



Embryonic water uptake during pregnancy is stage- and fecundity-dependent in the snake *Vipera aspis*



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ABSTRACT

Water is a crucial resource that can profoundly impact the biology of terrestrial organisms. Early life stages are particularly sensitive to hydric constraints because water uptake is an important component of embryonic development. While amniotic eggs constitute a key innovation to terrestrial life, many vertebrates are viviparous wherein the mother must be the source of water for her developing embryos. Since most viviparous squamates are lecithotrophic (i.e., energy is supplied to the offspring as yolk deposited into pre-ovulated follicles), water is the predominant resource allocated from the mother to the offspring during development. Contrary to energy that can be stored (e.g., as fat reserves), water typically cannot be acquired in advance. Therefore, the embryos' need for water can impose significant constraints on the pregnant female. We detailed water flux during pregnancy in a viviparous snake, the aspic viper (*Vipera aspis*). We found that embryonic water uptake occurred mostly during the second half of pregnancy—a period dominated by somatic growth. We also found that, somewhat unexpectedly, changes in female plasma osmolality were negatively related to fecundity. This latter result suggests that water consumption by the female is especially important for large litter sizes, and thus may suggest an important sensitivity of reproductive females to environmental water availability.

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1. Introduction

Variation in environmental resources can have profound influences on physiological traits, tradeoffs, and life history traits (O'Connor et al., 2006). Water is a critical resource that can exhibit important seasonal fluctuations and is known to affect terrestrial organisms' physiology (Bradshaw, 1997), growth (Lorenzon et al., 2001), and survival (Shine and Brown, 2008; McKechnie and Wolf, 2010). Embryonic life is particularly sensitive to water availability, and eggs are sensitive to dehydration (Du, 2004; Stein and Badyaev, 2011). For instance, water supply is critical for the conversion of stored energy reserves (yolk) to embryonic mass (Vleck, 1991; Thompson and Speake, 2003; Belinsky et al., 2004). Therefore, water limitation can profoundly alter embryonic development and affect offspring quality or result in embryonic death (Brown and Shine, 2005; Lourdais et al., 2007). Such negative impacts have favored the emergence of multiple adaptations, including the selection of an appropriate nesting site and/or parental care of the eggs to minimize water loss (Shine, 2004a; Shine and Brown, 2008; Stahlschmidt and DeNardo, 2010). The eggshell's structure can also be modified either to minimize water loss (hard-shell eggs are laid in a desiccating atmosphere) or, conversely, favor water uptake (parchment shell eggs

laid in a humid environment or substrate) (Deeming and Ferguson, 1991; Shine and Thompson, 2006).

While most terrestrial organisms are oviparous, viviparity has emerged on multiple occasions in amphibians, non-avian reptiles, and mammals (Blackburn, 2000; Shine, 2004b). These repeated transitions are associated with a diversity of embryonic nutrition strategies that have attracted considerable interest (Blackburn, 2006, 1999). Despite the focus on energy allocation, a viviparous female must also be the source of water for embryonic development (Thompson, 2007), and this facet of maternal resource provisioning has multiple implications to consider (Oftedal, 2002). For instance, contrary to energy that can be stored (as body fat) to prepare for energy investment during reproduction (Lourdais et al., 2002b), water typically cannot be accumulated in advance. Therefore, water availability in the environment must match the timing of the reproductive requirement for water. In many environments, water may well be more constraining than energy during reproduction because gestation often occurs during dry summer months (Lourdais et al., 2004a; Le Gaillard et al., 2012). Water limitation may alter reproductive success either by inducing embryonic mortality or by affecting offspring quality (Dauphin-Villemant and Xavier, 1986; Ross and Desai, 2005). Importantly, water demand during reproduction should be directly related to reproductive effort since an increase in the number of developing embryos increases water demand. Therefore, increased fecundity should either increase female water acquisition from the environment or cause greater hydric deficit in the female.

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Despite the likely importance of water in influencing female behavior, physiology, and fecundity, maternal water flux remains largely overlooked. Squamate reptiles are unique among vertebrates for the vast number of times that viviparity has independently evolved (>100, Blackburn, 2000; Stewart and Thompson, 2000). While some species show complex placentotrophy, most lizards and snakes are lecithotrophic where yolk reserves support the energy demands during embryonic requirement (Stewart and Thompson, 2000; Thompson and Speake, 2003; Van Dyke et al., 2014). Therefore, maternal resource demand during pregnancy is primarily focused on water. This situation offers a simple context to address the implication of water allocation independent of nutritional aspects. A previous study in the common lizard, *Zootoca vivipara*, suggested that water uptake is greatest during late embryonic stages when most somatic growth occurs (Dauphin-Villemant and Xavier, 1986). The same study also revealed an important impact of experimental water deprivation on reproductive success. To improve our understanding of water allocation, it is also critical to consider the impact of pregnancy and fecundity on female water balance.

We used a viviparous snake, the asp viper, *Vipera aspis*, to describe water flux during pregnancy. We used high-resolution ultrasonography to monitor embryonic volume and estimate water intake during development. We also monitored relevant maternal traits including body mass and plasma osmolality over pregnancy. Our main hypothesis was that embryonic water requirements should influence maternal water intake and water balance.

We tested the following predictions:

- (1) Water uptake by the embryos should mainly occur during the second half of gestation when embryonic somatic growth occurs.
- (2) Maternal mass change during pregnancy should reflect water intake and be closely related to the number of developing embryos.

2. Material and methods

2.1. Study species and maintenance

The asp viper, *V. aspis*, is a small viviparous snake of the western Palearctic region. This species is a typical capital breeder and thus accumulates energy needed for reproduction over an extended period before engaging in a reproductive effort (Bonnet et al., 2002; Lourdais et al., 2002b). Pregnancy is a long process, lasting up to three months, and it is associated with an increase in thermal preference and precision, which necessitates an increase in thermoregulatory activities (Saint Girons, 1952; Naulleau, 1979; Ladyman et al., 2003) and modified escape tactics (Lorioux et al., 2013a). Reproduction in females is also associated with reduced movement and food intake (Saint Girons, 1952). The study species is lecithotrophic, and it has been shown that food intake after ovulation has no influence on reproductive output (Lourdais et al., 2002a)

2.2. Experimental design

We collected 42 pregnant females (12 in 2009 and 30 in 2010) in neighboring districts (Loire Atlantique, Maine et Loire, Vendée,) of west-central France. Reproductive status (late stages of vitellogenesis) was first determined by manual palpation of the abdomen and then confirmed in the laboratory with high-resolution ultrasonography (SonoSite microMaxx, Inc., Bothell, WA, USA). Body mass (± 1 g) and snout-vent length (± 5 mm) were recorded, and scale clipping was used to identify individuals.

Female vipers were housed in cages (100 cm \times 30 cm \times 35 cm) that had a thermal gradient (18 °C–41 °C) created by placing a 75 W incandescent light bulb over one side of the cage. The light was on for 6 h per day enabling basking from 10:00 to 16:00 h, but forcing body temperatures to drop to room temperature (18 °C) at night. Each cage contained a main shelter (half cylinder of polyvinyl chloride (PVC) pipe,

diameter = 15 cm, length = 37 cm, with two 3-cm circular openings in the sides) located at one end of the cage opposite from the basking zone and three secondary shelters (half cylinder PVC pipe, diameter = 15 cm, length = 25 cm) scattered throughout the cage to facilitate concealed movements to the basking zone.

Females were randomly assigned to one of the cages, some housing two females and some housing three as a result of space limitations. Importantly, no agonistic behavior was observed in co-housed individuals. Females were provided water *ad libitum*, but were not fed until parturition, since they typically do not eat during gestation (Lourdais et al., 2002a). Importantly, a pre-ovulation ecdysis occurs and provides a reliable indicator of the onset of gestation in this species (Lorioux et al., 2013b).

2.3. Variables measured

2.3.1. Embryonic volume

We monitored the change in volume of the embryonic unit (i.e., embryo, yolk, and extra-embryonic membranes) to estimate embryonic water uptake during pregnancy. Using high-resolution ultrasonography (see Lorioux et al., 2013b), we determined total volume at three different stages of embryonic development (Hubert and Dufaure, 1968): “ovulation” (-1.9 ± 0.7 days before ecdysis), mid-pregnancy (29.8 ± 0.9 days after ecdysis; $37.6 \pm 1.2\%$ of developmental duration), and late pregnancy (55.5 ± 1.3 days after ecdysis; $70.6 \pm 1.5\%$ of developmental duration). For each female, we collected sagittal view images of the most cranial and most caudal embryonic units and measured their heights (H), lengths (L), and general shape (see Maritz and Douglas, 1994). We estimated embryonic volume (cm^3) following the method described in Maritz and Douglas (1994), which considers embryonic unit L , H , and a coefficient associated with its form (F_{emb}): Embryonic volume = $\pi \times L \times H^2 \times F_{\text{emb}}$. For analysis, we averaged the volume of the two embryonic units measured at each stage for each female. For one female, the initial volume was not recorded and this individual was removed from the analysis.

2.3.2. Body mass (BM)

Change in BM has been well established as an estimator of water loss in squamate reptiles that are food-restricted (DeNardo et al., 2004; Lillywhite et al., 2008a, 2008b; Dupoué et al., 2014). BM was collected during early and late pregnancy stages and also after parturition. We calculated BM changes (g) between early and late pregnancy stages for all individuals.

2.3.3. Osmolality

We also measured changes in female plasma osmolality (Osmo), as this parameter is an effective indicator of hydration state (Peterson, 2002). For safety reasons, each snake was encouraged to enter its head and upper body into a clear plastic tube, and then blood was collected via cardiocentesis (Saint Girons et al., 1993). This variable was only measured in the 12 pregnant females collected in 2009, which had substantial variation in litter size (1 to 11). We collected blood samples (100 μL) using a 1 mL heparinized syringe and a 27-gauge needle. Blood samples were collected at early, mid, and late pregnancy and after parturition. Plasma osmolality ($\text{mOsm}\cdot\text{kg}^{-1}$) was measured from 10 μL triplicates (intra-individual variation <1%) as described in (Wright et al., 2013).

2.3.4. Litter traits

For each female, we recorded litter size (number of undeveloped ova, stillborn, and neonates), fit litter size (number of neonates only), litter mass (mass of undeveloped ova, stillborn, and neonates), and fit litter mass (mass of neonates only) (see details in methods of Lourdais et al., 2002a).

2.4. Statistics

All statistics were performed with Statistica 6.0. Changes in embryonic volume, body mass, and osmolality over time were analysed using repeated measures ANOVA using the successive measurement sessions as the repeated factor. We also analysed the variation in embryonic volume over pregnancy by fitting embryonic volume with developmental time. We tested three functions (i.e., linear, square, and exponential) and used Akaike's information criterion (AIC) to select the best model. When two models differed by less than two AIC, they were considered equivalent. Changes in body mass and in osmolality were examined using simple correlations analysis. Tukey's *post hoc* tests were conducted for 2×2 comparisons. Unless otherwise stated, values are reported as means \pm standard deviation.

3. Results

3.1. Embryonic volume

We detected significant variation in embryonic volume over time ($F_{2,80} = 151.68, p < 0.001$) with a clear increase over the three consecutive periods (mean embryonic volume $2.71 \pm 0.10, 4.90 \pm 0.25$, and $8.42 \pm 2.6 \text{ cm}^3$ for early, mid, and late pregnancy measures, respectively). All three stages were significantly different from each other (Tukey's *post hoc* tests, all p values < 0.001). Total volume change represented, on average, an increase of 210% of initial volume. When considering time from ovulation rather than period, we found that the increase in embryonic volume was non-linear and the best fit was obtained using an exponential curve (AIC = 476.5, 451.6, and 448.2, respectively, for linear, square, and exponential functions; Fig. 1).

3.2. Body mass

We observed a significant increase in body mass over pregnancy (107.79 ± 6.49 vs. $112.24 \pm 6.94 \text{ g}$ for initial and late measures; $F_{1,41} = 13.48, p < 0.001$). However, important variations were observed between females and mass change ranged from -11.2 g to $+30.2 \text{ g}$ (Fig. 2). Mass change was positively related to litter size

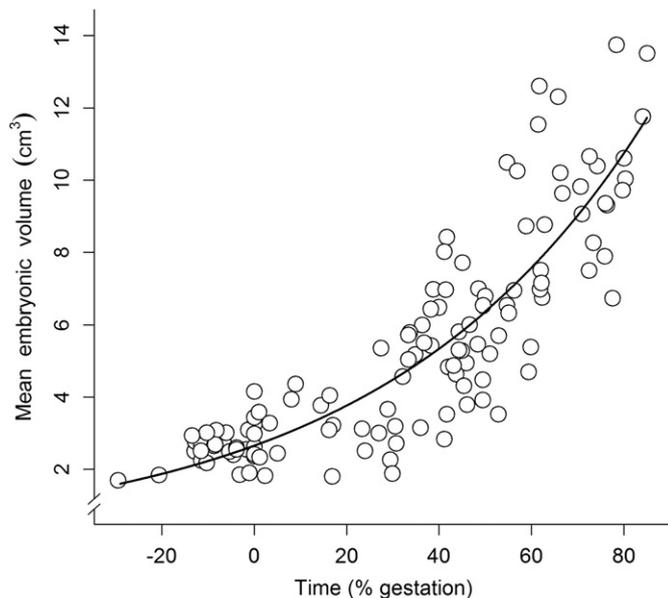


Fig. 1. Relationship between embryonic volume (cm^3) and time expressed as a proportion of gestation duration (%) from ovulation (0%) to parturition (100%) in 41 female aspik vipers (3 measures per female). Negative values correspond to pre-ovulation (vitellogenesis). This relationship was best described with an exponential function (equation: $y = 2.66 e^{0.017x}$). See text for statistics.

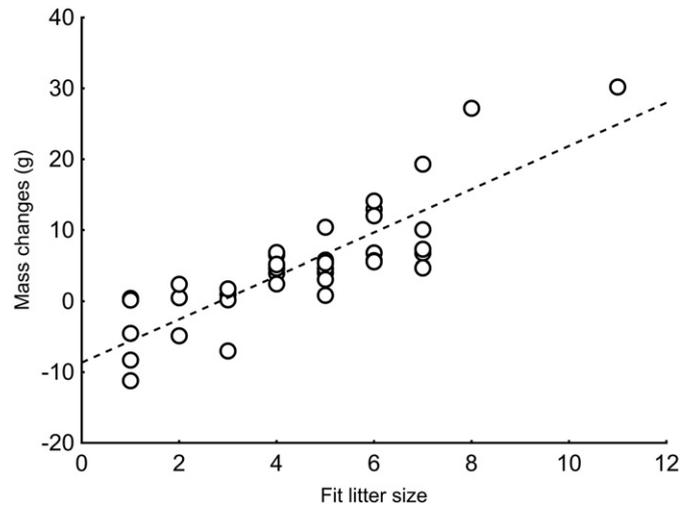


Fig. 2. Relation between fecundity (fit litter size) and changes in body mass during pregnancy ($r^2 = 0.71$, equation: $y = 0.84x - 6.87$).

($F_{1,40} = 25.71, p < 0.001, r^2 = 0.38$), but was better explained when considering fit litter size ($F_{1,40} = 94.77, p \leq 0.001, r^2 = 0.71$; Fig. 3). Mass change was also closely related to litter mass ($F_{1,40} = 43.32, p < 0.001, r^2 = 0.51$) and fit litter mass ($F_{1,40} = 74.39, p < 0.001, r^2 = 0.64$).

3.3. Osmolality

No significant differences were observed in absolute osmolality ($F_{1,11} = 1.47, p = 0.25$; 303.5 ± 9.9 vs. $309.9 \pm 14.4 \text{ mOsm.kg}^{-1}$, respectively, for early and late pregnancy). However, the change in osmolality over pregnancy was quite variable among females with values ranging from -24.0 to $+37.5 \text{ mOsm.kg}^{-1}$. Osmolality change was negatively related to body mass change ($F_{1,10} = 9.54, p < 0.010, r^2 = 0.49$). It was also negatively related to fit litter size ($F_{1,10} = 5.54, p = 0.04, r^2 = 0.36$) and fit litter mass ($F_{1,10} = 10.24, p = 0.009, r^2 = 0.51$; Fig. 3).

4. Discussion

Our study on the aspik viper provides significant insight into the dynamics of water transfer between mother and offspring, but also

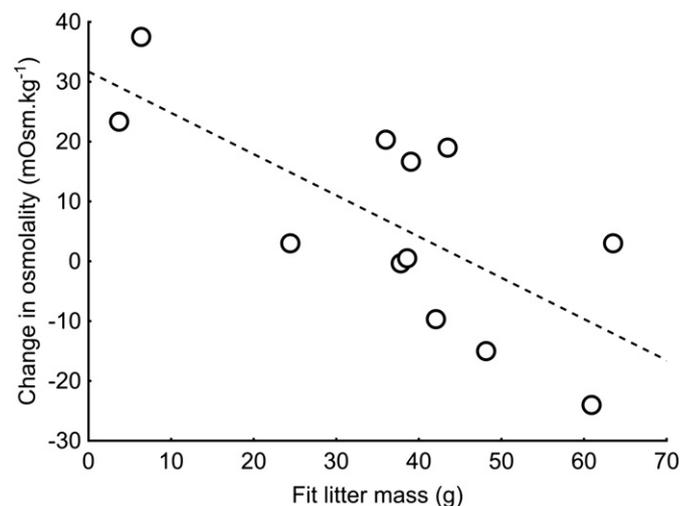


Fig. 3. Relation between the mass of neonates produced (fit litter mass) and changes in osmolality (mOsm.kg^{-1}) during pregnancy ($r^2 = 0.51$, equation: $y = -0.71x + 31.7$).

into the potential impact of embryonic water demands on maternal physiology and drinking behavior. We found that water uptake occurred mostly during the second half of pregnancy and was closely related to the number of developing embryos. We discuss our results in details below.

High-resolution ultrasonography has been previously used to assess reproductive output in oviparous snakes (Stahlschmidt et al., 2011) and lizards (Gilman and Wolf, 2007). Using this technique in *V. aspis*, we revealed massive changes in embryonic volume over pregnancy (+210%). Because the study species is lecithotrophic and most of the nutrients are allocated prior to ovulation, changes in embryonic volume can therefore be attributed to water uptake. Examining embryonic volume relative to developmental time revealed that water uptake was not linear but rapidly increased during the second half of pregnancy. This pattern is likely related to exponential somatic growth that occurs mainly during the last third of development (Dauphin-Villemant and Xavier, 1986; Andrews, 2004) and requires water uptake to convert yolk reserves to embryonic tissue (Vleck, 1991; Thompson and Speake, 2003). Our final measures of embryonic volume were made at, on average, 70% of developmental time. Therefore, we likely underestimate total water uptake due to massive uptake that occurs during preparturition stages.

Estimates of embryonic water uptake during gestation are only available for a few lecithotrophic vertebrates. For example, Guallart and Vicent (2001) reported significant water uptake during gestation (+99% to 101%) in the gulper shark. In the common lizard, *Z. vivipara*, Dauphin-Villemant and Xavier (1986) reported a +266% increase in embryonic wet mass that was directly related to changes in water content. In six other lecithotrophic squamates, (Blackburn, 1999) reported somewhat smaller wet mass increases of the conceptus (from +51% to 142%) while dry mass decreased over time. Two studies reported significant changes in egg volume in oviparous squamates at late stages of gravidity (Gilman and Wolf, 2007; Stahlschmidt et al., 2011). Therefore, water flux may also be relevant in oviparous species during the portion of embryonic development that occurs within the maternal body. Vleck (1991) suggested that the magnitude of water uptake by reptilian embryos exceeds the amount required for somatic growth, and that water uptake is also necessary in other embryonic compartments (i.e., amniotic fluids and allantois). Our measure of total embryonic volume likely encompasses all of these components.

Changes in body mass revealed a significant increase during gestation but also important variation among females (females with small litter sizes tended to lose mass). Changes in mass were closely related to the number or mass of developing embryos (see Fig. 3) underlying that water uptake in the embryonic compartment is responsible for the observed increase in mass. Meanwhile, pregnancy induces important metabolic costs in squamates (Dupoué and Lourdais, 2014; Foucart et al. 2014) and females often mobilize their remaining fat stores and structural proteins (Bonnet et al., 2001; Lourdais et al., 2004b; Dupoué and Lourdais, 2014). Mass change during pregnancy should be interpreted cautiously, since it integrates opposite processes (embryonic water intake and maternal mass loss). In contrast, plasma osmolality provides a reliable index of water balance in vertebrates (Peterson, 2002; Davis and Denardo, 2009). Our values were very similar to those reported in the field in the study species (Bradshaw, 1997). We found that females that gained body mass also experienced a significant drop in osmolality while the opposite pattern was observed in females that lost mass. Osmolality change was also negatively related to the number and mass of developing embryos, suggesting that females with high fecundity drank relatively more during pregnancy. In turn, important osmolality increases (+37.5 and +23.33 mOsm.kg⁻¹) were detected in two individuals with only one developing embryo (Fig. 3). These results could reflect variation in phenotypic quality (Beauplet et al., 2004; Stahlschmidt et al., 2013) with enhanced maternal water intake in more fecund individuals.

Maternal care has attracted considerable attention and encompasses crucial aspects such as provisioning nutrients and developmental temperature (Bernardo, 1996; Blackburn, 1999; Mousseau et al., 2009). While provisioning water is inherent to vertebrate viviparity, this facet of the female–offspring relationship remains largely understudied (Brace, 1995; Brace et al., 2014). Our study underlines the magnitude of water requirement and the impact of pregnancy on maternal water balance in non-constraining conditions. Contrary to energy that can be accumulated (e.g., fat reserves) to anticipate environmental variations (Bonnet et al., 1998), long-term storage options for water are limited (Davis and DeNardo, 2007). Water deprivation during pregnancy may induce reproductive failure and even result in maternal death (Hirschhorn et al., 1969). Therefore, a possible conflict for water may exist between maternal and embryonic needs (Dupoué et al., 2015) as previously documented for energy (Crespi and Semeniuk, 2004). Such a conflict is more likely at the end of gestation when embryonic water requirements are peaking.

Finally, our work is relevant in the framework of understanding the impacts of ongoing climatic change. The impact of temperature increase on activity budget and energy acquisition has attracted considerable interest (e.g., Sinervo, 2010). While precipitation changes are more complex to predict, the frequency of extreme climatic hydric events (prolonged drought) is expected to increase in the coming decades (IPCC 2014). We predict that reduced water availability during pregnancy will have a profound impact on reproductive performance in many viviparous vertebrates.

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