Steroids and Reproductive Biology in the Blotched Blue-tongued Lizard, *Tiliqua nigrolutea*

by

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Declaration

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

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Abstract

This thesis documents the annual profiles of the primary reproductive steroids testosterone (T), 17β-oestradiol (E2) and progesterone (P4), in the reproductive cycles of male and female blue-tongued lizards, *Tiliqua nigrolutea*. Data collected from a large captive population over three consecutive reproductive seasons are included. Reproductive cycles are discussed in the context of other viviparous squamate reptiles, while a broader comparative approach is used to consider patterns of steroid biosynthesis and peripheral metabolism.

The annual patterns of circulating concentrations of T, E2 and P4 have been characterised for both sexes. In males, peak plasma T (10.9 ± 3.00 ng ml^{-1}) and E2 (778.0 ± 120.00 pg ml^{-1}) concentrations occur coincident with late spermatogenesis and observations of mating, respectively. Plasma P4 concentrations remain basal (< 1.2 ng ml^{-1}) throughout the annual reproductive cycle. In females, increasing plasma E2 concentrations (275.2 ± 33.87 pg ml^{-1} – 715.1 ± 106.68 pg ml^{-1}) are associated with vitellogenesis and plasma T peaks (6.3 ± 0.63 ng ml^{-1}) in the mating and peri-ovulatory period. In pregnant females, plasma P4 concentrations are elevated for the first two thirds of gestation, peaking in the second trimester at 12.7 ± 1.27 ng ml^{-1} and falling rapidly prior to parturition. Concurrently, plasma P4 concentrations in non-reproductively active adult females remain basal (1 – 2 ng ml^{-1}) throughout the year.

There is good circumstantial evidence for a multiennial reproductive cycle in females. Parturition occurs late in the active season, presumably leaving little time for females to store sufficient fat reserves to become vitellogenic in the following spring: reproductive opportunities are effectively missed in at least one year following a reproductive effort. Observed reproductive behaviours, including agonistic male – male interactions, mating, and parturition, are documented.

An investigation of gonadal steroid biosynthetic pathways in this viviparous squamate is presented. This compares variation in the relative contributions of the delta-4 and delta-5 steroidogenic pathways according to sex and reproductive condition. The delta-4 pathway

predominates in both sexes, aligning this species phylogenetically with other reptiles. However, there are clear differences between sexes and with changing reproductive condition in the patterns of production of pathway intermediates and end-products. Additionally, detection of a possibly novel polar steroid as a major end-product of steroid biosynthesis in both sexes is reported.

Peripheral (extragonadal) metabolism of T and E2 in a number of reproductively relevant steroid target tissues is compared at times of year chosen to represent three clearly distinctive reproductive conditions in each sex. There are differences both between sexes, between tissue types and with changing reproductive condition in the relative proportions of steroid conjugates and non-conjugated derivatives produced. Biosynthetic pathway activity and peripheral steroid metabolism both appear to be plastic in response to changing reproductive condition in *Tiliqua nigrolutea*.

With a comprehensive database of information about the reproductive endocrinology and physiology of *Tiliqua nigrolutea*, this species is now available as a model to further examine selected aspects of the steroid hormone control of reproductive physiology and behaviour in a cool temperate, viviparous reptile.

Ethics permits

This project was conducted with the approval of the University of Tasmania animal ethics committee under ethics permit numbers 95046 and 98015.

No permit or licence is required in Tasmania to collect (with the exception of collection from national parks) or keep reptiles.

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Table of contents

Abbreviations used in the text

Animals
SVL snout-vent length

Chemicals
DCM dichloromethane
DEE diethylether
EtOH ethanol
MeOH methanol
$ChCl_3$ chloroform
UV ultraviolet

Methods
GSI gonadosomatic index
HPLC high performance liquid chromatography
RIA radioimmunoassay
Rf elution coefficient
TLC thin layer chromatography
RCM relative clutch mass
GC-MS gas chromatography-mass spectroscopy

Statistics
ANOVA analysis of variance
(M)ANOVA repeated measures analysis of variance

Steroids
AD androstenedione
Δ4/4-ene delta 4 pathway
Δ5/5-ene delta 5 pathway
DHA dehydroepiandrosterone
E1 oestrone
E2 17-beta-oestradiol
E3 oestriol
EpiT epitestosterone
^{3}[H] tritiated
P4 progesterone
P5 pregnenolone
T testosterone
3β-HSD 3-beta-hydroxysteroid dehydrogenase
17β-HSD 17-beta-hydroxysteroiddehydrogenase
5α-DHT 5-alpha-dihydrotestosterone
6α-OH-E2 6-alpha-hydroxyoestradiol
6β-OH-E2 6-beta-hydroxyoestradiol
11β-HSD 11-beta-hydroxysteroid dehydrogenase
11β-OH-T 11-beta-hydroxytestosterone

11-KT 11-ketotestosterone
17α-OH-P4 17-alpha-hydroxyprogesterone
17α-OH-P5 17-alpha-hydroxypregnenolone

Tissues
AR androgen receptor
CE columnar epithelium
CL corpus luteum
E epithelium
ER oestrogen receptor
HPG hypothalamic-pituitary-gonadal axis
L lumen
PR progesterone receptor
SER smooth endoplasmic reticulum
SS renal sexual segment
ZP zona pellucida
ZR zona radiata

Chapter 1 General introduction

1.1 The evolution of endocrine systems

It seems likely that regulatory molecules were essential to the survival of the earliest single-celled organisms, for the co-ordination both of internal events (autocrine regulation) and for necessary cell-cell interactions such as aggregation (Norris, 1997). Chemical communication systems used in extant organisms are likely to have developed from a simple, common origin that predates multicellularity (Scott, 1985; Bückmann, 1987). The evolution of receptor molecules capable of recognising these signals would also have been necessary. This concept of a common origin for all biological communication systems may help to explain the highly conserved nature of biologically active hormones in extant organisms.

1.2 Reproductive steroids

The conservative nature of vertebrate hormone systems is well illustrated by the similarity of structure of the gonadal steroids. Steroids are all lipoidal compounds derived from cholesterol, sharing a basic four-ring structure known as the steroid nucleus (Norris, 1997). The skeleton consists of three six-membered rings (A, B and C) and a five-membered ring (D) (Schulster *et al*., 1976). While rings A, B and C can adopt strain-free conformations, the whole structure is constrained to a rigid formation and cannot undergo ring flips (McMurray, 1988). A variety of functional groups and steric configurations afford differing chemical properties to the steroid molecules produced by various endocrine organs in the body, including the testis, ovary and adrenal glands. In most cases, the important gonadal sex steroids are testosterone (T), 17β-oestradiol (E2) and progesterone (P4), but in some vertebrate groups these are converted to more biologically active derivatives in either the gonads or the peripheral tissue (Kime, 1987) (See Appendix 1.1 for the structures of some common steroids).

In higher vertebrates, the same molecules that began as signal chemicals are applied to a much broader range of regulatory functions than those for which they are likely to have been intended originally. For example, in mammals, P4 has important roles in the maintenance of pregnancy (Kime, 1987), while in some reptiles, the same molecule is implicated in stimulating male copulatory behaviour (Witt *et al*., 1994). That a single

molecule, identical in each of these organisms, can serve these rather different reproductive functions reflects the conservative nature of sexual reproduction, and the early evolution of an efficient system of chemical signalling which could benefit little from subsequent modification (Bentley, 1976). This variety of functions may partly reflect the evolution of the receptor molecules with which the steroids bind, and so facilitate the induction of cellular effects. Variation in the tissue types in which the receptors are located determines which parts of the body respond to a hormone and differences in receptor density can affect the magnitude of response (Owen *et al*., 1982). Structural changes in the conformation of the receptor proteins through mutation can allow a particular steroid to bind receptors originally specific for another (Schuurmans *et al.*, 1990; Kemppainen *et al.*, 1992; Veldscholte *et al.*, 1992). Rather than the hormones themselves being altered, it has been the target tissue receptors and tissue responses that have evolved to afford steroid hormones a broader spectrum of regulatory functions (LeRoith *et al.*, 1986; Bückmann, 1987).

1.3 Reproductive steroids in reptiles

As the first vertebrate class to undertake a largely terrestrial existence, reptiles were faced with several problems directly related to reproduction (Cogger, 1993). Away from an aquatic environment, fertilisation must be internal and offspring are held inside the body for some or all of their development (Withers and O'Shea, 1993). Many female reptiles provide substantial energy reserves in the form of yolk for their offspring, and are gravid for an extended period. Both processes require the hormonal support of reproductive steroids. Adaptation to terrestrial reproduction is likely to have been associated with important endocrine system changes in both males and females, to regulate not only gamete maturation and vitellogenesis, but also mate location and recognition, and synchrony of courtship and copulatory behaviours between sexes. Such functions are likely to involve steroid hormones.

There are many different naturally occurring steroids, although not all are biologically active (Kime, 1987). The gonadal steroids are traditionally classified as androgens, oestrogens and progestogens, according to the primary physiological processes they mediate. Biosynthesis and/or modification of these molecules occurs in many reptilian body tissues (Kime, 1987; Norris, 1997).

1.3.1 Androgens

Androgens are steroids of 19 carbon atoms (C19), usually possessing a double bond between carbons four and five. Androgens are the male sex steroids, and stimulate both the development of male physiological and secondary sexual characteristics (Norris, 1997) and male aggressive and sexual behaviours (Kime, 1987). In addition to their reproductive hormonal roles, the anabolic effects of androgens have been documented in birds (Fennell and Scanes, 1992a), livestock (Ina, 1991; Claus and Weiler, 1994) and in humans (Kuipers *et al.*, 1991). Androgen receptors (AR) have been identified in a number of reptilian tissues, indicating that, among others, the testis (Cardone *et al.*, 1998) and oviduct (Smith *et al.*, 1995) are potential target tissues for androgens. The most biologically active androgens are T and its metabolite 5α-dihydrotestosterone (5α-DHT); these and other androgens are synthesised in the testes and adrenal glands of male vertebrates, and in the ovaries and adrenal glands of females (Kime, 1987).

1.3.2 Oestrogens

Oestrogens are 18 carbon (C18) steroids produced from androgens, primarily in the vertebrate ovary (Norris, 1997). Oestrogens are characterised by an aromatic A ring in the steroid nucleus (McMurray, 1988). They are responsible for the development of female sexual characteristics in many vertebrates, and the induction of vitellogenesis in non-mammalian vertebrates, including reptiles (Ho *et al.*, 1982; Ho, 1987). Additionally, oestrogens play a vital part in the sexual differentiation of the vertebrate brain (Kime, 1987). The most active naturally occurring oestrogen in reptiles is E2: oestrogen receptors (ER) have been identified in reptilian oestrogen target tissues including the gonad (Bergeron *et al.*, 1998), the oviduct (Paolucci and Di Fiore, 1994; Vonier *et al.*, 1997), the liver (Riley and Callard, 1988; Yu and Ho, 1989) and the hypothalamus (Godwin *et al.*, 1996).

1.3.3 Progestogens

Twenty-one carbon (C21) progestogens are steroids which function to maintain pregnancy in mammals (Kime, 1987). They also have the ability to delay ovulation (Frieden and Lippner, 1971). The most common progestogen is progesterone (P4) (Norris, 1997) which is also an active androgen antagonist in mammals, competing for androgen binding sites (Rhoades and Pflanzer, 1992). Progestogens are secreted by the corpora lutea in the ovary following ovulation, as well as by the testes and adrenal glands in both males and females (Kime, 1987). While many are biologically active themselves, progestogens such as P4 and pregnenolone (P5) also act as intermediates in the formation of most other steroids and are, consequently, synthesised in all steroidogenically active tissues of the body (Frieden and Lippner, 1971). Progesterone receptors (PR) have been detected in tissues including the oviduct (Kleis-San Francisco and Callard, 1986; Paolucci and Di Fiore, 1994; Vonier *et al.*, 1997) and the hypothalamus (Godwin *et al.*, 1996) implying that these are target tissues for this steroid.

1.4 Roles of reproductive steroids in squamate reptiles

Many squamates are oviparous, laying single or multiple clutches each year (Withers and O'Shea, 1993). Viviparity, however, has evolved numerous times in both snakes and lizards (Shine, 1985; Guillette, 1987). Regardless of the mode of parity, the gonadal sex steroids have many similar regulatory functions throughout the squamates.

1.4.1 Males

In males, seasonal changes in plasma T concentrations are well correlated with reproductive events. Usually, both plasma T concentration and testis mass increase during spermatogenesis to peak in the final stages of gamete maturation, coincident with mating, and falling rapidly thereafter (Bona-Gallo *et al*., 1980; McKinney and Marion, 1985; Ando *et al*., 1992; Bonnet and Naulleau, 1996).

Testosterone has also been implicated in the stimulation of reproductive and agonistic behaviours in male squamates. In many species, plasma T remains elevated during the mating period, beyond the completion of gamete maturation (Courty and Dufaure, 1979; Bonnet and Naulleau, 1996) and can stimulate reproductive behaviours directly (Crews, 1975; Crews *et al*., 1978; Adkins-Regen, 1981; Tokarz, 1986). However, in contrast to this pattern, in other male squamates plasma T concentrations fall prior to the mating period (Johnson *et al*., 1982; Ando *et al*., 1992); it is not known which steroids are

responsible for stimulating reproductive behaviour in these animals. Additionally, many male squamates fight agonistically with other males immediately prior to and during the mating period; such behaviour has also been attributed to stimulation by T (Ando *et al*., 1992; Schuett *et al*., 1997). A causal relationship between T and agonistic behaviour in males has been well documented in many squamates (Greenberg and Crews, 1990; Gobbetti *et al.*, 1994; DeNardo and Sinervo, 1994; Wade, 1997).

Males of some species exhibit two peaks in plasma T concentration during an annual cycle, coincident with the timing of different stages of spermatogenesis or with first and second mating opportunities. In species that mate twice yearly these peaks in plasma T concentration are not always associated with an increase in testicular mass (Flemming, 1993a; Swain and Jones, 1996; Jones *et al.*, 1997; Schuett *et al.*, 1997). Schuett *et al.* (1997) suggest that the source of the second plasma T peak in such species is likely to be extragonadal, possibly adrenal in origin.

Data on plasma steroids other than T in male reptiles are few. In those species in which plasma P4 or E2 have been examined, little attempt has been made to find causal relationships between temporally coincident behaviours and steroid physiology. Some studies suggest that P4 may be important in stimulating reproductive behaviour in male squamates (Lindzey and Crews, 1988; 1992; Young *et al.*, 1991; Witt *et al.*, 1994). Progesterone has been shown to stimulate sexual behaviours in males of the lizard *Cnemidophorus inornatus* (Lindzey and Crews, 1986). This is contrary to the usual findings in male vertebrates: P4 typically inhibits male sexual behaviours (Moore and Lindzey, 1992). Saint Girons *et al*. (1993) provided one of the few published studies in which the timing of behaviours associated with reproduction is correlated with changes in concentrations of all three primary gonadal steroids in the plasma of a male squamate. While plasma P4 concentrations were highly variable, sexually inactive male *Vipera aspis* exhibited elevated plasma E2 and low plasma androgens, and in breeding males the situation was reversed. However, in male *Podarcis sicula sicula*, plasma E2 concentrations increase post-reproductively (Ando *et al.*, 1992).

1.4.2 Females

Elevated plasma E2 concentrations in female squamates generally correlate very well with vitellogenesis and follicular development, dropping rapidly at ovulation (Yaron, 1972a; Bona-Gallo *et al*., 1980; Kleis-San Francisco and Callard, 1986; Bonnet *et al*., 1994; Diaz *et al*., 1994; Jones and Swain, 1996; Jones *et al*., 1997). Elevated plasma E2 has also been correlated with the hypertrophy of the female genital tract (Yaron, 1972a; Jones and Guillette, 1982; Gavaud, 1986) and mating (Joss, 1985; Saint Girons *et al*., 1993). However, in the viviparous garter snake, *Thamnophis sirtalis parietalis*, elevated plasma E2 is associated only with the period immediately after mating, falling rapidly to basal concentrations that are maintained during hibernation and subsequent vitellogenesis and ovulation (Whittier *et al*., 1987). Mating can also be temporally dissociated from peak plasma E2 concentrations (Jones and Swain, 1996; Jones *et al*., 1997). A second oestrogen, oestrone (E1), has been identified in ovarian extracts of the lizard *Lacerta sicula* (Lupo di Prisco *et al*., 1968) and Joss (1985) proposes a role for E1 in mating in females of the lizard, *Lampropholis guichenoti*. Moreover, the ovary is not always the major source of circulating E2. In the viper, *Trimeresurus flavoviridis*, the adrenal gland is credited with the majority of E2 production (Yokoyama and Yoshida, 1994).

There is also variation in patterns of P4 production. Ovulation typically results in increased P4 in the ovary and plasma of oviparous and viviparous reptile species (Yaron, 1972a). In oviparous squamates, plasma P4 often becomes elevated during follicular development, peaking after ovulation during the gravid period, then falling to basal concentrations at oviposition (Arslan *et al*., 1978a; Bona-Gallo *et al*., 1980; Joss, 1985; Diaz *et al*., 1994). Postovulatory plasma P4 concentrations, particularly in viviparous squamates, are also usually elevated, but the timing of the P4 peak varies. In some species, P4 remains elevated until parturition (Callard *et al*., 1972a; Xavier, 1982; Xavier *et al*., 1985), while in others, plasma P4 concentration is greatest in the second trimester of pregnancy, decreasing prior to parturition (Kleis-San Francisco and Callard, 1986; Nalleau and Fleury, 1990; Van Wyk, 1994; Jones and Swain, 1996; Jones *et al*., 1997). Progesterone is described as having an important role in ovulation (Chieffi and Pierantoni,

1987) and as an anti-oestrogen or inhibitor of vitellogenesis during pregnancy (Callard *et al*., 1972b; Ho *et al*., 1982; Callard and Ho, 1987; Nalleau and Fleury, 1990). It is assumed that the corpus luteum (CL) is the source of P4 during pregnancy (Highfill and Mead, 1975a; Arslan *et* al., 1978a; Kleis-San Francisco and Callard, 1986), although Guillette *et al*. (1981) suggest that this is not the case for all of gestations in the lizard, *Sceloporus jarrovi*. In *T. s. parietalis* also, P4 does not appear to be associated with pregnancy at all, being low or undetectable at this time (Whittier *et al*., 1987).

Androgens are sometimes also considered in the context of the reproductive cycle in female squamates. In *V. aspis* significantly elevated 5α-DHT occurs at oestrus (Saint Girons *et al*., 1993). In other species plasma T concentrations can be variously elevated during late vitellogenesis and ovulation (Whittier *et al*., 1987), until oviposition (Bona-Gallo *et al*., 1980), only during gravidity (Arslan *et al*., 1978a) or may be low to undetectable throughout the cycle (Lupo di Prisco *et al*., 1968; Moore *et al*., 1985a).

Despite the highly conserved nature of the steroid molecules themselves, and the commonality of regulatory functions ascribed to them throughout all reptiles, there are still fundamental differences in many aspects of steroid physiology at ordinal, and even generic, levels. For example, in males of the snake *Agkistrodon contortrix* plasma T concentration is elevated during spermatogenesis and mating (Schuett *et al*., 1997). In the related *A. piscivorus*, plasma T concentration peaks coincident with maximal spermiogenesis but then falls; sperm are stored by males over winter and mating occurs after spring emergence when circulating T concentrations are low (Johnson *et al*., 1982). In contrast to both these patterns is the garter snake *T. s. parietalis*, in which plasma T concentrations are low throughout both the mating period and postnuptial spermatogenesis (Krohmer *et al*., 1987). Courtship behaviour in this species is independent of androgens (Mendonca *et al*., 1996).

Comprehensive studies of complementary physiological and behavioural aspects of function within a single species of reptile are lacking, and there remain substantial gaps in basic knowledge regarding the gonadal steroid control of numerous aspects of

reproductive physiology and behaviour in reptiles (Schuett *et al*., 1997). The availability of such information about a single species would reduce problems with invalid assumptions and extrapolation based on work involving other species and so provide high quality data: these would provide a meaningful and valuable contribution to the database of endocrine studies on reproductive physiology and behaviour. The research presented in this thesis contributes to this knowledge base, using a species of the genus *Tiliqua*.

1.5 The genus *Tiliqua*

Lizards belonging to the genus *Tiliqua* are large, viviparous skinks confined, with the exception of one species, to Australia (Cogger, 1992). Known collectively as blue-tongued lizards, at least one representative of the genus can be found in most areas of Australia, from arid to cool temperate regions (Cogger, 1992). All eight species are diurnal and omnivorous (Greer, 1989). The extensive literature base on the genus is dominated by work on one species, *T. rugosa*, the sleepy lizard. The population dynamics, home range and activity patterns of *T. rugosa* have all been documented (Satrawaha and Bull, 1981; Fergusson and Algar, 1986; Bull, 1987; 1995; Henle, 1990; Bull *et al*., 1991; Dubas and Bull, 1992). Other species have received far less attention.

Studies of the reproductive biology of *Tiliqua* species are relatively few and, again, concentrate on *T. rugosa*. Reproductive cycles have been detailed for all species, providing information on the timing of reproductive events, but are based on museum specimens and lack endocrinological data (Shea, 1989; 1992; 1993). Mechanisms of mate location and recognition have been considered in *T. rugosa*, largely from a behavioural perspective (Bull, 1990; 1994; Bull *et al*., 1993a). Studies on reproductive steroid hormones in *T. rugosa* have concentrated on male lizards. The annual plasma androgen cycle is dominated by epitestosterone (epiT) rather than T (Bourne *et al*., 1985; 1986a); biosynthetic pathways of both epiT and T have been studied *in vitro* (Huf *et al*., 1989). Patterns of testicular steroid biosynthesis are known to vary with season (Bourne and Seamark, 1978) and temperature (Bourne *et al*., 1986b), and conjugated T has been detected in the plasma of males (Huf *et al*., 1987).

In female *T. rugosa*, plasma P4 concentrations peak in the second trimester of pregnancy (Bourne *et al.*, 1986c) and CLs are probably the major source of circulating P4 (Fergusson and Bradshaw, 1991). In combination with the decline in plasma P4 prior to birth, E2 may be involved in the onset of parturition (Fergusson and Bradshaw, 1992). Data on gestation length and a detailed description of parturition have also been published (Bull *et al*., 1993b).

Despite this information, the mechanisms of endocrine control of reproduction in this genus remain largely unknown. Several aspects of reproductive biology have received attention, but little attempt has been made to draw the information together into a coherent and complete picture of the seasonal and sex differences of the steroid hormone regulation of reproductive physiology and behaviour in any member of this genus.

1.6 The study animal, *Tiliqua nigrolutea*

The animal chosen for such an extensive examination of reproductive endocrinology was the southern or blotched blue-tongued lizard, *Tiliqua nigrolutea*. There is great scope in the field of endocrinology for consideration of data, not only within a reptilian context, but also for a broader appreciation of the functioning of vertebrate hormone systems. *Tiliqua nigrolutea* is an abundant, relatively large lizard, providing ample tissue for *in vitro* studies and, while quite cryptic and secretive, is extremely easy to catch when found. Housing and feeding requirements are simple, and this species adapts quickly to handling and life in captivity. It is perhaps surprising, then, that little scientific knowledge exists about the reproductive biology of such a common animal.

1.7 Project aims

This project was designed to provide a comprehensive understanding of the roles of the primary reproductive steroids T, E2 and P4 in regulating reproductive physiology and behaviour in a viviparous reptile, *Tiliqua nigrolutea*. Both males and females were considered throughout their reproductive cycles. Additionally, other aspects of the species' biology associated with reproduction, such as gestation length, reproductive frequency, relative clutch mass and the repertoire of reproductive behaviours in both sexes, were examined opportunistically. Specific project aims were as follows.

Aim 1

To characterise the reproductive cycles in both sexes, by monitoring the changes in plasma T, E2 and P4 concentrations, gonadal histology, and the timing of reproductive events and behaviours, as a basis for experimental manipulation.

Aim 2

To characterise the preferred route of steroid biosynthesis by the gonads, considering the possibility of a difference in pathway emphasis between sexes and at times of gonadal activity and inactivity.

Aim 3

To examine the extragonadal metabolism of T and E2 by various steroidogenically active peripheral tissues, considering the differences in the emphasis of peripheral conversions between sexes and with changing reproductive status, and correlating these differences with observed histological variations.

Aim 4

To collect general biological data relevant to reproduction and husbandry in a species about which little is known.

1.8 Thesis format

This thesis is divided into several sections. This general introduction to the topic and the experimental animal comprises *Chapter 1*. *Chapter 2* outlines the general methods used to maintain the captive lizard population, and in the collection of data. *Chapters 3* to *6* are concerned with physiological aspects of reproduction. *Chapter 3* has been peer reviewed for submission to *Journal of Herpetology* and reviewers' comments are incorporated. It is included in the form prepared for publication, including American spelling, with minor concessions to the thesis format, such as figure labelling, chapter cross-referencing and the removal of the reference list. It is acknowledged that a reduction in the number of Figures will be necessary before resubmission of the chapter as a manuscript. *Chapters 3* and *4* present characterisations of the male and female reproductive cycles, respectively, describing annual patterns of change in plasma steroid concentrations and timing of reproductive events. *Chapter 5* examines steroid biosynthesis in the gonads and details variations in steroid production according to sex and reproductive condition. *Chapter 6* considers peripheral conversions of primary steroids in a selection of reproductively relevant body tissues, again considering variation with sex and reproductive condition. Finally, *Chapter 7* provides a general discussion of the results, and some ideas for future research.

Chapter 2 General materials and methods

General procedures and other information relevant to more than one experimental chapter are included here. Any techniques particular to a single experiment are included in the appropriate chapter. However, some repetition is unavoidable.

2.1 Animals

Source and identification

Lizards were captured opportunistically by hand throughout southeastern Tasmania, the island state south of mainland Australia, during their active season (spring – mid-autumn). In Tasmania there are no other species with which *Tiliqua nigrolutea* can be confused. Upon arrival at the laboratory, animals were tentatively sexed by examining relative head width, overall body size and shape: males have relatively broader heads with smaller, more slender bodies. Sex was confirmed by visual examination of the cloacal opening for the presence or absence of hemipenis musculature. Successful extrusion of the hemipenes was rarely accomplished in adults and was only possible during the spring mating season (Figure 2.1), although eversion of the hemipenes in male neonates was easily accomplished. During late spring, adult males were also characterised by the display of aggressive behaviour toward other adult and sub-adult males. Sub-adult males were identified subjectively by size (SVL < 25 cm) and their sex was confirmed by whether or not they elicited an agonistic response from known adult males during spring (Oct-Nov). Very few sub-adult females were encountered, but the smallest reproductively active female had a SVL of 26 cm. Juveniles were rarely encountered in the wild.

Housing

Animals were housed in roofed enclosures 1.9 x 3.4 x 2.1 m. These were wire-fronted, allowing access to UV light and a natural photoperiod (Figure 2.2). During

Figure 2.1 Eversion of a hemipenis in male *Tiliqua nigrolutea* is only possible during the mating period.

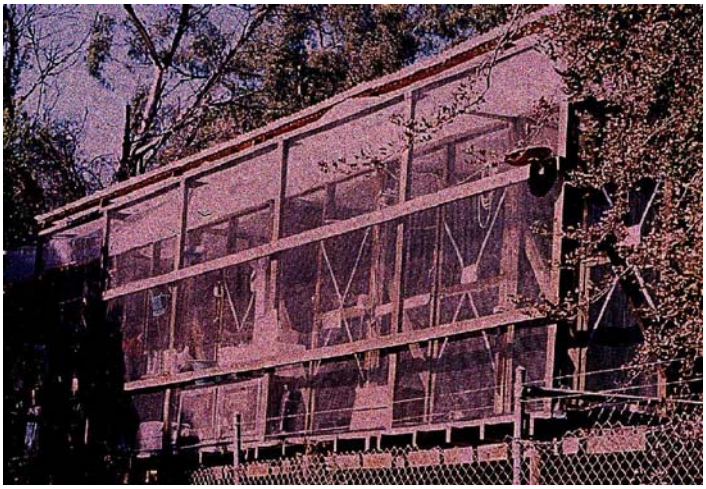

Figure 2.2 Animals were housed in roofed, wire-fronted enclosures that allowed access to direct sunlight and a natural photoperiod.

the lizards' active season, direct sunlight and a 120 W globe as an additional heat source suspended 30 cm above the floor at the front of each cage provided a thermal gradient across which the lizards could thermoregulate. Bark and leaf litter, in which the animals could hide, were also provided. Mixed-sex groups of approximately five animals were maintained in each cage, although individual males only were housed with females from mid spring (Oct) to mid summer (Feb) to avoid male-male conflicts during the reproductive season.

Animals remained in captivity for varying lengths of time. A base population was kept for three reproductive seasons to allow repeated blood sampling and assessment of reproductive frequency: any other individuals not killed during the course of the experiment in which they were involved were released at the conclusion of that experiment. Neonates were released shortly after parturition.

Diet

Lizards were fed two to three times weekly during the active season (Sept – Apr). Their diet consisted of tinned catfood (meat varieties), snails when available, and a selection of fresh fruits including apple and banana. Water was available *ad libitum* at all times. Neonates received an identical diet.

2.2 Data collection

Morphological measurements

At the time of capture, and thereafter at monthly intervals, each lizard was weighed to the nearest 1 g. However, from approximately 3 - 4 weeks before parturition (mid-Feb) females were weighed every three to four days to ensure an accurate pre-partum weight was obtained. Snout-vent length (SVL) to the nearest 1 mm was determined using a plastic ruler, and head width at the broadest point was measured using vernier callipers.

Blood Sampling

Blood samples were collected on the day of capture and then at monthly intervals; however, throughout spring (Sept-Dec) females were sampled fortnightly. The lizards were brought into the laboratory prior to sampling. Samples were taken routinely between 0930 and 1230. Blood was collected from the caudal artery (without anaesthesia) using a heparinised syringe, and held on ice until centrifuging at 6400 rpm. Plasma was stored frozen at -20 °C until analysis. Up to 1 ml of blood was taken from each animal, although some samples were much smaller. From a few blood samples insufficient plasma was obtained to complete all three steroid assays and, occasionally, no blood was obtained.

Opportunistic Data Collection

Throughout the project, other biological data were recorded opportunistically. The dates of mating, parturition and skin moults were recorded. At parturition, post-partum females were weighed and a blood sample taken for analysis of plasma progesterone (P4), 17β-oestradiol (E2) and testosterone(T) concentrations. All offspring were weighed at birth, measured (as previously described) and their sex determined by whether or not hemipenes could be everted. The timing of other events related to reproduction, such as emergence from hibernation, was noted, and all reproductive and parturient behaviours were detailed.

2.3 Plasma steroid analyses

Plasma concentrations of T and P4 were measured using established radioimmunoassay (RIA) techniques based on a tritiated steroid as the radiolabel. Plasma E2 concentrations were measured using a commercially prepared kit. Details of the assays are given below. The limit of detection for all three assays was 10 pg of authentic steroid. Intra- and interassay coefficients of variation for each assay are given in Table 2.1. In all assays, serial dilutions of lizard plasma ran parallel to the standard curves.

Table 2.1 Intra- and interassay coefficients of variation for steroid hormone radioimmunoassays.

assay	Coefficient of variation intra	inter
T	6	< 10
P4	8.4	12.1
E2	8	13

Analytical reagent grade isooctane, hexane and ethanol were purchased from Biolab Scientific Pty, Ltd (Victoria, Australia). Scintillation fluid (Ecolite +) came from ICN (Costa Mesa, CA). [1,2,6,7-^{3}H]Testosterone (spec. act. 100 Ci/mmol) and [1,2,6,7-^{3}H]progesterone (spec. act. 80-110 Ci/mmol) were purchased from Amersham Life Sciences (UK).

Progesterone assay

The progesterone antiserum was a gift from A.J. Bradley to whom it was provided originally by J. Malecki (details in McDonald *et al*. (1988)). The antibody cross-reacts with 11α-hydroxyprogesterone (44 %) and 5α-pregnane-3, 20-dione (16 %), but not with corticosterone or dehydroepiandrosterone (McDonald *et al.*, 1988). The assay is described in detail in Jones and Rose (1992). Briefly, P4 is extracted from 50 µl of plasma in isooctane through Chromosorb W (Alltech) -packed columns topped with acid-washed sand. The extraction efficiency is 93 %. The P4 assay has been validated for skink plasma using pooled samples from *Niveoscincus metallicus* (Jones and Swain, 1996). The plasma extracts are evaporated to dryness and incubated overnight at 4 ºC with 50 µl [^{3}H]-progesterone and 100 µl antiserum. The unbound fraction is removed by centrifuging with 500 µl dextran-coated charcoal (0.125 %) and the radioactivity remaining in the supernatant is measured using a Beckman LS 5801 liquid scintillation system for radioactive counting as outlined in Jones and Rose (1992). There was a minor modification for this project: P4 was eluted from columns in 3 ml isooctane, rather than 3.5 ml.

Testosterone assay

The T antiserum was also a gift from A.J. Bradley (details in Bradley, 1990). The T assay was validated for skink plasma in a previous study using *T. nigrolutea* plasma (Swain and Jones, 1994). Plasma T concentrations were extracted from 50 μl of plasma and assayed by a modification of the method of Castro *et al*. (1974) as detailed in Swain and Jones (1994). Extraction efficiency for this assay is 67 % and the cross-reactivity of the antiserum is: 5α-dihydrotestosterone (5α-DHT) 28.6 %, androstenedione (AD) 6.2 %, and epitestosterone (epiT) < 2.5 %. Testosterone is extracted in 500 μl of 2 % ethanol in hexane, evaporated to dryness and incubated overnight at 4 ºC with 50 μl [^{3}H]-testosterone and 100 μl antiserum. The unbound fraction is removed by centrifuging with 500 μl dextran-coated charcoal (0.125 %) and the radioactivity remaining in the supernatant is measured using a Beckman LS 5801 liquid scintillation system for radioactive counting as outlined in Jones and Rose (1992).

17β-Oestradiol assay

Plasma E2 concentrations were measured using commercially prepared Spectria coated-tube ^{125}I RIA kits purchased from Orion Diagnostica. Cross-reactivities with other steroids, provided by the manufacturer, were very low: E2 (100 %), oestrone (E1) (0.97 %), oestriol (E3) (0.44 %), P4 (< 0.05 %), T (< 0.001%). 17-oestradiol was assayed directly from plasma, so no extraction step was required. Half (50 μl) the recommended volume of plasma was assayed, and reagent volumes were halved accordingly. These modifications and the use of this kit were validated for *T. nigrolutea* plasma by Jones and Swain (1996). The radioactivity was counted on a Roche MR 252 automatic gamma counting system.

2.4 Sacrifice of animals

Lizards were killed by ketamine injection (0.4 ml IM, (1 ml/kg)) and simultaneous inhalation of halothane gas, in accordance with ANZCCART recommendations for reptile euthanasia (ANZCCART, 1993). At autopsy, various tissues were harvested for particular experiments. Additionally, samples of liver, kidney, gonad, epididymis or oviduct, and pericloacal tissue were preserved in Bouin's fixative for histological examination. A blood sample was collected by cardiac puncture at point of death.

2.5 Histology

Fixed tissues were decoloured in 70% ethanol (EtOH) saturated with $LiCO_3$. Tissues were then dehydrated and embedded in paraffin wax, sectioned at 6 μm, and stained with Ehrlich's haematoxylin and eosin B before examination by light microscopy. Numerous characteristics were measured in both male and female lizard tissues; details are provided in the relevant chapters. Forty to fifty determinations of each characteristic were made, unless fewer examples were present in the section, in which case all were measured.

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Plasma Steroid Concentrations, Spermatogenesis and Reproductive Behavior Throughout the Annual Reproductive Cycle in the Viviparous Blue-tongued Skink, Tiliqua nigrolutea, (Scincidae), in Tasmania

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Key Words: Tiliqua, reproduction, skink, steroid, mating, spermatogenesis

Abstract.— Few published studies have detailed comprehensively the correlations between plasma steroid hormone peaks and the timing of reproductive events in male squamate reptiles. We examined the patterns of plasma testosterone (T), 17β-estradiol (E2) and progesterone (P4) concentrations in males of the viviparous blue-tongued skink, Tiliqua nigrolutea, throughout the annual cycle. Plasma T concentrations varied through the spermatogenic cycle, peaking at 10.9 ± 3.00 ng / ml during spermiogenesis, coincident with aggressive male–male encounters, but falling prior to the mating period. Mean plasma T concentrations were basal (2 – 3 ng / ml) during reproductive quiescence. Mean plasma E2 concentrations were significantly elevated (778.0 ± 120.00 pg / ml) during the mating period; mean concentrations remained basal (< 300 pg / ml) both before and after mating. Mean plasma P4 concentrations peaked during the mating period (1.1 ± 0.17 ng / ml) and declined significantly after mating. Detailed descriptions of mating behavior and seasonal changes in the histology of the testis, epididymis and sexual segment of the kidney are provided. We propose a role for E2 in the stimulation of reproductive behavior during the mating period.

INTRODUCTION

The gonadal steroids which control reproduction in reptiles are assumed to be testosterone (T), progesterone (P4), and 17β-estradiol (E2) (Kime, 1987). However, there are few comprehensive published descriptions of the annual profiles of steroid concentrations, particularly E2 and P4, in the plasma of male reptiles. Most studies of reproduction in male squamates have described simply the annual cycle of hypertrophy and regression of reproductive organs (Sanyal and Prasad, 1967; Nilson, 1980; Krohmer and Aldridge, 1985; Flemming, 1993b; Shea, 1993; Aldridge and Brown, 1995), or have combined such information with descriptions of cycles of plasma androgen concentrations only (Arslan et al., 1978b; Courty and Dufaure, 1979; 1980; Johnson et al., 1982; McKinney and Marion, 1985; Bourne et al., 1986a; Moore, 1986; Flemming, 1993a; Swain and Jones, 1994; Schuett et al., 1997). Thus, in many cases, supposition about the roles of these gonadal steroids in the regulation of reptilian reproduction is based on analogy with mammals rather than experimental evidence (Ozon, 1972a; Wiebe, 1985; Chieffi and Pierantoni, 1987).

Courtship and copulatory behaviors are also known to be mediated by steroids in most vertebrates (Wade, 1997). Androgens have been implicated in the stimulation of reproductive behavior in male vertebrates, including reptiles (Crews, 1975; Crews et al., 1978; Lindzey and Crews, 1986; Moore, 1987); for example, administration of exogenous testosterone stimulates reproductive behavior directly, rather than following conversion to 17β-estradiol, in the lizards Anolis carolinensis (Adkins-Regen, 1981; Crews, 1975; Crews et al., 1978) and A. sagrei (Tokarz, 1986). Elevated plasma T concentrations are correlated with reproduction and mating in many squamates (Lance,

1984). However, male mating behavior in the garter snake Thamnophis sirtalis parietalis is at least partially (Crews, 1991), if not completely (Mendonca et al., 1996), independent of androgens. Several studies have suggested that in male reptiles, estrogens may influence reproductive behavior through aromatisation of androgens in the brain. Aromatase activity has been detected in the brains of the turtle Chrysemys picta (Callard et al., 1977) and males of the lizard Podarcis sicula sicula (Gobbetti et al., 1994). In the lizard A. carolinensis, exogenous E2 more successfully reinstates reproductive behavior in male castrates than T (Crews and Morgentaler, 1979). However, aromatisation of T to E2 does not appear to be necessary for the induction of the reproductive behaviors that occur coincident with increases in plasma testosterone in male A. sagrei (Tokarz, 1986).

An increasing number of published studies suggest that P4 may also be important in stimulating reproductive behavior in some male squamates (Lindzey and Crews, 1988; 1992; Young et al., 1991; Witt et al., 1994). Exogenous progesterone has been shown to stimulate sexual behaviors in males of the lizard Cnemidophorus inornatus (Lindzey and Crews, 1986). This finding is contrary to the usual in male vertebrates; typically P4 inhibits male sexual behaviors (Moore and Lindzey, 1992).

Given these often conflicting reports, it is surprising that annual changes in plasma concentrations of P4 or E2 in male reptiles have not been documented in more species; such information is vital for us to better understand the hormonal control of reproduction in reptiles. Saint Girons et al. (1993) provided one of the few published studies in which the timing of behaviors associated with reproduction is correlated with changes in concentrations of all three primary gonadal steroids in the plasma of a male squamate reptile. They reported that sexually active male Vipera aspis exhibited elevated

plasma androgen and E2 concentrations, while non-breeding males in autumn had low circulating androgen concentrations and significantly higher plasma E2 concentrations. Plasma P4 concentrations were highest during the spring and autumn mating periods (Saint Girons et al., 1993). However, more studies of this nature are required.

Tiliqua nigrolutea is a large, viviparous skink distributed throughout southeastern Australia (Cogger, 1992). Adult males can range from 25–29 cm snout–vent length (SVL) and weigh between 300 and 450 g, with females somewhat larger and heavier. In Tasmania, where this study was conducted, T. nigrolutea occurs in low altitude heath, savanna woodland and dry sclerophyll forest in the cool temperate regions of the state (Rawlinson, 1974). Presented here is a comprehensive examination of annual cycles of plasma T, E2 and P4 concentrations in male T. nigrolutea, and details of the spermatogenic cycle. Observations of the behaviors associated with reproduction in males are also described.

MATERIALS AND METHODS

Animals.— Lizards were captured opportunistically by hand throughout southeastern Tasmania from Sep – Jan. Males were distinguished from females by their relatively broader heads (our unpublished data) and an examination of the cloacal opening for the musculature of the hemipenes. Animals were housed in roofed outdoor enclosures 1.9 x 3.4 x 2.1 m; these were wire-fronted, allowing access to UV light and a natural photoperiod. The direct sunlight and a 120 W floodlight globe as an additional heat source at the front of each cage provided a temperature gradient across which the lizards could thermoregulate during their active season of spring (Sept) to mid-autumn (Apr). We provided bark and leaf litter in which the animals could hide. Mixed-sex groups of approximately five animals were maintained in each cage from early autumn (Mar) to early spring (Sept); during this period animals were not breeding and few interactions were observed between individuals. However, during the breeding season (mid–spring (Oct) to mid-summer (Jan)), males were separated from each other in similar, but smaller, cages to prevent agonistic interactions and their possible effects on plasma hormone concentrations. The lizards were maintained on a varied diet of fresh fruits, snails and tinned catfood, provided two to three times weekly. Water was available ad libitum. The number of male animals held varied from 12–19 over the period of the study.

Blood sampling.— Blood samples were collected at monthly intervals. Samples were taken routinely between 0930 and 1230 without anaesthesia from the caudal artery, using a heparinised syringe. Samples were held on ice until centrifuging at 6400 rpm and plasma was stored frozen at -20 C until analysis. Up to 1 ml of blood was taken from

each animal, although some samples were much smaller and occasionally no blood was obtained. Twelve samples were collected from each animal for the measurement of plasma T and P4 concentrations and 9 samples were collected from each animal for the measurement of E2.

Histology.— Three premating (late Oct), two postmating (early Dec) and two non–reproductive (late Apr) adult male lizards were killed by ketamine injection (0.4 ml IM) and simultaneous inhalation of halothane gas in accordance with ANZCCART recommendations for reptile euthanasia (ANZCCART, 1993). At autopsy, the testes, epididymides and the renal sexual segments were dissected out and weighed. Body mass (g) and snout – vent length (SVL) (mm) were also recorded. These tissues were preserved in Bouin's fixative and embedded in paraffin wax. Sections were cut at 6 μm and stained with Ehrlich's haematoxylin and eosin B; they were examined by light microscope. In the testicular tissue, seminiferous tubule diameter, lumen diameter and the height of the seminiferous epithelium were measured using an eyepiece graticule. An estimation of the stage of spermatogenesis was also made. In the epididymal sections, tubule and lumen diameter and epididymal epithelial cell height were measured. Finally, for the kidney tissue, measurements of the tubule and lumen diameter and epithelial cell height of the sexual segment of the uriniferous tubules were made. Determinations of 40–50 instances of each characteristic were made, unless fewer examples were present in the section, in which case all were measured.

Radioimmunoassays.— Analytical reagent grade isooctane, hexane and ethanol were purchased from Biolab Scientific Pty. Ltd. (Victoria, Aust.). Scintillation fluid (Ecolite +) came from ICN (Costa Mesa, CA.). [1,2,6,7-^{3}H]Testosterone (spec. act. 100

Ci/mmol) and [1,2,6,7-^{3}H]progesterone (spec. act. 80-110 Ci/mmol) were purchased from Amersham Life Sciences (UK). Testosterone antiserum was a gift from A. J. Bradley (details in Bradley, 1990). Plasma testosterone concentrations were assayed by a modification to the radioimmunoassay of Castro et al. (1974) as detailed in Swain and Jones (1994). Inter- and intraassay coefficients of variation for the testosterone assay were < 10% and 6%, respectively (Swain and Jones, 1994). Progesterone antiserum was from J. Malecki (details published in McDonald et al., 1988). The progesterone radioimmunoassay method was described in Jones and Rose (1992) with a minor modification for this study: progesterone was eluted from the columns in 3 ml isooctane. Intra- and interassay coefficients of variation for the progesterone assay were 12.1% and 8.4% respectively. All testosterone and progesterone assay samples were measured as outlined in Jones and Rose (1992). Plasma oestradiol was measured using Spectria coated-tube radioimmunoassay kits as in Jones and Swain (1996). Intra- and interassay coefficients of variation for the oestradiol assay were 13% and 8%, respectively. The limit of detection for all three assays was 10 pg authentic steroid. Assays were validated using T. nigrolutea plasma (T and E2 assays) or pooled skink plasma (P4 assay): in all cases serial dilutions of plasma ran parallel to the standard curves.

Statistics.— Mean monthly plasma hormone concentrations were compared by repeated measures analysis of variance ((M)ANOVA) using SYSTAT 8.0 (Wilkinson et al., 1998). A significance level of $\alpha = 0.05$ was used throughout. All data were log transformed prior to analysis to satisfy assumptions of normality and homogeneity of variance and all values are presented as mean ± 1 standard error (SE). The original data sets were reduced to include only those individuals for which samples from all (or most)

sample periods were available. Occasional missing individual date points resulted from collecting insufficient plasma to complete all three hormone assays. These missing points were assigned the mean value for animals in the same sample period, although no more than two such values were assigned to any individual or any sample period (Mundry, 1999; D. Ratkowsky, pers. comm.). As multivariate output cannot be generated from repeated measures data sets in which there are more samples than cases (L. Barmuta, pers. comm.), the univariate output was examined and an *a posteriori* Student t test was conducted for each hormone profile. The periods targeted were either the animals' emergence from hibernation or the mating period, as the precise timing of these events was unknown before hormone assays were conducted. Visual examination of completed hormone profiles and concurrent behavioral observations of the captive sample population was used to identify relevant successive pairs of sample sets.

RESULTS

Behavioral observations.— Reproductive behaviors were first observed in the captive population in mid–spring (Oct). Interactions between males placed together at this time were agonistic; powerful bites were regularly delivered to the head and body during an encounter (Fig. 3.1), often drawing blood and sometimes severing digits. If males were housed together, similar behaviors continued through the mating period (Oct–Nov), and until mid-summer (Dec–Jan).

Mating was observed on several occasions in mid to late spring (Oct-Nov). A male began by approaching a female, walking with jerky, irregular movements with his body raised above the ground; at the same time tongue flicks were directed toward her head, body and cloacal region (Fig. 3.2). He then initiated a chase around the cage, attempting to grasp a resisting female on the torso behind the front legs with his mouth (Fig. 3.3). It often took several attempts for a male to secure his grip, as the female ran away or struggled violently. Once in position, the male curled his body around the female's, positioning his cloaca alongside hers (Fig. 3.4). Using the established grip, he then tilted the female's whole body forcibly until her cloaca became exposed. At this point, the female occasionally broke free and ran, only to be chased and recaptured by the male. The male then used one of his hind feet to scratch the female dorsally at the base of the tail (Fig. 3.5). A receptive female responded by gaping her cloaca and allowing the male to intromit one of his hemipenes (Fig. 3.6). Males would test the receptivity of a female repeatedly early in the mating period. The duration of copulation was highly variable; one male was observed to maintain his grasp on a female for approximately six hours, with intromission only occurring towards the end of that time, while an interaction

involving two other lizards lasted only about 30 minutes. More usually, the chase and actual copulation were relatively rapid, with the male then maintaining a post-mating grip on the female for at least one hour (Fig. 3.7), preventing her from leaving despite her attempts to do so. Males showed no loyalty to any particular female; most attempted, often repeatedly, to copulate with two or more different females during the mating period. Females became unreceptive immediately following intromission and responded to additional copulatory attempts by lunging and biting, hissing and gaping at the approaching male (Fig. 3.8). However, one male was observed to successfully copulate twice with the same female using first the right and then the left hemipenis, but this occurred without the animals separating after the first intromission. Several matings were observed in which intromission was successful, but from which females did not conceive. No copulatory plugs were observed.

Histology.— Due to their cryptic and secretive behavior, animals were difficult to obtain in large numbers. Therefore, only a limited number of specimens were available for histological examination of testicular, epididymal and sexual segment tissues. However, distinct seasonal patterns of hypertrophy and regression were obvious in all three tissue types. In autumn (Apr), when spermatogenesis was initiated (Fig. 3.9), the testes were small (mean GSI = 0.84) and ovoid. The lumina of the seminiferous tubules were largely occluded by vast numbers of primary and secondary spermatocytes and some spermatids. In early spring (Sept) the males emerged from hibernation to complete gamete maturation. By mid spring (Oct), spermatogenesis was complete: seminiferous tubules were filled with mature spermatozoa and motile sperm were observed (Fig. 3.10). The testes were fully hypertrophied at this time (mean GSI = 1.53). Tubule lumina had

highly convoluted epithelia forming convolutions in which the heads of many mature spermatozoa were embedded. By the start of summer (Dec) the testes were regressed (mean GSI = 0.50), with only primary spermatocytes and a few mature spermatozoa visible in the seminiferous tubules, and the luminal epithelia were no longer convoluted (Fig. 3.11). Testicular quiescence continued from mid–summer (Jan) to mid–autumn (Apr) when a new cycle of spermatogenesis commenced.

In mid–autumn, epididymal tubules were lined by cuboidal epithelium and had relatively large lumina (Apr) (Fig. 3.12); the surrounding connective tissue was extensive. By mid–spring (Oct), the tubules were hypertrophied and the surrounding connective tissue was no longer visible. Tubules were lined by non-ciliated, columnar, epithelial cells containing small, round, basal nuclei and the tubule lumina were filled with stored mature spermatozoa (Fig. 3.13). By early summer (Dec), the columnar epithelium was greatly reduced in height, although tubules remained hypertrophied and filled with sperm (Fig. 3.14); little connective tissue was visible.

The renal sexual segment (SS) tubules were indistinguishable from other kidney tubules in mid–autumn (Apr). However, by mid spring (Oct), clusters of greatly hypertrophied tubules were obvious (Fig. 3.15). Sexual segment tubules were lined with non-ciliated columnar epithelium containing basal nuclei, and maintained this differentiated appearance into early summer (Dec).

Gonadosomatic index.— Using those animals killed for other experiments and some relatively intact road-killed individuals, the annual variation in the gonadosomatic index (GSI) for male T. nigrolutea was observed (Fig. 3.16). Gonadosomatic index was calculated as (testis mass (g) / body mass (g)) x 100. It was considered ethically

inappropriate to kill animals specifically for this purpose, so sample sizes are too small in some cases to allow statistical analysis. In male T. nigrolutea, GSI is lowest during reproductive quiescence (summer (Dec), post-mating) and increases gradually to peak coincident with peak spermiogenesis and mating in spring (Oct).

Plasma steroids concentrations.— Mean monthly plasma testosterone (T) concentrations in male T. nigrolutea from November 1995 to October 1996 are shown in Fig. 3.17. A distinct unimodal annual cycle was evident, with considerable uniformity between males in both the timing of the seasonal plasma T pattern and the magnitude of plasma T concentrations. Mean plasma T concentration varied significantly throughout the annual cycle ((M) ANOVA: $F = 12.504$, df = 11, $P = 0.000$). Plasma T concentrations decreased significantly from 8.8 ± 0.79 ng / ml during the mating period (Nov) to 5.3 ± 0.62 ng / ml in early summer (Dec) when post-mating male lizards were reproductively quiescent ($t = 3.097$, df = 9, $P = 0.013$). Plasma T concentrations were basal (approx. 1–3 ng / ml) from mid–summer to mid–winter (Jan–Jul), before increasing significantly ($t = -2.450$, df = 9, $P = 0.037$) to 8.0 ± 3.12 ng / ml in late winter (Aug), when male lizards emerged from hibernation, about four weeks earlier than females. Mean plasma T concentration peaked at 10.9 ± 3.05 ng / ml in mid spring (Oct), coincident with the completion of spermiogenesis and the observation of the onset of agonistic male-male interactions. There was no correlation between peak (Oct) plasma T concentration and SVL.

As with the plasma T profile, the annual pattern of changing E2 concentrations in plasma of male blue-tongued lizards plasma varied significantly throughout the reproductive cycle (Fig. 3.18) ((M)ANOVA: $F = 6.267$, df = 8, $P = 0.000$). Mean

plasma E2 concentration was basal (< 300 pg / ml) in emergent animals in early spring (Sept), but became significantly elevated to 460.8 ± 55.41 pg / ml from mid spring (Oct) (t = -6.721, df = 8, P = 0.000), when spermatogenesis had been completed, through to late spring and early summer (Nov–Dec), when mating was observed, peaking at 778.0 ± 120.99 pg / ml in December and dropped sharply to 396.9 ± 53.44 pg / ml by mid summer (Feb), when males were reproductively quiescent. Concentrations remained low (approximately 300 pg / ml) until males began hibernating in late autumn (May).

Mean monthly plasma P4 concentrations in male T. nigrolutea from November 1995 to October 1996 were low (< 1.2 ng /ml) but above the limit of detection of the assay throughout the year, and varied significantly over time (Fig. 3.19) ((M)ANOVA: F = 4.556, df = 11, P = 0.002). Plasma P4 concentration peaked during the mating period (Nov) at 1.1 ± 0.17 ng / ml and fell significantly to 0.8 ± 0.09 ng / ml by early summer (Dec) (t = 4.334, df = 8, P = 0.002). Mean plasma P4 concentration fell significantly to 0.3 ± 0.08 ng / ml in mid-winter (Jun) (t = 2.458, df = 8, P = 0.039) and rose significantly (t = -2.94, df = 8, P = 0.019) when males emerged at the end of winter (Aug).

DISCUSSION

In cool temperate areas, temperature is the most important climatic factor influencing reptilian reproduction (Marion, 1982). An annual spermatogenic cycle is the most common pattern, and reptiles must reproduce during the warmer months, because completion of spermatogenesis typically requires a period of eight to ten weeks with body temperatures above 20 C (Saint Girons, 1985). In Tasmania, the active season for non-alpine reptiles extends from early spring (Sep) to late autumn (May).

Males of the viviparous skink Tiliqua nigrolutea exhibit a peak in plasma T coincident with peak testis mass, spermiogenesis, and agonistic male–male interactions associated with spring mating (Oct – Nov). Spermatogenesis is prenuptial, commencing in autumn (Mar – Apr) and being completed the following spring. This type III reproductive cycle sensu Heatwole and Taylor (1987) conforms to the pattern exhibited by other members of the genus living in milder climatic regions on the Australian mainland (Shea, 1992), but is in sharp contrast the cycles exhibited by two other Tasmanian viviparous skinks, both of which display a type VI reproductive pattern (Heatwole and Taylor, 1987) of autumn spermatogenesis and mating, with a second mating in spring (Swain and Jones, 1994; Jones et al., 1997).

Male T. nigrolutea in Tasmania commence spermatogenesis in autumn (Mar – Apr), following a period of reproductive quiescence (Dec – mid Feb). Spermatogenesis is probably arrested during winter. Males emerge in early spring (Sept), approximately four weeks before the females (our unpubl. data), so that sperm maturation is completed by mid spring (Oct). This presumably allows mating to occur as early in spring as possible (usually late Oct – Nov). In the wild, male T. nigrolutea become increasingly

mobile toward the completion of spermatogenesis (Oct), presumably seeking females; a corresponding restlessness was observed in our captive males. Males are often captured, or seen killed on the roadside at this time of year, but females are rarely encountered (Edwards and Jones, unpubl. data.). This observation is in agreement with a study on the related species, T. rugosa in South Australia which showed that, in most cases, it was the male lizard which located the female for mating by following her scent trail, tongue-flicking at airborne signals or searching familiar sites (Bull et al., 1993a). Bonnet and Naulleau (1996) reported a similar phenomenon in the snake Coluber viridiflavus, describing a significantly increased likelihood of encountering either a live or road-killed male during the mating period. Such observations imply the use of chemical signals (pheromones) for mate location, probably produced by the female and detected by the male.

The jerky, irregular walk observed as a male approached a female to attempt copulation has been described previously in this species as part of the limited courtship ritual (Clutterbuck, pers. comm, cited in Shea, 1992). However, we observed this type of movement in several additional contexts, including male –male interactions, during both the mating period (Oct – Nov) and the less frequent agonistic male – male fights encounters in summer (Dec – Feb) when males were reproductively quiescent. During the mating period, however, the awkward walk signalled the interest of a male in a female and usually preceded a chase. This limited courtship display is similar to that documented for male *Lacerta vivipara,* a lizard in which there is little preliminary activity before mating (Bauwens et al., 1989).

Captive male T. nigrolutea did not appear to pair-bond with a cagemate female. During the mating period we regularly observed caged males chasing and attempting to copulate with up to four different females. Both reproductive and non-reproductive (no vitellogenic follicles on palpation) females were pursued vigorously, suggesting that males may be unable to identify the reproductive condition of a female prior to courtship. This is in direct contrast to T. rugosa which pair-bond monogamously for the mating period (Bull et al., 1993a), with the same pairs often reforming over consecutive reproductive seasons (Bull, 1994). Experiments testing the use of urine and cloacal secretions as a source of chemical information about sex and reproductive condition in T. nigrolutea are currently in progress.

Bourne et al. (1986a) suggested that in Tiliqua rugosa long-term captivity may negatively affect the expression of reproductive behavior and depress plasma steroid concentrations. Captive T. rugosa males do not display a seasonal plasma androgen cycle (Watson et al., 1987). All males used in our study, however, continued to cycle normally in comparison with opportunistically sighted and wild-caught individuals in the timing and magnitude of plasma steroid peaks, the timing of regular skin moults and in the expression of agonistic and mating behaviors. There was little difference in mean plasma T concentration in November between males in the captive population and eight wild individuals that were opportunistically captured at that time (Edwards and Jones, unpubl. data). Any stress caused by handling and blood sampling is unlikely to have had a significant impact on plasma steroid concentrations. Kreger and Mench (1993) considered the impact of handling and restraint on Tiliqua scincoides and found no significant chronic effect on plasma concentrations of the stress steroid hormone,

corticosterone. Additionally, Moore et al. (1991) observed that the effects of acute handling stress in the lizard Urosaurus ornatus are rapidly dissipated. Tiliqua nigrolutea is a placid animal that adapts quickly to captive life. Our animals were housed under conditions of natural temperature and photoperiod with only an additional heat source provided for basking, and underwent a normal hibernation. We are confident that the mean plasma steroid concentrations reported here reflect those in wild populations.

Male reptiles commonly display an annual pattern of plasma T concentrations with low concentrations during the early stages of spermatogenesis and a peak during spermiogenesis, corresponding with peak testicular hypertrophy (Lance, 1984). Tiliqua nigrolutea exhibits this typical pattern of plasma T concentrations mirrored by the patterns of regression and hypertrophy of SS and epididymal tissues, which are, presumably, androgen-linked. Plasma T concentrations are low during autumn (Mar–Apr) when spermatogenesis commences, but rise during spermiogenesis, peaking at 10.9 ± 3.05 ng / ml in mid–spring (Oct) and declining through the second half of the mating period. This pattern closely resembles that of the plasma T cycle of T. rugosa (Bourne and Seamark, 1975), although peak concentrations of plasma T (approximately 40 ng / ml) are higher in T. rugosa. However, the major androgen in T. rugosa is epitestosterone (epiT), not found in T. nigrolutea (Bourne et al., 1985), and epiT is present in much higher concentrations (approximately 140 ng / ml) in the blood (Bourne et al, 1986a). However, much variation occurs in the magnitude of the T peaks between species. Plasma T peaks at 80-100 ng / ml in the snake, Agkistrodon contortix, (Schuett et al., 1997) and 51.7 ± 1.6 ng / ml in the viviparous lizard, Niveoscincus metallicus (Swain and

Jones, 1994), but only approximately 3 ng / ml in the snake A. piscivorus (Johnson et al., 1982).

Given that plasma T declines during the mating period in male T. nigrolutea, we suggest that another steroid is required for stimulating reproductive behaviors in males of this species. Circulating concentrations of E2 also follow an annual pattern, although the values reported here could be higher than actual plasma E2 concentrations. The possibility of an oestrogen metabolite cross-reacting with the E2 assay antiserum is discussed in *Chapter 5 Section 5.4.5* in the context of alternative oestrogens. Regardless of absolute concentrations, throughout the annual cycle plasma E2 concentrations in male T. nigrolutea are elevated during the mating period (Oct – Dec) and drop rapidly to basal concentrations for the rest of the active season. This implies a role for E2 in the induction of sexual behaviors. However, although males of other lizards exhibit annual cycles of E2 production, elevated plasma E2 does not always coincide with mating. Saint Girons et al. (1993) demonstrated that sexually active male Vipera aspis have high plasma T and low E2 during the mating period, with E2 being elevated to approximately 520 pg / ml in non-mating males. In male Podarcis s. sicula plasma E2 concentrations increase in the post-reproductive refractory period to about 1.5 ng / ml (Ando et al., 1992). Intracranial implants of oestrogen in castrated male A. carolinensis restore sexual behaviors (Crews and Morgentaler, 1979), but later studies on anoles suggest that the expression of reproductive behavior in Anolis species is under the direct control of androgens (Adkins-Regen, 1981; Tokarz, 1986).

We also examined plasma concentrations of P4 in male T. nigrolutea. Plasma P4 concentrations showed a seasonal pattern in male T. nigrolutea: mean concentrations

were low (< 1.2 ng / ml) throughout the year with a small but significant elevation prior to emergence (Aug). This significant change observed in male plasma P4 concentrations may be, simply, a function of an overall increase in metabolism as a result of higher temperatures during the animals' active season, such as has been documented in other ectothermic vertebrates (Kime, 1979; 1987; Kime and Hyder, 1983). However, P4 has been shown to stimulate male reproductive behavior in some lizards (Lindzey and Crews, 1986; 1988; 1992; Young et al., 1991; Moore and Lindzey, 1992; Witt et al., 1994) acting as a progestin rather than through conversion to other steroids (Moore and Lindzey, 1992). Plasma P4 concentrations have also been measured in males of the lizard P. s. sicula, in which there was a post-reproductive increase which peaked at approximately 8 ng / ml (Ando et al., 1992). The lack of a pronounced annual pattern in T. nigrolutea, however, implies that P4 may not have a primary role in the induction of reproductive behaviors in males of this species.

Further experimental studies are required to elucidate the hormonal control of reproductive behaviors in males of T. nigrolutea and other squamates as there appears to be variation in the plasma steroid hormones that are elevated during mating. This study has addressed the lack of published work in which data on reproductive behaviors and coincident steroid hormone profiles are available.

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FIGURE LEGENDS

FIG. 3.1. An agonistic male – male interaction during spring.

FIG. 3.2. A male lizard approaches a female during the mating period, tongue-flicking down the side of her body. M = male, F = female.

FIG. 3.3. A male lizard achieves a bite-hold on the shoulder of a female. M = male, F = female.

FIG. 3.4. A male lizard attempts to position a female for copulation. M = male, F = female.

FIG. 3.5. A male lizard taps with his foot at the base of the female's tail to encourage her to permit intromission. M = male, F = female.

FIG. 3.6. Intromission. M = male, F = female.

FIG. 3.7. A male retains a post-mating bite hold on a female. M = male, F = female.

FIG. 3.8. A female becomes unreceptive rapidly following copulation, gaping and hissing at a male. M = male, F = female.

FIG. 3.9. Seminiferous tubules of the testis of T. nigrolutea during early spermatogenesis (autumn). Lumina of tubules are occluded by large numbers of primary and secondary spermatocytes and some spermatids (S).

FIG. 3.10. Seminiferous tubules of the testis of T. nigrolutea during late spermatogenesis (spring), containing mature spermatozoa (MS) with tails extending into the tubule lumen (L).

FIG. 3.11. Seminiferous tubule of the testis of T. nigrolutea during the post-mating period (summer). Only sertoli cells (SC) and primary spermatocytes (P) are present.

FIG. 3.12. Tubule of the epididymis of T. nigrolutea during early spermatogenesis (autumn). Tubules are lined by cuboidal epithelium (E), no spermatozoa are visible and lumina (L) are empty.

FIG. 3.13. Tubule of the epididymis of T. nigrolutea during late spermatogenesis (spring). The tubule is lined by non-ciliated, columnar epithelium (CE) and is packed with mature spermatozoa (MS).

FIG. 3.14. Tubule of the epididymis of T. nigrolutea during the post-partum period (summer). Columnar epithelium (E) is reduced and tubules remain packed with mature spermatozoa (MS).

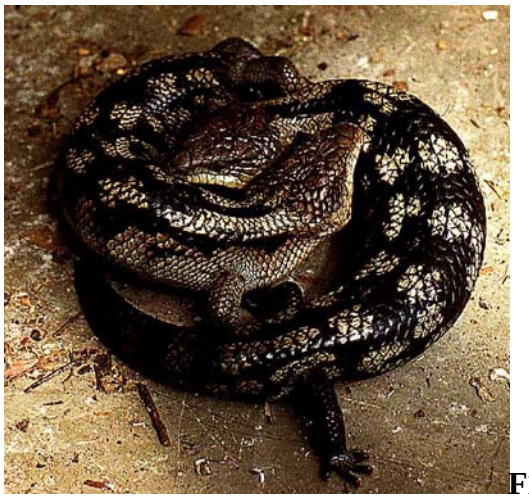
Fig. 3.15. Renal sexual segment of T. nigrolutea during late spermatogenesis (spring). Tubules are hypertrophied and lined with non-ciliated, columnar epithelium (CE).

FIG. 3.16. Gonadosomatic index (GSI) for adult male T. nigrolutea. Values are means ± 1 standard error. Sample sizes: April (early spermatogenesis), N = 2; October (late spermatogenesis), N = 7; December (post-mating), N = 3; February (quiescence), N = 4. GSI = (total testicular mass (g) / intact body mass (g)) x 100.

FIG. 3.17. Changes in mean monthly plasma testosterone concentrations in male T. nigrolutea throughout the annual reproductive cycle. Sampling was from November 1995 to October 1996. Values are means ± 1 standard error, N = 10.

FIG. 3.18. Changes in mean monthly plasma 17β-estradiol concentrations in male T. nigrolutea throughout the annual reproductive cycle. Sampling was from September 1996 to May 1997. Values are means ± 1 standard error, N = 8.

FIG. 3.19. Changes in mean monthly plasma progesterone concentrations in male T. nigrolutea throughout the annual reproductive cycle. Sampling was from November 1995 to October 1996. Values are means ± 1 standard error, N = 9



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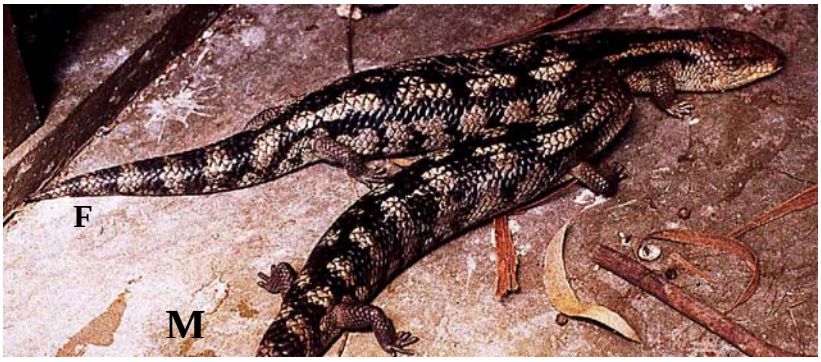

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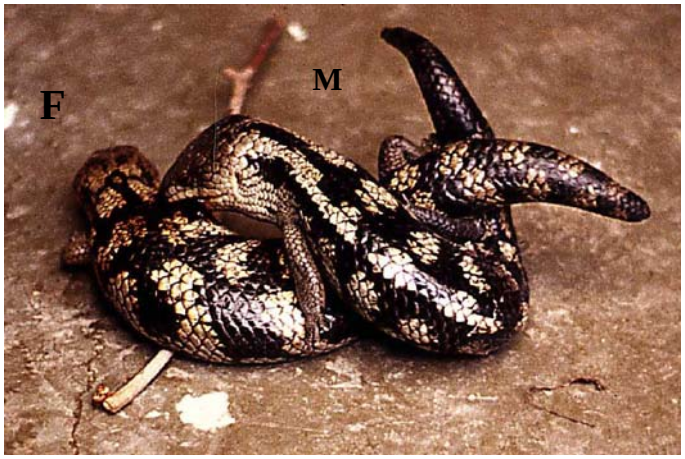
FIG. 3.5



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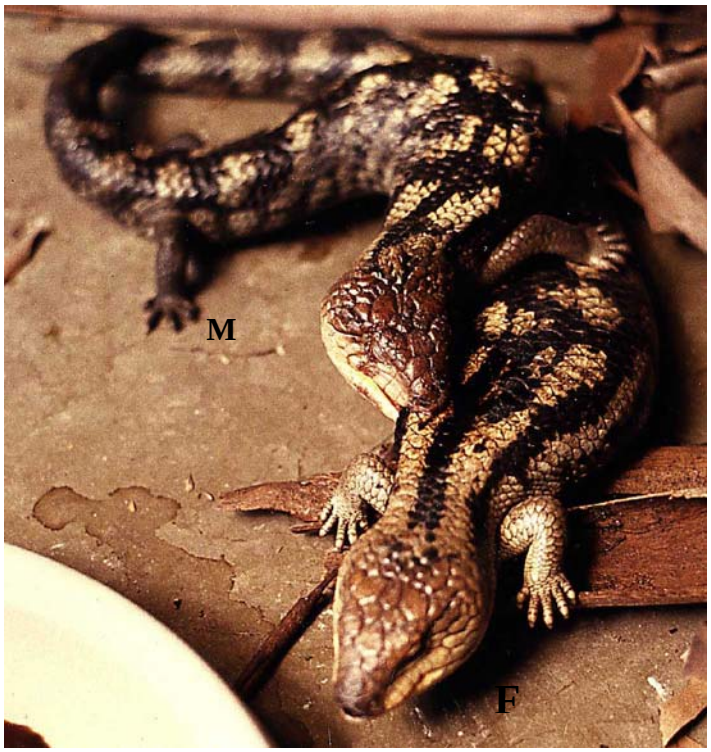

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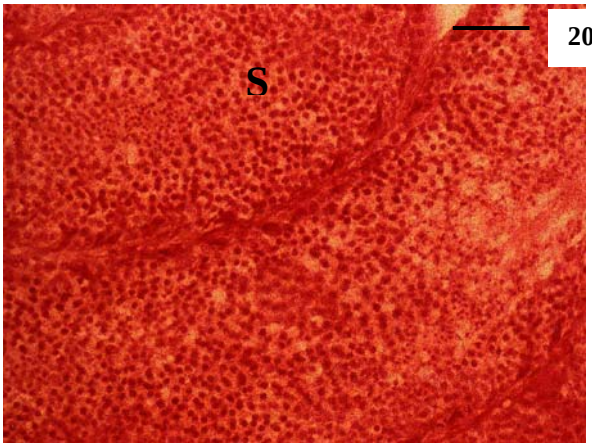

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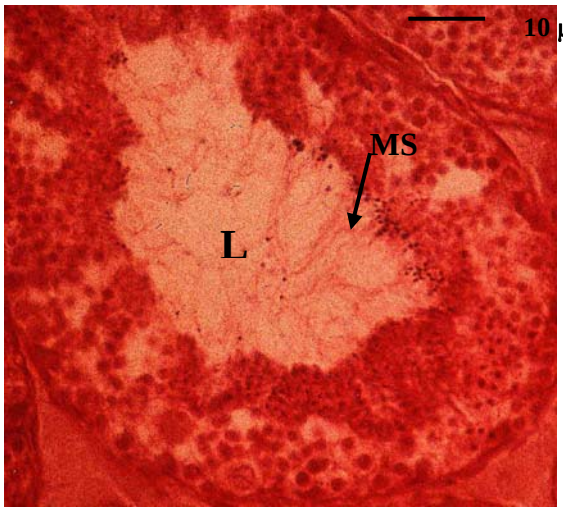

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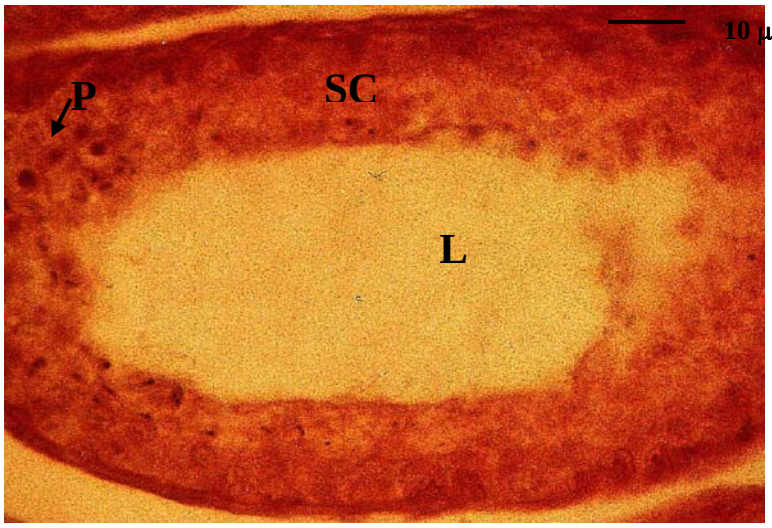

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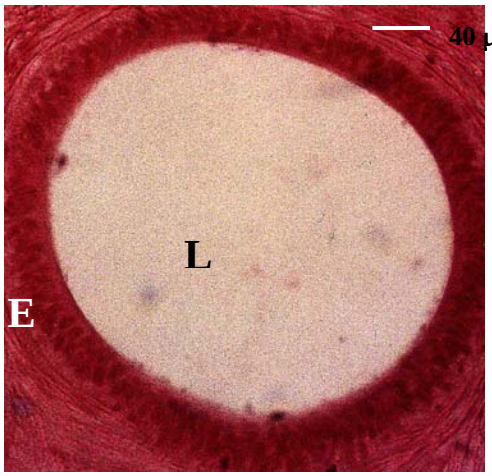

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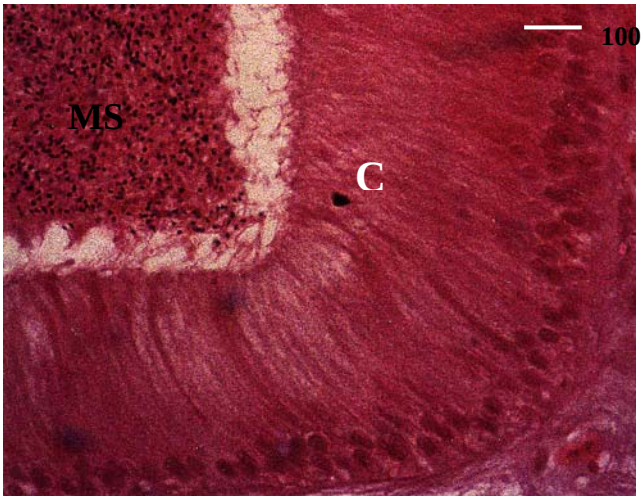

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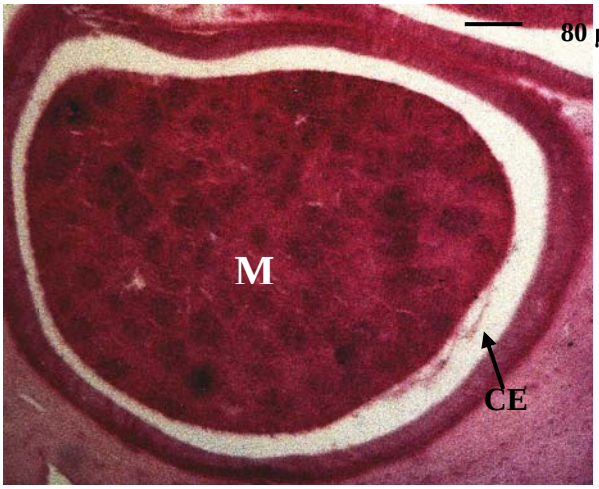

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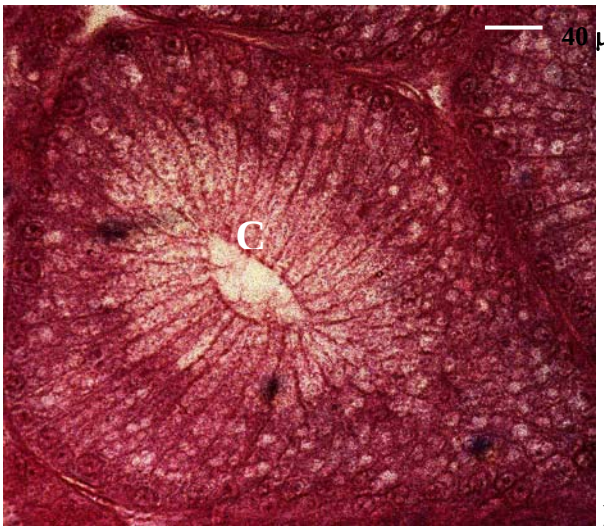

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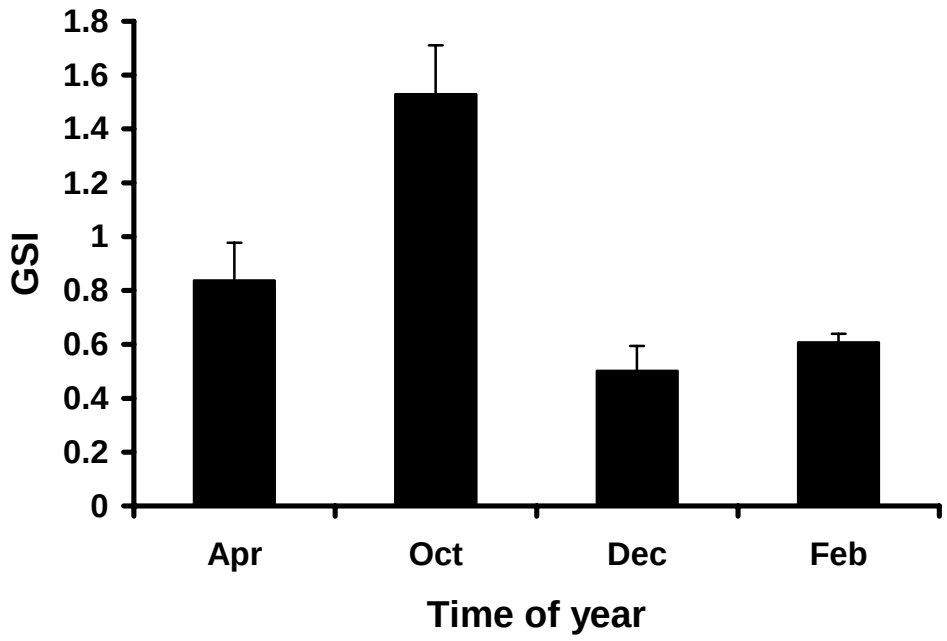
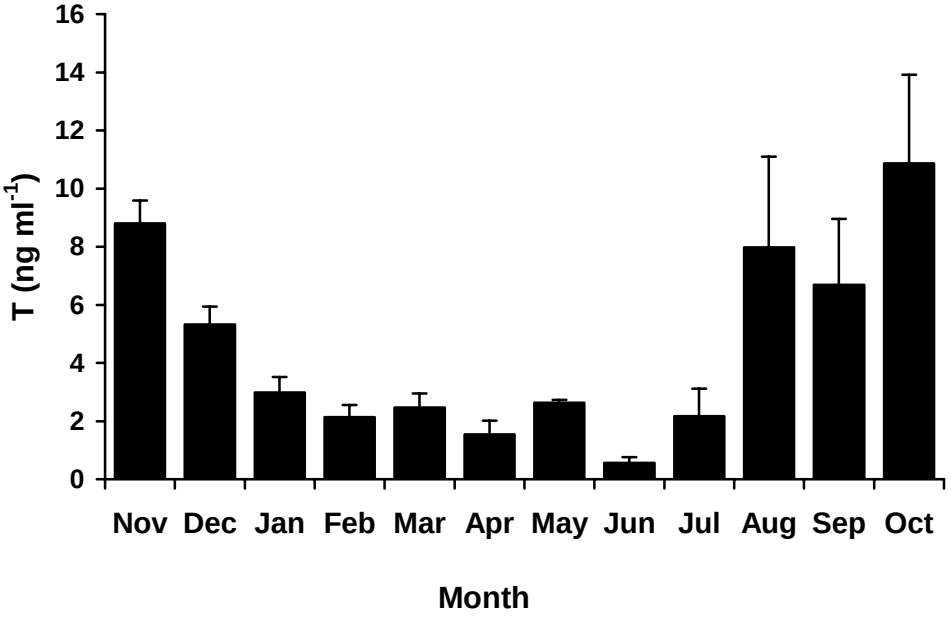


FIG. 3.17



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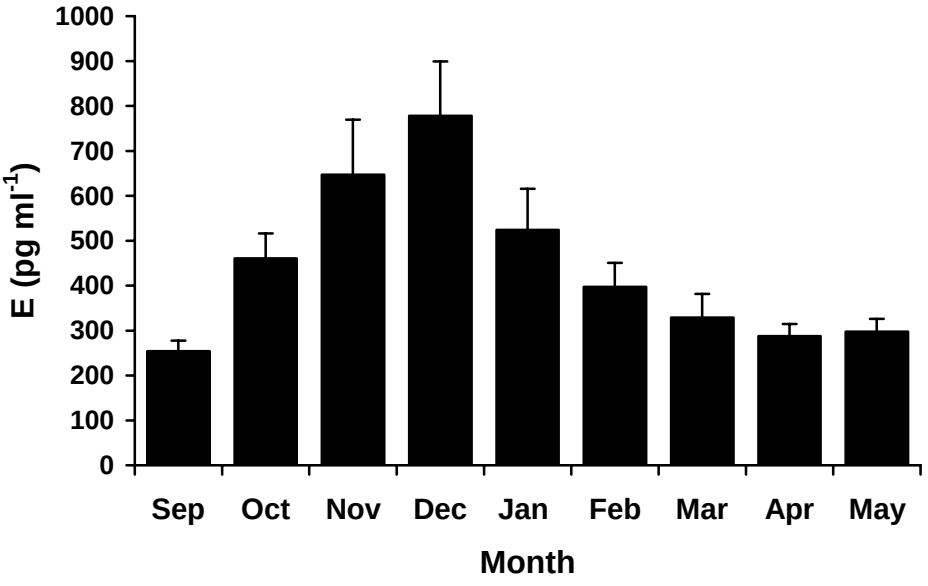

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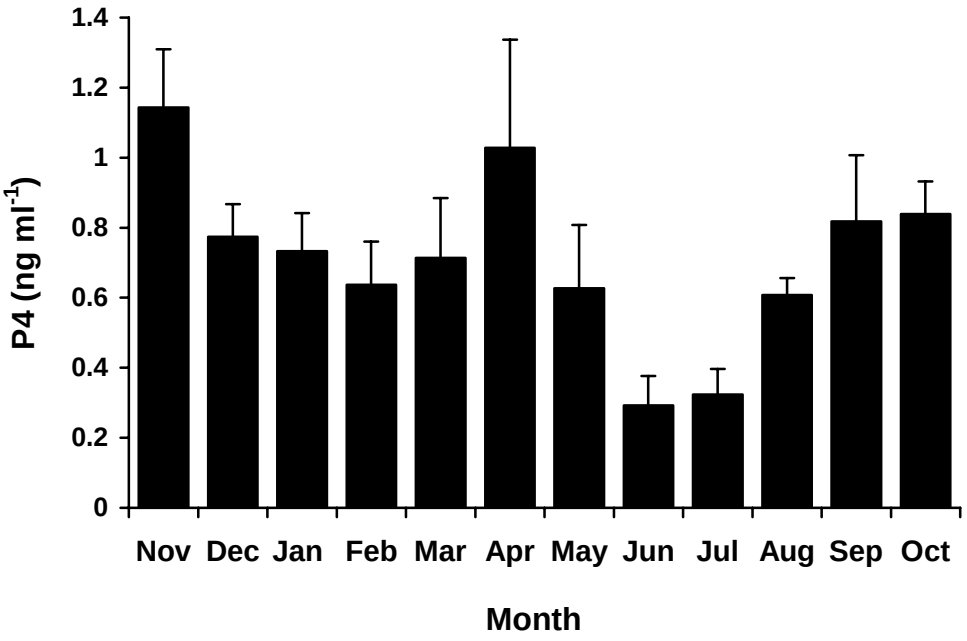

Chapter 4 Reproductive cycle of female *Tiliqua nigrolutea*

4.1 Introduction

4.1.1 Timing of reproduction

In viviparous squamates parturition usually occurs at the time of year that provides optimal conditions for growth and survival of neonates (Goldberg, 1971; Callard *et al.*, 1972c; Vitt and Lacher, 1981; Abts, 1988; Olsson and Shine, 1997). In temperate regions, this is usually summer or early autumn (Whittier *et al*., 1987; Naulleau and Fleury, 1990; Van Wyk, 1994; Jones and Swain, 1996; Jones *et al*., 1997). The timing of ovulation and the length of gestation are key variables determining when parturition occurs in viviparous squamates (Heatwole and Taylor, 1987), and a variety of reproductive strategies have evolved to ensure that young are born into favourable conditions.

The most common reproductive pattern in temperate zone squamates is one in which vitellogenesis, ovulation, gestation and parturition are all completed within one year (Gavaud, 1983; Shine, 1985; Yokoyama and Yoshida, 1994; Jones and Swain, 1996; Jones *et al*., 1997). Squamate ovarian follicles undergo a period of early growth known as primary vitellogenesis, in which RNA and nutrients are transferred into the follicle but no yolk is deposited (Aldridge, 1982; Motta *et al.*, 1995; Uribe *et al*., 1995). This is followed by more rapid growth (secondary vitellogenesis) as yolk protein accumulates around the oocyte (Aldridge, 1979; Saint Girons, 1985; Estrada-Flores *et al*., 1990). Two patterns of yolk deposition have been described in temperate zone squamates (Aldridge, 1979; Yokoyama and Yoshida, 1994): type I, in which vitellogenesis is initiated and completed wholly after spring emergence prior to ovulation as occurs in some snakes (Aldridge, 1979; Whittier *et al*., 1987; Yokoyama and Yoshida, 1994); and type II vitellogenesis, which begins in late summer or autumn following parturition and is completed the following spring (Aldridge, 1979; Yokoyama and Yoshida, 1994). The latter pattern has been described in several lizards (Flemming, 1993c; Van Wyk, 1994; Cree and Guillette, 1995) and snakes (Krohmer and Aldridge, 1985; Seigel *et al*., 1995).

Ovulation usually occurs in spring (Xavier, 1982; Semlitsch and Moran, 1984; Taylor, 1985; Naulleau and Fleury, 1990; Shine *et al*., 1996a), presumably to allow gestation to begin as early as possible once thermal conditions become favourable in a potentially limited active season. Females are usually pregnant during the warmer part of the active season, as the length of gestation is largely determined by temperature (Beuchat, 1986; Shine and Harlow, 1993; Mathies and Andrews, 1995; Castilla and Swallow, 1996). The rate of embryonic development increases with rising temperature, and so gestation length is influenced by the body temperature and thermoregulatory opportunities of the pregnant female (Beuchat, 1988; Schwarzkopf and Shine, 1991; Méndez-de la Cruz *et al.*, 1998; Wapstra, 1998). A prolonged gestation is undesirable, resulting in offspring which might be less fit (Schwarzkopf and Shine, 1991; Wapstra, 1998) and leaving the female less time before hibernation in the post-partum period to feed and store energy reserves for winter (Saint Girons, 1985).

However, the timing of the mating period is less rigidly constrained and need not correspond with any particular physiological stage of the reproductive cycle. Mating may coincide with spring ovulation (Taylor, 1985; Shea, 1992; Rostal *et al.*, 1998; this study), or be temporally dissociated from it, occurring in late summer or autumn with fertilisation accomplished later using sperm stored by the female (Joss and Minard, 1985; Whittier *et al.*, 1987; Whittier and Limpus, 1996; Jones *et al.*, 1997). In such species, the hormonal control of gonadal development may become dissociated from the hormonal control of courtship and copulatory behaviours (Whittier *et al.*, 1987; Crews and Gans, 1992).

4.1.2 Reproductive behaviour

Female reproductive behaviour in reptiles is likely to be under the influence of steroids (Adkins-Regan, 1981; Flores and Crews, 1995; Wade, 1997). Many authors note an elevation of plasma steroid concentrations coincident with the occurrence of reproductive behaviours (Bona-Gallo *et al.*, 1980; Joss, 1985; Saint Girons *et al.*, 1993; Jones *et al.*, 1997; Rostal *et al.*, 1998). Sexual receptivity, copulatory and rejection behaviours have been described for some species (Ferguson, 1966; 1970; Carpenter, 1978; Secor, 1987; Martins, 1994; Schuett and Duvall, 1996), but only occasionally in conjunction with endocrinological data (Cooper *et al.*, 1986a; Saint Girons *et al.*, 1993). However, causal relationships between elevated plasma 17β-oestradiol (E2), progesterone (P4) or testosterone (T) and observed reproductive behaviours are difficult to demonstrate (Moore and Lindzey, 1992). In those species in which peak gonadal development is temporally dissociated from displays of reproductive behaviour there is an intuitive paradox, whereby maximal gonadal development is associated with high plasma steroid concentrations without the induction of mating behaviour. For most reptiles, the mechanism of this differential control is yet to be elucidated. Hence, there is a basic need for studies including both observations of reproductive behaviours and coincidental changes in plasma steroid hormone concentrations.

4.1.3 Steroid hormones

Significant increases in plasma steroid hormone concentrations are often correlated with the timing of important events in the reproductive cycle. In females of seasonally cycling squamates an annual pattern of plasma concentrations is usually evident for each of the three primary gonadal steroids, P4, E2 and T, suggesting a role for each steroid in the regulation of various stages of the annual reproductive cycle. In addition to the direct individual actions of each hormone, it is important to consider potential interactions between steroids: many aspects of reproductive physiology are known to be under multihormone control (Guillette *et al.*, 1981; Ho *et al.*, 1982; Ho, 1987; Callard *et al.*, 1992).

As in many other vertebrates, P4 has a role in the maintenance of gestation in viviparous reptiles (Highfill and Mead, 1975b; Naulleau and Fleury, 1990). This may be an indirect role through the antigonadal properties ascribed to P4 (Callard *et al*., 1972a): elevated P4 is thought to inhibit E2-induced follicular growth during pregnancy (Callard *et al*., 1972b; Guillette *et* al., 1981; Ho, 1987), ensuring that vitellogenesis and gestation are mutually exclusive (Callard *et al.*, 1992). This is particularly important in viviparous temperate zone reptiles, which usually produce only a single clutch each year (Dunham *et al.*, 1988).

The major source of P4 during gestation is widely believed to be the corpus luteum (CL) (Callard *et al*., 1972b; Arslan *et* al., 1978a; Xavier, 1982; Kleis-San Francisco and Callard, 1986), the transitory endocrine organ formed from each post-ovulatory follicle (Weekes, 1934; Guillette and Jones, 1985; Xavier, 1987). The life span of the CL has been positively correlated with P4 production in some species (Callard *et al*., 1972b; Bona-Gallo *et al*., 1980), but not in others (Guillette *et al.*, 1981; Guarino *et al*., 1998). There is, in fact, considerable variation in patterns of circulating P4 concentrations among viviparous squamates, especially regarding the proportion of gestation during which P4 remains elevated (Xavier, 1982; Van Wyk, 1994; Jones *et al.*, 1997).

In most snakes and lizards, elevated plasma E2 concentrations are associated with the vitellogenic phase of the reproductive cycle (Bona-Gallo *et al*., 1980; Joss, 1985; Moore and Crews, 1986; Van Wyk, 1994; Jones *et* al., 1997). 17β-Oestradiol is almost exclusively the hormone responsible for the stimulation of hepatic vitellogenesis in reptiles (Callard *et al*., 1972b; Callard and Ho, 1987; Ho, 1987; Kime, 1987; Diaz *et al.*, 1994). It may also have a role in the uptake of vitellogenin by the follicle, as it is known to induce cellular endocytosis (Callard and Ho, 1987). 17β-Oestradiol is synthesised by the growing follicles (McNicol and Crews, 1979; Etches and Petitte, 1990) and plasma concentrations fall rapidly at or around the time of ovulation (Callard *et al*., 1978; Bona-Gallo *et al*, 1980; Yokoyama and Yoshida, 1994). As with circulating P4 concentrations, annual patterns of circulating E2 concentrations vary among viviparous squamates.

Little is known about the functional significance of T in reproduction in female reptiles beyond its importance as a precursor in the synthesis of oestrogens (Staub and De Beer, 1997). Published studies focus on oviparous species, in which a cyclic pattern of plasma T concentrations is apparent (Arslan *et al*., 1978a; Callard *et al*, 1978; Bona-Gallo *et al*., 1980; Callard and Kleis, 1987; Whittier *et al*., 1987; Cree *et al*., 1992; Saint Girons *et al*., 1993; Rostal *et al*., 1998). The T in the plasma is likely to be of both ovarian and adrenal origin (Staub and De Beer, 1997; Wade, 1997). A survey of the literature suggests the involvement of T in vitellogenesis in the snakes *Thamnophis sirtalis parietalis* (Whittier *et al.*, 1987) and *Naja naja* (Bona-Gallo *et al.*, 1980), and in the hypertrophy of the oviduct in the lizards *Anolis carolinensis* (Jones and Guillette, 1982) and *Hemidactylus flaviviridis* (Prasad and Sanyal, 1969). Plasma T concentrations are significantly elevated above basal concentrations in the periovulatory period in *T. s. parietalis* (Whittier *et al.*, 1987), *N. naja* (Bona-Gallo *et al.*, 1980) and in the lizard *Uromastix hardwicki* (Arslan *et al.*, 1978a). Mean plasma T also rises during the mating period in the turtle *Lepidochelys kempi* (Rostal *et al.*, 1998), the tuatara *Sphenodon punctatus* (Cree *et al.*, 1992) and the viviparous snake *Vipera aspis* (Saint Girons *et al.*, 1993). In addition, T is known to work synergistically with P4 to inhibit E2-induced vitellogenesis in reptiles (Ho, 1987; Ho *et al.*, 1982). The mechanism of action of T in female reptiles is yet to be elucidated.

4.1.4 This study

Reptiles exhibit conservation of the structures and functions of the steroid hormones that regulate reproduction. Numerous studies covering various aspects of the reproductive biology of a wide range of taxa have contributed to our understanding of the steroid hormone control of reproductive physiology and behaviour in female reptiles. However, generalisations from these studies sometimes prove inaccurate. Many examples of significant deviations from expected patterns of reproduction have been documented. For example, one population of a viviparous gecko *Hoplodactylus maculatus* gestates young for 14 months, while in other populations gestation extends for only three to five months (Cree and Guillette, 1995). Some populations of the lizards *Saiphos equalis* (Smith and Shine, 1997) and *Lerista bougainvillii* (Qualls and Shine, 1995) are viviparous while others are oviparous. The genera *Cnemidophorus* (Moore *et al*., 1985a; Moore and Crews, 1986) and *Heteronotia* (Whittier *et al*., 1994) contain both parthenogenetically and sexually reproducing species. Such intraspecific variations in life history are likely to be related to fundamental differences in endocrinology. Additionally, and from an endocrine perspective, the garter snake *T. s. parietalis* displays an aberrant P4 profile; plasma P4 concentrations are never elevated above basal concentrations throughout the annual cycle, even during gestation (Whittier *et al*, 1987). In the oviparous viper, *Trimeresaurus flavoviridis*, plasma E2 is synthesised not by the ovaries, but by the adrenal glands (Yokoyama and Yoshida, 1994).

There are surprisingly few squamate species for which both detailed endocrinological and behavioural observations have been reported, even though such studies are vital to our understanding of the role of steroid hormones in the control of behaviour. This type of investigation will enhance our understanding of the links between steroid hormones and reproductive behaviour. There are many apparent paradoxes between the control of reproductive physiology and the display of mating and other reproductive behaviours, particularly in those squamates in which the mating period is dissociated from the gonadal cycle, which warrant closer examination. Such studies will be aided by first examining species such as *Tiliqua nigrolutea*, in which maximal gonadal activity is synchronised with mating activity.

Furthermore, an accumulation of related information about a single species requires that fewer assumptions be made from trends described in the literature. To this end, I have comprehensively examined the reproductive cycle of females of the viviparous lizard, *T. nigrolutea*. The timing of reproductive events and annual cycles of plasma P4, E2 and T are presented, with correlated histological changes and basic life history information. I also describe reproductive behaviours (receptivity, copulation and rejection) and other behaviours observed throughout the reproductive cycle.

4.2 Materials and methods

General methods are described in *Chapter 2*, but information specific to the work in this chapter is presented here.

4.2.1 Blood sampling

A captive population of 12 reproductively active and 10 non-reproductive female *T. nigrolutea* was used for this study, although the data set of hormone values was reduced because blood samples were missed from some animals. During the vitellogenic, mating and ovulation periods, blood samples were collected fortnightly from all females, at the start of the first (A) and third (B) weeks of each month (Sept 1997 - Dec 1997). Monthly sampling of all individuals continued until the end of the reproductive season (Jan 1998 - Apr 1998). An additional blood sample was collected from each reproductively active female within 24 hr of parturition.

4.2.2 Histology

No animals were killed solely to provide material for histological examination of reproductive tissues. The ovaries of vitellogenic (spring, N = 2), pregnant (summer, N = 2) and post-partum (autumn, N = 2) were available for histological examination when animals were killed for another experiment. Ovarian tissues from non-vitellogenic (spring, N = 1), and non-reproductive (summer, N = 1) females were also available for comparison. (See *Section 2.5* for details regarding preparation of samples.). For each sample measurements (N = 25) of theca and granulosa thickness (pyriform cell diameter) and widths of hyaline band and zona radiata (ZR) regions of the zona pelucida (ZP) were made. Points of measurement were distributed evenly around the largest follicle present in each preparation. Follicles were staged according to Uribe *et al*. (1995; 1996).

4.2.3 Life history characteristics

During the three year study period a variety of life history data were collected. Some of these data are reported here:

1) *Mean relative clutch mass* (RCM). This was determined, first, separately for the most successful (97/98) reproductive season and, second, for the entire study. Relative clutch mass was calculated as:

fresh neonate mass (g) / maternal post-partum mass (g)

This method avoids the statistical problem of the maternal mass occurring in both the numerator and the denominator (Shine, 1980). This represents a conservative estimate of female reproductive effort as the mass of amniotic fluid expelled at birth is not included.

2) *Reproductive frequency*. Many females in the captive population were held for two or more reproductive seasons (N = 19), so frequency of reproduction could be assessed directly for these individuals.

3) *Reproductive condition*. Wild-caught and road killed individuals (N = 73) were also assessed for reproductive condition throughout each active season (by dissection of dead animals or by the occurrence or absence of later parturition events) as a measure of reproductive frequency in the wild population.

4.2.4 Behavioural observations

All behavioural observations were made on an informal basis. During the mating period (approximately mid-Oct to the end of Nov) all individuals were observed almost continuously each day from the time they became active each morning (approx. 1030) until they submerged in the afternoon (approx. 1500 – 1700). During gestation all animals were again observed every day, for 10 min at approximately one hour intervals. During the parturition period, cages holding gestating females were checked every morning for the presence of neonates, and all pregnant animals were checked every 30 to 60 min from 1030 to 1600 for the onset of parturition.

4.2.5 Statistics

All statistical analyses were performed using SYSTAT 5.2 for the Macintosh (Wilkinson *et al.*, 1992). A significance level of $\alpha = 0.05$ was used throughout. All data points were initially log-transformed to satisfy the assumptions of normality and homogeneity of variance. Occasional missing individual data points resulted from collecting insufficient plasma to complete all three assays. These were assigned the mean value for animals of like reproductive status in the same sample period, although no more than one such value was assigned to any sample set or any individual animal (Mundry, 1999; D. Ratkowsky, pers. comm.). During analysis, several data points were flagged as outliers by the statistics package. These data were re-examined and were retained for analysis unless a valid reason could be found to exclude them (e.g. plasma clotting prior to the assay). Data from the initial sampling period (Sept A) was not included in analyses as many animals had not yet emerged from hibernation at that time. Annual patterns of mean plasma steroid concentrations were examined in both pregnant and non-pregnant female *T. nigrolutea* by two-way repeated measures Analysis of Variance ((M)ANOVA). The multivariate output of these analyses (Pillai trace statistic) tested both for changes in mean plasma steroid concentration through time and for time-state interactions, where "state" was the pregnant or non-pregnant condition of the lizards. Multivariate analysis was used because the univariate output from the SYSTAT programme is unsuitable, due to a lack of independence of the data through time (C. Johnson, pers. comm.). As Tukey's *post hoc* test is unsuitable for pairwise comparison of multivariate output, *a posteriori* unpaired Student *t* tests were conducted to compare values for pregnant and non-pregnant animals for each steroid hormone. These targeted either the mating (E2) or ovulation (T, E2) periods because the precise timing of these events was unknown before hormone assays were conducted. Visual examination of completed hormone profiles and concurrent behavioural observations of the captive sample population determined target sample sets.

Regression analysis was used to examine relationships between mean peak plasma steroid concentrations and both female SVL through the active season and the number of offspring produced by each reproductively active female.

Chi squared analysis was used to assess whether the frequency of reproduction varied significantly from an annual pattern of reproduction in which all females reproduce each year.

4.3 Results

4.3.1 Behavioural observations

Mating

No agonistic interactions between females were ever observed. Captive adult females were unreceptive toward males at spring emergence (Oct A) despite persistent and daily attention. Mating was observed in full on seven occasions. Females either remained still or moved a short distance away in response to male tongue-flicking around the cloaca, body and neck. During the mating period (Nov), females moved around the cage, closely followed by a single male. Often a female would run away and subsequently other males in the cage would begin to chase her, or other females. This attention was non-preferential, directed at both reproductively active and inactive females, and continued throughout the mating period (approximately four weeks). When a male attempted to gain a copulatory bite hold above the shoulder, the female fought, struggled and bit the male on the head and body. Both receptive and unreceptive females behaved in this way, arching and rounding their bodies to prevent the male obtaining a bite hold. Often the female escaped and the male gave chase. A receptive female eventually allowed the male to obtain a bite hold and remained passive as he moved her body into position, with cloacae aligned. When the male tapped and scraped with his foot at the base of her tail, she raised her tail and gaped her cloaca, allowing the male to intromit a hemipenis. In contrast, an unreceptive female would not allow cloacal alignment, nor would she gape her cloacal opening for the male. The time from the final chase and obtaining a bite hold to the end of intromission was usually approximately 30 minutes. Males retained a post-copulatory bite hold on the female for an hour or more, which may constitute some form of mate guarding. During this time many females displayed rhythmic sinusoidal lower abdominal contractions, although it is not known if these were the result of ovulation taking place immediately, or simply to assist the movement of sperm along the reproductive tract. Females struggled vigorously to escape while being held by the male and moved away immediately they were released. Females became sexually unreceptive rapidly following this release. Further attention from males elicited biting, hissing and gaping, lunging and butting, presumably to deter any subsequent mating attempts. Males

often responded to this by crouching (possibly submissive) and a jerky, stilted retreat from the female. Over three seasons, only one female was observed to copulate more than once, and in this case the male did not allow the female to move away between intromissions. Photographs of the mating sequence are included in *Chapter 3 Results*.

Gestation

The mating period occupied approximately four weeks in spring (late Oct - mid Nov). Thereafter, females received no attention from males, but basked on all sunny days and fed regularly. The proportion of time spent basking was not recorded, but did increase noticeably throughout gestation. During late gestation (late Jan - Mar), feeding by gravid females was greatly reduced; in some cases females fed little or not at all for several weeks prior to parturition. Respiratory depth and rate were also altered, from regular, relatively shallow breaths during early gestation, to sporadic and very deep inhalations that caused the whole upper torso to jerk. Late gravid females moved around very little, emerging as sun entered the cages in the morning, basking throughout the day, and returning to cover in the mid - late afternoon (1500 – 1700 hrs). Shuttling between sun and shade involved moving only 30 - 50 cm, several times each day, while non-reproductive females and males foraged actively for much of the day. Gestation length was calculated from the day each female was observed mating to the day of parturition. For those individuals for which copulation was not observed, the date of mating was estimated as the mid-point between the date an individual was observed being chased by male lizards and the date that rejection behaviour towards males was first noticed. All animals were observed each day during the mating period, ensuring that these estimates were accurate. Mean length of gestation was 130.7 ± 4.66 days (1 SE) (N = 20), but ranged from 104 to 184 days.

Parturition

Parturition was observed on many occasions (N = 11) and occurred from late summer (late Feb) to late autumn (late May). There was no change in a female's behaviour in the days before birth, or on the day of parturition itself, until birthing actually began. Following several hours of basking the gravid female became restless and began moving around, both in the leaf litter at the back, and in the open area at the front of the cage.

Sporadic lower abdominal contractions were observed, with the female also repeatedly undulating her whole body sinusoidally. Stronger contractions of the lower abdomen followed, with the female raising her back legs and lifting her lower abdomen off the substrate. Several such efforts were required for the neonate to be positioned ready for birth. Even stronger contractions followed (approximately 5) with the female also arching her tail to expel the neonate.

Young were born surrounded by embryonic membranes and attached to a yolk sac containing a small amount of residual yolk. Young were often born curled in a circle, with head and tail overlapping (Figure 4.1); they wriggled vigorously to free themselves. Sometimes, however, the embryonic membranes burst during expulsion and the neonate emerged headfirst (Figure 4.2). Often, the female began walking away as the neonate was emerging, dragging it behind her for a metre or more until it was completely expelled. Time between births varied from only a few seconds, when two neonates were expelled by the same set of contractions, to approximately 30 min. Between births, the female wandered around the entire cage. She paid no direct attention to neonates after they were born, as they tore off and consumed their birth membranes and the residual yolk contained within the yolk sac (Figure 4.3). Occasionally a neonate had great difficulty bursting the membranes and struggled more and more feebly as it became cold and tired

Figure 4.1 Some neonates are born curled, enclosed in membranes.

Figure 4.2a Typical leg-raising and tail-arching posture during parturition.

Figure 4.2b Neonate emerging headfirst.

Figure 4.3 Neonate consuming residual yolk and membranes.

Figure 4.4 A neonate which required assistance tearing off and consuming birth membranes.

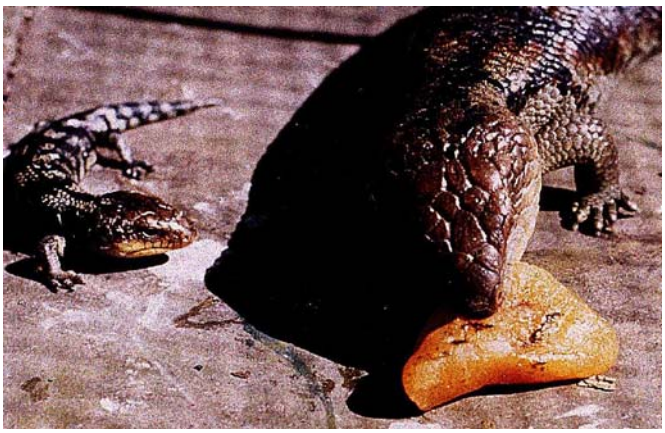

Figure 4.5 Post partum female consuming unfertilised yolk mass.

(Figure 4.4); this attracted no attention or assistance from the mother. In several parturition events that were not observed, young that had failed to escape their embryonic membranes were found dead. Thereafter, I assisted any others I saw in great difficulty by piercing membranes for them. While most females completed parturition in a single day, several females gave birth to an extra neonate the following day. This was usually stillborn, or did not successfully emerge from embryonic membranes, and was often found dead at the back of the cage. Unfertilised eggs were also passed as large yolky masses, sometimes between live births, but more often at the completion of parturition. The female later consumed these (Figure 4.5). While females did not tongue-flick, sniff, touch or approach their newborns, some were extremely aggressive towards observers entering the cage during the parturition period and up to 60 min following the birth of the last neonate. Attempts to approach or handle young or remove stillborn babies were met with aggressive lunging and biting attacks.

4.3.2 Histology

Histological features observed at each stage of the ovarian cycle are summarised in Tables 4.1 and 4.2. The thecal layer was visible throughout the ovarian cycle, but only as a single layer. In both reproductive and non-reproductive females, the granulosa layer was polymorphic (pyriform, intermediate and stem cells visible) throughout the year (Figure 4.6), with the exception of a monomorphic granulosa (stem cells only) in the post-partum individuals (Figure 4.7). The zona pellucida (ZP) became differentiated into an outer hyaline and an inner striated zona radiata (ZR) in both reproductive and non-reproductive females throughout the spring vitellogenic period. However, during this time, yolk granules were deposited only in the ooplasm of reproductive females. Vacuoles (Figure 4.8) were present throughout the ooplasm during hibernation, but became localised peripherally by emergence and were situated in the centre of the follicle during gestation.

Table 4.1 Features of ovarian follicles throughout the reproductive cycle in reproductive female *Tiliqua nigrolutea.* Data collected from autopsied animals and histological examination of ovarian tissue. ZR = zona radiata.

season	winter	early spring	late spring	summer	autumn

	Jun-Aug	Sep	Oct-Nov	Feb	Mar-Apr
activity	hibernation	emergence	mating period	gestation	post-parturition
thecal width (μm)	single layer (17.9)	single layer (27.4)	-	single layer (24)	single layer (39.4)
granulosa (μm)	polymorph. (21.0)	polymorph. (53.1)	-	polymorph. (50.7)	monomorph. (20.7)
zona pellucida (μm)	homogen.	differentiated (hyaline: 2.5) (ZR: 2.7)	-	homogen. (hyaline 2.5)	homogen. (hyaline: 4.2) (N = 1)
ooplasm	vacuolated throughout	vacuolated peripherally	-	vacuolated centrally	vacuolated throughout
yolk granules	absent	present peripherally	-	absent	absent
ovarian stage	previtell.	early vitell.	late vitell.	previtell.	previtell.
diameter of largest follicle (cm)	< 0.5	0.6 - 1.0	1.5 - 2.3	< 0.5	< 0.5
N	1	2	4	2	2

Table 4.2 Features of ovarian follicles throughout the reproductive cycle in non-reproductive female *Tiliqua nigrolutea.* Data collected from histological examination of ovarian tissues. ZR = zona radiata.

season	**late spring Oct-Nov**	**summer Feb**
activity	mating period	Quiescent
thecal width (μm)	single layer (28.9)	single layer (76.1)
granulosa (μm)	polymorph. (51.1)	polymorph. (44.0)
zona pellucida (μm)	differentiated (hyaline: 1.2) (ZR: 1.8)	homogen. (hyaline: 5.1)
ooplasm	vacuolated peripherally	vacuolated throughout
yolk granules	absent	present peripherally
ovarian stage	previtell.	previtell.
diameter of largest follicle (cm)	< 0.5	< 0.5
N	1	1

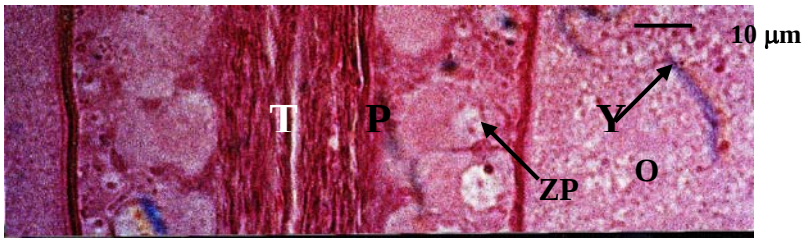

Figure 4.6 Walls of two adjacent early vitellogenic-stage follicles. The theca (T) appears as a single layer, the granulosa is polymorphic (PG) and the zona pellucida (ZP) is undifferentiated. Yolk (Y) droplets are visible in the ooplasm (O).

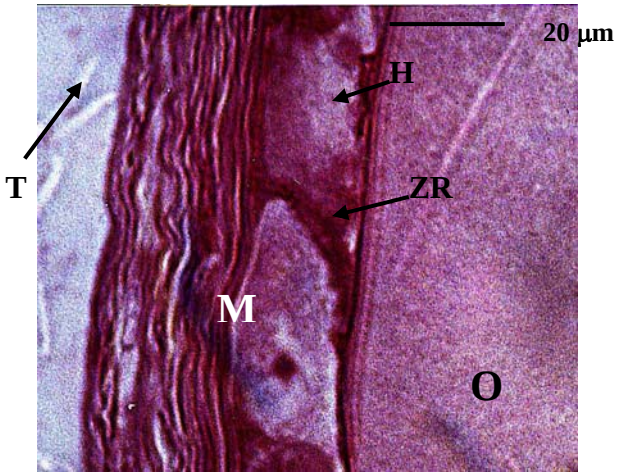

Figure 4.7 Follicle from a post partum individual. The theca (T) appears as a single layer, the granulosa layer is monomorphic (MG) and no yolk is visible in the ooplasm (O). The zona pellucida is differentiated into a hyaline band (H) and the zona reticularis (ZR).

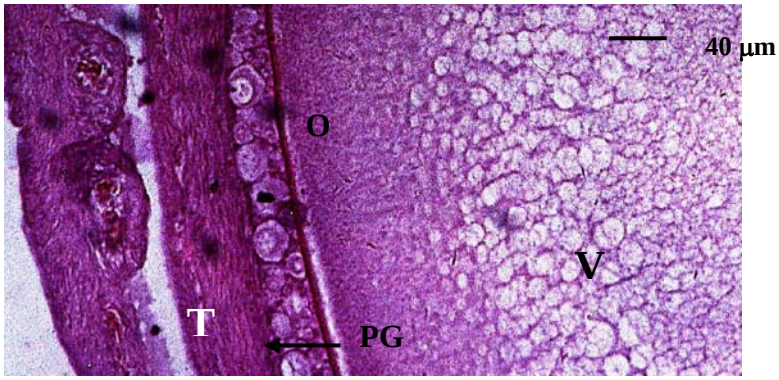

Figure 4.8 A non-vitellogenic follicle during gestation. The theca (T) appears as a single layer and the granulosa is polymorphic (PG). Vacuoles (V) are located centrally in the ooplasm (O).

4.3.3 Gonadosomatic index

A series of gonadosomatic indices (GSI) were generated (GSI = (total ovarian mass (g) / total body mass (g)) x 100) for females killed for other experiments and recent, intact road-killed individuals (Figure 4.9). While small sample sizes and opportunistic collection of data preclude statistical analysis, a distinctive pattern is evident.

Ovaries were regressed during hibernation, but increased in diameter and mass during early spring (Sept) as vitellogenesis began. Ovarian mass relative to body mass was greatest at the conclusion of vitellogenesis (Oct - Nov) shortly before mating. Following ovulation ovaries remained regressed throughout gestation (Dec - Feb), the post-partum period (Mar - Apr) and hibernation (Apr - Sept). Data from a non-reproductive female with regressed follicles during summer have also been included.

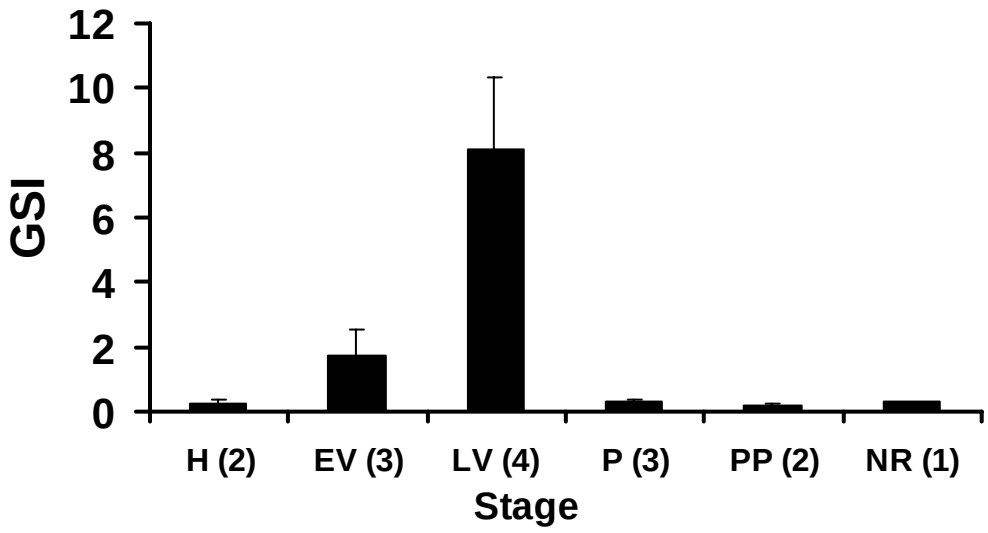

Figure 4.9 Gonadosomatic index (GSI) for female *Tiliqua nigrolutea*. GSI = (total ovarian mass (g) / total body mass (g)) x 100. Sample sizes in parentheses. (H: hibernation, EV: early vitellogenesis, LV: late vitellogenesis, P: pregnancy, PP: post-parturition, NR: non-reproductive (summer)).

4.3.4 Plasma steroid concentrations

Progesterone

A seasonal pattern of variations in plasma P4 concentrations was evident. A comparison of mean plasma P4 concentrations in reproductively active and inactive female *T. nigrolutea* throughout the reproductive season (spring (Sept) 1997 - autumn (Apr) 1998) is shown in Figure 4.10. In inactive females, P4 concentrations were low (1.1 ± 0.20 ng ml^{-1}) at emergence in early spring (Sept B) and remained basal (1 - 2 ng ml^{-1}) throughout the active season. In reproductive females, mean plasma P4 concentration became significantly elevated above that of inactive females in mid spring (Nov A) ((M)ANOVA: F = 52.600, df = 10, P = 0.000) and remained high, peaking in the second trimester (Jan) of gestation (12.7 ± 1.27 ng ml^{-1}). Mean plasma P4 concentrations fell by late summer (Feb) and returned to basal concentrations by early autumn (Mar).

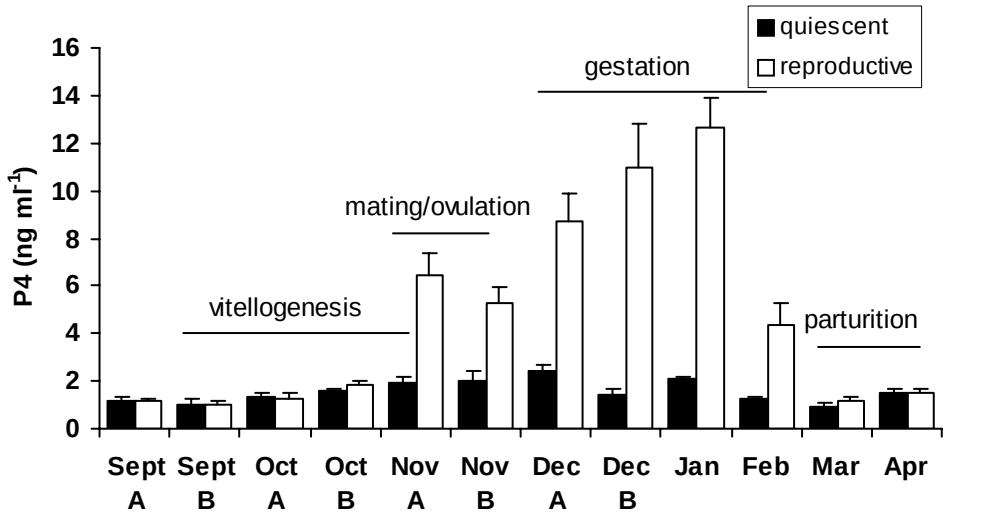

Figure 4.10 Plasma P4 concentrations in reproductively active (N = 8) and inactive (N = 8) female *Tiliqua nigrolutea* throughout the active season. Sampling was fortnightly from September 1997 to December 1997 and then monthly until April 1998. Values are means ± 1 standard error. (Sample to the left of the vertical broken line was not included in the statistical analysis: see text).

Multivariate analysis also revealed a significant interaction effect between time and condition of females (reproductive or non-reproductive) ((M)ANOVA: F = 11.634, df = 10, P = 0.007). There was no significant change in mean plasma P4 concentration

between late pregnancy and within 24 hr of parturition (Appendix 4.1) and there was no correlation between peak mean plasma P4 concentration (mid summer (Jan)) and the SVL or the number of offspring of reproductively active females.

17β-*Oestradiol*

Mean plasma E2 concentration varied significantly throughout the active season ((M)ANOVA: $F = 354.117$, df = 10, $P = 0.000$) (Figure 4.11). Multivariate analysis also revealed a significant interaction effect between time of year and reproductive condition of females ((M)ANOVA: $F = 5.903$, df = 10, $P = 0.032$).

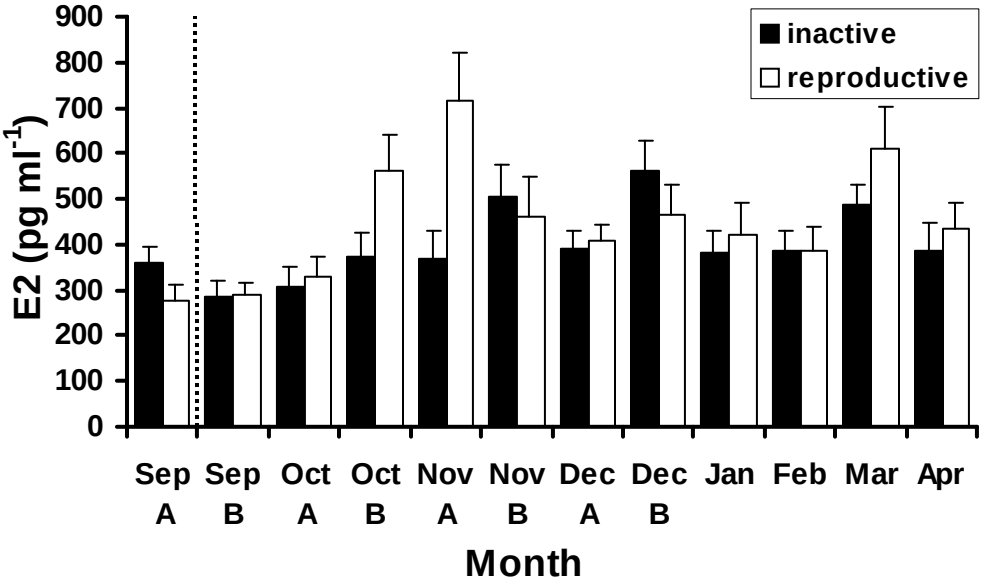

Figure 4.11 Plasma E2 concentrations in reproductively active (N = 8) and inactive (N = 8) female *Tiliqua nigrolutea* throughout the active season. Sampling was fortnightly from September 1997 to December 1997 and then monthly until April 1998. Values are means ± 1 standard error. (Sample to the left of the vertical broken line was not included in the statistical analysis: see text).

While plasma E2 concentrations in non-reproductive females fluctuated throughout the active season, there were no significant changes with time. In contrast, mean plasma E2 concentration in reproductively active females was low (275.2 ± 36.87 pg ml^{-1}) at spring emergence (early spring (Sept B)) and increased during mid spring (Oct A and B), peaking later in spring (Nov A) at 715.1 ± 106.68 pg ml^{-1}. *A posteriori* unpaired *t* tests demonstrated that mean plasma E2 concentrations in reproductively active females were

elevated significantly above those of inactive females at times corresponding to late vitellogenesis and mating (mid spring (Oct B)) (*t* test: $t = -2.337$, df = 14, $P = 0.035$) and to ovulation (late spring (Nov A)) (*t* test: $t = -3.032$, df = 14, $P = 0.009$). There was no significant change in mean plasma E2 concentration between late parturition and within 24 hr of parturition, and there was no correlation between peak mean plasma E2 concentration (late spring (Nov A)) and the SVL or number of offspring of reproductively active females.

Testosterone

Significant variations in mean plasma T concentrations in reproductive females were observed through the active season ((M)ANOVA: $F = 46.339$, df = 10, $P = 0.000$) and a similar pattern was observed in inactive females (Figure 4.12). The dominant feature of the cycle was a peak in plasma T concentrations in late spring (Nov B) (reproductive: 6.3 ± 0.63 ng ml^{-1}, non-reproductive: 4.7 ± 0.31 ng ml^{-1}) and a marked decline to < 1 ng ml^{-1} by early summer (Dec A) in all individuals. An *a posteriori* unpaired *t* test showed a significant elevation in mean plasma T concentration in reproductively active females above that of inactive animals in late spring (Nov B) (*t* test: $t = -2.415$, df = 14, $P = 0.030$); this corresponded to the ovulation period in reproductively active females. There was no significant change in mean plasma T concentration between late gestation and within 24 hr of parturition, and there was no correlation between peak mean plasma T concentration (late spring (Nov B)) and the SVL or number of offspring of reproductively active females.

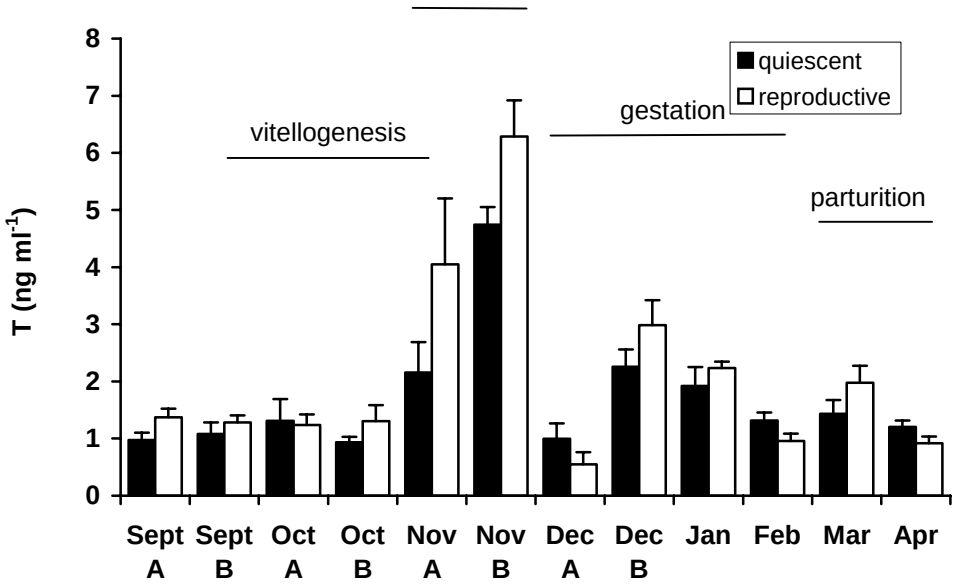

Figure 4.12 Plasma T concentrations in reproductively active (N = 8) and inactive (N = 8) female *Tiliqua nigrolutea* throughout the active season. Sampling was fortnightly from September 1997 to December 1997 and then monthly until April 1998. Values are means ± 1 standard error. (Sample to the left of the vertical broken line was not included in the statistical analysis: see text).

4.3.5 Life history characteristics

Relative clutch mass

The mean relative clutch mass for all females across the combined three seasons of this study was 0.40 ± 0.040 (N = 17), and the mean RCM for the most successful season (with the greatest number of clutches produced) (97/98) was 0.45 ± 0.050 (N = 12). Clutch size varied from one to 15 (mean = 7.9 ± 0.80, N = 17) and was not correlated with maternal body length (SVL).

Frequency of reproduction in captivity

Five individuals in the captive population produced a clutch in the 95/96 reproductive season, no clutch in 96/97, and either became vitellogenic (determined at autopsy) or produced a second clutch in the 97/98 season. Of the individuals held for two seasons or more (N = 19), no female produced offspring in consecutive years. Many captive females successfully produced young following at least one quiescent year (N = 12), but some

females of adult size failed to reproduce after two (N = 3) or three (N = 4) seasons in captivity.

Frequency of reproduction in the wild

The proportion of wild-caught female *T. nigrolutea* that was reproductively active in each season and for the entire study is summarised in Table 4.3. Over the three reproductive seasons of this study 39.7 % of females captured or dissected during the reproductive season were pregnant or gave birth to young. However the proportion of females in the wild that were reproductively active varied between seasons, at least for the sample observed. Chi squared analysis indicated that the proportion of females that was reproductively active in a given season differed significantly from an annual reproductive pattern (100 % of adult females pregnant each year) for every season of this study (95/96: $P < 0.005$, 96/97: $P < 0.0005$, 97/98: $P < 0.050$).

Table 4.3 Proportion of reproductively active and inactive wild-caught female *T. nigrolutea* in each reproductive season and for the entire study.

active season	females caught or dissected (N)	reproductive females (%)
1995/1996	21	33.3 (N = 7)
1996/1997	24	20.8 (N = 5)
1997/1998	28	60.7 (N = 17)
combined 1995-1998	73	39.7 (N = 29)

4.4 Discussion

4.4.1 Timing of reproduction

Cool-temperate zone reptiles are constrained by the need to reproduce during the warm months of the year (Saint Girons, 1985; Shine, 1985). If the active season is short, there may be insufficient time to complete vitellogenesis, ovulation, gestation and parturition in a single season (Saint Girons 1985). Viviparity itself is believed to have evolved as an adaptation to cold climates (Shine 1983; Mathies and Andrews, 1995; Méndez-de la Cruz *et al.*, 1998), and several different strategies have developed among viviparous squamates to facilitate reproduction in cool or cold environments. These include the extension of the period of vitellogenesis (Cree and Guillette, 1995) or gestation (Vial and Stewart, 1985; Hutchinson *et al.*, 1989; Cree and Guillette, 1995) such that a reproductive cycle extends over more than one active season at the expense of the ability to reproduce annually. However, some viviparous lizards with a cool temperate distribution are able to reproduce each year (Jones and Swain, 1996; Jones *et al.*, 1997).

Females of the viviparous skink *Tiliqua nigrolutea* exhibit a reproductive cycle that is completed within a single active season (late Sept – Apr), despite their cool temperate zone distribution in Tasmania, Australia. The ovarian follicular cycle begins in early spring (late Sept) with the initiation of vitellogenesis and a marked increase in the GSI over the vitellogenic period (Figure 4.9), which is completed prior to late spring (Nov) mating and ovulation. However, gestation (approximately 4 – 4.5 months (Shea, 1989; this study)) is long by comparison with sympatric viviparous species (3.5 – 4 months (Jones and Swain, 1996; Jones *et al.* 1997) and parturition usually occurs late in the active season (autumn: Mar – Apr). This pattern is identical to a description for female *T. nigrolutea* throughout their range (Tasmania and the south-eastern Australian mainland) (Rawlinson, 1974), and is also very similar to that described for other *Tiliqua* species, based on an examination of museum specimens (Shea, 1992).

Histological examination of the ovaries of *T. nigrolutea* throughout the year revealed a sequence of follicular development correlated with the timing of spring emergence, mating, gestation and parturition, although small sample sizes precluded statistical analysis of the data. The sequence of events is similar in most respects to those previously described for other squamates (Etches and Petitte, 1990; Whittier *et al.*, 1994; Motta *et al.*, 1995; Uribe *et al*., 1995; 1996). In the ovarian follicles of emerging *T. nigrolutea* females, the zona pellucida (ZP) surrounding the follicle is already differentiated into an outer homogeneous hyaline band and an inner striated zona radiata (ZR). These structures have been observed in other squamates (Etches and Petitte, 1990; Whittier *et al.*, 1994; Motta *et al.*, 1995; Uribe *et al*., 1995; 1996). Cytoplasmic bridges that form between the follicular ooplasm and the cells of the granulosa (Whittier *et al.*, 1994) cause the striations of the ZR. Squamates are unique among reptiles in that the ovarian granulosa differentiates to become a polymorphic structure consisting of pyriform, intermediate and basal cell types (Etches and Petitte, 1990). Basal cells differentiate, via intermediate cells, into pyriform cells (Motta *et al.*, 1995); the latter extend from the theca to the ZP (Etches and Petitte, 1990). The cytoplasmic bridges across the ZP are used to transport RNA and nutrients into the previtellogenic follicle, assisting with early follicular growth (Motta *et al.*, 1995). When vitellogenic growth begins, the cytoplasmic bridges disappear (Etches and Petitte, 1990) and the pyriform cells degenerate, leaving the maturing follicle with a monomorphic cuboidal granulosa (Neaves 1970; Etches and Petitte, 1990; Motta *et al.*, 1995).

However, the striated ZR and polymorphic granulosa persist for varying periods of time. In the lizards *Podarcis sicula* (Motta *et al.*, 1995) and *Heterontia binoei* (Whittier *et al.*, 1994), the ovarian granulosa layer does become monomorphic at the end of the previtellogenic period, but the ZR is still visible at this time (Whittier *et al.*, 1994), implying that cytoplasmic bridges persist at least into early vitellogenesis. In other lizards, such as *Sceloporus torquatus torquatus* and *Ctenosaura pectinata*, the granulosa remains polymorphic until early or mid vitellogenesis and the striations of the ZR are visible throughout the vitellogenic period (Uribe *et al.*, 1995; 1996).

In the ovarian follicles of *T. nigrolutea*, striations in the ZR were only observed until the early stage of vitellogenic growth. However, follicles of this species differed from histological descriptions of other squamate follicles in the persistence of a polymorphic granulosa for all but the post-partum period of the reproductive cycle; this observation warrants further investigation. Small sample sizes preclude statistical analysis of changes in thecal and granulosa thickness (pyriform cell diameter) and hyaline and ZR band widths. However, the maintenance of a constant thecal layer width for all but the hibernation period is worthy of mention. Changes to the thickness of the granulosa layer are also noteworthy: despite being polymorphic in cell type during winter, the granulosa is markedly narrowed in comparison with the active season. These changes may relate to some steroid secretory function.

4.4.2 Steroid hormones

Progesterone

The changes in mean plasma P4 concentration described for female *T. nigrolutea* throughout the active season are typical of those observed in many other temperate zone viviparous squamates (Chan *et al.*, 1973; Kleis-San Francisco and Callard, 1986; Van Wyk, 1994; Jones and Swain, 1996; Jones *et al.*, 1997). The magnitude of peak mean plasma P4 concentration shows little variation between species with similar plasma P4 profiles (*Cordylus giganteus*: 5 ng ml^{-1} (Van Wyk, 1994), *Niveoscincus ocellatus*: 6.5 ng ml^{-1} (Jones *et al.*, 1997), *Nerodia* sp.: 10 ng ml^{-1} (Kleis-San Francisco and Callard, 1986), *Niveoscincus metallicus*: 11.5 ng ml^{-1} (Jones and Swain, 1996) and all are comparable with *T. nigrolutea* (12.7 ± 1.27 ng ml^{-1}).

The sudden and significant elevation of P4 concentrations in the plasma coincident with ovulation in female *T. nigrolutea* suggests that circulating P4 in early gestation is largely luteal in origin. Changes in CL activity and appearance during the reproductive cycle were not considered for this study, as this would have necessitated killing pregnant females at regular intervals throughout gestation. However, the CL as a major source of post-ovulatory P4 has been described in numerous squamate species (Callard *et al.*, 1972b; Highfill and Mead, 1975a; Arslan *et al.*, 1978a; Bona-Gallo *et al.*, 1980; Xavier,

1982; Yaron, 1985; Kleis-San Francisco and Callard, 1986). It is probable that the CL is also the major source of circulating P4 in female *T. nigrolutea*, although mean plasma P4 concentration was not correlated with the number of CLs present (extrapolated from the number of offspring produced). It is possible, however, that in *T. nigrolutea* there is an additional non-luteal or extra-gonadal source of P4.

There are several known alternative non-ovarian sources of P4 in viviparous squamates. The chorioallantoic (CA) placenta of two viviparous lizards, *Sceloporus jarrovi* (Guillette *et al.*, 1981) and *Chalcides chalcides* (Guarino *et al*., 1998) produces P4 after CLs degenerate in mid gestation. Guarino *et al.* (1998) describe a highly complex type III placenta (Yaron, 1985) in *C. chalcides*, but in contrast, the CA placenta of female *S. jarrovi* is of the most simple type (I) seen in reptiles (Yaron, 1985), suggesting that P4 secretion is not related to placental complexity. Female *T. nigrolutea* have a type I placenta (Weekes, 1930; 1935), and plasma P4 concentrations do not remain elevated until parturition. It is, therefore, unlikely that the CA placenta contributes significantly to circulating P4 during pregnancy in female *T. nigrolutea*.

In some squamates, the adrenal glands also produce and secrete P4 (Highfill and Mead, 1975a; b; Dauphin-Villemant and Xavier, 1985; Bourne *et al.*, 1986c). The adrenals are the source of low concentrations of circulating P4 present in non-pregnant females of the lizard *Lacerta vivipara* (Dauphin-Villemant and Xavier, 1985) and the snake *Thamnophis elegans* (Highfill and Mead, 1975a; b). If an extragonadal source of P4 exists in pregnant female *T. nigrolutea*, it is most likely to be the adrenal glands. In the closely related skink *Tiliqua rugosa* the adrenals have been proposed as an alternative minor source of P4 during pregnancy (Bourne and Seamark, 1972; Bourne *et al.*, 1986c). Future studies should include incubations of adrenal tissue from both reproductively active and inactive female *T. nigrolutea* with ^{3}[H]-P5 to assess the capacity of the adrenals to synthesise and secrete P4.

The sustained elevation of mean plasma P4 concentrations during the first two thirds of gestation suggests that P4 plays an important role in the maintenance of normal gestation

in female *T. nigrolutea*, as it does in many viviparous squamates. The dependence of gestation on P4 has been demonstrated in several species (Yaron, 1985). Early – mid gestation in species from two viviparous snake genera, *Nerodia* and *Storeria*, is completely dependent on the presence of intact ovaries: ovariectomy resulted in resorption or abortion of all embryos (Clausen, 1940). Ovariectomy or luteectomy in *Sceloporus cyanogenys* resulted in significantly delayed parturition (Callard *et al*., 1972c), demonstrating a degree of dependence on intact gonads. None of these studies included measurements of plasma P4 concentrations before or after surgery and P4 may have been produced elsewhere. However, CLs are the major source of P4 during gestation in *T. elegans* (Highfill and Mead, 1975a) and luteectomy during gestation in this species resulted in fewer, lighter offspring following an extended gestation, demonstrating a level of dependence on intact CLs for normal gestation (Highfill and Mead, 1975b). In contrast, in the viviparous snake *T. s. parietalis*, mean plasma P4 concentrations remained low or undetectable throughout the reproductive period (Whittier *et al.*, 1987), implying that P4 is unnecessary during pregnancy in this species. Similarly, pregnancy in the viviparous lizards *Chalcides ocellatus*, *Mabuya capensis* ((Badir, 1967a, 1968) cited in Yaron, 1985) and *Mabuya carinata* was unaffected by luteectomy (Sekharappa and Devaraj Sarkar, 1978), although in *M. carinata*, at least, plasma P4 was not remeasured following surgery, so, again, the possibility of an extragonadal source of P4 cannot be dismissed.

These observations raise the question of the exact nature of the role of P4 in gestation. Rather than exerting a direct effect at some point in the embryonic sequence of development, P4 may function to slow the rate of ovarian development, delaying ovulation and the start of gestation, so that young are born into conditions optimal for their survival (Callard *et al.*, 1992). Progesterone may act by influencing the *rate* of development (Gemmell, 1995), or by delaying parturition by reducing oviductal contractility (Guillette *et al.*, 1991). It has been proposed that the evolution of viviparity as a response to cold climates was facilitated by the effects of P4 on uterine motility (Guillette *et al.*, 1981; Guillette and Jones, 1985), gestation length (Callard *et al.*, 1972a; b; Highfill and Mead, 1975b) and oviductal hypertrophy (Guillette and Jones, 1985), resulting in prolonged gestation (Callard *et al.*, 1972a; b; Shine and Guillette, 1988).

Elevated plasma P4 concentrations during gestation also inhibit E2-stimulated follicular growth (Yaron and Widzer, 1978), such that vitellogenesis is not initiated during pregnancy (Callard *et al.*, 1992). This effect of P4 on follicular growth is termed "antigonadal" (Callard *et al.*, 1972a; Guillette *et al.*, 1981; Ho, 1987) and is important for temperate zone viviparous reptiles which are usually constrained by a limited active season to a single reproductive effort each year (Shine, 1985; Dunham *et al.*, 1988). The decline in mean plasma P4 seen well before parturition in *T. nigrolutea* is likely to be related to the antigonadal actions of this hormone, and to reflect the multihormone control of gestation and parturition, which will be discussed later.

17β-Oestradiol

In reproductively active female *T. nigrolutea*, annual changes in mean plasma E2 concentrations occur coincident with important physiological events in the reproductive cycle. Mean plasma E2 concentration is basal at emergence and becomes elevated during late vitellogenesis, coincident with the presence of large yolky follicles. Mean plasma E2 concentration peaks at the time of ovulation, then returns to basal concentrations during gestation. A similar plasma E2 profile is displayed in many other viviparous squamates (Callard *et al.*, 1972c; Kleis-San Francisco and Callard, 1986; Callard and Kleis, 1987; Bonnet *et al.*, 1994; Van Wyk, 1994; Jones and Swain, 1996; Jones *et al.*, 1997). With very few exceptions, E2 is the primary natural inducer of hepatic vitellogenesis in reptiles (Kime, 1987; Ho, 1987; Diaz *et al.*, 1994) and also stimulates endocytosis of vitellogenin by ovarian follicles (Callard and Ho, 1987).

Mean plasma E2 in reproductively active female *T. nigrolutea* rises again at parturition, although it does not become significantly elevated above concentrations in non-reproductive females. A similar trend has been reported in two other viviparous squamates, the lizard *C. giganteus* (Van Wyk, 1994) and the snake *Nerodia* sp. (Kleis-San Francisco and Callard, 1986). The possible mechanism for this non-significant elevation in mean plasma E2 concentrations may also be related to the multihormone control of reproduction, which will be discussed later.

There is considerable variation in the magnitude of peak mean plasma E2 concentrations among viviparous squamates. Circulating E2 is usually of ovarian origin in reptiles (Yaron, 1972b; Staub and de Beer 1997; Wade, 1997), although Yokoyama and Yoshida (1994) report the production of E2 by adrenal glands, in the oviparous snake *T. flavoviridis*. This conclusion was based on the observation that an antibody to E2 reacted positively to adrenal tissue in an immunohistochemical study (Yokoyama and Yoshida, 1994). Such a result is questionable, as the chemicals to which the tissue was exposed during wax embedding should remove any steroids present (Lance, pers. comm.).

Peak E2 concentrations at ovulation range from 700-1300 pg ml^{-1} in *T. nigrolutea* (this study), *N. metallicus* (Jones and Swain, 1996), *N. ocellatus* (Jones *et al.*, 1997) and *Nerodia* sp. (Kleis-San Francisco and Callard, 1986), to 4 ng ml^{-1} in *V. aspis* (Saint Girons *et al.*, 1993; Bonnet *et al.*, 1994) but rarely as high as concentrations seen in *C. giganteus*, in which plasma E2 peaks above 600 ng ml^{-1} (Van Wyk, 1994). Absolute plasma E2 concentrations presented in the present study may be higher than actual values. The possibility of a metabolite of E2 cross-reacting with the E2 antiserum in the assay used for this work is discussed in *Chapter 5 Section 5.4.5* in the context of alternative oestrogens. However, regardless of the differences in the magnitude of plasma E2 peaks between species, the patterns of change throughout the reproductive cycle remain the same.

Testosterone

In female reptiles, T appears to be an important secretory product of the ovary (Arslan *et al.*, 1978a; Callard *et al.*, 1978; Owens, 1997; Staub and De Beer, 1997). The presence of T at physiologically relevant concentrations in plasma implies a biological function for this hormone in females (Staub and De Beer, 1997), although it could also act as a reserve pool of steroid for rapid oestrogen production or further metabolism.

Both reproductively active and inactive female *T. nigrolutea* display variation in mean plasma T concentrations throughout the active season. The changes observed in reproductively active females are also shown, but at lower concentrations, in non-

reproductive animals. This has also been reported in the viviparous snake *T. s. parietalis* (Whittier *et al.*, 1987). In reproductive female *T. nigrolutea* mean plasma T increases through late vitellogenesis, peaking at 6.3 ± 0.63 ng ml^{-1} at ovulation and then declining rapidly. The magnitude of this T peak is relatively high for a female reptile. By comparison, plasma T in females peaks between 300 and 400 pg ml^{-1} in the oviparous snake *N. naja* (Bona-Gallo *et al.*, 1980) and the turtles *Caretta caretta* (Wibbels *et al.*, 1990) and *L. kempi* (Rostal *et al*., 1998). Peak plasma T is higher in female *Alligator mississippiensis* (1.12 ng ml^{-1}) (Guillette *et al.*, 1997), *T. s. parietalis* (2 ng ml^{-1}) (Whittier *et al.*, 1987) and the turtle *Chrysemys picta* (4.5 ng ml^{-1}) (Callard *et al.*, 1978). Only the tuatara *S. punctatus* (11.4 ng ml^{-1}) (Cree *et al.*, 1992) is reported to have a higher peak plasma concentration of T in females than *T. nigrolutea*. Such differences may reflect species-specific variation in the specificity or capacity of sex steroid binding proteins (Paolucci *et al.*, 1992).

The pattern of seasonal changes in mean plasma T concentrations in *T. nigrolutea* is very similar to that seen in many other female reptiles. Although the majority of studies have examined non-squamate species, a correlation between elevated plasma T concentrations and vitellogenesis and ovulation is commonly reported (Callard *et al.*, 1978; Wibbels *et al.*, 1990; Cree *et al.*, 1992; Guillette *et al.*, 1997; Rostal *et al.* 1998). Testosterone may have a role in preparation for ovulation in the lizard *S. jarrovi* (Moore, 1986). However, few studies of plasma T concentrations in female viviparous squamates are available for comparison. The oviparous snake *N. naja* (Bona-Gallo *et al.*, 1980) and the viviparous snakes *T. s. parietalis* (Whittier *et al.*, 1987), *Nerodia sipedon* (Callard and Kleis, 1987) and *V. aspis* (Saint Girons *et al.*, 1993) also display an annual plasma T profile similar to that of *T. nigrolutea.*

Testosterone is known to stimulate oviductal hypertrophy (Jones and Guillette, 1982) and uterine development in ovariectomised lizards (Yaron, 1972a). The profile of mean plasma testosterone in female *T. nigrolutea* provides circumstantial evidence that T may also be involved in the regulation of vitellogenesis and ovulation in this species. The observation that circulating concentrations of T are considerably greater than plasma E2

concentration also suggests a physiological role for T in female *T. nigrolutea* beyond its role as a precursor for the synthesis of oestrogens. However, while a temporal correlation between elevated plasma T and vitellogenesis and ovulation has been demonstrated in a number of female reptiles, a causal relationship between the two is yet to be proven for any species. It is also currently uncertain whether T acts directly, or following peripheral conversion to E2; however, T is likely to be involved in some aspect of the multihormone control of reproduction (discussed later).

Steroid hormone control of mating behaviour

Several criteria must be met before a functional link between a hormone and an observed behaviour is proven.

1. The expression of the behaviour is at least loosely correlated with elevated circulating levels of the hormone.
2. Expression of the behaviour is abolished by chemical or surgical ablation of the source of the hormone and restored by replacing the hormone.
3. The hormone binds in regions of the brain that can be shown by lesion studies, by electrical stimulation, or by electrical recording to be involved in the control of the behaviour.
4. Very localised implants of the hormone in this same region of the brain restore the behaviour whenever other sources of the hormone have been ablated. (Taken from Moore and Lindzey, 1992).

The aim of this study was to satisfy the first of these criteria. Adult female *T. nigrolutea* exhibit an associated reproductive cycle: peak gonadal development coincides temporally with the mating period. Mean plasma P4, E2 and T concentrations all increase through late vitellogenesis and the mating period. Female reproductive behaviours in reptiles are probably regulated by steroids (Adkins-Regen, 1981; Flores and Crews, 1995; Staub and De Beer, 1997; Wade, 1997), and P4, E2 and T are likely to be involved in the stimulation of female receptivity and copulatory behaviour in *T. nigrolutea*.

Traditionally, E2 is perceived as the steroid most likely to influence female reproductive behaviour (Crews, 1975; Wade, 1997). In the viviparous lizard *S. jarrovi* plasma E2 concentration are only elevated when females are sexually receptive (Moore, 1986). In the lizards *A. carolinensis* (McNicol and Crews, 1979) E2 and P4 act synergistically to induce sexual receptivity in females. However, in the lizards *Lampropholis guichenoti* and *Lampropholis delicata*, another oestrogen, oestrone (E1), may stimulate mating behaviour (Joss, 1985). Oestrogens other than E2 have been identified in other reptiles, for example *T. s. parietalis*, in which the presence of a 6-substituted E2 has been confirmed (Whittier and Hess, 1992).

Androgens may also influence reproductive behaviour in female reptiles. For example, in *V. aspis* oestrous females display elevated plasma E2 and 5α–DHT (Saint Girons *et al.*, 1993). At the onset of mating in the turtle *L. kempi*, T and E2 are at peak concentrations in the plasma and decline rapidly post-mating (Rostal *et al.*, 1998). Plasma T is also elevated in female *N. naja* during the mating period (Bona-Gallo *et al.*, 1980). These androgens are likely to be of ovarian origin (Staub and De Beer, 1997; Wade, 1997) and may act directly (Rostal *et al.*, 1998) or following aromatisation to E2 (Adkins-Regen, 1981) to stimulate female reproductive behaviours. In view of the observed rise in circulating T concentrations in female *T. nigrolutea* at ovulation, an examination of the effects of sexual receptivity following exogenous application of T may provide useful information about the control of reproductive behaviours in this species.

4.4.3 Frequency of reproduction

A multiennial reproductive cycle is proposed for female *T. nigrolutea* in Tasmania. Deviations from an annual pattern of reproduction can be achieved in several ways (Ibargüengoytia and Cussac, 1998). The ovarian cycle may be prolonged, by extending vitellogenesis and/or gestation, as occurs in the viviparous biennially breeding lizards *Barisia monticola* (Vial and Stewart, 1985), *Hoplodactylus maculatus* (Cree and Guillette, 1995) and *Niveoscincus microlepidotus* (Hutchinson *et al.*, 1989). In *B. monticola* and *H. maculatus*, ovarian activity is effectively continuous (Vial and Stewart, 1985; Cree and

Guillette, 1995). The lengthening of the ovarian cycle in either of these ways is most common in squamates in cool or cold temperate regions (Hutchinson *et al.* 1989; Cree and Guillette, 1995). A second strategy for achieving a multiennial cycle is exemplified by the viviparous lizard *Liolaemus pictus*; this species responds to short and cold activity seasons by assigning vitellogenesis and gestation to separate activity seasons with a period of gonadal latency during the intervening winter (Ibargüengoytia and Cussac, 1996). Several other squamate species are reported to display multiennial reproductive cycles (Prestt, 1971; Goldberg and Bezy, 1974; Larsen *et al.*, 1993; Cree, 1994; Whittier and Limpus, 1996), but the way in which this is achieved in each species is uncertain. A reproductive effort only every second (or third) year may also be achieved by actually "skipping" a year. Such cycles are usually restricted to females of temperate zone reptile species which produce moderate to large clutches (Bull and Shine, 1979).

In squamates, multiennial reproduction is associated with longer-lived species (Van Wyk, 1991), and with those which are viviparous or egg-brooding (Bull and Shine, 1979). Late parturition and lower autumn temperatures do not allow such females time to store sufficient reserves to endure winter hibernation and become vitellogenic in the following spring (Saint Girons, 1985). Additionally, a reduction or cessation in feeding during late gestation may occur as a result of less space being available in the body cavity for food due to the presence of eggs or embryos (Schwarzkopf, 1996). Some or all of these factors may regularly result in missed opportunities for reproduction (Shine, 1980) as is reported for the lizards *Eulamprus tympanum* (Schwarzkopf, 1996), *C. giganteus* (Van Wyk, 1991) and *Anguis fragilis* (Patterson, 1983).

It is into this final category that the reproductive cycle of female *T. nigrolutea* from Tasmania best fits. *Tiliqua nigrolutea* is a long-lived, late-maturing and viviparous skink with a cool-temperate zone distribution. Parturition occurs very late in the active season (autumn (Mar-Apr)) following a long (4 - 4.5 month) gestation. Most females feed little, or not at all, during the final weeks of gestation and all individuals, including males and non-reproductive females, cease feeding in late autumn (late Apr-May) ahead of the start of the hibernation period (late May). This presumably leaves post-partum females

insufficient time to store the fat reserves necessary to survive hibernation as well as to begin vitellogenesis in spring; in this study no captive females became vitellogenic in the spring following the production of a clutch. This pattern is in sharp contrast to that of two other Tasmanian viviparous lizard species, *N. metallicus* and *N. ocellatus*, which mate in autumn during vitellogenesis and store sperm until spring ovulation (Swain and Jones, 1994; Jones *et al.*, 1997). This allows ovulation to occur earlier in spring (Sept) than in *T. nigrolutea* and parturition can then occur in mid-summer (Jan) (Swain and Jones, 1994; Jones *et al.* 1997).

On the Australian mainland, female *T. nigrolutea* are also likely to be biennial breeders while other members of the genus usually reproduce annually (Shea, 1992). There is, however, a single unverified record of an individual captive female *T. nigrolutea* producing clutches in consecutive years (D. Clutterbuck, pers. comm., cited in Shea, 1992). Some viviparous female squamates display a plastic reproductive cycle, in that they have the ability to breed annually if conditions are good. In the snakes *V. aspis*, *Elaphe longissima* (Bonnet and Naulleau, 1990) and *T. s. parietalis* (Whittier and Crews, 1990) stored energy is the limiting factor in the regulation of reproduction; females do not become vitellogenic in a given year if their stored fat reserves are below a threshold level. The lizard *Liolaemus elongatus* displays an annual or biennial reproductive cycle that is plastic in response to climate (Ibargüengoytia and Cussac, 1996). It is unfortunately beyond the scope of this study to determine whether the reproductive cycle of female *T. nigrolutea* displays this type of plasticity.

The proportion of wild-caught females that were reproductively active varied between seasons but in each year, reproductive frequency was inconsistent with a pattern of annual reproduction. The low mean proportion of reproductively active wild females (39.7 %) and the infrequent reproduction of captive females are unlikely to be a reflection of embryos being resorbed either spontaneously in the wild or as a consequence of capture. There is, indeed, little evidence for embryo resorption in squamate reptiles (Blackburn, 1998). Captive female *T. nigrolutea* in this study were observed, on several (N = 4) occasions to pass and consume large yolky masses (presumably unfertilised ova)

during parturition, implying that such masses are not resorbed during gestation. This has been previously reported for *Tiliqua scincoides* (LeBreton, 1990). These yolky masses were also observed in the oviducts at autopsy in pregnant female *T. nigrolutea.*

A mean RCM of 0.40 ± 0.040 for female *T. nigrolutea* over the three seasons of this study (0.45 ± 0.050 in the 97/98 season) suggests that the effort of reproduction is high. This mean RCM is comparable with other viviparous squamates which are regarded as having a high RCM (0.52 in *L. bougainvillii* (Qualls and Shine, 1995) and 0.482 in *Aspidelaps scutatus* (Shine *et al.*, 1996b)) and is also very similar to the RCM of 0.4 reported for *T. rugosa*, although these authors did not provide details of the calculation used (Fergusson and Algar, 1986). Thus, although *T. nigrolutea* does not reproduce annually, once the commitment to produce a clutch is made, a large effort is allocated, perhaps offsetting the cost to life-time fecundity of not producing a clutch each year.

It is interesting to note that while RCM in *T. nigrolutea* and *T. rugosa* is similar, two different reproductive strategies operate. *Tiliqua rugosa* females are thought to reproduce twice every three years, depending on conditions (e.g. temperature, food availability) (Bull, pers, comm.) and produce two (approx. 130 g each) to five (approx. 50 g each) young (Shea, 1992; Green, 1995). Juvenile mortality in the first year in this species is estimated at 84 % (Bull, 1995). In contrast, *T. nigrolutea* females, also displaying a multiennial pattern of reproduction, produce up to 15, much smaller (15 – 20 g), young. Juvenile mortality in *T. nigrolutea* has not been assessed.

4.4.4 Multihormone control of reproduction

It is important to consider the combined effects of gonadal steroids in the regulation of reproductive physiology. Several features of the plasma steroid profiles of P4, E2 and T in reproductively active female *T. nigrolutea* are best explained by the hypothesis of a multihormone control of reproduction. The seasonal profiles of plasma P4, E2 and T in female *T. nigrolutea* are shown overlaid in Figure 4.14.

In *T. nigrolutea*, mean plasma E2, T and P4 concentrations rise during the late vitellogenic period. In other reptiles, E2 and T are synthesised by growing follicles: plasma concentrations increase as follicles enlarge (Wade 1997; Staub and De Beer, 1997) and then fall in the periovulatory period (Callard and Kleis, 1987; Wibbels *et al.*, 1990; Guillette *et al.*, 1997; Rostal *et al.*, 1998). Estrogens and progestins secreted by the ovary in the pre-ovulatory period act synergistically to stimulate maturation of the genital tract in female reptiles (Yaron, 1972b). The release of the ovum is thought to deactivate the enzyme systems responsible for the further conversion of P4 to E2 (Yaron, 1972b). Correspondingly, at ovulation in *T. nigrolutea*, plasma E2 and T concentrations decrease and mean plasma P4 concentration rises. All three steroid hormones are likely to be interacting at this time. Both P4 and T have marked antagonistic effects on E2-induced vitellogenin synthesis in reptiles (Giannoukos and Callard, 1995) and may function to terminate vitellogenesis after ovulation (Ho *et al.*, 1982; Callard *et al.*, 1992). This is accomplished at two levels; the liver is the site of one such antigonadal effect, where T regulates the stimulatory action of E2 (Callard and Ho, 1987). Simultaneously, at the follicular level P4 inhibits the incorporation of vitellogenin into oocytes (Yaron and Widzer, 1978). In this species, mating occurs immediately prior to ovulation, coincident with peak plasma E2, and a small peak in plasma P4. However, as described in *Chapter 4 Section 4.1.1*, mating behaviour may be temporally dissociated from the gonadal cycle. The hormonal control of mating behaviour in females was not examined in this study.

During gestation in *T. nigrolutea*, elevated plasma P4 and T (presumably of ovarian origin) may inhibit E2 production and prevent further follicular development, as described in other reptiles (Callard *et al.*, 1972a; Ho *et al.*, 1982; Callard *et al.*, 1992). Alternatively, inhibition may be accomplished at the receptor level; P4 causes downregulation of E2 receptors in the brain of the lizards *Cnemidophorus inornatus* and *Cnemidophorus uniparens* (Godwin *et al.*, 1996). The rise in plasma E2 concentration associated with the onset of parturition seen in *T. nigrolutea* has been observed in the related species *T. rugosa* (Fergusson and Bradshaw, 1992) and occurs coincident with falling P4 and low T concentrations in the plasma.

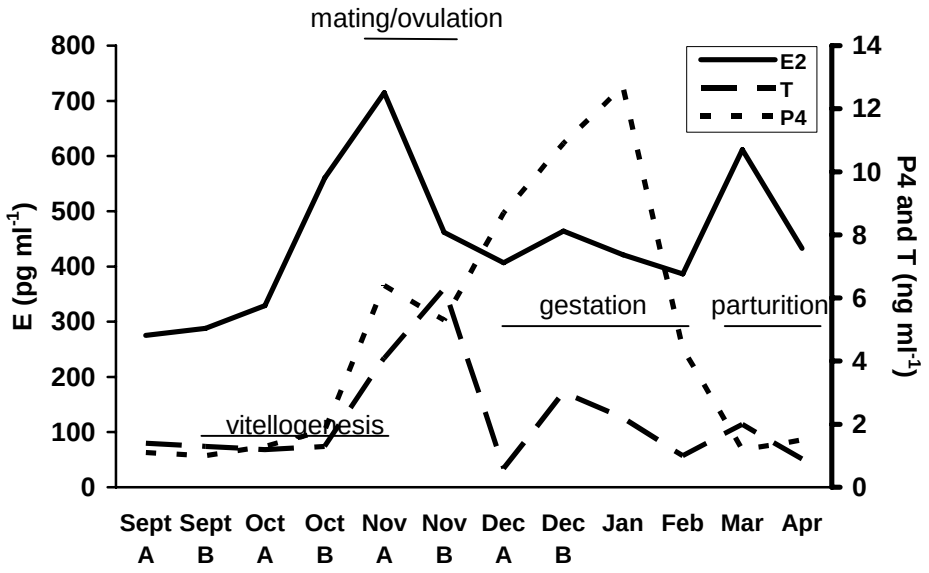

Figure 4.14 Mean plasma P4, E2 and T concentrations in reproductively active female *Tiliqua nigrolutea* (N = 8) throughout the active season. Standard errors not shown.

The plasma steroid profiles of P4, E2 and T in reproductively active female *T. nigrolutea* (Figure 4.14) suggest that vitellogenesis, ovulation, gestation and parturition are under multihormonal control in this species, although experimental evidence is still required to support this hypothesis. Such multihormonal control of reproductive cycles is likely to occur in most reptiles. It is, therefore, vital to consider the potential interaction effects of steroid hormones in the interpretation of results from castration or other hormone manipulation studies.

Chapter 5 Biosynthesis of steroid hormones in male and female Tiliqua nigrolutea

5.1 Introduction

5.1.1 Steroid biosynthetic pathways

The *de novo* synthesis of all steroid hormones begins with the production of cholesterol, a 27 carbon (C27) molecule, from acetate, a two carbon (C2) molecule (Kime, 1987). Steroidogenesis, which is the formation of this steroid nucleus from acetate, occurs in many tissues of the body (Norris, 1997). In male vertebrates both the interstitial (Leydig) and Sertoli cells (Wiebe, 1985) of the testis are steroidogenically active; in females it is the thecal and granulosa cells of the ovarian follicles that synthesise the majority of steroid hormones (Chieffi and Pierantoni, 1987). Additionally, the ovarian interstitial gland tissue is steroidogenically active in mammalian species (Duke, 1978; Lance and Callard, 1978). However, such interstitial tissue is thought to be absent from the ovaries of non-mammalian vertebrates, including reptiles (Duke, 1978). Other steroidogenic tissues include the adrenal glands and the placenta (Kime, 1987).

Following steroidogenesis, cholesterol undergoes further conversions to produce a variety of hormonally active steroids. Located within the mitochondria of a steroid-producing cell is a P450-21, 22-lyase enzyme that converts cholesterol (C27) to pregnenolone (P5) (C21) (Kime, 1987) by cleaving the cholesterol side chain (Norris, 1997). Pregnenolone then moves to the cell's smooth endoplasmic reticulum (SER) where it undergoes further conversion (Kime, 1987). The production of P5 is common to all steroidogenic pathways.

Reproductive steroids are usually produced from P5 via two established pathways. The first is the 5-ene, or delta-5 (Δ5), pathway (Figure 5.1). Action on P5 by a P450-17-hydroxylase enzyme results in the production of 17α-hydroxypregnenolone (17α-OH-P5). The C17 side chain is then cleaved by a

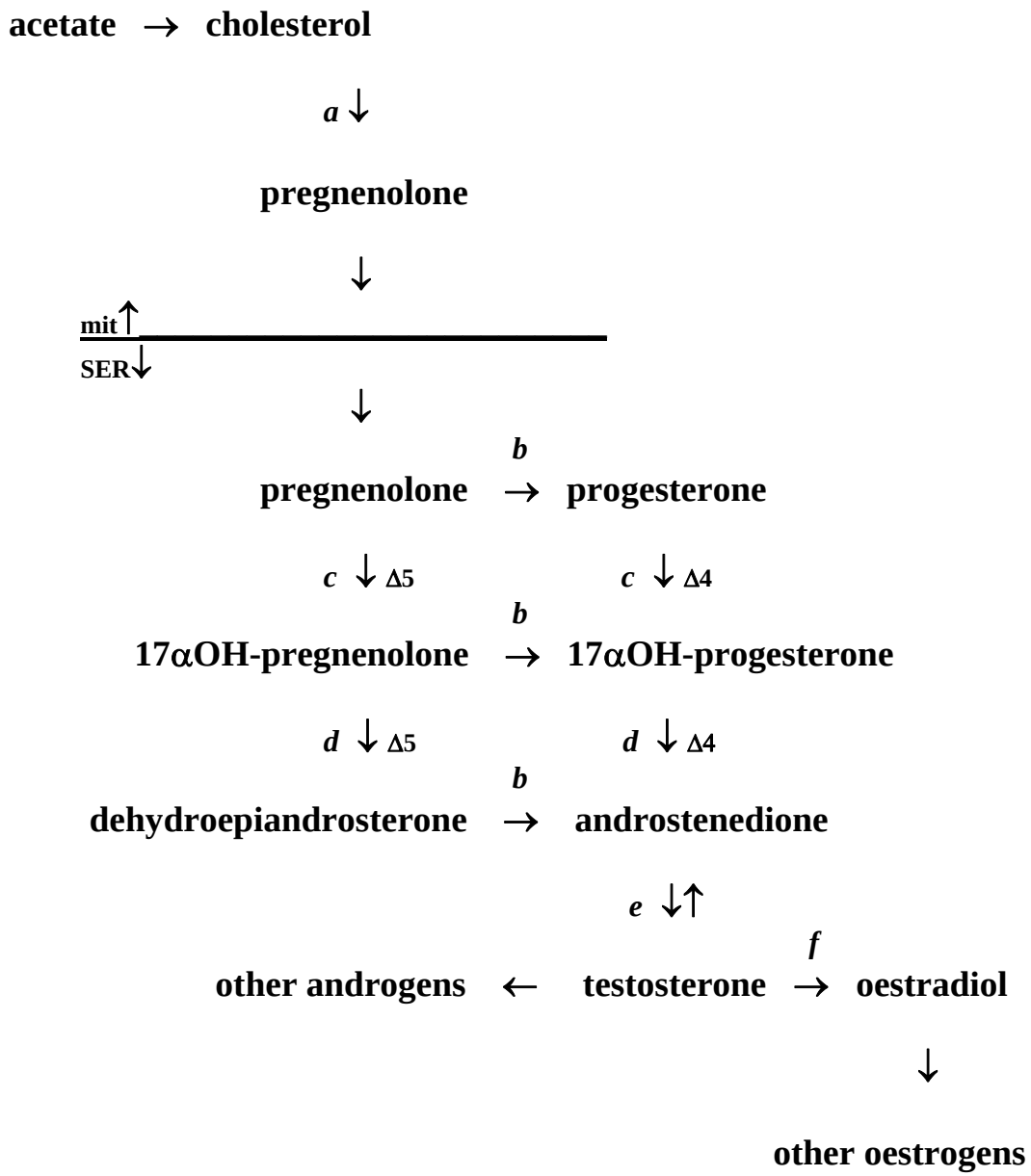

Figure 5.1. Biosynthetic pathways for the production of testosterone and 17β-oestradiol in vertebrates. Mit = mitochondrion; SER = smooth endoplasmic reticulum; *a* = P450-21, 22-lyase; *b* = 3β-hydroxysteroid dehydrogenase; *c* = P450-17-hydroxylase; *d* = P450-17, 20-lyase; *e* = 17β-hydroxysteroid dehydrogenase; *f* = P450-aromatase. Adapted from Norris, 1997 and Kime, 1987.

P450-17, 20-lyase, forming dehydroepiandrosterone (DHA). Subsequent oxidation at the third carbon (C3) by 3β-hydroxysteroid dehydrogenase (3β-HSD) results in the formation of androstenedione (AD). This is then acted upon by 17β-hydroxysteroid dehydrogenase (17β-HSD), resulting in testosterone (T).

The alternative 4-ene or delta-4 (Δ4) pathway (Figure 5.1) involves the oxidation of P5 at the C3 position by 3β-HSD to produce progesterone (P4) (C21) (Kime, 1987). A P450-17α-hydroxylase then converts P4 to 17α-hydroxyprogesterone (17α-OH-P4). The C17 side group is then removed by a P450-17, 20-lyase to give AD, which is reduced by 17β-HSD to form T.

Oestrogens (C18) are synthesised from androgens (C19), usually through 17β-oestradiol (E2) (Figure 5.1): through the action of a P450-aromatase enzyme located in the SER, T loses one carbon atom and an aromatic A-ring is formed in the steroid nucleus to form E2 (Norris, 1997; Kime, 1987). Oestrogens are, therefore, effectively metabolites of T.

5.1.2 Phylogenetic variation in pathway preference

The pathways by which the major reproductive steroids are synthesised in vertebrates are generally well conserved (Dawson, 1998) in that both Δ4 and Δ5 pathways operate in all vertebrate classes from agnathans to mammals (Kime, 1987; Norris, 1997). However, the relative contributions of each biosynthetic pathway to total production of reproductive steroids can vary considerably both between and within vertebrate classes (Kime, 1987). In the testes of the fishes *Betta splendens* (Leitz and Reinboth, 1987) and *Gobius paganellus* (Colombo *et al.*, 1970), intermediates from both pathways have been isolated, suggesting that both the Δ4 and Δ5 pathways contribute to the production of androgens. However, the Δ4 pathway predominates in the testes of the anuran and urodele amphibians *Rana nigromaculata* (Kobayashi *et al.*, 1993), *R. catesbeiana* (Dale and Dorfman, 1967) and *Pleurodeles waltlii* (Ozon, 1967).
In those mammals that have been studied, pathway preference appears to be variable. In the guinea pig (Hoschoian *et al.*, 1991), the monkey *Macaca fascicularis* (Blacker *et al.*,

1991) and in human males (Yanaihara and Troen, 1972; Ruokonen, 1978; Dehennin, 1993) both pathways are active but the Δ5 route is dominant. However, a conflicting report has suggested more recently that, as in the laboratory rat (Blacker *et al.* 1991), the Δ4 pathway plays the more active role in the production of testicular androgens in human males (Lombardo *et al.*, 1993). These inconsistencies may be a reflection of the design of studies conducted in the past, as discussed in *Chapter 5 Section 5.1.5*. Moreover, pathway preference has actually been examined in only a relatively small number of vertebrate species. The suggested phylogenetic patterns of conservation of steroidogenic pathway preferences within vertebrate classes are, therefore, based on limited results only. There are insufficient data available from avian species to allow an assessment of any trend in steroidogenic pathway preference, although in the duck *Gallus domesticus* the Δ4 pathway is known to predominate (Boucek and Savard, 1970). Pathway preference in reptiles is discussed separately in *Chapter 5 Section 5.1.5*.

5.1.3 End-product variation

While their relative contributions may vary, the two pathways by which reproductive steroids are synthesised are active in most, if not all, vertebrates examined to date. There is, however, greater conservation of the major end-products of steroid biosynthesis within vertebrate classes than there is in the dominant pathway by which they are produced. Among the reptiles, birds and mammals, T and E2 are usually the major products of gonadal steroidogenesis (Kime, 1987), although in mammals T is often converted peripherally to the more androgenic 5α-DHT (Norris, 1997). However, in several classes of lower vertebrates, the primary gonadal steroids, T and E2, are not the major end-products of gonadal steroid biosynthesis; these hormones are instead converted to more biologically active derivatives. For example, the agnathan *Myxine glutinosa* produces 6- and 7-hydroxylated derivatives of E2 and T in the ovaries (Kime and Hews, 1980) and testes (Kime *et al.*, 1980), while 15α- and 15β-hydroxylated derivatives of these hormones are synthesised by both sexes of *Petromyzon marinus* (Kime and Callard, 1982) and *Lampetra fluviatilis* (Kime and Rafter, 1981). Many teleost fishes produce E2 as their major ovarian oestrogen, while the males convert T to 11-ketotestosterone (11-

KT) in the testis (Idler *et al.*, 1971; Leitz and Reinboth, 1987; Borg *et al.*, 1992). In addition, some fish species produce testicular 11β-hydroxytestosterone (11β-OH-T) (Kime and Hews, 1978a; Huang *et al.*, 1985; Leloup-Hatey *et al.*, 1985). Within the Amphibia, males and some females of several urodele species secrete gonadal 11-KT (Lupo Di Prisco *et al.*, 1971; 1972; Bolaffi *et al.*, 1979), and in females of the newt *Trituris cristatus carnifex*, oestrone (E1), but not E2, is detected following incubation of ovarian tissue with T (Lupo Di Prisco *et al.*, 1971). However, among anurans, E2 is again the major oestrogen, while T and 5α-dihydrotestosterone (5α-DHT) appear to be the most important testicular end-products (Kime, 1987).

Within each vertebrate class, there are several species-specific deviations from these patterns of preferred steroid end-product biosynthesis. For example, in male lungfish *Neoceratodus forsteri* the major testicular androgen is the T characteristic of higher vertebrates, rather than the expected 11-KT of fish and some amphibians (Joss *et al.*, 1996). In the lizard *Tiliqua rugosa* the major testicular androgen is not T but epitestosterone (epiT) (Bourne *et al.*, 1985) and in females of the snake *Thamnophis sirtalis parietalis* substantial quantities of 6α- and 6β-hydroxylated oestradiol (6α- and 6β-OH-E2) have been isolated from plasma (Whittier and Hess, 1992). Such deviations may provide important information about the adaptive evolution of the usually well-conserved steroidogenic pathways within vertebrate groups.

5.1.4 Intraspecific variation in pathway preference

Some studies of the pathways of steroid biosynthesis have suggested the possibility of differences in pathway preference within a species. In many vertebrates, reproductive condition affects the relative contributions and overall activity of the steroid biosynthetic pathways (reptile: Lofts, 1972; Bourne and Licht, 1985; fish: Borg *et al.*, 1992; Joss *et al.*, 1996; elasmobranch: Kime and Hews, 1982; Callard and Leathem, 1965; mammal: Bedrak *et al.*, 1983). Steroidogenic activity can also depend on sex (fish: Kime and Groves, 1986) and age (mammal: Wiebe, 1985; Hoschoian *et al.*, 1991; fish: Sakai *et al.*, 1989) while temperature, particularly in ectothermic species, can drastically alter steroidogenic end-products and their further metabolism (conversion or conjugation) (fish: Kime, 1979; Kime and Hyder, 1983; Manning and Kime, 1985; Lofts, 1987; reptile: Xavier, 1982; Huf *et al.*, 1989). Even substrate concentration (hagfish: Kime and Hews, 1980; fish: Kime and Abdullah, 1994), incubation length (fish: Delrio *et al.* 1985) and tissue type (mammal: Payne, 1980; amphibian: Lupo Di Prisco *et al.*, 1971; 1972; agnathan: Kime and Callard, 1982) have been shown to alter the activity of steroid biosynthetic pathways.

5.1.5 This study

For many of the published studies, it is difficult to assess the relative activity of each steroid biosynthetic pathway for a number of reasons. Often pathway intermediates are examined following incubation of steroidogenic tissue using only P4 as a substrate (Ozon, 1967; Dale and Dorfman, 1969; Tam *et al.*, 1969; Lofts and Choy, 1971; Kime and Hews, 1980; Kime *et al.*, 1980; Kime and Rafter, 1981;). Progesterone is itself a Δ4 pathway intermediate, with no link back to the Δ5 route; conclusions drawn from such work regarding the dominance of the Δ4 pathway in the biosynthesis of reproductive steroids may, therefore, be invalid. Additionally, some studies examining the production of reproductive steroids have used immature or larval animals (Rao *et al.*, 1969; Leloup-Hatey *et al.*, 1985; Cooke, 1992; Guillette *et al.*, 1995; Yeoh *et al.*, 1996). Extrapolating conclusions from these data to sexually mature animals is likely to be unsound, given the number of recent publications reporting changes in steroidogenic patterns with different

stages of development or maturation (Sakai *et al.*, 1989; Feist *et al.*, 1990; Hoschoian *et al.*, 1991; Yeoh *et al.*, 1996). Finally, most examinations of vertebrate steroid biosynthetic pathways have used animals of one sex only, usually males (Fevold and Eiknes, 1963; Hews and Kime, 1978b; Bourne and Licht, 1985; Huf *et al.*, 1989; Kobayashi *et al.*, 1993). The biosynthesis of oestrogens is often not considered because oestrogens are regarded as metabolites of T, the assumption being that preference to that point in the biosynthetic pathways will be the same in both sexes. There has been a widespread failure to acknowledge the possibility of variations in pathway preference between sexes of one species.

There is little information available on steroid biosynthesis in reptiles. The question of pathway preference for reproductive steroid production by reptilian gonads has been partially addressed in only a few species, and usually only in animals of one sex (Bourne and Licht, 1985; Hews and Kime, 1978; Chan and Callard, 1974; Lofts, 1972; Callard, 1967). Cautious extrapolation from the available literature suggests that the Δ4 pathway is more active, but some authors report the detection of small amounts of Δ5 pathway intermediates (Huf *et al.*, 1989; Bourne and Licht, 1985; Bourne, 1981; Chan and Callard, 1974; Lupo Di Prisco, *et al.*, 1968).

This study aims to examine several aspects of steroid biosynthesis in a reptile, the viviparous skink, *Tiliqua nigrolutea*. This species was chosen because a sound information base on the annual reproductive cycle and patterns of change of plasma steroid concentrations has already been established (See *Chapter 3 Results* and *Chapter 4 Section 4.3.4*). Questions addressed by the present study are as follows.

1) Do the steroid biosynthetic pathways used by the gonads of this species conform to the general patterns of vertebrate steroid biosynthesis in the use of the Δ4 and Δ5 pathways?

2) If so, is there a clear preference for either the Δ4 or the Δ5 pathway in *Tiliqua nigrolutea*?

3) Are there differences between the sexes in:

a) the relative contribution of each pathway to steroid biosynthesis?

b) the emphasis on the synthesis of particular end-products?

4) Does pathway preference vary with changing reproductive condition within either sex?

5.2 Materials and methods

General methods are described in *Chapter 2 Section 2.1*, but information specific to the work in this chapter is presented here.

5.2.1 Tissue collection

Testicular or ovarian tissue was collected from adult male and female *T. nigrolutea*, respectively, at two different stages of gonadal activity. These represent reproductively active and inactive conditions as outlined in Table 5.1.

Table 5.1 Sampling regime for collection of gonadal tissue from male and female *Tiliqua nigrolutea* for *in vitro* incubation with ^{3}[H]-P5.

	Sex	Hypertrophied gonads (active)	Regressed gonads (inactive)
male:	**gonadal stage**	late spermatogenesis	Quiescence
	season	spring (early Oct)	summer (late Feb)
	sample size	N = 4	N = 4
female:	**gonadal stage**	late vitellogenesis	post-parturition
	season	spring (late Oct)	autumn (mid-Mar)
	sample size	N = 2	N = 2

5.2.2 Incubation

Males

These experiments were completed on two consecutive days each in late summer (late Feb) (N = 4) and mid-spring (early Oct) (N = 4), 1998. Tissues from each male were treated separately. Animals were killed as described in *Chapter 2 Section 2.4*. At autopsy, testicular tissue was removed and weighed. Left and right testes from each animal were combined and minced finely with scissors. Testicular tissue from each animal was then divided evenly between 10 incubation flasks (200 mg per flask) each containing ^{3}[H]-pregnenolone (^{3}[H]-P5) (15 μCi) as substrate. HEPES-buffered Leibovitz culture medium

(pH = 7.6) (5 ml) was added to each flask: these were held on ice until all flasks were prepared. Samples were incubated at 35 °C in an air environment in a gently rocking waterbath. The temperature of the waterbath reflected the previously published preferred body temperature of this species (34.8 °C, Rawlinson, 1974). Incubations (N = 2) were terminated at 10, 30, 60, 120 and 180 min by rapid freezing and samples were stored at -20 °C. Free (non-conjugated) steroids were later extracted from thawed samples in two volumes of dichloromethane (DCM). These DCM washes were combined, and stored at -20 °C until further analysis. Following the extraction of free steroids, a 100 μl aliquot of the original medium from each incubation was assayed for radioactivity in 3 ml scintillation cocktail. This allowed the determination of the proportion of originally labelled steroid that became conjugated during the incubation.

Females

Procedures were similar to those for the male incubations. Samples of active gonadal tissue were collected in spring (mid-Oct, 1998) from late vitellogenic stage females (N = 2) over two consecutive days; yolk was expressed and follicles rinsed with incubation medium prior to incubation. This provided 300 mg of ovarian tissue (both thecal and granulosa cells) per flask. Samples of inactive gonadal tissue were collected in summer (late Feb – Mar) from post-partum females (1997: N = 1, 1998: N = 1) within one week of parturition, providing 60 mg of ovarian tissue (thecal, granulosa and corpora lutea (CLs)) per flask. As with male tissues, each flask contained HEPES-buffered Leibovitz culture medium (5 ml) and ^{3}[H]-P5 (15 μCi). Both thecal and granulosa (and CL when present) tissues were included in the incubations in an attempt to gain an accurate picture of the total biosynthetic ability of the ovaries. Only two females were used in each sample period, as reproductively active females were available in only limited numbers. The proportion of radioactivity remaining in the incubation media was assayed to allow determination of the proportion of originally labelled steroid that became conjugated during the incubation.

5.2.3 Thin layer chromatography

Steroids were tentatively identified using thin layer chromatography (TLC). The DCM washes containing free steroids extracted from the incubation medium were evaporated to dryness, redissolved in 2 x 200 μl DCM washes and spotted onto precoated Merck (0.2 mm silica gel) 20 x 20 cm plastic 60 F_{254} plates using a 10 μl capillary tube. Initially, steroids were coeluted with authentic steroids in system I (DCM: diethyl ether (DEE) (5:2 v/v)). Plates were air-dried and standards were visualised using UV light (254 nm) or sublimed iodine crystals. The visualisation method and Rf (distance travelled by steroid / distance travelled by solvent front) in each solvent system for the routinely used standard steroids are summarised in Table 5.2.

After this initial separation, fractions from the TLC plate corresponding to the standards and the spaces between them were scraped off the TLC plate separately and eluted through columns packed with cotton wool and a thin layer of acid-washed Celite® in two column volumes of methanol (MeOH) (approximately 20 ml). Eluates were evaporated to dryness and redissolved in 5 ml MeOH. To identify peaks of radioactivity from sample runs, a 100 μl aliquot was assayed for radioactivity in 3 ml scintillation cocktail (Ecolite (+), ICN) in a Beckman LS 5801 counter. Each fraction containing a radioactive peak was evaporated to dryness, spotted onto a second TLC plate in 200 μl DCM and the procedure repeated using system II (chloroform ($CHCL_3$)/MeOH) (95:5 v/v)). Individual radioactive peaks that had coeluted twice with authentic standard were stored in MeOH at -20 °C until further (HPLC) analysis. Any radioactive peaks isolated from regions of the TLC plate that did not coelute with an authentic standard were also rechromatographed in the second solvent system; peaks were kept separate for HPLC analysis.

5.2.4 High performance liquid chromatography with radiometric detection

Steroids isolated as peaks in the TLC systems were presumptively identified using high performance liquid chromatography (HPLC) with on-line radiometric detection. The HPLC system used was a Waters Alliance 2690 liquid chromatograph attached to a Packard 500TR Series radiometric detector. The column used was a Waters Nova-Pak® C18 reversed phase column (dimensions: 3.9 x 150 mm, 4 μm particle size). The mobile phase was initially (four min) isocratic (MeOH: water (70:30)) and ramped to 85% MeOH over the next eight min at a flow rate of 0.8 ml min^{-1}. Run time was 12 min per sample and at the conclusion of each run the system was equilibrated back to MeOH: water (70:30) for six min at the same flow rate. Authentic steroid standards were run through the system to determine the elution time for each steroid. Samples in MeOH from TLC analysis were concentrated to 1.2 ml and filtered through 2 μm filters into HPLC vials from which 15 μl was injected for HPLC analysis. Radiometric detection of HPLC runs allowed comparison of elution times between authentic standards and the tentatively identified products from incubation with ^{3}[H]-P5. A Waters 996 Photodiode Array Detector (resolution 1.2 nm) scanned UV absorbance patterns for each run from 190 – 300 nm. Chromatograms at specific wavelengths were subsequently extracted from the three-dimensional data (time x wavelength x absorbance). Those steroids that coeluted isopolar with authentic steroid in two TLC systems and had an elution time corresponding with that of the same authentic steroid in this HPLC system were considered to be presumptively identified. Elution times in the HPLC system for steroids used as standards are presented in Table 5.2. Recrystallisation of steroids to constant specific activity was not attempted due to the low amounts of steroids produced.

Table 5.2 Rf values in TLC solvent systems I and II, method of visualisation and HPLC retention time for unlabelled authentic steroids used as standards.

Steroid	Symbol	TLC System I Rf	TLC System II Rf	TLC Visualisation Method	HPLC retention time (min)
Pregnenolone	P5	0.41	0.57	I_2	10.660
Progesterone	P4	0.53	0.73	UV 254 nm	8.619
Androstenedione	AD	0.47	0.73	UV 254 nm	3.948
Testosterone	T	0.34	0.57	UV 254 nm	4.716
Dehydroepi-androsterone	DHA	0.41	0.43	I_2	5.305
17β-Oestradiol	E2	0.36	0.29	I_2	3.702

5.2.5 The unidentified steroid

An unidentified steroid (See *Chapter 5 Section 5.3.7*) was detected consistently in TLC separations of extracted incubation media, and appeared as a single peak in both solvent systems. To generate a larger amount of this steroid for identification by HPLC and GC-MS, an additional two late vitellogenic-stage female lizards were killed the following spring. Their ovarian tissue was incubated in 20 flasks containing 300 mg tissue, 5 ml incubation medium and a mixture of 500 μg untritiated P5 with 5 μCi ^{3}[H]-P5 (to allow detection in TLC systems) per flask. All other procedures were as described in *Chapter 5 Section 5.5.2.* Late vitellogenic-stage female lizards were selected because that sex had synthesised the greatest amount of the unidentified steroid in the earlier incubations. In view of the observed highly polar nature of the unidentified steroid, oestriol (E3) was purchased, and samples of two trihydroxylated oestrogens, 6α-and 6β-hydroxyoestradiol (6α-OH- and 6β-OH-E2) were obtained through kind donation by Associate Professor Joan Whittier (University of Queensland), for comparison of their Rfs and retention times in the TLC and HPLC systems used in this study.

5.3 Results

Peaks of radioactivity that coeluted with authentic standard in both TLC solvent systems were regarded as presumptively identified if, in HPLC analysis, the retention time of the radioactive peak corresponded to that of the authentic standard. The identities of P5, P4, AD, DHA and T were established in this way. Yields of these metabolites after incubation with ^{3}[H]-P5 as substrate are expressed as a % of the total radioactivity (i.e. the sum of the radioactivity extracted as free steroids and the radioactivity remaining in incubation medium as conjugates). Losses on glassware were negligible. It was not appropriate or meaningful to analyse these results statistically.

5.3.1 Proportion of steroids conjugated

It can be assumed that any tritiated steroid remaining in the incubation medium following DCM extraction of free steroids was conjugated to either a glucuronide or a sulphate moiety and, therefore, resisted extraction with the organic solvent (Kime and Abdullah, 1994). The mean proportions of the original radioactivity that were conjugated during *in vitro* incubations of ^{3}[H]-P5 with active or inactive ovarian or testicular tissue are summarised in Figure 5.2.

In both sexes conjugated steroids were produced more rapidly by active than inactive gonadal tissue, and formed a greater proportion of the total radioactivity after 180 min of *in vitro* incubation in those cases. Active ovarian and testicular tissue continued to produce conjugated steroids throughout the 180 min incubations, while production of steroid conjugates by inactive ovarian tissue was greatest at 120 min (48.5 % of total tritiated steroid) and declined slightly by 180 min. Incubations of inactive testicular tissue rapidly conjugated approximately 30 % of the original ^{3}[H]-P5 (by 10 min), but subsequently showed little change in the proportion of steroid conjugated during the incubation.

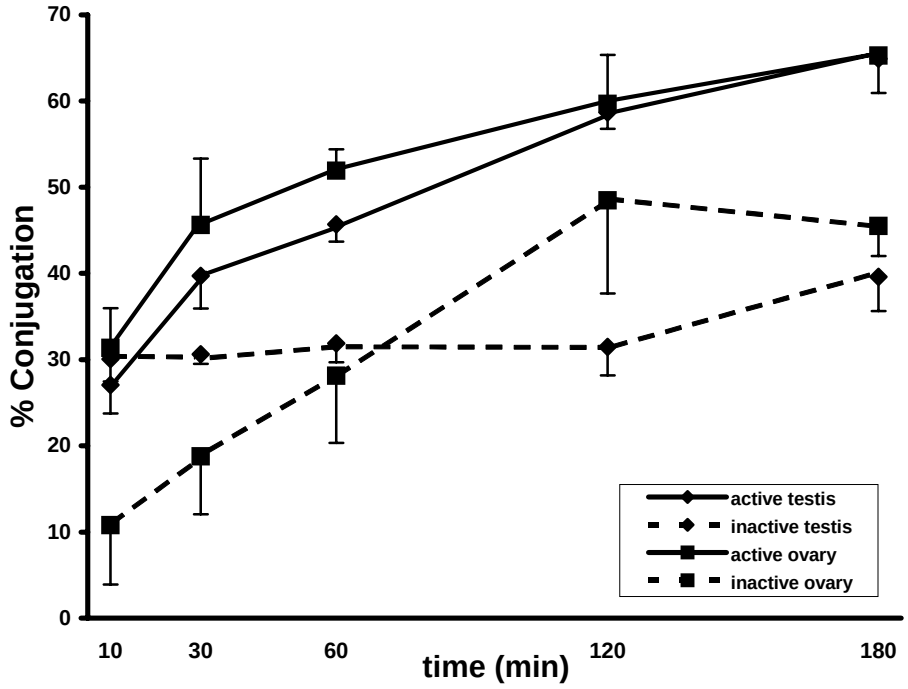

Figure 5.2 Mean proportion (%) of the original tritiated steroid conjugated *in vitro* over time by active and inactive gonadal tissue of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4; active and inactive ovary, N = 2). Values are means ± 1 SE. Some error bars removed to aid clarity.

5.3.2 Metabolism of pregnenolone

The metabolism of ^{3}[H]-P5 by the active and inactive gonadal tissue of male and female lizards is illustrated in Figure 5.3. ^{3}[H]-Pregnenolone was most rapidly and most completely metabolised in the incubations of active male gonads, in which only 5.0 % of the original ^{3}[H]-P5 remained after 180 min. The proportion of ^{3}[H]-P5 metabolised by active testes was greater than that from equivalent inactive tissue samples at 10 min of incubation and remained so until the incubations were completed (180 min). The active gonadal tissue from both males and females metabolised ^{3}[H]-P5 more rapidly and more completely than inactive tissue from the same sex.

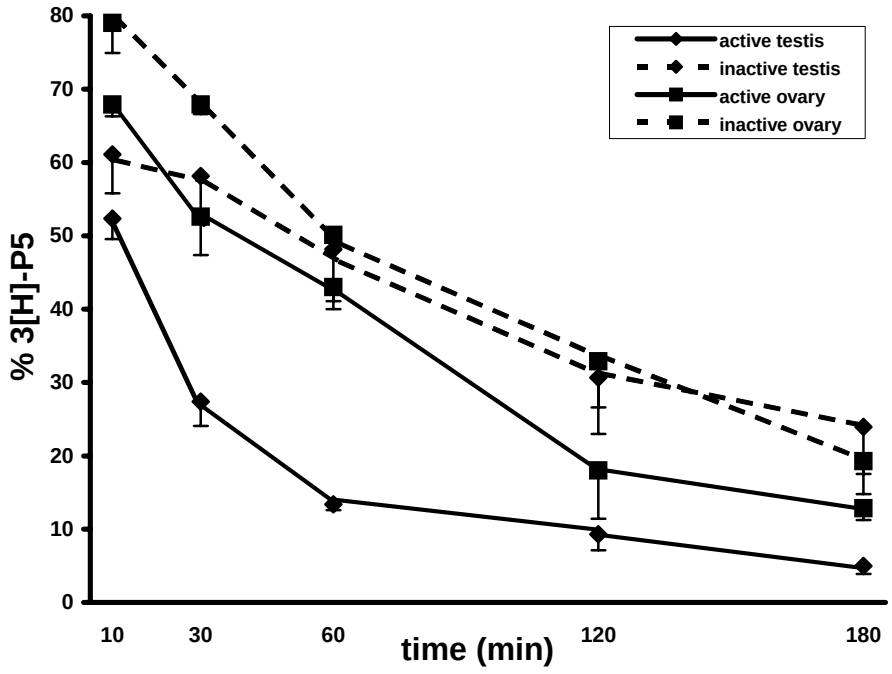

Figure 5.3 Mean proportion (%) of ^{3}[H]-P5 metabolised *in vitro* over time by active and inactive the gonadal of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4; active and inactive ovary, N = 2). Values are means ± 1 SE. Some error bars removed to aid clarity.

5.3.3 Progesterone

Seasonal patterns of *in vitro* P4 biosynthesis by gonadal tissue from both sexes are presented in Figure 5.4. Ovarian tissue collected from post-parturient females displayed the greatest and most rapid production of P4, which comprised 28.6 % of the total tritiated steroids present by 180 min. Conversely, late vitellogenic-stage ovarian tissue rapidly (by 10 min) converted more than 10 % of the total ^{3}[H]-P5 to P4, but showed no increase in the proportion of tritiated steroid present as P4 over time. By 10 min, the proportion of tritiated steroid present as P4 in incubations of both active and inactive ovarian tissue was greater than in incubations of testicular tissue from either group and remained so until 180 min of incubation. At no time during any incubation of male tissue was tritiated P4 detected as more than 3.0 % of the total tritiated free steroids metabolised from ^{3}[H]-P5.

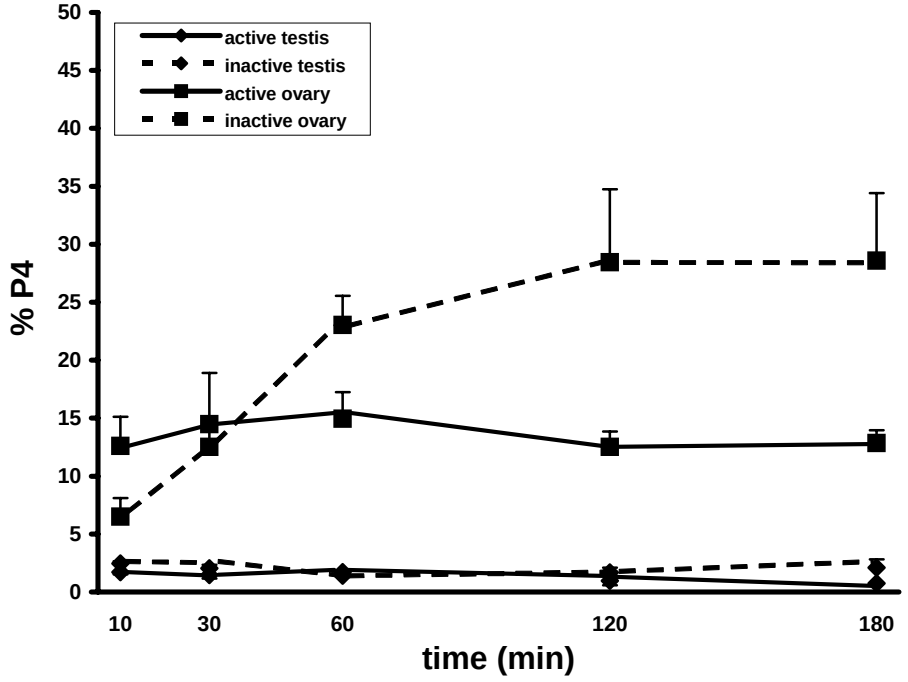

Figure 5.4 Mean proportion (%) of ^{3}[H]-P5 converted to P4 over time in *in vitro* incubations with the active and inactive gonadal tissue of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4; active and inactive ovary, N = 2). Values are means ± 1 SE. Some error bars removed to aid clarity.

5.3.4 Androstenedione

The patterns of AD production *in vitro* by active and inactive gonadal tissue of both sexes are presented in Figure 5.5. There were no clear seasonal differences in the proportion of radioactivity present as AD in the incubation of gonadal tissue of either sex. The proportion of total radioactivity present as AD fluctuated over time, but was never greater than 8.0 % in any incubation. As expected, more AD was detected in incubations of testicular than ovarian tissue in each season, and the proportion of AD was greater in incubations of inactive testicular tissue. There was a greater difference between the sexes in AD production by inactive gonadal tissue than in incubations using active gonads, except at 180 min.

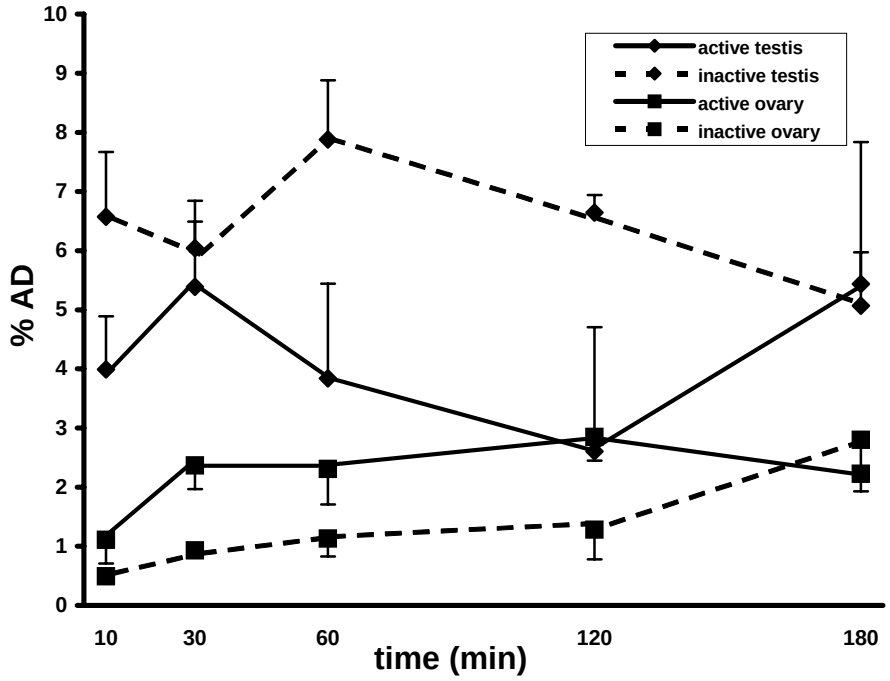

Figure 5.5 Mean proportion (%) of ^{3}[H]-P5 converted to AD over time in *in vitro* incubations with the active and inactive gonadal tissue of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4, active and inactive ovary, N = 2). Values are means ± 1SE. Some error bars removed to aid clarity.

5.3.5 Testosterone

The *in vitro* production of T from tritiated precursors over time by active and inactive gonadal tissue of male and female lizards is illustrated in Figure 5.6. Testosterone production was greater by gonadal tissue of males than females throughout the incubations at both times of year. Testosterone continued to accumulate through time in the incubation of inactive testicular tissue, rapidly until 120 min of incubation and then more slowly, until, after 180 min, 31.7 % of the radioactivity originally added as ^{3}[H]-P5 was present as T. The proportion of T present in incubations of active testicular tissue increased rapidly until 60 min then declined from 60 – 180 min. Testosterone was also synthesised by female gonads, to a greater extent by active than inactive gonads, reaching a maximum of 10.9 % of the total counts present following 180 min of incubation with late vitellogenic-stage, active, ovarian tissue.

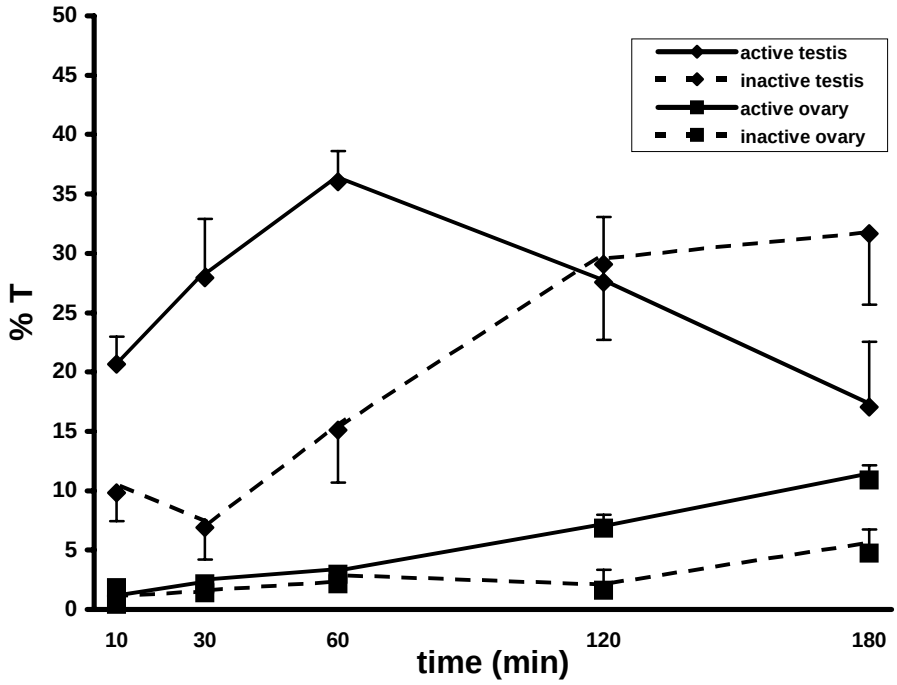

Figure 5.6 Mean proportion (%) of ^{3}[H]-P5 converted to T over time in *in vitro* incubations of with the active and inactive gonadal tissue of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4, active and inactive ovary, N = 2). Values are means ± 1 SE. Some error bars removed to aid clarity.

5.3.6 Dehydroepiandrosterone and 17β-oestradiol

Dehydroepiandrosterone was detected only in small amounts in incubations of inactive testicular tissue: DHA comprised 4.4 % of tritiated free steroids in the incubation by 180 min. No DHA was detected in incubations of active testis, or from incubations using ovarian tissue at either time of year. 17β-Oestradiol was not detected as a metabolite of ^{3}[H]-P5 from either male or female tissues at any stage of any incubation during either season.

5.3.7 Unidentified steroid

One unidentified steroid was synthesised in relatively large proportions by gonads of both males and females. The behaviour of this fraction isolated from all incubations was identical and so it is assumed that this unidentified steroid is identical for all incubations. Accumulation of this unidentified steroid over time is illustrated in Figure 5.7. In incubation of ^{3}[H]-P5 with inactive testicular tissue, only approximately 5-6 % of total radioactivity was present as the unidentified steroid at all time periods sampled. In contrast, the proportion of tritiated steroid present as the unidentified steroid in incubations of active testicular tissue was elevated by 10 min and remained so throughout the incubation. By 180 min, 28.1 % of the original tritiated precursor was present as the unidentified steroid. There was no difference between seasons in the proportion of original ^{3}[H]-P5 converted to the unidentified steroid following incubation of active or inactive ovarian tissue; 45.3 % and 33.9 %, respectively, of the original tritiated precursor was present as the unidentified steroid by 180 min of incubation.

The unidentified steroid was very polar, with an Rf of 0.07 in TLC system I and 0.17 in TLC system II. These Rfs corresponded with the authentic steroid 6α-OH-E2 in system I but with no available authentic standard in system II. The HPLC elution time of the steroid was 3.196 min, and the radioactive peak did not coelute with any available authentic standard in the HPLC system used. When E2 was not detected as a product of incubation with ^{3}[H]-P5, the possibility that this steroid is an oestrogen other than E2 was considered. The Rfs of the unidentified steroid indicated that it is more polar than E2, suggesting that it may be a trihydroxylated oestrogen. Three triols were obtained as

standards and run through the TLC and HPLC systems. However, this suggested that the unidentified steroid was less polar than oestriol (E3), 6α-hydroxyoestradiol (6α-OH-E2) and 6β-hydroxyoestradiol (6β-OH-E2). The results of TLC and HPLC runs using these three triols in comparison with the chemical properties of E2 and the unidentified steroid are summarised in Table 5.3. Ultraviolet (UV) absorbance spectra for E2, 6β-OH-E2, testosterone (T), progesterone (P4) and the unidentified steroid are included for comparison in Appendix 5.1**.**

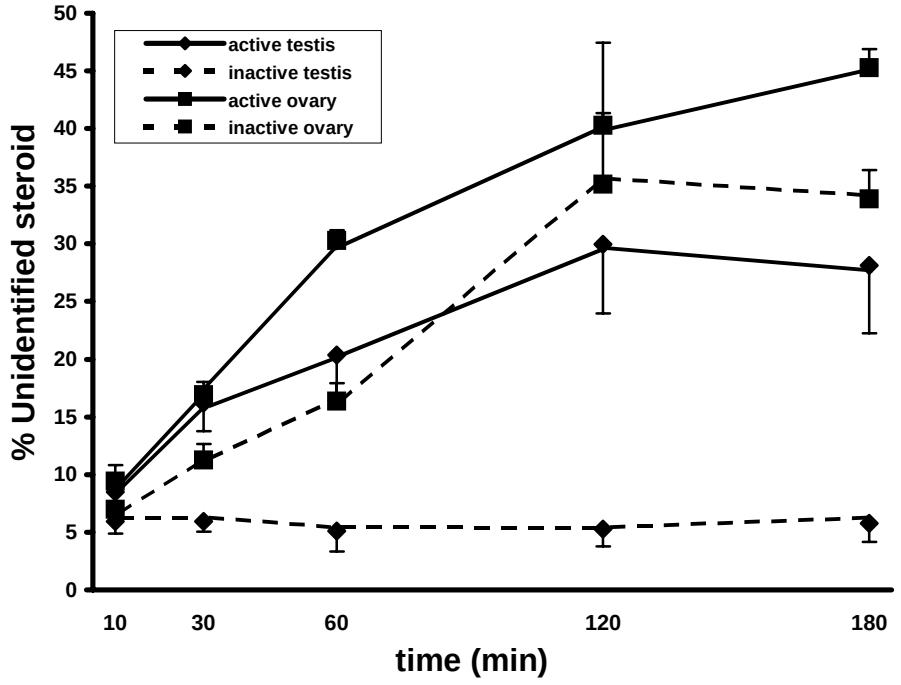

Figure 5.7 Mean proportion (%) of ^{3}[H]-P5 converted to the unidentified steroid over time in *in vitro* incubations with the active and inactive gonadal tissue of male and female *Tiliqua nigrolutea* (active and inactive testis, N = 4; active and inactive ovary, N = 2). Values are means ± 1 SE. Some error bars removed to aid clarity.

Table 5.3 The Rfs in TLC Systems I and II, and HPLC elution times of four oestrogens for comparison with the unidentified steroid.

Steroid	TLC System I (Rf)	TLC System II (Rf)	HPLC elution time (min)
E3	0.03	0.1	1.913
6α-OH-E2	0.07	0.1	1.820
6β-OH-E2	0.05	0.07	2.100
E2	0.36	0.29	3.702
Unidentified steroid	0.07	0.17	3.196

5.4 Discussion

This study was based on the assumption that the ^{3}[H]-P5 used as substrate, and all the tritiated metabolites derived from it, behaved in the same way as their endogenous equivalents in all chemical procedures. This premise has formed the basis of many previous *in vitro* studies of vertebrate endocrine physiology (Kime *et al.*, 1980; Huf *et al.*, 1989; Asahina *et al.*, 1990; Kobayashi *et al.*, 1993; Yeoh *et al.*, 1996). Several authors acknowledge that results obtained from *in vitro* studies may not accurately represent physiological processes *in vivo* in all respects (Colombo *et al.*, 1970; Ozon, 1972; Weisbart and Yousen, 1977; Kime and Abdullah, 1994), but not for reasons related to the radio-labelling of the molecules. Nonetheless, this type of experiment remains an extremely useful tool for examining the physiology of steroid hormones.

5.4.1 Steroid biosynthetic pathways

There are few recent studies of steroid biosynthetic pathways in reptiles. The intermediates of both the Δ4 and Δ5 pathways identified in this study show that *Tiliqua nigrolutea* conforms to the typical steroid biosynthetic patterns observed in most higher vertebrates (Kime, 1987; Norris, 1997). Steroid biosynthetic pathways are well conserved throughout the vertebrates (Dawson, 1998), possibly reflecting the early evolution of an efficient system of chemical communication (Bentley, 1976). Consequently, the same two pathways (Δ4 and Δ5), their intermediate metabolites, and several of their end-products have been identified in phylogenetically distant vertebrate groups (Kime, 1987; Norris, 1997).

Use of substrates below the point in the biosynthetic cascade at which the Δ4 and Δ5 pathways diverge has been a confounding factor in some previous studies (Ozon, 1967; Dale and Dorfman, 1969; Tam *et al.*, 1969; Lofts and Choy, 1971; Kime *et al.*, 1980; Kime and Hews, 1980; Kime and Rafter, 1981), meaning that pathway preferences could not be determined. In this study, pregnenolone was selected as the precursor for metabolism because it is the starting point to the biosynthetic cascade of steroid hormone production, and occurs at the point of divergence of the Δ4 and Δ5 pathways. In *Tiliqua*

nigrolutea, several intermediates of both the Δ4 (P5, P4 and AD) and Δ5 (DHA and AD) steroid biosynthetic pathways were identified following the incubation of gonadal tissue with ^{3}[H]-P5. The amount of P5 present declined over the duration of the incubations as it was converted to other pathway intermediates. Correspondingly, relative proportions of metabolites (such as P4, AD, and DHA) further down the biosynthetic pathways varied during the incubations as they were first synthesised, and then further metabolised.

5.4.2 Pathway preference

Some AD (a Δ4 pathway intermediate) was produced in all incubations. However, DHA (a Δ5 pathway intermediate) was detected only in those incubations using inactive testicular tissue: DHA was present as a considerably smaller proportion of total radioactivity than AD throughout the incubations. This implies that in *T. nigrolutea* the Δ4 pathway predominates in the biosynthesis of gonadal steroids. This is consistent with the small number of studies on other reptile species that have used ^{3}[H]-P5 or ^{3}[H]-cholesterol as a substrate for *in vitro* incubation (Callard, 1967; Chan and Callard, 1974; Hews and Kime, 1978; Huf *et al.*, 1989). For example, testicular tissue from the snake *Natrix sipedon pictivorus* (Callard, 1967) and the lizard *Lacerta viridis* (Hews and Kime, 1978) synthesised AD from ^{3}[H]-P5, but no DHA was detected, suggesting exclusive activity of the Δ4 pathway. However, in the lizard *Dipsosaurus dorsalis* and the turtle *Pseudemys scripta elegans*, small amounts of Δ5 pathway intermediates were isolated from incubations using ovarian tissue, in addition to the much larger quantities of Δ4 metabolites detected (Chan and Callard, 1974).

In contrast, extracts of non-incubated ovarian tissue of the lizard *Lacerta sicula* contained all the pathway intermediates from both the Δ4 and Δ5 biosynthetic pathways in amounts which suggest an approximately equal contribution by each pathway, although the possibility that some intermediates had been synthesised elsewhere was not eliminated (Lupo Di Prisco *et al.*, 1968). Intermediates of both pathways have also been detected in the lizard *Tiliqua rugosa*, as determined by incubation of testicular tissue (Bourne and Seamark, 1978) and by intravenous injection (Bourne, 1981) with ^{3}[H]-P5 and ^{3}[H]-P4,

although other incubation studies indicate that the Δ4 route is the more active in the production of T in that species (Huf *et al.*, 1989). Only one of these studies considered variation in steroid production with changing reproductive condition, although this was restricted to males (Bourne and Seamark, 1978).

The isolation of both DHA and AD from incubations of testicular tissue indicates that both the Δ4 and Δ5 pathways can operate in the gonads of *T. nigrolutea*. However, the shape of the %-yield curves did not allow unambiguous determination of the dominant steroid biosynthetic pathway. This is partly because I used a static incubation system. This meant that pathway intermediates were synthesised and metabolised simultaneously in the same flask, and it was not possible to separate these two processes. Additionally, I took samples at recommended time intervals from 10 to 180 min (Kime, pers. comm.), as has been done in numerous published studies (Dale and Dorfman, 1967; Leitz and Reinboth, 1987; Kime, 1990); thus each measurement indicates only the relative proportions of each steroid present at each time sampled.

The detection of DHA only from incubations of ^{3}[H]-P5 with inactive testicular tissue may well indicate that DHA was synthesised only at that time, and only by males. Additionally, the very small amount of DHA detected suggests that the Δ5 pathway may make only a small contribution to total steroid production by the gonads of *T. nigrolutea*. However, these observations may, instead, be a reflection of *increased* Δ5 pathway activity in spring, with DHA being produced but metabolised too rapidly to be detected by the sampling regime used here. Future experiments to determine conclusively the preferred pathway for gonadal steroid biosynthesis in this species should involve the *in vitro* incubation of gonadal tissue with a mixture of ^{14}C and ^{3}H labelled substrates such as P5 or cholesterol, so that the contributions of each pathway to total end-products can be determined.

5.4.3 Metabolism of P5

^{3}[H]-Pregnenolone was more rapidly and more completely metabolised to other pathway intermediates by active than inactive gonadal tissue of both sexes. This is likely to reflect increased biosynthetic activity and greater production of T and E2 by the gonads during spring. This observation correlates well with the changes reported in plasma steroid concentrations throughout the reproductive season in males (See *Chapter 3 Results*) and females (See *Chapter 4 Section 4.3.4*), in which plasma T and E2 concentrations in males and plasma E2 concentrations in females are elevated in reproductively active animals during spring. Additionally, there was a greater seasonal difference between incubations of male than female gonadal tissue in the amount of ^{3}[H]-P5 that was metabolised throughout the incubations.

5.4.4 Intersexual and seasonal variation in the biosynthesis and metabolism of pathway intermediates

Progesterone

There was a clear difference between the sexes in the extent of the production and metabolism of P4. The shapes of the curves illustrated in Figure 5.4 imply that P4 is physiologically important in female *T. nigrolutea*, but acts only as an intermediate in testicular steroid biosynthesis. Very little (< 3.0 %) of the total radioactivity was present as P4 during *in vitro* incubations of testicular tissue in any sample from either season. This presumably reflects the position of P4 as a precursor for T or E2 production and correlates with the lack of a pronounced annual pattern of changes in plasma P4 concentrations described for males (See *Chapter 3 Results*), in which plasma P4 concentrations are low throughout the annual reproductive cycle. In contrast, 12.9 % (active) – 28.6 % (inactive) of the original tritiated P5 was present as P4 following 180 min of *in vitro* incubation of ovarian tissue. Correspondingly, plasma P4 concentrations in females (See *Chapter 4 Section 4.3.4*) change significantly throughout the annual reproductive season correlated with the timing of ovulation (spring) and the first two thirds of gestation. Additionally, the clear seasonal variation observed between the *in vitro* incubations of preovulatory (spring) and post-partum (autumn) ovarian tissue is probably a reflection of increased pathway activity in spring, with P4 being more rapidly

metabolised to other pathway intermediates at this time. This is supported by the increased concentration of both E2 and T in the plasma during spring (see *Chapter 4 Section 4.3.4*). Corpora lutea (CL) were included in incubations of ovarian tissue from post-partum females. The plasma P4 profile suggests that CLs remaining in the post-partum period are degenerating, although this was not confirmed histologically. The presence of CLs may provide an alternative explanation for the greater proportion of P4 present in autumn incubations of ovarian tissue.

Androstenedione and dehydroepiandrosterone

Androstenedione was synthesised from ^{3}[H]-P5 by the gonadal tissue of both sexes. Although the proportion of tritiated steroid present as AD was low in all cases, males tended to produce more AD than females. Androstenedione exists in equilibrium with T (Kime, 1987), a major pathway end-product in male vertebrates. Higher circulating plasma T concentrations in males would, therefore, result in a correspondingly elevated presence of AD, while in females, E2 rather than T, is the major end-product of gonadal steroid biosynthesis.

The synthesis of DHA was only detected in incubations of testicular tissue. This suggests an interesting variation in pathway preference between males and females in that the Δ5 pathway appears to contribute to steroid biosynthesis in males only. Moreover, production as discussed in *Chapter 5 Section 5.4.2*, DHA was only isolated from incubations using tissue collected in summer, suggesting a seasonal change in the contribution of each biosynthetic pathway to total steroid. Contribution to steroid biosynthesis by both Δ4 and Δ5 pathways has been reported for several reptile species, but seasonal variation has not often been considered (Lupo Di Prisco, *et al.*, 1968; Chan and Callard, 1974; Bourne, 1981; Bourne and Licht, 1985; Huf *et al.*, 1989).

5.4.5 Intersexual and seasonal variation in the biosynthesis and metabolism of pathway end-products

Testosterone

Bourne *et al.* (1985) determined that, while males of the related lizard *T. rugosa* produce epiT in large quantities, this androgen is not present in *T. nigrolutea*. Thin layer chromatography and HPLC of blood and testicular extracts have demonstrated that T is the major testicular androgen in *T. nigrolutea* (Bourne *et al.*, 1985), as in other reptiles (Kime, 1987).

The proportion of ^{3}[H]-P5 converted to T, and the shape of the %-yield curve for the production of T by testicular tissue *in vitro* in summer, suggests that T is an end-product of ^{3}[H]-P5 metabolism in male gonads at that time of year. Plasma E2 and P4 concentrations during summer are basal; presumably there is no need for the further metabolism of T at this time. In spring, the proportion of tritiated steroid present as T peaks after 60 min of *in vitro* incubation and then declines markedly. Correspondingly, plasma T concentrations are elevated during spring, but plasma E2 concentrations are at a peak. The shape of the %-yield curve (Figure 5.6) for the accumulation of T in spring suggests that T is synthesised early and rapidly metabolised further, probably to E2. It is possible that the unidentified steroid may also be synthesised from T, contributing to the rapid decline in the proportion of radioactivity present as T throughout the 180 min *in vitro* incubations of tissue from either sex. The channelling of T toward this unidentified steroid might also explain why E2 is only detected in [pg ml^{-1}] quantities in the plasma, compared with [ng ml^{-1}] of T.

The proportion of labelled steroid present as T during *in vitro* incubations of ovarian tissue continued to increase from 10 to 180 min but never reached more than 4.8 - 10.9 % of the total radioactivity present (Figure 5.6). However, given that circulating T [ng ml^{-1}] concentrations are also several orders of magnitude greater than those of circulating E2 [pg ml^{-1}] concentrations in female *T. nigrolutea* (See *Chapter 4 Section 4.3.4*), T cannot be eliminated as a physiologically important metabolite of steroid biosynthesis by the ovaries. Testosterone may be a minor end-product of ovarian steroid biosynthesis as well as an intermediate for oestrogen production. In view of this, a physiological role for T in ovulation in female *T. nigrolutea* is proposed in an earlier chapter of this thesis (See *Chapter 4 Section 4.3.4*).

17β-Oestradiol

Interestingly, E2 was not isolated as a metabolite of ^{3}[H]-P5 in *T. nigrolutea* from any incubation of ovarian or testicular tissue. It is probable that there was insufficient immediate precursor present to allow detectable amounts of E2 to be synthesised as an end-product (J. Joss, pers. comm.). This is supported by the observation that relatively small amounts of T were isolated from *in vitro* incubations of ^{3}[H]-P5 with ovarian tissue (Figure 5.6). However, in the lizard *Dipsosaurus dorsalis* and the turtle *Pseudemys scripta elegans* both E2 and oestrone (E1) were detected after *in vitro* incubation of ovarian tissue using experimental conditions similar to those employed in this study (Chan and Callard, 1974). Additionally, both E2 and E1 have been identified from extracts of ovarian tissue from the lizard *Lacerta sicula* (Lupo Di Prisco *et al.*, 1968) suggesting that E2 is indeed a product of ovarian steroidogenesis in some lizards. It is, therefore, more likely that E2 is produced by the ovaries of *T. nigrolutea*, but in quantities too small to be detected, than that it is not synthesised at all. Unfortunately most *in vitro* studies of steroid biosynthesis reported in the literature have used male animals only and do not report any attempts to identify oestrogens as incubation products (Callard, 1967; Tam *et al.*, 1969; Lofts and Choy, 1971; Bourne and Seamark, 1978; Bourne and Licht, 1985).

17β-Oestradiol cannot yet be eliminated as a product of ovarian or testicular steroid biosynthesis in *T. nigrolutea*. Further investigation using T or AD as a substrate for steroid production is warranted, given that plasma E2 concentrations appear to cycle annually in both sexes (See *Chapter 3 Results* and *Chapter 4 Section 4.3.4*). 17β-Oestradiol has been isolated from incubation of ovarian, testicular and fat body tissue from other vertebrates with ^{3}[H]-P5, ^{14}[C]-cholesterol or ^{14}[C]-P4 under similar experimental conditions to those used in this study (Ozon, 1967; Lupo Di Prisco *et al.*, 1971; Chan and Callard, 1974). The possibility that the failure to detect E2 reflects a genuine absence of this oestrogen rather than an artifact of the experimental design is discussed later in this section in the context of the production of alternative oestrogens. The unlikely prospect that E2 production occurs peripherally, rather than in the gonads, is considered in *Chapter 6*. Future studies examining the gonadal and peripheral distribution

of the P450-aromatase enzyme, coupled with the isolation of metabolites from the *in vitro* incubation of peripheral tissues with T, would eliminate this possibility.

Unidentified steroid

A considerable proportion (%-yield at 180 min: spring, male 28.1 %; female 45.3 %: autumn, female 34.0 %) of the original radio-labelled P5 was converted to an unidentified polar steroid, clearly not 17β-oestradiol, by tissue from both sexes, suggesting that it may be a physiologically important steroid. In incubations using both active and inactive ovarian tissue, and using active testicular tissue collected in spring, the proportion of tritiated steroid present as the unidentified steroid continued to increase throughout the incubations. It is likely, therefore, that the unidentified steroid is an end-product of gonadal steroid biosynthesis in *T. nigrolutea*. Plasma concentrations of this unidentified steroid have not been examined.

Polar steroids that could not be positively identified have been isolated following similar incubation studies in several species of lower vertebrates. For example, the testes of *Myxine glutinosa* produce unidentified polar metabolites from P4 and T *in vitro* (Kime and Hews, 1980; Kime *et al.*, 1980). Testicular tissue from the dogfish *Scyliorhinus caniculus* also synthesises unidentified polar steroids from tritiated P5 and tritiated T in relatively high yields (30-35 %) *in vitro* (Kime and Hews, 1982). The injection of juvenile trout, *Oncorhynchus mykiss*, with P5, T or E2 resulted in the detection of low yields of a suite of polar metabolites (Yeoh *et al.*, 1996). Such metabolites often remain unidentified due to low yields or, if the steroid is unusual, to the absence of sufficient authentic standard for derivatisation and recrystallisation procedures (Kime and Hews, 1980; Yeoh *et al.*, 1996).

Given the failure to detect E2 as a metabolite of ^{3}[H]-P5 in this study, the possibility that the unidentified steroid might be an alternative oestrogen was considered. Alternatives to E2 have been reported in the plasma and from incubation and biosynthetic pathway studies in other vertebrates. For example, six- and seven- (Kime and Hews, 1980) and

15α- and 15β- (Kime and Callard, 1982; Kime and Rafter, 1981) hydroxylated derivatives of E2 are the predominant oestrogens in some lower vertebrates.

Initially, several lines of evidence supported the possibility of an alternative oestrogen in *T. nigrolutea*. The unidentified steroid is more polar than E2, but is isopolar with 6α-OH-E2 in one of the TLC solvent systems used in this study. Based on HPLC elution times the polarity of the unidentified steroid is greater than that of E2, but less that that displayed by any of the triols examined (Table 5.2). Conjugation with a sulfate or glucuronide moiety to a non-oestrogenic steroid might explain this strong polarity, but the extraction technique used eliminates this possibility; the unidentified steroid was extracted into the organic DCM, which extracts non-conjugated steroids, meaning that the unidentified steroid is free (non-conjugated).

The unidentified steroid is synthesised *in vitro* by ovarian tissue in both active and inactive tissue in approximately equivalent amounts. It is also synthesised by testicular tissue, but the yield is far greater in spring than summer, corresponding to the time of year during which plasma E2 is elevated (See *Chapter 3 Results*). 17β-Oestradiol was not detected as an end-product of steroid biosynthesis in the gonads of either sex, although it is measurable in plasma in concentrations that change significantly in a clear annual cycle of production in both sexes (See *Chapter 3 Results* and *Chapter 4 Section 4.3.4*). It is possible that, if the unidentified steroid is an oestrogen, it cross-reacted with the antibody used to measure plasma E2 concentrations, contributing to this observed annual variation. Should this be the case, the annual plasma E2 profiles presented in *Chapter 3 Results* (males) and *Chapter 4 Section 4.3.4* (females) might actually be a representation of the additive effect of E2 and the unidentified steroid, masking changing proportions of each throughout the season. Such a phenomenon has, indeed, been described in another reptile, the snake *Thamnophis sirtalis parietalis*; 6α– and 6β-OH-E2 were detected in plasma in significant quantities using a commercially prepared E2 radioimmunoassay (RIA) kit (Whittier and Hess, 1992). The procedures used to generate the antibody for that kit involved manipulating the steroid nucleus at the C6 position, thus facilitating cross-reactivity with 6α– and 6β-OH-E2 (J. Whittier, pers. comm.). For the Spectria kits used

in my study, the antibody to E2 was generated in a similar manner, by manipulating the steroid nucleus at the C6 position. It is, therefore, possible that the unidentified steroid could have cross-reacted with the E2 antibody in this case.

The decline in the proportion of tritiated steroid present as T from 60 min in incubations using testicular active tissue in spring introduces the possibility that T may act as a precursor for the production of the unidentified steroid. In incubations of inactive testis collected in summer, when the proportion of unidentified steroid present remains low, T appears to behave as an end-product of steroidogenesis (Figure 5.6), in that the proportion of radioactivity present as T does not decline during the later stages of the incubation. However, the unidentified steroid is detected early (by 10 min) in the incubation time course (Figure 5.7). It may, therefore, be at least partially synthesised from a precursor that occurs earlier than T in the biosynthetic cascade.

Unfortunately, despite the relatively high abundance of the unidentified steroid, the absolute yield was insufficient to allow positive identification. I attempted to generate a greater yield of the unidentified steroid for identification by GC-MS through a supplementary *in vitro* incubation using larger amounts of tissue and substrate. This was not as successful as expected, with little or no conversion of ^{3}[H]-P5 to the unidentified steroid, highlighting the effect of changing substrate concentration on the products of *in vitro* incubation studies (Kime and Hews, 1980; Kime and Abdullah, 1994). Following discussions with Prof. Robert Mason (Oregon State University), a small sample of the unidentified steroid was sent to the National Institutes of Health (NIH) in the USA. A single GC-MS FAB+ analysis was carried out. The results suggested that the single radioactive peak detected by the radiometric detector attached to the HPLC system used in this study consists of two different molecules with molecular ions at 385 and 371 (L. Pannell, pers. comm.), although these do not correspond to any common steroid derivatives. However, the signal was weak, and this avenue could not be pursued further. The splitting pattern produced by the single GC-MS FAB+ injection is included as Appendix 5.2. Further speculation on the structure and possible roles of the unidentified steroid would be premature at this time.

5.4.6 Pathway plasticity

The literature suggests a strong phylogenetic conservation in pathway preference for the biosynthesis of reproductive steroids in vertebrates (Kime, 1987; Norris, 1997; Dawson, 1998), while the conservation of the major end-products of gonadal steroid biosynthesis within each vertebrate class gives a misleading impression that all aspects of steroid production are similarly highly conserved. A critical review of the limited literature available suggests that there is marked variation within vertebrate classes in the relative contributions of each biosynthetic pathway to steroidogenesis (See *Chapter 5 Section 5.1.2*). There is also evidence of plasticity within species in the activity of each of the two biosynthetic pathways in response to changing reproductive condition, temperature and other factors (See *Chapter 5 Section 5.1.4*).

There are two alternative mechanisms by which such a shift in the activity of one or both of the biosynthetic pathways might be achieved. The vehicle by which both of these mechanisms accomplish change, particularly in ectothermic species, is likely to be the differential effects of temperature on the activity of biosynthetic pathway enzymes (Kime, 1979; Kime and Hews, 1982).

The first of these mechanisms for plasticity is variation in the proportions of steroid conjugates produced. A common pattern has emerged among those fish and reptile species that have been examined. Steroid conjugates are preferentially formed at higher temperatures in the teleost fishes *Salmo gairdneri* (Manning and Kime, 1985), *Saratherodon mossambicus* (Kime and Hyder, 1983), *Salmo gairdneri* (Kime, 1979), the elasmobranch *Scyliorhinus caniculus* (Kime and Hews, 1982), and the lizard *Tiliqua rugosa* (Bourne *et al.*, 1986b). The removal of incubation substrate and products as their conjugates is reflected in decreased circulating concentrations of free steroids and acts to restrict free steroid production to times of year during which the cooler temperatures are favourable for reproduction.

The second mechanism is through the production of inactive or alternative steroids; these may act as a reserve pool that can readily be converted to the required steroid molecule when needed. It is noteworthy that in both species in which this strategy has been described a steroid other than T is used as the major testicular androgen, with T instead acting as a "biological shunt" molecule. This phenomenon has been described in the goldfish *Carassius auratus*, in which T (and 11-KT-glucuronide) is formed instead of the more biologically active free androgen 11-KT at incubation temperatures too high for successful reproduction (Kime, 1980). The shunting alternative has also been adopted in the lizard *T. rugosa*, in which both T and epiT are synthesised by the testis, but epiT is the major testicular androgen (Bourne and Seamark, 1978). Bourne *et al.* (1986b) demonstrated that seasonal temperature variation did not affect testicular T production *in vitro*, but that testicular biosynthesis of epiT increased with increasing temperature. The seasonal switch in the T:epiT ratio may arise because the enzymes specific to epiT production are more temperature sensitive than those associated with T production (Bourne *et al.*, 1986b). In another study, *in vitro* incubation of *T. rugosa* testicular tissue with ^{3}[H]-P5 and ^{14}[C]-P4 demonstrated that the yield of epiT was maximal during summer, while the yield of T was greater in spring (Bourne and Seamark, 1978), when the latter may be responsible for initiating mating behaviour (Bourne and Seamark, 1975). Additionally, while T is synthesised via the Δ4 pathway only, both Δ4 and Δ5 routes contribute to the production of epiT in this species (Huf *et al.*, 1989). All incubations described in the current study were conducted at the same temperature (35 °C) to eliminate this potential source of variation.

This study using the lizard *T. nigrolutea*, reports the detection of intermediates of both the Δ4 and Δ5 steroid biosynthetic pathways. Delta-5 pathway activity was only detected in males, suggesting a variation in pathway preference between the sexes. This study also reports clear variations in steroid biosynthetic pathway preference between gonadal tissue collected during reproductively active (spring) and inactive (summer: males; autumn: females) times of year. Additionally, a potentially novel end-product of steroid biosynthesis was detected, which warrants further investigation.

Changes in the mass of gonadal tissue available were noted (See *Chapter 3 Results* and *Chapter 4 Section 4.3.3* for GSI). Sufficient testicular tissue was collected from all males to allow a comparable mass of tissue per flask for all incubations. Changes in ovarian mass were extreme and very little "inactive" tissue was available from each female killed (tissue per animal: vitellogenic – 60 g; post partum – 6.6 g). However, such a difference in the mass of tissue incubated reflects the total tissue available *in vivo* for each reproductive condition. The contributions of different ovarian tissues to steroid biosynthesis were not considered by this study. Relative proportions and absolute amounts of thecal, granulosa and luteal tissue types vary during the reproductive cycle, in contrast with testicular tissue, the presence of which varies in absolute terms only. *In vitro* incubation of single tissue types will be required to identify the site of synthesis of the unidentified polar steroid.

The extent of the contribution of various extragonadal tissues to circulating steroid concentrations was not assessed in this study, but is worthy of further investigation and would complement the data presented in the next chapter, which examines the metabolism of T and E2 by extragonadal (peripheral) tissues. The response of the two steroid biosynthetic pathways to temperature is clearly another avenue for further research and would add another dimension to this study. Gonadal tissue incubated at a range of physiologically relevant temperatures is likely to provide greater sensitivity in detecting differences in the activity of the two pathways, both between sexes and with changing reproductive condition.

Contrary to some established literature, steroid biosynthesis appears to be a dynamic and plastic process, with scope for variation according to physiological conditions. It is likely that as more in-depth studies of this type are completed, an alternative will emerge to the traditional view of steroid biosynthetic patterns as being phylogenetically conserved; it is likely that there are greater, albeit subtle, variations in the patterns and products of steroid biosynthesis, both within and between species, than previously suspected.

Chapter 6 Peripheral steroid metabolism in male and female *Tiliqua nigrolutea*

6.1 Introduction

6.1.1 Sites of steroid metabolism

Following biosynthesis in the gonads or other endocrine organs, steroids are released into the circulatory system, via which they travel to peripheral target tissues (Ozon, 1972). In these tissues, testosterone (T) and 17β-oestradiol (E2) bind with specific receptors to induce a biological effect (Evans, 1988). Steroid receptors belong to the nuclear receptor superfamily (Koelle *et al*., 1991), which also includes receptors for thyroid and retinoid hormones (Laudet *et al*., 1992; Seagraves, 1991). All steroid receptor proteins are intracellular (Norris, 1997), but steroids are lipid soluble and diffuse easily through cell membranes (Bentley, 1976). Within a cell, either in the cytosol or within the nucleus, the hormone and receptor form a complex (Evans, 1988), which migrates to interact with the DNA upstream from the promoter region of a target gene (Norris, 1997). This process stimulates DNA transcription, and the resulting mRNA codes for protein products which bring about the biological effects associated with steroid hormones (Evans, 1988). It is in this way that a steroid modulates gene expression. A steroid molecule can bind directly with its specific receptor within a target cell; however, as it diffuses towards a receptor it may first be altered by enzymes located within the mitochondria, smooth endoplasmic reticulum (SER) or cytosol (Kime, 1987). The way in which a steroid is derivatised, conjugated, or both, depends on the specific enzymes located within the cells of the target tissue with which it comes in contact prior to reaching a receptor. This is determined largely by the vertebrate class to which the animal belongs and the type of tissue concerned (Kime, 1987). Should a steroid become conjugated at a target tissue, it is then unable to interact with the receptor (Cuevas *et al.*, 1992).

Peripheral (extragonadal) metabolism of the primary reproductive steroids, T and E2, has been documented *in vitro* in an enormous 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), gastrointestinal mucosa (mammals: Grimmer *et al.*, 1992) and liver (fish: Kime and Saksena, 1980; Snowberger and Stegeman, 1987, mammals: Payne, 1980). The paucity of reptile data should be noted.

6.1.2 Types of steroid metabolism

Derivatisation

Conversion of the original steroid molecule to a derivative often results in the formation of a more biologically active steroid with greater affinity for the steroid receptor (Johnson and Everitt, 1988). There are a number of enzymes located within different parts of a target cell which act to make specific modifications to the steroid molecule. For example, 5α-reductase, located in the SER (Kime, 1987), is the enzyme responsible for the conversion of T to 5α-dihydrotestosterone (5α-DHT) in many androgen target tissues (Johnson and Everitt, 1988) such as mammalian prostate and skin. This conversion is often regarded as the first step in the mechanism of action of T at target cells (Ozon, 1972). 5α-Reductase is also active in the brain and skin of birds (Balthazart *et al.*, 1979; Schlinger *et al.*, 1989), the seminal vesicles of the anuran *Discoglossus pictus* (Ozon and Fouchet, 1972) and the ovary of the urodele *Necturus maculosus* (Callard and Leathem, 1966).

Another enzyme located in the SER of steroid metabolising cells is aromatase, which produces oestrogens from androgens (Kime, 1987). Aromatase activity has been detected in the brains of species from all vertebrate classes (Callard *et al.*, 1977; 1978; 1980). Such localised production of oestrogens is likely to mediate the expression of T-stimulated aggressive behaviour in the bird *Coturnix coturnix japonica* (Schlinger and Callard, 1990) and is likely to be important in the activation of female reproductive behaviour in mammals (Adkins-Regen, 1981). Other tissues, including the kidney and the testes in the agnathan *Petromyzon marinus* (Callard *et al.*, 1980), the skin of the bird *Gallus gallus* (Sebright bantam) (Wilson *et al.*, 1987) and the ovaries and fat bodies of

the amphibian *Triturus cristatus carnifex* (Lupo Di Prisco *et al.*, 1971), also have the ability to synthesise oestrogens from androgens, indicating the presence of the aromatase enzyme.

Testosterone is converted to 11β-hydroxytestosterone (11β-OH-T) by 11β-hydroxylase and then to 11-ketotestosterone (11-KT) by 11β-hydroxysteroid dehydrogenase (11β-HSD) in species from several different vertebrate classes. 11-Ketotestosterone is known to be more androgenic than T in the kidney of *Gasterosteus aculeatus* (Borg *et al.*, 1992) and in the skin of *Salmo trutta* (Pottinger and Pickering, 1985), although its site of production is unclear.

The gonads of many vertebrates both synthesise and further metabolise major steroids (Ozon and Stocker, 1974; Kime and Hews, 1980; Huf *et al.*, 1989; Cuevas *et al.*, 1992). Conversion of T to 11-KT may actually occur following biosynthesis of T in the testis in these species, as it does in *Salmo salar* (Idler *et al.*, 1971). 11-Ketotestosterone is also produced by both gonadal and fat body tissue of males and females of a urodele amphibian *Triturus cristatus carnifex* (Lupo Di Prisco *et al.*, 1971; 1972).

Conjugation

The second way in which a steroid molecule may be altered is by conjugation with either a sulphate or glucuronide moiety, involving sulphatase or glucuronidase enzymes, respectively. In mammals, this occurs most extensively in the liver as a way of deactivating and solubilising steroids (Kime, 1987; Norris, 1997) for later excretion in faeces or urine (Heistermann *et al.*, 1993; Wasser *et al.*, 1996; Velloso *et al.*, 1998). Steroid conjugation is also known to occur in the mammalian placenta, gonads and adrenals (Scott and Vermierssen, 1994). Most non-mammalian vertebrates also conjugate some steroids in the liver to facilitate excretion (Kime, 1987).

However, conjugation of steroids for reasons other than deactivation has been described in some non-mammalian vertebrates. In teleosts, the role of conjugated steroids as pheromones, when released into the water with urine or milt, has been well documented

(Scott and Vermierssen, 1994; Stacey *et al*., 1994; Stacey and Sorensen, 1986). Conjugated steroids are known to stimulate milt production (Stacey *et al.*, 1989; Van Der Kraak *et al.*, 1989) and spawning behaviour (Sherwood *et al.*, 1991; Sorensen and Stacey, 1991; Carolsfeld *et al.*, 1997a; b) in fish. These conjugated steroids are largely gonadal in origin (Stacey *et al.*, 1986; Van Den Hurk *et al.*, 1987; Scott and Vermierssen, 1994), rather than being excretory products generated by the liver as occurs in mammals (Kime, 1987). However, whether gonadal conjugation involves newly synthesised steroids prior to their release into the circulation, or whether the gonads are conjugating already circulating steroids, and so acting much like an additional peripheral tissue, is unclear. In teleosts, skin (Stacey *et al.*, 1986) and gills (Kime and Ebrahimi, 1997) have also been implicated in the conjugation of steroids for use as pheromones.

Among other aquatic vertebrates the testis of the elasmobranch *Squalus acanthias* (Cuevas *et al*., 1992), the Australian lungfish, *Neoceratodus forsteri* (Joss *et al*., 1996) and the liver of the amphibians *Pleurodeles waltlii* (Ozon and Breuer, 1966) and *Dicoglossus pictus* (Ozon and Stocker, 1974) produce conjugated steroids, although it is not yet clear if any of these molecules function as pheromones.

Additionally, there are differences between the vertebrate classes in the dominant conjugates that are formed. Tissues from mammalian species usually form steroid sulphates (Ruokonen and Vihko, 1974; Payne, 1980; Dehennin, 1993), while glucuronidated steroids predominate in teleost species that use conjugates as pheromones (Kime, 1980; Schoonen and Lambert, 1986a; Van Den Hurk *et al.*, 1987; Sherwood *et al.*, 1991). However, some fish species partition steroid conjugate production. For example, in the trout *Oncorhynchus mykiss* glucuronides are produced by the liver for excretion in faeces, while sulphates are released in the urine (Vermierssen and Scott, 1996). In the testis of *S. acanthias* (Cuevas *et al.*, 1992) and the ovary of the goldfish *Carassius auratus* (Scott and Sorensen, 1994), both glucuronidated and sulphated steroids are produced, for which separate paracrine regulatory, excretory or pheromonal functions are proposed. Recently, differential routes of steroid excretion and different roles for

particular steroid molecules have also been documented in some mammalian species (Wasser *et al.*, 1996; Velloso *et al.*, 1998).

6.1.3 Variation in the patterns of steroid metabolism

Numerous *in vitro* and *in vivo* studies of steroid metabolism in vertebrates have documented variations in the patterns of production of conjugates and derivatives according to the reproductive condition of the animal (Ozon and Fouchet, 1974; Kime, 1987; Schlinger *et al.*, 1989; Borg *et al.*, 1992). In ectothermic vertebrates such variations can be mediated by the effects of temperature on enzyme activity (Kime, 1979; Kime and Hyder, 1983; Manning and Kime, 1985; Lofts, 1987) (Refer to *Chapter 5 Section 5.4.6* for more information). Seasonal variations in several other factors, including receptor density, which is itself up or down regulated in response to steroid concentrations in lizards (Paolucci and Di Fiore, 1994; Cardone *et al.*, 1998), steroid substrate concentration (Kime and Hews, 1980; Kime and Abdullah, 1994) and binding protein concentration, could also conceivably alter ratios of free to conjugated steroids, although such factors are often not considered.

6.1.4 This study

The effects of reproductive steroids on some peripheral tissues have been observed in several reptile species. For example, epididymal function (Shivanandappa and Devaraj Sarkar, 1987) and hypertrophy of the sexual segment of the kidney (Prasad and Sanyal, 1969) are both regulated by androgens, and exogenous application of E2 can stimulate the hypertrophy of cloacal glands (Cooper *et al.*, 1986b) in lizards. Moreover, hemipenal tissue in *Lacerta vivipara* is insensitive to T in adult animals (Dufaure and Chambon, 1978), but in *Calotes versicolor* it is regulated by androgens (Ananthalakshimi *et al.*, 1991). This tissue may, therefore, be a candidate as a site for the action of a metabolite of testosterone. However, links between gonadal steroids and their effects on peripheral tissues are often inferred rather than proven, and are, furthermore, generally assumed to be the direct effects of T or E2 rather than their metabolites.

The detection of particular steroid metabolising enzymes in target tissue cells goes some way towards strengthening the link between a steroid, its metabolites, and the physiological or behavioural responses attributed to it. The presence of enzymes including aromatase and 5α-reductase, as well as sulphating and glucuronidating enzymes have all been detected in, or inferred by the presence of appropriate metabolites, in several different tissues in reptiles including the brain (Callard *et al.*, 1977; 1978; Crews and Morgentaler, 1979; Huf *et al.*, 1987a; Gobbetti *et al.*, 1994; Wade, 1997; Winkler and Wade, 1998) and renal sexual segment (Crews *et al.*, 1978). Some authors have acknowledged that observed changes in tissue morphology or physiology in response to the application of exogenous steroid may actually occur following localised metabolism of the steroid at the target tissue (Dufaure and Chambon, 1978; Abell, 1998). Indeed, variations in responsiveness to steroids may, at least partly, be due to differences in the patterns of steroid metabolism in different peripheral tissues. The presence or absence of appropriate enzymes is, in no small way, responsible for this additional level of tissue specificity.

No studies, to my knowledge, have examined potential changes in the patterns of peripheral steroid metabolism throughout the annual reproductive cycle in reptiles, according to reproductive condition or between sexes. This study examines the ability of a range of reproductively important tissues to metabolise a primary steroid (T or E2) as a measure of the activity of steroid metabolising enzymes in those tissues, in the viviparous lizard *Tiliqua nigrolutea* and considers intersexual and seasonal differences in patterns of steroid metabolism. The possibility of a role for steroid metabolites as semiochemicals in a lizard is considered. This study was conducted prior to the experiments presented in *Chapter 5* so the possibility of an alternative to E2 as the major ovarian oestrogen in this species had not yet been raised. Testosterone and E2 were chosen as the basis for this metabolic study based on a survey of the literature.

Although not peripheral tissues, ovarian and testicular tissues were included in this study. The ability of gonadal tissue to modify T and E2 post-biosynthetically by conjugation or

derivatisation has been well documented in vertebrates (Cuevas *et al.*, 1992; Joss *et al.*, 1996; Scott and Vermierssen, 1996).

6.2 Materials and methods

General methods are described in *Chapter 2 Section 2.1*, but information specific to the work in this chapter is presented here.

6.2.1 Tissue collection

Peripheral tissues potentially capable of steroid metabolism were collected from male and female *Tiliqua nigrolutea* at autopsy of freshly killed individuals between April 1995 and February 1996. Male and female lizards were sampled at times of year that corresponded to distinct phases of the annual reproductive cycle (summarised in Table 6.1). Tissues collected for incubation were skin (lateral abdominal body surface), muscle (abdominal wall), liver, cloaca (surrounding the cloacal opening), adrenal and kidney (both sexes), ovary (including corpora lutea (CLs) when present), oviduct (females only), and testis, epididymis and sexual segment (SS) of the kidney (males only). Oviductal tissue was collected in gestating females from regions adjacent to developing embryos, and from an similar position in post-parturient and preovulatory animals). Kidney tissue from early spermatogenic-stage males in autumn was not collected for incubation. In males of the sympatric species, *Niveoscincus metallicus*, the renal SS is clearly identifiable (by hypertrophy and colour change) during the autumn mating period as the anterior third of each kidney (Jones, pers. comm.); a similar region was selected in *T. nigrolutea*.

Skeletal muscle was used as a (presumably) non-reproductively relevant control tissue. Gonadal tissue from each sex was included, despite its not being a peripheral tissue, because it is also a potential site of post-biosynthetic modification of primary gonadal steroids. Samples of liver, kidney and sexual segment, adrenal gland, oviduct, epididymis and cloacal tissues were preserved in Bouin's fixative and examined histologically (details in *Chapter 2 Section 2.5*). Details of testicular and ovarian histology are presented in *Chapter 3 Results* and

Table 6.1 Sampling regime for collection of peripheral tissues from male and female *Tiliqua nigrolutea* for *in vitro* incubation.

	Male	Female
reproductive stage	early spermatogenesis (active)	post-parturition (inactive)
month	mid-Apr 1995	mid-Apr 1995, 1996
sample size	N = 2	N = 2
reproductive stage	late spermatogenesis (active)	late vitellogenesis (active)
month	mid-Sep 1995	early Oct 1995
sample size	N = 2	N = 2
reproductive stage	post-mating quiescence (inactive)	late gestation (active)
month	late Dec 1995	mid-Feb 1996
sample size	N = 2	N = 2

Chapter 4 Section 4.3.2, respectively. Histological changes were not analysed statistically.

6.2.2 Incubation

All tissues from each animal were incubated separately. At autopsy samples of each tissue type (200 mg, with the exception of adrenal tissue (50 – 100 mg)) were collected and minced finely with scissors. Duplicates of each tissue were prepared when sufficient material was available, and additional tissue was fixed for histological examination as described in *Chapter 2 Sections 2.4* and *2.5*. Minced tissue samples 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, respectively, with ^{3}[H]-testosterone (^{3}[H]-T) (5 μCi) or ^{3}[H]-17β-oestradiol (^{3}[H]-E2) (5 μCi) as substrate.

Each flask was held on ice until all flasks were prepared. Samples were then incubated at 35 °C in an air environment in a gently rocking waterbath for 180 min. The temperature of the waterbath 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 further analysis. Free (non-conjugated) steroids were later extracted

from thawed incubation media in two volumes of dichloromethane (DCM). Dichloromethane washes were combined and stored at -20 °C until further analysis.

6.2.3 Thin layer chromatography

Steroid incubation products were tentatively identified using thin layer chromatography (TLC). The DCM washes containing extracted free steroid metabolites were evaporated to dryness and spotted onto precoated Merck (0.2 mm silica gel) 20 x 20 cm plastic 60 F_{254} plates in 200 µl DCM using a 10 µl capillary tube. Steroids were coeluted with authentic steroids in Solvent System II (dichloromethane: diethyl ether (5:2 v/v), see *Section 5.2.3*). Plates were air-dried and standards were visualised using UV light (254 nm) or sublimed iodine crystals. The visualisation method and Rf (distance travelled by steroid / distance travelled by solvent front) in each solvent system for the commonly used standard steroids are summarised in Table 6.2. After this separation, fractions from the TLC plate corresponding to the standards and the spaces between them were scraped off the TLC plate separately, and eluted through columns packed with cotton wool and a thin layer of acid-washed Celite® in two column volumes of methanol (MeOH) (approximately 20 ml). Eluates were evaporated to dryness and redissolved in 5 ml MeOH. To identify peaks of radioactivity from sample runs, a 100 µl aliquot was assayed for radioactivity in 3 ml scintillation cocktail (Ecolite (+), ICN) in a Beckman LS 5801 counter. Each fraction containing a radioactive peak was stored in methanol at -20 °C until further (HPLC) analysis.

Any radioactive peaks isolated from regions of the TLC plate that did not coelute with an authentic standard were also kept separate for HPLC analysis. A 100 µl aliquot of the original medium from each incubation was assayed for radioactivity in 3 ml scintillation cocktail following the extraction of free steroids. This allowed the determination of the proportion of originally labelled steroid that became conjugated during the incubation. 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.

6.2.4 High performance liquid chromatography with radiometric detection

Steroids were presumptively identified using high performance liquid chromatography (HPLC) with on-line radiometric detection. The HPLC system used was a Waters Alliance 2690 liquid chromatograph attached to a Packard 500TR Series radiometric detector. The column used was a Waters Nova-Pak® C18 reversed phase column (dimensions: 3.9 x 150 mm, 4 μm particle size). The mobile phase was initially (four min) isocratic (MeOH:water (70:30)) and ramped to 85% MeOH over the next eight min at a flow rate of 0.8 ml/min. Run time was 12 min per sample, and at the conclusion of each run the system was equilibrated back to MeOH: water (70:30) for six min at the same flow rate. Authentic non-tritiated steroid standards were run through the system to determine the elution time for each steroid. Samples in methanol from TLC analysis were concentrated to 1.2 ml and filtered through 2 μm filters into HPLC vials from which 15 μl was injected for HPLC analysis. Radiometric detection of HPLC runs allowed comparison of elution times between authentic standards and the tentatively identified products from incubation with ^{3}[H]-T or ^{3}[H]-E2. A Waters 996 Photodiode Array Detector (resolution 1.2 nm) scanned UV absorbance patterns for each run from 190 – 300 nm. Chromatograms at specific wavelengths were subsequently extracted from the three-dimensional data (time x wavelength x absorbance). Those steroids which coeluted isopolar with authentic steroid in TLC solvent system II and also had an elution time corresponding with that of the authentic steroid in this HPLC system were considered to be presumptively identified. High performance liquid chromatography elution times for steroids used as standards are presented in Table 6.2. Recrystallisation to constant specific activity was not attempted due to the low amounts of steroids produced.

Table 6.2 Elution distances (Rf) in TLC solvent system II, methods of visualisation and HPLC retention time for unlabelled authentic steroids used as standards.

	Symbol	**TLC System II Rf**	**TLC visualisation method**	**HPLC retention time (min)**
17β-oestradiol	E2	0.29	I_2	3.702
testosterone	T	0.57	UV 254 nm	4.716
5α-dihydrotestosterone	5α-DHT	0.60	I_2	6.400
androstenedione	AD	0.73	UV 254 nm	3.948

6.3 Results

Results are presented as mean proportions of the total original substrate (^{3}[H]-T in incubations with male tissues, ^{3}[H]-E2 in incubations with female tissues), which were either derivatised and left free, conjugated, or remained unmetabolised as the original tritiated substrate following 180 min of *in vitro* incubation. The proportion of tritiated steroids regarded as conjugated was calculated as the percentage of the original substrate that remained in the incubation medium following extraction with DCM. This could have included both conjugated original substrate and any derivatised steroids that were then conjugated during the incubation. This means that the proportions of metabolised steroids from each incubation are likely to be conservative estimates of the degree of metabolism occurring during the *in vitro* incubations.

Free steroids were defined as those that were not conjugated to a glucuronide or sulphate moiety and 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 calculated as a percentage of the total free steroids extracted.

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. Rather, the relative proportions of conjugates and derivatives produced, and the substrate remaining after incubation were used as indicators of the overall activity of each tissue. A sample chromatograph of some of the numerous polar metabolites that were detected by the HPLC system used is presented in Appendix 6.1.

6.3.1 Incubation of tissues

Liver

The majority (63 - 93 %) of the tritiated substrate became conjugated by 180 min in all incubations (Figure 6.1), with only small proportions of tritiated steroids present as substrate (4 - 26 %) or free derivatives (3 - 16 %). Of the liver samples, liver from gestating females conjugated the tritiated substrate to the greatest extent. Thin layer chromatography and HPLC analysis indicated that the small proportion of tritiated steroid detected in the derivatised fractions of media from incubations of female liver was comprised of several highly polar metabolites. Androstenedione was detected in incubation media from all three incubations with male liver, while 5α-DHT was produced only by the incubations of liver collected from early spermatogenic-stage males. Several polar metabolites were also detected, but no E2 was produced as a metabolite of ^{3}[H]-T in any incubation with liver. There were no qualitative histological differences in liver tissue from animals of different reproductive conditions or sexes.

Muscle

Only a small proportion (11 - 20 %) of tritiated substrate was converted to free steroid derivatives following 180 min of *in vitro* incubation of muscle tissue (Figure 6.2). Androstenedione and 5α-DHT were not detected in media from any incubation with tissue from males. A range of polar metabolites was detected in media from all incubations from both sexes, and small amounts of T and 5α-DHT were detected in media from incubations with muscle collected from late vitellogenic females. With the exception of gestating females, 46 - 63 % of original substrate remained unmetabolised and < 39 % became conjugated. Muscle tissue from gestating females conjugated a greater proportion (64 %) of the original tritiated substrate than muscle from late vitellogenic or post-partum animals. Muscle was not examined histologically.

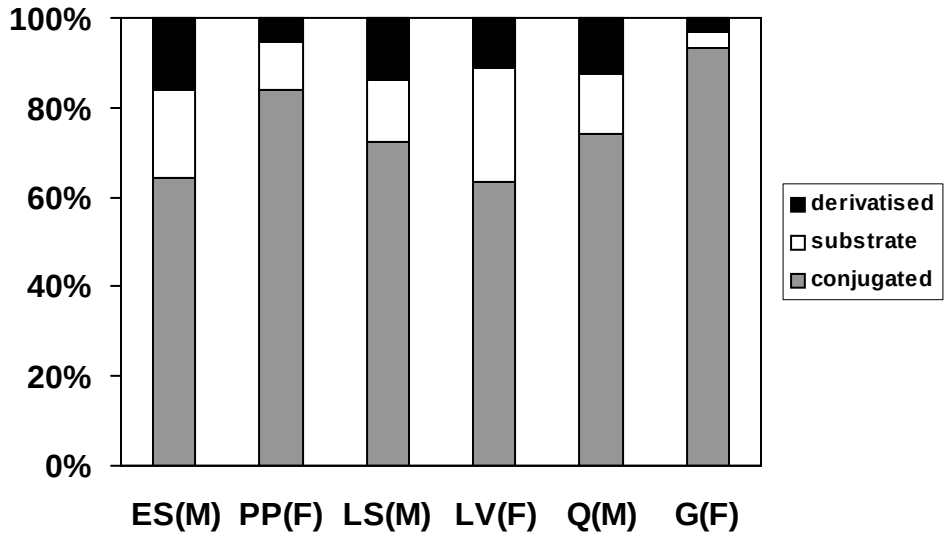

Figure 6.1 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with liver tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

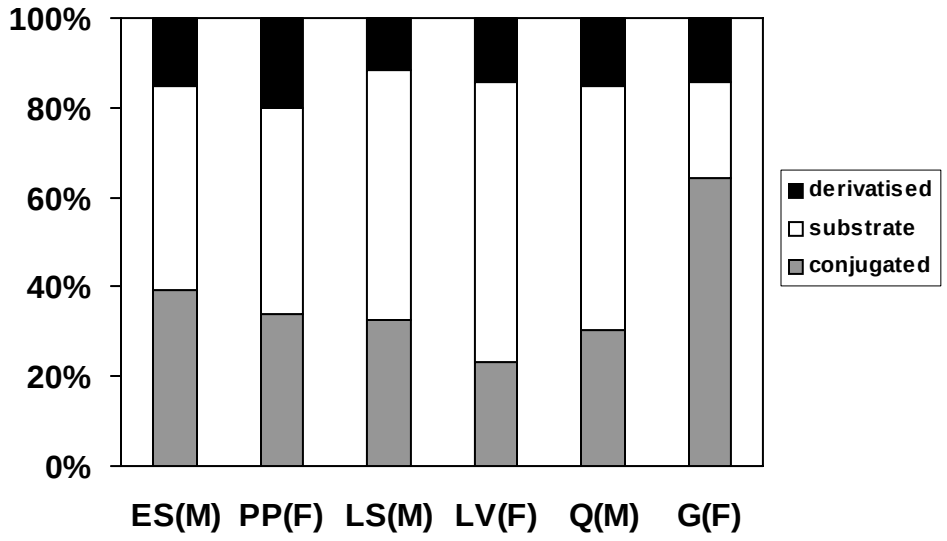

Figure 6.2 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with muscle tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

Skin

Incubations of skin with tritiated substrate (Figure 6.3) resulted in the detection in the media of similar proportions of derivatised, conjugated and unmetabolised steroids to those observed using muscle tissue. Only 16 - 27 % of the substrate became derivatised in any sample: the derivatised fractions included small amounts of 5α-DHT in all incubations of male tissue and also in incubations of skin collected from late vitellogenic females. A range of polar metabolites was produced in all incubations from both sexes. With the exception of skin from gestating females, 25 - 35 % of the original substrate was conjugated and 39 - 59 % remained unmetabolised. Skin collected from gestating females produced a greater proportion (69 %) of conjugated steroids with a correspondingly lower

proportion of unmetabolised substrate present. No E2 or AD was detected in medium from any incubation in which ^{3}[H]-T was substrate. Skin was not examined histologically.

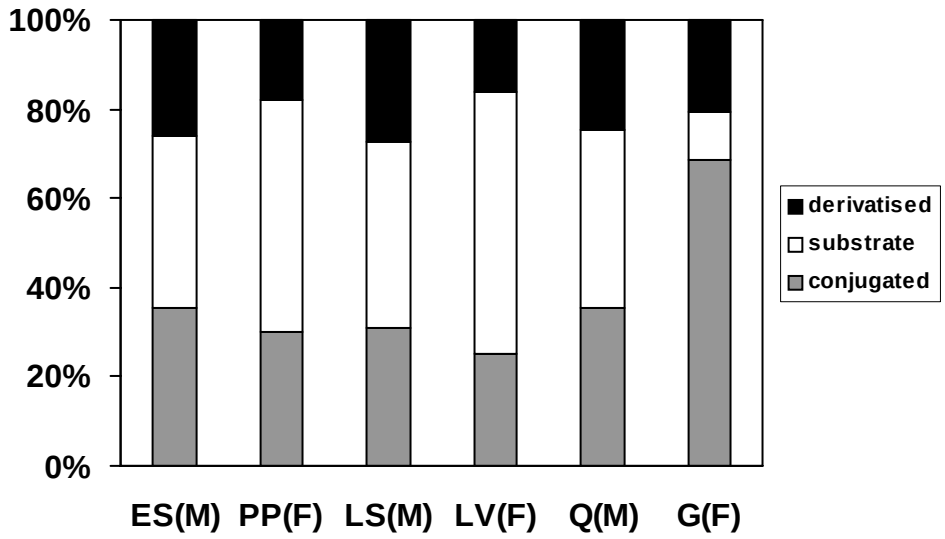

Figure 6.3 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with skin tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

Adrenal gland

In vitro incubation of male adrenal tissue from all three reproductive conditions sampled resulted in approximately equal proportions of derivatives (36 - 37 %), conjugates (26 - 38 %) and unmetabolised tritiated substrate (25 - 36 %) being present after 180 min (Figure 6.4). Free 5α-DHT was detected in media from incubations of tissue from early spermatogenic-stage and quiescent males, and AD was produced only by 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 (8 - 14 %) (Figure 6.4); this consisted entirely of a number of polar metabolites. Adrenals collected from late vitellogenic females produced the smallest proportion of conjugated steroids (18 %) and, correspondingly, returned the greatest proportion of substrate unmetabolised (73%). The largest proportion of conjugated steroids was produced

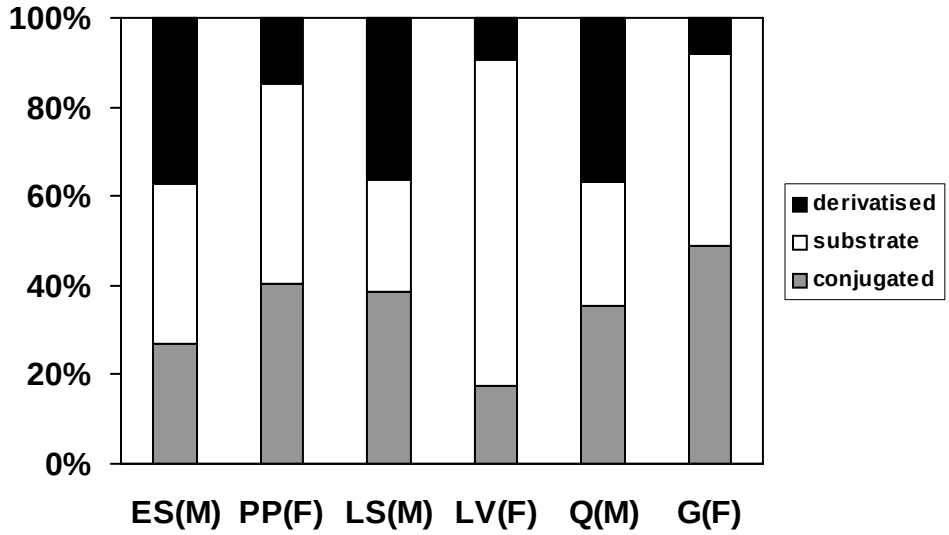

Figure 6.4 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with adrenal tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

by tissue from gestating females (49 %). Tissue from both males and females appeared as shown in Figure 6.5a, with the cortical tissue clearly arranged into cords and separated from the chromaffin tissue. The exception to this was adrenal tissue collected from

vitellogenic females, in which cortical cell nuclei were no longer basally located, but instead migrated towards the middle of the cortical cells (Figure 6.5b).

Kidney

Non sexual segment kidney tissue from late spermatogenic-stage and quiescent males only was incubated with tritiated substrate (Figure 6.6). Only 9 - 11 % of the original substrate remained unmetabolised, while approximately 48 - 51 % 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 39 - 41 % of the original tritiated substrate became conjugated by kidney tissue from males. *In vitro* incubations using female kidney tissue from animals of differing reproductive conditions resulted in variation in the patterns of metabolism after 180 min. The greatest proportion of steroid conjugates was produced in incubations of kidney from gestating females. Only 13 - 36 % of the original tritiated substrate remained unmetabolised. There was markedly less derivatisation by kidney tissue collected from females (9 - 33 %) of all three reproductive conditions than by either male kidney sample. Polar metabolites were produced during all incubations, and a small amount of T was identified in media from incubations of tissue from post-parturient females. Kidney tissue collected from females was similar in histological appearance to kidney tissue collected from early spermatogenic-stage males (Figure 6.7). Changes in male kidney histology were similar to those observed in the renal SS (See *Chapter 3 Results*).

Renal sexual segment

In vitro incubation using the sexual segment (SS) of the kidney (not present in females) with ^{3}[H]-T resulted in the production of similar proportions of derivatised (40 - 54 %), conjugated (34 - 41 %) and unmetabolised (7 - 19 %)

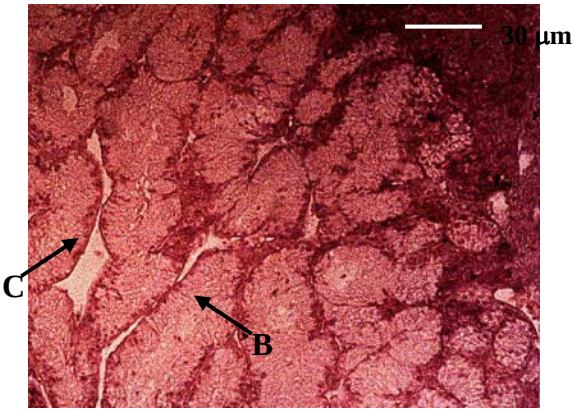

Figure 6.5a Adrenal tissue with cortical tissue arranged into cords (C), with basal nuclei (B).

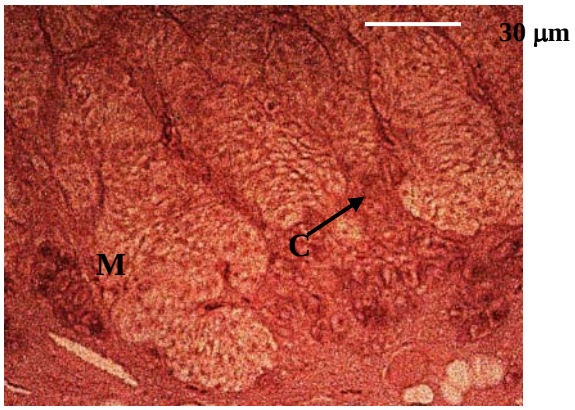

Figure 6.5b Adrenal tissue with cortical tissue arranged into cords (C), with medial nuclei (M)

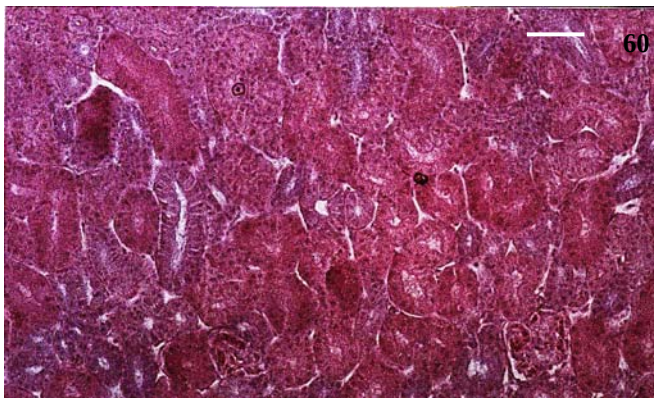

Figure 6.7 Kidney tissue from female *Tiliqua nigrolutea* maintained a similar appearance for all reproductive conditions.

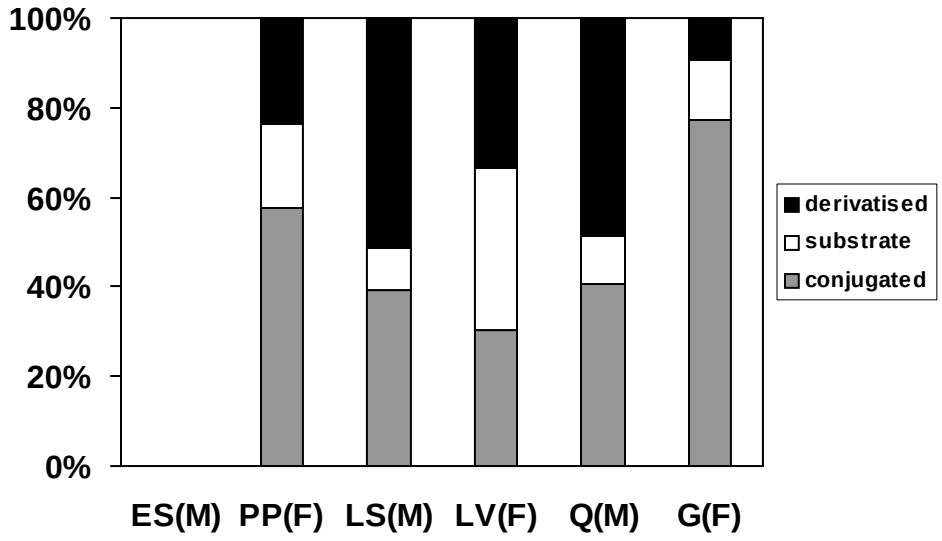

Figure 6.6 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with kidney tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

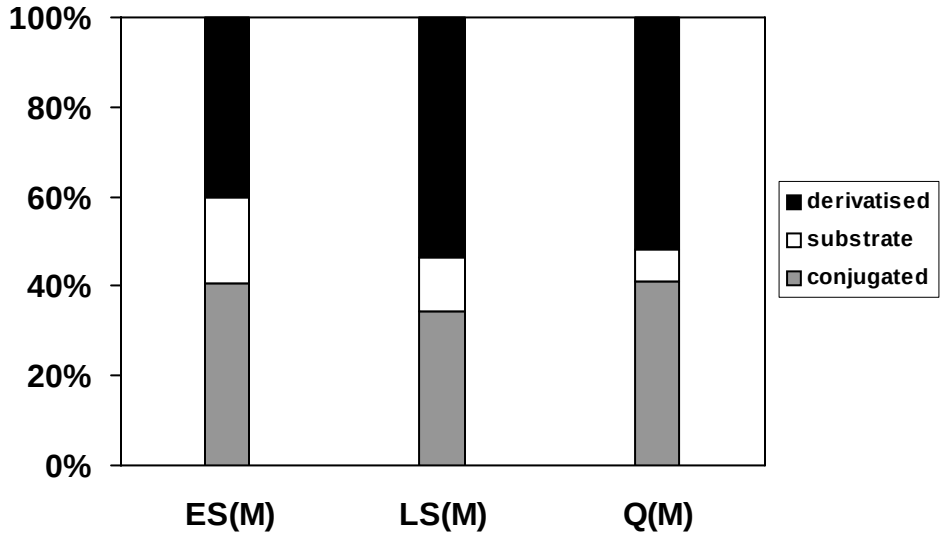

Figure 6.8 Proportions of original tritiated substrate (^{3}[H]-T) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with renal sexual

segment tissue from male *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, ES = early spermatogenic, LS = late spermatogenic, Q = quiescent).

steroids (Figure 6.8) to 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.

Cloaca

Following incubation of male and female cloacal tissue collected at three different stages of reproduction (Figure 6.9), 31 - 39 % of the original tritiated substrate had been conjugated, with the exception of tissue collected from gestating females, in which the proportion of substrate conjugated was substantially greater (64 %). Cloacal tissue from males produced a smaller proportion of steroid derivatives during early spermatogenesis (28 %) than in other stages of their reproductive cycle (43 - 45 %), while tissue from females at all three stages of reproduction produced low proportions of derivatives (5 - 21 %). The derivatised fractions of media from all incubations from both sexes consisted largely of a range of polar

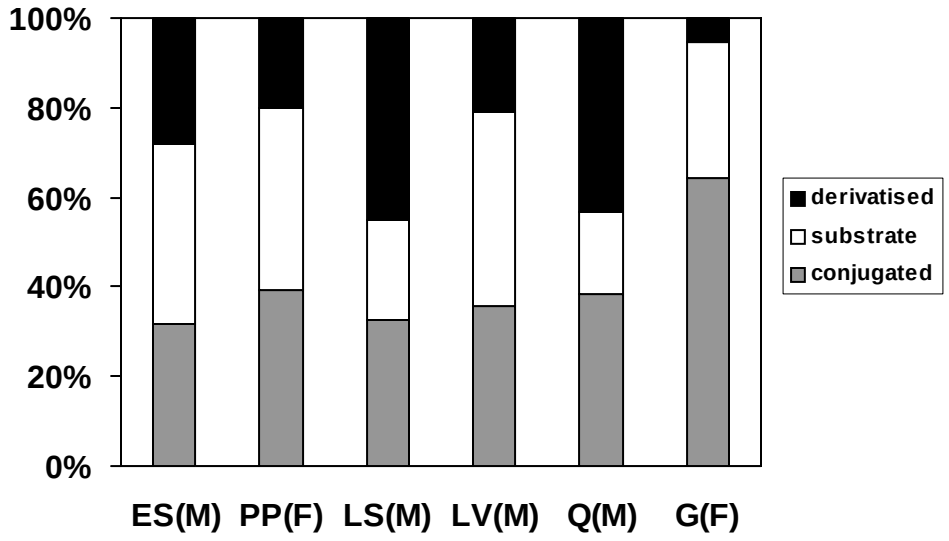

Figure 6.9 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with cloacal tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

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metabolites. However, 5α-DHT was detected in incubation media of tissue from late spermatogenic-stage and quiescent males. Additionally, a small amount of E2 was identified as a product of the incubation of ^{3}[H]-T with late spermatogenic-stage male cloacal tissue. The most notable histological change was the transformation of the epithelial lining of the cloaca from cuboidal in early spermatogenic-stage males and post-parturient females (not illustrated), to columnar in late vitellogenic-stage and gestating females, and late spermatogenic-stage males (Figure 6.10a). Secretory granules were observed within the columnar epithelial cells of the cloaca in late-spermatogenic-stage males and their apparent release was observed from the epithelial cells of gestating females (Figure 6.10b).

Epididymis and oviduct

The results for incubation of epididymis and oviduct tissues are conveniently presented together (Figure 6.11) because they are both accessory reproductive tissues (Fox, 1977; Norris, 1987; Ananthalakshimi *et al.*, 1991; Perkins and Palmer, 1996). However, while they are homologous structures of coelomoductal origin (Fox, 1977), they are embryologically derived from Wolffian (epididymis) or Müllerian (oviduct) ducts. Thus, they are not the *same* tissue, and were incubated with different substrates (^{3}[H]-T or ^{3}[H]-E2), so they are not directly comparable. For this reason, they are grouped on the graph by tissue instead of by reproductive condition.

In vitro incubation of epididymal tissue with tritiated substrate resulted in large proportions of conjugated (37 - 46 %) or derivatised (34 - 49 %) steroids being produced throughout the year. 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 epididymal tissues collected from both early and late spermatogenic-stage males. Only a small proportion of original substrate (4 - 25 %) remained following 180 min of incubation for all epididymal samples. Changes in epididymal histology with reproductive stage are described in *Chapter 3 Results*.

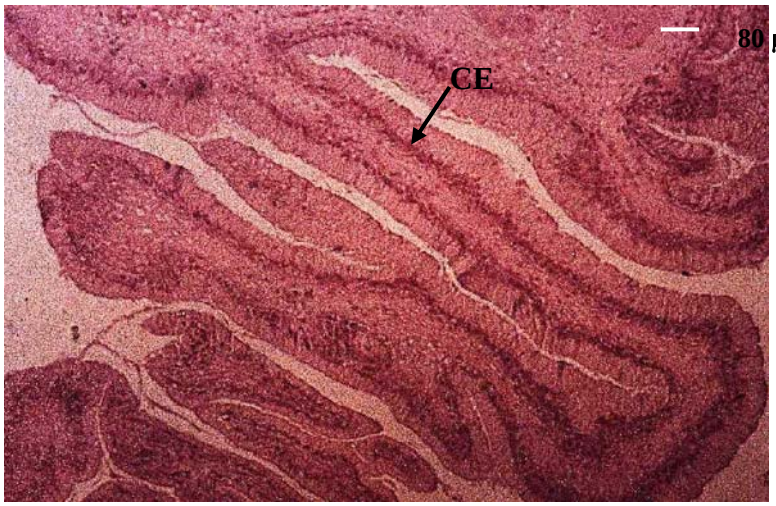

Figure 6.10a Extensive columnar epithelium (CE) in the region surrounding the cloacal opening in gestating females (identical in late vitellogenic-stage and late spermatogenic-stage male *Tiliqua nigrolutea*).

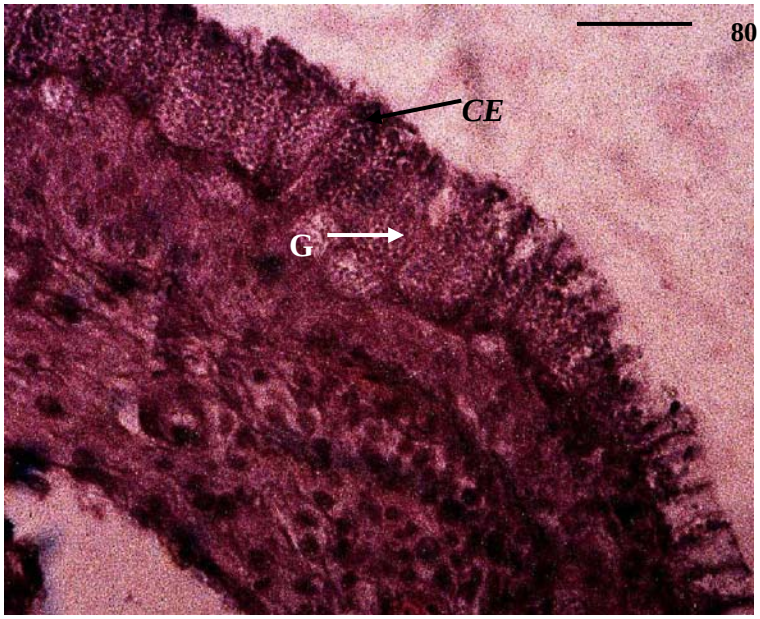

Figure 6.10b Secretory granules (G) within columnar epithelial (CE) cells of the cloaca in gestating females (identical in late spermatogenic-stage male *Tiliqua nigrolutea*).

Compared with oviductal tissue from post-parturient and late vitellogenic-stage animals, that collected from gestating females produced the greatest proportion of conjugated steroids (74 %) following 180 min of *in vitro* incubation with tritiated substrate. In samples collected during the late vitellogenic-stage, a large proportion of the original substrate remained unmetabolised (51 %), and the proportion of steroids conjugated was smallest at this time (24 %). The proportion of original substrate derivatised during the incubation (8 - 26 %) represented only polar metabolites. Histological changes in the oviduct were evident. During the vitellogenic period, oviductal crypts were small, but epithelial cells were beginning to hypertrophy (Figure 6.12a). During gestation, the oviduct was lined by columnar epithelium (Figure 6.12b) which began to degenerate within one week of parturition.

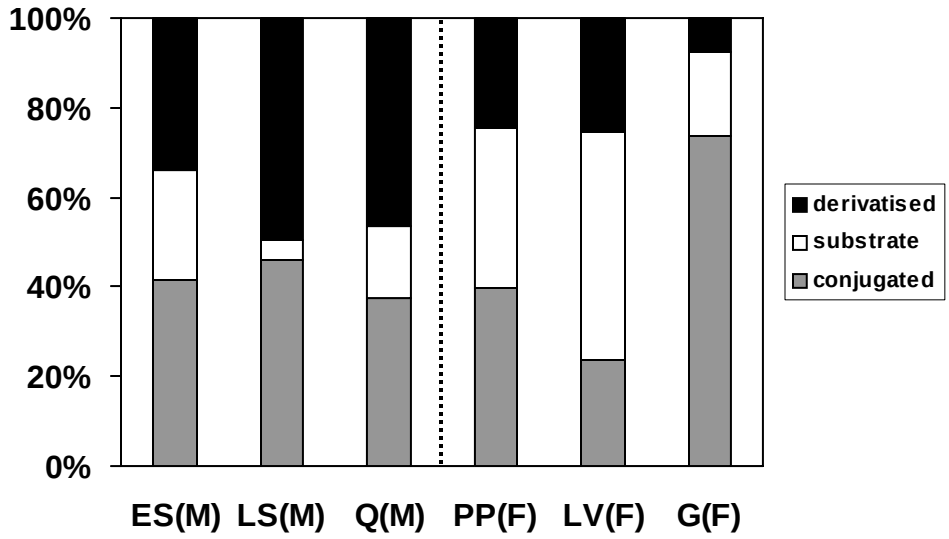

Figure 6.11 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with epididymal (male) or oviductal (female) tissue from *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

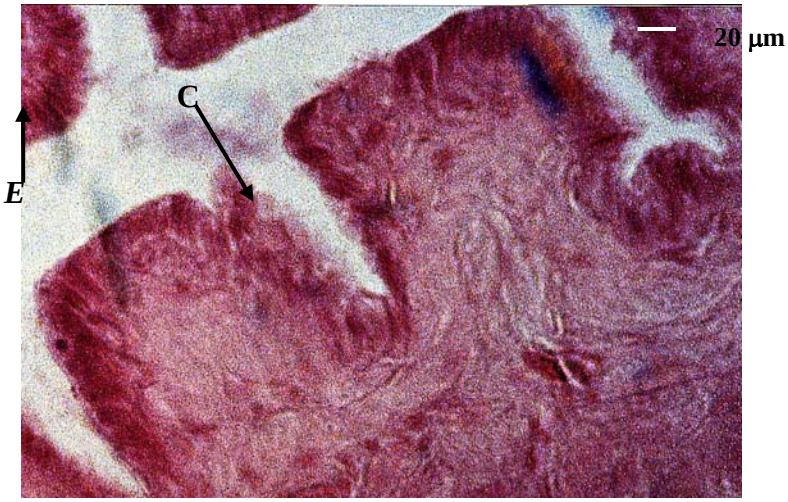

Figure 6.12a Oviductal crypts (C) in vitellogenic females are small, and epithelial cells (E) are beginning to hypertrophy.

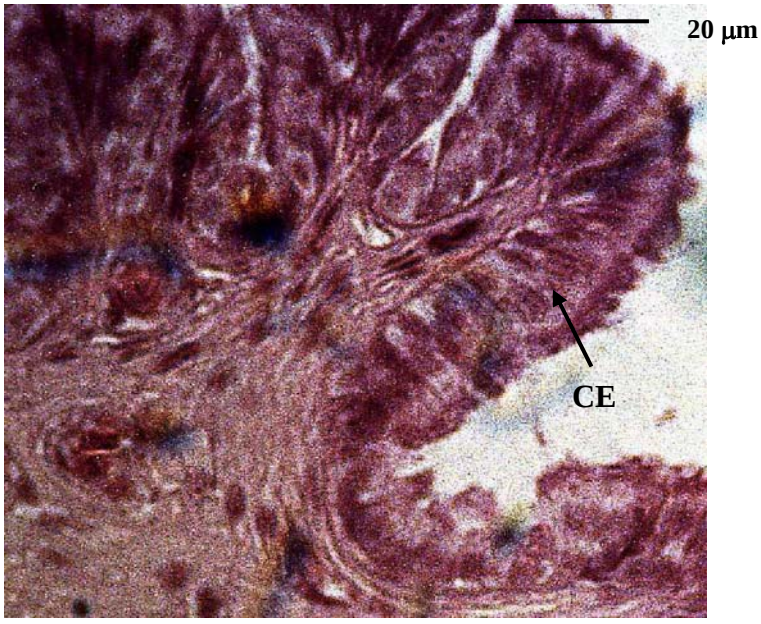

Figure 6.12b Oviductal epithelium in gestating females is convoluted and columnar (CE).

Testis and ovary

Results for these two tissues are also presented together, although they were incubated with different substrates (testis:^{3}[H]-T, ovary: ^{3}[H]-E2) (Figure 6.13). It must be emphasised that they are not directly comparable; the results are grouped by tissue instead of by reproductive condition.

In incubations of testicular tissue, 36 - 53 % of the original substrate became conjugated; the largest proportion of conjugated steroids was produced by testicular tissue from quiescent males. Only 23 - 40 % of the ^{3}[H]-T remained unmetabolised after 180 min of *in vitro* incubation, while 24 - 37 % became derivatised, largely through conversion to polar metabolites. Androstenedione was identified from media from all testicular incubations, but no 5α-DHT or E2 was detected. Illustrations of testicular histology are presented in *Chapter 3 Results*.

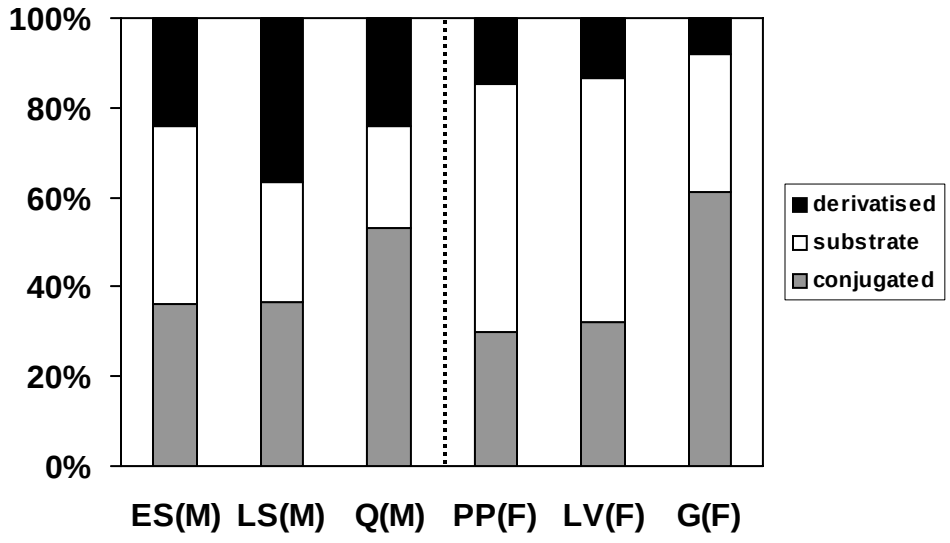

Figure 6.13 Proportions of original tritiated substrate (males: ^{3}[H]-T, females: ^{3}[H]-E2) which was derivatised, conjugated or remained unmetabolised following 180 min of *in vitro* incubation with gonadal tissue from male and female *Tiliqua nigrolutea* at three different stages of the reproductive cycle. (M = male, F = female, ES = early spermatogenic, PP = post

parturient, LS = late spermatogenic, LV = late vitellogenic, Q = quiescent, G = gestating).

In incubations using ovarian tissue, the greatest proportion of conjugated steroids was produced by tissue from gestating females (61 %). Throughout the year, only small proportions of derivatised steroids (8 – 15 %) resulted from incubations of ovarian tissue and 30 - 55 % of the original substrate remained unmetabolised. The derivatised fractions of all incubations with ovarian tissue were small (8 - 15 %). Polar metabolites were identified in all three cases. Ovarian histology is described in *Chapter 4 Section 4.3.2*.

6.4 Discussion

All tissues examined in this study displayed the ability to metabolise primary steroids *in vitro* by conjugating or derivatising tritiated substrate. Not surprisingly, the liver displayed the greatest ability to conjugate ^{3}[H]-T and ^{3}[H]-E2. Incubations of several other tissues resulted in varying proportions and types of substrate metabolism, either between sexes or between reproductive conditions.

While results are presented grouped by reproductive condition, it should be reiterated that differences between male and female cycles (See *Chapters 3* and *4*) mean that male and female tissues were regarded as active or inactive at different times of year, as summarised in Table 6.1. Additionally, incubation of ovarian tissue from gestating and post-parturient females included luteal tissue, which was not present in samples from preovulatory animals.

6.4.1 General trends

For all tissue types examined, including muscle, the control tissue, samples collected from gestating females produced noticeably greater proportions of conjugated steroids than samples collected for animals of both sexes at other stages of reproduction. This may simply be because the greater circulating steroid concentrations during gestation (See *Chapter 4 Section 4.3.4*) have primed the maternal tissues by up-regulating receptor numbers or enzyme concentrations (Paolucci and Di Fiore, 1994; Cardone *et al.*, 1998). However, a similar phenomenon was not observed in samples from male lizards collected in spring, when circulating T and E2 concentrations also become elevated.

Increased conjugation by gestating females could reflect an attempt by the maternal system to shield developing young from inadvertent exposure to either the organisational or activational actions of steroid hormones (described by Moore and Lindzey (1992)) during their development. This would be akin to the steroid metabolising actions of the human foeto-placental unit (Johnson and Everitt, 1988). The foetal liver and adrenals sulphate steroids coming across the placenta and the placenta itself desulphates and

further metabolises them as they leave the foetus (Jones, 1991). This allows the maternal system to maintain the high circulating steroid concentrations necessary for the maintenance of gestation, without exposing the developing foetus to the effects of the hormones (Johnson and Everitt, 1988). However, there is little published data available to support this hypothesis.

The derivatised fraction of all tissues included a range of unidentified polar steroids with HPLC elution times from 1.400 to 3.400 min (See Appendix 6.1 for an example). None of these times corresponded with the elution time of the unidentified steroid discussed in *Chapter 5*. However, the same group of chromatographic peaks and, therefore, possibly the same group of steroid metabolites was present in most tissues examined. This suggests that these steroids represent a group of biologically important molecules. However, insufficient amounts of these molecules were generated by *in vitro* incubation to allow their identification.

6.4.2 Metabolism by peripheral tissues

Liver

Production of conjugates was very high in incubations of liver from both sexes and from all reproductive conditions considered. This suggests that the liver has an important role in solubilising reproductive steroids for excretion in either urine or faeces, as has been documented in numerous vertebrate species (Payne, 1980; Kime, 1987; Vermierssen and Scott, 1996) including reptiles (Kime, 1987). In the closely related lizard *Tiliqua rugosa*, the liver is an important site of conjugate production (Bourne, 1981; Huf *et al.*, 1987b). Mainly steroid sulphates are produced for excretion via the gut and kidney (Bourne, 1981) and their formation may control the availability of biologically active steroids in the body (Huf *et al.*, 1987b).

There was little variation in the proportion of original substrate conjugated with changing reproductive condition. This suggests that in *T. nigrolutea* the function of conjugation in liver tissue is to aid in steroid excretion, rather than to synthesise molecules that might have biological roles such as chemical communication. However, without proper identification of the individual components of the conjugated steroid fractions and knowledge of how relative proportions of these may vary with reproductive condition, the latter possibility cannot be eliminated.

Compared with the proportions of steroids conjugated, there was far less emphasis on the production of steroid derivatives in incubations with liver tissue, with little variation between sexes or with reproductive condition. Interestingly, 5α-DHT was detected in small proportions (compared with the polar metabolites) following incubation of liver from early spermatogenic-stage males. However, it is difficult to ascribe a role to 5α-DHT from this result alone. For example, it is unlikely that the products of hepatic androgen metabolism could function in sperm maintenance in the testis.

Skin

Patterns of steroid metabolism by the skin in *T. nigrolutea* varied little between all samples incubated in the proportions of conjugates and derivatives produced, with the exception of gestating females (discussed previously). Small amounts of 5α-DHT were detected in the derivatised fractions from incubations with male skin samples from all three reproductive stages, suggesting the presence of the 5α–reductase enzyme in the skin of that sex. The conversion of T to 5α-DHT in the skin has been described in other vertebrates (Ozon, 1972; Ozon and Fouchet, 1972; Hays *et al.*, 1976; Schlinger *et al.*, 1989), including reptiles (Fergusson *et al.*, 1985; Hews and Moore, 1995). In the lizards *Amphibolurus ornatus* (Fergusson *et al.*, 1985) and *Urosaurus ornatus* (Hews and Moore, 1995), production of 5α-DHT is associated with the androgen-dependent stimulation of secretion of semiochemicals by epidermal skin glands (femoral pores) at particular stages of the reproductive cycle.

In males of *T. nigrolutea*, no such seasonal pattern of 5α-DHT production by skin tissue was evident. They do not possess femoral pores and no gross change in epidermal structure was ever observed in animals of either sex. This suggests that the conversion of T to 5α-DHT may simply be part of the normal mechanism of action of androgens in the skin, rather than having a semiochemical function associated with reproductive condition. The detection of small amounts of 5α-DHT in the derivatised fraction of muscle (control tissue) incubation extracts supports this idea. However, pheromonal communication in skinks is highly developed (Cooper and Trauth, 1992) and many lizards are able to gain information about the identity and reproductive condition of conspecifics from their skin (Maderson and Chiu, 1985; Cooper *et al.*, 1986b; Alberts, 1990).

While 5α-DHT does not appear to be involved in chemical communication in this species, one of the unidentified polar metabolites or conjugated steroids produced in incubations of skin tissue may provide such information, either purposefully or inadvertently: a distinction can be made between pheromones which have evolved as discrete chemical signals and those which are primarily excreted metabolic products (Liley, 1982).

Adrenal gland

This study demonstrates the ability of the adrenal glands of both sexes of *T. nigrolutea* to conjugate and derivatise sex steroids. Steroid conjugates produced by the adrenal glands of mammals including the laboratory rat (Payne, 1980) and human foetus (Johnson and Everitt, 1988) may act as intermediates of steroid metabolism (Cuevas *et al.*, 1992). Regions of the mammalian adrenal cortex are known to synthesise androgens (Kime 1987; Norris, 1997) and aromatase activity has been detected in the interrenal tissue of the amphibian *Rana esculenta* (Gobbetti and Zerani, 1995). However, previous studies of adrenal steroid metabolism in reptiles have considered only synthesis of corticosteroids (Gist and DeRoos, 1966; Vinson *et al.*, 1975; Duggan and Lofts, 1978) and the distribution of the relevant enzymes (Sandor *et al.*, 1972).

In this study, the substantially greater production of steroids derivatised by male rather than female adrenal tissue suggests intersexual variation in adrenal sex steroid metabolism that warrants further investigation. Such variation may be influenced by

variations in corticosteroid production, which were not investigated in this study. Corticosterone has been detected in the plasma of the related lizard *T. rugosa* (Bradshaw and Fontaine-Bertrand, 1970; Bourne and Seamark, 1973), although seasonal variations in circulating concentrations were not considered.

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). The adrenals of females of the lizard *Lacerta vivipara* are unable to synthesise androgens but do produce P4 (Dauphin-Villemant and Xavier, 1985), as do the adrenals of *T. rugosa* (Bourne and Seamark, 1972; Bourne *et al.*, 1986c) and the snakes *Hydrophis cyanocinctus* (Duggan and Lofts, 1978) and *N. naja* (Huang *et al.*, 1969).

The detection of 5α-DHT only in media from incubations of adrenal tissue from early spermatogenic-stage males suggests that 5α-DHT may be a vehicle by which spermatogenesis can be initiated without the induction of the reproductive behaviours observed in spring when plasma T concentrations are elevated. The final stages of spermatogenesis and the display of reproductive behaviours are correlated with elevated plasma T and E2 concentrations (*Chapter 3 Results*). If lower concentrations of androgens are required to stimulate the initiation of spermatogenesis in the preceding autumn, it may be advantageous to produce extratesticular (adrenal) 5α-DHT, which then acts on the testis while it is regressed. The production of 5α-DHT would also prevent the inadvertent stimulation of E2-induced reproductive behaviours because it cannot be aromatised (Adkins-Regen, 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 to be confirmed in *T. nigrolutea*.

The histological difference observed in adrenal tissue collected from late vitellogenic-stage females occurred coincident with the greatest proportion of tritiated substrate remaining unmetabolised of all adrenal tissue incubations. The migration of the cortical cell nuclei from basal to medial positions within the cells may be related to steroid metabolising activity.

Inevitably, the mass of adrenal tissue available for incubation was small compared with that of other tissues. However, obtaining a mass of adrenal tissue comparable with those of other incubations would have necessitated killing more that twice as many animals of each sex from the captive population at each sample period, specifically to obtain adrenal tissue. Reduced tissue mass is unlikely to result in the production of different metabolites during incubations. Adherence to the general pattern of the tissue collected from gestating females conjugating the largest proportion of the original tritiated substrate suggests also that the relative proportions of derivatives and conjugates produced were not affected by the small tissue masses used. However, several authors have reported variation in the proportions of incubation products with changing substrate concentration (Kime and Hews, 1980; Kime and Abdullah, 1994), so comparison of adrenal and other incubations should be made with caution.

Kidney and renal sexual segment

Histological changes observed in the kidney and renal SS tissues are inconsistent with changes in steroid metabolising activity. No seasonal difference was observed in the proportions of steroids conjugated by kidneys in male *T. nigrolutea*, and it is most likely that conjugation by this tissue serves an excretory function. 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.*, 1987b), although it is not clear from this study whether the kidneys themselves act as a site of conjugation. However, the kidneys of the lizard *Lacerta vivipara* are thought to both metabolise and excrete androgens (Dufaure and Chambon, 1978). The kidneys of the fish *Gasterosteus aculeatus* are also able to glucuronidate steroids for excretion (Borg *et al.*, 1992).

In female *T. nigrolutea*, the kidneys displayed seasonal variation in the proportion of tritiated substrate conjugates produced. Conjugation was least in incubations of kidney of late vitellogenic-stage females, possibly because there is a greater need for the direct action of E2 at that time, in preparation for ovulation and mating, or in readying the

reproductive tract for gestation. However, there is little literature available about other female vertebrates for comparisons of renal steroid metabolism.

Derivatisation of tritiated substrate was greater by kidney collected from male lizards. 5α-Dihydrotestosterone was detected in both late spermatogenic-stage and quiescent samples, but not from any incubation using female kidney tissue. This implies a sex-specific role for this steroid, although the nature of this role is unknown.

The kidneys of other vertebrates are also known to derivatise steroids. 5α-Reductase, 6β- and 16-hydroxylase and 17-hydroxysteroid dehydrogenase/ oxidoreductase activities have all been detected in the kidney of the fish *Salmo gairdneri* and enzyme activities vary in different regions of the kidney (Pesonen *et al.*, 1990). The concept of functional domains within the kidney is also observed in male squamate reptiles, which possess the specialised SS region of the kidney. This area is known to be stimulated to hypertrophy during the mating season by androgens (Prasad and Sanyal, 1969; Prasad and Reddy, 1972; Fox, 1977; Nilson and Andren, 1982; Weil, 1984). Secretions produced by the reptilian SS may function to attract females (Prasad and Reddy, 1972), nourish or activate sperm (Cuellar *et al.*, 1972; Fox, 1977; Depeiges and Dufaure, 1982) or transport sperm (Weil, 1984).

In incubations of SS from male *T. nigrolutea*, proportions of steroids conjugated or derivatised did not differ from patterns of steroid metabolism by non-SS kidney tissue. The renal SS is inconsistently described in the literature as being the caudal portion of the kidney (Prasad and Sanyal, 1972), the hypertrophied collecting ducts of the kidney (Prasad and Sanyal, 1969), and in the terminal, preterminal, distal or intermediate region of the uriniferous tubules (Fox, 1977). In the sympatric Tasmanian skinks, *Niveoscincus ocellatus* and *N. metallicus*, the renal SS is easily distinguishable as a hypertrophied lobe comprising the anterior one third of each kidney (Jones, pers. comm.): a similar lobe was observed in the same region in male *T. nigrolutea*, although the possibility that the SS region of the kidney in male *T. nigrolutea* was incorrectly identified must be considered.

Cloaca

In many amphibian and reptile species which rely on chemical means to transfer information regarding location, identity, sex and reproductive condition, cloacal tissue and cloacal glands are often the sources of the compounds which provide this information (Cooper *et al.*, 1986b; Belvedere *et al.*, 1988; Cobbetti and Zerani, 1992; Cooper and Trauth, 1992; Toyoda *et al.*, 1994; Weldon and Leto, 1995). In several amphibian species reproductive steroids involved in chemical communication are released from this region and a pheromonal role is suggested for free P4 (Belvedere *et al.*, 1988; Cobbetti and Zerani, 1992; Toyoda *et al.*, 1994). Many reptiles also use chemical signals released from cloacal glands to communicate; 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 *et al.*, 1986b; Trauth *et al.*, 1987; Cooper and Garstka, 1987). Some female vertebrates also respond to male cloacal products (Belvedere *et al.*, 1988).

Tissue collected from around the cloacal opening of both male and female *T. nigrolutea* actively metabolised ^{3}[H]-T or ^{3}[H]-E2 in all samples incubated. Chemical communication is highly developed in skinks (Cooper and Trauth, 1992) and it is likely that the products of cloacal steroid metabolism in *T. nigrolutea* carry information relevant to reproduction. The transformation of cuboidal to columnar secretory epithelium observed histologically occurs coincident with the final stages of spermatogenesis in males, and vitellogenesis and late gestation in females. However, these changes are not consistently correlated with the production of high proportions of either steroid derivatives or conjugates from *in vitro* incubations. It is likely that cloacal tissue is a target for reproductive steroids, but that the relationship is complex. 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.*, 1993a), suggesting the use of both volatile and non-volatile chemicals. In view of this, further investigation of possible cloacal involvement in the production or release of semiochemicals related to reproduction in *T. nigrolutea* is currently being undertaken.

The detection of 5α-DHT and E2 in incubations of cloacal tissue from late spermatogenic-stage male *T. nigrolutea* suggests a dual function and two mechanisms of action for T in this tissue (via reduction or aromatisation). Whether either of these derivatives is used to convey information to, or to stimulate a response from, females, is currently unknown and awaiting identification of the conjugates and polar derivatives also produced by this tissue.

Epididymis

Based on the small amounts of tritiated substrate remaining after incubation, epididymal tissue in *T. nigrolutea* appears to be one of the most metabolically active of all tissue types examined in this study. 5α-Dihydrotestosterone was detected after incubations of epididymal tissue collected from both early and late spermatogenic-stage males, but was not produced by quiescent males. This could indicate a role for 5α-DHT in sperm maintenance.

Accessory sex organs in other male vertebrates are also known to metabolise reproductive steroids (Ozon and Fouchet, 1972; Hay *et al.*, 1976; Schoonen and Lambert, 1986b; 1987). The production of 5α-DHT by the seminal vesicles of the frog *D. pictus* (Ozon and Fouchet, 1972) and by the clasper, sperm sac and vas deferens of the elasmobranch *Squalus acanthias* (Hay *et al.*, 1976) have been reported. Additionally, the seminal vesicles of the anuran *D. pictus* are responsive to both T and 5α-DHT (N'Diaye *et al.* 1974).

In reptiles, epididymal function in known to be regulated by androgens, although the distinction between the actions of T and of its metabolites is rarely considered (Dufaure and Chambon, 1978; Shivanandappa and Devaraj Sarkar, 1987; Morel *et al.*, 1993). Epididymal tubule epithelium was maximally hypertrophied in late spermatogenic-stage male *T. nigrolutea* and this corresponded to the sample in which *in vitro* steroid metabolising activity was greatest. 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 large proportions of original

tritiated substrate detected as conjugates or derivatives in this study suggest that steroid metabolism and modes of androgen action in epididymal tissue warrant further investigation.

Oviduct

Oviductal histology (Palmer and Guillette, 1988; Aldridge, 1992; Palmer *et al.*, 1993; Perkins and Palmer, 1996; Girling *et al.*, 1998) and sperm storage (Halpert *et al.*, 1982; Adams and Cooper, 1998; Gist and Congdon, 1998) have been examined in a number of reptiles, but few authors consider the effects of reproductive steroids on the oviduct (Yaron, 1972a; Mead *et al.*, 1981; Masson and Guillette, 1987; Whittier, 1992). In the snake *Thamnophis sirtalis*, both T and 5α-DHT affect oviductal morphology (Whittier, 1992). In the lizard *Xantusia vigilis* (Yaron, 1972a) and the snake *Thamnophis elegans* (Mead *et al.*, 1981) P4 and E2 stimulate the maturation of the pre-ovulatory genital tract and in three snake species, maximal plasma P4 concentrations are correlated with peak oviductal vascularity (Masson and Guillette, 1987). Despite the oviduct being an obvious target tissue for steroid hormones in reptiles and other vertebrates, steroid metabolism by this tissue has rarely been examined.

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). Correspondingly, the greatest proportion of unmetabolised ^{3}[H]-E2 was detected in incubations of oviduct from LV (pre-ovulatory) females. This suggests that in *T. nigrolutea* 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, so the steroids are unlikely to function in sperm maintenance. Rather, I suggest that these molecules may act as semiochemicals, providing information on reproductive condition to male conspecifics.

Oviductal hypertrophy was maximal during gestation, the time when production of conjugated steroids predominates over derivatives or unmetabolised substrate after *in*

vitro incubation. Histological changes in oviductal vascularity in three oviparous lizards have previously been correlated with changes in circulating steroid (P4) concentration during the reproductive cycle (Masson and Guillette, 1987). The metabolites detected in the current study may, likewise, have a role in some aspect of the maintenance of the oviduct itself during gestation in this viviparous species.

Testis

The testicular tissue of many vertebrates synthesises conjugated steroids (Idler *et al.*, 1971; Ruokonen and Vihko, 1974; Kime, 1978; Payne, 1980; Stacey *et al.*, 1986; Cuevas *et al.*, 1992; Joss *et al.*, 1996). Often conjugates are not formed for excretion, but as pheromones (Stacey *et al.*, 1986; Scott and Vermiersson, 1996) or as intermediates of testicular steroid metabolism (Cuevas *et al.*, 1992; Payne, 1980). In the elasmobranch *Squalus acanthias* steroid sulphates may have paracrine intratesticular regulatory function (Cuevas *et al.*, 1992).

In some species, seasonal variation occurs in testicular steroid conjugate production (Huf *et al.*, 1987b; Joss *et al.*, 1996). In the lungfish *Neoceratodus forsteri* little testicular conjugation occurs except during spawning, when glucuronides of both T and AD are detected in *in vitro* incubations (Joss *et al.*, 1996). In contrast, *in vitro* conjugation of T by *T. nigrolutea* is 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 is described as low, and less than occurs in liver or kidney tissues (Huf *et al.*, 1987b), although seasonal variation was not examined.

Testicular derivatisation of T in *T. nigrolutea* was greatest in incubations of testis collected in spring, at which time testes were maximally hypertrophied Interestingly, no 5α-DHT or E2 was detected. Testes of the anuran *D. pictus* (Ozon and Stocker, 1974) and the laboratory rat (Folman *et al.*, 1973) both produce 5α-DHT, possibly to maintain mature sperm. Additionally, aromatase activity (Bolaffi and Callard, 1979) and the presence of an oestrogen receptor (Mak *et al.*, 1983) have been reported in the testis of

the urodele *Necturus maculosus*. Numerous products of androgen metabolism, including 11β-hydroxyandrostenedione (11β-OH-AD), 11-ketotestosterone (11-KT), 11β-hydroxytestosterone (11β-OH-T) and 7α-hydroxyandrogens (Inano *et al.*, 1970; Idler *et al.*, 1971; Lupo Di Prisco *et al.*, 1972) have been detected in the testes of other vertebrates. 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). This androgen was not isolated in my study and was not detected in the plasma in a previous study using *T. nigrolutea* (Bourne *et al.*, 1985). It should be noted that sex steroid receptors have been identified in rat spermatid cells (Galena *et al.*, 1974; Wright and Frankel, 1980; Vornberger *et al.*, 1994) and spermatozoa may be able to metabolise steroid hormones (Ozon, 1972). Contributions of the male gametes to testicular steroid metabolism in *T. nigrolutea* were not examined in this study.

Ovary

17β-Oestradiol is the major end-product of ovarian steroid biosynthesis in many vertebrates (Norris, 1997; Kime, 1987) and few authors report the further metabolism of E2 by the ovary. With the position of T as an immediate precursor in the biosynthesis of oestrogens, examinations of steroid metabolism by the vertebrate ovary have more often been studies of androgen metabolism, considering products generated by the ovary *in vitro* from AD or T (Callard and Leathem, 1966; Kime and Callard, 1982). However, in light of the proposed role for T in ovulation in female *T. nigrolutea*, the incubation of ovarian and adrenal tissue could prove useful in understanding the mechanism of ovulation in this species.

Ovarian steroid metabolism in *T. nigrolutea* resulted in only a small proportion of substrate derivatised or conjugated, (with the exception of gestating females), compared with other tissues. Some variation in ovarian steroid metabolism may be explained by the changing relative proportions of thecal, granulosa and luteal material present in samples collected from animals of differing reproductive conditions. The results suggest one of two things. First, it is most probable that E2 is required to induce activity in this tissue, as in most vertebrates (Kime, 1987), and that further metabolism is largely unrequired. 17β-

Oestradiol is a major ovarian oestrogen produced by the anuran *Rana tempororia* (Ozon and Breuer, 1964) and has been isolated from ovarian extracts in the lizard *Lacerta sicula* (Lupo Di Prisco *et al.*, 1968). However, it is also possible that E2 is not the end-product of steroid biosynthesis in the ovary of *T. nigrolutea*. If an alternative oestrogen to E2 predominates in this species, it may act in this tissue without peripheral metabolism. This would negate the need for the presence of any enzymes which may have been expected to act on the tritiated E2 provided to the tissue during *in vitro* incubation (See *Chapter 5*, particularly *Section 5.4.5* for a discussion of the possibility of an alternative oestrogen in this species.). In agnathans, 15α- and 15β-hydroxylated (Kime and Rafter, 1981) and 6- and 7-hydroxylated oestrogens (Kime and Hews, 1980) appear to be more important the oestrogens. Additionally, in the urodele *Triturus cristatus carnifex* E1, but not E2, is detectable as a product of *in vitro* incubation of ovarian tissue (Lupo Di Prisco *et al.*, 1971). Until the presence of an alternative oestrogen to E2 is confirmed or the possibility is eliminated, it is not possible to comment on the metabolism of E2 by ovarian tissue, beyond the observation that some derivatisation and conjugation does occur. The strong likelihood that an alternative oestrogen acts via a generic oestrogen receptor intensifies the need to identify the molecule itself, rather than using receptor-based studies.

Chapter 7 General discussion

This study employed a multifaceted approach to examine the roles of steroid hormones in the regulation of reproduction in the viviparous lizard, *Tiliqua nigrolutea*. This animal was selected as an appropriate model because it is a large skink that adapts well to captivity. It is also amenable to repeated blood sampling and collection of relatively large quantities of most tissue types for *in vitro* studies. These features allowed me to undertake a comprehensive experimental programme.

I have characterised the reproductive cycle of each sex with respect to the timing of physiological and behavioural events. This information is correlated with changes in plasma concentrations of three key steroid hormones: testosterone, (T), 17β-oestradiol (E2) and progesterone (P4) and the histological changes in reproductive tissues. The results are discussed in the context of present knowledge of the steroid hormone control of reproduction in reptiles.

Male *T. nigrolutea* exhibit an annual reproductive cycle typical of most cool temperate zone lizards. Prenuptial spermatogenesis commences in autumn, prior to hibernation, and males emerge before females in the following spring to complete spermatogenesis and prepare for mating. Peak plasma testosterone (T) occurs coincident with the final stages of spermatogenesis and agonistic interactions between males, but concentrations decline during the mating period (Figure 3.18). While many studies have considered only the traditional "male" hormone, T, I also examined annual changes in plasma E2 and P4 concentrations in male *T. nigrolutea*. Plasma 17β-oestradiol (E2) concentrations are elevated only during spring, and probably have a role in the induction of reproductive behaviours (Figure 3.19). A preliminary study characterising a dose regime to elevate plasma T and E2 concentrations in males to physiologically relevant concentrations is described in Appendix 7.1; this will form the basis of further studies examining the roles of T and E2 in the activation of reproductive behaviours in *T. nigrolutea.* In males of *T. nigrolutea* plasma P4 concentrations are greatest in late spring (Nov), during the mating period (Figure 3.20). The question of the role of progesterone (P4) in the male

reproductive cycle is raised by this study. Together with the hypothesised involvement of E2 in the stimulation of mating behaviour, this will be an exciting avenue for further research.

In contrast to the annual reproductive pattern displayed by male *T. nigrolutea*, and in contrast to females of some sympatric, small, viviparous species, such as *Niveoscincus metallicus* and *Niveoscincus ocellatus* (Jones and Swain, 1996; Jones *et al.*, 1997), female *T. nigrolutea* do not reproduce every year. Their multiennial pattern of reproduction is achieved by 'skipping' seasons between reproductive efforts. This contrasts with the biennial cycle of another Tasmanian viviparous skink, *N. microlepidotus*, in which gestation is prolonged throughout hibernation (Swain, 1972; Hutchinson *et al.*, 1989). Multiennial cycles with skipped years have been noted in other cool temperate zone viviparous squamates which are long-lived (Van Wyk, 1994), and those that produce large clutches (Bull and Shine, 1979). *Tiliqua nigrolutea* fulfils both of these criteria. Relative clutch mass (RCM) is high in this species in comparison with sympatric viviparous lizards (Swain and Jones, in prep.; Wapstra, 1998), but is comparable with that of the related lizard *T. rugosa*, which is also believed to exhibit a multiennial cycle.

Evidence that reproduction in female in *T. nigrolutea* might be limited by resource availability comes from the observation that females of this species are believed to also display a multiennial pattern of reproduction throughout the rest of their southeastern Australian mainland distribution (Shea, 1992). Consideration of the possibility of a plastic reproductive cycle that varies in response to environmental conditions was beyond the scope of this project, but warrants further investigation. Additionally, although not quantified here, there may exist a threshold body condition in this species, below which reproduction does not occur. A large reproductive investment in one year may breach this threshold in *T. nigrolutea*. Such a body condition index (BCI) has been described in the viviparous snakes *Vipera aspis* (Bonnet and Naulleau, 1994; Bonnet *et al.*, 1994) and *Thamnophis sirtalis parietalis* (Whittier and Crews, 1990).

However, once the decision to reproduce is made, the cycle is completed within a single active season. Vitellogenesis begins at spring emergence (approx. four weeks after male emergence) and is completed relatively rapidly, in time for mid spring mating. Gestation is long (4 – 4.5 months) and young are born in mid autumn, shortly before the next winter hibernation. Increasing plasma E2 concentrations are detected during the vitellogenic period (Figure 4.11), but fall at ovulation, coincident with peak plasma T concentrations (Figure 4.12). Plasma P4 concentration rises at ovulation, peaking in the second trimester of gestation (Figure 4.10). As P4 concentrations decline, a small peak in plasma E2 concentrations occurs around the time of parturition.

As discussed in *Chapter 4 t*hese observations are consistent with the concept of the multihormone control of reptilian reproduction by steroids (Ho *et al.*, 1982; Ho, 1987). This illustrates the importance of considering *in vivo* steroid hormone interactions and of measuring hormones other than the one in question when interpreting the meaning of patterns of change in circulating steroid hormone concentrations. Further understanding of the interactions between reproductive steroids *in vivo* will require examinations of changes in the concentrations of steroid binding proteins and of steroid receptor numbers and specificities. Few reptilian studies of this nature are available. Additionally, as part of the hypothalamic-pituitary-gonadal (HPG) axis, gonadal steroids are themselves under higher order endocrine control. An understanding of the endocrine regulation of the HPG axis in reptiles awaits further research.

The second major focus of this thesis was the biosynthesis and peripheral metabolism of reproductive steroids, targeting various stages of the reproductive cycle in animals of both sexes. Here the results were considered primarily from a phylogenetic perspective, as the literature suggests that patterns of steroidogenesis vary between vertebrate classes. *In vitro* studies using gonadal tissue incubated with a tritiated steroid precursor showed that patterns of steroid biosynthesis conform to the most common vertebrate patterns (Kime, 1987; Norris, 1997), in that both the main steroid biosynthetic pathways, the $\Delta 4$ and $\Delta 5$, operate. As in the few other reptile species that have been studied (Callard, 1967; Chan and Callard, 1974; Huf *et al.*, 1989), the $\Delta 4$ route appears to predominate. In each

sex, seasons of greater gonadal steroid biosynthetic activity *in vitro* correlate well with increased circulating steroid concentrations *in vivo*. However, considerable seasonal and intersexual variation in pathway preference and activity are evident. In contrast to the traditional view of the conservative nature of steroid hormone biosynthesis, the relative activity of biosynthetic pathways appears to be plastic in response to reproductive condition. Such seasonal variation in steroid biosynthetic pathway activity is described in males of the related lizard *Tiliqua rugosa* (Bourne and Seamark, 1978). I suggest that this is likely to be a widespread phenomenon amongst the vertebrates.

In keeping with the plasticity of patterns of steroid biosynthesis is the variation I describe in peripheral steroid metabolism in male and female *T. nigrolutea*. The ability of peripheral tissues to metabolise primary reproductive steroids also varies with sex and reproductive condition and, additionally, between tissue types. It would be interesting to extend this aspect of the project to include the biosynthetic ability of other, extragonadal tissues, such as liver and adrenal, to assess their contributions to circulating concentrations of reproductive steroids. The adrenals, in particular, have been postulated to play a key role in reproductive steroid production in squamates (Tam *et al.*, 1972; Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1985; Bourne *et al.*, 1986).

Like many other researchers, when designing experiments I was forced to make some assumptions based on the available literature. The *in vitro* incubation experiments, presented in *Chapter 6* were based on the assumption that E2 was the most important oestrogen in female *T. nigrolutea*, as it is in most female vertebrates. The basis for this assumption is, however, questioned by the results presented in *Chapter 5*. Here I examined the possibility that an alternative oestrogen may be more important in this species. I detected an unidentified polar steroid as an end-product of gonadal biosynthesis from pregnenolone (P5), but not from peripheral or gonadal metabolism of T or E2, and was unable to isolate E2 following incubation of gonadal tissue with P5. This provides a tantalising indication of a divergence from expected reptilian and vertebrate patterns of steroid production and metabolism. This observation is apparently in keeping with the precedent set by the related lizard *T. rugosa*: males of that species appear to be unique

among vertebrates in the production of epitestosterone (epiT) as the major testicular androgen (Bourne *et al.*, 1985). Again, such results emphasise the danger of extrapolating indiscriminately, even from closely related species.

The isolation of this unidentified polar steroid, the revelation that mean plasma E2 concentrations in males are elevated during the breeding period, and the characterisation of a multiennial reproductive cycle in females, highlight the very reasons for this study. The more information available about a single species, the fewer inadvertently spurious assumptions will be made in designing experiments, resulting in higher quality data. With a comprehensive database of information about the reproductive endocrinology and physiology of *Tiliqua nigrolutea*, this species is now available as a model to further examine selected aspects of the steroid hormone control of reproductive physiology and behaviour in a cool temperate, viviparous reptile.

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