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# Patterns of peripheral steroid metabolism vary with sex, season, and tissue type in blotched blue-tongued lizards (*Tiliqua nigrolutea*)

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**Abstract**

We examined sexual and seasonal variation in the ability of reproductively relevant tissues (liver, skin, adrenal gland, cloaca, kidney, renal sexual segment, epididymis, oviduct, muscle, testis, and ovary) to metabolise a primary steroid [testosterone (T) or estradiol (E2)] in the scincid lizard, *Tiliqua nigrolutea*. We observed considerable variation between sexes and across seasons in the patterns of conjugation and derivatisation of the primary steroids by these tissues. All tissues demonstrated the ability to conjugate the relevant primary steroid. Other general trends included increased conjugation by all tissues of gestating females, reduced metabolism of E2 by female tissues during late vitellogenesis, and reduced metabolism of T by males during early spermatogenesis. 5α-Dihydrotestosterone was the most commonly detected derivative in males, and production varied with season and tissue type. We suggest that seasonal variation in the ability of reproductively relevant tissues may be important in the physiological regulation of reproduction in this species.
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## 1. Introduction

Following biosynthesis in the vertebrate gonad the primary steroids, testosterone (T) and 17β-estradiol (E2), are released into the circulatory system, via which they travel to peripheral (extragonadal) target tissues (Ozon, 1972a,b) to induce a biological effect (Evans, 1988). Many studies of steroid hormones in vertebrates have, therefore, measured concentrations of these hormones in plasma, and considered their correlations with physiological changes. However, this approach, while useful, overlooks a key factor in steroid biochemistry and action: while a primary steroid may bind directly with a specific cytoplasmic or nuclear receptor within a target cell, alternatively, it may first be modified through further metabolism (Kime, 1987).

The mode of modification depends on the specific enzymes located within the cells of the target tissues (Baker, 2004). These modifications are usually conserved within a tissue type, within a particular vertebrate class, or both (Kime, 1987). Peripheral metabolism of T and E2 may involve either derivatisation or conjugation. In mammals, derivatisation, such as the reduction of T to 5α-dihydrotestosterone (5α-DHT), or aromatization of T to E2, usually results in the formation of a more biologically active steroid with greater affinity for its specific receptor (Johnson and Everitt, 1988). Although in non-mammalian vertebrates it has generally been assumed that T or E2, rather than their metabolites, are responsible for the induction of observed physiological changes, links between primary gonadal steroids and their putative effects on peripheral tissues are often inferred rather

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than proven. For example, hemipenal tissue in *Lacerta vivipara* is insensitive to T in adult animals (Dufaure and Chambon, 1978), but hemipenal hypertrophy in *Calotes versicolor* is regulated by androgens (Ananthalakshmi et al., 1991). Different metabolites of testosterone may therefore act upon this tissue in different species.

A steroid may be conjugated to either a sulphate or a glucuronic acid moiety. In mammals, conjugation occurs most extensively in the liver to deactivate and solubilise steroids (Kime, 1987; Norris, 1997) for excretion in faeces or urine (Heistermann et al., 1993; Velloso et al., 1998; Wasser et al., 1996). Non-mammalian vertebrates also conjugate some steroids. In teleosts, for example, the role of conjugated steroids as pheromones has been well documented (Scott and Vermeirssen, 1994; Stacey and Sorensen, 1986; Stacey et al., 1994). In male *T. nigrolutea*, conjugated T forms the majority (80%) of detectable T in faeces during spring (Atkins et al., 2002), but similar studies have not been undertaken using females.

In vitro derivatisation and/or conjugation of steroids have, indeed, been observed in a wide variety of vertebrate body tissues including muscle (birds: Fennell and Scanes, 1992a,b), skin (fish: Pottinger and Pickering, 1985), brain (birds: Schlinger et al., 1989; Schlinger and Callard, 1990; reptiles: Callard et al., 1977), gonads and/or fat bodies (lungfish: Joss et al., 1996; amphibians: Lupo Di Prisco et al., 1971, 1972; mammals: Folman et al., 1973), blood (fish: Schulz, 1986; mammals: Milewich et al., 1982), and liver (fish: Kime and Saksena, 1980; Snowberger and Stegeman, 1987; mammals: Payne, 1980). Moreover, in vitro and in vivo studies of vertebrate steroid metabolism have demonstrated variations in the patterns of production of conjugates and derivatives according to the reproductive condition of the animal (Borg et al., 1992; Kime, 1987; Ozon and Fouchet, 1972; Schlinger et al., 1989). In ectothermic vertebrates, such variations may, at least partly, be mediated by the effects of temperature on enzymic activity (Kime, 1979; Kime and Hyder, 1983; Lofts, 1987; Manning and Kime, 1985).

Very few studies, to our knowledge, acknowledge the significance of variation in the patterns of peripheral steroid metabolism with sex and reproductive condition in a reptile (e.g., Ananthalakshmi et al., 1991; Dufaure and Chambon, 1978), and none of those studies have investigated a broad spectrum of relevant tissues. The current study examines the metabolism of a primary steroid (T or E2 in males and females, respectively) in a range of reproductively relevant tissues in the viviparous, cool temperate-zone lizard *Tiliqua nigrolutea*. A large body of information is already available about endocrine aspects of reproduction in this species, including plasma steroid concentrations (Edwards and Jones, 2001a,b); patterns of reproduction (Edwards et al., 2002a); and patterns of gonadal steroid biosynthesis: steroid biosynthetic pathway preference, the extent of steroid conjugation by the gonads and the end-products of biosynthesis all vary with sex and season in *T. nigrolutea* (Edwards et al., 2002b, 2003). The present study considers variations in patterns of steroid metabolism between sexes and reproductive states.

## 2. Materials and methods

### 2.1. Incubations

Animals were maintained as previously described (Edwards and Jones, 2001a,b). Lizards were killed by ketamine injection [0.4 ml im; (1 ml $kg^{-1}$)] and simultaneous inhalation of Halothane gas, in accordance with ANZCCART recommendations for reptile euthanasia (ANZCCART, 1993). All procedures were approved by the University of Tasmania Animal Ethics Committee (Approval numbers 95046 and 98015). Peripheral tissues (see below) were collected from male and female *T. nigrolutea* at autopsy of freshly killed individuals between April 1995 and February 1996. Two male and two or four female lizards were sampled at each of three times of year that corresponded to distinct phases of the annual reproductive cycle (summarised in Table 1). Tissues collected for incubation were skin (lateral abdominal body surface), muscle (abdominal wall), liver, cloaca (surrounding the cloacal opening), adrenal gland, kidney, ovary (including corpora atretica (CA) in mid April and corpora lutea (CL) in mid February), oviduct, testis, epididymis, and sexual segment of the kidney (SS) (males only). Kidney tissue was not collected from early spermatogenic-stage males in autumn. Oviductal tissue was collected in gestating females from regions adjacent to developing embryos, and from a similar position in postparturient and non-virginal preovulatory animals (as determined by palpating females of known reproductive histories). In male *T. nigrolutea*, the sexual segment of the kidney is not clearly distinguishable during the reproductive season, but in males of the sympatric species, *Niveo-*

Table 1
Sampling regime for collection of peripheral tissues from male and female *Tiliqua nigrolutea* for in vitro incubation

| | Male | Female |
|---|---|---|
| Reproductive stage (Autumn) | Regressed testes, ES mid April ($N = 2$) | Regressed ovaries, PP mid April ($N = 4$) |
| Reproductive stage (Spring) | Hypertrophied testes, LS mid September ($N = 2$) | Hypertrophied ovaries, LV early October ($N = 2$) |
| Reproductive stage (Summer) | Regressed testes, Q late December ($N = 2$) | Regressed ovaries, G mid February ($N = 2$) |

ES, early spermatogenic; LS, late spermatogenic; Q, reproductive and gonadal quiescence; PP, post parturient; LV, late vitellogenic; and G, late gestation.

*scincus metallicus*, the SS is clearly identifiable (by hypertrophy and colour change) during the autumn mating period as the anterior third of each kidney (Edwards and Jones, pers. obs.). The corresponding region was selected in males of *T. nigrolutea*. Skeletal muscle was used as non-reproductively relevant control tissue.

Although not peripheral tissue, gonadal tissue from each sex was included, because of the potential for post-biosynthetic modification of primary gonadal steroids (Cuevas et al., 1992; Joss et al., 1996; Scott and Vermeirssen, 1994). Yolk was expressed from all vitellogenic follicles, and follicles were rinsed with incubation medium prior to incubation.

All tissues from each animal were incubated separately. At autopsy, samples of each tissue type (200 mg, with the exception of adrenal tissue: as adrenal glands were small and mass varied between 50 and 100 mg, each incubation comprised a single minced gland to allow meaningful comparisons) were collected and minced finely with scissors. Duplicates of tissues were prepared whenever sufficient material was available. Minced tissues were added to individual flasks each containing 5 ml of Hepes-buffered Leibovitz culture medium (pH 7.6). Tissues from males and females were provided with [$^3$H]T (5 μCi) or [$^3$H]E2 (5 μCi), respectively, as substrate. Each flask was held on ice until all flasks were prepared. Samples were then incubated for 180 min at 35 °C in an air environment in a gently rocking waterbath. The incubation temperature reflected the preferred body temperature of this species (34.8 °C: Rawlinson, 1974). Incubations were terminated by rapid freezing, and samples were stored at −20 °C until analysis.

Free (non-conjugated) steroids were later extracted from thawed incubation media in 2 × 10 ml of dichloromethane (DCM). These DCM washes were combined and stored at −20 °C until further analysis as described below. A 100 μl aliquot of the original medium from each incubation was then assayed for radioactivity in 3 ml scintillation cocktail. This allowed the determination of the proportion of tritiated substrate that became conjugated during the incubation.

### 2.2. Thin layer chromatography

Steroids synthesized from [$^3$H]T or [$^3$H]E2 were first quantified and identified tentatively using sequential thin layer chromatography (TLC) solvent systems, as previously described in detail (Edwards et al., 2002b, 2003). Any regions of radioactivity that were located behind the most polar standard steroid (E2) in the TLC system used were grouped as polar steroids for further analysis. Free steroids were defined as those that were not conjugated to a glucuronic acid or sulphate moiety and which were, therefore, successfully extracted from the aqueous incubation medium into DCM following the incubations. This proportion was also calculated as a percentage of the original tritiated substrate. The proportion of substrate that was derivatised in the incubations of each tissue includes all products of the conversion of either [$^3$H]T or [$^3$H]E2 to other free steroids. This was also calculated as a percentage of the total original tritiated substrate.

### 2.3. High-performance liquid chromatography with radiometric detection

Steroids isolated using TLC were next presumptively identified using high-performance liquid chromatography (HPLC) with on-line radiometric detection as described in detail in Edwards et al., 2003, 2002b. Those steroids for which radioactive peaks coeluted with authentic standards using TLC were regarded as presumptively identified if the HPLC elution time (±1 SE) also corresponded to that of the authentic standard. The unmetabolised [$^3$H]T or [$^3$H]E2 recovered from each incubation served as a positive control. The chemical properties of 5α-DHT in our TLC and HPLC systems [TLC $R_f$ in DCM:DEE (5:2 v/v = 0.60), HPLC retention time = 6.400 ± 0.03 ($N$ = 15)] were established using these techniques; the characteristics of other steroids used (i.e., pregnenolone, progesterone, dehydroepiandrosterone, testosterone, androstendione, 17β-estradiol, estriol, 6α-, and 6β-hydroxyestradiol) have been previously published (Edwards et al., 2002b, 2003). Given the extensive suite of free steroid metabolites, particularly polar steroids, and the very small amounts of the individual steroids produced in incubations of some tissues, it was not possible to identify every metabolite. Instead, the relative proportions of conjugates and derivatives produced, and of the substrate remaining after incubation, were used as indicators of the overall activity of each tissue. As in Edwards et al. (2003), results were not analysed statistically due to small sample sizes.

## 3. Results

### 3.1. Liver

There was no distinct seasonal or sexual variation in patterns of steroid ([$^3$H]T, males; [$^3$H]E2, females) metabolism by the liver. In all incubations, the majority (mean 75.2%, range 63.4–93.2%) of the tritiated substrate became conjugated, with only small proportions of tritiated steroids remaining as substrate (mean 14.5%, range 3.8–25.5%) or converted to free derivatives (mean 10.3%, range 3.1–16.0%) (Fig. 1). The derivatised steroid fraction was comprised of several highly polar metabolites. Androstenedione was detected in incubation media from all three incubations with [$^3$H]T, while 5α-DHT was produced liver from early spermatogenic-stage males. Several polar metabolites were detected, but no E2 was produced as a metabolite of [$^3$H]T in any incubation with liver.

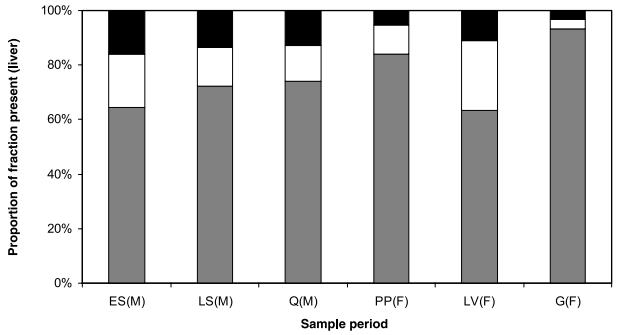


Fig. 1. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars), or remained unmetabolised (white bars) following 180 min of in vitro incubation with liver tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post-parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

### 3.2. Skin

Incubations of skin with tritiated substrate (Fig. 2) resulted in the detection in the media of similar proportions of derivatised, conjugated, and unmetabolised steroids to those observed using muscle tissue (control) (see below). Only 22.1% (range 16.2–27.1%) of the substrate became derivatised in any sample: the derivatised fractions included small amounts of 5α-DHT in all incubations of male skin. A range of polar metabolites was produced in all incubations of skin from both sexes. With the exception of skin from gestating females, 31.4% (range 25.1–35.4%) of the original substrate was conjugated and 46.3% (range 38.6–58.7%) remained unmetabolised. Skin collected from gestating females produced a greater proportion (68.9%) of conjugated steroids with correspondingly lower proportions of unmetabolised (10.6%) substrate and derivatised products (20.8%) present. No E2 or AD was identified in medium from any incubation in which [$^3$H]T was the substrate.

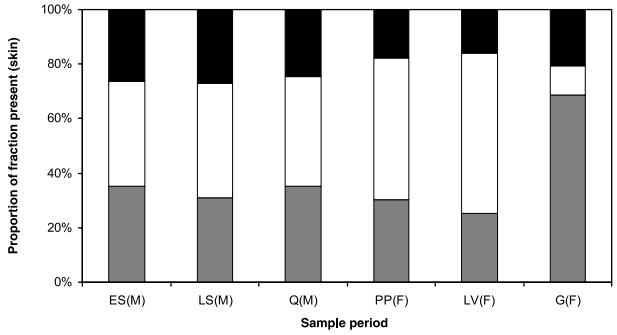


Fig. 2. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars), or remained unmetabolised (white bars) following 180 min of in vitro incubation with skin tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

### 3.3. Adrenal gland

In vitro incubation with [$^3$H]T of male adrenal tissue from all three reproductive conditions sampled resulted in the production of approximately equal proportions of derivatives (mean 36.7%, range 36.2–37.3%), conjugates (mean 33.5%, range 26.7–38.4%) and unmetabolised tritiated substrate (mean 29.8%, range 25.4–36.0%) being present after 180 min (Fig. 3). Free 5α-DHT was identified in media from incubations of tissue from early spermatogenic-stage and quiescent males, and AD was present only in incubations of tissue from late spermatogenic-stage males. Numerous polar metabolites were detected, but no E2 was identified as a product of any incubation using male adrenal tissue. In contrast, incubations using female tissue resulted in the production of only a small proportion of derivatised steroids (mean 10.6%, range 7.9–14.7%) (Fig. 3), which consisted entirely of a number of polar metabolites. Adrenals collected from late vitellogenic-stage females produced the smallest proportion of conjugated steroids (17.6%) and, correspondingly, returned the greatest proportion of unmetabolised substrate (73.1%). The largest proportion (48.8%) of conjugated steroids was produced by tissue from gestating females.

### 3.4. Cloaca

Following incubation of male or female cloacal tissue (Fig. 4), 40.3% (range 31.6–64.1%) of the tritiated substrate was conjugated. When the tissue collected from

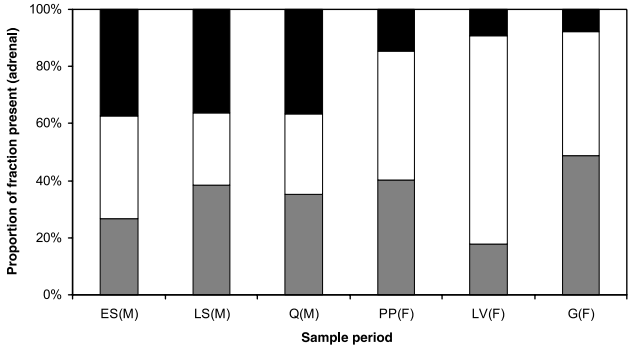


Fig. 3. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with adrenal tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post-parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

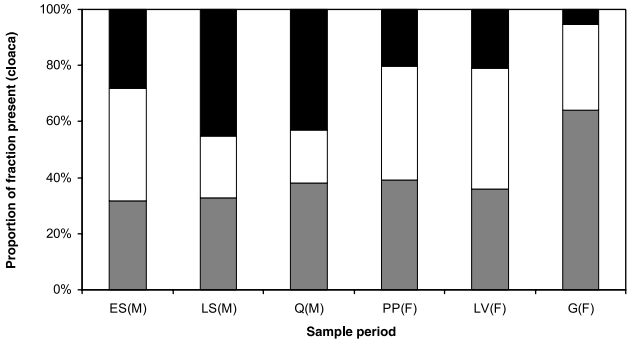


Fig. 4. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with cloacal tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

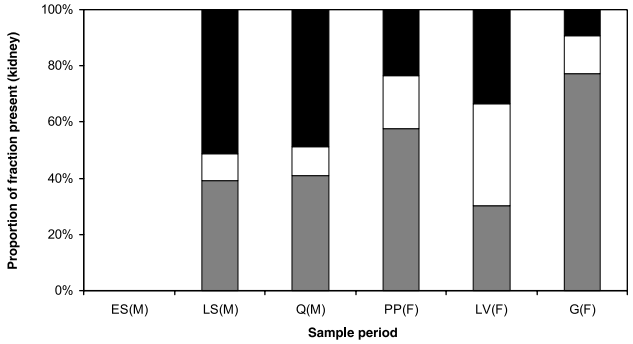


Fig. 5. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with kidney tissue from male and female *Tiliqua nigrolutea* at different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post-parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

gestating females (in which the proportion of substrate conjugated was substantially greater (64.0%)) are excluded, the results appear more uniform (mean 35.5%, range 31.6–39.2%). Cloacal tissue from early spermatogenic males produced a smaller proportion of steroid derivatives (28.2%) than at other stages of the reproductive cycle (mean 44.1%, range 43.1–45.0%), while tissue from females at all three stages of reproduction produced low proportions of derivatives (mean 15.6%, range 5.5–20.2%). The derivatised fractions from all incubations from both sexes consisted largely of a range of polar metabolites. However, 5α-DHT was produced by late-spermatogenic and quiescent males. No E2 was detected in any samples.

### 3.5. Kidney and SS

Only kidney tissue from late-spermatogenic and quiescent males was incubated with tritiated substrate (Fig. 5): 10.1% (range 9.7–10.6%) remained unmetabolised, while approximately 50.0% (range 48.7–51.2%) became derivatised. Numerous polar metabolites and small amounts of 5α-DHT were detected in the derivatised fractions of media from both incubations with male tissue. Approximately 40.0% (range 39.1–40.8%) of the substrate became conjugated by male kidney tissue. In vitro incubations using kidney tissue from females resulted in variation in the patterns of metabolism across differing reproductive conditions. The greatest proportion of conjugates was produced in incubations of kidney from gestating females (55.0%), while only 22.9% (range 13.5–36.4%) of the original tritiated substrate remained unmetabolised. There was markedly less derivatisation by kidney tissue from females (mean 22.0%, range 9.3–33.3%) of all three reproductive conditions than by either male kidney sample. Polar metabolites were produced during all incubations.

In vitro incubation of SS tissue (males only) with [$^3$H]T resulted in the production of proportions of derivatised (mean 48.6%, range 40.3–53.6%), conjugated (mean 38.5%, range 34.2–41.0%), and unmetabolised (mean 12.9%, range 7.2–19.2%) steroids (Fig. 6) similar to those seen in incubations of kidney tissue from males. Free 5α-DHT and several polar metabolites were detected in the derivatised fractions of media from all three incubations of SS tissue; no E2 or AD was identified.

### 3.6. Epididymis and oviduct

The results for incubations of epididymal (Fig. 7) and oviductal (Fig. 8) tissues are discussed together because

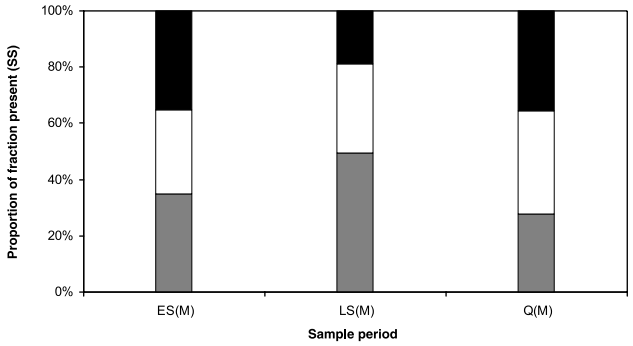


Fig. 6. Proportions of original tritiated substrate (males: [$^3$H]T) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with renal sexual segment tissue from male *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); and Q, quiescent (mid summer).]

they are both accessory reproductive tissues (Ananthalakshmi et al., 1991; Fox, 1977; Norris, 1987; Perkins and Palmer, 1996). However, while they are homologous structures of coelomoductal origin (Fox, 1977), the epididymis and oviduct are embryologically derived from Wolffian (epididymis) and Müllerian (oviduct) ducts, respectively. Thus, these results are not directly comparable.

In vitro incubation of epididymal tissue with tritiated substrate resulted in large proportions of conjugated 41.0% (range 37.5–46.0%) or derivatised 43.2% (range 34.0–49.4%) steroids being produced throughout the reproductive cycle. A large proportion of the derivatised fractions of media from each incubation of male tissue consisted of polar metabolites. Free 5α-DHT was produced by incubation of epididymis collected from both early- and late-spermatogenic males. Only a small proportion of the original substrate 15.2% (range 4.7–24.7%) remained after of incubation for all epididymal samples.

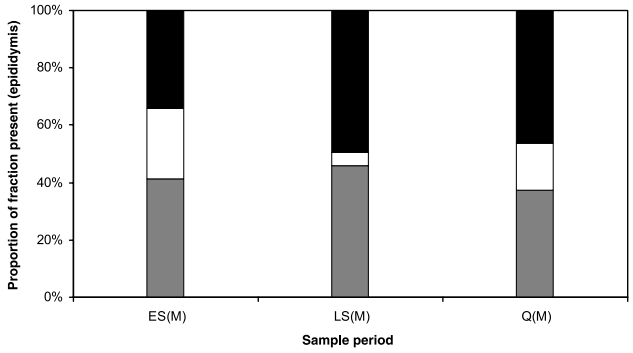


Fig. 7. Proportions of original tritiated substrate (males: [$^3$H]T) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with epididymal tissue from male *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); and Q, quiescent (mid summer).]

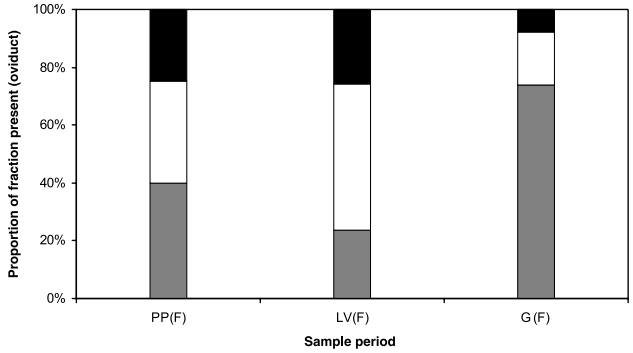


Fig. 8. Proportions of original tritiated substrate (females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with oviductal tissue from female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [F, female; PP, post-parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

Oviductal tissue from gestating females produced the greatest proportion of conjugated steroids (73.7%) following 180 min of in vitro incubation with [$^3$H]E2, compared with tissue from post-parturient and late vitellogenic animals (mean 31.8%, range 23.8–39.8%). In samples collected during late vitellogenesis, a large proportion of the substrate remained unmetabolised (51.0%), and the proportion of steroids conjugated was smallest at this time (23.8%). The only derivatives produced during the experiment (mean 19.3%, range 7.7–25.6%) were polar metabolites.

### 3.7. Testis and ovary

Results for the testicular (Fig. 9) and ovarian (Fig. 10) tissues are also discussed together, although it must be emphasised that they are not directly comparable. In incubations of testicular tissue, 41.9% (range 36.1–53.0%) of the original substrate became conjugated; the largest proportion of conjugated steroids was produced by testicular tissue from quiescent males (53.0%). Only 28.2% (range 22.8–40.0%) of the [$^3$H]T remained unmetabolised after 180 min of in vitro incubation, while 28.2% (range 23.9–36.5%) became derivatised, largely through conversion to polar metabolites. 5α-DHT was not detected in any incubation, but E2 was identified in media from all testicular incubations as a product of incubations with [$^3$H]T, demonstrating that this system *does* allow the detection of E2, if and when it is produced.

In incubations of ovary, tissue from gestating females produced the greatest proportion of conjugated steroids from E2 (61.3%). Only small proportions of derivatised steroids (mean 12.0%, range 81–14.7%) were produced during all incubations of ovarian tissue and 46.8% (range 30.6–55.4%) of the original substrate remained

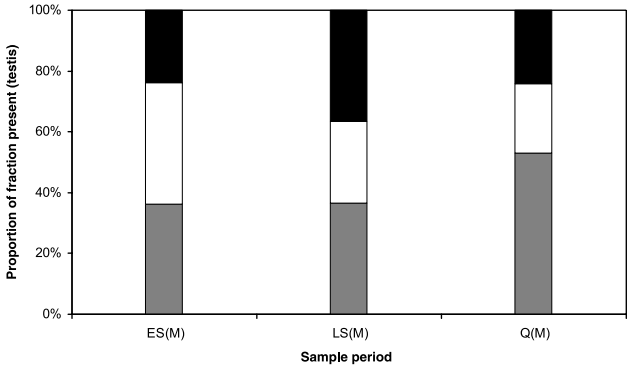


Fig. 9. Proportions of original tritiated substrate (males: [$^3$H]T) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with testicular tissue from male *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); and PP, post parturient (mid autumn).]

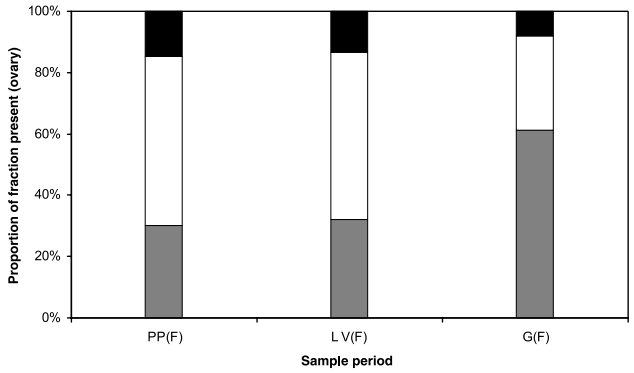


Fig. 10. Proportions of original tritiated substrate (females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with ovarian tissues from female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [F, female; PP, post parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

unmetabolised. Polar metabolites were produced in incubations carried out in all three sample periods.

### 3.8. Muscle

Only 15.0% (range 11.4–20.3%) of original substrate was converted to free steroid derivatives during in vitro incubation of male or female muscle tissue (Fig. 11), but AD and 5α-DHT were not detected in media from any incubation with male muscle. A range of polar metabolites was detected in media from all incubations from both sexes. With the exception of gestating females, 52.9% (range 45.8–62.6%) of original substrate remained unmetabolised, and 38.1% (range 23.3–39.1%) became conjugated. Muscle tissue from gestating females conjugated a greater proportion (64.3%) of the original tritiated substrate than muscle from late vitellogenic or post-parturient females, with 21.6% remaining as unmetabolised substrate.

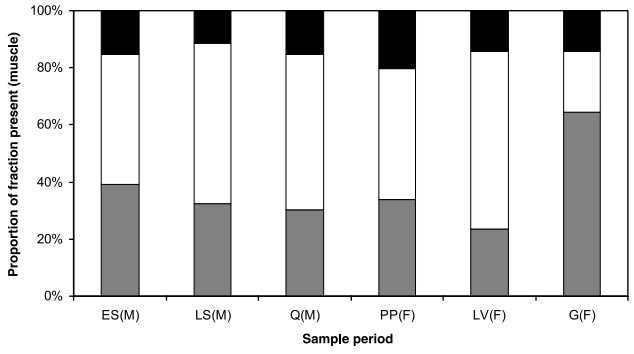


Fig. 11. Proportions of original tritiated substrate (males: [$^3$H]T, females: [$^3$H]E2) which was derivatised (black bars), conjugated (grey bars) or remained unmetabolised (white bars) following 180 min of in vitro incubation with control (skeletal muscle) tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. [M, male; F, female; ES, early spermatogenic (mid autumn); LS, late spermatogenic (early spring); Q, quiescent (mid summer); PP, post parturient (mid autumn); LV, late vitellogenic (mid spring); and G, gestating (mid summer).]

## 4. Discussion

All tissues examined in this study, including testis and ovary, displayed the ability to metabolise a relevant primary steroid (T in males and E2 in females) in vitro. Baseline levels of steroid metabolism in all tissues probably incorporate a contribution from blood, which has been reported in some vertebrates to possess steroid metabolising ability (Jakob et al., 1997; Milewich et al., 1982, 1983 ; Schulz, 1986). However, most tissues showed considerable variation in patterns of substrate derivatisation and conjugation with sex and time of year, although, as in previous studies Edwards et al., 2002a,b, 2003, these data were not analysed statistically.

Steroid conjugates were produced by all tissues, including skeletal muscle. Conjugation by the vertebrate liver plays an important role in solubilising reproductive steroids for excretion in either urine or faeces (Kime, 1987; Payne, 1980; Vermeirssen and Scott, 1996). In *T. nigrolutea* liver produced a greater proportion of conjugates than other tissues with the least variation across reproductive states. In the closely related lizard, *Tiliqua rugosa*, the liver is also an important site of steroid conjugation (Bourne, 1981; Huf et al., 1987) for excretion via the gut and kidney (Bourne, 1981). Conjugate formation may also help to control the availability of biologically active steroids in the body (Huf et al., 1987). However, in *T. nigrolutea*, there was little variation in the proportion of original substrate conjugated with changing reproductive condition, suggesting that the function of conjugation by the liver is to aid excretion, rather than to synthesise biologically active molecules as occurs in fish (Scott and Vermeirssen, 1994; Stacey and Sorensen, 1986; Stacey et al., 1994). All kidney incubations also produced conjugated steroids, probably as an aid to excretion. Similarly, the kidneys are known to be a major route of excretion of sulphated steroids in the related lizard *T. rugosa* (Bourne, 1981; Huf et al., 1987). It is not clear from those studies whether the kidneys themselves acted as the site of conjugation; however, the kidneys of the lizard *Lacerta vivipara* are thought to both metabolise and excrete androgens (Dufaure and Chambon, 1978).

Samples from gestating females metabolised E2 to produce the greatest proportion of conjugated steroids, regardless of tissue type. This could be a mechanism for removing E2 from the maternal system during gestation, as circulating estrogen concentrations are low at this time (Edwards and Jones, 2001a). In contrast, we observed that for the majority of female tissues, the

largest proportion of unmetabolised E2 remained after incubations with tissues from the late vitellogenic stage: published literature documents a causal relationship between E2 and vitellogenesis in vertebrates (Ho, 1987; Kime, 1987) and plasma estrogen is elevated during this period (Edwards and Jones, 2001a). In males, a similar, but less pronounced trend was apparent: tissues from the early spermatogenic-stage metabolised the lowest proportion of T substrate. This result was unexpected: it has been suggested that elevated plasma T (which may reflect decreased metabolism) is responsible for the later, rather than the earlier, stages of spermatogenesis in reptiles (Saint Girons, 1985). Indeed, circulating T concentrations in male *T. nigrolutea* rise markedly between early and late spermatogenesis (Edwards and Jones, 2001b).

The detection of 5α-DHT in incubations of both late spermatogenic-stage cloacal tissue and male skin samples from all three reproductive stages implies the presence of the 5α-reductase enzyme in these tissues. The conversion of T to 5α-DHT occurs in the skin of other reptiles (Fergusson et al., 1985; Hews and Moore, 1995), and 5α-DHT is generally accepted to be the hormonally active metabolite of T in many vertebrates (Kime, 1987; Norris, 1997). Reptiles operate in a largely terrestrial environment, and many use chemical signals released from cloacal glands or across the epidermis to communicate with conspecifics; non-volatile lipids can be released by one sex to create scent trails for the other (Cooper and Vitt, 1986). For example, males of the lizard genus *Eumeces* respond strongly to the cloacal region of conspecific females, or to cloacal odours from them (Cooper and Garska, 1987; Cooper et al., 1986; Trauth et al., 1987). Chemical communication is highly developed in skinks (Cooper and Trauth, 1992) and products of cloacal or epidermal steroid metabolism in *T. nigrolutea* may therefore carry information relevant to reproductive condition or identification of individuals. Adult *Tiliqua scincoides* use cloacal and skin odours to distinguish conspecifics from self (Graves and Halpern, 1991) and *T. rugosa* males and females are able to locate partner lizards by following scent trails and sniffing for airborne chemical signals (Bull et al., 1993), suggesting the use of both volatile and non-volatile compounds. Whether 5α-DHT of either cloacal or epidermal origin is used to convey similar information to, or to stimulate a response from, females of *T. nigrolutea*, is currently unknown, but the seasonal and sexual variation in derivative formation observed in this study suggests that such functions are possible. 5α-Dihydrotestosterone or one of the unidentified polar metabolites (see Edwards et al., 2002b, 2003) or conjugates produced in incubations of skin tissue may provide such information.

5α-Dihydrotestosterone was also detected in the incubations of male adrenal tissue from the early spermatogenic stage. Adrenal tissue can synthesise androgens in males of the snake *Naja naja*, although production is low and does not vary seasonally (Tam et al., 1972). In males of *T. nigrolutea*, plasma T concentrations are low during early spermatogenesis; however, the final stages of spermatogenesis and the display of reproductive behaviours are correlated with elevated plasma T and E concentrations (Edwards and Jones, 2001b). The presence of extragonadal 5α-DHT may serve to prevent the inappropriate stimulation of estrogen-induced reproductive behaviours because 5α-DHT cannot be aromatised (Adkins-Regan, 1981). Examples of similar differential roles for androgens have been documented in *Anolis carolinensis* (Crews et al., 1978) and *Urosaurus ornatus* (Hews and Moore, 1995), but require additional study in *T. nigrolutea*.

Epididymal tissue in *T. nigrolutea* appears to be one of the most metabolically active of all tissues examined. The production of 5α-DHT by epididymal tissue from early and late spermatogenic, but not quiescent, males may indicate a role for 5α-DHT in sperm production, or possibly sperm maintenance. Accessory sex organs in other male vertebrates are also known to metabolise reproductive steroids (Hay et al., 1976; Ozon and Fouchet, 1972; Schoonen and Lambert, 1986, 1987). In reptiles, epididymal function is known to be regulated by androgens, although any distinction between the actions of T and of its metabolites is rarely considered (Dufaure and Chambon, 1978; Morel et al., 1993; Shivanandappa and Devaraj Sarkar, 1987). In the lizard *Lacerta vivipara* epididymal epithelial cells are secretory in response to androgens during the final stages of sperm maturation, which occur in the epididymis (Morel et al., 1993). The demonstrated ability of this tissue to metabolise androgens could well demonstrate localised production for localised function and warrants further investigation.

The oviduct of *T. nigrolutea* actively metabolises E2, producing both steroid conjugates and free derivatives. However, E2 itself is probably the most important regulator of oviductal function in reptiles (Mead et al., 1981). In the lizard *Xantusia vigilis* (Yaron, 1972) and the snake *Thamnophis elegans* (Mead et al., 1981), P4 and E2 stimulate the maturation of the pre-ovulatory genital tract. Correspondingly, in *T. nigrolutea* the greatest proportion of unmetabolised E2 was detected in incubations of oviduct from late vitellogenic (pre-ovulatory) females. This suggests that in *T. nigrolutea* unmetabolised E2 has an important direct effect on the oviduct at this time of year. While the function of the numerous steroid conjugates and derivatives produced by the oviduct is not certain, *T. nigrolutea* females do not store sperm (Edwards, 1999), so the steroids are unlikely to function in sperm maintenance by females.

In vitro conjugation of T by *T. nigrolutea* was greater in testicular tissue of quiescent males than in those

undergoing spermatogenesis. This may simply indicate a changing seasonal requirement for T in a non-conjugated form. In the related lizard *T. rugosa* steroid conjugation in the testes was described as low, and less than occurs in the liver or kidney (Huf et al., 1987), although seasonal variation has not been examined. Testicular derivatisation of T in *T. nigrolutea* was greatest in incubations of testis collected in spring, at which time testes are maximally hypertrophied (Edwards and Jones, 2001b). Interestingly, no 5α-DHT or E2 were detected. In the testis of *T. rugosa*, androgens are metabolised to the unusual androgen epitestosterone (epiT), which may act as a mechanism controlling androgen availability (Huf et al., 1989). However, T is clearly the major androgen in *T. nigrolutea* (Bourne et al., 1985; Edwards et al., 2002b, 2003).

17β-Estradiol is the major end-product of ovarian steroid biosynthesis in many vertebrates (Kime, 1987; Norris, 1997) and few authors report the further metabolism of E2 by the ovary. Ovarian steroid metabolism in *T. nigrolutea* resulted, with the exception of gestating females, in only a small proportion of the original E2 substrate becoming derivatised or conjugated, compared with other tissues. It is quite likely that E2 is required to induce activity in this tissue, as in most vertebrates (Kime, 1987), and that further metabolism is largely unrequired. 17β-Estradiol has been isolated from ovarian extracts in the lizard *Lacerta sicula* (Lupo Di Prisco et al., 1968). However, it is possible that an alternative estrogen, and not E2, is the end-product of steroid biosynthesis in the ovary of *T. nigrolutea* (Edwards et al., 2002b, 2003). If an alternative estrogen predominates in this species, it may act on the oviduct without peripheral metabolism.

These results demonstrate the extent to which sex, season and tissue type can influence steroid metabolism in a reptile from a temperate environment. The patterns illustrated here highlight the importance of considering these variables in exploring possible functions for circulating primary steroids in experimental design and in comparisons with published studies. The results also suggest that steroid derivatives and/or conjugates may have a role in physiological regulation in this reptilian species.

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