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Exploring Sexual Differentiation in the Tammar Wallaby Using Transcriptomics

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Exploring Sexual Differentiation in the Tammar Wallaby Using Transcriptomics

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ABSTRACT

Sex determination and sexual differentiation are some of the most well studied topics in human development. Sex determination is the point where the undifferentiated gonad becomes committed to the ovarian or testicular pathway. This event can be triggered genetically or environmentally. Sexual differentiation occurs after sexual determination and involves the maturation of the gonad, which in turn, coordinates the corresponding physical and behavioral phenotypes. Disorders of sexual development (DSD) are among the most common congenital abnormalities seen in humans and are increasing at an alarming rate. Congenital DSDs can be caused by genetic, hormonal, and/or environmental stimuli. Increased exposure to environmental endocrine disrupters (EEDs) especially those that affect estrogen signaling have been shown to cause DSDs. In addition to the mouse, the tammar wallaby, *Macropus eugenii*, has become a novel model for studying gonadal differentiation in mammals. Unlike mice, sexual determination and differentiation occurs postpartum in the tammar. Furthermore, pouch young are easily accessible for surgical and hormonal manipulation. More is known about testicular differentiation than ovarian differentiation. Thus, there is a substantial need to create transcriptomes as a resource in discovering conserved and novel mechanisms in gonadal development. This thesis explores the creation and analyses of these transcriptomes using normal male and female developing gonads and estrogen induced ovarian development in male marsupial gonads. It will also show that the tammar wallaby is a viable model for studying gonadal differentiation and the effects of estrogen in sexual development in mammals.

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LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
AMH	Anti-Müllerian hormone
DES	Diethylstilbestrol
DHH	Desert hedgehog
DMRT1	Doublesex and mab-3 related transcription factor 1
DSD	Disorders of sexual development
EED	Environmental endocrine disrupters
ER	Estrogen receptor
ESD	Environmental sex determination
FOXL2	Forkhead box L2
GSD	Genetic sex determination
NR0B1	Nuclear receptor subfamily 0, group B, member 1
PDGFA	Platelet-derived growth factor alpha polypeptide
PTGDS	Prostaglandin D2 synthase
RSPO1	R-spondin 1
SOX9	Sry-type HMG box 9
SRY	Sex-determining region on the Y chromosome
TESCO	Testis-specific enhancer of SOX9
WNT4	Wingless-type MMTV integration site family member 4

*Note: Uppercase letters refer to genes in humans and lowercase letters refer to genes in mice e.g. Sry.

Introduction

Sex Determination and Sexual Differentiation

Sex determination and sexual differentiation are some of the best described aspects of human development. Sex determination is the event that triggers an undifferentiated gonad to follow either a male or female developmental pathway. The mechanisms for sex determination are highly variable among the vertebrates and can be either triggered genetically or by environmental conditions. Genetic sex determination (GSD) is when the undifferentiated gonad depends on intrinsic genetic elements, such as a gene or chromosome to determine its trajectory, where as environmental sex determination (ESD) depends on extrinsic environmental cues [1]. All mammals including humans have a genetic sex determining system, where a single gene on the Y chromosome triggers male development. Sexual differentiation occurs following the sex determination event and coordinates development of the mature gonad and the appropriate urogenital and behavioral phenotype. Disorders of sexual development (DSD) arise when there are problems associated with either the sex determination or sexual differentiation event and are the most common congenital conditions in humans. DSDs can be caused by an abnormal chromosomal complement, defective gonadal or anatomical development stemming from a combination of genetic, hormonal, and/or environmental origins [2,3,4,5]. These disorders range from Turner's syndrome and Klinefelter's syndrome to gonadal dysgenesis, cryptorchidism and hypospadias [2,4].

Increasing cases of DSD have been linked to our increasing exposure to environmental endocrine disrupters (EEDs). EEDs can be natural or synthetic chemicals that affect the endocrine system and can be detrimental to human health especially the process of sexual differentiation. Exposure of EEDs such as antiandrogens, estrogens and progestins particularly during pregnancy can

cause masculinization in females, feminization in males, decreased sperm count and cancer [6,7,8]. For example, diethylstilbestrol (DES), a synthetic estrogen, was used by millions of pregnant women during the 1940s to 1970s [6,7]. Exposure of DES to the fetus resulted in many forms of DSD ranging from undescended testes, hypospadias, decreased sperm count, vaginal hypoplasia, vaginal adenosis, infertility, and other reproductive issues. Additionally, exposure increased risk of testicular cancer and clear cell vaginal adenocarcinoma [6,7]. The damaging effects of EEDs are not only seen in humans but also in other mammals, fish, birds, reptiles, amphibians and crustaceans. Similar effects of DES and other EEDs in humans have been demonstrated in mice, rats, rabbits, goats and marsupials. There has been a rise of intersex fish and frogs, increased mortality rate and decreased reproductive capacity in fish, birds and alligators, and feminization of males and masculinization of females in birds and mollusks [8]. Thus, there is a great need to understand the impact these chemicals have on the molecular pathways regulating normal sexual development.

The Marsupial Model

Many studies have been done utilizing mouse models especially knockout mice to explore testicular and ovarian differentiation and the effects of estrogen on these systems. More recently marsupial mammals like the gray short-tailed opossum, *Monodelphis domestica*, and the tammar wallaby, *Macropus eugenii*, are becoming significant model species in the field of sexual development. Similar to humans and other eutherian mammals, the tammar wallaby has a XY genetic sex determining mechanism. The tammar diverged from humans around 130 to 148 million years ago [9,10]. Unlike the tammar wallaby, sexual differentiation in humans and other eutherian mammals occurs *in utero*. However, the tammar wallaby gives birth to altricial young where sexual differentiation and development occurs postnatally. Easy access to pouch young

allows for hormonal and surgical manipulation without the interference of the placenta [10,11,12,13]. Many laboratories that use mice to study sexual differentiation have a difficult time with hormonal administration due to placental metabolism of hormones. Moreover, sexual differentiation occurs at a more rapid rate *in utero* in comparison to the tammar wallaby, and germ cell and somatic cell development occurs simultaneously [1]. Even though mice reproduce more rapidly, difficult and time consuming intrauterine surgery must occur to obtain access to the fetuses. The tammar wallaby's unique ability to give birth to altricial young allows for easy sex reversal using estradiol and hormonal and surgical manipulation during significant developmental time points [14]. All these factors make it advantageous to model DSD using the tammar wallaby.

The majority of the key genes involved in sex determination and sexual differentiation in mice have been investigated in marsupials [15,16,17]. While the current data suggests that there are some differences in gene expression, the majority of genes behave in an identical manner in establishing mouse and marsupial gonadal development. In both eutherian and marsupial mammals, sex is determined by the Y-linked *SRY* gene. The presence of *SRY* triggers and upregulates the expression of *SOX9*, Sry-type HMG box 9, in both eutherians and marsupials. This in turn triggers the development of the testis and initiates the expression of *AMH*, Anti-Müllerian hormone. Compared to eutherians, the marsupial X and Y chromosomes are relatively small. The tammar X is homologous to the long arm and pericentric region of humans while the tammar Y shares just four genes with the human Y [9,10].

Ovarian Development

Early research into sexual development including Alfred Jost's pioneer experiment in rabbits in the 1930s suggested that ovarian development was the default sexual developmental pathway. In

other words, if the gonad did not receive a dominant signal from the Y-chromosome (*SRY*) to develop a testis, it would passively follow the developmental pathway of the ovary and subsequently trigger female sexual development. This led to the Z-factor theory, which hypothesizes that a single gene in the female gonad prevents testicular differentiation and activates ovarian differentiation. However, recent research suggests otherwise and reveals the presence of several pathways working in parallel pathways to coordinate female development. R-spondin 1 (*RSPO1*) is part of the protein R-spondin family, which have been linked to various developmental and oncogenic pathways. All R-spondins have an N-terminal signal peptide, two furin repeats, one thrombospondin type 1 domain and a C-terminal domain with a nuclear localization signal [18]. *RSPO1* is particularly important in ovarian differentiation as it represses testicular development by activating the canonical WNT/β-catenin signaling pathway [19]. In humans, *RSPO1* is located on chromosome 1p34.3 and mutations cause female-to-male sex reversal, variations of testicular DSD, lack of Müllerian ducts, and palmoplantar hyperkeratosis with squamous cell carcinoma of the skin [2,3,4,18,20,21]. Studies of *Rspo1* KO in XX mice exhibit partial female-to-male sex reversal with the formation of ovotestis and the presence of both Wolffian and Müllerian ducts [18,19,22]. The *Rspo1* KO gonad showed increased expression of the testis markers *Sox9*, *Amh*, *Fgf9*, and *Ptgds* reiterating its role in suppressing male development [22]. The presence of the Wolffian and Müllerian ducts in *Rspo1* knockouts suggests that *RSPO1* is not involved in the development of the mesonephros, which is a temporary kidney during embryogenesis and later becomes the Wolffian and Müllerian ducts. In addition, it indicates the failure of Sertoli cell development since *Amh* is not fully produced to regress the Müllerian ducts. Knockouts for both *Rspo1* and *Wnt4* resulted in the inactivation of the WNT/β-catenin signaling pathway and the activation of β-catenin rescued *Rspo1* KO mice

from phenotype mentioned above [18,19]. Deactivating any part of the WNT/β-catenin signaling pathway results in female-to-male sex reversal and vice versa when activating the pathway in males. During early development, *RSPO1* expression levels were both low in male and female wallabies until day 4 postpartum where levels increased in females and decreased in males. This follows with ovarian differentiation occurring in the tammar on day 4. Results in tammar wallaby were consistent with those in mice and humans except that *RSPO1* could be acting downstream of *WNT4* and another ovarian gene *FOXL2* [16].

Forkhead box L2 (*FOXL2*) is a forkhead/winged helix nuclear transcription factor that was first discovered by studies on sex reversed XX goats with polled-intersex syndrome. This led to *FOXL2* being mapped to human chromosome 3q23 and associated with Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome (BPES), premature ovarian failure (POF) and XX sterility. Unlike in goats, *FOXL2* mutations do not cause sex reversal in humans, but 1-3% of all women are affected by POF [23,24,25]. *FOXL2* is involved in primordial follicle development and maintenance suggesting its relevance in ovarian morphogenesis and homeostasis during female reproductive life [3,23,26,27,28,29]. *Foxl2* mutations in XY mice have no effect on testis differentiation, but overexpression inhibits testis formation [24,29]; however, any sort of *Foxl2* mutation in XX mice causes partial female to male sex reversal, granulosa cells to convert into sertoli-like cells, and the expression of genes involved in testicular development [24,27,28,29,30,31]. Double loss of function mutations of *Foxl2* with *Wnt4* or *Rspo1* still resulted in partial sex reversal, but increased the masculinization of ovaries and boosted expression of testis-specific genes including *Sox9* [24,26]. The interaction between estrogen receptor 1 and *FOXL2* in mammals, including the tammar wallaby, represses the expression of *SOX9* through binding to the testis-specific enhancer of *SOX9*, TESCO [1,24,31]. Along with

mice, female wallabies exhibit higher levels of *FOXL2* than males suggesting its conserved role in ovarian differentiation. During day 5 to 10 postpartum, *FOXL2* was upregulated during ovarian development in female wallabies. Estrogen exposure in male tammar wallabies resulted in sex reversal, decreased expression of testis-specific genes *SRY* and *AMH*, lack of nuclear *SOX9*, and increased expression of ovarian genes *FOXL2* and *WNT4* [16].

Wingless-type MMTV integration site family member 4 (*WNT4*) is part of a family of signaling molecules that are involved in embryogenesis. In humans, *WNT4* is located on chromosome 1p35 and duplication in males disrupts testis differentiation and causes sex reversal, hypospadias, the presence of Müllerian ducts, and ambiguous external genitalia. Loss of function mutations in females induces Müllerian aplasia, hyperandrogenism, and SERKAL syndrome, which is described as female to male sex reversal, gonadal dysgenesis, and developmental problems in the kidneys, lungs and heart [2,3,17,31]. In conjunction with *RSPO1* and β-catenin, *WNT4* represses testicular differentiation and promotes ovarian differentiation by activating the canonical WNT/β-catenin signaling pathway and initiates *FOXL2* transcription. This pathway suppresses testicular differentiation by preventing the expression of *SOX9* and in turn *AMH*. *WNT4* plays significant roles in ovarian steroidogenesis, oogenesis, and the development of the urogenital system especially the mesonephros and Müllerian ducts [17,25,32,33,34]. Numerous studies have observed partial female to male sex reversal in *Wnt4* knockout mice indicating that there are more genes involved in promoting ovarian differentiation and blocking testicular differentiation. Lack of Müllerian ducts was also observed reiterating *Wnt4*'s importance in its development. Double knockout *Foxl2* and *Wnt4* mice have increased expression of *Sox9*, *Fgf9* and *Amh* and have a higher degree of sex reversal than just *Wnt4* KO mice [21,25,28,35,36]. *WNT4* is highly conserved between eutherian mammals and the tammar wallaby; the tammar

shares 93.2% amino acid homology with humans and has conserved roles in gonadal development. Consistent with the female tammar developmental timeline, *WNT4* expression is significantly upregulated after day 4 postpartum in comparison to males. XY gonads exposed to estrogen exhibited sex reversal, decreased expression of *SRY*, *AMH* and *FGF9*, lack of nuclear *SOX9* and increased expression of *FOXL2* and *WNT4* [16,37,38].

Estrogen Induced Ovarian Development

Several different mouse models have been used to study the effects of estrogen on gonadal development. Aromatase knockout (ArKO) mice are deficient for the gene *CYP19* that encodes for aromatase, an enzyme that converts androgens to estrogens. αβERKO mice lack both estrogen receptor 1 (ER1 or ERα) and estrogen receptor 2 (ER2 or ERβ) which mediate estrogen signaling. Both are required for normal ovarian function and are highly conserved among vertebrates [11]. Female (XX) mice from both models exhibit an increase in circulating testosterone, increased expression of genes involved in testicular differentiation, presence of Sertoli and Leydig cells, and transdifferentiation of the ovary [11,39,40,41]. ArKO mice still have intact ERs and can be rescued with exogenous estrogen. Estrogen exposure in non-mammalian vertebrates like birds, reptiles, amphibians and fish that follow environmental sex determination causes male to female sex reversal. This indicates that estrogen is necessary and sufficient to cause ovarian development in these vertebrates [11,42]. Studies on eutherian mammals reveal estrogen's importance in ovarian development and differentiation especially during folliculogenesis [40,41].

As previously mentioned, exogenous estrogen exposure to the marsupial XY gonad results in complete sex reversal and ovarian development. Easy accessibility to the tammar pouch young and gonadal differentiation occurring right after birth allows for exogenous estrogen treatment. A

study published in 2010 by Pask *et al.* explored the effects of estrogen induced ovarian development in the tammar wallaby. Undifferentiated XY tammar gonads were cultured in the presence of estrogen. The expression levels of 'female' genes *WNT4*, *FOXL2*, *RSPO1*, and *FST* and 'male' genes *SRY*, *SOX9*, *AMH*, and *FGF9* were evaluated in XX gonads and treated and untreated XY gonads from day 25 prenatal to day 10 postpartum. All these genes involved in gonadal development are conserved between eutherian and marsupial mammals. During normal ovarian differentiation, XX gonads had high expression levels of 'female' genes around day 4 postpartum and low expression levels of 'male' genes. During normal testicular differentiation, XY gonads had high expression levels of 'male' genes around day 1 postpartum and low expression levels of 'female' genes. These high expression time points represent when ovarian differentiation and testicular differentiation, respectively, occurs during tammar gonadal development.

As expected, expression levels of genes involved in ovarian differentiation especially *WNT4* and *FOXL2* were upregulated and testicular genes were downregulated in the estrogen treated XY gonads. However, *SOX9* expression levels remained the same in both the untreated and treated estrogen XY gonads. Immunohistochemistry revealed ovarian-like structures such as cortex and medulla in the treated gonads and lack of testicular cord formation. In addition, *SOX9* was found in the cytoplasmic regions of gonadal cells unlike the control gonads which had nuclear *SOX9*. This nuclear exclusion of *SOX9* in the estrogen cultured gonads prevents the upregulation of *SOX9* and *AMH* leading to ovarian development. As previously mentioned, *FOXL2* along with estrogen receptors blocks the expression of *SOX9* and *AMH* to induce ovarian differentiation and repress testicular differentiation. These findings reveal a potential mechanism by which estrogen could prevent testicular development and a conserved role of estrogen in vertebrates in thwarting

SOX9 nuclear translocation in the ovary. The absence of estrogen results in nuclear *SOX9* and therefore the initiation of testicular development. However, the precise mechanism by which estrogen prevents nuclear translocation of *SOX9* is still unclear [16]. This study offers some insight into the effects of environmental estrogen exposure on gonadal development and differentiation in humans.

The tammar wallaby can be used a novel model to understand ovarian development, which is still poorly understood compared to testicular development. These marsupials can also be used to define the effects of estrogen on the development of the gonad. Recently, the genome has been sequenced enhancing the utility of the tammar as a genetic model by Renfree *et al*. 2010. Due to the increase in disorders of sexual development in humans, there is a considerable need to understand gonadal development. Thus, we performed transcriptomics of the normal developing gonads in both males and females as well as for gonads undergoing estrogen induced sex reversal. This transcriptome resource will be especially important in identifying novel and conserved mechanisms of sexual development. This thesis describes the creation, validation and analyses of these libraries. It also shows that the tammar wallaby is a viable alternative model for studying gonadal differentiation and the effects of estrogen in sexual development in mammals.

Table 1. A Selection of Key Genes with Significantly Affected Expression in Sexual Development in Mammals

Gene	Role(s)	Human diseases/conditions	References
AMH	regression of the Müllerian ducts; supports testicular differentiation; development of epididymis, vas deferens, and seminal vesicle	Persistent Müllerian duct syndrome, gonadal dysgenesis, ovotesticular DSD	2,14,15,35,43
FOXL2	primordial follicle development and ovarian homeostasis; represses testicular differentiation	Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome and premature ovarian failure, XX sterility	3,23,26,27,28
RSPO1	activation of WNT/β-catenin signaling pathway in ovarian differentiation; represses testicular differentiation	sex reversal, testicular DSD, palmoplantar hyperkeratosis with squamous cell carcinoma	2,3,4,18,19,20, 21
SOX9	Sertoli cell development; promotes testicular differentiation	Campomelic dysplasia, sex reversal, gonadal dysgenesis	3,21,25,44,45
SRY	initiates and supports testicular differentiation	sex reversal, gonadal dysgenesis	2,3,5,20,21,31, 46,47
WNT4	activation of WNT/β-catenin signaling pathway in ovarian differentiation; represses testicular differentiation; oogenesis; ovarian steroidogenesis; urogenital development	SERKAL syndrome, sex reversal, testicular DSD, Müllerian aplasia, hyperandrogenism	2,3,17,25,31,32, 33,34

Materials and Methods

Animals

Tammar wallabies (*Macropus eugenii*) from Kangaroo Island of South Australia were maintained in our breeding colony in grassy outdoor enclosures. Fetal sex was determined through PCR by *SRY*; pouch young sex was determined through the presence of scrotal bulges or mammary primordial [48]. Male and female fetal tammar wallaby gonads were dissected and

snap frozen in liquid nitrogen from fetal day 24, 25, and 26 embryos and from days 0, 1, 2, 4, and 8 postpartum pouch young. Husbandry, handling and experiments were in accordance with the National Health and Medical Research Council of Australia (2004) guidelines and approved by the University of Melbourne Animal Experimentation Ethics Committees.

Treatment of Estradiol in Culture

The gonads were cultured at the University of Melbourne as described as follows. Pairs of gonadal ridges were cultured from day 25 of gestation from XY-male tammar wallaby fetuses. The tammar wallaby gonadal ridge first develops on day 21 of the 26.5-day gestation period [11,16]. Testicular differentiation occurs around day 2 postpartum, and ovarian differentiation occurs around day 8 postpartum [11,16]. Pairs of gonads were isolated and placed, one into control media and the other into estrogen media for five days. The control media contained Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) and 50 mg/ml ampicillin. The estrogen media used the control media with the addition of estradiol benzoate (to a final concentration of 100 ng/ml). Estradiol benzoate was diluted in 100% ethanol (without estradiol) was added to control cultures. After five days, the gonads were snap-frozen and stored at -80°C until needed for RNA extraction.

RNA Extraction

Total RNA was extracted from snap frozen tammar wallaby gonads using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Cat. no. RTN10) as per manufacturer's instructions. Total RNA was DNase treated using the TURBO DNA-free™ Kit (Ambion Cat. no. AM1907) as per manufacturer's instructions. Total RNA was stored at -80°C until needed.

Concentrating RNA

The total volume of each RNA sample for each developmental time point and each sex was pooled and concentrated into 25 µl using the RiboMinus™ Concentration Module (Invitrogen Cat. no. K1550-05) as per manufacturer's instructions. Concentrated total RNA was stored at -80°C until needed.

Removal of Ribosomal RNA

Ribosomal RNA was removed from 24 µl of concentrated total RNA using the Ribo-Zero™ Magnetic Kit (Human/Mouse/Rat; Epicentre Cat. no. MRZH11124) as per manufacturer's instructions. While treating the samples with rRNA Removal Solution, 0.5 µl of 1:10 dilution of ERCC RNA Spike-In Control Mixes (Ambion Cat. no. 4456740, 4456739) was added to each sample. After rRNA removal, the samples were purified using the RNeasy MinElute Cleanup Kit (Qiagen Cat. no. 74204) as per manufacturer's instructions. The concentration of the sample was determined using the NanoDrop 1000 Spectrophotometer. Total RNA was stored at -80°C until needed.

Table 2. List of Samples Used for RNA Sequencing Library

Sample	Number of gonads extracted	ERCC RNA Spike-In Control Mix	Concentration (ng/µl)	Amount (µl)	Total RNA (ng)
d24♀	6	1	10.4	9	93.6
d25♀	7	2	13.2	9	118.8
d26♀	8	1	10.8	9	97.2
D0♀	7	1	16.7	9	150.3
D4♀	16	2	10.8	9	97.2
D8♀	6	2	8.1	9	72.9

d24♂	7	1	27.5	11	302.5
d25♂	9	1	5.5	11	60.5
d26♂	7	1	12.8	11	140.8
D0♂	7	1	12.2	11	134.2
D1♂	6	2	12.1	10	121
D2♂	14	1 and 2	13.7	10	137
D4♂	6	2	5.9	10	59
control d25♂ untreated	6	1	33.19	9	298.7
E_2 d25♂ treated	6	2	5.63	9	50.7

Note: Samples with a lowercase 'd' indicate developmental time points during gestation. Samples with an uppercase 'D' indicate developmental time points postpartum.

Library preparation and sequencing

RNA sequencing and preparation was outsourced to PerkinElmer in Branford, Connecticut. The Illumina HiSeq 2000 sequencing system was used with HiSeq Flow Cell v3, TruSeq SBS Kit v3, TruSeq PE Cluster Kit v3-cBot-HS, and TruSeq Multiplex Sequencing Primer Box as per manufacturer's instructions.

Data Analysis

FastQC was used to check the quality of the raw high throughput mRNA sequencing reads obtained from PerkinElmer as per manufacturer's instructions.

The Bowtie suite published by Trapnell *et al.* 2012 was used to annotate the transcriptomes, discover novel genes and to test for differential gene expression. The reads were adapter trimmed and quality filtered using TrimGalore. Reads that were smaller than 20 bp were discarded. The samples were then mapped to rRNA sequences using Bowtie2, and the remaining reads were

then mapped to an unpublished unannotated tammar assembly as a reference genome using TopHat2. The aligned reads from TopHat2 were then independently assembled to identify putative genes using Cufflinks using the option for stranded reads. Cuffmerge was used to merge the independent assemblies. Genes were identified using BLASTX to search RefSeq, with the match with the lowest e-value being selected as the identity of the gene. Differentially expressed genes and transcripts were identified utilizing Cuffdiff. Lastly, CummbeRbund provided visualized expression data supplied by Cuffdiff [49].

In addition to the Bowtie suite, BLASTX aided in the search for protein coding genes and Blast2GO annotated the results as per manufacturer's instructions.

Results

Table 3. Summary of Sequencing Results

Sample	Yield (GB)	# of Reads (million)	% Reads Q >= 30	Mean Q Score
d24♀	13.39	133.9	84.29	33.74
d25♀	13.14	131.44	84.44	33.79
d26♀	13.88	138.78	84.4	33.77
D0♀	12.51	125.14	84.57	33.8
D4♀	15.04	150.38	85.14	34.01
D8♀	11.69	116.93	84.79	33.9
d24♂	11.63	116.28	82.32	33.14
d25♂	14.86	148.59	82.59	33.24
d26♂	14.53	145.3	82.46	33.19
D0♂	14.46	144.62	84.82	33.86
D1♂	12.18	121.8	84.56	33.8
D2♂	13.14	131.43	84.69	33.83
D4♂	12.24	122.41	82.29	33.15
control d25♂ untreated	14.66	146.64	81.59	32.93
E_2 d25♂ treated	12.9	128.95	82.16	33.1

This table summarizes the RNA sequencing results obtained from PerkinElmer using the Illumina HiSeq 2000 sequencing system. The number of reads for each sample ranged from 116.28 to 146.64 million base pairs. The percentage of those reads with Phred quality score or Q score of 30 or higher ranged from 81.59 to 85.14. This Q score is used to measure base calling accuracy. The mean Q score given to each base ranged from 32.93 to 34.01. This indicates that base call accuracy of this data set is between 99.9 to 99.99% and the probability of incorrect base call is 1 in 1,000 to 1 in 10,000. This table confirms that this transcriptome data set is very accurate and should be used for data analysis.

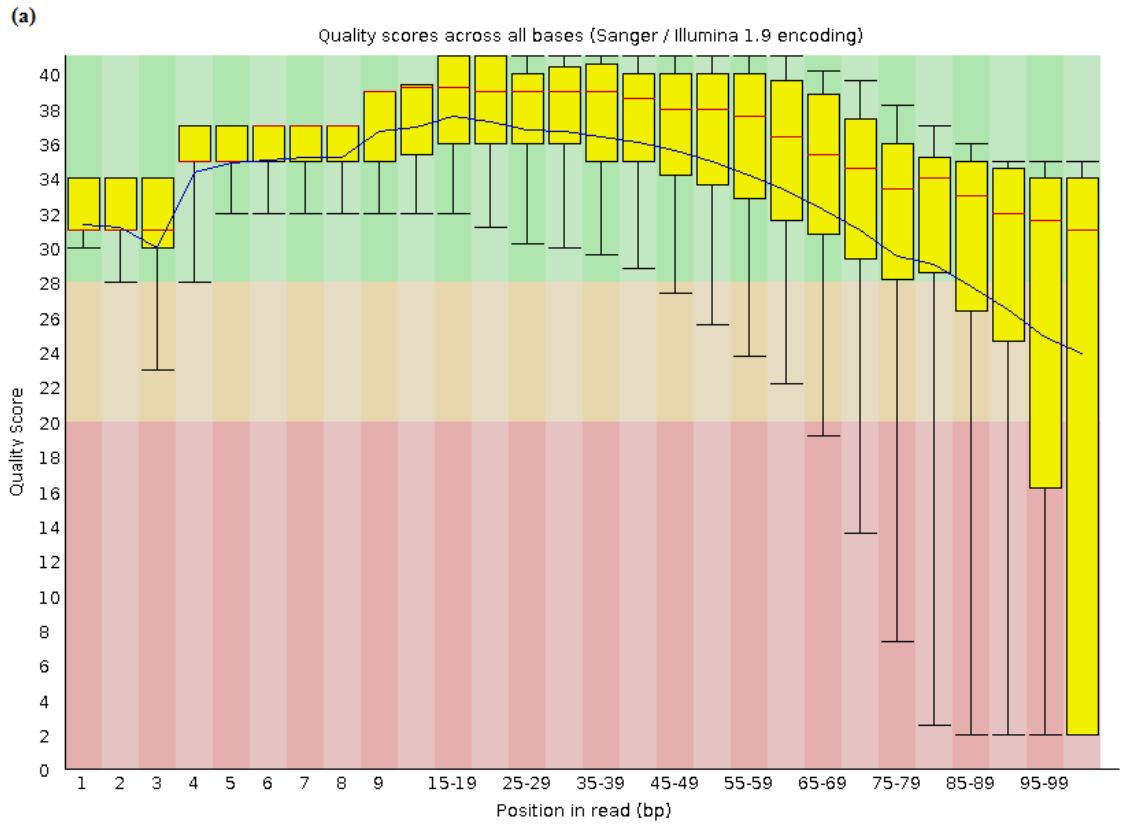
FastQC

Table 4. FastQC Report Basic Statistics

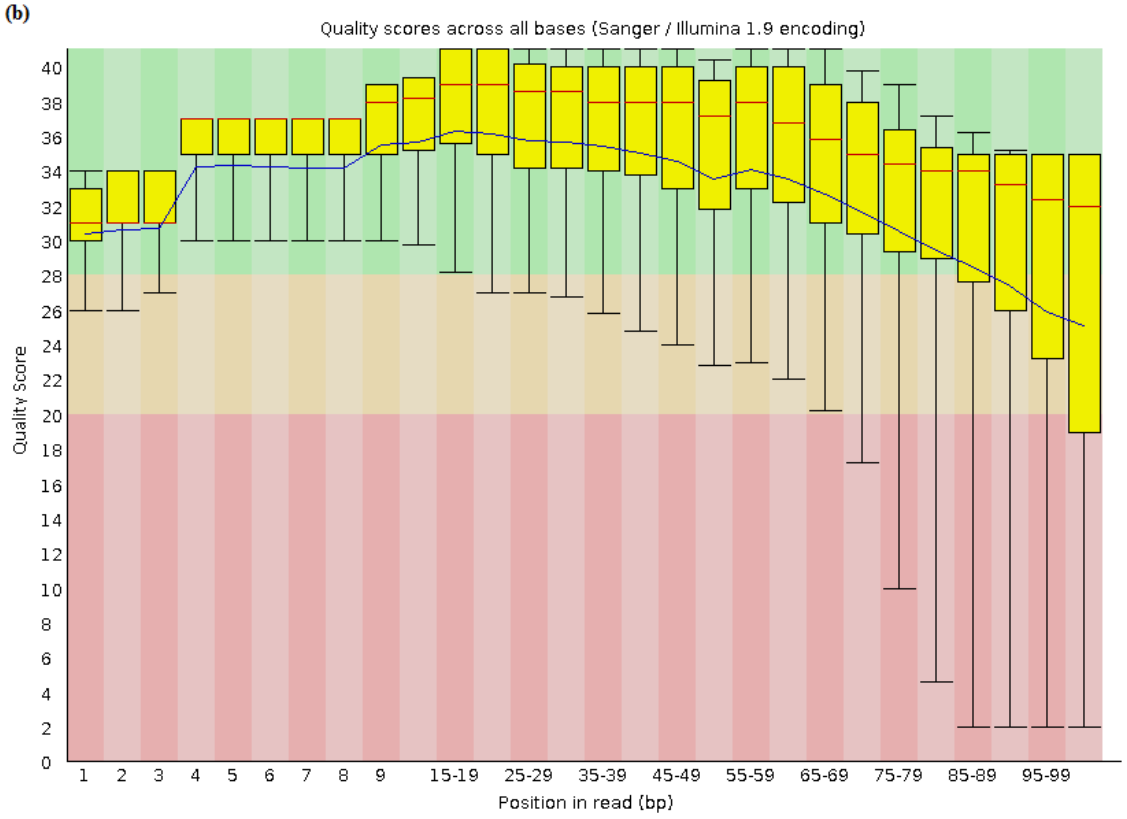
Sample	Total Sequences	% Mean GC Content
d24♀	66950752	45
d25♀	65721535	45
d26♀	69390000	45
D0♀	62548792	45
D4♀	75191503	45
D8♀	58464804	45
d24♂	58137974	45
d25♂	74293998	45
d26♂	72648754	45
D0♂	72310073	46
D1♂	60898922	45
D2♂	65715502	46
D4♂	61203170	45
control d25♂ untreated	73321177	46
E_2 d25♂ treated	64474138	46

FastQC was used to check the quality of the raw high throughput mRNA sequencing reads obtained from PerkinElmer. All sequences were not filtered and sequence length was 100 base pairs. For every sample, the basic statistic parameters were set by FastQC. Total sequences for

each sample ranged from 58 to 75 million. The mean percentage of GC content of the sequences ranges from 45 to 46. High GC content indicates coding regions in these sequences.



(b)


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Quality Score
0
2
4
6
8
10
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24
26
28
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40
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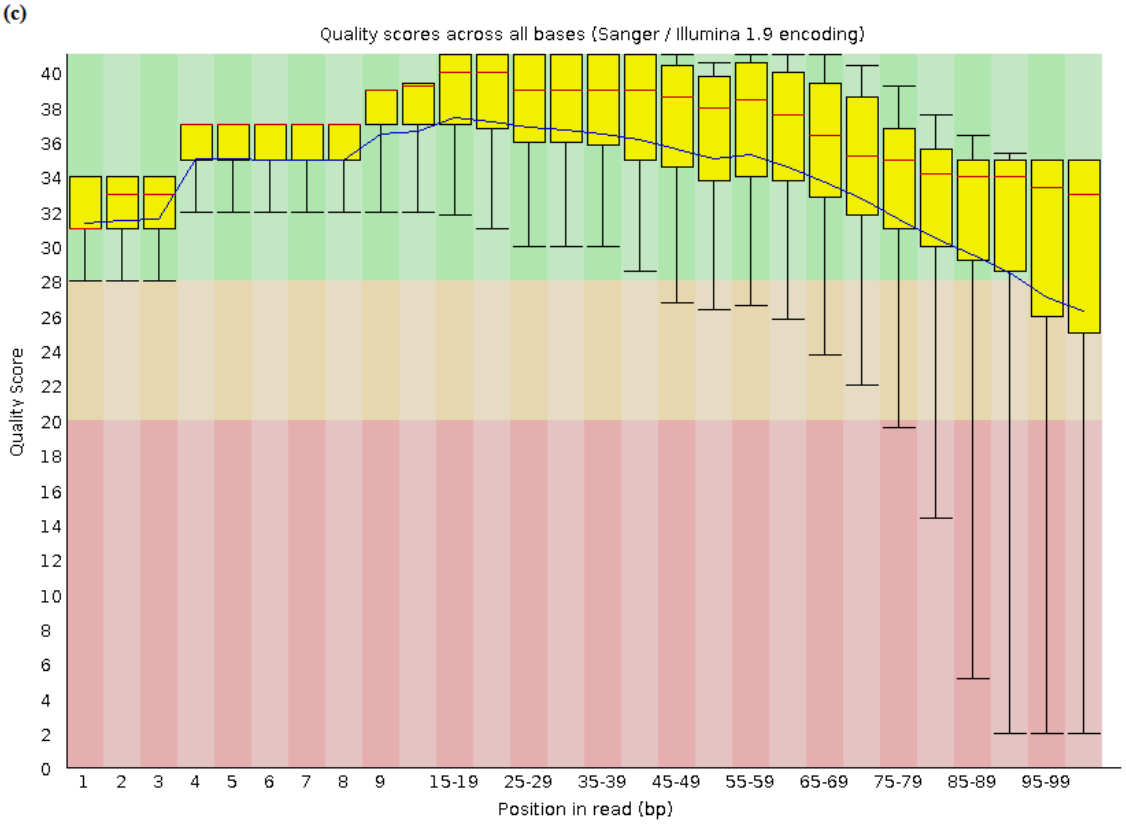
8
9
15-19
25-29
35-39
45-49
55-59
65-69
75-79
85-89
95-99
Position in read (bp)

(c)


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Quality Score
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
1
2
3
4
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85-89
95-99
Position in read (bp)

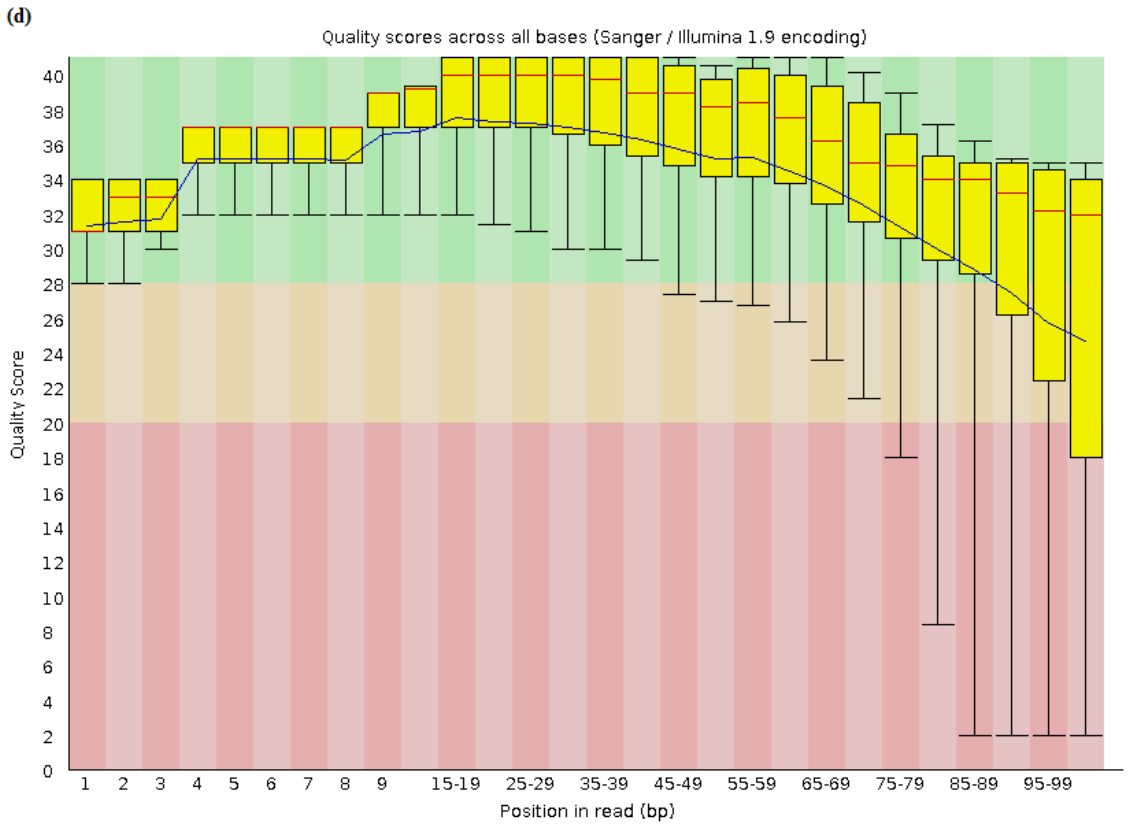
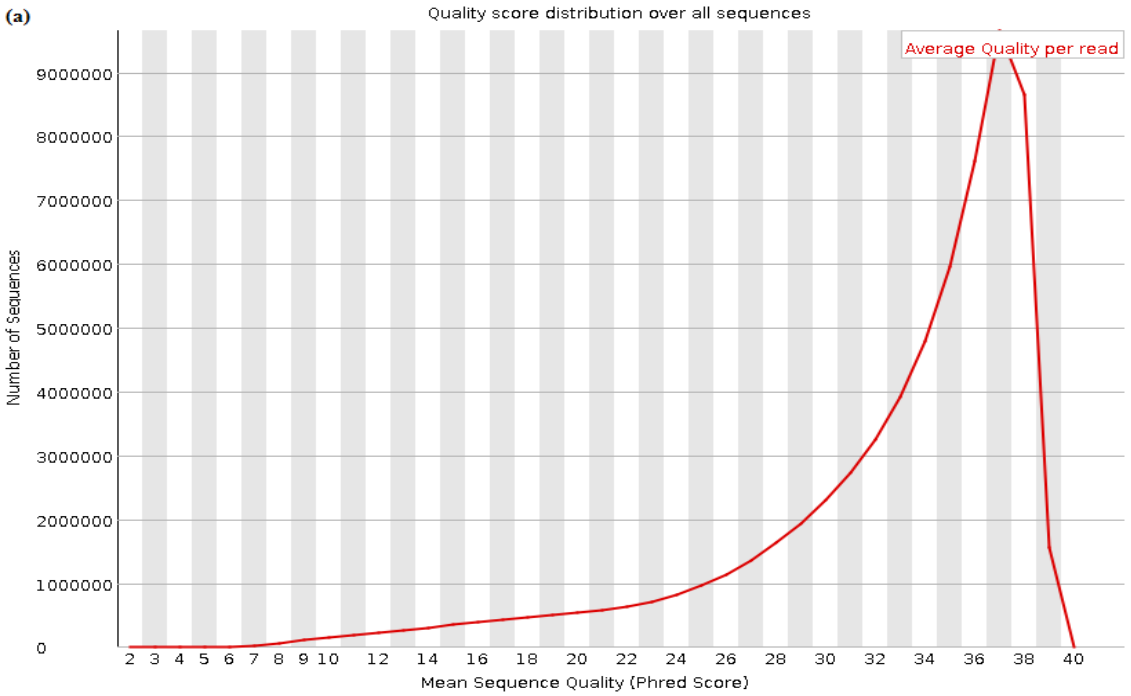
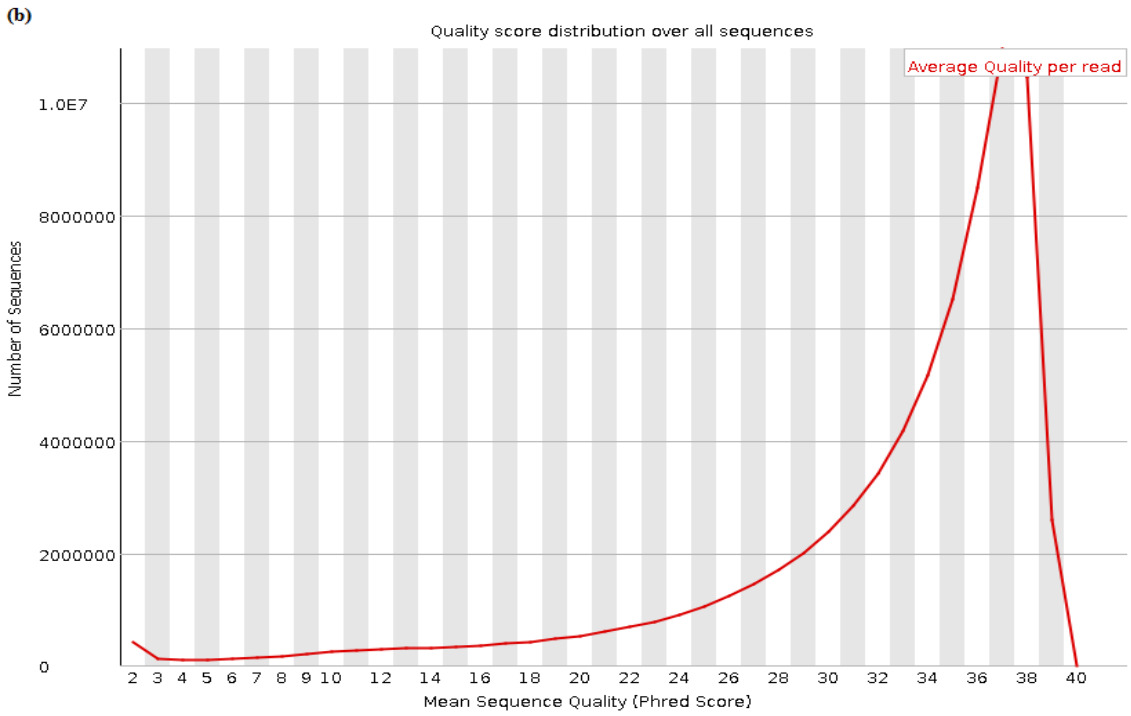


Figure 1. Overall Representation of FastQC Report Quality Scores Across All Bases: (a) **E_2 d25♂ treated,** (b) **control d25♂ untreated,** (c) **D0♂,** (d) **D0♀**

Part (a) of this figure shows a failed per base sequence quality graph according to FastQC. However, the lack of quality in the first few base pairs of the sequence is normal, and there is not a huge difference between (a) and all the other parts of the figure. In addition, the low quality base pairs towards the end of the sequence are expected because of the sequencing chemistry. Parts (b), (c) and (d) all passed according to FastQC standards. Regardless of FastQC qualifications of passed and failed data sets, all of the scores are still very good and are within normal parameters. The difference between passed and failed per base sequence quality graphs is very slight and is discounted. The blue line in all four figures corresponds to the mean quality

score (Q score). These are representative QC reports from control untreated, E_2 treated, and normal male and female gonad samples. All samples showed similar quality scores as the passed and failed figures shown here.





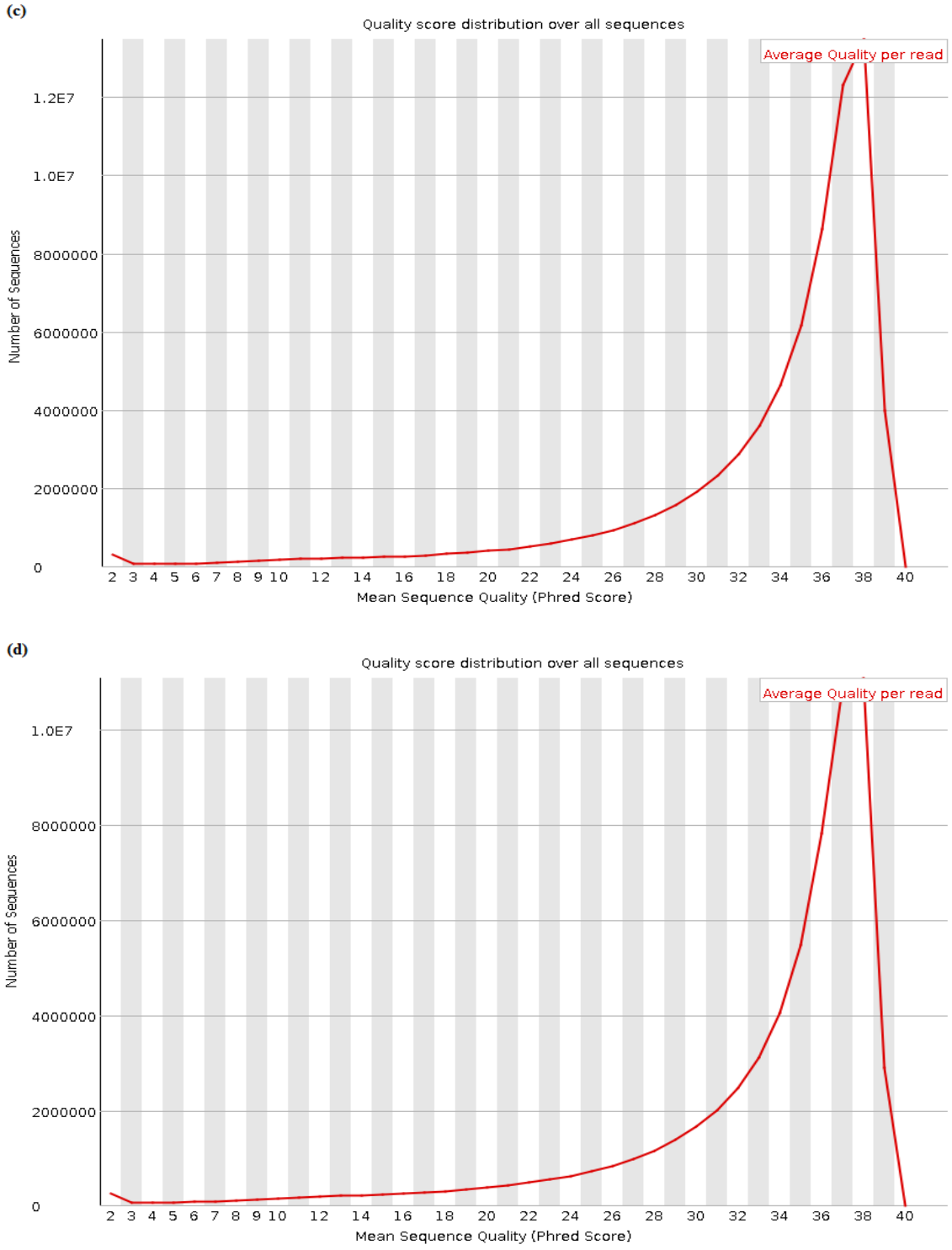


Figure 2. Overall Representation of FastQC Report Quality Score Distribution Across All Sequences**:** (a) **E_2 d25♂ treated,** (b) **control d25♂ untreated,** (c) **D0♂,** (d) **D0♀**

This figure shows a great representation of the mean sequence quality of each sample. Every sample passed FastQC's per sequence quality check with average scores around 37 to 38. As previously mentioned, Q scores are used to measure base calling accuracy. FastQC indicates that base call accuracy of all samples are between 99.9 to 99.99% and the probability of incorrect base call is 1 in 1,000 to 1 in 10,000.

From all these FastQC reports, these transcriptomes are of considerable high quality and accuracy to be utilized for discovering differentially expressed genes and transcripts.

Gene Expression Analyses

Table 5. Potential Gene Targets of Estrogen in Ovarian Development

Gene	**D4 Male (average FPKM)**	**Control Male (average FPKM)**	**E_2 Treated Male (average FPKM)**	**Upregulation Fold by Estrogen**
DIO1	–	34.6153	122.152	3.529
ABCA5	–	17.9194	51.80455	2.891
SDSL	–	50.3308	129.641	2.576
NPHS2	8.50532	14.1093	36.0758	2.557
GREB1	–	12.6886	27.8067	2.191
IFIT3	4.18097	38.064	72.03505	1.892
CFH	–	69.3539	131.191	1.892

Differential expression is quantified by fragments per kilobase of transcript per million mapped fragments (FPKM). FPKM is used by Cufflinks and Cuffdiff to standardize expression levels of each transcript. This takes into consideration the length and abundance of each transcript. Then FPKM values can be utilized to compare expression levels of different genes and transcripts [49]. Table 5 lists genes from highest to lowest upregulation fold by estrogen treatment. This upregulation fold value was obtained by dividing the E_2 treated male FPKM values by the control male FPKM values. These genes are induced by exogenous estrogen treatment and could

be potential targets of estrogen during ovarian differentiation. This table provides a glimpse into novel genes involved in ovarian differentiation. *DIO1*, *ABCA5*, and *SDSL* show the highest upregulation in differential expression. These genes were further examined in the mouse GenitoUrinary Development Molecular Anatomy Project (GUDMAP), and show upregulation during ovarian development in mice in comparison to testes at the same stage. Thus, these genes are potentially highly conserved mammalian ovarian development factors and are likely to be estrogen responsive.

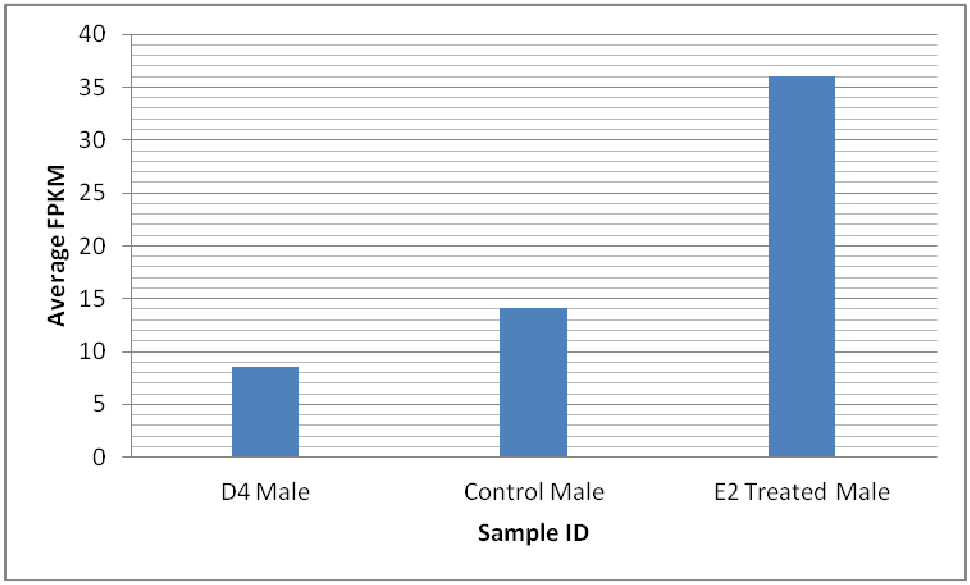


Figure 3. Differential Expression Levels of *NPHS2* gene

Figure 3 shows an example of significantly changed differential expression of the *NPHS2* gene. The expression level at day 4 postpartum in males is almost equivalent to male gonads cultured in control media but considerably less than expression levels of male gonads cultured in estrogen. This figure shows *NPHS2* as a potential target of estrogen during ovarian

differentiation. *NPHS2* expression in E_2 treated males is induced by exogenous estrogen treatment and not by culture.

Table 6. Top 10 Changed Gene Expression Levels from Male vs. Female at Each Key Developmental Stage

Blue indicates genes increased in expression in males while pink shows those increased in females.

Pre-gonadal Differentiation	**During Gonadal Differentiation**	**Post-gonadal Differentiation**
AMH	*ALAS2*	*ALAS2*
ALAS2	*ALDH1A1*	*ALDH1A1*
CALB1	*CLU*	*CLU*
CGA	*GSTM3*	*GSTM3*
CLU	*HBA2*	*HSD3B1*
CPA1	*NDNL2*	*NDNL2*
HBA2	*PDZK1IP1*	*PTGDS*
NDNL2	*RNASEH1*	*RNASEH1*
SERPINA3	*SERPINA3*	*SERPINA3*
TMEM52B	*TNNC1*	*TNNC1*

Note: Pre-gonadal differentiation refers to day 24 of gestation to day 2 postpartum in females and day 24 of gestation to day 0 postpartum in males. During gonadal differentiation refers to day 4 postpartum in females and day 1 to day 2 postpartum in males. Post-gonadal differentiation refers to day 8 postpartum in females and day 4 postpartum in males.

This table shows the comparisons male vs. female for each gene listed. Genes shaded in light blue indicate higher differential expression in males than females. Genes shaded in light pink indicate higher differential expression in females than males. For example, in comparison to males, the gene *ALAS2* is downregulated in females during all stages of sexual differentiation. The genes *AMH* and *PTGDS* are especially important due to their roles in testicular

differentiation. *AMH* is significantly upregulated in males during pre-gonadal differentiation. *PTGDS* is significantly upregulated in males during post-gonadal differentiation.

Table 7. Known Testicular and Ovarian Genes with Significant Differential Expression at Each Stage of Gonadal Differentiation

	♂ pre	♂ during	♂ post	♀ pre	♀ during	♀ post
AMH	↑	↑	↓	↑		
DHH		↑				
DMRT1	↑	↑	↑			
FOXL2					↑	
NR0B1				↑		
PTGDS	↑	↑	↑	↑		↑
SOX9	↑	↑	↓			

Note: Pre-gonadal differentiation refers to day 24 of gestation to day 2 postpartum in females and day 24 of gestation to day 0 postpartum in males. During gonadal differentiation refers to day 4 postpartum in females and day 1 to day 2 postpartum in males. Post-gonadal differentiation refers to day 8 postpartum in females and day 4 postpartum in males.

Table 7 indicates whether each gene is upregulated or downregulated during each stage of gonadal differentiation with an up arrow or a down arrow respectively. *AMH*, *DHH*, *DMRT1*, *PTGDS*, and *SOX9* all show significant gene expression in males during gonadal differentiation. This reaffirms their roles in testicular differentiation and validates our transcriptome analyses. *FOXL2* shows significant gene expression in females during gonadal differentiation. This confirms its role in ovarian differentiation, which is consistent with previous studies in the tammar, mouse and human, again confirming our transcriptome analyses.

The genes in the following four figures show very little expression in female wallabies. No ovaries were collected for day 1 and day 2 postpartum. No testes were collected for day 8 postpartum. There are notable differences in expression levels between females and males.

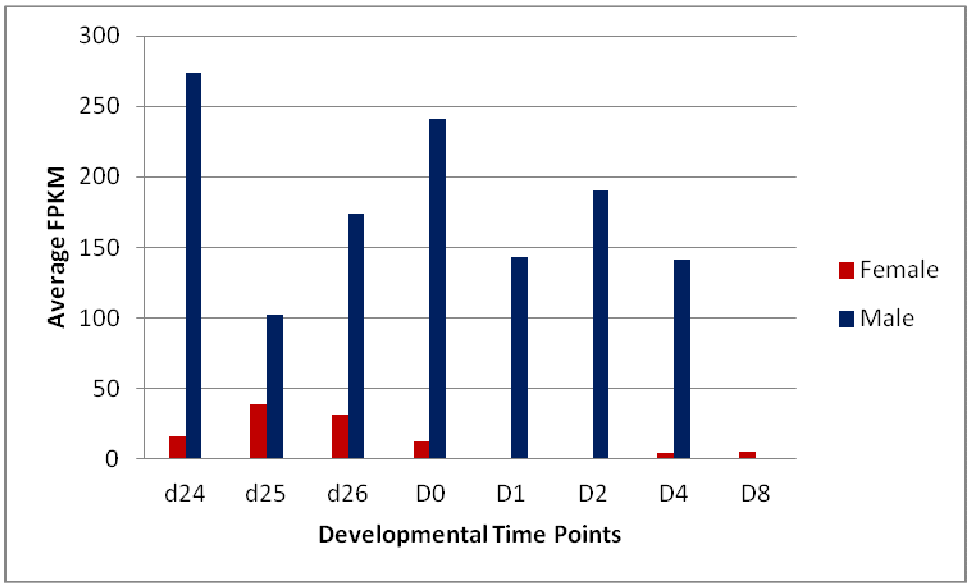


Figure 4. Differential Expression Levels of *AMH* gene

AMH expression levels were significantly higher at day 24 of gestation and day 0 postpartum in males. Levels were low at day 25 of gestation and day 4 postpartum in males. Differential expression fluctuates in males and is almost nonexistent in females. This figure reiterates this gene's role in testicular differentiation.

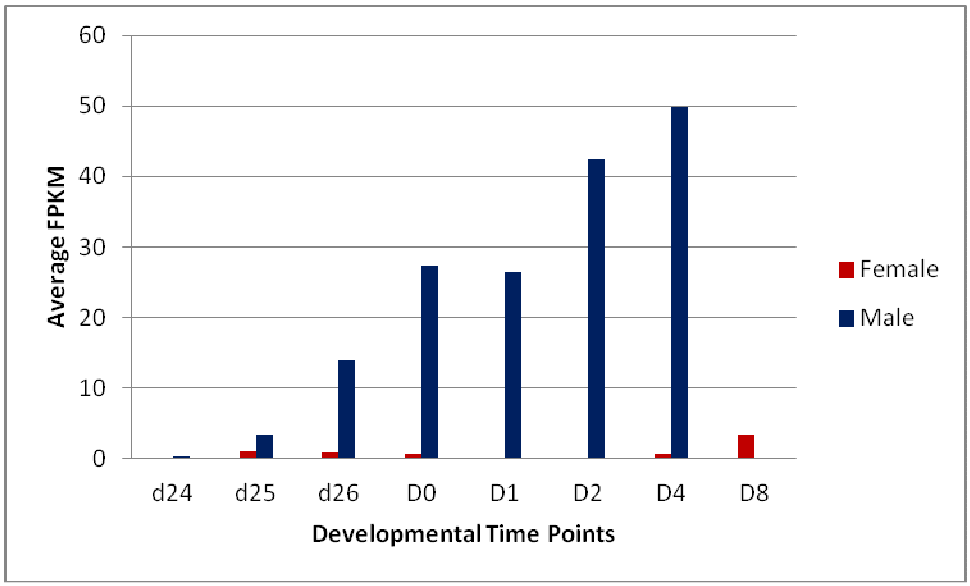


Figure 5. Differential Expression Levels of *DMRT1* gene

DMRT1 expression levels were significantly higher at day 2 and day 4 postpartum in males. Levels were low at day 24 and day 25 of gestation. Differential expression is upregulated at a linear rate. This figure reiterates this gene's role in testicular differentiation.

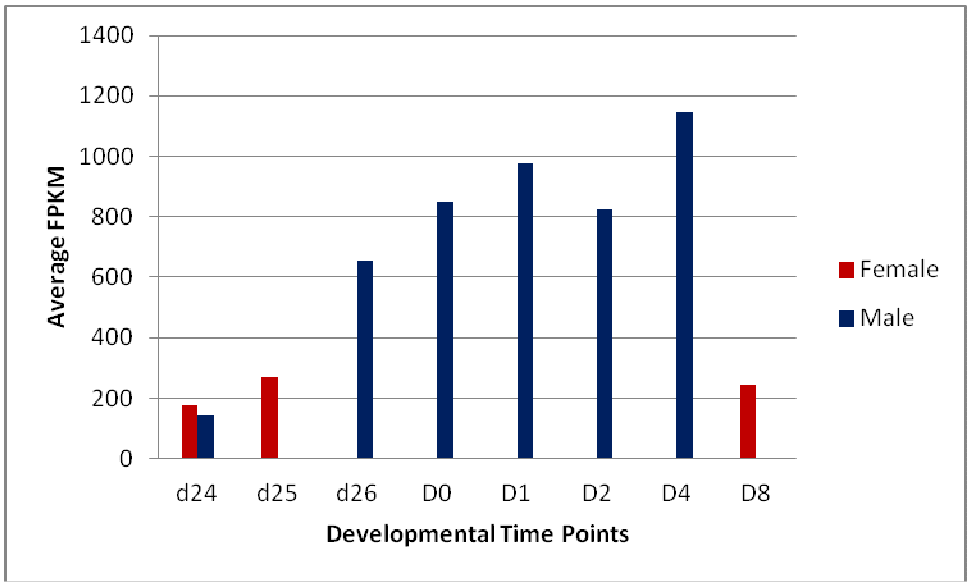


Figure 6. Differential Expression of *PTGDS* gene

PTGDS expression levels were significantly higher at day 1 and day 4 postpartum in males. Levels were low at day 24 of gestation. Differential expression is upregulated at a linear rate. This figure reiterates this gene's role in testicular differentiation.

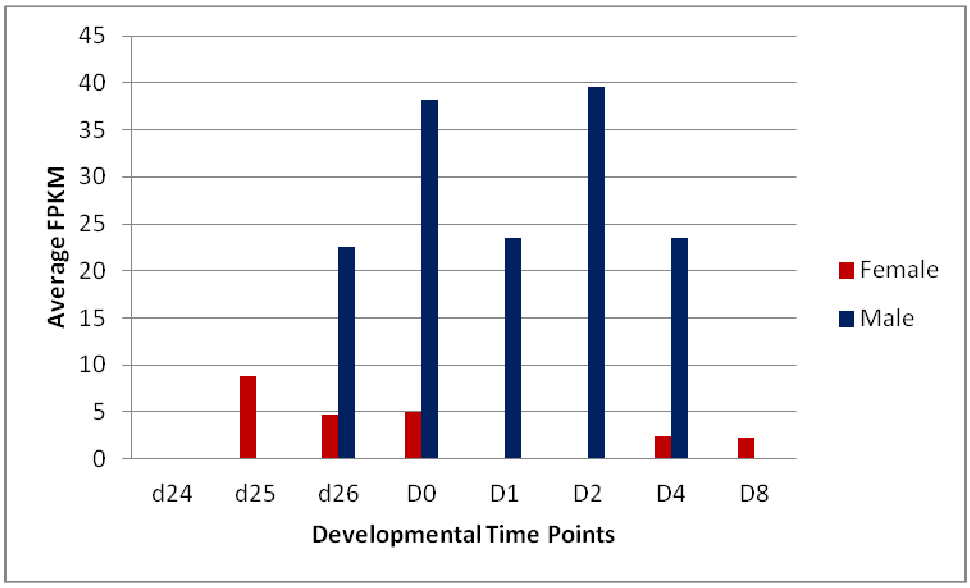


Figure 7. Differential Expression of *SOX9* gene

SOX9 expression levels were particularly high at day 0 and day 2 postpartum in males. Levels were low at day 26 of gestation and day 1 and day 4 postpartum in males. Differential expression fluctuates in males and is almost nonexistent in females. This figure reiterates this gene's role in testicular differentiation.

Table 8. Potential Novel Genes Involved in Ovarian and Testicular Differentiation

Genes		
AFP	*GSTM3*	*PDGFA*
ALAS2	*NDNL2*	*SERPINA3*

These genes were chosen through analyses of FPKM values for all stages of gonadal differentiation and focused on expression levels during gonadal differentiation for both males and females. *AFP* and *PDGFA* differential expression levels were investigated further.

The genes in the following two figures show very little expression in female wallabies. No ovaries were collected for day 1 and day 2 postpartum. No testes were collected for day 8 postpartum. There are notable differences in expression levels between females and males.

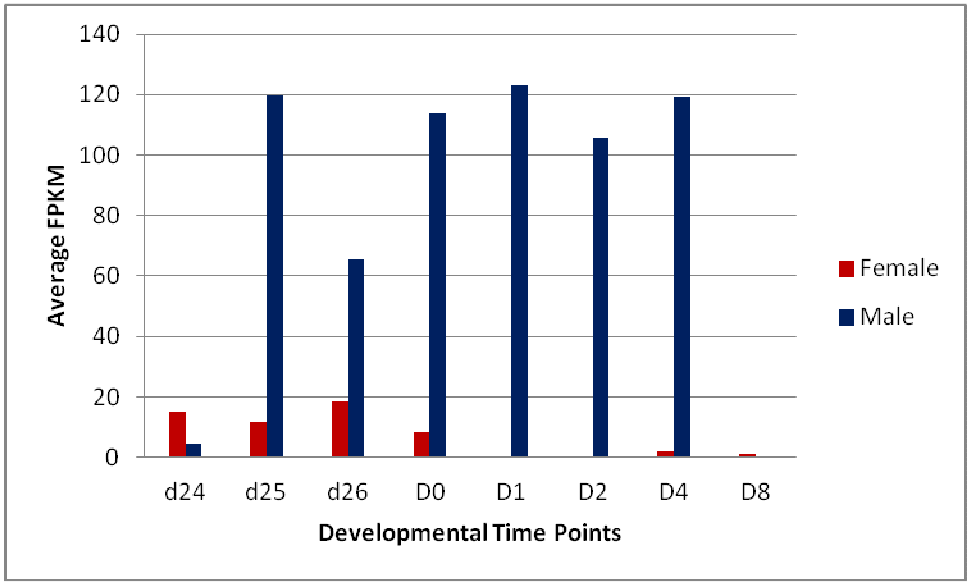


Figure 8. Differential Expression of *AFP* gene

AFP expression levels were particularly high at day 25 of gestation and day 1 postpartum in males. Levels were at its lowest at day 24 of gestation and low at day 26 of gestation in males. Differential expression essentially plateaus from day 25 of gestation to day 4 postpartum in males and is almost nonexistent in females.

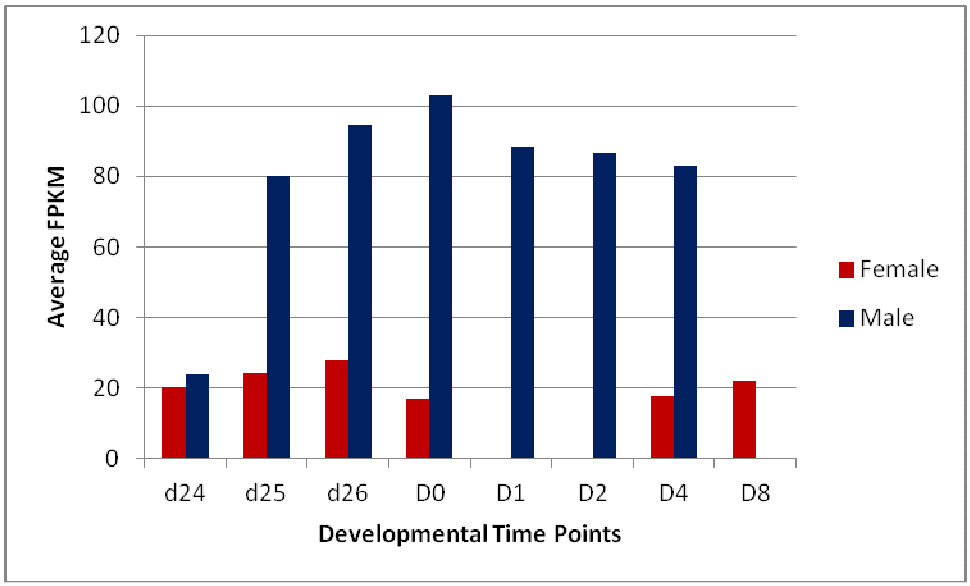


Figure 9. Differential Expression of *PDGFA* gene

PDGFA expression levels were particularly high at day 26 of gestation and peaks on day 0 postpartum in males. Levels were at low at day 24 of gestation in males. Differential expression is upregulated from day 24 of gestation to day 0 postpartum and is downregulated from day 0 to day 4 postpartum in males. In females, differential expression stays pretty level.

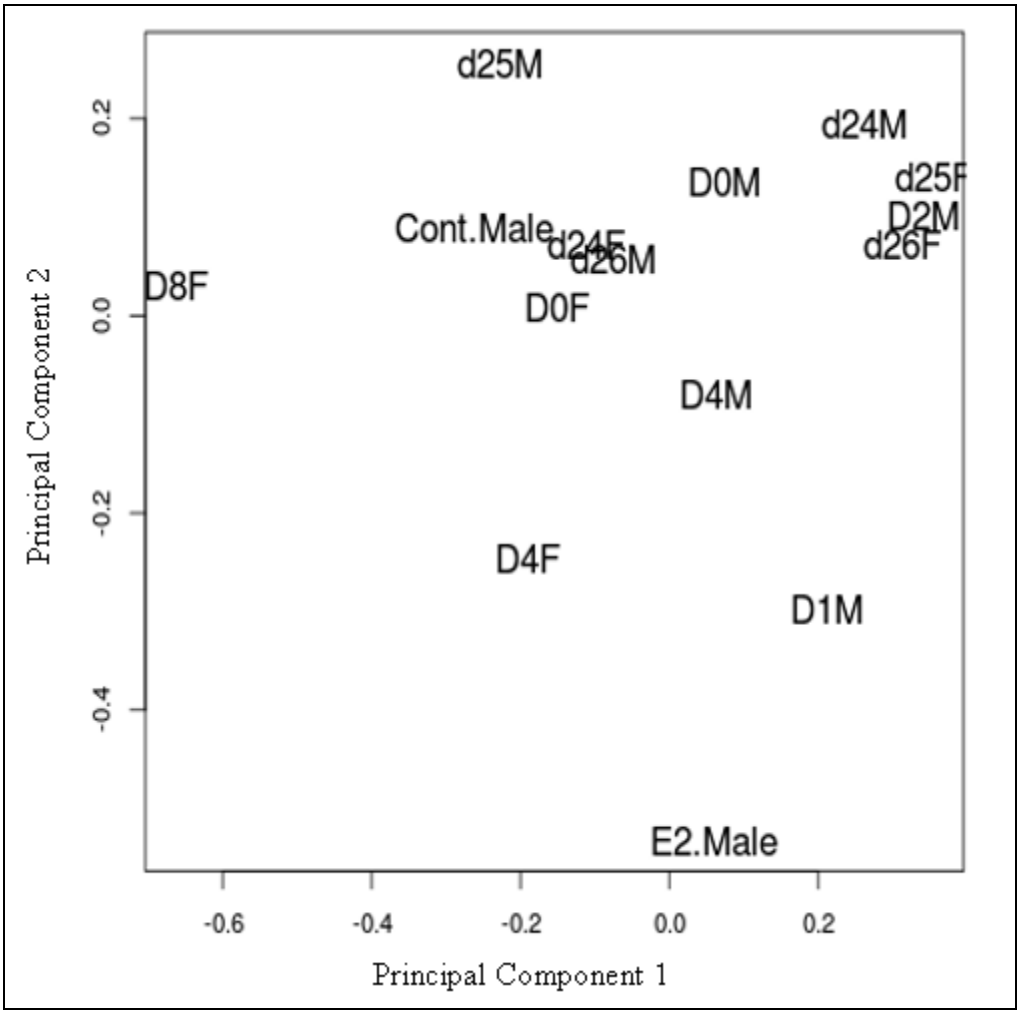


Figure 10. Principal Component Analysis (PCA) Graph of Transcriptome Samples

This figure was composed to determine the relationships between each of the transcriptome samples by grouping them together. There are a few outliers that are especially interesting: day 4 and day 8 females postpartum, E_2 treated male and day 1 male postpartum. Day 4 and day 8 females follow a different trajectory than the rest of the samples, which adheres to previous differential expression studies. E_2 treated male also follows a different trajectory and lies close to day 4 female postpartum. Control male lies close to day 4 male postpartum as expected. During day 1 male postpartum, the gonad is known to undergo major transcriptional changes since this is around the time it commits to the testis pathway.

Functional Annotation and Gene Ontology from Blast2GO

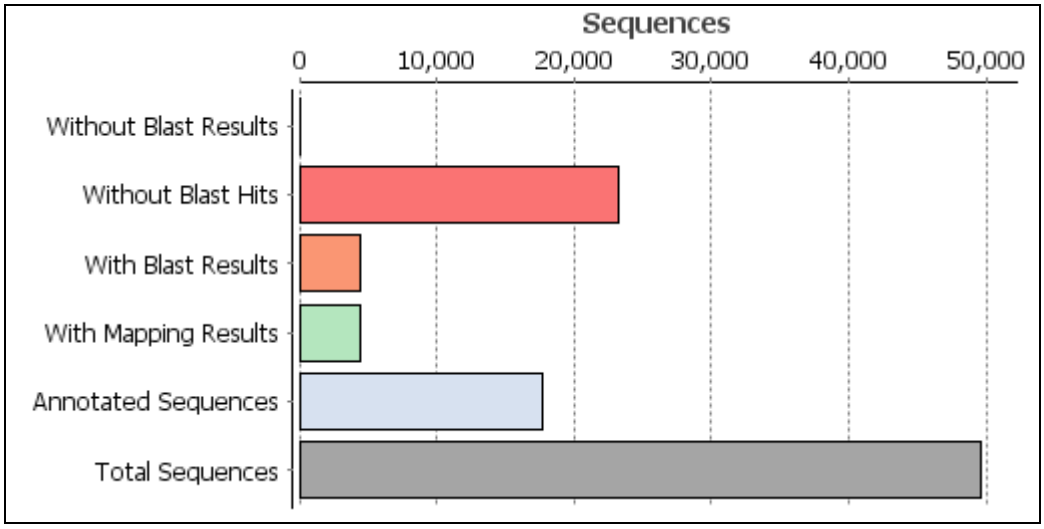


Figure 11. Annotation Results from Blast2GO

This figure was created by Blast2GO. No sequences were without Blast results indicating that all sequences were processed. There were about 25 thousands sequences without Blast hits after the BLAST step. There were about five thousand successful sequences with Blast results after the BLAST step. There were about five thousand successful sequences with mapping results after the mapping step. There were almost 20 thousand successful sequences that were annotated. There were 50 thousand sequences entered into Blast2GO for annotation.

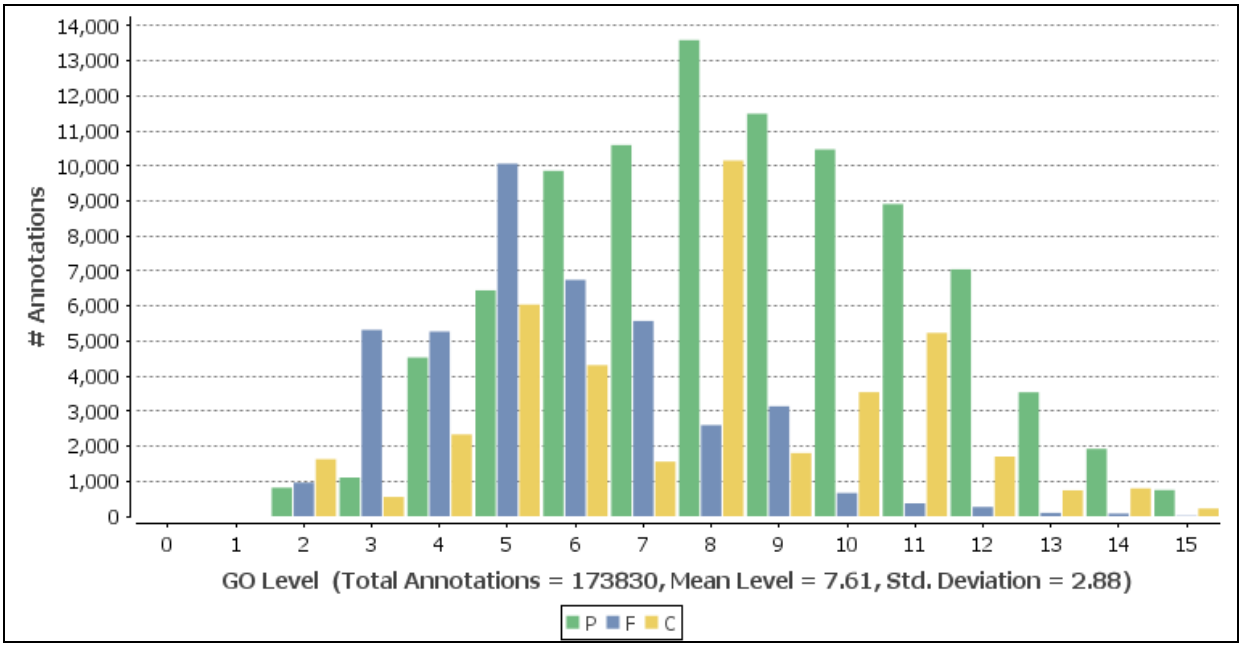


Figure 12. GO-level distribution of Total Annotations from Blast2GO: P = biological process, F = molecular function, C = cellular component

This figure was created by Blast2GO. It shows the overall distribution of gene ontology terms for each GO level for 173,830 annotations. A GO level is assigned by Blast2GO and shows the maximum depth of each GO term. Each term is divided into three different categories: biological process, molecular function, and cellular component. GO terms are created by the Gene Ontology Consortium to standardize gene function and product descriptions.

The following tables list GO terms from Blast2GO in their respective categories for genes of interest.

Table 9. Gene Ontology Terms for Top Changed Gene Comparisons

Gene	Molecular Function	Biological Processes	Cellular Component
ALAS2	5-aminolevulinate synthase activity coenzyme binding glycine binding protein binding pyridoxal phosphate binding	cellular iron ion homeostasis erythrocyte differentiation heme biosynthetic process hemoglobin biosynthetic process oxygen homeostasis porphyrin-containing compound metabolic process protoporphyrinogen IX biosynthetic process response to hypoxia small molecule metabolic process	mitochondrial inner membrane mitochondrial matrix mitochondrion
ALDH1A1	Ras GTPase activator activity aldehyde dehydrogenase (NAD) activity androgen binding retinal dehydrogenase activity	cellular aldehyde metabolic process ethanol oxidation positive regulation of Ras GTPase activity retinol metabolic process small molecule metabolic process xenobiotic metabolic process	cytoplasm cytosol extracellular vesicular exosome
AMH	Growth factor activity Hormone activity Receptor binding Transforming growth factor beta receptor binding	Müllerian duct regression Aging Cell-cell signaling Gonadal mesoderm development Positive regulation of: NF-kappaB transcription factor activity, gene expression Preantral ovarian follicle growth Response to: drug, organic cyclic compound Sex determination Sex differentiation Urogenital system development	Cytoplasm Extracellular region Extracellular space
APOE	antioxidant activity beta-amyloid binding	G-protein coupled receptor signaling pathway N-methyl-D-aspartate receptor clustering	Golgi apparatus blood microparticle

	cholesterol transporter activity heparin binding hydroxyapatite binding identical protein binding lipid binding lipid transporter activity lipoprotein particle binding low-density lipoprotein particle receptor binding metal chelating activity phosphatidylcholine-sterol O-acyltransferase activator activity phospholipid binding protein binding protein homodimerization activity tau protein binding very-low-density lipoprotein particle receptor binding	aging alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate selective glutamate receptor clustering artery morphogenesis cGMP-mediated signaling cell death cellular calcium ion homeostasis cellular response to: cholesterol, growth factor stimulus, interleukin-1 cholesterol: catabolic process, efflux, homeostasis, metabolic process chylomicron remnant clearance cytoskeleton organization fatty acid homeostasis high-density lipoprotein particle: assembly, clearance, remodeling intracellular transport lipoprotein biosynthetic process lipoprotein catabolic process lipoprotein metabolic process long-chain fatty acid transport low-density lipoprotein particle remodeling maintenance of location in cell negative regulation of: MAP kinase activity, beta-amyloid formation, blood coagulation, blood vessel endothelial cell migration, cholesterol biosynthetic process, cholesterol efflux, dendritic spine development, dendritic spine maintenance, endothelial cell proliferation, inflammatory response, lipid biosynthetic process, lipid transport across	chylomicron cytoplasm dendrite early endosome endocytic vesicle lumen extracellular matrix extracellular region extracellular space extracellular vesicular exosome extrinsic component of external side of plasma membrane high-density lipoprotein particle intermediate-density lipoprotein particle late endosome low-density lipoprotein particle membrane neuronal cell body nucleus plasma membrane very-low-density lipoprotein particle vesicle

		blood brain barrier, neuron apoptotic process, neuron death, phospholipid efflux, platelet activation, postsynaptic membrane organization, presynaptic membrane organization nitric oxide mediated signal transduction oligodendrocyte differentiation peripheral nervous system axon regeneration phospholipid efflux phototransduction, visible light positive regulation of: axon extension, beta-amyloid formation, cGMP biosynthetic process, cholesterol efflux, cholesterol esterification, dendritic spine development, dendritic spine maintenance, lipid biosynthetic process, lipid transport across blood brain barrier, low-density lipoprotein particle receptor catabolic process, membrane protein ectodomain proteolysis, neurofibrillary tangle assembly, neuron death, nitric-oxide synthase activity, phospholipid efflux, postsynaptic membrane organization, presynaptic membrane organization protein import receptor-mediated endocytosis regulation of: Cdc42 protein signal transduction, axon extension, beta-amyloid clearance, neuron death, neuronal synaptic plasticity, tau-protein kinase activity response to: dietary excess, ethanol, insulin, reactive oxygen species, retinoic acid retinoid metabolic process	

		reverse cholesterol transport small molecule metabolic process synaptic transmission, cholinergic triglyceride metabolic process vasodilation very-low density lipoprotein clearance very-low density lipoprotein remodeling	
C3	C5L2 anaphylatoxin chemotactic receptor binding endopeptidase inhibitor activity protein binding receptor binding	G-protein coupled receptor signaling pathway complement activation complement activation, alternative pathway complement activation, classical pathway fatty acid metabolic process immune response inflammatory response innate immune response negative regulation of endopeptidase activity positive regulation of: G-protein coupled receptor protein signaling pathway, activation of membrane attack complex, angiogenesis, apoptotic cell clearance, glucose transport, lipid storage, protein phosphorylation, type IIa hypersensitivity, vascular endothelial growth factor production regulation of: complement activation, immune response, triglyceride biosynthetic process signal transduction	blood microparticle extracellular region extracellular space extracellular vesicular exosome plasma membrane
CA3	carbonate dehydratase activity nickel cation binding zinc ion binding	bicarbonate transport one-carbon metabolic process response to ethanol response to oxidative stress small molecule metabolic process	Cytosol
CALB1	calcium ion binding	cellular response to organic substance	Axon

	protein binding vitamin D binding zinc ion binding	cytosolic calcium ion homeostasis locomotory behavior long-term memory metanephric collecting duct development metanephric connecting tubule development metanephric distal convoluted tubule development metanephric part of ureteric bud development regulation of synaptic plasticity retina layer formation short-term memory	cytosol dendrite extracellular vesicular exosome neuronal cell body nucleus synapse
CFD	serine-type endopeptidase activity serine-type peptidase activity	blood coagulation complement activation complement activation, alternative pathway innate immune response platelet activation platelet degranulatioon proteolysis	extracellular region extracellular space extracellular vesicular exosome platelet alpha granule lumen
CGA	hormone activity protein binding	cell-cell signaling cellular protein metabolic process cellular response to hormone stimulus developmental growth follicle-stimulating hormone secretion gonad development luteinizing hormone secretion negative regulation of organ growth peptide hormone processing positive regulation of cell migration positive regulation of cell proliferation positive regulation of transcription from RNA polymerase II promoter signal transduction	Extracellular region

		thyroid gland development thyroid hormone generation	
CLU	NOT ATPase activity misfolded protein binding protein binding ubiquitin protein ligase binding	blood coagulation cell morphogenesis central nervous system myelin maintenance chaperone-mediated protein complex assembly chaperone-mediated protein folding complement activation complement activation, classical pathway innate immune response intrinsic apoptotic signaling pathway lipid metabolic process microglial cell activation microglial cell proliferation negative regulation of beta-amyloid formation negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage negative regulation of protein homooligomerization platelet activation platelet degranulation positive regulation of: NF-kappaB transcription factor activity, apoptotic process, beta-amyloid formation, intrinsic apoptotic signaling pathway positive regulation of neurofibrillary tangle assembly, neuron death, nitric oxide biosynthetic process, proteasomal ubiquitin-dependent protein catabolic process, tau-protein kinase activity, tumor necrosis factor	apical dendrite blood microparticle chromaffin granule cytoplasm cytosol endoplasmic reticulum extracellular matrix colocalizes with extracellular matrix extracellular region extracellular space extracellular vesicular exosome mitochondrial membrane mitochondrion neurofibrillary tangle nucleus perinuclear region of cytoplasm platelet alpha granule lumen spherical high-density lipoprotein particle

		production protein import protein stabilization regulation of: beta-amyloid clearance, neuron death, neuronal signal transduction release of cytochrome c from mitochondria response to misfolded protein response to virus reverse cholesterol transport	
CPA1	Metallocarboxypeptidase activity Zinc ion binding	proteolysis	Extracellular space
CTRB2	serine-type endopeptidase activity	cobalamin metabolic process digestion extracellular matrix disassembly extracellular matrix organization proteolysis small molecule metabolic process vitamin metabolic process water-soluble vitamin metabolic process	extracellular region extracellular space
DIO1	selenium binding thyroxine 5'-deiodinase activity	Cellular nitrogen compound metabolic process hormone biosynthetic process oxidation-reduction process small molecule metabolic process thyroid hormone generation	endoplasmic reticulum membrane integral component of membrane plasma membrane
GSTM3	enzyme binding glutathione binding glutathione transferase activity identical protein binding protein homodimerization activity	cellular detoxification of nitrogen compound establishment of blood-nerve barrier glutathione derivative biosynthetic process glutathione metabolic process nitrobenzene metabolic process response to estrogen small molecule metabolic process xenobiotic catabolic process	Cytoplasm cytosol extracellular vesicular exosome nucleus sperm fibrous sheath

		xenobiotic metabolic process	
HBA2	Contributes to haptoglobin binding heme binding iron ion binding oxygen binding oxygen transporter activity contributes to peroxidase activity protein binding	Biocarbonate transport Hydrogen peroxide catabolic process Oxidation-reduction process Oxygen transport Positive regulation of cell death Protein heterooligomerization Response to hydrogen peroxide Small molecular metabolic process	Blood microparticle cytosol cytosolic small ribosomal subunit endocytic vesicle lumen extracellular region extracellular vesicular exosome haptoglobin-hemoglobin complex hemoglobin complex membrane
HSD3B1	3-beta-hydroxy-delta5-steroid dehydrogenase activity Steroid delta-isomerase activity	androgen biosynthetic process estrogen biosynthetic process glucocorticoid biosynthetic process mineralocorticoid biosynthetic process oxidation-reduction process small molecule metabolic process steroid biosynthetic process steroid metabolic process	endoplasmic reticulum membrane integral component of membrane mitochondrial inner membrane mitochondrial intermembrane space smooth endoplasmic reticulum membrane
IGFBP3	fibronectin binding insulin-like growth factor I binding insulin-like growth factor binding metal ion binding protein binding protein tyrosine phosphatase activator activity	apoptotic process cellular protein metabolic process negative regulation of: cell proliferation, protein phosphorylation, signal transduction, smooth muscle cell migration, smooth muscle cell proliferation osteoblast differentiation positive regulation of: MAPK cascade, apoptotic process, catalytic activity, insulin-	extracellular region extracellular space extracellular vesicular exosome insulin-like growth factor binding protein complex nucleus

		like growth factor receptor signaling pathway, myoblast differentiation protein phosphorylation regulation of cell growth regulation of glucose metabolic process type B pancreatic cell proliferation	
MGP	calcium ion binding extracellular matrix structural constituent structural constituent of bone	cartilage condensation cell differentiation ossification regulation of bone mineralization	colocalizes with extracellular matrix extracellular vesicular exosome proteinaceous extracellular matrix
NDNL2	Protein binding	DNA recombination DNA repair positive regulation of protein ubiquitination regulation of growth	Smc5-Smc6 complex Chromosome, telomeric region Cytoplasm nucleus
PDZK1IP1			Extracellular vesicular exosome Integral component of membrane
PTGDS	Fatty acid binding Prostaglandin-D synthase activity Retinoid binding Transporter activity	Arachidonic acid metabolic process Cyclooxygenase pathway Prostaglandin biosynthetic process Regulation of circadian sleep/wake cycle, sleep Response to glucocorticoid Small molecule metabolic process transport	Golgi apparatus Endoplasmic reticulum membrane Extracellular region Extracellular space Extracellular vesicular exosome Nuclear membrane Perinuclear region of cytoplasm Rough endoplasmic

			reticulum
RNASEH1	RNA binding RNA-DNA hybrid ribonuclease activity Magnesium ion binding Nucleic acid binding Protein homodimerization activity Ribonuclease activity	RNA catabolic process RNA phosphodiester bond hydrolysis RNA phosphodiester bond hydrolysis, endonucleolytic Mitochondrial DNA replication	Mitochondrion nucleus
SAA1	G-protein coupled receptor binding heparin binding	acute-phase response innate immune response lymphocyte chemotaxis macrophage chemotaxis negative regulation of inflammatory response neutrophil chemotaxis platelet activation positive regulation of: cell adhesion, cytokine secretion, cytosolic calcium ion concentration, interleukin-1 secretion	endocytic vesicle lumen extracellular region extracellular vesicular exosome high-density lipoprotein particle
SERPINA3	DNA binding protein binding serine-type endopeptidase inhibitor activity	acute-phase response inflammatory response maintenance of gastrointestinal epithelium negative regulation of endopeptidase activity regulation of lipid metabolic process regulation of proteolysis	blood microparticle extracellular region extracellular space extracellular vesicular exosome intracellular nucleus
TMEM52B			extracellular vesicular exosome integral component of membrane
TNNC1	actin filament binding calcium ion binding calcium-dependent protein binding	cardiac muscle contraction diaphragm contraction muscle filament sliding	actin cytoskeleton cytosol mitochondrion

	protein binding protein homodimerization activity troponin I binding troponin T binding	regulation of ATPase activity regulation of muscle contraction regulation of muscle filament sliding speed response to metal ion ventricular cardiac muscle tissue morphogenesis	NOT nucleolus nucleus troponin complex

Table 10. Gene Ontology Terms for Potential Novel Genes

Gene	Molecular Function	Biological Processes	Cellular Component
AFP	metal ion binding	SMAD protein signal transduction ovulation from ovarian follicle progesterone metabolic process transport	Cytoplasm extracellular space
PDGFA	Collagen binding Growth factor activity Platelet-derived growth factor binding Platelet-derived growth factor receptor binding Contributes to platelet-derived growth factor receptor binding Platelet-derived growth factor receptor binding Protein binding Protein heterodimerization activity Protein homodimerization activity	Fc-epsilon receptor signaling pathway Actin cytoskeleton organization Angiogenesis Blood coagulation Cell activation Cell projection assembly Cell-cell signaling embryo development Epidermal growth factor receptor signaling pathway Extracellular matrix organization Fibroblast growth factor receptor signaling pathway Hair follicle development Innate immune response Inner ear development Lung alveolus development Negative chemotaxis	Golgi membrane Cell surface Endoplasmic reticulum lumen Extracellular region Extracellular space Microvillus Platelet alpha granule lumen

		Negative regulation of phosphatidylinositol biosynthetic process Negative regulation of platelet activation Neurotrophin TRK receptor signaling pathway Organ morphogenesis Phosphatidylinositol-mediated signaling Platelet activation Platelet degranulation Platelet-derived growth factor receptor signaling pathway Positive regulation of: DNA replication, ERK1 and ERK2 cascade, MAP kinase activity, MAPK cascade, cell division, cell migration, cell proliferation, fibroblast proliferation, mesenchymal cell proliferation, metanephric mesenchymal cell migration by platelet-derived growth factor receptor-beta signaling pathway, phosphatidylinositol 3-kinase signaling, protein autophosphorylation, protein kinase B signaling NOT regulation of DNA biosynthetic process Regulation of actin cytoskeleton organization Regulation of branching involved in salivary gland morphogenesis by epithelial-mesenchymal signaling NOT regulation of glomerular mesangial cell proliferation Regulation of pepitdyl-tyrosine phosphorylation Regulation of smooth muscle cell migration Response to: drug, estradiol, hypoxia, substance, retinoic acid, wounding Skin development	

		Transforming growth factor beta receptor signaling pathway Wound healing	

Discussion

There is a tremendous need for comparative transcriptome analyses focusing on mammalian sexual development. Even though there is a vast amount of knowledge on this subject, many aspects of gonadal differentiation, especially ovarian development, have yet to be discovered. This thesis presents an important new comparative resource for the sex determination community with the analyses of high-depth transcriptomes during marsupial gonadal development. Due to time constraints, the vast amount of work went into extensive RNA sample prep and QC, and there was only a limited amount of time for data analyses. Even though the data analyses are rather rudimentary at this stage, results confirm previous findings on sexual differentiation, validating the utility of this data set. Analyses also revealed novel gene information and uncovered novel pathways induced by exogenous estrogen exposure.

Testicular development in the tammar wallaby occurs during day 1 and day 2 postpartum. However, ovarian development occurs around day 4 postpartum at the molecular level and around day 8 postpartum histologically. Due to the window of time in which gonads were collected for these analyses, most of the differential expression data obtained pertains to genes involved in the testicular developmental pathway. Nevertheless, analyses did reveal significant upregulation of differential expression levels of *FOXL2* in females during day 4 postpartum, which is during ovarian differentiation. This reaffirms the conserved role of *FOXL2* in ovarian differentiation in mice, humans, and the tammar wallaby and its noninvolvement in testicular differentiation [16,24,29]. There was significant upregulation of differential expression of desert hedgehog *(DHH)*, during day 1 postpartum in males, which is during testicular differentiation. This data is consistent with previous research stating the importance of *DHH* in testicular differentiation especially the early development of Leydig cells in mice and humans. In addition,

it confirms the conserved role of *DHH* in the tammar wallaby and eutherian mammals in the male gonadal pathway [10,17,21]. Nuclear receptor subfamily 0, group B, member 1 (*NR0B1*) was upregulated during day 24 of gestation in females. The role of *NR0B1* during gonadal differentiation is unclear due to its characteristic of acting in a dose-dependent manner in mice and humans [3,9,15,36]. Previous studies have opposing views on whether it plays any role in gonadal development and whether it has roles in both testicular and ovarian differentiation [14,15,25]. Unlike mice and humans, *NR0B1* is located on autosomal chromosome 5 in the tammar wallaby and not on the X-chromosome [14,17]. This gene can act as a pro-ovary gene consistent with its early upregulation in the ovary in the tammar. Nevertheless, more studies need to be done to clarify *NR0B1*'s role in sexual differentiation.

Table 7 and Figure 4 show considerable differential expression levels of *AMH* throughout each stage of testicular differentiation and during pre-ovarian differentiation. These figures recapitulate the conserved importance of *AMH* in supporting male urogenital differentiation and development [2,17,44,50]. Furthermore, these data corroborate those seen previously for *AMH* in the tammar wallaby [16]. Results also revealed significant upregulation of doublesex and mab-3 related transcription factor 1 *(DMRT1)* in males, especially from day 26 of gestation to day 4 postpartum growing at an increasing rate. Prior studies indicate that *DMRT1* upregulation in males is highly conserved among all vertebrates in the testes, and show it has a vital role in mice during postnatal testicular differentiation [15,25,28,30]. These studies are consistent with expression levels found in the tammar wallaby and highlight the relevance of this gene in male development in all vertebrates including the tammar. Similarly to *DMRT1*, the expression levels of *PTGDS*, prostaglandin D2 synthase, increased at linear rate in males. This follows with research on the importance of *PTGDS* in testicular differentiation and the development of Sertoli

cells [21,36,47]. Lastly, *SOX9* is differentially expressed during all stages of male gonadal differentiation and is barely expressed in females. This data stresses the established and conserved role of *SOX9* in promoting testicular formation and differentiation in all vertebrates including the tammar wallaby [1,9,14,21,28,45,47]. Previous studies done on the tammar wallaby show similar expression patterns, which reiterates this gene's role conservation in eutherians and marsupials [15,16,45]. Taken together, these analyses validate the transcriptomes as a viable resource and a great representation of key sexual differentiation genes.

Besides known sexual differentiation data, information on potential novel genes involved in gonadal differentiation in the tammar wallaby can be obtained from expression analyses. Based on analyses of significant expression levels during periods of testicular and ovarian differentiation, a list of potential novel genes was attained. These include *AFP*, *ALAS2*, *GSTM3*, *NDNL2*, *PDGFA*, and *SERPINA3*. For instance in Figure 9, platelet-derived growth factor subunit A (*PDGFA*) had substantially high expression levels during testicular differentiation and low levels in females. This indicates the likelihood of *PDGFA* being relevant in male gonadal development. A few studies briefly mention *PDGF* and *PDGFA* potentially being part of *SOX9* localization and the development of Leydig cells [21,25,47,51]. Moreover in Figure 8, alpha-fetoprotein (*AFP*) also had particularly high expression levels during all stages of male gonadal differentiation and very low levels in females especially during ovarian differentiation. This data illustrates the prospect of *AFP* being a component of the male developmental pathway. None of the GO terms listed in Table 10 indicate any role in gonadal development for *AFP* and *PDGFA*. More studies such as immunohistochemistry need to be done for both *PDGFA* and *AFP* to confirm their importance in testicular differentiation.

Additionally, differential expression data were obtained from male gonads cultured in control media and in estrogen media. Preliminary data suggest that a plethora of genes were affected by exogenous estrogen exposure. In comparison to expression levels of gonads treated with control media, various genes were upregulated and downregulated on many levels due to exogenous estrogen exposure. These genes could potentially play a significant part of gonadal differentiation and could provide clues to how exogenous estrogen can activate estrogen receptors leading to the ovarian phenotype. As previously mentioned, exogenous estrogen exposure of the marsupial XY gonad results in complete sex reversal and ovarian development. Both estrogen receptors in the tammar are shown have a high degree of sequence homology to humans, mice and other vertebrates [11]. Therefore, further analyses can be performed on mice and humans to verify the effects of exogenous estrogen on the expression levels of the affected genes and their relevance in sexual differentiation. Many of the genes I identified to be upregulated in the estrogen cultured gonad were also known to be significantly differentially expressed and upregulated during ovarian development in the mouse (using GUDMAP). Such genes are highly conserved and are novel factors in the female developmental pathway that will form the topic of future investigations in the development of the mammalian ovary.

Although a great deal of information was obtained, there are limitations to how much insight the initial data analyses will reveal about ovarian differentiation. Despite the enormous coverage of the reference tammar wallaby genome, there are some reads from the created transcriptomes that will not be mappable. To reduce the amount of unmapped reads, the genomes of two other marsupials, the opossum and Tasmanian devil, can be used to aid in read annotation, as well as the human and mouse genomes. However, it will only align those reads that are highly conserved across these species. In addition, the gene *SRY* was not able to be pulled from the data analyses

due to different alignment tools calling it different genes. This could potentially be the case for other divergent genes that could not be mapped. A new annotation of the tammar genome is pending release in 2015 and will significantly improve the utility of this data in future analyses. Furthermore, the data analyses only provides information about tammar wallaby transcriptomes and therefore more work will need to be done to create transcriptomes for the aromatase knockout mouse (ArKO) and the human cell line NT2/D1. The current tammar transcriptomes described in this thesis show normal gonadal development, the effects of estrogen in the gonad, and mentions some possible targets of environmental endocrine disrupters (EEDs) in the gonads. However, the specific interests of this thesis pertain to genes that are somatic cell specific meaning the supporting cell lineage and totipotent germ cells of the ovary and testis. NT2/D1 is a human pluripotent embryonal carcinoma cell line that was found to express both estrogen receptors, to be an excellent model for male gonadal development, express *SRY* and *SOX9*, and can respond to exogenous estrogen [5,52]. By developing transcriptomes with exogenous estrogen treatment in the tammar wallaby, NT2/D1 cell line and ARKO mouse, developmental researchers can perform comparative analyses to isolate somatic cell specific estrogen responsive genes. From those studies, potential genes involved in mammalian ovarian differentiation that trigger disorders of sexual development (DSDs), and are targets of estrogenic EEDs could be discovered. With this valuable data, prospective treatments could possibly be developed to treat DSDs and prevent future exposure of humans to EEDs.

Conclusion

The tammar wallaby, *Macropus eugenii*, is a novel and important new genetic model for research in human development especially sex determination and sexual differentiation. With the ever increasing rate and occurrence of disorders of sexual development (DSDs), comparative studies

such as this one are going to be critical in determining the key events in sexual differentiation in all mammals including humans. Transcriptomes from this study could potentially lead to treatments for DSDs and reveal new information about ovarian differentiation in mammals. These results show a lot of similarities, but also some interesting differences from mouse and human studies. Overall, this is a viable transcriptome resource and a good comparative data set to uncover conserved and novel genes regulating mammalian gonad formation. This thesis provides the first transcriptome analyses investigating the role of estrogen in sex reversal in the mammalian gonad.

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