

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

by

Ashley Edwards BSc (Hons)

**Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy, School of Zoology, University of Tasmania
(July, 1999)**

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.

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Signature:

Date:

Abstract

This thesis documents the annual profiles of the primary reproductive steroids testosterone (T), 17 β -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 ($10.9 \pm 3.00 \text{ ng ml}^{-1}$) and E2 ($778.0 \pm 120.00 \text{ pg ml}^{-1}$) concentrations occur coincident with late spermatogenesis and observations of mating, respectively. Plasma P4 concentrations remain basal ($< 1.2 \text{ ng ml}^{-1}$) throughout the annual reproductive cycle. In females, increasing plasma E2 concentrations ($275.2 \pm 33.87 \text{ pg ml}^{-1} - 715.1 \pm 106.68 \text{ pg ml}^{-1}$) are associated with vitellogenesis and plasma T peaks ($6.3 \pm 0.63 \text{ ng ml}^{-1}$) 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 $12.7 \pm 1.27 \text{ ng ml}^{-1}$ and falling rapidly prior to parturition. Concurrently, plasma P4 concentrations in non-reproductively active adult females remain basal ($1 - 2 \text{ ng ml}^{-1}$) 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.

Acknowledgments

First, I must thank **Sue Jones**. What an amazing person my supervisor is! For treating me as friend, student and equal all in one, for all your support, faith and encouragement (and just the right amount of heavy-handedness!), thank you! You came all the way to Queensland to get me, and as soon as I heard you were a rattie sympathiser, I just knew we would get along! Thank you, also, for not using red pen – my self-esteem is so much the better for soothing colours, smiley faces, and let's not forget the gold star! (Why was there only one of those? – sigh!) I strongly advocate the chocolate reward system you initiated, and see no reason for it to stop now! For all the coffee and chats, project-related and otherwise, again, thank you! You have put such a lot of time and effort in to me - I couldn't have wished for a better role model.

Many thanks also go to **Roy Swain**. Here is a remarkably resilient man who has allowed me to make him look foolish almost continuously, and yet retains enormous self-confidence and a very silly grin! My hat off to you, Sir! I am definitely coming back for the retirement party! I always feel welcome in your office, except when you are doing the crossword! Thanks for all the help, advice, editing, support and entertainment! Do you know where your glasses are right now?

Thank you to my number one chief volunteer lizard holder and all round helper, **Fiona “they're my lizards too” Reardon** - never again will I ask you to do anything so smelly, no matter how much disinfectant there is! For all the stuff I couldn't do alone, for *trying* to keep me sane at home as well (can't win 'em all, hey?), for lizard caring above and beyond the call, thank you so much. I'm really sorry F14 bit you! Thanks for help with all those late night forays into peoples' gardens with a torch to hunt for snails in the rain. I'm still surprised no one ever called the police. There is no one with whom I would rather wrestle a lizard to the ground.

Special thanks go to **Noel Davies** for letting me be the guinea pig on the new HPLC, and for all your time, effort and help identifying steroids. Thanks also to **Joan Whittier** for the samples of oestrogens and to **Adrian Bradley** for the gifts of antisera for the RIAs.

Thanks to **Robert Mason** for advice and for volunteering the services of **Lew Pannell** in an attempt to identify a tricky steroid.

Thanks also to **Erik Wapstra** for talks and coffee, oh, and the messy lab. Thanks to **Julianne O'Reilly** for trying to make Erik less messy, and to both of you for reading thesis drafts in your tiny bits of spare time. I've tried not to hold the overseas trip and all the postcards against you, while I was back here, plodding along in the lab.

To **Brett Gartrell**, who turned out to be the best person for coffee breaks I have ever met! Thank you too, for all your support while I was writing up, for reading bits of thesis, all your help with Rattie, with my computer troubles (except the ones you were responsible for!), for company at lunch and generally propping me up at regular intervals!

To **Tamara Kincade**, thanks for heaps of help bleeding lizards, even if they were not as interesting as bats!

Thanks to all those people who ever caught me a lizard. There are so many of you who thought of me and wrestled lizards to the ground in the name of science! Special mentions to **Ineke Kasteel** for her efforts in the middle of the Midlands Highway, to **Robert Barbour** for regular contributions ranging from alive to sort-of-alive to dead, **Fiona Reardon** for the one that came flying through her classroom window and to Sue's dog **Hannah the blue-tongue-seeking hound**, who teamed up with various Joneses for a superior lizard-catching effort.

Thanks to **Warren, Sarah** and **Penny Jones**, for welcoming me that first Easter. I trust none of you will use the negatives to my detriment! You have all continued to help me feel so extremely "special" since I arrived in Tasmania. I actually think you are all pretty "special" yourselves!

To **Robert White**, thank you for working so hard on my scholarship, and of course, for all the snails – the lizards certainly appreciated them!

Thanks to everyone else who brought in snails for the lizards as well, especially **Di Moyle**, who donated them by the bucketful.

Thank you to all the technical staff in the School of Zoology, **Kate Hamilton, Sherrin Bowden, Wayne Kelly, Barry Rumbold, Richard Holmes, Adam Stephens, Alan Dumphy** and **Ron Mawbey** for all your help along the way.

Thanks to the people who tried to explain all that statistics stuff to me, **Roy Swain, Leon Barmuta, Craig Johnson, David Ratkowsky**, and **Alistair Richardson**. I feel like I won a war but was trampled on most of the battlefields.

Thank you to **Brita Hansen** who showed me all sorts of little tricks to make my life easier – computers can be fun after all! At least, it's possible to get them to do what you want some times!

To my mother, **Judi Edwards**, thank you for your support all the way through uni, nervous course change and all – I think it turned out to be the right decision! I will try not to come and live at home again. Thanks to my father, **Peter Edwards**. Having a car made winters in Tasmania almost bearable! To my brother, **Spencer Edwards**, I can't just think what you did to help, Spency, but thought I would mention you anyway.

And of course, special thanks to **Grant Davis**, alias Shag Nastie. For walks on the beach, for all those hot dinners, for letting me be stupid and hugging me anyway, thank you. I'm glad you decided to come along for the ride, and then so considerately stayed right out of the way! You're a bit good! In return, I promise to rapidly become extremely rich and support you so that you can quit work and play golf. I will feed you jelly beans and yellow snakes whenever your heart desires, and even massage your scurvy feet at night! Maybe.

Table of contents

Declaration	ii
Abstract	iii
Ethics permits	v
Acknowledgments	vi
Table of contents	x
Abbreviations used in the text	xv
1 General introduction	1
1.1 The evolution of endocrine systems	1
1.2 Reproductive steroids	1
1.3 Reproductive steroids in reptiles	2
1.3.1 Androgens	3
1.3.2 Oestrogens	3
1.3.3 Progestogens	4
1.4 Roles of reproductive steroids in squamate reptiles	4
1.4.1 Males	5
1.4.2 Females	6
1.5 The genus <i>Tiliqua</i>	9
1.6 The study animal, <i>Tiliqua nigrolutea</i>	10
1.7 Project aims	11
1.8 Thesis format	12
2 General materials and methods	13
2.1 Animals	13
<i>Source and identification</i>	13
<i>Housing</i>	13
<i>Diet</i>	15
2.2 Data collection	15
<i>Morphological measurements</i>	15

	<i>Blood sampling</i>	16
	<i>Opportunistic data collection</i>	16
2.3	Plasma steroid analyses	16
	<i>Progesterone assay</i>	17
	<i>Testosterone assay</i>	18
	<i>17β-Oestradiol assay</i>	18
2.4	Sacrifice of animals	19
2.5	Histology	19
3	Plasma steroid concentrations and reproductive behavior throughout the annual reproductive cycle in the viviparous blue-tongued skink, <i>Tiliqua nigrolutea</i>, (Scincidae), in Tasmania.	20
	Abstract	21
	Introduction	22
	Materials and methods	25
	<i>Animals</i>	25
	<i>Blood sampling</i>	25
	<i>Histology</i>	26
	<i>Radioimmunoassay</i>	27
	<i>Statistics</i>	28
	Results	29
	<i>Behavioural observations</i>	29
	<i>Histology</i>	30
	<i>Gonadosomatic index</i>	32
	<i>Plasma steroid concentrations</i>	32
	Discussion	35
	Figure legends	42
	Figures	45
4	Reproductive cycle of female <i>Tiliqua nigrolutea</i>	55
	4.1 Introduction	55
	4.1.1 Timing of reproduction	55
	4.1.2 Reproductive behaviour	57
	4.1.3 Steroid hormones	57

4.1.4	This study	60
4.2	Materials and methods	62
4.2.1	Blood sampling	62
4.2.2	Histology	62
4.2.3	Life history characteristics	63
4.2.4	Behavioural observations	63
4.2.5	Statistics	64
4.3	Results	66
4.3.1	Behavioural observations	66
	<i>Mating</i>	66
	<i>Gestation</i>	67
	<i>Parturition</i>	68
4.3.2	Histology	71
4.3.3	Gonadosomatic index	75
4.3.4	Plasma steroid concentrations	76
	<i>Progesterone</i>	76
	<i>17β-Oestradiol</i>	77
	<i>Testosterone</i>	78
4.3.5	Life history characteristics	79
	<i>Relative clutch mass</i>	79
	<i>Frequency of reproduction in captivity</i>	79
	<i>Frequency of reproduction in the wild</i>	80
4.4	Discussion	81
4.4.1	Timing of reproduction	81
4.4.2	Steroid hormones	83
	<i>Progesterone</i>	83
	<i>17β-Oestradiol</i>	86
	<i>Testosterone</i>	88
	<i>Steroid hormone control of mating behaviour</i>	89
4.4.3	Frequency of reproduction	91
4.4.4	Multihormone control of reproduction	94
5	Biosynthesis of steroid hormones in male and female <i>Tiliqua nigrolutea</i>	98
5.1	Introduction	98
5.1.1	Steroid biosynthetic pathways	98
5.1.2	Phylogenetic variation in pathway preference	100
5.1.3	End-product variation	101
5.1.4	Intraspecific variation in pathway preference	103
5.1.5	This study	103

5.2 Materials and methods	106
5.2.1 Tissue collection	106
5.2.2 Incubation	106
<i>Males</i>	106
<i>Females</i>	107
5.2.3 Thin layer chromatography	108
5.2.4 High performance liquid chromatography with radiometric detection	109
5.2.5 The unidentified steroid	110
5.3 Results	111
5.3.1 Proportion of steroids conjugated	111
5.3.2 Metabolism of pregnenolone	113
5.3.3 Progesterone	114
5.3.4 Androstenedione	115
5.3.5 Testosterone	116
5.3.6 Dehydroepiandrosterone and 17β-oestradiol	117
5.3.7 Unidentified steroid	117
5.4 Discussion	119
5.4.1 Steroid biosynthetic pathways	119
5.4.2 Pathway preference	121
5.4.3 Metabolism of pregnenolone	123
5.4.4 Intersexual and seasonal variation in the biosynthesis and metabolism of pathway intermediates	123
<i>Progesterone</i>	123
<i>Androstenedione and dehydroepiandrosterone</i>	124
5.4.5 Intersexual and seasonal variation in the biosynthesis and metabolism of pathway end-products	125
<i>Testosterone</i>	125
<i>17β-Oestradiol</i>	126
<i>Unidentified steroid</i>	127
5.4.6 Pathway plasticity	130
6 Peripheral steroid metabolism in male and female <i>Tilapia nigrolutea</i>	134
6.1 Introduction	134
6.1.1 Sites of steroid metabolism	134
6.1.2 Types of steroid metabolism	135
<i>Derivatisation</i>	135
<i>Conjugation</i>	136
6.1.3 Variation in the patterns of steroid metabolism	138
6.1.4 This study	138

6.2 Materials and methods	141
6.2.1 Tissue collection	141
6.2.2 Incubation	142
6.2.3 Thin layer chromatography	143
6.2.4 High performance liquid chromatography with radiometric detection	144
6.3 Results	146
6.3.1 Incubation of tissues	147
<i>Liver</i>	147
<i>Muscle</i>	147
<i>Skin</i>	149
<i>Adrenal gland</i>	150
<i>Kidney</i>	151
<i>Renal sexual segment</i>	151
<i>Cloaca</i>	154
<i>Epididymis and oviduct</i>	155
<i>Testis and ovary</i>	159
6.4 Discussion	161
6.4.1 General trends	161
6.4.2 Metabolism by peripheral tissues	162
<i>Liver</i>	162
<i>Skin</i>	163
<i>Adrenal gland</i>	164
<i>Kidney and renal sexual segment</i>	166
<i>Cloaca</i>	168
<i>Epididymis</i>	169
<i>Oviduct</i>	170
<i>Testis</i>	171
<i>Ovary</i>	173
7 General discussion	175
References	180
Appendices (numbered by reference chapter)	208
Appendix 1.1	208
Appendix 4.1	209
Appendix 5.1	210
Appendix 5.2	213
Appendix 6.1	214
Appendix 7.1	215
Appendix 7.2	230
Appendix 7.3	231

Abbreviations used in the text

Animals

SVL snout-vent length

Chemicals

DCM dichloromethane

DEE diethylether

EtOH ethanol

MeOH methanol

ChCl₃ 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
17 α -OH-P4 17-alpha-hydroxyprogesterone
17 α -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