Development and testing of gene expression biomarkers for gonadal dysgenesis in conjunction with the US EPA Endocrine Disruptor Screening Program's Tier 2 Larval Amphibian Growth and Development Assay

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Dedication

This thesis is dedicated to my loving and supportive wife who is now carrying our first child... and to my parents, in-laws, siblings, family, friends, mentors, Earth – thank you all for being part of this life.

Abstract

The Endocrine Disrupter Screening Program of US EPA has recently developed a Tier 2 testing guideline using model amphibian species *Xenopus laevis*. The Larval Amphibian Growth and Development Assay assesses a chemical's endocrine-related effects *in vivo* and generates concentration-response information for ecological risk-assessment. Currently, the assay relies on histopathological evaluations for identifying endocrine-related reproductive effects. However, histopathology can seldom define the chemical mode of action and is not easily interpreted in the context of risk assessment when the effects are minimal to moderate. This study explores the use of gene expression biomarkers in the gonad that could potentially inform a chemical's mode of action and detect adverse reproductive effects that are otherwise uncharacterized by histopathology. To identify candidate biomarkers, global expression was analyzed in differentiating ovary and testis tissues of *Xenopus tropicalis*. Genetic programs responsible for reproductive maturation in gonad tissues were characterized and provided a foundation from which specific genes could be selected proximal to a model chemical's known mode of action. Four genes were selected within the putative androgen molecular network to evaluate as biomarkers of the anti-androgen mode of action characteristic of a common fungicide, prochloraz. Following continuous exposure to prochloraz throughout embryonic, larval and juvenile development in *Xenopus laevis*, assessments of growth, liver and kidney pathology, and reproductive development were made. To evaluate the predictive capabilities of the candidate genes, one gonad was kept *in situ* for histopathological evaluations while the other was processed for mRNA analyses. Results indicate that prochloraz exposure caused metabolic toxicity in the liver and kidney; it caused testis degeneration coincident with the onset of androgen-mediated spermatogenesis and inhibited regression of Müllerian ducts. Two of the four candidate genes showed increases in expression at the high test concentration and appeared to be predictive of an anti-androgen-induced adaptive response. The behavior of these biomarkers stimulated valuable discussion and generated testable hypotheses to better understand the evolution of molecular mechanisms driving gonad development in a cross-species context. This study provides a model for expression-based biomarker development in endocrine tissues and offers direction toward enhanced ecological risk-assessment.

Table of Contents

Acknowledgements i
Dedication ii
Abstract iii
Table of Contents iv
List of Tables vi
List of Figures vii
Overview 1
Background 1
Problem 2
Objectives 2
Approach 3
Chapter 1: Global gene expression analysis during early differentiation of *Xenopus (Silurana) tropicalis* gonad tissues. 5
1. Introduction 5
2. Materials and methods 8
2.1 Animal care and culture 8
2.2 Experimental design 9
2.3 Total RNA target preparation and hybridization 9
2.4 Data analysis 10
3. Results 12
3.1 Differential expression and clustering 12
3.2 Functional analyses 14
4. Discussion 18
4.1 Ovary 19
4.2 Testis 23
5. Conclusion 27
Chapter 2. Measuring testis dysgenesis using gene expression biomarkers in *Xenopus laevis* following exposure to prochloraz. 29
1. Introduction 29
2. Materials and methods 33
2.1 Experimental design 33
2.2 Test concentrations 33
2.3 Environmental conditions 34
2.4 Exposure system 34
2.5 Chemical stock preparation 35
2.6 Analytical chemistry 35
2.7 Test initiation 36
2.8 Feeding 37
2.9 Larval sub-sampling 37
2.10 Cull 38
2.11 Juvenile sampling (test termination) 38
2.12 Histological procedures 39
2.13 Genetic sexing 40

2.16 Testis RNA processing 40
2.17 Quantitative PCR 41
2.18 Statistics 42
3. Results 43
3.1 Test concentrations 43
3.2 Generalized toxicity (survival, growth, liver and kidney pathology) 43
3.3 Endocrine toxicity (gonad, duct, gene expression) 46
4. Discussion 51
5. Conclusion 57
Chapter 3. Conclusion and future directions 58
Bibliography 60
Appendix A: Python programming code for enhanced array annotation 72
Appendix B: Transcript IDs and functional information corresponding to Figure 2 heatmap. 99

List of Tables

Table 1.1. Numbers of transcripts differentially expressed between tissues at each developmental stage. 13
Table 2.1. Measured water quality parameters throughout the duration of prochloraz exposure.. 34
Table 2.2. Accession numbers, primer and probe sequences used for quantitative PCR of selected gene transcripts. 42
Table 2.3. Mean ± standard deviation for growth and LSI measurements in control and prochloraz-treated *Xenopus laevis*. 44
Table 2.4. Incidences of histopathological findings in control and prochloraz-exposed testis tissue of two month post-metamorphosis male *Xenopus laevis*. 47
Table 2.5. Male and female oviduct stage distributions in control and prochloraz-exposed two month post-metamorphosis *Xenopus laevis* 49

List of Figures

Figure 1.1. Histological sections of gonad tissues at NF58, NF66, and 2WPM.....6
Figure 1.2. Hierarchical clustering of the 2WPM, ≥ 5-fold transcript list.....14
Figure 1.3. Cellular distributions of gene products by cluster.....15
Figure 2.1. Histological sections of control liver tissue at two months post-metamorphosis.....44
Figure 2.2. Histological sections of 180 μg/L prochloraz-treated livers at two months post-metamorphosis.....45
Figure 2.3. Histology sections of control and prochloraz-treated kidneys at two months post-metamorphosis.....46
Figure 2.4. Histology sections of control and prochloraz-treated testis tissues at two months post-metamorphosis.....48
Figure 2.5. Histological section of asynchronous cyst development in testis tissue of prochloraz-treated male.....49
Figure 2.6. Gene expression levels in testis tissue of two month post-metamorphosis control and prochloraz-exposed *Xenopus laevis*.....50

Overview

Background

The 1996 Food Quality Protection Act and an amendment to the Safe Drinking Water Act mandates the EPA to implement a screening program to determine whether certain substances interact with human endocrine systems causing adverse hormonal effects. Following the passage of this legislation, EPA responded by chartering the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) made up of representative scientists who recommended a tiered approach to endocrine disruptor testing that addresses effects in both humans and wildlife. EDSTAC also recommended that screening and testing should include estrogen, androgen and thyroid-related processes. The EPA's Endocrine Disruptor Screening Program (EDSP) has been actively developing assays to identify endocrine disrupting chemicals (EDC) using a two-tiered approach. Tier 1 screening assays are designed for high-throughput and to quickly identify whether a chemical will interact with some aspect of the endocrine system. Tier 2 testing systems are designed to assess Tier 1-active chemicals for endocrine-related effects *in vivo* and generate concentration-response information suitable for ecological risk assessment.

As part of the Tier 2 assay design effort, a chronic toxicity testing procedure has been developed with amphibian species *Xenopus laevis* that considers growth and development from fertilization through the early juvenile period. The Larval Amphibian Growth and Development Assay (LAGDA) involves a water-borne exposure of early life stage *Xenopus laevis* embryos with continuation of exposure until approximately two months following completion of metamorphosis, when the gonads are fully differentiated. Amphibian metamorphosis is a thyroid hormone-mediated process, so developmental assessments at metamorphic climax (Nieuwkoop and Faber stage 62) including growth, rate of somatic development, and thyroid gland histopathology allow the assay to identify potential thyroid-specific effects. Continuation of the assay post-metamorphosis allows further evaluations of growth and reproductive development to help identify a substance's potential to cause gonad dysgenesis or adverse effects on reproductive ducts.

Histopathological assessments of liver and kidney are also made at test termination as measures of overt toxicity.

Problem

Tier 2 testing is designed to be a chronic toxicity testing paradigm. This requires constant dosing of aquatic test chambers for up to six months while maintaining steady chemical concentrations and acceptable water quality. The in-life portion of the LAGDA is resource-intensive in terms of man-hours for husbandry, water quality monitoring, mechanical maintenance of dilution systems, and weekly analytical chemistry. Subsequent to completion of a successful test, hundreds of fixed tissue samples are processed and evaluated for histopathology. Needless to say, each attempt at this bioassay for a single chemical is extremely expensive and can take up to a year to acquire a completed study report.

As the LAGDA currently stands, histopathology evaluations are the most powerful and informative tool for assessing effects of chemical exposure on organs and tissues. However, these types of assessments cannot inform the molecular mode of action of the chemical, nor are these assessments easily interpreted in the context of risk assessment when the effects present as minimal to moderate. The research presented in this thesis explores the use of gene expression biomarkers as a supplement to LAGDA histopathology, specifically in the gonad, that could potentially inform the chemical mode of action and detect adverse reproductive effects in tissues presenting with minimal to no observable pathologies. This work also provides a model for development of biomarkers in other tissues susceptible to perturbations due to xenobiotic exposure.

Objectives

The objectives of this research are to (1) identify constitutive genes and genetic programs unique to each ovary and testis tissues during differentiation, (2) choose a subset of candidate genes to evaluate as biomarkers of gonadal dysgenesis mediated

through a particular mode of action, and (3) test candidate genes' predictive capabilities using gonad tissues adversely affected by exposure to an endocrine disrupting chemical.

Approach

Anuran sexual development and toxicology research has identified that many amphibian species are susceptible to chemical-induced gonad dysgenesis presenting as complete sex reversal or mixed sex gonads (Hayes, 1998; Kloas, 2002). Exposure to exogenous estrogens generally cause either complete male-to-female sex reversal, or at lower doses, males will present with mixed sex gonad tissues exhibiting characteristics of both ovary and testis tissues (Bögi *et al*., 2002; Kloas *et al*., 1999; Pettersson *et al*., 2006; Villalpando and Merchant-Larios, 1990; Witschi and Allison, 1950). Amphibian exposure to androgenic substances has presented mixed results differing by compound and species. *Xenopus laevis* larvae exposed to testosterone did not change sex ratios, but dihydrotestosterone and methyltestosterone had similar masculinizing effects (Bögi *et al*., 2002). *Xenopus tropicalis* larval exposure to a synthetic androgen, 17β-trenbolone, caused significant masculinization and mixed sex gonad tissue at sub-lethal concentrations, but was unable to cause complete female-to-male sex reversal (Olmstead *et al*., 2012). However, in *Rana* spp., androgen treatment produced 100% males (Kawamura and Yokota, 1959; Richards and Nace, 1978). Complete female-to-male sex reversal was demonstrated in *Xenopus tropicalis* when the enzyme that converts androgen to estrogen (aromatase) was chemically inhibited, effectively diminishing intrinsic levels of estrogen (Olmstead *et al*., 2009a). These modes of action occur through modulation of steroid hormone levels that translate to more or less activation of their respective cellular receptors (*e.g*. androgen receptor, estrogen receptor). One alternative mode of action is antagonism of the androgen receptor itself, inhibiting proper signaling within target tissues. Fungicides vinclozolin and prochloraz have been identified as anti-androgens causing altered sex differentiation (Gray *et al*., 1994) and impaired growth of androgen-dependent reproductive tissues (Vinggaard *et al*., 2002) in male rats respectively. All of these modes of action are germane to a comprehensive understanding of molecular pathways leading to gonadal dysgenesis.

As a first step toward understanding the molecular interactions associated with gonad dysgenesis in the context of amphibian Tier 2 testing, a thorough understanding of constitutive genetic programs in both testis and ovary tissues was needed - especially during the time at which the gonad is actively differentiating. Knowing which genetic programs are unique to ovary and testis morphogenesis in *Xenopus* would provide a foundation from which specific hypotheses could be tested regarding targeted chemical-induced perturbations. To this end, global gene expression analysis was performed on ovary and testis tissues during gonad differentiation in *Xenopus tropicalis*. This species was used (as opposed to *Xenopus laevis*) because its genome was sequenced and fully represented on a commercially available microarray chip. Details regarding this descriptive study are presented as Chapter 1.

Following the gene array study, potential biomarkers of gonadal dysgenesis were evaluated in conjunction with development of the EDSP amphibian Tier 2 bioassay. An anti-androgenic imidazole fungicide, prochloraz, was one chemical to be tested in the validation effort of the Tier 2 test guideline. Based on the anti-androgenic mode of action of prochloraz, four genes were chosen to evaluate as potential biomarkers of testis dysgenesis. These genes were chosen based on several criteria: (1) according to the gene array study, they had to exhibit robust expression profiles that were statistically testis-biased, (2) they had to be well-annotated and well-characterized in gonadal development within and between species, and (3) they had to have high likelihood of informing the chemical mode of action. Of the candidate genes that met these criteria, four were chosen to be evaluated as potential biomarkers using quantitative PCR. Details regarding the prochloraz exposure and results are presented as Chapter 2.

Chapter 1: Global gene expression analysis during early differentiation of *Xenopus (Silurana) tropicalis* gonad tissues.

1. Introduction

African clawed frog *Xenopus* sp. has been used extensively for developmental biology and toxicology research. With mounting concerns about wildlife exposure to environmental pollutants and potential endocrine disrupting compounds (reviewed by Kloas, 2002), efforts to better understand the mechanisms by which xenobiotics cause reproductive harm are warranted. Many studies have demonstrated the susceptibility of amphibians to altered gonadal development due to xenobiotic or exogenous hormone exposure resulting in sex reversal or altered sex ratios (Bögi *et al*., 2002; Olmstead *et al*., 2009a; Othani *et al*., 2000; Pettersson *et al*., 2006; Reeder *et al*., 1998; Villalpando and Merchant-Larios, 1990). The molecular processes of sex determination in amphibians vary by species, but many of the downstream molecular processes driving differentiation (*e.g.,* steroid hormones) are similar even across taxa (reviewed by Hayes, 1998). With the latest technological advances in global gene expression analysis, it may be possible to characterize the genetic programs responsible for gonad differentiation so similarities and differences can be identified between species. These similarities and differences could then be exploited in biological and toxicological research to enable better cross-species extrapolations following targeted experimentation involving genetic manipulations.

Three events are operative sequentially in determining the sex of most vertebrates: fusing of the gametes at conception (*e.g.,* XX/XY or ZZ/ZW sex chromosomes), sex determination by a molecular switch during embryonic or larval development, and finally, the cascading molecular expression patterns set in motion by the sex determination switch. Prior to sex determination in Anurans, the undifferentiated, bipotential gonad consists of a cortex and medulla (Merchant-Larios and Villalpando, 1981). In *Xenopus tropicalis*, the first signs of testicular differentiation at the histological level occur around Nieukoop and Faber ([NF] 1994) stage 48, while the first evidence of ovarian differentiation occurs around NF stage 51 based on differences in laminin

localization patterns (El Jamil *et al*., 2008). The primordial germ cells (PGC) reside in the cortex of the bipotential gonad and continue to remain in the cortex of the female gonad while the medulla recedes to an inner epithelial layer forming a lumen (Ogielska, 2009a). In the testis, the PGCs migrate from the cortex to the medulla; the cortex eventually disappears in lieu of the peritoneal epithelium. These processes are initiated and orchestrated by genetic control, but the exact mechanisms have not been elucidated in Anuran species (Hayes, 1998). By NF58, the gonad is fully distinguishable histologically as either an ovary or a testis based on the presence or absence of the cortex and medulla (Figure 1.1).

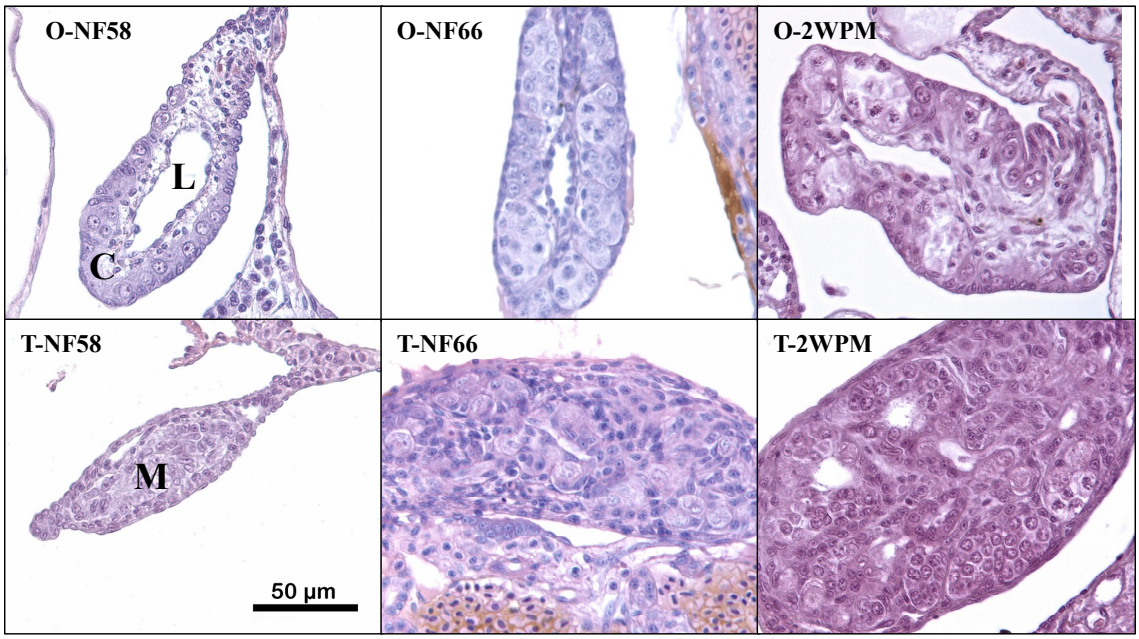

Figure 1.1. Histological sections of ovary and testis tissues at NF58, NF66, and two weeks post-metamorphosis (2WPM). Stained with H&E. O: ovary; T: testis; C: cortex; M: medulla; L: lumen.

Following NF58, many noticeable changes take place histologically that indicate substantial up-regulation of differentiation processes. In the ovary, primary oogonia are actively dividing mitotically and form germ cell nests of secondary oogonia (Ogielska, 2009b). These germ cell nests are surrounded by somatic pre-follicular cells that will eventually support development of the diplotene oocytes. Blood vessels and mesenchyme cells migrate into the cortex through the thin space between the cortex and what was previously the medulla. These blood vessels will eventually deliver yolk

precursors to the oocytes from the liver where they are synthesized. Secondary oogonia become primary oocytes and proceed through meiosis to the diplotene stage, when follicular cells proliferate and envelop each oocyte to form follicles. Within each follicle, oocytes begin to protrude microvilli that interlace with follicular cell macrovilli, and gap junctions are formed between the oocyte and follicular cell villi which allow more surface area for passage of hormones and signaling molecules between cells to direct oocyte meiosis and maturation. The microvilli of the oocyte begin to branch out at their tips, running parallel with the oocyte surface creating an envelope (zona pellucida) where glycoproteins are localized to mediate the acrosome reaction and resist polyspermy. An extensive network of cytoskeletal components are assembled in the diplotene oocyte to traffic molecules and prepare for resumption of meiosis when triggered by progesterone much later in development. By two weeks following the completion of metamorphosis, the ovary contains pre-meiotic germ cells and meiocytes representing every stage of meiosis up to the arrested diplotene stage.

The histological hallmarks of testis differentiation throughout this period of development are mainly cellular organization and proliferation, as spermatogenesis does not commence until seminiferous tubules are fully developed closer to sexual maturity. However, notable activities include invasion of blood vessels and mesenchyme cells into the testis between the cortex and medulla (as in the ovary), primary spermatocytes become encapsulated by somatic cells and organize into primordial seminiferous cords that eventually become tubules, and mesenchyme cells differentiate into Leydig cells that will produce steroid hormones (Ogielska, 2009c). Overall, the ovary undergoes more activity in germ cell development while the testis experiences more cellular movement and organization for duct formation.

The objective of this study was to characterize gene expression patterns associated with *Xenopus tropicalis* gonad differentiation and to identify candidate genes having robust associations with gonad development. A secondary objective was to compare the candidate gene expression profiles to existing knowledge of the orthologous gene expression in other species during gonadal development, to broaden the understanding of functional significance in a cross-species context. To that end, temporal

global expression profiles were measured in four different stages of differentiating *Xenopus tropicalis* ovary and testis tissues by microarray. Resulting expression profiles were compared between tissues at each developmental stage and enriched gene sets were analyzed further for functional associations. As part of the analysis process, human orthologs were identified for as many probe sets as possible so the extensive mammalian molecular interaction databases could be used for functional analyses and cross-species comparisons.

2. Materials and methods

2.1 Animal care and culture

The in-house *X. tropicalis* breeders used to generate animals for this study were of the "golden strain" and the female was confirmed to have the W-linked polymorphism identified by Olmstead *et al*. (2010). This sex-linked marker and a housekeeping marker were used to determine genetic sex of the offspring following sampling (described later). The animal care and culture conditions were the same as described by Olmstead *et al*. (2009b) and were as follows. Adult breeding pairs were maintained on a 12:12 light:dark photoperiod at 25^{o}C in 7 L glass aquaria with 50ml/min flow-through conditions and were fed 3/32″ premium sinking frog food pellets (Xenopus Express, Plant City, FL, USA). Water was from nearby Lake Superior (Duluth, MN, USA) and was ozone treated and filtered prior to use. Mating of breeding pairs was induced by injections of human chorionic gonadotropin (Sigma Aldrich, St. Louis, MO, USA). An initial injection of 20 IU gonadotropin followed by a second injection of 100 IU gonadotropin 5 hours later were administered to both the male and female breeder. Following mating, the adult breeders were moved to another tank and embryos were allowed to hatch in flow-through conditions. A single spawn was disbanded to a density of 40/tank (12:12 photoperiod, 25^{o}C, 7L tank, 25ml/min flow) and fed a diet consisting of homogenized cooked carrot and spinach, Sera Micron (Sera North America, Toronto, Canada), and brine shrimp three times daily until removed for sampling.

2.2 Experimental design

Four developmental time-points were considered for global expression analysis: NF58 (pro-metamorphosis), NF66 (completion of metamorphosis), one week post-metamorphosis (1WPM), and two weeks post-metamorphosis (2WPM). Gonad tissues were time-matched and stage-matched, so collection occurred on a single day for each developmental time-point and all animals used were from the same spawn. For the 1WPM and 2WPM time-points, all animals reached NF66 on the same day and were separated from the rest of the spawn until sample collection one and two weeks later respectively. On the day of tissue collection, animals were developmentally staged (Nieuwkoop and Faber, 1994) and euthanized by submersion in a lethal concentration of tricane methanesulfonate ([MS-222], Argent Chemical Laboratories, Redmond, WA, USA) buffered with sodium bicarbonate. Gonad tissues were then excised and transferred to a separate vial for each individual, homogenized in lysis buffer from an RNeasy micro kit (Qiagen Inc., Valencia, CA, USA), and immediately frozen on dry ice until samples could be moved to -80^{o}C storage. Tail or toe tissue was also collected from each individual for genetic sex determination using methods previously described by Olmstead et al. (2010). Once genetic sex was determined for each individual, testes and ovaries were randomly assigned to pools of four individuals (eight gonads per pool) to make a total of five pools of testis tissue and five pools of ovary tissue at each developmental time-point, with the exception of NF58 which had four pools of testis tissue and four pools of ovary tissue.

2.3 Total RNA target preparation and hybridization

Homogenates corresponding to the four individuals to be pooled were combined and then total RNA was extracted from each pool according to RNeasy kit instructions (Qiagen Inc., Valencia, CA, USA). RNA concentrations were measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., http://www.nanodrop.com) and quality was checked using a 2100 Bioanalyzer with an RNA 6000 pico assay

(Agilent, Santa Clara, CA, USA). Samples were then frozen at -80°C and shipped to the US EPA Genomics Research Core in Research Triangle Park, NC, where each RNA pool was amplified and labeled using the 3' IVT Express kit (Affymetrix, Santa Clara, CA, USA) according to kit instructions. Subsequent hybridization, washes, staining, and array scanning were performed according to Affymetrix GeneChip® *Xenopus tropicalis* Genome Array Expression Analysis instructions. CEL files containing feature intensities were generated using Affymetrix GeneChip® Command Console® software.

2.4 Data analysis

2.4.1 Data summarization

The data files (CEL) were imported into GeneSpring GX 12.6 (Agilent) software where they were summarized using the PLIER16 algorithm including a baseline adjustment to the median of all samples. The PLIER16 algorithm assumes multiple-array analysis, uses quantile normalization and a weighted approach to background correction using perfect match and mismatch probe pairs (Hubbell, 2004, 2005). Quality control was performed on all arrays using principle component analysis and hybridization control comparisons. The series of biotin-labeled hybridization control levels for one sample replicate (NF58 testis) were indicative of faulty hybridization. This sample was determined to be compromised based on these hybridization controls and was removed from subsequent analyses. As a result, the NF58 testis sample set consisted of three replicates while there were four replicate NF58 ovary samples.

2.4.2 Enhanced annotation

In order to utilize Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA, USA) developed for mammalian gene network, pathway and functional analysis, putative human orthologs were identified for as many probe sets as possible. This was performed by using Python programming language (version 2.7) to search the UniGene database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/unigene) with each UniGene ID provided in the *Xenopus*

tropicalis GeneChip® annotation file. If a human alignment was present for a particular *X. tropicalis* UniGene ID, the human protein accession number was appended to the annotation file for the associated probe set ID. Some probe sets had multiple associated UniGene clusters and therefore had multiple UniGene IDs. For cases where multiple human orthologs were found for a single probe set, the human ortholog with the highest alignment score was used for any subsequent analyses. The Python code written for this procedure is provided as Appendix A. For details on UniGene cluster assignments and protein similarity criterion, see UniGene FAQ (http://www.ncbi.nlm.nih.gov/UniGene/help.cgi?item=FAQ). The updated annotation file was then imported into GeneSpring so post-analysis gene lists including human ortholog accession numbers for IPA recognition could be exported.

2.4.3 Differential expression analysis and clustering

T-tests assuming unequal variance (Welch) were performed to compare the testis versus ovary tissues at each of the developmental stages (Table 1.1). P-values were computed asymptotically and the Benjamini Hochberg FDR multiple testing correction was applied (corrected p-value cutoff = 0.01). This analysis generated a gene list for each developmental stage which exhibited ≥ 2-fold, ≥ 5-fold, and ≥ 10-fold differential expression between tissues. The 2WPM, ≥ 5-fold gene list was chosen for functional analyses due to the 2WPM gonadal tissues being most differentiated and exhibiting the most differentially expressed transcripts. Additionally, using the ≥ 5-fold cutoff as opposed to the ≥ 2-fold cutoff was meant to target transcripts with more robust differences, but to also remain more inclusive than the ≥10-fold cutoff. Hierarchical clustering was performed on the 2WPM, ≥ 5-fold transcript list using Euclidian similarity measure and Ward's linkage rule. Similarity was determined using the transcript profile across all eight conditions (each gender and developmental stage) (Figure 1.2).

2.4.4 Functional analyses

Five clades from the hierarchical dendogram were chosen for functional analyses based on the similarity of their expression profiles. The testis-enriched transcripts

represented two clades while the ovary-enriched transcripts represented three clades. These five clades were exported from GeneSpring as individual gene lists including human orthologs and were then imported into IPA separately for functional analyses. Transcripts not having a human ortholog were filtered out during the import by default. Also, not all of the recognized ortholog accessions were considered for analyses because interaction data is not yet available for every molecule in the IPA database. Therefore, the resulting number of molecules considered in each analysis was always less than the number of transcripts in the original lists.

3. Results

Xenopus tropicalis ovary and testis transcriptomes were analyzed at four different developmental stages during active tissue differentiation using Affymetrix GeneChip technology. Each of the arrays contained 59,021 probes representing approximately 51,000 transcripts. The objective of this study was not only to identify transcripts differentially expressed between the two tissue types, but to identify transcripts showing robust differences between the tissues attributable to the genetic programs associated with gonad differentiation processes. Therefore, stringent cutoffs were applied including a corrected p-value cutoff of 0.01 (Benjamini Hochberg FDR) and fold difference cutoff of ≥ 5-fold for functional analyses.

3.1 Differential expression and clustering

According to the corrected p-value cutoff of 0.01, there were very few differentially expressed transcripts between tissue types at NF58 and all were testis-enriched (*i.e.,* elevated expression levels in the NF58 testis as compared to the NF58 ovary) (Table 1.1). Between NF58 and the completion of metamorphosis (NF66), there was an appreciable increase in the number of differentially expressed transcripts, with subsequent increases throughout the remaining developmental stages. In most cases, aside from NF58, the testis-enriched transcripts make up approximately 1/3 of the total differentially expressed transcripts regardless of fold-differences.

Table 1.1. Numbers of transcripts differentially expressed between tissues at each developmental stage. (T-test assuming unequal variance; Benjamini Hochberg FDR corrected $p < 0.01$).
Format of each cell: Testis-enriched / Ovary-enriched

	Fold difference		
Dev. Stage	≥2-fold	≥5-fold	≥10-fold
NF58	7 / 0	7 / 0	7 / 0
NF66	588 / 1079	88 / 91	27 / 19
1WPM	692 / 1626	95 / 196	41 / 54
2WPM	1487 / 2617	143 / 292	53 / 111

As explained in methods section 2.4.3, a decision had to be made regarding which transcript list to carry forward for functional analysis in order to set limits on the scope of the analysis. Gonad tissues at 2WPM are most differentiated histologically as compared to the earlier developmental stages and the differential expression data in Table 1.1 corroborates this fact. With regard to the fold-change cutoff, use of transcripts ≥ 5-fold different between tissues was intended to include only those transcripts showing robust differences between tissues, but at the same time, remain more inclusive than the ≥ 10-fold cutoff. Therefore, clustering and functional analyses were performed on the 2WPM, ≥ 5-fold differentially expressed transcript list.

Hierarchical clustering revealed a distinct separation between ovary and testis expression profiles (Figure 1.2). For interpretation, a coarse threshold was set to divide the dendogram into five clusters, which were each later analyzed for function using IPA software. Generally, testis-enriched transcripts in both clades are already being expressed at NF58 and continue to be highly expressed throughout the developmental periods examined. Transcript profiles in the ovary, particularly clade A, indicate that expression is initiated for many genes starting between NF58 and NF66 and levels of expression increase throughout this period of development, whereas many other ovary-enriched genes, most notably clade C, are induced sometime between one and two weeks post-metamorphosis.

3.2 Functional analyses

3.2.1 Ovary Clade A

Transcript expression profiles in this cluster are increasing substantially in the ovary throughout this period of development starting sometime between NF58 and NF66 (Figure 1.2, Appendix B). Each of the three ovary clusters show different timing of general transcript up-regulation. Ovary A generally shows up-regulation following Ovary B and prior to Ovary C. Many transcripts in the Ovary A cluster are functionally associated with gonadogenesis, gametogenesis, meiosis, and fertility including *FIGLA, BRDT, TDRD5, PABPC1L, HORMAD1, TEX11,* and *MSH5*. Interestingly, *BRDT, TDRD5, HORMAD1* and *TEX11* are associated with testis development in mammals and are being highly expressed in differentiating ovary tissue of *Xenopus tropicalis*. Several other transcripts are functionally associated with ovary development and oocyte maturation (*LHX8, NPM2, GTSF1, CGA, ZGLP1*). Additionally, many transcripts are associated with DNA replication, recombination, and repair, of which some are also associated with cell cycle and meiosis of germ cells (*DMC1, HORMAD1, MSH5, BRDT, TEX11, NPM2, TBP, SASS6, BANF1, SYCE2*). Another interesting result from this cluster is that many

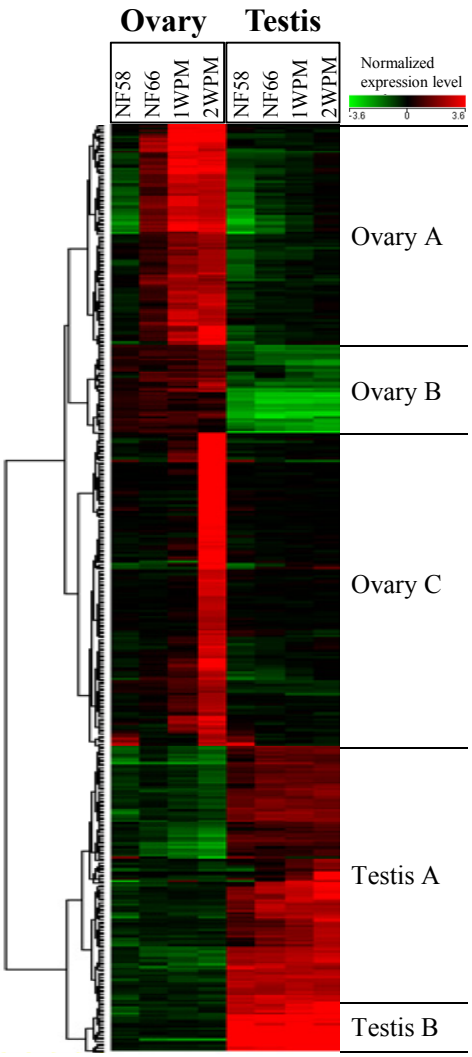

Figure 1.2. Hierarchical clustering of the 2WPM, ≥ 5-fold transcript list using Euclidian similarity measure and Ward's linkage rule. On the heatmap, red indicates the highest normalized expression level, green indicates the lowest normalized expression level and black indicates expression levels close to the median of all samples. Cluster designations are listed down the right side and correspond to specific clades within the dendogram on the left side of the heatmap.

transcripts are functionally associated with cancer, but lack information about more specific functions (see Appendix B). This is likely an artifact of the context in which these genes have been studied and the metadata associated with them in the IPA database. Nonetheless, these genes are likely associated with cell proliferation. The majority of gene products in this cluster reside in the nucleus (40%) and cytoplasm (32%) indicating an emphasis on intracellular processes (Figure 1.3).

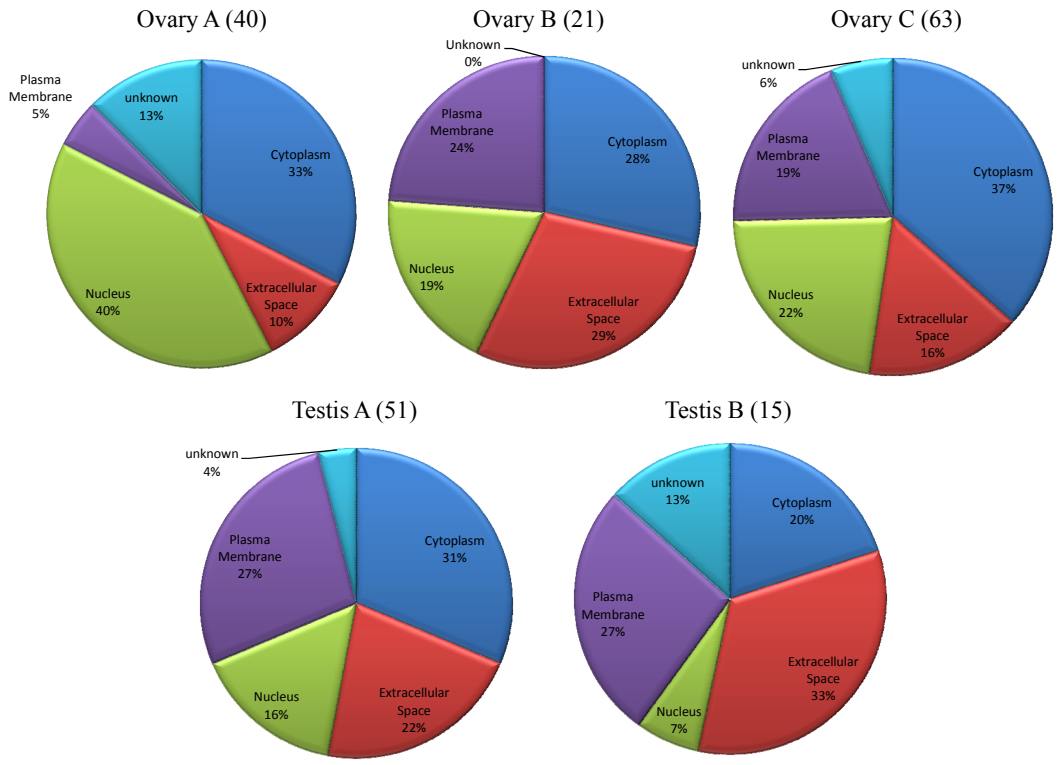

Figure 1.3. Cellular distributions of gene products by cluster. Total numbers of unique gene products per cluster are in parentheses next to cluster ID. Cellular location information was derived from IPA output.

3.2.2 Ovary Clade B

This cluster shows the earliest differential expression of the three ovary clusters starting at NF58 and remaining differentially expressed throughout this period of development, with some profiles showing increasing expression throughout this period of

development (Figure 1.2, Appendix B). Transcripts in this cluster are mainly associated with gonad morphology (*CHST8, EMX2, CYP26A1, GGT1, IRX5*), cell proliferation (*OLFML2A, LUM, NPPC, ESRP1, CLDN6, CLDN7, TACSTD2*) and primary sex determination (*EMX2, CYP19A1*). Several transcripts are also associated with nervous system development (*NTN1, KAL1, IRX5, A2M, IRX3*). Interestingly, several of the upstream regulators predicted to be controlling expression in this cluster are being expressed in testis tissue throughout this period of development (Testis Clade A: *FGF10, RARRES1, DAX-1*). Other predicted upstream regulators of genes in this cluster include β-estradiol, progesterone, and FSHβ. Gene products in this cluster primarily reside in the extracellular space (29%) and plasma membrane (24%) indicating an emphasis on intercellular signaling/activity (Figure 1.3).

3.2.3 Ovary Clade C

Transcripts in this cluster are substantially up-regulated between 1WPM and 2WPM. Many of them have very similar temporal expression profiles and similar magnitudes of up-regulation at 2WPM. Functionally, these genes are associated with reproductive system development and gametogenesis (*BMP15, CCNA1, COMT, GJA4, H2AFX, NANOS2, OCA2, SOX2, ZP3*), cell cycle and meiosis (*BMP15, BTG3, BTG4, CCNA1, CCNB1, EIF4E, LGALS3, MAD1L1, SOX2, WEE2*), transcription (*BMP15, BTG3, CAPRIN2, FOXH1, GSTP1, GTF3A, H2AFX, MAD1L1, SALL4, SOX2, TDGF1, ZFP36, ZFP36L2, ZIC2, ZP3*), and RNA binding, stabilization and trafficking (*CCNB1, EIF4E, ZFP36, ZFP36L2*). Possible upstream regulators of genes in this cluster are *NOBOX, MYBL2, SALL4* and *FIGLA*. *FIGLA* is present in the Ovary A cluster and shows a substantial increase in expression starting between NF66 and 1WPM, which precedes up-regulation of many genes in this cluster making it a particularly strong candidate transcriptional regulator. Gene products in this cluster mainly reside in the cytoplasm (37%) and nucleus (22%) indicating an emphasis on intracellular processes and is consistent with cell cycle regulation, transcription, and RNA binding, stabilization, and trafficking occurring for developing meiotic oocytes (Figure 1.3).

3.2.4 Testis Clade A

Gene expression profiles in this cluster generally show less differential expression between tissue types overall as compared to the Testis B cluster (Appendix B). Additionally, most of the transcripts in this cluster become more testis-biased between NF66 and 2WPM. This is due in part to general decreases in ovary transcript expression while testis expression remains constant (upper half of cluster) and general increases in testis transcript expression while ovary expression remains constant (lower half of cluster) (Figure 1.2). Many transcripts in this cluster are involved in cardiovascular system development (*TPM2, GAS6, CNN1, FBLN5, KCNJ8, MYH11*), vasculogenesis (*FHL2, COL13A1, ENPP2, SEMA3B, COL8A1, TGM2*), and connective tissue development (*INHA, INHBA, DHH, NLK, COL9A1, FHL2, FGF10*) (Appendix B). There are also many transcripts involved in reproductive system development and testis morphology (*NKX3-1, SRD5A2, INHA, INHBA, DHH, CYP26B1, FGF10, DAX-1*, *SNAI2*). Some of the same genes associated with testis morphology are also associated with sex determination (*SRD5A2, INHA, DHH, DAX-1*). In addition, there was significant enrichment of transcripts associated with steroidogenesis and androgen biosynthesis in this cluster (*AKR1C3, CYP17A1, SRD5A2, DKK3, INHBA, KITLG*). Upstream regulation analysis identified TGFβ and androgen receptor as potential activators based on enrichment of transcripts involved in those pathways. Gene products in this cluster show fairly even representation of possible cellular locations with a slight majority occurring in the cytoplasm (31%) (Figure 1.3).

3.2.5 Testis Clade B

Gene expression profiles in this cluster exhibit substantial expression levels in the testis throughout this period of development and are markedly higher than ovary expression levels, which are ostensibly absent (Figure 1.2, Appendix B). Functional analysis of this cluster indicates significant contributions to cardiovascular, connective tissue, and nervous system development as in the Testis A cluster. Specifically, transcripts are associated with blood vessel development (*ACTA2, MYL4, AQP1,*

FGFBP3), angiogenesis (*AQP1, GPC1, NPTX1*), collagen development (*BMP1*), and cell signaling and nervous system development (*NPTX1, SLITRK4, HTR2C, CLEC4M*). Three transcripts in this cluster are functionally associated with reproductive system development (*INSL3, ASTL, AQP1*) with the first two being associated with fertility. Functional analysis of this cluster also identified laminin as a potential upstream regulator. Laminin is localized to the basal membrane surrounding seminiferous tubules in *Xenopus tropicalis* testis tissue (El Jamil *et al*., 2008) and is known to influence cell migration, differentiation, and tissue organization (Hadley *et al*., 1990; Sasaki *et al*., 2004). The majority of gene products in this cluster are located in the extracellular space (33%) and plasma membrane (27%) indicating an emphasis on intercellular signaling and tissue development.

4. Discussion

Gonad tissues are unique from other vertebrate tissues in that the indifferent gonad is triggered, through genetic programs, to differentiate into two morphologically distinct mature tissues that both support germ cells and ultimately support reproduction. Gonad phenotype is determined genetically in some vertebrate species and by environmental triggers in others (*e.g.,* temperature). However, the sequence of events following sex determination and the molecular factors responsible for developing these tissues are similarly orchestrated in gonads across many vertebrate species, all in support of germ cells. Since gonad differentiation is susceptible to chemical perturbations leading to infertility or sex reversal at the individual level, efforts are necessary to understand the vulnerable targets and pathways leading to aberrant gonad development potentially causing adverse effects at the population level.

To contribute to the knowledge base at the molecular level, we employed microarray technology to identify gender differences in the genetic programs associated with gonadal development and differentiation in model Anuran species *Xenopus tropicalis*. To date, global gene expression analysis has not been performed strictly on ovary and testis tissues this early in *Xenopus* development, as they are difficult to excise

and close to impossible to differentiate visually. Recent advances in *Xenopus* research allows for the genetic determination of gender in individual animals (Olmstead *et al*., 2010; Yoshimoto *et al*., 2008) making it possible to extract ovary and testis tissues from larvae prior to these tissues becoming visibly dimorphic. By collecting tissues at four different time-points through development, temporal profiles of gonad gene expression specific to each sex were acquired.

Differentially expressed transcripts were clustered by their temporal profiles, and bioinformatic analyses of gene function and pathway enrichment were performed to associate transcripts with tissue processes and to identify potential upstream regulators. Performing these types of analyses with molecular interaction, pathway, and functional annotations specific to *Xenopus* proved to be both bioinformatically cumbersome and not easily interpreted. In contrast, the mammalian-based Ingenuity Pathway Analysis software provided a reasonably comprehensive analysis that facilitated more meaningful interpretation of results. However, human orthologues had to be acquired from the NCBI UniGene database in an automated fashion using Python scripting language. The assumption underlying the analysis is that orthologous genes play similar roles in frogs and humans. While it is recognized that there may be exceptions, the expression profiles of well-characterized gender-biased genes in gonad tissue including *CYP19A1* and *SRD5A2* provided confidence that this assumption was generally valid.

4.1 Ovary

At larval somatic stage NF58, the ovary (stage IV according to Ogielska and Kotusz' 2004 staging criteria for Anuran ovary development) exhibits a cortex containing germ cells and germ cell nests separated by a layer of prefollicular cells. At this stage of ovarian differentiation, the germ cells are dividing mitotically while blood vessels and mesenchyme cells invade the cortex (Ogielska, 2009b). The suite of genes being expressed at this time are in Ovary Clade B which includes, most notably, those coding for enzymes CYP19A1 (aromatase) and CYP26A1. Aromatase catalyzes the production of 17β-estradiol, which has been studied extensively for its role in sex determination and

sexual differentiation. It is no surprise aromatase is being expressed in the ovary as it is widely accepted to be a determining factor for whether the bipotential gonad becomes a testis or ovary; its expression profile supports the quality of these results. CYP26A1 is primarily involved in retinoic acid regulation and has been shown to be estrogen-responsive in human endometrium (Deng *et al*., 2003). Retinoic acid has also been identified as the key factor for initiating sex-specific timing of germ cell entry into meiosis in mammals (reviewed by Bowles and Koopman, 2007). Based on the timing of expression of other meiosis-specific transcripts in this dataset, it is highly plausible that retinoic acid, and regulator CYP26A1, are responsible for initiating *X. tropicalis* oocyte meiosis in a similar fashion.

Oocyte meiosis begins during ovary stage V which is approximately concomitant with the completion of metamorphosis (NF66). Many transcripts in Ovary Clade A are associated with meiosis and are up-regulated around NF66 including *HORMAD1, SPDYA, MSH5, SYCE1, BRDT, TEX11, DMC1, TDRD5,* and others. Interestingly, *HORMAD1, BRDT, TEX11,* and *TDRD5* have been primarily associated with testis tissue in mammals and were found to be playing roles in male germ cell meiosis (Chen *et al*., 2005; Gaucher *et al*., 2012; Jones *et al*., 1997; Wang, P.J. *et al*., 2001; Yabuta *et al*., 2011), but it is suggestive here that these genes also play significant roles in *X. tropicalis* oocyte meiosis. This superficial inconsistency in functional analysis output is another example of an artifact of the context in which genes have been studied and the metadata associated with them in the functional database. Upon further investigation, *HORMAD1, BRDT,* and *TEX11* have been found to play roles in both spermatocyte and oocyte meiosis (Houmard *et al*., 2009; Kogo *et al*., 2012; Paillisson *et al*., 2007). In mouse spermatogonial cell lines *TEX11* has been shown to modulate effects of estrogen receptor-β, and overexpression resulted in enhanced transcription of an estrogen-responsive reporter gene (Yu *et al*., 2012). Given the importance of estrogenic influence on gonadal development, *TEX11* could be a critical factor in *Xenopus* ovary and germ cell development ultimately affecting fertility.

Prior to the appearance of diplotene oocytes, which occurs during ovary stage VI, transcripts coding for notable transcription factors including *TBP, LHX8*, and *FIGLA* are

ovary-enriched. During this time, early folliculogenesis takes place and is orchestrated by signaling between the oocyte and cumulous, or granulosa cells. *FIGLA* has been shown to directly regulate transcription of a series of glycoproteins localized to the oocyte zona pellucida (Soyal *et al*., 2000). Notably, *ZP3* and *ZP4* are up-regulated subsequent to the up-regulation of *FIGLA* in this dataset. In mouse oocytes *FIGLA* appears to repress expression of testis-associated genes including *CYP17A1, DKKL1, TDRD1, TDRD6, TDRD7,* and others, while activating factors associated with oocyte development (Hu *et al*., 2010). Expression profiles of *CYP17A1* and *DKKL1* isoform *DKK3* in the present dataset are consistent with the findings of Hu *et al*. (2010) whereas the tudor-domain isoform *TDRD5* expression profile is ovary-enriched. This difference could be species related, temporally related, or isoform related, all of which would require further investigation. However, *FIGLA* has been well-characterized in mammalian reproductive development and plays a critical role in ovary development and fertility. These data support the structural and functional conservation of this transcription factor and its importance to ovary development across species.

Transcription factor *LHX8* has also been implicated in mammalian ovary development and fertility (Choi *et al*., 2008). Deficiencies in *LHX8* cause misexpression of *GDF9, POU5F1, NOBOX, KIT,* and *KITL*. *NOBOX* and paralog *POU4F1* were both predicted upstream regulators of ovary-enriched genes in clades B and C. *NOBOX* has been shown to regulate germ-cell specific genes such as *BMP15* and *ZAR1* (Rajkovic *et al*., 2004), both of which are highly ovary-enriched here at 2WPM. *BMP15* and *GDF9* are close homologs in the TGF-β superfamily of growth factors and are both responsible for activation of primordial follicle development in the human ovary *in vitro* (Kedem *et al*., 2011). Although *GDF9* was not represented in Appendix B, it was ovary-enriched at 2WPM by over 2-fold (corrected $p < 0.01$) supporting its role in follicle development across species. In *X. tropicalis*, *KITL* message is continually increasing in testis tissue over this period of development. While in the ovary, expression of this transcript continually decreases. This appears inconsistent with what is reported in mammalian primordial follicle development (Driancourt *et al*., 2000), as KIT (receptor) together with KITL (ligand) control oocyte growth and theca cell differentiation, and protects preantral

follicles from apoptosis. However, *KITL* has been well-characterized as an essential factor for mammalian spermatogenesis and male fertility (Vincent *et al*., 1998) consistent with expression levels in *X. tropicalis* testis tissues here. Furthermore, this emphasizes the potential importance of its proper expression and uninhibited function for reproductive success in mammals and amphibians alike.

In addition to transcription factor signaling during early folliculogenesis, maternal mRNAs begin to be transcribed in maternal cells and are localized to the developing germ cells. In *Xenopus laevis* embryos, maternal stores of *TBP* mRNA become translated leading up to the mid-blastula transition of embryogenesis and are translationally masked by association with a repressive protein prior to this event (Veenstra *et al*., 1999). This increase in *TBP* protein coincides with an increase in RNA polymerase I protein to which *TBP* associates in order for active translation to take place during embryogenesis (Bell and Scheer, 1999). *TBP* appears to be one of many maternal mRNAs that begin to be localized to the soon diplotene oocytes. However, *TBP* expression is up-regulated here around NF66, which is well before many of the other maternal mRNAs are up-regulated indicating a possible role in translation both in primordial follicle development and embryogenesis.

During ovary stage VI and around 2WPM, oocytes reach the diplotene stage of meiosis, and oocyte maturation processes begin including maternal RNA transport and localization within the oocyte, arrangement of the cytoskeleton and microvilli in the oocyte cortex, and incorporation of sperm receptor glycoproteins in the zona pellucida (Ogielska, 2009b). Components of these processes are evident in this data set, especially in Ovary Clade C, as most of these gene profiles show substantial expression levels starting at the 2WPM time-point. Notable maternal mRNAs in this dataset previously identified as oocyte-specific are *ZAR1, NANOS1, FOXI2, H1FOO,* and *VEGT* to name a few that play key roles in embryogenesis following fertilization (Cha *et al*., 2012; Furuya *et al*., 2007; Lai *et al*., 2012; Wu *et al*., 2003). Translational control of mRNAs is evident by several regulators that are highly ovary-enriched here (*ZFP36, ZFP36L2, EIF4E, PABPCL1*). These may play roles in maternal mRNA translational regulation and/or

regulate translation of factors involved in follicle development, but in either case show strong gender-biased expression profiles.

Oocyte meiosis is arrested at the diplotene stage and awaits resumption of meiosis during the later stages of progesterone-mediated oocyte maturation. *CCNB1, CCNB2,* and *CCNA1* all show robust expression around 2WPM when more oocytes are reaching the diplotene stage. *CCNB* complexed with a serine/threonine kinase (*CDC2*) mediates the G2/M transition when triggered upstream by progesterone (Nebreda and Ferby, 2000). Other genes involved in resumption of meiosis are *MAPK* and *WEE1*. Both of these genes are represented in this data set by putative homologs showing robust expression levels in the ovary starting around 1WPM and 2WPM respectively (*MAPK13* and *WEE2*). All of these transcripts are presumably being localized within the diplotene oocytes and inactivated prior to the events triggering resumption of meiosis.

4.2 Testis

Stage IV of Ogielska and Kotusz' (2004) staging criteria for Anuran gonad development is the point at which the undifferentiated gonad becomes either an early ovary or early testis. Therefore, similar to the ovary at somatic stage NF58, the testis is also at stage IV. This stage of testis development exhibits migration of germ cells and mesenchyme cells to the medullar space while the cortex recedes and eventually disappears (Ogielska, 2009a). The mesenchyme cells subsequently differentiate to become connective tissue and Leydig cells. Much uncertainty exists about what factor(s) signal germ cell migration toward the medullar space in Anurans ultimately differentiating into spermatogonia. Experimentation with parabiosis, transplanted gonad grafts, and steroid hormone exposure brought about a theory that the Anuran testis produces substance(s) other than steroid hormones that induce medullary development and is also capable of transforming ovaries of genetic females into testes (reviewed by Hayes 1998). Furthermore, the putative inducer substance(s) were later suggested to be blood-bound. This theoretical substance, termed "medullarin" by Chang and Witchi (1956), has not yet been identified nor has the mechanism been elucidated. Interestingly,

the mRNA showing the greatest magnitude of differential expression in the entire data set is a testis-biased relaxin family protein (rflcii) (Appendix B). The putative human ortholog for this gene is *INSL3*, which is a Leydig cell-specific insulin-like protein. Both of these proteins belong to the relaxin family of peptide hormones. This family of peptide hormones and their receptors have been implicated in various reproductive functions in humans and mice (Bathgate *et al*., 2013), but their roles in other vertebrates have not been well-studied. Based on our results, we propose that this *X. tropicalis* relaxin peptide hormone is a likely candidate for the unidentified "medullarin" substance promoting Anuran testis development.

Given that *INSL3* is Leydig cell-specific in mammals, it is likely that Leydig cells are present in the testis as early as NF58 despite being in the pre-spermatogenesis phase. Although fetal Leydig cells have been identified in 7-day-old rat testis tissues by immunohistochemical analyses (Svechnikov *et al*., 2010), histological studies in amphibians have not yet specifically identified how early Leydig cells differentiate and become influential in testis development. Our results now provide a precedent for "larval" Leydig cells being the amphibian equivalent to mammalian fetal Leydig cells. Also supporting the presence of Leydig cells at NF58 is the expression of genes coding for androgen-producing enzymes including *CYP17A1* and *SRD5A2*. Expression of these genes become more testis-enriched throughout this period of development. Another notable testis-enriched transcript codes for the *CYP26B1* enzyme. As discussed in relation to ovary-enriched *CYP26A1* during this period of development, CYP26 enzymes are responsible for retinoic acid regulation, which ultimately affects timing of germ cell meiosis (reviewed by Bowles and Koopman, 2007). *CYP26B1* expressed in Sertoli cells has been found to delay retinoic acid-mediated germ cell meiosis in mice testis tissue leading to development of spermatogonia later in reproductive maturation whereas Cyp26b1-knockouts exhibited precocious meiosis of germ cells similar to the timing of oocyte meiosis (Bowles *et al*., 2006). The observed patterns of CYP26 expression in *X. tropicalis* are consistent with what has been found in mammalian embryonic development research, emphasizing the conserved critical role of retinoic acid-mediated germ cell development.

In a very general sense, the testis-enriched transcripts are expressed at relatively constant levels as compared to the ovary-enriched transcripts that show time-dependent increases in expression throughout this period of differentiation. This can be attributed to the cellular organization and tissue morphology processes set in motion prior to NF58, and continuing until sex cords and ductules of *rete testis* are fully formed in the weeks and months following this period of differentiation. Orchestration of the various morphological changes in the differentiating testis involves signaling within and between cells involving extracellular proteins and growth factors (*FBLN5, DHH, FGFBP3, BMP1, ASTL, DKK3, NPTX1, FGF10, GAS6, INHA, INHBA, KITLG*), plasma membrane proteins (*ADCYAP1, COL13A1, RARRES1, INTGA2B*), and transcription factors (*DAX-1, FHL2, OSR2, SNAI2*).

Among the aggregation of primary spermatogonia into clusters that eventually become sex cords, mesenchymal cells begin to form connective tissue. Transcripts showing some of the highest magnitude of differential expression between testis and ovary tissue are *BMP1* and a similar gene *ASTL*, which are both pro-collagen metalloproteases (reviewed by Muir and Greenspan, 2011). Evidence of collagen production is further supported by the expression of various collagen isoforms *COL8A1, COL9A1,* and *COL13A1*. The recruitment of mesenchymal cells for the formation of connective tissue and Leydig cells involves the epithelial-mesenchymal transition (EMT) (reviewed by Thiery and Sleeman, 2006). This process has been shown to involve a complex network of signaling involving a series of genes well-represented by this data set's testis-enriched transcripts including a transcription repressor of E-cadherin *SNAI2*, an integrin cell surface receptor *INTGA2B*, fibroblast growth factor *FGF10,* and its putative activator *FGFBP3*. One component of the EMT is Wnt signaling, which is regulated in part by three members of the Dickkopf gene family (Krupnik *et al*., 1999). *DKK3* is testis-enriched here but has been shown not to function like the other Wnt antagonist Dkk isoforms. Instead, *DKK3* is related to a novel secreted protein *SGY* (*DKKL1*) that has been shown to localize to the acrosome in mammalian spermatocytes (Kohn *et al*., 2005). However, upon reviewing the original data, *DKK1* was testis-enriched by approximately 2-fold (corrected $p < 0.01$), supporting antagonist action on

the Wnt pathway allowing the EMT to take place in the testis. During the EMT, mesenchymal cells migrate and differentiate into the cells they are fated to become. This cell migration requires cytoskeletal components actin, myosin, and tropomyosin, which are all testis-enriched during this period of development (*MYH11, TPM2, ACTA2, MYL4*). However, whether the cells differentiate into connective tissue or support cells for spermatogenesis depends on other signaling factors.

One essential signaling factor being expressed in the testis is the desert hedgehog gene (*DHH*). This gene is expressed in Sertoli cells and is required for proper formation of sex cords by interacting with the peritubular myoid cells and inducing Leydig cell differentiation (Clark *et al*., 2000; reviewed by Franco and Yao, 2012). In fetal mice, Leydig cell differentiation is suggested to be triggered by Dhh-mediated steroidogenic factor 1 (*SF1*) up-regulation in cells outside the sex cords (Yao *et al*., 2002). A lack of Dhh protein in male Dhh-null mice has been shown to cause sterility, while having no effect on reproduction in females (Bitgood *et al*., 1996). This severity of response emphasizes the importance of *DHH* and associated signaling pathway to testis development; its expression pattern in *X. tropicalis* appears to be consistent with what is observed in mammals. Further investigation into modulation of *DHH* expression in Anurans is necessary as inhibition of this pathway could have major implications on successful reproduction.

Other notable testis-enriched transcripts in *X. tropicalis* that have been well-characterized in mammalian reproductive development include inhibin subunits *INHA* and *INHBA*, and also *DAX-1* (*NR0B1*). Inhibins belong to the TGF-β protein superfamily and are multifunctional hormones involved in control of FSH secretion from the pituitary and also paracrine/autocrine signaling within the human testis (Luisi *et al*., 2004). In the fetal human testis, *INHA* is expressed in Sertoli cells and both *INHA* and *INHBA* are expressed in Leydig cells. However, inhibin B is the subunit expressed later in development that is used clinically as a diagnostic indicator of Sertoli cell function, spermatogenesis, and fertility (Pierik *et al*., 1998). Therefore, it is clear *INHA* and *INHBA* are playing roles in early gonadogenesis, presumably consistent with mammalian testis development, but currently lack correlation with fertility.

Finally, *DAX-1* is a multifunctional orphan nuclear receptor protein that, with duplication, is capable of causing male to female sex reversal in mammals with a normal *SRY* gene (Lalli and Sassone-Corsi, 2003). It has been shown to play many regulatory functions within steroidogenic tissues through various mechanisms including DNA binding to transcription activation sites, recruitment of co-repressors, and interaction with the androgen receptor and estrogen receptors α and β. DAX-1 has been shown to directly interact with steroidogenic factor 1 (SF1) negatively regulating transcription of steroidogenic enzymes including CYP17, CYP19, and others (Gurates *et al*., 2002; Hanley *et al*., 2001; Wang, Z.J. *et al*., 2001). Inhibin α (*INHA*) discussed previously has also been shown to be negatively regulated either directly or indirectly by *DAX-1* (Achermann *et al*., 2001a). Therefore, the expression pattern of *DAX-1* in *X. tropicalis* testis tissue during these stages of development is very curious, as the sex determining gene has not been found in this species (it is not known to have functional *SRY*) and expression levels of *CYP17* and inhibin α appear to be uninhibited. However, *CYP19* expression in the testis is low to absent suggesting a plausible mechanism for maintaining a masculine tissue during this period of development. The *DAX-1* expression pattern in *X. tropicalis* testis tissue is consistent with *DAX-1* expression patterns in *Rana rugosa* (Sugita *et al*., 2001) indicating the importance of this transcript in frog testis development despite it being an "anti-testis" gene in mammals. This gene's key activities and diverse mechanisms, within and between species, make it a particularly interesting candidate for further investigations regarding cross-species differences in reproductive development.

5. Conclusion

The wealth of expression data was generated by this experiment. While the analysis and discussion was focused on transcripts with greater than 5-fold (corrected $p < 0.01$) differential expression between tissues, many other transcripts showing gender-biased expression remain to be explored. Robust expression profiles of key transcripts previously characterized in gonad development and the comprehensive representation of the various well-known developmental processes involved in gonad differentiation

emphasize the quality of this data set. Although limited to consideration of transcriptional differences, these data provide many insights into cross-species similarities of expression profiles specific to gonadal development, differentiation, and fertility. This work has generated numerous testable hypotheses and provides a foundation for subsequent investigations of chemical-induced perturbations of pathways proximal to molecular initiating events. Linking these molecular initiating events to adverse effects on the reproductive organs will identify vulnerable targets of environmental pollutants, aid in elucidation of adverse outcome pathways, and have potential for cross-species extrapolation and population modeling.

Chapter 2. Measuring testis dysgenesis using gene expression biomarkers in *Xenopus laevis* following exposure to prochloraz.

1. Introduction

The anti-androgenic effects of prochloraz in other species provided precedent to utilize this model chemical to evaluate expression-based biomarkers of testis dysgenesis. Prochloraz is an imidazole fungicide shown to have multiple mechanisms of action within the HPG axis. *In vitro* studies have shown that prochloraz has characteristics of both an antagonist of the androgen receptor and inhibitor of aromatase activity (Noriega *et al*., 2005; Villeneuve *et al*., 2007; Vinggaard *et al*., 2002). In adult fish, *in vivo* exposure to prochloraz produces effects that are consistent with inhibition of aromatase (Ankley *et al*., 2005). Further, developmental exposure in rats produces a spectrum of male reproductive tract malformations which have been attributed to anti-androgenic properties (Noriega *et al*., 2005; Blystone *et al*., 2007; Vinggaard *et al*., 2002). Interestingly, females were not affected by developmental exposure in the aforementioned studies.

In an attempt to better elucidate the anti-androgenic mode of action, careful selection of specific genes proximal to the androgenic regulatory network was warranted. The gene array study presented in Chapter 1 provided a foundation for selection of candidate genes exhibiting robust, statistically testis-biased expression profiles as a starting point. Specific genes were then identified as potential indicators of biological response at different points within and around the putative androgen molecular network. Specifically, genes were selected that have precedent for being (1) directly regulated by androgen/androgen receptor (Insl3/rflcii), (2) mainly responsible for producing endogenous androgen (Cyp17a1), (3) an endogenous suppressor of androgen receptor activity (Dax-1), and (4) suppressed by active androgen/androgen receptor signaling (Sox9b).

Relaxin family locus C type 2 (rflcii) is orthologous to the mammalian insulin like protein INSL3. INSL3 is a Leydig cell-specific peptide hormone responsible for testis

descent in mammals and plays a role in follicle selection in the adult ovary (Bathgate *et al*., 2013). In the gene array study presented in Chapter 1, *Xenopus tropicalis* rflcii was the transcript showing the highest normalized expression level overall, which was also the most differentially expressed transcript between ovary and testis tissue. This profound difference in expression between tissues provides early evidence of this gene's importance in *Xenopus* testis development. Furthermore, mammalian Insl3 has been shown to be positively regulated by testosterone/androgen receptor. It also contains an androgen responsive element in its promoter region and anti-androgen mono-(2-ethylhexyl) phthalate inhibits testosterone-induced *Insl3* expression (Laguë and Tremblay, 2008). Given this information, I hypothesized that the anti-androgen prochloraz would cause a decrease in testis expression levels of rflcii.

Cytochrome P450 (CYP) c17α-hydroxylase/17,20-lyase (CYP17) is an enzyme necessary for the production of steroid hormones. This key steroidogenesis enzyme is highly conserved across species. The Cyp17a1 transcript exhibited testis-biased expression in the gene array study (Chapter 1) and is generally associated with testosterone production, even though estrogen production proceeds through this same pathway. Prochloraz not only inhibits androgen receptor, but also inhibits CYP17 (Blystone *et al*., 2007; Noriega *et al*., 2005; Vinggaard *et al*., 2002) and other CYPs due to the imidazole interaction with iron. The inhibition of ergosterol synthesis by this interaction with 14α-demethylase (CYP51) is what gives prochloraz its antifungal properties (Van den Bossche *et al*., 1978). Therefore, CYP17 inhibition by prochloraz could be a major contributor to its anti-androgenic effects. Given the importance of this enzyme for testosterone production and the potential for inhibition by prochloraz, I hypothesized that prochloraz exposure would increase expression of CYP17 in the testis due to a compensatory response.

Dax-1 (NR0B1) is a nuclear receptor that has been shown to play a substantial role in mammalian gonad development as a transcriptional repressor of steroidogenesis (Zazopoulos *et al*., 1997). In mammals, duplication and over-expression of this gene causes male-to-female sex reversal, but loss of DAX-1 function in knockout mice does not affect normal ovary development, which is why it has been referred to as an "anti-

testis" gene (Muscatelli *et al*., 1994; Yu *et al*., 1998a; Zanaria *et al*., 1994). Early in *Xenopus tropicalis* gonad development, Dax-1 expression is testis-enriched (Chapter 1), and is consistent with expression profiles in *Rana rugosa* (Sugita *et al*., 2001) indicating that Dax-1 also plays an active role in anuran testis development. In addition to its repressive effects on steroidogenesis, mammalian DAX-1 protein has been shown to bind to androgen receptor inhibiting its transcriptional activity (Holter *et al*., 2002), presenting as an endogenous anti-androgen. However, steroidogenic factor 1 (SF-1), a transcriptional regulator of many genes involved in steroidogenesis and key determinant of endocrine differentiation and function (Parker *et al*., 2002), has been shown to bind the murine Dax-1 promoter, stimulating its expression (Yu *et al*., 1998b). DAX-1 protein then antagonizes SF-1-mediated gene activation (Guarates *et al*., 2003; Nachtigal *et al*., 1998), presenting as an SF-1 self-regulatory mechanism. Other mechanisms of Dax-1 regulation have also been demonstrated but are cell/tissue-specific and developmentally-specific. Down-regulation of Dax-1 transcription in adult rat Sertoli cells has been shown to be regulated by pituitary FSH through the cAMP signaling pathway (Tamai *et al*., 1996) and up-regulated in the differentiating ovary through the Wnt4/β-catenin pathway (Mizusaki *et al*., 2003). Because of the potent suppressive capabilities of Dax-1 protein on androgen receptor and SF-1-mediated steroidogenesis, up-regulation of this gene by prochloraz through an unknown mechanism could be a contributing factor to apical manifestations of anti-androgenic action. However, without precedent for a plausible mechanism by which this could occur, I hypothesize that expression of Dax-1 will not be affected by prochloraz exposure.

SRY (sex-determining region Y)-box 9 (Sox9) is an important transcription factor that regulates testis development (reviewed by Koopman *et al*., 2001). Although this gene was discovered in the XY sex determination system, it is conserved across species and is also active in the ZW sex determination system (*Xenopus*). The weight of evidence surrounding Sox9 behavior in testis development across species implies that it is a pivotal sex-determining gene, regardless of the genetic switch that activates its expression. In mammalian Sertoli cells it has been shown to interact with steroidogenic factor 1 (SF-1) protein as a cofactor for anti-Müllerian hormone (AMH) gene activation

(De Santa Barbara *et al*., 1998). It has also been shown that androgen/androgen receptor signaling has a repressive effect on Sox9 expression *in vitro* (Lan *et al*., 2013). Supporting this relationship, circulating AMH in humans during pubertal development has been shown to be negatively correlated with circulating testosterone (Rey *et al*., 1993) and lack of androgen receptor expression in human Sertoli cells during early testis development was found to account for high levels of AMH (Boukari *et al*., 2009).

AMH mediates Müllerian duct regression in male reproductive development and absence of AMH causes "default" maturation of Müllerian ducts in females with subsequent disappearance of male-specific Wolffian ducts (Kelley, 1996). Normal *Xenopus laevis* males exhibit Müllerian duct (oviduct) regression around two months post-metamorphosis implying that AMH expression is active – possibly mediated by SOX9. Although the Sox9 expression profile is not presented in Chapter 1, it was indeed differentially expressed in two week post-metamorphosis *Xenopus tropicalis* testis tissue compared to ovary tissue by almost 3-fold (Welch's t-test, $p < 0.01$). If prochloraz inhibits androgen receptor and testosterone production, one would expect uninhibited Sox9 expression with possible dose-dependent acceleration of AMH-mediated oviduct regression. However, accelerated oviduct regression would generally be considered an androgenic or anti-estrogenic effect, inconsistent with the previously observed anti-androgenic effects of prochloraz. Regardless of this contradiction, I hypothesize that inhibition of androgen receptor and testosterone production will cause Sox9 expression to increase with prochloraz treatment.

Gonad differentiation and development is orchestrated by the complex interplay of many different genes and gene networks. The selection of these four genes to evaluate as expression markers of testis dysgenesis following exposure to an anti-androgen is an initial step toward better understanding this chemical mode of action in amphibian gonad development. These genes are not well-characterized in amphibians and much is unknown about their developmental expression or molecular interactions within *Xenopus*. Ideally, the results from this experiment will not only inform the anti-androgenic mode of action in *Xenopus*, but will also inform species differences in regulation of these genes

important to gonad development and help to identify species-specific targets susceptible to chemical perturbations.

2. Materials and methods

2.1 Experimental design

Xenopus laevis embryos (≤ NF stage 15) were exposed to four different chemical concentrations and control water until two months after the median time to NF stage 66 in controls (See Nieuwkoop and Faber 1994 for staging details). There were five replicates in each test concentration with eight replicates for the control. Endpoints evaluated during the course of the exposure included those indicative of generalized and metabolic toxicity: mortality, abnormal behavior, growth determinations (length and weight) and histopathology of the juvenile liver and kidney. In addition, endpoints indicative of endocrine-specific toxicity were evaluated including larval thyroid histopathology, and juvenile histopathology of gonads and reproductive ducts. Either the right or left gonad was chosen randomly for histopathological evaluations while total RNA was extracted from the contralateral gonad for gene expression measurements. Genotypic/phenotypic sex ratios were also determined.

2.2 Test concentrations

Test concentrations were chosen deliberately to perturb the endocrine system and cause measurable effects on organ morphology and/or levels of endocrine-mediated biochemical indicators. The high test concentration was set based on range finding experiments conducted by MED. In those studies conducted with *X. tropicalis,* concentrations in excess of 200 µg/L resulted in mortality in early larval organisms. Therefore, the high test concentration was set at 180µg/L with a 0.33 dilution factor resulting in nominal test concentrations of 180, 60, 20 and 6.7µg/L.

2.3 Environmental conditions

Organisms were cultured in Lake Superior Water (LSW) initially filtered through sand, UV sterilized and filtered again to 5 µm before entering the in-house culture or exposure aquaria. Source water was tested on a yearly basis for contaminants including copper, chlorine, chloramine, estrogenic or androgenic chemicals, fluoride, perchlorate and chlorate as measurable levels of any of these compounds could confound results of the bioassay. Larvae were held at 21°C throughout the duration of the exposure by controlling the temperature of the delivery/dilution water and also by a water bath in which the exposure tanks were partially submerged. Dissolved oxygen, pH and conductivity were measured in all exposure tanks once per week throughout the duration of the test whereas alkalinity and hardness were measured once per month in one replicate tank per treatment (Table 2.1). There were no treatment-related effects on any water quality parameters. A 12 hour light and 12 hour dark cycle was used with fluorescent lighting at intensities between 600 and 2,000 lux (lumens/m^2) at the water surface. Exposure tanks were randomly assigned to positions within each exposure system to reduce potential effects manifested by systematic differences in environmental conditions including slight temperature variations or light intensity.

Table 2.1. Measured water quality parameters throughout the duration of prochloraz exposure. Mean ± SD.

Temperature (°C)	D.O. (% sat.)	pH	Alkalinity (mg/L $CaCO_3$)	Hardness (mg/L CaCO3)	Conductivity (µS)
21.0 ± 0.4	76.9 ± 15.2	7.45 ± 0.44	42.4 ± 1.7	47.0 ± 0.8	98 ± 2

2.4 Exposure system

The exposure dilution system consisted of a series of single drive/dual head metering pumps (Fluid Metering Inc., Syosset, NY) through which both diluent and toxicant flowed to make a single concentration in a stainless steel mixing cell. Flow-through conditions were maintained by delivering toxicant and control LSW solutions to the 6.5L exposure tanks at a constant rate of 44 mL/min with multichannel peristaltic

pumps in order to meet or exceed 9.5 tank turnovers per day. Wetted surfaces were strictly glass, Teflon®, Pharmed® tubing, or stainless steel.

2.5 Chemical stock preparation

18 mg/L prochloraz stock solutions were prepared by dissolving the neat material (Sigma-Aldrich, CAS 67747-09-5, purity: 99.1%) in 20 mL of high purity acetone and quantitatively transferring to a 19 L carboy. The side walls of the carboy were rinsed with acetone and it was carefully tipped onto its side and placed on a roller. Filtered and desiccated compressed air was gently blown into the bottle as it was rolled to evaporate the acetone and "shell coat" the carboy with prochloraz. Once the acetone was completely evaporated, the carboy was filled with reverse osmosis water and stirred overnight to dissolve the prochloraz.

2.6 Analytical chemistry[1]

Water samples (~1 mL) were collected weekly from the prochloraz exposure tanks (0, 6.7, 20, 60 and 180 µg/L) and placed into amber sample vials. The water samples were amended with 0.1 mL methanol, vortex mixed and immediately analyzed using an Agilent 1100 series high performance liquid chromatograph (HPLC) with diode array detection (λ = 205 nm). An aliquot (150 µL) of sample was injected directly into a Zorbax SB-C18, 2.1 x 75 mm column (Agilent, Wilmington, DE, PN# 866735-902) that was maintained at 25°C. The auto sampler temperature was maintained at 5°C. Prochloraz eluted from the column under isocratic conditions with 22% Burdick & Jackson (B&J) HPLC grade water and 78% methanol at 0.3 mL/min. Prochloraz concentrations were determined by the external standard method of quantification using linear regression. Prochloraz standards range from 5 to 200 µg/L and were prepared in 90% B & J water and 10% methanol. Quality assurance samples were conducted with each sample set and included Lake Superior water (LSW) blanks, duplicate samples and matrix spiked samples at each of the prochloraz treatment levels. No prochloraz was detected in the LSW blanks (n = 15). Mean ± SD (n = 15) recoveries of spiked samples

were 102.2 ± 3%, 99.5 ± 2.5%, 99.3 ± 2.5% and 100.7 ± 2.2% at the 6.5, 20, 60 and 180 µg/L spiked levels, respectively. Mean ± SD (n = 15) agreement among duplicate samples were 99.8 ± 1.9%, 99.3 ± 3.3%, 99.4 ± 1.5% and 100.3 ± 1.5% for the 6.7, 20, 60 and 180 µg/L treatments, respectively. The detection limit was 2.5 µg/L.
[1]Analytical chemistry was performed by Patricia Kosian (USEPA, Duluth, MN)

2.7 Test initiation

All animal care and use procedures for these experiments were reviewed and cleared by the division Animal Care and Use committee. Adult in-house breeding pairs were induced by injection of 750 IU human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO, USA) into the dorsal lymph sac of each male and female and were allowed to enter amplexus in static 10L tanks filled with 6L of culture water. Eggs were collected and jelly coats were removed by L-cysteine treatment approximately 14-16 hours after pairing. A 2% L-cysteine solution was prepared and pH adjusted to 8.1 with 1N NaOH. This 21^{o}C solution was added to a 500 mL Erlenmeyer flask containing the eggs from a single spawn and swirled gently for one to two minutes and then rinsed thoroughly 6-8 times with culture water. The spawn was transferred to a crystallizing dish and determined to be >70% viable with minimal abnormalities in embryos exhibiting cell division. Embryos were carefully screened for any cellular or superficial abnormalities and then sorted by stage and distributed evenly into each of 28 - 250 mL glass beakers containing approximately 20 mL of culture water. Each beaker contained 26 embryos to be added to each exposure tank. An attempt was made to initiate exposure at or before NF stage 8, but screening and distribution efforts delayed initiation of exposure until no later than NF stage 15. At test initiation, beakers containing the embryos were carefully submerged in the exposure tank solution and set on the bottom of the tank. This technique kept the embryos localized within the beaker until they were able to swim out into the tank on their own and allowed for simple observations of embryonic development. The beakers were positioned directly under the test solution delivery line to facilitate solution renewal and flow through the beaker. When all of the organisms had

left the beakers, the beakers were removed from the exposure tanks. Observations of arrested embryonic development were recorded prior to hatch, and mortality and abnormal behavior were recorded daily throughout the duration of the exposures.

2.8 Feeding

Larvae were fed a liquid diet three times per day on weekdays and once per day on weekends starting on day 4 post-fertilization. The diet consisted of Silver Cup Trout Starter (Nelson and Sons, Murray, UT, USA), *Spirulina* algae discs (Wardley, Secaucus, NJ, USA) and TetraFin® flakes (Tetra Sales, Blacksburg, VA, USA) blended together in culture water. Tadpoles were also fed 24-hour old live brine shrimp (Bio-Marine® Brand, Bio-Marine, Hawthorne, CA, USA) twice daily on weekdays and once daily on the weekends starting on day 8 post-fertilization. Brine shrimp nauplii were hatched overnight and separated from cyst casings with appropriately sized screen filters before use. Feeding was increased weekly until the majority of individuals in any given tank developed to NF stage 62 at which time food was decreased proportionately. When individuals completed metamorphosis (NF66), the diet was changed to 3/32" premium sinking frog food pellets (Xenopus Express, Plant City, FL, USA) until test termination.

2.9 Larval sub-sampling

Larval sub-sampling was performed by dividing the exposure tank and separating individuals that reached NF66 from individuals that had not yet reached NF66 and recording the date on which each individual completed metamorphosis. One out of each group of four consecutive individuals completing metamorphosis in any given tank was randomly sampled for growth metrics including weight and snout-to-vent length, and thyroid histopathology. At any given time during metamorphosis, developmental stages of organisms within an experimental unit are generally normally distributed. This larval sub-sampling method was intended to reduce bias toward sampling individuals on the leading or tailing edges of development in a particular tank by spreading the sampling out over time. That way all individuals in a tank had equal likelihood of being sampled upon

reaching NF66. Sub-sampled individuals were euthanized by submersion in 200 mg/L tricaine methanesulfonate ([MS-222], Argent Chemical Laboratories, Redmond, WA, USA) buffered with 400 mg/L sodium bicarbonate. Following growth measurements, a transverse cut was made at the posterior margin of the arms for decapitation and the head was fixed in Davidson's solution for thyroid histopathology. Individuals not sampled were left in the divided tank until all individuals had reached NF66 or day 70, at which time the tank dividers were removed and any larvae that hadn't reached NF66 were euthanized by immersion in MS-222. The purpose of the larval sub-sample is to evaluate the chemical's effect on the hypothalamic-pituitary-thyroid axis, which is outside the scope of this thesis. Therefore, methods or results related to the larval sub-sample endpoints will not be discussed.

2.10 Cull

Two to three weeks following the time at which all individuals completed metamorphosis (NF66), a cull was performed to reduce tank density and ideally result in five males and five females remaining in each tank until test termination. Toe clips were taken from every individual on test in a manner where they could be uniquely identified based on tank ID and toe clip scheme. These toe clips were analyzed for the presence or absence of DM-W in order to determine genetic sex (described in later section). An even number of males and females were randomly selected to remain in the tank and the remaining juveniles were euthanized by i.p. injection of 100 mg/mL MS-222 dissolved in phosphate buffer (pH 7.4).

2.11 Juvenile sampling (test termination)

When the study was terminated, endpoint measurements were collected on all juveniles immediately following euthanasia by i.p. injection of MS-222. Juveniles were weighed and measured for snout-to-vent length; the abdomen was opened and the liver was carefully removed intact, weighed, placed in a labeled cassette and transferred into Davidson's fixative. Subsequently, the remaining internal viscera was removed and

discarded with taking care to leave the gonads, kidneys and reproductive ducts undamaged and *in situ* for fixation. One of the gonads was randomly chosen to be removed for gene expression analysis, homogenized in lysis buffer from an RNeasy Plus kit (Qiagen Inc., Valencia, CA, USA), and immediately frozen on dry ice until samples could be moved to -80^{o}C storage. Gonad phenotype was recorded and a foot was removed and frozen for genetic sex determination. The torsos containing a gonad, kidneys and reproductive ducts were placed in individually labeled cassettes and transferred to Davidson's fixative.

2.12 Histological procedures

All tissues preserved for histological analyses were initially fixed in Davidson's fluid for 48 hours with constant mixing and at least 10x the volume of fixative relative to the volume of tissue. After approximately 48 hours, tissues were removed from Davidson's, rinsed with tap water and stored in 10% neutral buffered formalin. Tissues were shipped in neutral buffered formalin to Integrated Laboratory Systems, Inc. (ILS®), Research Triangle Park, NC, USA, where they were processed, embedded, sectioned, mounted and stained with hematoxylin and eosin (H&E) using typical histological procedures. Histopathologic findings were scored for severity by an ILS® pathologist according to the following grading system: X = not remarkable, Grade 1 = minimal (less than 10% of tissue affected), Grade 2 = mild/slight (≥10% but less than 40% of tissue affected), Grade 3 = moderate (≥40% but less than 80% of tissue affected), and Grade 4 = severe (≥80% of tissue affected). However, for the purposes of this thesis, data is presented as incidence, regardless of severity. The juvenile livers were embedded in their entirety and 200µm step sections were taken until the largest possible cross-sectional area was reached. Two 4µm thick sections were mounted on a single slide per individual, stained with H&E and evaluated by brightfield microscopy for pathology. The juvenile gonad, kidney and reproductive ducts were left *in situ* and were processed, sectioned and evaluated within the same sections. Torsos were trimmed transversely at the anterior and posterior margins of the gonads, and then the gonads were measured on the anterior-

posterior axis and bisected transversely in the exact center to yield two equally sized sections – one anterior and one posterior. Each section was embedded in a separate mold in order to efficiently obtain sections representative of the anterior, middle and posterior regions of the gonad. This was done to evaluate differences in sensitivity to chemical exposure between regions of the gonad due to evidence of the gonad differentiating along an antero-posterior gradient in *Xenopus tropicalis* (El Jamil et al. 2008). This regional comparison is beyond the scope of this thesis and will not be discussed. Data presented herein are incidences of a particular pathology in any of the three sections.

2.13 Genetic sexing

A hind limb from each individual was frozen at -20^{o}C until DNA could be purified using DNeasy kits (Qiagen Inc., Valencia, CA, USA) according to kit instructions. Testing for the presence or absence of the *Xenopus laevis* W-linked marker (DM-W) was performed by PCR based on the method described by Yoshimoto et al. (2008). DMRT1 was also amplified in duplexed PCR reactions as a housekeeping gene.

2.16 Testis RNA processing

Total RNA was isolated from testis homogenates using RNeasy Plus mini kits (Qiagen Inc., Valencia, CA, USA) according to kit instructions. RNA concentrations were initially measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., http://www.nanodrop.com). However, several factors warranted a more accurate determination of RNA concentrations: (1) low and variable yield, (2) the need to pool tank samples with equal representation of each individual, and (3) the use of total RNA concentrations to normalize expression values. To this end, a fluorescence-based RNA quantitation assay was performed on three samples randomly chosen per tank (Quant-iT^{TM} Ribo-green$^{®}$, Life Technologies, Grand Island, NY, USA). Samples showing less than 2ng/µl concentrations according to NanoDrop results were avoided if possible. One tank from the 20µg/L prochloraz treatment was excluded from subsequent analyses due to extremely low yields from all testis samples. RNA quality was checked

on all chosen samples using a 2100 Bioanalyzer with an RNA 6000 pico assay (Agilent, Santa Clara, CA, USA). If samples had obvious issues with integrity, a new sample was randomly chosen from that tank and its concentration and integrity were measured prior to pooling. The three samples per tank were then diluted to between 0.5 and 1ng/µl (depending on the lowest concentration of the three samples to be pooled) and combined in equal amounts to create one pool per tank. Each pool was divided into multiple aliquots to avoid freeze/thaw cycles and a new total RNA concentration was determined for each pool using the same fluorescence method previously described. All subsequent gene expression levels were normalized to the total RNA concentration in each respective pool.

2.17 Quantitative PCR

X. tropicalis accession numbers from four testis-enriched genes were aligned with the NCBI EST database for *X. laevis* using the nucleotide Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The highest scoring sequences were used to design primers and probes for use in a TaqMan® EZ RT-PCR assay (Applied Biosystems®) (Table 2.1). Primers and probes were designed using Primer Express version 3.0 (Applied Biosystems®). Standard RNA templates were generated by first generating cDNA and amplifying the target sequence using outer primers with a one-step reverse-transcription PCR kit (Enhanced Avian HS RT-PCR kit; Sigma-Aldrich). Next, target cDNA was amplified using separate primers that flanked the qPCR primers and contained the T7 promoter region for subsequent *in vitro* transcription using a MEGAScript kit (Ambion®) according to kit instructions. Transcribed RNA template concentrations were determined using the mean of three measurements from a NanoDrop ND-1000 Spectrophotometer. Pooled samples were run in 96-well plates according to TaqMan® kit instructions on an Agilent Strategene Mx3005P real-time quantitative PCR instrument. Standard curves and data were acquired from MxPro –Mx3005P9® software version 4.10 Build 389, Schema 85 (Stratagene).

Table 2.2. Accession numbers, primer and probe sequences used for quantitative PCR of selected gene transcripts.

Gene symbol	*X. tropicalis* accession	*X. laevis* accession	Primers (5' -> 3')	Probe (5' -> 3')
Cyp17a1	NM_001127045.1	NM_001097071.2	For: CCACGTGCTGTGACAACAGATA Rev: GCACTACCTTGCGGTGAAACT	ACATTGCTTTCGCCAACTACAGCCCA
rflcii	NM_001126835.1	CV081081.1	For: TGACACCATGGAACAGTTGCA Rev: CCGCACTCCTCCTCATCCT	TAGGTCAAGAACCACGGAGCACCAGC
Sox9b	NM_001016853.2	NM_001094473.1	For: TCCGTGATGTGGACATTGGT Rev: GGCAGGTACTGGTCAAATTCATT	AGTGAGGTCATCTCCACCATCGAAACCTT
Dax-1	XM_002933615.2	AB273178.1	For: GGCTTGCACTGCATCCAGTA Rev: GTGAATCATCTTGATGTGTTCGTTT	CAAGGACTACAGCATGAAGCTCAACAAGCA

2.18 Statistics

All data from juveniles were analyzed separately according to genetic sex. Continuous data (*e.g.* weight, length, LSI, mortality, gene expression) was checked for monotonicity by rank transforming the data, fitting to an ANOVA model and comparing linear and quadratic contrasts. All data was monotonic, so a step-down Jonckheere-Terpstra trend test was performed on replicate medians. Mortality data was analyzed for the time period encompassing the full test and was expressed as percent that died in any particular tank. Tadpoles that did not complete metamorphosis in the given time frame, those tadpoles that were in the larval sub-sample cohort or that were culled, and any animal that died due to experimenter error was treated as censored data and not included in the denominator of the percent calculation. Prior to any statistical analyses, mortality ratios were arcsin-square root transformed and analyzed as described above. Gene expression data was expressed as copies mRNA per ng of total RNA and was only log-transformed prior to analysis if values spanned multiple orders of magnitude. Otherwise, these data were not transformed prior to analyses.

3. Results

3.1 Test concentrations

Mean ± SD (n = 75) measured concentrations of the 6.7, 20, 60 and 180 µg/L treatments were 6.10 ± 0.69, 18.11 ± 2.14, 52.83 ± 5.90 and 169.76 ± 14 µg/L, respectively, over the duration of the experiment. Among-tank variability was ≤ 12% with the coefficient of variation (averaged across the duration of the experiment) of 11.4%, 11.8%, 11.2% and 8.2 %, respectively for the 6.7, 20, 60 and 180 µg/L treatments. No prochloraz was detected in the control water treatments.

3.2 Generalized toxicity (survival, growth, liver and kidney pathology)

Prochloraz treatment did not affect survival in either gender (data not shown) and did not significantly affect growth in females (Table 2.3); although, mean female weights with prochloraz treatment were all greater than the control mean weight. Male mean weights in all prochloraz treatments were slightly less than the mean control weight and the 180 µg/L treatment was statistically lower than control ($p < 0.05$, Jonckheere-Terpstra; Table 2.3). Liver-somatic indices were significantly lower in the 60 µg/L and 180 µg/L treatments in both genders ($p < 0.05$, Jonckheere-Terpstra; Table 2.3). Histopathological evaluations identified an increase in incidence and severity of hepatocellular degeneration, consistent with the decrease in liver-somatic indices. Minimal to moderate hepatocellular degeneration occurred in prochloraz exposed frogs at an incidence of 10% (2/20 frogs) for the 20 µg/L treatment, 35% (7/20 frogs) for the 60µg/L treatment and 100% (20/20 frogs) for the 180 µg/L treatment (Figures 2.1 & 2.2). In kidneys of 30 (60%) animals from the 180 µg/L treatment, large numbers of renal tubules contained ~12µm, irregularly oval, amphophilic intracytoplasmic bodies (inclusions) (Figure 2.3). Minimal (infrequent) accumulations of protein within the Bowman's capsule were observed in all study groups with a slightly higher percentage of animals affected in the 180 µg/L treatment: 1% (1/80) of control frogs, 4% (2/51) of frogs from the 6.7 µg/L treatment, 4% (2/46) of frogs from the 20 µg/L treatment, 4%

(2/50) of frogs from the 60 µg/L treatment, and 16% (8/50) of frogs from the 180 µg/L treatment (Figure 2.3).

Table 2.3. Mean ± standard deviation for growth and LSI measurements in control and prochloraz-treated *Xenopus laevis*. Asterisk (*) represents statistical significance at $p < 0.05$ (Jonckheere-Terpstra)

Male	Control (n = 8)	6.7 µg/L (n = 5)	20 µg/L (n = 5)	60 µg/L (n = 5)	180 µg/L (n = 5)
Weight (g)	13.2 ± 2.2	12.5 ± 2.6	11.8 ± 2.8	12.5 ± 2.3	12.5 ± 2.7*
Snout-to-vent length (mm)	47 ± 3	46 ± 3	45 ± 4	46 ± 3	46 ± 4
Liver-somatic index	0.059 ± 0.010	0.056 ± 0.012	0.055 ± 0.012	0.051 ± 0.007*	0.050 ± 0.004*
Female					
Weight (g)	12.9 ± 2.6	13.8 ± 3.4	14.1 ± 2.3	13.9 ± 2.5	13.3 ± 2.8
Snout-to-vent length (mm)	47 ± 3	48 ± 4	48 ± 2	48 ± 3	48 ± 4
Liver-somatic index	0.061 ± 0.007	0.056 ± 0.013	0.06 ± 0.007	0.052 ± 0.005*	0.052 ± 0.005*

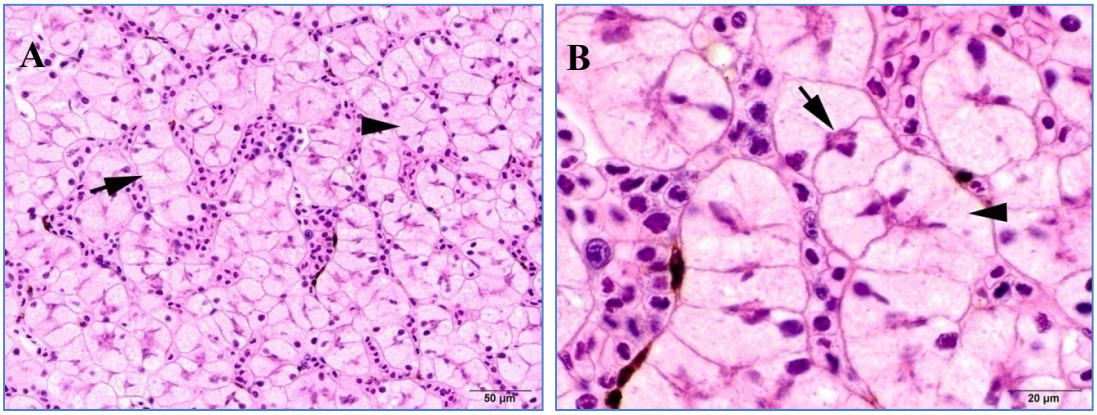

Figure 2.1. Histological sections of control liver tissue at two months post-metamorphosis. (A) 20X normal control liver. Hepatic cords are two cells thick (arrow) separated by bile canaliculus; hepatocyte cytoplasm is wispy and eosinophilic (arrowhead). (B) 60X normal control liver, higher magnification. Note wispy eosinophilic cytoplasm (arrowhead) and condensed (basophilic) cytoplasm adjacent to bile canaliculus (arrow).

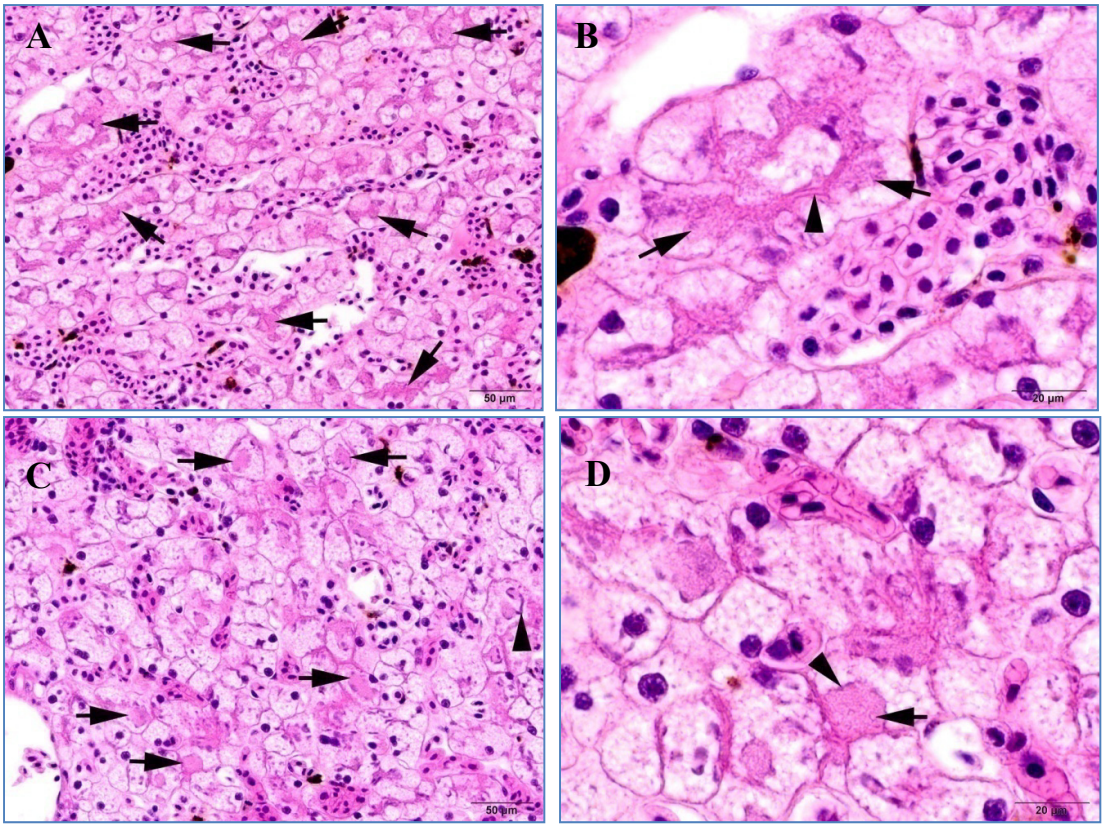

Figure 2.2. Histological sections of 180 µg/L prochloraz-treated livers at two months post-metamorphosis. (A) 20X 180 µg/L treated liver exhibiting moderate hepatocellular degeneration. Cytoplasmic inclusions are present within many hepatocytes (arrows); inclusion location is often pericanalicular (arrows). (B) 60X – higher magnification of (A). Degenerative hepatocytes are smaller and contain pericanalicular inclusions (arrows); note prominent bile canaliculus (arrowhead). (C) 20X – another example of a 180 µg/L treated liver exhibiting moderate hepatocellular degeneration. Degenerative hepatocytes containing cytoplasmic inclusions may be swollen (arrows); hepatic cords appear jumbled. (D) 60X – higher magnification of (C). Inclusions often consist of "punctuate" granular material (arrow); note smudgy basophilic material at periphery of inclusion (arrowhead).

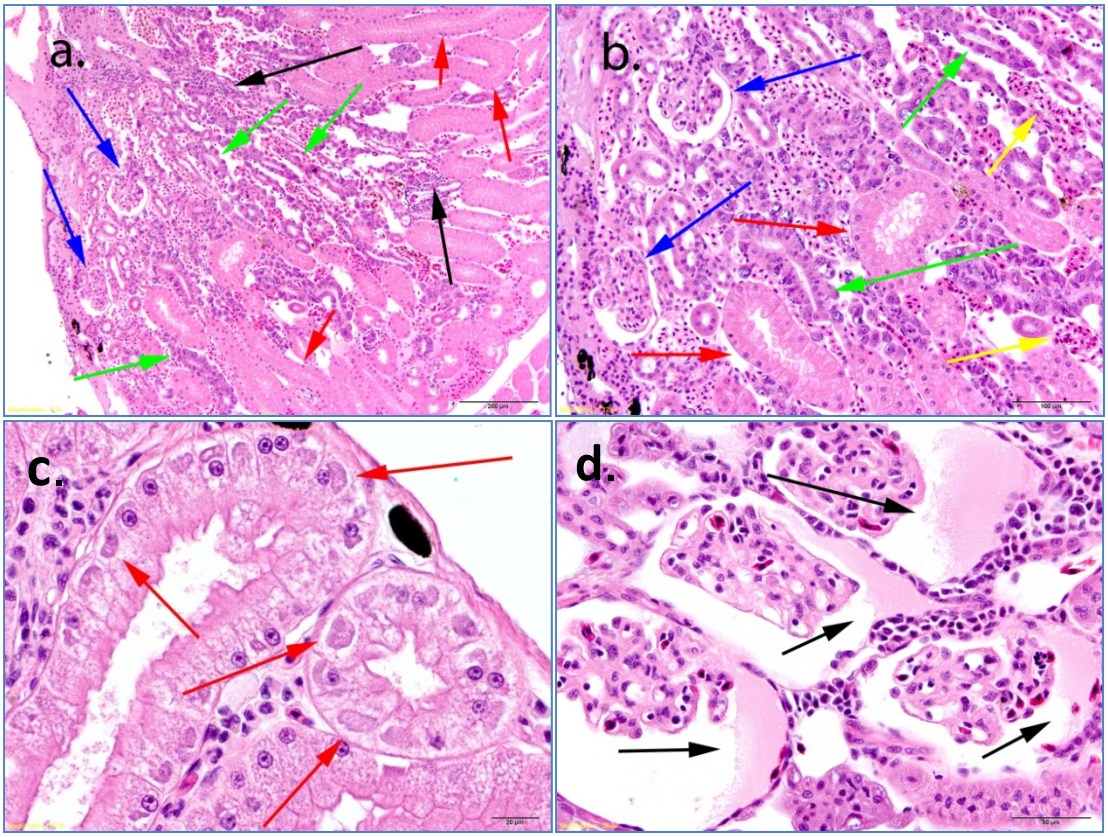

Figure 2.3. Histology sections of control and prochloraz-treated kidneys at two months post-metamorphosis. Control kidney, 10X(a) and 20X(b). Normal kidney. Profiles of the thick limb of the loop of Henle (red arrows) fill the cortex; glomeruli (blue arrows) and collecting tubules (green arrows) are largely confined to the cortex. Small cluster are myeloid cells are present (black arrows) and large numbers of red blood cells (yellow arrows) fill the interstitium. (c) 180µg/L study group, 60X. Intracytoplasmic amphophilic inclusions. Renal tubular cells contain large, irregular, intracytoplasmic, amphophilic inclusions (red arrows). (d) 60µg/L study group, 40X. Glomerular protein, Grade 1. The Bowman's capsule of several glomeruli contains small accumulations of pale, eosinophilic material (black arrows).

3.3 Endocrine toxicity (gonad, duct, gene expression)

Testis histopathology evaluations revealed a series of effects from prochloraz exposure mainly in the 180 µg/L treatment (Table 2.4). Separate assessments were made for gonadal degeneration that presented as germinal epithelial thinning/loss without apoptosis or necrosis as opposed to gonadal degeneration specifically presenting with evidence of apoptosis and necrosis. Although incidences of these diagnoses occurred across all treatments and control, a higher percentage of individuals were affected in the higher treatments (Figure 2.4). During normal testis development, all germ cells

associated with a single spermatocyst mature at the same rate and the spermatocyst generally maintains a circular or ovoid shape. Prochloraz treatment caused asynchronous development of germ cells within a spermatocyst and caused some spermatocysts to become irregular in shape (Figure 2.5); this occurred in a concentration-dependent manner. The 180 µg/L treatment experienced increased incidences of interstitial fibrosis, interstitial cell hypertrophy and hyperplasia, and mononuclear cellular infiltrate indicating inflammatory and compensatory responses to prochloraz insult in the testis. The ovaries of prochloraz-treated females were generally unaffected except for a slight increase in occurrence of ovarian hypoplasia and ovarian germinal epithelial loss indicating interference of maturation (data not shown).

Table 2.4. Incidences of histopathological findings in control and prochloraz-exposed testis tissue of two month post-metamorphosis male *Xenopus laevis*. NR = not remarkable; T* = total.

Chemical Dose (µg/L)	**0.0**	**6.7**	**20**	**60**	**180**	**T***
Number examined	36	25	24	24	25	134
Missing tissue	1	0	0	2	0	3
Gonadal degeneration apoptosis/necrosis						
NR	33	16	23	20	8	100
Incidences	3	9	1	4	17	34
Gonadal degeneration thinning/loss						
NR	33	21	22	13	3	92
Incidences	3	4	2	11	12	42
Testicular interstitial cell hypertrophy/hyperplasia						
NR	30	17	23	12	4	86
Incidences	6	8	1	12	21	48
Increased spermatogonia						
NR	34	24	22	21	19	120
Incidences	2	1	2	3	6	14
Interstitial fibrosis						
NR	27	13	17	18	3	78
Incidences	9	12	7	6	22	56
Asynchronous development within cysts						
NR	36	20	18	16	10	100
Incidences	0	5	6	8	15	34
Mononuclear cellular infiltrate						
NR	33	23	19	21	14	110
Incidences	3	2	5	3	11	24

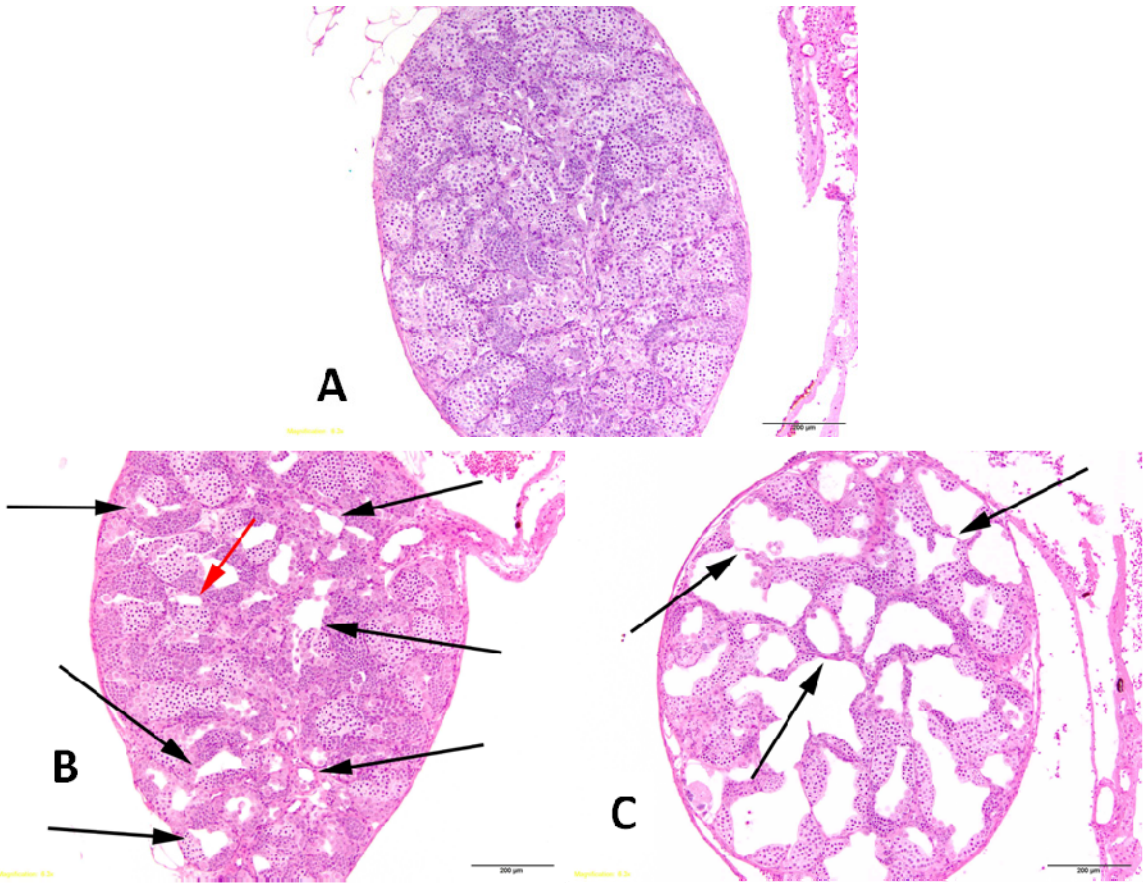

Figure 2.4. Histology sections of control and prochloraz-treated testis tissues at two months post-metamorphosis. A) Control group male from prochloraz study, provided for reference, 10X. B) Prochloraz 180 µg/L treatment group male, 10X. Gonadal degeneration thinning/ loss Grade 2. The germinal epithelium lining of numerous spermatic tubules is thin to absent (black arrows) with occasional exposure of the underlying basement membrane (red arrow). C) 180 µg/L treatment group male, 10X. Gonadal degeneration thinning/loss, Grade 4. Diffusely, the germinal epithelium is missing or thin with frequent exposure of the underlying basement membranes (black arrows).

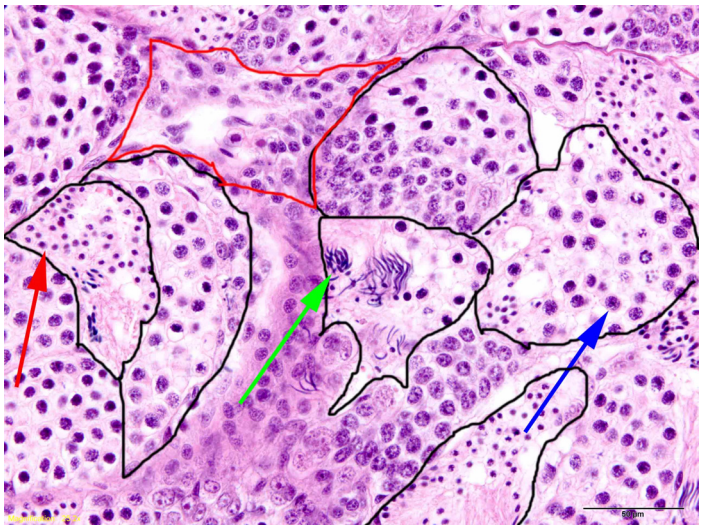

Figure 2.5. Histological section of asynchronous cyst development in testis tissue of prochloraz-treated male. 180µg/L study group male, 40X. Asynchronous development within cysts, Grade 3. Individual spermatocysts (outlined in black) are irregular in shape and contain cell types of markedly differing maturity: spermatocytes (blue arrow), spermatids (red arrow) and spermatozoa (green arrow). There are increased numbers of interstitial cells in the tubular interstices (red outline).

Both male and female oviduct development were affected by prochloraz treatment. Male oviduct regression was generally inhibited, and in some cases, oviducts matured to be characteristic of female oviducts at this stage of development (Table 2.5). Female oviducts exhibited accelerated development with prochloraz treatment. The effects on the oviduct in both genders with prochloraz treatment is indicative of a feminizing effect similar to the feminization of secondary sex characteristics of male rats during perinatal prochloraz exposure (Noriega et al., 2005; Vingaard et al., 2005).

Table 2.5. Male and female oviduct stage distributions in control and prochloraz-exposed two month post-metamorphosis *Xenopus laevis*. T* = total.

Chemical Dose (µg/L)	**0.0**	**6.7**	**20**	**60**	**180**	**T***
Number examined	37	25	24	26	25	137
Male oviduct development						
Fibrous tag = 1	31	22	12	8	1	74
Rudimentary = 2	6	2	10	15	23	56
Partial = 3	0	1	2	3	1	7
Female oviduct development						
Number examined	43	26	22	24	25	140
Fibrous tag = 1	0	0	0	0	0	0
Rudimentary = 2	19	0	2	1	3	25
Partial = 3	24	26	20	23	22	115

Of the four genes chosen to evaluate the anti-androgenic effects of prochloraz, rflcii was the only transcript that showed any substantial changes across treatments. The 60 and 180 µg/L treatments were significantly different than control ($p < 0.05$, Jonckheere-Terpstra) and show 3 to >5-fold increases respectively (Figure 2.6A). Cyp17a1 was expressed at fairly high levels in all treatments and control relative to the other transcripts (Figure 2.6B) and the 180 µg/L treatment was statistically higher than control ($p < 0.05$, Jonckheere-Terpstra). The remaining two transcripts, Sox9b and Dax-1, did not change with prochloraz treatment (Figure 2.6C & D).

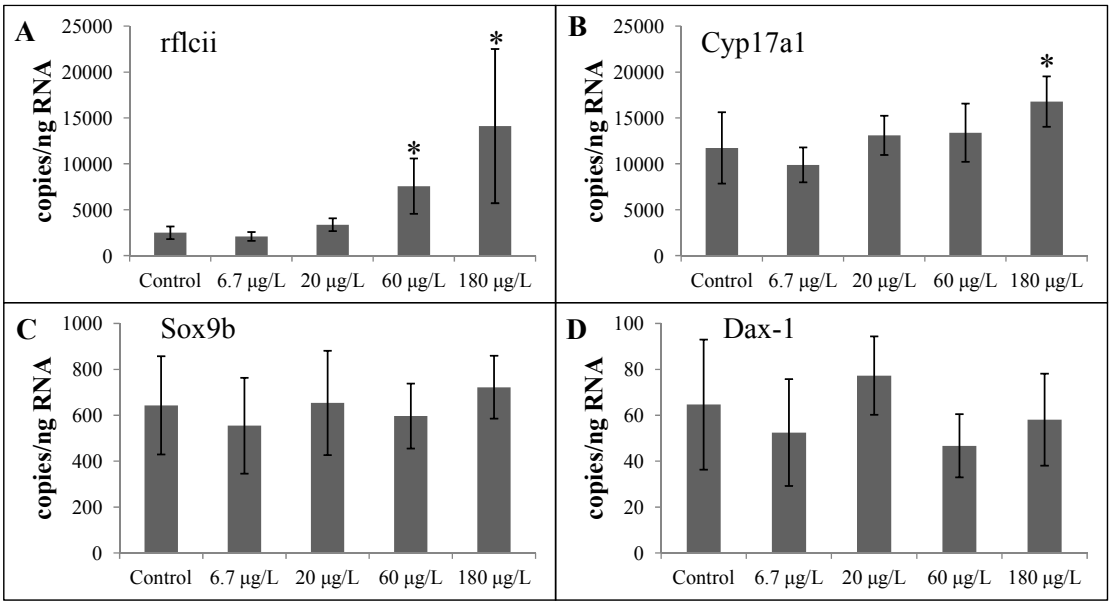

Figure 2.6. Gene expression levels in testis tissue of two month post-metamorphosis control and prochloraz-exposed *Xenopus laevis*. Means ± SD (error bars) are expressed as copies mRNA per ng total RNA. Asterisks (*) indicate treatments statistically different than control ($p < 0.05$, Jonckheere-Terpstra).

4. Discussion

Prochloraz exposure throughout *Xenopus laevis* embryonic, larval and juvenile development resulted in a range of toxicological effects. The observed hepatocellular degeneration, together with measured decreases in liver-somatic indices and intracytoplasmic inclusions in renal tubular cells are all indicative of metabolic toxicity in the 60 and 180 µg/L treatments. The histologic appearance of the hepatic inclusions is suggestive of excess protein and ribo-nucleic acid aggregations presumably related to metabolism of prochloraz (Figure 2.2). A study by Needham and Challis (1991) reported that one of the major prochloraz metabolites in rats was a glucuronide conjugate and high concentrations of prochloraz metabolites were found in liver tissue. In the same study, high concentrations of prochloraz metabolites were also found in kidneys. The similar appearance of hepatic and renal tubular cell inclusions in the current study is suggestive of metabolism, storage and excretion of prochloraz metabolites consistent with observations in rats (Needham and Challis, 1991).

Histopathological evaluations of testes revealed degeneration of the germinal epithelium from prochloraz exposure mainly in the high test concentrations. Although the severities of the degenerative effects were quite variable within the high treatments, one individual exhibited such severe thinning of the germinal epithelium that the tissue presented with many large voids throughout the sections (Figure 2.4). Germinal epithelial thinning was generally accompanied by several other sub-acute testicular lesions (*e.g.* interstitial cell hyperplasia, interstitial fibrosis, asynchronous spermatocyst development) in the higher treatments. This was in contrast to the lower treatments that, more often, presented with one or very few different lesions of minimal to mild severity. These chronic and sub-acute anti-androgenic effects of prochloraz are consistent with what has been reported in fathead minnows (Ankley *et al*., 2005) and rats (Blystone *et al*., 2007; Noriega *et al*., 2005). Interestingly, the appearance of the testis sections suggest that normal morphogenesis was not initially inhibited by prochloraz, as the ultrastructure was generally completely formed. More specifically, overall sizes of testes in the high prochloraz treatment were similar to control. Also, testis cords and spermatocysts were formed and appeared to be progressing through spermatogenesis,

albeit atypically. The degenerative process appears to occur either concurrently with unabated growth and formation of testis ultrastructure, or is allowed to take effect through some mechanism following the completion of testis morphogenesis. The most plausible explanation for this is antagonism of androgen-mediated spermatogenesis that is initiated in the later phases of testis morphogenesis.

Chapter 1 indicates that the transcriptional activity in the testis around completion of metamorphosis is primarily associated with morphogenesis including organization of the seminiferous tubules, differentiation of interstitial cells and production of connective tissue. Once testis cords are formed, pre-spermatogenesis takes place during late larval development and the early juvenile period. In two species of *Rana*, pre-spermatogenesis has been shown to occur at different points in the mating season independently of hormonal activity in interstitial tissue (reviewed by Ogielska, 2009). Androgens become essential for the second meiotic division of germ cells, which convert secondary spermatocytes to spermatids. Control testes in this study exhibited spermatocysts often containing spermatids and in some cases containing spermatozoa, indicating androgen-mediated "active" spermatogenesis is taking place at two months post-metamorphosis. Therefore, critical levels of androgen are produced by steroidogenesis enzymes in the Leydig cells beginning at some point between the completion of metamorphosis and two months post-metamorphosis, presumably mediated by gonadotropins released by the pituitary. The point at which androgens become necessary to support spermatogenesis is when the testis becomes sensitive to the anti-androgenic action of prochloraz, arresting meiosis and resulting in degeneration of the already formed germinal epithelium.

Cyp17a1 gene expression levels in testes (Figure 2.6B) also support the proposal that steroidogenesis is active and prochloraz treatment may cause compensatory induction of the enzyme's transcript. This was not a strong response, as there appears to be high levels of the Cyp17a1 transcript already present in all treatments and controls. Therefore, the weak response exhibited by this gene could be due to high levels of CYP17 already present. Also, there were spermatids and spermatozoa in testes of the animals in the 180 µg/L treatment indicating that prochloraz was not capable of completely arresting testosterone production by CYP17 inhibition at this level of

exposure. In a study by Ankley *et al*. (2009), male fathead minnows exhibited significantly reduced circulating testosterone levels with a corresponding significant increase in testis Cyp17 mRNA. While it is acknowledged the fish were exposed to 300 µg/L prochloraz for only eight days, the response is consistent with the apparent response seen here. It is possible that up-regulation of testis Cyp17 is an indicator of impaired testosterone production across species. However, the weak, and perhaps questionable, response seen in this study indicates its lack of usefulness as a biomarker in this particular system.

In addition to its ability to impair testosterone production, prochloraz has been shown to interfere with the androgen receptor (Vinggaard *et al*., 2002). Androgen receptor is highly expressed in the testis and to a much lesser degree in many peripheral tissues for regulation of secondary sexual maturation (Sassoon and Kelley, 1986; Sassoon *et al*., 1987; Tobias *et al*., 1991; Variale and Serino, 1994; Tobias and Kelley, 1995). An attempt was made in the current study to assess androgen/androgen receptor signaling in the testis using a series of gene transcript expression profiles. Rflcii (relaxin-like factor) in *Xenopus* is orthologous to the mammalian Insl3 (insulin-like 3) Leydig cell-specific growth factor peptide hormone. Insl3 expression in mammalian Leydig cells is regulated by testosterone in an androgen receptor-dependent manner (Laguë and Tremblay, 2008). In the same study, Laguë and Tremblay reported antagonism of testosterone-mediated Insl3 expression by anti-androgen mono-(2ethylhexyl) phthalate. Therefore, I hypothesized that Rflcii in *Xenopus laevis* would be down-regulated as a result of disruption of testosterone production and androgen/androgen receptor signaling. However, the results were the opposite of this hypothesis, with significant induction of Rflcii seen after exposure to 60 or 180 µg/L prochloraz (Figure 2.6A). To understand this unexpected relationship, it is necessary to understand the putative role that this relaxin-like factor plays in the testis and the mechanisms by which it may be regulated.

Insl3 has been well-characterized in mammalian reproductive development; however, this peptide has only recently been considered in teleosts and is essentially uncharacterized in amphibians (Bathgate *et al*., 2013; Good-Avila *et al*., 2009). The relaxin family of genes within and between species shares very little sequence similarity,

but the resulting peptides share structural similarity with insulin and confer similar functionality (Wilkinson *et al*., 2005). Insl3 in mammals during early reproductive development is highly expressed in fetal Leydig cells and is responsible for testis decent by acting on the gubernaculum (Zimmermann *et al*., 1999; Nef and Parada, 1999). Prevention of germ cell apoptosis by paracrine signaling has also been a suggested role of INSL3 in the adult rat testis (Anand-Ivell *et al*., 2006; Kawanamura *et al*., 2004). Protection of male germ cells by RFLCII (INSL3) seems more plausible in *Xenopus* as gonads in amphibians do not descend or migrate from the location of the original anlage. Further support of the protective role of *Xenopus* RFLCII is the response of its expression levels elicited by exposure to higher concentrations of prochloraz, together with the observed pathologies related to germinal epithelial degeneration. Induction of this gene in the current study appears to be an adaptive response, and in this case may act as an exceptional biomarker of impaired testis function.

The question remains as to how this gene is regulated in *Xenopus* if not in part by androgen/androgen receptor signaling as in mammalian Insl3. Without performing an analysis on the *Xenopus* rflcii promoter, alternate mechanisms of regulation demonstrated in mammalian models is all that is available for speculation. As such, SF-1-mediated transcriptional regulation of the relaxin-like factor promoter has been well-documented (Koskimies, *et al*., 2002; Zimmerman *et al*., 1998; Sadeghian *et al*., 2005; Robert *et al*., 2006; Tremblay and Robert, 2005). Relevant to the current study, DAX-1 has been shown to antagonize SF-1 regulation of the murine relaxin-like factor promoter (Koskimies *et al*., 2002). Dax-1 expression in the testis across all treatments and control is very low in comparison to expression levels of all three other genes and is not influenced by prochloraz exposure (Figure 2.6D). This supports the possibility that absence of DAX-1 allows uninhibited positive regulation of rflcii by SF-1. However, Leydig cell-specific Sf-1 null mice were still able to produce low levels of Insl3 and testis descent was not affected to the degree it was affected in the Insl3 knockout mouse model (Jeyasuria *et al*., 2004). This means mammalian Insl3 is regulated by multiple alternative mechanisms.

NR4A1 is an orphan nuclear receptor and transcription factor expressed in Leydig cells and has been shown to be rapidly induced in response to LH and cAMP analogs (Tremblay and Robert, 2005; Martin and Tremblay, 2005; Song *et al*., 2001). Tremblay and Robert (2005) demonstrated through transfection assays that NR4A1 was a much stronger activator of the Insl3 promoter than SF-1. The observation that androgen-mediated "active" spermatogenesis is taking place (explained earlier) in the current study supports the notion of LH-mediated activation of steroidogenesis in the Leydig cells. There is evidence from receptor expression profiles in different mammalian tissues that relaxin-like factors communicate with the brain through circulatory feedback (Bathgate *et al*., 2013). This supports a potential adaptive signaling response to the gonadotropic cells in the hypothalamus and pituitary possibly playing a role in modulation of circulating gonadotropins.

A more local explanation could involve Insl3-mediated paracrine regulation of androgen production through bone morphogenic proteins (BMP), as was demonstrated in ovarian androgen production (Glister *et al*., 2013). Glister *et al*. (2013) showed that knockdown of INSL3 or its receptor, RXFP2, in cultured bovine theca cells resulted in 77% and 81% reductions of Cyp17 mRNA respectively, along with reduced androstenedione secretion. While it is acknowledged this work was done with bovine thecal cells, they are the Leydig cell equivalent steroidogenic cell in the ovary that exhibits many of the same regulatory mechanisms of steroidogenic genes. Also, the gene array study presented in Chapter 1 showed that bmp1 was expressed at a similar level as rflcii in the testis and was highly testis-enriched. The expression profiles of rflcii and Cyp17a1 in the current study support the possibility that RFLCII may locally regulate androgen production by regulating Cyp17 expression. Further targeted experiments to test these relationships are necessary to fully understand the mechanism by which the anti-androgen prochloraz causes such a robust positive response in rflcii expression.

Dax-1 was also chosen to assess the androgen/androgen receptor regulatory network in the current study and did not respond to prochloraz treatment (Figure 2.6D). Dax-1 has been shown to be developmentally regulated in different tissues and exhibits particularly dynamic functionality (Achermann *et al*., 2001b; Jadhav, *et al*., 2011; Tamai

et al., 1996). Its testis-biased expression in *Xenopus tropicalis* during gonad differentiation (Chapter 1) is likely due to its suppressive effect on SF-1-mediated Cyp19 aromatase expression (Guarates *et al*., 2003; Wang *et al*., 2001). This ultimately inhibits production of β-estradiol during the time the testis is most susceptible to estrogen-induced dysgenesis or sex reversal (Villalpando and Merchant-Larios, 1990). Shortly after the completion of metamorphosis in Anurans, the hypothalamus and pituitary become able to secrete gonadotropins (reviewed by Ogielska, 2009c). Presumably, release of FSH and LH and subsequent cAMP production in Sertoli and Leydig cells down-regulate Dax-1 in the testis when steroid hormone production becomes necessary for spermatogenesis (Song *et al*., 2004; Tamai *et al*., 1996). With steroidogenesis activated through gonadotropin hormones and SF-1, DAX-1 interference with SF-1 appears to no longer be necessary in the testis at two months post metamorphosis. This is consistent with the low expression levels in the current study and makes Dax-1 only a potential indicator of active gonadotropin signaling. However, this would require further targeted experimentation.

Sox9b was also unaffected by prochloraz treatment (Figure 2.6C). This suggests that Sox9b is not regulated by androgen/androgen receptor, at least during this period of development. Interestingly, the Sox9b expression profile across treatments and control is very similar to the Dax-1 expression profile, but Sox9b expression is approximately 10-fold higher. This apparent relationship supports their close proximity within a regulatory network. As discussed earlier, they both have been shown to interact with SF-1, but play opposite roles. The putative interaction of SOX9 with SF-1 upregulates AMH, which mediates regression of the oviducts in male *Xenopus*. In the current study, oviduct regression was attenuated in prochloraz-treated males and oviduct maturation was accelerated in prochloraz-treated females (Table 2.5). Regardless of the direct molecular mechanism triggering oviduct regression or maturation, its fate is highly sensitive to exogenous androgenic and estrogenic substances respectively (USEPA, 2013; Porter *et al*., 2011). Therefore, it appears androgen is necessary, but may not be sufficient, for oviduct regression. In females, it appears as though constitutive levels of androgen slow oviduct maturation and reduction of androgen levels by prochloraz allows unabated

maturation. In this case, Sox9b is not a good indicator of this androgen-mediated process.

5. Conclusion

Overall, the anti-androgenic effects of prochloraz exposure were present in *Xenopus laevis* and consistent with the anti-androgenic effects observed in other species. The onset of testis degeneration coinciding with the androgen-mediated phase of spermatogenesis emphasizes the higher probability of adverse reproductive effects with exposure during the juvenile period of development, as opposed to the periods of embryonic or larval development. This supports the idea that the testes did not experience "dysgenesis." Rather, they experienced impaired spermatogenesis and resulted in testicular degeneration. Prochloraz is readily metabolized and the likelihood of constant environmental exposure at the levels tested here is low, especially because most amphibians transition to terrestrial life after completing metamorphosis. However, the need for concern arises during the mating season when acute aquatic exposure to prochloraz could interfere with spermatogenesis, even though the acute effects are likely reversible upon depuration. This critical window for seasonal breeders is clearly the highest risk time period for prochloraz exposure. Exposure to prochloraz (or anti-androgens) around breeding times must be given a higher level of consideration than other life stages for amphibian ecological risk assessment.

While the gene expression biomarkers did not provide a wealth of information regarding the chemical mode of action as intended, their expression profiles and regulation in other species stimulated valuable speculative discussion and generated a series of testable hypotheses. Subsequent investigations to elucidate rflcii regulation and mechanisms of action in amphibians could solidify its utility as a biomarker of Leydig cell function, gonadotropin signaling, and steroidogenesis. This work establishes this gene as an important factor in amphibian reproductive development. Further characterization of this protein may discover that it is essential for amphibian reproduction.

Chapter 3. Conclusion and future directions

The approach taken herein to develop expression-based biomarkers of impaired gonad function involved many complicated techniques. The microarray time-course in testis and ovary tissue lent numerous layers of complexity and decision making regarding normalization strategies, statistical comparisons, multiple comparison corrections, fold-change cutoffs, and biological references. Due to the status of *Xenopus* (or amphibian) molecular bioinformatic resources, pathway/network analyses were difficult to generate, and even more difficult to interpret. Therefore, it was decided to pursue the well-developed mammalian bioinformatic resources by translating as many transcripts as possible into human orthologs. This required computer programming skills to automate UniGene database navigation and acquisition of human accession numbers for thousands of *Xenopus tropicalis* array transcripts. Gene lists could then be recognized by Ingenuity Pathway Analysis software that allowed functional analyses and direct access to references providing precedent for molecular interactions. This was the basis from which genes were chosen to evaluate the particular anti-androgenic mode of action of prochloraz. The foundation of information provided by the gene array study lends well toward biomarker development and elucidation of other modes of action in ovary tissue also.

Following the gene array study and selection of candidate biomarkers, bioinformatic analyses were employed to identify gene homologs in *Xenopus laevis* using the EST database. Subsequently, primers and probes were designed for development of quantitative PCR assays for each of the four genes. Results from the gene expression analyses in testis tissues exposed to prochloraz were generally not consistent with what was expected, but all had plausible explanations for their behavior. In the current study design, rflcii appears to be predictive of impaired spermatogenesis. However, further experimentation is necessary in order to confirm its predictive capabilities with different anti-androgens in *Xenopus*, and to assess its predictive capabilities across species. Understanding the mechanism by which it is regulated would be the first step. This could be accomplished by culturing juvenile testes *ex vivo* and exposing the tissue to various

compounds or hormones and measuring the resulting gene expression response. For instance, addition of LH would indicate whether this gene is regulated via hypothalamic-pituitary gonadotropins through cAMP signaling and possibly a NR4A1 ortholog. Locating this protein's receptor with fluorescence *in situ* hybridization would help to understand its target cell population. Purifying the RFLCII protein and generating antibodies would allow development of an ELISA to measure secretion of this factor into the culture media to understand the relationship between gene expression and *de novo* protein synthesis. This could lead to an assay capable of measuring blood levels of this protein *in vivo* potentially indicating Leydig cell function. Relaxin-like factors could be a key indicator of Leydig cell function even across species, opening up the possibility of making field assessments on high-risk populations.

The goal of modern day ecotoxicology is to link a molecular initiating event to an adverse organismal outcome by using the least amount of animal and financial resources possible. Understanding the molecular basis for reproductive differentiation, manifestations of gonad pathologies and gene regulation will expedite the understanding of molecular initiating events. Also understanding species differences in these molecular programs will provide a spectrum of species susceptibilities, allowing focused efforts toward preservation of higher-risk populations. The work presented in this thesis provides direction toward attaining this goal.

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Appendix A: Python programming code for enhanced array annotation

```
#################################################################
#################
"""Creates a dictionary of UniGene IDs as keys and
corresponding human orthologues as values.  Need UniGene
Xx.data file for species of interest (
ftp://ftp.ncbi.nih.gov/repository/UniGene/)"""

from Bio import UniGene
handle = open("C:\Python27\Doc\Str.data")
ugparser = UniGene.parse(handle)
UG_Dict = {}

for record in ugparser:
    for protsim in record.protsim:
        if protsim.org=="9606":
            UG_Dict[record.ID]=record.ID, protsim.protid,
            protsim.pct, protsim.aln
            print record.ID, "has human orthologue"
handle.close

#################################################################
#################
""" Writes the UniGene dictionary to .csv file so it can be
built faster than rebuilding from database file."""

import csv
with open('UG_Dict.csv', 'wb') as f:
    writer = csv.writer(f)
    for item in UG_Dict:
        writer.writerow(UG_Dict[item])
f.close()


#################################################################
#################
"""Creates a dictionary of the annotation file so accessions
can be called and human orthologues can be appended to each
probe annotation"""
#Call Representative Public ID with Annot_Dict[Probe Set ID][8]
#Archival UniGene Cluster = [9]
#UniGene ID = [10]
#Gene Symbol = [14]
#Ensembl = [17]
```

```
#Entrez Gene = [18]
#SwissProt = [19]
#Annotation transcript cluster = [38]
#Updated UniGene ID = [41]

import csv
Annot_Dict = {}
with open('C:\Python27\Doc\X_tropicalis.na32.annot.csv', 'rb')
as f:
    reader = csv.reader(f, delimiter=',', quotechar='"')
    for row in reader:
        if not '#' in row[0]:
            Annot_Dict[row[0]] = row[0:]
f.close()

#####################################################################
#################
"""Updates UniGene IDs using Representative Public ID and
appends to each probe annotation.  Database needed: Str.retired
(UniGene) or other species of interest"""

update_UniGene_ID = "C:\Python27\Doc\Str.retired"
UGcount = 0
UGnone_count = 0
incompatible = 0
for item in Annot_Dict:
    if 'AFFX' in Annot_Dict[item][8]:
        incompatible +=1
        Annot_Dict[item].append('---')
        UGnone_count +=1
        print Annot_Dict[item][8], "incompatible ID.", ID

    elif 'ENSXETT' in Annot_Dict[item][8]:
        ID = ""
        if '(' in Annot_Dict[item][38]:
            for s in Annot_Dict[item][38]:
                if s != '(':
                    ID +=s
                else:
                    break
        else:
            for s in Annot_Dict[item][38]:
                if s != ',':
```

```
                ID +=s
            else:
                break
        if 'ENSXETT' in ID:
            incompatible +=1
            Annot_Dict[item].append('---')
            UGnone_count +=1
            print Annot_Dict[item][8], "incompatible ID.", ID
        else:
            infile = open(update_UniGene_ID)
            for line in infile.xreadlines():
                flag = 0
                if ID in line:
                    fields = line.split()
                    new_UGID = fields[1]
                    Annot_Dict[item].append(new_UGID)
                    flag = 1
                    UGcount +=1
                    break
            if flag == 0:
                Annot_Dict[item].append('---')
                UGnone_count +=1
            print ID
            print UGcount, "updated UniGene IDs and",
            UGnone_count, "unrecognized IDs."
            infile.close()

    elif 'jgi|' in Annot_Dict[item][8]:
        ID = ""
        if '(' in Annot_Dict[item][38]:
            for s in Annot_Dict[item][38]:
                if s != '(':
                    ID +=s
                else:
                    break
        else:
            for s in Annot_Dict[item][38]:
                if s != ',':
                    ID +=s
                else:
                    break
        if 'jgi|' in ID:
            incompatible +=1
```

```
            Annot_Dict[item].append('---')
            UGnone_count +=1
            print Annot_Dict[item][8], "incompatible ID.", ID
        else:
            infile = open(update_UniGene_ID)
            for line in infile.xreadlines():
                flag = 0
                if ID in line:
                    fields = line.split()
                    new_UGID = fields[1]
                    Annot_Dict[item].append(new_UGID)
                    flag = 1
                    UGcount +=1
                    break
            if flag == 0:
                Annot_Dict[item].append('---')
                UGnone_count +=1
            print ID
            print UGcount, "updated UniGene IDs and",
            UGnone_count, "unrecognized IDs."
            infile.close()

    elif 'RPTR' in Annot_Dict[item][8]:
        ID = ""
        if '(' in Annot_Dict[item][38]:
            for s in Annot_Dict[item][38]:
                if s != '(':
                    ID +=s
                else:
                    break
        else:
            for s in Annot_Dict[item][38]:
                if s != ',':
                    ID +=s
                else:
                    break
        if 'RPTR' in ID:
            incompatible +=1
            Annot_Dict[item].append('---')
            UGnone_count +=1
            print Annot_Dict[item][8], "incompatible ID.", ID
        else:
            infile = open(update_UniGene_ID)
```

```
            for line in infile.xreadlines():
                flag = 0
                if ID in line:
                    fields = line.split()
                    new_UGID = fields[1]
                    Annot_Dict[item].append(new_UGID)
                    flag = 1
                    UGcount +=1
                    break
            if flag == 0:
                Annot_Dict[item].append('---')
                UGnone_count +=1
            print ID
            print UGcount, "updated UniGene IDs and",
            UGnone_count, "unrecognized IDs."
            infile.close()

    else:
        if '.' in Annot_Dict[item][8]:
            ID = ""
            for s in Annot_Dict[item][8]:
                if s != '.':
                    ID +=s
                elif s == '.':
                    break
        else:
            ID = Annot_Dict[item][8]

        infile = open(update_UniGene_ID)
        for line in infile.xreadlines():
            flag = 0
            if ID in line:
                fields = line.split()
                new_UGID = fields[1]
                Annot_Dict[item].append(new_UGID)
                flag = 1
                UGcount +=1
                break
        if flag == 0:
            Annot_Dict[item].append('---')
            UGnone_count +=1
        print ID
        print UGcount, "updated UniGene IDs and", UGnone_count,
```

```
            "unrecognized IDs."
            infile.close()
        if Annot_Dict[item][41]=='---':
            ID = ""
            if '(' in Annot_Dict[item][38]:
                for s in Annot_Dict[item][38]:
                    if s != '(':
                        ID +=s
                    else:
                        break
            else:
                for s in Annot_Dict[item][38]:
                    if s != ',':
                        ID +=s
                    else:
                        break
            if ID != Annot_Dict[item][8]:
                infile = open(update_UniGene_ID)
                for line in infile.xreadlines():
                    flag = 0
                    if ID in line:
                        fields = line.split()
                        new_UGID = fields[1]
                        del Annot_Dict[item][41]
                        Annot_Dict[item].append(new_UGID)
                        flag = 1
                        UGnone_count -=1
                        UGcount +=1
                        print "UGID found with annotation
                        transcript cluster"
                        break
                infile.close()
                print ID
                print UGcount, "updated UniGene IDs and",
                UGnone_count, "unrecognized IDs."
Annot_Dict['Probe Set ID'][41]='Updated UniGene ID'
print UGcount, "updated UniGene IDs,", UGnone_count,
"unrecognized IDs, and", incompatible, "incompatible IDs."

#################################################################
#################
"""Writes updated annotation dictionary to .csv file so
dictionary can be re-created faster without having to run
```

```
UniGene update over again."""

import csv
with open('new_annot_w_new_UGID_update_method.csv', 'wb') as f:
    writer = csv.writer(f, delimiter=',', quotechar='"')
    writer.writerow(Annot_Dict['Probe Set ID'])
    for item in Annot_Dict:
        writer.writerow(Annot_Dict[item])
f.close()

#####################################################################
#################
"""Rebuilds annotation dictionary and UniGene dictionary using
.csv files rather than from databases."""

import csv
Annot_Dict = {}
with open('new_annot_w_new_UGID_update_method.csv', 'rb') as f:
    reader = csv.reader(f, delimiter=',', quotechar='"')
    for row in reader:
        Annot_Dict[row[0]] = row[0:]
f.close()
print "Annot_Dict import successful."
import csv
UG_Dict = {}
with open('UG_Dict.csv', 'rb') as f:
    reader = csv.reader(f, delimiter=',', quotechar='"')
    for row in reader:
        UG_Dict[row[0]] = row[0:]
f.close()
print "UG_Dict import successful."

#####################################################################
#################
"""Uses annotation dictionary for UniGene IDs and searches
UniGene dictionary for human orthologues which are appended to
probe annotation if available."""

count = 0
none_count = 0
Annot_Dict['Probe Set ID'].append('Human Ortholog Accession1')
Annot_Dict['Probe Set ID'].append('Hs_Orth1 PCT')
Annot_Dict['Probe Set ID'].append('Hs_Orth1 ALN')
```

```
Annot_Dict['Probe Set ID'].append('Human Ortholog Accession2')
Annot_Dict['Probe Set ID'].append('Hs_Orth2 PCT')
Annot_Dict['Probe Set ID'].append('Hs_Orth2 ALN')
Annot_Dict['Probe Set ID'].append('Human Ortholog Accession3')
Annot_Dict['Probe Set ID'].append('Hs_Orth3 PCT')
Annot_Dict['Probe Set ID'].append('Hs_Orth3 ALN')
for i in Annot_Dict:
    if Annot_Dict[i][41]==Annot_Dict[i][10]==Annot_Dict[i][9]:
        flag = 0
        ID = Annot_Dict[i][41]
        print ID
        if ID == '---':
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            none_count +=1
            print "No UniGene ID."
        else:
            for j in UG_Dict:
                if ID==UG_Dict[j][0]:
                    Annot_Dict[i].append(UG_Dict[j][1])
                    Annot_Dict[i].append(UG_Dict[j][2])
                    Annot_Dict[i].append(UG_Dict[j][3])
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    count +=1
                    flag = 1
                    print count, "Human orthologues found and",
                    none_count, "probes without ortholog."
                    break
            if flag==0:
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
```

```
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                none_count +=1
                print "No Record!"

    elif Annot_Dict[i][41]==Annot_Dict[i][9]:
        flag = 0
        ID1 = Annot_Dict[i][41]
        ID2 = Annot_Dict[i][10]
        prot1 = 0
        prot2 = 0
        for j in UG_Dict:
            if ID1==UG_Dict[j][0]:
                protID1 = UG_Dict[j][1]
                pct1 = float(UG_Dict[j][2])
                aln1 = float(UG_Dict[j][3])
                score1 = pct1 * aln1
                flag +=1
                prot1 = 1
            if ID2==UG_Dict[j][0]:
                protID2 = UG_Dict[j][1]
                pct2 = float(UG_Dict[j][2])
                aln2 = float(UG_Dict[j][3])
                score2 = pct2 * aln2
                flag +=1
                prot2 = 1
            if flag==2:
                break
        if flag==2:
            if protID1==protID2:
                Annot_Dict[i].append(protID1)
                if score1>score2:
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
```

```
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                count +=1
                print count, "Human orthologues found and",
                none_count, "probes without ortholog."
            else:
               if score1>score2:
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
               else:
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
               count +=1
               print count, "Human orthologues found and",
               none_count, "probes without ortholog."
        if flag==1:
            if prot1==1:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
```

```
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID2)
                Annot_Dict[i].append(pct2)
                Annot_Dict[i].append(aln2)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."
        if flag==0:
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            none_count +=1
            print "Two IDs and no Record!", ID1, ID2

    elif Annot_Dict[i][41]==Annot_Dict[i][10]:
        flag = 0
        ID1 = Annot_Dict[i][41]
        ID2 = Annot_Dict[i][9]
        prot1 = 0
        prot2 = 0
        for j in UG_Dict:
            if ID1==UG_Dict[j][0]:
                protID1 = UG_Dict[j][1]
                pct1 = float(UG_Dict[j][2])
                aln1 = float(UG_Dict[j][3])
```

```
                score1 = pct1 * aln1
                flag +=1
                prot1 = 1
            if ID2==UG_Dict[j][0]:
                protID2 = UG_Dict[j][1]
                pct2 = float(UG_Dict[j][2])
                aln2 = float(UG_Dict[j][3])
                score2 = pct2 * aln2
                flag +=1
                prot2 = 1
            if flag==2:
                break
        if flag==2:
            if protID1==protID2:
                Annot_Dict[i].append(protID1)
                if score1>score2:
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                count +=1
                print count, "Human orthologues found and",
                none_count, "probes without ortholog."
            else:
               if score1>score2:
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
```

```
                Annot_Dict[i].append(aln2)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID2)
                Annot_Dict[i].append(pct2)
                Annot_Dict[i].append(aln2)
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."
    if flag==1:
        if prot1==1:
            Annot_Dict[i].append(protID1)
            Annot_Dict[i].append(pct1)
            Annot_Dict[i].append(aln1)
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
        else:
            Annot_Dict[i].append(protID2)
            Annot_Dict[i].append(pct2)
            Annot_Dict[i].append(aln2)
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
        count +=1
        print count, "Human orthologues found and",
        none_count, "probes without ortholog."
    if flag==0:
        Annot_Dict[i].append('---')
```

```
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            none_count +=1

    elif Annot_Dict[i][10]==Annot_Dict[i][9]:
        flag = 0
        ID1 = Annot_Dict[i][41]
        ID2 = Annot_Dict[i][10]
        prot1 = 0
        prot2 = 0
        for j in UG_Dict:
            if ID1==UG_Dict[j][0]:
                protID1 = UG_Dict[j][1]
                pct1 = float(UG_Dict[j][2])
                aln1 = float(UG_Dict[j][3])
                score1 = pct1 * aln1
                flag +=1
                prot1 = 1
            if ID2==UG_Dict[j][0]:
                protID2 = UG_Dict[j][1]
                pct2 = float(UG_Dict[j][2])
                aln2 = float(UG_Dict[j][3])
                score2 = pct2 * aln2
                flag +=1
                prot2 = 1
            if flag==2:
                break
        if flag==2:
            if protID1==protID2:
                Annot_Dict[i].append(protID1)
                if score1>score2:
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
```

```
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                count +=1
                print count, "Human orthologues found and",
                none_count, "probes without ortholog."
            else:
               if score1>score2:
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
               else:
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
               count +=1
               print count, "Human orthologues found and",
               none_count, "probes without ortholog."
        if flag==1:
            if prot1==1:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
```

```
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID2)
                Annot_Dict[i].append(pct2)
                Annot_Dict[i].append(aln2)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."
        if flag==0:
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            none_count +=1
    else:
        ID1 = Annot_Dict[i][41]
        ID2 = Annot_Dict[i][10]
        ID3 = Annot_Dict[i][9]
        flag = 0
        prot1 = 0
        prot2 = 0
        prot3 = 0
        for j in UG_Dict:
            if ID1==UG_Dict[j][0]:
                protID1 = UG_Dict[j][1]
                pct1 = float(UG_Dict[j][2])
                aln1 = float(UG_Dict[j][3])
```

```
                score1 = pct1 * aln1
                prot1 = 1
                flag += 1
            if ID2==UG_Dict[j][0]:
                protID2 = UG_Dict[j][1]
                pct2 = float(UG_Dict[j][2])
                aln2 = float(UG_Dict[j][3])
                score2 = pct2 * aln2
                prot2 = 1
                flag += 1
            if ID3==UG_Dict[j][0]:
                protID3 = UG_Dict[j][1]
                pct3 = float(UG_Dict[j][2])
                aln3 = float(UG_Dict[j][3])
                score3 = pct3 * aln3
                prot3 = 1
                flag += 1
        if flag==0:
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            none_count +=1
        elif flag==1:
            if prot1==1:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            elif prot2==1:
                Annot_Dict[i].append(protID2)
                Annot_Dict[i].append(pct2)
                Annot_Dict[i].append(aln2)
```

```
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID3)
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."

        elif flag==2:
            if prot1==1 and prot2==1:
                if protID1==protID2:
                    Annot_Dict[i].append(protID1)
                    if score1>score2:
                        Annot_Dict[i].append(pct1)
                        Annot_Dict[i].append(aln1)
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                    else:
                        Annot_Dict[i].append(pct2)
                        Annot_Dict[i].append(aln2)
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                else:
```

```
                if score1>score2:
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append(protID1)
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."
        elif prot1==1 and prot3==1:
            if protID1==protID3:
                Annot_Dict[i].append(protID1)
                if score1>score3:
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    Annot_Dict[i].append(pct3)
                    Annot_Dict[i].append(aln3)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
```

```
                Annot_Dict[i].append('---')
        else:
            if score1>score3:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append(protID3)
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID3)
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            count +=1
            print count, "Human orthologues found and",
            none_count, "probes without ortholog."
    elif prot2==1 and prot3==1:
        if protID2==protID3:
            Annot_Dict[i].append(protID2)
            if score2>score3:
                Annot_Dict[i].append(pct2)
                Annot_Dict[i].append(aln2)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
```

```
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                else:
                    if score2>score3:
                        Annot_Dict[i].append(protID2)
                        Annot_Dict[i].append(pct2)
                        Annot_Dict[i].append(aln2)
                        Annot_Dict[i].append(protID3)
                        Annot_Dict[i].append(pct3)
                        Annot_Dict[i].append(aln3)
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                    else:
                        Annot_Dict[i].append(protID3)
                        Annot_Dict[i].append(pct3)
                        Annot_Dict[i].append(aln3)
                        Annot_Dict[i].append(protID2)
                        Annot_Dict[i].append(pct2)
                        Annot_Dict[i].append(aln2)
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                        Annot_Dict[i].append('---')
                count +=1
                print count, "Human orthologues found and",
                none_count, "probes without ortholog."
        elif flag==3:
            if protID1==protID2==protID3:
                Annot_Dict[i].append(protID1)
                if score1>score2 and score1>score3:
                    Annot_Dict[i].append(pct1)
                    Annot_Dict[i].append(aln1)
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                    Annot_Dict[i].append('---')
                elif score2>score1 and score2>score3:
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append('---')
```

```
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
        elif protID1==protID2:
            if score1>score2:
                newprot = protID1
                newpct = pct1
                newaln = aln1
                newscore = score1
            else:
                newprot = protID2
                newpct = pct2
                newaln = aln2
                newscore = score2
            if newscore>score3:
                Annot_Dict[i].append(newprot)
                Annot_Dict[i].append(newpct)
                Annot_Dict[i].append(newaln)
                Annot_Dict[i].append(protID3)
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID3)
                Annot_Dict[i].append(pct3)
                Annot_Dict[i].append(aln3)
                Annot_Dict[i].append(newprot)
                Annot_Dict[i].append(newpct)
                Annot_Dict[i].append(newaln)
                Annot_Dict[i].append('---')
```

```
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
    elif protID1==protID3:
        if score1>score3:
            newprot = protID1
            newpct = pct1
            newaln = aln1
            newscore = score1
        else:
            newprot = protID3
            newpct = pct3
            newaln = aln3
            newscore = score3
        if newscore>score2:
            Annot_Dict[i].append(newprot)
            Annot_Dict[i].append(newpct)
            Annot_Dict[i].append(newaln)
            Annot_Dict[i].append(protID2)
            Annot_Dict[i].append(pct2)
            Annot_Dict[i].append(aln2)
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
        else:
            Annot_Dict[i].append(protID2)
            Annot_Dict[i].append(pct2)
            Annot_Dict[i].append(aln2)
            Annot_Dict[i].append(newprot)
            Annot_Dict[i].append(newpct)
            Annot_Dict[i].append(newaln)
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
            Annot_Dict[i].append('---')
    elif protID2==protID3:
        if score2>score3:
            newprot = protID2
            newpct = pct2
            newaln = aln2
            newscore = score2
        else:
            newprot = protID3
            newpct = pct3
            newaln = aln3
```

```
                newscore = score3
            if newscore>score1:
                Annot_Dict[i].append(newprot)
                Annot_Dict[i].append(newpct)
                Annot_Dict[i].append(newaln)
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
            else:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                Annot_Dict[i].append(newprot)
                Annot_Dict[i].append(newpct)
                Annot_Dict[i].append(newaln)
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
                Annot_Dict[i].append('---')
        else:
            if score1>score2 and score1>score3:
                Annot_Dict[i].append(protID1)
                Annot_Dict[i].append(pct1)
                Annot_Dict[i].append(aln1)
                if score2>score3:
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    Annot_Dict[i].append(protID3)
                    Annot_Dict[i].append(pct3)
                    Annot_Dict[i].append(aln3)
                else:
                    Annot_Dict[i].append(protID3)
                    Annot_Dict[i].append(pct3)
                    Annot_Dict[i].append(aln3)
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                count +=1
                print count, "Human orthologues found and",
                none_count, "probes without ortholog."
```

```
                elif score2>score1 and score2>score3:
                    Annot_Dict[i].append(protID2)
                    Annot_Dict[i].append(pct2)
                    Annot_Dict[i].append(aln2)
                    if score1>score3:
                        Annot_Dict[i].append(protID1)
                        Annot_Dict[i].append(pct1)
                        Annot_Dict[i].append(aln1)
                        Annot_Dict[i].append(protID3)
                        Annot_Dict[i].append(pct3)
                        Annot_Dict[i].append(aln3)
                    else:
                        Annot_Dict[i].append(protID3)
                        Annot_Dict[i].append(pct3)
                        Annot_Dict[i].append(aln3)
                        Annot_Dict[i].append(protID1)
                        Annot_Dict[i].append(pct1)
                        Annot_Dict[i].append(aln1)
                    count +=1
                    print count, "Human orthologues found and",
                    none_count, "probes without ortholog."
                else:
                    Annot_Dict[i].append(protID3)
                    Annot_Dict[i].append(pct3)
                    Annot_Dict[i].append(aln3)
                    if score1>score2:
                        Annot_Dict[i].append(protID1)
                        Annot_Dict[i].append(pct1)
                        Annot_Dict[i].append(aln1)
                        Annot_Dict[i].append(protID2)
                        Annot_Dict[i].append(pct2)
                        Annot_Dict[i].append(aln2)
                    else:
                        Annot_Dict[i].append(protID2)
                        Annot_Dict[i].append(pct2)
                        Annot_Dict[i].append(aln2)
                        Annot_Dict[i].append(protID1)
                        Annot_Dict[i].append(pct1)
                        Annot_Dict[i].append(aln1)
                    count +=1
                    print count, "Human orthologues found and",
                    none_count, "probes without ortholog."
print "Done"
```

```
print count, "Human orthologues found and", none_count, "probes
without ortholog."

#################################################################
#################
"""Writes annotation file to .csv containing human ortholog
accessions and corresponding alignment data"""

import csv
with open('new_annot3.csv', 'wb') as f:
    writer = csv.writer(f)
    writer.writerow(Annot_Dict['Probe Set ID'])
    for item in Annot_Dict:
        writer.writerow(Annot_Dict[item])
f.close()
```

Appendix B: Transcript IDs and functional information corresponding to Figure 2 heatmap.

Fold difference in expression between tissues was calculated based on raw expression values and is colored according to magnitude of difference. Red indicates ovary-enriched, blue indicates testis-enriched, yellow indicates moderate difference and green indicates no difference. Gene symbols in parenthases are human gene symbols.

			|Fold-difference|					
Probe ID	Human Accession	Cluster	NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
Str.24079.1.A1_at	NP_060439.2	Ovary-A	1	1	14	40	(iws1)	IWS1 homolog (S. cerevisiae)	Cancer-related
Str.10336.1.S1_at	NP_653277.2	Ovary-A	1	2	17	34	tmed6	transmembrane emp24 protein transport domain containing 6	
Str.50399.1.S1_at	NP_002745.1	Ovary-A	1	2	13	16	mapk13	mitogen-activated protein kinase 13	Cancer-related, cell proliferation
Str.1727.3.A2_a_at	NP_031397.1	Ovary-A	1	1	65	47	brdt (brd3)	bromodomain containing 3	
Str.31845.1.S1_at	NP_000896.1	Ovary-A	1	4	25	57	npy	neuropeptide Y	Vascularization, secretion of hormone, fertility
Str.7244.1.S1_at	NP_001008779.1	Ovary-A	1	5	29	57	(spdya)	speedy homolog A (Xenopus laevis)	Oocyte maturation
Str.32211.1.A1_at	NP_001096637.1	Ovary-A	1	7	30	26	(zglp1)	zinc finger, GATA-like protein 1	Gonadogenesis, gametogenesis, oogenesis, ovary development
Str.34301.2.S1_at	NP_115508.2	Ovary-A	1	14	29	6	(hormad1)	HORMA domain containing 1	Gonadogenesis, gametogenesis, meiosis, fertility (male)
Str.1727.3.A1_at	NP_001717.2	Ovary-A	1	3	13	8	brdt	bromodomain, testis-specific	Gonadogenesis, chromatin remodeling (male)
Str.27519.1.S1_at	NP_003185.1	Ovary-A	1	4	9	11	tbpl2 (tbp)	TATA box binding protein	Transcription, embryonic development
Str.34382.1.A1_at	NP_751898.1	Ovary-A	1	4	15	8	msh5	mutS homolog 5 (E. coli)	Gonadogenesis, gametogenesis, meiosis, fertility
Str.32425.1.A1_at	NP_570140.1	Ovary-A	1	3	16	9	(syce1)	synaptonemal complex central element protein 1	Meiosis
Str.33708.1.A1_at	NP_001128320.1	Ovary-A	1	3	17	9	(ccdc152)	coiled-coil domain containing 152	
StrAffx.106.1.S1_s_at	NP_694999.3	Ovary-A	2	4	13	12	mamdc2	MAM domain containing 2	
Str.38722.1.S1_at	NP_872365.1	Ovary-A	1	6	23	10	tmprss12	transmembrane (C-terminal) protease, serine 12	Cancer-related
Str.33833.1.A1_at	NP_079335.2	Ovary-A	1	2	10	7	(ccdc170)	coiled-coil domain containing 170	Cancer-related
Str.21686.1.S1_at	NP_000726.1	Ovary-A	1	3	9	9	(cga)	glycoprotein hormones, alpha polypeptide	Gonadogenesis, morphology of ovary, quantity of hormone, fertility
Str.37820.1.S1_at	NP_001130042.1	Ovary-A	2	3	7	6	(fam47e)	family with sequence similarity 47, member E	
Str.31912.1.A1_at	NP_689820.2	Ovary-A	2	4	7	6	(c1orf177)	chromosome 1 open reading frame 177	
Str.38593.1.S1_at	NP_001026918.2	Ovary-A	1	2	9	5	(c12orf40)	chromosome 12 open reading frame 40	
Str.33738.1.A1_at	NP_001017975.3	Ovary-A	2	4	11	7	hfm1	HFM1, ATP-dependent DNA helicase homolog (S. cerevisiae)	Cancer-related
Str.34278.1.A1_at	NP_001003811.1	Ovary-A	1	5	11	7	(tex11)	testis expressed 11	Gonadogenesis, gametogenesis, meiosis, fertility (male)
Str.34311.2.A1_at	NP_001008777.2	Ovary-A	2	4	9	6	fbxo47	F-box protein 47	Cancer-related
Str.34301.3.A1_x_at	NP_115508.2	Ovary-A	1	8	13	6	(hormad1)	HORMA domain containing 1	Gonadogenesis, gametogenesis, meiosis, fertility (male)
Str.34301.1.S1_a_at	NP_115508.2	Ovary-A	1	8	16	8	(hormad1)	HORMA domain containing 1	Gonadogenesis, gametogenesis, meiosis, fertility (male)
Str.34301.1.S1_at	NP_115508.2	Ovary-A	1	11	18	7	(hormad1)	HORMA domain containing 1	Gonadogenesis, gametogenesis, meiosis, fertility (male)
Str.42327.1.A1_at	NP_001099048.1	Ovary-A	1	7	19	11	(syce2)	synaptonemal complex central element protein 2	DNA recombination, synapsis, meiosis
Str.49258.1.A1_at	NP_008999.2	Ovary-A	2	13	27	9	(dmc1)	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	Gonadogenesis, gametogenesis, oogenesis, meiosis, fertility
Str.51692.1.S1_at	NP_444505.1	Ovary-A	2	5	3	7	(rplp0)	ribosomal protein, large, P0	Cancer-related
Str.20782.1.S1_a_at	NP_060369.3	Ovary-A	1	2	4	5	tesc	tescalcin	Neurological disease, Na+ homeostasis
Str.32672.1.A1_at	NP_001138400.1	Ovary-A	1	2	4	5	(nkiras2)	NFKB inhibitor interacting Ras-like 2	Cancer-related
Str.30634.1.A1_at	NP_009131.2	Ovary-A	1	2	4	5	(akap13)	A kinase (PRKA) anchor protein 13	Cancer-related, decondensation of chromatin
Str.15244.2.S1_a_at	NP_079197.3	Ovary-A	2	2	4	5	pof1b	premature ovarian failure, 1B	Cancer-related, premature ovarian failure
Str.8393.1.S1_at	NP_001118228.1	Ovary-A	2	2	3	5	pabpc1l	poly(A) binding protein, cytoplasmic 1-like	Gonadogenesis, gametogenesis, oogenesis, meiosis, fertility
Str.52229.1.S1_at	NP_000976.1	Ovary-A	3	3	4	5	(rpl17)	ribosomal protein L17	Cancer-related
Str.26910.1.S1_at	NP_001164666.1	Ovary-A	3	3	4	6	elavl2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	Cancer-related
Str.19885.1.S1_at	NP_006863.2	Ovary-A	3	3	4	5	gtf2a1l///ston1	general transcription factor IIA, 1-like	
Str.32359.1.A1_at	NP_003869.1	Ovary-A	1	2	5	5	(ggh)	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	Amino acid metabolism
Str.47632.1.S1_x_at	NP_001137457.1	Ovary-A	2	1	3	5	baf-l (banf1)	barrier to autointegration factor 1	DNA recombination
Str.15950.1.S1_s_at	NP_005660.4	Ovary-A	2	2	4	5	reep6 (reep5)	receptor accessory protein 5	
Str.1623.1.S1_at	NP_690048.1	Ovary-A	2	3	5	5	stk35 (pdik1l)	PDLIM1 interacting kinase 1 like	Organization of actin stress fibers
Str.38422.2.A1_a_at	NP_079356.3	Ovary-A	2	2	6	8	(asrgl1)	asparaginase like 1	Cancer-related
Str.49275.1.S1_at	NP_775804.2	Ovary-A	2	5	7	5	tdrd5	tudor domain containing 5	Gonadogenesis, gametogenesis, meiosis, fertility (male)

Probe ID	Human Accession	Cluster	|Fold-difference| NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
Str.6726.1.S1_at	NP_653195.2	Ovary-A	3	6	6	5	gtsf1	gametocyte specific factor 1	Germ cell development
Str.38810.1.S1_at	NP_056002.2	Ovary-A	1	5	5	5	sel1l3	sel-1 suppressor of lin-12-like 3 (C. elegans)	Cancer-related
Str.5073.1.S1_at	NP_065392.1	Ovary-A	2	4	6	5	c20orf3 (apmap)	adipocyte plasma membrane associated protein	Cancer-related
Str.4267.1.S1_at	NP_919268.1	Ovary-A	2	2	6	6	sass6	spindle assembly 6 homolog (C. elegans)	DNA replication
Str.27191.2.S1_at	NP_001013682.2	Ovary-A	1	2	6	8	(frrs1)	ferric-chelate reductase 1	
Str.1727.2.A1_at	NP_001717.2	Ovary-A	1	2	7	7	brdt	bromodomain, testis-specific	Gonadogenesis, chromatin remodeling (male)
Str.710.2.S1_at	NP_076957.3	Ovary-A	1	1	5	8	c17orf39 (gid4)	GID complex subunit 4, VID24 homolog (S. cerevisiae)	
Str.4536.1.S1_at	NP_543026.1	Ovary-A	1	1	6	7	(stk35)	serine/threonine kinase 35	Cancer-related, organization of actin stress fibers
Str.8311.1.S1_at	NP_542195.1	Ovary-A	1	3	9	5	(tldc2)	TBC/LysM-associated domain containing 2	
Str.34311.1.S1_at	NP_001008777.2	Ovary-A	1	2	9	6	fbxo47	F-box protein 47	Cancer-related
Str.30824.1.A1_s_at	NP_689551.1	Ovary-A	1	4	10	7	zg16	zymogen granule protein 16	Cancer-related
Str.37576.1.S1_s_at	NP_079335.2	Ovary-A	1	4	12	8	(ccdc170)	coiled-coil domain containing 170	Cancer-related
Str.32445.1.S1_s_at	NP_006000.2	Ovary-A	2	3	4	18	tuba4 (tuba1a)	tubulin, alpha 1a	Nervous system development, microtubule dynamics
Str.32445.1.S1_at	NP_006000.2	Ovary-A	2	3	3	12	(tuba1a)	tubulin, alpha 1a	Nervous system development, microtubule dynamics
Str.7111.1.S1_at	NP_877724.1	Ovary-A	1	2	5	21	npm2	nucleophosmin/nucleoplasmin 2	Morphology of ovary, morphology of oocytes, fertility
Str.19394.1.S1_at	NP_001004311.2	Ovary-A	2	3	9	27	figla	folliculogenesis specific basic helix-loop-helix	Gonadogenesis, ovary development, meiosis of germ cells, fertility
Str.11490.1.S2_at	NP_001028253.1	Ovary-A	2	2	7	14	mycl1	v-myc myelocytomatosis viral oncogene homolog 1	Cancer-related
Str.11490.1.S1_at	NP_001028253.1	Ovary-A	2	2	7	14	mycl1	v-myc myelocytomatosis viral oncogene homolog 1	Cancer-related
Str.38460.2.A1_at	NP_001093392.1	Ovary-A	1	1	4	13	otud4		Cancer-related
Str.27686.1.S1_at	NP_001001933.1	Ovary-A	1	1	5	14	lhx8	LIM homeobox 8	Gonadogenesis, ovary development, nervous system development
Str.4078.1.S1_a_at	NP_001298.3	Ovary-B	4	5	5	10	cldn7	claudin 7	Ovarian cell proliferation, quantity of steroid hormone
Str.27610.1.S1_at	NP_002344.2	Ovary-B	3	4	4	8	epcam (tacstd2)	tumor-associated calcium signal transducer	Ovarian cell proliferation, cellular movement
Str.51982.1.A1_s_at	NP_077312.2	Ovary-B	3	6	5	7	irx3	iroquois homeobox 3	Organ development, nervous system development
Str.39298.2.S1_a_at	NP_000005.2	Ovary-B	2	5	5	6	(a2m)	alpha-2-macroglobulin	Nervous system development, cellular movement
Str.21092.1.S1_at	NP_005844.4	Ovary-B	2	4	4	5	irx5	iroquois homeobox 5	Organ development, morphology, nervous system development
StrJgi.3092.1.S1_at	NP_005089.2	Ovary-B	3	3	3	5	msc	musculin	Muscle development
Str.8649.2.S1_x_at	NP_067018.2	Ovary-B	3	3	5	11	(cldn6)	claudin 6	Cell proliferation, differentiation
Str.8649.2.S1_at	NP_067018.2	Ovary-B	3	3	5	11	(cldn6)	claudin 6	Cell proliferation, differentiation
Str.16327.1.S1_at	NP_005256.2	Ovary-B	2	1	4	5	ggt1	gamma-glutamyltransferase 1	Morphology of gonad, fertility, cell proliferation
Str.13287.1.S1_at	NP_056122.1	Ovary-B	2	5	5	6	fam189a1	family with sequence similarity 189, member A1	
Str.5962.2.A1_at	NP_060167.2	Ovary-B	2	4	5	15	esrp1	epithelial splicing regulatory protein 1	Ovarian cell proliferation, RNA splicing
Str.5962.1.S1_at	NP_060167.2	Ovary-B	2	3	6	20	esrp1	epithelial splicing regulatory protein 1	Ovarian cell proliferation, RNA splicing
Str.10951.1.S1_at	NP_065826.2	Ovary-B	3	3	3	12	(kiaa1324)	KIAA1324	
Str.34852.1.A1_at	NP_077720.1	Ovary-B	5	5	11	21	nppc	natriuretic peptide C	Reproductive system development, cell signaling, cell proliferation
Str.41205.1.A1_at	NP_000207.2	Ovary-B	6	13	9	8	kal1	Kallmann syndrome 1 sequence	Guidance of axons, cell movement, gonad development
Str.34732.1.A1_at	NP_000094.2	Ovary-B	7	12	8	11	cyp19a1	cytochrome P450, family 19, subfamily A, polypeptide 1	Morphology of gonad, primary sex determination
Str.8337.1.S1_at	NP_002099.1	Ovary-B	5	13	12	14	hal.2	histidine ammonia-lyase	Amino acid metabolism
Str.52013.1.S1_at	NP_000094.2	Ovary-B	5	8	5	6	cyp19a1	cytochrome P450, family 19, subfamily A, polypeptide 1	Morphology of gonad, primary sex determination
Str.19828.1.S1_at	NP_689648.2	Ovary-B	6	5	5	6	amdhd1	amidohydrolase domain containing 1	
Str.28022.1.A1_at	NP_001138578.1	Ovary-B	8	13	17	17	fibcd1	fibrinogen C domain containing 1	
Str.24572.1.A1_s_at	NP_000774.2	Ovary-B	8	22	15	27	cyp26a1	cytochrome P450, family 26, subfamily A, polypeptide 1	Organ development, morphology, metabolism
Str.12223.1.S1_at	NP_000742.1	Ovary-B	7	11	13	7	chrnd	cholinergic receptor, nicotinic, delta (muscle)	Muscle growth
Str.15551.1.S2_at	NP_002336.1	Ovary-B	6	7	6	5	lum	lumican	Cell proliferation, morphology, fertility
Str.15551.1.S1_at	NP_002336.1	Ovary-B	7	7	7	6	lum	lumican	Cell proliferation, morphology, fertility
Str.28084.1.S1_at	NP_004089.1	Ovary-B	5	7	9	6	emx2	empty spiracles homeobox 2	Morphology of gonad, primary sex determination
StrEns.9618.1.S1_a_at	NP_872293.2	Ovary-B	4	7	6	6	olfml2a	olfactomedin-like 2A	Endometrial cell proliferation
Str.38277.1.A1_at	NP_001121367.1	Ovary-B	5	4	5	5	chst8	carbohydrate (N-acetylgalactosamine 4-0) s	Morphology of gonad, concentration of hormone
Str.13722.1.S1_at	NP_000541.1	Ovary-B	3	6	8	6	tyrp1	tyrosinase-related protein 1	

Probe ID	Human Accession	Cluster	|Fold-difference| NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
StrAffx.70.1.S1_s_at	NP_004813.2	Ovary-B	21	61	43	64	ntn1	netrin 1	Nervous system development, cellular movement
Str.6910.1.S1_at	NP_002088.2	Ovary-C	1	1	2	85	42Sp43 (gtf3a)	general transcription factor IIIA	Confirmational DNA modification, transcription
Str.7712.1.S1_a_at	NP_002096.1	Ovary-C	1	1	1	56	hist1h2aa (h2afx)	H2A histone family, member X	Gametogenesis, meiosis, DNA repair
Str.4034.1.S1_at	NP_001104516.1	Ovary-C	1	2	3	71	ccna1	cyclin A1	Gametogenesis, cell cycle progression
Str.27228.1.S1_at	NP_004692.1	Ovary-C	1	1	2	34	(ccnb2)	cyclin B2	Cell proliferation, fecundity
Str.1760.2.S2_at	NP_071370.2	Ovary-C	1	1	2	42	cdca7	cell division cycle associated 7	Regulation of cell proliferation and transcription
Str.3994.1.S1_a_at	NP_114172.1	Ovary-C	1	1	1	45	ccnb1	cyclin B1	Cell cycle progression, mitosis
Str.6258.1.S1_at	NP_001167607.1	Ovary-C	1	1	2	50	cfc1 (tdgf1)	teratocarcinoma-derived growth factor 1	Embryonic development, mesoderm development
StrEns.9022.1.S1_s_at	NP_054879.3	Ovary-C	1	1	2	36	mkrn3 (mkrn2)	makorin ring finger protein 2	
Str.6468.4.S1_at	NP_054879.3	Ovary-C	1	1	3	101	mkrn3 (mkrn2)	makorin ring finger protein 2	
Str.8156.1.S1_at	NP_006073.2	Ovary-C	1	1	2	63	tuba4 (tuba1b)	tubulin, alpha 1b	
Str.26862.1.S1_at	NP_783318.1	Ovary-C	1	1	1	25	zar1	zygote arrest 1	Oocyte-specific maternal mRNA, embryogenesis
Str.32186.1.A1_at	NP_009067.2	Ovary-C	1	1	1	18	(znf80)	zinc finger protein 80	
Str.3682.1.S1_at	NP_060059.1	Ovary-C	1	1	2	28	btg4	B-cell translocation gene 4	Cell cycle progression, cell proliferation, mitosis
Str.17082.2.S1_x_at	NP_003541.2	Ovary-C	1	1	2	29	(mad1l1)	MAD1 mitotic arrest deficient-like 1 (yeast)	Cell cycle progression, cell proliferation, transcription
Str.1617.1.S1_at	NP_067009.1	Ovary-C	1	1	1	30	zp4.2	zona pellucida glycoprotein 4	Oocyte-specific, cell signaling, activation of protein kinase
Str.3.1.S1_a_at		Ovary-C	1	1	1	32	nanos1		Oocyte maternal mRNA regulation of embryonic development
Str.6457.1.S1_at	NP_997309.2	Ovary-C	1	1	1	31	foxi2	forkhead box I2	Oocyte maternal mRNA activator of embryonic development (Cha et al.,1 2012)
Str.20406.1.S1_at	NP_001130043.1	Ovary-C	1	1	1	33	(zar1l)	zygote arrest 1-like	Oocyte-specific maternal mRNA, RNA regulation embryogenesis
Str.5107.1.S1_at	NP_067009.1	Ovary-C	1	1	1	36	zp4	zona pellucida glycoprotein 4	Oocyte-specific, cell signaling, activation of protein kinase
Str.27898.1.S1_at	NP_003398.1	Ovary-C	1	1	1	36	zfp36l2.1 (zfp36)	ZFP36 ring finger protein	Cell proliferation, mRNA stabilization, transcription
Str.5859.1.S1_a_at	NP_001099028.1	Ovary-C	1	1	1	41	wee2	WEE1 homolog 2 (S. pombe)	Cell cycle, meiosis I, prophase I
Str.754.1.S1_at	NP_690851.2	Ovary-C	1	1	1	40	(prss33)	protease, serine, 33	
Str.1668.2.S1_at	NP_061897.1	Ovary-C	1	1	1	49	(heatr5b)	HEAT repeat containing 5B	
Str.30111.1.S1_at	NP_001122098.1	Ovary-C	1	1	1	48	(spire1)	spire homolog 1 (Drosophila)	
Str.3366.1.S1_at	NP_005940.1	Ovary-C	1	1	1	167	(mt1f)	metallothionein 1F	Cell death and survival
Str.117.1.S1_at	NP_009086.4	Ovary-C	1	1	1	120	(zp3)	zona pellucida glycoprotein 3 (sperm receptor)	Oocyte development, gametogenesis, fertility
Str.8649.1.S1_at	NP_067018.2	Ovary-C	1	1	2	91	cldn6.1	claudin 6	Cell proliferation, differentiation
Str.21536.1.S1_at	NP_008818.3	Ovary-C	1	1	1	495	zfp36l2.2	ZFP36 ring finger protein-like 2	Cell proliferation, mRNA stabilization, transcription
Str.3467.1.S1_at	NP_006797.3	Ovary-C	1	1	1	250	(btg3)	BTG family, member 3	Cell cycle progression, cell proliferation, transcription
Str.52147.2.S1_x_at	NP_001514.2	Ovary-C	1	1	1	15	(has1)	hyaluronan synthase 1	Cell proliferation, cellular movement
StrEns.4910.1.S1_at	NP_002051.2	Ovary-C	1	1	1	14	(gja4)	gap junction protein, alpha 4, 37kDa	Reproductive system development, gametogenesis, fertility
Str.3569.1.S1_at	NP_001154945.1	Ovary-C	1	1	1	14	aldh3b1	aldehyde dehydrogenase 3 family, member B1	
Str.6507.1.S1_at	NP_722575.1	Ovary-C	1	1	1	12	h1foo	H1 histone family, member O, oocyte-specific	Oocyte-specific maternal mRNA
Str.17082.1.S1_s_at	NP_000214.1	Ovary-C	1	1	1	11	xk81a1 (krt12)	keratin 12	Disruption of keratin filaments
Str.27268.1.S1_at	NP_003097.1	Ovary-C	1	1	1	12	sox3 (sox2)	SRY (sex determining region Y)-box 2	Reproductive system development, cell proliferation
Str.39270.1.A1_at	NP_005439.2	Ovary-C	1	1	1	13	(bmp15)	bone morphogenetic protein 15	Reproductive system development, gametogenesis, fertility
Str.31989.1.A1_at	NP_001002259.1	Ovary-C	1	1	2	9	caprin2	caprin family member 2	Cell proliferation, transcription
Str.10367.1.S1_at	NP_001135877.1	Ovary-C	1	1	2	17	slmo1	slowmo homolog 1 (Drosophila)	
Str.51662.1.A1_at	NP_001159766.1	Ovary-C	1	1	2	22	(ssx2ip)	synovial sarcoma, X breakpoint 2 interacting protein	Cell proliferation
Str.7206.1.S1_at	NP_001949.1	Ovary-C	1	1	1	18	42Sp50 (eef1a2)	eukaryotic translation elongation factor 1 alpha 2	Oocyte vegetal mRNA transport (Loeber et al. 2010)
Str.1153.1.S1_at	NP_001167022.1	Ovary-C	1	1	1	11	tmprss4	transmembrane protease, serine 4	
Str.27710.1.S1_at	NP_073587.1	Ovary-C	1	1	1	8	parp12	poly (ADP-ribose) polymerase family, member 12	
Str.10709.1.S1_at	NP_001073935.1	Ovary-C	1	1	1	7	btbd17	BTB (POZ) domain containing 17	
StrEns.9654.1.S1_s_at	NP_001002259.1	Ovary-C	1	1	1	7	caprin2	caprin family member 2	Cell proliferation, transcription
Str.15673.1.S1_at	NP_009294.1	Ovary-C	1	1	1	9	comt.2 (comt)	catechol-O-methyltransferase	Reproductive system development, metabolism, regulation of neurotransmitter
StrJgi.8914.1.S1_s_at	NP_543154.1	Ovary-C	1	1	1	7	(itln2)	intelectin 2	
Str.5899.1.S1_at	NP_653194.1	Ovary-C	1	1	1	8	rhebl1	Ras homolog enriched in brain like 1	Regulation of cell growth and proliferation

			|Fold-difference|					
Probe ID	Human Accession	Cluster	NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
Str.9274.1.S1_at	NP_001035189.1	Ovary-C	1	1	1	9	sptssb	serine palmitoyltransferase, small subunit B	
Str.51964.1.S1_s_at	NP_001004343.1	Ovary-C	1	1	1	6	map1lc3c	microtubule-associated protein 1 light chain 3 gamma	
Str.24662.1.S1_a_at	NP_009060.2	Ovary-C	1	1	1	6	zic2	Zic family member 2	Nervous system development
Str.2712.1.S1_at	NP_203125.1	Ovary-C	1	1	1	7	(casp7)	caspase 7, apoptosis-related cysteine peptidase	Cell death and survival, tissue morphology
Str.51683.1.S1_at	NP_057480.2	Ovary-C	1	1	1	6	(ctdspl2)	CTD (carboxy-terminal domain, RNA pol. II, polypep. A) small phosphatase like 2	Phosphatase activity
Str.22168.2.S1_at	NP_057405.1	Ovary-C	1	1	1	6	(rhcg)	Rh family, C glycoprotein	Morphology of gonad, molecular transport
Str.15798.1.S1_at	NP_001123886.1	Ovary-C	1	1	1	6	(apba2)	amyloid beta (A4) precursor protein-binding, family A, member 2	Exocytosis of vesicles
Str.19585.1.S1_at	NP_005906.2	Ovary-C	1	1	1	5	mcm6	minichromosome maintenance complex component 6	Confirmational DNA modification
Str.4026.1.S1_a_at	NP_003914.1	Ovary-C	1	1	1	5	foxh1	forkhead box H1	Embryonic development, patterning
Str.29857.1.A1_at	NP_689929.2	Ovary-C	1	1	1	5	patl2 (patl1)	protein associated with topoisomerase II homolog 1 (yeast)	
Str.49374.1.A1_at	NP_000843.1	Ovary-C	1	1	1	5	(gstp1)	glutathione S-transferase pi 1	Cell proliferation, conversion of beta-estradiol
Str.33548.1.S1_at	NP_001852.1	Ovary-C	1	1	1	6	(cox4i1)	cytochrome c oxidase subunit IV isoform 1	Cell cycle progression
Str.46745.1.A1_s_at	NP_005389.1	Ovary-C	1	1	2	7	ppp1r3c.2	protein phosphatase 1, regulatory subunit 3C	Insulin regulation, glycogen homeostasis
Str.27229.1.S1_at	NP_005389.1	Ovary-C	1	1	2	7	ppp1r3c.2	protein phosphatase 1, regulatory subunit 3C	Insulin regulation, glycogen homeostasis
Str.9274.1.S2_at	NP_001035189.1	Ovary-C	1	1	2	7	sptssb	serine palmitoyltransferase, small subunit B	
Str.8371.1.S1_a_at	NP_001130043.1	Ovary-C	1	1	1	6	(zar1l)	zygote arrest 1-like	Oocyte-specific maternal mRNA, RNA regulation embryogenesis
Str.15996.2.S1_at	NP_056530.2	Ovary-C	1	1	1	8	proca1	protein interacting with cyclin A2	Lipid metabolism
StrEns.5801.1.S1_s_at	NP_056530.2	Ovary-C	1	1	1	7	proca1	protein interacting with cyclin A1	Lipid metabolism
Str.37482.1.S1_at	NP_065109.1	Ovary-C	1	1	1	8	pnpla3	patatin-like phospholipase domain containing 2	Lipid metabolism
Str.6496.1.S1_at	NP_065169.1	Ovary-C	2	1	2	9	sall4	sal-like 4 (Drosophila)	Cell proliferation, growth
Str.5899.1.S2_at	NP_653194.1	Ovary-C	1	1	2	8	rhebl1	Ras homolog enriched in brain like 1	Regulation of cell growth and proliferation
Str.7388.1.S1_at	NP_001959.1	Ovary-C	1	1	2	15	eif4e	eukaryotic translation initiation factor 4E	Cell proliferation, cell death and survival, RNA trafficking
StrEns.410.1.S1_at	NP_542449.1	Ovary-C	1	2	3	12	vegt	T-box protein VegT	Oogenesis, germ layer specification of embryo
Str.4776.1.S1_at	NP_079206.2	Ovary-C	1	2	3	8	elovl7	ELOVL fatty acid elongase 7	Cell proliferation, synthesis of hormone
Str.27817.1.S1_at	NP_060179.2	Ovary-C	1	2	2	5	fam46c	family with sequence similarity 46, member C	
Str.8000.1.S1_a_at	NP_004692.1	Ovary-C	3	3	2	6	ccnb2	cyclin B2	Cell proliferation, fecundity
Str.11491.1.A1_at	NP_060546.2	Ovary-C	2	4	3	7	armc4	armadillo repeat containing 4	
Str.28193.1.A1_at	NP_000266.2	Ovary-C	2	2	4	8	(oca2)	oculocutaneous albinism II	Reproductive system development, gametogenesis
Str.5962.1.S2_at	NP_060167.2	Ovary-C	1	2	3	8	esrp1	epithelial splicing regulatory protein 1	
Str.287.1.S1_at	NP_002297.2	Ovary-C	2	2	3	5	lgals3	lectin, galactoside-binding, soluble, 3	Cell proliferation, expansion of mesangium
Str.15225.1.S1_at	NP_001091089.1	Ovary-C	2	2	2	6	tmem184a	transmembrane protein 184A	Protein localization
StrAffx.141.1.S1_at	NP_001005340.1	Ovary-C	1	1	3	10	gpnmb	glycoprotein (transmembrane) nmb	Cell proliferation
Str.31340.2.S1_at	NP_859062.1	Ovary-C	1	1	2	5	grasp	GRP1 (general receptor for phosphoinositides 1)-assoc. scaffold prot.	Cell proliferation, localization, sprouting of mossy fibers
Str.31947.1.A1_at	NP_001137501.1	Ovary-C	1	1	2	5	cntd2	cyclin N-terminal domain containing 2	
Str.13533.1.A1_at	NP_000736.2	Ovary-C	1	1	3	6	chrna5	cholinergic receptor, nicotinic, alpha 5 (neuronal)	Cell signaling, nervous system development
Str.1304.1.S1_at	NP_001129688.1	Ovary-C	2	1	3	6	tmem51	transmembrane protein 51	
Str.116.1.S1_at	NP_067009.1	Ovary-C	1	1	2	6	zpax (zp4)	zona pellucida glycoprotein 4	Cell proliferation, cell signaling, activation of protein kinase
Str.33670.1.S1_at	NP_001798.2	Ovary-C	1	1	1	6	(cel)	carboxyl ester lipase	Lipid metabolism
Str.19439.1.S1_at	NP_775761.4	Ovary-C	1	1	5	13	tmem171	transmembrane protein 171	
Str.31112.1.A1_at	NP_877435.2	Ovary-C	1	1	6	8	wdr72	WD repeat domain 72	Cell death and survival
Str.27291.1.S1_at	NP_005718.2	Ovary-C	1	1	4	16	tspan1	tetraspanin 1	
Str.9495.1.S1_at	NP_005846.1	Ovary-C	1	1	4	6	ramp1	receptor (G protein-coupled) activity modifying protein 1	
Str.48992.1.A1_at	NP_002395.1	Ovary-C	1	1	5	12	(mfap4)	microfibrillar-associated protein 4	
Str.6678.1.S2_at	NP_008883.2	Ovary-C	1	1	1	5	upk1b	uroplakin 1B	
Str.20226.1.S1_at	NP_055135.1	Ovary-C	1	1	1	5	hebp2	heme binding protein 2	
Str.10989.1.S1_a_at	NP_006399.1	Ovary-C	2	1	1	6	agr2	anterior gradient 2 homolog (Xenopus laevis)	Cell proliferation, cellular movement
Str.3249.1.S1_s_at	NP_001296.1	Ovary-C	1	1	1	9	cldn4	claudin 4	Cell proliferation, cellular movement

Probe ID	Human Accession	Cluster	|Fold-difference| NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
Str.12245.1.S1_at	NP_112191.2	Testis-A	6	3	5	10	kazald1	Kazal-type serine peptidase inhibitor domain 1	
Str.31614.1.S1_at	NP_006761.1	Testis-A	4	3	5	7	(marco)	macrophage receptor with collagenous structure	Cellular organization of cytoskeleton, microtubule dynamics
Str.29149.1.A1_at	NP_074035.1	Testis-A	3	3	5	6	(myh11)	myosin, heavy chain 11, smooth muscle	Vascular development, muscle contraction
Str.31855.1.S1_at	NP_001129944.1	Testis-A	10	12	16	9	(litaf)	lipopolysaccharide-induced TNF factor	Cellular movement
Str.37641.1.S1_at	NP_004973.1	Testis-A	3	3	4	5	kcnj8	potassium inwardly-rectifying channel, subfamily J, member 8	Vascular development
Str.6049.1.A1_at	NP_004604.2	Testis-A	2	2	3	6	tgm2	transglutaminase 2 (C polypeptide, protein	Vasculogenesis, angiogenesis, concentration of hormone
Str.31333.1.S1_a_at	NP_612379.2	Testis-A	3	3	3	6	pkdcc.2	protein kinase domain containing, cytoplasmic	Connective tissue development
Str.28107.1.S1_at	NP_003059.1	Testis-A	3	4	4	6	snai1 (snai2)	snail homolog 2 (Drosophila)	Gonadogenesis, morphology of testis, cell movement
Str.26739.1.S1_at	NP_612454.1	Testis-A	2	2	3	5	gpr146	G protein-coupled receptor 146	
Str.22594.1.A1_at	NP_116121.2	Testis-A	2	3	4	7	(il17rc)	interleukin 17 receptor C	
Str.44816.1.A1_at	NP_000466.2	Testis-A	4	6	9	6	(nr0b1/dax-1)	nuclear receptor subfamily 0, group B, member 1	Gonadogenesis, morphology of testis, sex determination, fertility
Str.31923.1.S1_at	NP_001841.2	Testis-A	3	5	6	9	(col8a1)	collagen, type VIII, alpha 1	Vasculogenesis, angiogenesis
Str.37100.1.A1_at	NP_699174.1	Testis-A	4	3	4	8	(enpp6)	ectonucleotide pyrophosphatase/phosphodiesterase 6	Hydrolysis
Str.15452.1.S1_at	NP_004627.1	Testis-A	4	4	3	5	sema3b	sema domain (semaphorin), immunoglobulin domain (Ig) 3B	Vasculogenesis, cellular movement, cell signalling
Str.15379.1.S1_at	NP_004456.1	Testis-A	3	4	5	5	fgf10	fibroblast growth factor 10	Gonadogenesis, morphology of testis
StrJgi.3117.1.S1_s_at	NP_848600.2	Testis-A	5	5	5	5	tmem26	transmembrane protein 26	
Str.15388.1.A2_at	NP_004568.2	Testis-A	8	4	4	5	psph	phosphoserine phosphatase	
Str.6594.1.S1_at	NP_001035181.1	Testis-A	11	6	7	6	enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	Vasculogenesis, cellular movement
Str.52251.1.S1_at	NP_003730.4	Testis-A	10	6	5	10	akr1c2 (akr1c3)	aldo-keto reductase family 1, member C3	Steroid synthesis and metabolism
Str.31895.1.S1_at	NP_542988.3	Testis-A	1	3	4	5	(col13a1)	collagen, type XIII, alpha 1	Vasculogenesis, angiogenesis
Str.15223.6.S1_at	NP_001003760.2	Testis-A	2	3	4	5	klhl31	kelch-like family member 31	
Str.33336.1.A1_at	NP_000410.2	Testis-A	1	3	3	5	itga2b.1	integrin, alpha 2b	Morphology of vascular system, cellular movement
Str.24395.1.A1_at	NP_063938.1	Testis-A	1	2	4	7	cyp26b1	cytochrome P450, family 26, subfamily B, polypeptide 1	Gonadogenesis, morphology of testis
Str.10824.1.S1_at	NP_000890.1	Testis-A	2	3	5	8	kitlg	KIT ligand	Gonadogenesis, development of testis, steroid synthesis
Str.25601.1.A1_at	NP_000798.2	Testis-A	3	3	6	9	(gabra2)	gamma-aminobutyric acid (GABA) A receptor, alpha 2	
Str.5581.1.S1_at	NP_068733.1	Testis-A	3	3	4	6	cfl2	cofilin 2 (muscle)	
Str.15616.1.S1_at	NP_002914.1	Testis-A	3	4	6	7	rgs2	regulator of G-protein signaling 2, 24kDa	Vascular development, development of muscle
Str.32447.1.S1_at	NP_963849.1	Testis-A	3	5	6	8	fhl5 (fhl2)	four and a half LIM domains 2	Angiogenesis, cellular movement, muscle cell development
Str.5047.1.S2_at	NP_001918.3	Testis-A	2	3	4	6	des.1	desmin	Morphology of vascular system
Str.28002.1.S1_at	NP_001071642.1	Testis-A	1	3	5	7	(slc29a1)	solute carrier family 29 (nucleoside transporters), member 1	Cellular proliferation
Str.22906.1.S1_at	NP_511040.2	Testis-A	1	2	2	5	col9a1	collagen, type IX, alpha 1	Connective tissue development
Str.5759.1.S1_at	NP_001303.1	Testis-A	1	1	2	7	crip2	cysteine-rich protein 2	Cellular proliferation, cellular differentiation
Str.1843.2.S1_at	NP_005621.2	Testis-A	1	1	3	6	slco2a1	solute carrier organic anion transporter family, member 2A1	Morphology of vascular system, molecular transport
Str.38324.1.S1_at	NP_057315.3	Testis-A	1	2	2	5	nlk	nemo-like kinase	Morphology of vascular system
Str.17054.1.S1_at	NP_001437.1	Testis-A	1	2	4	24	fabp7	fatty acid binding protein 7, brain	Cellular proliferation, cellular movement
Str.27613.3.S1_at	NP_005323.1	Testis-A	3	2	2	8	hba-l2 (hbz)	hemoglobin, zeta	Morphology of vascular system
Str.12190.1.S1_at	NP_003751.2	Testis-A	2	8	11	17	fstl5 (eif4g3)	eukaryotic translation initiation factor 4 gamma, 3	Cellular organization of cytoskeleton, microtubule dynamics
Str.39178.2.A1_at	NP_001452.2	Testis-A	5	7	10	15	fmo5	flavin containing monooxygenase 5	
Str.39178.1.S1_a_at	NP_001452.2	Testis-A	5	6	8	14	fmo5	flavin containing monooxygenase 5	
Str.13088.1.S1_at	NP_004725.1	Testis-A	5	6	7	14	dclk2 (dclk1)	doublecortin-like kinase 1	Cellular organization of cytoskeleton, microtubule dynamics
Str.25076.1.A1_at	NP_004168.1	Testis-A	5	8	10	8	(stx3)	syntaxin 3	Cellular organization of cytoskeleton, microtubule dynamics
Str.21675.1.A1_at	NP_000521.2	Testis-A	6	5	9	11	mpz	myelin protein zero	Nervous system development, cellular movement
Str.39202.1.S1_at	NP_006320.2	Testis-A	6	7	8	17	fbln5	fibulin 5	Vascular development, cellular movement
Str.26721.1.S1_at	NP_002183.1	Testis-A	2	2	4	10	inhba	inhibin, beta A	Morphology of testis, fertility, steroidogenesis, cellular movement
Str.24498.1.S1_at	NP_001161146.1	Testis-A	2	2	3	6	cadm2	cell adhesion molecule	
Str.15475.1.S1_at	NP_689917.2	Testis-A	3	4	5	7	fam123a (amer2)	APC membrane recruitment protein 2	
Str.21927.1.S1_at	NP_056965.3	Testis-A	4	3	5	10	dkk3	dickkopf 3 homolog (Xenopus laevis)	Steroid hormone synthesis, cellular movement, cellular organization
Str.38097.1.A1_at	NP_066382.1	Testis-A	3	4	4	6	(dhh)	desert hedgehog	Gonadogenesis, morphology of testis, sex determination, steroid synthesis

			|Fold-difference|					
Probe ID	Human Accession	Cluster	NF58	NF66	1WPM	2WPM	Gene Symbol (Human)	Entrez Human Gene Name	Mammalian Function
Str.51661.1.S1_at	NP_001329.1	Testis-A	2	3	4	8	cxadr	coxsackie virus and adenovirus receptor	Vascular development, cellular movement
Str.8537.1.S1_at	NP_001290.2	Testis-A	3	6	6	9	cnn2 (cnn1)	calponin 1, basic, smo	Vascular development, cellular movement, morphology of testis
Str.51373.1.A1_at	NP_001137417.1	Testis-A	3	5	7	7	(gas6)	growth arrest-specific 6	Vascular development, cellular movement
Str.39201.1.S1_at	NP_002182.1	Testis-A	3	3	3	6	inha	inhibin, alpha	Morphology of testis, fertility, steroidogenesis, sex determination
Str.37326.1.A1_x_at	NP_006158.2	Testis-A	4	10	12	14	(nkx3-1)	NK3 homeobox 1	Morphogenesis of testis
Str.17189.1.S1_at	NP_000339.2	Testis-A	5	8	11	16	srd5a2	steroid-5-alpha-reductase, alpha polypeptide 2	Steroidogenesis, fertility, sex determination, morphology of testis
StrJgi.3167.1.S1_x_at	NP_006158.3	Testis-A	6	12	16	16	(nkx3-1)	NK3 homeobox 1	Morphogenesis of testis
Str.37326.1.A1_s_at	NP_006158.2	Testis-A	6	12	15	15	(nkx3-1)	NK3 homeobox 1	Morphogenesis of testis
Str.5526.1.S1_at	NP_003280.2	Testis-A	6	10	12	22	tpm2	tropomyosin 2 (beta)	Vascular development, muscle contraction
Str.8998.1.S1_at	NP_001034933.1	Testis-A	15	23	10	8	acbd7	acyl-CoA binding domain containing 7	
Str.38286.1.A1_at	NP_002227.2	Testis-A	8	7	7	7	kcnf1	potassium voltage-gated channel, subfamily F, member 1	
Str.7705.5.S1_at	NP_001135934.1	Testis-A	7	9	13	11	osr2	odd-skipped related 2 (Drosophila)	Cellular proliferation, growth
Str.38466.1.S1_at	NP_000093.1	Testis-A	11	8	9	11	cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1	Steroidogenesis, fertility, sex determination
Str.11952.1.S1_at	NP_001120645.1	Testis-A	10	7	9	10	(cadm3)	cell adhesion molecule 3	
Str.21742.1.S1_at	NP_001109.2	Testis-A	8	6	8	6	adcyap1r1	adenylate cyclase activating polypeptide 1 (pituitary) receptor type I	Cellular proliferation, concentration of hormone, fertility
Str.52145.1.S1_at	NP_003730.4	Testis-A	6	6	7	8	akr1cl1 (akr1c3)	aldo-keto reductase family 1, member C3	Steroidogenesis
StrJgi.5073.1.S1_s_at	NP_003730.4	Testis-A	6	6	6	7	akr1cl1 (akr1c3)	aldo-keto reductase family 1, member C3	Steroidogenesis
Str.19735.1.S1_at	NP_003586.3	Testis-A	6	5	7	7	tpst2	tyrosylprotein sulfotransferase 2	Cellular movement, fertility
Str.11990.1.S1_at	NP_996846.1	Testis-A	13	9	10	11	rarres1	retinoic acid receptor responder (tazarotene induced) 1	Cellular movement
Str.5014.1.S1_at	NP_001135417.1	Testis-B	9	12	12	23	acta2	actin, alpha 2, smooth muscle, aorta	Vascular development, muscle contraction
Str.21784.1.A1_s_at	NP_002072.2	Testis-B	13	11	17	22	gpc1	glypican 1	Vascular development, cellular movement
Str.21784.1.A2_at	NP_002072.2	Testis-B	12	12	16	19	gpc1	glypican 1	Vascular development, cellular movement
Str.20614.1.S1_at	NP_932766.1	Testis-B	17	5	12	19	aqp1	aquaporin 1 (Colton blood group)	Vascular development, permeability of blood vessel, molecular transport
Str.22031.1.S1_at	NP_001877.1	Testis-B	22	18	21	21	cryba4	crystallin, beta A4	
Str.38708.1.A1_at	NP_001138378.1	Testis-B	19	14	12	20	(clec4m)	C-type lectin domain family 4, member M	Cell-to-cell signaling and interaction
Str.28004.1.A1_at	NP_000859.1	Testis-B	16	24	25	36	(htr2c)	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled	Cell-to-cell signaling and interaction
Str.16705.1.A1_at	NP_570844.1	Testis-B	26	30	41	65	(opa1)	optic atrophy 1 (autosomal dominant)	Mitochondrial organization, synthesis of pregnenolone
Str.33400.1.S1_at	NP_005723.2	Testis-B	22	25	21	21	(rad50)	RAD50 homolog (S. cerevisiae)	Cell death of embryonic stem cells, DNA repair
Str.28021.1.A1_at	NP_775101.1	Testis-B	17	17	22	19	slitrk4	SLIT and NTRK-like family, member 4	Formation of cellular protrusions, nervous system development
Str.21273.1.A1_at	NP_002513.2	Testis-B	70	36	53	47	nptx1	neuronal pentraxin I	Angiogenesis, cellular movement, nervous system development
Str.7898.1.S1_at	NP_002467.1	Testis-B	17	88	97	98	myl4	myosin, light chain 4, alkali; atrial, embryonic	Vascular development, muscle contraction
Str.38523.1.S1_at	NP_001002036.3	Testis-B	26	59	68	186	(astl)	astacin-like metallo-endopeptidase (M12 family)	Protein synthesis, fertility
Str.12662.1.S1_at	NP_689642.3	Testis-B	37	98	110	151	fgfbp3	fibroblast growth factor binding protein 3	Growth factor regulation, angiogenesis
Str.2374.2.A1_at	NP_001190.1	Testis-B	19	38	107	930	(bmp1)	bone morphogenetic protein 1	Collagen development, protein synthesis
Str.34508.1.S1_s_at	NP_001190.1	Testis-B	8	12	27	161	(bmp1)	bone morphogenetic protein 1	Collagen development, protein synthesis
Str.24477.2.S1_at	NM_001265587.1	Testis-B	222	302	314	289	rflcii (insl3)	insulin-like 3 (Leydig cell)	Testis development, fertility