruitment (Aubret, 2013; Warner *et al.*, 2012; While *et al.*, 2015), testing these effects in reproductive females and their developing offspring is critical to predict the dynamics of colonization in common wall lizards in mountainous environments affected by climate change.

Methods

Source populations and husbandry

The common wall lizard (Lacertidae: *Podarcis muralis*) is widespread across southern Europe in a variety of habitats (Arnold *et al.*, 1988; Speybroeck *et al.*, 2016). Its geographic distribution is restricted by the thermal environment because embryos are incapable of completing developing at cold temperatures (Strijbosch *et al.*, 1980; Van Damme *et al.*, 1991; While *et al.*, 2015). However, wall lizards have recently been observed at higher areas of mountainous regions in the south of France, extending up to 2,600 m above sea level [ASL] (Pottier, 2012). At low elevations, females produce two or three clutches per season with an average of six eggs per clutch (Le Henanff, 2011). We monitored populations in the Pyrénées Ariégeoises (France) from the beginning of the active season and sampled adult female lizards during their first reproductive event of the season in April and May 2018 at low (382-472 m ASL) and high (1402-1795 m ASL) elevations. A total of 47 gravid females were sampled from three low-elevation and two high-elevation populations (see Table S1 for sampling details). Individuals were caught using the lasso method (Blomberg and Shine, 1996; McDiarmid, 2012) and marked using a cautery pen (Vervust and Van Damme, 2009). On the day of capture, we measured mass to the nearest 0.01 g with a precision balance (mean ± SD: 5.73 ± 1.06 g) and snout-vent length (SVL) with a digital caliper to the nearest 0.01 mm (mean ± SD: 61.97 ± 4.08 mm). Neither mass nor SVL at capture differed between lizards from low- and high-elevation populations (Wilcoxon rank-sum test, Mass: $W = 206$, $P = 0.28$; SVL: $W = 176$, $P = 0.082$). On the day of capture, we transported animals to facilities either at the Station d'Ecologie Théorique et Expérimentale du CNRS à Moulis (low elevation treatment: 42°57'26.8"N 1°05'08.3"E; 436 m ASL; PO_2 ~20.1 kPa) or in the commune of La Mongie (high-elevation treatment: 42°54'34.8"N 0°10'53.6"E; 1735 m ASL; PO_2 ~17.4 kPa; See Fig. 1 for experimental design). We maintained females individually in identical plastic enclosures (38 × 26 × 23 cm) containing a thin layer of substrate, a water bowl, a plastic shelter/basking platform (15 × 5 × 3.5 cm), and a nest box (18 × 14 × 10 cm) filled with wet sand. Enclosures were misted with water 3-4 times a week. Every two days, 5 live mealworms (*Tenebrio* sp. larvae) were distributed to each female and standing water was supplied ad libitum. Light was provided by UV lamps for 12 h per day and the enclosures were warmed by heat lamps (42 W) for 5 h per day at one-hour intervals, providing a gradient of ca. 25-40ºC. Additionally, we monitored temperatures in the enclosures with iButton thermal data loggers (Model DS1921G-F5, Maxim Integrated, San Jose, CA) placed in the cool end of two of the enclosures in each treatment. Temperature regimes were nearly identical across labs, with a diel range from 15 ºC at night to 25 ºC during the day. Furthermore, we rotated the enclosures on their shelves every several days to ensure no position effects.

Maternal metabolic rate

We measured resting metabolic rate after two days in captivity and approximately every week thereafter until one week after egg-laying (measures were skipped when lizards were actively nesting; mean time between measures ± SD: 8.5 ± 2.9 d) Lizards were offered their first meal after their initial measurement (2 d after capture) and food was withheld for 2 d before each subsequent measure. Each individual was placed in a custom-made 250 mL opaque metabolic chamber, which was then placed in an incubator set at 32° C for 1 h before measurement, a temperature intermediate between thermal preference (32.6°C) and field body temperatures (30.6°C) for gravid females in this area (Trochet *et al.*, 2018; F. Aubret unpublished data). This acclimation time allows body temperature to equilibrate and reduces effects of handling stress (Braña, 1993; Tosini and Avery, 1996). We used pull-mode respirometry (Foxbox-C Field O2 and CO2 Analysis System, Sable Systems, Inc., Las Vegas, Nevada, USA) to measure gas exchange (oxygen consumption [$\dot{V}O_2$] and carbon dioxide production [$\dot{V}CO_2$], corrected for barometric pressure) continuously for 30 min. Air was pumped at a rate of 500 mL / min through the metabolic chamber, dried of water vapor with Drierite, then measured for both CO_2 and O_2 content. Data were analyzed with ExpeData software (version 1.7.30, Sable Systems, Inc.) to calculate the rates of carbon dioxide production ($\dot{V}CO_2$) and oxygen consumption ($\dot{V}O_2$). We extracted the average of the lowest values of gas exchange over a 10-minute interval for our analysis of metabolic rate, which allows the elimination of elevated metabolic rates during the measurement period (presumably because of activity). We recorded the body temperature of each lizard immediately following measurement via cloacal thermometer (Miller & Weber T-6000, Ridgewood, NY). All metabolic rate measures were conducted during the normal activity period for these diurnal lizards (7h30- 20h00), and so represent resting metabolic rate (Andrews and Pough, 1985).

Blood sampling and hematological measures

The day after metabolic rate measurements, blood samples were collected by placing a heparinized glass capillary in the retro-orbital sinus of lizards (MacLean *et al.*, 1973; Meylan *et al.*, 2003). We collected 15 to 25 µL of blood in less than three minutes (bleed time mean ± SD: 1.66 ± 0.62 min) and stored on ice until processing. Haematocrit values are the volume of packed red blood cells relative to the total volume as measured with a caliper after spinning 10-15µL of whole blood for 5 min at 5000 *g*. Haemoglobin concentration was measured with the colorimetric cyanmethemoglobin method using 5 µL whole blood, following manufacturer's instructions (Drabkin's reagent, Sigma-Aldrich, St. Louis, MO, USA; Catalog Number D5941). Samples were run in duplicate on five plates, with a pooled sample run repeatedly on each plate to provide estimates of intra- and interplate variation of 6.5% and 5.7%, respectively. Additionally, we quantified the amount of hyperoxides in blood plasma with the Reactive Oxygen Metabolite test (d-ROMs kit; MC001, Diacron International, Italy) as a proxy for overall plasma oxidative status (Costantini, 2016). After centrifugation of whole blood at 3000 *g* for 5 min, we removed plasma by pipette and stored plasma at -80°C until the time of assay. We followed manufacturer's instructions, modified for use with a 4 µL lizard plasma sample (Dupoué *et al.*, unpublished data). We calculated the amount of ROMs corrected for control blanks and report values in equivalents of mg H_2O_2 / dL. Samples were run on three plates, with a pooled sample run repeatedly on each plate to provide estimates of intra- and interplate variation of 7.0% and 8.6%, respectively. In some cases, plasma volume limitations and other logistical constraints precluded all measurements on each sample, so the number of observations varies slightly for each measure (see Table 1).

Reproductive measures

The nest boxes were checked every 24-48 h and eggs were immediately removed upon discovery. Eggs were individually weighed with a digital scale and the female was immediately re-weighed to obtain a post-oviposition mass. We calculated the relative clutch mass (RCM) as the ratio of total clutch mass to female post-oviposition mass. One egg from each clutch was randomly selected for dissection and staging. This egg was kept in moist vermiculite at room temperature for 1-4 d before we removed approximately 1/3 of the yolk with a syringe needle to facilitate embryo isolation and fixed the remaining embryo and tissue in 10% buffered formalin. Fixed eggs were sequentially transferred to 40 and 70% ethanol and then washed in a Tween 20/phosphate-buffered saline solution (PBS-T), following the embryo dissection protocol of Cordero and Janzen (2014). Incisions were made on extreme longitudinal poles of the egg and egg contents were gently removed and placed in PBS-T. The embryo was then located, transferred to 70% ethanol, and placed on a dissecting microscope for observation and staging following the criteria of Dufaure and Hubert (1961). Since stage at oviposition is generally invariable within a clutch (Mathies and Andrews, 1995), we can infer the stage of the randomly-selected embryo to be representative of all eggs in the clutch.

We recognize that an ideal design would have included more frequent checks of nest boxes and an immediate fixing of the randomly-selected embryo. However, this was impossible due to logistical constraints. We combated bias by checking nest-boxes in both treatments at the same intervals and through an analysis of the potential effect of the number of possible days the eggs were incubating in the nest boxes before being discovered. The number of potential days in the nest box did not affect mass of eggs at oviposition ($F_{1,46.1} = 2.99$, $P = 0.09$) nor did the number of days before fixation affect embryo stage at oviposition ($F_{1,37} = 0.57$, $P = 0.46$).

Egg incubation and treatments

The remaining eggs of each clutch (N = 209) were individually incubated in plastic cups containing moist vermiculite (1:5 water to vermiculate by volume; Cordero *et al.*, 2017a; While *et al.*, 2015) and sealed with plastic film to retain moisture. Incubators contained an open tray of water to maintain high humidity. We verified the total cup mass weekly and added water to maintain this ratio throughout incubation. Eggs were incubated at a constant 28°C, which provides optimal hatching success in this species (Van Damme *et al.*, 1991). Within the first week after oviposition (mean ± SD: 1.8 ± 2.2 d), eggs were dispatched to the three different incubation treatments (low, high, and extreme high-elevation). Within each maternal treatment, mass of eggs allocated to different incubation treatments did not differ (all $P > 0.15$). Low- and high-elevation treatments matched the locations of the maternal treatments (see above) while the extreme high-elevation treatment was conducted at the laboratory of Pic du Midi de Bigorre (42°56'11.0"N 0°08'32.9"E; 2,877 m ASL; PO_2: ~15.3

kPa; See Fig. 1 for complete experimental design and sample size). We rotated egg position in incubators 2x/week to ensure no position effects.

Embryo heart and metabolic rates

Beginning one week post-oviposition, we measured the heart rate of all embryos repeatedly throughout incubation (up to four times per embryo, mean interval between measures ± SD: 12.8 ± 4.2 d), spacing our measurements to ensure an equal sampling across the incubation period and amongst treatment groups. We measured the heart rate with a Buddy digital egg monitor (MK2; Avitronics, Cornwall, UK) placed inside the incubator at 28°C. Eggs were quickly moved from their incubation cup to the instrument and we used a stopwatch to measure the time from egg removal to when a consistent heart rate was observed (mean time ± SD: 121.6 ± 73.7 s). If a reliable measurement could not be made in under 5 min, we replaced the egg in the incubator and re-measured later in the day to reduce any effects of handling or temperature change (Cordero *et al.*, 2017a; Hulbert *et al.*, 2017).

In a subset of eggs, we also measured metabolic rate on the same day as we measured heart rate. Each egg was placed in a small cup with moist vermiculite and inside a 250 mL metabolic chamber. We replaced the chamber in the incubator at 28°C and used closed-system respirometry to measure gas exchange. We flushed the chamber for 15 min at a flow rate of 300 mL/min then closed valves to seal the chamber for 90 min. We then opened the valves to re-establish air flow, dried air of water vapor with Drierite, and measured O_2 and CO_2 as above. We used ExpeData software to calculate $\dot{V}O_2$ and $\dot{V}CO_2$ by integrating the change in instantaneous gas concentrations over the period the chamber was sealed (Lighton 2008).

Hatchling morphology

Eggs were checked for hatching every 48 h. After emergence from the egg, we measured SVL using digital calipers (mean ± SD: 25.1 ± 1.2 mm) and mass using an electronic balance (mean ± SD: 0.363 ± 0.053 g). Of the 209 eggs incubated, 184 hatched (88.0% hatching success rate).

Field sampling and experimental protocols were conducted under permit provided by the *Direction régionale de l'environnement, de l'aménagement et du logement* (DREAL) Midi-Pyrénées (Arrêté Préfectoral No: 2017-s-02 du 30 mars 2017), under current ethical committee approval (APAFIS#16359-201808011445465 v4), and in accordance with Directive 2010/63/EU on protection of animals used for Scientific Purposes.

Statistics

For our measures of the reproducing females, we assessed the influence of native elevation and gestation elevation treatments, time relative to oviposition, and reproductive investment on five physiological parameters (haematocrit, haemoglobin concentration, $\dot{V}O_2$, $\dot{V}CO_2$, and d-ROMs) using linear mixed models with the *lme4* package (Douglas *et al.*, 2015) in the programming language R (R Core Team, 2017). We verified normal distributions of residuals in all cases and assessed the relative importance of fixed effects using type III sums of squares, correcting denominator degrees of freedom for *F*-tests (Kenward and Roger, 1997). Models included the fixed effect of experimental treatment group, which combined origin elevation and gestation elevation (low-low, low-high, or high-high), relative oviposition date (both linear and quadratic to account for non-linear changes over this time period), and the interaction between treatment group and relative oviposition date (both linear and quadratic).

We also included a categorical fixed effect indicating whether the measure occurred before or after oviposition (Y/N). To test for the effect of reproductive investment, we included the covariate of size-corrected clutch mass (calculated as the residuals of the total clutch mass on SVL regression). Initial models also included the covariate of body size (SVL for haematocrit, haemoglobin concentration, and d-ROMs; $\log_{10}$-transformed mass for $\dot{V}O_2$ and $\dot{V}CO_2$). We accounted for non-independence of samples from each population and the repeated measures on individuals by including population of origin and individual as additive random effects. To simplify models, we used a backward selection procedure, removing non-significant interactions and main effects (all $P > 0.10$) except for the factor of treatment, which addresses our main biological hypotheses. To meet assumptions of normal distribution of residuals, $\dot{V}O_2$, $\dot{V}CO_2$, and d-ROMs data were $\log_{10}$-transformed before analysis.

For the reproductive parameters (stage at oviposition, clutch size, egg mass, relative clutch mass, post-oviposition body condition), we used linear mixed models with a fixed effect of treatment (as above) and size (SVL) as a covariate. We included the random effects of population of origin for all models and also maternal identity in the model of egg size to account for covariance among eggs in the same clutch. Body condition was calculated as the residual of the linear regression of $\log_{10}$-transformed mass on $\log_{10}$-transformed SVL. We reduced models following a backward-selection process as above. Because of the limited range of embryonic stages observed at oviposition, we also assessed the effect of treatment on stage at oviposition with an ordinal logistic regression using the *polr* function of the MASS package (Venables and Ripley, 2002), which gave results qualitatively identical to the linear mixed model analysis.

We analyzed aspects of embryo physiology (heart rate and oxygen consumption throughout incubation), development (incubation time and survival), and hatchling morphology (mass, and SVL) using the same linear model structure and backward selection approach as described above. The analysis of survival utilized a generalized linear mixed model with a binomial error distribution. We categorized embryos into one of seven treatment groups based on the combination of population of origin, maternal gestation treatment, and egg incubation treatment (low-low-low, low-low-high, low-low-extreme high, low-high-high, low-high-extreme high, high-high-high, or high-high-extreme high). All models included the fixed effect of treatment group. Initial models also contained the covariate of egg mass at the time of measurement (for heart rate and oxygen consumption) or egg mass at oviposition (for survival, hatchling mass, and hatchling size) to account for variation in maternal energetic investment (Uller and While, 2015; Van Damme *et al.*, 1991; Warner and Lovern, 2014), as well as the interaction of egg mass with treatment group. We also included the covariate of stage at oviposition in initial models of incubation duration to test if the developmental stage at which an egg was laid affected time to hatching. Initial models of embryo heart rate and oxygen consumption also included the linear and quadratic effects of time (days past oviposition) to account for the expected non-linearity these parameters over time (Burggren and Warburton, 1994; Cordero *et al.*, 2017a; Sartori *et al.*, 2017), as well as the interaction of treatment and the linear and quadratic effects of time. As above, models included the random effect of population of origin and maternal identity. Models of heart rate and oxygen consumption also included the random effect of individual to account for covariation of measures on the same individual. For models which demonstrated a clear effect of treatment, we then tested our specific biological hypotheses by using linear contrasts of estimated marginal means using the *emmeans* package (Russel, 2016; Russel, 2019), correcting for multiple comparisons with the Šidák method (see Table 4 for details). All data figures were created with the ggplot2 package (Wickham, 2009).

Results

Maternal physiology

Reproducing lizards from low-elevation populations increased both haematocrit and haemoglobin concentration in response to translocation to high elevation (by 23.9% and 31.8% compared to low-elevation values, respectively) and values for both parameters decreased over time in all groups. Additionally, haematocrit changed non-linearly over time and was greater before oviposition (Table 1, Fig. 2A, 2B).

As the results of the $\dot{V}CO_2$ and $\dot{V}O_2$ analyses are qualitatively identical, we will present and discuss the results of $\dot{V}O_2$ only. Resting metabolic rate increased after oviposition and did not differ among the treatment groups (Table 1, Fig. 2C). As expected, resting metabolic rates were positively dependent on both body mass and body temperature. Reactive oxygen metabolites in blood plasma were affected by time differently among the treatment groups: d-ROMs decreased linearly over time for lizards from low- and high-elevation populations kept at native elevations, while remaining elevated for lizards from low elevation transplanted to high elevation. Despite that lizards at native elevations exhibited higher levels of ROMs early in reproduction, this interaction resulted in transplanted lowland lizards exhibiting higher ROMs later in reproduction (Table 1, Fig. 2D). Additionally, d-ROMs after oviposition were higher than predicted by the change across oviposition date. Finally, d-ROMS were negatively correlated with size-corrected total clutch mass, indicating that individuals investing more in reproduction also produced less reactive oxygen metabolites (Table 1, Fig. 3).

Reproductive measures

Larger lizards had larger clutches and invested more in reproduction. After accounting for this variation, egg mass at oviposition differed slightly among the maternal origin-treatment groups, though none of the pairwise differences were significant. Lizards originating from populations at different elevations did not differ in any other reproductive parameter, nor did transplanting low-elevation lizards to high elevation affect reproduction. Female *P. muralis* laid eggs with embryos that were on average at stage 27 (range: 25 – 28.5) of Dufaure and Hubert (1961). Finally, we saw no effect of treatment group on post-oviposition body condition (Table 2, Fig. 4A-D).

Embryo heart and metabolic rates

Embryonic heart rates varied amongst treatment groups in a manner dependent upon the interactions with both linear and quadratic age post-oviposition (Table 3, Fig. 5A). Embryos from low-elevation populations generally showed a concave response, whereby heart rates were lowest early and late in incubation. Conversely, embryos from high-elevations showed a convex response, whereby heart rates were highest early and late in incubation. Using linear contrasts from the mixed models to test our specific *a priori* hypotheses demonstrates that embryos from low-elevation populations exhibited higher heart rates at their native elevation compared to embryos from high-elevation populations at native elevation, on average by 15.3 beats min^{-1} (Table 4). Furthermore, embryos from low-elevation populations reduced heart rates when incubated at high elevation (by 18.9 beats min^{-1}) or extreme high elevation (by 9.6 beats min^{-1}). Notably, the change was greatest in the high-elevation treatment, in which heart rates were lower than the extreme-high treatment by 9.3 beats min^{-1}. Embryos from high elevation populations increased heart rates when incubated at extreme high elevation compared to native (high) elevation, on average by 9.6 beats min^{-1}.

Embryo metabolic rate (oxygen consumption; $\dot{V}O_2$) increased across incubation, though this response was non-linear and dependent on a quadratic function of embryo age (Table 3; Fig 5B). Embryos from the different treatments exhibited different patterns of oxygen consumption, with this response dependent on a linear interaction with embryo age post-oviposition. The interaction of treatment and embryo age is driven by differences in embryos incubated at extreme high elevation: embryos for high-elevation populations increased oxygen consumption across incubation, whereas embryos from low-elevation populations exhibited lower levels later in incubation. No test of our *a priori* hypotheses for oxygen consumption demonstrated a clear difference between treatment groups. Furthermore, oxygen consumption was not clearly dependent on egg mass as we would expect (Vleck and Hoyt, 1991) most likely due to the strong effect of days post-oviposition, which is correlated with egg mass.

Embryo survival and hatchling morphology

We found no difference among treatment groups in probability of hatching (χ^2_6 = 5.45, P = 0.49) or an effect of oviposition egg mass on survival to hatching probability (χ^2_1 = 2.83, P = 0.09). Among embryos that survived to hatching, incubation duration was affected by incubation treatment (Table 3; Fig. 6A): embryos native to low-elevation populations took an average of 1.5 days longer to hatch at high elevation and 1.4 days longer to hatch at extreme high elevation. Egg mass at oviposition strongly and positively influenced hatching mass and body size (Table 6).

Discussion

Our results demonstrate that translocation to high elevation alters the physiology of lowland gravid lizards compared to lizards kept at native elevations (from both low- and high-elevation populations) but does not affect short-term reproductive investment or output. Importantly, the concentration of reactive oxygen metabolites did not decrease during the reproductive period in transplanted lizards, as it did in lizards kept at native elevation. This suggests that there may be a deleterious effect of gestating at high elevation for non-native lizards, even if that cost is not evident in current reproduction. Interestingly, reactive oxygen metabolite concentration was highest in females exhibiting the lowest relative clutch mass, contrary to assumptions of a reactive metabolite cost of reproduction. Nonetheless, at least in the short-term there appears no indication that lowland *P. muralis* reproduction is limited at higher elevations within the range of the species, assuming the environment is otherwise suitable. Furthermore, the conditions experienced by females during gestation did not affect the physiology, development, or hatchling morphology of embryos post-oviposition. Thus, we did not find evidence that maternal hypoxia exposure may 'prime' embryos from the constraints of post-oviposition hypoxia. Embryos post-oviposition demonstrated a clear physiological and developmental response to hypoxia, including reduced heart rate at high elevation, altered patterns of oxygen consumption indicating diminished late-stage metabolic rate, and increased incubation duration. These adjustments in embryo physiology and developmental timing at least partially buffer these lizards from potentially negative effects of reduced oxygen availability; development at these elevations did not affect fitness-related traits including embryo survivorship to hatching or hatchling morphology. Taken together, these results suggest that this widespread and common species, a successful invader elsewhere (Beninde *et al.*, 2018; Deichsel and Gist, 2001; Michaelides *et al.*, 2015), may likely successfully colonize higher-elevation habitat in future climate scenarios.

Maternal physiology and reproduction

As predicted, gravid females from low-elevation populations transplanted to high elevation had higher haematocrit and haemoglobin concentration compared to low-elevation lizards kept at low elevation. This response is concordant with other studies showing that lizards from high-elevation populations feature higher haematocrit and haemoglobin concentration compared to lowland populations (González-Morales *et al.*, 2015, Lu *et al.*, 2015). Haematocrit and haemoglobin concentration was increased in lowland lizards transplanted to high elevation to match lizards native to high elevations, which does not support the prediction that lizards native to different elevations would exhibit different blood chemistry profiles. The current data do not allow us to distinguish between plasticity and local adaptation in these traits in lizards native to high elevations, but in either case this could illustrate an adaptive response of lizards to facilitate oxygen transport at high elevation.

In parallel with these results, we did not observe differences in resting metabolic rate among the treatment groups. Even during a state of elevated energy exchange such as reproduction (Angilletta and Sears, 2000; Foucart *et al.*, 2014), oxygen demands are met at high elevation, possibly due to the observed plasticity in blood biochemistry. While energetic demands are increased in reproductive compared to non-reproducing females, it is important to note that this increased demand occurs during vitellogenesis (Foucart *et al.*, 2014; Van Dyke and Beaupre, 2011), before the start of this experiment. Nonetheless, the question of long-term effects of increased haemoglobin concentration and haematocrit remains because a higher concentration of red blood cells increases blood viscosity. This phenomenon could then carry an important cost for blood circulation (Dunlap, 2006; Hedrick *et al.* 1986) and may not be maintained, as was observed in lowland males of this species transplanted to extreme high elevation (Gangloff *et al.*, 2019). Increasing red blood cell concentration is especially constraining during gravidity when haematocrit drops, likely to reduce viscosity and facilitate circulation and gas transport to developing embryos (e.g., Dupoué *et al.*, 2015).

We found little evidence of important differences among treatment groups in indicators of reproductive investment, including clutch size and relative clutch mass. After accounting for variation due to maternal size, we identified a trend whereby lizards at high elevations (either native or transplanted) produced eggs with a greater mass at oviposition. This finding is counter to our prediction that lizards at higher elevations would demonstrate reduced reproductive output. Indeed, through changes in physiological parameters females appeared to compensate for reduced oxygen availability and maintain reproductive output across treatments. It is important to recognize that this experiment encompassed only the first reproductive event of a single year. Lowland lizards transplanted to high elevation were able to maintain reproductive output without an apparent cost in terms of body condition in this bout, but there may be a trade-off with future reproduction such as via ROMs damage. Although stage at oviposition did not differ among treatment groups and was within the expected range for most oviparous lizards (reviewed in Mathies and Andrews, 1999), our findings broadly support the prediction that gestation duration is a highly stable trait whose population mean might undergo gradual intergenerational change as an adaptive evolutionary response to high-altitude conditions (e.g., temperature, Mathies and Andrews, 1995). Thus, because stage at oviposition might be highly canalized in *P. muralis*, variation in this trait may not manifest until several generations are exposed to oxygen-limited conditions, similar to the adaptive evolution of prolonged egg retention in cool environments (Telemeco *et al.*, 2010; While *et al.*, 2015).

We provide two important results regarding reactive oxygen metabolite production in response to both reproduction and hypoxia. First, we found that females with increased reproductive investment (size-corrected total clutch mass) had lower levels of reactive oxygen metabolites in blood plasma. Broadly, this result does not support the common assumption that reproduction incurs an oxidative cost and thereby mediates life-history trade-offs (Dowling and Simmons, 2009; Metcalfe and Monaghan, 2013; Speakman and Garrat, 2014). Previous work in lizards concurs with our results: no such oxidative cost of reproduction was found in two other lizard species (*Niveoscincus ocellatus*, Isaksson *et al.*, 2011; *Ctenophorus pictus*, Olsson *et al.*, 2009; Olsson *et al.*, 2012). This result suggests that both reactive oxygen metabolite production and reproductive investment could be correlated via some unmeasured physiological parameter representing overall “individual quality”: higher-quality individuals are able to both invest more in reproduction and meet energetic demands while producing fewer potentially detrimental by-products (Wilson and Nussey, 2010). On the contrary, Webb *et al.* (2018) found that increased reproductive investment (as measured by follicle number) was associated with increased reactive oxygen metabolites in the lizard *Cyclura cychlura inornata*. Importantly, the study of Webb *et al.* (2018) measured this effect during vitellogenesis, thus pointing to this period as being key to understanding the relationship between reproductive investment and reactive oxygen metabolite production. A similar pattern of higher reactive metabolite production earlier in the reproductive cycle has also been found in snakes (*Antaresia children*, Stahlschmidt *et al.*, 2013) and birds (*Sula leucogaster*, Montoya *et al.*, 2016). Our results agree with this pattern, whereby reactive oxygen metabolites decreased during the gestation period, indicating that such potentially harmful byproducts are greatest earlier in the reproductive process.

Our second significant finding is that both low- and high-elevation lizards kept at native elevations decreased reactive oxygen metabolite production across the reproductive period, while lowland lizards transplanted to high elevations did not. We might expect that the reactive oxygen cost of reproduction will likely be context-dependent, perhaps because of resource limitations and subsequent allocation decisions (Dowling and Simmons, 2009). In our case, it appears that the reduced oxygen availability at higher elevations elicits such a constraint and prohibits females from decreasing reactive oxygen metabolite production across the reproductive period. Our results demonstrate higher reactive metabolite production later in gravidity and after oviposition in transplanted lizards. These elevated levels of reactive oxygen metabolites have the potential to bear costs for future fitness in both the mothers and their offspring, given the potential damaging effects on proteins, membranes, and DNA (Blount *et al.*, 2015; Constantini, 2016; Speakman and Garrat, 2014). Unfortunately, plasma volume limitations precluded us from also measuring antioxidant capacity, which would provide valuable data to quantify the response to these damaging molecules. For example, antioxidant capacity may change across the reproductive period, thus mitigating the potential negative impacts of increased reactive oxygen metabolite production. Future work is needed to understand patterns of reactive oxygen metabolites mediating physiological trade-offs at high elevations by measuring additional tissues, measuring antioxidant and repair capacity, and working to quantify the long-term impacts of increased reactive oxygen metabolite levels (Speakman and Garrat, 2014).

Embryo physiology and development

Developing *P. muralis* embryos appear to be robust to the effects of ecologically relevant hypoxia, at least until hatching and the beginning of the free-living stage. The most dramatic physiological response is the decreased embryonic heart rate in naturally occurring hypoxia at high elevation (Cordero *et al.*, 2017a), similar to many oviparous species exposed to laboratory-simulated hypoxia (Crossley and Altimiras, 2005; Du *et al.*, 2010; Nechaeva and Vladimirova, 2008; VanGolde *et al.*, 1997). This response is coupled with a relative increase in heart size in *P. muralis* (Cordero *et al.*, 2017a), which may facilitate the maintenance of oxygen delivery via increased stroke volume. This response is predicted under a scenario where hypervolemia increases the optimal haematocrit: oxygen delivery is maintained, despite reductions in heart rate, via increases in haematocrit and stroke volume (Birchard, 2015; Weibel *et al.*, 1991). While work is needed to test these specific relationships among parameters important to efficient oxygen delivery under hypoxia, we found that embryos at high and extreme high elevations maintained comparable metabolic rates (as measured by oxygen consumption). This concurs with previous studies finding that incubation under hypoxia had little effect on the metabolic rates of developing alligator embryos (*Alligator mississippiensis*, Crossley *et al.*, 2017; Warburton *et al.*, 1995).

While oxygen consumption generally increased across incubation, the pattern was dependent on treatment. Notably, lowland embryos incubated at extreme high elevation exhibited a pattern whereby oxygen consumption decreased late in incubation. This pattern is mirrored in the pattern of heart rate variation across incubation, suggesting a late-stage metabolic suppression resulting from oxygen limitation. This metabolic constraint on low-elevation *P. muralis* embryos developing at high and extreme high elevations could also explain the increased incubation duration of these embryos. That late-stage embryos native to high elevation did not experience this metabolic suppression and did not alter incubation times at extreme high elevation suggests a role for local adaptation in maintaining physiological and developmental robustness in hypoxia. Although late-stage embryos of sea-level chicken populations also exhibited reduced oxygen consumption when initially transplanted to high elevation, oxygen consumption rates exceeded sea-level values after > 9 generations of selective breeding (based on hatching success) at 3,100 m ASL (Beattie and Smith 1975; Wangensteen *et al.*, 1974). The fitness consequences of hypoxia-induced metabolic suppression in non-avian reptilian embryos awaits further experimentation.

While embryo physiology and development responded to high-elevation hypoxia, neither survival to hatching nor hatchling morphology were affected. This corroborates our previous work in this system in demonstrating that early-life stages are relatively resilient to hypoxia and therefore would not limit future colonization of high-elevation habitats beyond the current species range. We emphasize that even our extreme high elevation treatment represents a moderate and biologically relevant reduction in oxygen availability (approximately 72% of sea level equivalent), which explains why we did not find the clear negative effects of hypoxia as have other studies in reptiles exposed to more severe hypoxia, even for short periods of time (Andrews, 2002; Cordero *et al.*, 2017b; Iungman and Piña, 2013; Kam, 1993). The lack of effect of our maternal gestation treatments implies that embryos pre-oviposition did not face a mismatch between oxygen supply and demand. Our data suggest a maternal ability to maintain oxygen supply to tissues, including developing embryos, by adjusting oxygen carrying capacity (haematocrit, haemoglobin) to maintain metabolic rates. Furthermore, the early stages of embryonic development in lizards require low oxygen consumption (Thompson and Stewart 1997), as during this time embryos only constitute ~10% of their hatchling body mass (Andrews 2004). A fruitful direction for future experiments is to transplant reproductive females earlier in the reproductive process (i.e., before vitellogenesis) to test for trade-offs in reproductive investment between offspring size and number and also for earlier maternal exposure to affect offspring development, either via adaptive maternal effects (e.g., hormonal mechanisms) or because of energetic constraints imposed by hypoxia.

We report this apparent resilience of embryos to hypoxia with the important caveat that oxygen availability interacts with temperature to exacerbate the performance limitations and negative effects of hypoxia at higher temperatures (Gangloff and Telemeco, 2018). This may be most notable in the late embryonic stages, in which oxygen uptake depends on diffusion across the chorioallantoic membrane and behavioural responses to the environment are highly restricted (Cordero *et al.,* 2018; Telemeco *et al.*, 2016; but see Shine and Du, 2018). Work in other reptile taxa has demonstrated a clear pattern whereby the detrimental effects of hypoxia are more severe at higher temperatures (Flewelling and Parker, 2015; Iungman and Piña, 2013; Liang *et al.*, 2015; Smith *et al.,* 2015). The interaction of temperature and hypoxia likely also explains why we found longer incubation periods for low-elevation embryos incubated at higher elevations, while our previous work did not find such an effect. In the current study, we incubated eggs at 28°C, while Cordero *et al.* (2017a) used 24°C. The impact of hypoxia in prolonging development times was more pronounced at higher temperatures, likely due to limitations of the embryo to meet the corresponding increased oxygen demand, such as found previously in caimans (*Caiman latirostris*; Iungman and Piña 2013). While the effects of reduced oxygen availability and high temperatures interact to impact physiology, it is also possible that shared response pathways may offer protection against these simultaneous stressors (e.g., Teague *et al.*, 2017). Exploring the interrelation of these response pathways, as well as how responses may change across life-history stages, remains an important research avenue.

Our results concur with other recent work demonstrating the apparent flexibility of reptilian embryos in response to novel environments (e.g., Tiatragul *et al.,* 2017). This is not surprising, given that reptile nests are frequently exposed to low-oxygen conditions in natural settings (Ackerman and Lott 2004; Booth 1998; Packard and Packard 1988). Thus, evolution may have promoted physiological mechanisms to deal with hypoxia in embryos that can then facilitate development under situations of reduced oxygen partial pressure, as found at high elevations. Physiological plasticity to adjust to hypoxia could thus be stage-specific, explaining the apparent resilience of *P. muralis* embryos but not adults (Cordero *et al.*, 2017a; Gangloff *et al.*, 2019). The long-term effects of hypoxia exposure during development on phenotype and fitness at later life-history stages remain important research questions. For example, development in hypoxia has long-term effects on post-hatching cardiovascular phenotypes in snapping turtles (*Chelydra serpentina*; Wearing *et al.*, 2016, 2017), decreases swimming performance in snakes (*Natrix maura*; Souchet *et al.* in prep), and impacts cognitive ability in lizards (*Eremias argus*; Sun *et al.*, 2014). The adaptive significance of embryonic plasticity in response to hypoxia can only be evaluated by quantifying the long-term effects on individual fitness (Mitchell *et al.*, 2018).

Conclusion

This study addresses two important dimensions of reproduction with implications for understanding both life histories and range expansion in mountainous regions in common wall lizards: reactive oxygen metabolite production and the effects of hypoxia. That females transplanted to high elevation matched the reproductive output of lizards native to both low-elevation and high-elevation populations and that embryos are physiologically resilient to development at elevations even above the current range limits suggests that these life stages will not limit initial colonization of high-elevation habitats. Population establishment, however, will depend on long-term costs associated with life in reduced oxygen availability on adults (such as damage from increased ROM production) and the consequences of reduced performance (Gangloff et al., 2019). Colonization of high-altitude areas is important for conservation efforts as it will likely put at further risk high-elevation specialist lizards endemic to small mountaintop ranges, such as *Iberolacerta* spp., via competition for resources and direct interactions (Žagar *et al.*, 2015, 2017). With our work in this system, we hope to quantify the relative potential of physiological plasticity and local adaptation across life history stages to facilitate range expansion in a species likely to benefit under climate change scenarios.

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The authors declare no conflicts of interest.

Data Accessibility

Data are archived in Mendeley: http://dx.doi.org/10.17632/94xsf6wv6c.1

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Table 1. Results of linear mixed model analyses of physiological measures in gravid female *Podarcis muralis* lizards.

Source of Variation	Hct N = 186 obs.	[Hb] N = 187 obs.	$\dot{V}_{O_2}$ N = 175 obs.	d-ROMs N = 155 obs.
Treatment	__Low-High > High-High > Low-Low__	__Low-High > High-High > Low-Low__		__High-High > Low-High > Low-Low__
F (df_n, df_d)	9.91 (2, 6.9)	18.6 (2, 6.9)	1.37 (2, 9.68)	3.49 (2, 23.8)
Pr > F	__0.009**__	__0.0017**__	0.30	__0.047*__
Relative Oviposition Date Linear	__Negative__	__Negative__		__Negative__
F (df_n, df_d)	6.30 (1, 159.1)	28.0 (1, 161.4)	--	43.9 (1, 106.1)
Pr > F	__0.013*__	__<0.0001**__		__<0.0001**__
Relative Oviposition Date Quadratic	__See Fig. 2A__			
F (df_n, df_d)	8.43 (1, 154.5)	--	--	--
Pr > F	__0.0042*__			
Treatment × Relative Oviposition Date Linear				__See Fig. 2D__
F (df_n, df_d)	--	--	--	4.48 (2, 144.7)
Pr > F				__0.013*__
log₁₀(Mass)			__Positive__	
F (df_n, df_d)	--	--	9.97 (1, 82.7)	--
Pr > F			__0.0022*__	
Pre-Oviposition	__Pre > Post__		__Post > Pre__	__Post > Pre__
F (df_n, df_d)	14.88 (1, 157.2)	--	13.33 (1, 157.3)	41.9 (1, 128.5)
Pr > F	__0.00017**__		__0.0004***__	__<0.0001**__
Body Temperature			__Positive__	
F (df_n, df_d)	--	--	6.51(1, 156.1 1)	--
Pr > F			__0.012*__	
Size-corrected Clutch Mass				__Negative__
F (df_n, df_d)	__--__	__--__	--	13.98 (1, 33.9)
Pr > F				__0.0007**__

See text for statistical details. Significant factors shown in bold with one ($P < 0.05$) or two ($P < 0.001$) asterisks and directionality. Hct = Haematocrit; [Hb] = Haemoglobin concentration; $\dot{V}_{O_2}$ = resting oxygen consumption; d-ROMs = reactive oxygen metabolite concentration

Table 2. Results of linear mixed model analyses of reproductive parameters in gravid female *Podarcis muralis* lizards.

Source of Variation	**Stage at Oviposition N = 39 embryos**	**Clutch Size N = 47 clutches**	**Egg Mass N = 253 eggs**	**Relative Clutch Mass N = 47 clutches**	**Post-oviposition body condition N = 47 clutches**
Treatment			**See Fig. 4C**		
F (df_n, df_d)	0.32 (2, 9.1)	0.37 (2, 8.4)	4.80 (2, 7.5)	1.32 (2, 8.3)	1.32 (2, 7.2)
Pr > F	0.73	0.70	**0.046***	0.32	0.33
Snout-Vent Length		**Positive**	**Positive**	**Positive**	
F (df_n, df_d)	--	14.17 (1, 43.0)	5.58 (1, 43.2)	5.48 (1, 43.0)	--
Pr > F		**0.0005*****	**0.023***	**0.024***	

See text for statistical details. Significant factors shown in bold with one ($P < 0.05$) or two ($P < 0.001$) asterisks and directionality.

Table 3. Results of linear mixed models of embryo *P. muralis* heart rate and oxygen consumption during development, incubation duration, and hatchling morphology.

Source of Variation	**Heart Rate** **N = 414 obs.**	$\dot{V}_{O_2}$ **N = 136 obs.**	**Incubation Duration** **N = 184 embryos**	**Hatchling Mass** **N = 184 hatchlings**	**Hatchling Body Size (SVL)** **N = 184 hatchlings**
Treatment	**See Fig. 5A**	**See Fig. 5B**	**See Fig. 6A**		
F (df_n, df_d)	4.02 (6, 306.6)	6.14 (6, 91.3)	3.01 (6, 131.9)	1.97 (6, 144.4)	1.53 (6, 40.6)
Pr > F	**0.0007****	**< 0.0001****	**0.009***	0.074	0.19
Oviposition Egg Mass				**Positive**	**Positive**
F (df_n, df_d)	--	--	1.33 (1, 71.2)	42.5 (1, 79.3)	36.0 (1, 63.1)
Pr > F			0.25	**< 0.0001****	**< 0.0001****
Oviposition Mass × Treatment					
F (df_n, df_d)	--	--	2.10 (6, 136.4)	2.12 (6, 146.0)	--
Pr > F			0.057	0.055	
Days Post Oviposition Linear		**Positive**			
F (df_n, df_d)	1.35 (1, 300.1)	29.7 (1, 81.6)	--	--	--
Pr > F	0.25	**< 0.0001****			
Days Post Oviposition Quadratic		**See Fig. 5B**			
F (df_n, df_d)	1.41 (1, 305.0)	7.52 (1, 81.8)	--	--	--
Pr > F	0.24	**0.008***			
Treatment × Days Post Oviposition Linear	**See Fig. 5A**	**See Fig. 5B**			
F (df_n, df_d)	2.33 (6, 302.4)	6.55 (6, 89.9)	--	--	--
Pr > F	**0.033***	**< 0.0001****			
Treatment x Days Post Oviposition Quadratic	**See Fig. 5A**				
F (df_n, df_d)	2.35 (6, 307.1)	--	--	--	--
Pr > F	**0.031***				

See text for statistical details. Significant factors shown in bold with one ($P < 0.05$) or two ($P < 0.001$) asterisks and directionality. $\dot{V}_{O_2}$ = oxygen consumption; SVL = Snout-vent length

Table 4. Results of *a priori* hypothesis tests. Tests were constructed using linear contrasts of estimated marginal means. Estimate represents the differences in observed values between groups, taking into account covariates included in the model (see Table 3 for full model results and text for statistical details).

Hypothesis Test	**Heart Rate N = 414 obs.**	**$\dot{V}_{O_2}$ (log$_{10}$-transformed) N = 136 obs.**	**Incubation Duration N = 184 embryos**
Native low- vs. High-elevation at native elevation			
Estimate (SE)	-15.29 (4.11)	-0.047 (0.04)	0.058 (1.00)
t-statistic (*df*)	-3.72 (53.7)	-1.10 (6.27)	0.058 (4.4)
Pr > t	**0.0029***	0.89	> 0.99
Native low-elevation eggs at native elevation vs. high-elevation			
Estimate (SE)	-18.85 (3.58)	-0.076 (0.03)	1.49 (0.42)
t-statistic (*df*)	-5.26 (375.4)	-2.71 (56.7)	3.52 (166.6)
Pr > t	**< 0.0001****	0.052	**0.003***
Native low-elevation eggs at native elevation vs. extreme high-elevation			
Estimate (SE)	-9.60 (3.49)	-0.039 (0.03)	1.35 (0.47)
t-statistic (*df*)	-2.75 (363.1)	-1.35 (33.1)	2.86 (167.3)
Pr > t	**0.037***	0.71	**0.028***
Native low-elevation eggs at high elevation vs. extreme high-elevation			
F (*df$_n$*, *df$_d$*)	9.25 (2.92)	0.037 (0.03)	-0.14 (0.36)
Pr > F	3.17 (338.3)	1.25 (42.5)	-0.38 (162.3)
	0.0098*	0.77	> 0.99
Native high-elevation eggs at native elevation vs. extreme high-elevation			
Estimate (SE)	9.55 (3.60)	-0.009 (0.03)	-0.80 (0.41)
t-statistic (*df*)	2.65 (389.0)	-0.27 (66.6)	-1.95 (156.1)
Pr > t	**0.049***	> 0.99	0.28
Eggs from low-elevation females gestated at native elevation vs. high elevation			
Estimate (SE)	0.63 (3.41)	-0.001 (0.03)	-0.56 (0.41)
t-statistic (*df*)	0.19 (126.1)	-0.043 (35.42)	-1.37 (62.4)
Pr > t	> 0.99	> 0.99	0.69

See text for statistical details. Significant contrasts shown in bold with one ($P < 0.05$) or two ($P < 0.001$) asterisks and directionality. $\dot{V}_{O_2}$ = oxygen consumption

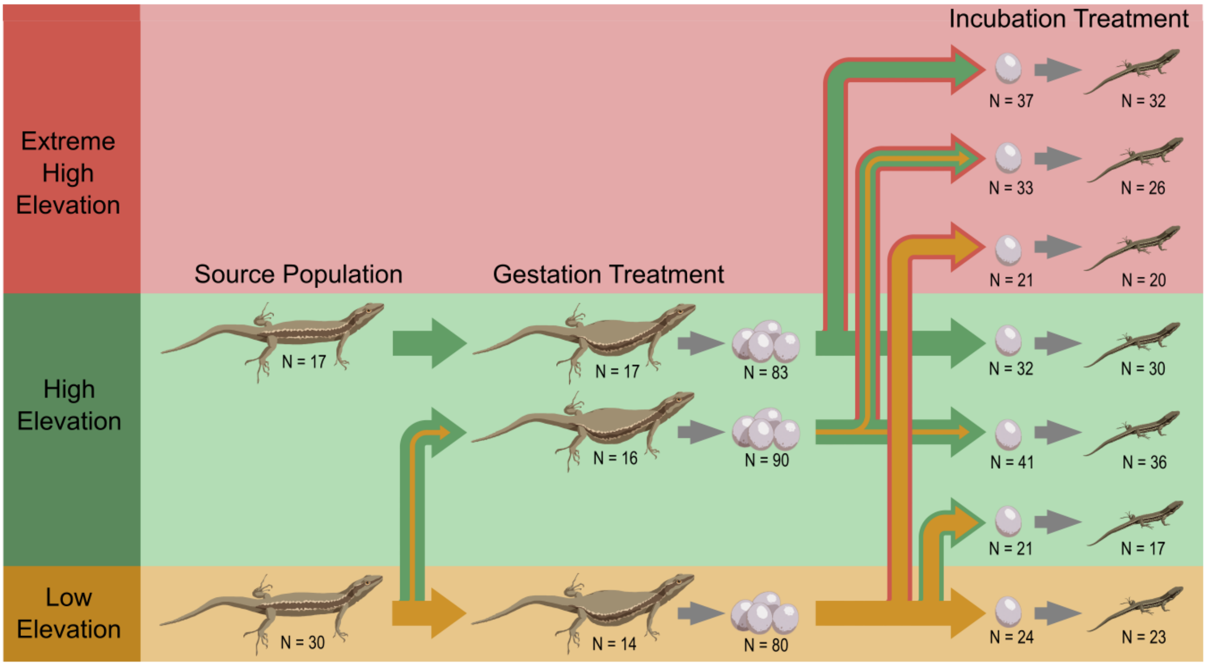

Figure 1. Experimental design. Gravid lizards were collected from three low-elevation (382-472 m ASL) and two high elevation (1402-1795 m ASL) populations in the Pyrénées of southern France (see Table S1 for details). We maintained all lizards in common-garden conditions in labs at low- (436 m ASL; PO_2 ~20.1 kPa) and high-elevation (1735 m ASL; PO_2 ~17.4 kPa), where they completed gestation and laid eggs. Eggs were then split within clutches and incubated at low, high, or extreme high elevation (2877 m ASL; PO_2 ~15.3 kPa). Illustration credit: Bea Angelica Andersson.

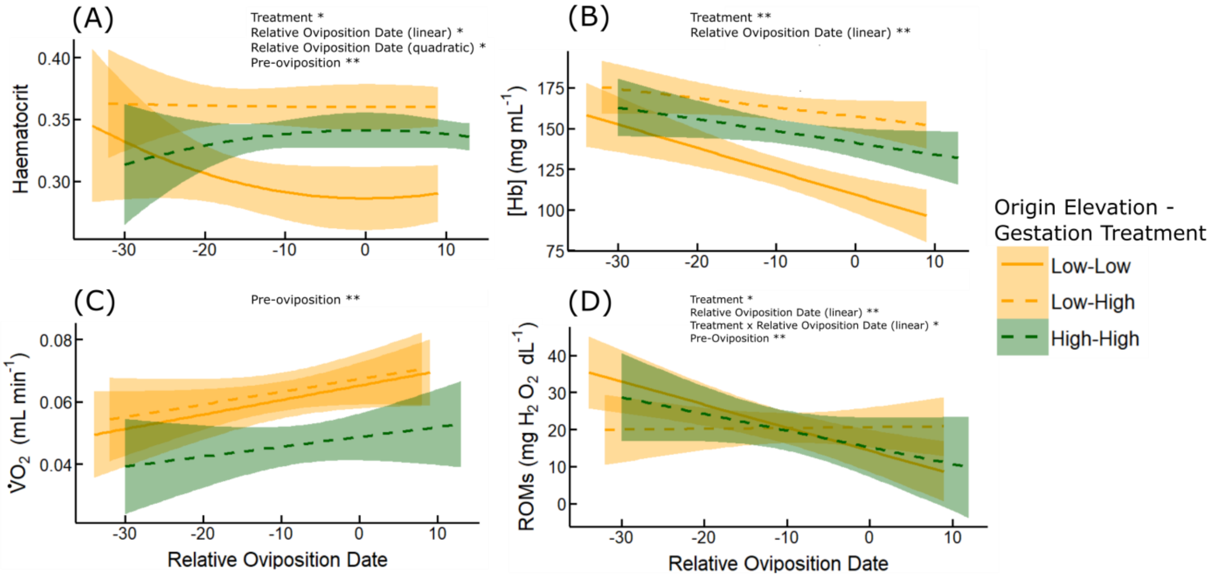

Figure 2. Response of haematocrit (Panel A), haemoglobin concentration (Panel B), resting oxygen consumption ($\dot{V}O_2$; Panel C), and reactive oxygen metabolites (d-ROMs; Panel D) over time in reproducing lizards from each experimental treatment. Lines are linear or quadratic regressions depending on the results of mixed effect models with shaded areas representing standard error of the estimate. Significant effects shown in inset with one ($P < 0.05$) or two ($P < 0.001$) asterisks (see Table 1 and text for statistical details). Relative oviposition date 0 = date of oviposition. Low-Low N =14 individuals; Low-High N = 16 individuals; High-High N =17 individuals

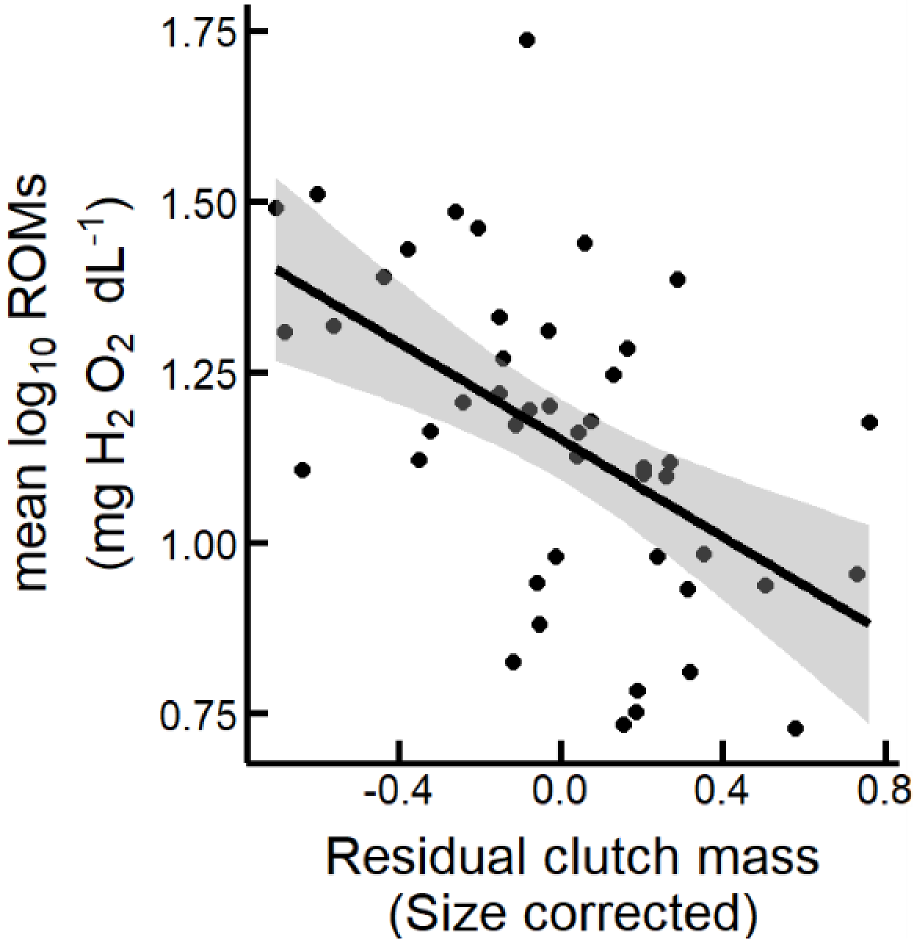

Figure 3. Scatterplot showing relationship between total clutch mass (corrected for body size) and mean reactive oxygen metabolites (ROMs) for all females (N = 47). Black line represents linear regression with standard error in gray shading. After accounting for other effects, ROMs decreases with increased relative clutch mass (see Table 2 for details).

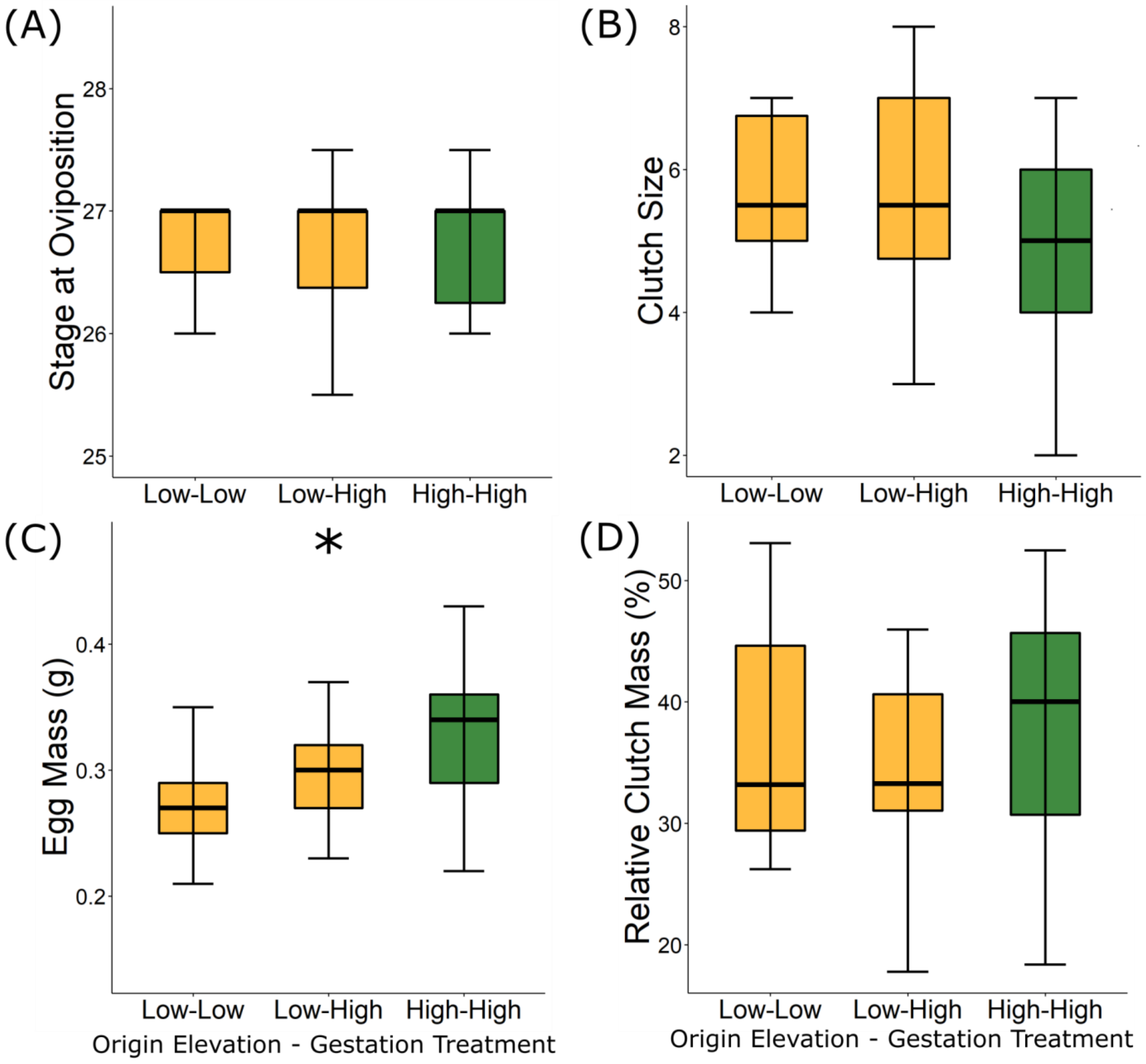

Figure 4. Reproductive parameters in females from three experimental treatments (combining origin elevation and gestation treatment): Stage at oviposition (A), clutch size (B), egg mass at oviposition (C), and relative clutch mass (D). Plots are Tukey boxplots showing median, interquartile range, and range of raw data values. Significant differences among treatment groups shown with one asterisk ($P < 0.05$; see Table 2 and text for statistical details). Low-Low N =14 individuals; Low-High N = 16 individuals; High-High N =17 individuals

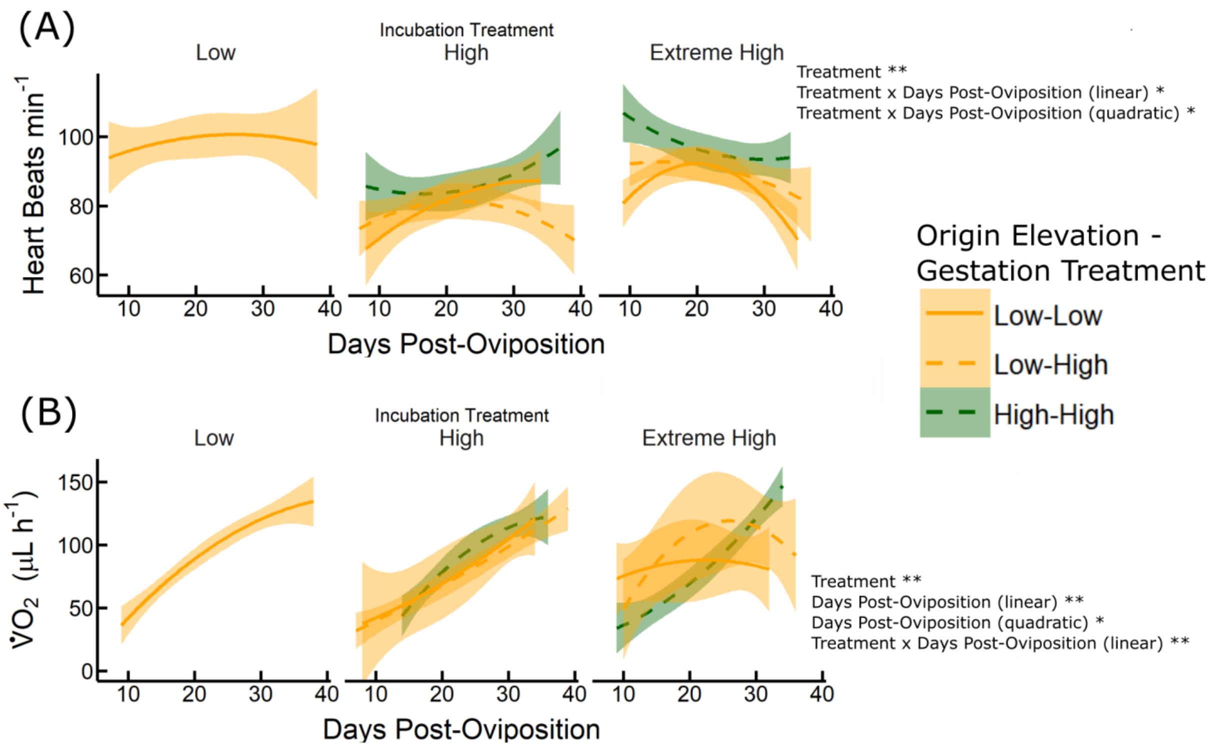

Figure 5. Heart rate (Panel A) and oxygen consumption ($\dot{V}O_2$; Panel B) over time in lizard embryos from each experimental treatment. Lines are quadratic regressions with shaded areas representing standard error of the estimate. Significant effects shown in inset with one ($P < 0.05$) or two ($P < 0.001$) asterisks (see Table 3 and text for statistical details). Embryos are from three maternal treatments (Low-Low N =14 clutches; Low-High N = 16 clutches; High-High N =17 clutches) incubated at three elevations. See Fig. 1 for complete sample size details.

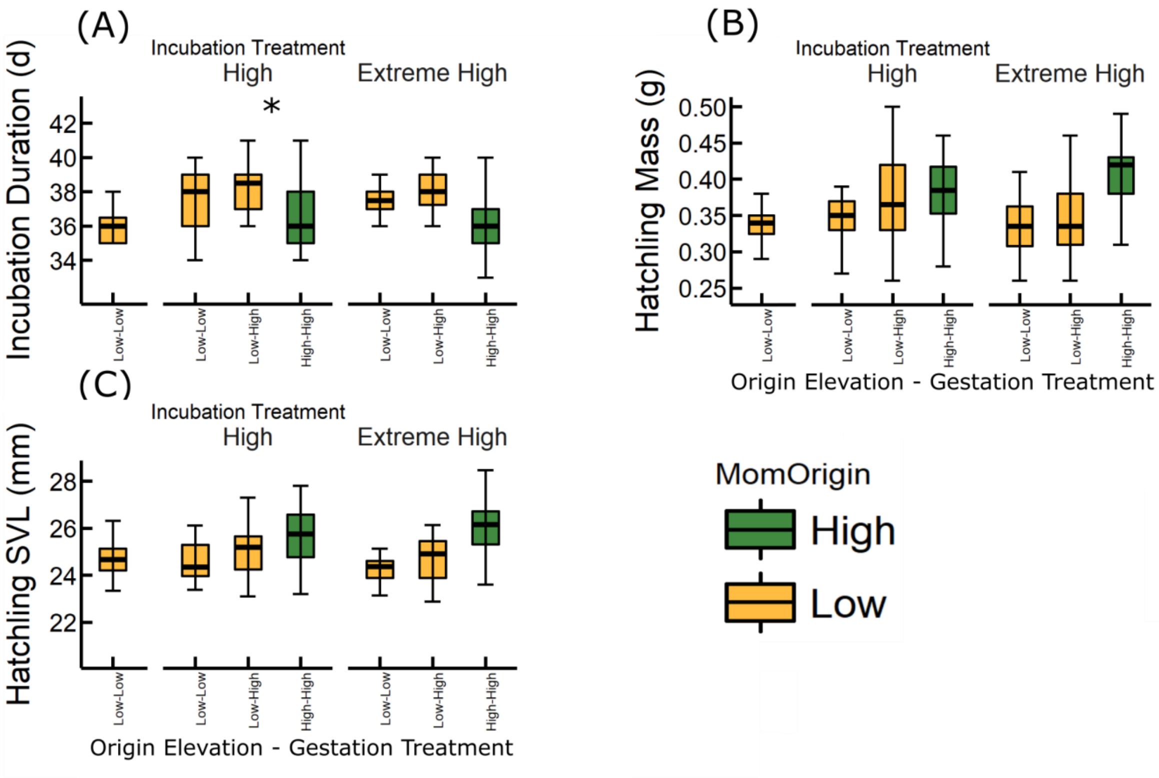

Figure 6. Incubation duration (A), hatchling mass (B), and hatchling snout-vent length (SVL) (C). Plots are Tukey boxplots showing median, interquartile range, and range of raw data values. Significant differences among treatment groups shown with one asterisk ($P < 0.05$; see Table 3 and text for statistical details). Hatchlings are from three maternal treatments (Low-Low N =14 clutches; Low-High N = 16 clutches; High-High N =17 clutches) incubated at three elevations. See Fig. 1 for complete sample size details.

Table S1. Descriptive details of sampled *Podarcis muralis* populations.

Name	Latitude Longitude	Altitude (m ASL)	Habitat Type	*N* Low-elevation *N* High-elevation	Sampling Dates
Alas[1]	42°56'58"N 001°2'47"E	472	Cemetery and rock wall	5 5	10 Apr. 2018 17 Apr. 2018
Luzenac[1,2]	42°57'22"N 001°5'1"E	443	Cemetery and rock wall	4 5	3-4 Apr. 2018 17 Apr. 2018
Mercenac	42°57'52"N 001°6'10"E	382	Cemetery	5 6	21 Apr. 2018 18 Apr. 2018
La Mongie	42°54'32"N 0°10'7" E	1795	Natural rock outcrops and rock walls	0 8	- 1-6 May 2018
Col de la Core	42°51'34.4"N 1°6'18.2"E	1402	Natural rock outcrop	0 9	- 26 May 2018

[1] Population also studied in Calsbeek *et al.* (2010) *J Evol Biol* 23:1138-47.

[2] Population also studied in Gangloff *et al.* (2019) *Physiol Biochem Zool* 92:189–200.