PLACENTAL TRANSFER OF ^{3}H- OLEIC ACID IN THREE SPECIES OF VIVIPAROUS LIZARDS: A ROUTE FOR SUPPLEMENTATION OF EMBRYONIC FAT BODIES?

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ABSTRACT: We hypothesize that facultative placentrotrophy evolved in viviparous squamates as a means of supplementing embryonic fat reserves. In this study, we aimed to demonstrate a capacity for placental transfer of lipid in three species of the genus *Niveoscincus* that differ in degree of placental complexity and in their ability to defer parturition after embryonic development is complete. In *Niveoscincus metallicus,* we injected gravid females at different stages of gestation with ^{3}H- oleic acid and studied transfer of the radio-label into maternal and embryonic lipid compartments over time. In a comparative study of *N. ocellatus* and *N. microlepidotus*, we measured transfer of ^{3}H- oleic acid after 240 min in females with embryos at stages 39-40 only. For *N. metallicus*, the time course experiment showed that transfer into the embryos tends to increase with time, and that the transfer ratio is greatest in late-stage embryos. Our results demonstrate that ^{3}H-oleic acid is transferred into embryos, and into embryonic fat bodies, of all three species, but that the magnitude of transfer does not appear to be correlated with placental complexity.

Keywords: Embryo; Lipid; Lizard; *Niveoscincus*; Placentotrophy; Viviparous

INTRODUCTION

Much of the literature surrounding the evolution of viviparity has considered the putative selective forces and constraints associated with live-bearing. A major outcome of this research has been the "Cold Climate Hypothesis", and a large number of descriptive and experimental tests, primarily dealing with proposed thermoregulatory advantages, provide support for this hypothesis (e.g.(Shine, 1995; Andrews and Mathies, 2000). However much less attention has been paid to the forces selecting for innovation in embryonic provisioning.

In viviparous squamates, the degree of placental complexity varies considerably, from the relatively simple Type I placentae to the more complex Type IV seen in species such as *Mabuya* (Thompson et al., 2004). As a corollary of this, the degree of placentotrophy also varies across a wide spectrum (Thompson, Stewart, and Speake, 2000). Some species of squamate rely almost entirely upon yolk to nourish the embryos, so that viviparity represents little more than prolonged egg retention. In such highly lecithotrophic species, embryonic nutrition depends on yolk laid down during vitellogenesis, sometimes months before gestation, and the placentae are required only for the transport of respiratory gases, water and, possibly, some inorganic ions. At the other extreme, in the few microlecithal squamates known, there is significant placental transport of organic nutrients to support the developing embryos (Blackburn, Vitt, and Beuchat, 1984; Blackburn, 2000). Across that spectrum, placental contributions of inorganic and organic nutrients may be either facultative or obligate (Stewart, 1989), where facultative contributions reflect an individual female's capacity to supply "bonus" nutrition during gestation to embryos already adequately supported by yolk.

Lecithotrophy is clearly an effective strategy in many viviparous squamates, but under ecological circumstances where lecithotrophy does have limitations, placentotrophy must

convey advantages that overcome the costs of producing and maintaining complex placentae. What, then, is the selective advantage of facultative placentotrophy? We have previously suggested that facultative placentotrophy evolved to supplement embryonic nutrition and to enhance offspring condition if environmental factors are favorable during gestation (Swain and Jones, 2000a). This hypothesis has been supported by experiments in which we have manipulated environmental conditions during gestation and examined the consequences for offspring fitness in the viviparous skinks *Niveoscincus metallicus* (Swain and Jones, 2000b), *N. ocellatus* and *N. microlepidotus* (Atkins, Swain and Jones, unpublished results).We further suggest that the major selective force for the evolution of facultative placentotrophy is the introduction of flexibility in the timing of parturition, the proximate mechanism being the supplementation of embryonic fat reserves that enhance neonatal condition, but may be utilized during gestation if parturition is delayed (Swain and Jones, 2000b).

What evidence is there that in squamates exhibiting facultative placentotrophy the placentae are indeed capable of transferring non-yolk lipids into the embryos? In a series of elegant studies comparing the composition of squamate yolks and neonates, Thompson and co-workers (reviewed by (Thompson, Stewart, and Speake, 2000) have determined that the degree of uptake of dry matter and of individual nutrients varies with placental complexity, with little or no uptake demonstrated in species with simple chorioallantoic placentae. For the highly placentotrophic *Niveoscincus ocellatus*, for example, there is evidence that "considerable lipid" crosses the placenta during embryonic development (Thompson et al., 2001). In contrast, for *N. metallicus,* there is no clear evidence for lipid uptake across the placenta (Thompson et al., 1999). Those authors do note that transfer of specific lipids cannot

be ruled out, and they describe the placenta of *N metallicus* as being functionally intermediate between Type I (simple) and Type III (complex) placentae.

Such studies are, however, limited in that they provide only indirect evidence of placental transfer, nor are they capable of assessing selective transport into different embryonic compartments. A more direct method of assessing maternal transfer of nutrients is to follow the entry of radio-labeled molecules into embryonic compartments: this technique has been used to demonstrate placental transfer of amino acids in squamates (Thompson, 1977; Yaron, 1977; Swain and Jones, 1997). In this study, we aim to assess the capacity of the placenta to transfer radio-labeled lipid into embryos, and, specifically, into embryonic fat bodies in *Niveoscincus metallicus*, *N. ocellatus*, and *N. microlepidotus*, three closely related viviparous species with different degrees of placental complexity, and different strategies for enhancing neonatal fitness. In *N. metallicus*, with a Type 2 placenta (Stewart and Thompson, 1994), the females are capable of deferring parturition for up to four weeks, and the embryos utilize their abdominal fat bodies during that period (Swain and Jones, 2000b), but females of *N. ocellatus* are less able to defer parturition in adverse environmental conditions (Atkins, unpublished results). *Niveoscincus microlepidotus* exhibits an unusual biennial reproductive cycle in which embryos fully developed in autumn are retained *in utero* for 6 to 7 months until parturition in spring. Again, the embryos utilize their abdominal fat bodies through this protracted gestation (Girling, Jones, and Swain, 2002), and this species has a type 2 placenta similar to that of *N. metallicus* (J. Stewart, pers. comm.), although a detailed anatomical study is not yet available.

We hypothesize that there will be differences between these species in the capacity of the placentae to transfer lipids into embryonic compartments. We chose to use radio-labeled

oleic acid for this study because this fatty acid is a major component of the triacylglycerol in both eggs and neonates of *N. metallicus* (Thompson et al., 1999) and of *N. ocellatus* (Thompson et al., 2001). We focused on the later stages of embryonic development (stages 35-40), because transformation of the embryo-maternal interface, and differentiation of the chorioallantoic placentae, begin at stage 32 in these species (Stewart and Thompson, 1994; (Stewart and Thompson, 2004).

MATERIALS AND METHODS

Animal collection.—We collected gravid females of *Niveoscincus metallicus*, *N. ocellatus*, and *N. microlepidotus* from, respectively, areas around Hobart, Orford or the Central Plateau, and Mt Wellington, in Tasmania, Australia. Stage of gestation was estimated by previous experience when documenting the reproductive cycles of these species (Jones and Swain, 1996; Jones, Wapstra, and Swain, 1997; Girling, Jones, and Swain, 2002), and females of *N. microlepidotus* carrying late stage embryos were collected in autumn. Animals were captured by mealworm "fishing", transported back to the laboratory in cloth bags, and housed communally overnight with access to water. Whenever possible, we carried out the experiments the day following capture of those animals.

Oleic Acid Transfer Experiments.— The time course of oleic acid transfer across the placenta was quantified using 70 gravid females of *Niveoscincus metallicus* collected during mid- and late gestation. We divided the females into groups according to embryonic stage as follows: Stages 31-34 (n = 7); Stages 35-38 (n = 30); Stages 39-40 (n = 20); Stage 40+ (n =

13). (Stage 40+ is the final, post-differentiation stage of gestation recognized in *N. metallicus* as the period when the yolk is very reduced and the embryos are ≥ 90% mean birth weight: Swain and Jones, 1997). For *N. ocellatus* (n = 16) and *N. microlepidotus* (n = 9) we collected animals only in late gestation, and killed all females at 240 min after injection. This allowed us to compare transfer ratios for oleic acid between these species and *N. metallicus* (n = 9) during stages 39-40 of embryonic development, when differentiation is almost complete.

We weighed each female and injected 1 μCi tritiated oleic acid ([9,10(n)-^{3}H] Oleic acid; Amersham TRK140) in 5 μl saline into the thigh muscle. Females were killed at 60, 120 or 240 min after injection so that the time course of oleic acid transfer and incorporation into various compartments could be assessed. As we could only assess embryo stage post-mortem, group sample sizes varied from 2 – 12, the small sample groups representing stages 31-34. At dissection, we collected maternal blood from the carotid arteries, liver, and abdominal fat bodies. The blood was centrifuged, and duplicate 20 μl plasma immediately added to scintillant (Ecolite, ICN Biochemicals) for radioactive counting in a Beckman Coulter LC5801 scintillation counter. Tissue samples were weighed and stored frozen until extraction. For each female, two embryos were dissected free of the uterus and processed. For each embryo, we took a 20 μl sample of amniotic fluid using a Hamilton syringe, and added this to scintillant for radioactive counting. The embryo was then dissected free of its membranes, staged (Dufaure and Hubert, 1961), rinsed in 0.9 % saline, weighed and stored frozen until extractions were performed. We processed the yolks as in Swain and Jones (1997), and re-weighed the female carcass after dissection to obtain the maternal weight and allow calculation of relative clutch mass (RCM = original weight – carcass weight/carcass weight).

For extraction of tritiated lipid, maternal and embryonic tissues were thawed, homogenised and extracted in methanol: chloroform (1: 2). We extracted maternal fat bodies in 1 ml solvent; liver in 2 ml; whole embryos in 3 or 6 ml depending on size. In each case, duplicate 100 µl aliquots of extract were added to scintillant for radioactive counting. As in Swain and Jones (1997), we expressed the results as transfer ratios (= dpm in 100 mg tissue or 100 µl liquid sample divided by dpm injected per 100 g maternal body weight) after correction for volume of sample counted for radioactivity. This calculation removes the necessity to compensate for clearance of radioactivity from maternal plasma during the experiment, and also corrects for different sample sizes. We averaged data for the two embryos from each mother, and used the means in our analyses. Unequal group sizes were inevitable because embryonic stage could only be determined when the mother was dissected.

Embryonic fat bodies.—For *N. microlepidotus* and *N. ocellatus*, we froze any excess embryos and, later, we dissected out their abdominal fat bodies, weighed and extracted them as described above. We extracted the embryonic fat bodies in 150 µl solvent, and counted duplicate 50 µl aliquots. For *N. metallicus*, such dissection was only possible for large (i.e. late stage) embryos. Because the decision to carry out such dissections was made after completing many of the individual experiments, data are available only for embryos of *N. metallicus* mothers killed at 60 min after injection.

Data analysis.—Comparisons between species of transfer ratios for tissue and fluid compartments were analyzed using ANOVA with Tukey's post-hoc tests; the significance level was $p = 0.05$ and data were checked for homogeneity of variances to ensure that the

assumptions of ANOVA were met. For the time course experiment with *N. metallicus*, we analyzed the data by two-way ANOVA (factors = stage and time). All statistical analyses were carried out using statistiXL v.1.5 (www.statistiXL.com).

RESULTS

Radio-labeled oleic acid was transferred into maternal blood and liver, and into amniotic fluid, yolk, embryo and embryonic fat bodies in all three species studied.

For *N. metallicus*, uptake of ^{3}H-oleic acid into maternal plasma (Fig. 1) and liver showed no significant change with time after injection, indicating that uptake into maternal compartments occurred rapidly, and at a constant rate. Mean transfer rate for maternal blood for the two animals with embryos at stages 31-34 sampled at 60 min was skewed by one very high value of 3.67, and those data were omitted from the figure. Comparison of transfer ratios (TRs) for whole embryos at 60, 120 and 240 min showed that uptake of ^{3}H-oleic acid was relatively low at 60 min, but tended to increase with time. Transfer into embryos was lowest in females with embryos at Stages 31-34; transfer rates increased with embryonic stage, and were greatest when embryos are at Stage 40+ (Fig. 2). Two-way ANOVA indicated that there was a significant difference between groups ($F_{11,57} = 2.528$; $p = 0.011$), reflecting a significant difference between stages ($F_{3,57} = 3.975$; $p= 0.012$) but not between times, and the interaction term was not significant. Transfer ratios for yolk tended to increase between 60 and 120 min, with little change between 120 and 240 min (Fig. 3), but two-way ANOVA indicated that the differences between groups were not significant. Note that the data for Stage 40+ are somewhat compromised by the very tiny mass of yolk remaining at this stage (for three out of the 12 females in this group, the embryos had no detectable yolk left). Transfer ratios for

amniotic fluid did not differ significantly with time or embryonic stage: three samples with very high TRs were excluded from the analysis due to suspected contamination with maternal blood, and we were unable to obtain amniotic fluid from eight stage 40+ embryos that burst out of their membranes before fluid could be collected.

The comparative study showed that there was no significant difference between species in the uptake into maternal blood or maternal liver after 240 min for females with embryos at Stages 39-40 (Table 1), giving us confidence that it is valid to compare transfer into embryonic compartments between our three study species. However, TRs for maternal fat did differ significantly between *N. metallicus* and both other species (ANOVA: $F_{2,32} = 4.771$, $p = 0.015$), with very low TRs for maternal fat in *N. ocellatus* and *N. microlepidotus* compared with all other tissues examined. The weight of the maternal abdominal fat bodies in *N. metallicus* was very variable within each group, with some females having no discernable fat, and others having fat bodies weighing up to 39.2 mg.

Mean relative clutch masses were 0.48 for *N .metallicus*, 0.52 for *N. microlepidotus* and 0.51 for *N. ocellatus*. Between species comparisons of transfer into embryonic compartments (for females with embryos at Stages 39-40, and at 4 h after injection) showed that there were no significant differences in the TR for yolk (Table 2). There was a marginally significant difference in mean TR for amniotic fluid ($F_{2,30} = 3.303$; $p = 0.051$), reflecting a significant difference (Tukey's post-hoc test: $p = 0.04$) in TR between amniotic fluids in *N. microlepidotus* and *N. ocellatus*; however after removal of one outlier (a high TR of 1.820 for one *N. microlepidotus* sample), we detected no significant differences between species (Table 2). However, there was a significant difference between species in TR for embryos ($F_{2,30} = 9.955$; $p = 0.001$): Tukey's posthoc tests indicated that TRs for *N. metallicus* were

significantly higher than those for both *N. ocellatus* ($p = 0.001$) and *N. microlepidotus* ($p = 0.001$). Examination of TRs for embryonic abdominal fat bodies showed that oleic acid was transferred into these fat bodies in embryos of all species, but there were no significant differences between species in TR, in mass of embryonic fat bodies or in mass expressed as a percentage of embryo body weight.

DISCUSSION

Placental transfer of both fatty acids and free amino acids has been extensively documented in mammals (Munro, Pilistine, and Fant, 1983) but little attention has been paid to the capacity of the placentae of viviparous reptiles to transfer organic nutrients. Yaron (1977) first used the technique we adapted for this paper to demonstrate the transfer of tritium-labeled leucine into embryo, yolk and amniotic fluids of *Xantusia vigilis*, while Thompson (1977) presented a brief report of the transfer of glycine and α- amino isobutyric acid into embryos of *Eulamprus quoyi*. Our previous work (Swain and Jones, 1997) has demonstrated maternal-fetal transfer of radio-labeled leucine in the viviparous skink *Niveoscincus metallicus*, and unlike the earlier studies, related transfer to embryonic stage and, therefore to placental development. Our current results enlarge on that work by demonstrating the capacity of the placenta of this, and the related species *N. ocellatus* and *N. microlepidotus*, to transfer oleic acid into embryonic compartments. Placental transfer of fatty acids occurs in some but not all, mammalian species studied (Munro, Pilistine, and Fant, 1983); ^{14}C-labelled oleic and linoleic acids and their trans-isomers cross the placenta in rats (Moore and Dhopeshwarker, 1981) and in humans, there is evidence for selective placental transfer of particular fatty acids, and for transfer in both

directions (Larque et al., 2002). Our study provides the first direct evidence for placental transfer of a fatty acid in a viviparous reptile.

In *N. metallicus*, the time-course experiment showed that radio-labeled oleic acid is rapidly transferred into maternal compartments, with little change in TR between times of sampling. This contrasts with the pattern we observed for leucine uptake, where blood TRs decreased in the first two hours (data for 30, 60, 90, 120, 350 min after injection), with a corresponding increase in incorporation of ^{3}H-leucine into maternal liver proteins (Swain and Jones, 1997): this may reflect differences in uptake of leucine and oleic acid from the injection site and/or the dynamics of protein and fat synthesis.

The time course experiment demonstrated that transfer ratios for yolk and for amniotic fluid, unlike those for leucine (Swain and Jones, 1997), did not change significantly between times, nor between stages: leucine TRs also did not vary with embryonic stage. We have previously discussed potential routes of transfer of maternally-derived nutrients into yolk, and have suggested that uptake via the boundary region between the omphaloplacenta and the chorioallantoic placenta, where the blood vessels are continuous with those of the allantoic and vitelline circulations; uptake into amniotic fluids may simply reflect intensive transfer at adjacent sites (Swain and Jones, 1997).

There was a trend for oleic acid transfer into the embryos to increase with time, although this was not significant. However, for ^{3}H-leucine, there was a clear relationship between time of sampling (at 30, 60, 120 min) and TR for embryonic protein (Swain and Jones, 1997). This difference may reflect our decision to alter our sampling times for the oleic acid experiment (to 60, 120, 240 min) based on the supposition that rates of protein synthesis would be faster than rates of lipid metabolism. Uptake of ^{3}H-oleic acid into embryos did

increase with embryonic stage, and transfer was highest for embryos at stage 40+. In contrast, TRs for ^{3}H- leucine were highest, and similar, for stages 35-38 and 39-40, but there was a significant decrease for late stage embryos (Swain and Jones, 1997) (Fig.4) that we attributed this to regression of the chorioallantoic placenta during embryonic stages 39-40 (Stewart and Thompson, 1994).

Leucine transfer into embryonic protein in *N. metallicus* presumably reflects the capacity for some placentotrophic contribution to embryonic biomass. In this species, the total amount of lipid in a neonate is approximately half the amount originally present in the yolk ((Jones, Bennett, and Swadling, 1998); Thompson et al., 1999), indicating that placental transfer of fatty acids is unlikely to contribute significantly to embryonic growth or energetic requirements. Thus our observations that transfer of oleic acid is maximal in the last phase of gestation may reflect the presence of a facultative mechanism for optimizing embryonic fat reserves in the later stages of gestation. We have demonstrated the importance of these reserves for embryonic survival if gestation is prolonged past the end of embryonic development (Atkins, Swain and Jones, unpublished results; Swain and Jones, 2000a, b). We suggest that the ability to optimize offspring fitness through supplementation of embryonic fat reserves represents a selective advantage for facultative placentotrophy: the substantial transfer ratios for the embryonic fat bodies in *N. metallicus* provide further support this hypothesis.

The comparative data are less easy to explain. Transfer into maternal plasma and liver indicated that the dynamics of uptake from the injection site were comparable in these closely related species of similar body size. We did, however, expect that transfer of oleic acid into embryos would be greatest for the most placentotrophic species, *Niveosciucus ocellatus*, and

least for *N. microlepidotus*. Assessment of the relative contributions of yolk and placentae to embryonic nutrition in viviparous squamates is traditionally based on a simple comparison of neonate dry weight to yolk dry weight. If that ratio is below one, the species is classed as primarily lecithotrophic, while ratios above one indicate substantial placental contributions to embryonic nutrition (Blackburn, 1994). In *N. microlepidotus,* the neonate dry mass to egg dry mass ratio is only 0.755, so lower TRs for embryonic compartments would be expected for this species.

The observation that TRs for embryos were highest in *N. metallicus* rather than *N. ocellatus* was surprising. Although anatomical studies (Stewart and Thompson, 1994; Thompson et al. 2004) are equivocal, it is possible that in *N. metallicus* the placentae remain functional later in gestation than in *N. ocellatus*. *Niveoscincus metallicus* does appear to differ from *N. ocellatus* in having a distinct period late in gestation, which we have termed stage 40+, when the yolk is utilized yet the fully differentiated embryos may still weigh ≈10% less than mean birth weight (Swain and Jones, 1997). In *N. ocellatus*, on the other hand, full-term, stage 40, embryos have small but obvious yolk masses, suggesting that this route of nutrient transfer is still important very late in gestation. Does this mean that the bulk of placental transfer of organic nutrients takes place earlier in gestation in *N. ocellatus*? We focused on the later stage of gestation in the comparative study because it is most likely that embryos accumulate metabolic reserves during this period: further studies should examine a range of embryonic stages.

The marked differences between species in TRs for maternal fat bodies suggest that there are fundamental species-specific differences in the dynamics of lipid uptake into storage sites which may also reflect differences in annual fat body cycling. In *N. microlepidotus*, fat

body mass is low (ca. 10 mg) relative to that of vitellogenic females (ca. 40 mg) during the period when we made our collections (Girling, Jones, and Swain, 2002) while in *N. ocellatus*, fat bodies reach their maximum at around the time of parturition (Wapstra and Swain, 2001).

This study has demonstrated a capacity for placental transfer of radio-labeled oleic acid in three closely related species of viviparous reptiles with different degrees of placentotrophy although it is difficult to correlate the degree of transfer with the degree of placental complexity, nor with previous estimates of the proportion of lipid derived from yolk and placenta (Thompson et al., 1999; 2001). There is, however, evidence for transfer of this fatty acid into embryonic fat bodies in all three species, demonstrating a potential route for supplementation of neonatal reserves via the placenta, with implications for neonatal fitness. Further studies are required on the ontogeny and dynamics of transfer of this and other lipid components, the interrelationships between yolk and placental transfer, and of the molecular mechanisms involved in transfer (Speake and Thompson, 2000).

Acknowledgments.—We thank A. Edwards and N. Atkins for unfailing support in field and laboratory. This work was approved by the Animal Ethics Committee, University of Tasmania (AE006940) and carried out under permit from the Nature Conservation Branch, Department of Primary Industry, Water and Environment, Tasmania (Permits no. 04129 and 04128) and a "Collection of fauna" permit from Wellington Park Management Trust. The research was funded by an Australian Research Council Discovery Grant to RS and SMJ.

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