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An experimental study of the gestation costs in a viviparous lizard: a hormonal manipulation

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SUMMARY

The trade-offs between reproduction and survival or future reproduction represent the costs of reproduction, which are central to the theory of life history traits evolution. In particular, different stages of the reproductive cycle may be associated with different costs and thus explain the evolution of alternative reproductive strategies. Viviparity (live-bearing) has evolved from oviparity (egg-laying) several times independently in vertebrates. To better understand these transitions, we aimed to specifically investigate gestation costs in a squamate reptile with a new experimental procedure. We reduced litter size during gestation in the common lizard (*Zootoca vivipara*) with a hormonal injection of arginin vasotocin. This method is less invasive than a surgical method and does not reduce the number of offspring of future reproductive events. We monitored body mass change, immune response, endurance capacity, thermoregulatory behavior, offspring characteristic at birth, female and offspring survival, female body mass gain after parturition, and offspring growth rate after birth. Maternal treatment did not significantly change the offspring characteristics measured. Thus, litter size reduction did not change offspring development during gestation. For the females, there is evidence that endurance capacity during gestation is modified because of the physical burden of the litter and because of physiological changes. With respect to gestation costs, we did not observe a trade-off between the investment during gestation and females' resources postparturition (female body mass) or survival, but there was a facultative trade-off with the immune response. It will be interesting to replicate this study to increase the robustness of these results and to confirm the effects on the endurance capacity and the immune response. Gestation costs seem to be limited in this species and they should be studied in more detail to evaluate their influence on the evolution of viviparity.

INTRODUCTION

Costs of reproduction are the trade-offs that exist between reproductive investment and survival and/or future reproduction (Stearns 1989; Roff 2002). A simple energetic link may explain such costs (Roff 2002): females that used more energy have fewer resources to invest in the following reproductive event. Moreover, carrying eggs may constitute a physical burden and directly reduce females' locomotor abilities and thus survival (e.g. Shaffer & Formanowicz 1996; Lee *et al.* 1996; Veasey, Houston & Metcalfe 2000; Miles, Sinervo & Frankino 2000). However, indirect effects may also exist, and the physiological basis of reproductive costs are increasingly studied (Zera & Harshman 2001; Harshman & Zera 2007). Indeed, some functions may be downregulated as a consequence of a high reproductive investment which may affect female survival or future reproduction. Major functions that can be downregulated are the immune system (e.g. Hanssen *et al.* 2005; French, DeNardo & Moore 2007; Cox *et al.* 2010), the oxidative defence (e.g. Dowling & Simmons 2009; Garratt *et al.* 2011) and growth (e.g. Berglund & Rosenqvist 1986; Landwer 1994; Cox 2006). Most experimental studies on reproductive costs are based on phenotypic engineering (Sinervo & Huey 1990; Sinervo *et al.* 1992) and more precisely on clutch/litter size manipulation. The strength of these studies is that the experimental approach allows causal conclusions to be drawn as opposed to correlations.

Different stages of the reproductive cycle may be associated with different costs, and thus the timing of litter size manipulation is important. For example, in birds clutch size is usually manipulated after laying (to study the costs of chick rearing), however egg production and incubation also incur fitness costs (Reid, Monaghan & Ruxton 2000; Visser & Lessells 2001). In mammals, lactation is more costly than gestation (e.g. Clutton-Brock, Albon & Guinness 1989; Michener 1989; Dufour & Sauter 2002). Disentangling the relative importance of each

stage may be important to assess the costs and benefits of different reproductive strategies. Viviparity (live bearing) has evolved from oviparity (egg laying) several times independently in vertebrates (Blackburn 1999a; b). In squamates (lizards and snakes) in particular, viviparity has evolved more than 100 times (Blackburn 1999a). To better understand these transitions we aimed to specifically investigate gestation costs in a squamate reptile. The main advantage of squamates is that, in most species, most nutrients for embryonic development are provided in the egg yolk (lecithotrophic viviparity (Blackburn 1999a)). This means that the period of egg formation (i.e. vitellogenesis) can be costly for females as in oviparous species and that few nutrients are transferred during gestation. The energy needed during gestation thus represents the costs to maintain and carry the developing embryos (gestation costs). In other words, the energetic cost in terms of egg formation (vitellogenesis cost) and the costs of gestation can be decoupled.

Clutch or litter size can be manipulated before egg laying by a surgical or hormonal manipulation. The removal of follicles (yolkectomy) (e.g. Sinervo & Licht 1991) or ovaries (e.g. Cox 2006; Cox *et al.* 2010) decreases or even suppresses the reproductive effort. An injection of the follicle stimulating hormone (FSH) increases the number of follicles and thus the reproductive effort (e.g. Sinervo & Licht 1991; Sinervo & DeNardo 1996; Swain & Jones 2000; Oksanen, Koskela & Mappes 2002; French, DeNardo & Moore 2007). These manipulations are a very powerful way to study reproductive costs but do not allow the researcher to quantify gestation costs *per se*: we need to manipulate litter size during gestation. Previous studies in squamates used a surgical procedure to decrease litter size during gestation: removal of one of the two oviducts containing developing embryos (“half-hysterectomy”). This method allows to study the development of the remaining embryos (Sangha *et al.* 1996; Swain & Jones 2000) and also gestation costs (Bleu *et al.* 2012b). However, such a procedure is invasive and permanent for the females. Thus it would be

interesting to develop a relatively non-invasive hormonal method to reduce litter size. The hormones oxytocin in mammals and the arginine vasotocin (AVT) in Reptilia are appropriate for these types of experiments because they induce early parturition or oviposition when injected into pregnant/gravid females (Guillette & Jones 1982; Guillette, Dubois & Cree 1991; Feldman 2007). Depending on species, on concentrations and on the timing of the injection, AVT induce partial or full parturition (oviposition) in viviparous (oviparous) squamates (e.g. Guillette 1979; Summers, Austin & Jones 1985; Atkins, Jones & Guillette 2006).

The aim of this study is to use AVT to manipulate litter size during gestation with a relatively noninvasive method and to assess the behavioral, physiological and survival gestation costs of large litters. To this end, we used the common lizard, *Zootoca vivipara*, and measured short-term effects of the manipulation: body mass change, immune response, endurance capacity, thermoregulatory behavior, offspring characteristics at birth and also long-term effects: female and offspring survival, offspring growth rate after birth and female body mass gain after parturition. An effect of the treatment on these traits would reveal gestation costs of large litters, whereas an effect of the initial litter size (litter size before the hormonal treatment) would suggest vitellogenesis costs (i.e. a correlation with the initial investment of the female). We also compared the results with previous ones from a study where litter size was surgically reduced during gestation in *Z. vivipara* (half-hysterectomy) (Bleu *et al.* 2012b). Thus we measured the same variables as in Bleu *et al.* (2012b) and we also measured the endurance capacity of the females (this measure is possible because this hormonal manipulation is less invasive than the surgical one). This study also aims to better understand the physiological control of parturition in a squamate.

MATERIALS AND METHODS

Model species, capture and rearing conditions

Zootoca vivipara is a small (adult snout-vent length 45-70 mm) ground-dwelling lizard, widely distributed across Eurasia. It includes both oviparous and viviparous reproductive forms in allopatric populations (Surget-Groba *et al.* 2001). We studied viviparous populations located in the Massif Central mountain range (South-eastern France). In this area, adults start to become active around mid-April (males) or early May (females). Mating may occur as early as 0-3 days after emergence and reproductive investment (vitellogenesis) occurs on average during the first three weeks after emergence (Bauwens & Verheyen 1985). During gestation, placentas formed from the chorioallantois and yolk sac allow respiratory, aqueous and mineral exchanges between mother and embryos (Panigel 1956; Stewart, Heulin & Surget-Groba 2004; Stewart, Ecaj & Heulin 2009). Parturition occurs after an average gestation period of 2 months. Mean litter size is five (range 1-12). Live offspring hatch immediately after parturition from their soft-shelled eggs and are thereafter autonomous. Lizards gradually enter into hibernation in September.

We captured 46 pregnant females in mid-June 2010 at Mont-Lozère (44°22'16''N, 03°47'47''E, 1260 m a.s.l.). The study site is ca. 5500 m² and is covered with a mixture of grass, heath, trees and rocks. Females were marked by toe-clipping, and brought to a field laboratory until parturition (July 14-24). Females were kept in individual terraria (18 cm x 12 cm x 12 cm) with a shelter and with damp soil as substrate. A 25-W spotlight provided opportunities for thermoregulation for 6 hours daily (from 9:00 to 12:00 and from 14:00 to 17:00), creating a thermal gradient from 24.7°C to 39.4°C in the terrarium. Water was provided *ad libitum* and 1 *Pyralis* sp. larva was offered per week. Immediately after parturition, mothers and their offspring were separated and measured. Within 4 days, the

females were released at their capture point and offspring were released randomly at 10 different points on the site. Offspring from the same family were released at the same point.

Experimental procedure

Litter size reduction during gestation.

The experimental reduction of litter size during gestation was achieved by an injection of AVT (Sigma-Aldrich, V0130). Previous studies on reptiles have successfully used this drug to induced parturition in viviparous lizards, at very different concentrations across species (Guillette, Dubois & Cree 1991; Girling, Jones & Swain 2002; Atkins, Jones & Guillette 2006; Bleu *et al.* 2012a). Females were randomly allocated to each treatment group: AVT-injected females ($n = 25$) and unmanipulated control females ($n = 21$). These two experimental groups did not differ in snout-vent length (SVL), body condition (i.e. body mass with SVL as a covariate in the statistical model) and litter size (fecundity) at the beginning of the experiment (all $P > 0.40$). On the July 5 or 6, females from the AVT-injected group received an intraperitoneal injection (20 μ L) of 0.02 μ g/ μ L AVT diluted in sterile phosphate-buffered saline (PBS, Sigma-Aldrich, reference D 5773). After the injection, 13 females gave birth to a partial litter and 12 females did not. In some of the females that did not give birth, we nonetheless saw contractions indicating that the AVT was active also in these females. There are no differences in initial SVL, body condition or fecundity between the females that reacted to the AVT injection and the females that did not (all $P > 0.60$). The manipulation was performed in the last third of gestation (embryos removed were at stages 34 to 37 (Dufaure & Hubert 1961)) because this drug is thought to be more efficient in late gestation (e.g. Guillette, Dubois & Cree 1991).

The experimental treatment resulted in three different groups: control females (hereafter called C, $n = 21$); females that received an AVT injection but did not react, i.e. they did not lay following the injection (hereafter called AC, $n = 12$); females that reacted to the injection

and laid a partial litter (hereafter called ALR, $n = 13$). The females that did not react to the AVT injection (AC) may allow us to control for the effects of AVT that are not related to parturition, if the AVT was nonetheless active in these females. These three groups did not differ in initial SVL, body condition or fecundity (all $P > 0.69$).

Short term effects of litter size reduction on female's performances.

Endurance was measured once on July 8 or 9. After warming up to approximately 27°C (natural activity temperature) for at least 30 min, we induced the females to run at the pace of the belt (0.5 km/h) by gently tapping on the hind leg (Le Galliard, Le Bris & Clobert 2003; Miles, Calsbeek & Sinervo 2007). A lamp suspended above the belt was used to maintain the body temperature of lizards. Our measure of endurance was the amount of time the lizards maintained their position on the treadmill until exhaustion. A lizard was determined to be exhausted if it failed to maintain its position on the belt after three attempts.

After the measure of endurance, the behavior of each lizard was measured seven times over 5 days, 1 or 2 times per day between 10:00-11:00 and/or 15:00-16:00, by an observer who did not know the treatment of the lizards. The observer noted whether the lizard was sheltering (under the shelter or the substrate), full basking or half-basking (the head under the light and the rest of the body hidden; as described elsewhere (Cote *et al.* 2010; Bleu *et al.* 2012a; b)). We distinguished half-basking from full basking, as the behaviors may differ in thermoregulatory efficiency (female body temperature) or predation risk.

After parturition (2.29 days, SE = 0.12 days), immunocompetence was estimated with the phytohaemagglutinin (PHA)-induced skin-swelling test as described by Bleu *et al.* (2012b). Briefly, we injected subcutaneously 0.04 mL of a solution of phosphate-buffered saline (PBS, Sigma-Aldrich, reference D5773) containing 2.5 mg/mL of PHA (Sigma-Aldrich, reference L8754) in the right posterior leg and measured the swelling response 12h later. We measured thickness of the leg to the nearest 0.01 mm with a spessimeter (ID-C Absolute digimatic,

Mitutoyo, France, reference 547-301). Although PHA swelling response is complex, including both innate and adaptive components of the immune system (Tella *et al.* 2008; Vinkler, Bainová & Albrecht 2010), it evaluates the general ability of an individual to mount an inflammatory response (Vinkler, Bainová & Albrecht 2010).

Parturition date, realized fecundity (litter size at parturition) and litter success (i.e. the presence or absence of non-viable embryos) were recorded. Initial fecundity was also calculated as the sum of the realized fecundity and the number of eggs removed by the AVT treatment. Offspring were marked by toe clipping, measured for SVL (to the nearest mm), weighed (to the nearest mg) and sexed by ventral scale count (Lecomte, Clobert & Massot 1992). Females were weighed after parturition.

Delayed effect of litter size reduction on growth and survival.

We recaptured the offspring and the adult females in late August of the year of release and in late May of the following year (Table 1). At each recapture, we weighed and measured SVL of all individuals. Juvenile growth rates before hibernation were calculated as the change in SVL (SVL at recapture – SVL at birth) divided by the time interval (date of recapture – date of birth). Adult body mass gain before hibernation was calculated as the change in body mass (body mass at recapture – postpartum body mass) divided by the time interval (date of recapture – parturition date).

Statistical analyses

All models were implemented in R 2.15.0 statistical software (<http://cran.r-project.org/>). They included the following additive fixed effects: (i) treatment (C, AC, ALR); (ii) female SVL; (iii) standardized initial fecundity (residuals from a regression between initial fecundity and female SVL, $R^2 = 0.32$, $P < 0.0001$), which represents the effect of the initial investment of females; and (iv) the first-order interactions with treatment. Models were simplified using backward elimination of the non-significant terms ($P > 0.10$). For the behavior, we first

analyzed the proportion of time spent basking (half- or full basking), and then we analyzed the proportion of time spent full basking when the lizard was basking. These analyses were conducted using generalized linear models with a quasibinomial family (to correct for overdispersion) and a logit function link (*glm* procedure). Since there was overdispersion, fixed effects were tested with F-tests (Venables & Ripley 2002). Litter success was analyzed as a binomial variable (litters with all viable offspring vs. litters with at least one failure) using the same *glm* procedure with a binomial family. In this case, fixed effects were tested with chi-square tests. Endurance (log transformed to achieve normality), postpartum body condition, PHA response and parturition date were analyzed with linear models (*lm* procedure and type III F-tests (Quinn & Keough 2002)). SVL and body condition at birth of offspring were analyzed with linear mixed effects models (*lme* procedure and marginal F-tests (Pinheiro & Bates 2000)). The random part of the models was maternal identity to account for a family effect. SVL of offspring at birth also included an effect of offspring sex. Offspring body condition at birth also included an effect of offspring sex and of offspring SVL.

Juvenile growth rates and female adult body mass gain before hibernation were analyzed with linear mixed effects models (*lme* procedure) and linear models (*lm* procedure) respectively. We tested the effects of the experimental treatment and the standardized initial fecundity. For the analysis of juvenile growth rate, we also included juvenile sex and initial SVL to control for decelerating growth curves (Andrews 1982). The random effect was maternal identity. For the analysis of female body mass gain, we also included female SVL and postpartum body condition (residuals from a regression between postpartum body mass and female SVL).

The assumptions of normality and homogeneity of variances were checked for all initial and final models. The values presented in the results section are the means for each group or the estimates of the models \pm the SEs.

Survival analyses

We tested the effect of the treatment on the probability of survival for adult females, juvenile males and juvenile females. Survival estimates were obtained independently of capture probabilities, using a capture-mark-recapture method based on the open population model of Cormack-Jolly-Seber. This model produces apparent survival estimates resulting from mortality and emigration. We used the program MARK version 6.0 to fit models (White & Burnham 1999), and models were compared with Akaike Information Criterion corrected for small sample size (AICc (White & Burnham 1999)). The best model is the most consistent with the data while using the fewest number of parameters, i.e. giving the lowest AICc. It is considered that two models differ when their difference of AICc is higher than 2 (Burnham & Anderson 1998). The goodness-of-fit of the time-dependent Cormack-Jolly-Seber models were tested with the bootstrap procedure (1,000 simulations) provided by the program MARK (White & Burnham 1999), and we did not find significant over-dispersion in the data (all $P > 0.18$).

RESULTS

Reproductive traits

The treatment affected the realized fecundity ($F_{2,42} = 15.12$, $P < 0.001$, see Figure 1). Post hoc comparisons show that on average ALR females gave birth to 2.90 ± 0.55 fewer offspring compared to C females ($t = -5.32$, $P < 0.001$) and to 2.52 ± 0.62 fewer offspring compared to AC females ($t = -4.07$, $P < 0.001$). Litter sizes of C and AC females were not significantly different ($t = -0.68$, $P = 0.499$). In the ALR group, the AVT injection reduced litter size by 52.34 ± 22.35 % (range = 14%-83 %, see Figure 1). The treatment had no effect on litter success ($\chi^2 = 3.85$, $df = 2$, $P = 0.146$). However, the AVT injection affected parturition dates of both ALR and AC females ($F_{2,43} = 3.68$, $P = 0.034$, Figure 2). All females gave birth in a

10-day period and post hoc comparisons show that ALR females gave birth on average on the same day as AC females ($t = -0.61$, $P = 0.547$) but 2.51 ± 0.99 days earlier than C females ($t = -2.55$, $P = 0.015$). The difference between C and AC females approached significance ($t = 1.81$, $P = 0.077$, Figure 2).

Concerning juvenile characteristics at birth, body condition (body mass statistically corrected by size) was not affected by the treatment ($F_{2,34} = 0.28$, $P = 0.754$, Table 2). As typical in *Z. vivipara*, male offspring were more corpulent than females ($F_{1,135} = 13.34$, $P < 0.001$). Also, offspring body condition was not significantly correlated to female standardized initial fecundity ($F_{1,36} = 2.03$, $P = 0.163$). The interactions and other variables tested were not significant (all $P > 0.29$). We observed the same pattern for offspring size at birth: there was no effect of the treatment ($F_{2,34} = 0.02$, $P = 0.984$, Table 2), but a significant effect of sex ($F_{1,136} = 52.46$, $P < 0.001$). Offspring size was not significantly correlated to female standardized initial fecundity ($F_{1,36} = 2.36$, $P = 0.133$).

Females' behavior and performance

Before parturition, ALR, AC and C females had different endurance capacity depending on their initial standardized fecundity (Table 3). In C females, there was a negative relationship between the endurance capacity and the initial standardized fecundity (Figure 3, $t = -2.47$, $P = 0.018$). This relationship was not observed in AC and ALR females ($t = 1.87$, $P = 0.070$ and $t = -0.44$, $P = 0.663$ respectively). Moreover, ALR females showed highest endurance (Figure 3). Concerning the thermoregulatory behavior, ALR, AC and C females were not significantly different (Tables 2 and 3): the proportion of time spent basking and the proportion of time spent full basking when the female was basking were not significantly affected by the treatment. After parturition, the response to PHA was dependent on the treatment and female size (Table 3, Figure 4). The response to PHA was positively correlated to female size only in ALR females such that longer ALR females had a stronger response than AC and C females

(Figure 4). The postpartum body condition of the females (body mass statistically corrected by size) was not significantly different between the experimental treatments (Tables 2 and 3).

Females and offspring growth rates and survival

We recaptured the adult females and the juveniles on two recapture sessions (see sample sizes in Table 1). None of our variables significantly explained female body mass gain between parturition and hibernation, and in particular it was not dependent on the treatment ($F_{2,21} = 0.99$, $P = 0.388$). The probability of female survival was not significantly affected by the treatment or by the time of recapture (best AICc for model Φ_{constant} and p_{constant} , Table A1). Juveniles' growth before hibernation was not dependent on the treatment of their mother (Tables 2 and 4). The probability of survival of juvenile females before hibernation was independent of the maternal treatment (best AICc for model Φ_{constant} and $p_{\text{treatment}}$, Tables 2 and A2 A). This best model and the other comparable models ($\Delta\text{AICc} < 2$, see Table A2 A) suggest that juvenile females had different capture probabilities depending on the maternal treatment ($C = 0.376 \pm 0.087$, $AC = 0.364 \pm 0.116$, $ALR = 1.00 \pm 0.01$). For juvenile males, the best model suggests that the probabilities of survival and capture were not dependent on the maternal treatment (best AICc for model Φ_{time} and p_{time} and model Φ_{constant} and p_{time} , Tables 2 and A2 B). However, other models have comparable AICc, including a model where capture probabilities and survival probabilities are dependent on the maternal treatment (Table A2 B).

DISCUSSION

Viviparity and oviparity are two reproductive strategies which entails specific costs and benefits. The main cost of viviparity is supposed to be the existence of higher reproductive cost due to gestation costs and the lower probability to produce several litters per season (Tinkle & Gibbons 1977; Blackburn 1999a). Gestation costs in viviparous squamates may be

attributed to an increase in metabolism, a shift in thermal preference and locomotor impairment (De Marco & Guillette 1992; Olsson, Shine & Bak-Olsson 2000; Ladyman *et al.* 2003; Le Galliard, Le Bris & Clobert 2003; Lin, Zhang & Ji 2008). However above-cited studies are based on comparisons between reproductive and nonreproductive females, or between reproductive and postreproductive females. Thus, they do not allow causal conclusions. In the first kind of comparison, females may differ according to other variables than their reproductive state: for example, in squamates, nonreproductive females often have lower body condition than reproductive females (e.g. Naulleau & Bonnet 1996). And when comparing performances of the same female before and after parturition, other confounding effects arise, such as seasonal variation in traits (e.g. Qualls & Shine 1998).

In this article, we report an experimental study that is complementary to a previous study where litter size of *Z. vivipara* females was reduced by half-hysterectomy (Bleu *et al.* 2012b). Concerning short term effects, we found no effect of the litter size reduction on offspring characteristics at birth, in line with results of the first study (Bleu *et al.* 2012b). It further supports the hypothesis that offspring development during gestation is not limited by maternal nutrient transfer or by the space available (Du, Ji & Shine 2005; Bleu *et al.* 2012b). We did not observe a trade-off between initial litter size and offspring mass or size. The detection of trade-offs in natural populations is difficult because it is obscured by the natural variability between females (e.g. variability in food acquisition) (van Noordwijk & de Jong 1986). In particular in the common lizard, it has been shown that the litter size-offspring mass trade-off is not observed every year (Bleu *et al.* 2013). We also found different results compared to the previous study (Bleu *et al.* 2012b): there was no effect of the litter size manipulation on the thermoregulatory behavior and on female postpartum body mass. Previously we showed that females with a reduced litter size spent more time half-basking and had a higher postpartum body mass (Bleu *et al.* 2012b). Metabolic rates increase with temperature, thus females which

have higher daily energetic demands may bask more and therefore lose a greater proportion of body mass after parturition compared to females with lower daily energetic demands. In this case, thermoregulatory behavior would reflect the energetic needs and thus reproductive costs (Bleu *et al.* 2012a; b). However in this study we did not detect different reproductive costs (energetic needs) between females with a reduced or an unmanipulated litter size (no difference in body mass), which may explain that there was no differences in the thermoregulatory behavior. The immune response was increased in females with a reduced litter although it was not affected in the previous study (Bleu *et al.* 2012b). This suggests a trade-off between reproductive investment and the immune function. However this effect was size dependent: only the longer females had a higher immunocompetence. This shows that it is not an obligatory response to gestation (French, DeNardo & Moore 2007). Larger females can store more resources (Avery 1974) and may thus be able to invest more in their immune response contrary to smaller females. It should be noted that the level of significance of this effect is not very strong ($p\text{-value} = 0.018$) and that, if we corrected the statistical analysis for multiple testing, this $p\text{-value}$ would not be considered significant. Thus, it will be necessary to replicate this study on more individuals and different populations to confirm this effect.

This study highlights some differences with the previous study of experimental litter size manipulation in *Z. vivipara* (Bleu *et al.* 2012b). We cannot exclude that this reflects annual (different years) or geographic (different populations) variations in reproductive costs (e.g. Qualls & Shine 1997). Also, we performed the manipulation of litter size slightly later during gestation (stages 34-37 compared to stages 29-34). However, the hormonal treatment itself might explain some of these differences. Indeed, AVT can induce parturition but it has also other effects. Most importantly, AVT affects sexual, social and aggressive behaviors (Goodson & Bass 2001; Godwin & Thompson 2012), thermoregulatory behavior (Bradshaw, Ladyman & Stewart 2007) and the salt and water balance (Bradshaw & Bradshaw 2002). The

AC females should allow us to detect these other effects, but we do not know why these females did not lay eggs in response to the AVT injection. The fact that parturition dates and endurance capacity of these females differed from control females is an indication that the AVT had some effects. However, we do not know to what extent the effects of AVT are similar in AC and ALR females and thus to what extent AC females are a relevant control group. For example, a change of the thermoregulatory behavior due to the AVT but only in the ALR females may counterbalance the effect of reproductive costs and result in the absence of differences between the females of the different experimental groups. This experiment shows that there is a need to increase our understanding of the actions of AVT in squamates, besides its classical effect on parturition.

In squamates, much attention has focused on the decrease of locomotor capacities of reproducing oviparous and viviparous females: a decrease of endurance (Cooper *et al.* 1990; Miles, Sinervo & Frankino 2000; Le Galliard, Le Bris & Clobert 2003; Zani *et al.* 2008) and/or sprint speed (Van Damme, Bauwens & Verheyen 1989; Cooper *et al.* 1990; Sinervo, Hedges & Adolph 1991; Qualls & Shine 1997; Le Galliard, Le Bris & Clobert 2003; Shine 2003b) have been documented. Only in some rare cases, locomotor capacities are not affected or are even increased during reproduction (Qualls & Shine 1997, 1998). Despite the interest in quantifying the changes of locomotor performance during reproduction, the underlying causes of these changes are not well understood. Experimental studies suggest that this cost may be both physical and physiological and may be species specific (oviparous: Miles, Sinervo & Frankino 2000; viviparous: Olsson, Shine & Bak-Olsson 2000; Shine 2003a). In *Z. vivipara*, litter size may be correlated with the decrease of locomotor performances (Van Damme, Bauwens & Verheyen 1989; Le Galliard, Le Bris & Clobert 2003), which suggest that it may be driven by the physical burden of reproduction. Moreover, the recovery of endurance capacity is quick (one week). However, the recovery of sprint speed is slower which suggests

physiological effects of gestation (Le Galliard, Le Bris & Clobert 2003). In control females there is a clear negative correlation between endurance and litter size, and females with reduced litters have a higher performance than control females. This confirms the importance of litter size and the physical burden on endurance capacity. However, AC females also show different endurance capacity than control females. In these females, the endurance capacity is not anymore correlated to litter size. This shows that a hormonal change (AVT injection) can affect endurance capacity despite a physical burden. AVT has been shown to increase activity in some species (Boyd 1991) but also to decrease activity in others (Thompson & Moore 2000; Nephew, Aaron & Romero 2005). This experiment suggests that it may increase endurance capacity in *Z. vivipara*. This may explain the quick recovery of endurance capacity after parturition (Le Galliard, Le Bris & Clobert 2003) since AVT is secreted before parturition (Guillette, Dubois & Cree 1991). Thus the physiological changes associated with gestation may also be important to shape locomotor impairment during this period. It will be interesting to confirm this result with additional studies because it is not robust to a correction for multiple testing ($p\text{-value} = 0.028$).

Finally, we also measured long-term effects of litter size reduction. There was no effect of the treatment on female characteristics before hibernation (survival, body mass gain). It seems that there is no important cost of gestation in terms of survival, as observed previously (Bleu *et al.* 2012b). However, it should be noted that our sample size for the survival analyses would not have allowed us to detect small effects on survival. We also measured offspring characteristics because reproductive effort can have intergenerational effects: offspring quality may decrease when reproductive investment increases, due to a trade-off between offspring number and quality (Roff 2002). Experiments have shown the existence of such a trade-off: for example, offspring from enlarged litters have been shown to have a lower survival (Sinervo 1999; Oksanen *et al.* 2007), and offspring from reduced litters have been

shown to grow faster after birth (in *Z. vivipara*: Bleu *et al.* 2012b). In this study, there was no clear effect of the maternal treatment on offspring growth or offspring survival. Offspring growth is very plastic and is influenced by parasite load (Uller & Olsson 2003), weather conditions (Lorenzon *et al.* 1999) or food availability (Le Galliard, Ferrière & Clobert 2005). These factors may help explain why we did not find an effect on growth contrary to a previous study (Bleu *et al.* 2012b). Again, we cannot exclude that the AVT treatment *per se* had an influence on juvenile growth (in juveniles from ALR or AC females). This hormonal manipulation can have effects beyond the effects on parturition. These effects need to be investigated further to better understand all the actions of AVT and to be able to develop this promising experimental method. In conclusion, with regard to gestation costs, we did not observe a trade-off between the investment during gestation and females' resources postparturition (female body mass) or survival.

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- 656

FIGURE LEGENDS

Figure 1. A. Litter size after the AVT injection (experimental parturition) and at the end of the gestation period (normal parturition). In order to reduce litter size during gestation, we intraperitoneally injected 25 females with AVT (0.02 μ g/ μ L). After the injection, 13 females gave birth to a partial litter (ALR females) and 12 females did not (AC females). We also had 21 control females (C females). The graph shows two values for each experimental group: on the left-hand side, the number of eggs laid after the injection (mean \pm SE), and, on the right-hand side, the number of offspring delivered on term (mean \pm SE). **B. Percentage of eggs laid in response to the AVT-injection in ALR females.** To calculate a percentage, the total litter size was calculated as the sum of the number of eggs laid at the moment of the injection and the number of offspring delivered at parturition.

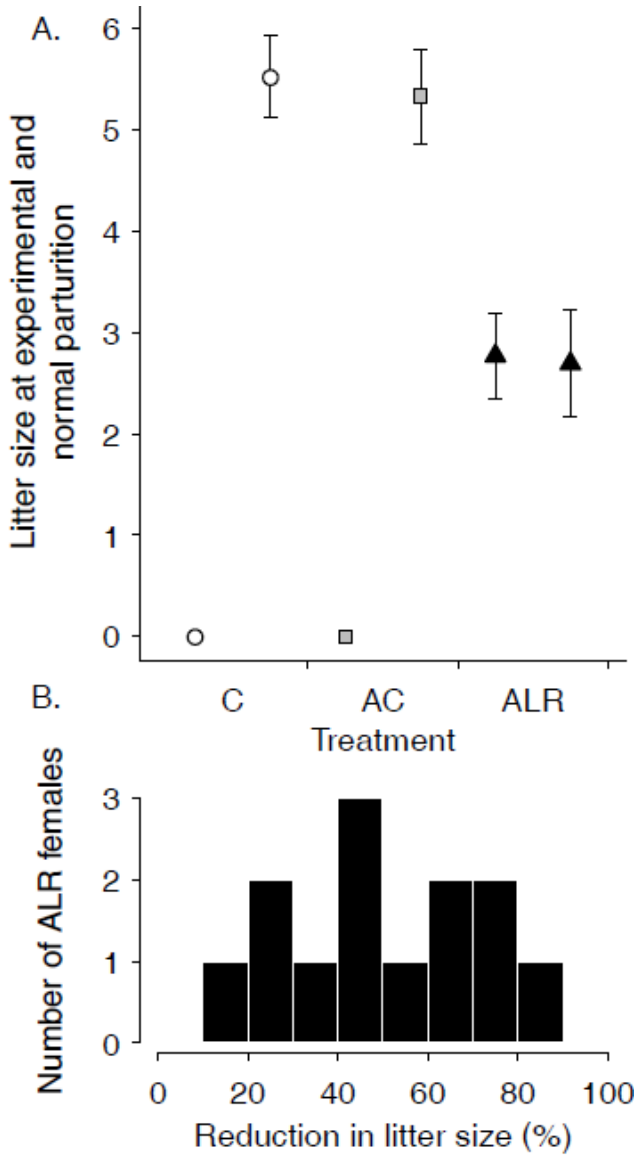
Figure 2. Parturition dates. Females gave birth to their offspring between July 14 and July 24. The mean \pm SE is represented for each experimental group: C females (control females), AC females (AVT-treated females with no litter size reduction) and the ALR females (AVT-treated females with a reduced litter).

Figure 3. Endurance. Endurance was measured before parturition as the time (s) spent running on a treadmill (0.5 km/h). The mean \pm SE is represented for C females (control females), AC females (AVT-treated females with no litter size reduction) and ALR females (AVT-treated females with a reduced litter). There was a significant interaction between the treatment and the standardized initial fecundity (see Table 3 for statistics). In this figure, we distinguished the female with a negative standardized initial fecundity (minus sign) and with a positive standardized initial fecundity (plus sign).

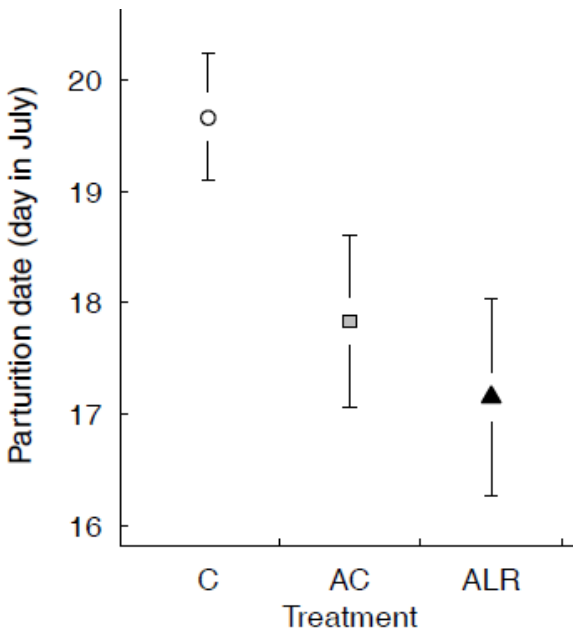
Figure 4. PHA swelling response. We measured thickness of the leg before and 12 h after the injection of PHA. PHA swelling response was calculated as the change in thickness of the leg between the two measurements. The white circles and the gray solid line represent C females (control females), the gray squares and the gray dashed line represent AC females (AVT-treated females with no litter size reduction), and the black triangles and the black dashed line represent ALR females (AVT-treated females with a reduced litter). There was a significant interaction between the treatment and the PHA response (see Table 3 for statistics). The slopes were estimated from the statistical model presented in Table 3.

FIGURES

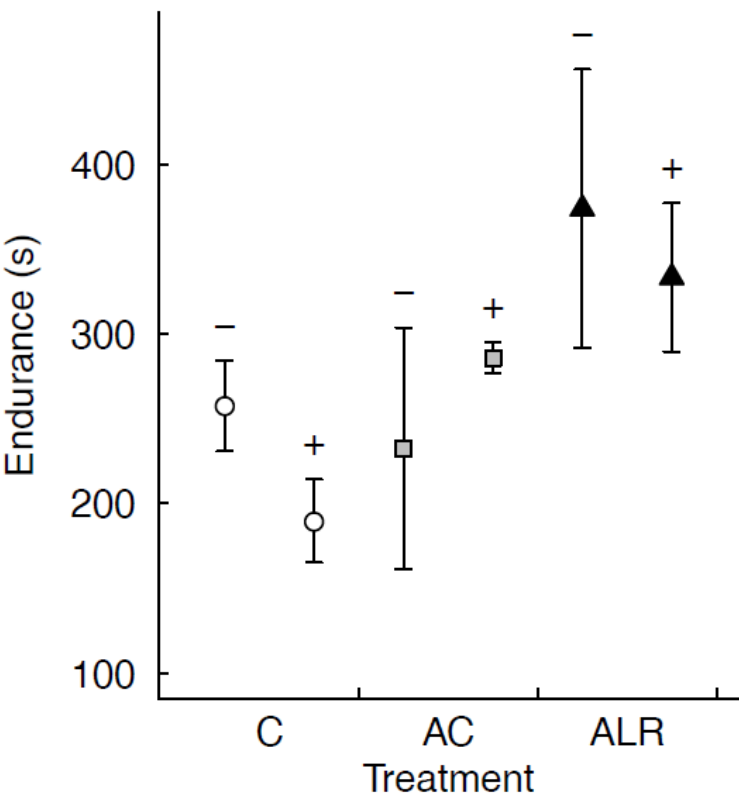
Figure 1



1 **Figure 2**

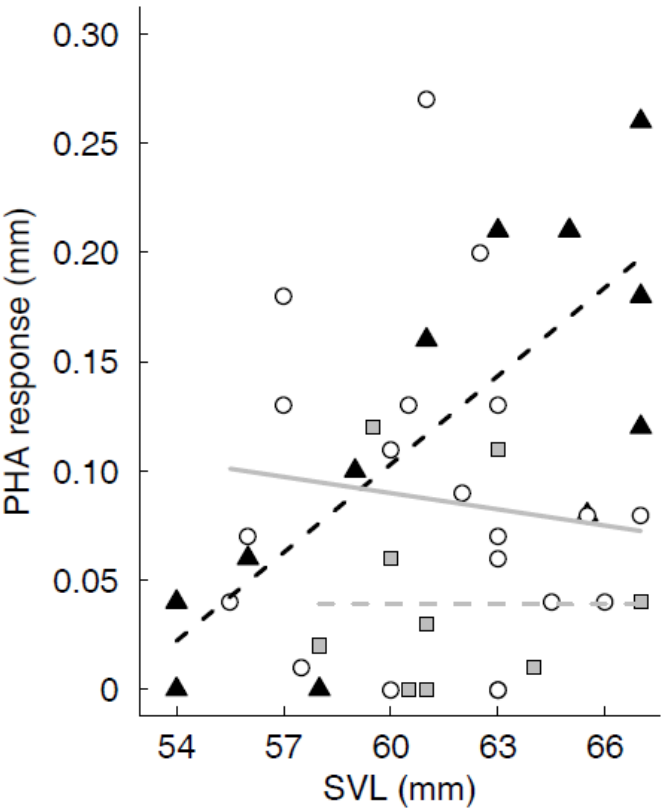


2
3 **Figure 3**



4
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8

Figure 4



TABLES

Table 1. Sample sizes during the experiment and the recapture sessions¹

| | | Adult females | | | Offspring | | |
|--------------|----------------|------------------------|--------------------------|-------------------------|------------------------|--------------------------|-------------------------|
| | | July 2010 (release) | Aug. 2010 (recapture) | May 2011 (recapture) | July 2010 (release) | Aug. 2010 (recapture) | May 2011 (recapture) |
| C | females | 21 | 10 | 8 | 54 | 14 | 12 |
| | males | | | | 46 | 14 | 14 |
| AC | females | 12 | 7 | 7 | 27 | 6 | 6 |
| | males | | | | 30 | 8 | 10 |
| ALR | females | 13 | 7 | 6 | 7 | 4 | 3 |
| | males | | | | 10 | 3 | 2 |
| Total | | 46 | 24 | 21 | 174 | 49 | 47 |

¹ We had three experimental groups: ALR females (AVT-treated females with a reduced litter), AC females (AVT-treated females with no litter size reduction) and C females (control females). Adult females and their offspring were released shortly after parturition (July 2010) and recaptured at two different times: at the end of August of the same year (before hibernation) and in May of the following year (after emergence from hibernation).

Table 2. Descriptive statistics of the parameters measured²

| | ALR females | AC females | C females |
|--|---|------------------|------------------|
| Before parturition | | | |
| Proportion of time spent basking when active | 0.53 ± 0.08 | 0.49 ± 0.07 | 0.48 ± 0.05 |
| Proportion of time spent half-basking when basking | 0.54 ± 0.09 | 0.57 ± 0.10 | 0.47 ± 0.08 |
| At parturition | | | |
| Postpartum body mass (g) | 2.49 ± 0.12 | 2.56 ± 0.09 | 2.45 ± 0.09 |
| Offspring SVL (mm) | M: 22.4 ± 0.3 | M: 22.0 ± 0.1 | M: 22.1 ± 0.2 |
| | F: 22.4 ± 0.5 | F: 23.0 ± 0.2 | F: 23.1 ± 0.2 |
| Offspring body mass (g) | M: 0.185 ± 0.006 | M: 0.175 ± 0.003 | M: 0.168 ± 0.003 |
| | F: 0.170 ± 0.013 | F: 0.178 ± 0.003 | F: 0.175 ± 0.004 |
| After parturition | | | |
| Female body mass gain (g/day) | 0.026 ± 0.003 | 0.034 ± 0.004 | 0.027 ± 0.004 |
| Offspring growth rate (mm/day) | 0.17 ± 0.02 | 0.19 ± 0.01 | 0.15 ± 0.01 |
| Female survival | 0.85 ± 0.09, confidence interval 95% = [0.57 ; 0.96] | | |
| Offspring survival | M: 0.58 ± 0.10, confidence interval 95% = [0.39 ; 0.75] | | |
| | F: 0.72 ± 0.09, confidence interval 95% = [0.52 ; 0.86] | | |

² The results are shown as mean ± se. For the offspring characteristics at birth M represents male offspring and F female offspring. Concerning the estimates of survival probabilities, they were obtained from the most parsimonious best models (see Tables A1 and A2), these models did not include an effect of the experimental group on survival. We also measured litter size, endurance capacity and immune response but these data are presented in figures.

Table 3. Short term effects of the litter size manipulation³

| Log(endurance) | | Time spent full | | | | Body mass | | PHA response | |
|----------------|--------------------------------------|--------------------|-------------------------------------|-------------------------------------|--------------------------------------|-----------|--------------------------------------|--------------|--------------------------------------|
| | | Time spent basking | | basking when the female was basking | | | | | |
| TRT | F _{2,37} =5.21, P=0.010* | TRT | F _{2,40} =2.42, P=0.102 | SVL | F _{1,42} =8.69, P=0.005* | TRT | F _{2,40} =2.54, P=0.091 | TRT | F _{2,38} =3.99, P=0.027* |
| FEC | F _{1,37} =3.48, P=0.070 | SVL | F _{1,40} =0.12, P=0.733 | | | SVL | F _{1,40} =6.04, P=0.018* | SVL | F _{1,38} <0.01, P=1.000 |
| TRTxFEC | F _{2,37} =3.94, P=0.028* | TRTxSVL | F _{2,40} =2.50, P=0.095 | | | TRTxSVL | F _{2,40} =2.52, P=0.093 | TRTxSVL | F _{2,38} =4.44, P=0.018* |

* P < 0.05

³ We had three experimental groups (treatment): ALR females (AVT-treated females with a reduced litter), AC females (AVT-treated females with no litter size reduction) and C females (control females). The full-models included an effect of the treatment (TRT), of female body length (SVL) and of standardized initial fecundity (FEC). Models were simplified using backward elimination of the non-significant terms (P > 0.10) and only the final models are presented. Measures of endurance were log-transformed to achieve normality. We have three missing values (2 AC and 1 ALR) for this measure. We analyzed the proportion of time spent basking (full basking + half basking) during the observations and also the proportion of time spent full basking when basking. Two females (1 C and 1 ALR) were always sheltering and were thus not included in the last analysis. Female body condition was analyzed as female body mass with female SVL as a covariate in the model. The PHA response was measured after parturition. We have two missing values (1 C and 1 AC) for this measure.

Table 4. Juvenile growth rates⁴

| | F | df | P |
|---------------------------|----------|-----------|----------|
| Maternal treatment | 2.16 | 2,23 | 0.1380 |
| Initial SVL | 3.12 | 1,21 | 0.0918 |
| Sex | 7.98 | 1,21 | 0.0102* |

*P<0.05

⁴ Growth rates before hibernation were calculated as the change in SVL (SVL at recapture – SVL at birth) divided by the time interval (date of recapture – date of birth). Sample sizes were 28 juveniles from control females (C juveniles), 14 juveniles from AC females (AC juveniles) and 7 juveniles from ALR females (ALR juveniles) as presented in Table 1. The full model included an effect of the maternal treatment, standardized initial fecundity, offspring sex and offspring SVL at birth (initial SVL). Maternal identity is included as a random effect. Models were simplified using backward elimination of the nonsignificant terms ($P > 0.10$) and only the final model is presented.