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Yolk contributes steroid to the multidimensional endocrine environment of embryos of *Niveoscincus metallicus*, a viviparous skink with a moderately complex placenta

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ABSTRACT

Maternally-derived testosterone (T) and 17-β-oestradiol (E_2) provide epigenetic mechanisms by which mothers can actively influence offspring phenotype. In amniotes, maternal steroids may be derived from yolk or transferred across the placenta according to parity mode. Viviparous reptiles utilise both a yolk and a placenta to support their developing embryos, but it has not yet been confirmed whether yolk is a source of maternal T and E_2 in such species. We investigated this question using the viviparous lizard *Niveoscincus metallicus* as our model species. We measured T and E_2 in the yolks during vitellogenesis, immediately post-ovulation and at progressive stages of gestation. Our results confirm that yolk is a substantial source of T and E_2 in *N. metallicus*. Contrary to the pattern seen in many oviparous species, we did not observe a marked decline in yolk concentrations of either T or E_2 after the initiation of sexual differentiation in the embryos. Rather, we found no statistically significant decline in yolk concentrations of both T and E_2 post-ovulation. In viviparous reptiles that utilise both yolk and placenta to nourish their embryos, yolk likely plays an important role in these dynamics but that role is not yet clear. Further research is warranted to understand the importance of yolk steroids in the endocrine environment of the developing viviparous reptile.

1. Introduction

The steroids testosterone (T) and 17-β-oestradiol (E_2) are critical to the differentiation of the reproductive system in vertebrates (Adkins-Regan et al., 1995; Kratochvíl et al., 2006; Radder, 2007; Arnold, 2009; Ramsey and Crews, 2009). From sex determination to the expression of diverse sexually dimorphic traits, exposure to T and E_2 profoundly influences life history (Vito and Fox, 1979; Clark and Galef, 1995; Schwabl, 1996; Wilson and Davies, 2007). During sexual differentiation, embryonic tissues are sensitive to exogenous steroids such as maternal hormones: maternally derived hormones are therefore a mechanism through which mothers can actively influence offspring phenotype (Schwabl, 1993, 1996; Fowden and Hill, 2001; Fowden and Forhead, 2004, 2009; Partecke and Schwabl, 2008).

In amniotes, the routes through which embryos are exposed to maternal hormone differ with parity mode. In therian mammals, embryos are exposed to maternal hormones via the placenta (Pasqualini, 2005). In oviparous reptiles and birds, the egg yolk is the source of maternal steroids (Schwabl, 1993; Janzen et al., 1998; Lovern and Wade, 2001). The influence of yolk steroids on the developing embryos has been repeatedly demonstrated in avian species. For example, size hierarchy in asynchronously hatching clutches is counteracted by increasing amounts of T and other androgens in the yolk with laying order (Schwabl, 1993, 1996; Cariello et al., 2006; Groothuis and Schwabl, 2008; Martin and Schwabl, 2008; Müller et al., 2012). The higher concentrations of androgens result in faster growth of the later-hatched offspring and more intense begging behaviour in the neonatal birds, and maximise their chances of becoming dominant in adulthood (Schwabl, 1993, 1996).

The subtle phenotypic effects of yolk T and E_2 have been less explored in oviparous reptiles compared with avian species. However E_2 and aromatisable androgens such as T have been demonstrated to play key roles in sex determination and differentiation in oviparous reptilian species (Wibbels and Crews, 1995; Crews et al., 1996; Conley et al., 1997; Janzen et al., 1998). Typically, yolk steroids decline rapidly at the time of sex differentiation and remain low for the remaining duration of development (Conley et al., 1997; Janzen et al., 1998; Paitz and Bowden, 2009) probably because the differentiating gonads require an external source of steroid. For example, T and E_2 incorporated into the yolk are critical to sex differentiation in *Alligator mississippiensis* (Conley et al., 1997) because the developing endocrine glands are not able to produce T and E_2 during the early phases of development (Smith et al., 1995).

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Although the more subtle phenotypic effects of yolk steroids on developing embryos are not fully understood, it is clear that the embryo actively utilises the T and E_2 rather than passively accepting T and E_2 as by-products of yolk utilisation. In eggs of some oviparous reptiles, the concentrations of hormones differ between layers within yolks (Lipar et al., 1999; Bowden et al., 2001). Such differential allocation suggests that hormones are strategically sequestered into the yolk, potentially to be utilised at specific stages of development. Differential allocation may be of particular significance for oviparous species because the connection between maternal and embryonic physiology is terminated upon oviposition. Maternally derived steroid signals are therefore predetermined and fixed during vitellogenesis in oviparous reptiles. What happens when the connection between maternal and embryonic physiology is extended for the duration of embryonic development?

Around 30% of reptilian species are viviparous, a parity mode which has evolved independently over 100 times in this taxon (Blackburn, 1982, 1985, 1992). Reptilian viviparity is most often achieved with a combination of yolk and placental support: embryonic exposure to maternal hormones could therefore occur via the placenta *and* the yolk. However, there is enormous variation in the degree of placental complexity and dependence on yolk support among viviparous reptiles (Blackburn, 1993, 2000; Stewart and Thompson, 2000; Thompson and Speake, 2006; Stewart, 2013). Therefore, four main placental types have been defined for viviparous reptiles: Type I lecithotrophic species have shell-less eggs that are retained in the oviduct. Embryos are sustained by a large yolk similar to that of oviparous species and a very simple placenta allows for water and respiratory gas exchange (Thompson and Speake, 2006; Stewart, 2013). Type IV placentrophic species support embryos with a microlecithal yolk and a complex placenta similar to that of mammals (Stewart and Thompson, 2000; Thompson and Speake, 2006). Types II and III placentae represent intermediates and exhibit varying degrees of complexity corresponding with differences in yolk size and composition (Stewart and Thompson, 2000; Thompson and Speake, 2006; Stewart, 2013).

Given that viviparity is a derived state, it is probable that the yolk of viviparous lizards of all placental types contain at least some steroid. Thus far, we know that steroid hormones are present in the yolk and can traverse the Type I placenta of *Scleroporus jarrovi* (Painter et al., 2002). Similarly, steroid hormones are present in the relatively small yolks (Parsley, Itonaga and Jones unpublished results) and can traverse the complex Type III placenta of *Pseudomoia entrecasteauxii* (Itonaga et al., 2011). Therefore there is evidence to suggest that the placenta and the yolk are both important sources of embryonic steroids, as well as organic and inorganic nutrients regardless of placental complexity (Painter et al., 2002; Itonaga et al., 2011; Stewart, 2013). We can speculate that the yolk is the primary route of maternal steroid in Type I species and the placenta the major route in Type IV species, however, these suppositions are yet to be tested. To further understand the routes of embryonic steroid exposure in viviparous lizards, we selected a study species with a moderately sized yolk and Type II placenta. In such species, is yolk a substantial source of steroid hormone despite the development of a reasonably sophisticated placenta?

Niveoscincus metallicus, a small (SVL ≤ 65 mm; mass 2–3 g) viviparous skink, provides an excellent model for exploring the endocrine environment of the embryos in viviparous lizards. The reproductive physiology of this species is well understood (Jones and Swain, 1996, 2006; Swain and Jones, 1997; Jones et al., 1998). In particular, the moderately complex Type II placenta and the composition of the moderately sized yolk of *N. metallicus* have been explored in several studies (Stewart and Thompson, 1994, 2003; Jones and Swain, 1996; Jones et al., 1998; Stewart and Thompson, 2000; Stewart, 2013) highlighting *N. metallicus* as an appropriate model for an initial study of the dependence upon the yolk as a source of steroid to developing embryos in viviparous reptiles.

We hypothesise that T and E_2 will be incorporated into the yolks of *N. metallicus* during vitellogenesis, that concentrations of both steroids in yolk will decline after sex determination, and that concentrations of both of T and E_2 in yolk will then remain low until parturition.

2. Material and methods

2.1. Ethical procedures

This research was conducted in accordance with the University of Tasmania Animal Ethics Committee under Permit A10797 and animals were collected with the permission of the Tasmanian Department of Primary Industries, Parks, Water and Environment: Permits FA10157, and FA09171.

2.2. Animal collection

To determine whether maternal hormones are sequestered into egg yolks, and to assess the importance of yolk hormones to developing embryos during development, T and E_2 were measured in the yolks of vitellogenic, post-ovulatory and gestating females. Eighty female *N. metallicus* were collected over two activity seasons during the periods described below from Old Farm Rd.: 42°53′38.33″S, 147°19′21.29″E in greater Hobart, Tasmania. Lizards were transported to the Herpetology facilities at the School of Zoology, University of Tasmania, where they were housed overnight with pureed fruit for food and water ad libitum. *N. metallicus* has a Type II reproductive cycle as defined by Taylor (1985). Vitellogenesis begins in post-partum females in mid-summer (January). Females enter winter torpor in May. Vitellogenesis is completed after spring emergence (September). Ovulation typically takes place in October. Gestation averages three months, after which time females give birth to between one and six young. In total, we had seven stages at which we sampled yolks from female *N. metallicus*: 'Vitellogenic' females were sampled in mid-autumn (i.e. April 2009), when yolks were about 1/3 of the size of a yolk at ovulation. All of the females in the vitellogenic group were collected on the same day, and all of the yolks had approximately the same diameter of around 2 mm. 'Post-ovulation': post-ovulatory females were defined as females having yolky eggs in the oviducts without any obvious signs of developing embryos. The post-ovulatory females were collected in mid spring (early October 2009 and 2010). The remaining five stages represent different progressive stages of gestation. Gestating females were collected when we anticipated embryos to have reached stages 29–30; 30–33 (the initiation of sexual differentiation: Neaves et al. 2006; Shine et al., 2007); 34–35; 36–38; and 39–40 as defined by Dufaure and Hubert (1961) in late October through December 2009 and 2010. An additional stage of embryonic development has been defined for *N. metallicus*. Stage 40+, uniquely described for *N. metallicus*, is characterized by embryos with internal hemipenes, little to no yolk remaining and development is ≥90% complete (Swain and Jones, 1997). We were able to sample yolks from embryos at stage 40+ for one female only captured in late December 2010. Embryos of *N. metallicus* cannot be staged until after dissection of the female, therefore we do not have equal numbers of embryos in each group.

2.3. Dissection and yolk collection

Adult female lizards were humanely killed by an overdose of sodium pentobarbital at a concentration of 500 ng/g. Yolks were dissected free from ovaries in vitellogenic females and from the oviducts in gestating females post-ovulation. Yolks associated with embryos were separated from extra-embryonic tissues and placentae and rinsed in Milli Q water; excess water was removed by blotting with clean paper towel. Isolated yolks were then placed into pre-weighed 1.5 mL microcentrifuge tubes, weighed to the nearest mg and covered with 200 μL of Milli Q water to prevent evaporative loss in the freezer (Painter et al., 2002): yolks were stored at −20 °C until analysis. Maternal and embryonic tissues were utilised for a separate study.