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RESEARCH ARTICLE

Stable isotope tracer reveals that viviparous snakes transport amino acids to offspring during gestation

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SUMMARY

Viviparity and placentation have evolved from oviparity over 100 times in squamate reptiles (lizards and snakes). The independent origins of placentation have resulted in a variety of placental morphologies in different taxa, ranging from simple apposition of fetal and maternal tissues to endotheliochorial implantation that is homoplasious with mammalian placentation. Because the eggs of oviparous squamates transport gases and water from the environment and calcium from the eggshell, the placentae of viviparous squamates are thought to have initially evolved to accomplish these functions from within the maternal oviduct. Species with complex placentae have also been shown to rely substantially, or even primarily, on placental transport of organic nutrients for embryonic nutrition. However, it is unclear whether species with only simple placentae are also capable of transporting organic nutrients to offspring. Among viviparous squamates, all of the snakes that have been studied thus far have been shown to have simple placentae. However, most studies of snake placentation are limited to a single lineage, the North American Natricinae. We tested the abilities of four species of viviparous snakes – *Agkistrodon contortrix* (Viperidae), *Boa constrictor* (Boidae), *Nerodia sipedon* (Colubridae: Natricinae) and *Thamnophis sirtalis* (Colubridae: Natricinae) – to transport diet-derived amino acids to offspring during gestation. We fed [^{15}N]leucine to pregnant snakes, and compared offspring ^{15}N content with that of unlabeled controls. Labeled females allocated significantly more ^{15}N to offspring than did controls, but ^{15}N allocation did not differ among species. Our results indicate that viviparous snakes are capable of transporting diet-derived amino acids to their offspring during gestation, possibly *via* placentation.

Key words: reproductive allocation, placentation, bioenergetics.

INTRODUCTION

Mechanisms by which resources are allocated to competing functions are key components of individual life histories and may explain variation in life-history traits (Dunham et al., 1989). In particular, mechanisms of nutrient provisioning to offspring are responsible for determining clutch size, offspring size and reproductive frequency, which contribute to offspring survival (Lack, 1954; Sinervo et al., 1992). As population growth rates are fundamentally tied to offspring survival (Cole, 1954), a thorough understanding of the mechanisms of reproductive allocation is necessary for prediction of population responses to environmental change (Boggs, 1997).

Various forms of matrotrophy, or maternal provisioning of offspring during embryogenesis, have been documented in mollusks (Glaubrecht, 2006), arthropods (Farley, 1999), echinoderms (Frick, 1998), chondrichthyan (Hamlett and Hysell, 1998) and osteichthyan fishes (Wourms et al., 1988), gymnophionan amphibians (Wake, 1977; Wake, 1993), squamate reptiles (Blackburn, 1992), mammals (Lillegraven et al., 1987) and embrophytic plants (Graham and Wilcox, 2000). Because of its apparently common, rapid and highly convergent evolution in diverse taxa (Wake, 1992), matrotrophy has been heralded as a useful model for studying the evolution of complex adaptations (Reznick et al., 2002). Within amniotes, viviparity has independently evolved over 100 times in squamate reptiles (lizards and snakes), and once in eutherian mammals (Blackburn, 1992). Because oviparous squamates exhibit substantial embryonic development prior to oviposition, squamates may even be preadapted for viviparity (Blackburn, 1992). Furthermore, matrotrophy *via* placentation has been hypothesized to evolve concurrently with viviparity in squamates (Blackburn, 1995; Blackburn, 2006).

Within all viviparous amniotes, placentae are responsible for matrotrophic allocation of nutrients (Blackburn, 1994). We follow Mossman (Mossman, 1937; Mossman, 1987) in defining placentae as organs formed through the apposition of embryonic and maternal tissues, and which function in physiological exchange. In viviparous reptiles, the maternal component of the placenta is the lining of the uterine oviduct (Blackburn and Stewart, 2011). The embryonic component is formed by the opposing extraembryonic membrane, which may be the chorion, chorioallantois or omphalopleure (yolk sac), depending on the region of the embryo examined (Stewart, 1997). The same structures also exist in the embyros of oviparous squamates (Blackburn et al., 2003). Because oviparity is ancestral to viviparity in squamates, extraembryonic membranes have been hypothesized to be exaptations co-opted for viviparity (Knight and Blackburn, 2008). In oviparous species, the chorioallantois is a thin, highly vascularized membrane that lies on the embryonic hemisphere of the egg and directly contacts the eggshell (Knight and Blackburn, 2008). Because of its dense vascularization, the chorioallantois is thought to function primarily in gas exchange (Andrews and Mathies, 2000), although the presence of calbindin-D_{28K} suggests that it might also absorb calcium from the eggshell (Ecay et al.,

2004; Stewart et al., 2004). In oviparous squamates, the omphalopleure is a non-vascularized bilaminar structure that overlays the abembryonic hemisphere of the egg. In all snakes that have been studied thus far, and some lizards (Stewart and Thompson, 2003), mesoderm invades the yolk sac early in development and separates a portion of yolk from the yolk body, thus forming both an isolated yolk mass and a yolk cleft (Hoffman, 1970). As development proceeds, the allantois expands into the yolk cleft and vascularizes the inner membrane of the isolated yolk mass (Blackburn et al., 2003; Stewart, 1993). The resulting structural combination of omphalopleure, isolated yolk mass and allantois has been termed the omphalallantois. In oviparous species, the omphalopleure and/or omaphallantois are thought to be responsible for taking up water from the surrounding environment (Weekes, 1935).

In viviparous squamates, the functions of extraembryonic membranes found in oviparous species are retained or even enhanced (Blackburn, 1993; Stewart, 1993). In species with simple placentae, the chorioallantois remains a highly vascularized membrane that surrounds the embryonic hemisphere of the egg. Chorioallantoic capillaries are closely aligned with maternal capillaries, so gas exchange is still thought to be its primary function in viviparous squamates, though transport of inorganic ions (Blackburn, 1993; Blackburn and Lorenz, 2003b) and histotrophic transfer (Stewart and Brasch, 2003) may also occur in natricid snakes. In squamates with complex placentae, such as the skink *Mabuya heathi*, the highly modified chorioallantois is also responsible for transport of organic nutrients (Blackburn and Vitt, 2002; Blackburn et al., 1984).

As in oviparous species, the omphalallantois of viviparous squamates remains avascular on its external surface, but is vascularized along the isolated yolk mass by the allantois (Stewart, 1993). Water uptake and sodium transport have been hypothesized to be the primary functions of the omphalallanatois in viviparous squamates (Stewart, 1992; Weekes, 1935), but electron microscopy has also revealed the potential for absorption of organic nutrients in natricid snakes (Hoffman, 1970; Stewart, 1992). Because the opposing uterine epithelium also appears to be specialized for secretion in some species, particularly natricid snakes, some authors have suggested that the omphalallantoic placenta (i.e. yolk-sac placenta) may be a site of histotrophic organic nutrient transport (Blackburn and Lorenz, 2003a; Hoffman, 1970; Stewart, 1992; Stewart and Brasch, 2003). In addition to maternal and embryonic tissues, many viviparous reptiles retain a reduced shell membrane (reviewed by Guillette, 1982), which exists as an acellular layer between the embryo and uterine lining (Hoffman, 1970). In the natricid snake *Virginia striatula*, Stewart and Brasch hypothesized that the shell membrane might function as a permeable dyalitic membrane that allows selective transport of material based on size (Stewart and Brasch, 2003).

Although viviparity has independently evolved in at least 30 snake lineages (Shine, 2003), most studies of placentation in snakes have focused on one lineage, the North American Natricidae (Hoffman, 1970; Stewart, 1989; Stewart, 1990; Stewart et al., 1990; Stewart and Brasch, 2003). As in other squamates, most examinations of placental function within natricid snakes have relied on mass balance comparisons, which have found placental transport of organic nutrients to be minimal or not apparent (Stewart, 1989; Stewart et al., 1990). However, Stewart and Thompson note that placental transfer of small quantities of organic molecules is not always estimable using mass balance (Stewart and Thompson, 2003). An alternative method has been to inject radiolabeled tracers into females during gestation and subsequently use the radioactivity of embryos or offspring as an estimate of placental transfer (Conaway and Fleming, 1960; Hoffman, 1970; Jones and Swain, 2006; Swain and Jones, 1997). Here, we used a similar approach to investigate the ability of viviparous snakes to transport diet-derived amino acids to their offspring during gestation. We fed [^{15}N]leucine-labeled diets to gestating females of four viviparous snake species (Boidae: *Boa constrictor*, Linnaeus; Natricidae: *Nerodia sipedon*, Linnaeus and *Thamnophis sirtalis*, Linnaeus; Viperidae: *Agkistrodon contortrix*, Linnaeus) during gestation, and compared resulting ^{15}N content of offspring with that of control groups that were not fed tracer. *Thamnophis sirtalis* has previously been reported to be capable of placental transport of amino acids (Blackburn and Lorenz, 2003a; Blackburn and Lorenz, 2003b; Blackburn et al., 2002; Hoffman, 1970), but placental transport of organic nutrients has not been documented in the other species we tested. Because viviparity is thought to have arisen independently in all three families studied here (Blackburn, 1992), we used a phylogenetic approach to test for differences in tracer transport among species. Importantly, our experiment only tests whether viviparous snakes are capable of transporting diet-derived amino acids to offspring, and cannot be used to discern the specific mechanism responsible for transport.

MATERIALS AND METHODS

This study was conducted with the approval of the University of Arkansas Institutional Animal Care and Use Committee (protocols 06003 and 09010). *Agkistrodon contortrix*, *N. sipedon* and *T. sirtalis* were collected under Scientific Collecting Permits 040420072 and 081620051 issued by the Arkansas Game and Fish Commission, and SO-FW-FY06-09 issued by the United States Department of Agriculture: Ozark/St Francis National Forest.

We studied placentotrophy in pregnant female snakes from four species: eight copperheads (*A. contortrix*), eight boas (*B. constrictor*), nine watersnakes (*N. sipedon*) and 12 garter snakes (*T. sirtalis*). We used at least six males of each species for mating purposes. All snakes were maintained in captivity at the University of Arkansas in large Rubbermaid^{TM} containers or glass aquaria, with newsprint bedding, at 30±2°C. Circadian light cycles were programmed to change in concordance with circannual changes in day length. Water was available *ad libitum*. Prior to pregnancy, *A. contortrix* and *B. constrictor* were fed rodents once per month, while *T. sirtalis* were fed rodents once per week. *Nerodia sipedon* were fed baitfish once per week. *Thamnophis sirtalis* and *N. sipedon* have high metabolic rates and activity levels, and therefore required more frequent feeding (Gibbons and Dorcas, 2004; Rossman et al., 1996). The timing of captive mating was species specific and coincided with the natural breeding seasons of each species. *Thamnophis sirtalis* and *N. sipedon* mated in spring (Trauth et al., 2004), and *A. contortrix* mated in both spring and autumn (Trauth et al., 2004). *Boa constrictor* mated at the end of their brumation period, during mid-late winter (De Vosjoli et al., 2005).

Once per month during the active season, females were weighed to the nearest 0.01 g (Sartorius, model BP3100S, Goettingen, Germany). We also assessed female reproductive condition using non-invasive ultrasonography (Bonnet et al., 2008) *via* an Aloka SSD-900V Veterinary Ultrasound Console with a 7.5 MHz linear transducer (Aloka, Wallingford, CT, USA). After ultrasonography indicated embryonic development (Fig. 1), we took scale clips from all female snakes to determine tissue background ^{15}N content (Pilgrim, 2005). While scale tissues are not likely to be catabolized for allocation to offspring, they should be representative of the isotopic composition of the female prior to artificial enrichment

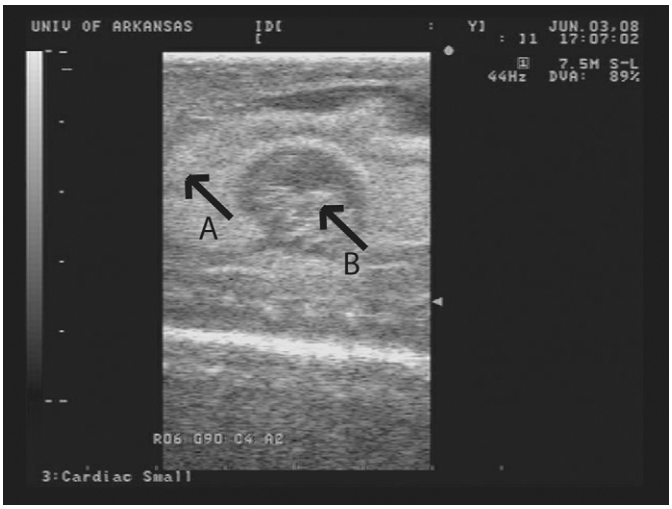

Fig. 1. An ultrasound image of an early-stage *Boa constrictor* embryo is presented to demonstrate how reproductive status was judged. Arrow A indicates the large yolk body that remains available in the embryo. Arrow B indicates the early-stage embryo. All [^{15}N]leucine supplements were made when embryos appeared similar to the one presented here.

(McCue, 2007). We lightly anesthetized females with isoflurane (0.1 ml kg^{-1} body mass), and used yolkectomy to remove at least 1 ml of yolk from a randomly chosen embryo (Van Dyke, 2011). After yolkectomy, we randomly divided females of each species into control and experimental groups. Some females were used for this study repeatedly in two consecutive years (*A. constrictor N*=1; *B. constrictor N*=2; *T. sirtalis N*=3). We switched treatments on all repeatedly sampled females, so that those in the control treatment in the first year were in the experimental treatment in the second, and *vice versa*.

Once pregnant, all females were given only a single meal until after parturition. The meal for experimental females was injected with a bolus of physiological saline containing at least 20 mg kg^{-1} body mass (mean ± s.e.m.=29.01±2.77 mg kg^{-1} body mass) of [^{15}N]leucine. Leucine was chosen as a tracer because it is a component of connective tissues, including muscle and integument (Nelson and Cox, 2000), and is therefore necessary for successful embryonic development. Control females were fed a meal injected with a bolus of physiological saline. At parturition, we took a second scale clip from all females in order to ensure that females assimilated dietary [^{15}N]leucine. All offspring were killed *via* isoflurane inhalation, and up to six from each litter were randomly chosen for isotopic analysis (<6 only if litter size <6). All offspring and maternal samples were frozen and stored at –20°C.

Maternal scale and yolk samples were lyophilized at –40°C and 0.2 mbar (1 mbar is 100 Pa) for at least 2 days in a Labconco Freeze-Dry System (Kansas City, MO, USA). Offspring were lyophilized for at least 7 days. We homogenized dried yolks and offspring in a Wig-L-Bug® amalgamator (Crescent Dental Company, Chicago, IL, USA). Homogenization was not necessary for scale tissues because whole scales were small enough for isotopic analysis. After homogenization, 0.3–0.7 mg subsamples of offspring, yolk and whole scales were weighed on a Sartorius SC-2 nanobalance and wrapped in airtight 3×5 mm pressed aluminium foil capsules for isotopic analysis.

We measured sample ^{15}N isotope signatures on a Finnigan Delta Plus continuous flow IR-MS and elemental analyzer located at the University of Arkansas Stable Isotope Laboratory (UASIL, Fayetteville, AR, USA). Measured ^{15}N isotope ratios were corrected for atmospheric N_2 standard and converted to δ-values using the following equation:

$$\delta^{15}\mathrm{N} = \left(\left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{STD}}}\right) - 1\right) \times 1000 , \quad (1)$$

where R_{sample} refers to the ratio of ^{15}N to ^{14}N in the sample and R_{STD} refers to the ratio of ^{15}N to ^{14}N in atmospheric N_2 gas. Because δ-values are not measures of absolute isotopic composition, but are relative to known standards, we converted all δ-values to atom per cent excess, and then multiplied that value by 1000 to calculate atom parts per million excess (p.p.m.), as atom per cent excess values were small in all cases. p.p.m. values are standard SI units of isotopic enrichment, and are therefore more appropriate for tracer studies (Slater et al., 2001). We used the following equation to convert δ-values to p.p.m.:

$$\mathrm{p.p.m.} = \left[\frac{100}{\left(\frac{1}{\left(\frac{\delta^{15}\mathrm{N}}{1000}+1\right)R_{\mathrm{STD}}}+1\right)}\right] \times 1000 , \quad (2)$$

Individual background scale ^{15}N p.p.m. values were subtracted from parturition-scale ^{15}N p.p.m. values to determine maternal assimilation of dietary [^{15}N]leucine. Offspring ^{15}N p.p.m. values were averaged within each litter to calculate mean ^{15}N p.p.m. values. Background yolk ^{15}N p.p.m. was then subtracted from mean litter ^{15}N p.p.m. to determine offspring (*via* placentotrophy) enrichment in each snake. This subtraction procedure may result in negative values for the change in ^{15}N p.p.m., particularly if offspring are depleted in ^{15}N relative to yolk. Scale and offspring enrichment of experimental and control treatments was then individually compared across species using factorial ANOVA, with a repeated statement to account for females utilized in consecutive years, in SAS (PROC MIXED) (Litell et al., 1996). Some female boas were known to be sisters, so we included maternal litter in the model as a random effect, and estimated the variance component of maternal litter (PROC VARCOMP; SAS Version 9, SAS Institute, Cary, NC, USA). The effects of body mass, leucine supplement mass, and timing of supplementation (no. of days prior to parturition) on offspring ^{15}N enrichment were further examined across species in experimental animals using ANCOVA (PROC GLM; SAS Version 9). We examined interaction effects to test for slope homogeneity prior to analysis of treatment and covariate effects. In all comparisons, data were log-transformed to meet assumptions of parametric statistics. Statistical significance was judged at the 0.05 Type I error level, and means are presented ±1 s.e.m.

RESULTS

Maternal enrichment

Post-partum scales of experimental females (61.64±10.24 ^{15}N p.p.m.) were significantly more enriched than those of control females (–0.27±1.24 ^{15}N p.p.m.) in all species (Fig. 2; $F_{1,4}$=58.95; P=0.0015). Scale ^{15}N enrichment did not vary significantly among species (Fig. 2; $F_{3,33}$=2.06; P=0.1245). Some females were used for

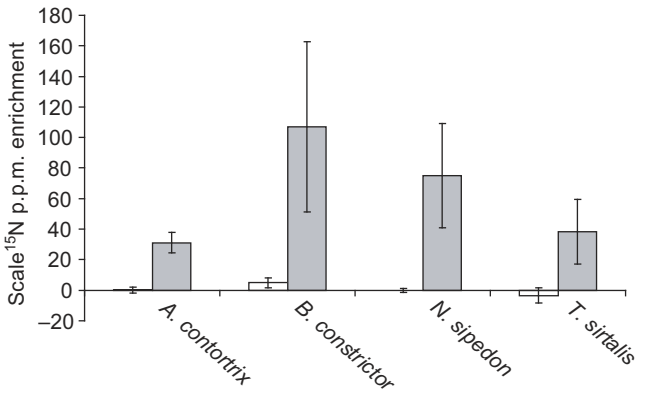

Fig. 2. Means and 95% confidence intervals of maternal scale ^{15}N p.p.m. enrichment in snakes fed control (white bars) and ^{15}N-labeled (gray bars) meals, where enrichment reflects the difference between pre- and post-supplementation scale ^{15}N p.p.m. Scale ^{15}N p.p.m. enrichment was significantly greater in snakes fed ^{15}N-labeled meals than those fed control meals. There were no significant differences in scale ^{15}N p.p.m. enrichment among species (*Agkistrodon contortrix*, *Boa constrictor*, *Nerodia sipedon*, *Thamnophis sirtalis*).

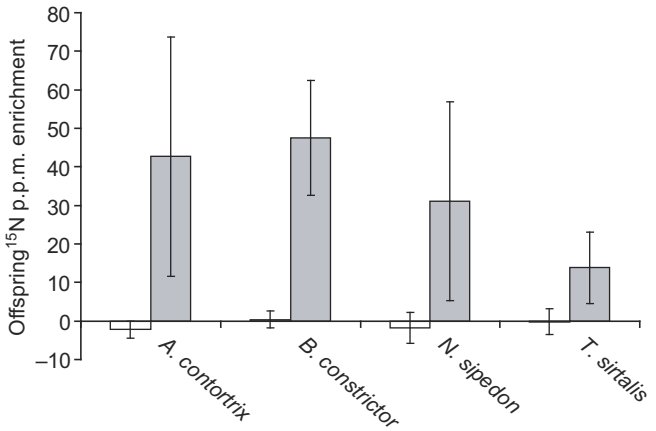

Fig. 3. Means and 95% confidence intervals of offspring ^{15}N p.p.m. enrichment in snakes fed control (white bars) and ^{15}N-labeled (gray bars) meals, where enrichment reflects the difference between pre-supplementation yolk and post-supplementation offspring ^{15}N p.p.m. Offspring ^{15}N p.p.m. enrichment was significantly greater in snakes fed ^{15}N-labeled meals than those fed control meals. There were no significant differences in offspring ^{15}N p.p.m. enrichment among species.

this study repeatedly in two consecutive years (see Materials and methods), but treatments were switched between years. Within these individuals, background scale ^{15}N enrichment did not vary significantly between years ($F_{1,4}$=1.24; P=0.3283). Although some of the *B. constrictor* in our study were sisters, their relatedness accounted for less than 1% of the variance in the model. No factorial interaction effects were significant. Thus, supplemented tracer was assimilated from diet and incorporated into maternal body tissues.

Offspring enrichment

Experimental offspring (32.84±5.70 ^{15}N p.p.m.) were significantly more enriched than control offspring (–0.71±0.81 ^{15}N p.p.m.) in all species (Fig. 3; $F_{1,4}$=59.32; P<0.0015). Offspring ^{15}N enrichment was not significantly different among species (Fig. 3; $F_{3,33}$=2.06; P=0.1246): *A. contortrix* 42.72±15.51 ^{15}N p.p.m.; *B. constrictor* 47.54±7.48 ^{15}N p.p.m.; *N. sipedon* 31.07±12.90 ^{15}N p.p.m.; and *T. sirtalis* 13.85±4.62 ^{15}N p.p.m. Neither did offspring ^{15}N enrichment vary significantly with year within individuals that were sampled in consecutive years ($F_{1,4}$=1.23; P=0.3297). Relatedness of sibling *B. constrictor* accounted for less than 1% of the variance in the model, and no factorial interaction effects were significant. Supplemented dietary tracer was therefore allocated to developing offspring during embryogenesis. Because there were no significant differences among species, it was not necessary to proceed with phylogenetically independent analyses. Within experimental animals, there were no significant relationships between offspring ^{15}N enrichment and maternal body mass ($F_{1,20}$=0.08; P=0.8006), the amount of labeled leucine supplemented ($F_{1,20}$=0.73; P=0.4833), or the timing of supplementation relative to parturition ($F_{1,20}$=0.46; P=0.5672). Offspring ^{15}N enrichment did not vary among species (Fig. 3; $F_{3,20}$=1.21; P=0.4818). No interaction effects were significant, so the assumption of slope homogeneity was met for ANCOVA.

DISCUSSION

All of the viviparous snake species we examined were equally capable of transporting dietary amino acids to their offspring during embryogenesis. We did not proceed with phylogenetically independent comparisons because transfer of dietary tracer was not significantly different among species. Because the species studied here represent three clades in which viviparity has independently evolved (Stewart, 1992; Stewart, 1993; Stewart et al., 2006), the ability to transport organic nutrients to developing offspring may be common, or even ancestral, in viviparous snakes. However, our experiment cannot explicitly identify placentation as the transporting mechanism. Based on Mossman's definition of placentae (Mossman, 1937; Mossman, 1987), the transport of amino acids must be a specific function of opposing embryonic and maternal tissues for it to be interpreted as placentation. The extraembryonic membranes of the oviparous cornsnake, *Pantherophis guttatus*, have been demonstrated to take up material, likely calcium, from the surrounding eggshell (Blackburn et al., 2003). Electron microscopy suggests that the mechanism of absorption may be phagocytosis or pinocytosis (Blackburn et al., 2003). Eggs of viviparous snakes may simply enhance the oviparous transport function to include amino acids as well as inorganic nutrients (e.g. Blackburn, 1993; Stewart, 1993). In addition, while the uterine linings of some viviparous natricid snakes are thought to be secretory (Blackburn and Lorenz, 2003a; Hoffman, 1970; Stewart, 1990; Stewart, 1992), uterine secretions have not been identified, and other species have not been investigated. Furthermore, the presence of placental structures has not been confirmed for each of the species in our study [*T. sirtalis* (Blackburn, 1998; Blackburn and Lorenz, 2003a; Blackburn and Lorenz, 2003b; Blackburn et al., 2002); *N. sipedon* (Conaway and Fleming, 1960)]. Because their placental morphologies have not been as thoroughly investigated as those of *T. sirtalis*, it is possible that *A. contortrix*, *B. constrictor* and *N. sipedon* exhibit a diversity of placental morphologies, or even lack sufficient apposition of maternal and fetal membranes to constitute placental specialization. If that is the case, then our results would lend support to the hypothesis that organic nutrient transport can be independent of placental complexity (Stewart, 1992; Stewart, 1993; Stewart et al., 2006). A possibility that has not been explored in the literature is that maternal uterine secretions maintain the shell membrane during gestation, and that the hypothesized absorptive function of the embryonic membranes consumes shell membrane material. In our experiment, we also cannot discount the possibility that diffusion is responsible for the transport of amino acids to developing

offspring. While leucine is strongly hydrophilic and not likely to diffuse across cell membranes, it is possible that ^{15}N may have been transaminated to other amino acids in the mother prior to being transported to offspring. If ^{15}N was transaminated to a hydrophobic amino acid, then that amino acid could have crossed to developing offspring *via* diffusion alone, and carried our ^{15}N label along with it. Further research is thus necessary to determine the mechanisms of maternal–fetal amino acid transport that might account for our observations.

All of the species studied here ovulate very large eggs with plentiful yolk, and are presumed to be primarily lecithotrophic (Stewart, 1992). Comparisons of egg and neonate content in other highly lecithotrophic viviparous reptiles have shown a net loss of lipid and protein quantities, and investigators have suggested that there is little or no net contribution of placental organic nutrients to embryonic tissues (Pilgrim, 2007). The same studies have shown that net increases in offspring water and inorganic ion quantities occur, likely *via* placental transfer (Stewart, 1989; Stewart and Castillo, 1984; Thompson et al., 1999). However, our results, in conjunction with similar tracer studies in other taxa (Stewart, 1989; Stewart and Castillo, 1984; Thompson et al., 1999), suggest that organic nutrients are transferred from mothers to developing offspring. If transport of organic nutrients occurs during gestation, why do mass balance studies not find a net gain in organic material from egg to neonate?

One possible answer is that mass balance studies do not account for catabolic waste. Lipids provide the bulk of energy for embryogenesis, so it is likely that both yolk- and placenta-derived lipids are catabolized during development, and resultant CO_2 waste is released by the mother (Hoffman, 1970; Jones and Swain, 2006; Swain and Jones, 1997). Thus, transport of lipids may not be detectable *via* common egg–neonate mass balance comparisons. Similarly, a net loss or lack of change in nitrogen content (indicative of protein content) may be explained by the catabolism of yolk- and/or placenta-derived protein for energy (Thompson and Speake, 2006). However, protein catabolism must result in nitrogenous waste, which would have to either be stored in the allantois of the embryo or be excreted to the mother (Thompson and Speake, 2003). Few mass balance studies have investigated either phenomenon (Blackburn, 1994). Embryonic excretion of nitrogenous waste to the mother has been documented in *Thamnophis* (Clark and Sisken, 1956), but neither the mechanism of excretion nor its frequency in other taxa is known. Carbohydrates might also be transported to offspring *via* placentotrophy, but they have received little experimental attention (Blackburn, 1994), and CO_2 waste from carbohydrate metabolism should be released to the mother, as described for lipid catabolism.

Another possibility is that the amino acid transport we observed is too small an amount to be detectable by mass balance. While we cannot calculate the mass of amino acids that were transported, our among-species estimate of mean ^{15}N enrichment in our labeled snakes (32.84±5.70 ^{15}N p.p.m., or less than 0.00004%) suggests a very small amount of ^{15}N label was transported to offspring. Furthermore, there was a wide variance around the mean in each of our species. If future studies do find further evidence that placentation is the mechanism of amino acid transfer in viviparous snakes, then our results suggest that placentation may be incipient in the species we studied.

Our investigation reveals that members of at least three lineages of viviparous snakes are capable of transporting diet-derived amino acids to developing offspring, including two (Boidae and Viperidae) in which it has not previously been documented. Because viviparity has evolved frequently in snakes and other squamates, amino acid transport during gestation may be far more widespread than previously thought, even among species presumed to be predominately lecithotrophic (e.g. Stewart, 1992). If placentation is the mechanism responsible for the amino acid transport we report here, then the apparently small amount of material transferred to offspring during gestation suggests that placentation may be incipient in highly lecithotrophic viviparous snakes. Alternatively, the possibility remains that greater transport may be masked by reciprocal transport of embryonic waste back to the mother. A further possibility is that predominately lecithotrophic viviparous squamates do not exhibit incipient placentation but instead retain or enhance the ability of oviparous squamates to absorb material external to their embryonic membranes. Further research on the ancestral oviparous condition (e.g. Blackburn et al., 2003; Ecay et al., 2004) is therefore crucial to distinguish between ancestral properties retained from oviparity and derived properties associated with placentotrophy (Blackburn, 2006). Answering these questions will create novel avenues of research in both bioenergetics and life histories, particularly into discovering the evolutionary forces that drive the switch from pre-ovulatory to post-ovulatory reproductive allocation.

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