

RESEARCH ARTICLE

Influence of reproductive mode on metabolic costs of reproduction: insight from the bimodal lizard *Zootoca vivipara*

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ABSTRACT

Examination of the selective forces behind the transition from oviparity to viviparity in vertebrates must include an understanding of the relative energy costs of the two reproductive modes. However, interspecific comparisons of reproductive mode are confounded by numerous other inherent differences among the species. Therefore, we compared oxygen consumption, as a reflection of energy costs, during reproduction in oviparous and viviparous females of the reproductively bimodal lizard *Zootoca vivipara* (Jaquin 1787). Female oxygen consumption progressively increased over the course of reproduction, peaking just prior to partition when it was 46% (oviparous form) and 82% (viviparous form) higher than it was at the pre-reproductive stage. Total increase in oxygen consumption (TIOC) during the pre-ovulation period was not different between the reproductive modes. Conversely, post-ovulation TIOC was more than three times higher in viviparous females, reflecting a dramatic increase in embryonic metabolism as well as maternal metabolic costs of pregnancy (MCP). MCP accounted for 22% of total metabolism in viviparous females, whereas it was negligible in oviparous females. Our results demonstrate that egg retention through the first third of development, as is typical of most oviparous squamates, entails minimal maternal energy demand, while extending retention imposes much greater metabolic constraints. Selection for transition from oviparity to viviparity must therefore provide benefits that outweigh not only the added burden associated with prolonged embryonic retention, but also the substantial additional energy costs that are incurred.

KEY WORDS: Reproduction, Oviparity, Viviparity, Squamate, Metabolic cost, Evolution

INTRODUCTION

Viviparity (live birth) may impart many fitness advantages under certain conditions (Shine, 1995). Accordingly, viviparity has independently evolved 150 times in vertebrates, including instances within all major taxa except birds, crocodylians, chelonians and agnathans (for reviews, see Blackburn, 1992; Blackburn, 2014). To decipher the selective forces involved in the transition from oviparity to viviparity, it is essential to understand the various costs and benefits to both the mother and offspring (Crespi and Semeniuk, 2004). Offspring benefits of intra-uterine retention have received considerable attention, with maternal, nutritional, phenological and offspring phenotypical effects all appearing relevant [for review in reptiles, see previous publications (Shine, 1985; Shine and

Schwarzkopf, 1992; Shine, 1995; Lorigou et al., 2013a; Lorigou et al., 2013b)]. Examination of maternal constraints and associated energy costs of reproduction has primarily focused on either the physical burden or the energy demands associated with providing nutrients for embryonic development (e.g. Shine and Schwarzkopf, 1992; Stewart, 2013). However, supporting the embryos *in utero* imposes a specific energy demand on the mother. While relatively unexplored, knowing to what extent the evolution from oviparity to viviparity alters maternal energy expenditure during reproduction is a crucial component of understanding the selective forces of viviparity.

An increase in the metabolic rate of females during reproduction has been documented in a variety of oviparous and viviparous amniotes (Hyttén and Leitch, 1971; Hoversland et al., 1974; Pernoll et al., 1975; Guillette, 1982; Birchard et al., 1984; Beuchat and Vleck, 1990; Beaupre and Duvall, 1998; Angilletta and Sears, 2000; Robert and Thompson, 2000; Nilsson and Raberg, 2001; Vézina and Williams, 2002; Schultz et al., 2008; Van Dyke and Beaupre, 2011; Yue et al., 2012). Available data suggest that metabolic changes during the pre-ovulation period vary among species depending on the extent of lecithotrophic energy provisioning (i.e. via yolk) (see Van Dyke and Beaupre, 2011). Besides direct energy provisioning, oviductal proliferation for ova implantation or egg-shelling may create additional pre-ovulation energy demands (for a review, see Van Dyke and Beaupre, 2011). Metabolic changes after ovulation (i.e. during pregnancy) are attributable to (i) embryonic metabolism and (ii) maternal metabolic demand of supporting the embryos (termed the metabolic cost of pregnancy, MCP). MCP may involve numerous maternal costs including but not limited to those associated with supplying oxygen to the fetus (Bader et al., 1959; Hyttén and Leitch, 1971; Grigg and Harlow, 1981; Birchard et al., 1984; Murphy et al., 2010; Parker et al., 2010), handling fetal wastes (Clark and Siskin, 1956), and, in matrotrophic species, supplying nutrients to the embryos (Stewart and Thompson, 2000).

While viviparity has evolved in most major vertebrate taxa, the preponderance of transitions to viviparity have occurred within the squamates (i.e. 115 times in lizards, snakes and amphisbaenians versus 35 times in all other taxa) (Blackburn, 1995; Blackburn, 2006; Blackburn, 2014). Despite the distinct difference in producing an egg versus giving live birth, oviparous and viviparous squamates share important similarities. Specifically, energy provisioning is predominantly lecithotrophic regardless of reproductive mode, and most oviparous squamates retain their eggs *in utero* for more than 25% of embryonic development (Shine, 1983; Blackburn, 1995; Andrews and Mathies, 2000). Therefore, viviparity in most squamates can be viewed as an extension of oviparous egg retention with associated complex physiological adaptation to support intra-uterine life (Shine, 1983, Van Dyke et al., 2014). Yet one important difference between oviparous and viviparous squamates is the pronounced development of uterine eggshell glands prior to ovulation in oviparous forms (Heulin et al., 2005) (see also

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List of symbols and abbreviations

ER1	egg retention 1, last measure before parition in oviparous females, with equivalent for viviparous females
ER2	egg retention 2, intermediate stage during viviparous gestation
ER3	egg retention 3, last measure before parition in viviparous females
M_b	body mass (g)
MCP	metabolic cost of pregnancy ($\text{ml O}_2 \text{ min}^{-1}$)
OV	ovulation stage, first measure after ovulation
PP	post-partition, measured 3 days after parition
PR	pre-reproductive stage, first measured after winter emergence
SVL	snout–vent length (mm)
TIOC	total increase of oxygen consumption (ml O_2)
VIT	vitellogenic stage, last measure before ovulation
\dot{V}_{O_2}	oxygen consumption ($\text{ml O}_2 \text{ min}^{-1}$)
$\Delta\dot{V}_{\text{O}_2}$	difference between pre-parition stage \dot{V}_{O_2} and post-parition \dot{V}_{O_2}

references therein). This difference could result in significantly higher maternal energy costs in oviparous species during the pre-ovulation period. In contrast, the extended embryonic retention experienced by viviparous species probably imparts considerably larger post-ovulation energy demand.

One major problem in examining differential maternal energy costs based on reproductive mode is that such studies typically require interspecific comparisons, where critical phylogenetic and physiological differences complicate the ability to attribute differences to reproductive mode (Birchard et al., 1984; Beuchat and Vleck, 1990; Demarco and Guillette, 1992; Demarco, 1993; Beaupre and Duvall, 1998; Angilletta and Sears, 2000; Robert and Thompson, 2000; Schultz et al., 2008; Van Dyke and Beaupre, 2011; Yue et al., 2012; Dupoué and Lourdais, 2014). However, a few squamate species are reproductively bimodal (Heulin et al., 1991; Heulin et al., 1993; Heulin et al., 1997; Qualls et al., 1995; Smith and Shine, 1997; Stewart, 2013) and therefore provide a rare opportunity for the examination of mode-based differences in reproductive energetics. *Zootoca vivipara* [formerly *Lacerta vivipara* (Jaquin 1787)] is one of three lizard species known to exhibit interpopulational variation in reproductive mode (Heulin et al., 1991; Stewart, 2013). Viviparous female *Z. vivipara* give birth to fully formed offspring (final stage 40) (Dufaure and Hubert, 1961), whereas oviparous females oviposit eggs with embryos between stages 30 and 35 (Braña et al., 1991; Heulin et al., 1991; Heulin et al., 2002). In the oviparous mode, the yolk (vitellus) and embryo are encased in an eggshell composed of a thick (40–65 μm) mat of fibrous protein overlaid by a thin (few micrometers) layer of calcium crystals (Heulin, 1990; Heulin et al., 2002; Heulin et al., 2005). The embryos of viviparous females are also lecithotrophic, but are enveloped in a very thin shell membrane (6–10 μm of fibrous protein without a calcium layer) throughout gestation (Panigel, 1956; Heulin, 1990). Under favorable climatic conditions, oviparous females can lay two clutches in a given year, while, even under optimal conditions, viviparous females are limited to a single brood each year (Heulin et al., 1991; Heulin et al., 1994; Heulin et al., 1997).

We compared, as a metabolic rate proxy, the oxygen consumption of oviparous and viviparous females throughout the reproductive cycle under standardized laboratory conditions. We limited our study to the first clutch in oviparous females. To identify possible phylogenetic (non-reproductive) differences between the two populations, we first assessed body characteristics: mass (M_b), snout–vent length (SVL), and oxygen consumption (\dot{V}_{O_2}) of both males and females at post-winter emergence, prior to any reproductive activity. We then monitored changes in \dot{V}_{O_2} throughout the reproductive cycle of females up to parition (term collectively

defining either oviposition or parturition) (Smith, 1975; Blackburn, 1992; Morafka et al., 2000). Increases in \dot{V}_{O_2} were integrated over time to provide synthetic estimates of total increase in oxygen consumption (TIOC) prior to and after ovulation. We also estimated the MCP just prior to parition. We hypothesized that metabolic demands of pregnancy are dependent on both the stage of reproduction and the reproductive mode being employed.

RESULTS**Oxygen consumption at winter emergence**

The mean oxygen consumption (\dot{V}_{O_2}) values were: $14.53 \pm 1.67 \mu\text{l min}^{-1}$ for the viviparous population males, $13.36 \pm 2.06 \mu\text{l min}^{-1}$ for the oviparous population males, $12.49 \pm 2.48 \mu\text{l min}^{-1}$ for the viviparous females, and $10.91 \pm 2.76 \mu\text{l min}^{-1}$ for the oviparous females. Individuals from the viviparous population were longer and heavier (SVL $F_{1,86}=6.88$, $P=0.010$; M_b $F_{1,86}=6.95$, $P=0.010$), and there was no effect of the interaction between sex and reproductive mode. Females were longer and lighter than males, independent of reproductive mode (SVL: $F_{1,86}=15.1$, $P<0.001$; M_b $F_{1,86}=12.13$, $P<0.001$). Due to differences in M_b and SVL between sexes and reproductive modes, we examined oxygen consumption relative to body mass ($\dot{V}_{\text{O}_2}=bM_b^a$; Fig. 1). We found a pronounced effect of M_b on \dot{V}_{O_2} ($F_{1,83}=81.52$, $P<0.001$) with a significant difference between males and females ($F_{1,83}=7.63$, $P=0.007$), but no difference between oviparous and viviparous individuals ($F_{1,83}=1.97$, $P=0.163$) and no interaction effects. Hence once controlling for sex and body mass, the oxygen consumption at emergence did not differ between the oviparous and viviparous populations of *Z. vivipara*.

Changes in body mass and comparative fecundity

In the subsample considered (i.e. reproductive females), viviparous females were larger than oviparous ones (mean initial body mass 2.85 ± 0.45 and 2.46 ± 0.55 g, respectively, t -tests: $t=-2.23$, d.f.=32, $P=0.033$). Body mass increased during reproduction for both reproductive modes (ANOVA; oviparous: $F_{4,80}=129.51$, $P<0.001$; viviparous $F_{6,72}=144.67$, $P<0.001$). In oviparous females, mass increased up to ovulation and remained constant thereafter [Tukey's

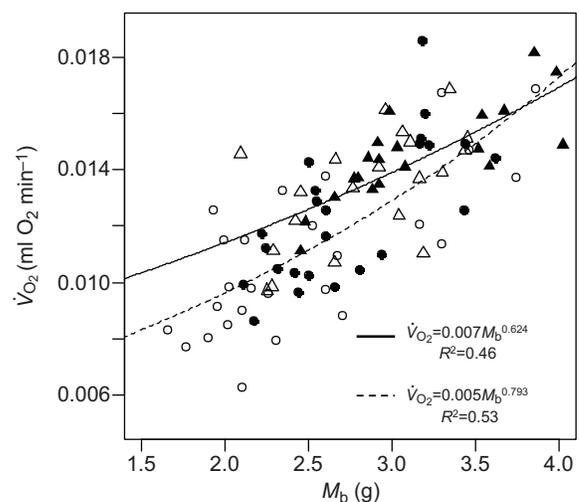


Fig. 1. Relationship between log-transformed \dot{V}_{O_2} and body mass (M_b) at post-winter emergence. Open triangles, oviparous population males; filled triangles, viviparous population males; open circles, oviparous females; filled circles, viviparous females. There is no difference between oviparous and viviparous individuals, but there is a significant difference between females (dashed regression line) and males (continuous regression line).

Table 1. Oxygen consumption (\dot{V}_{O_2}) of oviparous and viviparous females at various stages of reproduction

	Date (days after emergence)		Body mass (g)		\dot{V}_{O_2} ($\times 10^{-3}$, ml O ₂ min ⁻¹)	
	Oviparous	Viviparous	Oviparous	Viviparous	Oviparous	Viviparous
PR	0	0	2.46±0.55	2.85±0.45	10.21±2.78 ^a	13.01±2.65 ^{a,f}
VIT	28.6±8.5	31.2±7.9	3.13±0.75	3.44±0.49	13.88±2.74 ^b	15.07±2.31 ^{a,b}
OV	37.0±8.2	39.7±7.5	3.38±0.81	3.76±0.61	14.03±2.68 ^{b,c}	16.54±2.94 ^{b,c}
ER1	48.4±8.9	53.8±9.9	3.41±0.82	4.43±0.64	14.76±2.99 ^c	17.36±2.33 ^{c,d}
ER2	n.a.	62.7±9.4	n.a.	4.82±0.72	n.a.	19.39±3.21 ^d
ER3	n.a.	72.7±9.4	n.a.	4.89±0.85	n.a.	23.65±3.60 ^e
PP	54.1±8.4	84.2±7.7	2.34±0.47	2.76±0.38	9.53±1.88 ^a	12.24±1.50 ^f

Reproductive stages are defined as follows: PR, pre-reproductive; VIT, vitellogenesis–follicular differentiation; OV, ovulation; ER1 to ER3, intra-uterine development; PP, post-partition. The date is defined as the number of days after post-winter emergence. Superscript letters (a–f) refer to the results of Tukey's *post hoc* tests, with common letters indicating that the \dot{V}_{O_2} values do not differ between those stages within a reproductive mode (at $P>0.05$). n.a., data not available.

honestly significant difference (HSD) test, $P=0.990$]. In viviparous females, mass increased up to ER2 and remained constant thereafter (Tukey's HSD test, $P=1$; see Table 1). For each reproductive mode, body mass after reproduction (PP) was not different from body mass at emergence (PR, see Table 1) (Tukey's HSD test, $P=1$ and $P=0.39$, respectively, for viviparous and oviparous females).

Mean fecundity was significantly higher in viviparous (5.1±1.7 young per female) compared with oviparous females (4.0±1.2 eggs per female, $t=-2.10$, d.f.=32, $P=0.044$). When accounting for allometric variation, a positive influence of body mass was detected (ANCOVA: $F_{1,30}=5.85$, $P=0.022$), while no difference was found between oviparous and viviparous females (ANCOVA: $F_{1,30}=1.56$, $P=0.222$).

Changes in oxygen consumption during reproduction

We first studied oxygen consumption (\dot{V}_{O_2}) over reproduction separately in each reproductive mode with initial maternal body mass as a covariate. We considered five stages for oviparous females (pre-reproductive, PR; vitellogenesis, VIT; ovulation, OV; early egg retention, ER1; post-paritve, PP) and seven stages for viviparous females (same five plus two additional later stages during pregnancy, ER2 and ER3). In oviparous females, \dot{V}_{O_2} varied significantly during the reproductive cycle ($F_{4,80}=60.56$, $P<0.001$) with values being lowest pre-reproduction, and then elevating during the reproductive periods (VIT, OV, ER1), and finally returning to low levels after egg laying (PP) (Table 1). Similar variation was observed in viviparous females ($F_{6,72}=62.85$, $P<0.001$) with, however, further elevation in oxygen consumption during the prolonged egg-retention stages (ER2 and ER3) (Table 1, Fig. 2).

We then compared the two reproductive modes considering only the shared reproductive stages (i.e. PR, VIT, OV, ER1 and PP). We found no significant difference between oviparous and viviparous females for any shared stage (ANCOVA: $F_{1,31}=0.32$, $P=0.573$; Fig. 2). However, \dot{V}_{O_2} of viviparous females before partition (i.e. ER3) was twice that of oviparous females before partition (i.e. ER1)

(ANCOVA: $F_{1,34,88}=0.55$, $P<0.001$). \dot{V}_{O_2} before partition was 46% (oviparous females) and 82% (viviparous females) higher than pre-reproductive \dot{V}_{O_2} measures.

We used all \dot{V}_{O_2} measures collected to evaluate the increase in oxygen consumption over time (i.e. TIOC; see Materials and methods; see also Fig. 2). The mean raw TIOC were, respectively, for oviparous and viviparous females, 143±71 and 142±77 ml O₂ during the pre-ovulation period and 106±50 and 387±92 ml O₂ during the post-ovulation period (see Table 1 for duration of each period). A significant interaction was found between stage and reproductive mode (ANCOVA with body mass as a covariate, $F_{1,32}=50.26$, $P<0.001$). TIOC was higher during pre-ovulation than post-ovulation in oviparous females (Tukey's HSD test, $P=0.003$), while the opposite pattern was found in viviparous females (Tukey's HSD test, $P<0.001$). During the pre-ovulation period, TIOC did not differ between reproductive modes (Tukey's HSD test, $P=0.760$). Pre-ovulation TIOC represented 19.17±8.19% (range 4.10–36.60%) of the total pre-ovulation oxygen consumption in *Z. vivipara*. During the post-ovulation period, TIOC was higher in viviparous females than oviparous females (Tukey's HSD test, $P<0.001$). Interestingly, post-ovulatory TIOC represented similar percentages of total post-ovulation oxygen consumption (i.e. including non-reproductive demand) for oviparous and viviparous females (oviparous: 32.64±8.02%, range 20.24–55.97%; viviparous: 33.07±6.01%, range 20.63–44.35%; ANOVA, $F_{1,32}=0.09$, $P=0.769$).

During the pre-ovulation period, TIOC increased with fecundity (ANCOVA: $F_{1,29}=10.57$, $P=0.003$) but did not differ between oviparous and viviparous females (ANCOVA: $F_{1,29}=0.77$, $P=0.388$). During the post-ovulation period, TIOC increased with pre-reproductive (PR) maternal body mass (ANCOVA: $F_{1,29}=5.14$, $P=0.031$).

Metabolic costs of pregnancy

To calculate MCP we created linear regressions of oxygen consumption difference between pre- and post-paritve stages ($\Delta\dot{V}_{O_2}$)

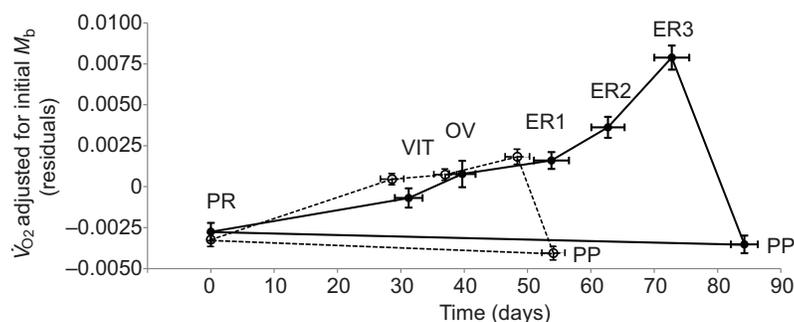


Fig. 2. Changes in oxygen consumption from emergence to partition in oviparous (open circles) and viviparous (filled circles) females. Oxygen consumption values are expressed as residuals from regression of oxygen consumption on body mass with data from all stages of followed females. Reproductive stages are as defined in Table 1. Error bars represent the standard error.

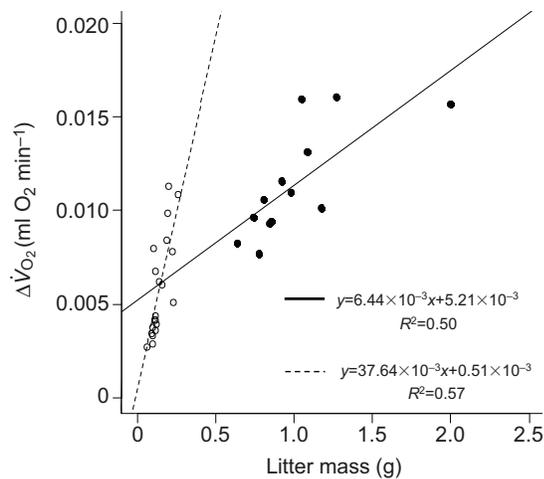


Fig. 3. MCP estimates derived from linear regressions of $\Delta\dot{V}_{O_2}$ against litter mass (embryos at oviposition or neonates at birth) at the end of egg retention in oviparous (open circles) and viviparous (filled circles) females. $\Delta\dot{V}_{O_2}$ is last pre-partition \dot{V}_{O_2} minus post-partition \dot{V}_{O_2} . The y-value at a null litter size represents the metabolic cost of pregnancy (MCP), i.e. the maternal energy expenditure to produce a null quantity of young.

on neonate (or embryo) masses (Fig. 3). Regressions were significant in the two groups of females (oviparous females: $F_{1,18}=26.41$, $P<0.001$; viviparous females: $F_{1,11}=12.85$, $P=0.004$). The MCP (intercept of the regression) was significantly different from zero for viviparous females (one-tailed t -test, $t=2.859$, $P=0.008$), but not for oviparous females (one-tailed t -test, $t=0.457$, $P=0.327$). The calculated intercept (MCP) was 10 times higher in viviparous females compared with oviparous females ($5.2\pm 1.8\times 10^{-3}$ ml O_2 min $^{-1}$ versus $0.5\pm 1.1\times 10^{-3}$ ml O_2 min $^{-1}$, respectively). These MCP values represented 46% of the mean $\Delta\dot{V}_{O_2}$ (or 22% of \dot{V}_{O_2} at stage ER3) in viviparous females and 9% of the mean $\Delta\dot{V}_{O_2}$ (or 3% of \dot{V}_{O_2} at stage ER1) in oviparous females.

DISCUSSION

Clarifying the energy implications of reproductive mode is a vital component in understanding selective pressures behind the evolution of viviparity (Shine and Schwarzkopf, 1992; Qualls and Shine, 1998; Bleu et al., 2012). Herein, we used the reproductively bimodal lizard *Z. vivipara* to compare the metabolic costs of reproduction between oviparous and viviparous individuals. Once controlled for body mass and sex, metabolic rate at post-winter emergence did not differ between oviparous and viviparous *Z. vivipara*.

During reproduction, oviparous and viviparous females exhibited similar increases in oxygen consumption up to the ER1 stage (last stage before oviposition in oviparous females). The most obvious difference between the two groups is that the prolonged gestation of viviparous females resulted in a further rise in metabolic rate that does not exist in oviparous females. Integration of \dot{V}_{O_2} over time provided additional insight: the total increase in oxygen consumption (TIOC) during the pre-ovulation period was similar between reproductive modes and was dependent on fecundity, but was 254% higher in viviparous females during the egg retention period. The greatly elevated TIOC of viviparous females during egg retention is not surprising, because this period is considerably prolonged compared with that of oviparous females (about 2 weeks versus 6 weeks, respectively, for oviparous and viviparous females; Table 1, Fig. 2) and because the last two-thirds of gestation corresponds to exponential embryonic growth and therefore

exponential increase in embryonic oxygen demand (Xavier and Gavaud, 1986).

Contrary to our first prediction, pre-ovulation TIOC was similar between oviparous and viviparous females. Previous dissection of recently ovulated eggs revealed that eggshells from oviparous females were eight times heavier (dry mass, 8 mg) than the embryonic membranes of viviparous females (1 mg), whereas yolk content of ovulated ova (mean dry mass, 55 mg) did not differ between the two reproductive modes (Stewart et al., 2009). Thus oviparous and viviparous female *Z. vivipara* are very similar in their pre-ovulation reproductive allocation except for the synthesis of the eggshell components that are stored in the uterine glands prior to ovulation and then deposited on the fertilized ova shortly after ovulation (Heulin et al., 2005). While synthesis of these shell components requires morphological modification of uterine epithelium (Adams et al., 2004), our results suggest that preparation for egg shelling has a negligible energy demand. Alternatively, preparation for shelling may occur over a rather short time frame and thus our measurement frequency may not have been sufficient to detect energy use associated with it. A significant increase in metabolic rate during the pre-ovulation period has previously been documented for six species of viviparous snakes, and the authors attributed the entire increase to the metabolic cost of vitellogenesis (MCV) and follicle differentiation (Beaupre and Duval, 1998; Van Dyke and Beaupre, 2011). Unfortunately, there are no comparable studies on related oviparous species with well-developed eggshells to test whether oviductal activity prior to ovulation (synthesis of components for subsequent egg shelling) may further increase pre-ovulation metabolic rate. We found significant MCV values that averaged 19% of the total metabolic demand, which is lower than those recently reported in snakes (30%) (Van Dyke and Beaupre, 2011). This highlights the need for further studies on pre-ovulatory metabolic rate.

Numerous studies have documented elevated metabolic rates near the end of reproduction in squamates, corresponding to a 50–200% increase in basal (non-reproductive) metabolism (Guillette, 1982; Birchard et al., 1984; Schultz et al., 2008; Van Dyke and Beaupre, 2011; Yue et al., 2012; Dupoué and Lourda, 2014). Most of these studies were performed on viviparous species, and the only one allowing for a direct comparison of oviparous versus viviparous characteristics is that of Guillette (Guillette, 1982), who found that the metabolic rate of females increased by 122% at the end of egg retention in the oviparous subspecies *Sceloporus aeneus aeneus* and by 199% at the end of pregnancy in the viviparous subspecies *Sceloporus aeneus bicanthalis*. The corresponding values calculated for *Z. vivipara* in the present study (\dot{V}_{O_2} increase of 46% in oviparous females, and 82% in viviparous females) are lower than those calculated for *S. aeneus*. Interestingly, the ratio of the metabolic increases (oviparous mode/viviparous mode: $122/199=0.61$) obtained in the study of Guillette (Guillette, 1982) is similar to that ($46/82=0.55$) obtained in our study of *Z. vivipara*. Therefore the proportion of metabolic change associated with viviparity is similar in these two squamate lineages, but further work is required to address the general value of this pattern.

Our study revealed that maternal costs of supporting early embryonic development (i.e. ER1 stage), were negligible in oviparous females, but significant in viviparous females at the end of pregnancy (i.e. at ER3 stage), when MCP represented $22\pm 8\%$ of the total metabolism of viviparous females. Several authors previously used the same regression method to test whether the MCP (intercept of the regression) at the end of pregnancy was significantly above zero for different viviparous squamates. Significant MCPs (representing between 12 and 26% of the total metabolism of late pregnant

females), were detected for some species (Robert and Thompson, 2000; Schultz et al., 2008; Yue et al., 2012; Dupoué and Lourdais, 2014), whereas non-significant MCPs were found for others (Beuchat and Vleck, 1990; Van Dyke and Beaupre, 2011). It is not clear whether these discrepancies result from phylogenetic differences or from methodological biases (see Schultz et al., 2008; Van Dyke and Beaupre, 2011). Before now, all these estimates of MCP in squamates were calculated at the end of pregnancy or egg-retention. It is, however, logical to expect changes in MCP over embryonic development, because of maternal physiological changes such as those needed to support the increasing oxygen demand of the growing embryos [e.g. changes in oxygen affinity (Grigg and Harlow, 1981); increased heart rate (Birchard et al., 1984); or for renal processing of the increased fetal nitrogenous wastes (Clark and Siskin, 1956)]. The possibility that MCP increases as egg retention progresses, though suggested by several authors (Demarco, 1993; Robert and Thompson, 2000; Van Dyke and Beaupre, 2011; Yue et al., 2012), has not yet been investigated. This is likely because it requires invasive approaches (surgical embryo removal) to compare the metabolic rates of females with and without their embryos *in utero* at different stages of pregnancy. Our data on *Z. vivipara* provide interesting, though indirect insight, as we found negligible (not significantly different from 0%) MCP in oviparous females (broadly at one-third of development) and higher MCP (22%) in viviparous females.

Most oviparous squamates lay their eggs at an embryonic stage of development that does not exceed that of oviparous *Z. vivipara* (stages 31–34) (Blackburn, 1995). Thus we suggest that, as reported in *Z. vivipara*, most oviparous squamates do not entail the additional maternal metabolic cost (MCP) that exists in viviparous species. This conclusion is important as it has recently been demonstrated that maternal thermoregulation early in development critically influences various fitness-related traits including date of birth, offspring morphology, behavior and survival (Lorioux et al., 2013a; Lorioux et al., 2013b). Hence a high thermal sensitivity at early embryonic stages may well explain the degree of egg retention (seldom prolonged after embryo stage 34) observed in most oviparous squamates. Prolonged retention provides enhanced maternal control of the embryonic thermal environment (i.e. access to preferred temperature) during critical developmental periods (neurulation, organogenesis) with minimal energy costs (our study) and over a rather short period (thereby limiting the duration of physical burden) (e.g. Le Galliard et al., 2003). Conversely, a surge in MCP that occurs at late development may help explain why so few species of oviparous squamates retain their eggs *in utero* beyond embryonic stage 34 (Blackburn, 1995; Lorioux et al., 2013a).

In conclusion, our study underlines post-ovulation metabolic consequences of prolonged gestation in viviparous females. We provide the first empirical results showing that MCP dramatically changes over time, being negligible at early retention stages (in the oviparous females) but becoming elevated at later stages (in the viviparous females). These findings are of broad interest to the understanding of the energy costs of development *in utero* and thus selective forces involved in the evolution of viviparity. Because of this value, research using other reproductively bimodal species, such as the Australian skinks *Lerista bougainvilli* and *Saiphos equallis*, would be very useful for validating and extending the conclusions drawn from our study on *Z. vivipara*.

MATERIALS AND METHODS

Study species

Zootoca vivipara [formerly *Lacerta vivipara* (Jaquin 1787)] is a small (adults 45–75 mm snout–vent length), ground-dwelling, reproductively

bimodal lacertid that generally lives in moist habitats. Life history and ecology of both oviparous and viviparous populations has been published elsewhere (Heulin et al., 1991; Heulin et al., 1997). Viviparous populations are widely distributed from the British Isles and central France to Scandinavia and eastern Russia, whereas two distinct oviparous populations (one in southern France/northern Spain, the other in northern Italy/Slovenia) are restricted to the southern margin of the range (Heulin et al., 1993; Ghielmi et al., 2001). Phylogenetic analyses and estimates of divergence time indicate that the differentiation of the oviparous and viviparous clades of this species occurred during recent (Pleistocene) geological time (Surget-Groba et al., 2001).

During the second half of August 2012, 20 males and 30 females of each reproductive mode were collected from an oviparous population near Louvie, southwest France (43°06'N, 0°23'W, 370 m above sea level), and a viviparous population near Paimpont, northwest France (48°01'N, 2°10'W, 155 m above sea level). Both of these populations have been used previously for studies of embryonic development (Stewart et al., 2004; Stewart et al., 2009). Captured lizards were transported to the Station Biologique de Paimpont. All experimental protocols were approved (authorization no. R-23012-BH-01) by the relevant ethics committee (Comité Rennais d'Ethique en matière d'Expérimentation Animale, no. 07).

Animal husbandry

Lizards were housed individually in plastic terraria. Each terrarium (30×20×20 cm) was equipped with a shelter, dishes of food and water, and a 40 W bulb that provided heat for 6 h day⁻¹. Large windows provided natural light for about 12 h day⁻¹.

In order to elicit reproduction and synchronize the reproductive cycles, the lizards were overwintered in the laboratory using a standard protocol (Gavaud, 1983; Heulin et al., 2005). On 1 October, lizards were placed in small boxes containing damp sand and wet moss, and these boxes were placed in a dark chamber (brand Liebherr, Dortmund, Germany) that was cooled progressively from 10 to 4°C during the first week, and then maintained at a constant 4°C thereafter. The lizards were removed from this chamber and placed in separate terraria after 3 months (males) or 4 months (females). At this time, all females are in a non-reproductive state (i.e. prior to the onset of vitellogenesis or uterine proliferation) (Heulin et al., 2005). During the third week post-emergence, females were allowed to copulate with males for 2–3 days.

Experimental design

First, to identify any phylogenetic (non-reproductive) differences in metabolism between the two populations, we measured, on the day following winter emergence and without having yet been fed, oxygen consumption (\dot{V}_{O_2} , in ml min⁻¹) of all lizards (26 oviparous females, 24 viviparous females, and 20 males from each population). Individuals used for \dot{V}_{O_2} measurements at winter emergence had the following body characteristics: viviparous population males ($N=20$; SVL=53.6±2.8 mm; $M_b=3.14±0.49$ g), oviparous population males ($N=20$; SVL=51.5±2.3 mm; $M_b=2.84±0.43$ g), viviparous females ($N=24$; SVL=56.1±3.7 mm; $M_b=2.75±0.45$ g), oviparous females ($N=26$; SVL=54.4±3.7 mm; $M_b=2.48±0.62$ g).

After these initial \dot{V}_{O_2} measurements, we focused solely on reproductive females. Several, mainly small individuals (five oviparous females and 11 viviparous females) did not reproduce or failed reproduction and were removed from this second analysis. Therefore, only a subset of 21 oviparous females (SVL=54.33±3.54 mm; $M_b=2.46±0.55$ g) and 13 viviparous females (SVL=56.38±3.95 mm; $M_b=2.85±0.45$ g) of the initial sample of females was used to survey oxygen consumption changes throughout reproduction. The second \dot{V}_{O_2} measurement (and an associated 48 h of fasting) was performed 3 weeks after the first measurement to avoid compromising the onset of reproduction (including copulation). During the following weeks up to ovulation, \dot{V}_{O_2} was measured approximately every 8–10 days. The total number of measures performed during the pre-ovulation period varied between females and ranged between 1 and 4 (1.94±0.89).

Ultrasound monitoring and identification of reproductive stages

After each \dot{V}_{O_2} measurement, the female underwent an ultrasound (Sonosite MicroMaxx, Inc., Bothell, WA, USA) examination to determine when the

female went from the vitellogenic stage (spherical follicles in the middle of the coelomic cavity) to the post-ovulation stage (ellipsoidal ova aligned in the uterus) (Gilman and Wolf, 2007). This enabled us to identify for each female a vitellogenic stage (VIT) and, about 8–9 days later, an early post-ovulation stage (OV). Subsequent \dot{V}_{O_2} measurements were performed during the early egg-retention stage (ER1, in both parity modes), prolonged egg-retention stages (ER2 and ER3, only in viviparous females), and a final post-parity stage (PP, in all females; see Table 1 for dates). PP measurements were obtained 3 days after partition (i.e. after 24 h of feeding followed by 48 h of fasting). Cages were inspected daily for egg laying and parturition. At egg laying, we dissected the embryo from one egg and weighed it (± 1 mg) to estimate the total embryo mass of each clutch (embryo mass \times number of eggs). We also weighed all viviparous neonates to calculate total litter mass at parturition for viviparous females.

Oxygen consumption

Lizards were fasted for 48 h prior to each metabolic measurement. We estimated standard metabolic rate by measuring oxygen consumption using closed-system respirometry. We performed all trials overnight (approximately 19:00–08:00 h) in a dark climatic chamber (brand Liebherr, Dortmund, Germany), because it was previously shown that *Z. vivipara* stop all activity when placed in the dark (Cragg, 1978). All trials were carried out at $30 \pm 0.5^\circ\text{C}$, which broadly corresponds to the preferred body temperature of reproductive females (LeGalliard et al., 2003; Rodríguez-Díaz et al., 2010). Lizards were placed individually into test containers (1000 ml) within the chamber and were allowed to acclimatize for 1 h before beginning a trial. A baseline air sample (two 140 ml syringes) was collected at the onset of the trial, and the test container was then carefully sealed. Trial duration was set to achieve adequate oxygen suppression based on preliminary trials (mean = 14 ± 1.13 h). A final sample of air was collected with two 140 ml syringes connected to a stopcock. Oxygen concentration (% O_2) of the samples was determined using an O_2 analyser (FOXBOX, Sable Systems, Las Vegas, NV, USA). Using an infusion pump (KDS 210, KD Scientific Inc., Holliston, MA, USA), samples were sent at a constant flow (60 ml min^{-1}) through a column of Drierite and then to the O_2 analyser, which was calibrated before each trial using outdoor air. Oxygen consumption (\dot{V}_{O_2} , in ml min^{-1}) was calculated as: (final % O_2 – initial % O_2) \times exact chamber volume (ml)/trial duration (min).

Metabolic parameters

In addition to \dot{V}_{O_2} , we calculated other relevant parameters. First, we estimated the increase in oxygen consumption attributable to reproduction (TIOC, in $\text{ml } O_2$) and total oxygen consumption ($\text{ml } O_2$), by integrating changes in oxygen consumption over time. TIOC corresponds to the surface area between a line joining successive \dot{V}_{O_2} measures (considering all measures for each individual) and a line that connects the pre-reproductive and post-parity values. Total oxygen consumption corresponds to the surface area between a line joining successive \dot{V}_{O_2} measures (considering all measures for each individual) and zero. We also calculated separately pre-ovulation TIOC (from winter emergence to ovulation) and post-ovulation TIOC (from ovulation to ER1 in oviparous females; from ovulation to ER3 in viviparous females). Our TIOC values are merely estimates because our measurements were not taken on the precise days of the onset of vitellogenesis, ovulation and partition, and we assume a linear change between our measurements. However, these estimates are sufficient for the comparative purposes for which they were used.

In order to estimate MCP (maintenance cost of pregnancy), we calculated for each female $\Delta\dot{V}_{O_2}$ ($\text{ml } O_2 \text{ min}^{-1}$) as the difference between pre-parity \dot{V}_{O_2} (ER1 in oviparous females; ER3 in viviparous females) and post-parity \dot{V}_{O_2} (PP). $\Delta\dot{V}_{O_2}$ corresponds to the fraction of oxygen consumption due to embryonic metabolism (EM, depending on the total amount of embryonic tissue) and also to a specific (non-somatic) maternal metabolism (MCP) required to support the embryos *in utero*. To estimate MCP, we regressed individual females' $\Delta\dot{V}_{O_2}$ against their litter mass (viviparous females) or embryo mass (oviparous females). MCP values correspond to the y -intercepts (when embryo mass is 0) of the regression lines (see Beuchat and Vleck, 1990; Schultz et al., 2008; Van Dyke and Beaupre, 2011; Yue et al., 2012; Dupoué and Lourdais, 2014).

Statistical analyses

All statistical analyses were performed with R software (The R Foundation for Statistical Computing, version 3.0.1). The data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (F -test) and log transformed when required to meet the assumptions of parametric testing prior to analysis. All linear models or linear mixed models were performed with packages lme4, car and multcomp.

Oxygen consumption at winter emergence

At winter emergence, we compared \dot{V}_{O_2} (and its allometric relationship to body mass $\dot{V}_{O_2} = bM_b^a$) between all individuals (males and females) from the oviparous and viviparous populations. We used a linear model on log-transformed data and performed analysis of covariance (with log body mass as a covariate) to test for differences between groups (sex and reproductive modes) and for interaction effects.

Changes in body mass and determinant of fecundity

We used a linear mixed model to analyse body mass change during reproduction. This analysis was conducted separately for each reproductive mode (considering all stages). Reproductive stage was treated as a fixed factor and female identity as a random factor to take into account the repeated measures on females. Degrees of freedom for the F -statistic were calculated using a Kenward–Roger correction, which is the most suitable for linear mixed models (Bolker et al., 2009). To compare fecundity between reproductive modes that had different masses, we used a linear model with reproductive mode as a fixed factor and body mass as a covariate (ANCOVA).

Changes in oxygen consumption during reproduction

We used a linear mixed model to analyse metabolic change during reproduction. This analysis was first conducted separately for each reproductive mode (considering all stages). Reproductive stage was treated as a fixed factor and female identity as a random factor to take into account the repeated measures on females. Degrees of freedom for the F -statistic were calculated using a Kenward–Roger correction.

To compare oviparous and viviparous females we used the same statistical approach as described above (linear mixed model, female identity as a random factor), treating reproductive mode and stages as fixed factors but restricting the analysis to stages shared by the two groups (i.e. PR, VIT, OV, ER1 and PP; see Table 1). We also compared oxygen consumption in the two groups just before partition (i.e. stage ER1 in oviparous females and ER3 in viviparous ones) using a similar procedure.

Female body mass changes dramatically over reproduction, and we were not able to evaluate the respective masses of their metabolically active (maternal soma, developing embryos) versus metabolically inactive (yolk, eggshell, water incorporated at various rate in the fertilized ova) compartments at each reproductive stage. However, we used initial body mass (which was not significantly different from post-reproductive body mass) as a co-factor in order to account for size variation within and between reproductive modes.

We compared pre- and post-ovulation TIOC between reproductive modes using a linear mixed model treating initial body mass as a co-variate, reproductive mode (oviparous versus viviparous) and stage (pre-ovulation versus post-ovulation) as fixed factors, and female identity as a random factor. We also used a linear model to test for an influence of fecundity on TIOC. Fecundity was calculated as residuals from the regression of clutch size or litter size against female initial body mass. We performed an ANCOVA for each reproductive stage (i.e. pre-ovulation and post-ovulation) with reproductive mode as a fixed factor and mass-adjusted fecundity and initial body mass as co-variables.

Metabolic costs of pregnancy

Finally, we evaluated MCP for each reproductive mode. To do so, we calculated the linear regression of the relationship between either litter mass (viviparous females) or embryo mass (oviparous females) and $\Delta\dot{V}_{O_2}$, and we then tested whether the MCP (intercept of the regression line) was significantly different from zero using a one-tailed t -test. All pairwise comparisons were conducted using Tukey's *post hoc* tests (package multcomp). All data are presented in the text and Table 1 as means \pm s.d. In

Fig. 2, data are represented as means±s.e.m. All results were tested for statistical significance at the $P>0.05$ level.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.F. conceived and designed the experiment, captured the experimental animals, executed the experiments, analysed and interpreted the findings and drafted and revised part of the article. O.L. conceived and designed the experiment and drafted and revised part of the article. D.F.D. conceived and designed the experiment and drafted and revised part of the article. B.H. conceived and designed the experiment, captured the experimental animals, analysed and interpreted the findings and drafted and revised part of the article.

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