Routes and mechanisms of embryonic hormone exposure and endocrine disruption in a viviparous lizard

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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October 2013

Declarations

Statement of originality

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or in any other institution, and to the best of my knowledge, this thesis contains no material previously published or written by another person, except where due acknowledgment is made in the text of the thesis.

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Four of the chapters in this thesis have been written as manuscripts for publication. I am the first author on all of the manuscripts; however, all aspects of the preparation of the manuscripts were supervised by Professor Susan Jones and Associate Professor Erik Wapstra. The contributions of my PhD supervisors are acknowledged by co-authorship on all of the manuscripts.

Acknowledgments

First and foremost, I have to thank my supervisor Sue Jones. I first became interested in endocrinology as a second year student, when Sue introduced me to the wonders of endocrine disruption and so, I found a way to combine my passion of organic chemistry and pesticides with zoology. Sue has continually neutralised my flighty, highly strung nature with her wisdom and calm headedness. Sue has stood by me, when many other wouldn't have. Sue has been a model supervisor, always happy to help, always quickly to respond and always giving me space to learn. Thank you Sue, I would never have made it this far without you. If I were half as clever as you I would be so lucky ☺ you are a very inspiring woman.

My gratitude of Erik Wapstra began long before he was my supervisor. Erik supported me like he would his own students even when he wasn't in any way required to. Erik has taught me invaluable lessons about myself, and thus has greatly contributed to my life. Thanks Erik for being so open, for always being supportive and allowing me to be honest, for being a wonderful supervisor and for finally admitting that pesticides are very interesting.

Heartfelt thanks to Ashley Edwards, for all of her help in the early stages of the project.

Thanks to Noel Davies for the support, the laughs, for helping me see a different perspective on things, and for teaching me lots. Thanks to John Ross and Laura Quittenden for the use of the lab, and all the rest. Sincere thanks to Sean Tracey, for teaching me how to make microscope slides, to Lisette Robertson for supporting me in the lab; especially for helping me work in the histology lab with Mat Russell, without passing out from xylene exposure ☺

So thanks Mat Russell, for being so funny and cute, and dancing with me when times got boring. Thanks to Richard, Flick, Kate, Wayne, Barry and Adam for the constant support. Thanks to Cynthia Awruch for her wisdom, support and friendship. Thanks To Gemma Morrow for fun and gossips in the lab. Thanks to Jo McEvoy, and to all of the BEER group members, for support and advice.

Thanks to all my friends especially Sinno, Beth, Shelly and Belinda for support, help catching lizards and infusions of vitamins G and T, to Damien for all the free lunches and to Neil and AST for the use of equipment.

Thanks to Ma and Pa Parsley for way too many things to list here.

Most of all, thanks to Steffen, for all the help with mite treatments and cage cleans, for being the best partner a girl could wish for, for supporting me and my horses, for buying me a beautiful home in the country and for continually making my dreams come true.

This project was made possible by funding from the Holsworth Wildlife Research Endowment, the Ecological Society of Australia and the Forest Practices Authority.

Abstract

The mechanisms of embryonic exposure to hormones in viviparous reptiles are speculative, despite detailed understanding of exposure in oviparous species and eutherian mammals. The yolk of oviparous species and the placenta of eutherian mammals are key sources of steroid to developing embryos. Importantly, most viviparous reptiles utilise a yolk *and* a placenta to support embryogenesis. Maternally-derived testosterone and oestradiol have major impacts on development. The yolk and the placenta are therefore both potential sources of testosterone and oestradiol to embryonic viviparous lizards, but this remains to be tested.

Many endocrine disrupting chemicals (EDCs) impair gonadal development by affecting testosterone and oestradiol concentrations. Some EDCs disrupt the activity of aromatase which catalyses the conversion of testosterone to oestradiol. Embryonic development can be affected either by alterations in maternal hormone concentrations or by direct exposure to EDCs. Embryonic viviparous reptiles are at particular risk due to the multiple routes of potential exposure. However, no study has examined the routes of embryonic exposure to EDCs or examined EDC effects in viviparous reptiles. The major aims of this study on the viviparous lizard, *Niveoscincus metallicus,* were to confirm that the yolk is a source of testosterone and oestradiol to developing embryos; to demonstrate the placenta and embryonic tissues as sites of aromatase; and to examine the effects of gestational exposure to two EDCs (diethylstilbestrol and the herbicide atrazine) on gonadal development.

Yolks of *N. metallicus* were sampled from vitellogenesis through to the final stages of gestation. As in in oviparous reptiles, the yolk of *N. metallicus* contains testosterone and oestradiol, however, the stages at which the steroids decline are not comparable to oviparous species, as the placenta also provides steroid. Aromatase activity was measured in the placenta and embryonic tissues during three progressive stages of development. Placental aromatase activity was highest in the early stages of development, suggesting oestrogen synthesis in *N. metallicus* supersedes yolk reserves. The effects of oestrogenic EDCs on gonadal development were characterised by dosing gestating females with diethlystilbestrol at 100 or 10 µg/kg and examining the gonads of

neonates. The effects of gestational exposure to a single dose of atrazine at 10 µg/kg on gonadal development were also characterised by examining the gonads of neonates. Diethlyslstilbestrol and atrazine disrupt gonadal development: by comparing the developmental effects observed from exposure to diethylstilbestrol and atrazine in males and females, I conclude that atrazine disrupts gonadal development in *N. metallicus* via increased aromatase activity. This thesis has expanded our understanding of the endocrine environment in which embryos of viviparous lizards develop, and has provided new evidence of the potential impacts of EDCs on viviparous vertebrates.

Table of Contents

Chapter 1: Introduction

In this thesis, I focus on the mechanisms of embryonic hormone exposure and disruption of gonadal development by endocrine disrupting chemicals (EDCs) in a viviparous (live-bearing) lizard. The endocrine environment of oviparous (egg-laying) reptiles and birds, and of the placenta supported eutherian mammals have been characterised in many studies. It is well known that the yolk of oviparous reptiles is a source of steroids to embryos, while the placenta synthesises and transfers hormones to embryos in mammals (Strauss et al. 1996; Freyer et al. 2003). The effects of EDCs and the mechanisms of disruption to the endocrine system have been explored in oviparous species and eutherian mammals and such studies have established a comprehensive understanding of endocrine disruption. Thus, current knowledge of the mechanisms of hormone exposure and disruption by EDCs in oviparous species and eutherian mammals is vast. Yet no study to date has investigated the mechanisms of hormone exposure or the effects of EDCs on embryonic development in viviparous reptiles. Viviparous reptiles are of special interest in that the majority of species utilise a yolk and a placenta to support developing embryos, providing two potential routes of embryonic steroid exposure. In the context of endocrine disruption, the yolk and the placenta of viviparous reptiles provide multiple routes of embryonic exposure to EDCs and the effects of EDCs: yolk contamination, altered placental function and altered maternal hormone concentrations. There is very little known about the endocrine environment of viviparous reptiles. In this thesis, I will fill some of the missing gaps in the current knowledge of the endocrine environment of viviparous reptiles, and examine the effects of gestational exposure to two EDCs.

Sexual development of the reproductive system and brain is largely controlled by steroid hormones in vertebrates (Crews et al. 1996; Glickman et al. 2005; Wilson and Davies 2007). After sex determination, development of the bipotent, undifferentiated gonad to form an ovary or a testis is controlled by several steroids produced by the Hypothalamic-Pituitary-Gonad (HPG) axis (Crews et al. 1996; Crews et al. 1996; Wilson and Davies 2007). Key steroid hormones of testis development are the aromatisable androgen testosterone (T) and the non-aromatisable androgen dihydrotestosterone (DHT) (Crews et al. 1996; Hughes 2001; Wilson and Davies

2007), while the estrogens estrone (E_1) and primarily, 17-β estradiol (E_2) are critical to ovarian development (Crews et al. 1996; Norris 2007; Jones 2011). Steroids are not only critical to the formation of functional gonads, but they also play a major role in the diversity of phenotype within the sexes. Variation of life-history traits, secondary sex characteristics, the ability to respond to environmental cues and to reproduce successfully are initiated during embryonic development via steroids such as T and E_2 (Vito and Fox 1979; Clark and Galef 1995; Brandenberger et al. 1997; Wilson and Davies 2007).

Although androgens and estogens are important in male and female development respectively, both male and female embryos synthesise androgens and estrogens. In all vertebrates, estrogens are synthesised from aromatisable androgens by the enzyme aromatase which converts T to E_2, and androstenedione (A_4) to E_1 (Simpson et al. 2002; Payne and Hales 2004). Aromatase is pivotal to sexual differentiation as the circulating *ratios* of T and E_2 are fundamental to the formation of the ovary or testis. Thus there are between sex differences in aromatase activity which in turn result in relative differences in the circulating concentrations of T and E_2 (Weniger 1993; Place et al. 2001; Blázquez et al. 2008; Nakamura et al. 2008), which drive sexual development.

Phenotypic differences within the sexes are mediated via several factors. One major factor that determines phenotypic diversity is exposure to externally produced (i.e. of non-embryonic origin) T and E_2 during key stages of embryonic development. For embryos of amniote vertebrates (Figure 1.1a), the origin of external steroids can be maternal production, or the production of extraembryonic membranes which form the chorioallantoic membrane (CAM) of oviparous species and (Figure 1.1b) the chorioallantoic placenta of viviparous species (Ferner and Mess 2011). In litter bearing eutherian species, embryos may be exposed to steroids produced by neighbouring siblings *in utero* (Schwabl 1993; Clark and Galef 1995; Pasqualini 2005; Cruze et al. 2013). The routes and mechanisms of embryonic exposure to external steroids differ with parity mode (figure 1.1).

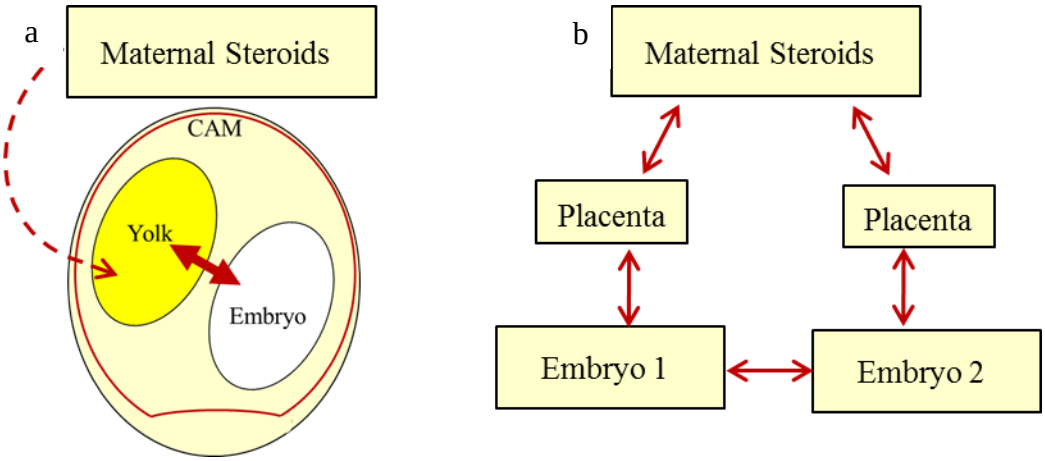

Figure 1.1 diagrammatic representation of the movement of steroids in an a) oviparous vertebrate embryo: the dashed line represents maternal steroids that are incorporated into the yolk during vitellogenesis, CAM is the chorioallantoic membrane. b) illustrates the movement of steroids during gestation in litter bearing eutherian mammals.

In oviparous species, maternal hormones are incorporated into the yolk (Schwabl 1993; Janzen et al. 1998; Lovern and Wade 2001). Yolk steroids may be associated with two major aspects of development in oviparous vertebrates. Firstly, maternal steroids deposited in the yolk during vitellogeneis and released during development are believed to play a major role in sexual differentiation of the reproductive tract, by providing an external source of steroid at a time when steroidogenesis in the undifferentiated gonad is low (Conley et al. 1997; Janzen et al. 1998; Paitz and Bowden 2009). Secondly, information regarding environmental conditions that the embryo will experience as a hatchling may be communicated via yolk steroid signals. Much of the understanding of the communicative role of yolk steroids has come from research on birds (Cariello et al. 2006; Martin and Schwabl 2008; Müller et al. 2012). In some avian species that exhibit size hierarchy in asynchronously hatching clutches, more androgen is allocated to later laid eggs (Cariello et al. 2006; Martin and Schwabl 2008; Müller et al. 2012). Increasing androgens with laying order counteracts the disadvantages of hatching last in competition with older siblings (Groothuis and Schwabl 2008). Chicks that are exposed to more androgens in the yolk exhibit faster growth, intense begging behaviour and are typically dominant as adults (Schwabl 1993; 1996). Although there are many potential species specific patterns, there is no

doubt that steroid and steroid derivatives in the yolk of oviparous vertebrates are the proximate mechanism underlying steroid mediated maternal effects. Furthermore, yolk androgen content relates to adult male attractiveness (Gil et al. 1999) and song quality (Gil et al. 2004), with females responding to attractive males and quality song by increasing androgens in the yolks of the entire clutch (Gil et al. 1999; Gil et al. 2004). Male chicks exposed to increased yolk T as an embryo present more striking plumage as adults (Strasser and Schwabl 2004), which presumably places them at an advantage for female selection in later life.

The yolk of oviparous reptiles is a substantial source of steroid, but, the phenotypic effects beyond gonadal differentiation are not yet clear (Conley et al. 1997; Janzen et al. 1998; Radder and Shine 2007; Paitz and Bowden 2009). Furthermore, the extraembryonic membranes the amnion, chorion and allantois that form the chorioallantoic membrane (CAM) of oviparous species is steroidogenic (Albergotti et al. 2009; Cruze et al. 2012; Cruze et al. 2013). The CAM of oviparous species has only recently been identified as an endocrine tissue (Albergotti et al. 2009; Cruze et al. 2012; Cruze et al. 2013) thus, the role of the CAM in the development of phenotype has not yet been established.

The role of the extraembryonic membranes as a source of hormones has been widely investigated in eutherian mammals (Strauss et al. 1996; Seckl 2001; Fowden and Forhead 2004)The amnion, chorion and allantois fuse to form the embryonic contribution to the chorioallantoic placenta of viviparous vertebrates (Ferner and Mess 2011). In eutherian mammals, the chorioallantoic placenta has replaced the yolk; therefore embryos are exposed to maternal hormones only via the placenta (Pasqualini 2005). The placenta of eutherian mammals plays an essential role in developmental programming (Strauss et al. 1996; Seckl 2001; Fowden and Forhead 2004). As in oviparous species, T and E_2 external to embryonic production influence sexual differentiation of the reproductive tract and the brain and control aspects of mating behaviours in adulthood (Vito and Fox 1979; Clark and Galef 1995; Brandenberger et al. 1997; Wilson and Davies 2007). The placenta of eutherian mammals is active in producing steroids (Strauss et al. 1996; Pasqualini 2005). In some eutherian mammals, the placenta synthesizes oestrogens but not androgens. The placenta relies on embryonic androgen as a substrate for oestrogen synthesis (Strauss et al. 1996).

As there are differences in the amount of androgen prodecued between the embryonic sexes, placental oestrogen synthesis is determined by embryonic sex (Strauss et al. 1996). The placenta of eutherian mammals is therefore involved in a sex-specific chemical dialogue in the production of steroids between mother and embryo and the ensuing 'chemical conversations' are key in the development of phenotype (Strauss et al. 1996; Seckl 2001; Harada 2004; Jones et al. 2007). Furthermore, in litter bearing species, embryos are exposed to steroids synthesised by neighbouring siblings *in utero* (Figure 1.1b). Female embryos positioned between two female embryos are exposed to higher concentrations of E_2 (vom Saal 1989; Clark and Galef 1995). In adulthood, females positioned between two females *in utero* are more attractive to males, have shorter oestrus cycles, and produce larger litters than females positioned between two males *in utero* (vom Saal 1989; Clark and Galef 1995). Thus embryo retention, creates a multidimensional endocrine environment, and provides extra scope for diversity in hormone mediated phenotypic effects.

The retention of embryos within the oviduct increases the potential for conflict between maternal and embryonic physiology. Although steroids are critical in processes in both the mother and the embryo, the physiological requirements of the mother and the embryo differ. The placenta therefore plays an additional role in mediating between the maternal and embryonic endocrine systems. An essential role of the placenta is therefore to 'buffer' effects of steroids between the mother and embryo (Strauss et al. 1996; Pasqualini 2005). The 'buffering' capacity of the placenta arises from the presence of enzymes that can modulate steroid potency by derivatising steroids to less potent compounds. Steroids can be conjugated to a glucuronic acid or sulphate moiety by the enzymes glucuronidase or sulphotransferase, or conjugated by steroid-specific β-hydroxysteroid dehydrogenases (Norris 2007). Conjugation increases aqueous solubility and prevents steroids binding with receptors and binding proteins, rendering the steroids biologically inactive (Norris 2007). The placenta of eutherian mammals is rich with steroid conjugating enzymes which 'buffers' both mother and embryo from steroid effects (Pasqualini 2005). In summary, the mammalian placenta is a complex endocrine organ that can synthesise hormones and hormone binding proteins, transport hormones between maternal and embryonic physiology,

and modulate steroids by chemical deactivation (Shozu et al. 1991; Yalcinkaya et al. 1993; Conte et al. 1994).

The placenta of eutherian mammals and the yolk of ovipsrous species provide embryos with an external source of steroid, but what are the functions of the yolk and the placenta in those species that utilize both a yolk and a placenta to support embryonic development? The majority of viviparous reptiles utilise a yolk and a placenta to support embryonic development. Amongst viviparous reptiles, there is enormous variation in the structure and complexity of placentae, reflecting over 100 independent origins of viviparity in this clade (Blackburn 1985; 1993; 2000; Thompson and Speake 2006; Stewart 2013). Such variation in structure and complexity relates to the degree of embryonic nutrient provisioning, and thus the dependence upon yolk to support embryonic development. The majority of viviparous reptiles are defined as lecithotrophic: these species are wholly dependent on yolk for embryonic nutrient provisioning, as the placenta transfers gasses and waste only. More rarely, viviparous reptiles are placentrophic: species with a microlecithal yolk that provides very little nutrition during development (Blackburn 1985; 1993; 2000; Thompson and Speake 2006; Stewart 2013). These two main forms of reptilian viviparity are at either end of a continuum. Four main types of chorioallantoic placentation have been defined:

Type I is the simplest placental type and is characterised by uterine apposition of the chorioallantois. The allantoic capillaries lie in close proximity to the uterus but do not actually interconnect (Weekes 1929, Blackburn 1993). A large yolk is required for embryonic development as the placenta is capable of gas and waste transport only.

Type II is characterised by vascularised uterine tissue in close connection with the chorionic epithelium (Weekes 1935, Blackburn 1993). Species with Type II placentation require a large yolk to support embryonic development; but, species within this group demonstrate significant variation in dependence on yolk, with varying degrees of placentrophy (Jones and Swain 2006, Swain and Jones 1997).

Type III placentae have extensive allantoplacental connections and specialised structures called placentomes, which are interconnected areas of maternal and embryonic tissue (Weekes 1935, Blackburn 1993); the reliance on yolk for embryonic development of these species is much reduced and the placenta provides organic nutrients to the embryo (Weekes 1935).

Type IV placentae are highly complex and bear similarities with the Type III placenta, but have highly complex placentomes and specialised chorionic areole (Blackburn 1993; Stewart and Thompson 2000). These species are microlecithotrophic, meaning they have minimal dependence on yolk to support embryonic development (Blackburn 1993).

Reptilian viviparity provides many opportunities in examining how placentae have evolved to provide embryos with T and E_2 with a reduction in the reliance of yolk for embryonic nutrition. The presence of both a yolk and a placenta and varying degrees of dependence upon the yolk allows for an examination of placental endocrine function. Yet no study to date has examined the involvement of the placenta in the development of T and E_2 mediated phenotype in any reptilian species. Furthermore, no study has confirmed that T and E_2 are present in the yolk of any viviparous reptile. In viviparous reptiles, there is the possibility that placental transfer, placental production, the yolk, or all three are sources of T and E_2 to developing embryos (Figure 1.2). The route of steroid transfer from mother to embryo may relate to placental complexity, with the yolks of Type I species as the dominant source of maternal steroid, and Type IV species relying upon placental transfer and production of steroids. However, this hypothesis currently lacks supporting evidence.

There is evidence, however, that progesterone is present in the yolk *and* can traverse the Type I placenta of *Scleroporus jarrovi* (Painter et al. 2002), and corticosterone can traverse the complex Type III placenta of *Pseudomoia entrecasteauxii* (Itonaga et al. 2011). Thus it is apparent that embryos of viviparous reptiles are exposed to *some* maternal steroids via both routes. Furthermore, there is evidence that placentae of viviparous reptiles are steroidogenic. Placental progesterone production has been demonstrated in *Eulamprus tympanium* (Adams et al. 2006), *Scleroporus jarrovi* (Guillette et al. 1989), and *Tiliqua nigrolutea* (Edwards and Jones 2001), all

of which possess a Type I chorioallantoic placenta. The Type II placentae of *Niveoscincus metallicus* and *N. microlepidotus* (Bennet and Jones 2002, Weekes 1935) and the complex Type III placenta of *Chalcides chalcides* all synthesis progesterone (Guarino et al. 1998, Blackburn 1993). Placental progesterone production in viviparous reptiles is thought to be associated with embryo retention (Adams et al. 2006), as it is in eutherian and metatherian mammals (Freyer et al. 2003; Norris 2007). There is also evidence to suggest that steroids from neighbouring siblings *in utero* can affect development in viviparous reptiles. Male *Lacerta vivipara* from female biased clutches exhibit differences in sexually dimorphic traits compared with males that developed in male biased clutches (Uller and Olsson 2003). Viviparous reptiles are therefore potentially exposed to hormones via multiple routes (Figure 1.2).

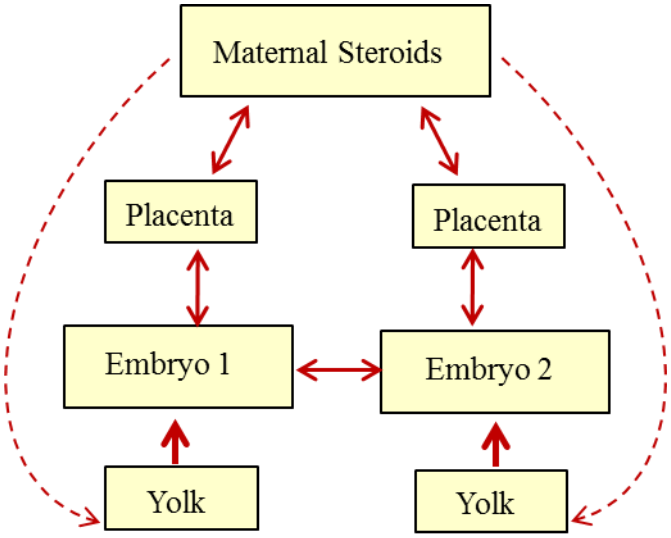

Figure 1.2 a schematic representation of the proposed routes of embryonic exposure to hormones such as T and E_2 in viviparous reptiles. The dashed lines indicate maternal steroids that are sequestered into the yolk during vitellogenesis, the solid lines indicate the movement of steroids throughout gestation.

A major aim of this study is to confirm the major routes of embryonic exposure to steroids in viviparous reptiles. No study to date has investigated the mechanisms controlling embryonic T and E_2 exposure in viviparous reptiles, or the potential for embryos to modulate T and E_2 to

which embryos are exposed. Therefore, the potential for placental buffering and derivatisation to less potent compounds by the embryo itself are major aims of this study.

The mechanisms controlling embryonic steroid exposure, synthesis and deactivation can be disrupted (Guillette et al. 1995; Guillette et al. 2000; Guillette and Gunderson 2001; Crews et al. 2003; Norris and Carr 2006). Disruption to the aforementioned endocrine processes leads to individuals with maladaptive phenotypes that persist into adulthood (Fry and Toone 1981; Colborn et al. 1993; Guillette et al. 2000; Norris and Carr 2006; Yang et al. 2011). A growing number of such individuals coincides with the persistence of hormonally active compounds in the environment.

Endocrine disruption is a broad term used to describe interference in any process of the endocrine system by a chemical of natural or synthetic origin (Norris and Carr 2006). Chemicals that have the capacity to interfere with any aspect of the endocrine system are termed endocrine disrupting chemicals (EDCs). It is estimated that there are over 15, 000 EDCs from several chemical classes. Examples of naturally occurring EDCs are phytoestrogens found in many plants and the androgenic substances released from processing wood in pulp mills (Norris and Carr 2006). Synthetic EDCs include several pharmaceuticals such as the contraceptive pill, additives found in cosmetics and personal care products, industrial additives such as polychlorinated biphenyls (PCBs), several herbicides, fungicides and insecticides, and products used in the manufacture of plastics (Fry and Toone 1981; Colborn et al. 1993; Guillette et al. 2000; Norris and Carr 2006; Yang et al. 2011).

Endocrine disrupting chemicals can interfere with any aspect of the endocrine system by mimicking, altering the production or metabolism of hormones or by interfering with the binding of hormones with plasma proteins or receptors (Guillette et al. 1995; Guillette et al. 2000; Guillette and Gunderson 2001; Crews et al. 2003; Norris and Carr 2006). Endocrine disruption affects vertebrates of all classes (Fry and Toone 1981; Giusti et al. 1995; Guillette and Moore 2006; Hayes et al. 2010; Jespersen et al. 2010; Guillette and Iguchi 2012), and can impair reproductive success via two main pathways. Firstly, *activational disruption*, is interference with

the proper functioning of a tissue. Activational disruption affects established organs and thus, commonly occurs in adulthood (Guillette et al. 1995; Silbergeld et al. 2002). Effects in males include reduced sperm count, sperm quality or sterility (Bayley 1999; Maczka et al. 2000; Kipfer et al. 2009; Hayes et al. 2011). In females, this form of disruption can lead to ovarian hypotrophy and ovarian, vaginal or mammary cancers (Maczka et al. 2000; Norris and Carr 2006). In both sexes, activational disruption can lead to altered mating behaviours (Bayley 1999; Maczka et al. 2000; Norris and Carr 2006; Hayes et al. 2010).

Secondly, *organisational disruption* is interference with the proper development and function of a tissue (Guillette et al. 1995; Silbergeld et al. 2002). This type of disruption occurs when the tissue is exposed to an EDC during a critical stage of development i.e. during embryonic development of early life (Guillette et al. 1995). Exposure to EDCs or abnormal concentrations of sex steroid as a result of EDCs during embryogenesis or early postnatal life can lead to permanent disruption to the development of tissues. Such effects may manifest as deformed gonads and/or gonaducts, which may cause gonadal dysfunction, sterility impaired neurological development, altered sex ratios or hermaphroditism (Guillette et al. 1994; Maczka et al. 2000; Newbold 2008; Hayes et al. 2010)

Current knowledge of endocrine disruption in amniotes has been largely obtained from studies on oviparous reptiles. Oviparous reptiles have been useful tools in identifying the effects and mode of action of EDCs. Reptiles inhabit terrestrial and aquatic ecosystems, prey on invertebrate species and demonstrate similar sensitivities to pollutants as some avian and mammalian species (Crain and Guillette 1998; Crews et al. 2003). Therefore, studies of endocrine disruption in reptilian species are relevant to many ecosystems and can shed light upon disruption in other taxa. Furthermore, reptiles can have temperature dependent sex determination (TSD), or genetic sex determination (GSD), where the sex of embryos is determined by incubation temperature or chromosomes respectively. Differences in mode of sex determination allow mechanisms of organisational effects to be elucidated (Crain and Guillette 1998; Crews et al. 2003). Exploiting TSD in laboratory studies has identified the EDC properties of many contaminants (Bergeron et al. 1994; Guillette et al. 2000; Milnes et al. 2002). For example, the identification of several

EDCs as estrogen agonists has been achieved by dosing eggs of TSD reptiles at male producing temperatures. Under control conditions, normal male phenotypes are formed, yet eggs exposed to estrogenic EDCs at male producing temperatures exhibit female phenotypes post-hatch (Bergeron et al. 1994).

Viviparity in reptiles allows the comparison of routes of embryonic exposure to EDCs of maternal origin to be assessed (Crain and Guillette 1998). Due to the lipophilic nature of many EDCs, high quantities of EDCs accumulate in adipose tissue and are mobilised during preparation for reproductive events (Guillette et al. 1995). For the oviparous amniotes contamination of the large yolky eggs with EDCs and altered concentrations of maternal hormones in the yolk due to EDC exposure are likely (van de Merwe et al. 2009; Hamlin et al. 2010). In eutherian mammals, embryos are likely exposed via placental transfer of contaminants (Shah and McLachlan 1976; Slikker et al. 1982). Viviparous reptiles are at particular risk due to the many potential routes of embryonic exposure to EDCs (Figure 3): altered maternal steroid signals via the yolk and the placenta, altered placental function and by direct contamination via the yolk and/or the placenta.

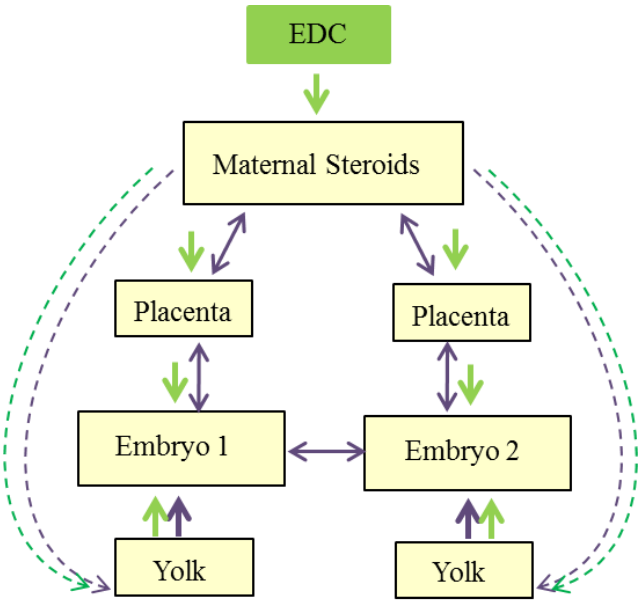

Figure 1.3 Diagrammatical representation of the proposed routes of embryonic exposure to EDCs in viviparous lizards. The green arrows indicate the movement of

EDCs from the mother to the embryo, the purple arrows indicate embryonic exposure to altered steroid signals (maternal activational disruption) following maternal EDC exposure. The dotted line indicates movement during vitellogenesis.

The timing of *embryonic* exposure to EDCs can determine their future effects (Guillette et al. 1995; Crain et al. 2008; Di Renzo et al. 2011; Gioiosa et al. 2013). For example, in laboratory mice, exposure to the potent EDC diethylstilbestrol (DES) results in the formation of polyovular follicles (POF) and the critical stage of exposure is post-natal day 3. Exposure outside of this time does not induce POF (Iguchi et al. 1986). In TSD reptiles, embryos are vulnerable to EDC exposure during the period of sex determination (Bergeron et al. 1994) as hormonal cues and temperature drive gonadal development (Crews et al. 1994; Crews et al. 1996; Pieau et al. 1999). This period of sexual lability is commonly in the early phases of development in reptiles (Wibbels et al. 1991; Shine et al. 2007). However, in some species of eutherian mammal such as the guinea pig, permanent organisational effects of steroids and thus EDCs occur immediately prior to or just after birth (Phoenix 2009).

The timing of transient *maternal* exposure to EDCs can determine the effects on embryonic development. In oviparous species, key phases of maternal exposure are during vitellogenesis, while in eutherian mammals gestational exposure is of particular significance. Importantly, in the majority of viviparous reptiles which utilise both a yolk and placenta, embryonic development can be affected by transient maternal exposure from vitellogenesis through to the completion of gestation.

Embryonic viviparous reptiles are at a potential greater risk of endocrine disruption due to the multiple routes of exposure, yet no study to date has utilised viviparous reptiles in any study of endocrine disruption. Thus, to examine the effects of EDCS on embryos of viviparous reptiles is one second major aim of this study.

For a first study of the routes of embryonic hormone exposure and endocrine disruption in viviparous reptiles, a suitable study species needed to be selected. *Niveoscincus* is a viviparous genus of lizard consisting of eight species, six of which are endemic to the island of Tasmania, at

the south-eastern end of Australia (Hutchinson et al. 1990; Melville and Swain 1997; 1999). Placentation and the composition of yolk in the context of nutrient provisioning has been extensively studied in members of this genus (Jones et al. 1998; Stewart and Thompson 2000; Swain and Jones 2000; Jones and Swain 2006; Stewart and Thompson 2009; Stewart 2013).

Niveoscincus metallicus is a small skink averaging 3-5 g, with a snout-vent length of 45-65 mm. *Niveoscincus metallicus* has been intensively studied and its reproductive physiology is well known (Swain and Jones 1994; Jones and Swain 1996; Girling et al. 2002; Stewart and Thompson 2009). The annual reproductive cycle and cycle of reproductive hormones has been established (Swain and Jones 1994; Jones and Swain 1996). Environmental influences on litter size, the effects of maternal body condition on development and the strategies utilised to maximise offspring success have also been determined (Jones and Swain 2000; Swain and Jones 2000; 2000). Importantly, placental structure and partitioning between nutrient provisioning of the yolk and the placenta is relatively well understood (Swain and Jones 1997; Jones et al. 1998; Thompson et al. 1999; Swain and Jones 2000; Stewart 2013), and some placental endocrine function has been established (Collins 2009). Furthermore, the distribution of *N. metallicus* is well known and reference sites free of EDCs such as pesticides allow collection of lizards suitable for laboratory studies (Melville and Swain 2000; Kookana et al. 2010; Department of Primary Industries 2013).

Laboratory studies examining the effects of EDCs are important to establish the effects of EDCs. Due to the complex nature of the mechanisms of disruption to the endocrine system, the effects of potential EDCs can be compared with effects of known EDCs, to establish common biological effects. Such comparisons allow for the identification of estrogenic or androgenic EDCs. One particularly useful EDC is the potent oestrogen mimic diethylstilbestrol (DES) which was used to prevent miscarriage up until 1971, when reproductive cancers were associated with developmental exposure to DES (Giusti et al. 1995). Presently, DES is utilised in laboratory studies as a positive control to understand more about estrogenic EDCs (Iguchi et al. 1990; Iguchi et al. 1991; Newbold 2004; Larocca et al. 2011; Newbold 2012; Simon et al. 2012). Diethylstilbestrol is a powerful tool to utilise in laboratory studies because the site of disruption

of the HPG axis and ensuing phenotypic changes are well known and accepted (Kuiper et al. 1997).

Atrazine (ATZ) is a selective herbicide used in forestry and agricultural practices and is a compound that has demonstrated endocrine disrupting effects, but the effects are widely disputed. Atrazine exposure in the African clawed frogs, *Xenopus laevis* resulted in demasculinisation of males and hermaphroditism (Hayes et al. 2002), yet when the same experiment was repeated by a different group, no developmental effects were observed (Kloas et al. 2009; Kloas et al. 2009). Hayes et al. (2010) went on to demonstrate such potent effects of ATZ that genetically male *Xenopus laevis* became so feminized and demasculinised by ATZ, that they developed functioning ovaries with viable oocytes. Furthermore, the effects of ATZ in embryonic oviparous reptiles are also conflicting. Abnormalities following ATZ exposure were observed in *Caiman latirostris*, but not in *Alligator mississippiensis* (Crain et al. 1997; Crain et al. 1999; Stoker et al. 2000; Rey et al. 2009). In laboratory rodents, *in utero* exposure to ATZ has very subtle reproductive effects, at high concentrations well above concentrations typically observed in the environment (Davis et al. 2011; Fraites et al. 2011). The subtle reproductive effects observed in laboratory rodents were therefore deemed as ecologically irrelevant: thus ATZ is presumed to pose no threat to wildlife or humans (Davis et al. 2011; Fraites et al. 2011), and is still legally used in many countries.

Atrazine increases aromatase activity in tissues which express the steroidopgenic factor 1 (SF-1) and the aromatase II promotor (ArPII) (Fan et al. 2007; Fan et al. 2007). Unlike some reptilian species that utilize SF-1 In gonadal development (Ramsey et al. 2007), the developing ovary of rodents does not utilize SF-1 for the majority of development (Schimmer and White 2010). Furthermore, rodents are the only eutherian mammal studied to date that do not exhibit placental aromatase (Simpson et al. 2002), and may indeed be the only amniote that do not express aromatase in the extraembryonic membranes (Albergotti et al. 2009; Cruze et al. 2012; Cruze et al. 2013). The subtle effects of ATZ observed in rodents could be a result of subtle differences in endocrine function. The ovary of rodents is not a site of ATZ disruption, as disruption occurs only via ArPII ans SF-1 (Fan et al. 2007; Fan et al. 2007). Despite these facts, rodents are the

only viviparous vertebrates in which laboratory studies on the effects of ATZ have been conducted.

I suggest therefore, that laboratory rodents are not the most suitable viviparous vertebrate in which to examine developmental effects of ATZ. Additional studies of the effects of developmental exposure to ATZ in other viviparous taxa should be conducted, prior to the assumption that ATZ persistent in the environment is not a threat to wildlife. I propose viviparous reptiles as particularly useful species to examine the effects of EDCs such as ATZ. Reptiles are often more susceptible to the effects of chemical contaminants, as many reptiles maintain optimum temperatures well below those of eutherian mammals, and therefore enzymatic activity is slower. This could result in less effective removal of toxicants from plasma (Campbell and Campbell 2000), and increase the potential of placental transfer to the embryo.

In this thesis, I aim to make a substantial contribution to the current knowledge of the routes of embryonic exposure to steroid hormones. Furthermore, I aim to establish a model species for the assessment of endocrine disruption in viviparous lizards, for future studies beyond this thesis. For the first time, I will examine endocrine disruption in a viviparous reptile.

1.1 Thesis structure

I have constructed this thesis to consist of a general introduction, four empirical chapters, a synthesis of the findings and an appendix. I first explore the endocrine mechanisms through which embryos could be subject to the influence of EDCs, by determining the yolk as a source of steroids and by identifying key sites and phases of aromatase activity in the placentae, maternal and embryonic tissues. I then carry out an experimental investigation of the endocrine disrupting effects of the potent EDC DES, to characterize the effects of estrogen mimicry in *N. metallicus* as a basis for future investigations. Finally, I examine the effects of ATZ upon gonadal development. Chapters 2-5 of this thesis are written as manuscripts submitted or intended to be submitted for publication, thus some repetition of concepts is unavoidable. Minor formatting of each chapter has been undertaken to streamline the thesis, therefore, specific journal formatting requirements have been altered.

1.1.1 Chapter 2: Yolk contributes steroid to the multidimensional endocrine environment of embryos of *Niveoscincus metallicus,* a viviparous skink with a moderately complex placenta

In chapter two, I identified the yolk as a source of T and E_2 for the developing embryos of viviparous lizards. Viviparity is a derived state, and in reptiles there is a progressive departure from the reliance on the yolk for embryonic nutrient provisioning which is reflected in each placental type . In a species with a moderately complex Type II placenta such as *N. metallicus*, the placenta and the yolk work in conjunction to provide embryonic nutritional support: the placenta and the yolk essentially contribute equally to provide nourishment to embryos (Stewart and Thompson 1994; Swain and Jones 1997; Jones et al. 1998; Swain and Jones 2000; Jones and Swain 2006; Stewart 2013). The yolk of *N. metallicus* is therefore mandatory in nutrient provisioning. The dependence on the yolk is an indication that although *N. metallicus* have developed a reasonably sophisticated placenta, the yolk maintains some functions analogous to oviparous reptiles. Therefore, it was expected that the yolk is a source of T and E_2. I therefore measured T and E_2 in the yolks of female *N. metallicus* from mid vitellogenesis through until the final stages of gestation. I confirm that the yolk is a substantial source of these steroids, but the decline in T and E_2 is unique compared to oviparous reptiles.

1.1.2 Appendix: No oestrogen derivatization by the placentae and embryonic tissues of a live-bearing reptile: an accurate result or a maladroit researcher?

Embryos of viviparous species are likely to be exposed to maternal hormones that traverse the placenta during gestation. The placenta of *N. metallicus* can metabolise corticosterone (Collins 2009), although, it is not yet known if the placenta can metabolise E_2. I therefore performed *in vitro* incubations with placentae, maternal and embryonic tissues to ascertain the ability of each tissue type to derivatise E_2 to free steroid metabolites and the biologically inactive steroid conjugates. I conducted a detailed chemical analysis to identify potential compounds that were produced following the *in vitro* incubation. Unfortunately no biologically significant results were generated, although analytical techniques were developed. I therefore present my findings in an

appendix, with the aim of demonstrating the advancements to steroid conjugate analysis that were made.

1.1.3 Chapter 3: Placental and embryonic tissues exhibit aromatase activity in the viviparous lizard *Niveoscincus metallicus*

Aromatase is central to embryonic development as aromatase directly controls circulating concentrations of T and E_2 (Simpson et al. 2002; Payne and Hales 2004). Many EDCs including ATZ disrupt aromatase (Sanderson et al. 2000; Sanderson et al. 2004; Hayes et al. 2006; Fan et al. 2007; Hayes et al. 2010), therefore characterizing aromatase activity during development provides the foundation for future studies on the effects of contaminants that disrupt aromatase activity. I demonstrated aromatase activity in the placentae, maternal and embryonic tissues of *N. metallicus* at three stages of gestation. I report aromatase activity in maternal and embryonic tissues that is comparable to other species; however, aromatase activity in placental tissue does not resemble activity in the placentae of eutherian mammals.

1.1.4 Chapter 4: *In utero* exposure to the oestrogen mimic diethylstilbestrol disrupts gonadal development in a live-bearing lizard

In chapter four I identified the effects of increased estrogen signalling on gonadal development in *N. metallicus.* Characterizing the effects of estrogenic EDCs was a necessary first step in using a new species for studies of endocrine disruption. Model contaminants are useful tools with which to examine the mechanisms of effects of EDCs, and in using new experimental systems to validate procedures. One EDC which demonstrates consistency in effects across vertebrate taxa is the synthetic estrogen diethylstilbestrol (DES) (Iguchi and Takasugi 1986; Iguchi et al. 1990; Yoshimura and Fujita 2005; Newbold 2008). I dosed gestating female *N. metallicus* to a single dose of DES at either 100 or 10 µg/kg, during the initial phase of embryonic sexual differentiation. I found *in utero* exposure to DES at both concentrations disrupted gonadal development in male and female neonates. I have found that the effects of increased estrogen signalling have effects on male *N. metallicus* that are similar to other vertebrate taxa. However, in

females I found few comparable effects to other taxa. I therefore developed a semi-quantitative scoring system to measure the extent of disruption to ovarian development.

1.1.5 Chapter 5: Atrazine is a potent endocrine disruptor to live-bearing lizards

In chapter 5, I identified the effects of the herbicide ATZ on gonadal development in *N. metallicus*, by exposing gestating females during the invitation of embryonic sexual differentiation. I dosed gestating females to a single ecologically relevant dose of ATZ (10 µg/kg). By utilising DES as a positive control I was able to identify the mechanism of disruption as an increase in oestrogen signalling. Atrazine is a potent EDC, but is still heavily used in most countries, as the ecological significance of ATZ as an EDC is still debated. My results suggest that ATZ should no longer be used, as clear developmental abnormalities were observed from a single dose at a low concentration.

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Chapter 2: Yolk contributes steroid to the multidimensional endocrine environment of embryos of *Niveoscincus metallicus*, a viviparous skink with a moderately complex placenta

Submitted to Comparative Biochemistry and Physiology Part A

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 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.

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). 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). Type 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 regardless of placental complexity. 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 this is 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 $\leq$ 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.

Methods

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'.33S, 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*. *Niveoscincus metallicus* have 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 till 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 $\geq$ 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 our groups representing the specified stages do not have equal numbers.

Dissection and yolk collection

One yolk from each female was randomly selected for steroid analysis. Where possible, a second randomly selected yolk from each female was used to obtain the dry weight of the yolk. The dry yolk mass was acquired by oven drying yolks for 24 hours at 60 ºC. The percentage of dry matter in the yolk was calculated (dry weight / wet weight *100). Steroids were extracted from wet yolks by modification of the protocol of Hamlin et al (2010). Homogenized yolks were topped up to 500 µl with Milli Q water and then extracted with 2 ml of AR grade diethyl ether by vormixing for 1 min. The aqueous and organic phases were allowed to separate for 1 min prior to snap freezing in dry-ice cooled methanol. Once the aqueous phase was frozen, the organic phase was decanted into a clean tube. This procedure was repeated three times. Due to the small volumes of yolk in *N. metallicus* extraction efficiency (87 %) was determined with yolk from the large viviparous skink *Tiliqua nigrolutea* spiked with 2,4,6,7-^{3}H-oestradiol or 1,2,6,7 ^{3}H-Testosterone respectively. Oestradiol and testosterone in yolk extracts were measured with radioimmunoassay (RIA).

Yolk extraction

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Radioimmunoassay

Duplicate aliquots (250 µl vitellogenic- stage 36, 500 µl post stage 37) of yolk extracts were incubated overnight at 4°C with 2,4,6,7-^{3}H-oestradiol or 1,2,6,7 ^{3}H-Testosterone (PerkinElmer, Victoria, Australia) and oestradiol or testosterone antiserum (Sirosera E_2;CAT C-6181, T; C-6050, North Ryde, New South Wales, Australia) in phosphate-buffered saline-gelatine. The standard curve ranged from 3.125 - 800 pg of authentic E_2 or T. The sensitivity was 1.5 pg for both T and E_2. Three assays were conducted for both T and E_2: the grouping of samples for assay was vitellogenic-stage 29, stages 30-36 and stages 37-40+. Assay parallelism was demonstrated by confirming that assaying different volumes of extract yielded equivalent results. The inter assay coefficient of variation was calculated by assaying replicate yolk extracts from *T. nigrolutea* and *N. metallicus* with previously determined T and E_2 content and yolks spiked with T and E_2. To further check assay variability, a third randomly selected yolk from a subsample of mothers at each stage was extracted and assayed for E_2 content. The inter assay coefficients of variation were 11.4% and 12.2% for E_2 and T respectively. All results were corrected individually for 87 % extraction efficiency.

Statistical analysis

Data analysis was performed using SAS 9.2 for Windows. Differences in adult female snout-vent length (SVL), litter size, wet yolk mass, dry yolk mass, total amount of E_2 and T (pg) and the concentration of E_2 and T (pg in the yolk / wet weight of the yolk in mg) of yolk were examined individually using general-linear-models (GLM). Normality of distribution was checked by examining plots of standardized residuals against predicted values and normal probability of the residuals. The data for litter size, the total amount of T and E_2 as well as the concentration of E_2 (pg/mg) required log transformation prior to analysis, while the concentration of T (pg/mg) had a normal distribution. Three outliers were removed from the analysis of the total amount of E_2 and

the concentration of E_2 in yolk because they were more than three standard deviations higher than the mean. Differences between the groups for all of the models were identified using Tukey's Honest Significant Difference.

Results

Adult female biometrics and yolk characteristics

There was no significant difference between adult female SVL or litter size in any of the seven groups ($F_{6,63} = 0.46$; $p = 0.8342$, $F_{6,73} = 1.10$; $p = 0.3710$ respectively). There was a significant difference in the dry weight of the yolks ($F_{5, 55} = 14.44$; $p < 0.0001$). The yolks from vitellogenic females weighed significantly less than the yolks at any other stage. There was no statistical difference between the dry weights of the yolks from the post ovulatory stage to stages 34-35. The mean dry weight of the yolks was significantly less at stages 36-38 compared with stages pre 35; however there was no significant difference between stages 36-38 and stages 39-40 (Fig 2.1a). There was a significant difference in wet yolk mass ($F_{6,73} = 38.73$; $p < 0.0001$): the yolks increased in weight from vitellogenesis to post ovulation, and then a successive decrease from post-ovulation to stages 30-33. There was no decrease in wet yolk mass between stages (fig 2.1a) 30-33 and 34-35, but the yolk mass decreased successively from stages 34-35 through until the final stages of gestation. Although we did not include the single sample we obtained from the one female at stage 40+ in the statistical analysis, we included that data in the Figures.

Oestradiol and testosterone

Oestradiol and T were present in yolks from vitellogenesis (ie pre ovulation) right until the final stages of gestation; the ratio of T: E_2 was consistently greater than 1 (Figure 2.1c, b and d). The total amount of E_2 in the yolk (pg) was lower in the vitellogenic group than at every other stage, but did not change significantly after ovulation ($F_{6,73} = 6.46$; $p < 0.0001$). The concentration of E_2 in the yolk (pg/mg) did not significantly change during gestation ($F_{6,73} = 1.88$; $p = 0.0974$). The total amount of T in the yolks (pg) was significantly lower during vitellogenesis than any of the other stages examined ($F_{6,72} = 22.17$; $p < 0.0001$). Yolks sampled from lizards with embryos at

stages 34-35 and 36-38 had higher total T than those at stages 39-40. The concentration of T (pg/mg) in yolk was lowest at stages 28-29, and highest during the final stages of gestation (39-40) ($F_{6,72} = 3.72$; $p = 0.0028$).

Discussion

Our results confirm that yolk is a substantial source of T and E_2 in *N. metallicus*. The concentrations of T and E_2 in the yolks were in the same order of magnitude as those measured in *Anolis carolinensis* (Lovern and Wade 2003) but lower than several other reptilian and avian species (Schwabl 1993; Bowden et al. 2001; Kratochvíl et al. 2006). The variation in our data is comparable to the variation in yolk steroids in chelonian species (Bowden et al. 2001; Elf et al. 2002). In *N. metallicu*s, the total amount of T in the yolks is higher than that of E_2. This pattern is consistent with observations in *A. carolinensis, Paroedura picta* and many avian species (Schwabl 1993; 1996; Lovern and Wade 2003; Kratochvíl et al. 2006). However, in chelonian and crocodilian species, the total amount of E_2 in yolk is most usually higher than the total amount of T (Conley et al. 1997; Janzen et al. 1998; Bowden et al. 2001, 2002; Bowden et al. 2002; Elf et al. 2002; Lovern and Wade 2003; Kratochvíl et al. 2006).

The difference in the ratio of T:E_2 is a potential indication of the function of these steroids deposited in the yolk (Lovern and Wade 2003). Lovern and Wade (2003) noted that bird and lizard species that have genetic sex determination (GSD) have a T:E_2 ratio of >1 (Schwabl 1993; Lipar et al. 1999; Lovern and Wade 2003; Kratochvíl et al. 2006), while reptilian species that have temperature dependent sex determination (TSD) have a T:E_2 ratio of <1 (Conley et al. 1997; Bowden et al. 2001, 2002; Elf et al. 2002; 2002). The different T:E_2 ratios could reflect the functional role of these steroids in the yolk, or perhaps relate to phylogenic history (Lovern and Wade 2003). Our data suggests that *N. metallicus* is a GSD species, however, the congeneric viviparous species *Niveoscincus ocellatus* has populations with GSD *and* populations with TSD (Pen et al. 2010). Investigating the ratio of T and E_2 in the yolks of *N. ocellatus* from different populations may help to determine the functional significance of variations in the ratio of T: E_2 in reptilian yolks.

Regardless of whether the species exhibits TSD or GSD, the typical pattern for oviparous species is that total amount of T and E_2 in the yolk are high during the early phases of development, but drop markedly after sex determination (Conley et al. 1997; Bowden et al. 2002; Elf et al. 2002; Lovern and Wade 2003), although exceptions have been reported recently (Ding et al. 2012; Huang et al. 2013). We hypothesised that *N. metallicus* would follow this pattern. As has been postulated for oviparous species, embryos of *N. metallicus* may have an increased requirement for yolk T and E_2 during the early phases of sex differentiation, when the steroidogenic capacity of embryos is low. Sex differentiation presumably occurs in *N. metallicus* around stages 30-32, as this is when the congeneric *N. ocellatus* (Neaves et al. 2006) and other species of lizard begin sexual differentiation (Shine et al. 2007). Contrary to our predictions, we did not observe a significant decline in either yolk T or yolk E_2 at stages 30-32. At no time during development did we observe a marked decline in either hormone to the very low levels reported in the yolks of oviparous species. We observed a significant increase in the total amount of both T and E_2 from vitellogenic yolks to yolks post ovulation, presumably reflecting the incremental addition of yolk to the vitellogenic follicle. However, there was no significant change in the total amount of E_2 in yolks during gestation. There was a reduction of total T in the yolks at stage 29-30, and the total amount of T was significantly higher at stages 34-38 compared with stages 29-30 and stages 39-40. Why do we observe such different patterns in the pattern of change in yolk T and E_2 in *N. metallicus* compared with oviparous reptiles at equivalent stages of development? There are several potential explanations for our results.

Firstly, steroids can be differentially allocated between layers in the yolk. For example, the majority of the E_2 could be in the inner layers of yolk, as in the turtles *Chrysemys picta marginata* and *Trachemys scripta elegans* (Bowden et al. 2001). In those species, the eggs are large enough to allow sub-sampling of yolk layers for separate analysis. We were unable to sub-sample layers of the small yolks of *N. metallicus*, however, we observed a significant increase in the *total amount* of both T and E_2 from vitellogenesis to post ovulation, and no difference in the *concentration* of either hormone. These results suggest that hormones are deposited at a consistent rate as yolk layers are built up throughout vitellogenesis.

Secondly, it is possible that the embryo synthesises steroids that are then deposited into the yolk (Lovern and Wade 2001; 2003). Embryonic contributions to yolk T are suggested to occur in *A. carolinensis.* In that species, a spike in yolk T occurs at day 24 of incubation, presumably reflecting embryonic production and transfer of lipophilic steroid to the yolk (Lovern and Wade 2003). Similarly, yolk T increases during development (from approximately the end of the first trimester to the end of the second trimester) in the oviparous *Gekko japonicus* (Ding et al. 2012), and in the oviparous leopard gecko (*Eublepharis macularis*) testosterone concentrations in the yolk-albumen are higher in late development compared with early development at the female-biased temperature 26 °C (Huang et al. 2013). However, it seems unlikely that accumulation of embryonic steroid into the yolk entirely explains our results. If the patterns of yolk T and E_2 that we report reflect initial maternal contributions followed by embryonic contributions, we would observe a marked decline in yolk T and E_2 as these steroids are utilised for gonadal development during sexual differentiation because the steroidogenic capacity of embryonic gonads is low in the early embryo (Smith et al. 1995; Girling and Jones 2006). Once the steroidogenic capacity of the embryonic gonads increases during later development (Smith et al. 1995; Girling and Jones 2006), an increase in yolk steroid content due to transfer into yolk might be observed, as reported for T in *A. carolinensis* (Lovern and Wade 2003). However, in *N. metallicus* at no point throughout embryonic development, did we observe a significant decline of either T or E_2 in the yolks. We certainly did not observe anything like the marked declines of steroid from the yolks reported for several oviparous reptiles (Conley et al. 1997; Bowden et al. 2002; Paitz and Bowden 2009). The increased yolk T we observed at stages 34-38 may reflect embryonic production of the hormone; however the pattern of yolk T and E_2 we report cannot be entirely explained by the potential for embryonic contributions to yolk T and E_2 content.

The consistently high E_2 and apparently fluctuating T content in the yolk could in part, be a result of *maternal* contributions to the yolk during gestation via the omphaloplacenta. Maternal contributions to yolk T post ovulation have been demonstrated in *A. carolinensis,* whilst the eggs were in the oviduct despite the presence of the egg shell (Lovern and Wade 2001; Cohen and Wade 2010). Dosing mothers with radiolabelled T resulted in higher proportions of labelled T in the yolks of oviducal eggs compared with vitellogenic follicles in *A. carolinensis* (Cohen and

Wade 2010). Similarly, experimentally increasing circulating maternal corticosterone during gestation in the viviparous lizard *Pseudomoia entrecasteauxii* resulted in significant transfer of corticosterone into the yolk (Itonaga et al. 2011). Collectively, the findings of Lovern and Wade (2001), Cohen and Wade (2010), and Itonaga et al. (2011) indicate that maternal steroids can become incorporated into the yolk of post-ovulatory eggs in both viviparous and oviparous lizards.

Nevertheless, maternal transfer of steroids into the yolk during gestation does not altogether explain our results. Embryos of viviparous reptiles are exposed to exogenous steroids via several potential routes: circulating maternal steroids via the placenta (Painter et al. 2002; Painter and Moore 2005; Itonaga et al. 2011), placental production (Girling and Jones 2003), production from neighboring siblings *in utero* (Uller and Olsson 2003; Uller et al. 2004, 2005), and maternal steroids that are sequestered into the yolk. These multiple possible routes of steroid exposure may mean that the embryos are not wholly dependent on yolk steroids in the early phases of gonadal differentiation. For steroids to move into the embryo as a by-product of yolk metabolism, yolk steroids would need to move against a concentration gradient from a lipophilic environment in which they have a high solubility to the hydrophilic circulation of the embryo (Moore and Johnston 2008). Moore and Johnson (2008) therefore reasoned that the marked declines in steroid content in yolks of oviparous species (Conley et al. 1997; Bowden et al. 2002; Elf et al. 2002; Lovern and Wade 2003) are not due to passive transfer of steroid to the embryo, but are the result of active transport of steroids from the yolk (Moore and Johnston 2008). If steroids are actively removed from the yolk during key phases of development, it is also possible that steroid uptake can be prevented by blocking such active transport, but this hypothesis remains to be tested.

In conclusion, the yolk of *N. metallicus,* a species with a moderately complex placenta, contains quantities of T and E_2 comparable to the yolks of oviparous lizards. Steroids have been demonstrated as important epigenetic agents by which mothers can influence offspring success (Schwabl 1996; Schwabl et al. 1997; Groothuis et al. 2005; Müller et al. 2012). In oviparous reptiles, such maternal effects are mediated via yolk steroids (Conley et al. 1997; Janzen et al. 1998; Lovern and Wade 2001; Paitz and Bowden 2009). In therian mammals, maternal steroids

can traverse the placenta (Pasqualini 2005). With the transition from oviparity to viviparity, a complex relationship between the maternal and embryonic endocrine systems has been established. In viviparous reptiles that utilise both yolk and placenta to nourish their embryos, yolk 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.

Acknowledgments

We thank Heather Hamlin and Louis J. Guillette Jr for their generous assistance with the yolk extraction procedure. Funding for this project was provided by the Holsworth Wildlife Research Endowment, the Forest Practices Authority, the School of Zoology UTAS and the ARC (EW: Future Fellowship). 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 FA 10157, and FA09171.

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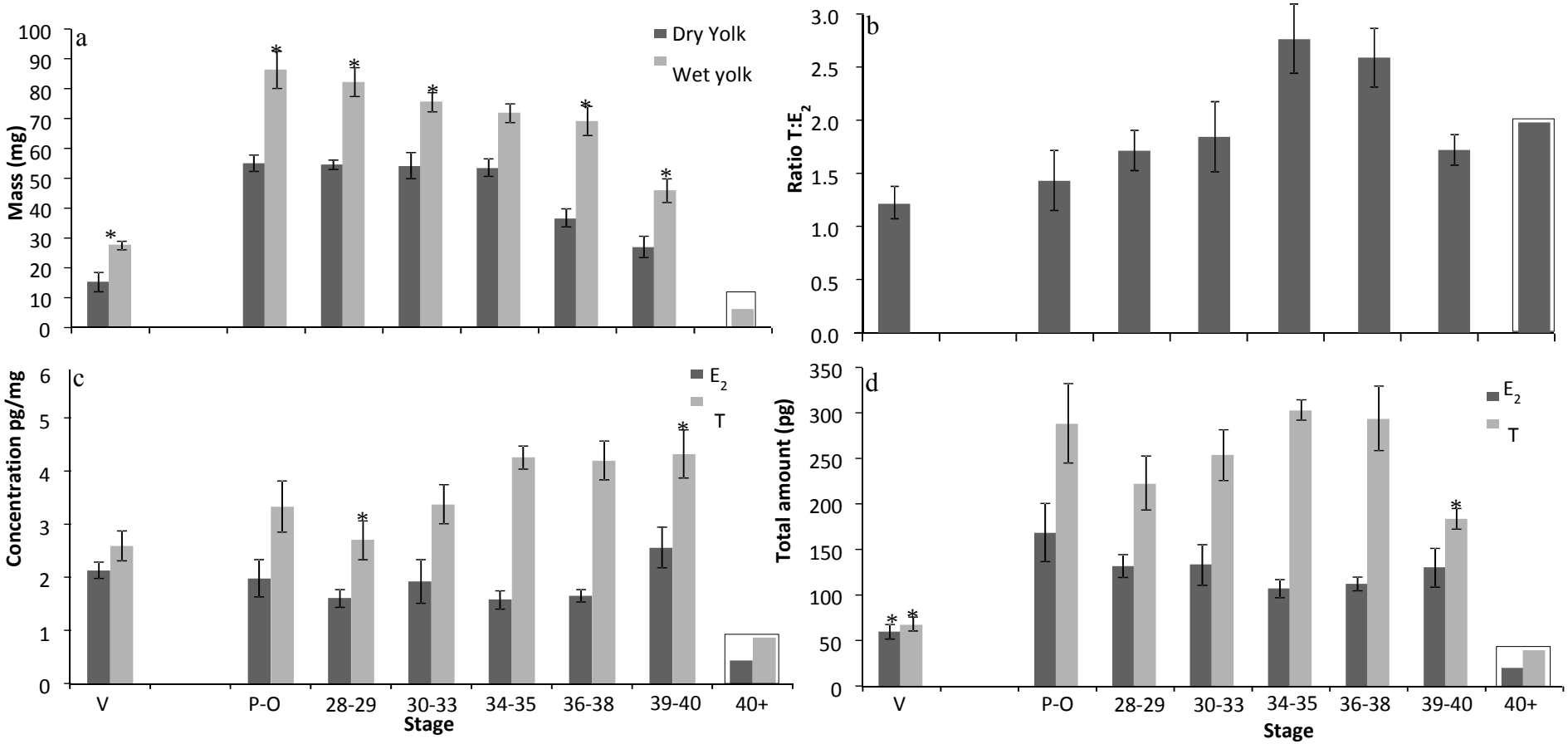

Figure 2.1 a) The wet and dry weight of yolks **b)** The ratio of T and E_2 in the yolks, **c)** The concentration of T and E_2 in the yolks and **d)** The total amount of T and E_2 in the yolks collected from N*iveoscincus metallicus* during V: vitellogenesis (n = 10), P-O: immediately post ovulation (n = 8) and at stages 29-30 (n = 11), 30-33 (n = 9), 34-35 (n = 12), 36-38 (n = 13), 39-40 (n = 17) and stage 40+ (n = 1) of gestation. Error bars represent the standard error of the mean. Asterisks indicate statistically significant differences. Boxes around bars indicate the single sample that was not included in the statistical analysis.

Chapter 3: Placental and embryonic tissues exhibit aromatase activity in the viviparous lizard *Niveoscincus metallicus*

In revision at General and Comparative endocrinology

Abstract

Aromatase is a key regulator of circulating testosterone (T) and 17-β-oestradiol (E_2), two steroids which are critical to the development, maintenance and function of reproductive tissues. The role of aromatase in sexual differentiation in oviparous (egg-laying) reptiles is well understood, yet has never been explored in viviparous (live-bearing) reptiles. As a first step towards understanding the functions of aromatase during gestation in viviparous reptiles, we measured aromatase activity in maternal and embryonic tissues at three stages of gestation in the viviparous skink, *Niveoscincus metallicus.* Maternal ovaries and adrenals maintained high aromatase activity throughout gestation. During the early phases of embryonic development, placental aromatase activity was comparable to that in maternal ovaries, but declined significantly at progressive stages of gestation. Aromatase activity in the developing brains and gonads of embryos was comparable with measurements in oviparous reptiles. Aromatase activity in the developing brains peaked mid development, and declined to low levels in late stage embryos. Aromatase activity in the embryonic gonads was low at embryonic stage 29-34, but increased significantly at mid-development and then remained high in late stage embryos. We conclude that ovarian estrogen synthesis is supplemented by placental aromatase activity and that maternal adrenals provide an auxiliary source of sex steroid. The pattern of change in aromatase activity in embryonic brains and gonads suggests that brain aromatase is important during sexual differentiation, and that embryonic gonads are increasingly steroidogenic as development progresses. Our data indicate vital roles of aromatase in gestation and development in viviparous lizards.

Introduction

The aromatase enzyme complex comprises of cytochrome P450 aromatase and nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase. The aromatase enzyme complex, hitherto referred to as aromatase, catalyses the conversion of androstenedione (A_4) and testosterone (T) to estrone (E_1) and 17-β-oestradiol (E_2) respectively (Conley and Hinshelwood 2001; Simpson et al. 2002; Payne and Hales 2004). Both the components and the functions of aromatase are highly conserved across vertebrate species (Conley and Hinshelwood 2001; Simpson et al. 2002; Payne and Hales 2004), highlighting the ubiquitous nature of estrogen synthesis in all vertebrates, and the pivotal role of aromatase in vertebrate reproductive function.

Aromatase is fundamental to embryonic development for several reasons. Aromatase activity influences circulating ratios of T and E_2 (Conley and Hinshelwood 2001; Simpson et al. 2002; Payne and Hales 2004), therefore, aromatase plays a fundamental role in sexual differentiation of phenotype. Testosterone and E_2 are critical to differentiation of the reproductive system and the expression of phenotypic diversity in sexually dimorphic traits (Clark and Galef 1995; Wilson and Davies 2007; Ramsey and Crews 2009). Although there are both species-specific and sex specific variations in aromatase activity during embryonic development, aromatase is expressed in the developing brains and gonads of male and female vertebrates (Krohmer and Baum 1989; Weniger 1990; 1993; Willingham et al. 2000; Blázquez et al. 2008).

Many studies have concluded that an external source of hormones including T and E_2, is required to initiate development of the embryonic gonads (Conley et al. 1997; Janzen et al. 1998; Paitz and Bowden 2009). In amniotes (mammals, birds and reptiles), parity mode influences the source of these external hormones. In oviparous (egg-laying) species, the egg yolk is the major source of steroids of maternal origin that are deposited in yolk during vitellogenesis (Schwabl 1993; Lovern and Wade 2003; Paitz and Bowden 2009): the chorioallantoic membrane (CAM), which is now known to be steroidogenic (Albergotti et al. 2009; Cruze et al. 2012, 2013), provides an additional source. In eutherian mammals, the amnion and chorion fuse to form the placenta which

allows transport of maternal hormones throughout gestation (Pasqualini 2005) and steroid hormones are also synthesised by the placenta (Leiser and Kaufmann 1994; Strauss et al. 1996).

The embryos of oviparous reptiles and eutherian mammals are clearly exposed to maternal hormones via very different routes (yolk versus placenta), but what do we know of those viviparous reptiles that utilise both a placenta and a yolk? Viviparity has evolved more than 100 times in reptiles and approximately 30 % of all reptilian species are viviparous (Blackburn 1982, 1985, 1993). Due to the number of independent origins of viviparity, there is considerable variation in the form and complexity of the chorioallantoic placenta, therefore, four main placental types have been defined in viviparous reptiles (Blackburn 1993, 2000; Stewart and Thompson 2000; Thompson and Speake 2006). Type I species are predominantly lecithotrophic: these species have shell-less yolky eggs that are retained in the oviduct and the very simple placenta allows for water and respiratory gas exchange (Thompson and Speake 2006). At the other end of the spectrum, Type IV species are highly placentrophic: a complex placenta provides the majority of the nourishment to developing embryos while a microlecithal yolk provides minimal nourishment to embryos (for review see Stewart and Thompson 2000; Thompson and Speake 2006; Stewart 2013). The intermediate Types II and III fall along a spectrum of placental complexity and degree of placentotrophy (Stewart and Thompson 2000; Thompson and Speake 2006).

To date, there is scant information on the steroidogenic capacity of any reptilian placentae. While it has been demonstrated that the delivery of nutrients varies with placental type, the capacity for synthesis of hormones may not. The demonstration of steroidogenesis by the CAM of the oviparous reptiles *Alligator mississippiensis* and *Pseudemys nelsoni*, as well as the bird *Gallus gallu*s, indicates that such steroidogenic function of the extraembryonic membranes is a conserved attribute of all amniotes (Albergotti et al. 2009; Cruze et al. 2012, 2013). This idea is further supported by progesterone production by representative reptilian species with three of the four placental types (Girling and Jones 2003; Guarino et al. 1998; Painter and Moore 2005). However, the presence of placental aromatase, and, therefore, placental capacity to synthesise oestrogens, has not been confirmed in any viviparous reptile.

This study therefore aims to identify key sites and activity levels of aromatase during gestation in the reproductive tissues of the viviparous lizard, *Niveoscincus metallicus*. This species provides an excellent model for a first investigation of the activity and putative functions of aromatase in viviparous lizards. The reproductive physiology of *N. metallicus* is well understood (Jones and Swain 1996; Swain and Jones 1997; Jones et al. 1998; Jones and Swain 2006) and the moderately complex Type II placenta has been extensively studied (Stewart and Thompson 1994; Jones and Swain 1996; Jones et al. 1998; Stewart and Thompson 2000; Stewart and Thompson 2003; Stewart 2013). We hypothesise that aromatase is present in placental tissue of *N. metallicus*, and that aromatase activity increases toward the later phases of gestation because placental development progresses with embryonic development (Stewart and Thompson 2003). We hypothesise that aromatase in the developing embryonic brains will peak during the mid-phases of embryonic development around the time of sexual differentiation. We also suggest that aromatase activity in embryonic gonads will increase from early to late development, because the steroidogenic potential of the gonads in early development in other reptilian species is very low (Girling and Jones 2006; Milnes et al. 2002; Smith1 and Joss; Willingham et al. 2000).

Methods

We measured aromatase activity in selected maternal and embryonic tissues of *N. metallicus*. *Niveoscincus metallicus* is a small lizard with snout-vent length (SVL) of ≤ 65 mm and body mass 2-3 g. *Niveoscincus metallicus* has a type II reproductive cycle as defined by Taylor (1985). Vitellogenesis is initiated in autumn and completed after spring emergence (September): ovulation typically takes place in mid spring (~ October in Tasmania) with gestation typically lasting ~ three months. Females give birth to between one and six young. We collected adult female lizards at three stages during gestation: 'early' (embryonic stages 29-34, n = 9); 'mid' (embryonic stages 35-37, n = 10) or 'late' (embryonic stages 38-40+, n = 11) gestation, as defined by Dufaure and Hubert (1961). In *N. metallicus*, an additional stage of embryonic development (stage 40+) has been defined: at this stage, embryos have internalised hemipenes, no yolk remaining and development is ≥ 90 % complete (Swain and Jones 1997). We collected gestating females when we anticipated embryos would have reached the aforementioned stages;

however, accurate staging of embryos is not possible in viviparous lizards until dissection, and thus we have unequal numbers of females (and embryos) for each stage.

Animal and tissue collection

Pregnant female *N. metallicus* were collected by mealworm fishing or noosing in and around the Sandy Bay campus of the University of Tasmania: 42°54'24.9"S, 147°19'21.89"E and Old Farm Rd: 42°53'38'.33S, 147°19'21.29"E in greater Hobart, Tasmania, Australia. The adult female lizards were collected from October through till December 2009 and 2010. Lizards were co-housed overnight in cages 200 x 600 mm with pureed fruit as a food source and water *ad libitum*.

Adult female lizards were weighed and measured (SVL) prior to humane sacrifice with an IP bolus injection of sodium pentobarbital, at a dose of 500 ng/g diluted 1:100 in saline solution. At dissection, litter size was recorded. Both ovaries and adrenal glands and samples of skeletal muscle tissue from a hind limb were removed and submerged in ice-cooled RPMI-1640 incubation medium (Sigma-Aldrich, Australia) while the dissections were completed. Maternal ovaries served as a 'positive control' tissue as we anticipated high aromatase activity in this primary steroidogenic tissue. Similarly, skeletal muscle tissue was included as a 'negative control' as we expected limited capacity for steroidogenesis in this tissue. Embryos were dissected free from the yolk and placental tissue. All embryos within each litter were utilised. Placental tissue (early: n = 23, mid: n = 35 and late: n = 37) was rinsed and placed in ice cooled RPMI-1640 incubation medium. The embryos were carefully dissected under a stereomicroscope. The small size of *N. metallicus* prevents isolation of the developing Adrenal-Kidney-Gonad complex (AKG) until after stage 37 so the AKG complex of embryos up to stage 36 (early: n = 32, mid: n = 34, late: n = 33) was sampled by removing the entire torso of the embryos (Girling and Jones 2003). Similarly, the entire head of embryos at or before stage 36 was used to assess aromatase activity in the brain (early: n = 31, mid: n = 35, late: n = 37). The AKG and the brain of embryos were dissected out in embryos post stage 37. The wet weight of maternal ovaries, skeletal muscle tissue and placentae were recorded. For maternal adrenal glands, embryonic heads, brains, trunks and AKGs tissue weight was not recorded, as an accurate weight could not

be obtained for maternal adrenals and embryonic AKGs. Embryonic trunks and heads contain non-steroidogenic tissue, thus the weight of such samples is not biologically meaningful and was also not included in the analysis. All tissues were finely minced prior to incubation with a fine pair of forceps.

Measurement of aromatase activity

Aromatase activity was measured with the tritiated water technique (Lephart and Simpson 1991) modified for turtle and alligator embryos (Willingham et al. 2000; Milnes et al. 2002) and adjusted to accommodate the small size of embryonic *N. metallicus*. All reagents used were AR grade and purchased from Sigma-Aldrich Australia unless specified otherwise. For each sample, 1.6 µM 1β-^{3}H-Androstenedione (A4) in 9:1 toluene ethanol (PerkinElmer, Melbourne, Australia) was added to a pre-weighed borosilicate test tube. The solvent was evaporated prior to the addition of 200 µl of buffer (RPMI-1640) supplemented with 25 mM Hepes, 1 mM dithiothreitol, 1 mM NADPH, 10 mM α-D-glucose-6-phosphate and 1U/ml glucose-6-phosphatedehydrogenase. Two 'tissue free' tubes containing A4 with RPMI-1640 supplemented media were included in the assay to track the non-specific release of 1β-^{3}H throughout the incubation process. The tubes containing A4 and media were equilibrated for 1 hr prior to the addition of the macerated tissues and then incubated on a constantly rocking water-bath for 6 h. The assay was conducted at 26˚C as this is the preferred body temperature of *N. metallicus* (Melville and Swain 2003).

After the incubation period, 750 µl of chloroform was added to halt the reaction and remove the hydrophobic A4 substrate from the reaction matrix. Two hundred and fifty microlitres of deionised water was added to each tube and pulse-vortexed before centrifugation at 1150 x *g*. Three hundred microliters of the aqueous phase (2/3 of the original aqueous phase) was isolated from the organic phase and placed into a clean boroscilicate glass test tube. To ensure complete removal of any unused A4 and remaining tissue, 300 µl of dextran-coated charcoal was added to each tube. The charcoal was then removed from the matrix by centrifugation at 1150 x *g.* Three hundred microlitres (1/3) of the aqueous phase was added to 2.5 ml of Ecolite scintillation

cocktail and counted on a Beckman LS6500 Multipurpose Scintillation counter. All tissues were discarded.

Aromatase activity was calculated by multiplying the sample disintegrations per minute (DPM) by 3 (1/3 of the aqueous phase was used for radiocounting), subtracting the amount of non-specific hydrogen release (tissue free tubes), dividing this number by the DPM of total count tubes (1.6μM 1β-^{3}H-Androstenedione in 2.5 ml scintillation cocktail), and multiplying by the mass of tritiated A4 (in pg) added to each tube (Willingham et al. 2000; Milnes et al. 2002).

Statistical analyses

Data analysis was performed using SAS 9.2 for Windows. Variation between stages in adult female SVL, litter size, the mean aromatase activity of both ovaries, skeletal muscle tissue (fmol/mg/hr) and the mean aromatase activity of both adrenal glands (fmol/adrenal/hr) were each examined by individual 1-way ANOVA. Normality of distribution was checked by examining plots of standardised residuals against predicted values and normal probability plots of the residuals. Analysis of aromatase activity in ovaries and maternal adrenal glands was performed on log-transformed data to ensure that a normal distribution was achieved prior to analysis. Significant differences were identified using Tukey's Honest Significant Difference (HSD).

Embryonic heads and brains, and embryonic trunks and AKGs, were treated as groups to represent activity throughout development in the brain and gonad respectively. Variation in aromatase activity between stages for placental tissue, heads/brains and trunks/AKGs were examined individually with a general linear mixed model. Maternal identity was included as a random factor, to statistically account for non-independence of samples of placentae and tissues from embryos of the same litter. We anticipated an effect of maternal identity on aromatase activity in placental and embryonic tissues. Therefore, the effect of maternal identity on aromatase activity in placental and embryonic tissues was examined individually by running the mixed models with and without maternal identity. The covariate parameter estimate was obtained together with model fit statistics. The significance of the effect of maternal identity was calculated by obtaining a χ^2 value (the difference between the -2 residual log likelihood of the

two models), and subsequent p value. In all cases, maternal identity improved the fit of the model (see results), but as this is not of biological relevance in this study, beyond reporting this result we don't discuss it further. The distribution of the data was checked as detailed above. Analysis of aromatase activity in placental tissue and developing gonads was performed on log-transformed data. Significant differences were identified with Tukey/Kramer corrected significant difference.

Results

There was no significant difference in adult female SVL ($F_{2,27} = 2.33$; $p = 0.1165$) or clutch size ($F_{2,27} = 0.67$; $p = 0.5194$) between each of the three sampling stages.

We measured detectable aromatase activity in the ovaries, adrenal glands, and skeletal muscle tissue of gestating female *N. metallicus* (Fig 3.1), and also in the placentae (Fig 3.2) brains and developing gonads of their embryos (Fig 3.3a and 3.3b). Maternal endocrine tissues were included in the *in vitro* incubation, not only to investigate their roles in gestation and the potential for endocrine disruption, but also to validate the incubation procedure. As predicted, maternal ovaries exhibited the highest activity of the maternal tissues examined because oestrogen synthesis is a primary function of the ovary (Jones 2011). Aromatase activity in ovarian tissue averaged 15 fmol/mg/hr, and in skeletal muscle tissue averaged 1 fmol/mg/hr), providing physiological validation of our technique. There was a marginally significant difference in aromatase activity in maternal ovaries between stages ($F_{2,25} = 3.40$; $p = 0.0508$), and no significant difference in aromatase activity of maternal adrenals between stages ($F_{2,26} = 3.00$; $p = 0.0685$), but for both tissues there was a trend for activity to be lower in 'late' gestation (embryonic stages 39-40+). Placental aromatase varied significantly between stages ($F_{2,67} = 28.46$; $p < 0.001$); activity was highest in the 'early' stage of gestation, significantly decreased by 'mid' gestation with a further significant decrease in 'late' gestation. Aromatase activity in skeletal muscle tissue differed significantly between stages ($F_{2,26} = 5.38$; $p = 0.0111$) with the difference arising because of a significant reduction in aromatase activity from the 'mid' to 'late' stage of gestation. Ovarian, placental and adrenal aromatase activities were all of the same order

of magnitude at all stages of gestation examined, while aromatase activity in skeletal muscle tissue was about ten times less than maternal endocrine tissues.

There was a significant effect of maternal identity on aromatase activity of placental tissue (estimate = 0.02; $\chi^2 = 5.8$; $p < 0.05$), aromatase activity in the developing gonads (estimate = 0.02; $\chi^2 = 8.8$; $p < 0.05$) and aromatase activity in the developing brains (estimate = 0.02; $\chi^2 = 35.8$; $p < 0.05$) of *Niveoscincus metallicus* embryos.

There was a significant difference between groups in aromatase activity in embryonic trunks/AKGs ($F_{2,70} = 6.48$; $p = 0.0026$). Activity in embryonic trunks and AKGs significantly increased from 'early' to 'mid' development, but did not change significantly between the 'mid' and 'late' stage of development. Similarly, there was a significant difference between stages in aromatase activity in embryonic heads/brains ($F_{2,73} = 5.05$; $p = 0.0089$) with a significantly lower aromatase activity observed at the 'late' stage of development compared to the 'early' and 'mid' stages. Aromatase activity in trunks/AKGs was around ten times higher than in the heads/brains.

Discussion

The major aim of this study was to demonstrate the presence of aromatase in maternal and embryonic tissues of *N. metallicus,* and investigate whether activity varied between stages of gestation. We measured aromatase activity in relevant maternal and embryonic tissues, and report activity levels comparable to those in other reptilian species (Willingham et al. 2000; Place et al. 2001; Milnes et al. 2002).

Aromatase activity in maternal ovaries did not change significantly between the 'early' and the 'mid' stages of gestation, although there was a marginally significant decrease in ovarian aromatase activity in 'late' gestation. Aromatase activity in maternal adrenals (reported as activity per gland) was not significantly different at any of the stages of gestation examined, but activity was comparable to that of the ovaries. Thus, our data suggests that maternal adrenals have significant capacity to contribute to oestrogen synthesis in *N. metallicus*. Adrenals are generally understood to produce a range of steroids, including sex steroids (Norris 2007). For

example, maternal adrenals are an accessory source of sex steroids in *Lacerta vivipara* (Dauphin-Villemant and Xavier 1985), while in *Niveoscincus microlepidotus*, maternal adrenals produce progesterone when incubated *in vitro* (Girling and Jones 2003).

For the first time, we report aromatase activity in the placental tissue of a viviparous reptile. Aromatase activity in placental tissue of *N. metallicus* is comparable to activity levels in maternal ovaries demonstrating a key role of placentae in oestrogen synthesis in both maternal and embryonic circulation. Contrary to our predictions, in *N. metallicus*, placental aromatase was highest in 'early' gestation, and then decreased to 'mid' gestation and again from 'mid' to 'late' gestation despite an increase in the complexity of the placenta during this period (Stewart and Thompson 2003). This suggests that the placenta provides an external source of steroids such as E_2 to the early gonad of *N. metallicus*, a role restricted to the yolk in oviparous species (Conley et al. 1997; Janzen et al. 1998; Paitz and Bowden 2009).

We investigated aromatase activity in the developing brains and gonads of embryos at three stages of gestation as a first step towards understanding the mechanism of sexual differentiation in this viviparous species. Aromatase activity in the AKG of *N. metallicus* followed a pattern similar to that previously reported in several oviparous reptilian species. Typically, aromatase activity in the embryonic gonad is low in the early stages of development, but as the gonads become more differentiated, aromatase activity increases (Desvages and Pieau 1992; Smith and Joss 1994; Smith et al. 1995; Willingham et al. 2000; Milnes et al. 2002). Furthermore, oestrogen production is one proxy for aromatase activity: the increase in aromatase activity of the developing AKGs between stages of gestation in embryos of *N. metallicus* parallels the pattern of *in vitro* E_2 production by AKGs of embryos of the related species *N. microlepidotus* (Girling and Jones 2006).

The similarity in patterns of aromatase activity levels in the AKG of *N. metallicus* and E_2 production in *N. microlepidotus*, together with data on many oviparous reptiles (Desvages and Pieau 1992; Smith and Joss 1994; Smith et al. 1995; Willingham et al. 2000; Milnes et al. 2002), suggest that aromatase activity and E_2 synthesis by embryonic adrenals and gonads are largely

comparable among reptiles at the early stages of development, regardless of parity mode. The initiation of sex differentiation presumably occurs in *N. metallicus* at stages 30-32 (defined as 'early' in this study) as it does in other species of lizard, including the closely related *N. ocellatus* (Neaves et al. 2006; Shine et al. 2007). Thus, once gonads have been exposed to an external source of steroid, steroidogenic potential increases and gonads drive their own development. However, contrary to data from oviparous reptiles (Smith and Joss 1994; Willingham et al. 2000; Milnes et al. 2002), aromatase activity in *N. metallicus* does not change significantly from the mid to the late-stages of development. This difference may reflect the presence of the yolk and a placenta and thus two contributing sources to embryonic steroid exposure in viviparous reptiles.

Aromatase activity in the brains of embryonic *N. metallicus* showed a similar pattern to activity in the brains of *T. scripta* (Willingham et al. 2000;) Like activity in *T. scripta*, in brains of embryonic *N. metallicus*, aromatase activity was high during the early and mid-stages and the lowest during late development. Thus, our data suggest that local activity of aromatase in the brain is most critical in earlier stages of development. It is well known that steroids play a key role in sexual differentiation of the brain, and thus local aromatase activity likely functions primarily in differentiation. In oviparous reptiles which exhibit temperature dependent sex determination, aromatase activity in the brain at the very early stages of development is potentially a trigger for gonadal differentiation (Place et al. 2001). However, the role of brain aromatase in sexual differentiation of viviparous reptiles remains largely speculative.

We have demonstrated that aromatase activity in the developing tissues of a viviparous reptile, *N. metallicus,* reflects activity in oviparous reptiles, but we suggest that the placenta plays a key role in providing an external source of steroid to embryos. This study has provided new insights into the embryonic endocrine environment and the contributing role of the placenta of viviparous reptiles, which to date has been largely speculative. Importantly, aromatase is susceptible to disruption from many chemical contaminants (Sanderson et al. 2000, 2004; Cantón et al. 2005), but there are tissue-specific factors that regulate aromatase which ultimately lead to differences in the responses of aromatase to endocrine signals and endocrine disrupting contaminants (Milnes et al. 2002; Kuhl et al. 2005; Fan et al. 2007). Therefore, while measuring aromatase activity in

maternal and embryonic tissues is the necessary first step in assessing the potential for endocrine disruption in *N. metallicus*, characterizing the tissue specific factors that regulate aromatase activity is the necessary next step. Further research is needed to achieve a greater understanding of the endocrine control of sexual differentiation in viviparous reptiles, and the potential for disruption to these processes by endocrine disrupting contaminants.

Acknowledgements

We thank Louis J. Guillette Jr and Matthew Milnes for generously providing their protocol for the tritiated water technique. We thank Ashley Edwards and Josiane Eve for assistance with dissections. Funding for this project was supplied by the Holsworth Research Wildlife endowment, The Forest Practices Authority, The Ecological Society of Australia, and the ARC (EW: Future Fellowship). This research was conducted in accordance with the University of Tasmania Animal Ethics Committee under Permit A10797. Lizards were collected with the permission of the Tasmanian Department of Primary Industries, Parks, Water and Environment: Permits FA 10157, and FA09171

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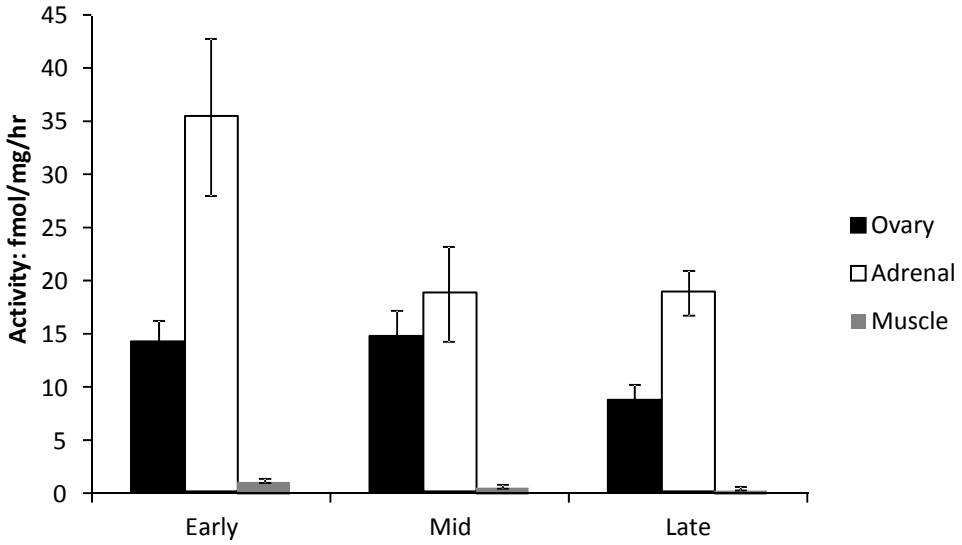

Figure 3.1 Aromatase activity in maternal ovaries, adrenal glands and skeletal muscle tissue of *Niveoscincus metallicus* during the early, mid and late stages of gestation. Error bars represent the standard error of the mean.

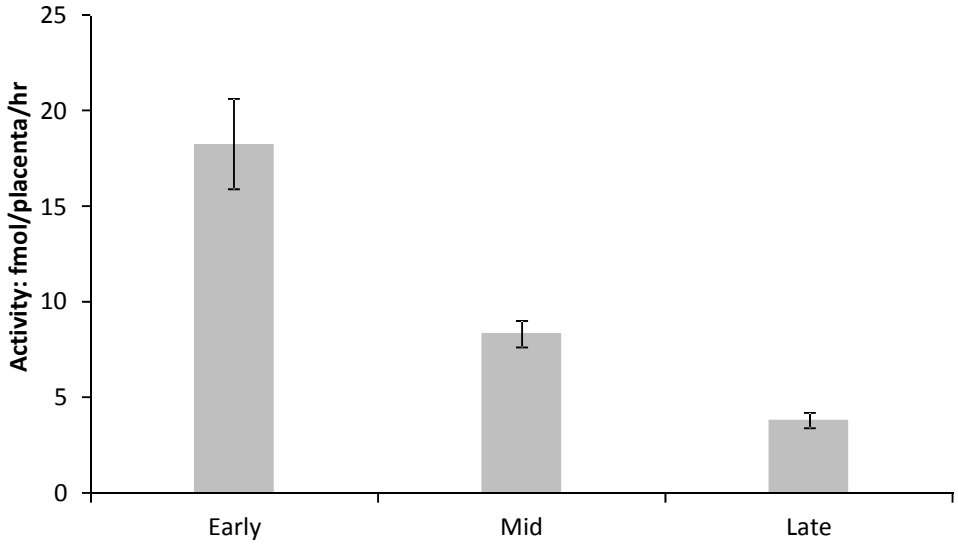

Figure 3.2 aromatase activity in placental tissue of *Niveoscincus metallicus* during the early (n = 23), mid (n = 35) and late (n = 37) stages of development. Aromatase activity decreased significantly at each progressive stage of gestaiton. Error bars represent the standard error of the mean.

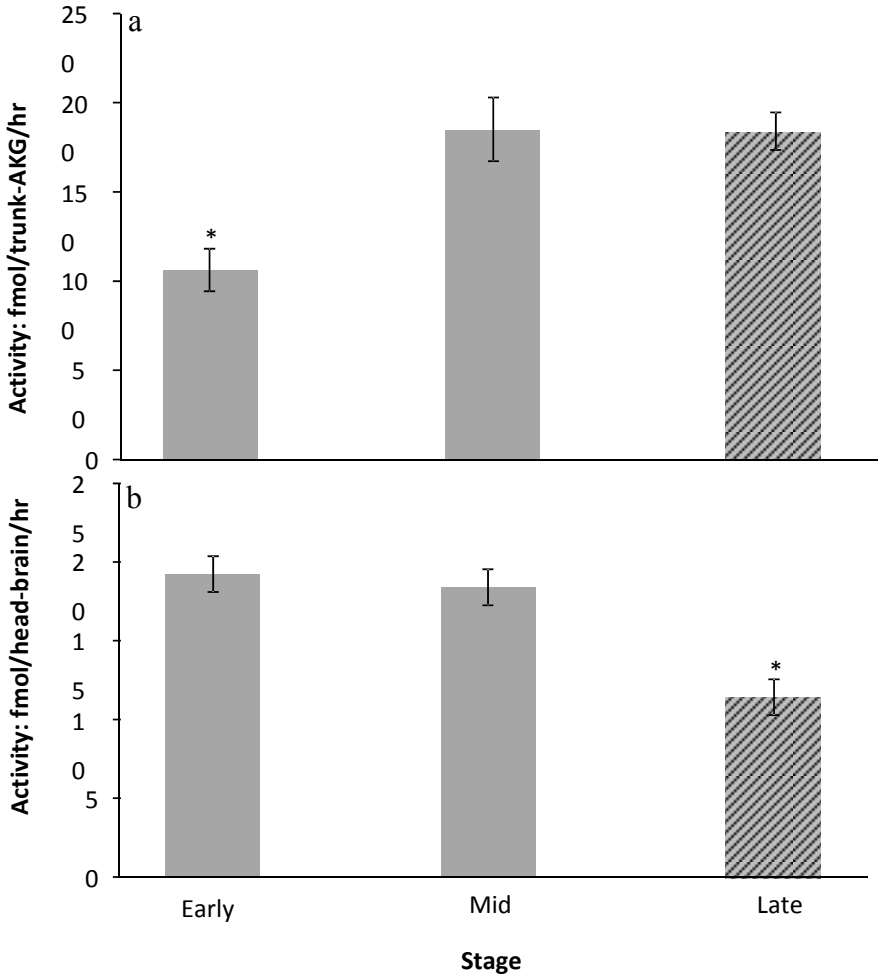

Figure 3.3 a, aromatase activity in embryonic 'trunks' or Adrenal-Kidney-Gonads of *Niveoscincus metallicus* during the early (n = 23), mid (n = 34) and late (n = 33) stages of development. b, aromatase acitvity in embryonic heads or brains of *Niveeoscincus metallicus* during the early (n = 31), mid (n = 35) and late (n = 37) stages of development. The solid bars represent aromatase activity in whole 'trunks' or 'heads' while shaded bars represent aromatase activity in the isolated 'adrenal-kidney-gonad' complexes or embryonic brains. The asterisk indicate the stage at which aromatase activity was statistically significantly different in the gonads or the brains compared with the other two stages. Error bars represent the standard error of the mean.

Chapter 4: *In utero* exposure to the oestrogen mimic diethylstilbestrol disrupts gonadal development in a viviparous reptile

Submitted to Reproduction, Fertility and Development

Abstract

The ubiquitous presence of endocrine disrupting contaminants (EDCs) in the environment is of major concern. Focus has been placed on the effects of estrogenic EDCs on reproduction and studies on oviparous reptiles have significantly advanced knowledge in this field. However, 30 % of reptilian species are viviparous (live-bearing), a parity mode in which both yolk and a placenta support embryonic development, thus exposure to EDCs may occur via multiple routes. In this first study of endocrine disruption in a viviparous lizard (*Niveoscincus metallicus*), the effects of estrogen mimic diethylstilbestrol (DES) on gonadal development were investigated. At the initiation of sexual differentiation, pregnant *N. metallicus* were exposed to a single dose of DES at 100 µg/kg or 10 µg/kg, a vehicle solvent, or received no treatment. The testes of male neonates born to DES-exposed mothers showed reduced organization of seminiferous tubules and a lack of germ cells compared with those from control groups. The ovaries of female neonates born to DES-exposed mothers exhibited phenotypic abnormalities of ovarian structure, oocytes and follicles compared to controls. The results indicate that in viviparous lizards, maternal exposure to estrogenic EDCs during gestation may have profound consequences for offspring reproductive fitness.

Introduction

The capacity of man-made chemical contaminants to interfere with the proper functioning of the endocrine system and thus impair reproductive success has been documented in many wildlife species (Guillette and Edwards 2008; Hayes et al. 2010; Jespersen et al. 2010). Endocrine disrupting chemicals (EDCs) interfere with many aspects of the endocrine system: EDCs affect the proper function of the endocrine system by mimicking, altering production or metabolism of hormones or by interfering with the binding of hormones with plasma proteins or receptors (Guillette et al. 2000; Guillette and Gunderson 2001; Crews et al. 2003; Norris and Carr 2006). Much of the focus on endocrine disruption has been on the feminizing effects of EDCs on reproductive tissues, and many estrogenic EDCs are reported to impair reproductive success in vertebrates (Fry and Toone 1981; Bergeron et al. 1994; Jespersen et al. 2010; Yang et al. 2011). Disruption can occur at any site of the hypothalamic pituitary gonadal (HPG) axis or from disruption to the transport, binding or secretion of gonadal steroids (Guillette et al. 2000; Guillette and Gunderson 2001; Crews et al. 2003; Norris and Carr 2006).

Endocrine disrupting chemicals can act via differing mechanisms but have comparable biological effects (Fry and Toone 1981; Bergeron et al. 1994; Jespersen et al. 2010; Yang et al. 2011). For example, an EDC can be broadly classed as ‘estrogenic’ because the biological effects of the EDC cause feminization. However, the mechanism of disruption could be by direct binding with estrogen receptors, or by increasing endogenous estrogen synthesis. Thus despite the differences in the mechanism of disruption, the biological effects are comparable (Fry and Toone 1981; Bergeron et al. 1994; Jespersen et al. 2010; Yang et al. 2011). However, the mechanisms of disruption by EDCs may differ between species, and some EDCs cause disruption in some species but not others. For example, p,p dichlorodiphenyldichloroethylene (p,p’-DDE), a metabolite of the insecticide dichlorodiphenyltricholoroethylene (DDT), causes estrogen-dependent sex reversal in some but not all oviparous reptiles (Guillette and Iguchi 2003) and inhibits androgen dependent sexual differentiation in rodents (Gray et al. 2001). The multiple mechanisms by which endocrine disruption occurs and the differing effects between species make prediction of the potency of EDCs difficult to assess. However, the ubiquitous presence of

EDCs in the environment and the impacts of such chemicals on wildlife highlight an urgent need for a greater understanding of endocrine disruption.

Model EDCs of known impact used as positive controls are powerful tools in understanding the effects and mechanisms of endocrine disruption (Larocca et al. 2011; Simon et al. 2012). One compound which consistently results in feminizing effects in those vertebrates so far tested is the synthetic oestrogen diethylstilbestrol (DES). Initially utilized in the prevention of miscarriage, DES was widely prescribed to pregnant women up until the early 1970s, when vaginal clear cell adenocarcinoma in young women was linked to DES exposure *in utero* (reviewed in Giusti et al. 1995). Since the discovery of this drug's adverse effects in humans, the effects and mechanisms of disruption by DES to the vertebrate endocrine system have been extensively studied, predominantly in laboratory rodents. Diethylstilbestrol is a non-steroidal oestrogen which binds to oestrogen receptors (ERs) with three times the affinity of native estradiol (E_2) to ER β and four times the affinity to ER α (Kuiper et al. 1997). Diethylstilbestrol readily traverses the placenta of several mammalian species (Shah and McLachlan 1976; Miller et al. 1982; Slikker et al. 1982; Newbold 2004) as the unconjugated parent compound (Slikker et al. 1982); thus DES poses a particular threat to developing embryos.

Prenatal exposure to DES affects the development of both male and female embryos. Male rodents born to DES treated mothers exhibit remnants of Müllerian ducts, testicular tumors, epididymal cysts and prostatic inflammation and increased probability of microphalli, hypospadias, hypoplastic and chryptorchid testes and infertility. Diethylstilbestrol exposed males may suffer lower sperm counts than non-exposed individuals (Newbold 1995, 2004). Specifically, demasculinisation of the testes manifests as disruption to the organization of seminiferous tubules (Newbold 1995, 2004). Effects on females include uterine tumors, abnormal ovarian hormone secretion and development, malformed cervical canal, cervical polyps, remnants of the mesonephros, malformed oviduct and impaired fertility (Newbold 1995, 2004). Polyovular follicles (POFs) are characteristically observed in females exposed to DES *in utero* and early life (Iguchi and Takasugi 1986; Iguchi et al. 1990; Kim et al. 2009; Kim et al. 2009). The well-characterized effects on the development of both male and female embryos in mammals highlight

DES as an obvious choice when selecting a contaminant to use as a positive control in assessing the developmental effects of EDCs which may be estrogenic (Larocca et al. 2011; Simon et al. 2012).

Reptiles are important models to study the effects of EDCs because they comprise a large percentage of faunal biomass in terrestrial and aquatic ecosystems, they exhibit similar sensitivities to pollutants as some birds and mammals and they typically live for a long time relative to mammalian species (Crain and Guillette 1998; Crews et al. 2003). Furthermore, reptiles can exhibit genetic sex determination or temperature sex determination, where the sex of embryos is determined by chromosomes or incubation temperature respectively (Crews et al. 1994; Pieau et al. 1999; Bull 2008): dosing the eggs of species with temperature dependent sex determination with EDCs at set temperatures has allowed the identification of several estrogen agonists (Bergeron et al. 1994). Importantly, reptiles may be oviparous (egg-laying) or viviparous (live-bearing), thus providing species with different parity modes as models with which to investigate the effects of EDCs (see Crain and Guillette 1998).

Typically, reptilian viviparity is achieved with a combination of yolk and placental support. Viviparity has evolved over 100 times in reptiles (Blackburn 1982; 1985; 1993), resulting in enormous variation in placental complexity and corresponding yolk size. Four main placental types have been defined (Blackburn 1993, 2000; Stewart and Thompson 2000; Thompson and Speake 2006). Type I species are predominantly lecithotrophic. These species have shell-less yolky eggs and a very simple placenta, which allows for water and respiratory gas exchange (Thompson and Speake 2006). At the other end of the spectrum, Type IV species are highly placentotrophic, and the microlecithal yolk provides minimal nutritional support for embryos (for review see Stewart and Thompson 2000; Thompson and Speake 2006; Stewart 2013). The placentae of the intermediate Type II and III species exhibit varying degrees of complexity that correspond with yolk size (Stewart and Thompson 2000; Thompson and Speake 2006; Stewart 2013).

In oviparous reptiles, the egg yolk functions as a major source of hormones that are necessary for gonadal development (Conley et al. 1997). Maternal exposure to EDCs can result in altered concentrations of steroids in the egg yolk (Hamlin et al. 2010), or the inclusion of EDCs into the yolk (Findholt 1984; van de Merwe et al. 2009). In eutherian mammals, the placenta mediates embryonic exposure to maternal hormones and synthesizes hormones critical to development (Strauss et al. 1996; Pasqualini 2005). Endocrine disrupting chemicals can traverse the mammalian placenta (Foster et al. 2002; Brown and Austin 2012; Edlow et al. 2012) and alter placental function (Susiarjo et al. 2013). In viviparous reptiles, then, the presence of both a yolk and a placenta increases the potential for multiple routes of embryonic exposure to maternal hormones and maternal contaminants. A capacity for placental steroidogenesis and maternal steroid transfer and the presence of substantial quantities of hormones in the yolk have been confirmed for species representing three of the four reptilian placental types (Guarino et al. 1998; Painter et al. 2002; Painter and Moore 2005; Itonaga et al. 2011). Thus in viviparous lizards, developing embryos are potentially affected by EDCs via several routes: altered maternal hormones via the yolk or placenta, altered placental function, or by direct exposure to EDCs transferred via the yolk, the placenta, or both.

No study investigating the effects of EDCs in a viviparous reptile has yet been conducted. We therefore examined the effects of the potent oestrogen mimic DES on gonadal development in a viviparous reptile with a moderately complex Type II placenta. The study species, *Niveoscincus metallicus,* is a small viviparous skink that provides a useful model for assessing the effects of EDCs because the reproductive ecology and physiology of this species are well known (Jones and Swain 1996; Jones et al. 1998; Girling et al. 2002; Stewart and Thompson 2009). Once estrogenic effects have been established in this species, future studies on EDCs of ecological significance to *N. metallicus*, or to other reptilian species, can be conducted.

Our main focus was on the effects of DES on gonadal development, however, other aspects of sexual development such as phallus size have been identified as important measures of endocrine disruption (Guillette et al. 1999; Newbold 2004). As in many species of skink, hemipenes develop in embryos of both sexes of *N. metallicus*, however, hemipenes regress in the female and

develop in the male at a species-specific time during development (Holmes and Wade 2005; Neaves et al. 2006; Inamdar et al. 2012). Regression or proliferation is governed by gonadal steroids (Holmes and Wade 2005; Inamdar et al. 2012) and thus disruption to development could occur in both sexes. We therefore included measures of the hemipenes of male and female neonates in our analysis. To assess the effects of DES on overall development, we measured locomotor performance to represent a range of physiological and morphological traits (Wapstra 2000; Le Galliard et al. 2004). We measured the weight of abdominal fat bodies, snout to vent length (SVL) and the weight of neonates as measures of the effects of DES on morphology and body condition.

Methods

Animal collection and husbandry

All experimentation was undertaken under the University of Tasmania Animal Ethics Committee permit A10797. Animals were collected with the permission of the Tasmanian Department of Primary Industries, Parks, Water and Environment: Permit FA09171.

Niveoscincus metallicus is a small (SVL ≤ 65 mm; mass 2-3 g) annually breeding skink. Ovulation typically occurs in October and gestation is approximately three months. Females give birth to between one and six offspring (SVL 20-24 mm; mass 0.2 g). In *Niveoscincus* species, as in other species of scincid lizard, sex differentiation in embryos begins around 1/3 of the way through development (Neaves et al. 2006; Shine et al. 2007). Female lizards were collected in late October 2009 during the early phases of gestation, to allow an acclimation period prior to dosing with DES during initial phases of sex differentiation. Eighty female *N. metallicus* were collected in greater Hobart, Tasmania (42°53’38’S, 147°19’21”E). Lizards were transported to the University of Tasmania Herpetology facilities. The lizards were housed in pairs in plastic terraria (200 x 300 x 100 mm) with terracotta saucers as a basking platform and a rock for shelter. Lizards were fed thrice weekly on *Tenebrio molitor* (mealworm) larvae and fruit puree; water was available *ad libitum.* The lizards were routinely treated for mites by bathing in vegetable oil and cages were cleaned once weekly. The laboratory was maintained at 15 °C; summer daylight

conditions were mimicked by fluorescent lights (20, 000 lux, and UV, 14L : 10D) and lizards were provided with thermoregulatory opportunity under basking lights from 8:00 to 16:00.

Treatment

Lizards were allowed to acclimatise to captivity for five days. After the five day period, the lizards were weighed (± 1 mg) and the snout to vent length (SVL) was measured (± 1mm). The lizards were then randomly allocated into treatment groups of 20. We selected two doses of DES based on a high concentration that has caused endocrine disruption in laboratory rodents 10 µg/kg (Newbold 1995, 2004) and a dose tenfold higher (100 µg/kg) to maximise the chances of endocrine disruption in *N. metallicus*. The 'High DES' group received an intraperitoneal bolus injection of DES dissolved in sesame oil, at a concentration of 0.016 mg/ml which was injected at 6.25 µl/g to yield a 100 µg/kg dose. Similarly, the 'Low DES' group were treated as described above, however, the DES solution was at a concentration of 0.0016 mg/ml to yield a 10 µg/kg dose. The 'Control-Injection' group received sesame oil alone at 6.25 µl/g. All injections were performed with a Hamilton gas-tight micro-syringe. The 'Control' group received no treatment. The lizards were housed as described above until parturition. Adult female lizards were checked thrice daily from the end of gestation (~ mid December), and the date of birth and clutch size were noted prior to separating neonates from the mother for testing as described below.

Locomotor performance testing, dissection and gonad collection

The day after birth, neonates were sprint tested as a measure of locomotor performance, which is a proxy for a range of physiological and morphological traits (Wapstra 2000; Le Galliard et al. 2004). Each neonate was placed in a sealed container in a water bath heated to 26 °C (the optimum body temperature of *N. metallicus* (Melville and Swain 2003) for 20 min to equilibrate (Wapstra and O'Reilly 2001). Each neonate was then sprinted individually along a 1 m long race track lined with infrared light beams placed at 25 cm intervals. The neonatal lizards were encouraged to sprint by consistent tapping on the tail with a soft paint brush. Each lizard was sprinted along the track twice in rapid succession (Melville and Swain 2003). The best of the eight sprint-times recorded was used in the data analysis as a measure of maximum performance.

The neonates were then humanely killed with an IP bolus injection of sodium pentobarbital at 500 ng/g diluted 1:100 in saline solution. After death, the weight (± 10 µg) total length, and SVL were measured (0.01 mm) prior to dissection and removal of the adrenal-kidney-gonad complex (AKG) and exposure of the hemipenises. The exposed hemipenes were photographed at 32 x on a Zeiss Stemi SV11 microscope with a Leica DFC 425 camera attachment. Photos were captured with Leica Application Suite V3 7.0. The width of the hemipenes of both male and female neonates was measured as another potential marker of endocrine disruption (Guillette et al. 1999; Newbold 2004). Both hemipenes were measured thrice in ImageJ and the average of the six measurements was utilised in the statistical analysis. The AKGs were carefully removed and placed directly into Bouin's fixative. Neonatal abdominal fat bodies were also removed and weighed as a measure of condition at birth (Atkins et al. 2006, 2007).

Gonad morphology

After 12 hr of preservation in Bouin's fixative, the AKGs were placed into 70 % ethanol solution. The tissues were then embedded in paraffin wax with a Tissue-Tek sampling processor. The AKGs (which consist of both gonads, both adrenal glands and the mesonephros) were serially sectioned at 6 µm, and stained with haemotoxylin and eosin. The serially sectioned AKGs were categorised from female neonates by the presence of Müllerian and Wolffian ducts and ovaries, or from male neonates by the absence of Müllerian ducts and presence of Wolffian ducts and testes. The microscope slides of the AKGs were then recoded to remove references to the experimental group to prevent biasing the subsequent data collection of ovary and testis morphology, which was performed by one operator (LMP).

Ovaries

At birth, the ovaries of female lizards have a well differentiated cortex of clearly defined germinal beds consisting of oogonia, primary oocytes and primordial follicles (Austin 1988; Hernández-Franyutti et al. 2005; Moodley and Van Wyk 2007; Jones 2011). The medulla contains large lacunae, is filled with stromal tissue, and the rete cords are absent (Jones 2011). The effects of DES on ovarian development in laboratory rodents are most often reported as an

increased occurrence of POF in the ovaries of exposed animals compared to the ovaries of non-exposed individuals (Iguchi and Takasugi 1986; Iguchi et al. 1990; Kim et al. 2009; Kim et al. 2009). However, when we compared the ovaries of neonatal *N. metallicus* with those of other species of reptile (Austin 1988; Hernández-Franyutti et al. 2005; Moodley and Van Wyk 2007), we noted other phenotypic differences in the structure and the developing germ cells and stromal tissue in the ovaries of our embryos. We therefore developed five criteria based on the known characteristics of the normal ovaries of other reptilian species (Austin 1988; Hernández-Franyutti et al. 2005; Moodley and Van Wyk 2007) which relate to the overall structure of the ovary, the oogonia, occytes, primordial follicles and the stroma. The details of these criteria are defined in Table 4.1. To produce a semi-quantitative assessment of the extent of disruption to ovarian development, we assigned a score of 'zero' to the 'normal' character state, and a score of 'one' to corresponding (presumed) abnormal states. Each section in every ovary was visualised and each ovary was scored according to the five criteria. Character scores were added cumulatively to produce a total score for each ovary, the maximum an ovary could score was five. All sections in the ovaries of each female neonate were checked three times to ensure the consistency of the result. Once all of the sections had been scored, the original sample code was restored allowing re-assignment of samples to treatment groups. The final data which were analyzed statistically were the mean total cumulative score of one ovary for each individual in each treatment group.

Testes

Disruption to the organisation of seminiferous tubules is a well-known marker of endocrine disruption in males (Pérez-Martínez et al. 1997; Yoshimura and Fujita 2005; Yamaguchi et al. 2009). We noted four main categories of seminiferous tubule organisation and thus defined four categorical scores relating to varying degrees of differentiation: score 0 = highly differentiated (normal), 1= moderately differentiated, 2 = poorly differentiated, and 3 = undifferentiated. The presence or absence of primordial germ cells was also recorded.

Statistical analyses

Data analysis was performed using SAS 9.2 for Windows. Normality of distribution was checked by examining plots of standardized residuals against predicted values and normal probability of the residuals. All GLM models on data from neonates were analyzed with maternal identity as a random factor. Variation between treatment groups in adult female SVL at the beginning of the experiment and litter size was examined individually using general-linear-models (GLM) to ensure there was no body size or litter size bias between treatment groups. Variation in the weight and SVL of neonates between treatment groups was analyzed with GLM separately using untransformed data. Variation in sprint speed and the weight of neonatal fat bodies was analyzed separately on square root transformed data. Variation in the cumulative score of ovarian abnormalities between groups was analyzed with GLM by adding a constant to all of the values (0.1) to account for the many zeros and allow square root transformation. Variation in the width of the hemipenes between groups was analyzed using a GLM on square root transformed data. Analysis of data of hemipenes included the sex of the neonates and maternal identity as random factors. Significant differences between treatment groups were identified using Tukey's Honest Significant Difference. In all cases, the data analyzed were from one randomly selected gonad from each neonate.

Variations between treatment groups in the sex ratios of neonates were analysed by calculating the number of males over clutch size and then performing a logistic regression. Differences in the treatment groups in the presence of POF in neonatal ovaries, the organization of seminiferous tubules and presence of primordial germ cells in neonatal testes and the presence of testicular cells with elongated nuclei and the presence of testicular lesions were all analysed separately using logistic regression. We randomly selected one neonate from each clutch to account for maternal identity, as small sample size did not allow for inclusion of maternal identity as a random factor for logistic regression analysis.

Results

Maternal and neonatal biometrics

There was no significant difference in maternal SVL between treatment groups ($F_{3,77} = 0.7$; $p = 0.4962$). There was no significant effect of treatment on the weight ($F_{3,114} = 0.17$; $p = 0.9171$) or SVL ($F_{3, 112} = 0.45$; $p = 0.7190$) of neonates. Similarly, there was no significant treatment effect on neonatal fat bodies ($F_{3, 78} = 1.92$; $p = 0.1333$) or sprint speed ($F_{3,93} = 0.97$; $p = 0.4162$), or sex ratio ($\chi^2 = 0.2143$; $p = 0.6434$).

Gonad histology and hemipene size

Exposure to DES *in utero* resulted in female neonates with phenotypically abnormal ovaries: examples of phenotypically abnormal ovaries are presented in Fig 4.1. There was a significant effect of treatment on ovarian development, with female offspring born to DES exposed mothers at both concentrations having higher 'cumulative scores' than those in control groups ($F_{3,31} = 24.08$; $p < 0.0001$; Fig 4.2). However, there was no significant difference between the high and low DES treatments or between the control-injection and control treatments. Very few female neonates (n = 6 from DES-treated mothers only) had ovaries with POF and the small number of female neonates with POFs were siblings. There was no significant difference in the occurrence of POF in female neonates between any of the treatment groups ($\chi^2 = 1.34$, $p = 0.2475$).

In testes, the incidence of cells with elongated nuclei (Fig 4.3) differed significantly between the four treatment groups ($\chi^2 = 11.08$; $p = 0.0009$) with a higher incidence of cells with elongated nuclei in males born to DES mothers compared to males born to control mothers (Fig 4.4). However, there was no significant difference between the occurrence of such cells in the testes of males born to females treated with high or low DES ($\chi^2 = 0.544$; $p = 0.4606$), or in the testes of males born to control injected or control females ($\chi^2 = 0.0036$; $p = 0.9520$). We observed four categories of seminiferous tubule organisation (Fig 4.3). There was a significant difference between treatment groups in the organisation of seminiferous tubules ($\chi^2 = 16.85$ $P < 0.0001$) with males born to DES treated mothers more likely to have a seminiferous tubule score of 1,

compared to those born to control mothers (Fig 4.5). However, there was no significant difference in seminiferous tubule organisation in the testes of males born to high and low DES exposed mothers ($\chi^2 = 0.0034$, $p = 0.9533$), or in control injected and control mothers ($\chi^2 = 0.0037$, $p = 0.9512$). There was a significant difference in the occurrence of testes without primordial germ cells between the treatment groups ($\chi^2 = 7.0127$, $p = 0.0081$) with males born to DES treated mothers more likely to have testes without primordial germ cells than males born to control mothers (Fig 4.6). There was no significant difference in the occurrence of primordial germ cells in testes of males born to females treated with high or low DES ($\chi^2 = 0.8934$, $p = 0.3446$), or in the occurrence of primordial germ cells in the testes born to females in the control-injection and control treatments ($\chi^2 = 1.4954$, $p = 02215$).

Discussion

The overall aim of this study was to investigate and document the effects of a known estrogenic agent on embryonic development as a first step towards understanding the potential impacts of EDCs in viviparous lizards. We exposed gestating female lizards to a single dose of the potent oestrogen mimic diethylstilbestrol (DES). We opted to utilize DES for this study as the effects on other species have been comprehensively investigated (Iguchi et al. 1990; Giusti et al. 1995; Newbold 2012), but effects on viviparous reptiles have not been examined. The mechanisms of DES effects are well understood (Kuiper et al. 1997; Kim et al. 2009), DES readily traverses the placenta of eutherian mammals, and is therefore likely to do so in viviparous lizards. Thus, we anticipated organizational effects of DES upon offspring exposed *in utero*.

There was no significant effect of DES at 100 or 10 µg/kg on the birthdate, or the directly observable morphological traits of size and whole body performance of the neonates, thus DES did not affect pregnancy success. However, there were significant effects of *in utero* exposure to DES at both doses on gonadal development, but there was no significant difference between our two DES treatments in any of the effects on gonadal development. Non-monotonic response curves such as this are characteristic of endocrine disruption (Vandenberg et al. 2012) and therefore this result is not surprising. In some biological systems a threshold dose is required to

induce a biological effect. Once the threshold is met, all doses above the threshold dose exert the same effect (Hayes et al. 2002). It is possible that a concentration of 10 µg/kg maximizes receptor binding. Increasing the dose tenfold does not therefore magnify the effect as maximum receptor binding has already been reached.

The effects of DES on the development of the testes in *N. metallicus* are consistent with the demasculinising effects of DES in other vertebrate species. Disruption to the organization of seminiferous tubules following exposure to DES during development has been observed in laboratory rodents (Pérez-Martínez et al. 1997; Yamaguchi et al. 2009) and in *Gallus gallus* (Yoshimura and Fujita 2005). The testes of male neonate *N. metallicus* born from DES-treated mothers were more likely to contain cells with elongated nuclei than those of males born from control mothers. Heavy granulation of these cells makes it difficult to discern cell type; however, *Alligator mississippiensis* from contaminated lakes exhibited primordial germ cells with elongated nuclei (Guillette et al. 1994). The elongation of such nuclei could be an indication that the cells are late stage spermatids initiating nuclei elongation, a process which can occur after E_2 and aromatizable androgen exposure (Ramaswami and Jacob 1965; Moore et al. 2010). Future analyses should include immunohistochemistry to allow the identification of the type of cells which exhibit elongation of nuclei in *N. metallicus*, and determine the future effects upon the function of the testis.

Male *N. metallicus* born to DES treated mothers also exhibited testes with no discernible germ cells. Exposure to DES during testicular development in rodents also results in germ cell loss (Godowidz et al. 1984; Newbold 1985; Nonclercq et al. 1996; Pérez-Martínez et al. 1997), and many studies associate exposure to DES *in utero* with impaired fertility and low sperm counts in males (Newbold 1995, 2004). In the Syrian hamster *Mesocricetus auratus*, germ cell loss following DES exposure was associated with a concomitant reduction in circulating follicle stimulating hormone and testosterone, which indicates DES is having a negative feedback effect on testosterone synthesis (Nonclercq et al. 1996). The loss of germ cells following contaminant exposure has been reported in other reptiles, amphibians and mammals (Hayes et al. 2011), and is therefore a reliable marker of endocrine disruption in males.

Exposure to DES affected ovarian development in female *N. metallicus* embryos and a range of abnormalities were identified. It is very well known that DES exposure during critical phases of development induces formation of POF (Iguchi and Takasugi 1986; Iguchi et al. 1990; Kim et al. 2009) and POF have been observed in the ovaries of a range of wildlife exposed to EDCs. For example, neonatal female *A. mississippiensis* from contaminated Lake Apopka had a higher incidence of POF than neonatal females from the relatively pristine Lake Woodruff (Guillette et al. 1994). Polyovular follicles, a known marker of DES effects on ovarian development, were observed in the ovaries of a small number of female *N. metallicus* born to mothers in both DES-treated groups only, but only females born to the same mothers. There are two possible reasons why we observed POFs in only a few of our neonatal ovaries. Firstly, in laboratory rodents, POFs are formed in the ovaries after several days of DES exposure, and secondly, the effects on POF formation in ovaries depends on the specific stage of gestation at which DES exposure occurs (Iguchi et al. 1986). Thus, we suggest that in *N. metallicus*, DES exposure during development results in the formation of POFs if embryos are exposed at some critical and short-lived stage of development.

We demonstrated that exposure to DES *in utero* significantly increases the incidence of ovarian abnormalities in neonatal female *N. metallicus*. Some of the ovarian criteria we have defined as 'abnormal' in neonates (e.g. the presence of medullary cords, a relatively indifferent medulla-cortex, and the absence of lacunae) are seen in the developing ovary of the congeneric *N. ocellatus* at stages 30-32 of development (Dufaure and Hubert 1961; Neaves et al. 2006). Similarly, in *N. ocellatus* seminiferous tubule formation is initiated in the testis at stages 30-32 (Dufaure and Hubert 1961; Neaves et al. 2006), again suggesting that the effects of DES we observed represent delayed gonadal development. The future effects of such development on reproductive success are unknown. Intersexuality in the turtle *Emys orbicularis* is rectified post hatch in some individuals (Pieau et al. 1998), however, the effects of EDCs are most commonly permanent (Iguchi et al. 1991; Guillette et al. 1994, 1995; Newbold 2008; 2012; Hamlin and Guillette 2011). Thus the potential for impaired reproductive success in *N. metallcus* needs to be addressed in future studies.

Reduced phallus size is a known marker of endocrine disruption in other vertebrate species and is associated with altered plasma steroid concentrations (Guillette et al. 1999). In many species of lizard, including *N. metallicus,* both male and female embryos initially develop hemipenes and sexual dimorphism of hemipenes is only evident in late stage embryos (Dufaure and Hubert 1961; Holmes and Wade 2005; Neaves et al. 2006; Inamdar et al. 2012). Gonadal steroids mediate hemipenis regression in the female and proliferation in the male at later stages of development (Holmes and Wade 2005; Inamdar et al. 2012), so disruption of the embryonic gonads might be expected to affect hemipenis development. However, the apparently delayed gonadal development observed in neonates exposed to a single dose of DES *in utero* was not associated with changes in the size of the hemipenes in either male or female neonates. This observation does not rule out EDC effects on hemipenis development as the timing and duration of dose may be critical.

We conclude that a single dose of DES, a potent oestrogen mimic, causes disruption of gonadal development in both male and female *N. metallicus* exposed via a single maternal dose during gestation. We are yet to assess the consequences of such DES exposure on reproductive success in *N. metallicus,* but typically, endocrine disruption during tissue development results in permanent effects which persist into adulthood (Guillette et al. 1995). We have identified the phenotypic effects of exposure to a feminizing EDC as a basis for future studies on the effects of EDCs with ecological significance in viviparous lizards. Previous work on EDC effects on reptiles have concentrated on oviparous species where effects on developing embryos are largely mediated via the egg yolk. Here we demonstrate that in viviparous species which comprise 30 % of reptiles, effects can occur during pregnancy as it does in eutherian mammals. This is the first such study in a viviparous reptile that nourishes its embryos using both yolk and placenta so that the embryos are potentially at risk of exposure to EDCs via multiple routes.

Acknowledgements

We thank Louis J. Guillette Jr for advising on experimental design. We would also thank Shelly Lachish, Belinda Yaxley and Beth Strain for helping with catching the lizards, and to Jo McEvoy

and Steffen Becker for assistance with lizard husbandry. Funding for this project was provided by the Holsworth Wildlife Research Endowment and the Australian Research Council (EW: Future Fellowship).

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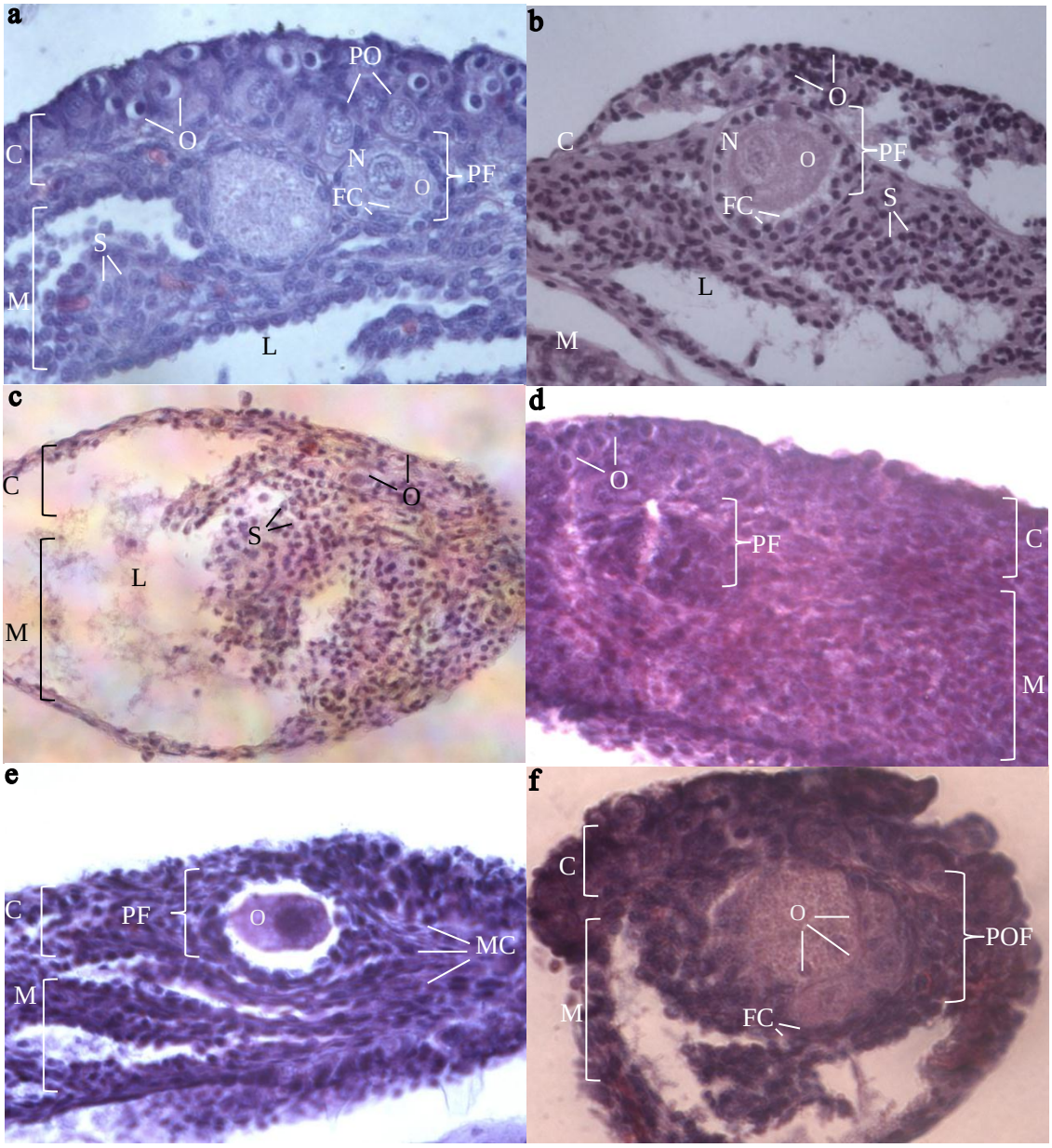

Figure 4.1 Ovaries from neonatal *N. metallicus* born from a) control mother, b and c mothers treated with DES at 100 µg/kg, d, e and f 10 µg/kg. a) Illustrates normal ovarian histology: C, cortex; M, medulla; O, oogonia; PO, primary oocyte; PF, primary follicle; N, nucleus; FC, follicular cell; S, stroma; L lacunae. b) Illustrates oocytes with granulated, elongated nuclei, granulated, elongated follicle cells, and granulated, elongated stroma. c) Illustrates granulated, crenated stroma. d) Illustrates oocytes with reduced vesicular nuclei, reduced lacunae, and indistinct medulla/cortex. e) Illustrates prominent medullary cords, atretic follicles and granulated elongated stroma. f) Illustrates a polyovular follicle, with four oocytes. All ovaries are stained with haematoxylin, eosin, sectioned at 6 µm, and magnified at 400 x.

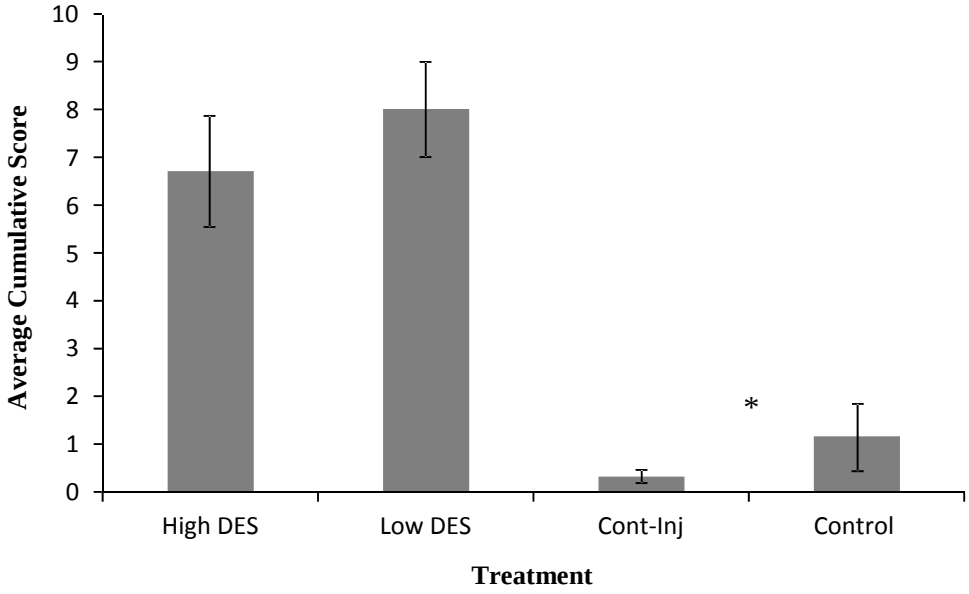

Figure 4.2 The average ovarian cumulative score of female neonatal *N. metallicus* born from mothers treated with high DES (100 µg/kg) n = 20, low DES (10 µg/kg) n = 26, Control Injected (sesame oil) n = 22 and Control (no treatment) n = 13. Error bars represent the standard error of the mean. The asterisk indicates the statistically significant difference.

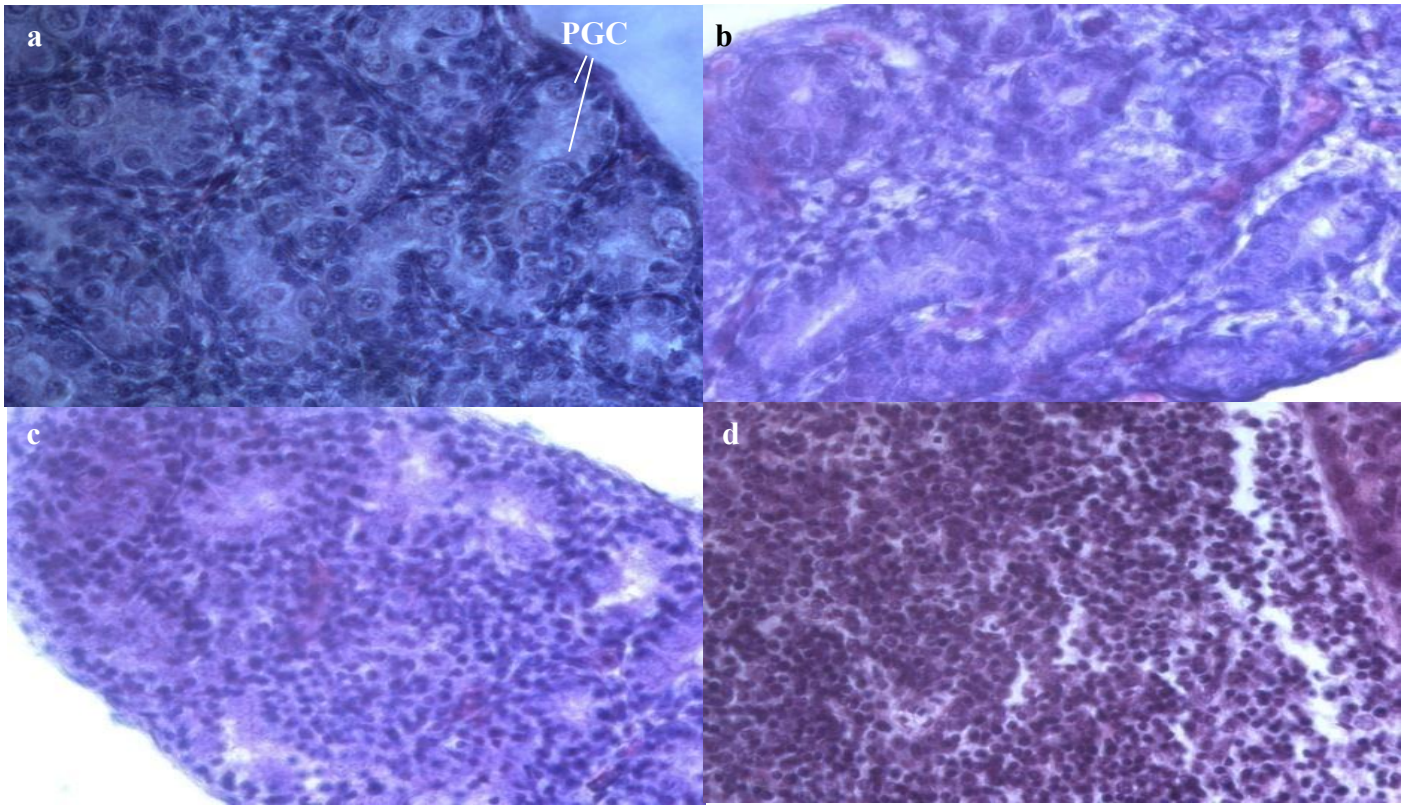

Figure 4.3 Testes of male neonatal *N. metallicus* demonstrating seminiferous tubule organisation scores. a) testis score = 0 (well differentiated, normal), b) testis score = 1 (moderately differentiated, c) testis score = 2 (poorly differentiated) and d) testis score = 3 (undifferentiated). Note the presence of primordial germ cells PGC in a) and b) and the reduction and absence of primordial germ cells in c) and d), and the elongation of nuclei in cells of c). All testes are stained with haematoxylin and eosin, sectioned at 6 µm, and magnified at 400 x.

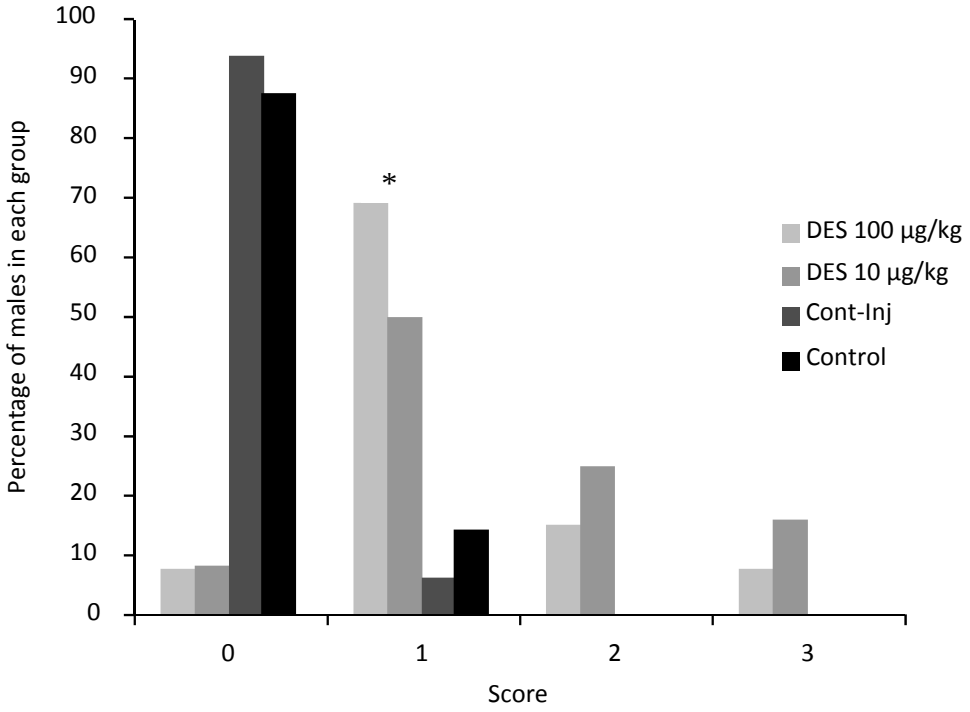

Figure 4.4 The percentage of male N. metallicus born from mothers exposed to high DES (100 µg/kg) n = 14, Low DES (10 µg/kg) n = 12, Control-Injected (sesame seed oil) n = 16 and Control (no treatment) n = 8 treated mothers, to receive a seminiferous tubule score of 0 (well differentiated, normal) 1= (moderately differentiated), 2 = (poorly differentiated) and 3 (undifferentiated). The asterisk indicates the statistically significant difference.

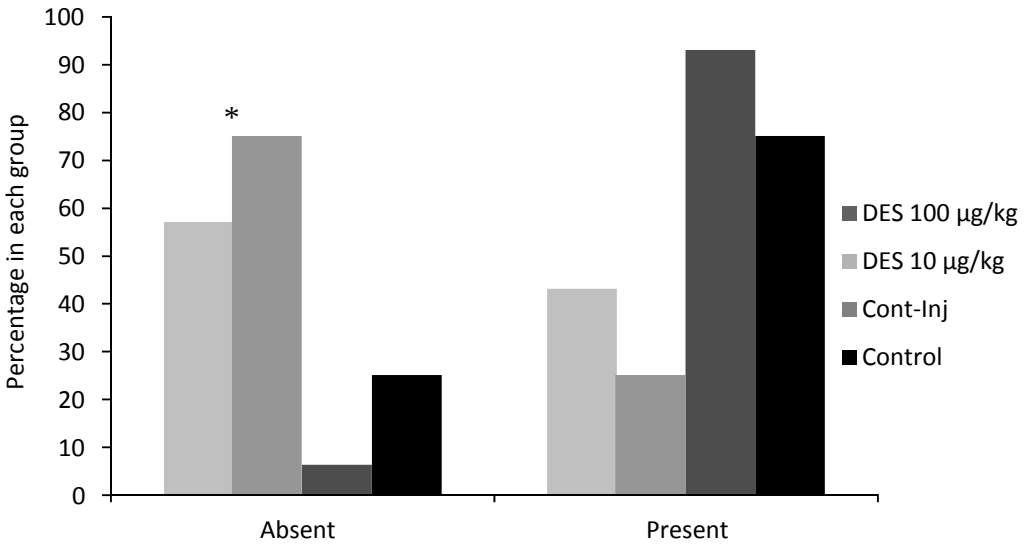

Figure 4.5 the percentage of male *N. metallicus* born from mothers exposed to high DES (100 µg/kg) n = 14, Low DES (10 µg/kg) n = 12, Control-Injected (sesame seed oil) n = 16 and Control (no treatment) n = 8 treated mothers, to have testes with or without primordial germ cells one day after birth. The asterisk indicates the statistically significant difference.

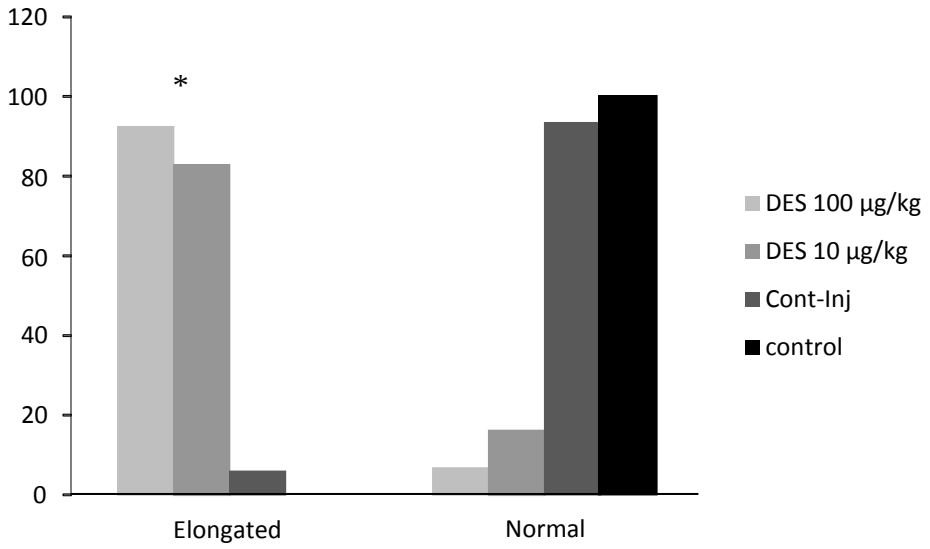

Figure 4.6 the percentage of male *N. metallicus* born from mothers exposed to high DES (100 µg/kg) n = 14, Low DES (10 µg/kg) n = 12, Control-Injected (sesame seed oil) n = 16 and Control (no treatment) n = 8 treated mothers, to have testes with cells exhibiting elongation of the nuceli one day after birth. The asterisk indicates the statistically significant difference.

Table 4.1 The five criteria used to determine the cumulative score of ovarian abnormalities in neonatal *Niveoscincus metallicus*.

The five criteria used to determine the cumulative score of phenotypic abnormalities. Each criterion relates to one aspect of the ovary and any of the listed abnormal phenotypes for each criterion results in a score of one for that criterion. Female *Niveoscincus metallicus* with ovaries with the normal phenotype for each criterion scored a zero, while neonates with one or several of the abnormal phenotypic traits for each aspect of the ovary scored one for that criterion. For each female, a cumulative score of all of the criteria was obtained. An ovary with a completely normal phenotype would have a total score of zero, while an ovary with a completely abnormal phenotype would score five.

Criterion	**The aspect of the ovary**	**Phenotype 1: normal, score = 0**	**Phenotype 2: abnormal score = 1**
1	Structure	Distinct medulla-cortex, lacunae present, no medullary cords	Indistinct medulla-cortex, or medullary cords present, or absent lacunae
2	Oogonia	Spherical or ovoid, prominent ooplasm, spherical nucleus	Ooplasm absent, or crenate, or elongated nuclei
3	Primary oocytes	Spherical or ovoid, lightly stained ooplasm, spherical nucleus	Heavily stained ooplasm, or granulated and elongated nuclei
4	Primordial Follicle	Cuboidal follicular cells with visible organelles, one oocyte, oocyte contacting follicular cells, not atretic,	Crenate elongated follicular cells, or poly ovular, or atretic receding oocyte
5	Stoma	Ovoid with visible organelles	Heavily granulated (organelles no longer visible), crenate or elongated cells

Chapter 5: Atrazine disrupts gonadal development in a live-bearing lizard

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Abstract

BACKGROUND: Atrazine (ATZ) is an endocrine disruptor that increases aromatase activity. In viviparous (live-bearing) vertebrates, the placenta is a critical to endocrine disruption because embryos may be exposed via this route. Studies of the effects of ATZ in viviparous amniotes have focused on rodents, but rodents appear to be relatively insensitive to ATZ. Thus, studies on other viviparous amniotes are required.

OBJECTIVE: To determine the effects of gestational exposure to a single low dose of ATZ on gonadal development in a viviparous skink, *Niveoscincus metallicus.*

METHODS: Pregnant females were exposed to ATZ, the synthetic estrogen diethylstilbestrol (DES) (positive control), vehicle solvent or received no treatment. Gonads were harvested from neonates at birth and examined histologically.

RESULTS: Females born to ATZ and DES exposed mothers were more likely to exhibit ovaries with abnormal oocytes compared with females born to control mothers. Males born to ATZ and DES exposed mothers exhibited testes devoid of germ cells with reduced organization of seminiferous tubules (ST) compared to males born to control mothers. Males born to ATZ exposed mothers were significantly more likely to exhibit testicular lesions than males born to DES and control treated mothers.

CONCLUSIONS: Atrazine disrupts gonadal differentiation in the viviparous lizard, *N. metallicus*. The similarity between the effects of DES and ATZ suggests that the developmental effects of ATZ in *N. metallicus* reflect increased estrogen signaling. We suggest that ATZ should be used with caution as exposure of wildlife to this EDC is likely to have adverse effects on reproductive health.

Introduction

The persistence of endocrine disrupting chemicals (EDCs) in the environment poses a threat to wildlife. A major effect of exposure to EDCs is impaired reproductive success, and disruption to the endocrine system can occur in any stage of the life cycle. However, the most profound effects of EDC exposure occur during sexual differentiation: disruption during organ development can result in permanent effects that persist into adulthood (Guillette et al. 1995; Newbold 2004; Hayes et al. 2010). Exposure to EDCs at critical stages of embryogenesis can result in impaired gonadal development of both male and female embryos (Guillette et al. 1994; Hayes et al. 2002; Newbold 2004), sex reversal (Bergeron et al. 1994; Milnes et al. 2005), impaired fertility (Guillette and Moore 2006; Guillette and Edwards 2008) or reproductive cancers (Giusti et al. 1995; Newbold et al. 2009) although these effects might not be apparent until later life.

Thousands of EDCs from many chemical classes are persistent in the environment and therefore present a significant risk to wildlife. The herbicide atrazine (ATZ) is highly debated as an EDC as the effects on vertebrates are disputed. Atrazine disrupts the hypothalamo-pituitary-gonadal (HPG) axis in multiple ways. Atrazine increases the expression of the enzyme aromatase (Faber and Thornburg 1986; Sanderson et al. 2000; Sanderson et al. 2001; Fan et al. 2007; Fan et al. 2007), which converts aromatisable androgens to oestrogens (Simpson et al. 2002). Atrazine also decreases circulating HPG hormones including gonadotrophin releasing hormone (GnRH), luteinizing hormone (LH) (Cooper et al. 2000; Stoker et al. 2000; Foradori et al. 2009) and the androgen dihydrotestosterone (DHT) (Hayes et al. 2011), and alters DHT availability (Danzo 1997).

Consistent effects of ATZ have been identified in representatives of all the major vertebrate groups (Hayes et al. 2011), but to date, analyses of the effects of most EDCs in vertebrates have focused on oviparous species. Studies on EDCs in viviparous vertebrates are required because the retention of embryos creates the potential for conflict between maternal and embryonic physiology. The placenta of viviparous vertebrates allows the transfer of maternal hormones to the embryo (Painter and Moore 2005; Pasqualini 2005; Itonaga et al. 2011), and placental

hormone synthesis is critical to embryonic development (Seckl 2001; Pasqualini 2005). Therefore the additive effects of disruption of maternal and placental endocrine function have the potential to magnify the effects of EDCs on the developing embryos of viviparous species.

The only studies of the effects of ATZ in viviparous vertebrates have been on laboratory rodents. Although laboratory rodents are useful models for assessment of the effects of some EDCs (Newbold 2004; Newbold et al. 2009), they may not be the most appropriate with which to study the effects of ATZ on viviparous vertebrates. Firstly, the placenta of rodents is unlikely to be a site of disruption of ATZ, because the placentae of rats and mice do not express aromatase (Strauss et al. 1996; Simpson et al. 2002; Pasqualini 2005). Secondly, ATZ disrupts aromatase expression in tissues which express aromatase via the ArPII promoter in cells that also express steroidogenic factor 1 (SF-1) (Fan et al. 2007; Fan et al. 2007) but in female rat embryos SF-1 is not active in the differentiating ovary until just before birth (Schimmer and White 2010). Thus disruption to various reproductive indices in laboratory rodents occurs only at concentrations of ATZ (1000 ppb-100 ppm) which are far above those of ecological relevance (Davis et al. 2011; Fraites et al. 2011). We therefore require other model species with which to examine the effects of ATZ in viviparous vertebrates.

Reptiles provide a variety of models with which to study EDCs as they exhibit a range of sex determining mechanisms and characterization of developmental stages is straightforward. Furthermore, reptiles exhibit differences in parity mode and some 30 % of reptilian species are viviparous. Most viviparous reptiles utilize a yolk and a placenta to support embryonic development and, accordingly, four main reptilian placental types have been defined (Blackburn 1993; Stewart and Thompson 1993; Blackburn 2000; Stewart and Thompson 2000; Stewart 2013). Type I species are entirely dependent upon yolk, while Type IV species are almost entirely placentotropic (Blackburn 1994; Herrel et al. 2001). The intermediates Types II and III exhibit varying degrees of placental complexity and dependence upon yolk as a source of embryonic nutrition (Blackburn 1993; Stewart and Thompson 1993; Blackburn 2000; Stewart and Thompson 2000; Stewart 2013). Viviparous reptiles are therefore of particular interest in EDC studies due to the multiple pathways through which EDCs may affect development: altered

maternal signals, via the yolk (Parsley et al submitted) or the placenta (Painter et al. 2002; Painter and Moore 2005; Itonaga et al. 2011), altered placental function (Parsley et al submitted) or via direct embryonic contamination via the yolk or the placenta. However, to date there have been no studies of the effects of EDCs on reproductive function in viviparous reptiles.

Niveoscincus metallicus is a small (adult mass ~ 3-5 grams, 45-65 mm snout vent length) viviparous skink with a moderately complex Type II placenta and a moderately sized yolk (Jones et al. 1998; Swain and Jones 2000; Stewart 2013). *Niveoscincus metallicus* is an excellent first model for use in EDC studies as yolk and placenta are both significant sources of embryonic nutrition. Optimising both yolk size and placental function maximises both potential routes of embryonic exposure to EDCs. Furthermore, details of the reproductive physiology of *N. metallicus* are well known (Stewart and Thompson 1994; Jones and Swain 1996; Stewart 2013). Importantly aromatase activity in placental tissue is high (comparable with that in maternal ovaries), at the time of embryonic sexual differentiation (Parsley et al. submitted: Chapter 3) although the specific promoters of aromatase have not yet been characterised for *N. metallicus*.

This study aimed to determine the effects of a single low dose of ATZ (10 ppb) on gonadal development of embryos exposed *in utero* at the time when embryonic sexual differentiation is initiated. We have previously established the effects of increased estrogen signalling during embryonic development using the potent estrogen mimic diethylstilbestrol (DES) (Chapter 4): DES was therefore utilised as a positive control in this study. We examined the effects of ATZ and DES on gonadal development in neonatal *N. metallicus* born to exposed mothers. We also measured locomotor performance as proxy for a range of physiological traits (Wapstra 2000; Le Galllard et al. 2004), the weight of abdominal fat bodies, the snout to vent length (SVL) and body weight of neonates, all of which relate to body condition (Atkins et al. 2007). Reduced phallus size is a known marker of endocrine disruption (Guillette et al. 1999; Newbold 2004). As in many other squamate reptiles, embryos of both sexes of *N. metallicus* develop hemipenes although in the females the hemipenes regress at a species specific stage of development (Holmes and Wade 2005; Neaves et al. 2006; Inamdar et al. 2012). Therefore, we measured the size of hemipenes of both male and female neonates as an additional marker of endocrine disruption.

Methods

Animal collection and husbandry

Eighty female *N. metallicus* were collected in late October 2010 from Old Farm Rd: 42°53'38.33"S, 147°19'21.29"E in greater Hobart, Tasmania. In *Niveoscincus* species, sex determination and subsequent differentiation in embryos occurs during the middle third of development (Neaves et al. 2006), which *in N. metallicus* occurs in late October. Lizards were transported to the University of Tasmania Herpetology facilities. The lizards were housed individually in plastic terraria (200 x 300 x 100 mm) containing a terracotta saucer as a basking platform and small square of wood for shelter. *Tenebrio molitor* larvae dusted with vitamins and fruit puree mixed with minerals were provided thrice weekly and water was available *ad libitum.* The ambient temperature was maintained at 15 °C, summer daylight conditions were mimicked by overhead lights (20, 000 lux, and UV, 14L: 10D). Basking lights were operative from 0800-1800 hr.

Atrazine exposure

Lizards were allowed to acclimatise to captivity for seven days. After acclimatisation the lizards were weighed (± 1 mg) and their snout to vent length (SVL) was measured (± 1mm). They were then randomly allocated into treatment groups of 20. The 'ATZ' group were dosed with ATZ (Sigma Aldrich, Australia) dissolved in sesame oil at a concentration of 0.0016 ppm: an intraperitoneal injection at 6.25 µl/g delivered a 10 ppb dose. The positive control 'DES' (Sigma Aldrich, Australia) group were dosed similarly with DES at 10 ppb in sesame oil, and the 'Control-Injection' group received sesame oil alone at 6.25 µl/g. All injections were performed with a Hamilton gas-tight micro-syringe. The 'Control' group received no treatment. The lizards were then maintained until parturition.

Locomotor performance testing, dissection, hemipenis measurement and gonad preparation

The locomotor performance of the neonates was assessed by sprint testing (Wapstra 2000) on the day following birth. The neonates were heated to the optimum body temperature of 26 °C (Melville and Swain 2003) in a sealed container in a water bath for 20 min (Wapstra and O'Reilly 2001). Neonates were sprinted individually along a 1 m race track lined with infrared light beams that recorded speed at quarterly intervals. Lizards were encouraged to sprint by consistent tapping on the tail with a soft paint brush. This procedure was repeated twice. The best of the eight sprint times was used in the data analysis (Chapple and Swain 2002).

After locomotor performance testing, the neonates were humanely killed with an intraperitoneal injection of sodium pentobarbital at 500 ng/g diluted 1:100 in saline solution. After death, the weight (± 10 µg) total length (TL), and SVL were measured (0.01 mm). The hemipenes were exposed by removal of the surrounding skin and muscle tissue and photographed on a Zeiss Stemi SV11 microscope with a Leica DFC 425 camera attachment at 32 x magnification. Photos were captured with Leica Application Suite V3 7.0. The width of hemipenes was measured thrice with ImageJ software, and the mean measurements for both hemipenes were used in the data analysis. The abdominal fat bodies adjacent to the AKG were removed and their wet weight obtained (± 10 µg) (Cale and Whitfield Gibbons 1972). The adrenal-kidney-gonad complex (AKG) was removed and placed directly into Bouin's fixative for 12 h prior to placement into 70 % ethanol solution which was periodically changed to remove all traces of the fixative. The tissues were then dehydrated and embedded in paraffin wax with a Tissue-Tek sampling processor. The AKGs were serially sectioned at 6 µm, and stained with haemotoxylin and eosin. The serially sectioned gonads were recoded to prevent biasing the collection of data prior to careful visual analysis of ovary and testis morphology. The gonads were identified as female by the presence of ovaries, Müllerian and Wolffian ducts, or male by the absence of Müllerian ducts and the presence of Wolffian ducts and testes.

Examination of ovarian histology

Estrogenic EDCs can cause polyovular (POF) follicles in developing ovaries, and POF is a marker of endocrine disruption in females (Iguchi et al. 1990; Guillette et al. 1994). However, we have previously described multiple phenotypic changes in the developing ovary as markers of endocrine disruption (Chapter 4; Jones 2011). Consequently, we developed a scoring system for characterizing the effects of exposure to 'estrogenic' EDCs *in utero* (Chapter 4). The criteria for scoring the phenotype of ovaries are presented in Table 1. All sections of the ovaries were examined three times to ensure accurate scoring of each criterion detailed in Table 1: the final data utilized for analysis were the total cumulative score of one randomly selected ovary of each female neonate.

Examination of testis histology

The effects of ATZ on testis development in other taxa include disruption to the organisation of ST and the presence of testicular lesions (Hayes et al. 2011). Similarly, we have identified the demasculinising effects of an estrogenic EDC (DES) on testis development in *N. metallicus* as the absence of primordial germ cells, and disruption to the organisation of ST (Chapter 4). We previously defined four levels of disruption to ST organisation: scored as 0 = highly differentiated (normal), 1= moderately differentiated, 2 = poorly differentiated, and 3 = undifferentiated (Chapter 4). We therefore scored the organisation of ST similarly, and noted the presence or absence of primordial germ cells and testicular lesions. We randomly selected data from one testis to include in the statistical analysis.

Statistical analyses

Data analysis was performed using SAS 9.2 for Windows. Differences in adult female SVL at dosing and parturition date were examined individually using general-linear-models (GLM). Data from all neonates were included in the analysis; maternal identity was therefore included as a random factor and thus data from neonates were analyzed with a general linear mixed model. Neonatal weight and SVL were analyzed separately on untransformed data. Sprint speed, the

weight of neonatal fat bodies, and the mean width of hemipenes were analyzed using log-transformed data. Neonate sex was included as a fixed factor in the analysis of hemipenis width. The cumulative score of ovarian abnormalities was analyzed after adding a constant (0.1) to all of the values to account for the many zero values in the dataset and log-transforming prior to analysis. Normality of distribution for all parametric models was identified by examining plots of standardized residuals against predicted values and normal probability of the residuals. Significant differences were identified using Tukey's Honest Significant Difference.

We included the occurrence of POF as a criterion in the average cumulative score, but POF are a known marker of disruption by estrogenic EDCs (Iguchi and Takasugi 1986; Guillette et al. 1994). Therefore we performed a separate analysis on the occurrence of POF in neonatal ovaries. The presence or absence of POF, the organization of ST, the presence or absence of primordial germ cells, the presence or absence of lesions and the occurrence of cells with elongated nuclei in neonatal testes were analyzed separately with maternal identity as a random factor by generalized linear mixed model with a Gaussian distribution.

Results

Maternal and neonatal biometrics

There was no significant difference in maternal SVL at dosing ($F_{3, 76} = 0.22$; $p = 0.8789$), nor was there a significant effect of treatment on parturition date ($F_{3, 58} = 0.44$; $p = 0.7254$). Similarly, there was no significant effect of treatment on the weight of neonates ($F_{3,130} = 2.18$; $p = 0.0938$), SVL ($F_{3, 161} = 1.78$; $p = 0.1525$), weight of fat bodies ($F_{3, 150} = 1.31$; $p = 0.2743$), or sprint speed ($F_{3, 107} = 0.07$ $p = 0.9767$) of neonates at birth.

Gonad morphology and hemipenis size

ATZ and DES treatment resulted in significant disruption of ovarian structure and the micro-anatomy of oocytes and follicles compared to controls: examples of abnormal ovarian structures are depicted in Figure 5.1. There was a significant effect of treatment on ovarian structure with

females born to ATZ and DES exposed mothers averaging higher cumulative scores compared to females born to control mothers, ($F_{3, 50} = 31.65$; $p < 0.0001$). However there was no significant difference between the cumulative scores of the females born to atrazine or DES treated mothers (Figure 5.2). We observed POF in the ovaries of some neonatal *N. metallicus* (Figure 5.3), however, there was no significant effect of treatment on the presence of POF ($F_{1,45} = 3.01$; $p = 0.0898$) although there was a trend with female neonates born to DES-treated mothers having higher incidence of POF.

There was a significant effect of both ATZ and DES treatment on ST formation ($F_{1, 47} = 28.38$; $p < 0.0001$) (see examples in Figure 4). Males born to ATZ and DES treated mothers were more likely to have a score of one compared to males born to control or control-injection mothers which were likely to score zero on this character. There was no significant difference in level of ST organization between males born to ATZ and DES treated mothers ($F_{1, 21} = 0.17$, $p = 0.6803$), nor between control-injection and control mothers ($F_{1, 26} = 0.15$; $p = 0.7039$) (Figure 5.5). There was a significant effect of treatment on the presence/absence of primordial germ cells ($F_{1, 45} = 22.31$; $p < 0.001$), but there was no difference between males born to ATZ and DES exposed mothers ($F_{1, 21} = 0.17$ $p = 0.6803$) or between males born from control-injection and control mothers ($F_{1, 26} = 0.15$, $p = 0.7039$) (Figure 5.6).

There was a significant effect of treatment on the presence/absence of testicular lesions ($F_{1, 43} = 11.00$, $p = 0.0019$), with more males born to ATZ treated mothers exhibiting testicular lesions compared with males born to DES or control-injection and control mothers ($F_{1, 17} = 5.91$ $p = 0.0265$; Figure 5.7). There was no effect of either ATZ or DES treatment on the width of hemipenis size ($F_{3,125} = 1.48$; $p = 0.2220$).

Discussion

We investigated the effect of atrazine (ATZ), a selective herbicide, on embryonic development in the viviparous lizard, *Niveoscincus metallicus*. Neonatal *N. metallicus* of both sexes born to mothers exposed to a single dose of ATZ at 10 ppb during sexual differentiation exhibited gonadal abnormalities. We also assessed the effects of ATZ on factors which contribute to fitness

and offspring survival in lizards, including birthdate (Warner and Shine 2007; Wapstra et al. 2010), locomotor performance (Wapstra 2000; Le Galllard et al. 2004), size of offspring (Ferguson and Fox 1984) and offspring body condition at birth (Atkins et al. 2006; Atkins et al. 2007). There was no effect of developmental exposure to ATZ on these surrogate measures of fitness: the effects we observed were on gonadal development. Female neonatal *N. metallicus* born to ATZ exposed mothers had a higher incidence of a range of developmental abnormalities of the ovary compared to females born to control mothers. Exposure to ATZ *in utero* increased the occurrence of polyovular follicles (POFs), atretic follicles and cells with aberrant nuclei. Furthermore, in contrast to females born to control mothers, females born to ATZ exposed mothers exhibited ovaries without a distinct medulla and cortex and lacunae. Ovaries of females born to ATZ treated mothers also retained meduallry cords, while medullary cords in the ovaries of females born to control-injection and control mothers had regressed.

Studies on the effects of ATZ on ovarian development in other taxa are limited in number and yield contradicting results. For example, Crain et al. (1999) reported no effect of *in ovum* exposure to ATZ on the developing ovary of *Alligator mississippiensis*, but *in ovum* exposure of female *Gallus gallus* resulted in persistence of the right gonad, which would normally regress (Matsushita et al. 2006). Exposure of embryonic *Caiman latirostris* to ATZ *in ovum* altered ovarian follicular dynamics such that follicles from exposed female neonates were at advanced stages compared with controls (Stoker et al. 2008). In contrast to the observations of Stoker et al. (2008), many of the abnormalities that we observed suggest a delay in ovarian development rather than an advance in maturation of follicular cells. For example, indistinct medulla and cortex, presence of medullary cords and absence of lacunae are all features observed in the developing ovary of other reptilian species, including the congeneric species *N. ocellatus* during early ovarian differentiation (Dufaure and Hubert 1961; Neaves et al. 2006; Jones 2011). Retarded ovarian development has also been reported in the fish *Coregonus lavaretus* exposed to estradiol during gonad development (Kipfer et al. 2009) and in Wistar rats exposed to ethynylestradiol *in utero* (Kummer et al. 2013). Similarly, exposure to estrogenic EDCs prevents follicle nest breakdown, thus delaying follicular maturation, and results in the formation of POF in laboratory rodents (Jefferson et al. 2006; Kim et al. 2009). We observed POF in the ovaries of

N. metallicus, supporting the idea that the effects we observed relate to delayed ovarian development.

We observed several markers of *in utero* exposure to ATZ on the testes of male *N. metallicus* which are in direct agreement with several other studies in male vertebrates. Testes with poorly defined ST, devoid of germ cells, containing testicular lesions were observed in male *Xenopus laevis* (Hayes et al. 2010) and *Caiman latirostris* (Rey et al. 2009) following developmental exposure to ATZ. Similarly, exposure to ATZ resulted in germ cell loss in reproductively mature rodents exposed during adulthood (Kniewald et al. 2000; Victor-Costa et al. 2010). Furthermore, testicular lesions following ATZ exposure were observed in reproductively mature *Carassius auratus* (Spanò et al. 2004), and laboratory rodents (Kniewald et al. 2000; Victor-Costa et al. 2010). Despite differences in life stages and class of the experimental subjects, ATZ appears to have consistent effects on the testes of vertebrates (Hayes et al. 2011): our results extend these findings to viviparous reptiles.

The mechanisms by which ATZ disrupts the vertebrate endocrine system have been extensively examined. Acceleration of aromatase activity in tissues which utilize the ArPII promotor and SF-1 is one major pathway of ATZ effects (Sanderson et al. 2000; Sanderson et al. 2001; Sanderson et al. 2004; Fan et al. 2007; Fan et al. 2007). Comparison of our data on the effects of ATZ and the effects of our positive control agent DES on gonadal development suggest that the majority of the effects that we observed are indeed mediated via an increase in estrogen signaling. We found no significant difference in any aspect of ovarian development between neonates exposed to DES or ATZ *in utero*. Furthermore, we found no significant difference between males born to DES or ATZ mothers in the organization of ST, the presence of germ cells or the presence of cells with elongated nuclei. Therefore we suggest that these measures of demasculinisation occur via increased estrogen signaling. Conversely, we observed a significant difference in the incidence of testicular lesions in males born to ATZ exposed mothers compared with males born to DES mothers. We therefore suggest that the formation of testicular lesions is not a result of increased aromatase activity and subsequent increase in estrogen signaling. Rather, we suggest that testicular lesions may reflect decreased availability in dihydrotestosterone (DHT). Atrazine

decreases androgen synthesis via multiple pathways (reviewed in Hayes et al 2001). Atrazine inhibits 5α reductase activity (Kniewald et al. 1995) which reduces dihydrotestosterone (DHT) synthesis (Hayes et al. 2011). Furthermore, ATZ inhibits DHT binding to binding proteins and receptors *in vitro* (Danzo 1997), and thus reduces DHT synthesis and potency (Hayes et al. 2011). Sex differences in the action and effects of ATZ are therefore likely, as testis formation is largely dependent on DHT (Pieau et al. 1982; Crews et al. 1996) but ovarian differentiation is not (Andrews et al. 1997; Pieau and Dorizzi 2004). Reduced DHT synthesis and potency is therefore likely to be of low consequence in ovary formation. Sex differences in the effects of EDCs have been reported in other taxa (Orlando and Guillette 2007) and we suggest that the effects relate to differences in the hormonal milieu in which males and females develop.

In males, reproductive success can be impaired by reduced phallus size. We found no significant effect of ATZ exposure on the size of hemipenes in neonates of either sex, which is consistent with the effects of the positive control agent DES in this and a previous study conducted by our group (Chapter 4). Hemipenis regression or proliferation is mediated via sex-specific production of gonadal steroids in other reptilian species (Holmes and Wade 2005; Inamdar et al. 2012). We exposed gestating females to a single dose of ATZ or the positive control agent DES during the early phases of sexual differentiation; however, hemipenes do not develop until much later in development and begin to be sexually dimorphic in the final stages before birth in *N. metallicus* (Parsley personal observations) and in the congeneric *N. ocellatus* (Neaves et al. 2006). The obvious effects of ATZ and DES on gonadal development in both sexes appear to have had no consequence for the development of hemipenes, potentially due to the timing of exposure in this study.

Conclusion

We have found that ATZ is a potent EDC in *N. metallicus*. A single dose of ATZ at 10 ppb during gestation demasculinises male gonads via two main potential routes and hyper-feminizes female gonads most likely via increased aromatase activity. The effects of low-level ATZ on *N. metallicus* are subtle phenotypic changes which relate to gonad histology; no other changes to

phenotype were observed. Assessment of the impact of herbicides such as ATZ should therefore examine gonad histology as the most relevant endpoint rather than ecological assessment of other parameters relating to reproductive fitness. We suggest that ATZ should be used with caution, as prolonged exposure of wildlife to this EDC is likely to have devastating effects on reproductive health.

Acknowledgements

We thank Louis J. Guillette Jr. for reviewing the experimental design and providing advice about the interpretation of gonad histology. We thank Jo McEvoy for fieldwork, Steffen Becker for lizard husbandry and Adam McKerinan for assistance with the measurement of hemipenes. Erik Wapstra acknowledges the support of the Australian Research Council. Funding for this project was provided by the Holsworth Research Wildlife Endowment, The Forest Practices Authority and the Ecological Society of Australia.

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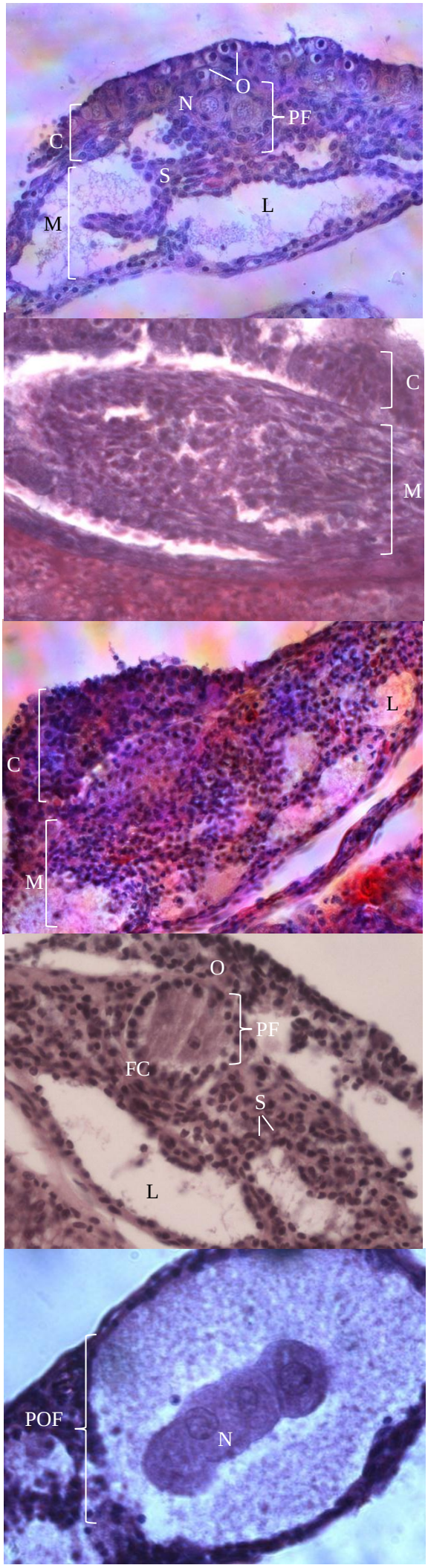

Figure 5.1 Ovaries from neonatal *N. metallicus* illustrating abnormal phenotypic traits which are utilised in the scoring system. a) illustrates normal phenotype, b), illustrates reduced lacunae and presence of medullary cords, c) illustrates crenated, elongated and heavily granulated stroma; d) illustrates oognia with reduced ooplasm, elongated nuclei and heavily granulated nuclei, and heavily granulated follicular cells . e) illustrates a POF with four oocytes. C, cortex; M, medulla; O, oogonia; PO, primary oocyte; PF, primary follicle; N, nucleus; FC, follicular cell; S, stroma; L lacunae. All ovaries are stained with haematoxylin, eosin and sectioned at 6 µm, and magnified at 400 x.

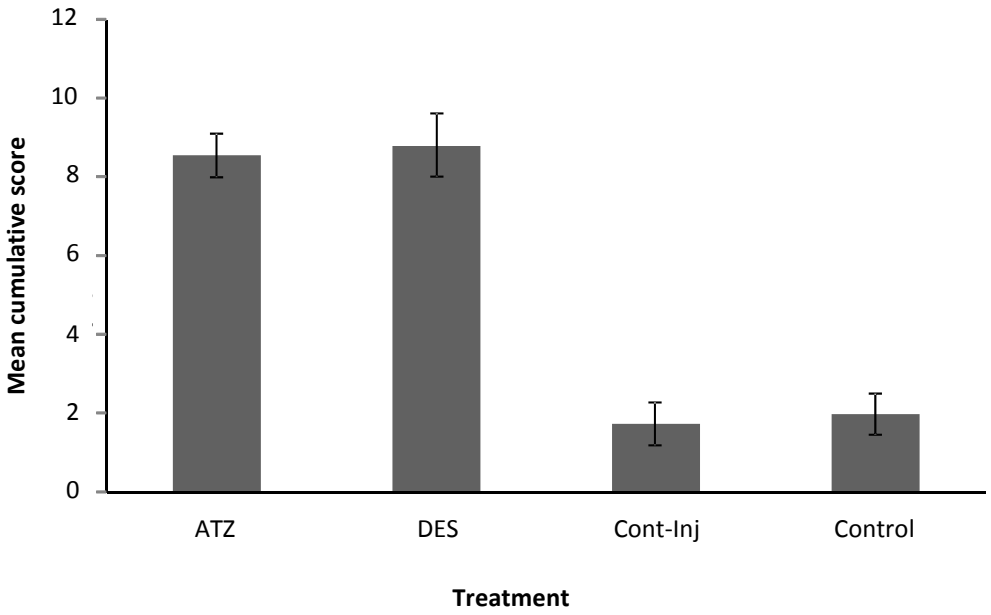

Figure 5.2 The accumulation of abnormal phenotypic traits relating to the cells and structure of the ovary comprising the 'cumulative score' of one randomly selected ovary of *N. metallicus*. The mean cumulative score of female *N. metallicus* born to ATZ (n= 32), DES (n = 28), Control-Injected (sesame oil n = 28) and Control (n= 29) treated mothers. The asterisk indicates the statistically significant difference, error bars are the standard error of the mean.

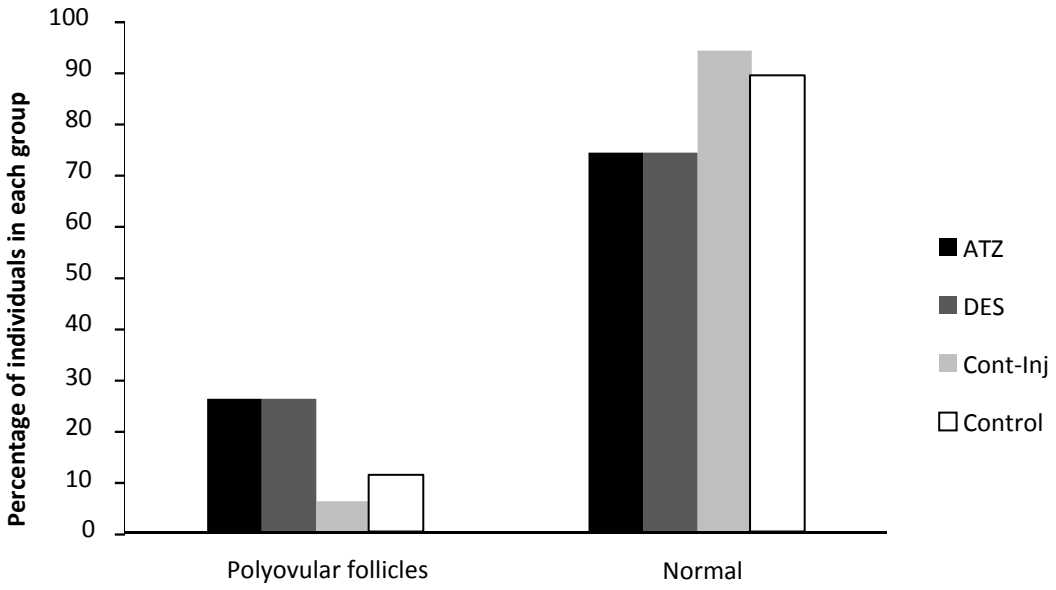

Figure 5.3 The occurrence of polyovular and normal follicles containing one oocyte in ovaries of neonatal *N. metallicus* born from ATZ, DES, Control-Injected (sesame oil) and control treated mothers.

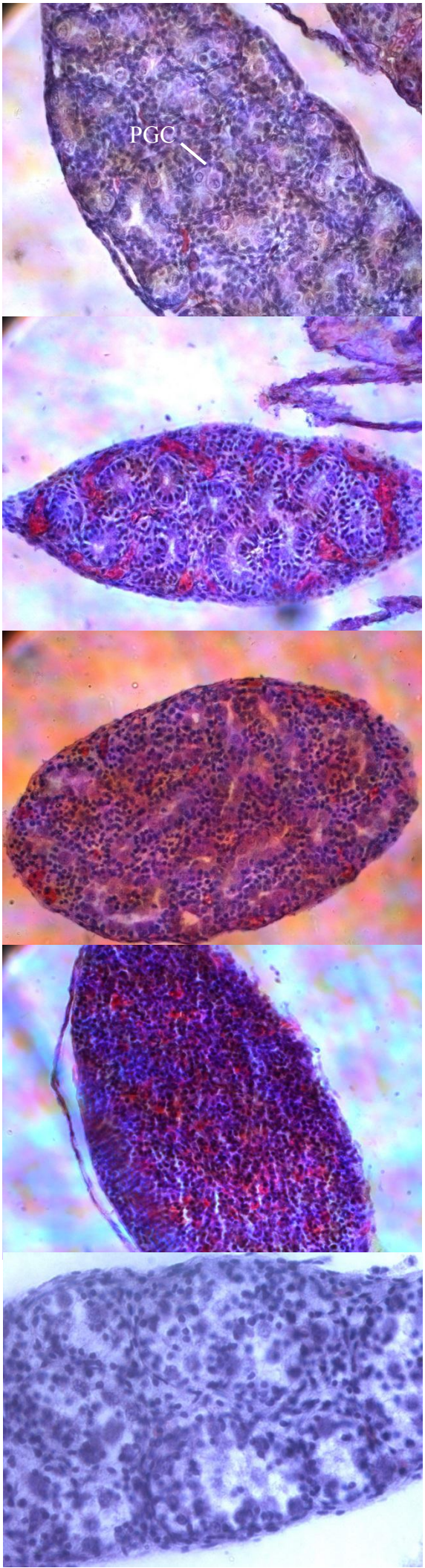

Figure 5.4 Testes from neonatal *N. metallicus* illustrating a) normal testis with well-defined seminiferous tubules (ST score = 0), b) testis with moderately defined seminiferous tubules (ST score = 1), c) testis with poorly defined seminiferous tubules (ST score 2), d) testis with no seminiferous tubules (ST score = 3), e) testis demonstrating testicular lesions. All testes were stained with eosin and haematoxylin, sectioned at at 6 µm, and magnified at 400 x

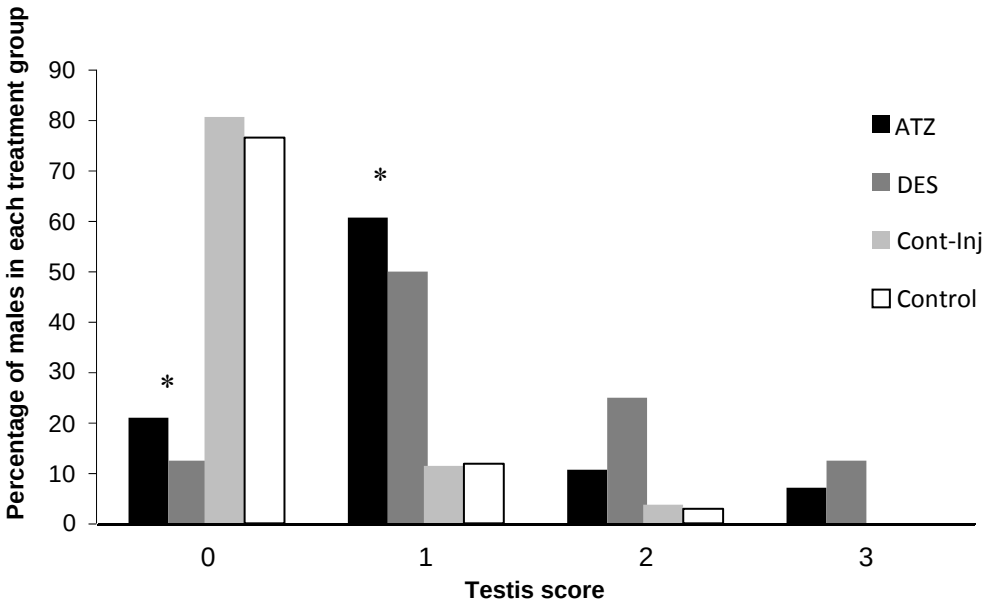

Figure 5.5 The percentage of male neonatal *N. metallicus* born to ATZ (n = 28), DES (n = 26) Control-injected (n = 24) and control (n = 34) treated mothers exhibiting seminiferous tubule scores of 0 (normal) 1, (moderately differentiated) 2, (poorly differentiated) and 3 (undifferentiated). Asterix indicate statistically significant differences.

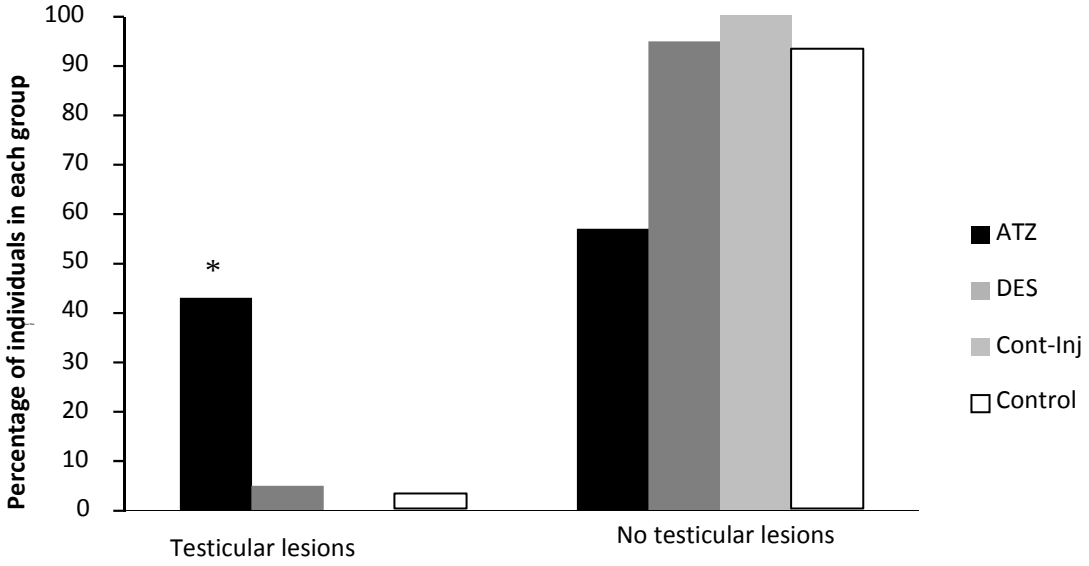

Figure 5.6 The percentage of male neonates born from ATZ (n = 28), DES (n = 24) Control-Injected (sesame oil, n = 26) and control (n = 34) treated mothers with testes exhibiting testicular lesions or neonates with a normal testicular phenotype. The asterisk indicates statistically significant differences.

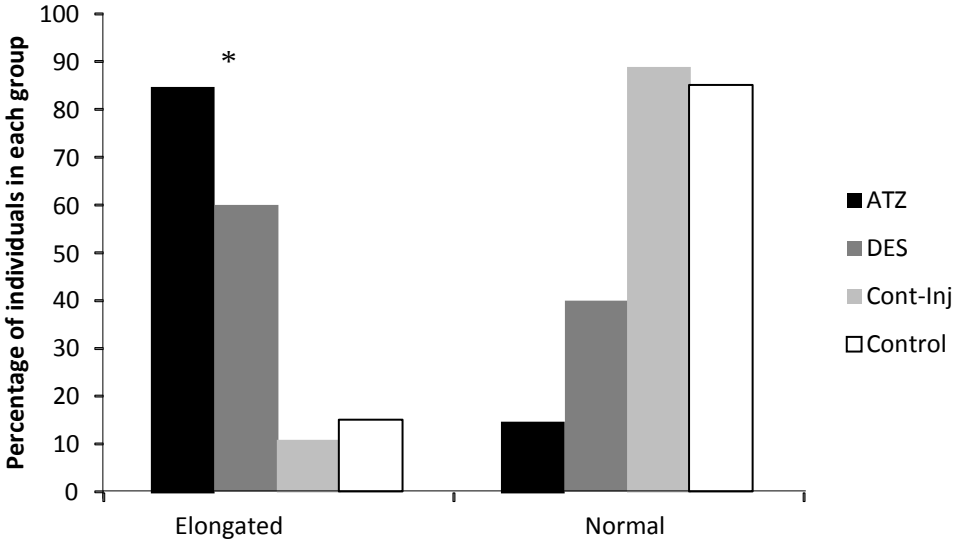

Figure 5.7 The percentage of male neonates born from ATZ (n = 28), DES (n = 24) Control-Injected (sesame oil, n = 26) and control (n = 34) treated mothers with testes exhibiting testicular cells with elongated nuclei or neonates with a normal testicular phenotype. The asterisk indicates statistically significant differences.

Chapter 6 Discussion

Discussion

In this thesis, I made a significant contribution to knowledge of the endocrine environment of embryonic viviparous reptiles. Prior to my research, it was only speculated that the yolk is a source of T and E_2 to embryonic viviparous reptiles. I have now confirmed that the yolk is a substantial source of T and E_2 in such species. Furthermore, I identified that the placenta is a key site of aromatase activity and thus, a site of T metabolism and E_2 synthesis. My findings therefore clearly demonstrate that the placenta and the yolk are both sources of steroid hormones that may impact on developing embryos of a viviparous lizard (Figure 6.1).

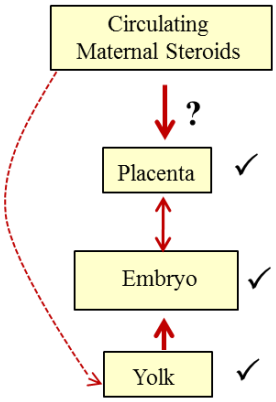

Figure 6.1 The confirmed routes of embryonic exposure to maternal steroids as demonstrated in chapters 2 and 3. The Dashed arrow indicates the movement of T and E_2 during vitellogenesis, solid arrows indicate movement during gestation. My findings confirm the yolk as a substantial source T and E_2 to embryos and also demonstrate significant placental aromatase activity in the viviparous lizard *Niveoscincus metallicus.*

I also investigated the potential for endocrine disruption of embryonic development in a viviparous lizard. I have demonstrated that the herbicide atrazine (ATZ) is a potent endocrine disrupting contaminant of embryonic *N. metallicus* and report significant effects on gonadal development in both males and females. By first characterising the effects of the known estrogen mimic diethlystilbestrol (DES) and comparing the effects of DES with the effects I observed following ATZ exposure, I was able to conclude that ATZ leads to increased estrogen signalling

in *N. metallicus* (Chapters 4 and 5). My study is the first to demonstrate endocrine disruption in a viviparous reptile. Furthermore, my study demonstrates endocrine disrupting effects of a single dose of ATZ at the lowest concentration utilised in any reptilian study.

The routes of embryonic exposure to T and E_2 differ with parity mode: I have provided evidence for two major sources of maternal steroid in a viviparous reptile (Figure 6.1). Viviparity is a derived state and in viviparous reptiles the four placental types correspond to a progressive decrease in dependence upon the yolk in embryonic nutrient provisioning. It is well known that the yolk of oviparous species serves a major purpose in providing embryos with steroids (Conley et al. 1997; Janzen et al. 1998; Paitz and Bowden 2009), while placental transfer and production serve to provide embryos with steroids in eutherian mammals. With the evolution of viviparity from oviparity in reptiles, the placenta of viviparous reptiles is likely to assume the role of the yolk to varying degrees. As placental complexity increases there is potential for a transition from the dependence on yolk to provide steroids to a dependence on the placenta to provide steroids. The moderately complex Type II placenta of *N. metallicus* has a facultative role in nutrient provisioning (Stewart and Thompson 1994; Swain and Jones 1997; Swain and Jones 2000; Stewart 2013) and the yolk of *N. metallicus* is therefore obligate in nutrient provisioning. However, prior to my thesis, the endocrine function of the placenta remained speculative. I anticipated that the role of the yolk in providing T and E_2 would be largely conserved. My data, however, suggested that it was not as straightforward and the decline of these key steroids in the yolk varied from oviparous species. The likely explanation for this key difference is the potential steroidogenic action of the placenta. Thus, I suggest that endocrine roles of the yolk and the placenta of *N. metallicus* are not likely to be directly comparable to the roles of the yolk of oviparous reptiles or the placenta of eutherian mammals that depend solely on a yolk or a placenta respectively.

In *N. metallicus*, I showed that yolk T and E_2 do not decline during sexual differentiation (Chapter 2): this result may be attributable to placental steroid production and transfer. *In N. metallicus*, placental aromatase activity is higher during the initiation of sexual differentiation than at any other stage of embryonic development (Chapter 3). Although I was not able to

measure the production of E_2 conjugates, and thus the 'buffering' capacity of the placenta of N. metallicus (Appendix) nor measure placental transfer of E_2, studies on other viviparous species may provide some clues regarding transfer of maternal steroids. For example, in *Pseudemoia entrecasteauxii*, experimentally increasing maternal plasma corticosterone with radiolabelled corticosterone resulted in higher transfer of radiation to embryos at the initiation of sexual differentiation compared with transfer at any other stage of development, and was lowest for late stage embryos (Itonaga et al. 2011). My results and the results of Itonaga et al. (2011) support the idea that yolk steroids may not be critical to gonadal development in the early embryo of viviparous reptiles such as N. metallicus, because the embryos are exposed to steroids via placental transfer and placental production (Figure 6.2).

I surmise that the opposite is also true: when placental steroidogenesis and transfer are both low (ie during the late stages of embryonic development), the yolk is the primary source of steroids to embryos in viviparous lizards that utilise both a yolk and a placenta to nourish their embryos (Figure 6.2). My data for placental aromatase activity fits this pattern because placental aromatase activity in females with embryos at stages 39-40+ was significantly lower than at any other of the stages examined (Chapter 3). However, the data from the yolks of N. metallicus are less straight forward, and need some reflection. Lizard embryos are traditionally staged on the Dufaure and Hubert (1961) scale of zero (= post-ovulatory egg) to Stage 40 (= fully developed embryo). However an additional embryonic developmental stage has been defined for N. metallicus: stage 40+ is characterised by fully developed embryos retained within the oviduct, with no remaining yolk (Swain and Jones 1997). In Chapter 2, I demonstrated that at the final stage at which I sampled yolks (Stages 39-40), *>100* pg of both *T* and E_2 remained in *an average* 40 mg of yolk (Chapter 2). The transition to stage 40+ therefore coincides with the mobilisation of significant quantities of steroid. The only other alternative is that the hydrophobic *T and* E_2 remain within the yolk membranes as undissolved crystals, but such an explanation does not seem plausible. Therefore, synthesising my results from Chapters 2 and 3, I suggest that the yolk and the placenta of N. metallicus work in concert as sources of *T* and E_2 to developing embryos (Figure 6.2). I propose that *yolk* T and E_2 are more critical in the *final* stages of gestation, when

placental transfer of maternal steroids (Itonaga et al. 2011) and placental aromatase activity are low (Chapter 3).

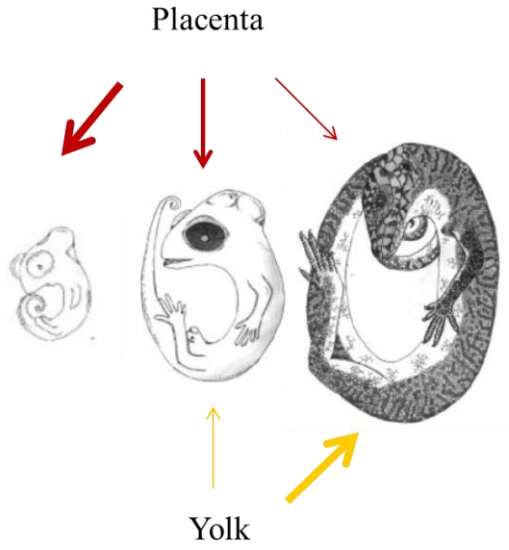

Figure 6.2 schematic representation of the roles of the placenta and the yolk as a source of hormones to embryos of *Niveoscincus metallicus*. In the early stages of development, the placenta is the primary source of hormones to embryos but becomes increasingly less important as development progresses. The yolk steroids start to decline in the later stage embryos, but are presumably used in the transition from 39-40 to 40+. The pictures of embryos utilised in this schematic are from Neaves et al. (2006).

If placental steroids are required in early development, and yolk steroids are required in late development in *N. metallicus*, would the same pattern be observed in reptiles with placentae of other types? Considering the large variation in placental complexity and nutrient provisioning in the four placental types of viviparous reptiles and the multiple independent origins of viviparity, the endocrine function of the placentae and the yolks are likely to vary with placental complexity. In species with simple Type I placentae, all of the nutritional support to embryos is provided via the yolk so steroids such as T and E_2 are expected to be deposited in the yolk as in oviparous species. However in microlecithal species with highly complex Type IV placentae, the yolk is very small and does not provide embryos with significant nutrition. Does this mean that the tiny

yolks of Type IV species do not contain T and E_2? Although my data on *N. metallicus* do not allow me to answer this question, I have preliminary data on yolks from two female *P. entrecasteauxii*. *Pseudomoia entrecasteauxii* has a Type III placenta and thus a significantly smaller yolk than N. metallicus despite the similarities in body size between the two species. I found that despite the significantly smaller size of the yolks, the total amount of E_2 in the yolks at ovulation equals that of the yolks of *N. metallicus* at ovulation. I therefore suggest that future investigations be directed toward understanding the differences in the endocrine function of the yolks in viviparous reptiles with a range of placental types. Based on my data on *N. metallicus* and preliminary data on *P. entrecasteauxii*, I hypothesise that T and E_2 are sequestered into the yolks of viviparous reptiles of all placental types by default.

Considering the species-specific differences in placental endocrine function in eutherian mammals in which viviparity has only one origin (Sharman 1976), there are enormous possibilities for species-specific placental function in viviparous reptiles, in which viviparity has evolved over 100 times (Blackburn 1982; 1985; 1993). Major differences in placental endocrine function in reptiles may relate more to placental type than to the repertoire of steroidogenic enzymes or the capacity of the placenta to function as an endocrine organ, features which underpin the major differences in placental endocrine function in eutherian mammals (Strauss et al. 1996). I suggest it is the *pattern* of placental production of steroids that could vary with placental complexity, reflecting patterns of yolk utilisation. The *stage* at which embryos are exposed to yolk steroids and steroids of placental production may differ with placental complexity. Future studies of the pattern of deposition and utilisation of yolk *T and* E_2 in viviparous reptiles of other placental Types (I, III, and IV) are a necessary next step in isolating the endocrine roles of the reptilian placenta and the yolk.

Regardless of parity mode and the routes of steroid exposure, embryos of many vertebrates do in part control their own endocrine environment. Embryos can modulate the effects of steroid hormones to which they are exposed by chemically derivatising and deactivating steroid hormones (Pasqualini 2005; Paitz and Bowden 2008; Paitz et al. 2012). If a surge of yolk hormones occurs in *N. metallicus* during the *final* stages of gestation, the activity of enzymes

such as sulfotransferase or glucuronidase, and steroid-specific β-hydroxysteroid dehydrogenases in the endocrine tissues of both mother and embryo may be higher at this phase than other phases of gestation. Unfortunately, I was not able to measure the production of steroid derivatives and conjugates and thus the capacity to 'buffer' steroid effects (see Appendix). However, obtaining such information will be important to future studies of endocrine disruption, firstly, because this information is important in understanding the endocrine environment of embryonic *N. metallicus* and, potentially, other viviparous reptiles, and secondly, because steroid derivatising enzymes are a key site of endocrine disruption (Kester et al. 2002).

Viviparous reptiles have been identified as useful models in studying the mechanisms of embryonic exposure to EDCs as most species utilize a yolk and a placenta (Crain and Guillette 1998). However prior to this thesis, no study had utilized viviparous reptiles in any study of endocrine disruption and knowledge of the pathways of embryonic exposure to EDCs were speculative only (Figure 6.3).

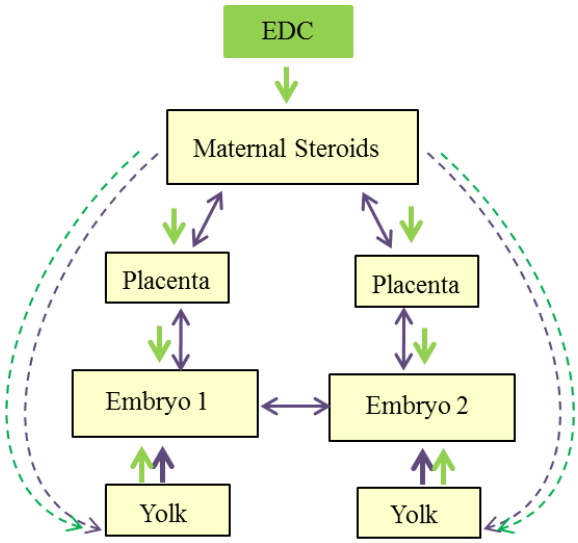

Figure 6.3 Diagrammatical representation of the proposed routes of embryonic exposure to EDCs in viviparous lizards. The green arrows indicate the movement of EDCs from the mother to the embryo, the purple arrows indicate embryonic exposure to altered steroid signals (maternal activational disruption) following maternal EDC exposure. The dotted lines indicate movement during vitellogenesis.

I have now demonstrated that embryonic viviparous lizards are at risk of EDC exposure via two major pathways, the yolk and the placenta (Figure 6.1). I examined the effects of *in utero* exposure to two EDCs on gonadal development in *N. metallicus* (Chapters 4 and 5). If female *N. metallicus* are dosed during gestation rather than during vitellogenesis, the placenta rather than the yolk is the most likely route to abnormal gonadal development in *N. metallicus* embryos (Figure 6.2). However, embryos of exposed mothers could be subject to altered maternal or placental endocrine signals, or embryos could be directly exposed to the EDCs via placental transfer (Figure 6.3). I did not trace the movement of either DES or ATZ from mother to embryo, so I cannot confirm the exact *route* of embryonic exposure to these two EDCs. However, I can deduce the *mechanism of embryonic exposure* from my observations.

It is well known that DES readily traverses the placenta of eutherian mammals (Shah and McLachlan 1976; Miller et al. 1982; Slikker et al. 1982; Newbold 2004), and most likely disrupts the endocrine systems of mothers and embryos by binding to both estrogen receptor subtypes (Kuiper et al. 1997) in all tissues where they are expressed. Therefore, the effects of DES on both male and female neonates of *N. metallicus* do not provide any real evidence as to whether embryos received altered maternal steroid signals, or were exposed directly to DES via placental transfer. However, the effects of ATZ that I observed suggest that the embryos were directly exposed to ATZ. In contemplating the effects of *in utero* ATZ exposure I observed in *N. metallicus*, the formation of testicular lesions provides a clue about the mechanisms of embryonic exposure to ATZ. I attributed testicular lesions in male *N. metallicus* (Chapter 5) to reduced dihydrotestosterone (DHT) synthesis and potency (Danzo 1997), as testicular lesion formation in other vertebrates have also been attributed to such effects of ATZ (Hayes et al. 2011). *Embryonic* production of DHT plays a major role in testis differentiation of the embryonic testis only (Pieau et al. 1982; Crews et al. 1996). Embryonic testes are therefore the only significant site of DHT production. Thus, disruption to DHT is likely to occur in embryonic testes only, suggesting direct embryonic exposure to ATZ, which I presume must only occur via placental transfer.

However the placenta is not the only possible route for endocrine disruption of embryonic development in *N. metallicus*. I have confirmed that maternal T and E_2 are incorporated into the

yolk during vitellogenesis (Chapter 2), thus activational disruption during vitellogenesis is likely to result in altered maternal steroid signals in the yolk (Figure 6.3). Furthermore, any lipid-soluble EDC would also become incorporated into the yolk if mothers were exposed during vitellogenesis (Figure 6.3). Therefore in *N. metallicus* there is potential for disrupted maternal endocrine signals in the yolk or for direct yolk contamination, both of which have been demonstrated in oviparous reptilian species (van de Merwe et al. 2009; Hamlin et al. 2010).

The stage at which embryos are exposed to EDCs can determine the ultimate effects of those EDCs (Guillette et al. 1995; Crain et al. 2008; Di Renzo et al. 2011; Gioiosa et al. 2013). Thus to further understand the effects of EDCs on embryonic development in *N. metallicus*, future studies of EDCs (including ATZ) should examine the impacts on embryos exposed to EDCs that are incorporated in the yolk and also investigate the effects of maternal exposure to EDCs during vitellogenesis. Maternal exposure to EDCs during vitellogenesis could potentially have very different effects than exposure during gestation. For example, exposure to ATZ during the initiation of gonadal development (ie embryonic stage 30-32) has profound effects on gonadal morphology (Chapter 5), but exposure prior to birth (stages 39-40+) may not affect the phenotype of the gonads.

How does the timing of embryonic exposure to yolk steroids affect the timing of embryonic exposure to disrupted maternal steroid signals in the yolk? My data indicate that during the initiation of gonadal differentiation in the viviparous lizard *N. metallicus*, yolk hormones are not yet mobilized, and may not become important to embryos until the final stages of gestation (Chapter 2). The timing of embryonic exposure to endocrine signals in the yolk is unlikely to change, thus embryos would be exposed to disrupted T and E_2 concentrations during the final stages of development. One potential role of the yolk T and E_2 is to control maturation of the hemipenes. In *N. metallicus* (Parsley personal observations) and the congeneric *N. ocellatus*, sexual dimorphism of hemipenes begins at stages 37-38 (Neaves et al 2006), which is when yolk T begins to be mobilised (Chapter 2). Sexual dimorphism of the hemipenes may be partially controlled by T and E_2 originating from the yolk. If so, this may explain why I did not observe any effect of either DES or ATZ on the size of hemipenes in male or female neonates exposed *in*

utero (Chapters 4 and 5). The phenotype of hemipenes of exposed embryos may have been preserved by the mobilisation of yolk steroids, which were unlikely to be affected by ATZ or DES exposure under my experimental regimes. However, if the mother was exposed to ATZ or DES during vitellogenesis, the phenotype of hemipenes of the embryos may have been affected, as I suggest that embryos would be exposed to disrupted T and E_2 concentrations during the final stages of development, when hemipenis maturation occurs. Furthermore, maternal exposure to EDCs during vitellogenesis may result in effects upon gonads and other reproductive parameters, which could occur via disrupted maternal endocrine signals.

Embryonic exposure to EDCs that are incorporated into the yolk may not necessarily occur at the end of development. Embryos potentially acquire yolk steroids via some kind of steroid-specific active transport (Moore and Johnston 2008). Therefore, depending on the properties of the EDC, embryos could potentially remove the EDC via the same steroid-specific mechanism. I speculate that EDCs such as DES that bind directly to steroid receptors are potentially removed from the yolk via the same mechanism and are therefore removed at the same time as steroids. However, other lipophilic EDCs that do not resemble steroids and do not have a high affinity for steroid receptors such as ATZ may not be acquired from the yolk via the same steroid-specific mechanism. Rather, embryos may be passively exposed to the EDC as a by-product of yolk metabolism, and are therefore potentially exposed for the duration of embryonic development. However, these ideas about the mechanisms of acquisition of yolk steroids and exposure to EDCs are hypothetical and future research is warranted. Firstly, because it is not yet known how embryos of oviparous or viviparous vertebrates remove steroids from the yolk, and secondly, because the timing of developmental exposure to EDCs determines EDC effects (Guillette et al. 1995; Crain et al. 2008; Di Renzo et al. 2011; Gioiosa et al. 2013).

Studies of the effects of hormones, embryonic hormone exposure and disruption of the endocrine system by EDCs are important to determine the potential effects on wildlife. Thus far, much advancement has been made in determining the effects of EDCs in many vertebrates, yet this is the first study to investigate endocrine disruption in viviparous reptiles. Although direct comparisons between laboratory and field studies cannot be made realistically, it is clear from the

results I obtained in Chapter 5, that ATZ has the potential to affect populations of *N. metallicus*. Atrazine use in Tasmania overlaps with populations of *N. metallicus* and several related species. Therefore, future studies should determine how populations of *N. metallicus* are affected by consistent use of ATZ to determine how spraying affects circulating concentrations of ATZ, to identify routes of maternal exposure, and the ultimate population-level effects.

Furthermore, future investigations of the effects of ATZ on embryonic development in *N. metallicus* should include other reproductive endpoints (other than gonadal development) including effects on aromatase expression in the brain (Krohmer and Baum 1989; Weniger 1990; 1993; Willingham et al. 2000; Blázquez et al. 2008). Atrazine potentially impacts the brain and, aromatase activity during brain development affects mating behaviours in adulthood (Bakker et al. 2002; Pierman et al. 2006). Disruption to sexually dimorphic brain function could impair mating behaviours in adulthood (Bakker et al. 2002; Pierman et al. 2006). Additionally, the effect of ATZ on steroid receptor expression in endocrine tissues is another endpoint that should be investigated. The endocrine system is an intricate system which relies upon many factors to function effectively. Circulating and local steroid production is only a small aspect of endocrine function; steroid receptors play a major role in endocrine function as receptors are the intermediate step between the steroid and the biological effect (Welshons et al. 2003). Disruption of receptor expression could therefore have major impacts on endocrine function. Receptor expression in the endocrine tissues of *N. metallicus* is therefore an important endpoint in studies of many EDCs.

Currently, the principles of endocrinology are rarely utilised in determining the safety of EDCs and many known EDCs are presently used despite potential harm to humans and wildlife. Studies of many EDCs have been discounted because of non-monotonic response curves, activity of EDCs at low concentrations and tissue specific effects (Vandenberg et al. 2012; Vandenberg et al. 2013). However, it is well known that hormones are present in circulation at very low concentrations which allows for many hormones to be present in circulation at the same time (Welshons et al. 2003). Furthermore, *hormones* regularly display nonlinear and/or non-monotonic response curves (Vandenberg et al. 2013). If hormone receptors are not saturated, a

ten-fold increase in hormone concentration will have a strong biological effect, but if the hormone receptor is saturated, a ten-fold increase in hormone concentration with have very little biological effect (Welshons et al. 2003). Hormones also have tissue specific responses due to the presence and concentration of steroid receptors which can vary significantly with tissue type and life-stage (Norris 2007). In two comprehensive reviews, Vandenberg et al. (2012; 2013) highlight the fact that EDCs operate in low concentrations, show non-monotonic response curves and have tissue specific effects because they function as part of the endocrine system, which as detailed above displays nonmonotonicity and operates at low concentrations.

Over two decades of research have led to logical explanations for what was initially thought to be peculiar responses to chemical exposure, yet very few EDCs are recognised by governing bodies and are still in use world-wide. I determined that a single dose of ATZ at 10 µg/kg disrupts gonadal development; however, studies that generate a comprehensive dose response curve are more powerful in assessing EDC effects. Future studies with *N. metallicus* should include multiple concentrations of ATZ to determine a comprehensive dose response curve, to identify the lowest concentration that disrupts gonadal development. Despite the use of one concentration at a single stage of gestation, the design of my ATZ experiment (Chapter 5) allows the results to be included in weight of evidence (WoE) approaches that determine the safety of a particular compound (Vandenberg et al. 2013). The WoE criteria suggested by Vandenberg et al. (2013) incorporate principles of endocrinology rather than toxicology to determine suitable studies that can be incorporated into the WoE approach to assessment of chemical safety. The WoE criteria suggested by Vandenberg et al (2013) incorporate low dose effects and nonmomotonic response curves rather than using them as a reason to discount the effects of an EDC. Clearly, action needs to be taken against the continued use of EDCs for the health of wildlife and humans. Thousands of studies have documented profound effects of EDCs on vertebrates of every class. Many EDCs are used in our daily lives and banning EDCs is not a practical option.

So what is the future for EDCs and what can we do to minimise EDC exposure to humans and wildlife? In my opinion, there are three major steps required in order to solve this problem. Firstly, as Vandenberg et al (2013) have suggested, regulatory decisions about EDCs need to

incorporate principles of endocrinology. There is far too much evidence of the danger of EDCs to continue on with current management practices. The issue of EDCs is enormous; ignoring the issues and denying EDC effects will not solve anything. Thus I suggest the first step is to embrace the issue and commit to solving the problem.

Secondly, environmental exposure to EDCs needs to be reduced. It is well known that EDCs such as some industrial waste products, pharmaceuticals, synthetic and natural hormones and additives in personal care products reach aquatic ecosystems via sewage treatment plants (Kolpin et al. 2002; Barber et al. 2013). With the issue of reduced clean water availability in many countries, advances in the removal of EDCs from drinking water have been made (Schafer and Waite 2002; Schröder et al. 2007). However there is a clear distinction between water destined for human consumption and water destined to be discarded or recycled for use in toilets (Schafer and Waite 2002). In my opinion, if the technology is available for removal of EDCs from drinking water, the technology must also be utilised in water destined for the environment. In the case of pesticides (herbicides, insecticides and fungicides) that are applied directly into the environment, efforts to reduce exposure to wildlife and humans should be made. It seems reasonable to suggest that chemicals with known endocrine disrupting effects, that are highly mobile in soil and have a long half-life such as ATZ (Kookana et al. 2010), should not be legal.

Thirdly, it is time to design new chemicals without endocrine disrupting properties. Schug et al. (2013) have devised a comprehensive five tiered testing protocol, designed to determine the endocrine disrupting effects and thus the safety of new compounds. The protocol 'Tiered Protocol for Endocrine Disruption' (TiPED) is designed to identify endocrine disrupting properties early on in the process of compound synthesis.

Tier 1 of the protocol utilises statistical, computer and mathematical models to predict EDC properties of molecules.

Tier 2 utilises high-throughput *in vitro* screens utilising cell based and cell free methods and allows for multiple endpoints such as estrogen and anti-androgen activity of a new compound.

Tier 3 utilises whole cell activity assessment and utilises cell division, differentiation or death as endpoints.

Tier 4 utilises amphibian and fish whole animal models to determine the endocrine disrupting properties of a new compound. The use of whole animal models allows for multiple endpoints to be assessed.

Tier 5, utilises mammalian whole animal assessment, similarly, many endpoints can be assessed utilising mammalian models.

The major benefits of TiPED are that initial testing is relatively inexpensive and that such testing eliminates the complication of working with animal models. Furthermore, the multi-level testing system allows for initial screening to occur shortly after the designing process. Thus TiPED is designed to identify EDCs early in the designing process which minimises wastage of time, effort and money (Schug et al. 2013).

Schug et al. (2013) stated that "TiPED is a scientific framework for progress" and they also anticipate additions to be made to the protocol to improve the protocol as it progresses. I therefore suggest viviparous reptiles such as *N. metallicus* as additional model species for future studies of endocrine disruption. I highlighted viviparous lizards as representing particularly important species in which to study endocrine disruption for several reasons: multiple routes of embryonic hormone and EDC exposure, embryo retention and therefore an extended period of conflict between embryonic and maternal physiology, and because reptiles are potentially more sensitive to chemical pollutants than mammalian species (Campbell and Campbell 2000). Furthermore, reptiles are relatively inexpensive and simple to maintain, and may provide more relevant models to study the effects of certain EDCs such as ATZ than laboratory rodents.

The issue of endocrine disruption is enormous and thus far strategies to reduce the harm caused by EDCs are inadequate. We must tighten legislation, reduce environmental exposure to EDCs and design endocrine disruption out of future chemicals to maintain the health of humans and wildlife.

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Appendix: No oestrogen derivatization by the placentae and embryonic tissues of a live-bearing reptile: an accurate result or a maladroit researcher?

1 Introduction

The sex steroids 17β-estradiol (E2) and testosterone (T) are important in sexual differentiation of the reproductive tract and the brain (Vito and Fox 1979; Clark and Galef 1995; Brandenberger et al. 1997; Wilson and Davies 2007). Exposure to E_2 and/or T during development has organisational effects on phenotype that persist into adulthood and profoundly influence life history traits (Vito and Fox 1979; Clark and Galef 1995; Brandenberger et al. 1997; Wilson and Davies 2007). Embryonic endocrine glands are sensitive to both endogenously produced hormones and hormones external to embryonic production, such as maternal hormones. Hormones are therefore a tool which mothers may utilise to actively influence offspring phenotype (Drummond et al. 2008; Groothuis and Schwabl 2008; Love and Williams 2008). For example, in some species of bird with hierarchical laying sequences, mothers differentially allocate androgens between eggs within clutches (Drummond et al. 2008). More androgen is sequestered into the yolk of later laid eggs, counteracting the disadvantages of hatching last in competition with older siblings (Groothuis and Schwabl 2008): exposure to higher concentrations of androgens helps those chicks compete with larger siblings (Schwabl 1993).

Although exposure to T and E_2 external to embryonic production is necessary for sexual differentiation, excessive or inappropriate exposure during development may be maladaptive. In rodents, the intra-uterine position in which an embryo develops can have a major impact on phenotype (Clark and Galef 1995) Female foetuses positioned between two males are more aggressive, have longer oestrus cycles, produce fewer litters and are less attractive to males in adulthood (vom Saal 1989; Clark and Galef 1995). Similarly, in viviparous lizards, male embryos from female biased clutches showed differences in sexually dimorphic traits compared with males that developed in male biased clutches (Uller and Olsson 2003). It seems that an intricate balance of exposure to T and E2 will determine whether phenotypic effects are adaptive or maladaptive, but how is the balance achieved?

Firstly, in amniotes, parity mode plays a major role in determining how, and when, embryos are exposed to hormones. Maternal hormones can be strategically placed within layers of yolk, with

release potentially corresponding with specific stages of development (Bowden et al. 2001). Mothers may attenuate their own endocrine system to prevent excessive transfer of steroids to the yolk (Moore and Jessop 2003). In oviparous species, the connection between maternal and embryonic physiology is terminated upon oviposition, thus alleviating conflict between maternal homeostasis and embryonic development. In eutherian mammals, the retention of embryos creates the potential for exposure of embryos to maternal hormones circulating via the placenta. The placenta of eutherian mammals is, however, a complex endocrine organ that acts as a buffer between maternal and embryonic physiology. The mammalian placenta can metabolise, deactivate and synthesise hormones (Strauss et al. 1996; Pasqualini 2005). A chemical dialogue between the embryo and the mother is mediated via the placenta throughout gestation, and, in litterbearing species such as rodents, the placenta overrides signals from the maternal pituitary (Strauss et al. 1996). Disruption to placental steroidogenesis results in disruption to embryonic development (Seckl 2001). The placenta is therefore a powerful endocrine organ which is pivotal to the development of sexually dimorphic traits in eutherian mammals.

Secondly, the potency of T and E_2 can be modulated by the enzymes sulphatase and glucuronidase. These enzymes derivatise free biologically inactive steroid to ionisable moieties by conjugating them with a glucose or sulphate molecule, rendering the derivatised steroid unable to bind to receptors and binding proteins (Norris 2007). Sulphatase in placental tissue reduces the concentration of biologically active hormone that is transferred from mother to embryo and *vice versa* (Pasqualini 2005). In the embryo, these enzymes modulate the effects of steroids such as T and E_2, thus buffering the embryo from the potential effects of maternal steroids. Furthermore, embryos of some mammalian species can metabolise hormones such as E_2 to less potent estrogens such as estrone (Raeside et al. 2009), which is a less potent than even E_2 in the active free form (Norris 2007).

There are clearly mechanisms in place through which the embryos of oviparous reptiles and eutherian mammals maintain the intricate balance of appropriate steroid signals, but how are steroid signals maintained at appropriate levels in viviparous reptiles, which utilise both yolk and placenta?

Embryonic viviparous reptiles may be exposed to hormones of maternal origin via the yolk (Chapter 2) or the placenta (Painter et al. 2002; Itonaga et al. 2011). Additionally, the embryonic viviparous lizard may also be exposed to hormones synthesised by the placenta (chapter 3) or by neighbouring siblings *in utero* (Uller and Olsson 2003; Uller et al. 2004; Uller et al. 2005), as the embryonic endocrine glands are active in the production of steroid hormones (Girling and Jones 2003; Raeside et al. 2009). Although our understanding of the endocrinology of reptilian placentation is growing, there are many unanswered questions regarding the routes of embryonic steroid exposure. For example, we do not yet know if the placenta of viviparous reptiles has the capacity to 'buffer' embryos from maternal hormones like the mammalian placenta does.

Characterising the endocrine role of the reptilian placenta is complex because viviparous reptiles exhibit a range of placental types defined by their capacity to deliver nutrients. Reptiles exhibit such variation in placentae that four overall placental Types have been defined. Most common are lecithotrophic species with a Type I placenta in which embryonic development is supported by a simple placenta and a large egg-yolk that supplies the majority of embryonic nutrients (Stewart and Thompson 2000; Thompson and Speake 2006). Less common are species with Type IV placentrophic placentae in which maternal nutrients are supplied to embryos via a complex placenta and there is minimal egg-yolk (Stewart and Thompson 2000). These two placental types are at either end of a continuum, with intermediates supporting embryos with moderately complex Types II and III placentae and moderately sized egg yolks (Thompson and Speake 2006).

Thus far, we know that experimentally increasing plasma progesterone concentrations in gestating *Sceloporus jarrovi*, a viviparous lizard with a simple Type I placenta, resulted in a tremendous increase in circulating maternal progesterone but only a moderate increase in progesterone in the embryonic circulation (Painter et al. 2002). This suggests that the placenta has considerable capacity to mediate maternal and embryonic physiology (Painter et al. 2002). Similarly, the Type II placenta of *N. metallicus* produces progesterone (Girling and Jones 2003) and can derivatise corticosterone to conjugated forms *in vitro* (Collins 2009). Thus there is some evidence that the 'reptilian placenta' does act as an endocrine organ and may 'buffer' embryos

from maternal hormones. However, the capacity of the placenta and of the embryos themselves to derivatise steroids has not been explored. I therefore set out to investigate the capacity for placentae and embryonic tissues of a viviparous lizard to biologically deactivate E_2 (Norris 2007). 17β-estradiol is at the end of the steroid metabolic pathway and is thus a product of T metabolism, it is the most potent oestrogen, it plays a critical role in gestation (Norris 2007) and is therefore likely to cause 'conflict' between mother and embryo.

I chose the metallic skink, *Niveoscincus metallicus* as my model species. *Niveoscincus metallicus,* a small (SVL ≤ 65 mm; mass 3.3g) viviparous skink, provides a suitable model for exploring the endocrine environment of the developing viviparous lizard. The reproductive physiology of this species is well understood (Jones and Swain 1996; Swain and Jones 1997; Jones and Swain 2006), and a great deal is already known about the composition of the yolk and the function of the moderately complex Type II placenta (Stewart and Thompson 1994; Swain and Jones 1997; Stewart and Thompson 2000; Swain and Jones 2000; Thompson and Speake 2006.). In this species, the ratio of neonate dry mass to egg dry mass is 0.91 and the developing embryo receives organic nutrients from both yolk and placenta (Thompson et al. 1999).

1.1 Justification for methodological approach

The experimental procedure for addressing my aims consists of two main phases: the *in vitro* tissue incubation where I aim to assess the capacity of placentae and embryonic tissues to derivitise E_2 and the chemical analysis where I aim to identify the derivatives. I chose an *in vitro* approach so that I could identify which tissues are active in modulating the effects of E_2, rather than an *in vivo* approach which would simply allow me to identify oestradiol metabolites and conjugates in maternal and embryonic circulation. I followed the *in vitro* protocol of Edwards et al. (2005) who identified patterns of peripheral E_2 metabolism in the large skink, *Tiliqua nigrolutea* and Collins (2009) who investigated placental metabolism of corticosterone in *N. metallicus*. Given the size difference between *T. nigrolutea* and *N. metallicus* (approx. 600 grams versus 4 grams), Collins (2009) successfully scaled the reagents to concentrations and volumes to accommodate for the smaller size of *N. metallicus.* The results generated by Edwards et al.

(2005) and Collins (2009) confirmed a robust and repeatable *in vitro* technique, and I therefore modified their protocol, but used E_2 rather than corticosterone.

Like many vertebrate species, *N. metallicus* has high circulating concentrations of E_2 (Jones and Swain 1996; Norris 2007), but the metabolic derivatives of E_2 were unknown. A technique with the capacity to detect multiple end-products at very low concentrations was required. Edwards et al. (2005) were able to presumptively identify some of the free steroid metabolites of E_2 by reversed-phased high-performance liquid chromatography (HPLC) with on-line radiometric detection (RD): HPLC-RD. High performance liquid chromatography with radiometric detection is a highly sensitive but limited technique: even with standards for every metabolite produced, a presumptive identification is all that can be obtained, and elution time is the only factor that can be compared between the samples and standards (Edwards et al. 2003; Edwards et al. 2005). I wanted the option of further investigating the identity of derivatives. Thus I needed a more comprehensive method for compound investigation if peak size permitted.

Gas-chromatography-mass spectrometry (GCMS) or high performance liquid chromatography mass spectrometry (HPLCMS) allow several compounds to be identified in a single sample by co-elution with standards and mass spectral analysis (Siggia and Dishman 1970; Yost and Enke 1978; Morrison 1991; Hoffman 1996), but without chemical derivatisation, sensitivity to free steroids is very low (Masse et al. 1989; Schanzer and Donike 1993; Jones and Swain 1996; Fedeniuk et al. 2004; Higashi and Shimada 2004; Jones and Bell 2004). High performance liquid chromatography requires ionic compounds for sensitive detection (Yost and Enke 1978; Harris 2000; Higashi and Shimada 2004), because steroids are typically poorly or non-ionisable (Higashi and Shimada 2004). Chemical derivatization to ionisable moieties increases detection limits (Fedeniuk et al. 2004; Higashi and Shimada 2004). The ionic steroid *conjugates* that are excreted by vertebrates (Norris 2007) can be detected in very low concentrations by HPLC-MS (Reddy et al. 2005). Conversely, steroid conjugates cannot be analysed by GC-MS without chemical or enzymatic dissociation to the free steroid (Masse et al. 1989; Schanzer and Donike 1993), which can then be derivatized to volatile compounds with sensitive detection limits (Fedeniuk et al. 2004). Considering the scope and limitations of each analytical technique, my

aim, and the small size of *N. metallicus*, HPLC-RD was the best analytical technique to use as my primary method of free steroid analysis. If I found free steroid metabolites in large enough quantities, I would then analyse the samples with HPLC-MS. I chose HPLC-MS over GC-MS because I could use the chromatography method of established by Edwards et al. (2005), and analyse conjugates without chemical derivatisation.

2 Methods

2.1 In vitro tissue incubation

2.1.1 Animal collection

Adult female lizards were captured by mealworm fishing and noosing in and around the Sandy Bay campus of the University of Tasmania: 42°54’24.9”S, 147°19’21.89”E and Old Farm Rd: 42°53’38’.33S, 147°19’21.29”E in greater Hobart, Tasmania. Lizards were co-housed in cages 200 x 600 mm overnight with pureed fruit as a food source and water *ad libitum*. The lizards were collected when embryonic development was in the early phases (embryos at stage 29-33), the middle phases (stages 34-38) and the late phases (stages 39-40+) of development (Dufaure and Hubert 1961).

2.1.2 Tissue harvesting and incubations

Adult female lizards were humanely killed by an over-dose of sodium pentobarbital at a concentration of 500 ng/g. From each adult female, both ovaries (positive control), the liver and a piece of skeletal muscle tissue from hind-limbs (negative control) were removed. The placentae were separated from the oviduct. All embryos were removed (litter sizes ranged from 1-6) from their membranes, staged (Dufaure and Hubert 1961) and dissected as follows. Due to the small size and degree of development of embryos at stages 29-36, the entire torso, containing the developing gonads, was removed for incubation. In embryos at stage 37, the gonads are not yet differentiated from the mesonephros and the developing adrenal glands. I therefore used the undifferentiated adrenal-kidney-gonad complex (AKG) as a proxy for the gonad. As I was

interested in the embryonic tissues that can reduce steroid potency, I collected liver tissue from embryos at or beyond stage 37. Embryos at stages earlier than 37 were too small to dissect; liver tissue was therefore not obtained from the early-stage embryos.

2.1.3 Incubation for radiometric detection with radio-labelled E_2

For every adult female in each of the three specified stages of embryonic development, one ovary, a portion of the liver, skeletal muscle tissue from one hind-limb (as negative control tissue not expected to have significant steroidogenic capacity), one placenta and corresponding piece of oviduct and the tissues from half of the embryos were incubated with 3 µl of 2,4,6,7 ^{3}H-E_2 250 µCi (PerikinElmer, Australia) and 3.0 ml of Leibovitz L-15 incubation medium (Sigma-Aldrich). The incubation medium was supplemented with 0.5 mM NADPH (Sigma-Aldrich) to support steroid metabolism (Painter and Moore 2005). 'Tissue free' tubes containing only media and 3 µl of 2,4,6,7 ^{3}H-E_2 250 µCi were included as a quality control measure. Total radioactivity per tube was 3,000 000 cpm (≈1 ng of E_2).

2.1.4 Incubation for mass spectral analysis with unlabelled E_2

For each adult female, one maternal ovary, half of the liver, skeletal muscle tissue from one hind-limb, and the tissues from half of the embryos were incubated with 1.0 ng of unlabelled E2 and 500.00 µl of Leibovitz L-15 incubation medium supplemented with 0.5mM NADPH. All samples were incubated at 26 ºC (the preferred body temperature of *N. metallicus* (Melville and Swain 2003)) with constant gentle rocking for 3 h. After completion of the incubation, incubation media were decanted into separate plastic vials and frozen at -20 ºC until analysis. Tissues were oven-dried, and dry weights were obtained.

2.2 Chemical analysis: method development, troubleshooting, and interpretation

My initial chemical analysis focussed on identifying any free steroid metabolites that were produced during the incubation. I therefore selected a sub-set of samples from incubation of

radiolabelled E_2 with maternal liver, ovaries and embryonic AKG (i.e. tissues deemed most likely to have produced derivatives) to analyse with HPLC-RD. I included Six tissue free samples as controls.

2.2.1 Liquid-liquid extraction and Thin Layer Chromatography – free steroid extraction and sample clean-up

Following Edwards et al. (2005), the free steroid component was extracted from incubation media by dichloromethane (DCM) liquid-liquid extraction using 1.0 ml of radiolabelled sample and 2 x 2.0 ml aliquots of DCM. The DCM extract was dried under a stream of air, resuspended in 200µl of DCM and loaded onto 20 x 20 cm Merck 0.2 mm silica gel plastic F_{254} plates (Sigma-Aldrich). The extracts were co-eluted with standards of E_2, oestrone and oestriol (Sigma-Aldrich) in AR grade chloroform and methanol (MeOH (95:5 v/v)). The plates were air-dried and sublimed with iodine crystals (Lupo Di Prisco et al. 1968) to visualise the location of the standards and the samples relative to the standards.

After visualisation, the individual fractions were isolated and loaded onto hand-made columns containing cotton wool and acid washed celite (Sigma-Aldrich). The fractions were then eluted with 2 x 10 ml aliquots of AR grade methanol, dried under a constant stream of air and re-suspended in 5 ml of methanol. A 100 µl aliquot of each fraction was then counted on a Beckman LS 5801 radiocounter. Any fraction of high radioactivity was evaporated to dryness, resuspended in 200 µl of methanol, filtered through a 0.2 µm syringe filter and assayed by HPLC-RD as described below.

2.2.2 High Performance Liquid chromatography with radiometric detection: sample analysis

Reversed phased ultra-performance liquid chromatography uses high pressure to force solvent through a column lined with tiny particles of silica bonded with any inert non-polar substance. The most widely used is the octadecyl carbon chain or C18 column. The particles that line the column make up the stationary phase (Harris 2000); the compounds of interest in the sample have a higher affinity for the stationary phase than the solvent in which they are dissolved, the mobile

phase. In reversed phase chromatography the stationary phase is nonpolar or weakly polar and the mobile phase is more polar. The mobile phase elutes compounds from the stationary phase: an isocratic (a single solvent or single mixture of solvents) mobile phase or gradient (continuous change of solvent composition to increase elution strength) can be used. The end result is high resolution separation and isolation of compounds of interest from unwanted compounds in the sample. The compounds are detected as they leave the column (e.g. with a photodiode array detector) and appear on chromatographs as peaks that occur at specific times based on their affinity to the stationary phase and the mobile phase (Harris 2000).

Samples were analysed by reversed phase liquid chromatography on a Waters Alliance 2690 liquid chromatograph attached to a Packard 500TR Series radiometric detector. The column, a Waters Nova-Pack C18 (3.9 x 150mm, 4µm particle size), was equilibrated with methanol: water 70:30 at 0.8 ml min^{-1} prior to sample injection. Samples were run for four min with the mobile phase at 70:30 MeOH: water; the mobile phase was then ramped up to 85 % MeOH for the final eight min. After the conclusion of each sample run, the column was equilibrated for six min at 70:30 MeOH: water. E2 elution time was compared with AR grade E_2 (Sigma-Aldrich), by HPLC analysis with a Waters 996 Photodiode Array Detector (resolution 1.2 nm) which recorded the UV spectra over the 190-300 nm range every second.

2.2.3 Results and interpretation: DCM extraction, TLC and HPLC

Oestradiol and three other distinct peaks occurred in every sample, including the tissue-free sample (figure 1). The occurrence of the peaks in the extracts from tissue -free and all of the tissue samples that were analysed suggests that the peaks are not metabolites that have resulted from *in vitro* incubation with E2. Iodine can associate with oestrogens on TLC plates during the sublimation process, so the unknown peaks in figure 1 may represent an association of iodine with E_2 that occurred during the sublimation process (N. Davies Pers. Comm).

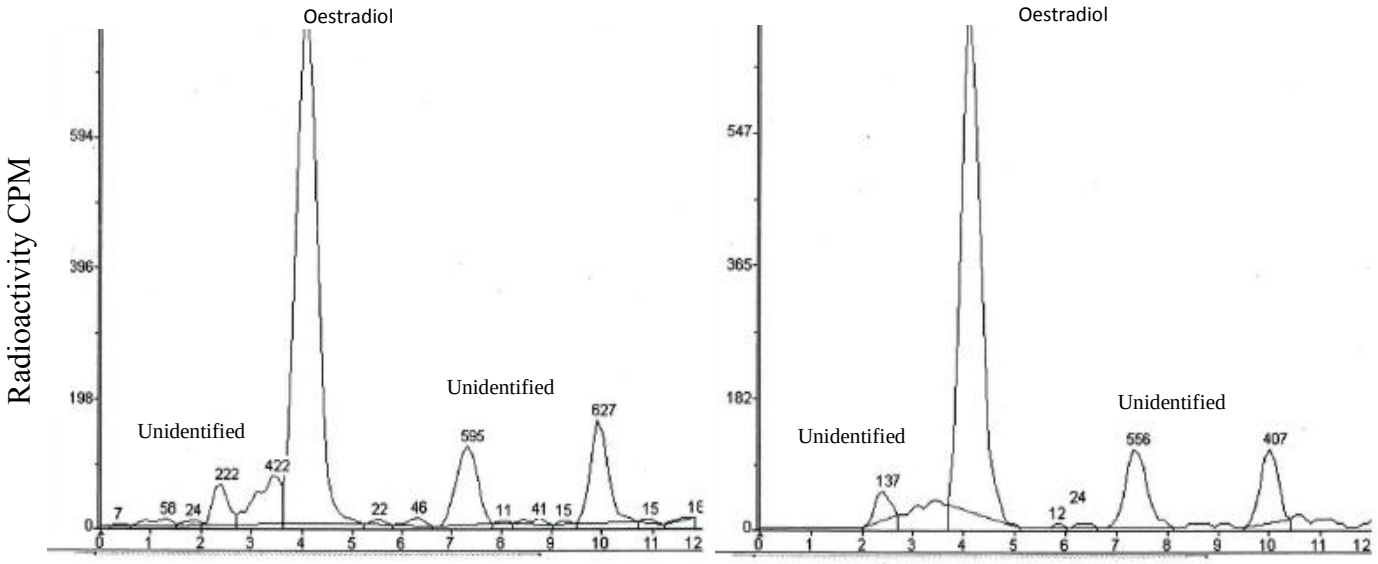

Figure 3 Chromatographs of E_2 and unknown metabolites extracted from incubation media after incubation with a) the adrenal-kidney-gonad complex (AKG) of an embryo of *Niveoscincus metallicus* at stage 39 of development, and b) tissue free incubation. Note the presence of three unidentified peaks at 3,7 and 10 min.

Typically TLC is used as a sample clean-up method. Compounds of interest can be isolated from unwanted contaminating compounds that would complicate HPLC analysis (Harris 2000). Despite the 3 large peaks that occur in all of the chromatographs in figure 1, the only other peak is the E_2 initially given to the tissues as a substrate. I decided that sample clean-up with TLC was not necessary as samples that had been extracted with DCM appeared to have very few contaminating compounds. The only observable contaminating compounds were most likely the result of iodine sublimation: I therefore removed TLC from the sample processing protocol.

I repeated the DCM extraction of the samples that I had run previously with TLC. Analysis of these same samples without TLC confirmed that the iodine was most likely the cause of the unidentified peaks (Figure 2). I then used DCM to extract all of the samples from incubations

with maternal liver, maternal skeletal muscle tissue, placenta, the torsos from embryos pre-stage 37, and AKG and liver from embryos at and beyond stage 37. I analysed these with HPLC-RD as detailed previously. I included tissue free samples to validate my results.

2.2.4 Results and interpretation: DCM extraction and HPLC

High performance liquid chromatography analysis revealed that none of the tissues had produced free steroid metabolites. Chromatographs for two of the 50 samples that were analysed are presented in Figure 2. All the chromatographs were generally 'clean' of contaminating compounds, yet there was one small peak which accounted for roughly 6 % of the total radioactivity observed at 2-3 min in the chromatographs from every sample.

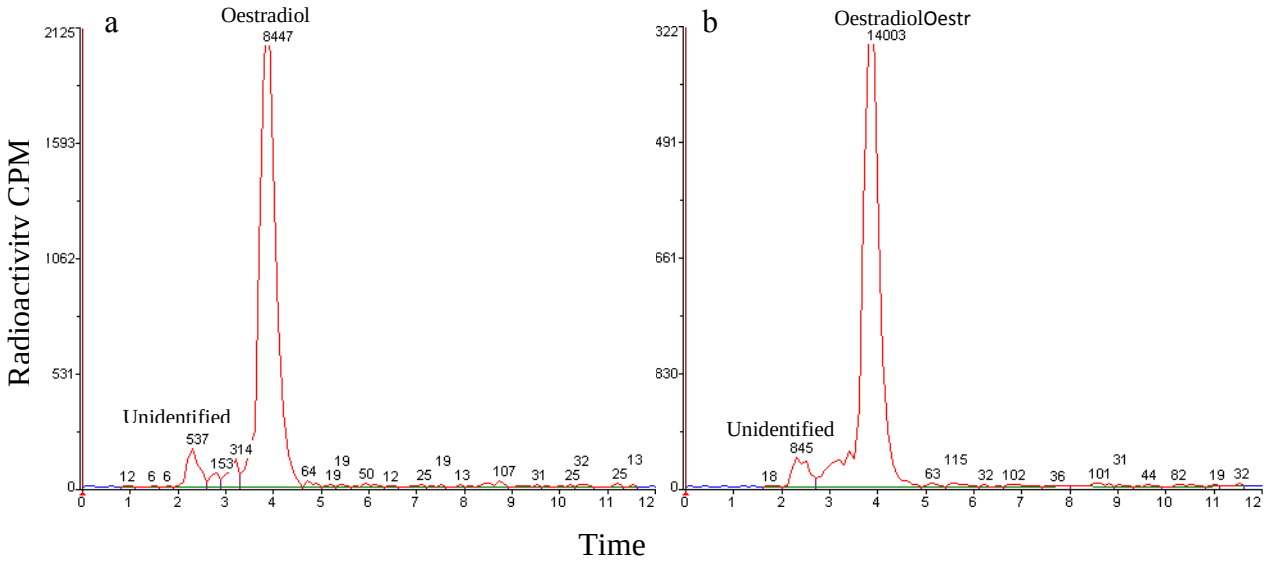

Figure 2 Chromatographs of Oestradiol and unknown compounds extracted from incubation media post incubation with a) a portion of maternal liver tissue, and b) tissue free incubation.

Dichloromethane extraction partitions the conjugates and the free steroid in the matrix. The free steroid is hydrophobic and is thus located in the DCM fraction, and any hydrophilic conjugates remain behind in the incubation medium. Finding no free metabolites of E_2 is an acceptable result because E_2 is at the end of the steroid metabolic pathway, and many vertebrate species remove free E_2 from circulation in conjugated forms only (Norris 2007). Despite this, the similarity of the

counts in the chromatographs from the tissue free samples and the liver tissue raised suspicion of the results. Even if the tissues did not produce free steroid metabolites, the liver was expected to produce steroid conjugates. If the tissues produced steroid conjugates, there should be a marked difference between the counts in the chromatographs from the liver and the tissue free incubations.

The similarity of the counts in the medium from tissue and tissue free tubes led me to trace the radioactivity of the incubation media incubated with maternal liver tissue, AKG, placentae and tissue free samples. I measured radioactivity in the incubation medium pre extraction, in the DCM post-extraction and in the incubation medium post-extraction on a Beckman LS 5801 radio counter. The tissue free samples had comparable counts to the media which had been incubated with tissues. The DCM extract contained 80 % of the radioactivity and the media post extraction contained 20 % of the radioactivity (figure 3). I assumed that the tissues were largely conjugating the E_2 (Painter and Moore 2005; Norris 2007; Paitz and Bowden 2008) so the radioactivity would represent water soluble compounds (Norris 2007; Piper et al. 2008; Piper et al. 2009) not present in the DCM extract (Edwards et al. 2003; Edwards et al. 2003; Edwards et al. 2005). Why was there no difference between the tissue free and tissue samples? There are two possible explanations: either the DCM extraction had low efficiency or none of the tissues had conjugated the E_2 during the *in vitro* incubation.

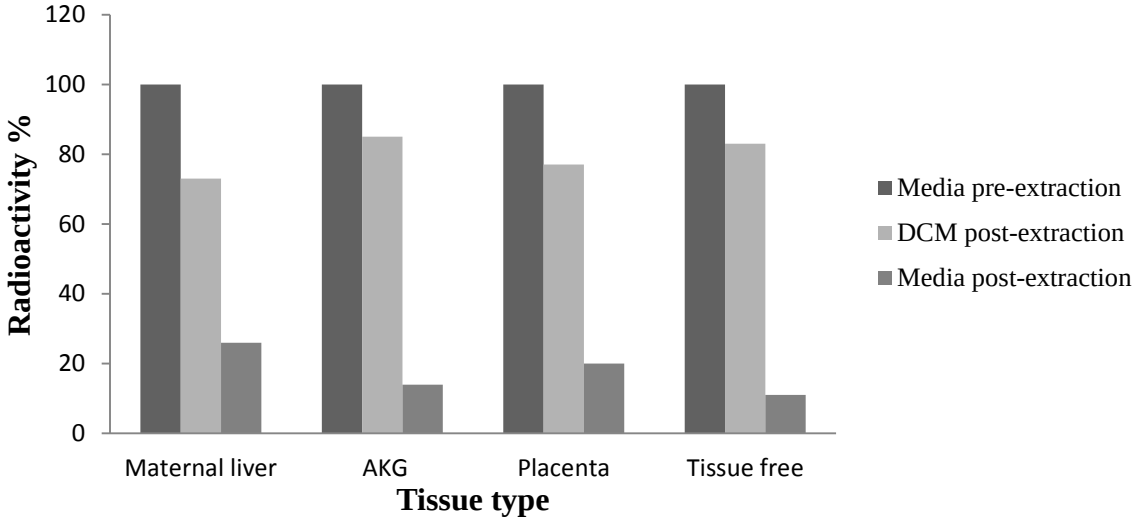

Figure 3 Percentage of initial radioactivity (added as 3 µCi 2,4,6,7 ^{3}H-E_2) in incubation media pre-DCM extraction, in the DCM extract and in incubation media post-DCM extraction.

I tested the efficiency of the DCM extraction by spiking incubation media with radiolabelled E_2. I performed the extraction as detailed previously (2.2.1) and counted the total radioactivity of the incubation media pre-extraction, the DCM extract post extraction and the incubation media post-extraction with a Beckman LS 5801 radio counter. The results indicated that 2 x 2.0 ml aliquots of DCM to 1.0 ml of incubation media extracted 80 % of the free E_2, suggesting that the remaining 20 % of the free E_2 was contributing to the counts in the incubation media. The results of the extraction efficiency were no different to the extractions that I had done on incubation media that had been incubated with the tissues. Thus, the counts that I had measured from the incubation medium previously were most likely not steroid conjugates, but were the remaining 20 % free E_2 which remained in the medium post DCM extraction.

2.2.4.1 Introduction of new analytical equipment

At this point of my investigation, I gained access to an ultra-performance liquid chromatograph (UPLC) with a triple quadrupole tandem mass spectrometer TQP MSMS. Although the extraction efficiency tests (see above) suggested that none of the tissues had conjugated the E_2, ,

it was still possible that there were small undetected concentrations of conjugates in the incubation media. I decided to re-analyse the samples I had incubated with unlabelled E_2 using the UPLC-MSMS.

A quadrupole mass spectrometer is made up of 4 metal rods connected electrically. A radiofrequency (RF) is applied between each of the two pairs of rods and a current voltage (CV) is applied over the RF (Hofman 2003). The quadrupole separates ions based on their mass and charge. Each ion has a unique mass to charge ratio (*m/z*) (Hofman 2003). A triple quadrupole mass spectrometer is a tandem mass spectrometer and consists of three quadrupoles in a line $Q_1q_2Q_3$. The first and third quadrupoles are mass filters whilst the second is used as a collision cell (Yost and Enke 1978; Morrison 1991). The collision cell uses RF and a desolvation gas such as nitrogen for collision induced dissociation (CID) of parent ions into new fragments or daughter ions (Yost and Enke 1978; Morrison 1991). By changing the applied voltages in the quadrupole, ions of different masses are selected to reach the detector (Harris 2000). The first and third quadrupoles can be set to a selected mass allowing only one daughter ion to be detected. This is called selective ion monitoring (SIM) and is a very targeted and sensitive mode. A scan of several daughter ions can be obtained by operating the instrument in multiple reaction monitoring (MRM) mode by setting Q_1 and Q_3 to more than one mass (Anderson and Hunter 2006). A compound can be identified by the daughter compound/s that are produced (Harris 2000).

The advantage of LCMS/MS is the potential for identification of compounds by both co-elution with known standards, and detection of daughter ions (Siggia and Dishman 1970; Yost and Enke 1978; Morrison 1991; Hoffman 1996). For my study, then, the advantage of the newly available LCMSMS was the potential of identifying conjugates as sulphate or glucuronic acid moieties (Norris 2007). The disadvantage of using LCMS/MS is the low sensitivity to free E_2. The mass analyser detects ions (Yost and Enke 1978; Morrison 1991; Harris 2000; Hofman 2003; Higashi and Shimada 2004; Anderson and Hunter 2006) and steroids are not ionic (Yost and Enke 1978; Morrison 1991; Harris 2000; Hofman 2003; Higashi and Shimada 2004; Anderson and Hunter 2006; Norris 2007). The detection limit for E_2 with UPLC with triple quadrupole MS is in the mg range (N. Davies Pers. Comm) which is far above circulating concentrations of E_2 in adult female

N. metallicus (Jones and Swain 1996). However, the ionic E_2 conjugates can be detected in concentrations as low as 1 pg and 100 fg for E_2 3 glucuronide and E_2 3 sulphate respectively (N. Davies Pers. Comm).

Contaminating compounds are less of as issue when using radiometric detection because the only compounds that are detected are radioactive, and thus have come from the initial substrate given to the tissues to derivatise. The mass spectrometer, however, identifies all compounds that are eluted from the HPLC column. I therefore had to develop a sample clean up method and determine the extraction efficiency of the E_2 conjugates. I chose to use Waters Sep-Pak C18 solid reversed phase extraction columns as they are effective in separating steroids from unwanted products (Supelco 1998; Harris 2000).

2.2.5 Solid phase extraction and sample clean-up

Free and conjugated E2 were isolated from other unwanted compounds in the incubation media by reversed solid-phase extraction on Sepak C18 columns. Columns were primed by running one column volume of AR grade Methanol followed by 3 column volumes of Milli Q water. Five hundred µl of incubation media was loaded onto the column and eluted with 3 column volumes of Milli Q water. The columns were then eluted with 3 column volumes of AR grade methanol. All extracts were evaporated to dryness in a Labconco Centrivap concentrator attached to a Dynavac condenser and Edwards vacuum pump. Samples were then resuspended in 200 µl of Milli Q water and centrifuged at 10,000 x *g* to remove particulates ≤0.2 µm. The supernatant was removed, placed into HPLC vials with 200 µl inserts and frozen at -20 ºC until analysis as described below. The C18 extraction method was validated with unlabelled E2 and the deuterated E_2 conjugate standards, sodium 17β-E2 16,16,17 ^{2}H 3-glucuronide and sodium 17β-E2 2,4,16,16 ^{2}H 3-sulphate stabilised with tris (CDN Isotopes, Canada). The C18 column yielded a 97 % recovery of free E_2 and 98 % recovery of both E_2 conjugate standards. The free E_2 in the extracted samples acts as an ‘internal standard’.

2.2.6 Ultra performance liquid chromatography with triple quadrupole mass spectrometry: sample analysis

The chromatography conditions were revised from those detailed previously (2.2.2) to include the free E_2 and the E_2 conjugates. Twenty five microlitre aliquots of the concentrated samples were injected using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 x 100 mm x 1.7 micron particles) was used, with mobile phases A= 5mM ammonium acetate at pH 5.4 and B= acetonitrile. The flow rate was 0.45 mL/min, with 90 % A 10 % B for 0.3 minutes, then a linear gradient to 60 %A and 40 % B at 5 minutes, then to 30 % A 70 % B at 8 minutes before immediate re-equilibration to initial conditions for 3 minutes.

Earlier results led me to anticipate that if any conjugates had been produced in my samples, then, they would be present in very low concentrations. I therefore chose a column method that was very sensitive and would allow detection of as many different types of conjugates as possible. To maximise detection sensitivity, the MS was operated in negative ion electrospray in selective ion monitoring (SIM) and multiple reaction monitoring (MRM) mode. Ions monitored by SIM were: E_2 disulphate 215.10, E_2 sulphate 351.3 and ^{2}H-E_2-3-sulphate 355.3 all with 0.087 sec dwell and cone voltage of 50.0, E_2 glucuronide 447.3 and E_2 ^{2}H-E_2 -3-glucuronide 450.3 with 0.087 sec dwell and cone voltage of 38.0. The ions monitored by MRM were: E_2 sulphate with a channel reaction of 351.3>97.00 and collision energy of 28.0 volts, E_2 sulphate with channel reaction 351.3>271.3 and collision energy of 34.0 volts ^{2}H-E2-3-sulphate 355.3>275.3 with collision energy of 34.0 volts; all with a 0.087 dwell and cone voltage of 50.0, E_2 glucuronide with channel reaction 447.3>271.1 and collision energy of 38.0 volts, and ^{2}H-E_2 -3-glucuronide with channel reaction 450.3>274.10 and collision energy of 38.0 volts. Both ions were set at at 0.087 sec and cone voltage of 36.0. The ion source temperature was 150°C, the desolvation gas was nitrogen at 1000 L/hr, the desolvation temperature was 350°C and the capillary voltage was 2.8 KV.

2.2.7 Results and interpretation: UPLC-MSMS

The revised chomatography conditions adjusted E_2 elution time from 4.5 min to 7.5 min. The expected E_2 conjugates should have occurred anywhere between 1-7 min. Initial LCMS revealed a small yet distinct peak (Figure 3 c) at 3.55 min. The elution time was close to that of the deuterated 3-E_2 glucuronide (figure 3 b): this could be the E_2 7-glucuronide isomer (Davies Pers. Comm), as there was no peak like this detected in incubation media. However, the peak was too small for MS/MS daughter scan analysis so I was unable to confirm that the peak was the E_2 7-glucuronide. Analysis HOW?? of the solution of unlabelled E_2 that I used as a substrate for the tissues indicated that the E_2 had somehow degraded . There was a possibility that the E_2 had degraded in the incubation media that had been incubated with tissue samples. I therefore abandoned MSMS analysis and switched to radiocounting because of the increased sensitivity.

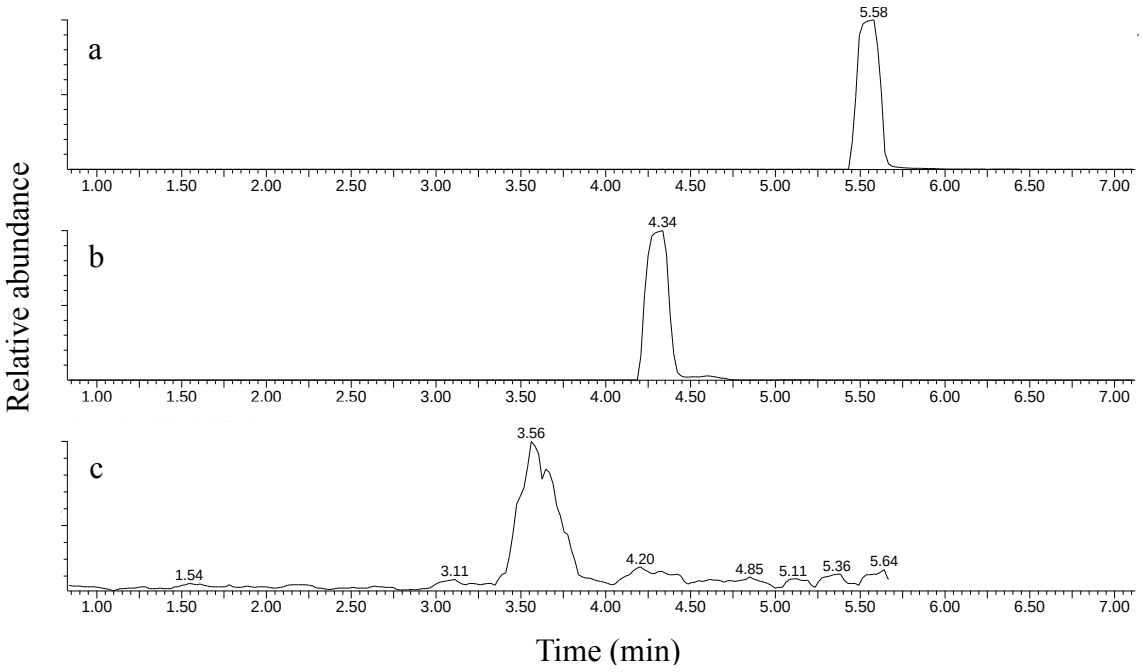

Figure 4 Chromatographs from a) deuterated oestradiol 3-sulphate standard at relative abundance 7.58 x10^{5}, b) deuterated oestradiol 3- glucuronide standard at relative abundance 7.45 x10^{4} and c) chromatograph of an unknown peak from an adult ovary incubated with unlabelled oestradiol at relative abundance 255.

2.2.8 Ultra performance liquid chromatography with radiometric detection: sample analysis

The interchangeablity of the radiometric detector and the mass spectrometer to the UPLC meant that I did not need to modify the chromatography conditions to switch to radiometric detection. I did, however, need to change the C18 extraction method by using 1.0 ml of incubation media rather than 500 µl as detailed in previously (2.2.5). I analysed all of the incubation media from placentae, AKG, Embryonic liver, oviducts, maternal liver and skeletal muscle tissue.

2.2.9 Resultsand interptretation: UPLC-RD

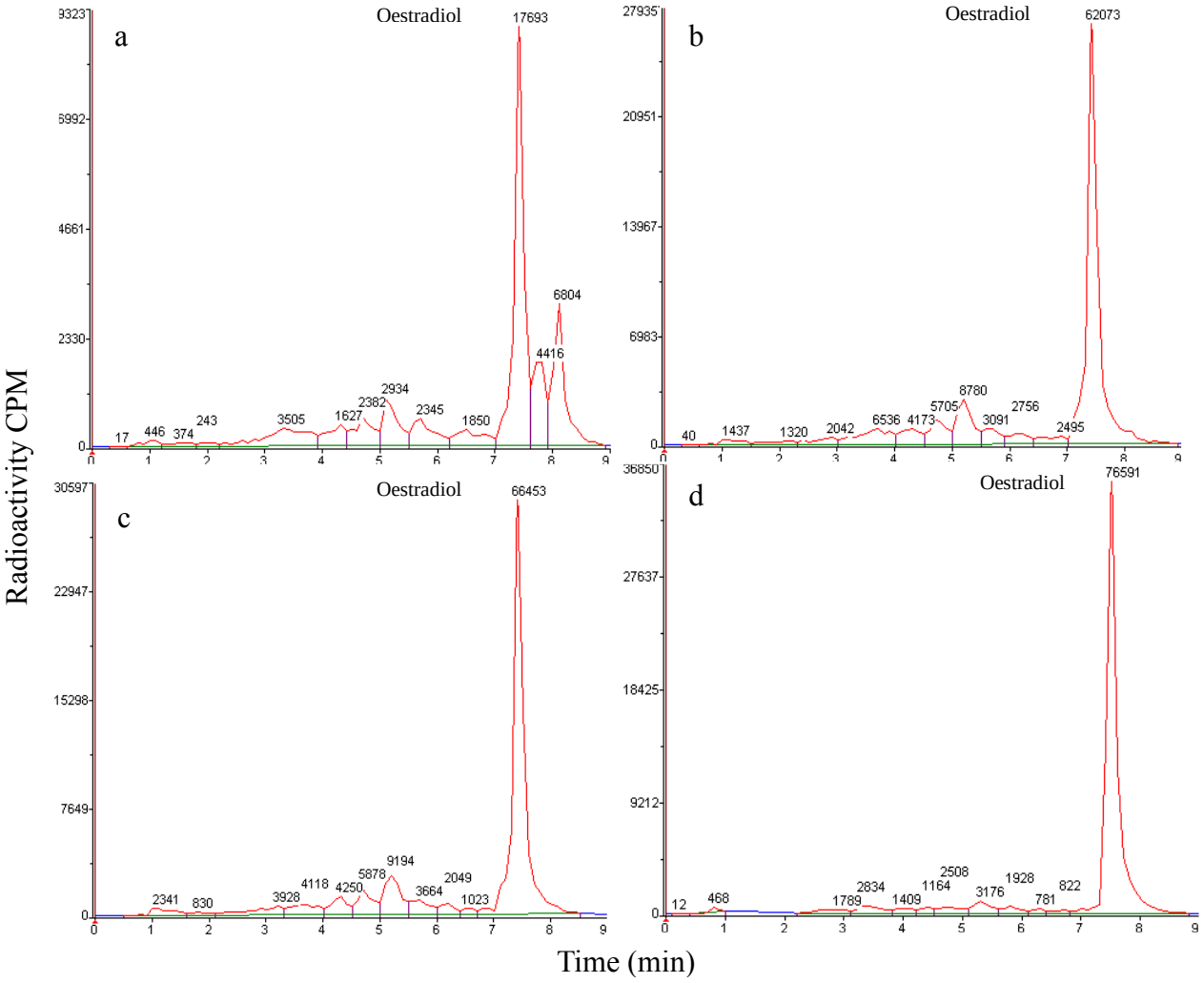

Figure 5 Chromatographs of oestradiol and unknown compounds extracted from a) incubation with a placenta from an embryo at stage 39 of development, b) incubation with a portion of the liver of an adult lizard, c) tissue free incubation and d) radiolabelled oestradiol that was not incubated.

Although the initial LC/MS suggested the potential presence of the 7 glucuronide (Figure 5 c: unidentified peak at 3.55 min), the radiometric detection did not support these findings as there were no clear peaks close to 3.55 min. The chromatographs in figure 5 clearly illustrated several small peaks in the media incubated with placental and liver tissue samples. The largest peak (figure 5a and b) corresponded to the elution time of E_2 3 sulphate (figure 5a); however, these peaks occured in all media including the radiolabelled E_2 standard, albeit lower than the peaks in the medium (figure 5 c and d). The unidentified peaks in the tissue free samples had higher counts than the samples incubated with liver and placental tissues; I was therefore unable to 'blank subtract' and correct for background noise. The presence of two large peaks 8 and 8.5 min in medium incubated with placental tissue indicates some derivatisation of E_2. The chromatography conditions under which these peaks occured indicate compounds more polar than E_2 and not the water soluble conjugtaes. The peaks occurred in all media incubated with placental tissue. The initial analysis of free steroid hormones (figure 1) did not indicate any free steroid metabolism; however the revised chromatography conditions increased the resolution of the compounds in the medium, and thus allowed detection of the two peaks described above.

Incubation media is a good sample matrix for solid phase extraction, but in some cases the analyte of interest is not freely solvated in the sample. Thus the analyte of interest is not completely free to bind to the SPE column (Supelco 1998; Harris 2000). Dilution of the incubation media in deionised water or buffer is sometimes necessary to ensure that the analyte binds to the column and is eluted successfully for analysis (Supelco 1998). Diluting the incubation media in deionised water had no effect in this case. Maternal liver samples were diluted in deionised water and run as previously described. As before, no water soluble conjugates were observed.

The results of the chemical analyses clearly indicate that the E_2 has not been derivatised to the biologcally inactive conjugted forms. Interestingly, the tissue free samples appeared to contain more new products than the tissue samples themselves. Why are the counts in the peaks of the tissue-free samples so high? Does the incubation process increase the proportion of the unidentified compounds present in the radiolabelled E_2? In order to find out if the E_2 was

associating with components of the incubation medium, I performed incubations as I had done previously with unlabelled E_2 in deionised water and Lebrovitz L15 incubation medium and then analysed the media with MSMS.

2.2.10 Results and interpretation: tissue free incubations with unlabelled E_2

An unidentified peak 355 at 4.07 min (Figure 5a) only occurred in *media* that had been incubated with E_2. This peak was not observed in incubations from E_2 and milli Q water. The largest peaks observed after the radiolabelled incubations were at 5-5.5 min, and there were several smaller peaks that were not seen in the incubations with the unlabelled E2. The MRM scan of peak 355 (figure 5b) revealed daughters with a mass to charge ratio of 156, 195, 197, 261 and 262. These daughter compounds are an indication that 355 does not relate to E_2. Figure 6b is an MRM daughter scan of E_2 under the same conditions. When comparing the two MRM scans it is obvious that 355 has no daughters that relate to E_2. We would expect to see at least *some* 271 (E_2) or 253 (dehydrated E_2) (Davies Pers. Comm), which is an interesting result considering the occurrence of peak 355 in media incubated with E_2 only.

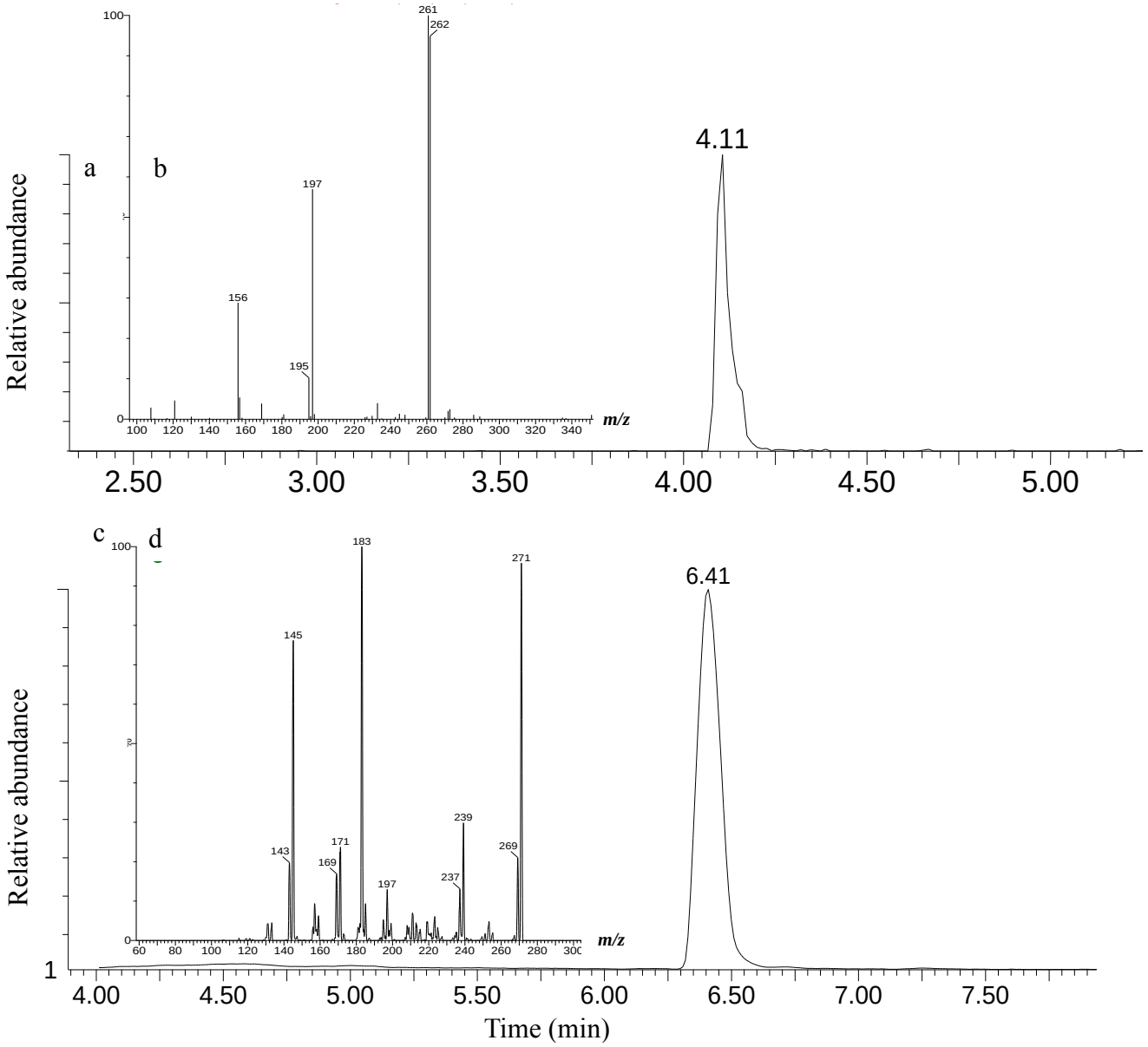

Figure 6 a) chromatograph and b) mass spectrum of unidentified peak from incubation of Lebrovitz L15 media with oestradiol and c) chromatograph and d) MRM mass spectrum of oestradiol. The mass spectrometer was operated in MRM mode.

The incubations with the unlabelled E_2 did not bring us any closer to understanding why tissue free samples have larger peaks than the tissue samples. This final incubation procedure suggested that there may be chemical processes occurring during the incubation that do not relate to E_2. It could be that the *impurities* in the tritiated E_2 associate with the incubation media. If I were to repeat the experiment, I would purify the radiolabelled E_2 prior to incubation by running the purchased product on a reversed phase HPLC column under the revised chromatography conditions identified here and isolating the compound of interest, E_2 for future experimentation (N. Davies Pers. Comm).

3 Discussion

The aim of this experiment was to assess the capacity of the placenta to 'buffer' embryos from maternal E_2 and to identify potential mechanisms that embryos may use to reduce steroid potency. I also aimed to characterise the modes of E_2 derivatisation from the potent free steroid to less potent free steroid derivatives and the conjugates.

Comprehensive chromatographical analysis revealed that, aside from placental tissue, none of the tissues, including the maternal liver and ovaries, which were included as a positive control, derivatised E_2 to any compound. The placental tissue was able to metabolise free E_2 to two compounds that are more polar than E_2. These (unknown) compounds were revealed only after modification to existing HPLC column conditions (Edwards et al. 2005) which increased the resolution and thus the detectability of the two peaks. The retention time and revised column conditions indicated that these two unidentified peaks could be lipophilic conjugates or polar derivatives of E_2 (Davies Pers. Comm). The identity of these two peaks was not confirmed, but the possibility of a metabolite that is less potent than E_2, or lipophilic conjugate of E_2 should not be completely discounted without further investigation.

The results from other analyses are confusing and somewhat contradictory. Liquid chromatography tandem mass spectrometry showed small peaks of an unidentified compound, potentially the E2-7-glucuronide isomer, in media from incubation of embryonic AKG, maternal liver and maternal ovaries (N. Davies Pers. Comm). Further analysis with HPLC-RD

contradicted these findings with the observation of peaks initially thought to be those of E_2 3-sulphate. It was later discovered that these peaks also occurred in the tissue free samples and the radiolabelled E_2 standard. Thus it seems that tissue free incubations with unlabelled E_2 resulted in the production of metabolites which appeared in the analysis as peaks that turned out to be unrelated to E_2.

In vertebrates, steroids are primarily excreted as hydrophilic conjugates (Norris 2007). Why were there no conjugates produced in this incubation system? It is possible that in *N. meallicus* the placenta and the embryonic AKG do not possess the enzymes necessary for steroid conjugation although the placentae and embryonic tissues of other reptilian species are able to do so (Painter et al. 2002; Painter and Moore 2005; Paitz and Bowden 2008; 2009). While the livers of the developing embryos may not possess the enzymes necessary to conjugate E_2, it is highly unlikely that maternal liver is unable to conjugate E_2: conjugatiopn of steroid hormones prior to excretion is a major role of the liver (Ternes et al. 1999; Edwards et al. 2005; Norris 2007).

A possible explanation for these results is that the liver tissue was damaged by pentobarbital, the anaesthetic used to kill the lizards (as approved by the UTAS Animal Ethics Committee) . Pentobarbital can cause tissue necrosis (J. Whittier Pers. Comm), and is metabolised by liver cells (Knodell et al. 1980). It could be possible that the pentobarbital has resulted in liver tissue necrosis and therefore inactivity: this could also explain the null results for the other tissues. However, I have used this same method of killing lizards elsewhere without compromising tissue productivity (chapter 3), and Collins (2009, (who demonstrated the capacity of the placenta to conjugate corticosterone *in vitro)* also used this method of humane sacrifice.

Another possible explanation is that I provided too much substrate for the tissues; although E_2 was reduced to accommodate for the small size of *N. metallicus.* Edwards et al. (2005) used 5 µCi of tritated E_2 for every 200 mg of tissue. But, the average wet weight of the placenta of *N. metallicus* mid-gestation is approximately 20 mg. Edwards et al. (2005) dosed 0.025 µCi/mg of tissue. If I were to accurately scale that to *N. metallicus* I should have incubated with 0.5 instead of 3 µCi of tritated E_2. The tissues may have been ‘over exposed’ to the E_2 Substrate. In their

description of E_2 receptor occupancy, Welshons et al. (2003) demonstrate that at low concentrations of E_2, a tenfold increase in E_2 can have a dramatic increase in receptor binding, whereas at high concentrations of E_2 an increase in the same order of magnitude can have very little effect on receptor binding (Welshons et al. 2003). This phenomenon occurs at concentrations above the dissociation for receptor-ligand binding kinetics (Welshons et al. 2003). However, 'over exposing' tissues to the E_2 substrate should result in *some* conjugation of E_2.

Despite the rigorous attention to experimental protocol and problem-solving, the results obtained from the tissue incubations do not allow me to address the original aims of this experiment. If the experiment were to be repeated, purified E_2 should be used and the incubation procedure should be modified by varying the amount of substrate and incubation times. The method of killing the lizards should also be investigated as a possible explanation for the null result. The duration of the chromatography 'runs' should be extended and the polar peaks identified from incubation with placental tissues should be investigated and identified where possible. The capacity of maternal and embryonic tissues of the viviparous lizard *N. metallicus* to buffer embryos from exogenous hormones remains to be resolved.

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