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Impact of cool *versus* warm temperatures on gestation in the aspic viper (*Vipera aspis*)

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ABSTRACT

Previous experimental data suggested that digestion and growth rates are not impaired under cool constant temperature (23 °C) in a viviparous snake (*Vipera aspis*). These results challenged the widespread notion that both elevated temperatures (e.g. 30 °C) and temperature fluctuations are required for digestion and growth in temperate climate reptiles. Here, we investigated the impact of constant cool temperatures on another physiological performance that is crucial to population persistence: gestation. At the time when reproductive females were midway through vitellogenesis, we placed ten reproductive and two non-reproductive female aspic vipers at each of two contrasted constant temperature conditions: cool (23 °C) *versus* warm (28 °C). Sixty percent of the females placed at 28 °C gave birth to healthy offspring, suggesting that constant warm body temperatures were compatible with normal offspring production. Conversely, none of the cool females gave birth to healthy offspring. A blister disease affected exclusively cool pregnant females. Apparently, the combination of cool temperatures plus gestation was too challenging for such females. Our results suggest that reproduction is more thermally sensitive than digestion or growth, indeed gestation faltered under moderately cool thermal constraints. This sensitivity could be a crucial factor determining the capacity of this species to colonize different habitats.

1. Introduction

In most reptiles, elevated and stable body temperatures are required to optimize demanding physiological functions, notably digestion, growth and reproduction (Huey and Bennett, 1987; Luiselli and Akani, 2002; Seebacher and Franklin, 2005). The ability exhibited by many species to maintain their body temperature into a narrow range of variations despite strong seasonal or daily temperature fluctuations promoted the widely accepted concept of preferred body temperature: PBT. Initially proposed in insects (Gunn, 1931) the PBT notion represents a paradigm in reptile ecology (Reynolds and Casterlin, 1979; Blouin-Demers and Weatherhead, 2001). As all functions cannot be optimized at a single temperature, access to environmental thermal gradient, and thus to different PBTs, is essential in free-ranging and captive individuals (Regal, 1967; Saint, 1982; Murphy and Campbell, 1987; Peterson et al., 1993; Blouin-Demers et al., 2000; Shine, 2005; Angilletta et al., 2006).

However, most studies of different thermal sensitivities of performances addressed acute functions (e.g., sprint speed, strike speed) rather than more chronic performances (e.g., digestion, growth, reproduction). A recent experiment (Michel and Bonnet, 2010) showed that digestive and growth performances were not different between vipers maintained under cool (23 °C) constant temperatures compared to vipers placed in a presumably more favorable thermal regime with access to elevated temperatures (28 °C). Moreover, using different thermally fluctuating regimes but similar average temperatures, this study failed to reveal any effect of temperature fluctuations. A sister study performed on newborn vipers showed that survival after 7 months was not compromised using cool constant temperatures (23 °C). Young vipers developed normally, albeit slowly, when exposed to cool constant conditions compared to warmer temperatures (Aïdam et al., 2013). Overall, these results do not fit well with the notion that temperature fluctuations and access to high PBT are essential in snakes from temperate climates.

In the current study, we addressed another physiological performance that is critical to species persistence: reproduction. Indeed, the two studies mentioned above were performed in captivity and focused on non-reproductive individuals. Therefore, the specific physiological constraints of female reproduction and associated costs (e.g. energy expenditure, survival) were overlooked. We exposed recently mated adult females to the same temperature regimes that were previously used on non-reproductive individuals (Michel and Bonnet, 2010), and we monitored the snakes until parturition.

In oviparous and viviparous reptiles, stable and elevated environmental conditions (temperature, humidity) are associated with optimal embryonic developmental trajectories (Fox et al., 1961; Vinegar, 1974; Lourdais et al., 2004; Aubret et al., 2005; Shine, 2005; Delmas et al., 2008; Lorioux et al., 2012). In this respect, viviparity offers important advantages because mothers can buffer environmental

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fluctuations through careful behavioral thermoregulation (Shine, 1983, 2004, 2005). However, in snakes viviparity also entails drastic physiological and behavioral costs during vitellogenesis, gestation, parturition, and during the post-parturition recovery phase (Birchard et al., 1984; Bonnet et al., 1999; Lourdais et al., 2002; Van Dyke and Beaupre, 2011). Overall, we expected that the impact of contrasted thermal regimes would be particularly marked in reproductive vipers. We emphasize that the different thermal regimes we employed (23 °C *versus* 28 °C) were not extreme and that they were well tolerated by non-reproductive individuals from both sexes and all age classes (Michel and Bonnet, 2010: Aïdam et al., 2013). Our objective was not to expose reproductive females to extremely challenging thermal conditions (i.e., under 15 °C digestion is impeded; Naulleau, 1983), but rather to examine the influence of stable albeit different (cool *versus* warm) thermal conditions on reproduction.

2. Material and methods

2.1. Studied species

The aspic viper (*Vipera aspis*) is a viviparous snake of the western Palearctic region. Mean adult body size and mean body mass are 55 cm (snout-vent length, SVL) and 100 g respectively. During the activity season (early March to October) free-ranging individuals experience marked fluctuations of ambient temperatures (0 °C - 45 °C) and exhibit important variations of body temperature (Naulleau, 1997). The females used in the current experiment originate from a single site (Forest of Chizé, 46°09 N, 0°24 W, 80 m elevation, France). Mating takes place during the first part of vitellogenesis, soon after female emergence in March–April (Vacher-Vallas et al., 1999; Aubret et al., 2002). Vitellogenesis extends until mid-June (Bonnet et al., 1994), ovulation occurs during the first two weeks of June (Naulleau, 1981), and parturitions are observed two to three months later (Bonnet et al., 2000). Overall, the production of offspring involves vitellogenesis plus pregnancy, and thus massive physiological and behavioral maternal investments during approximately 6 months, resulting in the depletion of maternal body reserves (Bonnet et al., 1994, 2001, 2002). Due to high energy costs of reproduction, most females reproduce every two years on average (Naulleau and Bonnet, 1996). During pregnancy, field and captivity data revealed that females select a PBT close to 33 °C (Naulleau, 1997); a temperature associated with optimal offspring development (Lorioux et al., 2013).

In the current study, reproductive and non-reproductive adult females were placed under two experimental thermal regimes, cool *versus* warm, from mid-vitellogenesis until parturitions (~12 weeks). The snakes were kept under standard conditions two years before the beginning of the experiment. Each individual was maintained into a transparent plastic boxes 42 cm * 34 cm * 19 cm (L*w*h), with artificial grass substratum, a shelter (concrete tile), and a water dish. A favorable thermal gradient was provided using heating cables (20 °C–38 °C during the day and 18 °C - 20 °C at night). The boxes were placed in a rack situated in an air-temperature-controlled room. The snakes were weighed every week and their body size (snout vent length, SVL) measured once a month. The snakes adapted well to captivity as indicated by their regular increase in size and mass.

2.2. Experimental design

2.2.1. Climatic chambers and thermal regimes

We used two climatic chambers (Michel and Bonnet, 2010) to control temperature and humidity independently (Voetsch ©, Pharmaclim 500 l, glass door, internal dimensions 58.5 cm * 65.5 cm * 133.5 cm, temperature stability 0.5 °C, relative humidity stability 3%). In each chamber we placed twelve snakes (N = 24 snakes) kept into an individual transparent plastic box (40 * 34 * 14 cm, fitted with an artificial grass substratum, a water dish, and a shelter). To randomize the general conditions experienced by the animals, every week we randomly displaced the snakes between the different shelves (N = 4) of each climatic chamber.

One climatic chamber was set to a constant temperature of 23 °C (cool regime), the other was set to 28 °C (warm regime). Humidity was maintained at 65% RH in both chambers. We used a temperature of 28 °C for the warm group, a value lower than the actual PBT of pregnant females (33 °C) as preliminary tests showed that the vipers tended to be over active (e.g. attempting to escape from their box) when ambient temperature was above 30 °C. Using a temperature of 28 °C, the vipers remained calm and spent most of their time retreated under their shelter (a normal behavior in this species).

2.2.2. Hibernation and mating prior thermal experimentation

Prior to experiment the 24 females were prepared as follow. They were individually hibernated during three months at 6 °C, from early November to early February. Following emergence, the snakes were weighed and measured. Then the females were placed with 15 adult males (also hibernated to stimulate mating) during 31 days in a large 4 m * 5 m * 1 m indoor enclosure, each female was observed copulating at least once. The 24 females were then returned into their standard individual box and they were monitored for three weeks. They were palpated every week to detect possible growing follicles (easily detected by abdominal palpation in this species). Among the twenty-four females, twenty became vitellogenic but in four females palpation failed to detect any growing follicle. Then, around mid-vitellogenesis, 12 females were randomly allocated to each climatic chamber (i.e. 10 pregnant plus 2 non-pregnant per thermal regime).

2.2.3. Data collection

Females were measured (SVL, ±0.5 cm) at the beginning and at the end of the experiment. The vipers were weighed (to the nearest 0.1 g) and palpated every week (the boxes were cleaned at the same time). We offered prey (dead mice, ~25% of snake mass) every two weeks. Cool snakes tend to refuse their meal compared to warm snakes. To avoid such undesired effect we first proposed prey to the cool females and randomly adjusted prey delivery to the warm females. Thus the same number of females in each group was fed, based on the number of cool females that ate. The boxes were carefully inspected every day. Parturition date was noted, the litter was carefully inspected, neonates and undeveloped eggs were counted and measured. The use of an artificial plastic grass substratum facilitated the detection and examination of small reproductive output items.

2.3. Analyses

Means are presented with standard error unless stated. Body condition was calculated as the residuals from the regression between body mass (log) against body size (log). We note that in vitellogenic or pregnant females, body mass also includes the (non-maternal) mass of the follicles or embryos. Timing of ovulation crudely assessed by palpation conformed to the information obtained using radiography (Naulleau, 1981). Snakes with a recently ingested prey were discarded from calculations. No departure from normally could be detected (not significant Shapiro–Wilk tests), so we used ANOVA in most comparisons. To analyze the influence of the temperature regimes on changes in body mass and body condition we used ANOVA for repeated measured (homoscedasticity assumption verified). All statistics were performed with Statistica 7.1 (StatSoft, Tulsa, OK, USA).

3. Results

Mean initial body size and body mass of the females were 48.5 ± 4.8 cm (range: 48.5–66.5 cm) and 200.8 ± 66.3 g (103.6–314.3 g), respectively. At the beginning of the experiment there was no difference between the two groups of snakes (ANOVA with thermal

regime as a factor, female body size, body mass and body condition as the dependant variables: $F_{1,\ 22} = 1.001$, $p = 0.328$, $F_{1,\ 22} = 0.011$, $p = 0.916$ and $F_{1,\ 22} = 0.032$, $p = 0.859$ respectively).

On average, females gained 4.8 ± 15.4 g in the course of the experiment and increased in size by 1.5 ± 1.3 cm. We found no difference between warm *versus* cool females regarding changes in body mass over time (ANOVA with repeated measures of mass as the dependant variable and thermal regimes as a factor: Wilk's lambda = 0.512; $F_{11,\ 12} = 1.039$, $p = 0.47$; effect of time $F_{11,\ 12} = 16.995$, $p < 0.001$) or changes in body size (ANOVA with repeated measures of size as the dependent variable and thermal regimes as a factor: $F_{1,\ 22} = 0.023$, $p = 0.881$; effect of time $F_{1,\ 22} = 31.46$, $p < 0.001$).

3.1. Fecundity one month after the onset of experiment

At the onset of the thermal treatment, around mid-vitellogenesis, palpations enabled us to count 4.5 ± 2.4 (range: 3–12) enlarged follicles (>2 cm in diameter) in the cool females (23 °C) and 5.6 ± 3.1 (6–13) in the warm females. These two values were not significantly different (one-way ANOVA with thermal regime as a factor and follicle number as the dependant variables: $F_{1,\ 22} = 0.923$ $p = 0.347$), suggesting an absence of divergence in the number of follicles recruited during vitellogenesis between the two groups of females prior to thermal treatment (Fig. 1).

3.2. Late fecundity and reproductive output

At the end of the experiment, the warm females gave birth to thirty-six offspring. The mean litter size was 5.2 ± 3.7 (1–11), the mean offspring mass was 6.6 ± 1.8 g (2.7 – 11.0 g), and the mean offspring SVL was 16.7 ± 1.7 cm (12.5 – 21.0 cm). The sex ratio was balanced (17 males and 19 females; $\chi^2 = 0.06$, $p = 0.84$). The cool females did not produce any healthy offspring: one female voided four stillborn, the other females (N = 4) expelled not-well developed items often impossible to count precisely (inform yolk mass with poorly developed embryos embedded), and the rest of the cool females (N = 5), although positively palpated several weeks before (at the onset of the experiment and then again one month later during early gestation), did not produce any "reproductive item", further palpation failed to detect remaining item into their oviducts (Fig. 1). The warm females gave birth 55 (±6 days) days after the beginning of the thermal treatment; the cool females voided undeveloped reproductive items 18 days later (±10 days).

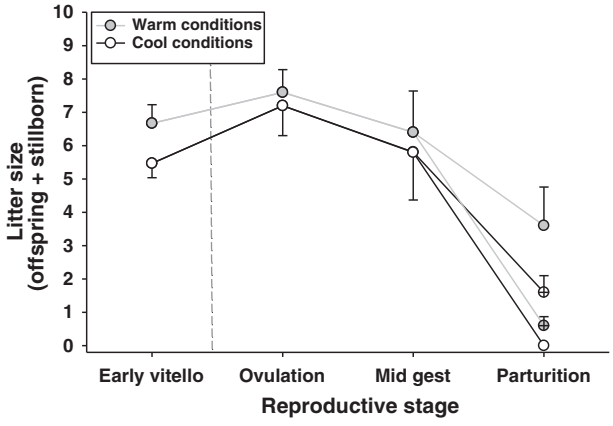

Fig. 1. Change of fecundity (e.g. number of reproductive items, follicles, embryos) over time recorded in females exposed to warm (grey circles, N = 10 females) *versus* cool conditions (open circles, N = 10 females). The vertical dashed grey line indicates the beginning of the thermal treatment. The main reproductive stages are identified as follow: early vitellogenesis (early vitello), near the time of ovulation (ovulation), mid-gestation (mid gest) and parturition. At parturition the number of reproductive items was split into viable offspring (open circles) and non-viable items (stillborns, undeveloped eggs) (crossed symbols). Means are expressed ±1SE. During early vitellogenesis, several small follicles were not easily detected leading to possible underestimation of early fecundity. Fecundity was accurately estimated via palpation at ovulation ("well developed follicles" were easily counted). During mid-gestation, non-developing ova (i.e. "undeveloped-eggs") were not easily counted. The difference between fecundity at ovulation and at parturition was due to the disappearance of "undeveloped-eggs" during gestation (see Bonnet et al., 2008 for details).

3.3. Skin disease

One month after the onset of the experiment, approximately during mid-gestation, we observed skin troubles exclusively in all cool pregnant females. The tip of the scales was slightly turned-up; the skin was shiny and moist in appearance. We took scale samples and sent them to a vet laboratory. Bacterial analyses revealed the presence of abundant *Escherichia hermannii*, *Enterococcus faecalis*, and *Staphylococcus epidermidis*. The cool vipers suffered from the "blister disease", a type of skin infection that occurs regularly on reptiles maintained in captivity under exaggeratedly humid and cool conditions (Brogard, 1992). We successfully treated the vipers with three subcutaneous injections of antibiotic (100 μL of Marbocyl, 2% = 20 mg/mL). None of the warm females and none of the cool non-pregnant females exhibited any sign of the blister disease, despite the fact that all the females were in contact to the bacteria involved in the blister disease (the snakes were not maintained in isolation in a sterile environment, instead the boxes were randomly displaced into the climatic chambers, the devices [caliper, electronic scale…] were not sterilized).

4. Discussion

Our results suggest that reproduction is more sensitive to thermal conditions compared to other functions such as food intake, digestion, or growth. Indeed, gestation faltered under moderately cool ambient temperatures whereas digestion and growth were not affected (Michel and Bonnet, 2010). Therefore, reproduction could be a crucial factor determining the capacity of this species to colonize different habitats; the specific thermal-sensitivity of gestation may well be a key climatic factor underlying geographical distribution of viviparous reptiles.

In the current experiment, non-pregnant females (either placed in cool or warm thermal regimes) did not exhibit any sign of disorder, reinforcing the notion that the vipers accustomed well to the experimental conditions. Our results also showed that females were able to produce healthy offspring (see Aïdam et al., 2013 for details) at a constant warm temperature of 28 °C in the absence of fluctuations and without possibility to reach their PBT (~33 °C). These results contrast to the widely admitted notion that high PBT and thermal fluctuations are essential for temperate climate reptiles (Fleury and Naulleau, 1987; Murphy and Campbell, 1987; Peterson et al., 1993).

Conversely, gestation under constant cool ambient temperature (23 °C) ended in reproductive failure. During vitellogenesis and early pregnancy we did not detect any trouble. Later however, repeated palpations indicated that the number of large follicles decreased and progressively softened to the touch (instead of hardening normally). Eventually, no cool female gave birth to any viable offspring. All the stillborn were malformed and embedded into abundant partly solidified yolk. Some stillborn exhibited anencephaly and/or showed an absence of closure of the neural tube. Other stillborn were very small, suggesting that their development stopped at an early stage. In the other cool females, late palpation failed to detect reproductive items, later we did not observe any undeveloped egg or stillborn in their box despite regular checking and cleaning. Such total disappearance of eggs during gestation (a process different from follicular atresia) has already been documented in this species using repeated sessions of magnetic resonance and ultrasound doppler-imaging; yet the underlying mechanisms remain unclear (Bonnet et al., 2008).

Approximately at mid gestation, the cool pregnant females suffered from blister disease. The bacteria involved (*Escherichia hermannii,*

Enterococcus faecalis, and *Staphylococcus epidermidi*) are usually found in the environment and they naturally occur on the skin of healthy snakes. These bacteria can provoke a disease when animals are kept in inappropriate captivity conditions (lack of cleaning, insufficient ventilation, etc.) and on individuals in poor conditions; none of these conditions applied to our study. As such disease occurred around mid pregnancy, when the embryos were not fully developed, we don't know the exact cause for the reproductive failure: disease *versus* cool temperature *per se*, or a combination of both factors. Perhaps that cool temperature of 23 °C is incompatible with correct embryo development. Alternatively, the physiological costs of pregnancy associated to low temperatures may have decreased immunity favoring skin disease, that later caused reproductive failure. Whatever the causality, the result was an absence of reproduction, whilst 23 °C does not prevent non-reproductive functions (e.g. digestion, growth) in the aspic viper (Michel and Bonnet, 2010; Aïdam et al., 2013).

Overall, our data suggest that pregnant females placed under cool temperatures were not able to simultaneously sustain reproductive effort and to maintain high immunity level. Physiological costs of immune function can be elevated and can negatively affect reproduction (Lochmiller and Deerenberg, 2000; Adamo et al., 2001; Martin et al., 2008). Experimental manipulation of reproductive effort in an oviparous lizard revealed physiological trade-offs between reproduction and immunity; notably under strong energetic constraints such as low food availability (French et al., 2007a,b). Further, immune defenses are degraded if individual body temperature remains low (Zapata et al., 1992; Mondal and Rai, 2001; Merchant and Britton, 2006; Zimmerman et al., 2009). Incidentally, the pregnant aspic vipers involved in the current experiment that failed to produce healthy offspring were subjected to both strong energetic constraints and low ambient temperatures.

This does not mean that the warm females were spared from costs of reproduction (Ladyman et al., 2003); however warm temperatures likely promoted correct embryonic development and adequate immunity. Overall, our results clearly suggest that successful reproduction requires higher temperatures compared to digestion for instance. Further experiments are required to explore this notion and to better understand species distribution, especially in the context of climatic changes.

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