Cross-talk between estrogen and thyroid hormones during amphibian development

by

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Dedicated to

My parents, Eduardo and Lia

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ABSTRACT

It is generally thought that in amphibians, thyroid hormones (THs) regulate metamorphosis, while sex steroids (estrogens and androgens) regulate gonadal differentiation. However, inhibition of TH synthesis in frogs alters gonadal differentiation, suggesting instead that these two endocrine axes interact during development. Specifically, THs may be involved in male development, while estrogens may inhibit tadpole metamorphosis. However, we do not currently know the mechanisms that account for these interactions, let alone how such mechanisms may differ between species. To develop and test new hypotheses on the roles of sex steroids and THs, I first examined transcriptional profiles (mRNA) of enzymes and receptors related to sex steroids and THs during embryogenesis and metamorphosis in *Silurana tropicalis*. Tadpoles were exposed to either an estrogen synthesis inhibitor (fadrozole) or TH (triiodothyronine, T3) during early larval or tadpole development. Acute exposures of *S. tropicalis* to fadrozole or T3 during early development resulted in increased expression of androgen- and TH-related genes in whole body larvae, while chronic exposure to fadrozole during metamorphosis affected gonadal differentiation but did not affect tadpole development. On the other hand, acute exposure to T3 during metamorphosis increased the expression of androgen-related transcripts both in the brain and gonad. In *S. tropicalis*, the results suggested that cross-talk is primarily in one direction (i.e., effect of THs on the reproductive axis) with a strong relationship between TH and androgen status. Lastly, I established developmental transcript profiles and investigated T3 regulation of brain and gonad transcripts in *Engystomops pustulosus*. I then compared these results with *S. tropicalis* and an earlier study in *Lithobates pipiens*. While each species developed with similar profiles, they differed in their response to T3. Exposure to T3 resulted in either an increase in

androgen-related genes (*S. tropicalis*) or a decrease in estrogen-related genes (*E. pustulosus* and *L. pipiens*). In conclusion, these data demonstrated that cross-talk mechanisms differ among these three evolutionary separate species, but in all cases, T3 appears to affect the balance of sex steroids, stimulating the androgen system and providing potential mechanisms of the masculinising effects of THs. These results will contribute to understanding the mechanisms of hormone interactions and their evolutionary basis in frogs.

RÉSUMÉ

Chez les amphibiens, les hormones thyroïdiennes (HTs) régulent la métamorphose, tandis que les stéroïdes sexuels (œstrogènes et androgènes) régulent la différenciation sexuelle des gonades. Cependant, l'inhibition de la synthèse des HTs altère la différentiation des gonades des grenouilles, suggérant que ces deux axes endocriniens interagissent au cours du développement. Plus précisément, les HTs semblent être impliquées dans le développement des mâles, tandis que les œstrogènes peuvent inhiber la métamorphose des têtards. Pourtant, nous ne connaissons pas les mécanismes qui peuvent expliquer ces interactions, ni comment ces mécanismes se comparent entre les espèces de grenouilles. Pour développer de nouvelles hypothèses sur le rôle des stéroïdes sexuels et des HTs chez les grenouilles, j'ai d'abord établi les profils d'expression des gènes (ARNm) reliés aux stéroïdes sexuels et les HTs au cours de l'embryogenèse et la métamorphose chez les têtards de *Silurana tropicalis*. Les têtards ont été exposés à un inhibiteur de la synthèse des œstrogènes (fadrozole) ou à l'HT (triiodothyronine, T3) au cours de différentes périodes de développement des têtards. Les expositions aiguës au fadrozole ou T3 pendant le développement embryonnaire ont abouti à l'augmentation de l'expression des gènes reliés aux androgènes et aux HTs, tandis que, l'exposition chronique au fadrozole pendant la métamorphose a affecté le rapport des sexes favorisant les mâles mais elle n'a pas affecté le développement des têtards. D'autre part, l'exposition aiguë au T3 des têtards pré-métamorphiques a augmenté l'expression des gènes reliés aux androgènes dans le cerveau et les gonades. Les résultats suggèrent que chez *S. tropicalis*, l'interaction est principalement dans une direction (i.e., les effets des HTs sur l'axe endocrinien de la reproduction) avec une forte relation entre l'état du système thyroïdien et celui des androgènes. Enfin, j'ai établi chez *Engystomops pustulosus*, les profils d'expression des même gènes que chez *S. tropicalis* et leur régulation par T3 dans le cerveau

et la gonade. J'ai ensuite comparé ces résultats avec ceux de *S. tropicalis* et *Lithobates pipiens* (étude antérieure). Bien que les profils des gènes soient similaires entre les espèces, ils diffèrent dans leur réponse au T3. L'exposition au T3 a soit augmenté les niveaux des gènes reliés aux androgènes (*S. tropicalis*) ou a diminué l'expression des gènes reliés aux œstrogènes (*E. pustulosus* et *L. pipiens*). En conclusion, ces résultats démontrent que les mécanismes d'interaction diffèrent entre ces trois espèces, mais dans tous les cas, les HTs semblent stimuler le système des androgènes. Cette thèse contribuera à la compréhension des mécanismes d'interaction des hormones et leur évolution chez les grenouilles.

TABLE OF CONTENTS

Dedication ii
Acknowledgements iii
Abstract iv
Résumé vi
Table of contents viii
List of tables xiii
List of figures xiv
List of abbreviations xvii
List of anuran species xix

Chapter 1:
General Introduction

1.1. Thesis rationale and approach 1
1.2. Hypotheses 2
1.3. Objectives 2
1.4. Sex steroids and the reproductive axis of anurans 4
1.4.1. Synthesis and action of sex steroid hormones 4
1.4.2. Gonadal differentiation 6
1.5. Thyroid hormone axis in anurans 8
1.5.1. Thyroid hormone synthesis and action 8
1.5.2. Anuran metamorphosis 9
1.6. Cross-talk between thyroid hormones and sex steroids 10
1.6.1. Effects of thyroid hormones on reproduction-related endpoints during development 11
1.6.2. Effects of estrogens on the thyroid hormone axis and metamorphosis 12
1.7. Anurans as biological models 15
1.8. The comparative project: rationale and description 16
1.9. Thesis outline 19

Chapter 2:
Expression and inhibition of aromatase to study the regulation of sex steroid- and thyroid hormone-related genes during *Silurana tropicalis* early development

2.1. Introduction 22
2.2. Materials and methods 23
2.2.1. Breeding and maintenance of Silurana tropicalis 23
2.2.2. Tissue collection for developmental profiles 24
2.2.3. Fadrozole exposure 24
2.2.4. RNA isolation and cDNA synthesis 25
2.2.5. Real-time RT-PCR in simplex 25
2.2.6. Real-time RT-PCR in multiplex 26
2.2.7. Real-time RT-PCR data analysis 27
2.2.8. Aromatase activity assay 28

2.2.9. Statistical analyses 29
2.3. Results 34
2.4. Discussion 38
2.4.1. Developmental profiles of cyp19 mRNA and activity 38
2.4.2. Effects of fadrozole exposure on sex steroid- and TH-related gene expression 39

Chapter 3:
Effects of the aromatase inhibitor fadrozole on gonadal differentiation, metamorphosis and gene expression in the frog *Silurana tropicalis*

3.1. Introduction 42
3.2. Materials and methods 44
3.2.1. Breeding and maintenance of Silurana tropicalis 44
3.2.2. Fadrozole exposure 44
3.2.3. Histology 45
3.2.4. RNA isolation and cDNA synthesis 45
3.2.5. Real-time RT-PCR assays 45
3.2.6. Aromatase activity 47
3.2.7. Statistical analyses 47
3.3. Results 47
3.3.1. Somatic growth and metamorphosis 47
3.3.2. Sex ratio and histological analysis 48
3.3.3. The effects of fadrozole on gene expression and cyp19 activity 48
3.4. Discussion 58
3.4.1. Effects of fadrozole on gonadal differentiation and sex ratios 58
3.4.2. Effects of fadrozole on liver gene expression 58
3.4.3. Effects of fadrozole on brain cyp19 activity and gene expression 59
3.4.4. Cross-talk between the estrogen and thyroid hormone axes 60
3.4.5. Comparison of fadrozole and finasteride gene expression results 62
3.4.6. Conclusions 62

Chapter 4:
Expression and triiodothyronine regulation of thyroid hormone- and sex steroid-related genes during *Silurana tropicalis* early development

4.1. Introduction 65
4.2. Materials and methods 66
4.2.1. Animals 66
4.2.2. Tissue collection for developmental profiles 67
4.2.3. Thyroid hormone exposure 67
4.2.4. RNA isolation and cDNA synthesis 67
4.2.5. Real-time RT-PCR 68
4.2.6. Data analyses 68
4.2.7. Statistical analyses 68
4.3. Results 69

4.3.1. Developmental profiles during embryogenesis 69
4.3.2. T3 exposure during embryogenesis 70
4.4. Discussion 75
4.4.1. Expression and regulation of thyroid hormone-related genes 75
4.4.2. Developmental profiles of sex-steroid receptors 77
4.4.3. Regulation of sex steroid-related genes by T3 78

Chapter 5:
Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in the brain of *Silurana tropicalis*

5.1. Introduction 81
5.2. Materials and methods 82
5.2.1. Animals 82
5.2.2. Brain collection for developmental profiles 83
5.2.3. Triiodothyronine exposure 83
5.2.4. RNA isolation and cDNA synthesis 84
5.2.5. Real-time RT-PCR 84
5.2.6. Data analysis 85
5.2.7. Aromatase activity assay 85
5.2.8. Statistical analyses 86
5.3. Results 86
5.3.1. Brain developmental profiles during metamorphosis and adulthood 86
5.3.2. Effects of T3 on brain transcript levels of thyroid hormone- and sex steroid-related genes 87
5.4. Discussion 95
5.4.1. Expression of thyroid hormone receptors and deiodinases during metamorphosis 95
5.4.2. Expression of estrogen- and androgen-related genes during metamorphosis 96

Chapter 6:
Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in the gonad-mesonephros of *Silurana tropicalis*

6.1. Introduction 101
6.2. Materials and methods 102
6.2.1. Animals 102
6.2.2. Sample collection for developmental profiles 102
6.2.3. Triiodothyronine exposure 103
6.2.4. RNA isolation and cDNA synthesis 103
6.2.5. Real-time RT-PCR 104
6.2.6. Data analysis 105
6.2.7. Statistical analyses 105
6.3. Results 106
6.3.1. Developmental profiles of transcript levels in the GMC 106

6.3.2. T3 exposure 106
6.4. Discussion 114
6.4.1. Profiles of sex steroid- and sex differentiation-related genes in the GMC during metamorphosis and in adult gonads 114
6.4.2. Profiles of TH-related genes in the GMC during metamorphosis and in adult gonads 116
6.4.3. Effects of T3 on transcript levels in the GMC 117

Chapter 7:
Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*. Part I: The brain

7.1. Introduction 120
7.2. Materials and methods 121
7.2.1. Animals 121
7.2.2. Cloning thyroid hormone and sex steroid-related genes 122
7.2.3. Phylogenetic analysis 123
7.2.4. Tissue collection for developmental profiles 124
7.2.5. Triiodothyronine exposure 124
7.2.6. RNA isolation and cDNA synthesis 125
7.2.7. Real-time RT-PCR 125
7.2.8. Data analysis 126
7.2.9. Statistical analyses 127
7.3. Results 130
7.3.1. Cloning of genes in E. pustulosus 130
7.3.2. Brain developmental profiles during metamorphosis 130
7.3.3. Effects of T3 on brain gene expression 131
7.4. Discussion 139
7.4.1. Developmental profiles and T3 regulation of TH-related genes 139
7.4.2. Developmental profiles and T3 regulation of sex steroid-related genes 140

Chapter 8:
Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*. Part II: the gonad

8.1. Introduction 145
8.2. Materials and methods 146
8.2.1. Animals and tissue collection for developmental profiles 146
8.2.2. Triiodothyronine exposure 147
8.2.3. RNA isolation and cDNA synthesis 147
8.2.4. Real-time RT-PCR 147
8.2.5. Statistical analyses 148
8.3. Results 149
8.3.1. Developmental profiles and sex differences in the GMC 149
8.3.2. Effects of T3 exposure in the GMC 151

8.4. Discussion 161
8.4.1. Developmental profiles and identification of sex markers in the GMC 161
8.4.2. Effects of T3 on transcript levels in the GMC 164

Chapter 9:
General Discussion

9.1. Cross-talk during *Silurana tropicalis* development 167
9.2. Comparison of developmental profiles and cross-talk mechanisms among three anuran species 171
9.2.1. Developmental profiles during metamorphosis 171
9.2.2. T3 regulation of thyroid hormone- and sex steroid-related genes 174
9.3. Future research directions 181
9.4. Implications and concluding remarks 183

References 185

Appendix A. Comparison of anuran developmental tables 200
Appendix B. Mating protocol for *Silurana tropicalis* in the laboratory 201
Appendix C. Histological methods for gonads 202
Appendix D. Brain transcript levels in *Lithobates pipiens* at juvenile stage 203
Appendix E. List of manuscripts not included in the thesis 205

List of Tables

Table 1.1 Summary of studies on the effects of thyroid hormones on sex steroid-related endpoints in vertebrates. 14

Table 1.2 Developmental and reproductive characteristics of *Silurana tropicalis*, *Engystomops pustulosus*, and *Lithobates pipiens*. 18

Table 2.1 SYBR-green real-time RT-PCR primers and assay conditions of genes of *Silurana tropicalis*. 30

Table 2.2 Multiplex real-time RT-PCR primers and dual-labelled fluorescent probes of genes of *Silurana tropicalis*. 31

Table 3.1 Somatic growth characteristics of *Silurana tropicalis* exposed chronically to fadrozole (2 µM) or to water (control). 50

Table 3.2 Comparison between hepatic and brain gene expression changes following a chronic exposure to fadrozole (2 µM) or finasteride (25 µM) during *Silurana tropicalis* development. 64

Table 7.1 Degenerate primers and conditions used for cloning thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*. 128

Table 7.2 SYBR-green real-time RT-PCR primers and assay conditions of target genes in *Engystomops pustulosus*. 129

Table 7.3 Percent nucleotide identity of *Engystomops pustulosus* target genes analysed in this study compared to sequences from other species belonging to different anuran families. 133

Table 8.1 Two-way analysis of variance (ANOVA) results for the developmental profiles in *Engystomops pustulosus*. 153

Table 8.2 Results of the discriminant analysis to determine sexing accuracy using stage of development, individual and combined GMC transcript levels in *Engystomops pustulosus*. 158

Table 9.1 Gene expression changes in the brain after T3 exposure in three frog species, *Silurana tropicalis*, *Engystomops pustulosus*, and *Lithobates pipiens*. 178

Table 9.2 Gene expression changes in the gonad-mesonephros complex after T3 exposure in *Silurana tropicalis* and *Engystomops pustulosus*. 179

List of Figures

Figure 1.1 (A) Main roles, potential roles and interactions of estrogens and thyroid hormones in frogs investigated in this thesis. (B) General approach of the thesis. 3

Figure 1.2 Schematic representation of the three main endocrine systems controlling amphibian development: the hypothalamus-pituitary-gonadal axis (A), hypothalamus-pituitary-thyroid axis (B) and hypothalamus-pituitary-prolactin axis (C). 13

Figure 1.3 Tree of relevant anuran taxa. 17

Figure 2.1 Developmental profile of the ribosomal protein L8 (*rpl8*) and ornithine decarboxylase (*odc*) during *Silurana tropicalis* embryogenesis and larval development. 32

Figure 2.2 Effects of fadrozole on *Silurana tropicalis* brain cyp19 activity *in vitro*. 33

Figure 2.3 Developmental profiles of cyp19 mRNA and activity during *Silurana tropicalis* embryogenesis. 35

Figure 2.4 Effects of fadrozole on cyp19 mRNA and activity, *eralpha* and *erbeta*, *ar*, and *srd5alpha1* and *srd5alpha2* mRNA levels in *S. tropicalis* larvae. 36

Figure 2.5 Effects of fadrozole on *tralpha* and *trbeta*, and *dio1*, *dio2* and *dio3* mRNA levels in *S. tropicalis* larvae. 37

Figure 3.1 Effects of fadrozole on gonadal histology and sex ratios in *Silurana tropicalis*. 51

Figure 3.2 Effects of fadrozole on cyp19 activity in the brain of NF60 *Silurana tropicalis*. 52

Figure 3.3 Effects of fadrozole on *eralpha*, *erbeta*, *ar*, *srd5alpha1* and *srd5alpha2* mRNA levels in NF60 *Silurana tropicalis* livers. 53

Figure 3.4 Effects of fadrozole on *tralpha*, *trbeta*, *dio*, and *dio3* mRNA levels in NF60 *Silurana tropicalis* livers. 54

Figure 3.5 Effects of fadrozole on *eralpha*, *erbeta*, *cyp19*, *ar*, *srd5alpha1*, and *srd5alpha2* mRNA levels in NF60 *Silurana tropicalis* brains. 55

Figure 3.6 Effects of fadrozole on *tralpha*, *trbeta*, *dio2*, and *dio3* mRNA levels in NF60 *Silurana tropicalis* brains. 56

Figure 3.7 Effects of fadrozole on *avt* and *odc* mRNA levels in NF60 *Silurana tropicalis* brains. 57

Figure 4.1 Developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* embryogenesis and larval development. 71

Figure 4.2 Developmental profiles of sex steroid-related genes during *Silurana tropicalis* embryogenesis and larval development. 72

Figure 4.3 Effects of T3 exposure on the expression of thyroid hormone-related genes during *Silurana tropicalis* larval development. 73

Figure 4.4 Effects of T3 exposure on the expression of sex steroid-related genes during *Silurana tropicalis* larval development. 74

Figure 4.5 Diagram summarizing the findings on thyroid hormone-related gene expression, THs levels, dio activity and anatomical and morphological changes during anuran embryonic and larval development. 80

Figure 5.1 Brain developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* metamorphosis and adulthood. 90

Figure 5.2 Brain developmental profiles of sex steroid-related genes during *Silurana tropicalis* metamorphosis and adulthood. 92

Figure 5.3 Effects of T3 exposure on the expression of thyroid hormone-related genes in *Silurana tropicalis*. 93

Figure 5.4 Effects of T3 exposure on the expression of sex steroid-related genes in *Silurana tropicalis*. 94

Figure 6.1 Developmental profiles of estrogen-related genes during *Silurana tropicalis* metamorphosis and adulthood. 108

Figure 6.2 Developmental profiles of sex differentiation- and androgen-related genes during *Silurana tropicalis* metamorphosis and adulthood. 109

Figure 6.3 Developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* metamorphosis and adulthood. 111

Figure 6.4 Effects of T3 exposure on the expression of sex differentiation- and sex steroid-related genes in the gonad-mesonephros complex in *Silurana tropicalis*. 112

Figure 6.5 Effects of T3 exposure on the expression of thyroid hormone-related genes in the gonad-mesonephros complex in *Silurana tropicalis*. 113

Figure 7.1 Phylogenetic tree of anuran *cyp19* (A) and *eralpha* (B) using nucleotide sequences from GenBank and sequences cloned from *Engystomops pustulosus*. 134

Figure 7.2 Brain developmental profiles of thyroid hormone-related genes during *Engystomops pustulosus* metamorphosis. 135

Figure 7.3 Brain developmental profiles of sex steroid-related genes during *Engystomops pustulosus* metamorphosis. 136

Figure 7.4 Effects of T3 exposure on the expression of thyroid hormone-related genes in the brain of *Engystomops pustulosus*. 137

Figure 7.5 Effects of T3 exposure on the expression of sex steroid-related genes in the brain of *Engystomops pustulosus*. 138

Figure 8.1 Developmental profiles of estrogen-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. 155

Figure 8.2 Developmental profiles of sex differentiation- and androgen-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. 156

Figure 8.3 Developmental profiles of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. 157

Figure 8.4 Effects of T3 exposure on the expression of sex differentiation- and sex steroid-related genes in the gonad-mesonephros complex (GMC) of *Engystomops pustulosus*. 159

Figure 8.5 Effects of T3 exposure on the expression of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) of *Engystomops pustulosus*. 160

Figure 9.1 Evidence for cross-talk between thyroid and sex-steroid axes in *Silurana tropicalis*. 170

Figure 9.2 Sex steroid- and thyroid hormone-related gene expression profiles in the brain during tadpole development. 173

Figure 9.3 Solutions to the regulation of the androgen system by thyroid hormones. 180

List of Abbreviations

The gene and protein nomenclature used in the thesis follows the guidelines published in Xenbase (available at: http://www.xenbase.org). Frog gene symbols are in lowercase and italicised, whereas protein designations follow the gene symbol but not italicised. mRNA uses the same formatting as gene symbols.

18S	-	18S ribosomal RNA
^{3}H-A	-	1β-^{3}H-androstenedione
5α-DHT	-	5α-dihydrotestosterone
5β-DHT	-	5β-dihydrotestosterone
actb	-	β-actin
ar	-	androgen receptor
ARE	-	androgen responsive element
avt	-	arginine vasotocin
cDNA	-	complementary DNA
CNS	-	central nervous system
CRH	-	corticotrophin releasing hormone
Ct	-	threshold cycle
cyp19	-	aromatase
dio1	-	type I iodothyronine deiodinase
dio2	-	type II iodothyronine deiodinase
dio3	-	type III iodothyronine deiodinase
dmrt-1	-	doublesex and mab-3 related transcription factor 1
DMSO	-	dimethylsulfoxide
DNA	-	deoxyribonucleic acid
E2	-	17β-estradiol
EDC	-	endocrine disrupting chemical
EE2	-	ethynilestradiol
ef1alpha	-	elongation factor 1α
eralpha	-	estrogen receptor alpha
erbeta	-	estrogen receptor beta
ERE	-	estrogen responsive element
FSH	-	follicle-stimulating hormone
G	-	Gosner developmental stage
gapdh	-	glyceraldehyde-3-phosphate dehydrogenase
gDNA	-	genomic DNA
GH	-	growth hormone
GMC	-	gonad-mesonephros complex
GnRH	-	gonadotropin-releasing hormone
hCG	-	human chorionic gonadotropin hormone

hpf	-	hours post-fertilisation
LH	-	luteinizing hormone
mRNA	-	messenger RNA
MS-222	-	3-aminobenzoic acid ethyl ester
NF	-	Nieuwkoop and Faber developmental stage
odc	-	ornithine decarboxylase
PCR	-	polymerase chain reaction
rpl8	-	ribosomal protein L8
RT-PCR		reverse transcriptase polymerase chain reaction
RXR	-	retinoic acid X receptor
srd5alpha1	-	steroid 5α-reductase type 1
srd5alpha2	-	steroid 5α-reductase type 2
srd5alpha3	-	steroid 5α-reductase type 3
srd5beta	-	steroid 5β-reductase
T	-	testosterone
T3	-	triiodothyronine
T4	-	thyroxine
TH	-	thyroid hormone
tralpha	-	thyroid hormone receptor alpha
trbeta	-	thyroid hormone receptor beta
TRE	-	thyroid hormone responsive element
TSH	-	thyroid stimulating hormone

List of Anuran Species

The following table presents current (used throughout the thesis) and former anuran species names (when applicable) with references for further taxonomic information.

Current species name	Former species name	Common name	Current Family
Amietophrynus rangeri	*Bufo rangeri*	Ranger's toad	Bufonidae
Bombina orientalis	-	Oriental fire-bellied toad	Bombinatoridae
Bufo bufo	-	Common toad	Bufonidae
Bufo japonicus	-	Japanese toad	Bufonidae
Engystomops petersi	*Physalaemus petersi*	Peters dwarf frog	Leiuperidae*
Engystomops pustulosus	*Physalaemus pustulosus*	Túngara frog	Leiuperidae*
Epidalea calamita	*Bufo calamita*	Natterjack toad	Bufonidae
Eupsophus insularis	-	Mocha island ground frog	Cycloramphidae*
Eupsophus migueli	-	Miguel's ground frog	Cycloramphidae*
Glandirana rugosa	*Rana rugosa*	Japanese wrinkled frog	Ranidae
Lithobates catesbeianus	*Rana catesbeiana*	American bullfrog	Ranidae
Lithobates clamitans	*Rana clamitans*	Green frog	Ranidae
Lithobates pipiens	*Rana pipiens*	Northern leopard frog	Ranidae
Lithobates sylvaticus	*Rana sylvatica*	Wood frog	Ranidae
Pelobates syriacus	-	Eastern spadefoot toad	Pelobatidae
Pelophylax kl. esculentus	*Rana esculenta*	Edible frog	Ranidae
Pelophylax perezi	*Rana perezi*	Iberian green frog	Ranidae
Pelophylax ridibundus	*Rana ridibunda*	Eurasian marsh frog	Ranidae
Rana temporaria	-	European common frog	Ranidae
Rhinella marina	*Bufo marinus*	Cane toad	Bufonidae
Scaphiopus couchii	-	Couch's spadefoot toad	Scaphiopodidae
Silurana tropicalis	*Xenopus tropicalis*	Western clawed frog	Pipidae
Spea multiplicata	-	New Mexico spadefoot toad	Scaphiopodidae
Xenopus laevis	-	African clawed frog	Pipidae

*Former family: Leptodactylidae

References: Cannatella and Trueb, 1988; Frost, 2010; Frost et al., 2006; Grant et al., 2006; Nascimento et al., 2005; Yu et al., 2007

CHAPTER 1

General Introduction

1.1. Thesis rationale and approach

In developing frogs, the main role of thyroid hormones (THs) is to regulate metamorphosis, while that of sex steroids (e.g., estrogens and androgens) is to regulate gonadal differentiation (Fig. 1.1A). There is increasing evidence that different hormone systems interact, not only in frogs, but also in mammals and fish. This is important to study because research indicates that inhibiting TH synthesis affects anuran gonadal differentiation, while exposure to estrogens can inhibit tadpole metamorphosis. However, the mechanisms of these interactions and whether different frog species display different mechanisms remain largely unknown.

In amphibians, basic information regarding the expression of important enzymes and receptors related to THs and sex steroids during development is also lacking. Therefore, I first established the profiles of sex steroid- and TH-related genes throughout development, from the egg stage until adulthood in the laboratory frog *Silurana tropicalis* (current taxonomy is used throughout the text; see Table p. xix). These profiles allowed me to develop and test new hypotheses about the role of THs and sex steroids (Fig. 1.1A) and to design experiments to modulate the synthesis of estrogens or the function of THs during anuran development. I then investigated hormone interactions by studying the regulation of TH- and sex steroid-related genes during different periods of development and in different tissues. Using this approach, I studied two directions of cross-talk in *S. tropicalis* (Fig. 1.1B). Next, to investigate how hormone interactions compared between frog species, I studied the effects of THs on the reproductive system during metamorphosis of the Neotropical species,

Engystomops pustulosus (Fig. 1.1B). Finally, I compared the results with *S. tropicalis* and previous research in the North American frog *Lithobates pipiens*.

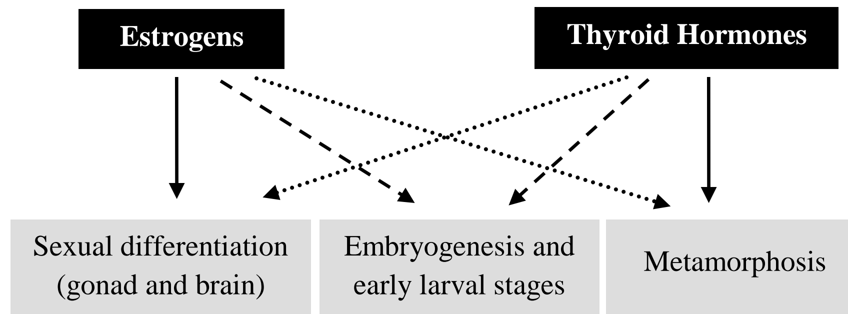
1.2. Hypotheses

i. Estrogen is required for tadpole development and metamorphosis and deficiencies in estrogen will affect development, gonadal differentiation, and TH-related transcripts levels in whole body larvae and in the brain and liver of metamorphs (Chapters 2 and 3);
ii. THs regulate sexual development in frogs and exposure to THs will affect TH- and sex steroid-related transcripts levels in whole body larvae (Chapter 4) and in brain and gonad of tadpoles (Chapters 5-8);
iii. The effect of THs on sex steroid-related transcripts is species-specific and will depend on the developmental and reproductive characteristics of the species (Chapters 7-9).

1.3. Objectives

i. To establish developmental profiles of sex steroid- and TH-related genes in *S. tropicalis* and *E. pustulosus*;
ii. To investigate the effects of inhibiting estrogen synthesis on development, gonadal histology, sex ratios and expression of sex steroid- and TH-related genes in *S. tropicalis*;
iii. To investigate the effects of TH exposure on transcripts levels of sex steroid- and TH-related genes in the brain and gonad of *S. tropicalis* and *E. pustulosus*.

A



B

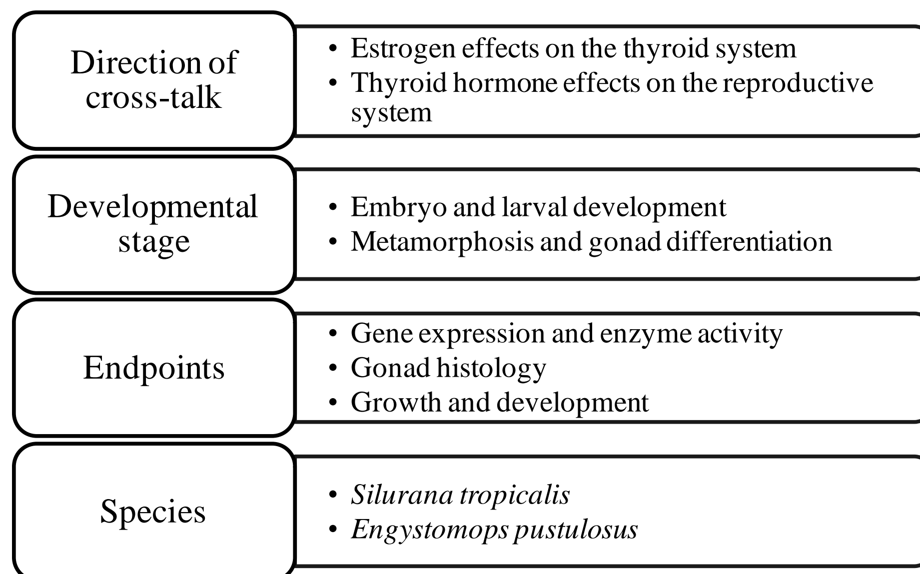


Figure 1.1. (**A**) Main roles (solid lines), potential roles (dash lines) and interactions (dotted lines) of estrogens and thyroid hormones (THs) in frogs investigated in this thesis. (**B**) General approach of the thesis. The direction of cross-talk involved investigating either the effects of estrogens on the TH system or the effects of THs on the reproductive system (both estrogen and androgen). Two periods of development were chosen for the experiments: early during development (i.e., the period before the larvae begin feeding) and during metamorphosis and gonad differentiation. Gene expression of TH- and sex steroid related transcripts was the common endpoint in all experiments. Measurement of enzyme activity was limited by the amount of tissue; therefore, it was measured when possible. Gonad histology and somatic growth were measured in the chronic experiment of Chapter 3. *Silurana tropicalis* tadpoles were used for all the experiments. In addition, the effects of TH on the reproductive system were also investigated during metamorphosis in *Engystomops pustulosus*.

1.4. Sex steroids and the reproductive axis of anurans

1.4.1. Synthesis and action of sex steroid hormones

Biosynthesis of estrogens and androgens involves a series of enzymatic steps, initiated and regulated by the transport of cholesterol from the outer to the inner mitochondrial membrane. Pregnenolone, an intermediate product first synthesised from cholesterol, can be converted to progesterone or used directly for the synthesis of corticosteroids. The alternative pathway is the conversion of progestagens into the androgens testosterone (T) and adrostenedione. These two androgens can either be: (1) converted to 5α-dihydrotestosterone (5α-DHT) by the enzymes 5α-reductases (srd5alpha1, srd5alpha2, srd5alpha3) or converted to 5β-dihydrotestosterone (5β-DHT) by the enzyme 5β-reductase (srd5beta; Hutchison and Steimer, 1984; Russell and Wilson, 1994) or (2) converted to the estrogens, estrone and 17β-estradiol (E2) by the enzyme aromatase (cyp19; Simpson et al., 1994). In most organisms, including frogs, cyp19 is encoded by one gene (*cyp19*) that is highly expressed in adult brain and gonad while at lower levels in liver, kidney and heart (Urbatzka et al., 2007). The isozymes srd5alpha1 and srd5alpha2 are encoded by two different genes and they have been the focus of most studies, since srd5alpha3 was only recently discovered in human prostate cells (Uemura et al., 2008). The isozymes differ in their tissue distribution. In rat and frog, srd5alpha2 is more associated with androgenic tissues (i.e., gonad and prostate) than srd5alpha1, which is more ubiquitously expressed (e.g., gonad, brain, liver, and kidney; Thigpen et al., 1993; Urbatzka et al., 2007). In contrast, the reproductive and developmental functions of srd5beta have been largely unexplored because of the long-held view that 5β-DHT is biologically inactive (Kokontis and Liao, 1999). Only recently, Langlois (2010; PhD thesis) demonstrated that srd5beta is present in *S. tropicalis* embryos and larvae and that it may play a role in frog sexual development. Because the roles

of srd5beta and srd5alpha3 in frog reproduction are not clearly understood, srd5alpha1 and srd5alpha2 were the two forms studied in this thesis.

Sex steroids exert their biological effects by binding to nuclear receptors that act as transcription factors to regulate gene expression. Two estrogen receptors (*eralpha* and *erbeta*) and one androgen receptor (*ar*) have been cloned in many vertebrates including amphibians (Katsu et al., 2010; Takase and Iguchi, 2007; Yokoyama et al., 2009). Once the receptor binds its ligand (i.e, estrogens or androgens), it homodimerises and binds to specific promoter regions of the DNA (i.e., estrogen or androgen responsive elements, ERE or ARE, respectively). In the case of androgens, 5α-DHT binds ar with higher affinity and has a stronger biological activity than T. Also, the 5α-DHT-ar complex is more stable than the T-ar complex (Heemers and Tindall, 2007; Nilsson and Gustafsson, 2002). An important characteristic of these nuclear receptors is that their expression can be induced by their own ligands (autoinduction) because of the presence of an ERE or ARE in the promoter region of the gene encoding the receptor. Autoinduction is usually observed during development (Tata et al., 1993). One of the best studied cases is *er* autoinduction in *X. laevis* (Shapiro et al., 1989; Tata, 1994); however there are indications that *ar* can also be autoinduced (Cardone et al., 1998; Esposito et al., 2002).

Synthesis of estrogens and androgens is under the control of hypothalamic and pituitary factors (Fig. 1.2A). Sex steroids regulate genes and proteins involved in multiple responses and functions such as tissue and bone growth, metabolism, development of secondary sexual characteristics, sexual behaviour, gonadal differentiation and maturation, in various tissues throughout the body including the central nervous system (CNS; Behl, 2002; Boon et al., 2010; Walters et al., 2010; Zuloaga et al., 2008). In mammals and birds, early exposure to T masculinises the developing brain through aromatisation into E2 and

subsequent ER activation (Morris et al., 2004). There is also evidence that activation of AR is required for some masculinising effects of brain structures and behaviour (Sato et al., 2004; Zuloaga et al., 2008) indicating that both androgen and estrogen systems play important roles in vertebrate brain development.

1.4.2. Gonadal differentiation

Gonadal (sex) differentiation refers to the development of the undifferentiated gonad into either ovaries or testes. In vertebrates, this process is under control of genetic factors (i.e., sex-determining genes) and/or environmental factors (i.e., temperature), directing the individual to develop into one of the sexes through developmental pathways. While all mammals display male heterogametic (XY/XX) genetic sex determination, all birds, display female heterogametic (ZZ/ZW) sex determination (Manolakou et al., 2006; Morrish and Sinclair, 2002). Most anurans do not have morphologically distinguishable sex chromosomes (Nakamura, 2009); however a number of sex reversal and breeding experiments have demonstrated that sexual determination in anurans is likely to be genetic (Chang and Witschi, 1956; Hayes, 1998; Kelley, 1996; Richards and Nace, 1978). Some species display a XX/XY genetic mechanism (e.g., *Bombina orientalis*, *Lithobates catesbeianus*, *L. pipiens*) while others display a ZZ/ZW mechanism of sex determination (e.g., *Bufo bufo*, *S. tropicalis*, *X. laevis*; Schmid and Steinlein, 2001; Sumida and Nishioka, 2000; Uno et al., 2008).

In mammals, sex determination is mainly controlled by one gene in the sex-determining region Y (SRY) of the Y chromosome, which is the initiator of the activation of a cascade of genes that induce the differentiation of the bipotential gonad into a testis. In non-mammalian vertebrates, no comparable gene to SRY has been found, and the molecular mechanism of sex determination remains poorly understood. Other genes, such as doublesex-

and mab-3-related transcription factor 1 (*dmrt-1*), DM-domain gene on the Y chromosome (*dmy*), SRY-box 9 (*sox9*), steroidogenic factor 1 (*sf1*), Müllerian-inhibiting substance (*amh*), and W-linked DM-domain (*dm-w*) seem to be involved in gonadal development and are the topic of extensive research in vertebrates (Eggert, 2004; Morrish and Sinclair, 2002; Nagahama et al., 2004; Nakamura, 2009). In contrast, sex determination in reptiles and some fish is under environmental control. For example, incubating snapping turtle eggs at high temperature (30-35ºC) results entirely in female offspring, while incubation at lower temperature (20-22ºC) results in males (Crews et al., 1989).

The development of the gonad is also dependent on epigenetic factors (internal and external) that act during development. Internal epigenetic factors include hormones and enzyme systems, and external epigenetic factors include xenobiotics (e.g., exogenous hormones; Hayes, 1998). In amphibians, reptiles and fish, both genetic and temperature sex determination can be overridden by treatment with sex steroid hormones, suggesting that regardless of the primary mechanism of sex determination, sex steroids are the natural inducers of gonad differentiation in these animals (reviewed in: Baroiller et al., 1999; Hayes, 1998; Uguz et al., 2003). The period of sexual differentiation can take place during larval development in species of the Ranidae and Pipidae families or after completion of metamorphosis in the case of species of the Bufonidae family (Hayes, 1998; Ogielska and Kotusz, 2004). In the former species, morphological sex can be assigned at the end of metamorphosis (by visual inspection of the gonads or using histological techniques when the gonads are not easily visualised) and the sensitive period of gonadal differentiation takes place early during tadpole metamorphosis. In *S. tropicalis*, the first signs of gonadal differentiation are observed during tadpole development, at Nieuwkoop and Faber (NF; 1994; a description and comparison of staging tables is presented in Appendix A) stage 48

and the gonads are differentiated by NF53 (El Jamil et al., 2008). In *X. laevis*, *L. pipiens*, and *Rana temporaria*, gonads are morphologically distinct by their equivalents of NF52 (Hayes, 1998; Humphrey et al., 1950; Ogielska and Kotusz, 2004).

1.5. Thyroid hormone axis in anurans

1.5.1. Thyroid hormone synthesis and action

The thyroid system in developing anurans is regulated by the hypothalamus-pituitary-thyroid gland axis (Fig. 1.2B; Denver, 1997; Denver, 1998). The two forms of TH, 3,5,3'-triiodothyronine (T3) and thyroxine (T4), mediate their respective physiological effects by binding to nuclear TH receptors (tr) encoded by two genes, *tralpha* and *trbeta* (Tsai and O'Malley, 1994). When TH binds to tr, the hormone-receptor complex dimerises with the retinoic acid X receptor (rxr) or with another tr. This complex regulates transcription by binding to a thyroid hormone receptor element (TRE) in the promoter region of target genes (Tsai and O'Malley, 1994). The metabolism of THs is regulated by three types of deiodinases. Deiodinase type 1 (dio1) catalyses outer-ring deiodination to produce T3 from T4 and also inner-ring deiodination to produce reverse T3 (rT3, inactive) from T4. Type 2 deiodinase (dio2) exclusively activates THs by catalysing the conversion from T4 to T3. In contrast, type 3 deiodinase (dio3) inactivates THs by inner-ring deiodination of T4 and T3 to produce rT3 and T2 (diiodothyronine), respectively (Galton, 2005). In vertebrates as a whole, THs are required for normal brain development and influence many processes such as metabolism, growth, and reproduction in adults (Bernal et al., 2003; Norris, 2007; Zoeller et al., 2007). In the case of amphibians, THs control tadpole metamorphosis.

1.5.2. Anuran metamorphosis

Metamorphosis is characteristic of amphibian species with distinct larval and adult stages. Anuran metamorphosis is characterised by the transition from an aquatic, fish-like and sometimes herbivorous larva to a tetrapodal, and often, terrestrial adult that is exclusively carnivorous. This period, also referred to as transformation, is characterised by a rapid and dramatic morphological remodelling. Different cell types proliferate, degenerate, and differentiate, with development of limbs, resorption of the tail, remodelling of the brain and gas exchange system, and reorganisation of the gastrointestinal system all taking place (Shi, 1994; Tata, 1993).

Anuran metamorphosis is tightly controlled by THs (reviewed in: Shi, 2000; Tata, 2006). The process is separated into three specific periods: premetamorphosis, prometamorphosis and metamorphic climax. Premetamorphosis (NF 46-54) is the period of early tadpole growth and development characterised by low levels of circulating THs. The growth of the hind limbs and toe differentiation is accelerated during prometamorphosis (NF 55-57), also characterised by a rapid increase in the concentrations of endogenous THs. During metamorphic climax (NF 58-65), TH levels peak and rapid morphological changes occur, such as the emergence of the forelimbs and tail resorption. THs are the primary regulators of metamorphosis; however, other hormones (e.g., corticosteroids, prolactin) and environmental factors (e.g., temperature, water availability, crowding, and diet) also play a role in this transformation (Fig. 1.2B-C; Shi, 2000; Denver, 1997).

During metamorphosis, every tissue and organ undergoes some remodelling but each change occurs at very distinct stages of development. This coordinated transformation ensures a successful transition from a tadpole to a frog (Shi, 2000). The main form of TH secreted from the thyroid gland is T4 and it is locally converted to T3, the more biologically

active form of THs (Brown, 2005; Galton, 1992). Localised activity of the different types of dio in each target tissue controls local TH levels which allow the various tissues to undergo independent and differentially-timed development during metamorphosis (Brown, 2005).

During tadpole development, *tralpha* and *trbeta* are differentially expressed. In *X. laevis*, *tralpha* is first detected after hatching (NF38; Yaoita and Brown, 1990) and continues to be expressed throughout metamorphosis. On the other hand *trbeta* is very low during premetamorphosis and only increases when THs begin to the increase (Wang et al., 2008; Yaoita and Brown, 1990). This difference in expression is explained by the dual-function model (Buchholz et al., 2006). Briefly, in the absence of the T3 ligand, tr suppresses the expression of target genes by interacting with transcriptional co-repressors such as SMRT (silencing mediator of retinoic acid and thyroid receptors) and N-CoR (nuclear receptor co-repressor). When T3 levels begin to increase, T3 binds to tr inducing the dissociation of tr from co-repressors and the recruitment of co-activators such as the steroid receptor co-activator (SRC). This activates the transcriptional machinery in target genes (Buchholz et al., 2006). One such direct response genes is *trbeta*, which contains at least one TRE in the promoter of the *X. laevis* gene. This model proposes that *tralpha* regulates early events during development. When THs begin to rise during prometamorphosis, *trbeta* is autoinduced and peaks at metamorphic climax. Autoinduction of *trbeta* regulates downstream target genes responsible for changes in phenotype during metamorphosis (Brown and Cai, 2007; Tata, 2006; Tata et al., 1993).

1.6. Cross-talk between thyroid hormones and sex steroids

Traditionally, the TH and reproductive axes have been studied in isolation (represented in Fig. 1.2). However, there is increasing evidence that hormones operate as part

of complex intracellular networks, sharing signaling pathways with other hormones and affecting other hormone systems. The interplay between hormones led to the concept of cross-talk and complicated our view of and research on hormone actions and endocrine disruption by environmental contaminants (Cooke et al., 2004; Cyr and Eales, 1996; Hogan et al., 2007; Myers et al., 2009; Tata, 1994; Ulisse and Tata, 1994; Vasudevan et al., 2002).

1.6.1. Effects of thyroid hormones on reproduction-related endpoints during development

General correlations between thyroid and reproductive status in adults first suggested that these two endocrine systems could interact. In vertebrates, hypo- and hyperthyroidism are associated with altered reproductive function (e.g., infertility, altered sex steroid levels, impaired testicular function and spermatogenesis, and altered menstrual cycles in humans; Jiang et al., 2001; Maran, 2003; Maruo et al., 1992; Swapna et al., 2006; Warren, 1940; Weng et al., 2007).

In vertebrates, THs seem to be involved in testicular development. In mammals, THs are important for the differentiation of the adult Leydig cell populations (Siril Ariyaratne et al., 2000) and the regulation of Sertoli cell development (Cooke et al., 1994). In anurans, there is evidence that THs are required for normal gonadal development. In *X. laevis*, blocking TH production during larval development (using thiourea or ammonium perchlorate) skews sex ratios towards females, relative to the 1:1 expected sex ratio (Goleman et al., 2002; Hayes, 1997a). Similar feminising results have been obtained in fish using perchlorate (Bernhardt et al., 2006; Mukhi et al., 2007). In zebrafish, treatment with perchlorate and co-treatment with T4 blocks the feminising effect of perchlorate, skewing the sex ratios towards males and advancing the onset of spermatogenesis (Mukhi et al., 2007). These studies suggest that THs induce male development; however, most of the research on

molecular mechanisms has concentrated on the actions of THs on mammalian gonads, especially testes (reviewed in: Cooke et al., 2004; Wagner et al., 2008). The effects of THs on sex steroid-related endpoints from the literature are summarised in Table 1.1. The results indicate that some of the masculinising effects of THs may be through the reduction of cyp19 and er and/or the increase of srd5alpha and ar. However, research on the developing frog gonad is lacking and additional targets in the brain (e.g., androgen-related endpoints) are required to better elucidate the mechanisms of cross-talk in anurans.

1.6.2. Effects of estrogens on the thyroid hormone axis and metamorphosis

The effects of estrogens on the TH system and metamorphosis are not completely understood. In some studies, exposure to estrogens inhibits tadpole metamorphosis (Gray and Janssens, 1990; Hayes, 1997b; Hogan et al., 2008; Richards and Nace, 1978), but mechanistically, there have been few studies that have investigated this interaction. Gray and Janssens (1990) found that E2 blocks the metamorphic effects of exogenous T3 in *X. laevis* tadpoles, in line with research in *L. pipiens* that found that the synthetic estrogen, ethinylestradiol (EE2), reduces the induction of *trbeta* and *dio3* mRNA by T3 in the tadpole brain (Hogan, 2006). Since autoinduction of *trbeta* is a direct metamorphic response gene, these results suggest that estrogens may inhibit metamorphosis by interfering with *trbeta* and/or other TH-related transcripts. However, other studies have found no effect of estrogens on metamorphosis. For example, chronic exposure of *S. tropicalis* to EE2, does not affect rate of metamorphosis or histology of the thyroid gland (Berg et al., 2009; Pettersson et al., 2006). Therefore, there is no clear consensus on the effect of estrogens on anuran development and the TH axis.

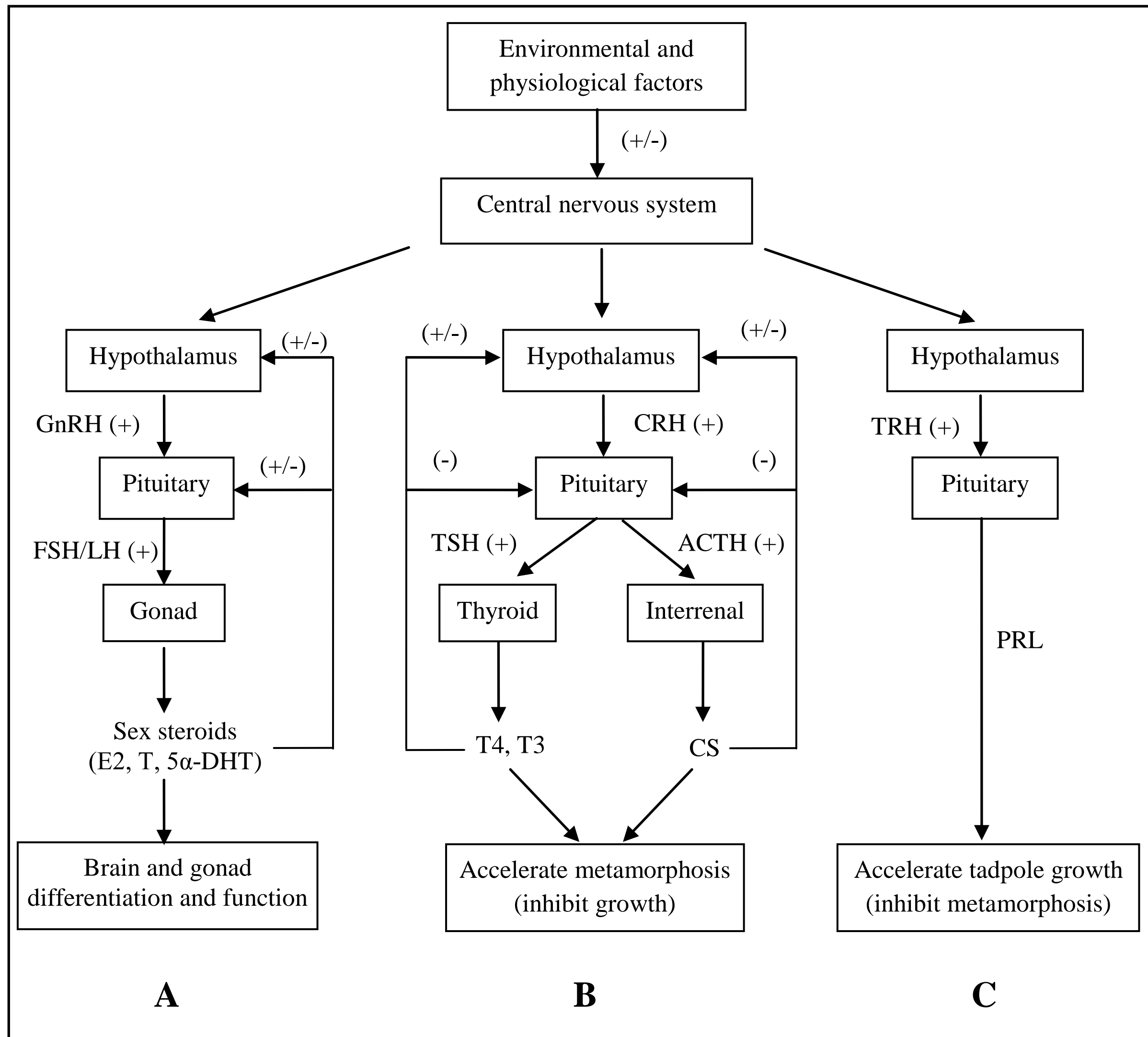


Figure 1.2. Schematic representation of the three main endocrine systems controlling amphibian development: the hypothalamus-pituitary-gonadal axis (**A**), hypothalamus-pituitary-thyroid axis (**B**) and hypothalamus-pituitary-prolactin axis (**C**). A plus sign indicates stimulatory and a negative sign indicates inhibitory. A plus and a minus together designate that the effect can be either stimulatory or inhibitory depending on the reproductive cycle (A) or developmental stage (B). ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; CS corticosteroids; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PRL, prolactin; T, testosterone; TSH, thyroid-stimulating hormone. Sources: Denver, 1997, 1998; Norris, 1997.

Table 1.1. Summary of studies on the effects of thyroid hormones on sex steroid-related endpoints in vertebrates. The literature results are first divided by tissue, then by target (transcript, enzyme activity [e], or protein [p]), and finally by organism. In the case of anurans, the species is indicated. For simplification, targets are written using anuran gene and protein nomenclature.

Tissue	Target	Effect	Compound	Type of exposure	Organism	Reference
Brain	*cyp19*	↓	T3	*in vivo* premetamorphic tadpoles	*L. pipiens*	[1]
	eralpha	↑	T3	*in vivo* premetamorphic tadpoles	*L. pipiens*	[1]
Liver	*eralpha*	↑	T3	*in vitro* adult hepatocytes	*X. laevis*	[2], [3]
	srd5alpha [e]	↑	Hypo*+T4	*in vivo* female and male adults	rat	[4]
	srd5alpha [e]	↑	T4	*in vivo* male adults	rat	[5]
Testis	*ar*	↑	T3	*in vivo* and *in vitro* adult tissue	lizard	[6]
	ar	↑	T3	*in vitro* Sertoli cell line	rat	[7]
	ar [p]	↑	T3	*in vitro* Sertoli cell line	rat	[8]
	cyp19	↓	T3	*in vivo* and *in vitro* adult tissue	goldfish	[9]
	cyp19	↓	T3	*in vitro* Sertoli cell line	mouse	[10]
	cyp19	↓	T3	*in vitro* Sertoli cell line	rat	[11], [12]
	cyp19 [e]	↓	T3	*in vitro* Sertoli cell line	rat	[11], [13]
	eralpha	↓	T3	*in vivo* and *in vitro* adult tissue	goldfish	[9]
	er [p]	↓	T3	*in vitro* Sertoli cell line	rat	[8]
Ovary	*eralpha*	↓	T3	*in vivo* and *in vitro* adult tissue	goldfish	[9]
	cyp19	↓	T3	*in vivo* adult tissue	goldfish	[9]
	cyp19	↓	T3	*in vitro* granulosa cells	rat	[14]
	cyp19	↓	T3	*in vitro* granulosa cells	mouse	[15]
	cyp19 [e]	↓	T3	*in vitro* granulosa cells	mouse	[15]
	cyp19 [e]	↓	T3	*in vit*ro thecal and granulosa cells	pig	[16]

* Hypothyroidism was induced with methimazole.

References: [1] Hogan et al., 2007; [2] Rabelo and Tata, 1993; [3] Ulisse and Tata, 1994; [4] Ram and Waxman, 1990; [5] Murray and Butler, 1999; [6] Cardone et al., 2000 ; [7] Arambepola et al., 1998; [8] Panno et al., 1996; [9] Nelson et al., 2010; [10] Catalano et al., 2003; [11] Ando et al., 2001; [12] Pezzi et al., 2001; [13] Ulisse et al., 1994 ; [14] Hatsuta et al., 2004; [15] Cecconi et al., 1999; [16] Gregoraszczuk et al., 1998.

1.7. Anurans as biological models

Anurans are excellent organisms to study the interactions between THs and sex steroids because much of their development (i.e., metamorphosis and sexual development) depends on these hormones and embryos develop externally without the hormonal influences of the mother. Most of the experiments in this thesis carried out using *S. tropicalis* of the family Pipidae (Fig. 1.3), native to Central West Africa. It is very closely related to the laboratory model *X. laevis* (family: Pipidae). However, *S. tropicalis* has been suggested as a better model for several reasons, but especially because it is diploid, it develops rapidly, and its genome has been sequenced (Hellsten et al., 2010; Hirsch et al., 2002). In the laboratory, adults can be induced by hormone injection to lay 1,000-3,000 eggs at a time (Amaya et al., 1998). Moreover, there are morphological and physiological studies on *S. tropicalis* (El Jamil et al., 2008; Wang et al., 2008) and it is also being used in amphibian toxicological studies (e.g., Berg et al., 2009).

There is a lack of research on the basic physiology and endocrinology of Neotropical anurans, therefore *E. pustulosus* was chosen as the representative of this large group of frogs. Commonly known as the túngara frog, *E. pustulosus* is found in Central America and parts of South America, from Mexico to eastern Panama and in northern Colombia and Venezuela. It belongs to the Leiuperidae family (Grant et al., 2006; Fig. 1.3). The adults can be easily kept and bred in captivity to provide tadpoles for experiments (Romero-Carvajal et al., 2009). *E. pustulosus* has been extensively used in studies on sexual selection, evolution of communication and neurobiology of vocalisation (reviewed in: Ryan, 1985; Ryan, 1998; Ryan and Rand, 2003); however, very limited information is available regarding tadpole development and the endocrine control of metamorphosis and sexual development.

1.8. The comparative project: rationale and description

Anuran species are very diverse, ranging from completely aquatic to almost completely terrestrial; some metamorphose from tadpole to frog while others exhibit direct development. The comparative project aimed at identifying and comparing mechanisms of hormone interactions in three evolutionary separate anuran species (Fig. 1.3). The first two species (described in section 1.7) are *S. tropicalis* (family: Pipidae) and *E. pustulosus* (family: Leiuperidae). The third species is *L. pipiens* (family: Ranidae), a semi terrestrial and cold tolerant anuran. It is native to North America where it has a wide range of distribution that spans most of Southern Canada and the Northern United States. Studies with *L. pipiens* were performed in collaboration with N. Hogan (Hogan, 2006) and results are further used herein to compare the mechanisms of cross-talk in the three different species.

All three species chosen for this project have a tadpole stage, but have different tadpole rates of development and reproductive characteristics (Table 1.2). The objective of this project was to compare the regulation of sex steroid- and TH-related genes by T3 in the three frog species. Since THs control metamorphosis, I hypothesised that T3 regulation of sex steroid-related genes would be different depending on the developmental rate of the tadpoles. Using the characteristics in Table 1.2, I predicted that *S. tropicalis* and *E. pustulosus* would have similar regulation of sex steroid-related genes while *L. pipiens* would show a different transcript profile. Since metamorphosis is similarly controlled in all studied anurans with a distinct tadpole stage (Shi, 2000), I hypothesised that T3 regulation of TH-related genes would be similar in the three species. The experimental approach was the same in all three studies and extreme care was taken to keep the protocols as similar as possible.

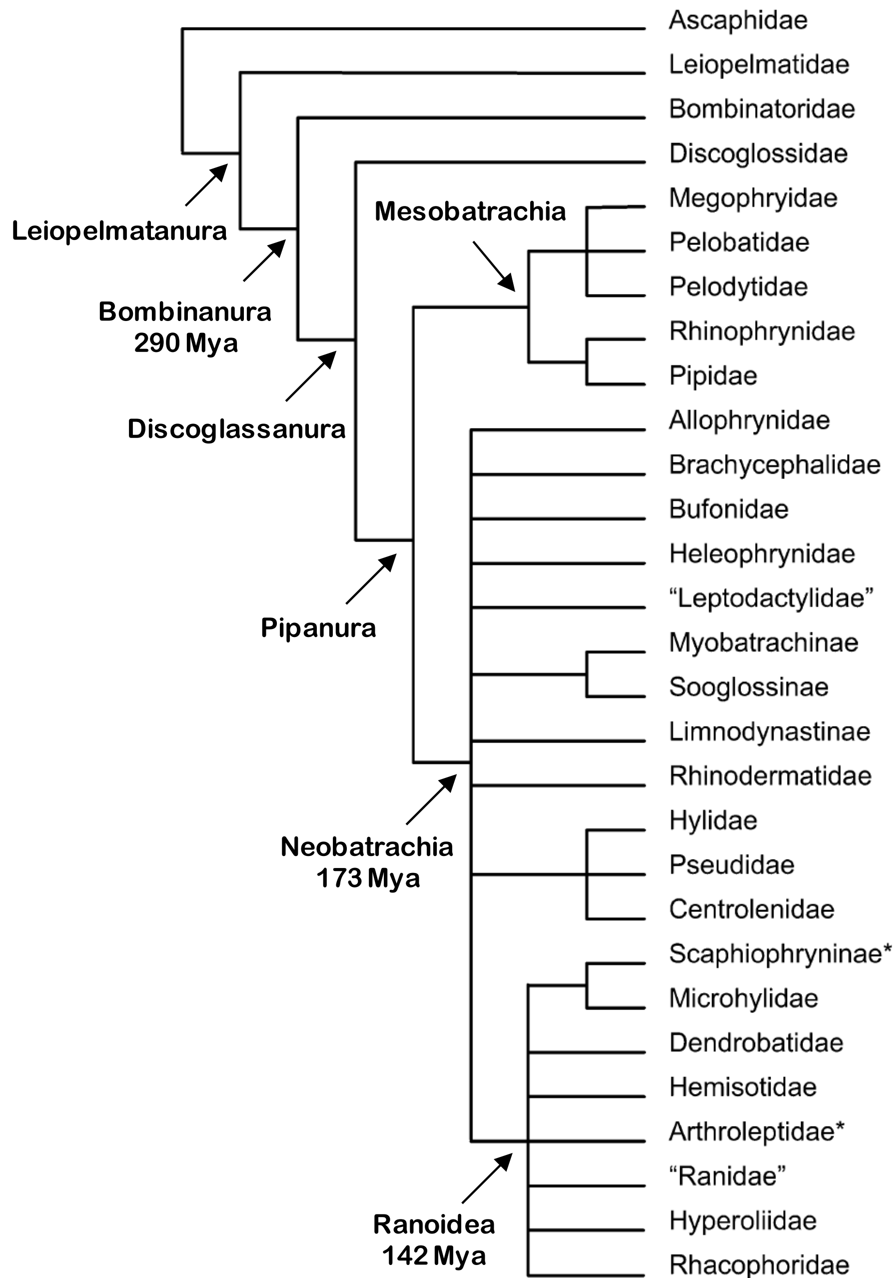


Figure 1.3. Tree of relevant anuran taxa determined by Ford and Cannatella (1993). An asterisk denotes a metataxon, and quotation marks denote non-monophyly. Since the study by Ford and Cannatella (1993), there have been taxonomic revisions and updates. Currently, *Silurana tropicalis* belongs to the Pipidae family, *Engystomops pustulosus* belongs to the Leiuperidae family broken out from the Leptodactylidae family (Grant et al. 2006) and *Lithobates pipiens* belongs to the Ranidae family. Estimated divergence time (in million years ago, Mya) were established by Zhang et al. (2005). Adapted from: Ford and Cannatella, 1993; Frost et al., 2006.

Table 1.2. Developmental and reproductive characteristics of *Silurana tropicalis*, *Engystomops pustulosus*, and *Lithobates pipiens*. Days to metamorphosis are based on laboratory observations in the Trudeau and Ryan laboratories. Information taken from: Amaya et al., 1998; Cannatella, 2008; COSEWIC, 2009; Force, 1933; IUCN, 2010; Kochan et al., 2003; Marsh, 2001.

	Silurana tropicalis	***Engystomops pustulosus***	***Lithobates pipiens***
Anuran family	Pipidae	Leiuperidae	Ranidae
Geographic location	Central Africa	Central & South America	North America
Days to metamorphosis	30-90	20-50	>90
Age at sexual maturity	~ 16-24 weeks	~ 8-12 weeks	~ 2-3 years
Sex-determining mechanism	ZZ/ZW	unknown	XX/XY
Reproduction	Rain season (prolonged breeder)	Rain season (prolonged breeder)	Spring (ephemeral breeder)
Hibernation (overwintering)	No	No	Yes

1.9. Thesis outline

This thesis is organised into seven data chapters (Chapters 2-8) followed by a General Discussion (Chapter 9). Each data chapter is adapted from one or two publications and involved work with various collaborators. In order to avoid repeating background information, each chapter contains a very concise introduction to explain the rationale of the chapter and its objectives. Chapters 2 and 3 examined one of the directions of cross-talk, the effect of inhibiting the synthesis of estrogens (using fadrozole) on TH-related endpoints in *S. tropicalis* (Fig. 1.1B). For the remainder of the thesis, I studied the other direction of cross-talk, i.e., the effect of THs (using T3) on sex steroid-related endpoints in *S. tropicalis* (Chapters 4, 5, and 6) and *E. pustulosus* (Chapters 7 and 8).

Chapter 2: I established the developmental profile of cyp19 mRNA and activity during *S. tropicalis* embryo and early larval stages. Based on these profiles, embryos were exposed to fadrozole (cyp19 inhibitor) and expression of TH- and sex steroid-related genes was measured in whole body larvae. I found that cyp19 transcript and activity were present during this period of development and inhibition of cyp19 increased the expression of androgen- and TH-related genes but it did not affect larval development.

Chapter 3: *S. tropicalis* tadpoles were chronically exposed to fadrozole during the entire metamorphic period in order to determine the effects of a lack of estrogens on gonadal development and metamorphosis. Chronic inhibition of cyp19 affected sex ratios and gonadal differentiation, but it did not affect tadpole metamorphosis. The experiments demonstrated that estrogens are required for gonadal development; however interactions with THs were only observed early during development (Chapter 2), rejecting part of my first

hypothesis (i). The experiments in Chapters 2 and 3 were performed in collaboration with a former PhD student (V. Langlois) and two Honours students (S. Ing and K. Hodgkinson).

Chapter 4: I established the transcript developmental profiles of *tr* and *dio* in embryos and early larvae of *S. tropicalis*. Developing embryos were exposed to T3 when *tralpha* and *trbeta* were both present and highly expressed. I found that T3 increased the expression of TH- and androgen-related genes, demonstrating that this direction of cross-talk is also present early during development (see other direction in Chapter 2). These results provided the first mechanistic support for the masculinising effects of THs in frogs.

Chapters 5 and 6: I investigated the effects of T3 in older tadpoles in order to dissect brain and gonad-mesonephros complexes (GMC). I began by establishing developmental profiles from early premetamorphosis until adulthood in *S. tropicalis* of TH- and sex steroid-related transcripts in the brain (Chapter 5) and GMC (Chapter 6). Premetamorphic tadpoles have low levels of THs but they are responsive to exogenous THs. I used this model system to examine the effects of T3 on brain and GMC transcripts. In the brain, T3 affected expression of estrogen- and androgen-related genes, demonstrating that a complex interplay between THs and sex steroids is present and important in the developing tadpole brain (Chapter 5). In the GMC, T3 increased the transcription of androgen synthesis enzymes and decreased *erbeta*, providing a possible mechanism of the masculinising effects of THs (Chapter 6). Taken together, the experiments using T3 supported my second hypothesis (ii).

Chapters 7 and 8: similar to Chapters 5 and 6 but performed in *E. pustulosus*. The same protocol of the *S. tropicalis* projects was used here to compare developmental profiles and

T3 regulation of transcripts in the brain (Chapter 7) and GMC (Chapter 8) between species. The exposures were performed in the laboratory of Dr. Michael Ryan at the University of Texas at Austin, where there is a captive breeding colony of *E. pustulosus* adult descendents of animals collected in Panama. Developmental profiles of sex steroid- and TH-related gene transcripts were similar across anuran species; however their regulation by T3 differed between species. The regulation of sex steroid-related genes in *E. pustulosus* was different to that of *S. tropicalis* but resembling that of *L. pipiens*, therefore rejecting hypothesis (iii).

Chapter 9: I summarised the main findings and proposed a model of cross-talk and TH action in frogs using the species comparison.

CHAPTER 2

Expression and inhibition of aromatase to study the regulation of sex steroid- and thyroid hormone-related genes during *Silurana tropicalis* early development

Adapted from:

Langlois VS[1], Duarte-Guterman P[1], Ing S[2], Pauli BD[3], Cooke GM[4], Trudeau VL[5]. 2010. Fadrozole and finasteride exposures modulate sex steroid- and thyroid-related gene expression in *Silurana tropicalis* early larval development. *General and Comparative Endocrinology*, 166: 417-427.

[1]Contributed equally to the project, i.e., designed research, performed research (developmental profiles, animal exposures, real-time RT-PCR optimisation and assays, enzyme activity optimisation and assays) and data analysis and wrote the manuscript. Note that only the fadrozole results are presented in this chapter.
[2]Contributed to animal exposures and gene expression assays
[3]Contributed gene expression materials, expertise in amphibian toxicology and to the revision of the manuscript
[4] Contributed enzyme activity expertise and material and to the revision of the manuscript
[5] Contributed to the design of the research and the revision of the manuscript

2.1. Introduction

Estrogens play a critical role during gonadal differentiation in amphibians (Hayes, 1998). In *Silurana tropicalis*, the first histological signs of gonadal differentiation are observed after the beginning of feeding during tadpole development at Nieuwkoop-Faber (NF; Nieuwkoop and Faber, 1994) stage 48 (~8 days post fertilisation; El Jamil et al., 2008). However, studies have reported the presence of estrogens before gonadal differentiation begins, during embryo and early larval development in frogs (Bogi et al., 2002) and other vertebrates (bird, Carere and Balthazart, 2007; mammal, Goldman-Johnson et al., 2008; fish, Iwamatsu et al., 2005) suggesting that estrogens may play a role early during development.

The synthesis of estrogens involves the actions of many enzymes, including aromatase (cyp19), catalysing the last step in their biosynthesis (i.e., converting testosterone [T] and androstenedione into estradiol [E2] and estrone, respectively; Lephart, 1996). The presence of cyp19 activity is an indicator of active estrogen synthesis; however, in frogs, the

presence and functionality of cyp19 during embryogenesis is unknown. T is also the substrate for steroid 5α-reductase (srd5alpha) enzymes involved in the synthesis of the potent androgen, 5α-dihydrotestosterone (5α-DHT), and expressed early during *S. tropicalis* development (Langlois, 2010). In addition, thyroid hormone (TH)-related-genes (i.e., TH-receptors, *tralpha, trbeta* and deiodinases, *dio1*, *dio2*, *dio3*) are also expressed and functional during anuran embryogenesis (Havis et al., 2006; Morvan-Dubois et al., 2006; Chapter 4), therefore, an interaction between the estrogen, androgen and TH axes may be present during early development.

The objectives of this study were to (1) establish the ontogenic expression of cyp19 mRNA and activity during *S. tropicalis* embryo and early larval stages, and (2) investigate the effects of inhibiting cyp19 (using fadrozole) on estrogen-, androgen-, and TH-related gene expression during early development.

2.2. Materials and Methods

2.2.1. Breeding and maintenance of Silurana tropicalis

Silurana tropicalis frogs were reared in dechlorinated and aerated water in the University of Ottawa Aquatic Care facility (Ontario, Canada). Fertilised eggs were obtained from eight pairs of frogs by injecting human chorionic gonadotropin hormone (hCG; Sigma) into the dorsal lymph sac of adult *S. tropicalis*. Both males and females received a priming injection of 12.5 IU hCG followed by a boosting injection of 100 IU hCG after 20 h (Appendix B). Staging was determined by following the Nieuwkoop and Faber developmental table (NF; Nieuwkoop and Faber, 1994). A 12:12h light:dark cycle was maintained with the light cycle occurring from 7 am to 7 pm at 24-25°C. The care and treatment of animals used in this study were approved by the Animal Care Committee,

University of Ottawa and adhered to the guidelines published by the Canadian Council on Animal Care for the use of animals in science.

2.2.2. Tissue collection for developmental profiles

Eggs and larvae were raised in petri dishes containing modified Ringer's solution (0.1 M NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 300 mg/L $NaHCO_3$; 1:9 v/v) and 0.04 mg/L antibiotic gentamycin (Sandoz Canada Inc.). Pooled embryos (before hatching; NF 2-34) and larvae (NF41 and 46) were used to ensure a sufficient amount of RNA for gene expression (20-25 per sample; n=6-8 pools) and protein for cyp19 (40-50 per sample; n=7-8 pools). For gene expression profiles, whole embryos and larvae were sampled at NF 2, 7, 16, 21, 26-27, 34, 41 and 46, which correspond to 1, 4.5, 13, 15, 20, 36, 44 and 72 hours post-fertilisation (hpf) under our husbandry conditions. Embryos were frozen immediately in dry ice and stored at -80ºC. Larvae at NF41 and 46 were anaesthetised by immersion in 3-aminobenzoic acid ethyl ester (MS-222; 0.01%; Sigma) before freezing. For enzyme activity analyses, whole embryos and larvae were also collected at NF 7, 21, 34, and 46. Embryos were frozen immediately in dry ice and stored at -80ºC.

2.2.3. Fadrozole exposure

Eggs were allowed to develop to NF6 at which point they were collected and dejellied with 2% (w/v) L-cysteine (pH 8.0; Sigma). The eggs were washed three times with modified Ringer's solution (1:9 v/v) following cysteine treatment and placed in petri dishes (containing modified Ringer's solution and antibiotic, as described above) at a density of 50 eggs per dish. Embryos were exposed from stage NF12 to 46 (8 to 72 hpf) to nominal concentrations of fadrozole of 0.5, 1.0, 2.0 µM (Novartis Pharma AG) dissolved in water.

During the exposure, the medium and antibiotic were refreshed daily. Whole NF46 larvae were sampled for gene expression (10 per sample; n=5-8 pools) and cyp19 activity (40 per sample; n=5 pools).

2.2.4. RNA isolation and cDNA synthesis

Homogenisation of the samples was achieved using an MM301 Mixer Mill (Retsch) at 20 Hz for 4 min. For the developmental profile samples, total RNA was obtained using the RNeasy Micro Kit (including the RNase-free DNase treatment), whereas for the fadrozole exposure, RNA was extracted using the RNeasy Mini Kit as described by the manufacturer (Qiagen). Isolated RNA was resuspended in RNase free water and stored at -80 ºC. Concentrations of RNA were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 2 μg total RNA and 0.2 μg random hexamer primers (Invitrogen) using Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). The cDNA products were diluted 80-fold prior to PCR amplification.

2.2.5. Real-time RT-PCR in simplex

Specific primer sets (Table 2.1) were designed for simplex real-time RT-PCR for *cyp19*, *srd5alpha1*, *srd5alpha2*, androgen receptor (*ar*), TH receptors (*tralpha* and *trbeta*), and deiodinases (*dio1*, *dio2* and *dio3*). Primers for the following reference genes were also designed (Table 2.1): ribosomal protein L8 (*rpl8*), β-Actin (*actb*), ornithine decarboxylase (*odc*), and elongation factor 1α (*ef1alpha*). Primers were designed using Oligoanalyzer 3.1 (available at: http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) and Primer 3 (available at: http://fokker.wi.mit.edu/primer3/input.htm). The specificity of every primer set

was confirmed by cloning and sequencing the single amplicon obtained. For all real-time RT-PCR assays, primer concentrations were optimised to obtain a minimum threshold cycle and a maximum change in fluorescence. The expression of individual gene targets was analysed using the MX Real-time Polymerase Chain Reaction system (Stratagene). The simplex reaction consisted of a 25 µL DNA amplification reaction containing 1.0 x PCR buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 µM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene, La Jolla, CA, USA), 1.25 U HotStarTaq (Qiagen), optimised concentrations of each primer set (Invitrogen), 0.25 x SYBR Green I Dye (Molecular Probes, Eugene, OR, USA), and 5 µL diluted cDNA template. The thermocycle program included an enzyme activation step at 95°C (15 min) and 40 cycles of 95°C (15 sec), 58-62°C (gene specific annealing temperature for 5 sec), 72°C (30 sec) and 80°C (8 sec). After this amplification phase, there was a denaturation step of 1 min (95°C) followed by 41 cycles starting at 55°C and increasing 1°C/30 sec to generate a dissociation curve to confirm the presence of a single amplicon.

2.2.6. Real-time RT-PCR in multiplex

A multiplex real-time RT-PCR assay was designed to measure transcript levels of estrogen receptors (*eralpha*, *erbeta*), *cyp19*, and *rpl8* for the fadrozole exposure samples. Gene specific primer and probe sets for multiplex were designed using Beacon Designer 2.1 (PREMIER Biosoft International; Table 2.2). Dual-labelled fluorescent probes for the multiplex assay were purchased from Integrated DNA Technologies (IDT). Primer and probe concentrations were optimised in simplex, duplex and multiplex for a minimum threshold cycle (Ct) and a maximum change in fluorescence. Each 25 µL DNA amplification reaction contained 1.0x PCR buffer (Qiagen), 4.0 mM $MgCl_2$ (Qiagen), 800 µM dNTPs (Invitrogen),

1.25 U HotStarTaq (Qiagen), optimised concentrations of each primer set (Invitrogen) and associated probe (Integrated DNA Technologies, Coralville, IA, USA), and 5 µL diluted cDNA template. The thermocycle program included an enzyme activation step at 95°C (15 min) and 45 cycles of 95°C (15 sec), 62°C (1 min).

2.2.7. Real-time RT-PCR data analysis

In every real-time RT-PCR assay, samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template) and a negative reverse transcriptase control (cDNA template for which water was added instead of Superscript II). Primer and probe concentrations were optimised to yield an efficiency of 90-110% and $R^2 \geq 0.990$. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. For the developmental profiles, the standard curves were generated using a serial dilution of NF46 cDNA mix since all of the target genes were expressed at that stage. For the fadrozole exposure, equal parts of cDNA from each treatment were used to produce a standard curve. Several reference genes were tested for the developmental profiles (*rpl8*, *actb*, *ef1alpha* and *odc*), but all of the genes changed significantly during development (*rpl8* and *odc* are shown in Fig. 2.1). Therefore, the developmental profile data are normalised to RNA content, as suggested by Sindelka et al. (2006), which consists of dividing the gene expression data obtained by real-time RT-PCR by the amount of RNA used in the cDNA synthesis reaction (2 µg; see section 2.2.4). Developmental data are presented as fold-change relative to NF2. Expression of *rpl8* did not change with fadrozole treatment; therefore, it was used as the endogenous control gene. Exposure data are expressed as fold-change relative to the water control.

2.2.8. Aromatase activity assay

Activity of cyp19 was measured using a modified radiometric method (Du et al., 2001), optimised for amphibian whole embryo and brain tissues, and determined by tritiated water release from the C-1β carbon of 1β-^{3}H-androstenedione (^{3}H-A) during its conversion to estrogen. The specificity of the assay was determined using fadrozole inhibition. In pilot studies, brain samples for *S. tropicalis* were incubated with one of the following concentrations of fadrozole: 0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM. Activity of cyp19 was inhibited with increasing concentrations of fadrozole in the brain (Fig. 2.2). Based on these results, three concentrations of fadrozole were chosen for the *in vivo* study: 0.5, 1.0 and 2.0 μM. Using the optimised assay, cyp19 activity was measured in whole embryos and larvae for the developmental profile and in NF46 larvae for the fadrozole exposure. Pools of 50 (for NF7 to NF34) or 40 (for NF46; all pools ranged from 20 to 40 mg) individuals were homogenised to measure enzyme activity. Samples were homogenised in 200 μL potassium phosphate buffer (100 mM KCl, 10 mM KH_2PO_4, 1 mM EDTA, 10 mM dithiothreitol, pH 7.4) and centrifuged at 1,000 g for 10 min at 4ºC. A 50 μL cofactor solution (100 mM KCl, 10 mM K_2HPO_4, 1mM EDTA, 10 mM dithiothreitol, 5 mM glucose-6-phosphate, 1 mM β-nicotinamide adenine dinucleotide phosphate, 10 U glucose-6-phosphate dehydrogenase, pH 7.4) and 0.3 μM ^{3}H-A (specific activity 23.5 Ci/mmol; Dupont Co., NEN Research Products) were incubated in a shaking water bath at 37ºC for 30 min. After this pre-incubation, 50 μL of the tissue homogenate was added to the cofactor and ^{3}H-A mix and incubated for 80 min in a shaking water bath at 25ºC. The reaction was stopped by adding 200 μL ice-cold charcoal solution (80 mg charcoal/ml in 10 % trichloroacetic acid) to the reaction tubes. The mix was incubated on ice for 10 min and centrifuged at 3400 rpm for 10 min at 4ºC. The supernatant was extracted again with the charcoal solution. The samples were run in

triplicate and each assay included negative substrate controls (sample containing buffer instead of tissue) and tritiated water controls (sample containing tritiated water instead of ^{3}H-A) used to calculate assay recovery. ^{3}H was measured as disintegrations per minute using a liquid scintillation counter (LS 6500 Beckman Coulter). Activity of cyp19 is expressed as fmol $^{3}H_2O$/h·mg protein. Total protein concentration was measured using the Bio-Rad protein assay kit as described by the manufacturer (Bio-Rad).

2.2.9. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corp., Seattle, Washington, USA) and a significance of $p<0.05$. Data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). One-way analysis of variance (one-way ANOVA) was used to analyse the results. If results were significant, the Bonferroni post-hoc multiple comparisons test was used to evaluate significant differences between groups. When data failed to meet assumptions even after being transformed (e.g., $\log_{10}$, square root), the nonparametric Kruskal-Wallis test on ranks was used.

Table 2.1. SYBR-green real-time RT-PCR primers and assay conditions of genes of *Silurana tropicalis*. Complete list of target genes, GenBank accession number, forward (F) and reverse (R) primer sequences (5'-3'), amplicon size (bp) and optimised primer concentration (nM).

Target Gene Accession No.		Primer Sequence (5'- 3')	Annealing Temperature (ºC)	Amplicon Size (bp)	Primer (nM)
cyp19	F	GAATCCCGTGCAGTATAACAGC	62	118	112.5
FJ644565	R	ACAGGTCTCCTCTTGATTCCATAG			112.5
srd5alpha1	F	GTTGAATGGTCTGGCTTTGC	62	96	350
BC076920	R	CTGTTGTGCCCTGGAAGTC			350
srd5alpha2	F	ACCAGAAGGGAAGCACACAA	60	120	650
NM_001017113	R	CCATAAGCAGCAGGATAAGTGA			650
ar	F	TGACAACAACCAACCAGACA	60	96	200
DT407367[a]	R	GCCTTTGCCCACTTTACAAC			200
dio1	F	GTAAGGACACCAACTGAGCAA	58	171	350
DT424550[a]	R	GCTGCAACCGTCACTAACAA			350
dio2	F	GTGTTGCCGACTTTGTGTTG	60	112	300
EF052283	R	CGTTCTTCTTGGTTTCTGTGCT			300
dio3	F	TCGGAACTGAGGATGTGGT	60	199	150
NM_001113667	R	ATGCCCAAGGAGATGAGTG			150
tralpha	F	TAAGTTCTCTGTTCCCCTTTCCG	62	77	300
AB244213	R	TCCTCTGATCTTCTTGCTGCTC			300
trbeta	F	ATCCAACACAGCAAAGGTATTTTC	58	106	300
AB244214	R	GTAATGACTGCCCCACATTGC			300
rpl8	F	CCCTCAACCATCAGGAGAGA	62	88	450
BC059744	R	TCTTTGTACCACGCAGACGA			450
odc	F	TGAATGATGGCGTGTATGGA	62	120	150
NM_001005441	R	GTCCCCAAATGCTGCTTG			150
ef1alpha	F	GGCAATGTGGCTGGTGATAG	62	98	150
NM_203970	R	GCACTAATCTGTCCTGGGTGA			150
actb	F	CTGCTTCTTCTTCATCATTGGA	62	88	200
CR855434	R	TCTGGACATCTAAACCGCTCA			200

[a] Expressed sequence tag (EST) accession number

Table 2.2. Multiplex real-time RT-PCR primers and dual-labelled fluorescent probes of genes of *Silurana tropicalis*. List of target genes, GenBank accession numbers, primer and probe sequences (5'-3'), amplicon size (bp), and optimised primer and probe concentrations (nM) and chemistry (dye, quencher).

Target Gene Accession No.	Element	Sequence (5'- 3')	Amplicon size (bp)	Primer/ Probe (nM)	Chemistry
eralpha	F Primer	CCCAACATTTTACAGGTCAAGTTC		112.5	
NM_203535	R Primer	GGCCCTTATCATTAGCTGATGTC	80	225	Cy5, BQH2
	Probe	TGCGTTCCCTTCCACTCTGCCTTC		200	
erbeta	F Primer	CAAGGAACTGGTTCACATGATAGG		700	
AY310903	R Primer	ATTGATCTCCACATTAGTCCCATC	135	700	FAM, BQH1
	Probe	ACCTCCAGCCAACAGCTCTCCAGC		150	
cyp19	F Primer	GAATCCCGTGCAGTATAACAGC		112.5	
FJ644565	R Primer	ACAGGTCTCCTCTTGATTCCATAG	118	112.5	ROX, BQH2
	Probe	ACTCTCGCACCTGCCACTACGGTT		100	
rpl8	F Primer	CCCTCAACCATCAGGAGAGA		450	
BC059744	R Primer	CTTTGTACCACGCAGACGA	88	450	HEX, BHQ1
	Probe	ACGACGGGCAGCAATAAGACCA		200	

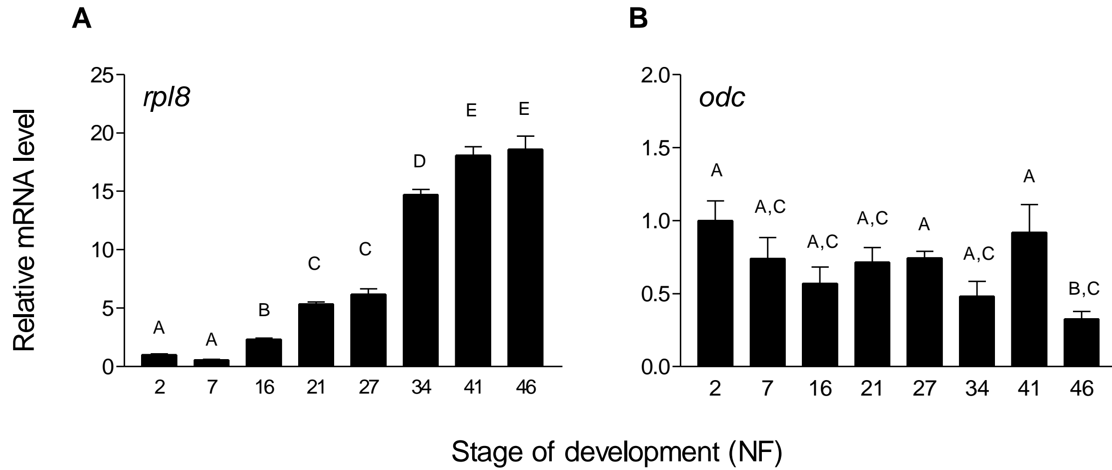


Figure 2.1. Developmental profile of the ribosomal protein L8 (*rpl8*; A) and ornithine decarboxylase (*odc*; B) during *Silurana tropicalis* embryogenesis and larval development. Transcript levels were measured using real-time RT-PCR from NF2 until NF46. Levels of mRNA levels are expressed relative to NF2. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (one-way ANOVA; n=6-8 pools; p<0.05).

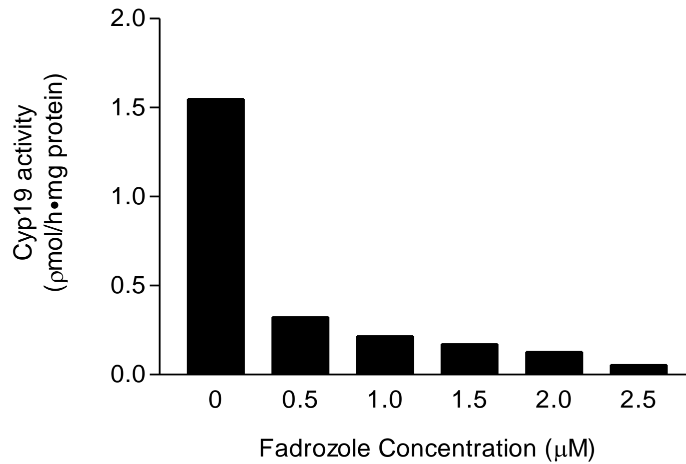


Figure 2.2. Effects of fadrozole on *Silurana tropicalis* brain cyp19 activity *in vitro*. Activity of cyp19 was measured in brain samples of NF66 juveniles incubated with increasing concentrations of fadrozole (0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM). A brain homogenate (pool of 14 brains; n=1 pool) was used to assess cyp19 activity for each fadrozole concentration. Activity is expressed in ρmole/h normalised to total protein content (mg).

2.3. Results

Real-time RT-PCR and a radiometric assay were used to determine the profiles of cyp19 mRNA and activity during *S. tropicalis* embryogenesis (Fig. 2.3). Transcripts and enzyme activity were detected at all stages of development studied, increasing toward NF41 (period of organogenesis) and NF46 (beginning of feeding) respectively. The mRNA levels of all reference genes tested (*rpl8*, *odc*, *actb*, *ef1alpha*) significantly changed during development. Among these, the levels of *odc* changed the least and were relatively stable until a decrease was detected at NF46 when compared to NF2 (ANOVA; $p<0.05$). The results for *odc* and *rpl8* are presented in Fig. 2.1 for comparative purposes. Since all of the potential reference genes changed (also reported during embryogenesis in *Xenopus laevis* by Sindelka et al., 2006), developmental data were normalised to RNA quantity (Huggett et al., 2005).

The effects of fadrozole on cyp19 expression and activity in NF46 larvae are shown in Fig. 2.4A. Fadrozole at 2.0 μM significantly inhibited the activity of cyp19 by 66%; whereas *cyp19* mRNA level increased with fadrozole concentrations but the effect was not statistically significant (1.5-fold; $p=0.056$). Treatment with fadrozole (1.0 and 2.0 μM) increased the expression of *ar* (2-fold; Fig. 2.4C) and *srd5alpha2* (1.5-fold; Fig. 2.4D) relative to control, whereas no changes were observed in the expression of *eralpha*, *erbeta* (Fig. 2.4B) and *srd5alpha1* (Fig.2.4D). Fadrozole increased the expression of *tralpha* and *trbeta* mRNA at 1.0 and 2.0 μM (Fig. 2.5A). Changes in the mRNA expression of *dio* were also detected following fadrozole treatments. In response to fadrozole, *dio1* mRNA significantly decreased in the 2.0 μM treatment group (Fig. 2.5B). In contrast, *dio3* increased following treatment with 1.0 and 2.0 μM fadrozole while no changes in *dio2* were observed (Fig. 2.5B).

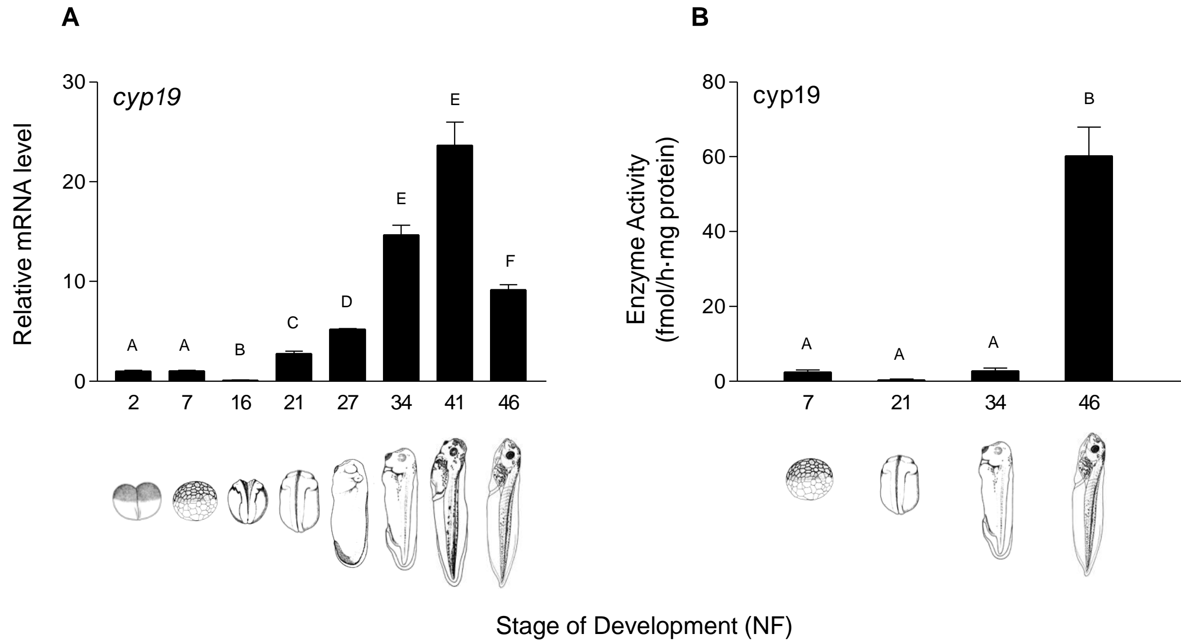


Figure 2.3. Developmental profiles of cyp19 mRNA (A) and activity (B) during *Silurana tropicalis* embryogenesis. Expression and activity were measured in whole embryos and larvae from NF2 (two-cell stage) to NF46 (beginning of feeding). The gene expression profiles are expressed relative to NF2 and normalised to RNA content. Enzyme activity is expressed in fmole/h normalised to total protein content (mg). Bars represent the mean + SEM. Data were analysed using the non-parametric Kruskal-Wallis rank test (n=6-8; $p<0.05$). Different letters indicate statistically significant differences between stages.

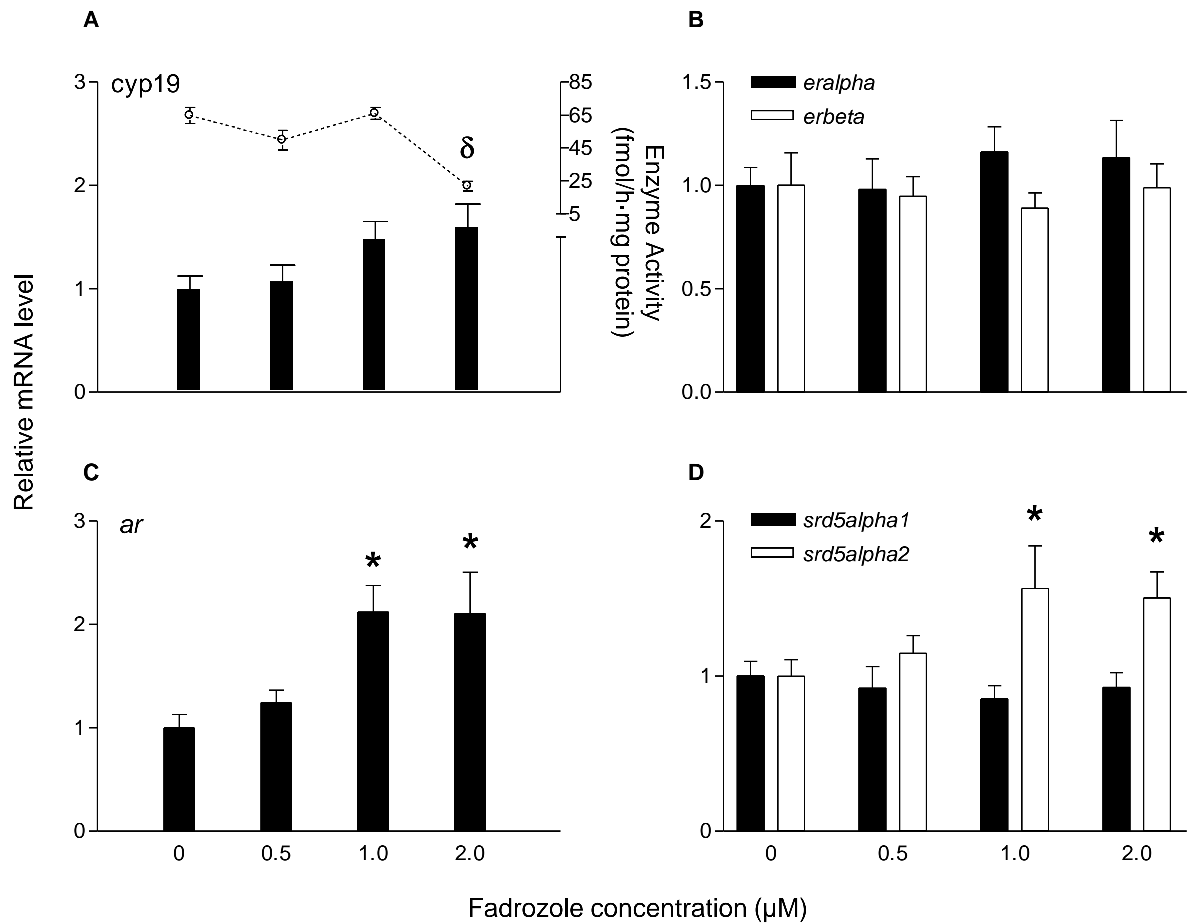


Figure 2.4. Effects of fadrozole on cyp19 mRNA and activity (A), *eralpha* and *erbeta* (B), *ar* (C), and *srd5alpha1* and *srd5alpha2* (D) mRNA levels in *Silurana tropicalis*. Larvae were exposed to fadrozole (0, 0.5, 1.0, 2.0 μM) from NF12 to NF46. Bars represent mean mRNA levels + SEM (y-axis on the left) expressed relative to the control groups and normalised to the expression of *rpl8*. Mean enzyme activity ± SEM (y-axis on the right; A) is expressed in fmole/h normalised to protein content (note the broken y-axis for A). Data were analysed using one-way ANOVA (n=5-8, $p<0.05$). Asterisks indicate significant differences from the control group for the gene expression data. Significant differences in enzyme activity levels are indicated by delta (δ) relative to the control group. Note that the scales of the y-axis vary among panels.

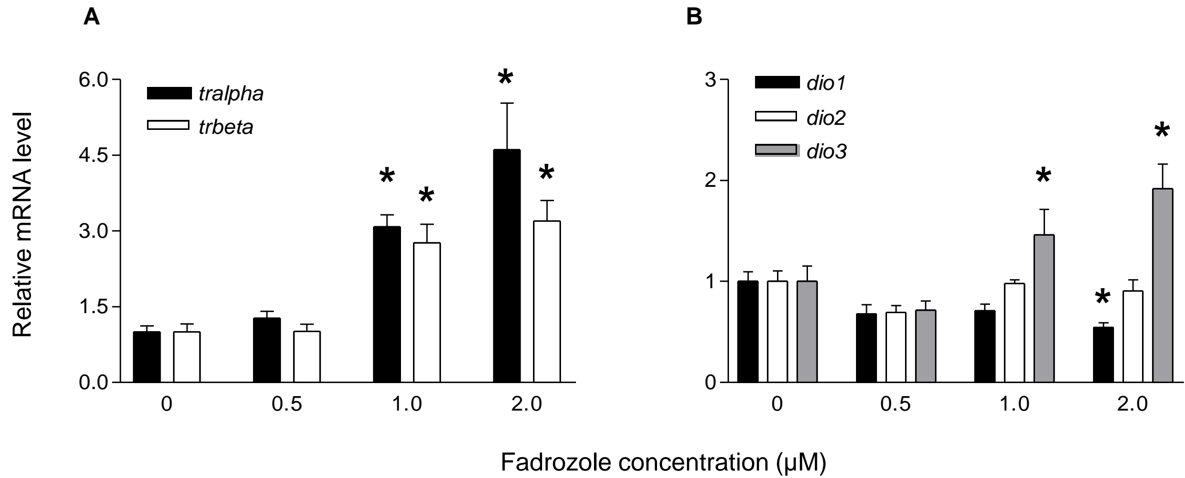


Figure 2.5. Effects of fadrozole on *tralpha* and *trbeta* (A), and *dio1*, *dio2*, and *dio3* (B) mRNA levels in *Silurana tropicalis*. Larvae were exposed to fadrozole (0, 0.5, 1.0, 2.0 µM) from NF12 to NF46. Bars represent mean mRNA levels + SEM expressed relative to the control groups and normalised to the expression of *rpl8*. Data were analysed using one-way ANOVA (n=8, $p<0.05$). Asterisks indicate significant differences from the control group for the gene expression data. Note that the scales of the y-axis vary among panels.

2.4. Discussion

2.4.1. Developmental profiles of cyp19 mRNA and activity

Estrogens regulate gonadal differentiation in vertebrates including amphibians; however, their role during embryogenesis has been largely unexplored. In this study, cyp19 mRNA and activity were measured during *S. tropicalis* embryogenesis. Developmentally-regulated increases in the transcription of *cyp19* were followed closely by an increase in enzyme activity. From these results, the time between mRNA induction and synthesis of cyp19 during *S. tropicalis* embryogenesis can be predicted. Major activity was detected at NF46 which is 24 h after a large increase in mRNA level was observed (NF41). Detection of enzyme transcripts in embryos at NF2 and NF7 indicates maternal transfer of mRNA to the egg. RNA and proteins of maternal origin have been shown to be important in the regulation of the earliest stages of embryonic development (Telford et al., 1990). The presence of cyp19 mRNA and activity in embryos suggests that estrogens are needed before embryonic transcription starts (NF12; gastrulation). In a complementary study from our laboratory, *srd5alpha1* and *srd5alpha2*, enzymes involved in the synthesis of 5α-DHT were also detected at NF2 and NF7 (Langlois, 2010). Sex steroids have been detected during embryogenesis in other vertebrates. For example, estrogens and androgens are important for survival, growth and sexual development in fish, bird and mammalian embryos (Adkins-Regan et al., 1995; Carere and Balthazart, 2007; Goldman-Johnson et al., 2008; von Engelhardt et al., 2006). Maternal transfer of T has also been implicated in offspring begging behaviour in birds (von Engelhardt et al., 2006). Steroid hormones in eggs can come directly from maternal transfer (Adkins-Regan et al., 1995); however, the present study detected cyp19 mRNA and activity in eggs suggesting that estrogens can also be synthesised in the embryo. The enzyme activity and mRNA profiles also indicate that estrogen production

increases throughout development. Endogenous sex steroids (E2, T and 5α-DHT) have been detected in early *X. laevis* (NF20) development (Bogi et al., 2002). In addition, the receptors of these hormones (*eralpha*, *erbeta*, and *ar*) are also present during *S. tropicalis* embryogenesis (Chapter 4) and follow similar profiles to the expression of the enzymes reported here (*cyp19*) and in Langlois (2010; *srd5alpha1* and *srdalpha2*). While transcripts were detected at NF2 and 7, mRNA levels of *cyp19* only began to increase at NF16 and this increase continued during organogenesis. A similar developmental study also detected *cyp19* in whole embryos in a related species *X. laevis* (Urbatzka et al., 2007), demonstrating that *cyp19* mRNA is present in early embryogenesis of at least two frog species.

The similar increases in *cyp19* (this study) and *srd5alpha1* and *srd5alpha2* (Langlois, 2010) after NF34 coincide with neurogenesis (Schlosser et al., 2002). In amphibians, little is known about the role of sex steroids in neurogenesis. However, exposure to exogenous estrogens can induce developmental abnormalities in *X. laevis* embryos including microcephaly (Sone et al., 2004). Estrogens are neuroprotective and neurotrophic (Behl, 2002). In fish, they have been implicated in neurogenesis (Mouriec et al., 2008) and in mammals, sex steroids play an important role in brain sexual differentiation during perinatal development (reviewed in Zuloaga et al., 2008). The presence of *cyp19* after NF34 suggests that it could be involved in amphibian neurogenesis along with *srd5alpha*. Therefore, the change in transcription and activity of cyp19 during embryogenesis could also be related to other steroid-sensitive biological processes occurring during organogenesis.

2.4.2. Effects of fadrozole exposure on sex steroid- and TH-related gene expression

Fadrozole, given to larvae between NF12 and 46 (the developmental period when transcription of *cyp19* increases) inhibited cyp19 activity which led to measurable effects on

ar and *srd5alpha2* mRNA levels. Changes in *ar* mRNA could be in response to the accumulation of androgens such as T because they are not being converted to estrogens in the presence of fadrozole and/or to a potential increase in 5α-DHT due to the increase in *srd5alpha2* after fadrozole treatment. In cyp19-deficient fish and mammals, increases in circulating T and 5α-DHT have been observed (Ankley et al., 2002; McPherson et al., 2001). Since *ar* is autoregulated by androgens (Cardone et al., 1998), increases in T and 5α-DHT could induce the transcription of *ar*. Depletion of cyp19 has been reported to lead to morphological and physiological defects. Chronic exposure to fadrozole for the entire developmental and metamorphic period (from stage NF12 to 66) in *S. tropicalis* affects gonadal differentiation skewing the sex ratio towards males (Olmstead et al., 2009; Chapter 3). Similar effects have been observed in fish (Navarro-Martin et al., 2009), reptiles (Wennstrom and Crews, 1995), and birds (Elbrecht and Smith, 1992). In the case of mammals, the functions of CYP19 have been studied using *Cyp19* knockout mice (ArKO; Fisher et al., 1998). CYP19 deficient-mice are phenotypically normal (Fisher et al., 1998), as were the frog larvae exposed to fadrozole in our study. However, ArKO female mice are infertile and have underdeveloped uteri and ovaries (Fisher et al., 1998; Toda et al., 2001). Moreover, male ArKO mice show impairment in sexual behaviour (Toda et al., 2001) and a disruption in spermatogenesis (Robertson et al., 1999). Together, studies in mammalian and non-mammalian model species provide evidence that a lack of cyp19 starting from embryogenesis can lead to reproductive defects later in life.

Exposure to fadrozole altered the expression of TH-related genes. There is strong evidence of the importance of tr and dio in early anuran embryogenesis (Havis et al., 2006; Morvan-Dubois et al., 2006; Tindall et al., 2007; Chapter 4). *X. laevis* embryos treated with NH-3 (a tr agonist/antagonist) present malformations and alterations in development (Havis

et al., 2006). In addition, inhibition of TH synthesis can also lead to developmental defects. *Silurana tropicalis* embryos exposed to methimazole (a thyroid peroxidase inhibitor) showed notochord malformations and an alteration in iodine uptake (Tindall et al., 2007). Iopanoic acid treatment (a dio1, dio2, and dio3 activity inhibitor) of *X. laevis* embryos resulted in developmental delays, oedema, mobility alteration, and deficiencies in eye development (Havis et al., 2006). Our results suggest that estrogens can regulate TH signalling during early frog development. *In vitro* studies using mammalian cell lines have demonstrated that tr and er can compete for the same estrogen responsive element in the promoter of certain genes and block the transcription of genes such vitellogenin, preproenkephalin, and oxytocin receptor (reviewed in Vasudevan et al., 2002). The increase in *tr* mRNA after fadrozole exposure could be the result of a reduced competition between tr and er in the promoter region of the *tr* gene. Nevertheless, the physiological consequences of these modulations on further tadpole development remain to be investigated.

Taken together, these results indicate interaction between the developing reproductive and TH axes during frog embryogenesis. This study also reports that cyp19 mRNA and activity is present during early amphibian development. In addition, pharmacological inhibition of cyp19 affected transcription of androgen- and TH-related genes which extends the role of sex steroids beyond sexual development. Although I demonstrated cyp19 is active during *S. tropicalis* early development, the precise role of estrogens remains to be elucidated. It will be important to establish the tissue location and time of expression of the steroid and TH-related genes and proteins that are implicated in early amphibian embryogenesis.

CHAPTER 3

Effects of the aromatase inhibitor fadrozole on gonadal differentiation, metamorphosis and gene expression in the frog *Silurana tropicalis*

Adapted from two publications:

Duarte-Guterman P[1], Langlois VS[1], Hodgkinson K[2], Pauli BD[3], Cooke GM[4], Wade MG[5], Trudeau VL[6]. 2009. The aromatase inhibitor fadrozole and the 5-reductase inhibitor finasteride affect gonadal differentiation and gene expression in the frog *Silurana tropicalis*. *Sexual Development*, 3: 333-341.

Langlois VS[1], Duarte-Guterman P[1], Trudeau VL[6]. 2011. Expression profiles of reproduction- and thyroid hormone-related transcripts in the brains of chemically-induced intersex frogs. *Sexual Development*, 5: 25-32.

[1]Contributed equally to this project, i.e., designed and performed research (animal exposures, real-time RT-PCR assays, enzyme activity assays, data analysis, and histological analysis), analysed the data and wrote the manuscripts for publication. Note that only the fadrozole results are presented in this chapter.
[2] Contributed to animal exposures, histology and gene expression assays
[3]Contributed gene expression materials, expertise in amphibian toxicology and to the revision of the manuscript
[4] Contributed enzyme activity expertise and materials and to the revision of the manuscript
[5] Contributed histology materials, expertise in toxicology and to the revision of the manuscript
[6] Contributed to the design of the research and the revision of the manuscript

3.1. Introduction

Estrogens are involved in gonadal differentiation in vertebrates including frogs (Hayes, 1998). Exposure to estrogenic compounds produces skewed sex ratios towards females and/or the presence of intersex individuals in frogs (Gyllenhammar et al., 2009; Hogan et al., 2008; Mackenzie et al., 2003; Pettersson et al., 2006). Intersex animals are characterised by the presence of both male and female gonadal tissue within the same individual and their presence in the laboratory and in the wild is linked to exposure to endocrine disrupting chemicals (EDCs) such as pharmaceuticals and pesticides (Hayes et al., 2003; Hayes et al., 2006; Mackenzie et al., 2003; McCoy et al., 2008). Interestingly, the molecular mechanisms and/or physiological characteristics behind the intersex condition are unknown.

Unlike gonadal development, the effects of estrogens on tadpole metamorphosis, the larval to juvenile transition, are not clear. There are reports that estrogens inhibit metamorphosis (Gray and Janssens, 1990; Hogan et al., 2008); however, their effects are not consistent (Chapter 1) and their mechanisms of thyroid hormone (TH) axis disruption are not understood. In *Silurana tropicalis*, gonadal differentiation takes place during tadpole development (El Jamil et al., 2008); therefore, during this vulnerable period of development, both the reproductive and TH axes are active and have the potential to interact.

In this study, I evaluated the effects of an anti-estrogenic exposure on both gonadal differentiation and metamorphosis in *S. tropicalis*. Tadpoles were exposed to fadrozole, a specific aromatase (cyp19) inhibitor in frogs (Chapter 2) during the entire metamorphic period. Histological analyses of the gonads were conducted to determine the effects of fadrozole on gonadal development and sex ratios. Somatic growth endpoints were used to determine the effects of fadrozole on tadpole metamorphosis. The liver and brain are major sites of synthesis and action of sex steroids and THs, expressing multiple genes regulated by and related to sex steroids and THs, such as sex steroid receptors, TH receptors, and hormone metabolising enzymes. In addition, studies have found molecular interactions between the reproductive and TH axes in the frog liver (Rabelo and Tata, 1993) and brain (Hogan et al., 2007; Chapter 5). Based on the histological sex of the gonads, sex steroid- and TH-related transcripts were measured in the brain and liver of males, females, and fadrozole-induced intersex individuals to investigate at the molecular level, the effects of fadrozole, the characteristics of the intersex phenotype and the interactions between the reproductive and TH-axes.

3.2. Material and Methods

3.2.1. Breeding and maintenance of Silurana tropicalis

Silurana tropicalis fertilised eggs were obtained from three pairs of frogs as described in Chapter 2. Staging was determined by following the Nieuwkoop-Faber developmental table (NF; Nieuwkoop and Faber, 1994). A 12:12h light:dark cycle was maintained where lights came on at 7 am. The water temperature was 25±1ºC. The care and treatment of animals used in this study were approved by the Animal Care Committee, University of Ottawa and adhered to the guidelines published by the Canadian Council on Animal Care for the use of animals in science.

3.2.2. Fadrozole exposure

Eggs were dejellied at NF6 with 2% L-cysteine (pH 8.0; Sigma) and placed in petri dishes as described in Chapter 2. Larvae were exposed from NF12 to NF60 to fadrozole (2.0 µM; Novartis Pharma AG) delivered in water. In the petri dishes, the medium and fadrozole were refreshed daily. At NF46, tadpoles were transferred to 5 L tanks containing activated carbon filtered and aerated water (pH=7.0-8.0, dissolved oxygen=80-85%, temperature= 25±1ºC, conductivity= 950-1050 µS) and fed Sera Micron twice a day. In the tanks, water and fadrozole were refreshed twice a week. At NF60, tadpoles were anaesthetised by immersion in 1% MS-222 (Sigma), and euthanised by decapitation. Body wet weight, snout-vent length, tail length, and number of days to metamorphosis were recorded. Brain and liver were dissected, frozen on dry ice and kept at -80ºC. The remainder of the body was fixed and decalcified in Cal-Ex II (Fisher Scientific) for 48 h and transferred into 70% ethanol for storage until histological processing.

3.2.3. Histology

The tadpole carcasses fixed with the gonad-mesonephros complex intact were trimmed, dissected and prepared for histological processing. Gonads were embedded in paraffin and serial 5 μm saggital sections were stained with hematoxylin and eosin (Appendix C). The stained sections were examined under a light microscope to assess the phenotypic sex as male, female, or intersex (defined as the presence of at least one oocyte in the testes). The investigator was blind to the treatment group of the animal being assessed. Pictures of the sections were taken by a Micro Publisher 3.3 Digital microscope camera (Qimaging Corp.).

3.2.4. RNA isolation and cDNA synthesis

Liver and brain samples were homogenised using an MM301 Mixer Mill (Retsch) at 20 Hz for 3 min. Total RNA was obtained from liver using the RNeasy Mini Plus Kit and from brain using RNeasy Micro Kit (Qiagen). Isolated RNA was resuspended in RNase free water and concentrations were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 2 μg (liver) and 1 μg (brain) of total RNA and 0.2 μg random hexamer primers using Superscript II reverse transcriptase (Invitrogen).

3.2.5. Real-time RT-PCR assays

All real-time RT-PCR assays were performed in an Mx3000P real-time PCR system (Stratagene) and details regarding specific gene primers, annealing temperatures, real-time RT-PCR conditions, and optimisation steps were described in Chapter 2. The expression of steroid 5α-reductases (*srd5alpha1*, *srd5alpha2*), androgen receptor (*ar*), TH-receptors

(*tralpha*, *trbeta*) and deiodinases (*dio1*, *dio2*, *dio3*) was analysed in simplex real-time RT-PCR assays using SYBR Green I Dye and primer sets and conditions described in Table 2.1. A multiplex assay was used to measure estrogen receptors (*eralpha*, *erbeta*), *cyp19* and ribosomal protein L8 (*rpl8*) gene expression using dual-labelled fluorescent probes using primer and probe combinations described in Table 2.2. In the brain, two additional targets were measured, ornithine decarboxylase (*odc*; see primers and conditions in Table 2.1) and arginine vasotocin (*avt*). Real-time RT-PCR primers for *avt* (forward 5'-3': tggacgacgagagcgaaa; reverse 5'-3': cataagccgcaggagaaagtc; product size: 92 bp; primer concentration: 300 nM each; annealing temperature: 58ºC) were designed based on the complete sequence published in GenBank (accession no. XM_002936358) and primers were optimised following the protocol described in Chapter 2.

Real-time RT-PCR standard curves contained equal parts of each treatment cDNA. Samples (n=8 per sex and treatment group) were run in duplicate along with no-template controls (RNase-free water was added to the reaction instead of the cDNA template) and no reverse transcriptase controls (RNase-free water was added to the cDNA synthesis reaction instead of the enzyme). All real-time RT-PCR assays achieved an efficiency of 100±10% and $R^2 \geq 0.990$. The expression of all the reference genes tested in the liver (*rpl8*, *actb*, *gapdh*, *and ef1alpha*) changed with fadrozole treatment (data not shown); therefore, liver data were normalised to RNA content as recommended by Huggett et al. (2005). Brain gene expression data are normalised to RNA content and *rpl8* which was not affected by fadrozole. Data are presented as fold changes relative to the control males.

3.2.6. Aromatase activity

Brain cyp19 activity was measured at NF60 using a radiometric method optimised for frog tissue (described in Chapter 2) in pools of two to four brains of animals of the same sex (n=2-5 pools) and is expressed as fmol ^3H_2O/h·mg protein.

3.2.7. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation) and statistical significance for all tests was set at $p<0.05$. Somatic growth and gene expression data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test) and analysed using one-way analysis of variance (ANOVA). The Bonferroni post-hoc multiple comparisons test was used to evaluate significant differences between groups. When data failed to meet assumptions even after transformation (e.g., $\log_{10}$, square root) the nonparametric Kruskal-Wallis test on ranks was used. Differences in the proportions of male, female, and intersex individuals were analysed using Fisher's exact test.

3.3. Results

3.3.1. Somatic growth and metamorphosis

The results of the somatic growth and developmental endpoints after chronic exposure to fadrozole during *S. tropicalis* development are presented in Table 3.1. The number of days to metamorphosis and snout-vent length were not affected by fadrozole treatment. At NF60, fadrozole-treated males had a significantly greater body weight (one-way ANOVA; $F_{4,157}=2.66$; $p=0.03$) and tail length (one-way ANOVA; $F_{4,157}=3.91$; p=0.005)

compared to control males. No differences in mortality were observed between treated and control after chronic exposure to fadrozole (data not shown).

3.3.2. Sex ratio and histological analysis

Treatment with fadrozole induced intersex gonads in *S. tropicalis* while no intersex animals were found in the control group (Table 3.1 and Fig. 3.1). Fadrozole significantly altered the sex ratio which resulted in 55% male, 30% female and 15% intersex (Fisher's exact test; $p<0.001$). In contrast, the control group exhibited 53% male, 47% female (Fig. 3.1D). Other than the presence of intersex gonads, male and female gonads in the fadrozole group did not show any obvious morphological differences when compared to control.

3.3.3. The effects of fadrozole on gene expression and cyp19 activity

Chronic exposure to fadrozole resulted in the strong inhibition (by approximately 93%) of cyp19 activity in the brain of fadrozole treated males, females, and intersex compared to controls ($p<0.01$; Fig. 3.2). The effects of fadrozole on sex steroid-related gene expression in livers at NF60 are shown in Fig. 3.3. The levels of expression of all targeted genes were similar between control males and females and fadrozole-treated males and females. In marked contrast, in the livers of fadrozole-induced intersex animals there was a 4.2-fold increase in *eralpha* mRNA ($p<0.05$; Fig. 3.3A), a 1.8-fold increase in *srd5alpha1* mRNA ($p<0.05$; Fig. 3.3D) and a 3.5-fold increase in *srd5alpha2* mRNA ($p<0.05$; Fig. 3.3E). In the case of hepatic *srd5alpha1* and *srd5alpha2*, fadrozole-induced intersex had similar gene expression responses to females of both fadrozole-treated and control groups but had different responses compared to males of fadrozole-treated and control groups. No changes were observed in hepatic expression of *erbeta* and *ar* (Fig. 3.3B-C) or in any of the

TH-related genes measured (*tralpha, trbeta, dio2* and *dio3*; Fig. 3.4). In the brain, exposure to fadrozole did not result in any changes in gene expression. Results for sex steroid- and TH-related genes are shown in Figs 3.5 and 3.6, respectively and results for *odc* and *avt* are shown in Fig. 3.7.

Table 3.1. Somatic growth characteristics of *Silurana tropicalis* exposed chronically to fadrozole (2 μM) or to water (control). Number of individuals (n) and mean (± SEM) of wet weight, snout-vent length, and tail length, as measured at stage NF60, and median number of days to metamorphosis (DTM) are presented. Bold indicates significant difference between treated and control males. M: male; F: female; I: intersex; n/a: not applicable.

Somatic growth endpoint	**Sex**	**Treatment**	
		Control	**Fadrozole**
Number of individuals	M	42	46
	F	37	25
	I	0	12
Wet weight (mg)	M	815 (25)	**924 (23)**
	F	893 (33)	906 (28)
	I	n/a	916 (41)
Snout-vent length (mm)	M	18.1 (0.2)	18.7 (0.2)
	F	18.5 (0.2)	18.3 (0.3)
	I	n/a	18.2 (0.4)
Tail length (mm)	M	36.4 (0.3)	**38.4 (0.3)**
	F	37.3 (0.5)	37.4 (0.4)
	I	n/a	37.0 (0.8)
DTM (d)	M	109	103
	F	103	99
	I	n/a	103

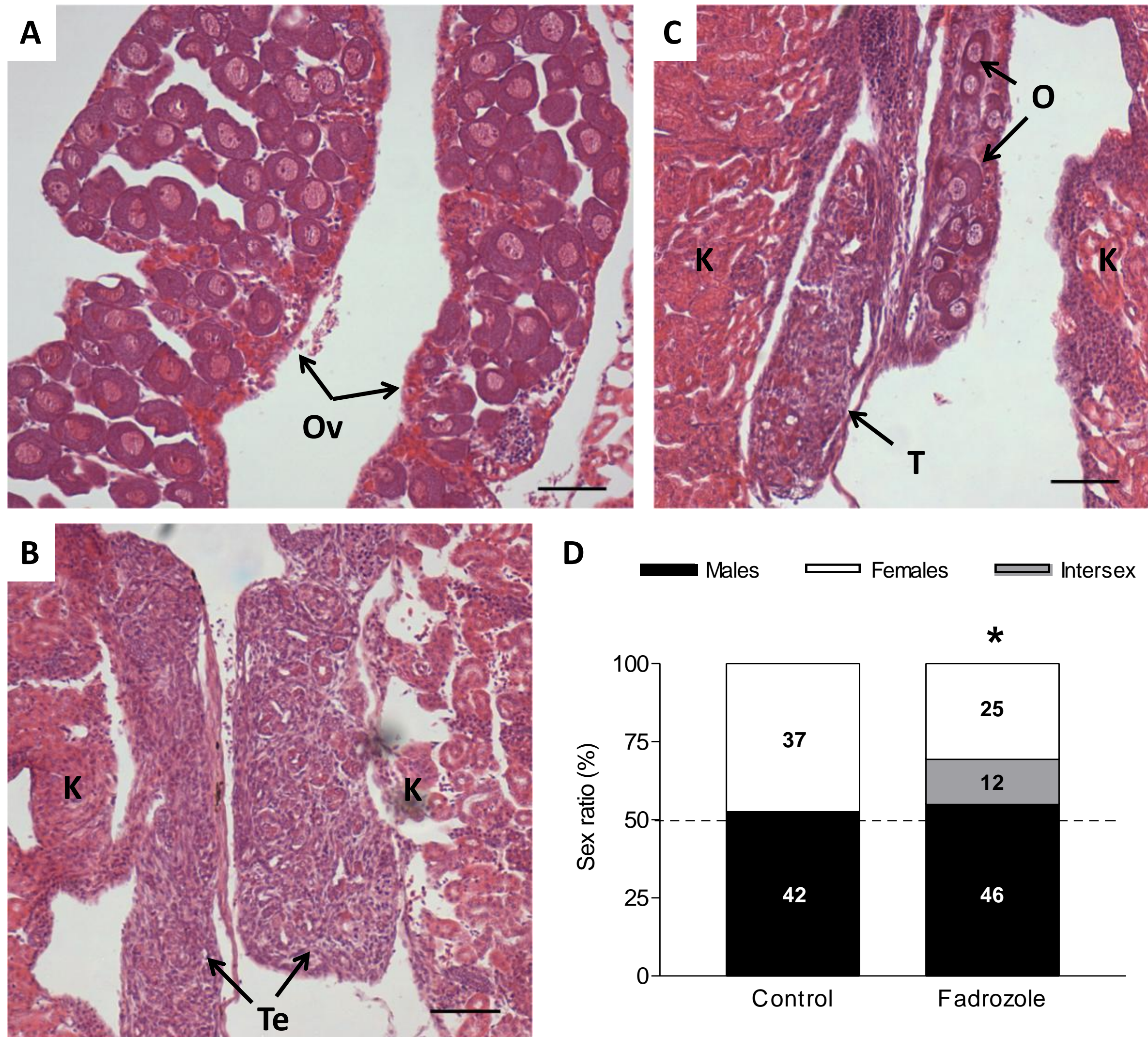


Figure 3.1. Effects of fadrozole on gonadal histology and sex ratios in *Silurana tropicalis.* Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 µM; delivered in water). Histological sections of NF60 gonads for control female (A), control male (B), fadrozole-induced intersex (C), and sex ratios (D) are presented. Sample size and percent sex ratio are reported. Asterisk indicates significant difference between control and fadrozole treatments (Fisher's exact test; $p<0.001$). Ov: ovary; Te: testes; O: ovarian follicle; T: testicular tissue; K: kidney. Bars represent 100 µm.

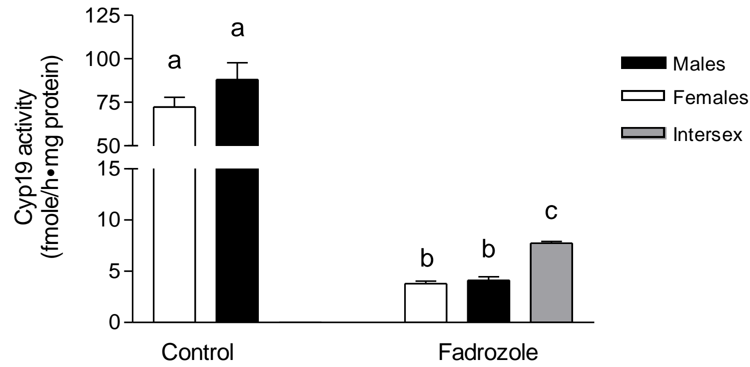


Figure 3.2. Effects of fadrozole on cyp19 activity in the brain of NF60 *Silurana tropicalis*. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 μM; delivered in water). Enzyme activity is expressed in fmole/h normalised to total protein content (mg). Bars represent the mean + SEM. Data were analysed using one-way ANOVA (2-4 brains per sample; n=2-5; $p<0.01$). Different letters indicate statistically significant differences between sex treatments.

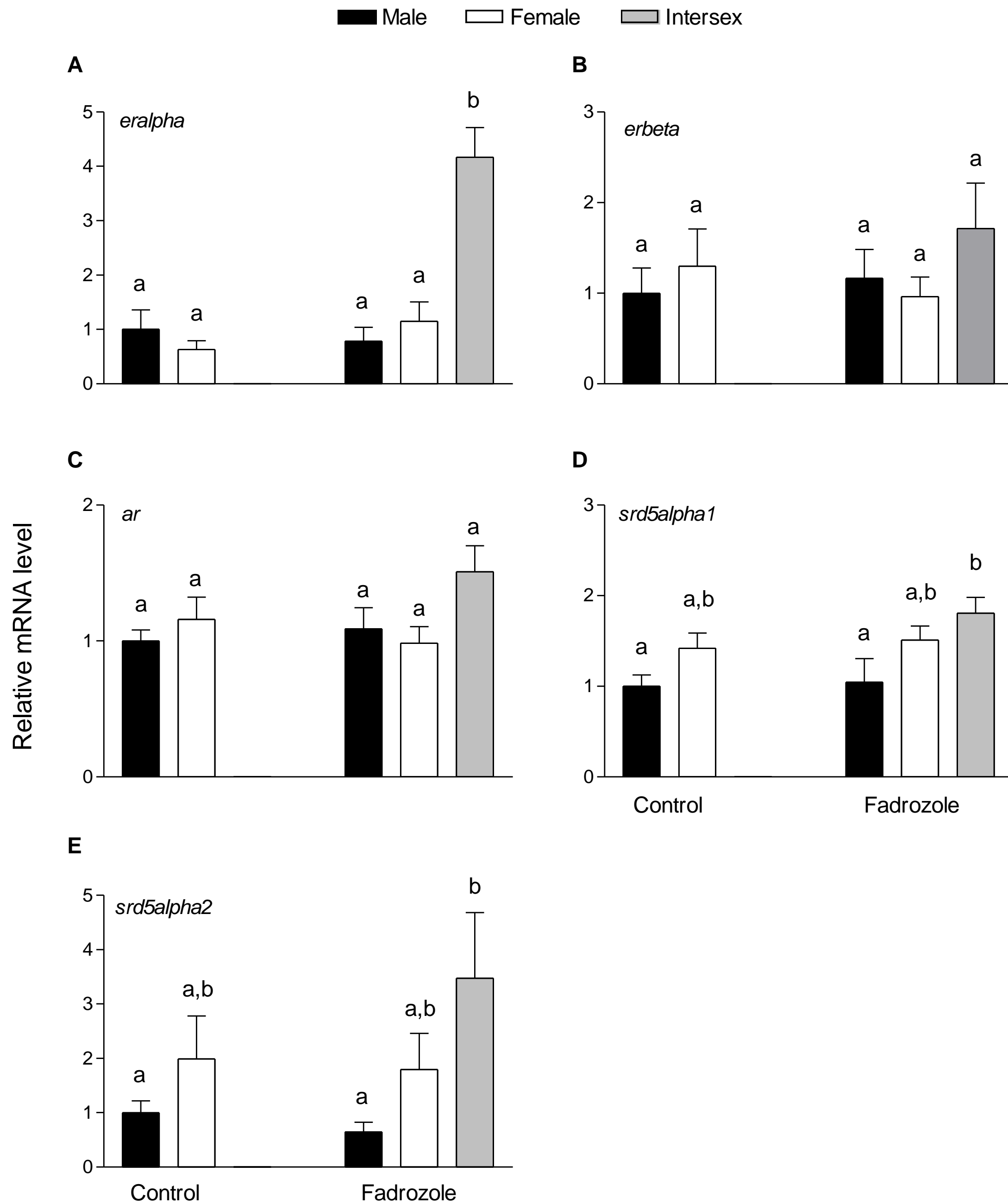


Figure 3.3. Effects of fadrozole on *eralpha* (A), *erbeta* (B), *ar* (C), *srd5alpha1* (D), and *srd5alpha2* (E) mRNA levels in NF60 *Silurana tropicalis* livers. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 µM; delivered in water). Levels of mRNA are expressed relative to the male control group and are normalised to RNA content. Bars represent the mean mRNA level + SEM. Data were analysed using one-way ANOVA (n=8, $p<0.05$). Different letters indicate statistically significant differences between sex treatments. The scales of the y-axis vary between graphs.

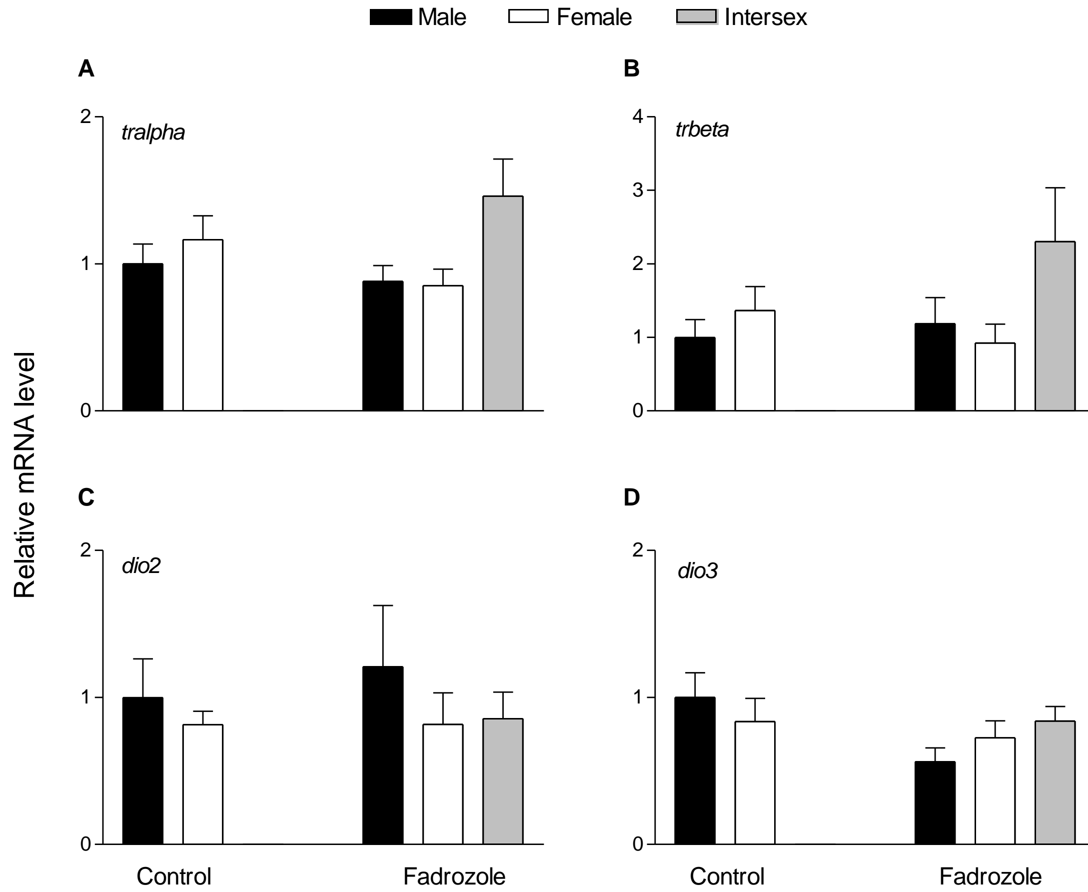


Figure 3.4. Effects of fadrozole on *tralpha* (A), *trbeta* (B) *dio2* (C), and *dio3* (D) mRNA levels in NF60 *Silurana tropicalis* livers. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 μM; delivered in water). Levels of mRNA are expressed relative to the male control group and are normalised to RNA content. Bars represent the mean mRNA level + SEM. No statistical differences between sexes and treatments were observed (one-way ANOVA; n=8; p set at 0.05). The scales of the y-axis vary between graphs.

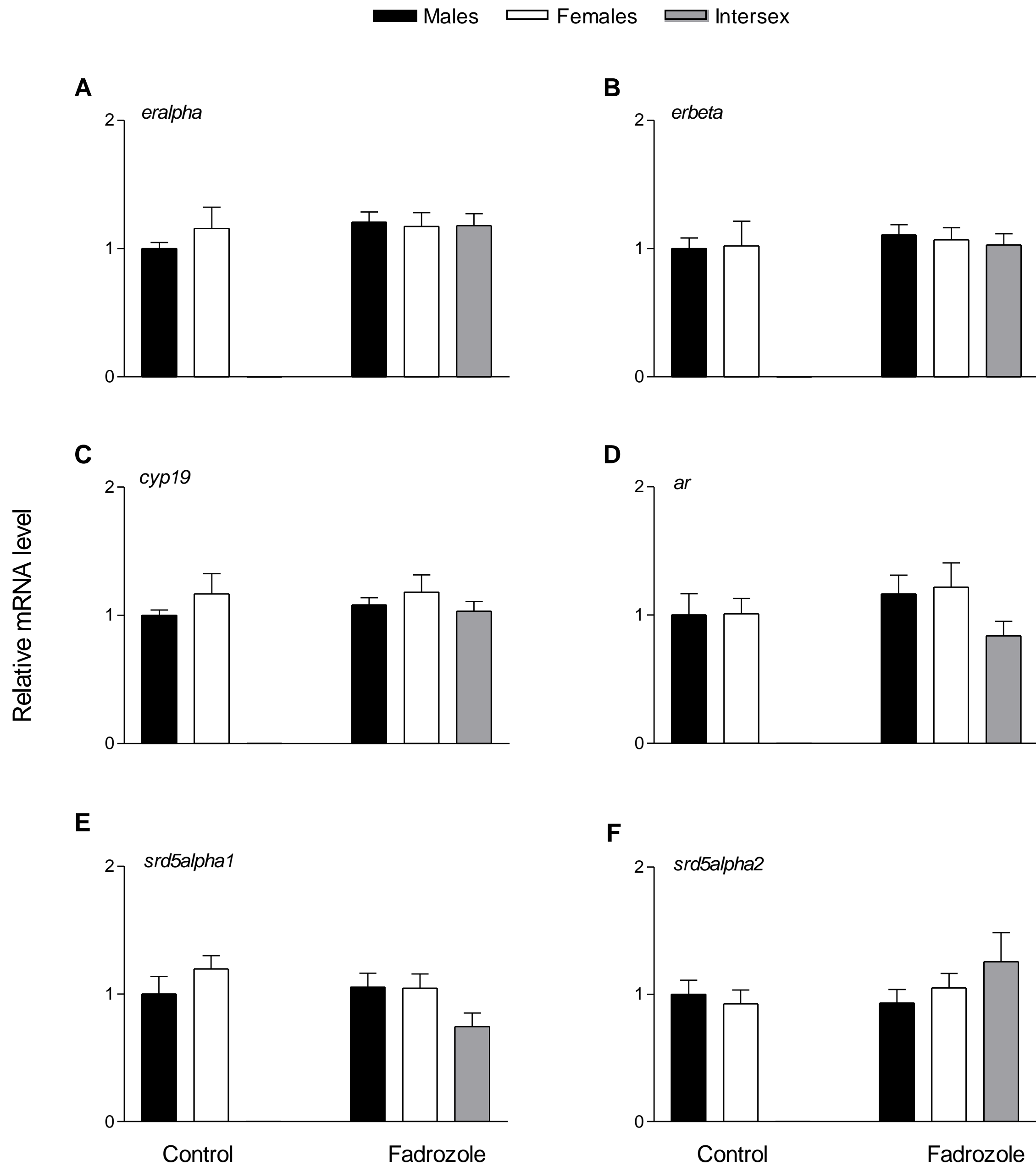


Figure 3.5. Effects of fadrozole on *eralpha* (A), *erbeta* (B), *cyp19* (C), *ar* (D), *srd5alpha1* (E), and *srd5alpha2* (F) mRNA levels in NF60 *Silurana tropicalis* brains. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 μM; delivered in water). Levels of mRNA are expressed relative to the male control group and are normalised to the ribosomal protein L8 (*rpl8*) mRNA levels. Bars represent the mean mRNA level + SEM. No statistical differences between sexes and treatments were observed (one-way ANOVA; n=8; *p* set at 0.05).

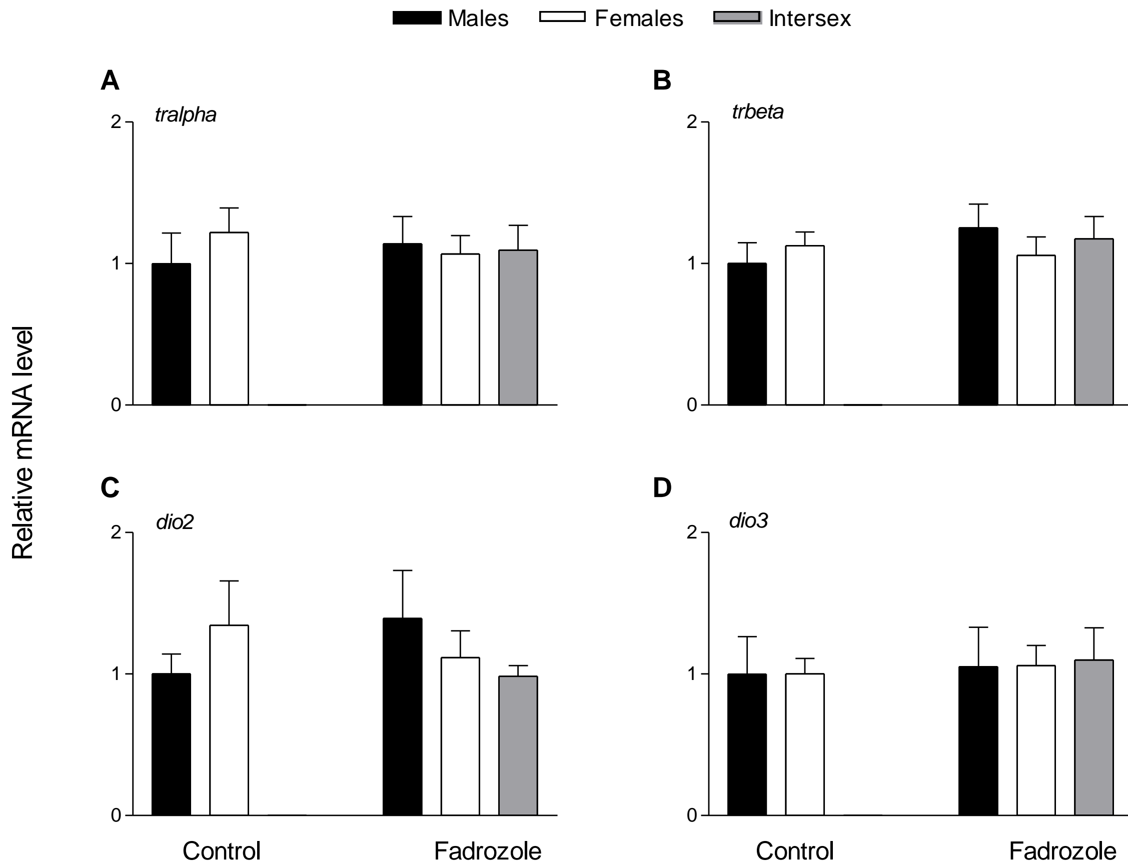


Figure 3.6. Effects of fadrozole on *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) mRNA levels in NF60 *Silurana tropicalis* brains. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 µM; delivered in water). Levels of mRNA are expressed relative to the male control group and are normalised to the ribosomal protein L8 (*rpl8*) mRNA levels. Bars represent the mean mRNA level + SEM. No statistical differences between sexes and treatments were observed (one-way ANOVA; n=8; p set at 0.05).

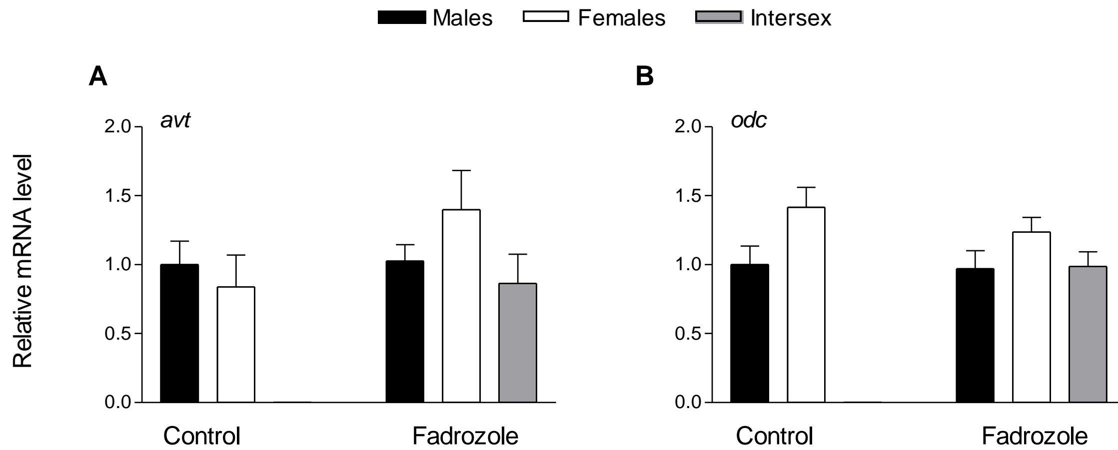


Figure 3.7. Effects of fadrozole on *avt* (A) and *odc* (B) mRNA levels in NF60 *Silurana tropicalis* brains. Tadpoles were chronically exposed from NF12 to NF60 to either water (control) or fadrozole (2 μM; delivered in water). Levels of mRNA are expressed relative to the male control group and are normalised to the ribosomal protein L8 (*rpl8*) mRNA levels. Bars represent the mean mRNA level + SEM. No statistical differences between sexes and treatments were observed (one-way ANOVA; n=8; p set at 0.05).

3.4. Discussion

3.4.1. Effects of fadrozole on gonadal differentiation and sex ratios

In frogs, gonadal differentiation and sex ratios can be affected by exposure to sex steroids. In general, treatment with estrogens leads to feminisation, while androgens induce masculinisation. In the present study, histological analyses demonstrated that blocking estrogen synthesis with fadrozole reduced the number of females compared to control and produced intersex tadpoles in *S. tropicalis*. These results are in agreement with the general effects of sex steroids in frogs. When exposed to fadrozole until NF66, Olmstead et al. (2009) demonstrated that *S. tropicalis* sex ratio resulted in 100% males and in the current study, I demonstrated that when exposed until NF60, 55% males, 30% females and 15% intersex were produced. Taken together these results suggest that intersex is a transition phase between female-to-male sex reversal in fadrozole-exposed *S. tropicalis.* According to El Jamil et al. (2008), gonads are still developing between NF60 to NF66; therefore this transition could still be taking place during this developmental period and along with differences in the concentrations of fadrozole and experimental design explain the differences between the two studies.

3.4.2. Effects of fadrozole on liver gene expression

In order to investigate the effects of fadrozole at the molecular level, sex steroid-related transcripts were measured in the liver and brain in males, females and intersex individuals. Real-time RT-PCR analysis revealed that fadrozole exposure altered hepatic sex steroid-related gene expression. Fadrozole induced the expression of *srd5alpha1* and *srd5alpha2*, enzymes responsible for the conversion of testosterone (T) into 5α-dihydrotestosterone (5α-DHT). In the liver of *S. tropicalis*, *cyp19* mRNA was not detected;

however, cyp19 activity measured in the brain of the same animals showed an almost complete inhibition (~93%) and the same concentration of fadrozole reduces cyp19 activity by 66% in whole body larvae of *S. tropicalis* (Chapter 2). The inhibition of cyp19 activity results in an increase in androgen-related gene expression (*srd5alpha1* and *srd5alpha2* mRNA in the liver; *srd5alpha2* and *ar* mRNA in whole body larvae, Chapter 2). Hence, I propose that fadrozole affects *S. tropicalis* sex ratio not only by reducing estrogen levels but also by favouring a male-like steroidogenesis pathway. Support for this hypothesis is found in the elevated circulating androgen levels and hydration of the fathead minnow testes (an androgen dependant response after fadrozole treatment; Ankley et al., 2002). Similarly, adult male transgenic mice lacking a functional CYP19 exhibit elevated circulating levels of T and 5α-DHT along with hypertrophied androgen-responsive tissues (McPherson et al., 2001).

3.4.3. Effects of fadrozole on brain cyp19 activity and gene expression

In contrast to the liver, fadrozole did not affect the expression of any of the genes analysed in the brain. This is also in marked contrast with previous studies in fish that have found that fadrozole affects brain gene expression (Villeneuve et al., 2009; Zhang et al., 2009). Interestingly, cyp19 activity was almost completely inhibited in the brain of fadrozole treated animals compared to controls; while none of the estrogen-responsive gene transcripts were affected (*cyp19*, *eralpha*, *erbeta* and *avt*). Research from our laboratory has shown that exposure to a synthetic estrogen (ethinylestradiol, EE2) increases the expression of estrogen-responsive genes (i.e., *cyp19* and *eralpha*) in the frog brain (Hogan, 2006). However, the current study suggests that a lack of estrogen (due to fadrozole) does not result in the opposite effect of an estrogen exposure in the tadpole brain (i.e., decrease in *cyp19* and *eralpha* mRNA levels). Future studies should investigate other endpoints (e.g., brain

morphology, sex steroid levels) in more specific areas of the brain to understand the consequences of a lack of estrogen during amphibian development. Furthermore, a 2-fold difference in cyp19 activity levels in the brain was observed between the fadrozole induced-intersex and the treated males and females. This difference in response between intersex and differentiated males and females (both fadrozole treated and control) was also observed in hepatic gene expression of the same animals. Together, these results suggest that intersex animals have a different physiology than normal and treated animals.

In vertebrate species, avt is an important neuropeptide regulating social behaviours such as vocalisation and parental and sexual behaviours (reviewed in Goodson and Bass, 2001). In amphibians, avt regulates reproductive behaviours (e.g., amplectic clasping of females and release calls; Moore and Miller, 1983). In this study, *avt* mRNA expression in the brain was used as an endpoint to assess whether this neuropeptide could be affected in intersex and sex-reversed individuals after exposure to fadrozole. Expression of avt was not affected after exposure to fadrozole. In adult amphibians, concentrations of avt are higher in certain brain regions such as the amygdala in males relative to females (Boyd and Moore, 1992; Boyd et al., 1992). In this study, *avt* did not show dimorphic expression in the brains of the control groups, which suggests that sex-specific regulation by avt may not be fully in place at the end of metamorphosis. Future research should investigate specific brain regions and the long term consequences of sex steroid synthesis inhibition on the avt system and other related endpoints in the tadpole brain.

3.4.4. Cross-talk between the estrogen and thyroid hormone axes

Following fadrozole treatment, the effects of estrogens on tadpole metamorphosis and development were assessed using somatic growth endpoints and expression of TH-related

genes in the liver and brain. Chronic exposure to fadrozole increased body weight and tail length in fadrozole-treated males relative to control males but did not affect the number of days to metamorphose. Chronic exposure to EE2 delays metamorphosis in *L. pipiens* (Hogan et al., 2008). In *Xenopus laevis*, estradiol inhibits triiodothyronine (T3) effects on metamorphosis, based on head and body weight measurements (Gray and Janssens, 1990), 1990). In adults, estrogen inhibits thyroid activity, depressing plasma TH levels and 5'-deiodination (dio1 and dio2) in the frog *Pelophylax ridibundus* (Vandorpe and Kuhn, 1989). Based on these previous observations, I would have expected that inhibition of estrogen synthesis would result in the acceleration of metamorphosis. However, this was not the case and the somatic growth measurements suggest the opposite effect could be occurring at least in one of the sexes; fadrozole exposure increased body weight and tail length in males which suggest a delay in male development. Differences on the effects of estrogen on metamorphosis may be due to species-differences and/or the high variability in developmental rates which make it difficult to detect small effects (e.g., Cheek et al., 1999; Helbing et al., 2006b). At the molecular level, I also did not observe any effects of fadrozole on the expression of TH-related genes in the liver or the brain. These results are in marked contrast with the findings in Chapter 2, where fadrozole increased whole body expression of *tr* and *dio*. Taken together, the present study suggests that chronic inhibition of estrogen synthesis does not affect the brain or liver TH system during metamorphosis in *S. tropicalis*; however, a shorter exposure to fadrozole in combination with T3 (i.e., a T3 challenge exposure previously used in Helbing et al., 2006b; Veldhoen et al., 2006) can reduce developmental variability and may provide more information about the role and potential mechanisms of estrogens during *S. tropicalis* metamorphosis.

3.4.5. Comparison of fadrozole and finasteride gene expression results

In a parallel experiment to the one presented here, tadpoles were exposed to finasteride, an inhibitor of srd5alpha1, srd5alpha2 and steroid 5β-reductase in vertebrates (Langlois, 2010). Although morphologically similar, the intersex animals produced by fadrozole and finasteride did not show similar hepatic or brain gene expression profiles (Table 3.2). For example, in the liver, finasteride-treated animals showed reductions in the mRNA levels of *srd5alpha1* and *srd5alpha2*; while fadrozole-induced intersex had an increase *srd5alpha1* and *srd5alpha2* expression. In the brain, finasteride affected the expression of sex steroid- and TH-related genes, whereas fadrozole did not affect transcription of any of the genes measured in the brain. The differences observed between fadrozole- and finasteride-induced intersex transcript profiles suggest that intersex conditions in frogs induced by exposure to EDCs will have different molecular signatures depending on the mode of action of the pollutant. These gene expression results are consistent with a study where *X. laevis* exposed to three other EDCs (i.e., estradiol, atrazine and cyproterone acetate) displayed different gonadal malformations (Hayes et al., 2006).

3.4.6. Conclusions

This study demonstrated that chronic exposure to fadrozole in *S. tropicalis* affects gonadal sexual differentiation; however, fadrozole did not appear to affect the TH axis, measured by developmental endpoints and gene expression. Hepatic sex steroid-related gene expression analyses revealed that chemically-induced intersex animals displayed different gene expression profiles than non-exposed animals. Even though intersex is a common morphological condition associated with the change from one sex to the other, the comparison of this study with the finasteride exposure indicates that differing

pathophysiologies can underlie the common morphology of animals with ovotestes. This study suggests that intersex individuals are physiologically different from normal males and normal females. Further characterisation of the intersex phenotype at the molecular level and determining whether the gene expression changes are the cause or the consequence of the intersex condition are required when studying the effects of EDCs that disrupt sexual development.

Table 3.2. Comparison between (A) hepatic and (B) brain gene expression changes following a chronic exposure to fadrozole (2 µM) or finasteride (25 µM) during *Silurana tropicalis* development. Letters indicate statistically significant fold changes along with arrows indicating the direction of change. Dashes indicate no changes; *nd*: not detectable.

A. Liver

Genes	Fadrozole[a]			Finasteride[b]		
	Male	Female	Intersex	Male	Female	Intersex
ar	-	-	-	-	-	-
srd5alpha1	-	-	1.8 ↑[d]	-	-	1.9 ↓[e]
srd5alpha2	-	-	3.5 ↑[d]	32 ↓[c]	26 ↓[c]	36 ↓[c]
eralpha	-	-	4.2 ↑[c]	4.1↑[c]	6.1↑[c]	-
erbeta	-	-	-	-	-	-
cyp19	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
tralpha	-	-	-	-	-	-
trbeta	-	-	-	3.1 ↑[c]	-	-
dio2	-	-	-	4.8 ↑[c]	-	6.1↑[c]
dio3	-	-	-	3.9 ↓[c]	-	16 ↓[c]

B. Brain

Genes	Fadrozole[a]			Finasteride[b]		
	Male	Female	Intersex	Male	Female	Intersex
ar	-	-	-	1.3 ↑[d]	-	1.2 ↑[d]
srd5alpha1	-	-	-	-	-	-
srd5alpha2	-	-	-	-	-	-
eralpha	-	-	-	-	-	1.7 ↑[c]
erbeta	-	-	-	-	-	1.6 ↑[d]
cyp19	-	-	-	-	-	1.7 ↑[c]
tralpha	-	-	-	-	-	-
trbeta	-	-	-	1.8 ↑[c]	-	2.2 ↑[c]
dio2	-	-	-	-	-	-
dio3	-	-	-	2.3 ↑[c]	-	3.8 ↑[c]
avt	-	-	-	-	-	-
odc	-	-	-	-	-	-

[a] Gene expression results reported in this chapter
[b] Results from Langlois, 2010
[c] Different from both control males and females ($p<0.05$)
[d] Different from control males ($p<0.05$)
[e] Different from control females ($p<0.05$)

CHAPTER 4

Expression and triiodothyronine regulation of thyroid hormone- and sex steroid-related genes during *Silurana tropicalis* early development

Adapted from:

Duarte-Guterman P[1], Langlois VS[2], Pauli BD[3], Trudeau VL[4]. 2010. Expression and T3 regulation of thyroid hormone- and sex steroid-related genes during *Silurana* (*Xenopus*) *tropicalis* early development. *General and Comparative Endocrinology*, 166: 428-435.

[1] Designed and performed research, analysed the data and wrote the manuscript for publication
[2] Contributed to gene expression assays and the revision of the manuscript
[3] Provided gene expression material and revised the manuscript
[4] Contributed to the design of the research and the revision of the manuscript

4.1. Introduction

A significant amount of research has been devoted to studying the functions and mechanisms of action of thyroid hormones (THs) during amphibian metamorphosis (e.g., Shi, 2000; Tata, 2006). However, the role of THs earlier during embryonic and larval development is less well understood. Since the thyroid gland becomes fully functional after feeding begins, for example, in *Xenopus laevis*, thyroid gland organogenesis begins at NF40, and iodine uptake is only detected at NF46 (beginning of feeding; Brown, 2005), it was generally believed that THs were not important during early embryo development. However, in *Bufo japonicus*, small amounts of THs are synthesised by G26 (equivalent to NF46); however, the gland is not fully formed until G33 (equivalent to NF53; Hanaoka et al., 1973). In addition, THs have been detected in eggs and embryos before the thyroid gland becomes active in *X. laevis* (Morvan-Dubois et al., 2006), *Rhinella marina* (Weber et al., 1994), *Pelophylax perezi*, and *Epidalea calamita* (Gancedo et al., 1997). These results led to the hypothesis that extrathyroidal THs are required for normal embryonic and early larval development (Morvan-Dubois et al., 2006; Tindall et al., 2007; Weber et al., 1994); however,

little is known about the regulation of TH receptors (*tr*) and deiodinases (*dio*) by THs during this period of development.

Sex steroid synthesis enzymes (aromatase, cyp19; steroid 5α-reductases, srd5alpha1 and srd5alpha2) are present in *Silurana tropicalis* embryos (Chapter 2; Langlois, 2010). In addition, exposure to a cyp19 inhibitor (fadrozole) affects the expression of *tr* and *dio* during early development (Chapter 2). Since TH- and sex steroid-related genes are present early in anuran embryos and larvae, the developmental period prior to metamorphosis represents an excellent opportunity to address the question of whether THs can regulate estrogen- and/or androgen-related genes early during development.

In this study, I first established complete profiles for *tr* (alpha and beta), *dio* (1, 2 and 3), estrogen receptors (*eralpha* and *erbeta*) and androgen receptor (*ar*) mRNA during early *S. tropicalis* development. Based on TH-related transcript profiles, I then investigated the regulation of the expression of these receptors and enzymes, as well as *cyp19* and *srd5alpha* by T3 early during development.

4.2. Materials and methods

4.2.1. Animals

Silurana tropicalis fertilised eggs were obtained from five pairs of frogs following the protocol described in Chapter 2. Staging was determined by following the Nieuwkoop-Faber developmental table (NF; Nieuwkoop and Faber, 1994). The care and treatment of animals used in this study were approved by the Animal Care Committee, University of Ottawa and adhered to the guidelines published by the Canadian Council on Animal Care for the use of animals in science.

4.2.2. Tissue collection for developmental profiles

Samples of whole embryos and larvae were collected at different stages of development as described in Chapter 2: NF 2, 7, 16, 21, 27, 34, 41, and 46 (see Fig. 4.5 for the main morphological characteristics and the corresponding hours post-fertilisation (hpf) for each stage of development under our husbandry conditions).

4.2.3. Thyroid hormone exposure

Embryos at NF6 were de-jellied (Chapter 2), placed in petri dishes at a density of 50 eggs per dish and exposed to three nominal concentrations of T3 (3,3′,5-triiodo-L-thyronine; 0.5, 5, 50 nM; Sigma) starting at NF 26-27 for 48 h. The final dimethyl sulfoxide (DMSO; Sigma) concentration in control and T3 petri dishes was 0.005%. The chemicals were renewed after 24 h of exposure when the medium was also refreshed. After 48 h whole tadpoles at NF46 were sampled from each treatment, placed in MS-222, then frozen in dry ice and stored at -80°C.

4.2.4. RNA isolation and cDNA synthesis

Total RNA for the developmental profiles and the T3 exposure samples was obtained from whole embryos and larvae using the RNeasy Micro Kit including the DNAse treatment (Qiagen). Sample homogenisation and disruption was achieved using a MM301 Mixer Mill (Retsch) at 20 Hz for 4 min. RNA was resuspended in RNase free water and stored at -80ºC. Concentrations of RNA were determined using NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 2 µg total RNA and 200 ng random hexamer primers using Superscript II reverse transcriptase (Invitrogen). The cDNA products were diluted 80-fold prior to PCR amplification.

4.2.5. Real-time RT-PCR

Levels of *eralpha*, *erbeta*, *cyp19*, *ar*, *tralpha*, *trbeta*, *dio1*, *dio2*, *dio3*, *srd5alpha1*, and *srd5alpha2* mRNA were measured in whole embryos and larvae during development and after T3 exposure using simplex real-time RT-PCR assays (using SYBR Green; Chapter 2). For the T3 exposure samples, *eralpha*, *erbeta*, *cyp19*, and ribosomal protein L8 (*rpl8*) mRNA were measured in a multiplex real-time RT-PCR assay (using dual-labelled fluorescent probes), as previously described in Chapter 2. For all the real-time RT-PCR assays, samples were run in duplicate using the Mx3000P Multiplex Quantitative PCR System (Stratagene) along with negative template controls where RNase-free water was added to the reaction instead of the template and a negative reverse transcriptase control where RNase-free water was added to the cDNA synthesis reaction instead of the enzyme.

4.2.6. Data analyses

The relative standard curve method was used to interpolate relative mRNA abundance of target genes within each sample. The standard curves were generated using a cDNA mix of NF46 embryos (for the developmental profiles), and using equal parts of cDNA from each treatment including controls (for the T3 exposure). Reaction efficiencies were determined by the MxPro software (Stratagene) using the slope of the standard curves and for all the genes efficiencies were 90-110% with $R^2 \geq 0.990$. Data for each target gene were averaged and normalised to RNA content. Data for the early developmental profiles are presented as fold change relative to NF2 (except for *tralpha*, which was first detected at NF16) and data for the T3 exposure are relative to the control group.

4.2.7. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation). Data for all the genes were first tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). When the assumptions were not met, the data were transformed as required (*e.g.* $\log_{10}$, square root). Data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons. When data failed to meet assumptions even after being transformed, the nonparametric Kruskal-Wallis test on ranks was used. Differences were accepted as significant when $p<0.05$.

4.3. Results

4.3.1. Developmental profiles during embryogenesis

The presence of mRNA prior to NF16 is indicative of a maternal origin. For most of the genes assessed, transcripts were detected throughout development and low levels of maternally-derived mRNA were observed at NF2 and NF7. The exception was *tralpha* which was first detected at NF16 (Fig. 4.1). The expression profiles of the receptors and enzymes showed distinct patterns and magnitude of change; however for all of the genes, mRNA levels increased during development (Figs. 4.1 and 4.2). Since all of the potential reference genes changed during early development (see Chapter 2 and Sindelka et al., 2006), developmental data were normalised to RNA quantity (Huggett et al., 2005).

During neurulation (NF16), *dio2* and *dio3* mRNA levels increased significantly (50- and 140-fold, respectively; $p<0.05$; ANOVA) and remained high during organogenesis, while levels of *dio1*, *eralpha*, *erbeta*, and *tralpha* remained low during this period (Figs. 4.1 and 4.2). In the case of *trbeta*, mRNA levels were low until NF21 then significantly increased 9-fold at NF27 ($p<0.05$; ANOVA) and remained high until NF46 (Fig. 4.1B). The period of tissue differentiation (NF34 to 46) showed a dynamic pattern of expression with

important increases for *dio2*, *dio3*, *eralpha*, and *ar* while more moderate increases for *dio1* and *tralpha* (Figs. 4.1 and 4.2). There were distinct differences in the expression patterns for *eralpha* and *erbeta*. For *eralpha*, transcripts increased 150-200 fold from NF34 ($p<0.05$; ANOVA; Fig. 4.2A), while *erbeta* levels remained low throughout embryonic development and only showed an 8-fold significant increase at NF46 ($p<0.05$; ANOVA; Fig. 4.2B).

4.3.2. T3 exposure during embryogenesis

Since TH- and sex steroid-related genes were detected during embryogenesis, I addressed the question whether T3 could regulate their expression. The first significant increase in the expression of *tralpha* and *trbeta* during embryogenesis was observed between NF21 and NF27 (2.5-fold and 11-fold, respectively; Fig. 4.1A-B). Based on these results, embryos were exposed to T3 (0, 0.5, 5.0, 50 nM) from NF 26-27 to NF46. At the end of the 48 h exposure, no changes in tadpole mortality or external morphology were observed. At NF46, exposure to T3 resulted in increases in *tralpha* (at 5.0 and 50 nM; 2.0- to 2.5-fold), *trbeta* (at all three concentrations of T3; 1.5- to 12-fold), *dio2* (at 5.0 and 50 nM; 1.3- to 2.5-fold), and *dio3* (at 5.0 and 50 nM; 1.5- to 4-fold) mRNA relative to control ($p<0.001$; ANOVA; Fig. 4.3). Transcript levels of *dio1* did not change significantly with treatment ($p=0.442$; ANOVA; Fig. 4.3C). The reference gene, *rpl8* was affected by T3 ($p<0.001$; ANOVA; Fig. 4.3F); therefore, the data are normalised to RNA content.

T3 also affected the expression of sex steroid-related genes (Fig. 4.4). Increases in *ar* (1.5- to 1.8-fold; $p<0.05$; ANOVA; Fig. 4.4E) and *srd5alpha1* mRNA (1.4- to 2.5-fold; $p<0.05$; ANOVA; Fig. 4.4D) were observed after 5.0 and 50 nM T3. In the case of *eralpha*, a 1.5-fold increase was observed at 50 nM T3 ($p<0.05$; ANOVA; Fig. 4.4A). There were no effects of T3 on the expression of *erbeta*, *cyp19* and *srd5alpha2* (Fig. 4.4).

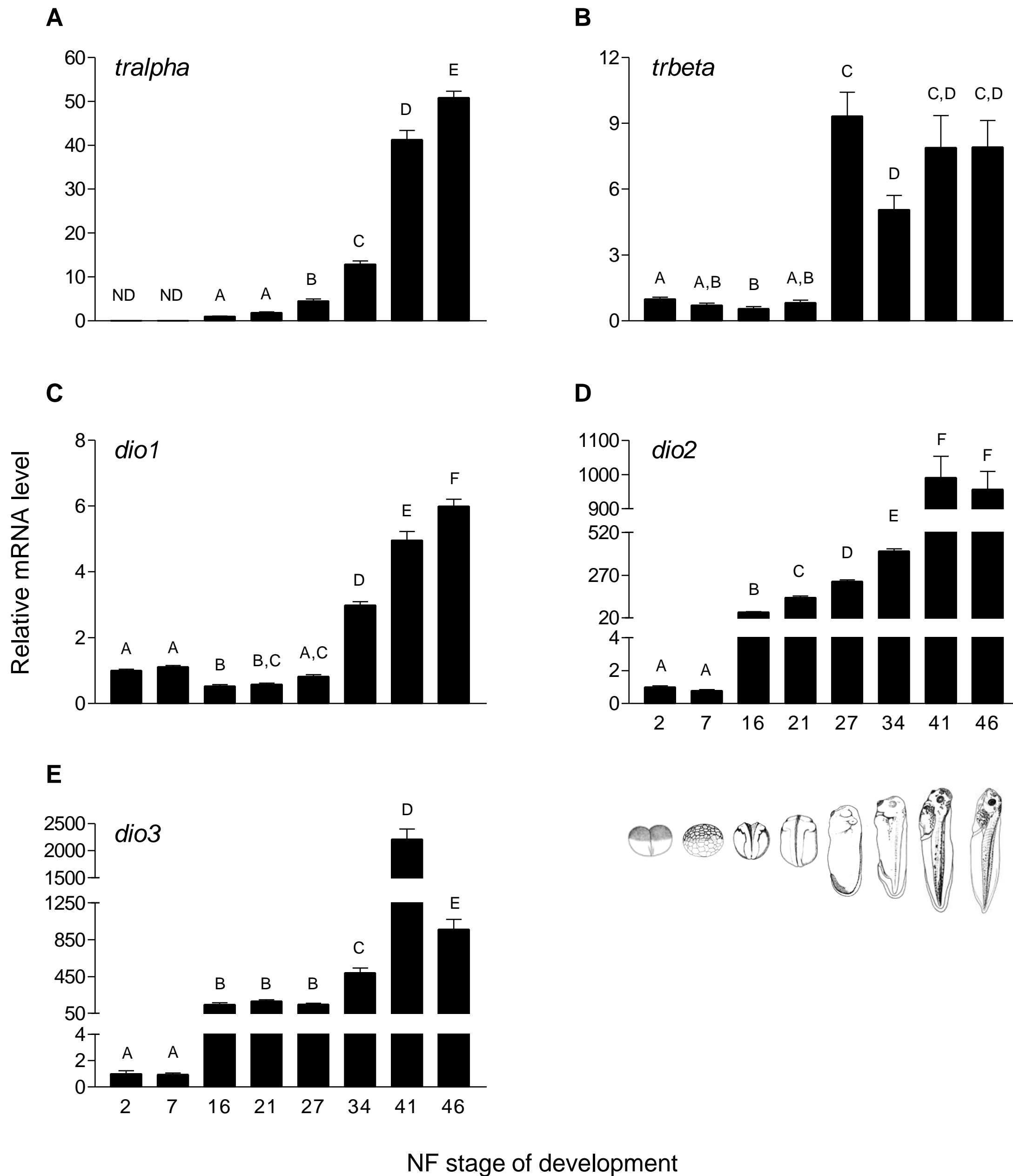


Figure 4.1. Developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* embryogenesis and larval development. Transcript levels of *tralpha* (A), *trbeta* (B), *dio1* (C), *dio2* (D), and *dio3* (E) were measured in whole embryos and larvae from NF2 (two-cell stage) to NF46 (beginning of feeding). Levels of mRNA are expressed relative to NF2 except for *tralpha* (relative to NF16) and are normalised to RNA content. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (one-way ANOVA; n=6-8 pools; $p<0.05$). Note that the scales of the y-axis vary among genes. ND= not detected.

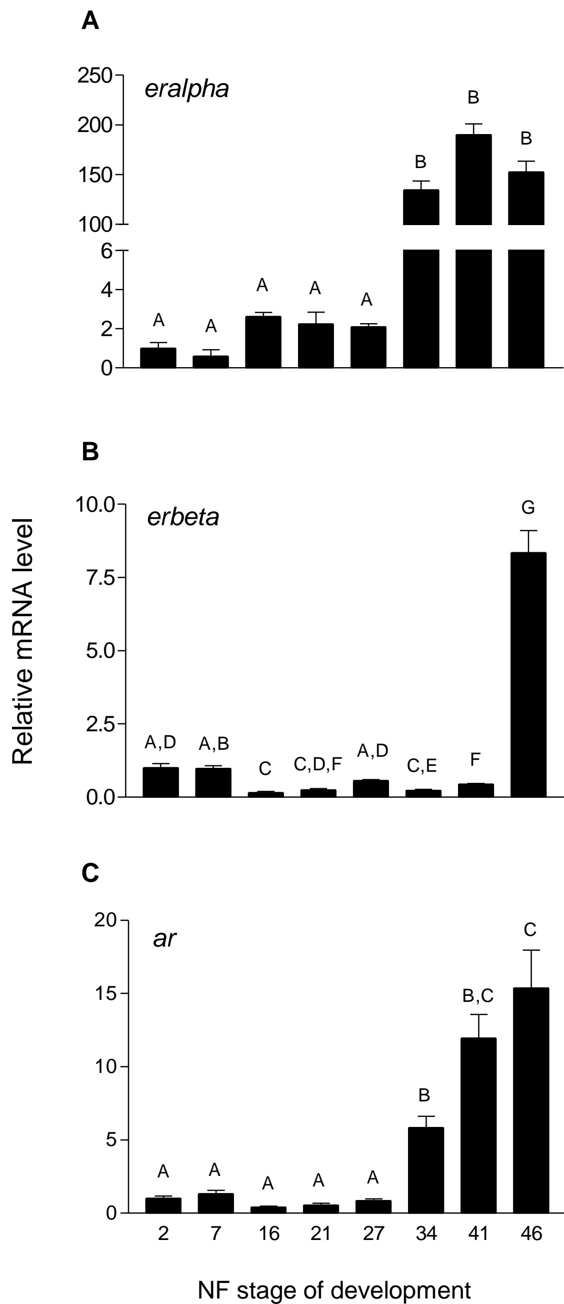


Figure 4.2. Developmental profiles of sex steroid-related genes during *Silurana tropicalis* embryogenesis and larval development. Transcript levels of *eralpha* (A), *erbeta* (B), and *ar* (C) were measured in whole embryos and larvae from NF2 (two-cell stage) to NF46 (beginning of feeding). Levels of mRNA are expressed relative to NF2 and are normalised to RNA content. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (one-way ANOVA; n=6-8 pools; $p<0.05$). Note that the scales of the y-axis vary among genes.

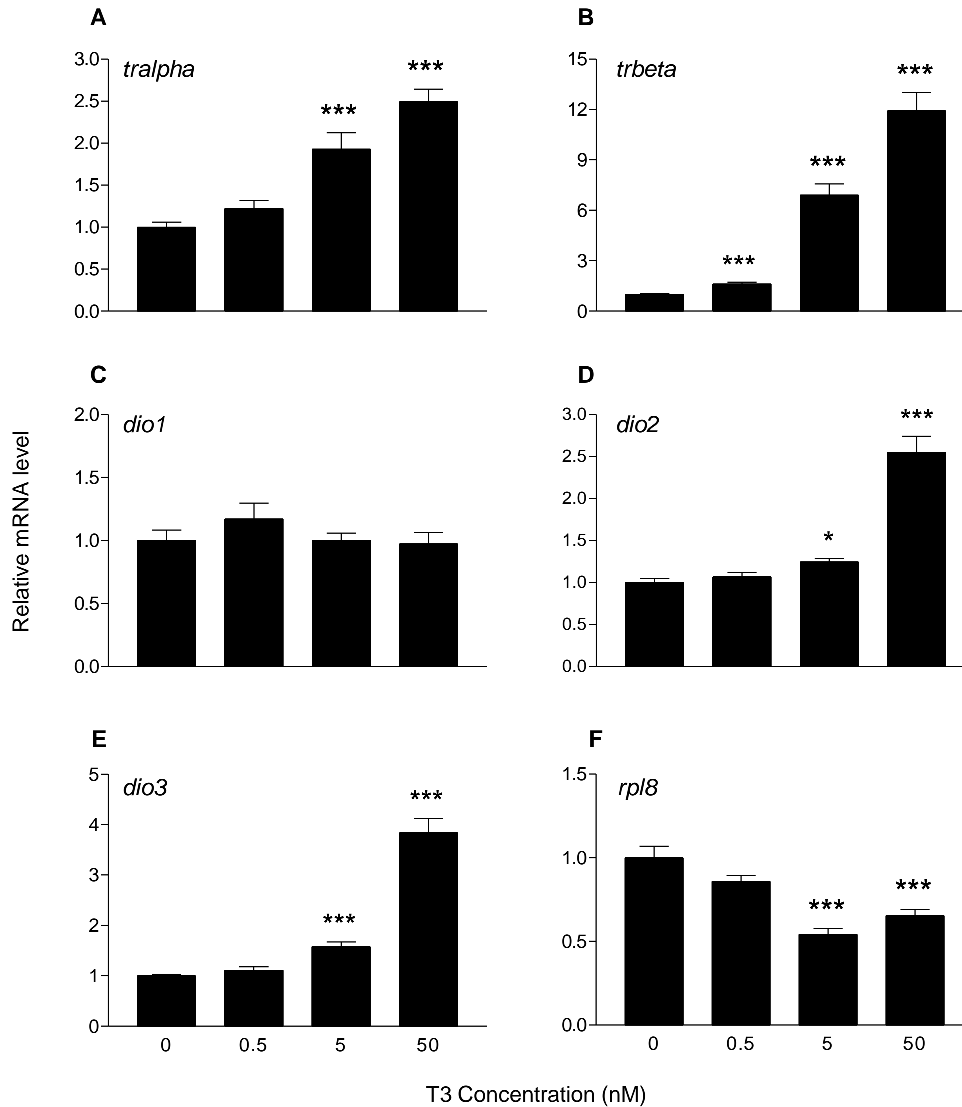


Figure 4.3. Effects of T3 exposure on the expression of thyroid hormone-related genes during *Silurana tropicalis* larval development. Larvae were exposed from NF 26-27 to NF46 to T3 (0, 0.5, 5, 50 nM). Effects of T3 on *tralpha* (A), *trbeta* (B), *dio1* (C), *dio2* (D), and *dio3* (E) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Results for the reference gene *rpl8* (F) are also presented. Bars represent the mean + SEM. Asterisks represent significant differences from the control group (one-way ANOVA; n=8 pools; * $p<0.05$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

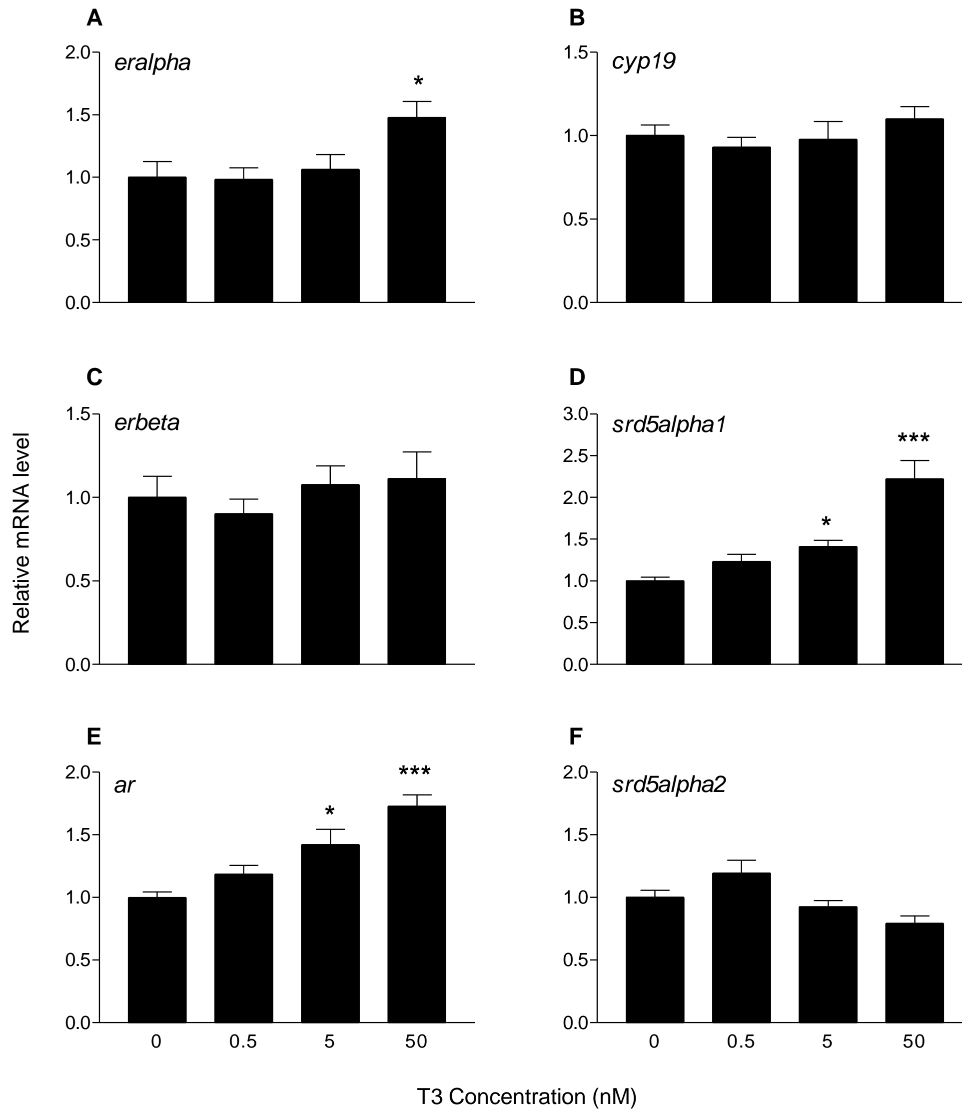


Figure 4.4. Effects of T3 exposure on the expression of sex steroid-related genes during *Silurana tropicalis* larval development. Larvae were exposed from NF 26-27 to NF46 to T3 (0, 0.5, 5, 50 nM). Effects of T3 on *eralpha* (A), *cyp19* (B), *erbeta* (C), *srd5alpha1* (D), *ar* (E), and *srd5alpha2* (F) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Bars represent the mean + SEM. Asterisks represent significant differences from the control group (one-way ANOVA; n=8 pools; * $p<0.05$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

4.4. Discussion

4.4.1. Expression and regulation of thyroid hormone-related genes

In this study, profiles of sex steroid receptors, TH receptors, and deiodinases were established during embryonic and early larval development in *S. tropicalis*. The expression of *tralpha* and *trbeta* during amphibian embryogenesis has been previously studied in *Xenopus laevis* (Banker et al., 1991; Havis et al., 2006). Here, similar profiles to those in *X. laevis* embryos (Havis et al., 2006) are presented, i.e., *tralpha* and *trbeta* mRNA increase significantly between NF21 and NF27, and remain high for *trbeta* but continue to increase for *tralpha*. Together these results suggest that the embryonic patterns of expression of *tr* are very similar between these anuran species. Few studies have investigated the role of tr in embryos; however it has been suggested that unliganded *trbeta* is important for amphibian embryonic head development since overexpression of *trbeta* in *X. laevis* results in abnormal head morphology and in eye deformities (Havis et al., 2006).

Local TH levels in tissues are regulated by dio. During embryogenesis, *dio2* and *dio3* mRNA levels increased significantly as early as NF16. Similar developmental profiles of *dio2* and *dio3* have been established in *S. tropicalis* (Tindall et al., 2007). Only recently *dio1* was found in frogs (Kuiper et al., 2006) and the developmental profile was first established for *X. laevis* (Morvan-Dubois et al., 2006). All three *dio* are mainly expressed in neurogenic areas and the retina in embryos (Morvan-Dubois et al., 2006; Tindall et al., 2007). Both T3 and T4 have been detected in eggs (NF0) and embryos (NF34) and activities of dio1 and dio2 have been measured at NF34 in *X. laevis* (Morvan-Dubois et al., 2006). This suggests that before the thyroid gland is active, THs can be activated in anurans and in the presence of tr, THs have the potential of regulating gene expression (Fig. 4.5), which is the question addressed in the second part of this study. In contrast to mammalian embryos, which can be

exposed to THs via the placenta, amphibian embryos develop externally with no exogenous source of T3; therefore, they represent a good model organism to study the roles and effects of THs during embryogenesis.

The levels of T3 used in this study included at least one physiologically relevant dose (0.5 nM; see Fig. 4.5 for levels found in *X. laevis* embryos). Exposure to T3 resulted in increases in *tralpha* and *trbeta* mRNA, demonstrating that T3 can auto-regulate the expression of both *tr* during early anuran development. A previous study by Cossette and Drysdale (2004) found no effect of T3 on *trbeta* expression in *X. laevis* embryos exposed from NF13 to NF35. These differences could be due to a potentially lower sensitivity of *in situ* hybridisations compared to real-time RT-PCR and/or because the exposure by Cossette and Drysdale (2004) took place earlier during development (beginning at NF13) when levels of *trbeta* are low and the exposure was not long enough to induce a transcriptional response. The present results suggest that *tr* can be autoregulated by T3 as early as NF46, before metamorphic changes begin.

Exposure to T3 resulted in an increase in *dio2* and *dio3* expression while *dio1* was not affected. This differential regulation of *dio* has also been observed in premetamorphic NF52 *X. laevis* tadpoles (Morvan-Dubois et al., 2006). A major function of dio3 is to protect organs and tissues from excessive levels of THs by their conversion to inactive metabolites (Galton, 2005). Therefore, the increase in *dio3* expression after T3 exposure indicates that this protective mechanism is already active in the early larva. Furthermore, in *X. laevis* and *Lithobates catesbeianus*, the promoter of the *dio3* gene contains a TH-responsive element (TRE) which supports the notion that *dio3* is a direct T3 responsive gene (Becker et al., 1995; St Germain et al., 1994). On the other hand, I would have expected a decrease in *dio2* expression in T3-treated larvae as previously observed in zebrafish embryos (Walpita et al.,

2007). However, these results are similar to T3-exposed tadpoles during metamorphosis where *dio2* mRNA is positively regulated by T3 (Brown, 2005; Hogan et al., 2007; Chapter 5-6). The present results show that T3 responsive genes typically related to metamorphosis, also respond in a similar way much earlier in tadpole development.

4.4.2. Developmental profiles of sex-steroid receptors

To the best of my knowledge, it is the first time that separate profiles for *eralpha* and *erbeta* have been established in anuran embryos and larvae. Using *er* primers and a non-specific PCR protocol that measured all subtypes of *er* mRNA in whole body of *X. laevis* beginning at NF20, Bogi et al. (2002) observed a gradual increase in transcript level during development. However, I was able to separately quantify both isoforms using specific primers and found that *eralpha* and *erbeta* have very different patterns of expression, suggesting that they play different roles during embryogenesis. In mammals, it is known that the er isoforms play different roles since *ERalpha* and *ERbeta* knockout (KO) mice exhibit different phenotypes. For example, both male and female alphaERKO mice are infertile while male betaERKO mice are fertile and female betaERKO mice are subfertile (infrequent pregnancies, small litter sizes; reviewed in Hewitt and Korach, 2002). In fish, little is known about the functions of each isoform during development. However, one study using a morpholino knockdown approach showed that *erbeta2* (*erbeta* paralog) is required for the development of the sensory system in zebrafish (Froehlicher et al., 2009). This suggests that *erbeta2* plays an important role in the brain, similarly to what has been found in betaERKO mice (Bodo and Rissman, 2006; Froehlicher et al., 2009).

In the case of *ar*, I also established the first complete developmental profile in embryos and larvae in amphibians. Expression of *ar* was detected throughout development,

only increasing significantly at the time of hatching (NF34). The profile established by Bogi et al. (2002) in *X. laevis* only starts at NF20 but shows an increase similar to that in *S. tropicalis*. Both er and *ar* transcripts were detected before gastrulation which indicates that *er* and *ar* mRNAs are maternally transferred. In addition, estradiol and testosterone have been detected in *X. laevis* embryos (Bogi et al., 2002) and sex steroid synthesis enzymes are present during embryogenesis (Chapter 2; Langlois, 2010) suggesting that the enzyme-hormone-receptor system is present early during development.

4.4.3. Regulation of sex steroid-related genes by T3

Exposure to T3 affected sex-steroid related gene expression in *S. tropicalis*. In *X. laevis*, there is evidence that THs are required for gonadal differentiation. Chronically blocking TH production with ammonium perchlorate during *X. laevis* development results in a female-biased sex ratio (Goleman et al., 2002). A similarly biased sex ratio was also obtained in zebrafish after exposure to sodium perchlorate (Mukhi et al., 2007). From these studies, it has been suggested that THs are required for testicular development. Here, I demonstrated that T3 increased *ar* and *srd5alpha1* mRNA in *S. tropicalis* at NF46 which is prior to the start of gonadal differentiation at NF 48-49 (El Jamil et al., 2008) but during the formation of the genital ridges (Fig. 4.5). In mammals, THs have been shown to play a role in testicular development and function (reviewed in Wagner et al., 2008). In Sertoli cells isolated from hypothyroid rats (3-4 weeks old), a decrease in the formation of 5α-dihydrotestosterone (5α-DHT) was reported (Panno et al., 1994). The srd5alpha enzymes convert testosterone into 5α-DHT; therefore, the results by Panno et al. (1994) suggest a decrease in Srd5alpha activity during hypothyroidism. Furthermore, T3 increases *Ar* mRNA and protein in rat Sertoli cells *in vitro* (Panno et al., 1996). Using cDNA microarrays, Tai

and colleagues identified the androgen receptor associated protein 70 (*ARA70*) as being regulated by T3 in different human cell lines (Tai et al., 2007). ARA70 is a nuclear receptor co-activator that increases *Ar* transcription in cotransfection assays. Future studies should investigate whether T3 can regulate *ar* and other androgen-related genes in the anuran gonad (Chapters 6 and 8).

The present results also demonstrate that T3 altered *S. tropicalis* gene expression in a similar manner to that observed in animals treated with the cyp19 inhibitor fadrozole (Chapter 2). For example, *tr*, *dio3* and *ar* similarly increased following both T3 and fadrozole exposures over similar periods of embryogenesis (see Chapter 9 and Fig. 9.1). Furthermore, by NF46, the genital ridges have formed (Fig. 4.5) and the gonad will begin to differentiate by NF 48-49 (El Jamil et al., 2008); therefore, T3 regulation of androgen-related genes observed at NF46 may have important consequences for gonadal differentiation. It remains a matter of speculation whether THs are directly involved in anuran masculinisation. However, the data on gene regulation in embryos (present study and Chapter 2), and observations of the masculinising effects of fadrozole on developing *S. tropicalis* tadpoles (Chapter 3; Olmstead et al., 2009) and feminising effects of TH synthesis inhibition in *X. laevis* (Goleman et al., 2002), all suggest that this mechanism exists and is further investigated later in the thesis (Chapters 6 and 8).

In conclusion, this study suggests that THs play important roles during early *S. tropicalis* development to regulate both TH- and sex steroid-related genes. However, whether these changes are functionally important during embryogenesis remains to be elucidated. In addition, analysis of the promoters of the TH- and sex steroid-responsive genes and direct effects of TH on tadpole gonads will help further elucidate interactions between these developmentally-regulated hormonal systems.

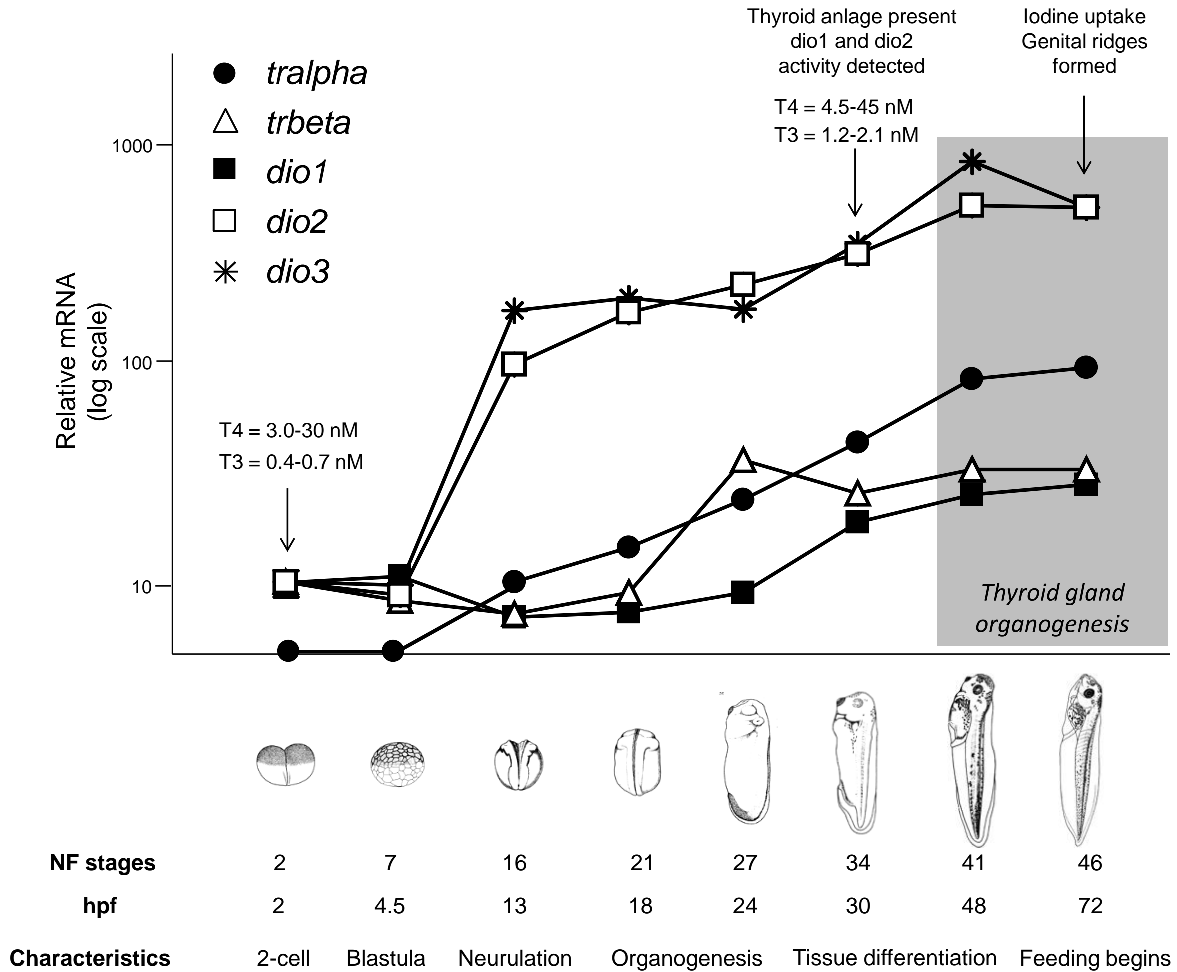


Figure 4.5. Diagram summarising the findings on thyroid hormone-related gene expression, THs levels, dio activity and anatomical and morphological changes during *Silurana tropicalis* and *Xenopus laevis* embryonic and larval development. The x-axis represents time of development in NF stages. Hours post-fertilisation (hpf) under our husbandry conditions are also presented along with the main morphological characteristics of each of the stages. The y-axis represents relative mRNA levels in a log scale. The results of the gene expression of *tr* and *dio* in *S. tropicalis* are those presented in Fig. 4.1. Levels of T3 and T4 and dio activity in *X. laevis* were taken from Morvan Dubois et al. (2006). Iodine uptake has been detected at NF46 in *X. laevis* by Brown (2005). The morphological and anatomical information was taken from Nieuwkoop and Faber (1994). Thyroid gland organogenesis begins around NF40 and the first follicles are visible around NF49 in *X. laevis*.

CHAPTER 5

Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in the brain of *Silurana tropicalis*

Adapted from:

Duarte-Guterman P[1] & Trudeau VL[2]. 2010. Regulation of thyroid hormone-, estrogen- and androgen-related genes by triiodothyronine in the brain of *Silurana tropicalis*. *Journal of Neuroendocrinology*, 22: 1023-1031.

[1] Designed and performed research, analysed the data and wrote the manuscript for publication
[2] Contributed to the design of the research and to the revision of the manuscript

5.1. Introduction

The central nervous system (CNS) in tadpoles is an important target of thyroid hormones (THs). During metamorphosis, the CNS is extensively remodelled, for example with re-structuring (disappearance of certain larval neuronal structures and the development of adult structures), axon guidance and growth, cell proliferation and death (Denver, 1998; Tata, 2006). The tadpole brain has one of the highest concentrations of TH receptor (*tr*) mRNA relative to other tissues (Kawahara et al., 1991) and deiodinase (*dio*) transcripts are also detected in the brain (Cai and Brown, 2004; Hogan et al., 2007; Morvan-Dubois et al., 2006; Tindall et al., 2007). Apart from being a target of THs, the CNS is also a target for sex steroids (i.e., estrogens and androgens) which regulate brain sexual development and reproduction. For example, estrogen is known for regulating and organising the neuroendocrine circuits and controlling reproductive functions in fish (Pellegrini et al., 2005). In rodents, testosterone (T) masculinises the brain via its conversion to estrogen by the enzyme aromatase (CYP19; reviewed in Roselli et al., 2009) and/or by binding directly to the androgen receptor (AR; reviewed in Matsuda et al., 2008). Previous research in our laboratory has shown that triiodothyronine (T3) regulates the expression of the estrogen-

responsive genes estrogen receptor alpha (*eralpha*) and aromatase (*cyp19*) in the brain of premetamorphic *Lithobates pipiens* (Hogan et al., 2007). In addition, in *Silurana tropicalis* larvae, T3 increases the whole body expression of androgen-related genes, steroid 5α-reductase type 1 (*srd5alpha1*) and *ar* (Chapter 4). Therefore, the objective of this study was to investigate whether TH- and sex steroid-related genes are also regulated by T3 in the brain of *S. tropicalis* tadpoles.

During premetamorphosis, tadpoles have very low levels of THs but they are responsive to exogenous THs; therefore this is an excellent developmental period to examine the functions and mechanism of action of THs (Tata, 2006). This model system was used to examine the effects of THs on the tadpole brain. First, developmental profiles of TH- and sex steroid-related genes were established in the brain during *S. tropicalis* natural metamorphosis and adulthood. Then, premetamorphic tadpoles were exposed to T3 and transcript levels of TH-, estrogen-, and androgen-related genes and activity of cyp19 were measured in the brain of *S. tropicalis*.

5.2. Materials and methods

5.2.1. Animals

Silurana tropicalis fertilised eggs were obtained from five pairs of frogs and raised in petri dishes as described in Chapter 2. At NF46 (beginning of feeding), tadpoles were transferred to 12 L tanks containing aerated water (pH=7.5-8.0, dissolved oxygen=80-85%, temperature=23-24°C, conductivity=850-900 µS) and fed Sera Micron twice a day. Staging was determined by following the Nieuwkoop-Faber (NF) developmental table (Nieuwkoop and Faber, 1994). A 12:12h light:dark cycle was maintained with the light cycle occurring from 7am to 7pm. The care and treatment of animals used in this study were approved by the

Animal Care Committee, University of Ottawa and adhered to the guidelines published by the Canadian Council on Animal Care for the use of animals in science.

5.2.2. Brain collection for developmental profiles

Brain was collected for gene expression measurement at different stages of development (Fig. 5.1): NF50 and 52 (premetamorphosis), NF55 (prometamorphosis), NF60 (metamorphic climax) and NF66 (juvenile). Tadpoles were anaesthetised by immersion into 1% MS-222 (Sigma) and euthanised by decapitation. The brain was then dissected, frozen on dry ice, and kept at -80°C. For NF 50-55, brains were pooled (2-5 per pool; n=8 pools) to ensure sufficient material for RNA isolation. For NF 60-66, brains were analysed individually (n=8). In addition, to compare the gene expression during development with adult gene expression, five mature males and five mature females from our colony were euthanised using 4% MS-222 and the brain was dissected out and frozen. For the cyp19 activity assay, whole brains were also collected for NF stages 52, 55, 60, 66 following the same procedure as for the gene expression samples.

5.2.3. Triiodothyronine exposure

Premetamorphic tadpoles (NF 52-54) were exposed to three nominal concentrations of T3 (0.5, 5 and 50 nM; 3,3′,5-triiodo-L-thyronine; Sigma) or a dimethyl sulfoxide (DMSO; Sigma) solvent control for 48 h. The final DMSO concentration in the tanks was 0.005% in all treatments. The density in all the tanks was 1 tadpole/L. Chemical additions were not renewed during the 48 h period. At the end of the exposure, tadpoles were anaesthetised by immersion into 1% MS-222 and euthanised by decapitation. The brain was dissected out, frozen on dry ice, and kept at -80°C.

5.2.4. RNA isolation and cDNA synthesis

Total RNA for the developmental profile and T3 exposure samples was obtained from whole brain using the RNeasy Micro Kit (including the DNase treatment set) as described by the manufacturer (Qiagen). Individual and pooled brains were homogenised and disrupted using a MM301 Mixer Mill (Retsch) at 20 Hz for 3 min. Isolated RNA was resuspended in RNase free water and stored at -80°C. Total cDNA was prepared from 1 μg (for the developmental profile) and 2 μg (for the T3 exposure) of total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II reverse transcriptase (Invitrogen). For the developmental samples, the reverse transcriptase reaction was modified to be carried out at 42°C for 90 min (instead of 50 min) to increase the cDNA yield. The cDNA products were diluted 20-fold prior to PCR amplification.

5.2.5. Real-time RT-PCR

Gene specific primers for real-time RT-PCR were designed and optimised in Chapter 2. Real-time PCR primers for additional reference genes were designed based on GenBank sequences: glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; accession no. CR760856; forward 5'-3': tcactgccacccagaagac; reverse 5'-3': ggatgactttcccaacagc; product size: 123 bp) and 18S ribosomal RNA (*18S*; accession no. X04025; forward 5'-3': tcaacacgggaaacctcac; reverse 5'-3': agacaaatcgctccaccaac; product size: 117 bp) and using Primer 3 (available at: http://fokker.wi.mit.edu/primer3/input.htm). Primer concentrations were optimised to obtain a minimum threshold cycle and a maximum change in fluorescence. The optimised primer concentration for *gapdh* was 200 nM and for *18S* it was 150 nM. Specificity of the primer sets was confirmed by cloning and sequencing the single amplicon obtained. Expression of target genes in the brain was analysed using dual-labeled fluorescent probes (for estrogen

receptors [*eralpha*, *erbeta*], *cyp19* and ribosomal protein L8 [*rpl8*]) and SYBR Green I (for TH-receptors [*tralpha*, *trbeta*], deiodinases [*dio1*, *dio2*, *dio3*], *ar*, *srd5alpha1*, *srd5alpha2*, β-actin [*actb*], elongation factor 1α [*ef1alpha*], *gapdh* and *18S*) real-time RT-PCR assays using a Mx3000P real-time polymerase chain reaction system (Stratagene) as previously described (Chapter 2).

5.2.6. Data analysis

The relative standard curve method was used to interpolate relative mRNA abundance of target genes within each sample. The standard curves were generated using a cDNA mix of NF 60-66 brain samples (for the developmental profiles), and using equal parts of cDNA from each treatment (for the T3 exposure). Reaction efficiencies were 90-110% with an $R^2 \geq 0.990$. Samples were run in duplicate along with negative template controls (RNase-free water instead of cDNA template) and negative reverse transcriptase controls (cDNA template for which water was added instead of Superscript II). Data for each target were averaged and normalised to RNA content (Huggett et al., 2005). Data for the developmental profiles are presented as fold change relative to NF50 and data for the T3 exposure are expressed relative to the control group.

5.2.7. Aromatase activity assay

Brain cyp19 activity during metamorphosis and after T3 exposure was measured by a radiometric method previously optimised for frog tissues (Chapter 2). Activity of cyp19 was measured in pools of 4-6 brains (n=5-6 pools) for the developmental profile and 6-7 brains (n=5 pools) for the T3 exposure and is expressed as fmol ^3H_2O/h·mg protein.

5.2.8. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation) and significance was set at $p<0.05$. Data for all the genes were first tested for normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test respectively. The data were transformed (e.g., $\log_{10}$, square root) when the ANOVA assumptions were not met. Data were analysed by one-way ANOVA except for NF66 and adult brain samples which were analysed using a two-way ANOVA to examine sex differences. Analyses were followed by the Bonferroni post-hoc multiple comparisons test. When data failed to meet assumptions, the nonparametric Kruskal-Wallis test on ranks was used.

5.3. Results

5.3.1. Brain developmental profiles during metamorphosis and adulthood

Developmental profiles of TH- and sex steroid-related genes were established by sampling at five NF stages of development (main morphological characteristics in Fig. 5.1): 50 and 52 (premetamorphosis; foot paddle stages), 55 (prometamorphosis; hind limb development), 60 (beginning of metamorphic climax; forelimb emergence) and 66 (juvenile frog; tail completely resorbed). For all of the genes assessed, transcripts were detected throughout metamorphosis and in the adult brain (Figs. 5.1 and 5.2). All the reference genes tested (*rpl8*, *ef1alpha*, *actb*, and *gapdh*) changed during development; therefore, the gene expression data were normalised to RNA content only (Huggett et al., 2005). Fig. 5.1F presents the brain developmental profile of *rpl8*, the reference gene that varied the least during metamorphosis. The expression profiles of the receptors and enzymes showed very distinct patterns and magnitude of change. Two genes that remained relatively constant throughout larval development were *tralpha* and *dio1* (Fig. 5.1A and C). For *tralpha*, the

only difference detected was at NF55 which showed a higher expression relative to NF52. For *dio1*, expression in the female brain decreased significantly after metamorphosis was complete (NF66 versus adult brain); however, these changes were relatively minor (1.5-fold). The genes that showed increases during development are *trbeta*, *dio3*, *eralpha*, *erbeta*, *ar* and *cyp19* (Figs. 5.1 and 5.2). Transcript levels of *trbeta* and *dio3* were low and steady during premetamorphosis (NF 50-52) and early prometamorphosis (NF55) and levels only increased significantly (30- and 20-fold respectively) at the beginning of metamorphic climax (NF60). In the case of *eralpha*, *erbeta*, *ar* and *cyp19*, mRNA levels gradually increased during development. Activity of cyp19 also increased during metamorphosis, following the mRNA profile very closely (Fig. 5.2B). Finally, expression of three genes (*dio2*, *sdr5alpha1* and *srd5alpha2*) decreased during development and remained low during adulthood (Fig. 5.1D and Fig. 5.2D and F). Sex differences in the brain of the expression of target genes were only detected at the adult stages for *erbeta* (males had 1.7 times higher expression than females) and *ar* (females had 1.7 times higher expression than males).

5.3.2. Effects of T3 on brain transcript levels of thyroid hormone- and sex steroid-related genes

In order to investigate whether T3 regulates the expression of TH- and sex steroid-related genes in the brain of *S. tropicalis*, premetamorphic tadpoles (NF 52-54) competent to respond to THs were exposed to exogenous T3 for 48 h. Treatment with T3 had no effect on mortality and 100% survivorship post-exposure was observed in all treatment groups. Exposure to T3 resulted in increases in *trbeta* (5.0 to 15-fold; ANOVA; $p<0.001$), *dio2* (2.0 to 3.0-fold; ANOVA; $p<0.01$), and *dio3* (2.5 to 120-fold; ANOVA; $p<0.01$) mRNA levels at all three concentrations tested relative to control (Fig. 5.3). Levels of *tralpha* remained

unchanged for the 0.5 and 5 nM groups but decreased significantly (2.0-fold; ANOVA; $p<0.05$) at the 50 nM T3 concentration (Fig. 5.3A). Transcript levels for *dio1* did not change with T3 treatment (Fig. 5.3B). The reference genes *rpl8*, *gapdh* and *18S* changed with T3 (Fig. 5.3C; ANOVA; $p<0.01$); therefore, the gene expression data was normalised to RNA content, as suggested by Huggett et al. (2005) and as previously reported in Chapters 2.

The effects of T3 on sex steroid-related gene expression and cyp19 activity are shown in Fig. 5.4. Exposure to 5.0 and 50 nM T3 resulted in increases in *eralpha* (1.8 to 2.0-fold; ANOVA; $p<0.001$) and *erbeta* (2.4 to 2.8-fold; ANOVA; $p<0.001$) mRNA levels (Fig. 5.4A). Expression of *cyp19* was not affected by T3; however, a significant increase (21% relative to control; ANOVA; $p<0.05$) in cyp19 activity was observed at the 50 nM concentration (Fig. 5.4B). Gene expression levels of *srd5alpha2* increased significantly (between 6.0 to 7.5-fold; ANOVA; $p<0.01$) at all concentrations of T3 while levels of *srd5alpha1* increased significantly at 0.5 nM T3 relative to the control group (1.5-fold; ANOVA; $p<0.05$; Fig. 5.4C). Transcript levels of *ar* did not change after exposure to T3 (Fig. 5.4D).

Figure 5.1. Brain developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* metamorphosis and adulthood. Transcript levels of *tralpha* (A), *trbeta* (B), *dio1* (C), *dio2* (D) and *dio3* (E) were measured in whole brain from NF50 until adulthood. Levels of mRNA are expressed relative to NF50 and are normalised to RNA content. Results for the reference gene *rpl8* (F) are also presented. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (n=5-8 pools; $p<0.05$). Brain samples for NF66 and adulthood were statistically analysed separately from NF52 to NF60 samples (see materials and methods for details). Main morphological characteristics (i.e., whole body and hind limb diagrams) are included for each NF stage of development. Note that the scales of the y-axis vary among genes. A= adult.

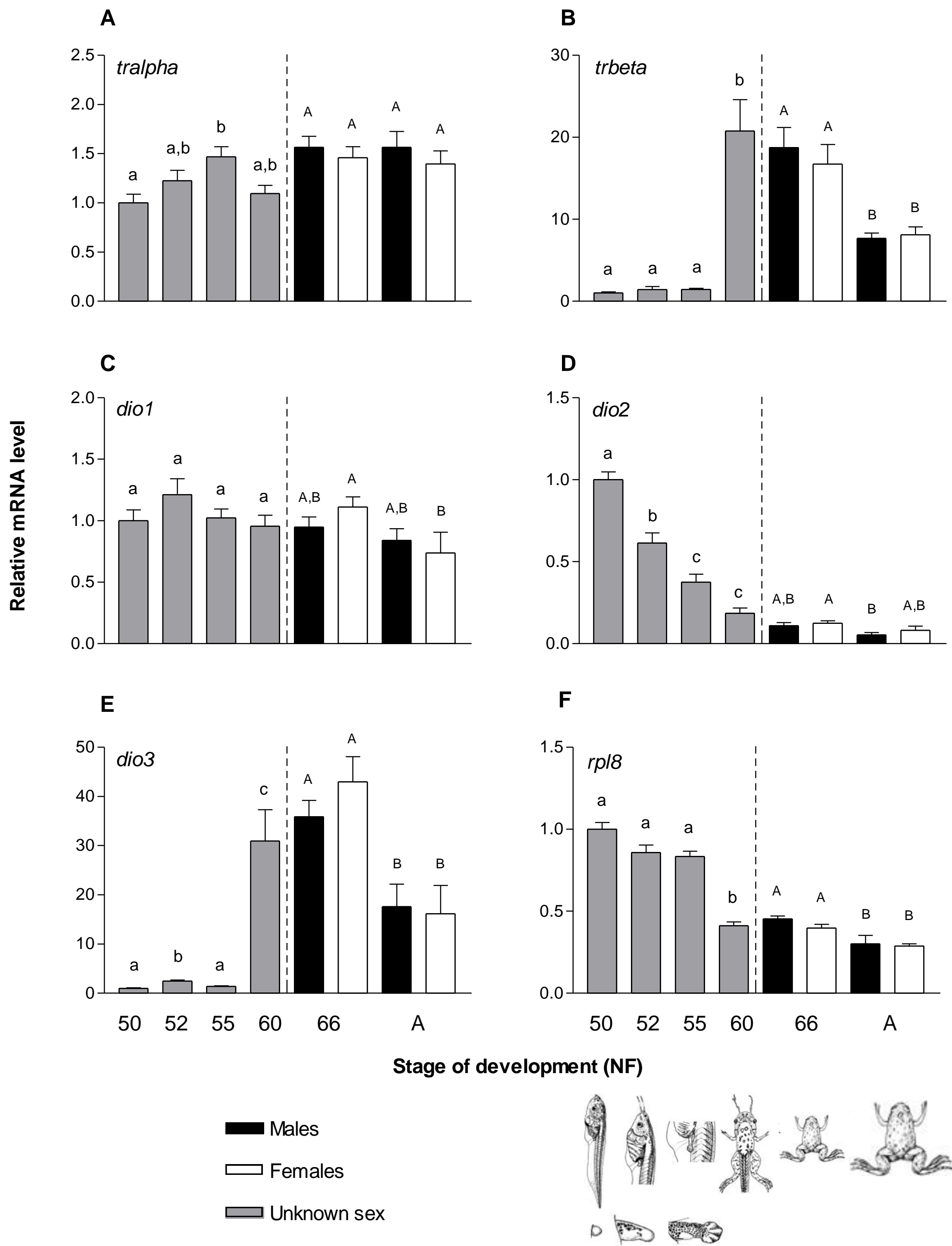
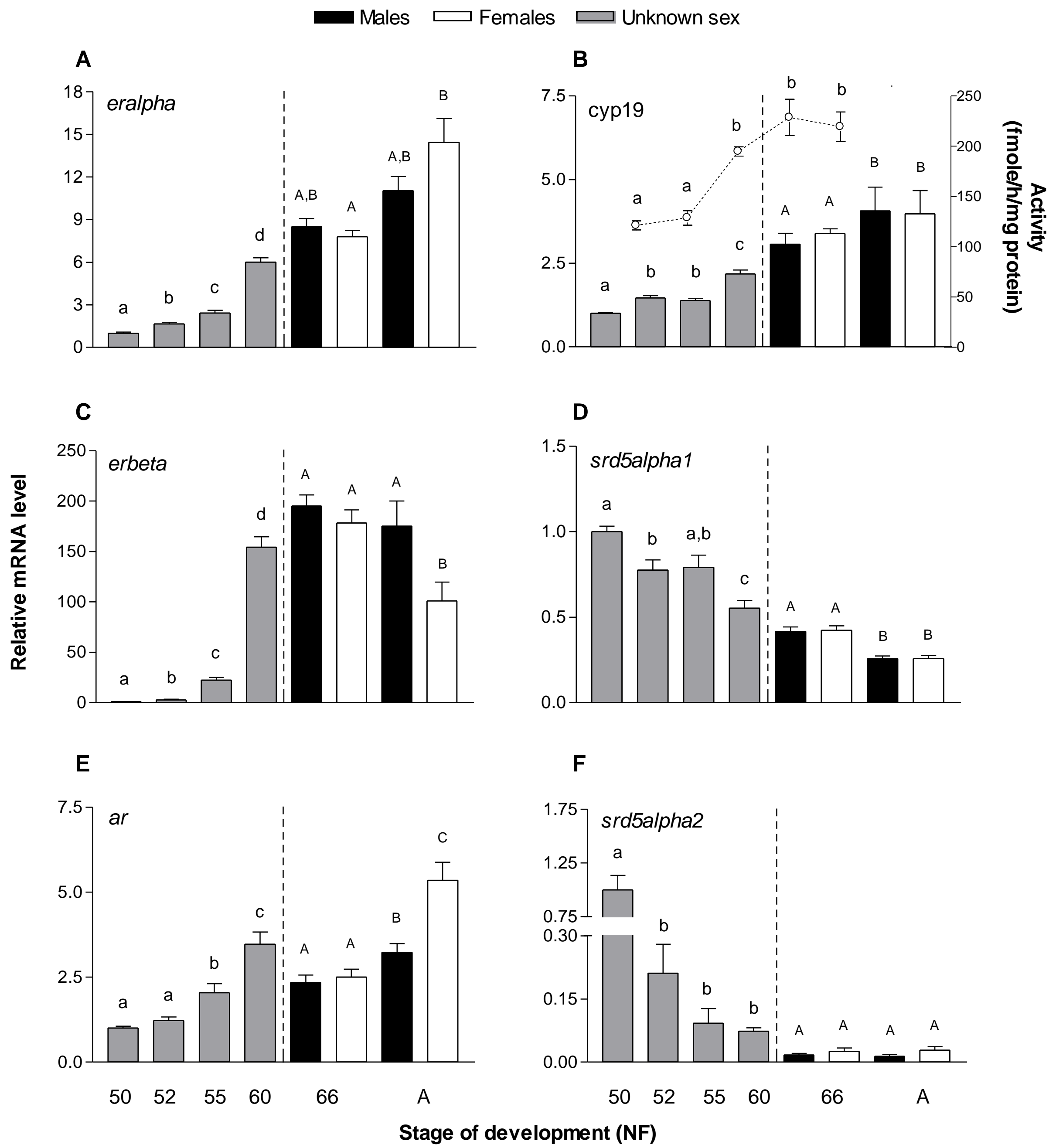


Figure 5.1 (caption on previous page)

Figure 5.2. Brain developmental profiles of sex steroid-related genes during *Silurana tropicalis* metamorphosis and adulthood. Transcript levels of *eralpha* (A), cyp19 mRNA and activity (B) *erbeta* (C), *srd5alpha1* (D), *ar* (E) and *srd5alpha2* (F) were measured in whole brains from NF50 until adulthood (y-axis on the left). Levels of mRNA are expressed relative to NF50 and are normalised to RNA content. Enzyme activity for cyp19 (B) was measured from NF52 to NF66 (y-axis on the right) is expressed in fmole/h normalised to protein content. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (n=5-8 pools; $p<0.05$). Brain samples for NF66 and adulthood were statistically analysed separately from NF52 to NF60 samples (see materials and methods for details). Significant differences in enzyme activity levels are indicated by small letters (n=5-6; $p<0.05$). Note that the scales of the y-axis vary among genes. A= adult.



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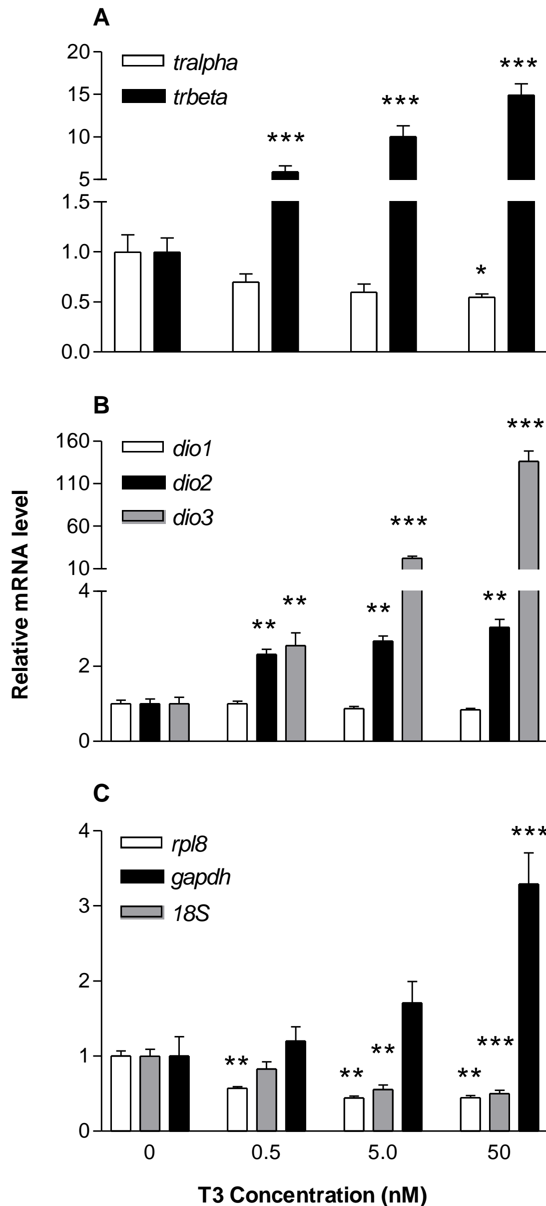


Figure 5.3. Effects of T3 exposure on the expression of thyroid hormone-related genes in *Silurana tropicalis*. Premetamorphic tadpoles (NF 52-54) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *tralpha* and *trbeta* (A), *dio1*, *dio2* and *dio3* (B) and the reference genes *rpl8*, *gapdh* and *18S* (C) are presented. Data are presented as fold changes relative to control and are normalised to RNA content (note the broken y-axis for A and B). Bars represent the mean + SEM. Asterisks represent significant differences from the control group (n=8 pools; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

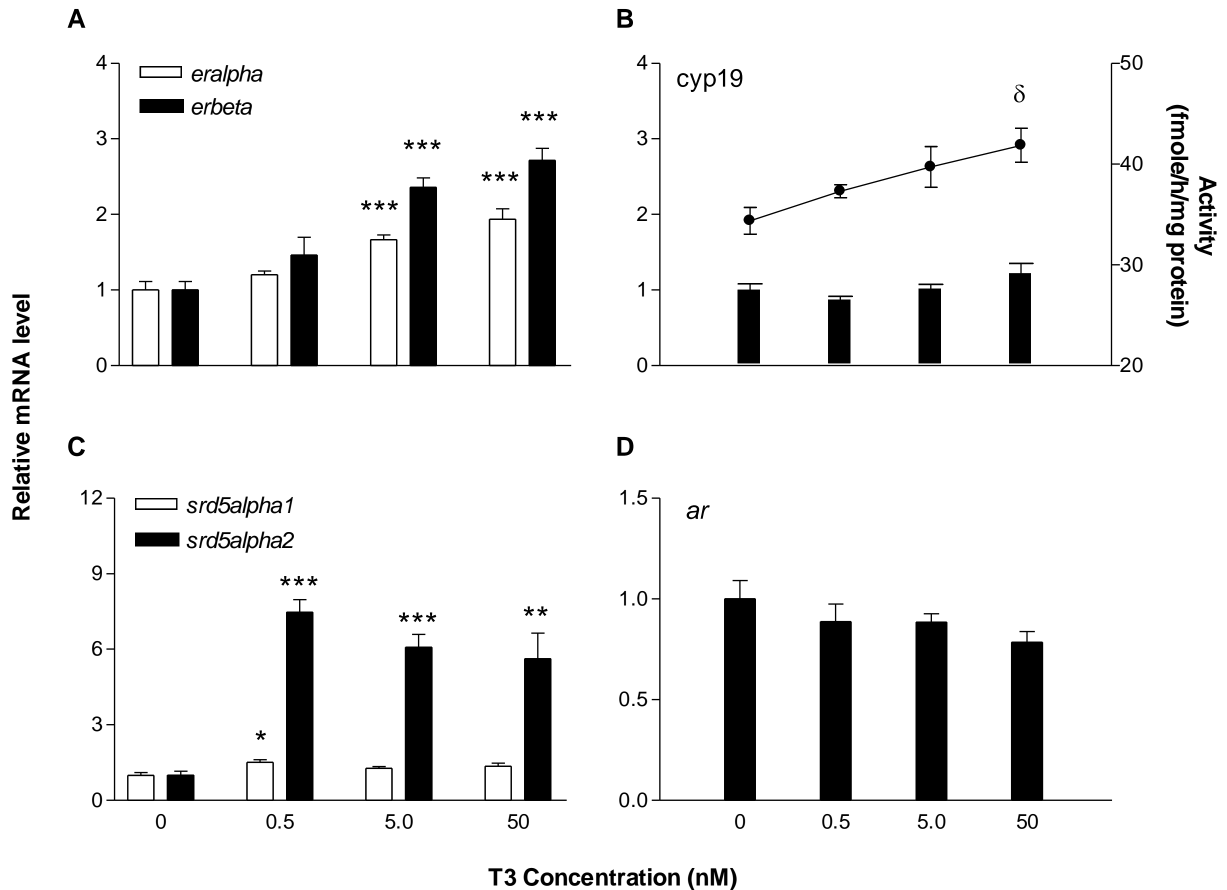


Figure 5.4. Effects of T3 exposure on the expression of sex steroid-related genes in *Silurana tropicalis*. Premetamorphic tadpoles (NF 52-54) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *eralpha* and *erbeta* (A), cyp19 mRNA and activity (B), *srd5alpha1* and *srd5alpha2* (C) and *ar* (D) are presented. Gene expression data (y-axis on the left) are presented as fold changes relative to control and are normalised to RNA content. Bars represent the mean + SEM. Enzyme activity (y-axis on the right; B) is expressed in fmole/h normalised to protein content. Asterisks represent significant differences in gene expression levels from the control group (n=8 pools; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Significant differences in enzyme activity levels are indicated by delta (δ; n=5; $p<0.01$) relative to the control group. Note that the scales of the y-axis vary among genes.

5.4. Discussion

In this study, I investigated the effects of T3 on brain gene expression in *S. tropicalis*. Premetamorphic tadpoles were exposed to three concentrations of T3 (0.5, 5.0 and 50 nM) for 48 h, following the same protocol (e.g., T3 concentrations, stages of tadpoles, duration of the exposure) as in the previous study using *L. pipiens* (Hogan et al., 2007) to allow valid comparisons between the two species. Here, I report that developmental expression and T3-regulation of TH-related genes in *S. tropicalis* are similar to those in other frogs. In contrast, this is not the case for estrogen responsive genes. Moreover, T3 can regulate expression of androgen synthesis enzymes in the tadpole brain.

5.4.1. Expression of thyroid hormone receptors and deiodinases during metamorphosis

Developmental profiles of *tr* and *dio* in the brain during natural metamorphosis have been previously established in *S. tropicalis* and other frog species. In the case of *trbeta*, the developmental profile and response to T3 obtained in this study are very similar to data from *Xenopus laevis* (Krain and Denver, 2004), *L. pipiens* (Hogan et al., 2007), and to those previously published for *S. tropicalis* (Wang et al., 2008). In *X. laevis*, *trbeta* mRNA increases in parallel with TH levels during metamorphosis and after T3 treatment (Yaoita and Brown, 1990).

This is the first time that expression profiles have been established for all three *dio* in the brain of *S. tropicalis*. To the best of my knowledge, this is the first developmental profile of *dio1* in a frog brain. In the case of *dio2* and *dio3*, the present results are similar to the profiles in *L. pipiens* (Hogan et al., 2007). Interestingly, *dio1* remained fairly constant during metamorphosis and after T3 exposure, suggesting that this enzyme does not play a major role in brain remodelling during metamorphosis. The results for *dio1* are in marked contrast to

those for *dio2* and *dio3* which show a very dynamic pattern of expression and T3 regulation. The positive regulation of *dio2* by T3 is surprising because I would expect *dio2* to decrease with T3 exposure (as observed in the developmental profile, *dio2* decreased at the beginning of metamorphic climax when T3 levels are highest). However, this positive regulation has been observed previously in whole larvae of *S. tropicalis* (Chapter 4) and *X. laevis* (Morvan-Dubois et al., 2006), and in the brains of *L. pipiens* (Hogan et al., 2007) and *S. tropicalis* tadpoles (Bonett et al., 2010) exposed to T3. The molecular mechanism underlying this response needs to be further investigated. Increases in *dio3* mRNA during development and after T3 exposure are also consistent with previous studies (Morvan-Dubois et al., 2006, Hogan et al., 2007; Chapter 4) and can be explained by the presence of a TH-responsive element in the promoter region of the *X. laevis* and *Lithobates catesbeianus dio3* genes (Becker et al., 1995; St-Germain et al., 1994). Low levels of *dio2* and high levels of *dio3* during metamorphic climax indicate that the brain has reduced T3 synthesis and has started inactivating THs, presumably because developmental remodelling of the brain has been completed (Cai and Brown, 2004).

5.4.2. Expression of estrogen- and androgen-related genes during metamorphosis

The present results indicate that the treatments were effective and the responses of TH-related genes in *S. tropicalis* brain to T3 were highly comparable to those in several other species. The second question addressed in this study was whether T3 could affect the expression of sex steroid-related genes in the brain during metamorphosis. For the first time, estrogen- and androgen-related genes were measured in the brain of *S. tropicalis* during metamorphosis. Although both *eralpha* and *erbeta* increased during development in the brain, the magnitude of change of their mRNA levels is very different. Of all the genes

analysed in this study *erbeta* showed the highest increase (150-fold) at NF60, while *eralpha* showed a more moderate increase (6.0-fold). Comparing the developmental profiles to the T3 exposure results helps elucidate whether endogenous THs are important regulators of gene expression. Interestingly, I found that both *er* mRNAs increased at the beginning of metamorphic climax and they were also positively regulated by T3. However, the relative increases were not as dramatic in the short T3 exposure compared to the metamorphic peak. Expression of *er* mRNAs has been shown to be regulated by estrogen and estrogenic compounds in frog and fish brain (Chandrasekar et al., 2010; Hogan, 2006; Marlatt et al., 2008). I suggest that part of the increase in *er* mRNAs during development could be due to the rising levels of THs during metamorphosis and the other part could be due to rising levels of estrogen in the brain via increasing cyp19 activity during development (present study). This study with *S. tropicalis* confirms the previous findings in the brain of *L. pipiens* where *eralpha* also increased during metamorphosis and after T3 exposure (Hogan et al., 2007). In the adult brain, I found that *erbeta* mRNA was higher in males than females, implying that there may be sex differences in the T3 regulation of *er* mRNA in the tadpole brain. However, this idea and the molecular mechanism by which T3 induces *er* expression need to be further investigated.

Both cyp19 mRNA and activity increased in the brain during *S. tropicalis* metamorphosis, similarly to gene expression profiles in *X. laevis* (Urbatzka et al., 2007) and *L. pipiens* (Hogan et al., 2007). T3 did not affect *cyp19* mRNA which is in marked contrast with results in *L. pipiens* where *cyp19* mRNA decreased with T3 concentration (Hogan et al., 2007). This difference between the two studies suggests there might be species differences in T3 regulation of *cyp19*; however, additional studies using other frog species will help further elucidate *cyp19* regulation in the frog brain (Chapters 7 and 9). Furthermore, I found that T3

increased cyp19 activity (21% at 50 nM relative to control) in the brain of *S. tropicalis*. To my knowledge, this is the first time that an increase in cyp19 activity has been reported after T3 treatment in the frog brain. In mammals, most of the research has focused on the effects of THs on gonadal development and function. Exposure to T3 decreases gonadal CYP19 activity *in vitro* in pigs (Gregoraszczuk et al., 1998), rats (Hatsuta et al., 2004; Ulisse et al., 1994) and mice (Cecconi et al., 1999). Therefore, the molecular mechanism and physiological consequences of T3 regulation of cyp19 activity in the brain are unknown at this point. One way T3 could affect cyp19 activity without affecting mRNA level is by regulating the enzyme at the post-translational level (e.g., phosphorylation of the enzyme; Balthazart et al., 2001) or by another indirect mechanism.

The profiles of *srd5alpha1* and *srd5alpha2* differ significantly with respect to the profiles of the other sex steroid-related genes. The expression of the two enzymes involved in the processing of T for 5α-dihydrotestosterone (5α-DHT) synthesis, decreased during development and remained low during adulthood in the brain. Urbatzka et al. (2007) measured *srd5alpha1* and *srd5alpha2* mRNAs by semi-quantitative RT-PCR in the brain of *X. laevis* and in general their profiles are very similar to the ones presented here for *S. tropicalis*. Immunohistochemical analyses have also shown that srd5alpha1 is present in the brain of *Pelophylax kl. esculentus* tadpoles during metamorphosis (Bruzzone et al., 2010). Exposure to all three concentrations of T3 resulted in a large increase (6.0 to 7.5-fold) in *srd5alpha2* but only a small increase (1.5-fold) in *srd5alpha1*. Previous studies have found that T3 induces *srd5alpha1* mRNA in whole larvae of *S. tropicalis* (Chapter 4) and in rats, hypothyroidism causes a decrease in hepatic Srd5alpha expression and activity, an effect that is restored with T4 treatment (Ram and Waxman, 1990). Srd5alpha2 is also involved in the conversion of progesterone into 5α-reduced metabolites such as allopregnanolone.

Allopregnanolone has been shown to be involved in neurogenesis, regulating cell death and proliferation *in vitro* in rat and human neuronal stem cells (Brinton and Wang, 2006) and in the developing sheep brain (Yawno et al., 2009). THs promote neurogenesis in the spinal cord of *X. laevis* (Schlosser et al., 2002), and are involved in neuronal proliferation and migration during vertebrate development (reviewed in Kress et al., 2009). Taken all together, the induction of *srd5alpha1* and *srd5alpha2* by T3 could be related to the roles of THs and neurosteroids in brain development. The comparison of developmental expression profiles and the results following T3 exposure of *srd5alpha1* and *srd5alpha2* reveal marked differences. During metamorphosis, *srd5alpha1* and *srd5alpha2* decreased in the brain, while they increased following T3 exposure. These results indicate that rising endogenous THs are not directly responsible for developmental decreases in *srd5alpha* expression in the tadpole brain. Whole body measurement of T and 5α-DHT indicates that androgens decrease during *X. laevis* development (Bogi et al., 2002). In *S. tropicalis* larvae exposed to finasteride (a 5α- and 5β-DHT synthesis inhibitor), a decrease in *srd5alpha2* is observed (Langlois, 2010). In rats, 5α-DHT induces *Srd5alpha* in the prostate (George et al., 1991) and *Srd5alpha2* in the adult brain (Torres and Ortega, 2003). These studies indicate that expression of *srd5alpha* can be regulated by its own enzymatic reaction product (i.e., feed forward control of 5α-DHT on *srd5alpha*). Therefore, the decrease in *srd5alpha* mRNA during development observed in this study could be linked to the decrease in 5α-DHT levels. On the other hand, rising TH levels likely counteract this to maintain or control the rate at which *srd5alpha* mRNA levels decrease as metamorphosis progresses. Another explanation involves the action of estrogens. Estradiol has been shown to inhibit Srd5alpha activity in rat skin (Dube et al., 1975) and adrenal tissue (Maynard and Cameron, 1973). In addition, in *S. tropicalis*, exposure to the aromatase inhibitor fadrozole increases whole body *srd5alpha2* (Chapter 2) and hepatic

srd5alpha1 and *srd5alpha2* mRNA levels (Chapter 3) and in fish fadrozole increases circulating androgen levels (Ankley et al., 2002). These studies suggest that estrogen can inhibit androgen synthesis in the tadpole as in adult frogs (Pierantoni et al., 1986). In the present study, I found that brain cyp19 activity increased during *S. tropicalis* metamorphosis; therefore, this putative increase in estrogen levels could inhibit srd5alpha activity in the brain leading to a decrease in 5α-DHT and *srd5alpha* mRNA due to the feed forward control. Future experiments are needed to test these hypotheses of the complex interplay between THs, androgens and estrogens as well as the physiological consequences of the regulation of *sr5alpha* by T3.

Taken together, the present results indicate that TH-related genes display very similar developmental profiles and T3 responses in the brains of different frog species. The brain was found to be an important site of TH regulation of not only TH-responsive genes but sex steroid receptor and enzyme genes. In the case of sex steroid-related genes, T3 regulation of *eralpha* seems to be common to both *S. tropicalis* and *L. pipiens*; however, this is not the case for *cyp19* and additional research is needed for the androgen-related genes. Finally, these results support the notion of a crosstalk between THs and sex steroids in the developing brain of *S. tropicalis* and provide an important baseline to determine the physiological consequences of this interaction during the remodelling of the frog brain.

CHAPTER 6

Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in the gonad-mesonephros of *Silurana tropicalis*

Adapted from:

Duarte-Guterman P[1] & Trudeau VL[2]. 2011. Transcript profiles and triiodothyronine regulation of sex steroid- and thyroid hormone-related genes in the gonad-mesonephros complex of *Silurana tropicalis*. *Molecular and Cellular Endocrinology*, 331: 143-149.

[1] Designed and performed research, analysed the data and wrote the manuscript for publication

[2] Contributed to the design of the research and to the revision of the manuscript

6.1. Introduction

Gonadal (sex) differentiation is the process by which undifferentiated gonads develop as either testes or ovaries. In amphibians and many other vertebrates this process can be affected by exposure to exogenous sex steroids (estrogens and androgens; Chapter 3; Bogi et al., 2002; Hogan et al., 2008; Mackenzie et al., 2003). Hormones of the thyroid gland can also affect gonadal differentiation. In *Xenopus laevis*, chronic exposure to perchlorate or thiourea (thyroid hormone (TH) synthesis inhibitors) results in feminisation (Goleman et al., 2002; Hayes, 1997a, respectively), leading to the hypothesis that THs are involved in testicular differentiation in frogs. However, the mechanisms by which THs could affect amphibian gonadal differentiation are currently unknown. Triiodothyronine (T3; the bioactive form of THs) increases the expression of two androgen-related genes in whole body larvae of *Silurana tropicalis*: androgen receptor (*ar*) and steroid 5α-reductase type 1 (*srd5alpha1*), one of the enzymes that converts testosterone (T) into the potent androgen 5α-dihydrotestosterone (5α-DHT; Chapter 4). These results led to the hypothesis that THs are involved in testicular development by inducing androgen-related genes.

In this study, I established developmental profiles of sex steroid- (estrogen receptors [*er*], aromatase [*cyp19*], *ar* and *srd5alpha*), sex differentiation- (doublesex- and mab-3-related transcription factor 1 [*dmrt-1*]) and TH- (receptors [*tr*] and deiodinases [*dio*]) related genes in the gonad-mesonephros complex (GMC) during development and in adult gonads. During premetamorphosis, tadpoles have low levels of circulating THs but they are responsive to exogenous THs. This period also coincides with gonadal differentiation in *S. tropicalis* (El Jamil et al., 2008). Therefore, I used this developmental period to examine whether T3 could regulate transcript levels in the GMC of *S. tropicalis* tadpoles during gonadal differentiation.

6.2. Materials and methods

6.2.1. Animals

Silurana tropicalis fertilised eggs were obtained from five pairs of frogs and raised in petri dishes at 24-25°C (as described in Chapter 2). Staging was determined using the Nieuwkoop-Faber (NF) developmental table (Nieuwkoop and Faber, 1994). When the tadpoles began feeding (NF46), they were transferred to 12 L tanks and raised as described in Chapter 5. The care and treatment of animals used in this study were approved by the Animal Care Committee, University of Ottawa and adhered to the guidelines published by the Canadian Council on Animal Care for the use of animals in science.

6.2.2. Sample collection for developmental profiles

The gonads of developing *S. tropicalis* tadpoles are very small and translucent; therefore, the gonads attached to the mesonephros and surrounding muscle tissue were dissected together, as reported in other frog studies (e.g., Maruo et al., 2008; Urbatzka et al.,

2007). Samples of GMC were taken at different stages of development (Fig. 6.1): NF52 (premetamorphosis; foot paddle stage), NF55 (prometamorphosis; hind limb development), NF60 (metamorphic climax; forelimb emergence) and NF66 (juvenile; tail completely resorbed). Tadpoles were anesthetised by immersion into 1% MS-222, euthanised by decapitation and GMC was dissected, frozen on dry ice and kept at -80°C. For all developmental stages, GMC samples were analysed individually (n=8-16 per stage). In addition, to compare transcript levels during development with adult expression levels, mature males and females from our colony were euthanised using 4% MS-222 and the testes and ovaries were dissected and frozen (n=6 per sex).

6.2.3. Triiodothyronine exposure

The same tadpoles (NF 52-54) as in Chapter 5 were exposed to three nominal concentrations of T3 (0.5, 5.0 and 50 nM; 3,3′,5-triiodo-L-thyronine; Sigma) or a dimethyl sulfoxide (DMSO; Sigma) solvent control for 48 h. At the end of the exposure, animals were euthanised and GMC were dissected out, frozen on dry ice and kept at -80°C. Pools of four GMC were analysed for gene expression (n=8 pools per treatment).

6.2.4. RNA isolation and cDNA synthesis

Total RNA for the developmental profile and T3 exposure samples was obtained from GMC and adult gonads using the RNeasy Mini Kit and RNeasy Micro kit respectively (including the DNase treatment set) as described by the manufacturer (Qiagen). GMC were homogenised and disrupted using a MM301 Mixer Mill (Retsch) at 20Hz for 4 min. Isolated RNA was resuspended in RNase free water and total cDNA was prepared from 1 μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II reverse

transcriptase (Invitrogen). For all the samples, the reverse transcriptase reaction was modified to be carried out at 42°C for 90 min (compared to the manufacturer's standard protocol of 50 min) to increase the cDNA yield. The cDNA products were diluted 40-fold prior to PCR amplification.

6.2.5. Real-time RT-PCR

Real-time RT-PCR primers were previously designed and optimised (Chapter 2) except for *dmrt-1*. Primers for *dmrt-1* were designed using Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm) based on a GenBank sequence (accession no. CX931409; forward 5'-3': agagagacagcgggttatgg; reverse 5'-3': caatgggcaaaggaatagga; product size: 98 bp). Primer concentrations were optimised to obtain a minimum threshold cycle and a maximum change in fluorescence. Specificity of the primer sets was confirmed by cloning and sequencing the single amplicon obtained. Target genes in the GMC and adult gonads were measured using dual-labeled fluorescent probes (*eralpha*, *erbeta*, *cyp19* and ribosomal protein L8, *rpl8*) and SYBR Green I (*tralpha*, *trbeta*, *dio1*, *dio2*, *dio3*, *ar*, *srd5alpha1*, *srd5alpha2*) real-time RT-PCR assays as described in Chapter 2. In the case of *dmrt-1*, the simplex reaction consisted of a 25 µL DNA amplification reaction containing 1.0 x PCR buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 µM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U HotStarTaq (Qiagen), 450 nM of each forward and reverse primer (Invitrogen), 0.25x SYBR Green I Dye (Molecular Probes), and 5 µL diluted cDNA template. The details of the thermocycle program are fully described in Chapter 2; the annealing temperature used for *dmrt-1* was 60°C.

6.2.6. Data analysis

Relative mRNA abundance of target genes within each sample was obtained using the relative standard curve method. The standard curves were generated using a cDNA mix of adult gonad samples (for the developmental profile), and using equal parts of cDNA from each treatment (for the T3 exposure). Samples were run in duplicate and reaction efficiencies were 90-110% with an $R^2 \geq 0.990$. Data for the developmental profiles are presented as fold change relative to NF52 and normalised to RNA content and *rpl8* mRNA levels. Data for the T3 exposure are expressed relative to the control group and normalised to RNA content (Huggett et al., 2005). In previous studies, mRNA levels of *rpl8* and other reference genes have been shown to be affected by T3 (Chapters 4 and 5); therefore, the T3 exposure data are normalised to RNA content only.

6.2.7. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation) and significance was set at $p<0.05$. Gene expression data were first tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test). When the assumptions were not met, the data were transformed as required (*e.g.* $\log_{10}$, square root). Data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc multiple comparisons test. When data failed to meet assumptions, the non-parametric Kruskal-Wallis test on ranks was used.

6.3. Results

6.3.1. Developmental profiles of transcript levels in the GMC

For all of the genes assessed, transcripts were detected throughout metamorphosis in the GMC and in adult gonads, with each of the genes showing a unique pattern of expression and magnitude of change (Figs. 6.1-6.3). The genes that significantly increased at metamorphosis (NF 60-66) compared to premetamorphosis (NF 52-55) were: *eralpha* (2.5-to 4.5-fold; Fig. 6.1A), *erbeta* (2.0- to 2.5-fold; Fig. 6.1B), *ar* (4.0- to 8.5-fold; Fig. 6.2B), *dmrt-1* (2.5- to 4.5-fold; Fig. 6.2D), *tralpha* (2.5- to 3.0-fold; Fig. 6.3A), *trbeta* (12- to 20-fold; Fig. 6.3C), *dio2* (2.0- to 3.0-fold; Fig. 6.3D) and *dio3* (3.5- to 6.5-fold; Fig. 6.3E). During development, the genes that remained relatively stable were: *cyp19* (Fig. 6.1C), *srd5alpha1* (Fig. 6.2A) and *srd5alpha2* (Fig. 6.2B). In the case of *cyp19*, within each stage, mRNA levels were very variable and for some individuals *cyp19* was not detected in the GMC. The only gene that decreased during metamorphosis was *dio1* (4.0- to 6.0-fold; Fig. 6.3B). Sex differences between adult testes and ovaries in the expression of sex steroid-, sex differentiation- and TH-related genes were detected for all the genes analysed in this study except *trbeta*. Expression was higher in testes compared to ovaries for: *eralpha* (4.5-fold), *erbeta* (8-fold), *ar* (6-fold), *dmrt-1* (750-fold), *srd5alpha2* (3-fold), *tralpha* (12-fold), *dio1* (3.5-fold), *dio2* (20-fold), *dio3* (10-fold); while expression was higher in ovaries compared to testes for *cyp19* (50-fold) and *srd5alpha1* (5-fold).

6.3.2. T3 exposure

Premetamorphic tadpoles (NF 52-54) were exposed to exogenous T3 for 48 h and transcript levels in the GMC were measured afterwards using real-time RT-PCR. Exposure to T3 resulted in a decrease in *erbeta* that was statistically significant at the 5 and 50 nM T3

relative to control (1.7- and 2.0-fold decrease; ANOVA; $p<0.01$; Fig. 6.4A). Expression of the androgen-related genes significantly increased at all levels of T3 (Figs. 6.4B and D): *ar* (1.4- to 1.8-fold; ANOVA; $p<0.01$), *srd5alpha1* (1.5-to 1.9- fold; ANOVA; $p<0.01$) and *srd5alpha2* (1.8- to 3.9-fold; ANOVA; $p<0.001$). Transcript levels of *eralpha*, *cyp19* and *dmrt-1* did not change with T3 treatment (Figs. 6.4A and 6.4C).

The effects of T3 on TH-related gene expression are shown in Fig. 6.5. Transcript levels of *trbeta* significantly increased at all three concentrations of T3 (6.0-fold; ANOVA; $p<0.001$; Fig. 6.5A) while *tralpha* showed more moderate increases (1.5- to 1.8-fold) that were only statistically significant at the 5 nM T3 (Fig. 6.5A). Exposure to T3 resulted in increases in *dio2* (at all three concentrations; 2.3- to 8.7-fold; ANOVA; $p<0.001$; Fig. 6.5B) and *dio3* (at 5.0 and 50 nM; 1.6- to 15-fold; ANOVA; $p<0.001$; Fig. 6.5B). Transcript levels of *dio1* decreased significantly at 5 and 50 nM T3 (1.7- and 1.9-fold; ANOVA; $p<0.05$; Fig. 6.5B) compared to control.

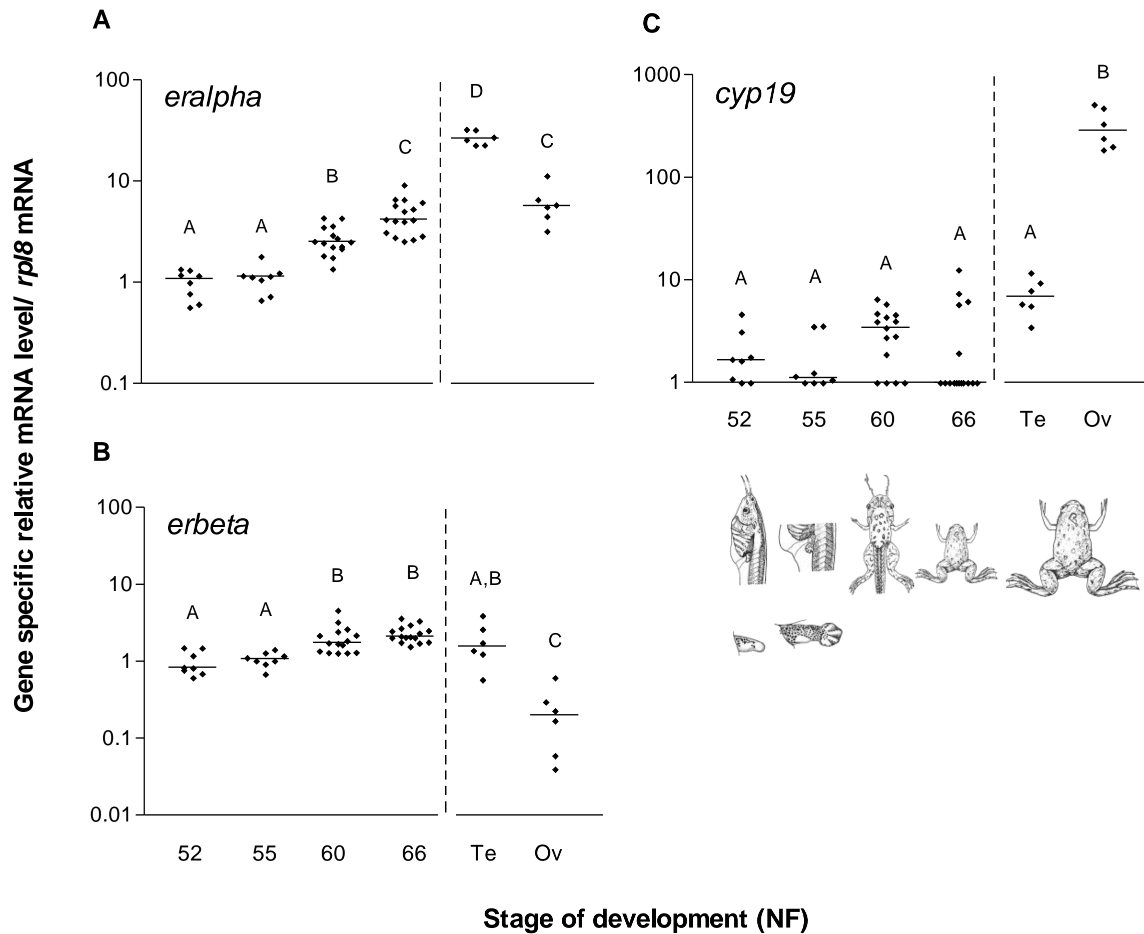


Figure 6.1. Developmental profiles of estrogen-related genes during *Silurana tropicalis* metamorphosis and adulthood. Transcript levels of *eralpha* (A), *erbeta* (B) and *cyp19* (C) were measured in the gonad-mesonephros complex from NF52 until NF66 and in mature adult gonads. Levels of mRNA are expressed relative to NF52 and are normalised to RNA content and *rpl8* mRNA. Individual sample points are presented along with the median. Different letters indicate statistically significant differences between stages and adult samples (n=6-16; $p<0.05$). Main morphological characteristics (i.e., whole body and hind limb diagrams) are included for each NF stage of development. A vertical dashed line separates the developmental GMC samples from the adult gonads. Note that the logarithmic scales of the y-axis vary among genes. In the case of *cyp19* a value equal to 1 corresponds to no detection of mRNA in the real-time RT-PCR. Te=adult testes; Ov=adult ovaries.

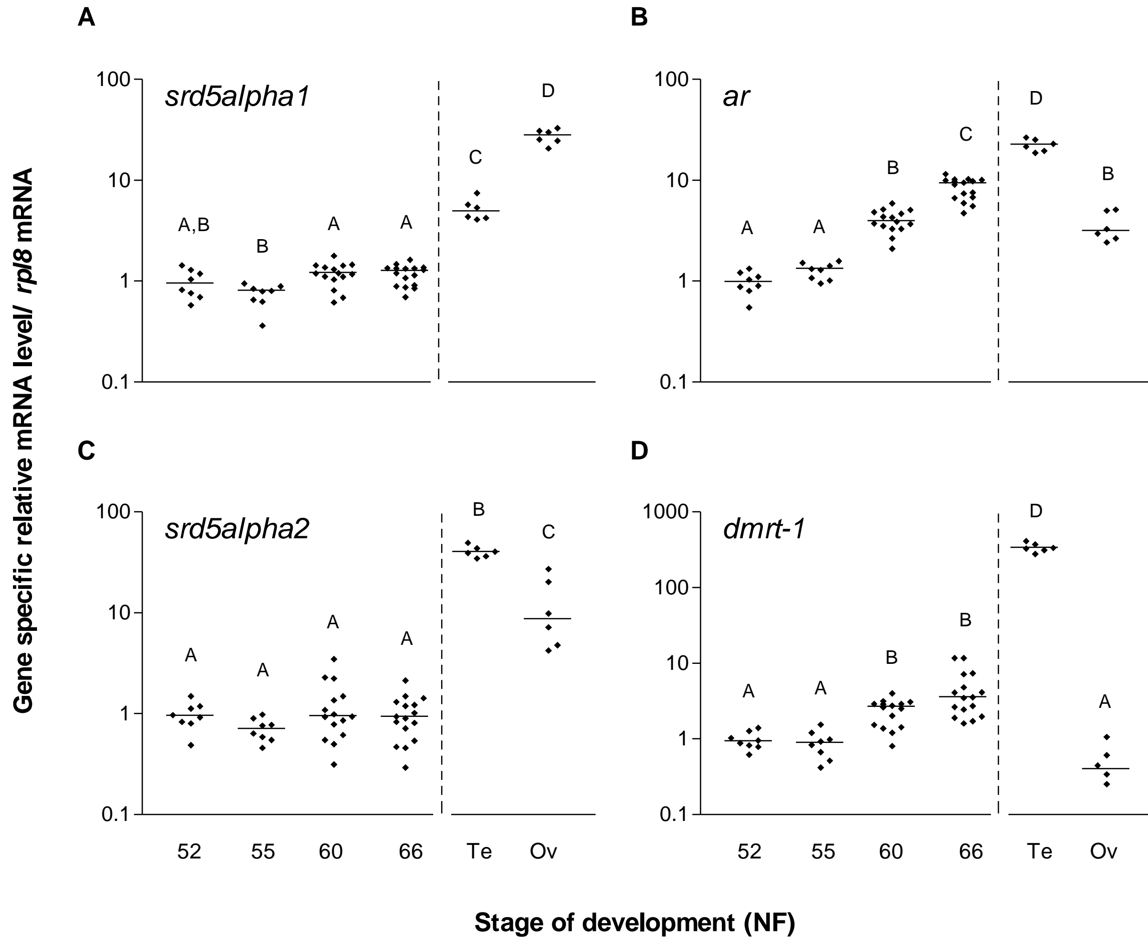


Figure 6.2. Developmental profiles of sex differentiation- and androgen-related genes during *Silurana tropicalis* metamorphosis and adulthood. Transcript levels of *srd5alpha1* (A), *ar* (B), *srd5alpha2* (C) and *dmrt-1* (D) were measured in the gonad-mesonephros complex from NF52 until NF66 and in mature adult gonads. Levels of mRNA are expressed relative to NF52 and are normalised to RNA content and *rpl8* mRNA. Individual sample points are presented along with the median. Different letters indicate statistically significant differences between stages and adult samples (n=6-16; $p<0.05$). A vertical dashed line separates the developmental GMC samples from the adult gonads. Note that the logarithmic scales of the y-axis vary among genes. Te=adult testes; Ov=adult ovaries.

Figure 6.3. Developmental profiles of thyroid hormone-related genes during *Silurana tropicalis* metamorphosis and adulthood. Transcript levels of *tralpha* (A), *dio1* (B), *trbeta* (C), *dio2* (D) and *dio3* (E) were measured in the gonad-mesonephros complex from NF52 until NF66 and in mature adult gonads. Levels of mRNA are expressed relative to NF52 and are normalised to RNA content and *rpl8* mRNA. Individual sample points are presented along with the median. Different letters indicate statistically significant differences between stages and adult samples (n=6-16; $p<0.05$). A vertical dashed line separates the developmental GMC samples from the adult gonads. Note that the logarithmic scales of the y-axis vary among genes. Te=adult testes; Ov=adult ovaries.

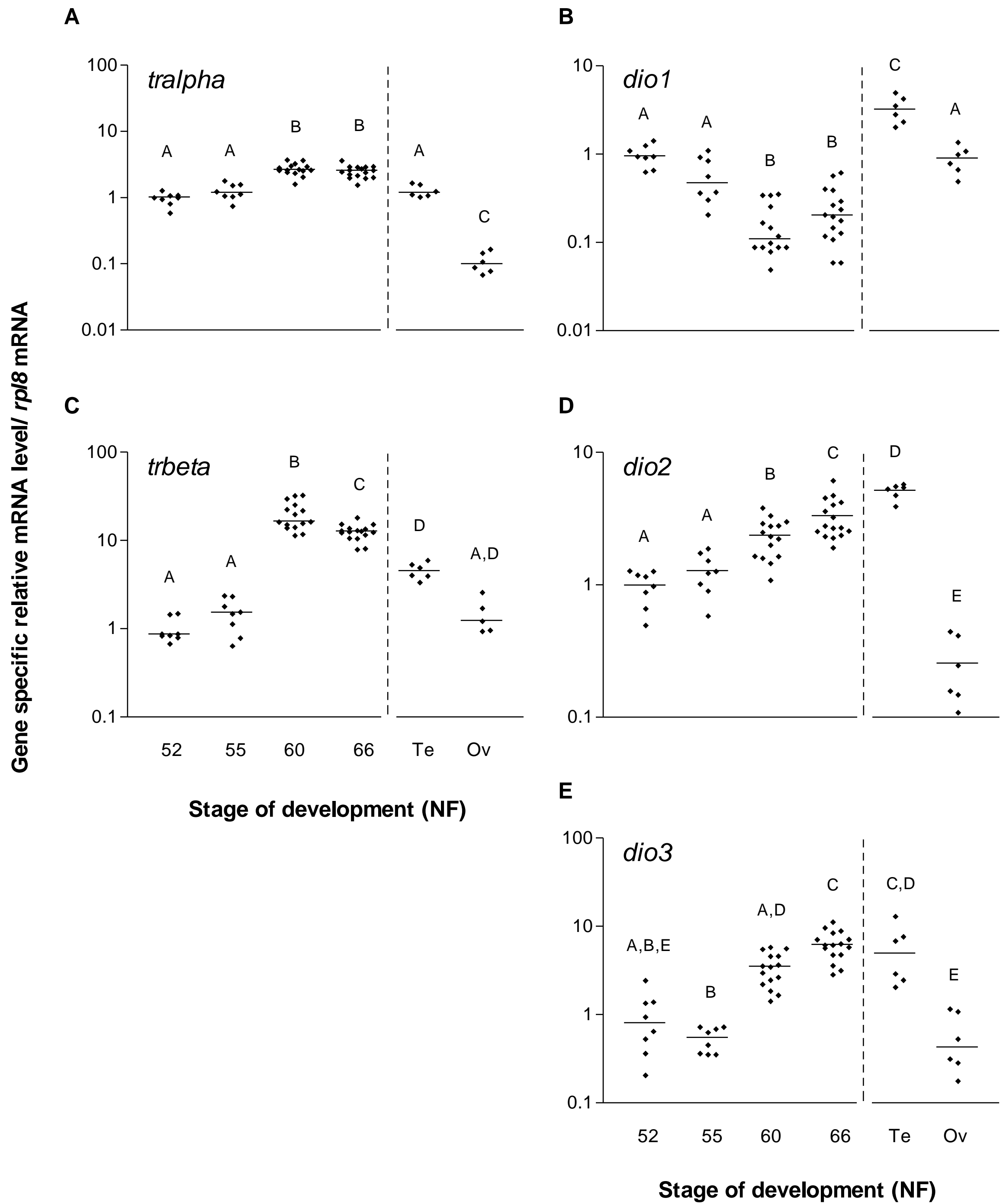


Figure 6.3. (Caption on previous page)

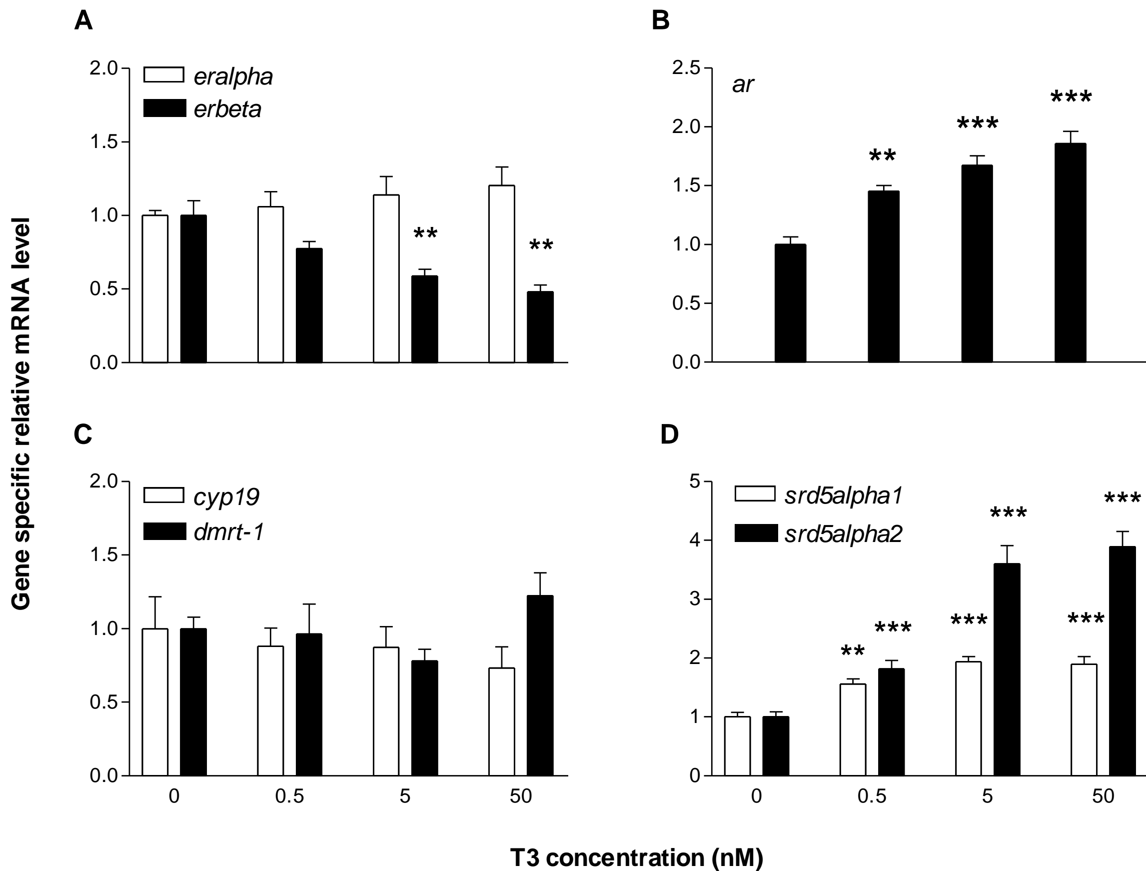


Figure 6.4. Effects of T3 exposure on the expression of sex differentiation- and sex steroid-related genes in the gonad-mesonephros complex in *Silurana tropicalis*. Premetamorphic tadpoles (NF 52-54) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *eralpha* and *erbeta* (A), *ar* (B), *cyp19* and *dmrt*-1 (C) and *srd5alpha1* and *srd5alpha2* (D) are presented. Gene expression data are presented as fold changes relative to control and are normalised to RNA content. Bars represent the mean + SEM. Asterisks represent significant differences in mRNA levels from the control group (n= 8; ** $p<0.01$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

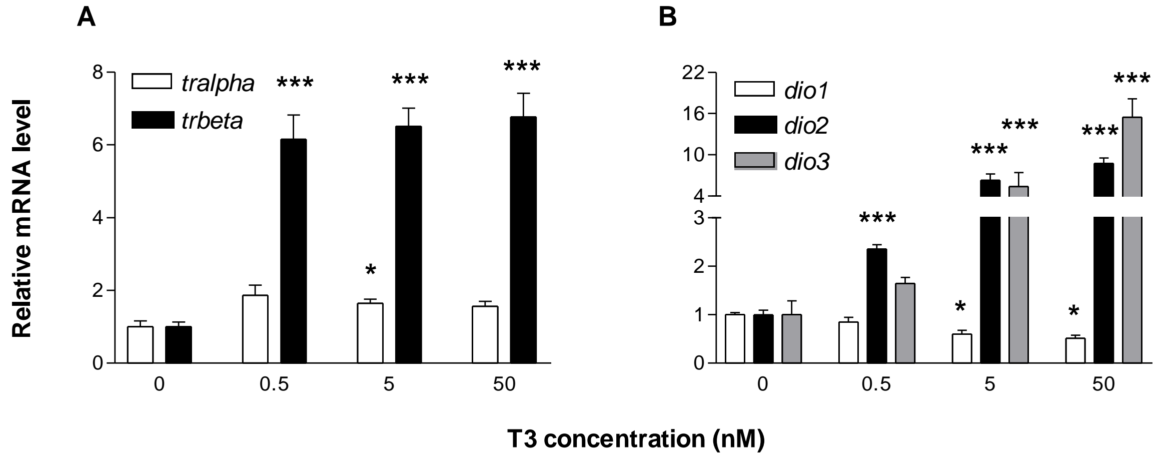


Figure 6.5. Effects of T3 exposure on the expression of thyroid hormone-related genes in the gonad-mesonephros complex in *Silurana tropicalis*. Premetamorphic tadpoles (NF 52-54) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *tralpha* and *trbeta* (A), and *dio1*, *dio2*, and *dio3* (B) are presented. Gene expression data are presented as fold changes relative to control and are normalised to RNA content. Bars represent the mean + SEM. Asterisks represent significant differences in mRNA levels from the control group (n=8; * p<0.05; *** p<0.001). Note that the scales of the y-axis vary among genes.

6.4. Discussion

6.4.1. Profiles of sex steroid- and sex differentiation-related genes in the GMC during metamorphosis and in adult gonads

In the present study, developmental profiles of sex steroid-, sex differentiation- and TH-related genes in the GMC of *S. tropicalis* were established from early tadpole until adulthood. The sex of a *S. tropicalis* tadpole cannot be unequivocally assigned by visual inspection until after metamorphosis is complete and no genetic sex marker has yet been found in this frog species; therefore, gene expression was measured at different stages of development in individual GMC samples. Interestingly, transcript levels during the period of gonadal sex differentiation and development (between NF 52-55; El Jamil et al., 2008) remained unchanged for all of the genes analysed. In addition, apart from *cyp19* (enzyme involved in the conversion of testosterone into estrogen), the individual samples did not show any obvious differences within stages which could be related to the sex of the tadpole. In the case of *cyp19*, in some individual GMC samples *cyp19* mRNA was not detected (i.e., some samples showed no amplification during the real-time RT-PCR assay) and this was observed at all four stages of development (NF 52, 55, 60 and 66). The adult gonad samples indicate that a high expression of *cyp19* is indicative of a female gonad. Therefore, this suggests that the GMC samples for which I could detect *cyp19* were presumptive female gonads and the gonads that had either very low or no expression of *cyp19* were most likely male. The results obtained in *S. tropicalis* for *cyp19* are similar to expression profiles in *X. laevis* and *Glandirana rugosa*. Urbatzka et al. (2007) and Maruo et al. (2008) found a higher expression of *cyp19* in female GMC compared to male GMC in both frog species. In several species of fish, sex differences in gonadal *cyp19* expression have also been observed during sex differentiation; a high expression of gonadal *cyp19* seems to be required for ovarian

development while very low or no expression is required for testicular differentiation (reviewed in Guiguen et al., 2010). In the GMC, levels of *srd5alpha1* and *srd5alpha2* (enzymes involved in the production of the potent androgen, 5α-DHT) mRNAs remained relatively constant during *S. tropicalis* development. These profiles are similar to GMC expression of *srd5alpha1* and *srd5alpha2* reported for *X. laevis* (Urbatzka et al., 2007) and *srd5alpha1* for *G. rugosa* (Maruo et al., 2008). Within each stage of development, expression of *srd5alpha* was not variable and clear sex differences were only detected at the adult stage. Sex differences in *srd5alpha2* in the GMC have been previously reported in only one study using *X. laevis,* where expression was found to be only 2-fold higher in males compared to females at metamorphic climax (Urbatzka et al., 2007). Differences between the two studies could be related to species differences; however, additional profiles of *srd5alpha2* need to be established in other frogs to address this possibility.

In many species from flies and worms to humans, *dmrt-1* has been proposed as one of the genes involved in testicular differentiation (Koopman, 2009) including the frog *G. rugosa* (Aoyama et al., 2003; Shibata et al., 2002). In the present study, expression of *dmrt-1* was higher (2000-fold) in adult testes compared to ovaries; however, during development, *dmrt-1* was detected in all of the GMC samples and expression levels did not show any differences that could be clearly related to sex. During metamorphosis, in *G. rugosa* and *Rhinella marina*, *dmrt-1* is expressed at the same levels in male and female gonads (Abramyan et al., 2009; Matsushita et al., 2007). Expression of *dmrt-1* seems to become sexually dimorphic at later stages of amphibian gonad development, but not during gonadal differentiation (Abramyan et al., 2009; Matsushita et al., 2007). The present results for *S. tropicalis* suggest that *cyp19* is involved in ovarian differentiation while *srd5alpha2* and *dmrt-1* may be involved in development and function of testes but not in their initial

differentiation. Chronic inhibition of cyp19 (using fadrozole) during *S. tropicalis* development affects the sex ratio at metamorphic climax, decreasing the number of females and inducing the presence of intersex (Chapter 3). Together, these results indicate that cyp19 plays an important role in gonadal differentiation in *S. tropicalis*.

Sex steroid receptor mRNA expression was also detected in the GMC during metamorphosis and in adult gonads. All receptors showed sexually dimorphic mRNA expression in adults: in all cases higher mRNA levels were found in the testes than in the ovaries. These results have also been observed for *ar* and *eralpha* in adult gonads of *G. rugosa* (Yokoyama et al., 2009) and for *eralpha* in *S. tropicalis* (Takase and Iguchi, 2007). Results are more variable between species for *erbeta* which was found to be higher in *G. rugosa* ovaries (Yokoyama et al., 2009), not sexually dimorphic in *S. tropicalis* gonads (Takase and Iguchi, 2007), and higher in *S. tropicalis* males (the present study). The consequences of sex differences in the expression of sex steroid receptors in adult gonads need to be further investigated.

6.4.2. Profiles of TH-related genes in the GMC during metamorphosis and in adult gonads

To the best of my knowledge, this is the first time that TH-related genes have been measured in the GMC during amphibian development. For all of the genes measured except *dio1*, I observed an increase during metamorphic climax and in the juvenile frog (NF 60-66). The profiles of TH-related genes indicate that the GMC is a target of TH during development. In addition, TH-related genes were also detected in adult testes and ovaries. Expression of *tr* and *dio* has been detected in gonads of fish (An et al., 2010; Filby and Tyler, 2007; Nelson and Habibi, 2006; Sambroni et al., 2001), alligator (Helbing et al., 2006a), chicken (Van der Geyten et al., 2002), and mammals (Brzezinska-Slebodzinska et al., 2000;

Palmero et al., 1992; Palmero et al., 1988). The only other study in frogs found a high expression of *tr* in oocytes in *X. laevis* froglets, using *in situ* hybridisation (Kawahara et al., 1991). In the present study, in the adult gonad, TH-related genes were higher in the testes than in the ovaries, except for *trbeta* for which the difference in expression was not statistically significant. In mammals, THs regulate the maturation and growth of the testes by regulating Sertoli and Leydig cell proliferation and differentiation (reviewed in Wagner et al., 2008). The present results suggest that in *S. tropicalis*, THs could also play a role in testicular function.

6.4.3. Effects of T3 on transcript levels in the GMC

Treatment with T3 during premetamorphosis resulted in changes in numerous transcript levels in the GMC. Exposure to T3 increased the expression of androgen-related genes (*ar*, *srd5alpha1*, *srd5alpha2*) and decreased the expression of the estrogen-related gene *erbeta*. These results suggest that T3 increases sensitivity to androgens while decreasing sensitivity to estrogens in the GMC. Similarly in rats, T3 decreased ER protein *in vitro* in Sertoli cells while the opposite is observed for AR (Panno et al., 1996) and *Ar* mRNA (Arambepola et al., 1998). The enzyme Srd5alpha seems to be also influenced by the TH axis in rats. In hypothyroid rats, hepatic *Srd5alpha* is reduced and treatment with T4 restores *Srd5alpha* mRNA levels (Ram and Waxman, 1990). I propose that the predicted decrease in estrogen sensitivity due to a decrease in *erbeta* mRNA, in combination with an increase in androgen synthesis and sensitivity underlies the masculinising effects of THs in anurans. All three doses of T3 resulted in increases in *trbeta* mRNA suggesting that some of the effects of T3 on the GMC are mediated by TH receptors. However, whether changes in sex steroid-related genes are mediated by *trbeta* remains to be elucidated.

Changes in transcript levels of TH-related genes in the GMC were very similar to changes observed in the brain of *S. tropicalis* tadpoles exposed to T3 (Chapter 5). However, differences were observed in the response of *er* mRNA to T3. In the tadpole brain, *eralpha* and *erbeta* increased with T3 while in the GMC, *erbeta* decreased yet *eralpha* was not affected by T3. These differences between brain and GMC in response to T3 treatment suggest that the mechanisms of cross-talk between the reproductive and TH axes are tissue-specific. One common gene, *srd5alpha2*, was found to be positively regulated in the brain and GMC by T3 at the lowest concentration tested (0.5 nM T3). Inhibiting srd5alpha by exposure to finasteride (a srd5alpha1, srd5alpha2 and 5β-reductase inhibitor) results in changes in the expression of TH-related genes: *trbeta*, *dio2* and *dio3* in the liver and *trbeta* and *dio3* in the brain of *S. tropicalis* metamorphs (Langlois, 2010). The present results provide further evidence for a close relationship between TH and androgen status in frogs.

The comparison between the profiles of sex steroid-related genes during development and after T3 exposure suggests that THs could be important regulators of some of the sex steroid-related genes expressed in the GMC. In the case of *erbeta*, mRNA levels increase in the GMC during metamorphosis while levels decrease after T3 exposure of NF 52-54 tadpoles. Estrogen positively regulates *er* mRNA in many tissues and species including frogs (reviewed in Bagamasbad and Denver, 2010). The present results suggest that rising TH levels (during metamorphosis) likely counteract the positive effects of estrogen to negatively control *erbeta* mRNA levels as metamorphosis progresses. On the other hand, levels of *srd5alpha1* and *srd5alpha2* remain fairly constant during development but both *srd5alpha* are positively regulated by exposure to T3. Estrogen has been shown to inhibit androgen production in the testis of the frog *Pelophylax kl. esculentus* (Pierantoni et al., 1986) and exposure to the estrogen synthesis blocker fadrozole induces hepatic *srd5alpha1* and

srd5alpha2 mRNA in *S. tropicalis* (Chapter 3). Therefore, I propose that levels of estrogen likely counteract the effects of rising THs levels to maintain constant *srd5alpha* mRNA levels during metamorphosis, thus promoting ovarian development. On the other hand, if estrogen levels are low, levels of *srd5alpha* mRNA can increase, promoting testicular development.

In conclusion, the present study demonstrates that the GMC is a target of sex steroids and THs and the developmental profiles suggest a critical role for cyp19 in regulating gonadal differentiation in *S. tropicalis*. I also present evidence of tissue-specific cross-talk between sex steroids and THs in *S. tropicalis*. Finally, this study also indicates that a fine balance between sex steroids and TH levels is required for normal gonadal sexual development in *S. tropicalis*.

CHAPTER 7

Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*
Part I: The brain

Adapted from:

Duarte-Guterman P[1], Ryan MJ[2], Hogan NS[3], Trudeau VL[4]. Developmental profiles and T3 regulation of brain transcripts in frogs: a comparative project with emphasis on *Engystomops* (*Physalaemus*) *pustulosus*. *Submitted.*

[1] Designed and performed research, analysed data, and wrote the manuscript

[2] Provided animals, tadpole rearing equipment, expertise in *Engystomops pustulosus* and contributed to the revision of the manuscript

[3] Contributed data for *Lithobates pipiens* and the revision of the manuscript

[4] Contributed to the design of the research and the revision of the manuscript

7.1. Introduction

Research on anuran species has demonstrated that thyroid hormones (THs) control the remodelling of the central nervous system (CNS) during metamorphosis (Denver, 1998). In vertebrates, the CNS is also a target of sex steroids to control brain sexual development and reproduction. During metamorphosis, these two hormone axes can interact to regulate gene expression (Chapter 5). THs affect brain transcripts during tadpole development but the regulation differs between species. Triiodothyronine (T3) positively regulates estrogen receptor (*er*) levels in the brain of *S. tropicalis* (Chapter 5) and *L. pipiens*, while T3 negatively regulates aromatase (*cyp19*) mRNA in *L. pipiens* only (Hogan et al., 2007). These results suggest that the regulatory effects of THs on sex steroid-related genes in the brain differ between anuran species. However, there are limitations about the conclusions that can be drawn from comparing only two species. While an important first step, these previous studies do not indicate the range of differences and how these may be distributed phylogenetically in anurans (Garland and Adolph, 1994). Therefore, the objective of this

study was to investigate the effects of THs on brain gene expression in a third anuran and to directly compare these data to my previous research in other species.

Research in frog developmental endocrinology, especially metamorphosis and sexual development has concentrated on species in the Pipidae (*Xenopus laevis*, *Silurana tropicalis*) and Ranidae (*Lithobates pipiens*, *Lithobates catesbeianus*, *Glandirana rugosa*) families (Hayes, 1998; Shi, 2000). The third species I have chosen is *Engystomops pustulosus* that belongs to the Leiuperidae family (Grant et al., 2006). It is evolutionary separated from the Pipidae and Ranidae species (Chapter 1, Fig. 1.3). Although *E. pustulosus* has been extensively used in sexual selection and communication studies (Ryan, 1985; Ryan and Rand, 2003), only very limited information is available regarding tadpole metamorphosis and sexual development.

I focused on characterising changes in transcript abundance in the developing tadpole brain. I cloned TH-related genes (TH-receptors [*tralpha*, *trbeta*] and deiodinases [*dio2*, *dio3*]) and sex steroid-related genes (*eralpha*, *erbeta*, *cyp19*, androgen receptor [*ar*], 5α-reductase [*srd5alpha1*, *srd5alpha2*]) and then examined their profiles in the brain during metamorphosis of *E. pustulosus*. In order to examine the influence of THs on the abundance of specific brain transcripts, I then treated premetamorphic tadpoles with T3.

7.2. Materials and methods

7.2.1. Animals

Romero-Carvajal et al. (2009) described the procedures for *E. pustulosus* care and reproduction in captivity; frogs in the current study were obtained from the same colony at the University of Texas at Austin. Fertilised eggs were obtained from three pairs of frogs, and developmental stages characterised according to the Gosner (G) developmental table

(Gosner, 1960). Tadpoles were reared in tap water treated with Kordon NovAqua water conditioner and Kordon Aquarium AmQuel instant water detoxifier, and were fed every two days with Jurassi diet (Jurassipet). Room temperature was maintained at 27-28ºC and tank water temperature was 25-26ºC. A 12:12 h light:dark cycle was maintained starting at 04:00 am local time. All the experiments were done at the University of Texas at Austin. Animal care and treatment protocols were approved by the Institutional Animal Care and Use Committee, University of Texas at Austin (IACUC protocol no. 08101701).

7.2.2. Cloning thyroid hormone- and sex steroid-related genes

Species-specific cDNA sequences for *tralpha*, *trbeta*, *dio2*, *dio3*, *eralpha, erbeta*, *cyp19, dmrt-1*, *srd5alpha1* and *srd5alpha2* were cloned from *E. pustulosus* cDNA or genomic DNA (gDNA). Total RNA was obtained from head, body or tail at G42 using the RNeasy Micro Kit (Qiagen). First strand cDNA synthesis was prepared from 1-2 μg total RNA and 200 ng random hexamer primers using Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). gDNA was obtained from a G42 tail following a standard phenol-chloroform-isoamyl extraction protocol. Mammalian and non-mammalian nucleic acid sequences for the target genes were obtained from GenBank (NCBI; available at: http://www.ncbi.nlm.nih.gov/genbank/) and aligned using ClustalW (EMBL-EBI; http:// www.ebi.ac.uk/Tools/msa/clustalw2/). Degenerate primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and are presented in Table 7.1. PCR amplification was performed using the Mastercycler® gradient Thermal Cycler (Eppendorf, Westbury, NY, USA). The PCR mixture (25 μL final volume) contained 1.0x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM forward and reverse primers, 1 U Taq® DNA Polymerase (Invitrogen), and 1 μL template (cDNA, gDNA or PCR product for nested PCR). The initial

denaturation step was performed at 94ºC for 3 min to activate the Taq enzyme. This was followed by 40 cycles with a denaturation step at 94ºC for 45 sec, an annealing step for 30 sec, and an extension step at 72ºC for 1 min. The final extension step was performed at 72ºC for 10 min and samples were immediately cooled at 4ºC. Table 7.1 presents the PCR conditions and the template (cDNA or gDNA) used to clone each gene. Amplification products were ligated directly into the 2.1 TOPO® vector (TOPO TA cloning kit, Invitrogen) and transformed into *Escherichia coli* (One Shot TOP 10 chemically competent cells, Invitrogen). Cells were then plated onto LB-agar plates containing standard concentrations of ampicillin and X-Gal and incubated at 37ºC for 16 h. Positive colonies were selected and an additional PCR was performed using M13 primers (included in the TOPO TA cloning kit) to ensure the correct insert size was present in the colonies before sequencing. Colonies were grown overnight at 37ºC in LB broth containing ampicillin, and plasmids (at least three per gene) were purified using the Qiaprep miniprep kit (Qiagen). The purified plasmids (10 μL) were sent to the Ontario Genomics Innovation Centre (Ottawa Health Research Institute, Ottawa, Canada) for sequencing. The resulting partial sequences were deposited on GenBank (accession numbers are presented in Table 7.1).

7.2.3. Phylogenetic analysis

Phylogenetic trees for *cyp19* and *eralpha* were constructed using anuran nucleotide sequences available on GenBank and the partial sequences I cloned from *E. pustulosus*. Sequence alignment was generated by Muscle (Edgar, 2004) and maximum-likelihood analyses were performed with PhyML (Guindon and Gascuel, 2003; Guindon et al., 2005). Statistical branch support was calculated using 500 bootstraps.

7.2.4. Tissue collection for developmental profiles

Brain samples were collected at different stages of development: G30 (premetamorphosis; foot paddle stages), G36 (prometamorphosis; hind limb development), and G42 (metamorphic climax; forelimb emergence) equivalent to Nieuwkoop-Faber (NF; Nieuwkoop and Faber, 1994) stage 51, 55, and 62, respectively (see Appendix A for a comparison between staging tables). Tadpoles were anesthetised by immersion in clove oil and euthanised by decapitation. Whole brains were dissected and preserved in RNAlater as described by the manufacturer (Ambion). For stages G30 and G36, brains were pooled (2-4 brains per pool, n=5-8 pools) but for G42, brains were analysed individually based on morphological sex (n=8), which was confirmed by the expression of *cyp19* in the gonad (Chapter 8).

7.2.5. Triiodothyronine exposure

In order to compare the T3 regulation of TH- and sex steroid-related genes between frog species, I used the same protocol and T3 concentrations described in Chapter 5 for *S. tropicalis* and in Hogan et al. (2007) for *L. pipiens*. Briefly, premetamorphic tadpoles (G 32-34 equivalent to NF 53-54) were exposed to three nominal concentrations of 3,3′,5-triiodo-L-thyronine (T3; 0.5, 5, 50 nM; Sigma) or a dimethylsulfoxide (DMSO; Sigma) solvent control for 48 h. The final DMSO concentration in the tank was 0.005% in all treatments. The density in all tanks was 1 tadpole/L of water. Chemical additions were not renewed during the 48 h period. At the end of the exposure, whole tadpole brains were dissected after the anaesthesia. Collected tissues were preserved in RNAlater (Ambion) and pooled (two brains per pool; n=8 pools) before RNA isolation.

7.2.6. RNA isolation and cDNA synthesis

Total RNA for the developmental profile and T3 exposure samples was obtained from brain using the RNeasy Micro Kit (including the DNase treatment set) as described by the manufacturer (Qiagen). Pooled and individual brain samples were homogenised with an MM301 Mixer Mill (Retsch) set to 20 Hz for 3 min. Isolated RNA was resuspended in RNase free water and concentrations were determined using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 1 μg total RNA and 0.2 μg random hexamer primers using Superscript II reverse transcriptase (Invitrogen). For all samples, the reverse transcriptase reaction was modified to be carried out at 42ºC for 90 min (compared to the manufacturer's standard protocol of 50 min) to increase the cDNA yield. The cDNA products were diluted 40 or 80-fold prior to PCR amplification.

7.2.7. Real-time RT-PCR

The partial sequences obtained from the cloning and the *ar* sequence published in GenBank (accession no. DQ320626) were used using Primer 3 to design gene-specific primer sets for real-time RT-PCR. Specificity of the primer sets was verified by PCR and gel electrophoresis. The appearance of a single product at the expected size and sequencing of these PCR products confirmed that the primers were amplifying the intended product. For all real-time RT-PCR assays, primer concentrations were optimised for a minimum threshold cycle (Ct) and a maximum change in fluorescence. Primer sequences and optimised conditions are presented in Table 7.2. The expression of individual gene targets was analysed using the Mx3005P real-time PCR system (Stratagene). The simplex reaction consisted of a 25-μL DNA amplification reaction containing 1.0 x PCR buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 μM dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U

HotStarTaq (Qiagen), optimised concentrations of each primer set (Invitrogen), 0.25x SYBR Green I Dye (Molecular Probes, Eugene, OR, USA), and 5 µL diluted cDNA template. The thermocycle program included an enzyme activation step at 95°C (15 min) and 45 cycles of 95°C (15 sec), 56-60°C (gene specific annealing temperature; 5 sec), 72°C (30 sec), and 80°C (8 sec). After the amplification phase, a denaturation step of 1 min (95°C) was followed by 41 cycles starting at 55°C and increasing 1°C/30 sec to generate a dissociation curve to confirm the presence of a single amplicon.

7.2.8. Data analysis

The relative standard curve method was used to interpolate relative mRNA abundance of target genes within each sample. The standard curves were generated using a cDNA mix of G42 samples (for the developmental profiles), and using equal parts of cDNA from each treatment including control (for the T3 exposure). Samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template) and a negative reverse transcriptase control (cDNA template for which water was added instead of Superscript II). Reaction efficiencies were 90-110% with an $R^2 \geq 0.990$. As also observed in *S. tropicalis* (Chapters 5 and 6), mRNA levels of the reference gene, ribosomal protein L8 (*rpl8*) decreased during development in *E. pustulosus* (Fig. 7.3F). Therefore, data for the developmental profiles are presented as fold change relative to G30 and normalised to RNA content. In experiments with *S. tropicalis*, common reference genes, including *rpl8*, used to normalise gene expression data were not available because their expression was affected by T3 (Chapters 4, 5 and 6) and therefore, normalising the data using reference genes was not possible. In the brain of *E. pustulosus* tadpoles, *rpl8* mRNA was not affected by T3 but in order to be consistent and compare data within this thesis, the data for the T3 exposure are

presented relative to the control group and normalised to RNA content only (Huggett et al., 2005), and the results for *rpl8* are shown separately in Fig. 7.5F.

7.2.9. Statistical analyses

Statistical analyses were performed using S-Plus 8.0 (Insightful Corporation) and a significance of $p<0.05$. Data for all the genes were first tested for normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test, respectively. When the assumptions were not met, the data were transformed as required (e.g., $\log_{10}$, square root). Data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparisons test between all stages (developmental profiles) or by Bonferroni's pair-wise comparison between treatment and control (T3 exposure). If the ANOVA assumptions could not be achieved, data were tested non-parametrically using Kruskal-Wallis' test.

Table 7.1. Degenerate primers and conditions used for cloning thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*. GenBank accession numbers, forward (F) and reverse (R) primer sequences (5'-3'), amplicon size (bp), template, and annealing temperature (°C) are presented.

Target Gene		Primer sequence (5'-3')	Amplicon size (bp)	Template	Annealing Temp. (°C)
tralpha	F	ACAAGATCACCCGVAACCAG	424	Tail G42 cDNA	58
HQ846626	R	TCMGAGAACATGGGCAGYTT			
trbeta	F	GRAAYCAGTGCCARGAATGT	739	Tail G42 cDNA	56
HQ846627	R	TCAAAWGCCARGAGGAAACC			
dio2	F	CACCTTTTAGACTTTGCCAGC	326	Head G42 cDNA	52
HQ846633	R	TAGGCMRCATTGGYRTTATT			
dio3	F	GHATGTGCACMTTGGAGT	388	Head G42 cDNA	52
HQ846634	R	GCCCCTTGRAGCATGAGY			
cyp19	F	GTGCATYGGSATGAATGAAA	501	Head G42 cDNA	56
HQ846628	R	WCTCCATGATTCTGKGCAAA			
eralpha	F	GMCCCTWCACAMCAAAACCWCT	725	Body G42 cDNA	54
HQ846629	R	TCATTCCMACTTCRTAGCAYT			
erbeta	F [a]	TTYTTYAARAGAAGCATTCAAGG	598	Head G42 cDNA	54
HQ846630	R[a]	CRCAYTTCCCYTCRTCCCTG			
dmrt-1	F1	TTCTGTATGTGGMGGGAHT	595	G42 gonad cDNA	54
HQ846631	R1	ARTGCATYCTGTACTGKGAGC			
	F2[b]	GCARCARGCYCAAGAAGA	451	PCR product[c]	54
	R2[b]	TTTCATCTGCCAYTGRBTTC			
srd5alpha1	F1	TGMGSTTGGCWTGGTTYRT	562	G34 brain cDNA	54
HQ846632	R1	TCAAGGTACCAYTTRTGATGCTG			
	F2[b]	TAYAAYGGBTACNTGCAGAGCAG	244	PCR product[c]	54
	R2[b]	ACCATTCARCWATTTCACCA			
srd5alpha2	F [d]	RGCMAAYTTCCTTGGDGARA	82	gDNA	54
HQ846635	R[d]	AAADGCAAAGGCAAATSCTG			

[a] Primers from Yokoyama et al., 2009
[b] Nested primers of set 1
[c] PCR product obtained with primer set 1
[d] Primers are located inside an exon (based on *Silurana tropicalis* genome sequence)

Table 7.2. SYBR-green real-time RT-PCR primers and assay conditions of target genes in *Engystomops pustulosus*. Forward (F) and reverse (R) primer sequences (5'-3'), amplicon size (bp), optimised primer concentration (nM) and annealing temperature (ºC) are presented.

Target Gene		Primer sequence (5'- 3')	Amplicon Size (bp)	Primer Concentration (nM)	Annealing Temp. (ºC)
tralpha	F	ATCGAAGTACCAACGCACAAG	90	350	60
	R	GCATTGACGCCATAGGAGA			
trbeta	F	TGCCAGAAGATATTGGACAGG	111	200	58
	R	CTCTTGTAATTGCTGGGGTGA			
dio2	F	AGAAACCTAGAAGATCGGTGTG	107	350	56
	R	GCATTGGCGTTATTGTCC			
dio3	F	ACACTGAGGTGGTCATGCTG	95	375	60
	R	GCAACTGCCAAAATTCACAA			
eralpha	F	CAGGTCAAGTTCAGACAACAGA	99	850	60
	R	CAATACCGAGTTTCCTTACTGG			
erbeta	F	TACATCTGCCCTGCCACA	111	450	58
	R	GGTTCCACATTTCATCATTCC			
ar	F	AGGGAGATGGCAGAGAAATG	117	350	58
	R	CAGCACAAACCACAACAGG			
cyp19	F	TTAGACACCTCCAACAACCTCTTC	91	400	58
	R	CCTGCCACGCATCAAAGTA			
dmrt-1[a]	F	GCTGCTCTCAGGCTACAAGTG	132	260	58
	R	CCACGACAAAGTCAGAAGTGC			
srd5alpha1	F	CTGGCCTCCCTATTAACTGGA	128	275	60
	R	CGTCTCCCCAGGTTGTCTC			
srd5alpha2	F	CTTGGGGAGATTGTGGAGTG	71	400	58
	R	AAGGCAAAGGCAAATGCTG			
rpl8	F	GAACCCTGTAGAACATCCCTTC	111	200	58
	R	CGAGCAGCAATAAGACCAAC			

[a] Measured in the gonad-mesonephros complex only (see Chapter 8)

7.3. Results

7.3.1. Cloning of genes in E. pustulosus

Gene fragments for TH- and sex steroid-related genes were cloned from *E. pustulosus* using degenerate primers designed to match motifs of the most conserved regions of these genes. The percent nucleotide identity of the genes obtained in this study in comparison to the homologous genes in other anuran species is presented in Table 7.3 and varies from 70 to 91%. TH receptors (*tralpha*, *trbeta*) are highly conserved (88-89% average identity). The deiodinases (*dio2*, *dio3*), sex steroid receptors (*eralpha*, *erbeta*, *ar*), *cyp19* and *srd5alpha2* have about 80-83% identity, while *sdr5alpha1* and *dmrt-1* are the least conserved sequences, with an average identity of 74%. A phylogenetic tree was constructed using the available anuran *cyp19* and *eralpha* sequences (6-7 sequences per gene) and the homologous sequence from the salamander *Pleurodeles waltl* as the outgroup (Fig. 7.1). Based on these two genes, *E. pustulosus* is most closely related to the Bufonidae, and more distantly related to the Pipidae (Fig. 7.1 and Table 7.3), which follows the currently accepted relationships between these species (Fig. 1.3; Ford and Cannatella, 1993).

7.3.2. Brain developmental profiles during metamorphosis

Developmental profiles of TH- and sex steroid-related genes were established by sampling at three stages of development (main morphological characteristics are shown in Fig. 7.2). For all of the genes assessed, with the exception of *srd5alpha2*, transcripts were detected in the brain throughout metamorphosis and no differences in mRNA levels were detected between males and females at metamorphic climax (Figs. 7.2 and 7.3). In the case of *srd5alpha2*, transcripts were not detected in the brain of *E. pustulosus* (i.e., no amplification was detected in the real-time RT-PCR after 45 cycles). For *tr* and *dios* (Fig.

7.2), and *erbeta* and *sdr5alpha1* (Fig. 7.3B and E), no significant differences in brain mRNA levels were observed between G30 and G36 tadpoles. Transcript levels of *eralpha* and *ar* showed similar gradual increases during tadpole development (1.7-fold at G36 and 5.0-fold at G42; Fig. 7.3A and D), while *cyp19* only increased 1.5-fold at G36 relative to G30, then remained constant between G36 and G42 (Fig. 7.3C). Large increases in mRNA levels at G42 were observed for *trbeta* (40-fold; Fig. 7.2B), *dio3* (44-fold; Fig. 7.2D) and *erbeta* (35-fold; Fig. 7.3B). The genes that remained unchanged throughout metamorphosis were *dio2* (Fig. 7.2C) and *srd5alpha1* (Fig. 7.3E). Expression of the reference gene *rpl8* decreased significantly during development (1.5-1.7-fold; Fig. 7.3F). Therefore, the gene expression data were normalised to RNA content and the results will contribute to further research and validation of *rpl8* as a reference gene in *E. pustulosus*.

7.3.3. Effects of T3 on brain gene expression

Premetamorphic tadpoles (G 32-34) were exposed to T3 for 48 h, as previously carried out in *S. tropicalis* (Chapters 5) and *L. pipiens* (Hogan et al., 2007), and transcript levels were measured in the brain. Treatment with T3 had no effect on mortality and 100% survivorship post-exposure was observed in all treatment groups. Real-time RT-PCR analyses revealed that mRNA levels of TH-related genes in the brain were affected by T3 (Fig. 7.4): *tralpha* ($F_{3,28}$=10.6; p<0.001), *trbeta* ($F_{3,28}$=74.7; p<0.001), *dio2* ($F_{3,28}$=35.87; p<0.001), and *dio3* ($F_{3,28}$=200.7; p<0.001). Transcript levels of *tralpha* showed the smallest changes with increases at 5 nM (1.2-fold; post-hoc test p<0.05) and 50 nM T3 (1.5-fold; post-hoc test p<0.001; Fig. 7.4A) while levels of *dio2* and *dio3* were more affected with larger increases at 5 nM (2.0-fold, post-hoc test p<0.001 and 4.0-fold, post-hoc test p<0.001 respectively) and 50 nM T3 (4.0-fold, post-hoc test p<0.001 and 30-fold, post-hoc test

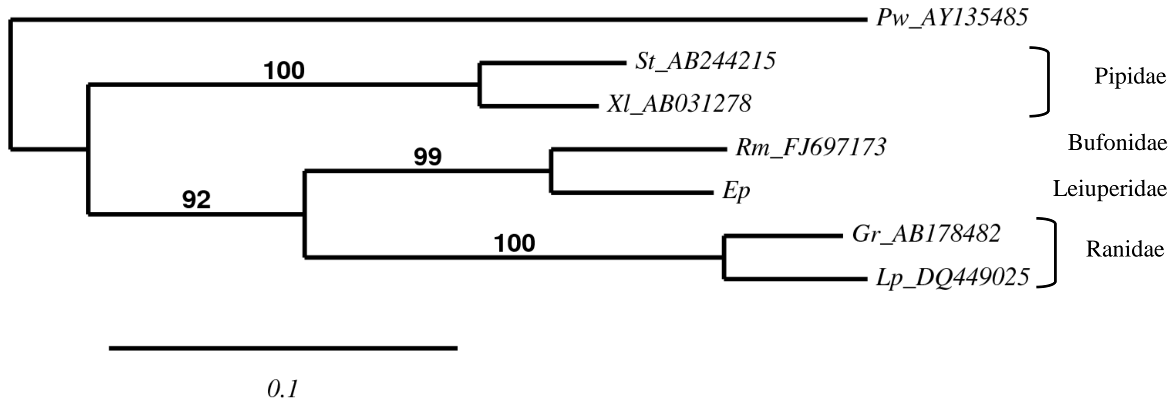
p<0.001 respectively; Fig. 7.4C and D). In the case of *trbeta*, T3 affected brain mRNA levels at all three concentrations (2.0-30-fold; post-hoc test *p*<0.01; Fig. 7.4B). Sex steroid-related genes were also measured after T3 exposure in the brain (Fig. 7.5). T3 exposure affected mRNA levels of *erbeta* ($F_{3,28}$=35.6; *p*<0.001), *cyp19* ($F_{3,28}$=3.54; *p*=0.03), and *ar* ($F_{3,28}$=3.16; *p*=0.04). In the case of *erbeta*, T3 significantly increased transcript levels at 50 nM T3 (3.5-fold; post-hoc test *p*<0.001; Fig. 7.5B) and for *ar*, mRNA levels increased at 5 and 50 nM (1.2-fold; post-hoc test *p*<0.05; Fig. 7.5D). Levels of *cyp19* significantly decreased with T3 exposure at 5 and 50 nM (1.4-fold; post-hoc test *p*<0.05; Fig. 7.5C). Transcript levels of *eralpha* ($F_{3,28}$=1.18; *p*=0.34) and *srd5alpha1* ($F_{3,28}$=1.16; *p*=0.34) were not affected by the treatment (Fig. 7.5A and E).

Table 7.3. Percent nucleotide identity of *Engystomops pustulosus* target genes analysed in this study to sequences from other species belonging to different anuran families. Available sequences in GenBank from all anuran species were selected for the comparison. Percent nucleotide identities were calculated using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Dash indicates the sequence is not available for that species.

	Xenopus laevis	***Silurana tropicalis***	***Lithobates catesbeianus***	***Lithobates pipiens***	***Glandirana rugosa***	***Rhinella marina***	***Amietophrynus rangeri***	***Bombina orientalis***	**Average**
tralpha	88	88	91	-	-	-	-	-	**89**
trbeta	86	86	90	-	89	-	-	-	**88**
dio2	82	82	86	-	-	-	-	-	**83**
dio3	82	82	78	79	-	-	-	-	**80**
eralpha	80	80	-	82	83	-	90	83	**83**
erbeta	79	79	-	-	82	-	88	-	**82**
cyp19	78	78	-	79	80	91	-	-	**81**
ar[a]	81	82	84	84	85	-	-	-	**83**
sdr5alpha1	73	74	-	-	75	-	-	-	**74**
sdr5alpha2	79	85	-	-	-	-	-	-	**82**
dmrt-1[b]	70	70	-	-	72	84	-	-	**74**
Range	**70-88**	**70-88**	**78-91**	**79-84**	**72-89**	**84-91**	**88-90**	**83**	

[a] Complete codons (GenBank accession DQ320626; Chakraborty and Burmeister, 2010)
[b] Measured in the gonad-mesonephros complex only (Chapter 8)

A: ***cyp19***



B: ***eralpha***

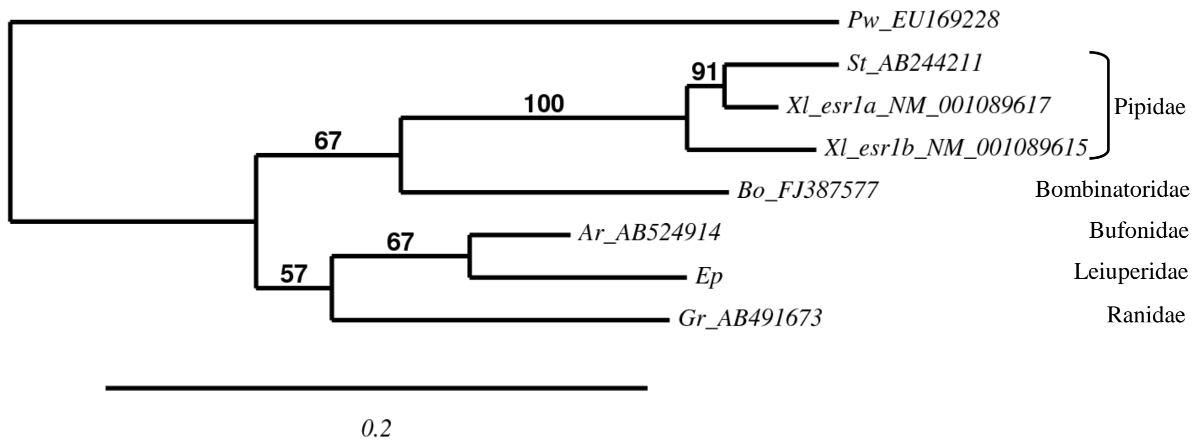


Figure 7.1. Phylogenetic tree of anuran (A) *cyp19* and (B) *eralpha* using nucleotide sequences from GenBank and sequences cloned from *Engystomops pustulosus*. Branch support values (%) and anuran families are presented. The sequences used in the analysis were >500bp. Species abbreviations are followed by GenBank accession numbers. *Pleurodeles waltl* (Pw) was used as the outgroup in both trees. Ar, *Amietophrynus rangeri*; Bo, *Bombina orientalis*; Ep, *Engystomops pustulosus*; Gr, *Glandirana rugosa*; Lp, *Lithobates pipiens*; Rm, *Rhinella marina*; St, *Silurana tropicalis*; Xl, *Xenopus laevis*. Note that the sequence Lp_*eralpha* (DQ398027) was not taken into consideration because it is <200bp long.

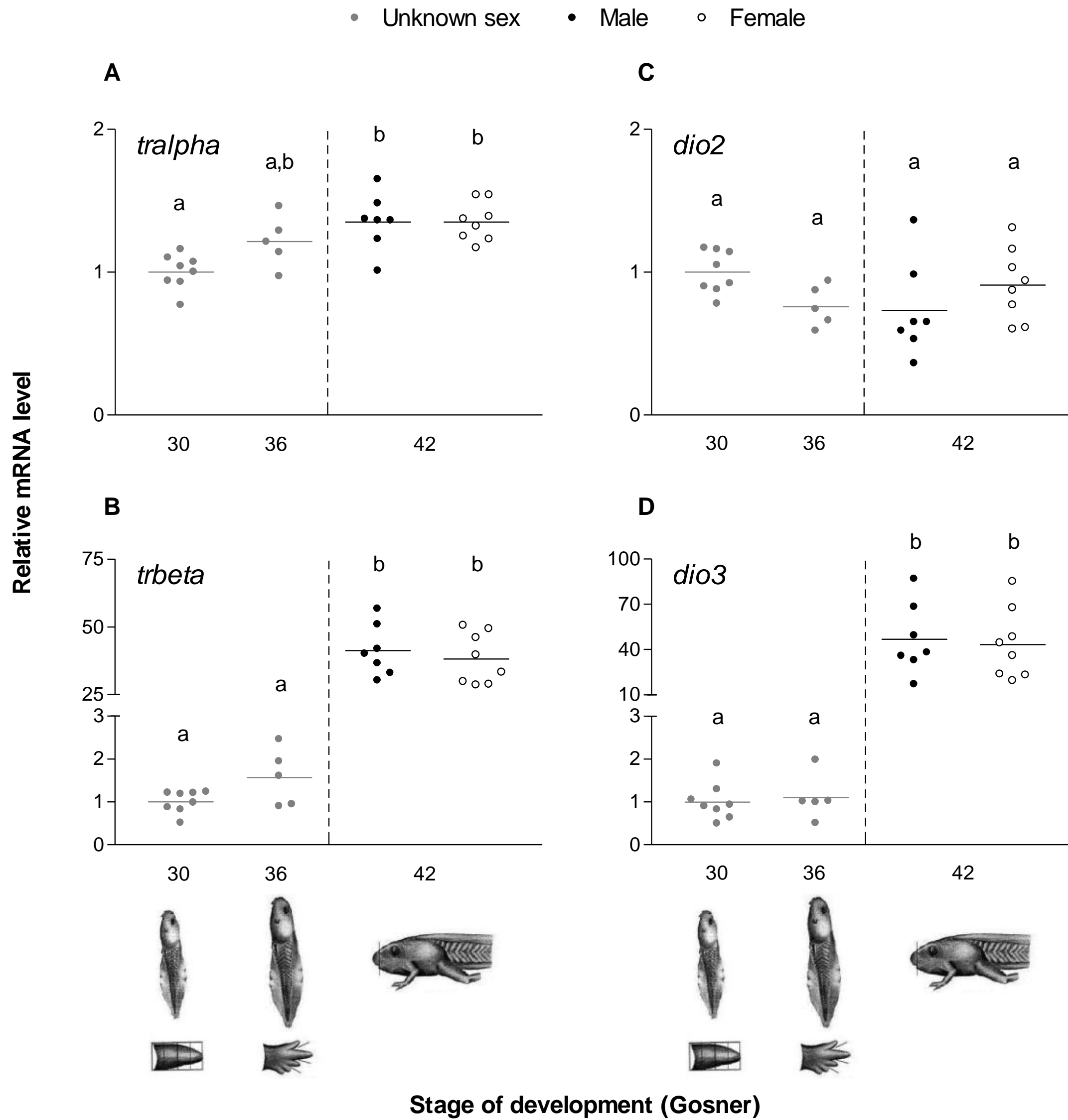


Figure 7.2. Brain developmental profiles of thyroid hormone-related genes during *Engystomops pustulosus* metamorphosis. Transcript levels of *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) were measured in the brain at G30, 36, and 42. Levels of mRNA are expressed relative to G30 and are normalised to RNA content. Individual sample points are presented along with the mean. Different letters indicate statistically significant differences among stages (one-way ANOVA; n=5-8; $p<0.05$). Main morphological characteristics (i.e., whole body and hind limb diagrams; Gosner, 1960) are included for each stage of development. A vertical dashed line separates the developmental samples from G42 male and female brains. Note that the scales of the y-axis vary among genes.

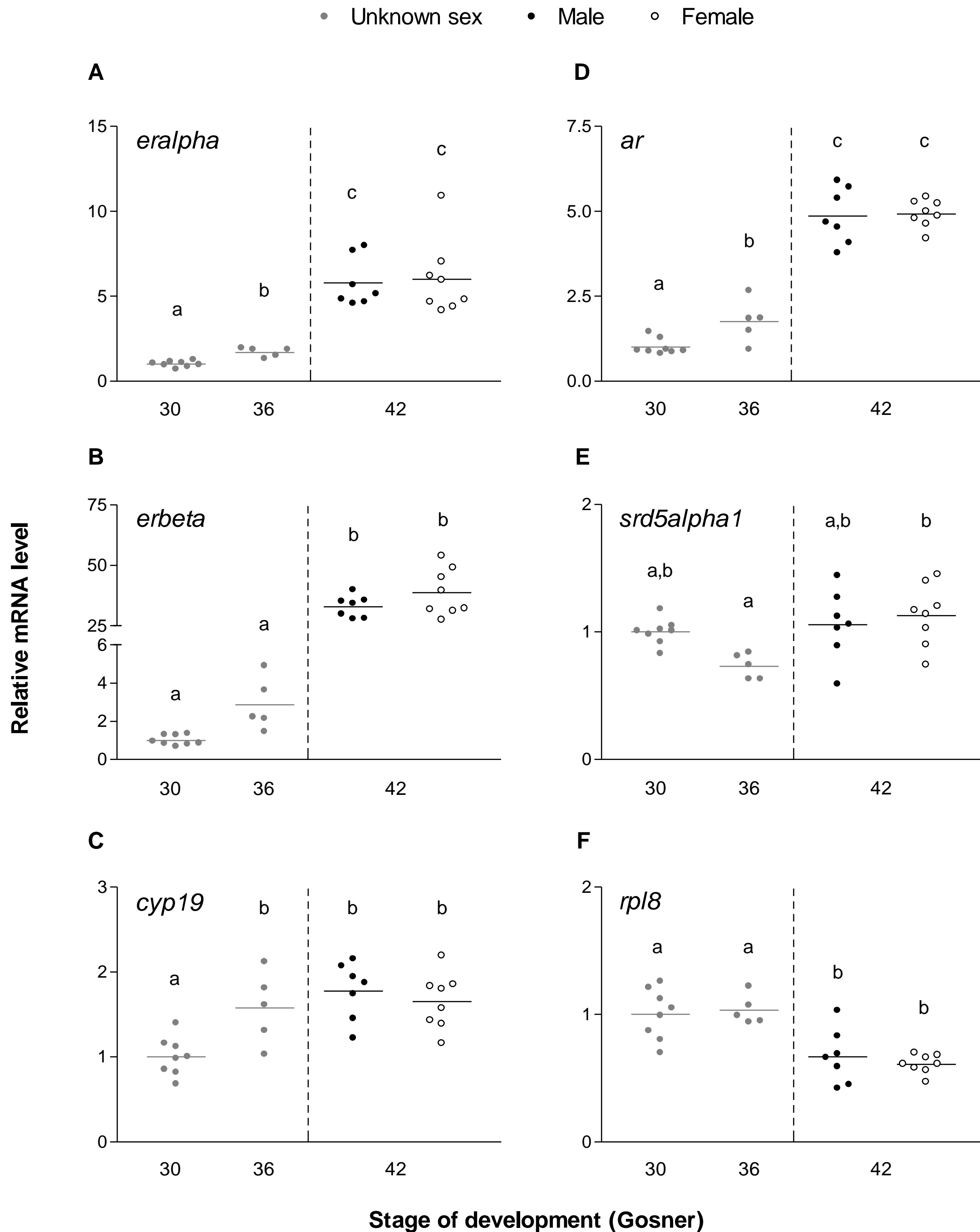


Figure 7.3. Brain developmental profiles of sex steroid-related genes during *Engystomops pustulosus* metamorphosis. Transcript levels of *eralpha* (A), *erbeta* (B), *cyp19* (C), *ar* (D), and *srd5alpha1* (E) were measured in the brain at G30, 36, and 42. Results for the reference gene *rpl8* are also presented (F). Levels of mRNA are expressed relative to G30 and are normalised to RNA content. Individual sample points are presented along with the mean. Different letters indicate statistically significant differences among stages (one-way ANOVA; n=5-8; $p<0.05$). A vertical dashed line separates the developmental samples from G42 male and female brains. Note that the scales of the y-axis vary among genes.

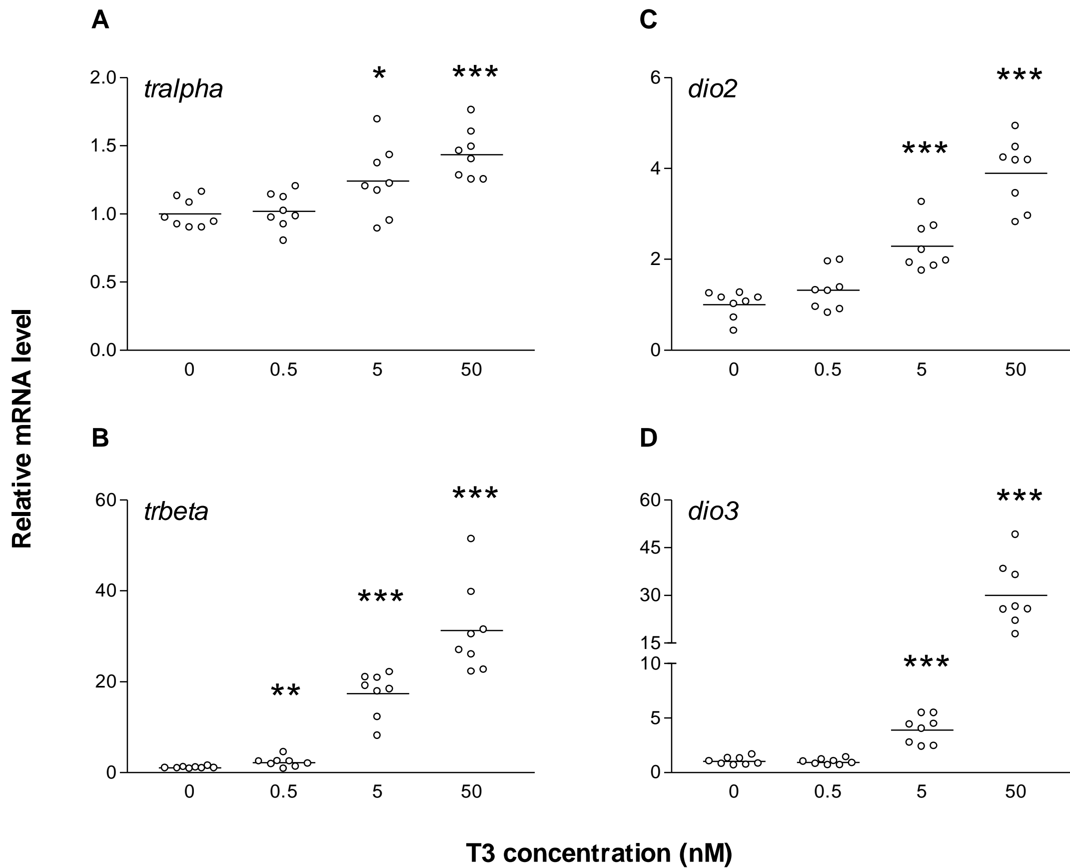


Figure 7.4. Effects of T3 exposure on the expression of thyroid hormone-related genes in the brain of *Engystomops pustulosus*. Premetamorphic tadpoles (G 32-34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h and transcript levels of *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) were measured in the brain. Data are presented as fold changes relative to control and are normalised to RNA content. Individual sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; n=8; * $p<0.05$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

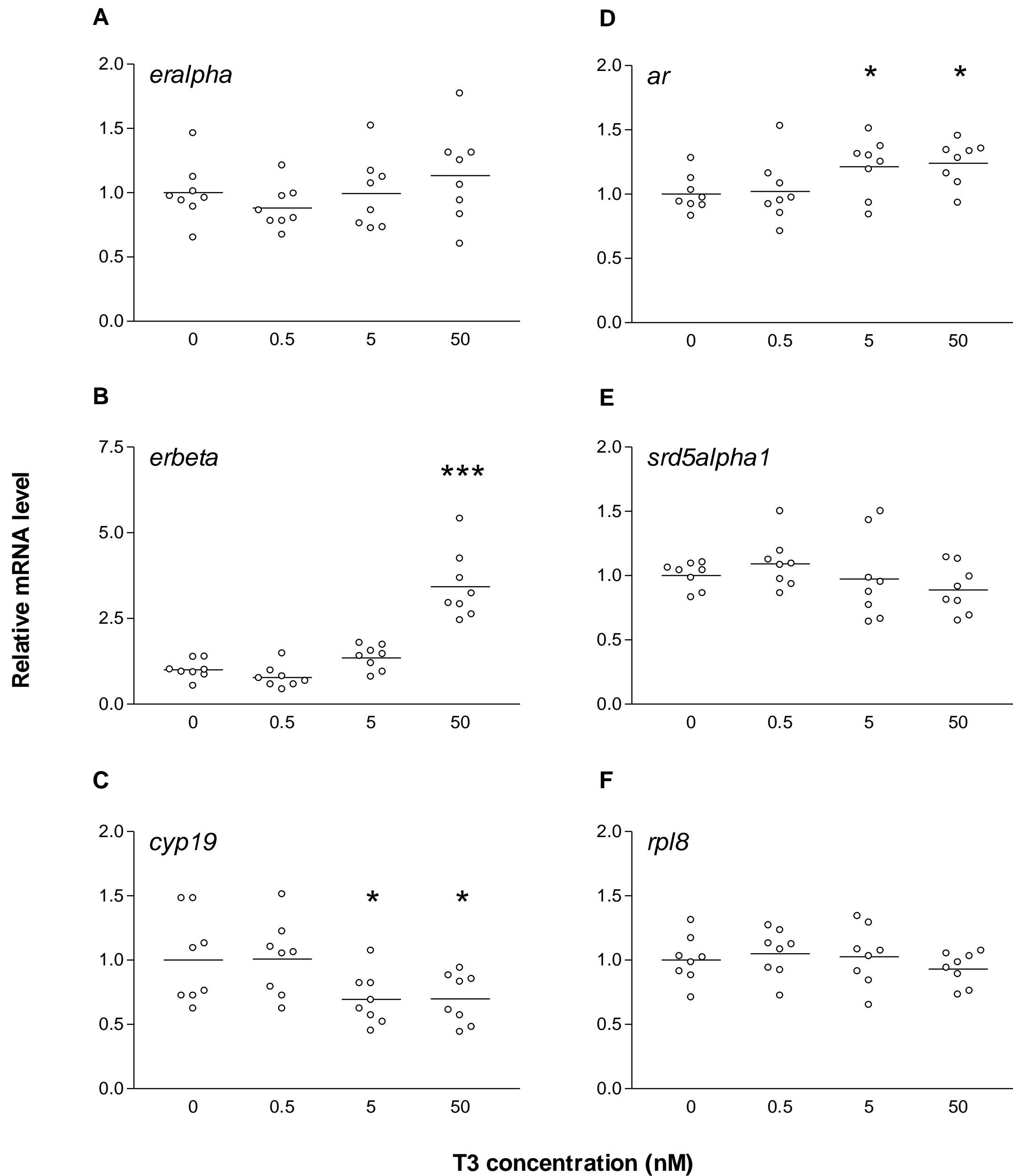


Figure 7.5. Effects of T3 exposure on the expression of sex steroid-related genes in the brain of *Engystomops pustulosus*. Premetamorphic tadpoles (G 32-34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h and transcript levels of *eralpha* (A), *erbeta* (B), *cyp19* (C), *ar* (D), and *srd5alpha1* (E) were measured in the brain. Results for the reference gene *rpl8* (F) are also presented. Individual sample points are presented along with the mean. Data are presented as fold changes relative to control and are normalised to RNA content. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; n=8; * $p<0.05$; *** $p<0.001$). Note that the scales of the y-axis vary among genes.

7.4. Discussion

7.4.1. Developmental profiles and T3 regulation of thyroid hormone-related genes

To the best of my knowledge, this is the first time that TH- and sex steroid-related transcripts have been measured during development in *E. pustulosus* and therefore, the discussion is directed at comparing the developmental profiles with published data for other anuran species. The profiles of *tralpha*, *trbeta*, and *dio3* during metamorphosis in *E. pustulosus* are similar to the trends observed in the brain of other anurans: *X. laevis* (Krain and Denver, 2004), *L. pipiens* (Hogan et al., 2007), and *S. tropicalis* (Chapter 5; Wang et al., 2008). Expression of *tralpha*, *trbeta*, and *dio3* increased during development and after T3 exposure suggesting that expression during development is regulated by rising TH levels. The induction of *trbeta* by T3 in *E. pustulosus* (Fig. 7.4B) has been observed in the brains of all studied premetamorphic tadpoles (*L. catesbeianus*: Helbing et al., 2006b; *L. pipiens*: Hogan et al., 2007; *X. laevis*: Krain and Denver, 2004; *S. tropicalis*: Chapter 5, Wang et al., 2008). This indicates that the autoinduction of *trbeta* by THs (Tata, 1994) is also present in *E. pustulosus*. Similarly, *dio3* was also induced by T3 in the *E. pustulosus* tadpole brain (Fig. 7.4D), which suggests the presence of a TH responsive element (TRE) in the *dio3* promoter, as has been found in *X. laevis* and *L. catesbeianus* (Becker et al., 1995; St-Germain et al., 1994).

In *E. pustulosus*, *dio2* remained relatively constant during development which is similar to results in two species of Ranidae (Becker et al., 1997; Hogan et al., 2007) but different from two species of Pipidae (Cai and Brown, 2004; Chapter 5). Becker et al. (1997) found that dio2 activity did not change in the brain during development of *L. catesbeianus* tadpoles and *dio2* mRNA did not change at metamorphic climax relative to premetamorphosis in *L. pipiens* (Hogan et al., 2007). However, at metamorphic climax, *dio2*

decreases in the brain of *X. laevis* (Cai and Brown, 2004) and *S. tropicalis* (~5-fold relative to premetamorphosis; Chapter 5, Fig. 5.1). On the other hand, the positive regulation of *dio2* by T3 is similar to what has been observed in other anurans (Bonett et al., 2010; Brown, 2005; Hogan et al., 2007; Chapter 5). Since endogenous THs increase during natural metamorphosis (Tata, 2006), comparing the developmental profiles to the T3 exposure results helps elucidate whether endogenous THs are important regulators of gene expression. In the case of *dio2,* expression increased after T3 treatment but remained constant during metamorphosis. These results suggest that additional factors regulate *dio2* levels in the brain during development. A possible candidate is prolactin, suggested to be an inhibitory factor of metamorphosis by counteracting the effects of THs and by promoting growth (reviewed in Shi, 2000). In *X. laevis* tadpoles, prolactin regulates the expression of another deiodinase, *dio3*, in tail and liver (Shintani et al., 2002) and it blocks the autoinduction of *trbeta* (Baker and Tata, 1992). Future research should investigate the possibility of prolactin controlling *dio2* expression in the tadpole brain.

7.4.2. Developmental profiles and T3 regulation of sex steroid-related genes

All the sex steroid-related transcripts were detected in the brain of *E. pustulosus* tadpoles, with the exception of *sdr5alpha2*. In contrast, *sdr5alpha2* mRNA has been detected during development in the brain of *S. tropicalis* (Chapter 5) and *X. laevis* (Urbatzka et al., 2007). The differences between the species could suggest that *srd5alpha2* expression patterns or biological activities differ between anurans. Previous attempts to clone *srd5alpha2* in *L. pipiens* (V. Langlois, pers. comm.) and *Lithobates sylvaticus* (L. Navarro-Martin, pers. comm.) have not succeeded, and to date no other sequences have been published for any other frog species. This could mean that the expression of *srd5alpha2* is

very low in these species and therefore difficult to amplify using degenerate primers. Indeed, *E. pustulosus srd5alpha2* could only be cloned using gDNA. In contrast, *srd5alpha1* was detected in the tadpole brain during metamorphosis. The regulation of *srd5alpha1* and *srd5alpha2* in the brain differs between species. In *S. tropicalis*, both *srd5alpha1* and *srd5alpha2* increased with T3, though *srd5alpha2* increased more dramatically (Chapter 5; Fig. 5.4). This is different from *E. pustulosus*, where brain *srd5alpha1* was not affected by T3. Future research should attempt to clone the full sequence of the *srd5alpha1* and *srd5alpha2* genes in *E. pustulosus* to better understand the differences in expression and regulation of these enzymes in frogs.

In *E. pustulosus* at stage G42, the sexes did not differ in brain transcript levels, similar to previous observations in *S. tropicalis* (Chapter 5) and *L. pipiens* metamorphs (Appendix D; Fig. A1). However, in adults, brain sex differences in the level of expression of *eralpha*, *erbeta* and *ar* have been observed in *E. pustulosus* (Chakraborty and Burmeister, 2010) and *erbeta* and *ar* in *S. tropicalis* (Chapter 5). These studies suggest that sexually dimorphic expression of sex steroid receptors only develops after metamorphosis is complete. Dimorphism in receptor expression in sexually mature frogs may contribute to the regulation of sex-specific behaviours (Chakraborty and Burmeister, 2010).

The developmental profiles and magnitude of changes of *eralpha*, *erbeta*, *ar* and *cyp19* were very similar between *E. pustulosus* and *S. tropicalis* (Chapter 5). For example, at metamorphic climax, *eralpha* increased 5-fold in *E. pustulosus* and 6-fold in *S. tropicalis*, relative to premetamorphic levels. In these two species, *cyp19* increased the least, only 1.7-fold in *E. pustulosus* and 2-fold in *S. tropicalis* at metamorphic climax relative to premetamorphosis. Expression of *erbeta* increased the most among sex steroid-related genes, with 35- and 150-fold at metamorphic climax relative to premetamorphosis in *E. pustulosus*

and *S. tropicalis*, respectively. In both species, there are marked differences in the profiles of *eralpha* and *erbeta* suggesting that each gene is differentially regulated during development. In amphibian species, *eralpha* and *erbeta* respond differently to a range of estrogens, indicating different ligand sensitivities in the autoregulation of these receptors (Katsu et al., 2010).

Even though the developmental profiles of sex steroid-related genes were overall very similar between species, their regulation by T3 seems to be more complicated. Using the same exposure protocol, I observed that T3 increases brain *eralpha* and *erbeta* in *S. tropicalis* (Chapter 5) and Hogan et al. (2007) observed an increase in brain *eralpha* in *L. pipiens*. Previously, it was suggested that T3 contributes to the increase in *eralpha* mRNA during development, in *S. tropicalis* and *L. pipiens* (Chapter 5; Hogan et al., 2007). In *E. pustulosus*, T3 increased *erbeta* but not *eralpha* expression which suggests that T3 does not have the same regulatory effect in this species. These results indicate that although expression patterns of *eralpha* during development are conserved across anuran species, the regulatory controls may differ. Indeed, Katsu et al. (2010) found that sensitivity of *er* to natural and synthetic ligands differs between amphibian species.

Treatment with T3 resulted in reduced expression of *cyp19* in the brain of *E. pustulosus* of the same magnitude as observed in *L. pipiens* (Hogan et al., 2007). In mammals, T3 suppresses Cyp19 mRNA and activity in gonad *in vitro* cultures (rats: Ando et al., 2001, Hatsuta et al., 2004, Ulisse et al., 1994; mice: Catalano et al., 2003, Cecconi et al., 1999; pigs: Gregoraszczuk et al., 1998). In mammals, one possible mechanism of TH action on *Cyp19* mRNA is competition between TH receptors (TR) and steroidogenic factor-1 (SF-1) to bind to the SF-1 response element, present in the *Cyp19* promoter (Catalano et al., 2003). In addition, TR can also compete with ER for the same estrogen responsive element

(ERE) to repress or induce transcription of some genes (reviewed in Vasudevan and Pfaff, 2005). To date, the promoter of the *cyp19* gene has been analysed only in *X. laevis* and *G. rugosa*. In *X. laevis*, the promoter contains two putative ERE half sites and a putative sf-1 binding site (Akatsuka et al., 2005); while in *G. rugosa*, only a putative sf-1 binding site has been reported (Oshima et al., 2006). Therefore, competition between tr and sf-1 and/or tr and er represent possible mechanisms regulating *cyp19* expression in anurans. However, since *S. tropicalis cyp19* was not affected by T3 (Chapter 5), there remains the possibility of a different mechanism at work in *E. pustulosus* and *L. pipiens*.

An increase in *ar* levels after T3 exposure was observed in the brain of *E. pustulosus*. Previously, I observed positive regulation of *ar* by T3 in *S. tropicalis* whole body larvae (Chapter 4) and in the gonad-mesonephros complex (Chapter 6). In *E. pustulosus*, it is possible that reduction of *cyp19* results in the accumulation of testosterone, which is also the substrate of srd5alpha enzymes. This could potentially increase the synthesis of 5α-dihydrotestosterone, known to autoregulate the expression of *AR* in lizards and hamsters (Cardone et al., 1998; Esposito et al., 2002). Indeed, this hypothesis is supported by data in Chapter 2. I found that inhibition of cyp19 by fadrozole resulted in an increase in the expression of the androgen-related genes, *srd5alpha2* and *ar*, in *S. tropicalis* whole body larvae (Chapter 2).

In conclusion, developmental expression and regulation of TH-related genes in *E. pustulosus* is similar to other species from divergent anuran families. This gives direct support to the hypothesis that THs control metamorphosis in all frog species. Profiles of sex steroid-related genes were generally comparable among anurans, with the important exception of the regulation of sex steroid-related genes by T3. Even though the developmental profiles of these transcripts are similar, the regulatory controls seem to be

different because T3 did not have the same effects in the various species tested. In this research, I sought to provide basic data on *E. pustulosus*. This represents an important contribution to the establishment of this phylogenetically divergent Neotropical species as a new laboratory model for studies on metamorphosis, sexual development and endocrine disruption. A model for TH action in the frog brain is presented and discussed in Chapter 9.

CHAPTER 8

Developmental profiles and T3 regulation of thyroid hormone- and sex steroid-related genes in *Engystomops pustulosus*
Part II: The gonad

Adapted from:

Duarte-Guterman P[1], Ryan MJ[2], Trudeau VL[3]. Developmental profiles and T3 regulation of gonad-mesonephros transcripts during metamorphosis of the Neotropical frog *Engystomops* (*Physalaemus*) *pustulosus*. *In preparation*.

[1] Designed and performed research, analysed the data, and wrote the manuscript
[2] Provided animals, tadpole rearing equipment, and expertise and contributed to the revision of the manuscript
[3] Contributed to the design of the research and the revision of the manuscript

8.1. Introduction

In amphibians, gonadal (sex) differentiation is influenced by endogenous sex steroids (estrogens and androgens). Both exposure to sex steroids (e.g., Bogi et al., 2002; Hogan et al., 2008) and inhibition of sex steroids synthesis (e.g., Langlois, 2010; Chapter 3) affects gonadal development and sex ratios in frogs. However, the molecular mechanisms that control differentiation of the gonad are not completely understood. The enzyme aromatase (cyp19) seems to be a key regulator of ovarian development in some frogs (e.g., Maruo et al., 2008; Urbatzka et al., 2007; Chapter 6), whereas there is no consensus about the genes driving testicular development. *Silurana tropicalis* has many advantages as a laboratory organism, however one important limitation is the inability to sex tadpoles by visual inspection because the gonads are small and translucent (Chapter 6). This limitation reduces the extent of analysis and conclusions about the mechanisms of gonadal differentiation. On the other hand, in *Engystomops pustulosus*, the morphology of the gonad is visible at metamorphic climax (G42; Chapter 7); therefore, *E. pustulosus* may be an amenable anuran species to examine gene expression in the GMC during development.

In some frogs, fish and mammals, thyroid hormones (THs) are thought to be involved in testicular development and function (Goleman et al., 2002; Mukhi et al., 2007; Wagner et al., 2008); still, the mechanisms of action of TH action in the gonad have only been investigated in one frog species, *S. tropicalis* (Chapter 6). In the gonad-mesonephros complex (GMC) of premetamorphic tadpoles, triiodothyronine (T3) increased expression of androgen- related genes (steroid 5α-reductase enzymes, *srd5alpha1* and *srd5alpha2*) involved in the synthesis of 5α-dyhydrotestosterone (5α-DHT) while decreasing expression of one of the estrogen receptors (*erbeta*), results that are in agreement with the masculinising effect of THs in frogs. However, there are species differences in the regulation of genes by T3 in the tadpole brain (Chapter 7) suggesting that the effects of T3 in the GMC might also be different between anuran species.

In this study, I first established transcript profiles of TH- and sex steroid related genes in the GMC during development to identify genes associated with gonadal development in *E. pustulosus*. In the second part of this project, I assessed the regulation of TH- and sex steroid related genes by T3 in *E. pustulosus* and directly compared the results to *S. tropicalis* (Chapter 6).

8.2. Materials and methods

8.2.1. Animals and tissue collection for developmental profiles

Fertilised eggs of *E. pustulosus* were obtained from three pairs of frogs from a colony at the University of Texas at Austin (see Chapter 7) and the staging followed the Gosner (G) developmental table (Gosner, 1960). The equivalent stages under the Nieuwkoop-Faber (NF; Nieuwkoop and Faber, 1994) staging method are provided for comparison in Appendix A. Gonad-mesonephros complex with surrounding muscle tissue (GMC) was dissected from the

same individuals in Chapter 7 at stages G30 (premetamorphosis; NF51), G36 (prometamorphosis; NF55), and G42 (metamorphic climax; NF62). GMC samples were preserved in RNAlater as described by the manufacturer (Ambion) and analysed individually for all stages of development (n=6-10). All the experiments were done at the University of Texas at Austin. Animal care and treatment protocols were approved by the Institutional Animal Care and Use Committee, University of Texas at Austin (IACUC protocol no. 08101701).

8.2.2. Triiodothyronine exposure

The same tadpoles (G 32-34 equivalent to NF 53-54) as in Chapter 7 were exposed to three nominal concentrations of T3 (0.5, 5, 50 nM; Sigma) or a dimethyl sulfoxide (DMSO; Sigma) solvent control for 48 h. At the end of the exposure, GMC were dissected, preserved in RNAlater (Ambion) and pooled (two GMC per pool; n=8 pools) before RNA isolation.

8.2.3. RNA isolation and cDNA synthesis

Total RNA extraction and cDNA synthesis procedures are identical to the ones described in Chapter 7, with the exception that GMC samples were homogenised for 4 min.

8.2.4. Real-time RT-PCR

Simplex real-time RT-PCR assays described in Chapter 7 were used to measure mRNA levels of estrogen receptors (*eralpha*, *erbeta*), aromatase (*cyp19*), androgen receptor (*ar*), TH-receptors (*tralpha*, *trbeta*), deiodinases (*dio2*, *dio3*), steroid 5α-reductases (*srd5alpha1*, *srd5alpha2*), doublesex and mab-3 related transcription factor 1 (*dmrt-1*) and ribosomal protein L8 (*rpl8*) in the GMC during development and after T3 exposure. Samples

were run in duplicate using the Mx3005P real-time PCR System (Stratagene) along with negative template controls where RNase-free water was added to the reaction instead of the template and negative reverse transcriptase controls where RNase-free water was added to the cDNA synthesis reaction instead of the enzyme.

Relative mRNA levels of target genes within each sample were obtained using the relative standard curve method. The standard curves were generated using equal parts of cDNA of G42 male and female samples (for the developmental profiles), and using equal parts of cDNA from each treatment (for the T3 exposure). Reaction efficiencies were 90-110% with an $R^2 \geq 0.990$. Expression of the reference gene *rpl8* decreased significantly during development and after T3 exposure (data not shown). Therefore, data for the developmental profiles are presented as fold change relative to stage G30 and normalised to RNA content and for the T3 exposure data are presented relative to the control group and normalised to RNA content (Huggett et al., 2005).

8.2.5. Statistical analyses

All statistical analyses were carried out using S-Plus (Insightful Corporation) with significance set at $p<0.05$. At stages G30 and 36, the gonads are very small and translucent; therefore, a morphological sex could not be assigneds by visual inspection at these early stages. In order to examine differences between sexes early during development, it was necessary to predict the sex of the tadpoles using a statistical method. A cluster analysis (using k-means) of *cyp19* was used to divide the data into two groups and assign one of the two sexes (putative male or female) to each tadpole at each stage of development (Insightful Corporation, 2007). Using these putative (G30 and 36) and morphological sexes (G42), differences in the expression of all the genes in the GMC with regards to sex and stage of

development were examined using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparisons test. In the case of *dio3*, the ANOVA assumptions could not be achieved; therefore data was analysed using the Scheirer-Ray-Hare test (non-parametric equivalent of a two-way ANOVA; Dytham, 2003) and differences between stages were analysed using the non-parametric Wilcoxon test (alpha value was adjusted using the Bonferroni correction for multiple comparisons). Finally, using the putative and morphological sexes, a discriminant analysis was performed to investigate which transcripts or combination of transcripts in the GMC could predict sexes in *E. pustulosus* tadpoles (Blázquez et al., 2009; Insightful Corporation, 2007). For the T3 exposure, statistical differences between treatment and control groups were examined using one-way ANOVA as described in Chapter 7.

8.3. Results

8.3.1. Developmental profiles and sex differences in the GMC

Developmental profiles of sex steroid- and TH-related genes were established in the GMC during tadpole development by sampling at three different stages: G30 (premetamorphosis), G36 (prometamorphosis), and G42 (metamorphic climax) (Figs. 8.1-8.3). The main morphological characteristics and changes over development are shown in Fig. 7.2. With the exception of *srd5alpha2*, all genes were detected in the GMC at each of the three stages of development. Levels of *srd5alpha2* were very low (real-time RT-PCR threshold cycle, C_t>37) and could not be measured using real-time RT-PCR.

Stage G42 tadpoles were visually sexed prior to sampling for gene expression. In the GMC, *cyp19* mRNA levels were clearly sexually dimorphic (250-fold greater in females compared to males; $p<0.001$) and the cluster analysis confirmed that the morphological sexes

belonged to two clearly differentiated groups (Fig. 8.1A). Because at G42, sexes were determined by *cyp19* levels, each tadpole at G30 and 36 was assigned a putative sex using the results of the cluster analysis of *cyp19*. Assigned and morphological sexes were used to test for sex and stage differences in all of the target genes (two-way ANOVA results are presented in Table 8.1). For *cyp19*, each sex showed different trajectories: female *cyp19* mRNA increased during development, while in males, it decreased. In the case of *srd5alpha1* (Fig. 8.2A), while mRNA levels remained relatively constant during metamorphosis, significant differences were detected between the sexes at stage G36 and 42, with higher levels in females relative to males (1.4-fold for both stages; ANOVA; $p<0.05$; Fig. 8.2A). In contrast, *eralpha* (1.7- and 6.0-fold; Fig. 8.1B), *dmrt-1* (3.5- and 7.0-fold; Fig. 8.2C), and *trbeta* (2.0- and 30-fold; Fig. 8.3B) increased with each developmental stage. In the case of *erbeta* (Fig. 8.1C), *ar* (Fig. 8.2B) and *dio3* (Fig. 8.3D), mRNA levels for both males and females remained relatively constant between stages G30 and 36 and only increased significantly at G42. Levels of *erbeta* increased 270-fold, *ar* 4.0-fold, and *dio3* 50-fold at stage G42 relative to G30. In the case of *eralpha*, even though mRNA levels in both males and females increased as development progressed, sex differences were also detected at all three stages of development (Fig. 8.1B), with higher *eralpha* mRNA levels in females compared to males (G30: 1.7-fold; G36: 2.8-fold; G42: 1.8-fold; $p<0.05$; Fig. 8.1B). The profile of *dio2* was unique among these genes, as levels for both sexes decreased from stage G30 to 36 (2.0-fold) and then increased from G36 to 42 (7.5-fold; Fig. 8.3C). Only *tralpha* (Fig. 8.3A) showed no appreciable change during development at the stages analysed. From these results, the genes that showed the maximum expression changes during development were *erbeta* followed by *dio3* and *trbeta*.

The assigned and morphological sexes were then used in a discriminant analysis to determine which genes, or which combination of genes, could be used as sex markers in *E. pustulosus* tadpoles (Table 8.2). Of the nine transcripts (not including *cyp19*), only *eralpha* and *srd5alpha1*, significantly predicted sex, each one alone and in combination with each other (Table 8.2). None of the TH-related genes predicted sex better than expected by chance (i.e., 50% sexing accuracy; $p>0.4$; data not shown). Expression of *eralpha* correctly predicted sexes in 60% of cases, while *srd5alpha1* predicted 75% of cases for both sexes. In no case was a combination of genes more effective than the individual genes alone at predicting sexes. When taking stage of development into account, sexing accuracy using *eralpha* improved from 60% to 87% for both sexes, while accuracy using *srd5alpha1* remained relatively similar (81% compared to 75% for both sexes).

8.3.2. Effects of T3 exposure in the GMC

Premetamorphic tadpoles (G 32-34) were exposed to T3 for 48 h to determine which transcripts are regulated by THs in the GMC. Exposure to T3 affected the expression of two sex steroid-related transcripts (Fig. 8.4), *erbeta* and *srd5alpha1* (*erbeta*: $F_{3,28}=18.59$; $p<0.001$; *srd5alpha1*: $F_{3,28}=4.12$; $p=0.015$) and the four TH-related genes measured (Fig. 8.5; *tralpha*: $F_{3,28}=20.31$; $p<0.001$; *trbeta*: $F_{3,28}=294.96$; $p<0.001$); *dio2*: $F_{3,28}=40.07$; $p<0.001$; and *dio3*: Kruskal-Wallis $\chi^2= 28.10$; $p<0.001$). Levels of *erbeta* significantly increased at 5 and 50 nM T3 relative to control (2.8- and 6.5-fold respectively; post-hoc test $p<0.01$; Fig. 8.4C), while *srd5alpha1* mRNA significantly decreased at 50 nM T3 (1.3-fold; post-hoc test $p<0.05$; Fig. 8.4D). Exposure to 5 and 50 nM T3 resulted in significant increases in *tralpha* (1.7- and 1.9-fold; post-hoc test $p<0.001$; Fig. 8.5A) and *dio2* (2.3- and 4.0-fold; post-hoc test $p<0.001$; Fig. 8.5C). Exposure to T3 (all three concentrations) resulted in increases in

trbeta (2.8- to 22-fold; post-hoc test $p<0.001$; Fig. 8.5B) and *dio3* (2.0- to 87-fold; post-hoc test $p<0.01$; Fig. 8.5D). Levels of *cyp19* ($F_{3,28}=1.93$; $p=0.15$; Fig. 8.4A), *eralpha* ($F_{3,28}=1.75$; $p=0.19$; Fig. 8.4B), *ar* ($F_{3,28}=0.78$; $p=0.51$; Fig. 8.4E), and *dmrt-1* ($F_{3,28}=0.12$; $p=0.94$; Fig. 8.4F) were not affected by exposure to T3.

Table 8.1. Two-way analysis of variance (ANOVA) results for the developmental profiles in *Engystomops pustulosus*. F-statistics and p-values are shown for the main factors (stage of development and sex) and the interaction term (stage:sex) for each gene.

Target	Stage		Sex		Stage:Sex	
	$F_{2,27}$	p-value	$F_{1,27}$	p-value	$F_{2,27}$	p-value
cyp19	4.59	0.019	523.58	<0.0001	63.74	<0.0001
eralpha	127.12	<0.0001	48.05	<0.0001	2.15	0.14
erbeta	456.26	<0.0001	1.48	0.23	2.90	0.072
srd5alpha1	13.03	<0.0001	22.09	<0.0001	0.39	0.68
ar	262.46	<0.0001	5.01	0.33	0.95	0.40
dmrt-1	79.48	<0.0001	2.26	0.14	3.20	0.057
tralpha	12.93	<0.001	0.42	0.52	0.28	0.75
trbeta	643.29	<0.0001	1.74	0.19	2.28	0.12
dio2	92.08	<0.0001	0.03	0.87	0.19	0.82
dio3	22.76*	<0.0001	0.64*	0.42	0.41*	0.82

* H-statistic (Scheirer-Ray-Hare test; Dytham, 2003)

Figure 8.1. Developmental profiles of estrogen-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. Transcript levels of *cyp19* (A), *eralpha* (B), and *erbeta* (C) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC samples are presented along with the grand mean (B, C). Grey circles (A) denote the two groups found with the cluster analysis of *cyp19* (each circle is centered on the mean of each group). Different letters indicate statistically significant differences between developmental stages and between sexes (two-way ANOVA; n=6-10; $p<0.05$; Table 8.1). In the case of *erbeta*, no differences were detected between sexes at any stage of development. Note that the logarithmic scales of the y-axis vary among genes.

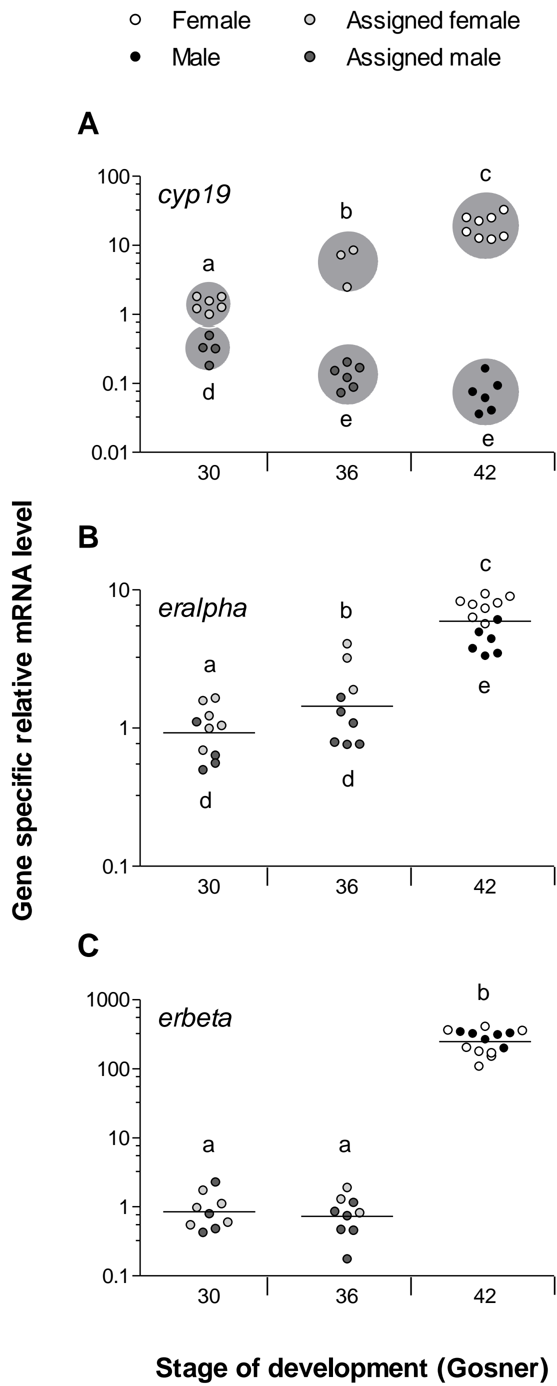


Figure 8.1. (caption on previous page)

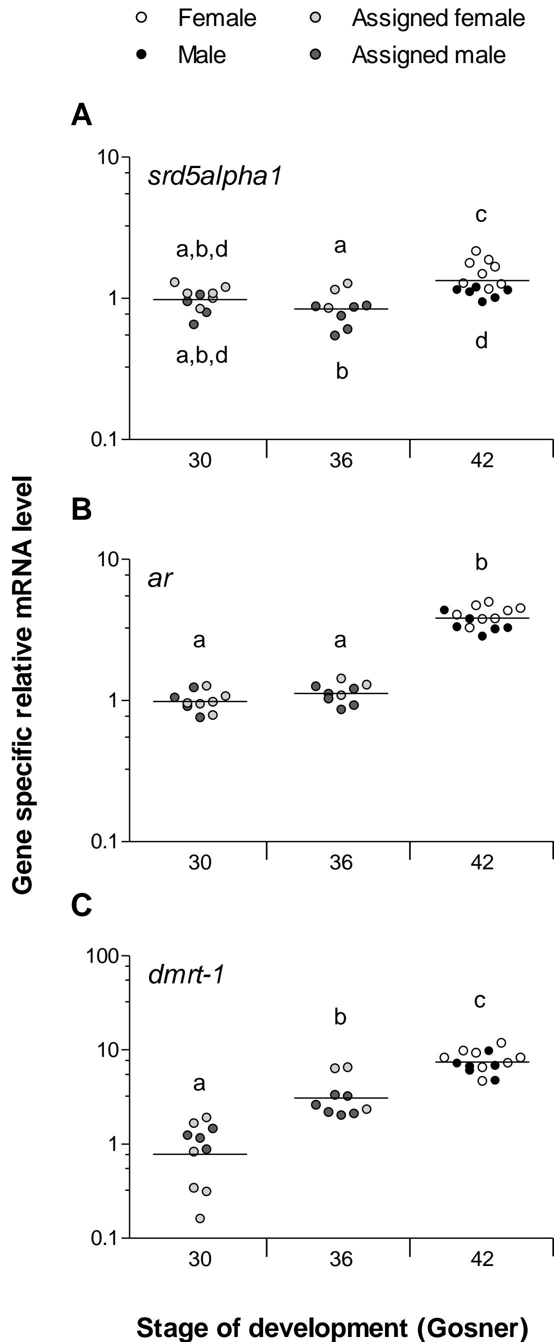


Figure 8.2. Developmental profiles of sex differentiation- and androgen-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. Transcript levels of *srd5alpha1* (A), *ar* (B), and *dmrt-1* (C) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC sample are presented along with the grand mean. Different letters indicate statistically significant differences between developmental stages and between sexes (two-way ANOVA; n=6-10; $p<0.05$; Table 8.1). In the case of *ar* and *dmrt-1*, no differences were detected between sexes at any stage of development. Note that the logarithmic scales of the y-axis vary among genes.

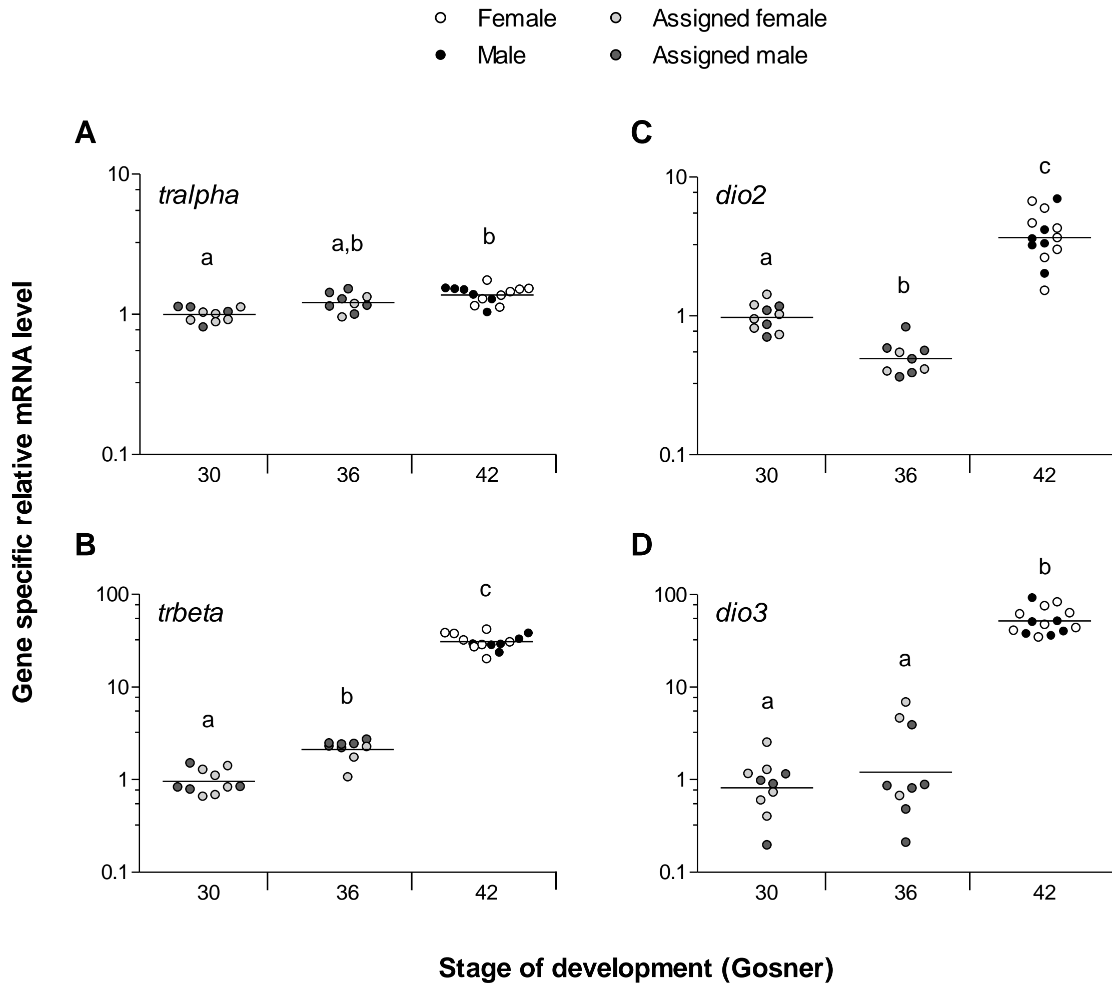


Figure 8.3. Developmental profiles of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) during *Engystomops pustulosus* metamorphosis. Transcript levels of *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) were measured from stage G30 until 42. Levels of mRNA are expressed relative to stage G30 and are normalised to RNA content. Individual GMC samples are presented along with the grand mean. Different letters indicate statistically significant differences between developmental stages. In the case of TH-related genes, no differences were detected between sexes at any stage of development (two-way ANOVA; n=6-10; $p<0.05$; Table 8.1). Note that the logarithmic scales of the y-axis vary among genes.

Table 8.2. Results of the discriminant analysis to determine sexing accuracy using stage of development, and individual and combined GMC transcript levels in *Engystomops pustulosus*. Sexes were assigned using a cluster analysis of *cyp19* levels and by visual inspection at G42.

Variable(s) in the model	Wilks' λ	*F* statistic	*p*-value	Sexing Accuracy	
				Females	**Males**
stage	0.94979	0.793	0.462	82% (14/17)	37.5% (6/16)
eralpha	0.8424	5.799	**0.022**	58.8% (10/17)	62.5% (10/16)
erbeta	0.97757	0.688	0.413	50% (8/16)	62.5% (10/16)
srd5alpha1	0.63933	17.488	**<0.001**	82% (14/17)	68.8% (11/16)
dmrt-1	0.9884	0.364	0.551	58.8% (10/17)	50% (8/16)
ar	0.96988	0.963	0.334	47% (8/17)	62.5% (10/16)
eralpha+stage	0.3859	15.384	**<0.001**	94% (16/17)	81.2% (13/16)
srd5alpha1+stage	0.51481	9.1104	**<0.001**	76.5% (13/17)	87.5% (14/16)
eralpha+srd5alpha1	0.63713	8.543	**0.0012**	82% (14/17)	68.8% (11/16)
eralpha+srd5alpha1+stage	0.3180	15.011	**<0.001**	88.2% (15/17)	81.2% (13/16)

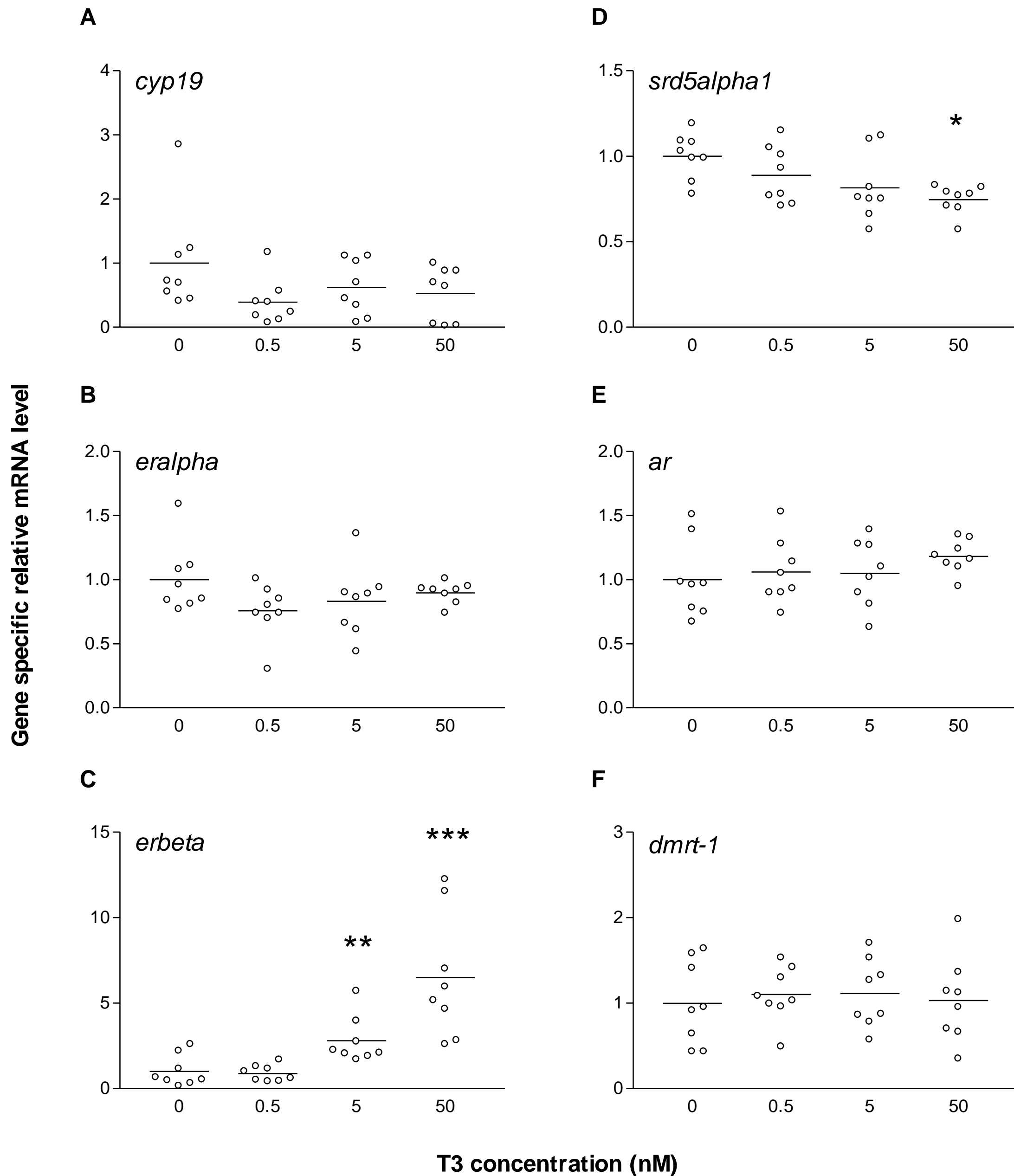


Figure 8.4. Effects of T3 exposure on the expression of sex differentiation- and sex steroid-related genes in the gonad-mesonephros complex (GMC) of *Engystomops pustulosus*. Premetamorphic tadpoles (G 32-34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *cyp19* (A), *eralpha* (B), *erbeta* (C), *srd5alpha1* (D), *ar* (E), and *dmrt-1* (F) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; two GMC per pool; n=8 pools; * $p<0.05$; ** $p<0.01$; *** $p<0.001$). Note that the scales of the y-axis vary between genes.

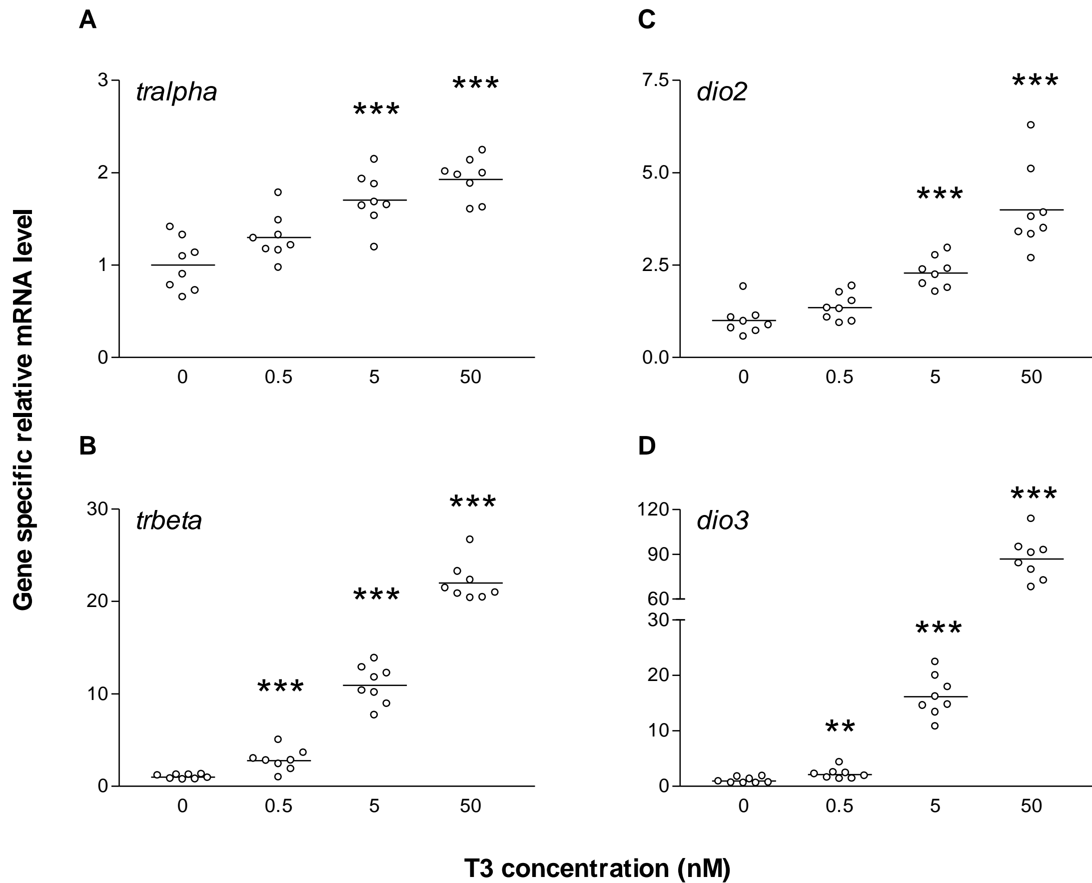


Figure 8.5. Effects of T3 exposure on the expression of thyroid hormone-related genes in the gonad-mesonephros complex (GMC) of *Engystomops pustulosus*. Premetamorphic tadpoles (G 32-34) were exposed to T3 (0, 0.5, 5, 50 nM) for 48 h. Effects of T3 on *tralpha* (A), *trbeta* (B), *dio2* (C), and *dio3* (D) are presented. Data are presented as fold changes relative to control and are normalised to RNA content. Sample points are presented along with the mean. Asterisks represent significant differences in mRNA levels from the control group (one-way ANOVA; two GMC per pool; n=8 pools; *** $p<0.001$). Note that the scales of the y-axis vary between genes.

8.4. Discussion

8.4.1. Developmental profiles and identification of sex markers in the GMC

Sex steroids have important effects on gonadal development in anurans; however, the molecular mechanisms and the sex differentiation genes involved are not completely understood and have only been studied in few frog species. In this study, sex steroid-related genes were measured in the GMC during tadpole development in the Neotropical frog *E. pustulosus*. At G42, the gonads were clearly visually differentiated into either male or female; therefore, gonadal differentiation in *E. pustulosus* seems to take place during tadpole development. This is also the case of frog species from the Pipidae and Ranidae families, for which histological analyses demonstrate that gonadal differentiation is complete at meta-morphic climax, and in many cases differentiation takes place during premetamorphosis between stage G26 and 36 (*S. tropicalis*: El Jamil et al., 2008; *Xenopus laevis*: Kelley, 1996; *Rana temporaria*: Ogielska and Kotusz, 2004; *Lithobates pipiens*: Richards and Nace, 1978).

Three transcripts (i.e., *cyp19*, *eralpha,* and *srd5alpha1*) in the GMC were identified as potential molecular markers of ovarian development and of the sex of a tadpole. At G42, *cyp19* mRNA was significantly higher in females than in males (~250-fold difference). The cluster analysis demonstrated that *cyp19* levels in the GMC were also sexually dimorphic as early as G30, a time when the gonads are not yet visible. In *S. tropicalis*, *cyp19* levels in the GMC showed significant variation between individuals (Chapter 6); however, morphological confirmation of the sexes was not available for comparison. This significant sexually dimorphic pattern of expression has been observed in other frogs; in *Glandirana rugosa*, *Lithobates sylvaticus*, and *X. laevis*, *cyp19* mRNA levels are significantly higher in females compared to males during tadpole development (Maruo et al., 2008; Navarro-Martin, unpublished results; Urbatzka et al., 2007; respectively). In addition, in *G. rugosa*, cyp19

activity has been confirmed to be higher in undifferentiated gonads of females compared to males (Isomura et al., 2011). An important difference among the frogs studied to date is the magnitude of the difference in *cyp19* levels between the sexes. At metamorphic climax, sex differences in *cyp19* are in the order of 10-fold in *X. laevis* (Urbatzka et al., 2007), 100-fold in *L. sylvaticus* (Navarro-Martin, unpublished results), and 250-fold in *E. pustulosus*. In sexually mature *S. tropicalis* adults, sex differences in *cyp19* are in the 50-fold range (Chapter 6). The physiological significance of these differences remains to be fully elucidated. Nonetheless, these studies strongly suggest that levels of *cyp19* mRNA can be used as a reliable indicator of the sex of a tadpole in different frog species from different families. This is not surprising since levels of gonadal *cyp19* seem to be required for ovarian differentiation across vertebrates, including in fish (Blázquez et al., 2008; Guiguen et al., 1999), newts (Kuntz et al., 2003), turtles (Ramsey et al., 2007), and birds (Kamata et al., 2004). Interestingly, a key role for cyp19 may set the anurans apart from other vertebrates (i.e., fish, chickens and mammals) and invertebrates (i.e., flies and nematodes). In the latter, homologous genes to the anuran *dmrt-1* have been found to be involved in testicular differentiation (Koopman, 2009). During anuran gonadal development, *dmrt-1* mRNA is not sexually dimorphic in species from different families, *G. rugosa* (Matsushita et al., 2007), *Rhinella marina* (Abramyan et al., 2009), *S. tropicalis* (Chapter 6), and *E. pustulosus*, suggesting that *dmrt-1* may not be involved in testicular differentiation in anurans. However, this does not necessary exclude *dmrt-1* from having a role in testicular function in adults (see discussion Chapter 6).

Levels of *eralpha* mRNA expression were higher in females than in males (putative and morphological). Higher levels of *cyp19* in females could result in higher estrogen levels which in turn could increase *eralpha* transcription and estrogenic effects relative to males.

Indeed, in other frog species, *eralpha* is autoinduced by estrogens (*X. laevis*: Bogi et al., 2002; Tata et al., 1993; *L. pipiens*: Hogan, 2006), and this regulation is mediated by the presence of an estrogen responsive element (ERE) in the promoter region of the *eralpha* gene (Lee et al., 1995). These results suggest that sex differences in *eralpha* are under the influence of estrogen synthesis (i.e., cyp19).

5α-Dihydrotestosterone (5α-DHT) in anurans induces male gonadal differentiation (e.g., *X. laevis*: Bogi et al., 2002; *Lithobates clamitans*: Coady et al., 2004), but surprisingly, mRNA levels of *srd5alpha1*, one of the enzymes responsible of 5α-DHT synthesis, were significantly higher in females than in males at G36 and 42. Sex differences in *srd5alpha1* were also observed in adult gonads of *S. tropicalis* and expression was higher in ovaries than in testes (5-fold; Fig. 6.2, Chapter 6). However, no significant differences in *srd5alpha1* were reported during development in *G. rugosa* and *X. laevis* (Maruo et al., 2008; Urbatzka et al., 2007). Interestingly, similar to the data for *E. pustulosus* where I documented a statistically significant 1.4-fold difference, in *G. rugosa* premetamorphic tadpoles, *srd5alpha1* expression in the GMC is ~2-fold higher in females compared to males, although this was not statistically significant according to the authors (Maruo et al., 2008). Another function of the srd5alpha enzymes is the conversion of progesterone into 5α-reduced metabolites such as allopregnanolone, a neurosteroid also produced in peripheral organs such as the gonads and adrenal gland in rats (Corpechot et al., 1993). In mammals, allo-pregnanolone is widely studied for its effects on brain function, such as the effects on mood and depression (van Broekhoven and Verkes, 2003) and neurogenesis (Brinton and Wang, 2006). Interestingly, in rats, plasma levels of allopregnanolone are higher in females compared to males (Corpechot et al., 1993). Measuring steroid hormone production in the

tadpole gonad will be required to determine the function of higher *srd5alpha1* expression in female gonads with respect to males.

In *E. pustulosus*, expression of *srd5alpha2* was very low and it was not possible to measure it in the GMC. Similarly, in the brain, *srd5alpha2* mRNA was not detected during metamorphosis (Chapter 7). These results are in marked contrast to previous studies in *X. laevis* (Urbatzka et al., 2007) and *S. tropicalis* (Chapter 6), the only studies that have measured *srd5alpha2* in the GMC and brain in amphibians. This suggests that *srd5alpha2* expression and regulation in *E. pustulosus* is different to what has been observed in the two species of the Pipidae family, and further work is needed to elucidate both the conserved and potentially divergent roles of srd5alpha enzymes in anurans.

8.4.2. Effects of T3 on transcript levels in the GMC

The second question addressed in this study was whether T3 could regulate sex steroid- and TH-related genes in the GMC in *E. pustulosus*. During development, cyp19 seems to be a key regulator of gonadal differentiation (e.g., Chapter 3; Elbrecht and Smith, 1992; Fenske and Segner, 2004; Guiguen et al., 2010; Olmstead et al., 2009). In *E. pustulosus*, exposure to T3 did not statistically affect *cyp19* mRNA in the GMC, even though *cyp19* levels in the T3 treatment groups were on average lower with respect to control (between 1.6- and 2.6-fold). In order to compare results with *S. tropicalis*, I followed the same protocol as in Chapter 6; tadpoles were exposed to T3 between stages G32 and 34 and GMC samples were pooled for gene expression analysis. However, according to the developmental profile, *cyp19* mRNA is already sexually dimorphic by G30 in *E. pustulosus*. The high variation in *cyp19* expression within T3 treatments could be due to the effect of pooling GMC samples and may indicate sex differences in response to T3. In goldfish

gonads, T3 decreases the expression of gonadal *cyp19* but the effects and mechanisms are different depending on the sex of the fish (Nelson et al., 2010). Future research should take into account sex differences when studying T3 actions on the developing gonads of *E. pustulosus*.

Exposure to 50 nM T3 decreased *srd5alpha1* levels. This is exactly opposite to the observed changes in *S. tropicalis*, where higher *srd5alpha1* and *srd5alpha2* mRNA levels were observed after T3 (Chapter 6). The developmental profile of *srd5alpha1* in *E. pustulosus* suggests that in addition to synthesising androgens (i.e., converting testosterone into 5α-DHT), the enzyme may be involved in other reduction reactions, such as those involving progesterone. Females at G36 and 42 had a higher *srd5alpha1* expression relative to males. After T3 exposure, *srd5alpha1* mRNA is reduced in the GMC of premetamorphic tadpoles, suggesting that the GMC after T3 treatment resembles more a male than a female GMC. These results in combination with a low *cyp19* in certain tadpoles could indicate that T3 may also promote some aspects of masculinisation in *E. pustulosus*.

T3 exposure increased the expression of *erbeta* in the GMC of *E. pustulosus* (Fig. 8.4C) similar to the brain (Chapter 7), but in marked contrast to the decrease observed in the GMC in *S. tropicalis* (Chapter 6). In rat pituitary cells, T3 increases transcription of *ERalpha* by interacting with the ERE in the promoter of the *ERalpha* gene (Fujimoto et al., 2004). Future studies should analyse the promoter of *erbeta* in anurans for the presence of functional EREs or TH responsive elements (TREs) and the potential direct regulation of *erbeta* by T3. The physiological consequences of a higher *erbeta* expression in the GMC after T3 exposure remains to be elucidated.

In the case of TH-related genes, all transcripts were expressed in the GMC during development and were positively regulated by T3. These results are very similar to the

effects of T3 in the brain of *E. pustulosus* (Chapter 7) and to the developmental profiles and the T3 regulation of TH-related genes in *S. tropicalis* GMC (Chapter 6). No sex differences were observed in the expression of TH-related genes either at metamorphic climax or in the putative sexes during development. These results suggest that the TH-related genes measured in this study are not direct regulators of gonadal differentiation. However, the present results indicate that the GMC is a target of THs and that some of the effects of T3 in the gonad could be exerted via its receptors.

In conclusion, the present study indicates that expression of *cyp19* can be used as indicator of ovarian development and the sex of developing *E. pustulosus* tadpoles. These results provide supporting evidence that levels of estrogen are important regulators of gonadal differentiation in frogs (e.g., Hogan et al., 2008; Ohtani et al., 2003; Olmstead et al., 2009; Yu et al., 1993; Chapter 3). On the other hand, none of the target genes associated with testicular development in vertebrates (i.e., *srd5alpha* and *dmrt-1*) showed a possible role in inducing male differentiation. The magnitude of *cyp19* sexual dimorphism suggests that *E. pustulosus* is a good Neotropical candidate species to study the process of gonadal differentiation. Along with the brain study (Chapter 7), these results provide important basic information for future studies on sexual development and metamorphosis. In the *S. tropicalis* GMC, T3 induced the expression of androgen-related genes while decreasing the estrogen-related gene *erbeta*, consistent with a masculinising role of THs. In contrast, in *E. pustulosus*, the T3 experiment did not provide a clear role for THs in gonadal development. After T3 exposure, a lower *cyp19* (in certain samples) and *srd5alpha1* in the GMC could contribute to male gonadal development. However, further research is needed to clarify the role of THs in gonad development. A model for the mechanisms of TH action in the frog GMC is presented in Chapter 9.

CHAPTER 9

General Discussion

Traditionally, thyroid hormones (THs) and sex steroids in frogs have been studied in isolation even though the thyroid and reproductive endocrine axes interact in vertebrates (cross-talk). I therefore investigated cross-talk mechanisms between THs and sex steroids in developing frogs. In addition, I presented novel roles of THs and estrogens in early development (Chapters 2 and 4), the molecular signature of the anuran (pathological) intersex condition (Chapter 3), and the importance of aromatase (*cyp19*) in anuran gonadal development (Chapters 3, 6 and 8). The following discussion summarises the different projects using *Silurana tropicalis* and presents a model of cross-talk in this species. I then conclude about the species comparison of developmental profiles and TH regulation of sex steroid- and TH-related genes in tadpoles and present a model of TH action in the brain and gonad. Finally, I discuss future research directions and implications of this research.

9.1. Cross-talk during *Silurana tropicalis* development

Acute exposures to triiodothyronine (T3) and fadrozole (cyp19 inhibitor) resulted in whole-body changes in androgen- and TH-related transcripts in *S. tropicalis* larvae (Chapters 2 and 4), providing the first evidence of cross-talk during early anuran development. Surprisingly, gene expression was altered in a similar manner when *S. tropicalis* were exposed either to an estrogen synthesis inhibitor or to T3; both resulted in increased whole-body mRNA of TH-receptors (*tr*), deiodinase type 3 (*dio3*), androgen receptor (*ar*), and steroid 5α-reductase (*srd5alpha*; Fig. 9.1A). Estrogen and T3 appear to have antagonistic functions in early larvae, but it is unclear which is the primary effector hormone. In the

future it will be necessary to carefully delineate the sequential activation of these different hormone systems. It is possible that estrogens physiologically oppose T3 action in early larvae, since inhibition of estrogen synthesis resulted in the induction of TH-related genes. Conversely, T3 may suppress the estrogen system via the increase of androgen-related transcripts. Based on these results, I propose a model in which interactions between THs and estrogens are mediated by the androgen system (Fig. 9.1B-C).

The androgen mediation model is supported by experiments performed during *S. tropicalis* metamorphosis. In the brain and gonad-mesonephros complex (GMC), T3 increased androgen-related gene expression (Chapters 5 and 6). These experiments demonstrated that there is a close relationship between expression levels of TH- and androgen-related genes, supporting the hypothesis that T3 is involved in male development in anurans. Both in the whole body (Chapter 4) and in the brain (Chapter 5), T3 also increased expression of estrogen receptors (*er*). This potential increase in estrogen sensitivity may be indicative of a negative feedback response to the opposing effect of T3 on the estrogen system.

On the other hand, chronically blocking estrogen synthesis (using fadrozole; Chapter 3) altered hepatic androgen-related transcript levels but not TH-related endpoints (growth and gene expression), thus only partially supporting my model. This was not likely the result of inefficient estrogen synthesis inhibition since cyp19 activity was almost completely inhibited in the brain (~90% compared to control). There may be several explanations for the apparent lack of effect of fadrozole on the TH system. The effects of estrogens on tadpole metamorphosis may be obscured by substantial variation in tadpole development. For instance, Pettersson and Berg (2007) observed that ethinylestradiol (EE2), an estrogen mimic, delayed *S. tropicalis* development in one study but not in another (Pettersson et al.,

2006). Another possibility is that chronic inhibition of cyp19 led to adaptations to low levels of estrogens, e.g., via feedback mechanisms. I might have missed some potentially early transcriptional effects of cyp19 inhibition on TH-related endpoints. The last possibility is that estrogens do not affect metamorphosis or the TH system in *S. tropicalis*. In goldfish, T3 affects sex steroid-related genes in the gonad (Nelson et al., 2010), but sex steroids (estradiol, testosterone and 11-keto-testosterone) do not affect *tr* expression in the gonads (Nelson and Habibi, 2009), suggesting that only one direction of cross-talk is present in the goldfish gonad. Future research, using shorter exposures to estrogens in combination with T3 (in order to reduce the variability in tadpole development), may help elucidate any molecular interactions that may exist (see discussion Chapter 3).

A.

Target genes	T3 (nM)			Fadrozole (μM)		
	0.5	5.0	50	0.5	1.0	2.0
take ocyp19	-	-	-	-	-	-
eralpha	-	-	↑	-	-	-
erbeta	-	-	-	-	-	-
ar	-	↑	↑	-	↑	↑
srd5alpha1	-	↑	↑	-	-	-
srd5alpha2	-	-	-	-	↑	↑
tralpha	-	↑	↑	-	↑	↑
trbeta	↑	↑	↑	-	↑	↑
dio1	-	-	-	-	-	↓
dio2	-	↑	↑	-	-	-
dio3	-	↑	↑	-	↑	↑

B.

⬇ Estrogen = **⬆ Androgen** = **⬆ Thyroid system**

↓ cyp19 activity (fadrozole)

↑ *ar*
↑ *srd5alpha*

↑ *tr*
↑ *dio3*

C.

⬆ Thyroid hormones = **⬆ Androgen** = **⬇ Estrogen**

(T3)
↑ *tr*
↑ *dio*

↑ *ar*
↑ *srd5alpha*

↑ *er*

Figure 9.1. Evidence for cross-talk between thyroid and sex-steroid axes in *Silurana tropicalis*. (**A**) Whole-body gene expression changes after treatment with triiodothyronine (T3; Chapter 4) or fadrozole (Chapter 2) during *S. tropicalis* early development. Arrows indicate statistically significant results ($p<0.05$) relative to control and dashes indicate no changes. (**B and C**) Proposed mechanisms of cross-talk in *S. tropicalis* based on these gene expression results. Interactions between thyroid hormone and estrogens are mediated by the androgen axis.

9.2. Comparison of developmental profiles and cross-talk mechanisms among three anuran species

9.2.1. Developmental profiles during metamorphosis

In this thesis, I presented profiles of gene expression in brain and GMC for two anuran species, *S. tropicalis* and *Engystomops pustulosus*. These developmental profiles were similar across species in comparison to what has been reported in the published literature (discussed in the individual chapters). I supplemented the two species comparative analysis with data originally reported by Hogan et al. (2007; Duarte-Guterman, third author) for *Lithobates pipiens* developmental profiles of sex steroid- and TH-related genes in the brain (Fig. 9.2). Even though the trends are similar for many of the genes analysed, developmental expression patterns in *S. tropicalis* and *E. pustulosus* are more similar and sometimes very different from *L. pipiens*. These results do not seem to be in agreement with the phylogenetic relationship among these three species, since *E. pustulosus* is more closely related to *L. pipiens* (Fig. 1.3; see also Chapter 7). In all three cases, *eralpha* and *trbeta* increase at metamorphic climax, though the fold changes with respect to premetamorphosis vary depending on the species: *eralpha* and *trbeta*, respectively, increase ~6-fold and ~30-fold in *S. tropicalis* and *E. pustulosus*, or 10-fold and 3.5-fold in *L. pipiens*. These differences may be related to the developmental and reproductive characteristics of each species (Table 1.2). In spadefoot toads, species with shorter larval periods (*Scaphiopus couchii*, *Spea multiplicata*) have greater *in vitro* tissue sensitivity to THs compared to species with slower developmental rates (*Pelobates syriacus*; Buchholz and Hayes, 2005). This also appears to be the case here since, compared to *L. pipiens*, *E. pustulosus* and *S. tropicalis* have both a shorter larval period, and increased sensitivity to THs, suggested by their greater *trbeta* mRNA increases in the brain at metamorphic climax relative to premetamorphosis

(Fig. 9.2D) and after T3 exposure (Table 9.1). It is thus an interesting hypothesis that TH physiology may affect the evolution of diverse anuran life histories (see Buchholz and Hayes, 2005; Buchholz et al., 2006). Unfortunately, until we have a better understanding of the roles of sex steroids in the tadpole brain, the physiological causes and consequences of potentially different brain estrogen sensitivities (due to differences in *eralpha* levels) remain unknown. It should be a priority to establish developmental profiles of the other estrogen receptor, *erbeta*, and androgen-related genes in *L. pipiens*, to help elucidate the differences in developmental expression and whether these can be related to reproductive or other characteristics of each anuran species.

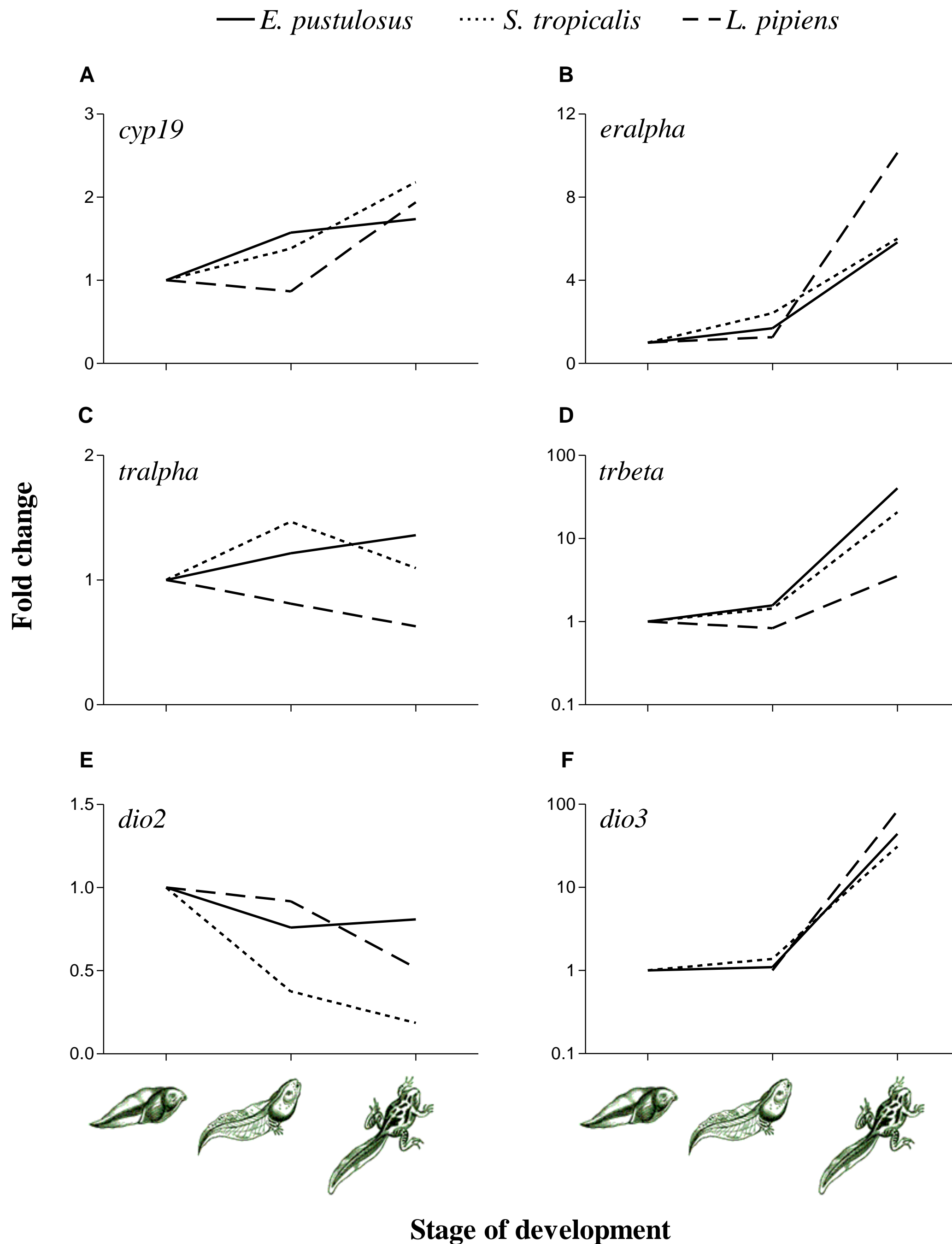


Figure 9.2. Sex steroid- and thyroid hormone-related gene expression profiles in the brain during tadpole development. Three anuran species are presented at three stages of development (premetamorphosis, prometamorphosis, and metamorphic climax). Data are normalised to RNA content only and presented as fold changes relative to the first stage of development (premetamorphosis), except for *dio3* in *Lithobates pipiens* which was not detected in the brain of premetamorphic tadpoles and is presented relative to pro-metamorphic levels. Note that the scale of the y-axes varies between genes and is logarithmic in the case of *trbeta* and *dio3*. Data for *S. tropicalis* and *E. pustulosus* comes from Chapters 5 and 7. Data for *L. pipiens* is modified from Hogan et al. (2007).

9.2.2. T3 regulation of thyroid hormone- and sex steroid-related genes

In order to compare cross-talk mechanisms between anuran species, I used a T3 exposure in premetamorphic tadpoles to investigate the regulation of TH- and sex steroid-related genes in three species, *S. tropicalis*, *E. pustulosus*, and *L. pipiens*. In Chapter 1, I hypothesised that T3 regulation of TH-related genes would be similar in all three species and the results strongly support this hypothesis (Tables 9.1 and 9.2). In all studied anurans with a distinct tadpole stage, THs regulate metamorphosis (Shi, 2000). In addition, the present results indicate that T3 similarly regulates transcription of genes involved in metamorphosis (both in the brain and GMC). However, one important difference that warrants further investigation is the magnitude of gene expression changes (discussed in section 9.2.1). Regulation of gene expression is one of the mechanisms underlying animal diversity (for review see: Chen and Rajewsky, 2007; Levine and Tjian, 2003). In the case of anurans, differences in the regulation of T3-responsive genes could be responsible for differences in larval period durations. Future studies could compare DNA regulatory sequences and elements (e.g., transcription factors, cofactors, chromatin remodelling complexes) of T3-responsive genes in anurans with different developmental rates. In addition, testing activation of transcription by the identified regulatory elements and by the T3-tr complex could also help understand the species differences in regulating gene expression.

In the case of sex steroid-related genes, the species differed not only in the magnitude, but also in the direction of changes (Tables 9.1 and 9.2). In the brain, one important difference is the negative regulation by ~1.5-fold of *cyp19* mRNA in *E. pustulosus* and *L. pipiens* but not in *S. tropicalis*. From the results, I propose a mechanism of TH action in the tadpole brain (Fig. 9.3A). In all three species, T3 exposure resulted in an induction of the androgen system in the brain. In the brain of *S. tropicalis*, T3 increased the expression of

androgen synthesis enzymes (*srd5alpha1* and *srd5alpha2*), whereas in *L. pipiens* and *E. pustulosus*, T3 reduced the expression of brain *cyp19*. The former could be a potential direct effect on the androgen system and the latter could be an indirect effect by reducing estrogen and affecting the balance of sex steroids.

Although only *E. pustulosus* and *S. tropicalis* were available to compare changes in the GMC, the overall results appear to be similar to the brain. Two-species comparisons are generally unreliable (Garland and Adolph, 1994), but I have nevertheless proposed a preliminary model of TH action in the GMC in Fig. 9.3B. In *S. tropicalis*, it is clear that T3 induced the androgen system by increasing expression of androgen-related genes (*srd5alpha1*, *srd5alpha2*, and *ar*) in the GMC. The picture is less clear in *E. pustulosus*, where T3 reduced mRNA levels of *srd5alpha1* and in some tadpoles, also reduced *cyp19* (Table 9.2). In the *E. pustulosus* GMC, levels of *cyp19* and *srd5alpha1* were greater in females than in males, suggesting that these genes can be markers of ovarian development (Chapter 8). As such, decreases in the expression of *cyp19* and *srd5alpha1* in the GMC after T3 treatment suggest altered or reduced ovarian development, possibly promoting testicular development through the androgen system (Fig. 9.3B). Moreover, since srd5alpha enzymes are involved in the synthesis of 5α-dihydrotestosterone, which is predominantly involved in male development, it was surprising that *srd5alpha1* expression was higher in females. Future studies in additional frog species are needed to clarify the roles of srd5alpha enzymes in anurans. In addition, it would be interesting to test whether the proposed model applies to *L. pipiens* in the GMC and/or other anuran species.

Both brain and GMC experiments indicate that anuran species may have alternative mechanisms to achieve a similar physiological endpoint, i.e., induction of genes related to the androgen system (Fig. 9.3A-B). In other vertebrates, exposure to THs also appear to

stimulate the gonadal androgen system, but this seems to be accomplished primarily by reducing estrogens, through cyp19 regulation (Fig. 9.3C-D); so far, there are no data for the brain. The overall similarities in strategies between these vertebrates could imply evolutionary convergence on the ultimate goal of linking THs and androgens. However, it seems more parsimonious to suggest that the link between THs and androgens is basal in vertebrates, and that there has been evolutionary divergence of the proximate mechanisms linking these two hormone axes. This is suggested by the similarities in mechanisms between rodents and goldfish (Fig. 9.3C-D). This will become clearer when a greater number of frogs and other vertebrates are surveyed to establish the extent of the link between THs and androgens and the frequency of alternate mechanisms.

Using the results of the comparative study, I rejected the hypothesis that the effects of T3 on sex steroid-related transcripts depend on the developmental and reproductive characteristics of the considered species (Chapter 1). It is clear that while *E. pustulosus* and *S. tropicalis* have similar developmental rates and reproductive characteristics (Table 1.2), they have different sex steroid-related transcript profiles after T3 exposure (Tables 9.1 and 9.2). One possibility is that the differences observed between the species are related to the sex-determining system of the species. In amphibians, the ancestral state of the sex-determining system is female heterogamety (ZZ/ZW), and male heterogamety (XX/XY) seems to have evolved independently several times (Hillis and Green, 1990). *S. tropicalis* has a ZZ/ZW sex-determining system, while *L. pipiens* has an XX/XY system (Table 1.2). The sex-determining system in *E. pustulosus* is not known (M. Ryan, pers. comm.). However, analysis of the chromosomes in the closely related frog, *Engystomops petersi*, shows that certain populations possess XX/XY heteromorphic chromosomes (Lourenco et al., 1999; Targueta et al., 2010). In addition, *Eupsophus migueli* and *Eupsophus insularis* from the

same family also have a XX/XY sex determining system (Cuevas and Formas, 1996; Hillis and Green, 1990; Iturra and Veloso, 1988). Therefore, if *E. pustulosus* has a XX/XY sex-determining system like its close relatives, this suggests that future work should examine whether the mechanism of sex determination determines the effect of THs on sex steroid-related endpoints. The difference in gene regulation could be related to the function of sex steroid-related genes during development. For example, *srd5alpha1* and *srd5alpha2* genes in Pipid species seem to be involved in male development (Urbatzka et al., 2007; Langlois, 2010); however, in the case of *E. pustulosus*, *srd5alpha1* seems to be a marker of female development and *srd5alpha2* is either very lowly expressed or not expressed at all (Chapter 8). Therefore, an interesting hypothesis would be that in frogs with the ZZ/ZW system, srd5alpha enzymes play a role in inducing male development, while in frogs with the XX/XY system, male development is achieved through the reduction of estrogen (by reducing cyp19), allowing srd5alpha enzymes to perform other functions (e.g., those involved in the progesterone pathway). Testing this hypothesis would help further understand the roles of sex steroids in gonad development and the mechanisms of cross-talk between THs and sex steroids in anurans.

Table 9.1. Gene expression changes in the brain after T3 exposure in three frog species, *Silurana tropicalis*, *Engystomops pustulosus*, and *Lithobates pipiens*. Premetamorphic tadpoles were exposed to T3 (0.5, 5, 50 nM) for 48 h. For each gene, the average fold change with respect to control (0 nM T3) is presented along with an arrow indicating the direction of change. An asterisk (*) denotes statistically significant differences from control (one-way ANOVA; $p<0.05$). Dashes indicate no differences, *na* = not measured, *nd* = not detected.

Target gene	***S. tropicalis*[a]**			***E. pustulosus*[b]**			***L. pipiens*[c]**		
	0.5	**5.0**	**50**	**0.5**	**5.0**	**50**	**0.5**	**5.0**	**50**
eralpha	-	1.7↑*	2.0↑*	-	-	-	-	-	1.8↑*
erbeta	1.5↑	2.4↑*	2.7↑*	-	1.3↑	3.4↑*	*na*	*na*	*na*
cyp19	-	-	-	-	1.4↓*	1.4↓*	-	1.7↓	2.0↓*
srd5alpha1	1.5↑*	-	-	-	-	-	*na*	*na*	*na*
srd5alpha2	7.5↑*	6.1↑*	5.6↑*	*nd*	*nd*	*nd*	*na*	*na*	*na*
ar	-	-	-	-	1.2↑*	1.2↑*	*na*	*na*	*na*
tralpha	1.4↓	1.7↓	1.8↓*	-	1.2↑*	1.4↑*	-	-	-
trbeta	6.0↑*	10↑*	15↑*	2.0↑*	17↑*	31↑*	-	-	4.5↑*
dio2	2.3↑*	2.7↑*	3.0↑*	1.3↑	2.3↑*	3.9↑*	-	-	3.5↑*
dio3	2.5↑*	22↑*	136↑*	-	3.9↑*	30↑*	-	-	7.5↑*

[a] Results from Chapter 5
[b] Results from Chapter 7
[c] Results from Hogan et al., 2007

Table 9.2. Gene expression changes in the gonad-mesonephros complex after T3 exposure in *Silurana tropicalis* and *Engystomops pustulosus*. Premetamorphic tadpoles were exposed to T3 (0.5, 5, 50 nM) for 48 h. For each gene, the average fold change with respect to control (0 nM T3) is presented along with an arrow indicating the direction of change. An asterisk (*) denotes statistically significant differences from control (one-way ANOVA; $p<0.05$). Dashes indicate no differences, *dl* = detected at very low levels.

Target gene	***S. tropicalis*** [a]			***E. pustulosus*** [b]		
	0.5	**5.0**	**50**	**0.5**	**5.0**	**50**
eralpha	-	-	-	-	-	-
erbeta	1.3↓	1.7↓*	2.1↓*	-	2.8↑*	6.5↑*
cyp19	-	-	-	2.6↓	1.6↓	1.9↓
dmrt-1	-	-	-	-	-	-
srd5alpha1	1.6↑*	1.9↑*	1.9↑*	-	1.2↓	1.3↓*
srd5alpha2	1.8↑*	3.6↑*	3.9↑*	*dl*	*dl*	*dl*
ar	1.5↑*	1.7↑*	1.9↑*	-	-	-
tralpha	1.8↑	1.6↑*	1.5↑	1.3↑	1.7↑*	2.0↑
trbeta	6.2↑*	6.5↑*	6.8↑*	2.8↑*	11↑*	22↑*
dio2	2.4↑*	6.3↑*	8.7↑*	1.4↑	2.3↑*	4.0↑*
dio3	1.6↑	5.4↑*	15↑*	2.0↑*	16↑*	87↑*

[a] Results from Chapter 6
[b] Results from Chapter 8

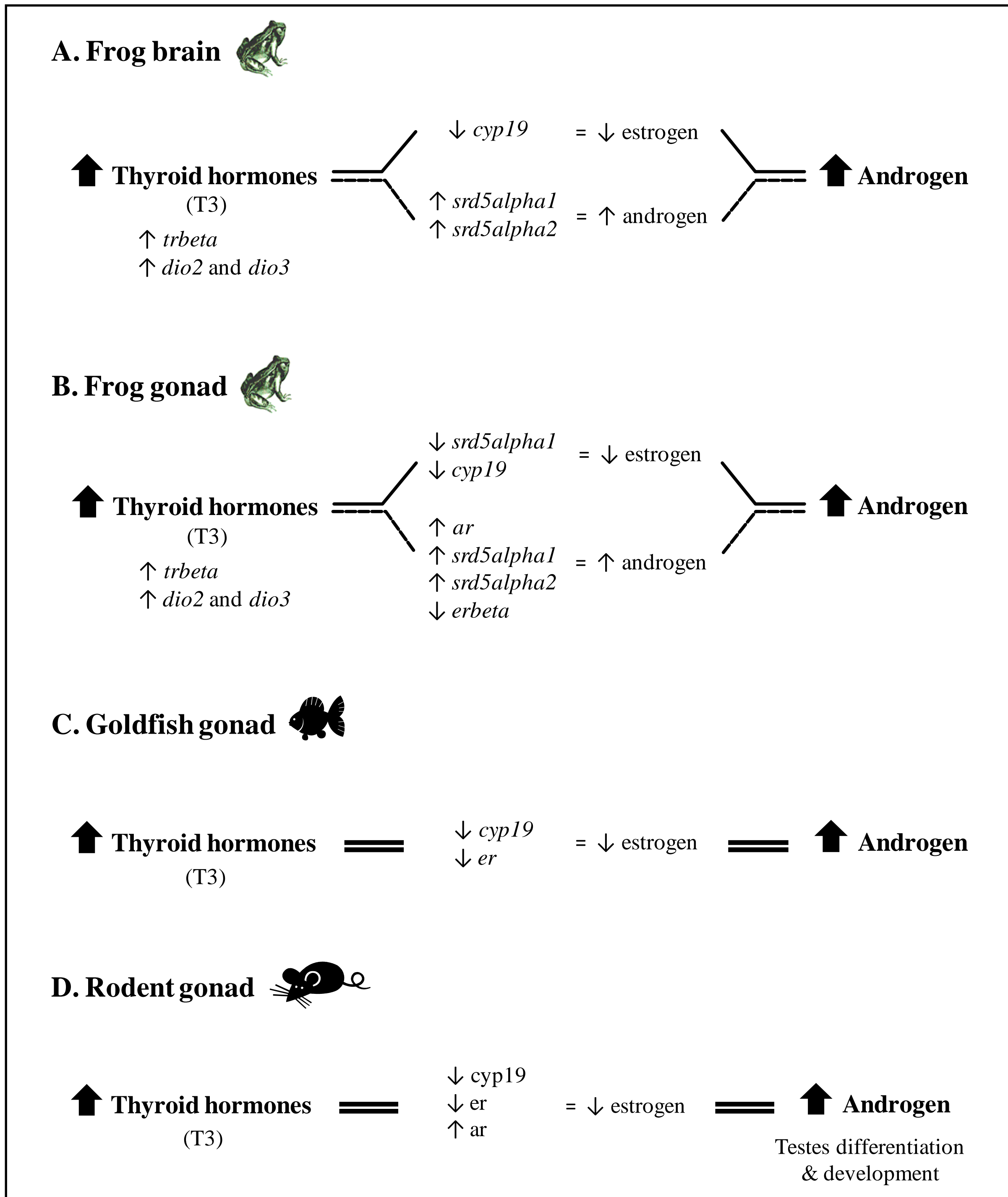


Figure 9.3. Solutions to the regulation of the androgen system by thyroid hormones (THs). Proposed models of TH action in (**A**) the frog brain, (**B**) the frog gonad-mesonephros, (**C**) the goldfish gonad, and (**D**) the rat and mouse gonad. The induction of the TH system (with T3) leads to the induction of the androgen system in the brain and gonad by either the increase in transcription of androgen-related genes (enzymes and receptors) in *S. tropicalis* (dashed lines) or by the decrease in estrogen synthesis enzymes in *E. pustulosus*, *L. pipiens*, goldfish and rodent models (solid lines). For details regarding gene expression changes, see Table 1.1 (goldfish and rodent data) and Tables 9.1 and 9.2 (frog data).

9.3. Future research directions

The results reported in this thesis lead to numerous questions and research directions; many of these have been pointed out in the individual chapters, including the present one. In this section, I emphasise three additional long-term research questions arising from my results. Two limitations in this work are first the use of GMC rather than only gonad, which potentially resulted in the measurement of gene expression from non-reproductive tissues. The analysis of GMC was used because in anurans, the developing gonads are very small and translucent. However, it is now possible to use laser microdissection to dissect pure gonad samples and measure gene expression. Nevertheless, in the case of *cyp19* mRNA, the analysis of GMC worked well since sexually dimorphic expression was observed in *S. tropicalis* (Chapter 7) and *E. pustulosus* (Chapter 8). The second limitation which will have to be addressed in future experiments is the lack of protein data (e.g., Western blots) to complement gene expression analyses. Presently, the antibodies available for anurans for the targets of interest are limited; the exceptions are antibodies for tralpha and trbeta (raised against *X. laevis*; Fairclough and Tata, 1997) and more recently srd5alpha1 (raised against human but used in *Pelophylax kl. esculentus*; Bruzzone et al., 2010) and cyp19 (raised against *Glandirana rugosa*; Kato et al., 2004). The optimised enzyme activity assay for cyp19 was an alternative solution to protein measurements, but required pooling many individual tissues. This situation may be improved soon, with a recent undertaking of designs for antibodies against *S. tropicalis* for proteins of sex steroid-related genes regulated by T3 (V. Trudeau, pers. comm.). Measuring protein levels will help elucidate the physiological significance of the interaction between sex steroids and THs.

An important and novel result was the regulation of GMC gene expression by T3 in developing tadpoles. Both the reproductive and thyroid endocrine axes are centrally

controlled by the brain-pituitary neuroendocrine axis (Fig. 1.2); therefore, there are multiple sites (i.e., brain, pituitary, and gonad) where these two axes can interact. Even though THs seem to induce the androgen system in anurans (Fig. 9.3A-B), it is not currently possible to determine whether the effects observed *in vivo* in the GMC reflect direct regulation in the gonad, or indirectly, the result of T3 effects on the brain and/or pituitary which then regulates the gonad (Fig 1.2). This could be investigated *in vitro* in isolated brain, pituitary, and gonad tissue cultures.

In the tadpole brain and gonad, T3 affected the expression of sex steroid-related genes (e.g., *srd5alpha*, *er*, *cyp19*, and *ar*), but the molecular mechanism is unknown in frogs. In vertebrates, *AR* mRNA can be induced by T3 (rats: Arambepola et al., 1998; lizards: Cardone et al., 2000; hamsters: Esposito et al., 2002; frogs: Chapters 6 and 7). In hamsters, the regulation is direct and involves the action of TRs which bind to a TH responsive element (TRE) in the promoter of the *AR* gene (Varriale and Esposito, 2005). It is unknown whether the promoters of the frog sex steroid-related genes regulated by T3 also contain such TRE, or other hormone responsive elements (e.g., estrogen responsive element [ERE], androgen responsive element [ARE]). In mammalian cell culture studies, ERs and TRs can bind to the same ERE and affect transcription (Vasudevan and Pfaff, 2005), presumably because of similarities in amino acid composition, protein structure and DNA recognition elements (Evans, 1988). Future research should investigate potential interactions between receptors in the promoter of anuran target genes (see also discussion Chapter 7).

Although research in developing tadpoles has focused on the classical genomic mechanisms of TH action in which its binding to tr affects gene transcription, THs can also exert effects through non-genomic pathways (Cheng et al., 2010; Davis et al., 2008). Studies using mammalian models have demonstrated that these pathways are very rapid (seconds,

minute to an hour) and depend upon cellular signal transduction systems and novel cell surface TH receptors or extranuclear TRs (reviewed in: Davis and Davis, 1996; Davis et al., 2008). Therefore, in addition to interaction with nuclear receptors, THs and sex steroids may interact via non-genomic mechanisms in frogs. One example of non-genomic cross-talk is the phosphorylation of ERalpha in human breast cancer cells; THs act at the cell surface to activate a mitogen-activated protein kinase (MAPK) signal transduction cascade (Tang et al., 2004). Non-genomic effects of THs may also include regulation of mRNA stability (Cheng et al., 2010; da Silva et al., 2010; Narayan and Towle, 1985). Intriguingly, this mechanism may be implied by the preliminary observation that elongation factor 1 alpha (*ef1alpha*) mRNA in the brain also appears to increase with all three T3 concentrations (data not presented). ef1alpha can regulate mRNA stability by binding RNAs to the cytoskeleton (da Silva et al., 2010; Liu et al., 2002; Mickleburgh et al., 2004). Consequently, increases in transcript levels in the brain with T3 exposure could be related to regulation of transcript stability by an enzyme like ef1alpha.

9.4. Implications and concluding remarks

Anuran gonadal differentiation and metamorphosis can be affected by exposure to environmental contaminants (Crump et al., 2002; Hayes et al., 2002; Hogan et al., 2008; Langlois et al., 2010; Pettersson et al., 2006; Veldhoen et al., 2006). In the field of endocrine disruption, the effects on the endocrine axes are usually studied in isolation from each other and chemicals are characterised based on their effects, e.g., estrogenic, androgenic, thyrogenic compounds. However, the boundaries of these axes are less well defined than previously thought; a thyrogenic chemical may also have androgenic properties by affecting the expression of androgen synthesis enzymes. This will have important implications in

endocrine disruption research and the evaluation of the effects of environmental contaminants on the endocrine system.

In conclusion, I have presented novel mechanisms of cross-talk during the entire developmental period, indicating that hormone interactions are required for normal embryo and tadpole development. Although the effects of THs on the reproductive system differed among the three anuran species, in all cases THs appeared to affect the balance of sex steroids towards androgens, suggesting that there is a close relationship between thyroid and androgen status in developing tadpoles. This suggests hitherto unexplored evolutionary and mechanistic hypotheses for hormone regulation in frogs specifically and in vertebrates in general.

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APPENDIX A

Comparison of Gosner (1960) and Nieuwkoop and Faber (1994) developmental tables

Stage of metamorphosis	Gosner	Nieuwkoop & Faber	Main morphological characteristics	
Premetamorphosis	26	46	Limb bud growth	
	27	47-48		
	28	49-50		
	29	51		
	30	51		
	31	52	Foot-paddle stages	
	32	53		
	33	53	Toe differentiation	
	34	54		
	35	54		
Prometamorphosis	36	55		
	37	55	Rapid hind limb growth	
	38	56		
	39-40	57-58		
Climax	40	59		
	41	60-61		
	42	62	Tail resorption begins	
	43	63		
	44	64		
	45	65		
Juvenile frog	46	66		

Sources: Denver et al., 2002; Shi, 2000.

APPENDIX B

Mating protocol for *Silurana tropicalis* in the laboratory

The mating protocol for *Silurana tropicalis* is based on the protocols of the Grainger (available online at: http://faculty.virginia.edu/xtropicalis/) and Harland laboratories (available online at: http://tropicalis.berkeley.edu/home/). Two injections with human chorionic gonadotropin hormone (hCG; Sigma; Catalogue No. C-1063) are used to induce the frogs to mate. Male and females are injected the same amount of hCG.

<u>The day before the breeding</u> (16-24 h before)

1. Adjust water pH to 5.8-6.0 in both tanks (male and female are kept separate)
2. A priming dose of 12.5 units of hCG (in a 50uL volume) is injected into the dorsal lymph sac of each frog (male and female).

<u>The day of the breeding</u>

1. Adjust water pH to 5.8-6.0 in the breeding tank
2. A boosting dose of 100 units of hCG (in a 250uL volume) is injected into the dorsal lymph sac of each frog (male and female).
3. Place the pair together in the breeding tank for clasping and cover to block light.

APPENDIX C

Histological methods for gonads

<u>Schedule for tissue processing and embedding in paraffin</u>

The Tissue-Tek® VIP™ Vacuum Infiltration Processor (Sakura E150/E300 Series) was used for dehydration, clearing and infiltration of tissues in paraffin:

Dehydration of tissues (45 minutes for each step)

1. 70% Ethanol at 40ºC
2. 80% Ethanol at 40ºC
3. 90% Ethanol at 40ºC
4. 95% Ethanol at 40ºC
5. 100% Ethanol at 40ºC
6. 100% Ethanol at 40ºC

Clearing tissues of ethanol

7. Xylene (three 45 min. cycles)

Infiltration of tissues in paraffin

8. Paraffin at 58ºC (four 45 min. cycles)

Embedding of tissues in paraffin

9. Processed tissues were embedded in paraffin blocks with the ventral side facing down for longitudinal ventral to dorsal sectioning

<u>Harris' Hematoxylin and Eosin Y staining procedure for gonad paraffin sections</u>

Deparaffinise and hydrate sections

1. Xylene (three 3 min. cycles)
2. 100% Ethanol (three 1 min. cycles)
3. 95% Ethanol (1 min)
4. Tap water (30 s)
5. Rinse sections in tap water

Stain sections with Hematoxylin and Eosin-Y

6. Hematoxylin (Richard Allan Scientific) (2.5 min)
7. Tap water (30 s)
8. Clarifier II (Richard Allan Scientific) (30 seconds)
9. Tap water (30 s, agitate)
10. Bluing agent (1 min)
11. Tap water (1 min)
12. 95% Ethanol (30 s)
13. Eosin-Y (Richard Allan Scientific) (1.5 min)

Dehydrate and clear sections

14. 100% Ethanol (three 1 min. cycles)
15. Xylene (three 1 min. cycles)
16. Mount slides with Permount (Fisher)

Blue (Hematoxylin basic dye binds to basophilic tissues): nucleus
Pink (Eosin Y acidic dye binds to eosinophilic tissues): cytoplasm

APPENDIX D

Brain transcript levels in *Lithobates pipiens* at juvenile stage

Methods:

Lithobates pipiens egg masses were collected from an isolated and permanent pond in Old Chelsea (QC, Canada). The eggs masses were maintained in aerated filtered water obtained from the University of Ottawa Aquatic Care Facility until they reached Gosner (G, 1960) stage 42. The water had the following characteristics: pH 7.0, dissolved oxygen 9.2-10 mg/L and temperature 21-22ºC. At stage G42, metamorphic tadpoles were taken out of the water to avoid drowning and once the tail was completely reabsorbed (G46), frogs were decapitated and brain was dissected and frozen on dry ice. Morphological sex was assigned by visual inspection of the gonads. Total RNA was isolated using the RNeasy micro kit (Qiagen) and cDNA synthesis was carried out using 2 μg total RNA and 0.2 μg random hexamer primers using Superscript II reverse transcriptase (Invitrogen). Expression of thyroid hormone receptors (*tralpha* and *trbeta*), and deiodinase enzymes (*dio2* and *dio3*) was measured using fluorescent probe-based real-time PCR. Levels of aromatase (cyp19), estrogen receptor α (*eralpha*) and β-actin (*actb*) were detected using SYBR Green I. Optimisation and reaction conditions of all the assays (except *actb*) are described in Hogan et al. (2007). Primers for *actb* were designed using Primer 3 (forward: 5`-cccaaagccaacagagaaa-3`; reverse: 5`-gaggcatacagggacagca-3`; product size: 101 bp) and optimisation was done as previously described in Chapter 2. The reactions were performed in triplicate, along with negative template controls (no template and no reverse transcriptase controls) and the data obtained were averaged and normalised to *actb* mRNA. Data are presented as individual points along with the mean (n=7). Statistical differences between males and females were assessed using unpaired t-tests (statistical significance, $p<0.05$) in S-Plus 8.0 (Insightful Corporation).

Results:

In *L. pipiens*, all of the genes measured were detected in the brain at stage G46 (Fig. A1). No statistical differences were observed between males and females in transcript levels at the juvenile stage.

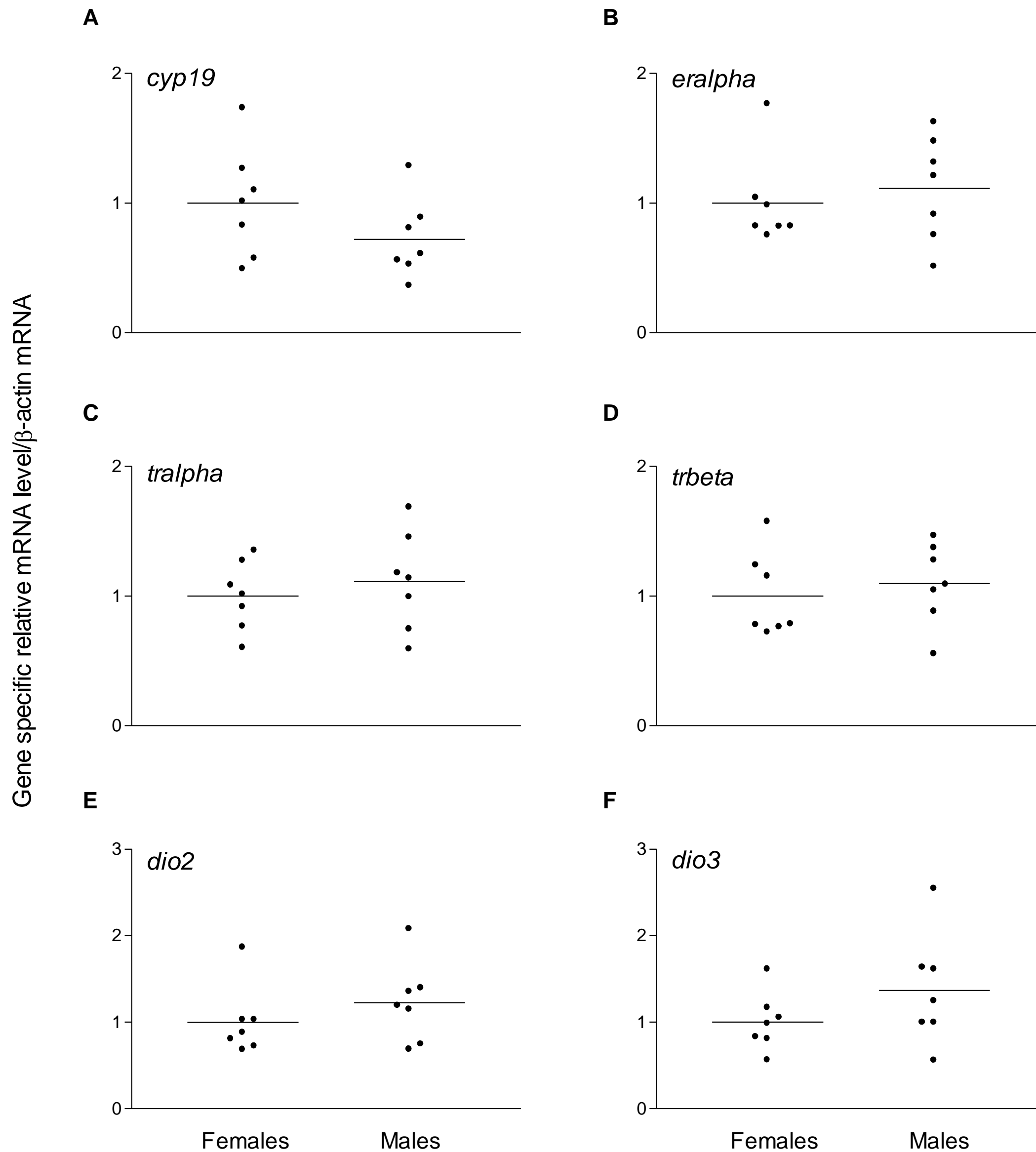

Figure A1. Brain transcript levels of estrogen- and thyroid hormone-related genes in *Lithobates pipiens* at Gosner stage 46. Aromatase (*cyp19*; A), estrogen receptor α (*eralpha*; B), thyroid hormone receptor α (*tralpha*; C), thyroid hormone receptor β (*trbeta*; D), deiodinase type 2 (*dio2*; E) and deiodinase type 3 (*dio3*; F) mRNA levels were measured in individual brains of females and males at Gosner stage 46. Individual samples are presented along with the mean (n=7). Levels of mRNA are expressed relative to females and are normalised to β-actin. No significant differences were observed between females and males for any of the genes analysed.

APPENDIX E

List of manuscripts not included in the thesis

Mennigen JA, Lado WE, Zamora JM, **Duarte-Guterman P**, Langlois VS, Metcalfe CD, Chang JP, Moon TW, Trudeau VL. 2010. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*. *Aquatic Toxicology*, 100: 354-364.

Croteau MC, **Duarte-Guterman, P**, Lean DRS, Trudeau, VL. 2010. Pre-Exposure to UVB radiation and 4-tert-octylphenol exposure affects the response of *Rana pipiens* tadpoles to 3,5,3'-triiodothyronine. *Environmental Toxicology and Chemistry*, 29: 1804-1815.

Zhang D, **Duarte-Guterman P**, Langlois VS, Trudeau VL. 2010. Temporal expression of piRNA pathway genes Mael, Piwi, Vasa, and their regulation by steroid hormones during *Silurana* (*Xenopus*) *tropicalis* embryogenesis. *Comparative Biochemistry and Physiology Part C*, 152: 202-206.

Zhang D, Popesku JT, Martyniuk CJ, Xiong H, **Duarte-Guterman P**, Yao L, Xia X, Trudeau VL. 2009. Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. *Physiological Genomics*, 38: 351-361.

Croteau MC, Davidson M, **Duarte-Guterman P**, Wade M, Popesku JT, Wiens S, Lean DR, Trudeau VL. 2009. Assessment of thyroid system disruption in *Rana pipiens* tadpoles chronically exposed to UVB radiation and 4-tert-octylphenol. *Aquatic Toxicology*, 95: 81-92.

Hogan NS, **Duarte P**, Wade MG, Lean DRS, Trudeau VL. 2008. Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (*Rana pipiens*): identifying critically vulnerable periods of development. *General and Comparative Endocrinology*, 156: 515-523.

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