

The Evolutionary Economics of Embryonic-Sac Fluids in Squamate Reptiles

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ABSTRACT: The parchment-shelled eggs of squamate reptiles take up substantial water from the nest environment, enabling the conversion of yolk into neonatal tissue and buffering the embryo against the possibility of subsequent dry weather. During development, increasing amounts of water are stored in the embryonic sacs (i.e., membranes around the embryo: amnion, allantois, and chorion). The evolution of viviparity (prolonged uterine retention of developing embryos) means that embryonic-sac fluid storage now imposes a cost (increased maternal burdening), confers less benefit (because the mother buffers fetal water balance), and introduces a potential conflict among uterine siblings (for access to finite water supplies). Our data on nine species of squamate reptiles and published information on three species show that the embryonic-sac fluids comprise around 33% of neonatal mass in viviparous species versus 94% in full-term eggs of oviparous squamates. Data on parturition in 149 vivipars (*Vipera aspis*, a viviparous species) show that larger offspring store more fluids in their fetal sacs and that an increase in litter size is associated with a decrease in fluid-sac mass per offspring. Overall, the evolutionary transition from oviparity to viviparity may have substantially altered selective forces on offspring packaging and created competition among offspring for access to water reserves during embryonic development.

Keywords: gestation, offspring number, offspring size, relative litter mass, trade-off.

Introduction

Phylogenetic transitions in reproductive mode may impose novel selective forces on not only traditional life-history traits, such as offspring size and number, but also accessory characteristics that are important for offspring viability. For example, most life-history analyses focus on energy content of offspring as the critical currency, but reproducing females

must also allocate water reserves. Without water, it is impossible for an embryo to develop and transform yolk into somatic tissue (Noble 1991; Packard 1991; Thompson and Speake 2004). Importantly, those water reserves may be substantial in mass and volume relative to the embryo itself (Belinsky et al. 2004). Commonly, increased physical burdening of the pregnant female imposes significant costs (e.g., in her mobility and thus vulnerability to predation; Seigel et al. 1987), or fecundity is limited by maternal body volume (e.g., Vitt and Congdon 1978; Shine 1992). Under both of these scenarios, we expect selection to fine-tune maternal decisions about total mass and volume of uterine progeny (and thus allocation of water as well as energy into the developing litter). Despite the extensive literature on allocation decisions based on the energy content of offspring, the equivalent challenge in terms of water allocation has attracted very little scientific attention (Brown and Shine 2009; Dupoué et al. 2015).

The frequent phylogenetic transition from oviparity (egg laying) to viviparity (live bearing) in squamate reptiles (Shine 1985; Blackburn and Stewart 2011) provides a suitable model system in which to investigate these issues because that shift can be expected to cause a profound shift in optimal water-allocation tactics by reproducing females. In all terrestrial vertebrates, fetal membranes (amnios, allantois, and chorion) form the embryonic sac that provides the aquatic environment essential to the embryo (Ferner and Mess 2011). Embryonic-sac fluids (ESFs) prevent desiccation and offer the physical environment required for the development of the organs by limiting compression from surrounding tissues on the germ layer folds and growing tissues (Ostergard 1970; Packard and Packard 1980; Ferner and Mess 2011). Therefore, the cleidoic egg (i.e., a closed egg capable of retaining water) is considered to be a key innovation for the evolutionary success of terrestrial vertebrates. Whatever their reproductive mode (oviparity, viviparity), habitat (marine, terrestrial), and lineage (reptiles, birds, mammals), embryos of tetrapods develop using ESF contained within embryonic

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sacs. Although the nutritional and respiratory roles of the embryonic-sac membranes are well documented (Ostergard 1970; Packard and Packard 1980; Burton and Tullett 1985; Ferner and Mess 2011), the functions of ESF per se have been less intensively studied. ESF may play important roles in nutrition, respiration, immunity, and waste regulation and offer cushioning against physical shock (Ostergard 1970; Brace 1997). In late pregnancy, fetuses drink ESF to activate the development of the gastrointestinal tract and the maturation of the lungs (Mulvihill et al. 1985; Brace 1997). Premature loss, deficit, or excess of ESF (oligohydramnios, polyhydramnios) entail higher mortality and morbidity at birth (Chamberlain et al. 1984a, 1984b; Mercer et al. 1984; Hadi et al. 1994; Fedakar et al. 2016). The retention of fluid-containing embryonic sacs in all tetrapod lineages suggests important functional roles for these structures and for the fluids that they contain.

In squamate reptiles, the transition from oviparity to viviparity involves a continuum of intermediate stages rather than an abrupt transition (Shine 1983). Consequently, the role of ESF functions likely remains broadly similar in species of each reproductive mode, as expected if an aquatic environment is required for organogenesis. In oviparous squamate species, the embryo undergoes a substantial proportion of total embryonic development in the oviduct prior to oviposition (in contrast to birds, turtles, and crocodiles), and thus the first quarter (at least) of embryogenesis occurs under relatively similar conditions in oviparous and viviparous species. Within several species, females exhibit oviparity as well as viviparity, and viable offspring have been obtained through oviparous \times viviparous breeding (Heulin et al. 1989; Smith and Shine 1997). Further emphasizing the broad similarities in developmental biology across the two modes of reproduction, ontogeny and structure of the fetal sac membranes are similar between oviparous and viviparous populations of the same species (Stewart et al. 2004). The vast majority of squamate reptiles (viviparous as well as oviparous) are lecithotrophic, with most or all yolk provided at ovulation rather than continuously through gestation, and thus substantial amounts of water are needed to convert yolk into neonatal tissue (Thompson and Speake 2004). The continuum between oviparity and viviparity allows us to focus on specific questions about the role of water supplies for embryogenesis rather than confronting the issue of fundamental divergences in the roles of that fluid supply. We propose that although water fulfils similar physiological roles in supporting embryogenesis in oviparous and viviparous squamates, the transition in reproductive modes has generated a strong shift in the costs and benefits associated with the storage of ESF.

An egg in an external nest benefits from the uptake of substantial water reserves that facilitate embryogenesis and buffer against abiotic challenges (Belinsky et al. 2004; Brown

and Shine 2005). Thus, increased egg size and mass are likely to enhance rather than reduce egg viability. In contrast, an embryo developing inside the maternal oviduct is under major size constraints. Finite space within the oviduct and body cavity means that total mass (or volume) of the clutch may limit maternal reproductive output (Olsson and Shine 1997) or mobility (Shine 2003a). Hence, the transition from oviparity to viviparity imposes a novel suite of pressures on per-offspring total mass and volume (rather than simply energy content, as in oviparous ancestors). Viviparity has evolved from oviparity in more than 100 lineages of lizards and snakes (Blackburn 2015a; Griffith et al. 2015; King and Lee 2015), providing an opportunity to examine this phenomenon in multiple taxa.

Below, we briefly review relevant literature on water relations of squamate eggs. Both field and laboratory data show that parchment-shelled eggs of squamate reptiles take up considerable volumes of water from the incubation medium (Vleck 1991; Deeming and Unwin 2004). That uptake is critical for embryonic survival (Vleck 1991; Warner and Andrews 2002; Aubret et al. 2003), and even minor variation in water availability can influence not only hatching success but also phenotypic traits of the offspring, such as body size, shape, and locomotor ability (Gutzke and Packard 1987; Vleck 1991; Brown and Shine 2005, 2009; Delmas et al. 2008). Eggs can take up a large volume of water during a brief period of moist conditions and use that stored water to support longer-term development even if nest conditions become dangerously dry (Badham 1971; Packard 1991; Ji and Du 2001; Brown and Shine 2005). Presumably reflecting the advantages of such insurance water (for buffering against thermal extremes as well as to support conversion of yolk to somatic tissue; Vleck 1991; Du and Shine 2008) and the lack of any disadvantage to large water reserves, full-term squamate eggs contain substantial fluids (e.g., >75% of the mass of the hatchling from that egg; Belinsky et al. 2004; Thompson and Speake 2004).

The evolution of viviparity changes the selective pressures involved in the allocation of water to embryos. First, the availability of moisture around the egg is now under maternal control, allowing reproducing females to buffer temporal shifts in soil moisture levels (Warner and Andrews 2002). Thus, the selective advantage of amassing and retaining a large volume of fluid around the embryo is reduced. Second, because maternal body volume constrains total clutch mass and volume (Vitt and Congdon 1978; Shine 1992; Qualls and Shine 1995; Qualls and Andrews 1999), any increase in water reserves (above the minimum needed for embryogenesis) either reduces the maximum clutch volume that can be produced or increases physical burdening on the pregnant female. In combination with the first factor (reduced benefit of large water reserves), this pressure (costs of increased water reserves to maternal fecundity or mobil-

ity) should favor an evolutionary reduction in the amount of water provided to each embryo for its development. Last, the shift to intrauterine development creates a potential conflict among siblings and between the offspring and their mother (Crespi and Semeniuk 2004; Dupoué et al. 2015). With total water allocation to the litter now constrained (by maternal abdominal volume or selection against excessive burdening), each offspring might benefit by obtaining a more than average share of those finite reserves.

Overall, although a minimal amount of ESF is vital for embryogenesis (Brace 1997), several factors could favor a reduction in the amount of water allocated to each embryo in viviparous species compared to their oviparous ancestors. Those factors broadly pertain to two main categories: a reduced need for insurance against external moisture fluctuations and a greater cost to increased maternal burdening. These ideas generate three testable predictions:

1. The amount (mass, volume) of embryonic-sac water per gram of offspring should be lower in full-term embryos of viviparous species of squamates than in embryos of oviparous taxa.

2. Larger body size of offspring should be positively associated with more water in embryonic sacs (because more water enhances embryogenesis, because larger offspring need more water, or because larger offspring can extract more water from the communal pool).

3. All else being equal, an increase in litter size should reduce per-offspring water reserves via competition among siblings.

We tested the first prediction by comparing the mean amounts of ESF in oviparous versus viviparous species. For that comparison, we complemented the limited information available in the literature with our own observations. We tested the two other predictions with a large data set ($N > 140$ females; $N > 1,000$ offspring) collected from a viviparous snake species. Our data on ESF mass at birth in >300 offspring enable us to quantify relationships between neonatal and ESF masses and to explore the consequences of possible conflict among offspring for water supplies during development.

Material and Methods

ESFs in Oviparous versus Viviparous Squamate Reptiles

We compiled published data on the mass of ESF (in grams) relative to neonatal mass at the time of birth or hatching in squamate reptiles (table 1). Data were derived from eggs artificially incubated from laying until hatching or through dissections of full-term embryos in utero in viviparous species. We also used unpublished data obtained by collecting free-ranging females and incubating their eggs in the laboratory (Deeming 1989; see table 1 for measurement details). Last, we used a large data set on reproduction in females of a

viviparous snake species (see below). Our data set includes species from a range of squamate lineages (e.g., pythons vs. geckos) sampled in very different places (Europe, Africa, Australia).

Relationships between ESF and Reproductive Output in the Aspic Viper

Study Species. The aspic viper (*Vipera aspis*) is a medium-sized (mean snout vent length = 60 cm) viviparous snake from Italy, Switzerland, and France. Females of this species do not reproduce every year; vitellogenesis takes place in spring following hibernation, with the 2.5-month gestation period occurring in summer and parturition occurring generally in late summer (Bonnet et al. 1994, 2001; Naulleau and Bonnet 1996). Mean litter size is six or seven offspring (range = 1–21; Naulleau 1976).

Data Collected at Parturition. A total of 149 gravid female *V. aspis* specimens were captured, marked, and monitored during long-term population surveys in the field in western-central France (at three sites over the period 1992–2015; see methods in Bonnet et al. 2003). Animals were brought to the laboratory before parturition and were released with their offspring at the exact place of capture. Several ($N = 14$) of these females were also recaptured when gravid 2 or 3 years later (2.4 ± 0.5 years later; total number of captures of gravid females = 163). The females were measured (snout vent length [SVL] and total length [TL] ± 0.5 cm), weighed (body mass [BM] ± 1 g with an electronic scale), maintained in captivity in individual cages (for 14 ± 11 days on average), and reweighed prior to and immediately after parturition.

In a study of water allocation, the availability of water for the pregnant female is critical. We provided water ad lib. (freshwater bowl), and most females drank as soon as they were placed in their individual cages and then regularly during captivity. Our rationale for providing water was severalfold. First, to withhold water would be ethically unacceptable. Freshwater availability is often low in our study areas: there is no standing water supply, and rainfall and dew are rare during the period of late pregnancy (July–August). Thus, withholding water may have put pregnant females under increased stress, possibly killing the embryos. Second, providing water to the females allowed them to regain hydromineral balance (this process takes only a few hours; Maughan et al. 1996), enabling us to focus on the fluid-volume constraint. Third, freshwater is occasionally available in the field during late pregnancy in our study area (e.g., due to heavy summer rainfall), and nearby populations of aspic vipers have access to permanent water bodies (e.g., ponds, rivers). Thus, our study design simulates the most favorable situation in terms of freshwater availability for preg-

Table 1: A compilation of published and unpublished data on the mass of extraembryonic fluids relative to neonatal mass at the time of birth or hatching in squamate reptiles

Reproductive mode and species	EM (g)	ESF (g)	ESF %	Source
Oviparous:				
<i>Natrix natrix</i> (27)	3.7 ± .3	2.1 ± .3	71 ± 15	X. Bonnet, personal observation
<i>Coluber viridiflavus</i> (19)	6.2 ± 1.0	4.5 ± .2	87 ± 36	X. Bonnet, personal observation
<i>Elaphe longissima</i> (74)	9.6 ± 1.6	6.0 ± 1.4	76 ± 21	X. Bonnet, personal observation
<i>Python regius</i> (14)	72.2 ± 6.3	49.2 ± 13.1	61 ± 64	X. Bonnet and F. Aubret, personal observation
<i>Python regius -bis</i> (14)	106 ± 102	...
<i>Natrix tessellata</i> (31)	6.74 ± .9	7.3 ± 4.7	146 ± 95	Dmi'el et al. 1993
<i>Elaphe obsoleta</i> (2)	15.6	14.6	122	Deeming 1989
<i>Eublepharis macularius</i> (5)	3.2 ± .2	2.7	96	Deeming 1989
Viviparous:				
<i>Vipera aspis</i> (315)	6.5 ± 2.7	2.1 ± 1.0	33 ± 15	This study
<i>Austrelaps ramsayi</i> (7)	5.5 ± .7	1.9 ± .7	34 ± 10	Shine 1978
<i>Suta dwyeri</i> (2)	3.7	1.21	32	Shine 1978
<i>Notechis scutatus</i>	6.2 ± 1.0	1.6 ± 1.0	25 ± 11	Shine 1978
<i>Pseudechis porphyriacus</i> (8)	22.9 ± 2.7	11.8 ± 2.7	51 ± 6	Shine 1978

Note: For seven oviparous species, data were derived from eggs artificially incubated from laying until hatching. For five viviparous species, data were collected at parturition (this study; *V. aspis*) or through dissections of full-term embryos in utero (raw data from Shine 1978). Sample sizes (number of litters or clutches) are indicated in parentheses. EM = egg mass prior to hatching; ESF = embryonic-sac water content (calculated as EM minus hatchling mass). Shell mass was excluded from the calculation of ESF in *E. obsoleta* and *E. macularius* (note that shell mass represented only 4%–6% of the EM). ESF % was expressed relative to hatchling mass. Artificially incubated eggs gained mass during incubation, except in pythons. Thus, ESF was calculated in two ways for *P. regius*: the usual method and also when water loss during incubation was taken into account (shown as *P. regius -bis*). EMs were recalculated from table 1 in Dmi'el et al. (1993) using the initial egg mass + water mass change during incubation. For the gecko (*E. macularius*), mean (\pm SE) was available for EM but not for ESF. Incubation conditions differed markedly among studies (see references).

nant females. Although water-deprived pregnant aspics allocate water to their embryos at the expense of their own reserves (Dupoué et al. 2015), water nonetheless remains a limited resource for the embryos due to space limitation (and their osmotic capacity to absorb and retain water in their embryonic sacs; Kilpatrick et al. 1991).

We obtained information on 1,030 offspring (on average 6.33 ± 2.29 per female; 826 healthy offspring, 92 stillborn eggs, and 112 undeveloped eggs). The mass of each reproductive item (e.g., offspring, egg) was recorded; the SVL and TL of healthy offspring were measured.

In a subset of 89 litters ($N = 82$ different females of which seven produced two litters each), we witnessed parturition directly and thus were able to collect 315 neonates that were still inside their fetal membranes (on average 3.54 ± 2.02 offspring per litter; range = 1–11). We first weighed the intact fetal sac (see Kim and Blackburn 2015 for anatomical details) containing the neonate (± 0.1 g) and then reweighed the neonate (patted dry with absorbent paper) after it ruptured the sac and emerged. Because the dry mass of the fetal sac membranes (+ umbilical cord) was negligible (<0.1 g), the difference between the two masses represents the fluid contained in the amnion and allantois (hereafter referred to as ESF). Where the amniotic and allantoic sacs were ruptured before we could weigh them, we estimated the total mass of the ESF of each litter by multiplying the

mean measured ESF of that litter by the total number of offspring (excluding undeveloped eggs). The structures represented by amnion, allantois, umbilical cord, or ESF are routinely qualified as “extraembryonic,” “accessory,” or “annexes.”

In the remaining 74 parturitions (i.e., 163 captures of gravid females minus 89 cases where parturition was witnessed), birth was not observed directly, and the litter was collected 1–12 h later. The mass of the ESFs was then estimated as follows: preparturition maternal mass – (postparturition maternal mass + litter mass). To evaluate the accuracy of this method, we performed the same calculation on the 89 litters for which direct measurements were also available. All data are deposited in the Dryad Digital Repository (Bonnet et al. 2017).

Analyses. Reproductive output was characterized using total litter size (LS), fit litter size (Fit-LS, excluding nonviable offspring; Bonnet et al. 2000), and offspring size (SVL, TL, and BM of offspring). Offspring mass was considered as the main dependent variable in most analyses because it offers a robust measure of offspring quality in snakes (Shine 2003b). Offspring mass and SVL were highly correlated ($r = 0.75$, $P < .001$), and analyses based on either variable gave similar results. Relative litter mass (RLM), a commonly used index of maternal reproductive effort (Vitt and Congdon 1978), was described as the ratio of litter mass divided

by postparturition maternal mass (Bonnet et al. 2003); however, maternal mass was included as a covariate in the analyses or total litter mass to avoid spurious results when using ratio. RLM was our second dependent variable examined.

We used generalized linear models (GLMs) to assess the relative importance of maternal body size, offspring number, and ESF on the dependent variables, offspring mass, and RLM. Maternal SVL strongly influences fecundity in snakes (Shine 2003b), so it was included in the null model; other traits (litter size, ESF) were then progressively added. We compared Akaike information criterion (AIC) values to select the best model. Because females were collected in three different sites and during different years, sites and years were also included as factors in the analyses. Because neither of these factors and their interactions contributed significantly to the model ($P = .96$ for years; $P = .45$ for sites), they were not retained in subsequent analyses. Similarly, mean annual precipitation during July and August (during gestation) in each site did not contribute significantly (directly or via interactions) to the results and was not included in the final models. Incomplete data (e.g., on maternal mass prior to parturition) for several females (litters) slightly reduced sample sizes for some analyses (see "Results"). GLMs were performed on reduced sample sizes for which all data were available. Removing data based on the few females represented more than once did not change any conclusions (e.g., when retaining data for only the first litter of each female); therefore, it was not necessary to control for maternal identity in the analyses (few females were represented more than once and thus did not bias the outcome). These analyses were performed with Statistica, version 12.0 (<http://www.statsoft.fr>).

Because maternal abdominal volume is finite, we expect to see a threshold effect when the total volume of the litter reaches this limit; ESF volume should be constrained in larger litters compared to small litters. The volumetric mass density of ESF is close to 1 (>99% water), and the volumetric mass density of neonates is slightly above 1 (reptile neonates consistently contain 78% of water [Belinsky et al. 2004], whereas in other main mineral compounds g/cm^3 is about 1.5). Therefore, we used mass (g) as a proxy of the volume (cm^3) occupied by the ESF.

Ethical Note. No animals were mistreated or injured during this study. All procedures were performed in accordance with French guidelines and regulations (permits and ethical approvals 09/346/DEROG, A79-001, and 79-157).

Results

Reproductive Mode and ESF in Squamates

The comparison between oviparous and viviparous squamate species revealed a consistent difference, with no over-

lap in the mean ESF values between reproductive modes (table 1). In all oviparous species, the mean proportion of ESF relative to offspring mass was above 60%, with an average of 94% (excluding one outlier record for the *Python regius-bis* value [106%]; table 1). In contrast, viviparous taxa had ESF values <51%, with an average of 35% (adjusted Mann-Whitney U -test $Z = 2.84$, $P < .01$). The variance in ESF was higher in oviparous than in viviparous species: 913.8 versus 92.5 (coefficients of variation were 32% and 27%, respectively).

ESF and Reproductive Output in the Asp Viper

Measured versus Estimated ESF. Total ESF per litter (directly measured in 315 neonates and calculated for 89 litters) strongly correlated with total ESF, as estimated from the difference between pre- and postparturition maternal mass plus litter mass ($r = 0.91$, $r^2 = 0.83$, $P < .001$; fig. 1). The coefficient ($b = 0.91 \pm 0.04$ [SE]) and intercept (1.40 ± 0.71 [SE]) suggest that both methods provided similar results, with the estimated total ESF slightly higher than the measured total ESF (fig. 1). We thus used estimated ESF as a proxy of the actual ESF.

Mean ESF per Neonate and per Litter. The mean ESF directly measured at parturition in 315 neonates was 2.10 ± 0.96 g and ranged between 0.30 and 7.70 g per neonate. On average, this comprised $32.75\% \pm 15.30\%$ of neonate mass (i.e., ESF/BM %). The mean ESF per neonate estimated in 147 litters was 2.74 ± 1.44 g.

For entire litters, the mean total measured ESF was 12.78 ± 7.44 g ($N = 89$) whereas the mean total estimated ESF was 14.70 ± 8.47 g ($N = 152$), representing $20.77\% \pm 11.49\%$ and $21.16\% \pm 11.71\%$, respectively, of postpartum maternal body mass (i.e., ESF/maternal postpartum BM %).

Determinants of Offspring Mass. Use of measured versus estimated ESF of each litter did not change any conclusions from our analyses of influences on mean offspring mass. For simplicity, we present only the results from 147 litters where ESF was estimated. Model selection based on AIC suggested that the best model included maternal body size (SVL), litter size (LS), and total ESF (table 2). Including mean ESF per neonate instead of total ESF per litter did not change the results (indeed, the contribution of LS then became nonsignificant because mean ESF per neonate already includes LS; see "Material and Methods").

The estimated parameters of the best model indicate that mean offspring mass was positively influenced by maternal SVL and total ESF and was negatively influenced by offspring number (LS; table 3). This negative relationship suggests a trade-off between offspring number and the overall

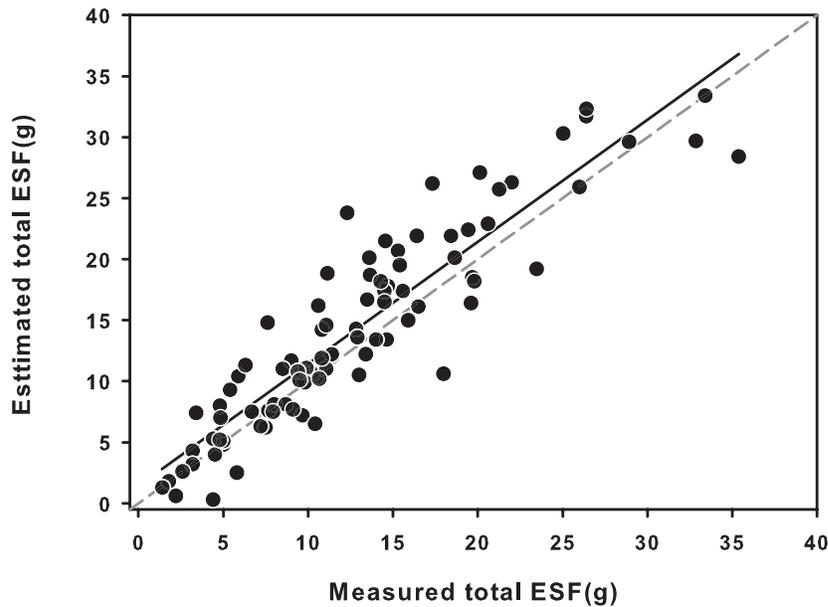


Figure 1: Correlation between measured embryonic-sac water content (ESF) and estimated ESF per litter for 88 litters of aspik vipers for which direct data were available. The solid black line indicates the fitted linear regression ($r = 0.91$, $P < .001$). On average, estimated ESF values were slightly above measured values of ESF (the dashed gray line indicates equivalence).

mean mass of the offspring (i.e., the combination of neonate body mass plus ESF). This issue is further explored below.

Influence of Offspring Number on Neonatal Mass and ESF.

We did not find a significant negative correlation between mean offspring mass and offspring number using either litter size ($r = -0.072$, $P = .395$) or fit litter size ($r = -0.043$, $P = .615$). Including maternal body size in the analysis had a significant effect (multiple $r = 0.252$, $P = .011$), but offspring number had much less of an effect ($b(\text{LS}) = -0.154$, $P = .079$) than did maternal SVL ($b(\text{SVL}) = 0.255$, $P = .004$).

Mean ESF of neonates was negatively correlated with offspring number regardless of whether we used total litter size ($r = -0.174$, $P = .040$) or fit litter size ($r = -0.222$, $P = .007$; fig. 2) as our measure of fecundity. Including maternal body size improved the model (multiple $r = -0.243$, $P = .015$) and increased the negative contribution of offspring number ($b(\text{Fit-LS}) = -0.250$, $P = .004$) compared to the effect of maternal SVL ($b(\text{SVL}) = 0.103$, $P = .227$). Overall, then, an increase in offspring number reduced the mass of ESFs more than it reduced offspring body mass. Including maternal SVL ($b = 0.181$), offspring number (Fit-LS; $b = -0.280$), and total ESF ($b = 0.299$) of the litter further improved the model to explain variations of neonate mass (multiple $r = 0.317$, $P < .003$).

Importantly, the range of variation in ESF decreased with increasing litter size (see the dotted line in fig. 2). Maximal

ESF per offspring was maintained above a line with a negative slope that delimited a wide empty space that progressively increased with litter size (fig. 2). Despite a large sample size, we found no embryos with high values of ESF from large litters. This threshold effect is exactly what we would expect from a constraint on maternal abdominal volume. Thus, we further explored this threshold effect by randomly reassigning values from ordinates to abscissas to calculate the probability (by chance) of observing a lack of data above

Table 2: Comparison of generalized linear models to explain variation in mean offspring mass and relative litter mass in aspik vipers

Dependent variable and model	df	AIC
Mean offspring mass:		
SVL	145	424.61
SVL + LS	144	422.98
SVL + ESF	144	425.46
SVL + LS + ESF	143	417.64
Relative litter mass:		
SVL	150	1,359.95
SVL + LS	149	1,254.79
SVL + ESF	149	1,288.18
SVL + LS + ESF	148	1,230.42

Note: $N = 147$ and 152 litters, respectively. AIC = Akaike information criterion; ESF = total embryonic-sac water content of the litter; LS = litter size; SVL = maternal snout vent length. Note that for relative litter mass, restricting the analysis to 147 litters (i.e., those with viable offspring only) did not change the results.

Table 3: Parameters estimated in the best models to explain variation in the mean mass of the neonates and relative litter mass in aspic vipers

Dependent variable and effect	Estimations	SE	Wald statistic	P
Mean offspring mass:				
Intercept	1.3956	.1563	79.7571	<.001
SVL	.0106	.0034	9.9998	.002
ESF	.0053	.0019	7.5406	.006
LS	-.0237	.0073	10.3768	.001
Relative litter mass:				
Intercept	5.2161	.2625	394.99	<.001
SVL	-.0416	.0060	47.0398	<.001
ESF	.0145	.0028	27.2291	<.001
LS	.0935	.0110	71.6726	<.001

Note: $N = 147$ and 152 litters, respectively. ESF = total embryonic-sac water content of the litter; LS = litter size; SVL = maternal snout vent length. Δ AIC between the best and the second-best models (table 2) was 5.3 for offspring mass and 24.4 for relative litter mass.

the maximal limit (Crowley 1992; Guillemain et al. 1997). For 10,000 random reassignments the probability was <0.001 . To ensure that these analyses were not compromised by a potential statistical artifact (using offspring number in both X- [Fit-LS] and Y- [total estimated ESF/Fit-LS in each litter] axes), we repeated the procedure using ESF as measured

in each of the 315 offspring where the intact fetal sac was weighted at birth (i.e., removing the Fit-LS term from the Y-axis). The threshold effect was confirmed: variation in ESF values was observed only in small litters, and no value exceeded the threshold, as revealed using mean ESF values (reassignment probability < 0.001 ; fig. 2).

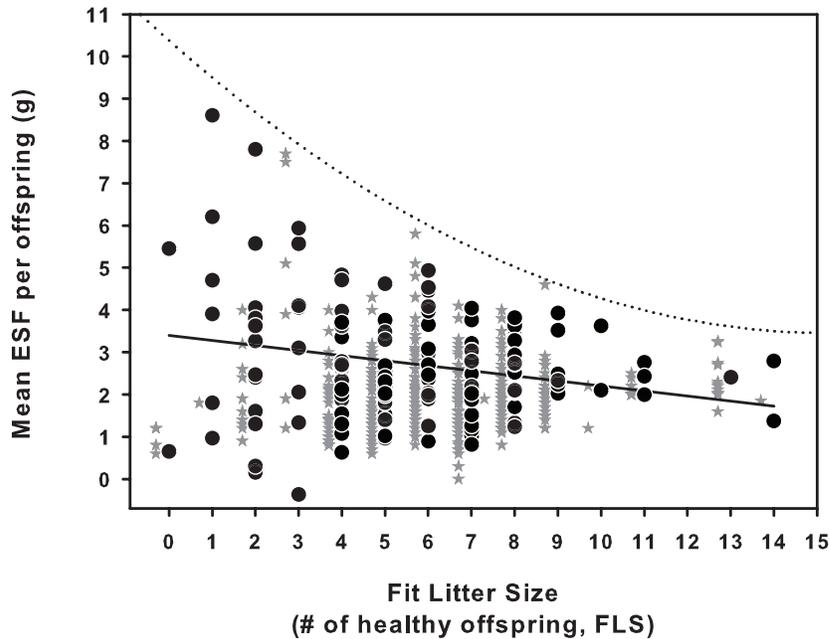


Figure 2: Relationship between offspring number (number of viable offspring at parturition; fit litter size [FLS]) and the mean embryonic-sac water content (ESF; black circles) estimated per neonate (offspring) in 147 litters of aspic vipers (one data point per litter). The solid black line indicates the linear regression fitted to these data ($r = -0.222$, $P = .007$). The dotted line indicates the threshold above which the mean ESF per offspring appears to be constrained by the abdominal volume of the mother. Gray stars show the values of ESF measured on 315 offspring at birth and hence depict the volume constraint on individuals without including offspring number in the calculation (thus controlling for possible statistical artifacts during ESF estimation). Note that measured values suggest that nonviable offspring (FLS = 0) do not accumulate ESF; thus, the estimated values likely included fluid lost at parturition in addition to ESF in litters with stillborn eggs.

Determinants of Relative Litter Mass. For 152 litters (including five additional litters with no viable offspring), we have complete data on all variables: RLM (our measure of reproductive effort), maternal SVL, LS, and estimated ESF. Mean RLM was $54.6\% \pm 20.9\%$. Model selection based on AIC suggested that the best model included maternal SVL, litter size, and total ESF (table 2). The estimated parameters of the best model indicate that RLM was positively influenced by LS (as expected) but also by total ESF (table 3). Using the more restricted sample size of 147 litters did not change the results.

Discussion

Broadly, the comparative information available on oviparous (seven species) and viviparous (five species) reptiles and our detailed data from parturition in a viviparous snake support the three main predictions posed in the introduction to this article. We were unable to gather accurate data on temporal changes in ESF during the course of pregnancy. Nonetheless, ESF at birth represents a critical measurement to test our predictions about constraints imposed by space within the maternal body cavity. In the asp viper (as in other species), exponential growth of the embryos from mid-pregnancy to parturition entails a concomitant increasing rate of water uptake into the amniotic and allantoic sacs (Beall et al. 2007; Lourdaï et al. 2015). Any constraints or trade-offs associated with water allocation to the developing litter should thus be most obvious at parturition. Moreover, the embryos are fully developed only at birth; therefore, the relationship between neonate mass and ESF can be accurately examined only at parturition. We acknowledge, however, that ESF measured at birth might not always represent the maximal value of ESF during pregnancy if embryonic fluids are lost before parturition (Hadi et al. 1994); this phenomenon may explain several low ESF values.

Absolute Investment of Water into Developing Offspring

A review of available data suggests that the fluids associated with hatchlings of oviparous species constitute a larger proportion of total egg mass than is the case with full-term neonates of viviparous squamates (table 1), as predicted by Thompson and Speake (2004, table 3.1). Although there is a continuum between oviparity and viviparity (Shine 1983), the difference between reproductive modes in ESF is clear. Marked variations of water exchange across the eggshell occur in oviparous species (Vleck 1991; Belinsky et al. 2004). Eggs lose or gain substantial amounts of water during incubation as a function of environmental conditions (Deeming 1989; Belinsky et al. 2004), with marked differences among species (e.g., pythons vs. small lizards or parchment vs. rigid eggshells; Deeming 1989). Whatever the case, ESF typically

represents more than 60% of neonatal mass (94% on average, ranging from 61% to 146%; table 1) and may reflect adaptations to local climatic conditions (Vleck 1991). For example, large initial water reserves might be crucial in eggs deposited in arid nests (e.g., royal pythons in Togo; Aubret et al. 2003; table 1) but be less important in mesic conditions, possibly helping to explain the high variance of ESF % values. Mean ESF relative to offspring mass was about three times lower in viviparous species than in oviparous taxa (35%, ranging from 25% to 51%; table 1), consistent with the idea that intrauterine constraints associated with the transition from oviparity to viviparity limit the amount of water that can be incorporated into amniotic and allantoic sacs during embryogenesis. The exchange of fluids, electrolytes, and nitrogenous wastes between embryonic and maternal tissues may further reduce the amount of ESF required for development (compared to oviparous embryos, which store these compounds until hatching). Because ESF is crucial to embryogenesis, these limitations may have consequences on the phenotypic traits of hatchlings.

Correlations between Mass of Embryo and Embryonic-Sac Water Content

In the vipers we studied, the total mass of offspring at parturition averaged 55% of maternal postpartum mass, and the total mass of fluids averaged about 21% of maternal postpartum mass. That is, broadly one-third of total litter mass (in a species exhibiting a very high relative litter mass; Bonnet et al. 2003) is composed of water supplies contained in the fetal sacs. This amount of water thus comprises a substantial proportion of the physical burden carried by a reproducing viviparous snake. An extensive literature on costs of reproduction and the evolution of reproductive tactics in squamates has largely ignored this factor. Instead, mass or energy content of neonates has been used as the measure of reproductive output in reptiles (Niewiarowski and Dunham 1994). That neglect is important given that the evolution of viviparity changes the costs and benefits associated with maternal investment into the hydration as well as nourishment of offspring. Spatial and seasonal variation in hydric attributes of nest sites, and in eggshell porosity, may also shift optimal water-allocation strategies of females to their developing offspring (Warner and Andrews 2002; Shine 2004). Our data are significant in not only revealing strong patterns in water allocation but also identifying the opportunity for future research on this neglected topic.

In asp vipers, larger offspring were associated with larger stores of water. This correlation might be due to at least three nonexclusive processes. First, larger offspring may possess larger amniotic or allantoic sacs and thus can store more ESF (neonate mass positively correlates with ESF; $r = 0.24$, $P < .001$, $N = 315$). However, this relationship was

weak ($r^2 = 0.06$). Thus, although each embryo needs a minimum amount of water to develop, other factors also influence ESF. Second, larger offspring may be more adept at intrauterine competition, thereby extracting more water from the maternal system than can their smaller siblings. Developing embryos uptake substantial amounts of water during gestation, especially during the exponential phase of growth during late development (Lourdais et al. 2015). Third, the correlation might reflect causation in the other direction, whereby increased water supply allows an offspring to grow larger. Possible mechanisms involve better exchanges between the mother and the embryo, better assimilation of the yolk (Deeming 1989; Noble 1991; Aubret et al. 2003), mechanical effects (compression of embryonic tissues may impede development, cushioning effect), or the use of ESF for storage of waste products (Packard et al. 1977). Increasing ESF may thus benefit an embryo in several ways. This physiological complexity may explain the high variation in ESF we found among neonate vipers. Future work could usefully tease apart those possibilities, perhaps by experimentally manipulating the water supply to eggs of different sizes or even to gravid females. We used only body mass to describe offspring quality, but future studies should examine whether ESF influences other major traits, such as locomotor performance, postbirth growth, or early behavior.

Correlations between Litter Size and Embryonic-Sac Water Content

Our data are consistent with the hypothesis that offspring compete for finite water supplies within the maternal oviduct. Notably, figure 2 suggests that the total ESF of the litter is constrained: variance in ESF is high in small litters but low in larger litters. Above a certain threshold imposed by intrauterine maternal volume, embryos in large litters may be unable to incorporate additional ESF. These fecundity-related effects on ESF may well translate into effects on offspring viability (perhaps via size effects or by changing other phenotypic traits). Trade-offs between litter size and offspring size are a central focus of life-history theory (e.g., Zera and Harshman 2001), but remarkably, the potential role of currencies other than energy in driving such trade-offs has been virtually ignored. Reproductive energy allocation is determined at parturition (vitellogenesis + pregnancy investments) in most viviparous squamates (Van Dyke and Beaupre 2011; Blackburn 2015a). Therefore, assessments of the fundamental trade-off between offspring size and offspring number should include ESF in addition to other constraints, such as intrauterine space (Du et al. 2005) and the balance between stored and recently acquired energy (Niewiarowski and Dunham 1994; Bonnet et al. 2001; Blackburn 2015b). Competition among siblings for finite water

supplies may also occur in oviparous species in which water can move between adjacent eggs (e.g., Radder and Shine 2007).

Experimental studies on oviparous reptiles suggest that clutch size may influence water relations in eggs as well as embryos. In large clutches, eggs may have more of their surface area in contact with adjacent eggs rather than the surrounding soil, modifying patterns of water exchange (Packard et al. 1987; Packard 1991). As a result, an increase in clutch size may reduce rates of water uptake of developing eggs (as in lacertid and scincid lizards; Marco et al. 2004; Radder and Shine 2007)—or, counterintuitively, increase those rates of water uptake (as in natricine snakes; Brown and Shine 2005, 2009). Further, these effects may depend on the water potential of the surrounding incubation medium (Marco et al. 2004). Intriguingly, an increase in water uptake per egg may increase offspring size in some species (Marco et al. 2004; Brown and Shine 2005) but decrease it in others (Radder and Shine 2007). The logistical ease of experimentally manipulating incubation conditions for eggs creates an opportunity for studies to comprehensively explore the way in which reptilian embryos react to subtle shifts in moisture availability.

Studies on oviparous reptiles could be invaluable for interpreting the responses of embryos in viviparous species, where experimental manipulations of intrauterine conditions (especially hydric factors) are logistically more challenging (Olsson et al. 2002). Nonetheless, recent studies have demonstrated the critical importance of uterine hydration for viviparous squamates. Notably, Dupoué et al. (2015) experimentally demonstrated that water-deprived pregnant females compromise their own hydromineral balance in favor of their embryos. Lourdais et al. (2015) found that water uptake increases during late pregnancy, attaining maximal values prior to birth. Therefore, our measure of ESF at parturition offers an index of maternal allocation of water at a critical phase in development as a consequence of complex opposing inflows and outflows (e.g., incorporation into embryonic somatic tissues, feto-maternal exchanges) during gestation.

Conclusion

In viviparous species, maintaining water balance may represent a significant challenge for the reproducing female, especially in arid environments or for marine reptiles. Under conditions where water is scarce, reproducing females may need to select microhabitats that provide access to water (e.g., Shine 1979) or compromise their thermal regimes (Ladyman and Bradshaw 2003). Even when water is periodically abundant (e.g., during rainfall following drought; Bonnet and Brischoux 2008), abdominal constraints limit the amounts that can be stored. More generally, the evolution

of viviparity in squamate reptiles may have introduced novel pressures on patterns of water subsidy to developing embryos, and future work could usefully expand the range of currencies with which we measure a mother's allocation of resources to her offspring. It would also be interesting to explore the possibility that viviparity has modified the physiological requirements of the embryos (e.g., Du and Shine 2015) in response to the constraints and benefits imposed or offered by the mother.

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Top, a female aspic viper (*Vipera aspis*) with her six offspring a few minutes after parturition. Litter size varies between 1 and 15 in this species with a mean value of 6. Litter mass (37 g on average) represents 55% (max. 111%) of the postmaternal mass (70 g on average). Fluids stored in embryonic sacs are vital for development; at parturition, they correspond to an additional burden of 15 g for the mother and represent 21% of the postmaternal mass and thus occupy an important intrauterine volume. Siblings compete for finite amounts of water and pose a strong hydromineral challenge for their mother. *Bottom*, a neonate *V. aspis* halfway through the process of birth. The small snake is expelled by the mother while still inside the embryonic sac. The fluids contained in the sac weigh approximately 2.5 g on average (range = 0.3–7.7 g) and represent 33% of the neonate mass. Photo credit: Xavier Bonnet.