Stage Dependence of Phenotypical and Phenological Maternal Effects: Insight into Squamate Reptile Reproductive Strategies

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Submitted April 17, 2012; Accepted February 13, 2013; Electronically published June 17, 2013

Dryad data: http://dx.doi.org/10.5061/dryad.14hj1.

ABSTRACT: Enhanced thermal conditions have been credited as a driving force for the evolution of viviparity, particularly in squamate reptiles, among which it has independently evolved more than 100 times. However, maternal thermoregulation is also a critical component of reproduction in oviparous squamates, for which considerable embryonic development occurs prior to oviposition. When carrying eggs, oviparous mothers modify thermoregulation in a manner similar to that of pregnant females. To further understand the role of temperature in influencing reproductive strategies, it is critical that we elucidate the degree to which thermal sensitivity varies across developmental stages. We studied stage-dependent embryonic sensitivity in a viviparous snake, the aspic viper (*Vipera aspis*). We manipulated female body temperature at different stages of pregnancy—early development, early embryonic growth, and late embryonic growth—by imposing two contrasting daily thermal cycles that mimicked reproductive (warm) and nonreproductive (cool) female temperature profiles. Thermal sensitivity of offspring phenotype was stage dependent, with offspring quality more negatively affected when exposure to cool temperatures occurred early in development. In contrast, developmental rate was slowed by the cooler cycle, independent of the timing of the exposure. Given the more persistent effect on phenology, phenological effects likely provide a greater driving force for complete embryonic retention (i.e., viviparity).

Keywords: development, embryo retention, snakes, temperature, thermoregulation, viviparity.

Introduction

Sensitivity of embryos to temperature has been relatively well studied in both vertebrates and invertebrates. While most studies have used constant thermal conditions throughout development (Deeming and Fergusson 1991; Johnston and Benett 1996; Farmer 2000), environmental conditions, as well as embryonic sensitivity to environmental conditions, vary over time (Andrews 2004; Henry and Ulijaszek 2009). Early development, when much of nervous-system development and organogenesis occurs, is particularly sensitive to environmental conditions, while later development, which is dominated by somatic growth, is less sensitive (Andrews 2004; Henry and Ulijaszek 2009; Mihaila et al. 2011). Thus, effects of maternal efforts to buffer developing progeny from environmental conditions should be investigated in the context of this temporally varying sensitivity. In most squamates, the energetic demands of offspring development are met through the provisioning of yolk deposited into follicles during vitellogenesis (Stewart and Thompson 2000; Blackburn 2005). With the need to provide energy during development thus eliminated, maternal effort during development can be focused on regulating the thermal environment.

Female squamates typically bask more and change both their body-temperature range and average thermal preference when carrying developing offspring, whether they are oviparous (Lourdais et al. 2008) or viviparous (Robert et al. 2006; Shine 2006). These behavioral adjustments result in considerable offspring benefits (Shine 1995; Wapstra 2000; Lorioux et al. 2012). In fact, the benefits of maternal thermophily are a fundamental component of multiple theories regarding the evolution of viviparity (Tinkle and Gibbons 1977; Shine 1995). To enhance our knowledge of reproductive strategies used by squamate reptiles (e.g., delayed oviposition, viviparity) and perhaps of driving forces that might lead to different strategies, a better understanding of embryonic thermosensitivity and its stage-dependent nature is required.

We investigated whether the effects of maternal thermal regulation on offspring phenotype and developmental duration are temporally variable in a temperate viviparous snake, the aspic viper (*Vipera aspis*). We imposed ecolog-

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Am. Nat. 2013. Vol. 182, pp. 223–233.
0003-0147/2013/18202-53791$15.00.
DOI: 10.1086/670809

ically relevant constraining (cooler) daily temperature cycles on pregnant females throughout gestation: only during early development, only during early embryonic growth, only during late embryonic growth, or not at all. This enabled us to test the following predictions: (1) Offspring phenotype is more thermally sensitive early in gestation, so a deviation from maternally selected temperatures during early development should induce irreversible developmental disruption. (2) Early and late embryonic growth make up the majority of gestation time, so thermal constraint during these periods will have a greater impact on gestation duration and thus delay the date of birth. (3) Compensatory embryonic responses to maternal thermal constraints are more likely during late gestation (fetal life), once major organizational processes are completed.

Material and Methods

Study Species

The aspic viper, *Vipera aspis*, is a small, viviparous snake of the western Palaearctic region. During gestation, females have a higher preferred temperature and substantially increase basking time relative to nonreproductive females (Saint Girons 1952; Naulleau 1979; Ladyman et al. 2003; Lourdais et al. 2004).

Capture and Maintenance

Sixty reproductive female aspic vipers were collected in west-central France from neighboring populations between May 25 and June 17, 2009. Reproductive activity was evaluated in the field using manual palpation and later confirmed in the lab using ultrasonography (SonoSite MicroMaxx, Bothell, WA).

Each female was measured (± 0.5 cm), weighed (± 0.1 g), marked, and then placed in an opaque plastic box ($10.5 \times 30 \times 16.5$ cm), which was then placed in a temperature-controlled climatic chamber (Vötsch VP 600, Balingen, Germany). Females were provided a shelter and water ad lib. but were not fed until parturition, since they typically do not eat during gestation (Lourdais et al. 2002). Among the 60 females, only 38 subsequently ovulated. Females were checked daily to determine date of birth.

Experimental Design

In order to address stage-dependent embryonic thermal sensitivity, we divided development into three broad but relevant periods according to available information (Hubert and Dufaure 1968; Andrews 2004; Lourdais et al. 2004):

1. *Early development.* Major embryonic events occurring, including blastulation, gastrulation, neural-tube development, and somite development.
2. *Early embryonic growth.* Development of the optic vesicles and olfactory bulb, appearance of the jaws, cloacal slit, genitalia, and trunk/tail scales; rapid growth of the embryo with the development of spiral coiling of the body.
3. *Late embryonic growth.* Fetal life; further embryonic growth and completion of development, including development of pigmentation and differentiation of head scales.

Daily Temperature Cycles. Pregnant females display a high preferred temperature ($T_{set} = 33$°C; Ladyman et al. 2003), but environmental constraints impose substantial thermal variation within and between days. We manipulated thermal conditions during gestation by applying ecologically relevant daily temperature cycles. Two thermal cycles (fig. 1*A*) were designed with SIMPATI software (ver. 2.06, Vötsch) and applied using four climatic chambers (two for each type of thermal cycle). Relative humidity was held constant at 85% throughout the experiment. The assignment of females between the two chambers that provided the needed thermal regime was random. The two thermal cycles were as follows:

Reproductive. Chamber temperature was 33°C (pregnant female T_{set}) for 8 h and then steadily declined over the next hour to 18°C, where it was maintained for 14 h before returning to 33°C over the course of another hour. This cycle mimicked a typical daily thermal profile of free-ranging reproductive females.

Nonreproductive. This cycle was similar to the reproductive cycle, except that the upper plateau temperature was set at 27°C rather than 33°C. This cycle reflects a typical daily thermal profile of a free-ranging nonreproductive, postabsorptive female.

Temporal Application of Temperature Cycles. We designed five experimental treatment groups that differed in the timing of thermal perturbations during gestation (fig. 1*B*). In the warm treatment (W), females ($n = 6$) were exposed to the reproductive cycle during the entire gestation period. In the C1 ($n = 8$), C2 ($n = 10$), and C3 ($n = 9$) treatments, females were exposed to the nonreproductive daily cycle only during early development, early embryonic growth, or late embryonic growth, respectively. In the last experimental group, C, females ($n = 5$) were exposed to the nonreproductive cycle for the entire gestation period. Females were allocated into each treatment after accounting for snout-vent length and litter size (as determined by ultrasonography).

Identification of Embryonic Stages and Female Transfer. Since this experiment relied on specific thermal cycles applied at three different periods of development, the identification of

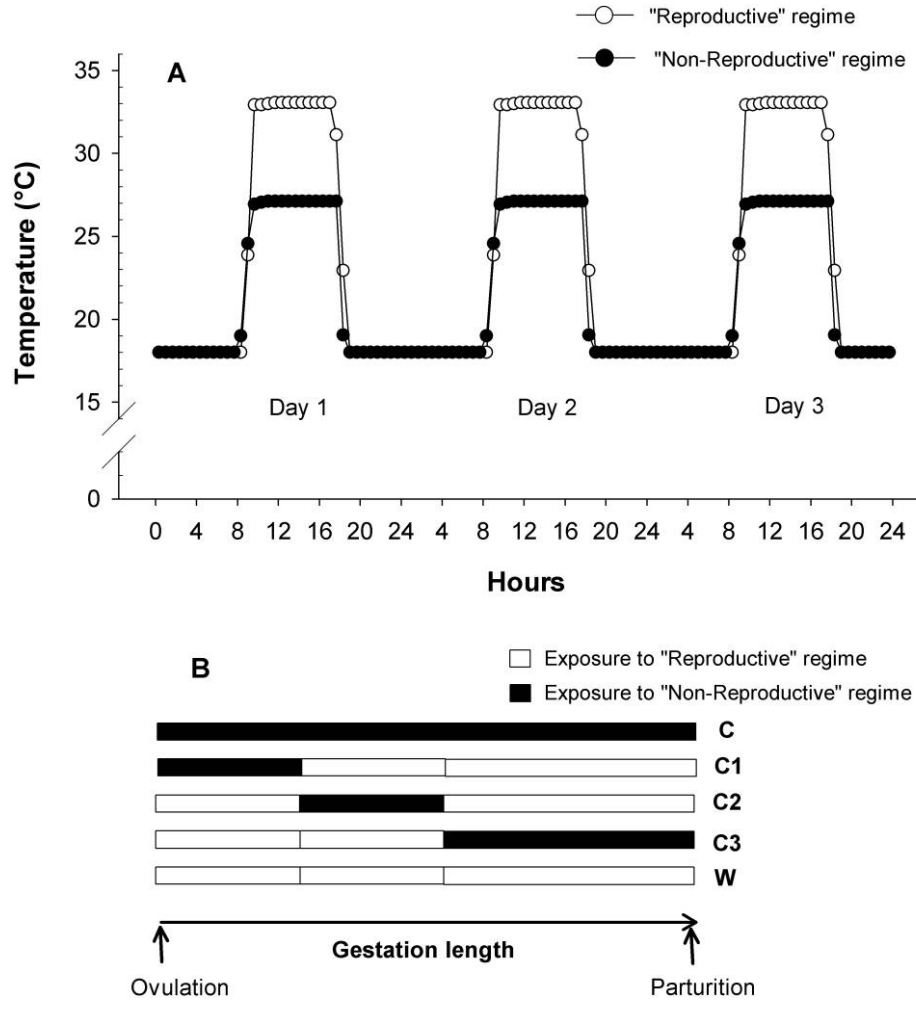

Figure 1: Experimental design used during gestation. Thermal treatments were based on two different daily temperature cycles applied at different times during gestation. *A*, In the warm daily cycle, which mimicked the thermal cycle of reproductive females in the wild (open circles), females maintained their preferred body temperature (T_{set} = 33.0°C) for 8 h, while the cool cycle mimicked the thermal regime maintained by nonreproductive females, which have a lower T_{set} (27.0°C). *B*, During gestation, the five thermal treatments varied in relative frequency and timing of the two daily thermal cycles. White and black bars represent exposure to the warm (reproductive) and cool (nonreproductive) daily temperature cycles, respectively.

embryonic stages was crucial for determining the boundaries of each period. Importantly, in aspic vipers, a preovulation ecdysis occurs and provides a reliable index for the onset of gestation (O. Lourdais, personal observation). The accuracy of this relationship was validated by ultrasonography. Ultrasonography was also used to monitor embryonic development by measuring embryos (longest diagonal line from the top of the skull), to evaluate the remaining vitellus, and to assess embryonic traits that are available in developmental tables (Hubert and Dufaure 1968) so as to estimate embryonic stages. Using these data, we determined the average stage of embryonic development when the C1, C2, and C3 treatments were transferred to the nonreproductive temperature cycle (table 1). Exposure duration to the nonreproductive cycle lasted 31, 28, and 54 days for the C1, C2, and C3 treatment groups, respectively.

Durations of cool-temperature exposure were unequal among treatments, because the relationship between embryonic stages and the proportion of development in reptiles is nonlinear (Andrews 2004). Embryonic development is a complex process in which cellular differentiation and proliferation occur simultaneously but at different rates (Gould 1977; Hall 1992). While developmental tables provide detailed embryo descriptions, no link has been made between embryonic stages, time, and temperature. Our main objective was to target relevant developmental pe-

riods; hence, we did not intend to divide gestation into periods of equivalent durations. Instead, we focused on contrasted steps in embryonic life as described above (early differentiation, early growth, and late growth). The implications of differential exposure durations are explored in "Discussion."

Variables Measured

Gestation Length and Litter Composition. Gestation length (from ovulation to parturition) was calculated for each female from the time interval (days) between shedding and parturition dates. The components of the litter were counted and weighed (± 0.1 g). Offspring and, when possible, stillborns were also measured (± 0.1 cm) and sexed. In sum, we gathered data from 37 litters composed of 108 healthy offspring, 28 stillborns, 17 dead embryos, and 29 undeveloped ova.

Offspring Quality. Neonates were housed individually in small plastic containers ($10.5 \times 30 \times 16.5$ cm) containing a polyvinyl chloride (PVC) shelter and provided water ad lib.

Size and growth. At birth, we collected snout-vent length (SVL), total length, jaw length, and body mass (BM). Body condition was derived from the residuals of log (BM) against log (SVL). These data were also collected 1 month after birth to assess growth patterns. During the first month of life, neonates were not fed and growth solely relied on energy stores at birth (Ar et al. 2004).

Abnormalities. Developmental temperature is known to affect the frequency of morphological abnormalities in neonate squamates, and such abnormalities are known to affect survival (Lindell et al. 1993; Forsman et al. 1994). Aberration of the ventral scales is a common temperature-sensitive developmental abnormality in snakes. Such aberrations have fitness implications and can be easily quantified (Shine et al. 2005; Löwenborg et al. 2010). The ventral surface of each individual was scanned (CanoScan 8800F, Canon) to determine the number of ventral scales and the proportion of individuals with split ventral scales. Also, as skin pattern provides multiple ecological signals (Wüster et al. 2004), we analyzed the total number of abnormalities in the dorsal zigzag and lateral patterns. Skin-pattern abnormalities were defined as irregularities and breaks in the general pattern. Additionally, a synthetic score of pattern irregularity was attributed to each individual as either 1 ($\leq$2 slight irregularities), 2 (3–6 irregularities), or 3 (>6 irregularities).

Defensive behavior. Since newborn snakes are particularly vulnerable and antipredator behaviors are relevant to fitness (Burger 1998), we measured defensive responses just after birth at room temperature (25° $\pm$ 1°C). Each neonate was manually restrained, allowing only the first third of its body to be free. For the duration of a 30-s trial, a predator mimic (head model with large eyes; Burger 1998) was moved in front of and made repeated contact with the snake. We categorized responses as "protective" (the snake tried to hide or escape), "none" (it expressed no obvious behavior), and "defensive" (it demonstrated strike behavior). We also recorded the number of strikes and tongue flicks.

Exploratory behavior. Neonate vipers are autonomous at birth, and individual exploratory behavior should be a good indicator of the individual's ability to find suitable microhabitats in a new environment. Within 2 days of birth, we assessed neonate exploratory behavior using a 70×70-cm square PVC arena divided into four compartments partially separated with 40-cm-high removable partitions. Two shelters were placed in each compartment. Two opposite compartments had a supplemental light and heat source (60-W bulb) 10 cm above the enclosure floor, while the other two compartments had no bulb. The light sources created a thermal gradient within the system (ranging from 37.7° $\pm$ 0.1°C under the heat source to 21.8° $\pm$ 0.1°C in the middle of the arena). Prior to a trial, each snake was acclimated in the middle of the arena under an opaque cover for 5 min. The cover was then removed, and the snake's behavior was video recorded (HDR XR100, Sony) for 15 min. We assessed time spent before initial movement, total time spent in "active exploration" (moving), in "scanning behavior" (motionless but tongue flicking and/or head moving), "passive" (motionless without tongue flicking or head moving), in "thermoregulation" (motionless under the heat source), and "hidden" (under a shelter). We also documented the number of visited compartments and tongue flicks. Between trials, the entire system was cleaned with 70% alcohol to remove residual odors.

Table 1: Characterization of embryonic stages during exposure to the "nonreproductive" thermal cycle in C1, C2, and C3 treatments

Treatment	Developmental stages during cold exposure	Embryo mean size (cm)	Estimated remaining vitellus (%)	Cold exposure (days)
C1	0 to 35–36	0–1.02	>60–70	31
C2	35–36 to 39–40	1.02–2.36	30–60	28
C3	39–40 to 43	≤2.36	<30	54

Note: Values based on *Vipera aspis* developmental tables (Hubert and Dufaure 1968) and estimations from ultrasonography.

Traction strength. Once the vipers reached 1 month of age, we used a force transducer (UFI, Morro Bay, CA) to assess offspring traction force (for methodology, see Stahlschmidt and DeNardo 2009). We conducted three consecutive 30-s trials at room temperature (25° ± 1°C). Between each trial, the snake was released for 10 s and then stretched again. For each trial, traction force was recorded every 0.1 s, and we calculated total traction effort (i.e., the sum of all recorded values).

Standard metabolic rate. We measured standard metabolic rate (SMR) 1 month after birth using closed-system respirometry (for details, see Lelièvre et al. 2010). Briefly, trials were conducted in 2-L opaque test chambers kept within a temperature-controlled environmental chamber (LMS, Sevenoaks, Kent, UK; 25° ± 1°C). Trials were conducted between 1800 hours and 0800 hours, with individuals being placed into the test chamber 2 h prior to testing and a trial duration (840 min) ensuring sufficient O_2 suppression (>0.1%).

Long-Term Effects. In the aspic viper, neonates often start hibernation without any food intake. We mimicked this situation by wintering individuals after the completion of all tests at 1 month of age. Snakes were kept at 8.0° ± 0.1°C (relative humidity 88.5%) in the climatic chambers for 3 months, and we assessed the influence of treatment on winter survival. Then, after overwintering, the chambers were warmed to 25°C. One month after the end of the wintering period (i.e., when they were 5 months of age), snakes were offered their first prey, a fresh-killed neonate mouse (mean mass = 1.6 ± 0.2 g, ~30% of the snake body mass). The prey was offered in the evening, and consumption was assessed the following morning.

Statistical Analyses

All statistical comparisons were performed with R software (ver. 2.12.1; R Development Core Team 2010) using linear models (lm models, stats package), mixed models (lme models, nlme package), and generalized linear mixed models for binomial distribution (glmer models, lme4 package). To test for the effect of thermal treatment in all glmer models, we used a model-selection procedure (since no *P* values are provided in R for glmer models) and selected the model with the smallest Akaike Information Criterion (AIC) values. When two models differed by less than 2, we chose the more parsimonious.

We analyzed the effect of thermal treatment on gestation length using a one-factor ANOVA. We addressed variation in the proportion of living neonates per litter using glmer. Due to the very low number of living neonates in the C treatment ($n = 2$), all analyses conducted on offspring traits excluded that treatment. We used linear mixed model analyses, with mother identity as a random factor, in all statistical tests that followed (Massot et al. 1994). Morphological traits at birth were analyzed, including effect of treatment, offspring sex, and appropriate linear covariates (body mass, SVL). Body condition was calculated using residual values from the regression of log (BM) on log (SVL) (Jayne and Bennett 1990). Growth (jaw length, SVL) and mass changes over the first month of life were analyzed using measurement change as the dependent variable, treatment as factor, and initial measures and initial body condition as linear covariates. Performance traits (metabolic rate, traction force) were analyzed, including effect of treatment and individual body mass. Binary variables (occurrence of split ventral scale, propensity to strike during antipredator behavior tests, feeding success, long-term survival) were analyzed using glmer models. Offspring behavior was analyzed using linear mixed models after a logit transformation of the data (Warton and Hui 2011) and treating the proportion of time spent in each behavior category as the dependent variable. All pairwise comparisons were realized using Tukey's post hoc tests (multcomp package). Significance was determined at $\alpha < 0.05$ for all tests.

Results

Gestation Duration and Litter Composition

Thermal treatment significantly influenced gestation duration ($F_{4,32} = 22.174$, $P < .001$; fig. 2). Gestation was shortest in females exposed to the warmer, reproductive female thermal cycle throughout gestation (W treatment: 82.2 ± 1.1 days; Tukey's post hoc test, W vs. all: $P < .01$) and longest in the females exposed to the cooler, nonreproductive thermal cycle (C treatment: 123.0 ± 2.8 days;

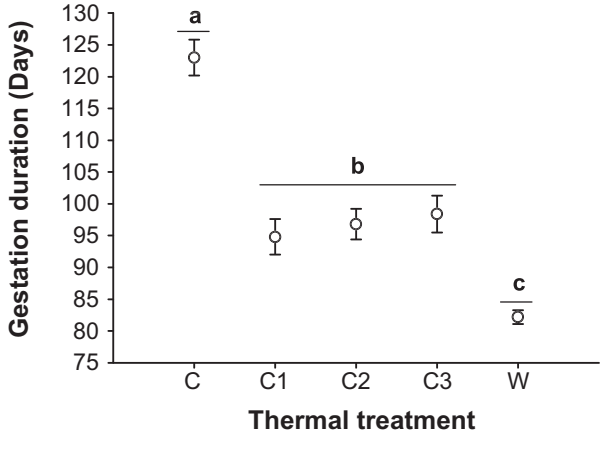

Figure 2: Impact of thermal treatment on gestation duration in *Vipera aspis*. Error bars represent SE. Symbols not connected by the same letter are significantly different.

Tukey's post hoc test, C vs. all: $P < .01$). Gestation durations in the partially constrained groups were intermediate, with no statistical difference among them: C1 (94.8 ± 2.8 days), C2 (96.8 ± 2.4 days), and C3 (98.4 ± 2.9 days) treatments (Tukey's post hoc tests: $P > .05$).

Proportion of living offspring in a litter was different among thermal treatments (glmer, $P < .05$). It was lowest for females from the C treatment (12.5% ± 8.5%; Tukey's post hoc test, C vs. all: $P < .05$), but there was no difference among the C1 (75.0% ± 7.8%), C2 (61.1% ± 8.2%), C3 (78.1% ± 7.4%), and W (94.6% ± 3.8%) treatments. Number of live neonates per treatment was 36 (W), 25 (C3), 27 (C2), 24 (C1), and 2 (C).

Offspring Quality

Morphology and Growth. SVL at birth was not influenced by sex ($F_{1,86} = 1.032$, $P = .312$) but was profoundly influenced by thermal treatment ($F_{3,28} = 3.580$, $P = .026$; fig. 3*A*), with neonates from the C1 treatment being significantly smaller than the other neonates (Tukey's post hoc tests, W vs. C1: $z = 3.229$, $P = .007$; all other pairwise comparisons: $P > .05$). Body mass at birth was not influenced by thermal treatment (table 2) or by sex ($F_{1,86} = 0.009$, $P = .925$). Body condition at birth was also not influenced by treatment (table 2) or sex ($F_{1,86} = 0.395$, $P = .532$). Relative jaw length was affected by thermal treatment ($F_{3,28} = 4.230$, $P = .014$; fig. 3*B*) but not by sex ($F_{1,80} = 0.110$, $P = .738$), with neonates from the C1 treatment having shorter jaws compared to neonates from the C3 and W treatments (Tukey's post hoc tests, W vs. C1: $z = 3.095$, $P = .011$; C3 vs. C1: $z = 3.051$, $P = .012$; all other pairwise comparisons: $P > .05$).

Growth during the initial month of life was influenced by body condition at birth ($F_{1,69} = 8.152$, $P = .006$) but not by initial SVL ($F_{1,69} = 0.734$, $P = .395$), and it was faster for neonates in the W treatment and slower for C1 neonates (table 2). Loss in body mass over the first month of life did not differ among treatments (table 2) but was influenced by body condition at birth ($F_{1,69} = 8.343$, $P = .005$) and initial body mass ($F_{1,69} = 5.751$, $P = .020$). Jaw-length growth was significantly influenced by thermal treatment (table 2), initial jaw length ($F_{1,72} = 14.878$, $P < .001$), and body condition at birth ($F_{1,72} = 10.061$, $P = .002$). Neonates from the W treatment had greater jaw growth compared to C3 treatment neonates (table 2).

Developmental Abnormalities. We found a significant effect of thermal treatment on the proportion of neonates exhibiting split ventral scales ($P < .05$; table 2) but no sex effect ($P > .05$). Split ventral scales were present in nearly all neonates from the C1 and C2 treatments, whereas hatchlings

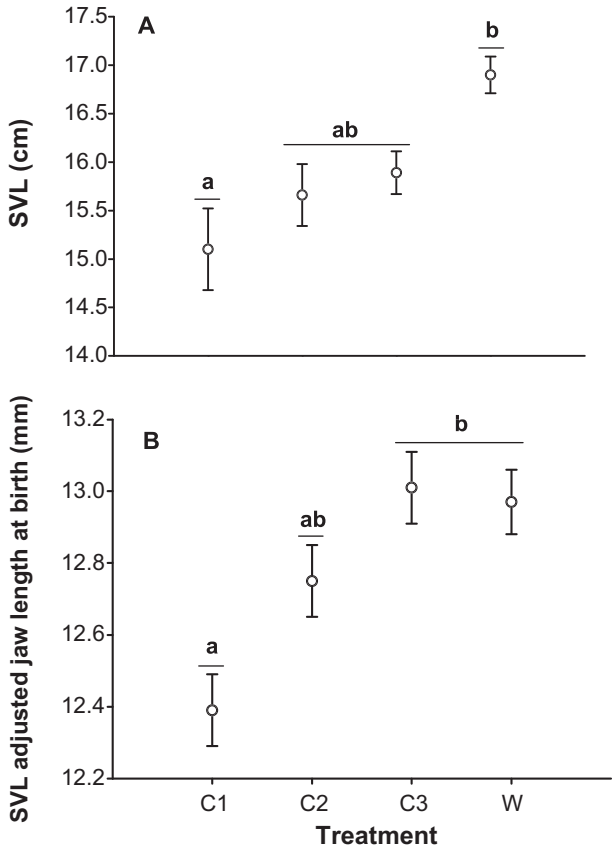

Figure 3: Impact of thermal treatment on (*A*) snout-vent length (SVL) and (*B*) SVL-adjusted jaw length at birth in neonate *Vipera aspis*. Error bars represent SE. Different letters above the symbols represent significantly different values.

from the W treatment had the lowest proportion of individuals with split ventral scales (Tukey's post hoc tests, W vs. C1: $z = -2.885$, $P = .019$; W vs. C2: $z = -3.324$, $P = .006$; all other pairwise comparisons: $P > .05$). Ventral-scale number was not influenced by thermal treatment (table 2), the mother's number of ventral scales ($F_{1,28} = 4.100$, $P = .053$), or sex ($F_{1,86} = 0.500$, $P = .485$).

Dorsal and lateral patterns were also affected by thermal treatment (table 2). The number of pattern abnormalities was highest among neonates from the C1 treatment and lowest among neonates from the C3 and W treatments. We obtained the equivalent results when considering pattern score (table 2).

Behavior. Antipredator behavior at birth was modified by thermal treatment. Neonates differed in total strike number (table 3): neonates from the C2 and W treatments were more prone to strike than neonates from the C1 treatment. The proportion of protective response and the

number of tongue flicks were not influenced by thermal treatment (table 2).

Exploratory behavior at birth was profoundly influenced by thermal treatment ($F_{3,25} = 3.051$, $P = .047$; fig. 4). The proportion of time spent in active exploration was less for neonates in the C1 treatment and greater for C3 neonates (Tukey's post hoc tests, C3 vs. C1: $z = 3.024$, $P = .013$; all other pairwise comparisons: $P > .05$; fig. 4*A*). Additionally, neonates from the C1 treatment spent more time motionless than neonates from the other treatments ($F_{3,26} = 5.003$, $P = .007$; Tukey's post hoc test, C1 vs. all: $P < .05$; fig. 4*B*). Neonates from the W and C3 treatments visited more compartments than neonates from the C1 treatment ($F_{3,25} = 3.608$, $P = .027$; Tukey's post hoc tests, W vs. C1: $z = 2.945$, $P = .017$; C3 vs. C1: $z = 2.575$, $P = .049$; all other pairwise comparisons: $P > .05$; fig. 4*C*). The number of tongue flicks was highest among neonates from the C2 and W treatments ($F_{3,25} = 3.220$, $P = .039$) and lowest for C1 neonates, which had approximately 50% fewer tongue flicks than the C2 neonates (Tukey's post hoc tests, W vs. C1: $z = 2.739$, $P = .032$; C2 vs. C1: $z = 2.639$, $P = .042$; all other pairwise comparisons: $P > .05$; fig. 4*D*).

Physiology and Performance. Oxygen consumption was influenced by thermal treatment (table 2) but also by body mass ($F_{1,65} = 5.498$, $P = .022$). With consideration for body mass differences, the standard metabolic rate was highest among neonates from the C3 treatment and lowest among those from the W treatment (table 2).

Total effort during traction trials differed among thermal treatments (table 3): neonates from the C3 treatment deployed a greater total force compared to the C1 and W neonates (table 3). Total force was also dependent on body mass ($F_{1,71} = 53.398$, $P < .001$) and decreased over trials ($F_{1,201} = 41.156$, $P < .001$).

Long-Term Impact

Overwintering Survival. Thermal treatment significantly influenced winter survival rate (glmer, $P < .05$). Survival was lowest for C1 neonates (60.9%), while there was no difference among the remaining treatments (100% for the C2 and W treatments, 96.0% for the C3 treatment).

Prey Consumption. Consumption of the first prey item offered differed significantly among groups (glmer, $P < .05$). Only 13.3% of the C1 juveniles accepted their first meal, while 69.4% of W juveniles ate. Food intake was intermediate in the C2 (54.5%) and C3 (50.0%) juveniles (Tukey's post hoc tests, W vs. C1: $z = 3.157$, $P = .008$; all other comparisons: $P > .05$).

Discussion

We examined stage-dependent developmental sensitivity by limiting thermal constraints to different stages of development—early development, early embryonic growth, and late embryonic growth (C1, C2, and C3 treatments, respectively). Our study clearly demonstrates temporal variation in embryonic sensitivity and suggests that maternal thermoregulation provides additive benefits by (1) decreasing development time and (2) avoiding developmental disruption. Interestingly, the effect on development time was similar regardless of the timing of our manipulation, while phenotypic effects were stage dependent, with the greatest sensitivity during early development. Finally, our results suggest that maternal thermal constraints may translate reliable environmental cues and induce adaptive phenotypic responses in late-stage developing offspring.

Maternal Control of Developmental Duration

Maternal thermoregulation significantly enhanced developmental rate. This phenological effect is likely adaptive in that early birth can influence hatching success and postbirth survival (Olsson and Shine 1998; Warner and Shine 2007). However, contrary to our second prediction and as documented in a study on lizards (Wapstra et al. 2010), the benefits of maternal regulation over development were continuous rather than stage dependent, despite the thermally constraining treatment being much longer in our C2 and C3 treatment groups compared to the C1 group.

Maternal Influence on Offspring Phenotype

The C treatment accounted for 25% of all undeveloped ova and, more importantly, 62.5% of all stillborn neonates. This high incidence of embryonic death, combined with the numerous morphological abnormalities seen in this group—smaller SVL (13.1 ± 0.5 cm) and 100% of the C neonates having split ventral scales (mean = 108.1 ± 36.9 split ventral scales per individual)—demonstrates the adaptive value of maternal thermophily during development (Webb et al. 2006; Rodríguez-Díaz et al. 2010). In addition, thermal influences on offspring quality were extremely dependent on the timing of exposure. Offspring exposed to the cool temperature cycle during early embryonic development and, to a lesser extent, during early embryonic growth exhibited pronounced phenotypic and behavioral alterations, suggesting an impact on coordinated expression of developmental genes (Forsman et al. 1994; Shine et al. 2005; Löwenborg et al. 2010).

Conversely, no such alterations were detected when treatment occurred during fetal life (C3). In fact, we de-

Table 2: Effects of the timing of thermal perturbations during gestation on morphological traits at birth, growth patterns, and metabolic rate in the viviparous snake *Vipera aspis*

Trait	Gestation treatment				Statistical test: effects of thermal treatment[a]	Tukey's post hoc tests[b]			
	C1	C2	C3	W		C1	C2	C3	W
Body mass (g)	4.88 ± .30	4.88 ± .30	5.34 ± .19	5.28 ± .13	NS	...	...	...	...
Body condition	.026 ± .023	−.013 ± .015	.029 ± .010	−.027 ± .009	NS	...	...	...	...
% individuals with split ventral scales	96.0 ± 4.0	93.3 ± 4.6	60.7 ± 9.4	44.4 ± 8.4	S	A	A	AB	B
Number of split ventral scales	145.8 ± 1.3	147.4 ± .7	145.5 ± .8	147.4 ± .8	NS	...	...	.....	...
Number of dorsal-pattern abnormalities	6.8 ± .5	5.0 ± .6	3.3 ± .5	3.5 ± .4	$F_{3,24} = 7.531$, $P = .001$	A	A	AB	B
Dorsal-pattern score	2.9 ± .2	2.3 ± .2	1.9 ± .2	1.7 ± .2	$F_{3,24} = 4.816$, $P = .009$	A	AB	B	B
Snout-vent length growth (mm)	.70 ± .12	1.09 ± .08	1.14 ± .11	1.16 ± .06	$F_{3,25} = 2.810$, $P = .060$	A	AB	B	B
Body mass (BM) change (g)	−.63 ± .10	−.49 ± .04	−.51 ± .05	−.40 ± .03	NS	...	...	...	...
Adjusted jaw growth (mm)	.40 ± .06	.52 ± .05	.34 ± .05	.62 ± .05	$F_{3,25} = 4.191$, $P = .016$	AB	AB	B	A
BM-adjusted O_2 consumption (mL h^{-1})	.27 ± .14	.26 ± .14	.37 ± .14	.13 ± .14	$F_{3,24} = 3.073$, $P = .047$	AB	AB	B	A

Note: Table shows mean values ± SE.

[a] S: significant. NS: not significant. No *P* values are available for glmer models in R. The effect of thermal treatment was determined using model selection on Akaike Information Criterion.

[b] Significantly different values are represented by different letters.

Table 3: Effects of the timing of thermal perturbations during gestation on behavioral traits at birth in the viviparous snake *Vipera aspis*

Trait	Gestation treatment				Statistical test: effects of thermal treatment[a]	Tukey's post hoc tests[b]			
	C1	C2	C3	W		C1	C2	C3	W
Antipredator behavior at birth:									
Proportion of strikers	.54 ± .10	.73 ± .10	.80 ± .08	.94 ± .04	NS	...	...	...	...
Number of strikes	2.9 ± .6	7.3 ± .9	5.4 ± .7	5.9 ± .7	$F_{3,24} = 3.754$, $P = .024$	A	B	AB	B
Proportion of protective response	.46 ± .10	.32 ± .10	.32 ± .19	.40 ± .08	NS	...	...	...	...
Number of tongue flicks	9.9 ± 1.5	9.0 ± 1.0	11.5 ± .8	12.1 ± .9	NS	...	...	...	...
Exploratory behavior at birth:									
Time to initiate the first movement (s)	117.5 ± 55.9	36.7 ± 22.6	11.8 ± 2.5	11.3 ± 2.5	NS	...	...	...	...
% time scanning	21.4 ± 4.1	16.6 ± 4.1	7.9 ± 1.3	18.6 ± 2.2	NS	...	...	...	...
% time under shelter	17.3 ± 4.9	22.2 ± 6.0	20.3 ± 3.3	20.2 ± 3.8	NS	...	...	...	...
Number of different visited shelters	.9 ± .1	2.1 ± .4	1.9 ± .2	2.2 ± .3	$F_{3,25} = 3.075$, $P = .045$	A	AB	AB	B
Contraction force:									
Total contraction force (g)	5,557.1 ± 617.4	7,448.9 ± 695.4	8,836 ± 644.5	6,039.0 ± 361.0	$F_{3,25} = 3.819$, $P = .022$	A	AB	B	A

Note: Table shows mean values ± SE.

[a] NS: not significant.

[b] Significantly different values are represented by different letters.

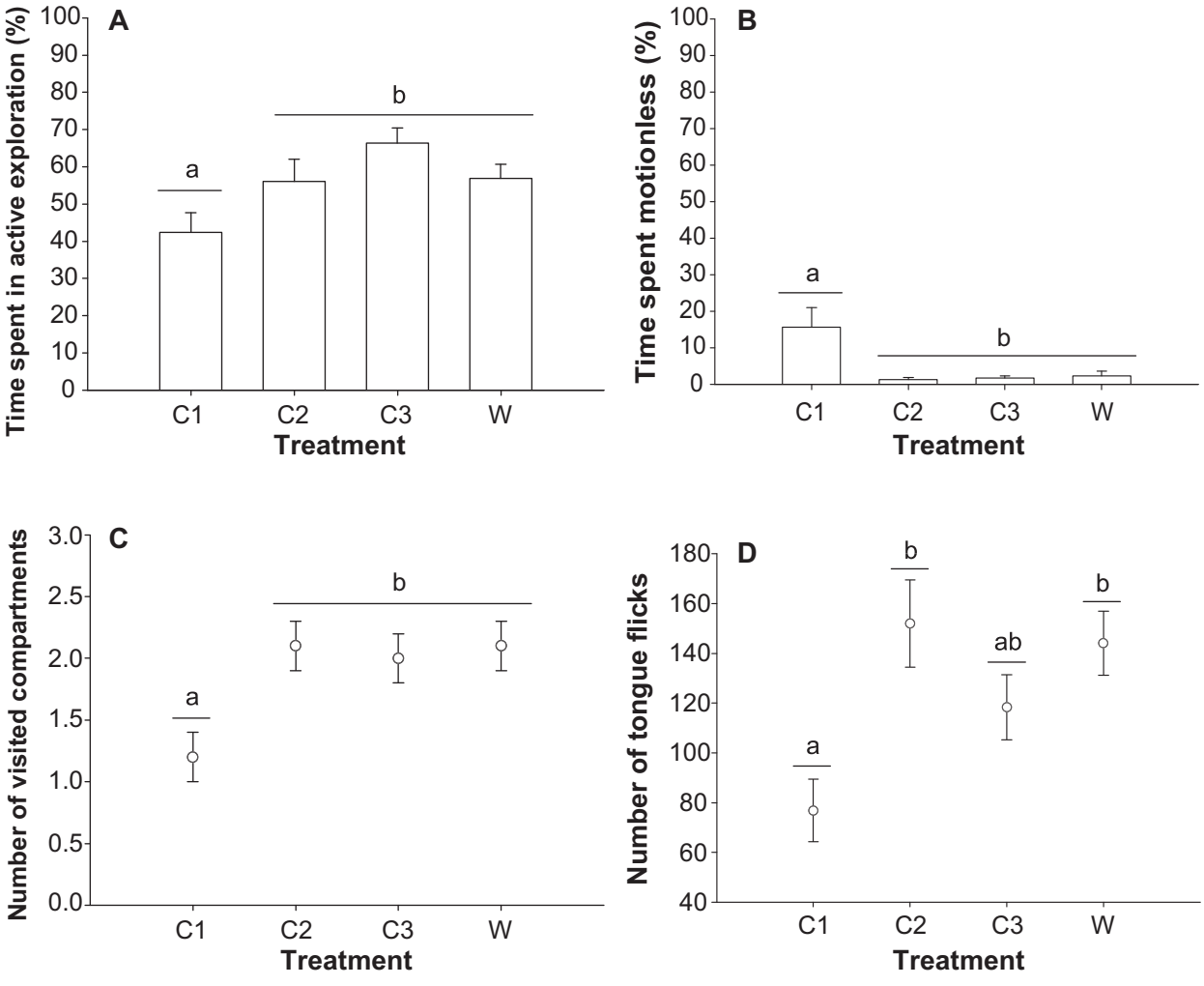

Figure 4: Impact of thermal treatment on exploratory behaviors in neonate *Vipera aspis*: time spent in active exploration (*A*), time spent motionless (*B*), number of visited compartments (*C*), and number of tongue flicks (*D*). Error bars represent SE. Different letters above the bars and symbols represent significantly different value.

tected evidence for compensatory fetal responses to maternal temperature. Neonates impacted late in development had the highest SMR and greatest traction strength among all groups. Since SMR was measured 1 month after birth, these differences do not represent short-term reversible embryonic acclimation (Du and Shine 2010). Low maternal temperatures in *Vipera aspis* typically reflect thermoregulatory constraints associated with weather conditions (Angilletta et al. 2005) and thus may transfer relevant information to the fetus, leading to a better phenotypic match to the postnatal environment (stage-dependent effect). Finally, intermediate stages of embryonic development (C2) appear sensitive to deviations from optimal temperatures, notably in terms of general organization (morphological features), but C2 neonates did not exhibit physiological or behavioral alterations.

Evolutionary Implications

Oviparous squamates universally retain their eggs for at least the first 25%–33% of embryonic development and routinely oviposit them in the limb-bud stages (Shine 1983; Blackburn 1995). This duration of egg retention includes the cool-temperature-exposure period of our C1 treatment. Thus, even oviparous squamates use maternal thermal regulation to buffer offspring during their most sensitive stage of development. Considering the limited thermal sensitivity of later developmental stages, benefits to offspring quality associated with extending maternal thermophily throughout development may be offset by the high costs inferred by viviparity (e.g., increased physical burden, reduced foraging opportunity). In contrast, given the stage-independent thermal sensitivity of developmen-

tal rate, viviparity, with its extended maternal thermoregulation, may greatly hasten development, leading to an earlier date of birth and associated benefits (Tinkle and Gibbons 1977; Olsson and Shine 1998; Wapstra et al. 2010). Since the phenological effects are not stage dependent and thus are additive across gestation, phenology may provide a better explanation for complete embryonic retention (i.e., viviparity) than offspring-quality benefits.

Acknowledgments

We thank G. Guiller and P. Quistinic, who helped in snake capture, and all students who helped with animal maintenance. Financial support was provided by the Agence Nationale de la Recherche (ECTOCLIM project), the Fyssen Foundation, the Programme opérationnel pluriregional Loire FEDER (#PRESAGE 30810), and the Etablissement Public Loire.

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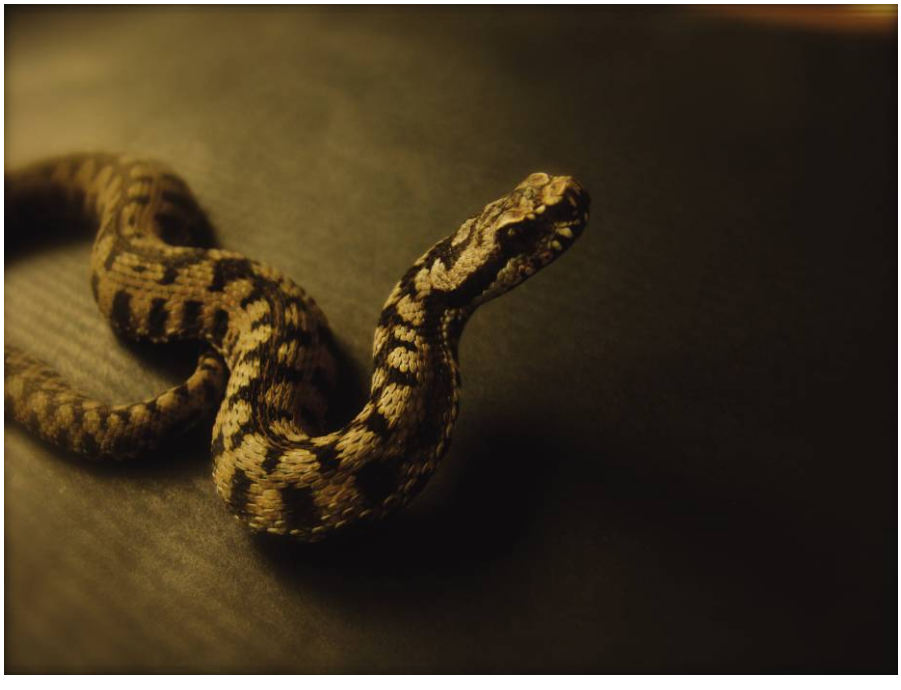
Associate Editor: Carlos Martínez del Rio
Editor: Judith L. Bronstein



Aspic viper (*Vipera aspis*) neonate. Photograph by Sophie Lorioux.