FUNCTIONAL GENOMIC ANALYSIS OF GONAD DEVELOPMENT IN THE PROTANDROUS ASIAN SEABASS

JIANG JUNHUI

B.Sc (First Class Honours), National University of Singapore

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES &

TEMASEK LIFE SCIENCES LABORATORY

NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Jiang Junhui

15 Sep 2014

Acknowledgements

I would like to thank my mentor and supervisor Prof. Laszlo Orban for accepting me as his student and making me part of the RGG family. Every PhD teacher-student bond is a special one and I am forever grateful and indebted to Laszlo for the kindness and generosity that he has given to me.

Next, it would not be possible for me to embark on this long learning journey without the support of my CEO, Ms. Tan Poh Hong, Group Director, Dr. Chew Hong and Director, Mr. Lim Huan Sein. I would also like to thank Mrs. Renee Chou and Dr. Ling Kai Huat for their encouragement and support.

I would also like to thank all the staff at MAC, in particular Liang Bing, Xiao Xu, Yazid, Syed Ali, Chua and Chee. My work on the Asian seabass would not have been possible without their help in taking care of the fishes at St John's Island. I would also like to thank RGG lab members Xueyan, Inna, Shubha, Keh-Weei Natascha, Datta, Jolly, Shawn, Purush, Doreen and ex-members Rajini, Preethi, Hsiao Yuen and Candy. I also appreciate ex-RGG PhD student, Dr. Wang Xingang, for passing down excellent histology and FISH protocols. I would also like to acknowledge my fellow AVA colleague, Amos Koh, and RGG PhD student, Liew Woei Chang, for their unreserved and forthcoming sharing of fish husbandry knowledge and laboratory techniques respectively with me. Without them, my learning journey would have been much more difficult. I also appreciate the work of TLL core facilities, especially the Sequencing Lab and Fish Facility, which have my work much easier.

Lastly, I thank my loving wife for her understanding and support when I needed to put in extra hours in the lab.

Table of Contents

Abstract

Sex differentiation in teleosts is highly pliable and sequential hermaphroditism is the epitome of this characteristic. The protandrous Asian seabass (*Lates calcarifer*) is such a hermaphrodite, undergoing male-to-female sex change during its sexual reproductive cycle. While there have been detailed histological descriptions of its sexual development, the molecular analysis of the sex change process is lacking. Natural sex change is a useful system to understand sex differentiation, a conserved process in vertebrates. In addition, a better understanding of sex change in Asian seabass could form the basis for future experiments to solve sex control issues in this aquaculture species.

In order to profile the transcriptomic changes that occurred during Asian seabass gonad development, next generation sequencing technology was utilized to determine the Asian seabass transcriptome. Using the information, a custom mid-throughput qPCR array and a high-throughput micorarray was generated. At the same time, gonad samples were collected from Asian seabass ranging from juveniles to adults.

The histological and transcriptomic results showed that testis differentiation occurred early at around nine months post-hatching and could be mandatory. During gonad transformation, 'pro-male' genes (*i.e.* those with a function supporting testis development or maintenance), such as *dmrt1* and *sox9*, were down-regulated while apoptosis was activated to clear the male germ cells. The early transforming gonad thus assumed a near-undifferentiated transcriptomic state. Subsequently, ovarian differentiation from the transforming gonad involved the activation of the 'pro-female' Wnt signaling pathway. In order to understand the role of the brain in the sex

change process, a microarray analysis was also carried out on the brain of adult male and female Asian seabass, but no widespread differences could be found. This indicated that any existing differences in expression were likely to be mild, localized and possibly transient. Separately, Asian seabass was found to be able to respond to gonadotropin-releasing hormone (GnRH) induction with a spike in the mucus 11-ketotestosterone level and the magnitude of this change was dependent on the gonadal maturation stage. Long-term treatment of GnRH could also promote the development of spermiating testis in juvenile seabass.

To test the hypothesis on the role of Wnt signaling in ovarian differentiation, the zebrafish model was used as it was previously shown by our laboratory that Wnt signaling genes were differentially expressed during zebrafish's 'juvenile ovary-to-testis' transformation. Transgenic down-regulation of Wnt signaling in the *Tg(hsp70l:dkk1b-GFP)w32* zebrafish line through induced activation of *dkk1b-GFP* expression resulted in an increased proportion of males with corresponding decrease in gonadal aromatase gene (*cyp19a1a*) expression. These results provided the first functional evidence that, similarly to mammals, Wnt/ß-catenin signaling is a pro-female pathway that regulates gonad differentiation in zebrafish and possibly Asian seabass.

The results from this study have led to a greater understanding of the sexual development of the Asian seabass at both the developmental and molecular level. The zebrafish has also proved itself to be a useful model system for the functional validation of genes and pathways involved in gonad differentiation. Results from both the Asian seabass and zebrafish have shown that despite the opposite direction of gonad transformation, the same set of genes was involved, albeit in the appropriate direction, reinforcing the notion that several aspects of sex differentiation is conserved during sex reversal.

List of Tables

List of Figures

List of Abbreviations

11-KT	11-Ketotestosterone
17MT	17α-Methyltestosterone
BLAST	Basic Local Alignment Search Tool
BPG	Brain-pituitary-gonadal axis
CSD	Chromosomal sex determination
DET	Differentially expressed transcripts
dpc	days post coitum
dpf	days post-fertilization
E2	17ß-estradiol
ESD	Environmental sex determination
FDR	False discovery rate
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GSD	Genetic sex determination
JO	Juvenile ovary
JOT	Juvenile ovotestis
LH	Luteinizing hormone
LHRHa	Luteinizing-hormone releasing hormone analog
MAS	Marker-assisted selection
mph	months post-hatching
PCA	Principal component analysis
PGC	Primordial germ cell
TSD	Temperature-based sex determination

List of [1]Gene Symbols

18S	*18S ribosomal RNA*
acvr1	*activin A receptor, type I*
amh	*anti-mullerian hormone*
ar	*androgen receptor*
axin1	*axin1*
bactin	*beta-actin*
bfar	*bifunctional apoptosis regulator*
bmp1/2	*bone morphogenetic protein 1/2*
c6/7	*complement component C6/7*
catd	*cathepsin D*
ck2a	*casein kinase 2 alpha*
cox1	*cytochrome c oxidase assembly homolog 1*
ctnnb1	*catenin beta 1*
ctnnbip1	*catenin beta interacting protein 1*
ctsk	*cathepsin K*
cyp11a1	*cytochrome P450, family 11, subfamily A, polypeptide 1*
cyp11c1	*cytochrome P450, family 11, subfamily C, polypeptide 1*
cyp17a1	*cytochrome P450, family 17, subfamily A, polypeptide 1*
cyp19a1	*cytochrome P450 aromatase*
cyp26a1	*cytochrome P450, family 26, subfamily A, polypeptide 1*
cyp26b1	*cytochrome P450, family 26, subfamily B, polypeptide 1*
Dhh	*Desert hedgehog*
dkk1b	*dickkopf 1b*
dkk3	*dickkopf 3*
dlc	*deltaC*
dmrt1	*doublesex and mab-3 related transcription factor 1*
DMW	*doublesex and mab-3 related transcription factor 1, W-linked*
dmy	*doublesex and mab-3 related transcription factor 1, Y-linked*
dnd	*dead end*
dvl2	*dishevelled, dsh homolog 2*

[1] All teleost gene and protein symbols are standardized to the zebrafish nomenclature: for gene symbol, all letters are italicized and lower case while the protein symbol is the same as the gene symbol, but non-italic and the first letter is uppercase.

ef1a	*elongation factor 1-alpha*
esr1/2	*estrogen receptor 1 /2*
foxl2	*forkhead box L2*
fshr	*follicle stimulating hormone receptor*
fsta	*follistatin a*
fzd1/8	*frizzled homolog 1/8*
gadph	*glyceraldehyde-3-phosphate dehydrogenase*
gcl	*germ cell-less*
gdf9	*growth differentiation factor 9*
gli1	*GLI-Kruppel family member 1*
gsdf1	*gonadal soma derived factor 1*
Hes1	*hairy and enhancer of split-1*
hsd11b2	*11-beta-hydroxysteroid dehydrogenase type 2*
hsd17b1	*17-beta-hydroxysteroid dehydrogenase type 1*
hsd3b	*3 beta-hydroxysteroid dehydrogenase*
hsp70	*heat shock cognate 70-kd protein*
Ihh	*Indian hedgehog*
ikbe	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon*
inhbb	*inhibin, beta B*
jag1b	*jagged 1b*
kiss2	*kisspeptin 2*
kiss2r	*kisspeptin receptor 2*
lef1	*lymphocyte enhancer binding factor 1*
lhr	*luteinizing hormone receptor*
LRP5/6	*low density lipoprotein receptor-related protein 5/6*
LRWD1	*leucine-rich repeats and WD repeat domain containing 1*
nfkb2	*NF-kappa-B 2*
nkap	*NF-kappa-B-activating protein*
npb	*neuropeptide B*
nr0b1	*nuclear receptor subfamily 0 group B member 1*
nr5a2/4	*nuclear receptor subfamily 5 group A member 2/4*
odf3	*outer dense fiber of sperm tails 3*
peli1	*pellino homolog 1*
piwil1	*piwi-like 1*
pr	*progesterone receptor*

psap	*prosaposin*
psen1	*presenilin 1*
Ptch1	*patched 1*
rdh3	*retinol dehydrogenase 3*
rpl8	*ribosomal protein L8*
rspo1	*R-spondin homolog*
rtkn1/2	*rhotekin 1/2*
rttn	*rotatin*
sdY	*sexually dimorphic on the Y-chromosome*
sema4e	*semaphorin 4e*
sept6	*septin 6*
Sf1	*splicing factor 1*
sh3rf1	*SH3 domain containing ring finger 1*
shh	*sonic hedgehog*
smad4	*MAD homolog 4*
sox9	*SRY-box containing gene 9*
Sry	*sex-determining region Y*
star	*steroidogenic acute regulatory protein*
stra6	*stimulated by retinoic acid gene 6 homolog*
sycp1/3l	*synaptonemal complex protein 1/3-like*
tac1/2	*tachykinin 1/2*
tcf4	*transcription factor 4 (T-cell specific, HMG-box)*
tdrd1/7	*tudor domain containing 1/7*
tekt1	*tektin 1*
tnks	*tankyrase*
tp53	*tumor protein p53*
ts1	*ts 1*
tuba	*alpha tublin 1*
ubq	*ubiquitin*
unc5a	*unc-5 homolog A*
vasa	*vasa homolog*
vtgr	*vitellogenin receptor*
wnt3/4a/16	*wingless-type MMTV integration site family, member 3/4a/16*
wt1	*wilms' tumor 1*
zp1/2/3	*zona pellucida glycoprotein 1/2/3*

1 Introduction

1.1 Hermaphroditism – a platform for the study of sex differentiation and implications for aquaculture

Sexual reproduction is a hallmark of life for the vast majority of vertebrates with very few exceptions. It involves the production of two gametes, one of the male sex (sperm) and the other of the female sex (ovum), each carrying half the genome. The subsequent fusion of two such gametes creates the next generation. The significance of this is that the processes of meiosis and fertilization result in the offspring being genetically different from the two parents and also between siblings and this generates the genetic diversity necessary for the selection and evolution of a species.

In most vertebrates, the two sexes are separated and this is brought about by the processes of sex determination and sex differentiation (**Figure 1**). In developmental biology, sex determination refers to the commitment of cells or tissues to the male or female developmental fate, while sex differentiation refers to the subsequent development of the testis and ovary from the bipotential gonad, the reproductive organs that produce the sperm and ovum respectively (Valenzuela 2008). Gonad differentiation is often used interchangeably with sex differentiation, although sex differentiation can also additionally imply the development of secondary sexual characteristics that are not part of the reproductive system. Decades of research on vertebrate sex have also led to the current notion that the upstream signals for sex determination are diverse while the downstream molecular regulators for sex differentiation are conserved (Wilkins 1995, Morrish and Sinclair 2002, Graham *et al.* 2003, Barske and Capel 2008, Scherer 2008).

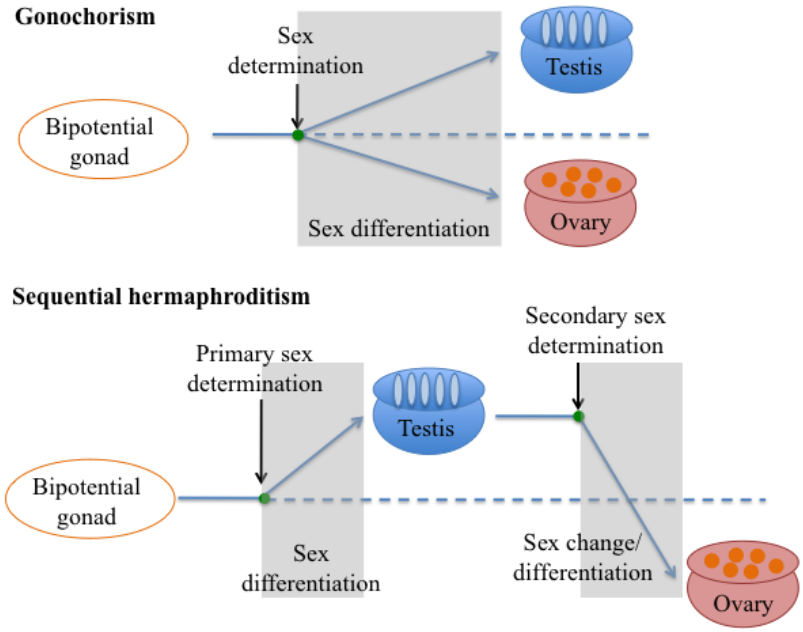

Figure 1. Sex determination and sex differentiation in gonochorists and sequential hermaphrodites.
In gonochoristic species, the bipotential gonad differentiates into the testis or ovary upon sex determination. However, in sequential hermaphrodites (*e.g.* male-to-female sex changers), the bipotential gonad differentiates into the testis upon the initial or primary sex determination signal and remains as testis until a secondary sex determination signal triggers the sex change or differentiation into the ovary.

Among vertebrates, teleosts are the most ancient class and they are unique in the sense that their representatives together possess almost all the known systems of sex determination (Barske and Capel 2008), exhibit the most plastic forms of sex differentiation (Devlin and Nagahama 2002) and present a wide diversity of sexual reproduction strategies ranging from gonochorism to hermaphroditism (Barske and Capel 2008). In contrast, in mammals, specifically eutherians, sex determination involves the Y-linked *Sry* gene and sex differentiation is less amenable to exogenous hormonal or steroidal manipulation (Ditewig and Yao 2005, Barske and Capel 2008). This sex-related diversity in fish is not unexpected, given that a third genome duplication event occurred in the teleost lineage of the ray-finned fish (Actinopterygia) (Meyer and Schartl 1999, Taylor *et al.* 2003, Christoffels *et al.* 2004, Jaillon *et al.* 2004) that provides the genetic material for divergence and speciation.

As a result, there are over 23,500 actinopterygians that make up about half of all vertebrate species and over 99% belong to the teleosts (Volff 2004).

The plasticity of sex differentiation in fish has been demonstrated by a plethora of experiments showing that sex ratios or gonad development can be easily manipulated with exogenous sex steroids or endocrine disruptors (Devlin and Nagahama 2002, Orban *et al.* 2009, Kobayashi *et al.* 2013). In this regard, natural sex change in sequential hermaphrodites found in teleosts is possibly the epitome of this plasticity, as the gonads have to retain the competency to undergo a dramatic physical transformation from a fully functional testis to a fully functional ovary or vice-versa. In a review by De Mitcheson and Liu (2008), functional hermaphroditism could be found in at least 27 teleost families in seven orders with tropical marine perciforms forming a significant group.

However, it is important to recognize that not all sequential hermaphrodites undergo sex change that is a real *de novo* differentiation of the gonad, whereby the entire gonad changes from one sex type to another sex type. Instead, in several hermaphrodites, the gonads comprised of both testicular and ovarian tissues simultaneously with the ovarian tissues in the regressed or non-functional form and the testicular tissue in the active and functional form during the male phase and vice-versa during the female phase. The protandrous gilthead seabream (*Sparus aurata*) (Chaves-Pozo *et al.* 2005) and the serial bi-directional sex changer, *Trimma okinawae*, are examples of sequential hermaphrodites with bisexual gonads (Kobayashi *et al.* 2009).

The protandrous Asian seabass (*Lates calcarifer*), is one perciform that is capable of sex change whereby the entire gonad changes from one sex type to another as depicted in **Figure 1** (Moore 1979, Davis 1982, Guiguen *et al.* 1994). In addition, it

is a popular aquaculture species in this region and its sexual reproduction strategy has brought about obstacles to the genetic improvement (selective breeding) of the species, namely in its long generation time and changing sex ratios. At the same time, this type of natural sex change offers an excellent opportunity to understand more about the basic molecular mechanisms involved in gonad differentiation.

Hence in this thesis, I will focus on uncovering the molecular mechanisms regulating gonad development by using the natural sex change of Asian seabass as a platform and concurrently, expand the existing knowledge regarding the sexual reproduction of this commercially important species. The characteristics of the Asian seabass and topics of sex determination, sex differentiation, sex change and the zebrafish model will be explored in the latter sections of the Introduction.

1.2 Characteristics of the Asian seabass

1.2.1 Distribution, diversity and environment

The Asian seabass belongs to the order Perciforms and can be found naturally in the tropical areas of the Indo-West Pacific region extending from the Indian subcontinent to Northern Australia (Nelson 1994). The Asian seabass is also commonly known by two other vernacular names, the barramundi and the giant perch. Several studies have been performed on the genetic diversity of the species in Southeast Asia and Australia (Keenan 1994, Chenoweth *et al.* 1998, Norfatimah *et al.* 2009). In particular, microsatellite-based analysis has shown that genetic differences exist between Australian and Southeast Asian stocks with the latter more genetically diverse than the Australian stocks (Yue *et al.* 2009). Given the large spread of the Asian seabass native range, DNA barcoding of the mitochondrial *cox1* gene has further shown that Asian seabass from Myanmar and Australia may be two different

species (Ward *et al.* 2008). A recent morphology-based analysis has even concluded that Asian seabass found at Myanmar (*Lates uwisara*) and Sri Lanka (*Lates lakdiva*) are two separate species different from the Asian seabass found in the Indo-Pacific region (Pethiyagoda and Gill 2012).

In the wild, Asian seabass of up to 20 kg could be found and it is a catadromous species that migrates from inland waters of low salinity to coastal waters of high salinity for spawning (Moore 1982). However, analysis of the barium and strontium levels in Asian seabass scales has suggested that there may also exist marine-only populations in Australia (Pender and Griffin 1996).

1.2.2 Commercial importance

The Asian seabass is commercially important both as a food fish and as a sport fish. The global aquaculture production of Asian seabass has risen rapidly from near naught in the 1970s to almost 70,000 tonnes in 2011 (**Figure 2**) and the majority of this production today comes from Southeast Asia, Taiwan and Australia (**Figure 3**). The rapid aquaculture expansion of this species has been primarily a result of the successful captive spawning of Asian seabass and the development of large-scale hatchery production techniques involving omega-3 fatty acids-enriched live feeds (rotifers and *Artemia*) during the early pre-weaning stages (Dhert *et al.* 1990, Dhert *et al.* 1992).

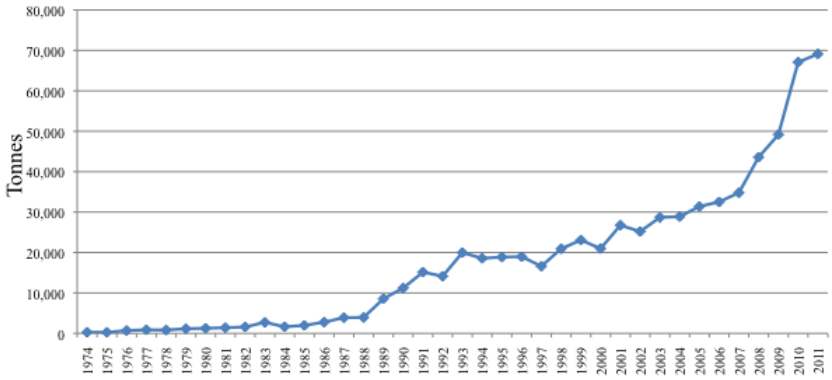

Figure 2. Global aquaculture production of Asian seabass.
Data downloaded from FAO - Fisheries and Aquaculture Information and Statistics Service – accessed on 20/02/2014.

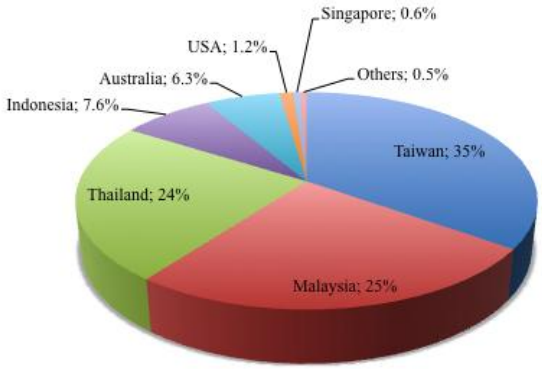

Figure 3. Major aquaculture producers of Asian seabass in 2011.
The global aquaculture production of Asian seabass was 69,116 tonnes in 2011 and was contributed mainly by countries from Southeast Asia, Taiwan and Australia. Data downloaded from FAO - Fisheries and Aquaculture Information and Statistics Service – accessed on 20/02/2014.

This species can be cultured in both freshwater and seawater environments and given the quality of its mild flavored white flesh, it has been promoted widely by industrial-scale farmers to be the "next big fish" (Pierce 2006). In addition, Asian seabass contain high levels of omega-3 fatty acids comparable to those of Chinook salmon, mackerel and menhaden but lower than Atlantic cod (Xia *et al.* 2014). However, it is also a carnivorous species requiring fishmeal and fish oil in its diet although these can be partially replaced with plant-based substitutes (Katersky and Carter 2009, Alhazzaa *et al.* 2011). The Asian seabass is also a cannibalistic species,

requiring frequent grading and separation of sizes to reduce cannibalism during the early culture period (Ribeiro and Qin 2013).

1.2.3 Male-to-female sex change

The most interesting biological aspect of Asian seabass is that it is a protandrous hermaphrodite and there have been extensive histological descriptions of its sexual development (Moore 1979, Davis 1982, Guiguen *et al.* 1994, Szentes *et al.* 2012). Through these studies, the Asian seabass is known to generally mature as males at 2-4 years of age before changing sex to females (Guiguen *et al.* 1994). In addition, the presence of young primary females suggests that there may exist individuals that have either skipped through or transited earlier from the male phase. Similarly, the presence of older males suggests that there may be terminal males that do not undergo sex change (Moore 1979, Davis 1982). In addition, no fixed size or age at sex change could be determined (Moore 1979, Davis 1982).

The interesting sexual reproduction strategy of Asian seabass has implications for aquaculture, a growing industry that is fast overtaking that of capture fisheries. At the same time, natural sex change is also a good platform to understand the molecular mechanisms of sex differentiation. As described previously, the late age at sexual maturity, two years for males and beyond for females, means that there is a long generation time. In addition, within the same generation of Asian seabass, a large percentage exists as males during the early years, while a large percentage exists as females during later years. This creates a problem in maintaining constant sex ratios required for the production of high parental contribution in selective breeding projects based on mass crosses.

1.2.4 Available molecular tools for Asian seabass

As the Asian seabass is an increasingly popular aquaculture species, a few selective breeding projects have begun on the species in recent years. One of them is the marker-assisted selective breeding project undertaken by the Temasek Life Sciences Laboratory (TLL) and Agri-Food and Veterinary Authority of Singapore (AVA) that started in 2003. The project is now at the F_2 generation and moving onto the F_3 generation.

The breeding value of an individual is defined as its genetic potential relative to a trait and is usually estimated by measuring the performance of its progeny (Gjerde 2005). In marker-assisted selection (MAS), the estimation of the breeding values of candidates are based on the analysis of the allelic variation of DNA markers located close to the genes that determine a quantitative trait (also known as quantitative trait loci or QTL). This has several advantages over classical phenotypic-based estimation of breeding values. For example, traits such as fillet yield and disease resistance that cannot be measured directly in the candidates by phenotypic methods can be measured using DNA marker genotyping. In addition, MAS is also unaffected by the variation of the environment.

As the Asian seabass is not a model research organism, several molecular tools had to be generated so that MAS could be carried out. Currently, over 1,200 microsatellite markers have been isolated and a high-resolution linkage map comprising of 790 microsatellites and SNPs (Wang *et al.* 2011) and a BAC-based physical map have been generated (Xia *et al.* 2010). A genotyping platform based on 10 microsatellites has also been developed (Zhu *et al.* 2010). So far, QTLs have been mapped for omega-3 fatty acids (Xia *et al.* 2014) and growth (Xia *et al.* 2013a) in the Asian seabass.

However, as of June 2012, the number of Asian seabass EST sequences deposited in NCBI was about 22,000, an order of magnitude less than other comparatively well studied aquaculture species such as the Atlantic salmon (528,251), channel catfish (357,011), rainbow trout (290,794) and Nile tilapia (121,224). Hence, for this study, there is need to generate more cDNA sequences in order to carry out whole transcriptome profiling analysis of the Asian seabass gonad development and this will be carried out through the use of next generation sequencing technology.

1.3 Diversity of vertebrate sex determination

1.3.1 Genetic and environmental sex determination

Vertebrate sex determination can be genetic or environmental. Genetic sex determination (GSD) can be further classified into chromosomal sex determination (CSD) such as the XX/XY system in mammals where males are the heterogametic sex (XY) (Wallis *et al.* 2008) and the ZZ/ZW system in birds (Smith and Sinclair 2004) where females are the heterogametic sex (ZW). GSD can also be polygenic, which is so far found only in fish, where several genes cumulatively decide on the sexual fate. Examples of polygenic GSD include the zebrafish (Bradley *et al.* 2011, Anderson *et al.* 2012, Liew *et al.* 2012) and the European seabass (*Dicentrarchus labrax*) (Vandeputte *et al.* 2007).

In environmental sex determination (ESD), abiotic factors such as temperature (TSD) decide the sexual fate. The best-known examples of TSD are reptiles such as the crocodiles and turtles, where egg incubation temperature is critical to the sex of the hatchling (Deeming *et al.* 1988). Other less well-studied environmental factors include density, for example in the freshwater eels, *Anguilla*, where high population

density was postulated to lead to male-biased populations (Krueger and Oliveira 1999).

In terms of the genetics of sex determination, only a few master sex determining genes have been discovered so far. The most well known sex determining gene is probably the mammalian *Sry* (review: McElreavey *et al.* 1993). Its transient expression from the Y chromosome peaking at 11.5 days post coitum (dpc) in the mouse (Hacker *et al.* 1995) leads to a feed forward activation of *Sox9*, thus 'switching on' the testis developmental pathway (Sekido and Lovell-Badge 2008) and at the same time represses the ovarian developmental pathway of Rspo1/Wnt/ß-catenin signaling (Lau and Li 2009).

Most of the other candidate master sex determining genes discovered to date are found in teleosts and all are male sex determining like the mammalian *Sry* (Table 1). While several of the master sex determining genes are homologous such as *dmy* of medaka, *dmrt1* of tongue sole, *DM-W* of *Xenopus* and *DMRT1* of chicken, other master sex determining genes have entirely different origins such as the immune-related *sdY* of the rainbow trout (Yano *et al.* 2012). In addition, several of these master sex determining genes actually evolved from the duplication of pro-male genes (*amh*, *dmrt1* and *gsdf*) that function to promote testicular differentiation (see later section).

The diversity of the sex determination signals hence indicates that sex determination is not conserved among vertebrates, especially within teleosts where most of the known sex determination systems can be found. Some teleost genera like the *Oryzias* (medakas) and *Oreochromis* (tilapias) can even have members possessing different CSD systems (*i.e.* XX/XY and ZZ/ZW could be found in species belonging to the same family) (Takehana *et al.* 2007, Cnaani 2013). On the other hand, species

like the zebrafish possess polygenic sex determination system (Liew *et al.* 2012) while cichlids from the genus *Apistogramma* possess TSD (Ospina-Álvarez and Piferrer 2008).

Table 1. Candidate master sex determining genes in non-mammalian vertebrates.

S/N	Gene Symbol	Gene Name	Species	References
1	*dmy (dmrt1bY)*	*doublesex and mab-3 related transcription factor 1, Y-linked*	Japanese medaka (*Oryzias latipes*)	Matsuda *et al.* 2002
2	*dmrt1*	*doublesex and mab-3 related transcription factor 1*	Tongue sole (*Cynoglossus semilaevis*)	Shao *et al.* 2014
3	*gsdfY*	*gonadal soma derived growth factor on the Y chromosome*	Coscosleng or Luzon ricefish (*Oryzias luzonensis*)	Myosho *et al.* 2012
4	*gsdf*	*gonadal somatic cell derived factor* (*gsdf*)	Sablefish (*Anoplopoma fimbria*)	Rondeau *et al.* 2013
5	*amhY*	*anti-Mullerian hormone Y-linked*	Patagonian pejerrey (*Odontesthes hatcheri*)	Hattori *et al.* 2012
6	*amhr2*	*anti-Mullerian hormone receptor, type II*	Japanese fugu (*Takifugu rubripes*)	Kamiya *et al.* 2012
7	*sdY*	*sexually dimorphic on the Y-chromosome*	Rainbow trout (*Oncorhynchus mykiss*)	Yano *et al.* 2012
8	*DM-W*	*Doublesex And Mab-3 Related Transcription Factor 1, W-linked*	African clawed frog (*Xenopus laevis*)	Yoshimoto and Ito 2011
9	*DMRT1*	*Doublesex And Mab-3 Related Transcription Factor 1*	Chicken (*Gallus gallus*)	Smith *et al.* 2009

1.3.2 *Primary and secondary sex determination in sequential hermaphrodites*

Sex determination is not necessarily a developmental concept reserved for gonochoristic species where the two sexes are separate. In the case of sequential hermaphrodites, primary and secondary sex determination have been used to describe the initial commitment of the protandrous black porgy (*Acanthopagrus schlegelii*) to the male fate and the subsequent sex change to become the female respectively (Wu and Chang 2013b). However, while the molecular mechanisms involved in the sex

change in several hermaphrodites have been described to some extent, few secondary sex determination mechanisms have been described so far. The only known secondary sex determination signal is the effect of social influence on several reef species (Wittenrich and Munday 2005). The secondary sex determination signal in the Asian seabass is currently unknown but the search for that is not an objective of this study.

1.4 Conservation of vertebrate sex differentiation

Although the upstream signals for sex determination are diverse, the downstream molecular regulation of sex differentiation is generally conserved across vertebrates. One reason is that the morphological organization of the testis (*i.e.* the testis cord structure) and ovary (*i.e.* the cortical-medullary structure) are similar across vertebrates with the same set of somatic and germ cells (DeFalco and Capel 2009). Secondly and more importantly, across vertebrates, the same genes are found to be involved in sex differentiation and often have the same function and sexual dimorphic expression that can be classified as pro-male or pro-female (reviews: Orban *et al.* 2009, Cutting *et al.* 2013).

Pro-male genes contribute to testicular differentiation and generally have higher expression in the testis compared to the ovary while pro-female genes contribute to ovarian differentiation and their expressions are typically up-regulated in the ovary compared to the testis. Besides individual genes, several signaling pathways have also been shown to be involved in the gonad differentiation process. However, it is important to note that the conservation of sex differentiation is not absolute as not all genes and pathways have identical functions across vertebrates.

1.4.1 Pro-male and pro-female genes of gonad differentiation

In mammals, the master sex determining gene, *Sry*, activates *Sox9* to initiate the testis developmental pathway (Sekido and Lovell-Badge 2008) while several other pro-male genes such as *Dmrt1*, *Amh*, *Wt1* and *Nr5a1* work further downstream in the signaling cascade to further promote testis differentiation (Eggers and Sinclair 2012). With the exception of the *Sry* gene that is found only in mammals, the orthologs of these pro-male genes have testicular differentiation function in teleosts as well. For example in the zebrafish, the corresponding othologs are *sox9a*, *dmrt1*, *amh*, *wt1a* and *nr5a4* and all these genes show higher expression level in the zebrafish testis than in the ovary (von Hofsten and Olsson 2005, Sreenivasan *et al.* 2008).

According to the classical theory, the differentiation of the ovary in the absence of the male master sex determining gene seemed to suggest that ovarian differentiation is a default and passive pathway. However, recent studies have shown that ovarian differentiation is instead an active pathway requiring the activation of several pro-female genes such as *Foxl2*, *Rspo1* and *Wnt4* (Lau and Li 2009, Veitia 2010). Similar to the pro-male genes, these pro-female genes have a role in ovarian differentiation in teleosts as well. Together, these pro-male and pro-female genes serve opposing roles in guiding the developmental fate of the bipotential gonad.

Dmrt1 is a transcription factor known to be involved in testicular differentiation from teleosts to mammals. *Dmrt1* is expressed in both murine Sertoli and germ cells with higher expression in the testis established at 12.5 dpc, shortly after the expression of the sex determining gene *Sry* (Lei *et al.* 2007). *Dmrt1* is dispensable for mammalian ovarian differentiation (Raymond *et al.* 2000) but required in testicular differentiation to inhibit *foxl2* (a pro-female gene) expression in order to maintain Sertoli cell identity (Matson *et al.* 2011). In teleosts, this gene has been shown in

several species to be testis-specific in its expression, including in the rainbow trout (Marchand *et al.* 2000) and in the Nile tilapia, where testis-specific expression of *dmrt1* precedes that of *sox9* and *amh* (Ijiri *et al.* 2008), two other pro-male genes. The disruption of *dmrt1* expression in Nile tilapia also resulted in increased *foxl2* and *cyp19a1* expression (Li *et al.* 2013). *Cyp19a1* or gonadal aromatase is an important steroidogenic gene that will be described in detail in a later sub-section. In addition, as described in the previous section, a duplicated paralog of *dmrt1* has become the master male sex determining gene in several teleosts.

Amh, another pro-male gene, is not a transcription factor but a member of the transforming growth factor-ß family and is expressed 20 hrs after *Sry* expression (Hacker *et al.* 1995) and activated by Sox9 (De Santa Barbara *et al.* 1998) in mouse to promote testis differentiation through the regression and apoptosis of the Mullerian duct (Allard *et al.* 2000). *Amh* acts by inhibiting FSH-stimulated expression of *Cyp19a1* expression (Rouiller-Fabre *et al.* 1998). In zebrafish and Nile tilapia, *amh* shows testis-enhanced expression during gonad differentiation (Rodriguez-Mari *et al.* 2005, Ijiri *et al.* 2008) and *amh* has been further suggested to also inhibit *cyp19a1a* expression in zebrafish (Wang and Orban 2007). Like those of *dmrt1*, *amh* and its receptor *amhr2* have also become the candidate male master sex determining gene in the Patagonian pejerrey and Japanese fugu respectively.

Recently, a new pro-male gene, *gsdf*, was described (Shibata *et al.* 2010). Like *amh*, *gsdf* is a member of the transforming growth factor-ß superfamily and was recently found to have a role in promoting the proliferation of PGC and spermatogonia in rainbow trout (Sawatari *et al.* 2007). *Gsdf1* has also been shown to be a marker for the onset of precocious puberty in European seabass (Crespo *et al.* 2013). Similar to *dmrt1* and *amh*, a paralog of *gsdf* has also evolved to become the

master sex determining gene in two fish species. Interestingly, while *gsdf* is conserved in teleosts, the gene could not be found in mammals and birds (Gautier *et al.* 2011).

On the other end, *Foxl2*, a pro-female gene, is a transcription factor with a dose-dependent function in mammalian ovarian differentiation (Garcia-Ortiz *et al.* 2009). In goat, loss-of-function mutation of *Foxl2* alone can lead to female-to-male sex reversal and has been described as the goat's female sex determining gene (Boulanger *et al.* 2014). However, in mouse, besides *Foxl2*, female-to-male sex reversal requires the additional loss-of-function mutation of *Wnt4*, another pro-female gene (Ottolenghi *et al.* 2007), suggesting some differences in the pro-female function of *Foxl2* in goat and mouse. *Foxl2* have also been shown in mammals and fish to directly activate the expression of *cyp19a1* (Pannetier *et al.* 2006, Wang *et al.* 2007a).

1.4.2 Signaling pathways involved in gonad differentiation

Among pro-female genes, *Rspo1* and *Wnt4* are known to be members of the canonical Wnt signaling pathway, also called Wnt/ß-catenin signaling (MacDonald *et al.* 2009). Wnt signaling has been shown to be a pro-ovarian pathway in mammals where loss-of-function mutation of *Wnt4* in XX mice can lead to masculinization (Vainio *et al.* 1999) and *WNT4* over-expression in XY human males can lead to sex reversal (Jordan *et al.* 2001). Recent gene expression studies have also pointed to the role of Wnt signaling in the ovarian development of teleost. In the zebrafish and medaka, *rspo1* has a higher expression in the ovary compared to the testis (Zhang *et al.* 2011b, Zhou *et al.* 2012). In the rainbow trout, Wnt signaling has been suggested to promote expression of *fst*, a gene required for ovarian development (Nicol *et al.* 2013).

Notch signaling (review: Artavanis-Tsakonas *et al.* 1999) has also been shown to be involved in mammalian ovarian differentiation by promoting the proliferation of granulosa cells (Zhang *et al.* 2011a). On the other hand, during testis development, the blocking of Notch signaling through the use of chemical DAPT or through the deletion of its target gene *Hes1* can result in increased Leydig cells numbers while the constitutive activation of Notch signaling results in severe loss of Leydig cells (Tang *et al.* 2008). This shows that Notch signaling also works to promote the ovarian differentiation by inhibiting the differentiation of the male soma, Leydig cells. However, Notch signaling has not been shown to be involved in teleost sex differentiation so far. Other signaling pathways shown to be involved in ovarian development include retinoic acid signaling that has also been shown in mammals and fish to regulate the onset of meiosis in oocytes (Rodriguez-Mari *et al.* 2010, Childs *et al.* 2011).

In mammalian testicular differentiation, NF-κB signaling (review: Hayden and Ghosh 2008) may play a role in spermatogenesis given that NF-κB proteins were found to be expressed in Sertoli cells and spermatocytes and the levels of NF-κB fluctuate according to specific stages of spermatogenesis (Delfino and Walker 1998). NF-κB has also been shown in humans to activate the testis-enriched expression of *LRWD1* gene, whose reduced transcript levels result in spermatogenic defects (Teng *et al.* 2012). However, in zebrafish, inflammation-induced or sodium deoxycholate-induced activation of NF-κB results in female-biased sex ratios, with the increased survival of oocytes due to modulation of apoptosis-related genes suggested as a possible mechanism (Pradhan *et al.* 2012).

Besides NF-κB signaling, Hedgehog signaling (review: Ingham and McMahon 2001) has also been shown to play a role in mammalian testis differentiation. *Dhh* and

its receptor *Ptch1* are required for Leydig cell differentiation through the activation of *Sf1* (Yao *et al.* 2002) and the signaling pathway is inactive in the fetal ovary (Barsoum and Yao 2011). However, Hedgehog signaling has also been shown to play a role in mammalian ovarian differentiation. *Shh* is required to promote oocyte maturation in pigs (Nguyen *et al.* 2009) while *Ihh* and *Dhh* were found to be expressed in murine granulosa cells to activate gene expression in neighboring theca cells (Wijgerde *et al.* 2005). Hence, Hedgehog signaling can have both pro-male and pro-female functions, possibly depending on the ligand types, messenger molecules, transcription factors and also stage of development. In teleosts, the role of Hedgehog signaling is not clear with conflicting data regarding the role of the signaling pathway in germ cell migration (Deshpande *et al.* 2001, Renault *et al.* 2009).

1.4.3 Steroidogenic genes – effectors of gonad differentiation

The process of steroidogenesis starts with the catabolism of cholesterol into pregnenolone by Cyp11a and this forms the rate-limiting step for the biosynthesis of all steroid hormones (Payne and Hales 2004) (**Figure 4**). Several of the products are important in the regulation of the spawning cycle. These include the progestagens, or maturation-inducing hormones (MIH), derivatives of the C21 steroid 17-hydroxyprogesterone which leads to the maturation of the gametes (spermiation and final oocyte maturation); 11-KT which stimulates spermatogenesis in males; and E2 which stimulates oocyte growth (Mylonas *et al.* 2010; Nagahama 1994) (**Figure 4**).

One of the key downstream target genes during gonad differentiation is the steroidogenic gene, the gonadal aromatase (*cyp19a1*). The pro-male gene, *Amh* has been suggested to inhibit expression of *Cyp19a1* in mammals (Vigier *et al.* 1989, Rouiller-Fabre *et al.* 1998) and fish (Wang and Orban 2007) while pro-female gene

Foxl2 has also been shown to directly activate *Cyp19a1* in mammals (Pannetier *et al.* 2006, Fleming *et al.* 2010) and fish (Wang *et al.* 2007a).

The gonadal aromatase is an important physiological effector of ovarian differentiation due to its role in the conversion of testosterone to estrogens (Baroiller *et al.* 1999). The inhibition of aromatase activity alone can result in the female-to-male sex reversal of several species including Nile tilapia, rainbow trout (Guiguen *et al.* 1999) and Japanese flounder (Kitano *et al.* 2000), while the application of estrogen alone can induce feminization in medaka (Hishida 1965).

On the other hand, 11ß-hydroxylase (*cyp11c1*, previously *cyp11b2*) converts testosterone to 11-ketotestosterone (11-KT) (Baroiller *et al.* 1999), the main and most potent androgen in teleosts (Hishida and Kawamoto 1970). Similarly, application of androgens alone such as 17α-methyltestosterone (17MT) can result in female-to-male sex reversal in teleosts such as Nile tilapia (El-Greisy and El-Gamal 2012) and Japanese flounder (Kitano *et al.* 2000). In several species such as zebrafish (Rodriguez-Mari *et al.* 2005, Wang and Orban 2007) and Nile tilapia (Ijiri *et al.* 2008), *cyp19a1* expression is high and *cyp11c1* expression is low during ovarian differentiation, while the opposite is true for testicular differentiation.

With regards to the sites of steroidogenesis, it is known generally that the Leydig cells of the testes are the major sites of androgen production (Devlin and Nagahama 2002). In the ovaries, the theca cell produce androgens that are transported to the granulosa cells where they are converted to estrogens and progesterone (Ungewitter and Yao 2013).

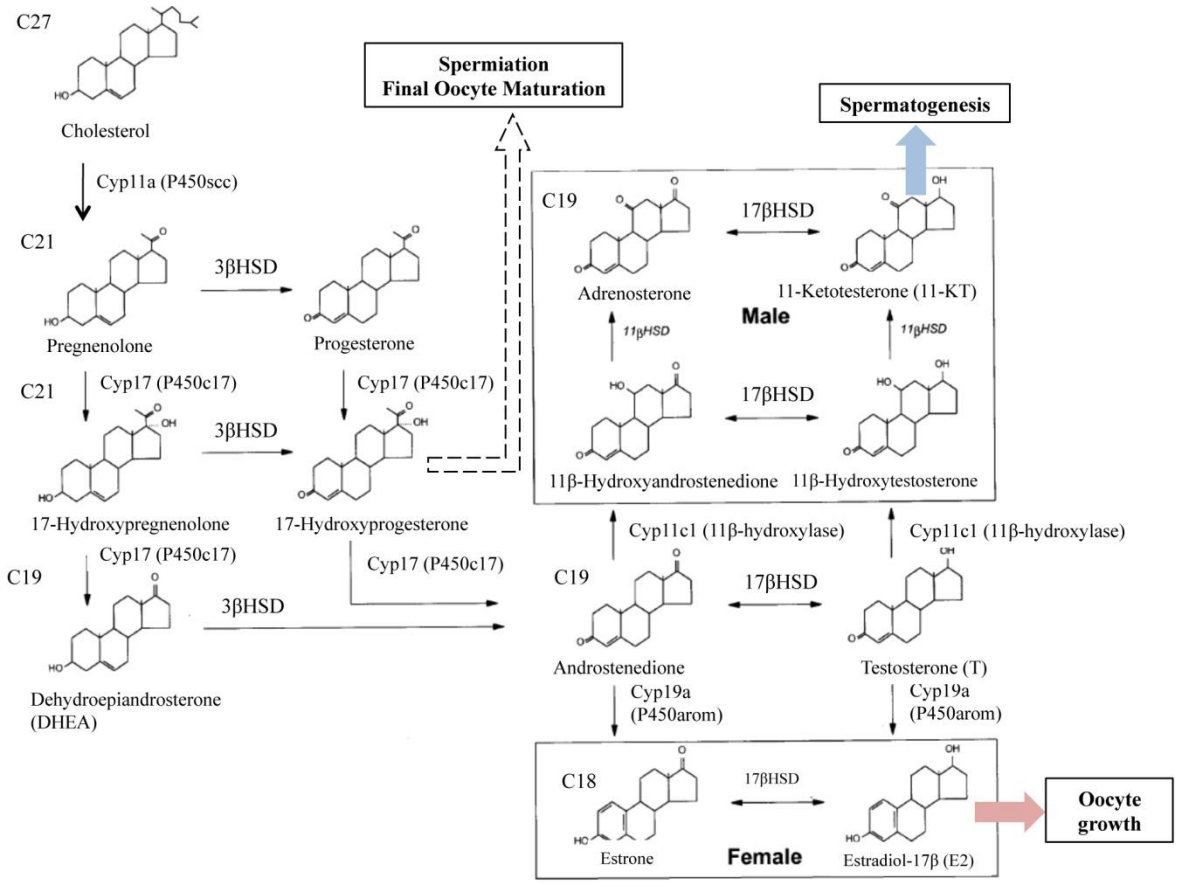

Figure 4. Overview of the synthesis of the sex steroids in fish.
Adapted from Baroiller *et al.* 1999.

1.5 Sex reversal in species with GSD

To understand sex differentiation, sex reversal in teleosts with GSD has been used as a method to identify and uncover the role of sex-related genes during the process. In several of these studies, temperature and hormonal manipulation are the two methods that can successfully induce sex reversal during the early sex differentiation phase of these species.

1.5.1 Temperature and its effect on DNA methylation and cortisol levels

In several teleosts with GSD such as zebrafish and Japanese medaka, high temperatures outside of the natural environmental range can result in sex reversal leading to male-biased sex ratios (Ospina-Álvarez and Piferrer 2008). In the European

seabass, higher temperatures have been shown to result in female-to-male sex reversal through reduced expression of gonadal aromatase (*cyp19a1*) caused by increased DNA methylation at the promoter region of the gene (Navarro-Martin *et al.* 2011). In the tongue sole (*C. semilaevis*) with ZZ/ZW female heterogametic GSD, males and females were found to have different DNA methylation profiles and ZW pseudo-males induced from high rearing temperatures had methylation patterns similar to ZZ males (Shao *et al.* 2014). In particular, the genetic and pseudo-males had lower methylation levels of the pro-male genes *dmrt1* and *gsdf* resulting in their higher expression and vice-versa for the pro-female steroidogenic *cyp19a1* gene (Shao *et al.* 2014).

In other species, like the pejerrey (*O. bonariensis*) (Hattori *et al.* 2009), Japanese flounder (*Paralichthys olivaceus*) (Yamaguchi *et al.* 2010) and medaka (Hayashi *et al.* 2010), increased cortisol was found to be a cause for female-to-male sex reversal induced by high temperatures. In the case of the Japanese flounder, the cortisol was also shown to directly suppress the expression of *cyp19a1* (Yamaguchi *et al.* 2010). However, it is not known if DNA methylation also plays a role in these three studies.

1.5.2 Steroidal treatments and changes to gene expression

In the sex reversal of genetic all female rainbow trout using androgen treatment (11ß-hydroxyandrostenedione), gene expression analysis showed that genes with granulosa cell-enhanced expression in normal females (*e.g. fst*, *cyp19a1*) were down-regulated before genes with potential function in oogenesis (*e.g. gcl* and *vtgr*), suggesting that the masculinization requires a de-differentiation of the granulosa cells (Baron *et al.* 2008).

In another experiment involving the Nile tilapia, it has been shown that even females with fully developed ovaries could be masculinized to form functional testes through long-term treatment of an aromatase inhibitor, Fadrozole (Sun *et al*. 2014). They showed that during sex reversal, the Sertoli and Leydig cells were trans-differentiated from the granulosa and interstitial cells of the ovary, respectively, while the earliest spermatogonia arose from germline stem cell (GSC)-like cells located at the germinal epithelium. Subsequently, the male somatic cells provided the micro-steroidal environment required for the proliferation of the spermatogonia.

In both studies, female-to-male sex reversal caused by steroidal manipulation resulted in a down-regulation of pro-female genes (*e.g. foxl2*) and up-regulation of pro-male genes (*e.g. dmrt1*). However, both studies have also shown that despite having classical testis histology, the sex reversed Nile tilapia and rainbow trout both possessed global gene expression profiles more similar to the control ovaries than to the control testes (Baron *et al.* 2008, Sun *et al.* 2014). According to their speculation, changes in the expression of sex-related genes affected by the hormonal treatments were only a small subset of the global transcriptome which comprised of a larger subset of genes unaffected by the hormonal treatments and expressed from the intrinsic sex chromosomes of both species.

1.6 Sex change in natural hermaphrodites

Besides the use of sex reversal, sex change in natural hermaphrodites has also been used as a platform to study gonad differentiation. Studies of gene expression changes during sex change in natural hermaphrodites have uncovered the involvement of several of the pro-male and pro-female genes described earlier, reinforcing the notion that sex differentiation is conserved in vertebrates. However, so far there is no

large-scale analysis of gene expression changes in any natural hermaphrodites during sex change.

1.6.1 The protandrous black porgy

The black porgy (*Acanthopagrus schlegeli)* is one of the best-studied hermaphrodites. This is possibly because sex change in the protandrous black porgy is highly predictable with 50% of the males changing into females during the third year of their lives (Wu and Chang 2013b). In addition, the black porgy is also an important food fish in China, Taiwan and Japan (Leu 1997, Mana and Kawamura 2002, Hong and Zhang 2003). Furthermore, sex change in the black porgy can be triggered by surgical removal of testicular tissue resulting in ovary with vitellogenic oocytes (Wu and Chang 2009) or by long term E2 administration resulting in ovary without vitellogenic oocytes (Wu *et al.* 2008).

During the male-to-female sex change, several genes have been found to be differentially expressed, including the up-regulation of pro-female *cyp19a1* and *nr5a4* and decreased transcript levels of pro-male, *nr0b1* (previously *dax*) (Wu *et al.* 2008). Wnt signaling has also been implicated as *wnt4* was found to be up-regulated in association with ovarian growth during early sex change (Wu and Chang 2009). *Dmrt1* was also found to be required to maintain the testis fate and its knockdown can result in testis regression and sex change (Wu *et al.* 2012). In addition, *dmrt1* was shown to be activated by gonadotropins, and serum luteinizing hormone (LH) levels was in turn detected to decrease during sex change (Wu *et al.* 2012). Hence the brain-pituitary-gonadal (BPG) axis was suggested to be the secondary sex determination switch in black porgy (Wu and Chang 2013b). The BPG axis was also found to be a key factor in the sex change of the serial bi-directional sex changer, *Trimma okinawae*

with the active tissue (testis or ovary) of the bisexual gonad expressing more of the gonadotropin receptors (*fshr* and *lhr*) (Kobayashi *et al.* 2009).

1.6.2 The protogynous groupers and wrasses

Other studied hermaphrodites include the groupers and wrasses. In the protogynous honeycomb grouper (*Epinephelus merra*), pro-female *foxl2* was down-regulated and pro-male *dmrt1* was up-regulated during ovary-to-testis transition (Alam *et al.* 2008) while in another protogyne, the threespot wrasse (*Halichoeres trimaculatus*), pro-male *gsdf* expression increases in the somatic cells of transforming gonads (Horiguchi *et al.* 2013). On the other hand, in the latter species, contradictory increased expression of pro-female *foxl2* was observed during ovary-to-testis gonad transformation (Kobayashi *et al.* 2010). Hence, exceptions do exist in the conservation of vertebrate sex differentiation.

1.7 Zebrafish as a model for gonad differentiation studies

The zebrafish is a small freshwater teleost that is easy to keep and attains sexual maturity at 10 to 12 weeks (Westerfield 2000). This species, pioneered by George Streisinger, has long been used as a model organism for developmental study of early processes such as neurogenesis and somitogenesis due to its transparent embryos. In addition, the zebrafish reference genome (Howe *et al.* 2013), its catalog of protein-coding genes (Kettleborough *et al.* 2013) and SNPs and insertions/ deletions (Patowary *et al.* 2013) are now available.

Besides the availability of whole genome and protein information, there are several functional tools that allow reverse genetics to be carried in the zebrafish, making it a good genetic model organism. Transgenic zebrafish lines can be easily

generated using the *tol2* transposon system (Suster *et al.* 2009) while modified oligonucleotides known as morpholinos can be used to transiently knock down gene expression via direct micro-injections into the zebrafish embryos (Nasevicius and Ekker 2000). Recently, site-specific zinc-finger nucleases and transcription activator-like effector nucleases (TALENs) have been developed to allow targeted gene inactivation in the zebrafish (Meng *et al.* 2008, Bedell *et al.* 2012).

Zebrafish is an undifferentiated gonochorist and has been described in the past as a juvenile hermaphrodite due to the gonadal transformation process observed in juveniles that are fated to be males. During early sexual differentiation, all zebrafish develop ovary-like gonads or juvenile ovaries (JO) before transforming into testes in the male zebrafish or in the case of the female zebrafish, the development continues into mature ovaries (Takahasi 1977, Uchida *et al.* 2002, Maack and Segner 2003). The use of various transgenic reporter lines and histological analysis showed that males vary among themselves in the onset of transformation and in the number of oocytes in the juvenile ovary prior to the transformation (Hsiao and Tsai 2003, Onichtchouk *et al.* 2003, Wang *et al.* 2007b). This analysis also revealed the presence of three types of males in the *Tg(vasa:vasa-EGFP)zf45* transgenic line (Wang *et al.* 2007b): Type I with very few oocytes during the juvenile ovary phase and corresponding weak activity of the reporter gene; Type II with low-medium oocyte count and intermediate transgene expression; and Type III with female-like oocyte numbers and high transgene expression. A transcriptome analysis of the zebrafish testis and ovary has similarly implicated the involvement of several pro-male and pro-female genes (Sreenivasan *et al.* 2008).

While the zebrafish is not a true hermaphrodite and in addition undergoes ‘sex change’ in the opposite direction as the Asian seabass, the obvious signs of

conservation of sex differentiation indicate that it is possible to use the zebrafish as a model for Asian seabass in the functional validation of sex genes.

1.8 Objective and aims of this study

Despite the extensive morphological, histological and biochemical descriptions of the Asian seabass sexual development, the molecular mechanisms underlying its sexual development especially during the sex change process are poorly understood. In addition, little is known about the sexual state of the Asian seabass during the long period prior to it becoming a sexually mature male. Furthermore, research on natural sex change in the Asian seabass could contribute to better understanding of the basic molecular regulation of sequential hermaphroditism and even the gonad differentiation process in gonochoristic teleosts.

Hence, the main objective of this study was to expand the current knowledge on sexual development of the Asian seabass and uncover the molecular mechanisms regulating the gonad development process in this species. To this end, the study aimed to:

i. Collect a repertoire of Asian seabass gonads ranging from juveniles to adults; classify them according to existing histological criteria developed previously for the species (Guiguen *et al.* 1994); and gather data on the sex and age of captive breeding stocks.
ii. Generate the molecular tools necessary for the study of global gene expression patterns in the gonads of this non-model organism by utilizing next generation sequencing technology (NGS).

iii. Profile and analyze the gene expression patterns of the various sexual maturation stages of Asian seabass gonads using the tools developed and functionally validate some of the data on the zebrafish.

iv. Test and develop methods that can solve aquaculture-related problems such as non-invasive sexing methods and/or promotion of early sexual maturation.

2 Materials and Methods

2.1 Ethics Statement

All experiments and procedures were approved by TLL Institutional Animal Care and Use Committee (approval ID: TLL(F)-10-001; TLL(F)-10-003) for experiments carried out at Temasek Life Sciences Laboratory and AVA Institutional Animal Care and Use Committee (approval numbers: AVA-MAC-2011-01, AVA-MAC-2012-01) for experiments carried out at Marine Aquaculture Centre, Agri-Food and Veterinary Authority of Singapore.

2.2 Fish stocks

Asian seabass individuals were collected from the Marine Aquaculture Centre of the Agri-Food and Veterinary Authority of Singapore and from farms in Singapore. The Asian seabass were reared in full seawater conditions of 29-31 parts per thousand (ppt) salinity and under ambient temperatures of 28-31ºC.

For the zebrafish-related experiments, wild-type zebrafish of the AB strain and the transgenic zebrafish strains, *Tg(vasa:vasa-EGFP)zf45*, *Tg(TOP:GFP)w25* and *Tg(hsp70I:dkk1-GFP)w32* were kept at TLL fish facility at 26-28°C and at a 14 hour light and 10 hour dark cycle in AHAB (Aquatic Habitats) recirculation systems.

2.3 Captive breeding of Asian seabass

Asian seabass broodstock were kept in 20-60 tons tanks and spawning was carried out either naturally or induced using gonadotropin-releasing hormone (GnRH) (LHRHa, Argent Laboratories). Fertilized eggs were incubated in 29-31 ppt seawater

at under ambient temperatures of 28-31ºC. The larvae were fed rotifers (*Brachionus plicatilis*) enriched with algae (*Nannochloropsis oculata*) from two days post fertilization (dpf) to 14 dpf while *Artemia* nauplii (INVE Aquaculture) were introduced from 12 dpf onwards. At 15 dpf, weaning was gradually carried out as artificial larvae feed (Otohime, Marubeni Nisshin Feed Co. Ltd) was introduced while the amount of live feeds was reduced until complete weaning at 15 dpf. As the fingerlings grow larger, pelleted feed (Skretting) of increasing size was introduced. Grading was subsequently carried out regularly in 5-7 days interval from about 30 dpf onwards to minimize cannibalism until the seabass were about three inches in length.

2.4 Histology and staging of Asian seabass gonads

Gonads were fixed in 10% formalin overnight at room temperature. After dehydration by ethanol, samples were embedded in plastic resin (Leica Biosystems), and then serial cross-sections of 5–10 μm were cut using a microtome (Leica Biosystems) and dried on slides at 42 °C overnight. The sections were stained with hematoxylin and eosin and then mounted in Permount (Thermo Fisher Scientific). The Asian seabass gonads were classified according to sexual maturation status as described previously (Guiguen *et al.* 1994).

2.5 RNA isolation and cDNA synthesis

In this study, total RNA was prepared using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. For fibrous tissues such as muscle, skin and intestine, an additional step of proteinase K treatment was included after the homogenization step. This step involved adding 10 μl of proteinase K solution

(Roche) and 590 µl of RNase-free water to the homogenized sample (comprising of 300 µl RLT) and incubated at 55 ºC for 10 min.

RNA quality and concentration were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop spectrophotometer, respectively. For samples sent for next generation sequencing, Qubit Fluorometer (Life Technologies) was used to determine the concentration.

All cDNA used in this study were reverse transcribed from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions.

2.6 Sample preparation for Asian seabass transcriptome sequencing

Transcriptome sequencing was carried out on three different platforms, SOLiD 3+ (Applied Biosystems), 454 FLX Titanium (Roche) and HiSeq 2000 (Illumina). Asian seabass RNA was prepared in this study with the library preparation and sequencing outsourced to service providers (AIT Biotech Pte Ltd, Macrogen Inc and YourGene Bioscience). The assembly was then carried out with the help of in-house laboratory bioinformaticians.

For SOLiD 3+ sequencing, total RNAs were obtained separately from Asian seabass testes and ovaries using Qiagen RNeasy mini kit and were separately depleted of ribosomal RNA using a RiboMinus Eukaryote Kit for RNA-seq (Invitrogen). The depletion was verified using Agilent 2100 Bioanalyzer (Agilent Technologies) before and after the depletion steps and quantity was determined using Qubit Fluorometer (Life Technologies). The two rRNA-depleted samples (testis and ovary) were then sent to a service provider (AIT Biotech Pte Ltd) for library preparation of two

sequencing libraries and the subsequent sequencing. The two libraries were prepared according to the SOLiD Whole Transcriptome Analysis Kit (ABI, PN 4425680).

For 454 sequencing, rRNA-depletion of total RNA was similarly carried out. This time, total RNA was extracted from more organs and tissues and these samples were grouped into seven different pools that were then separately depleted of rRNA using the same method described earlier (**Figure 5**). These rRNA-depleted pools of RNA were then combined in equal quantities into one sample and sent to a 454 sequencing service provider (Macrogen Inc). The single-end sequencing was carried out on one full PicoTiterPlate (PTP) run and the library was prepared by the service provider according to the cDNA Rapid Library Preparation Method (Roche, October 2009).

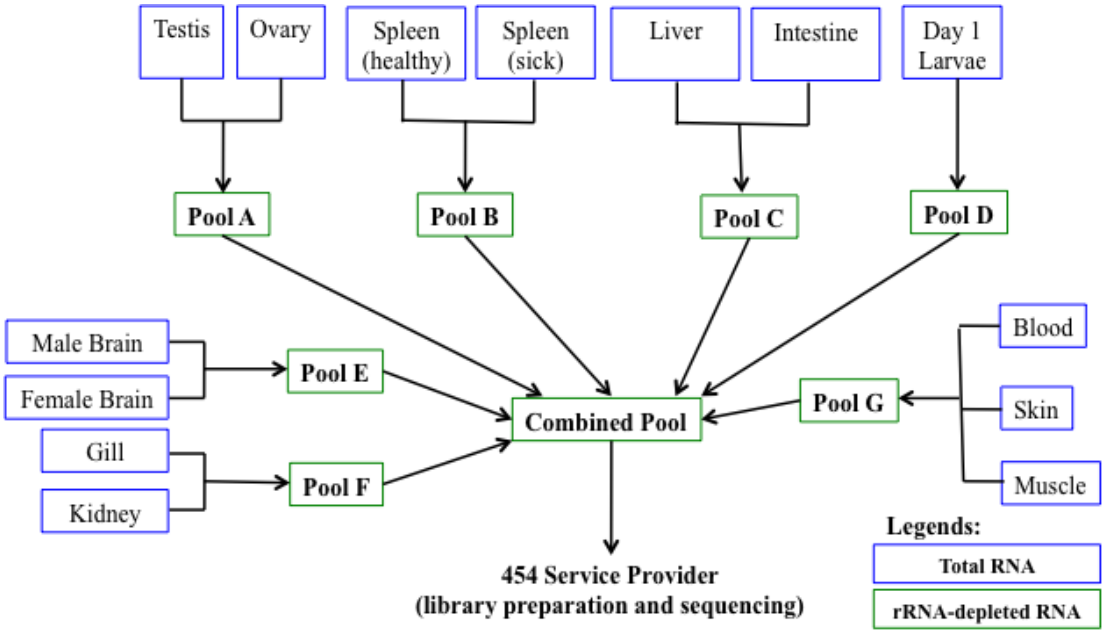

Figure 5. Overview of sample preparation steps for 454 FLX Titanium sequencing.
Total RNA extracted from various organs and tissues of Asian seabass were grouped equally into seven pools (A-G) and rRNA-depletion was carried out separately on each pool using Invitrogen's RiboMinus kit. The pools of rRNA-depleted RNA were then combined in equal quantities for sequencing as one library. Blue boxes indicate total RNA and green boxes indicate rRNA-depleted RNA.

For Illumina HiSeq sequencing, two combined pools of RNA were prepared, one that comprised of RNA extracted from stomach, pyloric caeca, intestine and whole larvae that may contain contaminating microorganisms. The other combined pool comprised of RNA extracted from other organs and tissues that were unlikely to contain contaminations (**Figure 6**). Two libraries (2x100bp paired-end), one normalized and one un-normalized were prepared by the service provider (YourGene Bioscience) from each of the two combined pools for a total of four libraries. Normalization was carried out using duplex-specific thermostable nuclease (DSN) protocols. All four libraries were mRNA-enriched and strand-specific protocols were also incorporated into the library preparation steps using TruSeq RNA Sample Preparation Kit v2 (Illumina) and ScriptSeq v2 Library Preparation kit (Epicentre) respectively.

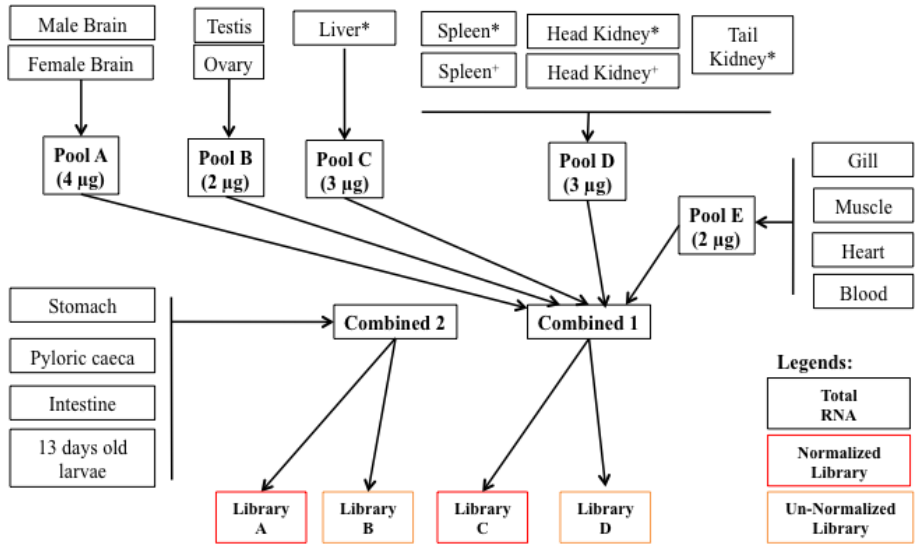

Figure 6. Overview of the library preparation steps for Illumina HiSeq 2000 sequencing.
Two combined pools of total RNA were prepared and sent to a service provider for generation of four libraries and subsequent sequencing. All four libraries were prepared from mRNA-enriched RNA with strand-specificity protocols incorporated into the 2x100bp paired-end libraries. Libraries A and C had additional processing that involved a normalization step. Black boxes indicate total RNA, red boxes indicate normalized library and orange boxes indicate un-normalized library. *Sample is obtained from seabass vaccinated with commercial *Streptococcus iniae* vaccine (Norvax Strep Si, MSD Animal Health). $^{+}$Sample is obtained from seabass infected with "big belly" disease (Gibson-Kueh *et al.* 2004).

2.7 De novo assembly of the Asian seabass transcriptome

Several *de novo* assemblies had been carried out prior to the availability of all sequencing data and in-house bioinformatics support, resulting in several versions of the Asian seabass transcriptome. In particular, the *de-novo* assembly of SOLiD-only data using Velvet (Zerbino and Birney 2008) and co-assembly of 454 and SOLiD data using the programs Velvet, CLC Genomics Workbench (CLC Bio, version 5.1) and Sequencher (Gene Codes), were carried out before the availability of HiSeq data. Both assemblies had been deposited into NCBI TSA database with the accession numbers GAML00000000 and GAMU00000000 respectively. Part of these assembled data had been used in the design of primers for the real-time qPCR carried out in this study. Subsequently, when Hi-Seq data was available, *de novo* assembly using Hi-Seq only data was carried by in-house bioinformaticians and deposited into NCBI TSA database (accession number GAQL00000000). The assembly workflow described below was the final assembly used for the subsequent design of the expression microarray (**Figure 7**).

Reads from 454 (SRR949061) and SOLiD (SRR944005, SRR944006) sequencing runs were co-assembled using the CLC Genomics Workbench (version 5.1), and then merged with about 22,000 Asian seabass EST sequences from NCBI Genbank as well as sequences from an Asian seabass 454-based intestine assembly (Xia *et al.* 2013b) using CAP3. In parallel, reads from HiSeq sequencing (SRR1032078, SRR1032087, SRR1032088, SRR1032089), following quality- and adapter-trimming and removal of reads that were potentially of contaminant origin, were assembled using Trinity. The resulting contigs were then further assembled using CAP3. The contigs from the 454, SOLiD and EST co-assembly were

subsequently merged with the contigs from the HiSeq assembly to result in a dataset of 362,369 contigs.

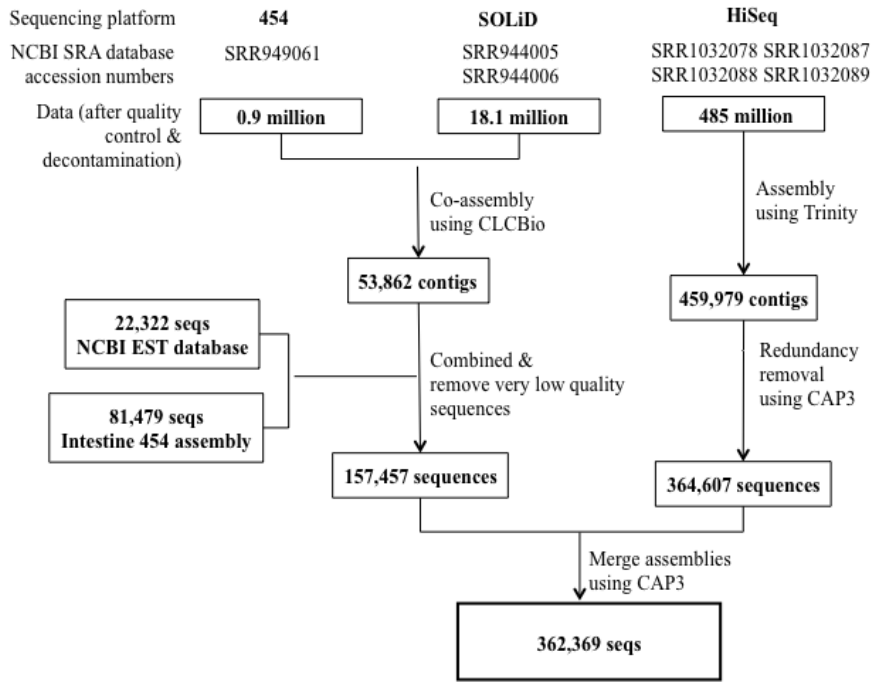

Figure 7. *De novo* assembly workflow for Asian seabass transcriptome.
Reads from 454 and SOLiD sequencing were co-assembled first before another co-assembly with sequences available from NCBI databases. HiSeq-only reads were assembled before co-assembling with that of 454, SOLiD and NCBI assembly. A total of 362,369 contigs were obtained.

2.8 Design of Asian seabass expression microarray

The Asian seabass expression microarray utilized the Agilent SurePrint G3 custom gene expression 8X60K oligonucleotide arrays platform (Cat No. G4102A). Each microarray slide consists of eight arrays, each containing 60,000 probes (60'mer oligo probe). Hence, eight different samples could be used on each microarray slide.

With this platform, one probe was sufficient to represent one transcript. Hence, 60,000 transcripts had to be shortlisted from the 362,369 assembled sequences (**Figure 8**). A blast search was first carried against NCBI Refseq Database (comprising of teleosts, *Xenopus* and human sequences) to shortlist sequences with

known identity. Only blast hits with e-value < 1e-4 and aligned length greater than 200bp (for Blastn) or 65 amino acids (for Blastx) were considered. The longest sequence among the multiple sequences that had the same top hit was selected for probe design to reduce redundancy. This amounted to 28,109 sequences.

Another 30,000 probes were then selected from sequences with no blast hits by mapping SOLiD reads obtained from the testis and ovary libraries to sequences without blast hits using CLC Genomics Workbench (version 5.1). Sequences that had coverage by the SOLiD reads at more than 25 and fold-change between ovary and testis at more than 10-fold were selected. This amounted to another 33,600 sequences. A total of 61,709 sequences were hence shortlisted. The online eArray software (https://earray.chem.agilent.com/earray/) from Agilent was then used to design probes using these sequences. A total of 60,080 sequences had probes designed and printed onto the microarray.

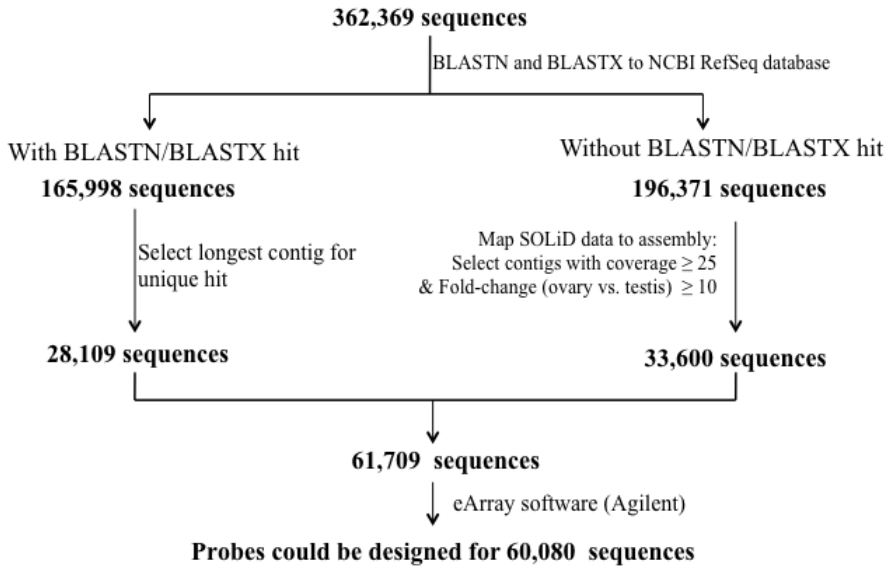

Figure 8. Workflow for the shortlisting of sequences for microarray probe design.
The microarray comprised of probes generated based on Asian seabass sequences with known identity (has similarity to deposited NCBI mRNA or protein sequences) and also of unknown identity (no similarity to any deposited NCBI mRNA or protein sequences).

2.9 Real-time qPCR using qPCR array

Real-time qPCR was performed using a BioMark HD system (Fluidigm Corporation). In this qPCR array system, real-time qPCR can be carried out simultaneously on 48 samples targeting 48 genes using a 48.48 Dynamic Array Integrated Fluidic Circuit (IFC) or 96 samples and 96 genes using a 96.96 IFC.

Specific target amplification was first carried out on each cDNA sample. This step involved mixing all the 48 or 96 primer pairs at a final concentration of 0.05 μM to the cDNA and the TaqMan PreAmp Master Mix (Life Technologies). The mixture was then placed into a thermal cycler for 14 cycles of amplification of the gene-of-interests (GOIs). The cDNA with enriched GOIs was then loaded onto the IFC according to Fluidigm's EvaGreen DNA Binding Dye protocols before being run by the BioMark machine.

Primers were designed using NCBI Primer-BLAST (Ye *et al.* 2012) by setting the optimal melting temperature to 60 °C. The primer sequences for the Asian seabass and zebrafish experiments are listed in appendices Table A 1 and Table A 2 respectively.

2.10 Microarray hybridization

The microarray experiments were carried out on Agilent SurePrint G3 custom gene expression 8X60K oligonucleotide arrays (Cat No. G4102A). All microarray data had been deposited into NCBI Gene Expression Omnibus database (Asian seabass gonadal analysis: GPL17855; Asian seabass brain analysis: GSE53901).

Total RNA (100 ng) was reverse transcribed and labeled using Agilent Low Input Quick Amp One Color labeling kit (Cat No. 5190-2305) according to the

manufacturer's instructions. cRNA samples were labeled with cyanine 3-CTP and the amount of cyanine 3-labeled cRNA was determined using NanoDrop. Labeled cRNA samples with cRNA yields > 0.825 µg and specific activity > 6 pmol Cy3 per µg cRNA were used for hybridization. Equal amounts of labeled target sample cRNA (600 ng) were hybridized to each individual array. Microarray hybridization conditions and washing procedures were performed as described in Agilent Gene Analysis protocols (One Color Microarray-Based Gene Expression Analysis, Version 6.5). The microarray slides were scanned using Profile AgilentG3_GX_1Color and the scan data were extracted using Agilent Feature Extraction Software.

2.11 Gonadotropin-releasing hormone (GnRH) induction of Asian seabass

GnRH was prepared by diluting LHRHa (1mg, Argent Laboratories) with sterile saline solution (0.9% NaCl). LHRHa is an analog of mammalian GnRH substituted with D alanine in position 6 and deletion and replacement of the tenth amino acid with ethylamide. Asian seabass were first sedated by 30 parts per million (ppm) of AQUI-S (50% isoeugenol; Aqui-S New Zealand Ltd) before injected with GnRH using a 1 ml Tuberculin syringe at the dorsal muscle region at a dose of 10 µg/kg body weight.

2.12 Sexing of Asian seabass

Asian seabass that were not sacrificed for sample collection were sexed by stripping (applying abdominal pressure) to check for the presence of milt and cannulation using a catheter to check for presence of eggs. The sex of the fish would be considered unknown if no gametes could be obtained. These Asian seabass were induced with GnRH a day before in order to facilitate the sexing.

2.13 ELISA measurement of mucus 11-KT

Levels of 11-KT in the Asian seabass body mucus were measured using the 11-KT EIA kit (Cayman Chemical). Mucus were collected from the seabass body using three cotton buds and stored in -80 ºC until ready for use. The cotton buds were thawed and vortexed briefly in 1.2 ml of 20 µM tris-buffered NaCl (0.9%), pH 7.4, in a 7 ml Bijou container to extract the mucus into the saline solution. 900 µl of the solution was then transferred to glass culture tubes where the sample is extracted twice with diethyl ether (Sigma-Aldrich), and after evaporation, reconstituted with 300 µl of EIA buffer. The purified sample is then used according to the manufacturers' instructions for the 11-KT EIA kit. The reminder of the saline solution was used for a Coomassie Plus (Bradford) assay (Pierce) according to the manufacturer's instructions. The measurements obtained from the 11-KT kit were adjusted for the dilution factor and normalized using the total soluble protein quantified by the Bradford assay.

2.14 Hormone implantation in Asian seabass juveniles

Slow release hormone pellets were made by dissolving poly(ethylene-co-vinyl acetate) (EVAc) (Sigma-Aldrich) in dichloromethane (CHROMASOLV® Plus, Sigma-Aldrich) (10%w/v) at 42 °C in a glass cylinder. The required amount of hormones was then added into the mixture, stirred and allowed to cool. Pellets (15mm x 1.5mm x 1mm) were then cut from the solidified mixture to be used for implantation using a passive integrated transponder (PIT) tag implanter. The pellets were implanted into the dorsal muscle of the fish. Four types of pellets were made: control pellet containing no hormones; 17MT pellet containing 2.1±0.2mg 17MT

(Sigma-Aldrich); Dom pellets containing 4.2±0.8mg domperidone (Argent) and GnRH pellets containing 0.118±0.016mg LHRHa (Argent).

2.15 Collection of zebrafish gonads

For the real-time qPCR validation of a previous laboratory microarray result, 35 dpf juvenile ovary (JO) and 35 dpf juvenile ovotestis (JOT) were removed from the *Tg(vasa:vasa-EGFP)zf45* zebrafish individuals with the aid of Egfp visualization under a dissecting fluorescent microscope and by using fine forceps. The juvenile ovaries had much stronger Egfp signal than the ovotestes (**Figure 9**).

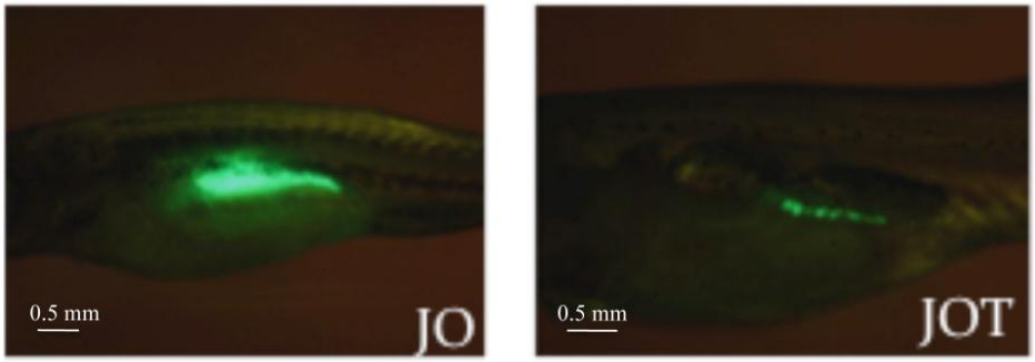

Figure 9. Egfp expression in *Tg(vasa:vasa-EGFP)zf45* transgenic zebrafish.
Egfp is strongly expressed in 35 dpf JO compared to the transforming JOT of *Tg(vasa:vasa-EGFP)zf45* zebrafish as observed under the dissecting fluorescent microscope. The JOT shown here is similar to the Type II and Type III males described by Wang *et al.* (2007b).

For the heat-shock experiments, trunk sections (without head and tail sections) of 36 dpf zebrafish from heat-shocked wild-type and heat-shocked transgenic F_1 (*Tg(hsp70I:dkk1-GFP)w32*) were collected, as in the absence of *Tg(vasa:vasa-EGFP)zf45*, direct gonad collection was impossible.

2.16 Immunohistochemistry on zebrafish gonads

Immunohistochemistry was carried out on cryosections of heat-shocked juvenile *Tg(hsp70I:dkk1-GFP)w32* testes and ovaries. Tissue sections were first incubated with primary antibody (chicken polyclonal anti-GFP antibody; Abcam) in blocking solution overnight at 4ºC followed by a one-hour room temperature incubation of secondary antibody (Alexa Fluor 488 Goat Anti-Chicken Antibody; Invitrogen) and DAPI (Sigma-Aldrich).

2.17 Chemical treatments of IWR-1-endo on zebrafish

IWR-1-endo (Chen *et al.* 2009) was used as a treatment to inhibit Wnt signaling during the zebrafish gonad differentiation period from 15 to 40 dpf. IWR-1-endo was dissolved in DMSO to make a 10 mM stock solution. For the treatment groups, 0.2 ml of the 10 mM stock was added to one litre of egg water to make 2 μM of IWR-1-endo. An equal volume of DMSO was added to the control group. The egg water with DMSO or IWR1-endo was changed daily.

2.18 Heat shock experiments on zebrafish

A single *Tg(hsp70l:dkk1b-GFP)w32*$^{+/-}$ hemizygous zebrafish (*Tg(dkk)/-*) was crossed to a single wild-type AB strain partner. The resulting offspring, consisting of about 50% wild-type and 50% *Tg(dkk)/-* F_1 siblings, were reared together. After about two weeks, the larvae were transferred to a wire gauze-cage measuring 15 cm x 10 cm x 5 cm and kept within an 800 ml water tank.

At 20 dpf, all the F_1 fish were heat-shocked for two hours by transferring the wire-gauze cage containing the fish from the original water tank to another 800 ml water tank pre-heated overnight in an incubator set at 39.0°C. After two hours, the

entire water tank together with the wire-gauze cage and fish were transferred to another incubator set at 28.5°C for gradual cooling to normal temperature. This process was repeated every day until 60 dpf, including and well beyond the period when zebrafish gonad differentiation is known to occur normally (Wang *et al.* 2007b).

At the end of the heat-shock treatment, the fish were transferred to an AHAB recirculation system and subsequently reared together until about 70 to 80 dpf when they were sorted according to size to prevent stunting. At about 100 to 120 dpf, the fish were sorted according to the phenotype presented by the dorsal fin and then sexed by visual analysis of the dissected gonads.

2.19 Statistical analyses

For the microarray experiments, Partek Genomic Suite (version 6.6) was used to analyze the data. The microarray data were quantile normalized and $\log_2$ transformed for statistical analysis. Principal component analysis (PCA) was first carried out as a preliminary analysis and quality control. PCA is a statistical method that linearly transforms the expression values of the 60,080 probes into three new variables, allowing the samples to be plotted into a three-dimensional graph (Jolliffe 1988).

Subsequently, to identify transcripts with differential expression between two gonad types, a two-way ANOVA was carried out. In this analysis, the gonad type was listed as the fixed effect experimental factor while the microarray chip was listed as the random effect experimental factor so as to remove the batch effect introduced by the use of multiple microarray chips. (For the microarray experiment on Asian seabass brains, one-way ANOVA was carried out as only one microarray chip was utilized.) In addition, false discovery rate (FDR; Benjamini and Hochberg multiple testing correction (Benjamini and Hochberg 1995)) was included into the calculation

of the p-value. A transcript was considered significantly differentially expressed when p-value FDR <0.01 and fold-change ≥1.5 or ≤-1.5. A one-way ANOVA analysis was also performed to identify genes that showed significant variation (p-value FDR < 0.0001) across the various gonad types and the expression profiles of these genes were used to generate a hierarchical clustering map using the Partek software.

For real-time qPCR carried out using the qPCR array, the Ct value of each gene in each replicate was automatically selected by the software, Fluidigm Real-Time PCR Analysis (version 3.0.2, Fluidigm Corporation). The relative quantity (RQ) of each gene in each sample (relative to the sample with the highest quantity of the gene) was calculated by the formula *2^(MinCt-AvgCt)*. The RQ of the candidate reference genes were used as the input data for GeNorm analysis (Vandesompele *et al.* 2002) to select for reference genes that showed the highest gene expression stability and the optimal number of reference genes to be used. Subsequently, normalization of RQ was carried out through the division of the geometric mean of the RQ of the selected reference genes. The normalized RQ values were subsequently $\log_2$ transformed and the Partek software described in the previous paragraph was used to generate a three dimensional PCA plot for preliminary analysis and quality control. A one-way ANOVA was also performed using the Partek software to identify genes that showed significant variation against the gonad type (p-value < 0.05) and the genes were then used to generate a hierarchical clustering map. The fold-change in expression of a gene between two gonad types was calculated through the division of the average normalized RQ while p-value was calculated using Microsoft Excel's two-tailed Student's T-Test assuming unequal variance on the $\log_2$ normalized RQ values. A transcript was considered significant differentially expressed when p-value < 0.05 and fold-change ≥1.5 or ≤-1.5.

3 Results

3.1 Identification of adult Asian seabass testes, transforming gonads and ovaries at various sexual maturation stages

To begin the analysis of the Asian seabass sexual development, gonads were collected from sacrificed captive-bred adult Asian seabass over a period of four years. A part of every gonad collected was analyzed by histology in order to classify the gonad according to sexual maturation stages described by Guiguen *et al.* 1994 while the remainder of the gonad was stored in -80ºC for RNA extraction.

In total, 77 gonads from adult Asian seabass were collected, of which 29 turned out to be testes, 14 transforming gonads and 34 ovaries at various sexual maturation stages based on their histological analysis (Table 2). Substantial portions of ovarian tissues could not be found in the testes and vice-versa for testicular ones in the ovaries (**Figure 10**, panels A-D, I-L). However, one of the adult testes contained small numbers of primary oocytes (see section 3.2). Similarly, the transforming gonads were not intersex gonads that contained significant amount of both testicular and ovarian tissues. Instead the transforming gonads seemed to have undergone testicular degeneration with gradual appearance of primary oocytes during the early transforming stages (T1-T2) before more substantial numbers of primary oocytes appeared in the late transforming stages (T3-T4) when no testicular tissues could be found anymore (**Figure 10**, panels E-H).

Table 2. Classification of gonads collected from adult seabass based on histological analysis.

Sex type	Index	Gonadal maturation stage	*Histological criterion	Average weight (kg)	No. collected
Male	M1	Testis gonia	Mostly gonia	3.0±1.2	4
	M2	Spermatogenesis	Mostly spermatocytes and spermatids	3.4±0.4	3
	M3	Spermiation	Mostly spermatozoa	2.8±0.5	20
	M4	Post-spawning	Absence of spermatozoa in testicular lobules	3.7±0.0	2
Transiting	T1-2	Early Transiting	Degeneration of testis with appearance of some primary oocytes	3.2±0.6	8
	T3-4	Late Transiting	Appearance of substantial primary oocytes with no testicular tissue	3.4±0.6	6
Female	F1	Pre-vitellogenesis	Mostly pre-vitellogenic oocytes	3.7±1.2	8
	F2	Early vitellogenesis	Vitellogenic oocytes less than half of section	4.1±0.8	5
	F3	Vitellogenesis	Vitellogenic oocytes more than half of section	3.5±1.5	13
	F4	Atretic	Presence of atretic oocytes	4.1±1.5	8
		Total number of samples			**77**

*Based on Guiguen *et al.* (1994).

3.2 Detection of 'juvenile testis', 'oocytes-in-testes', primary females and terminal males among captive-bred Asian seabass

The collection of gonads was extended to that of juvenile captive-bred Asian seabass ranging from 4.5 to 9 months post-hatching (mph) and despite their string-like appearances, the sexual maturation stages described in the previous section could be identified (Table 3). Gonads from two months old Asian seabass were also collected, but they were too small for histological analysis.

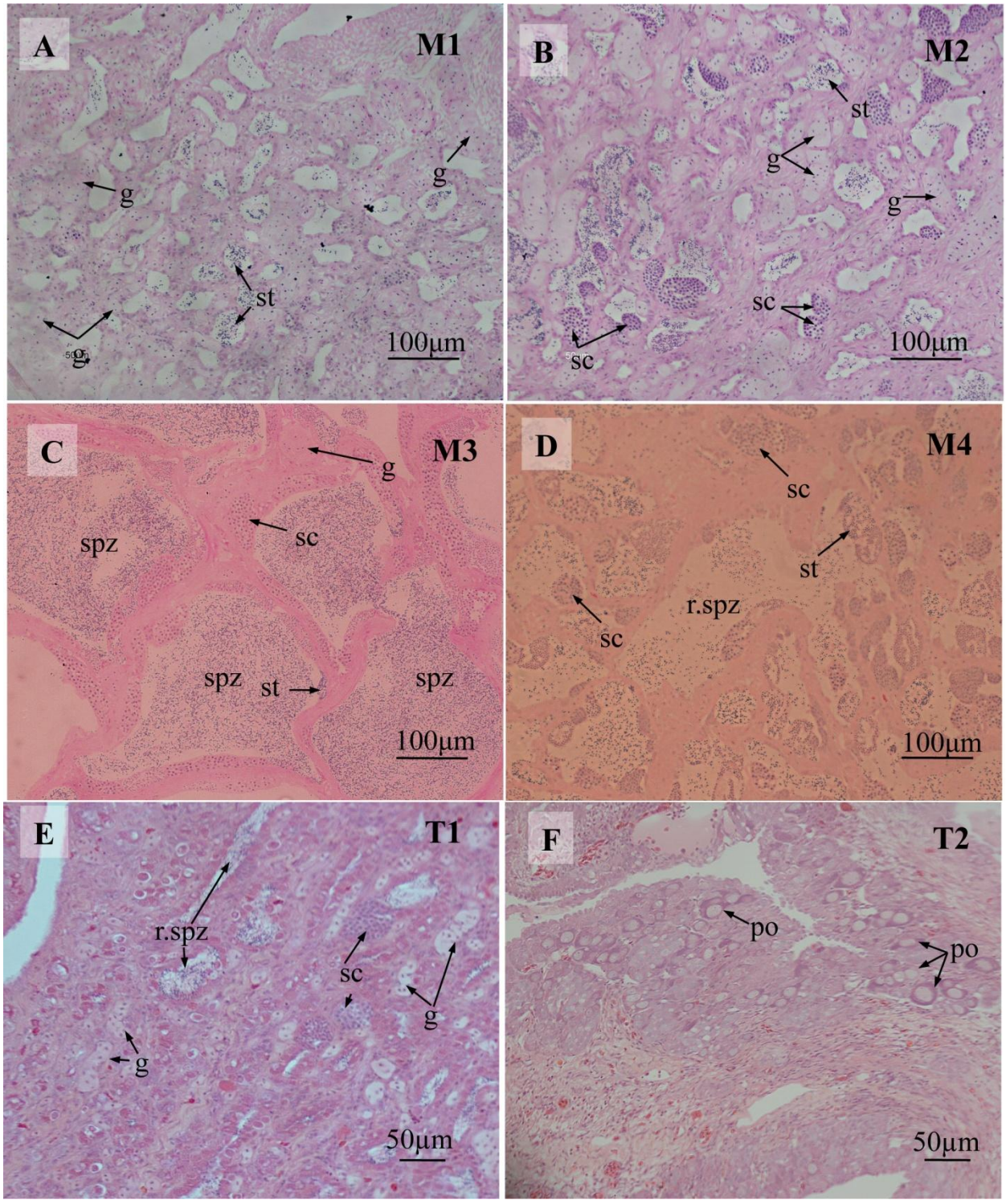

Figure 10. Stages of gonad maturation collected from adult Asian seabass based on the classification by Guiguen *et al.* 1994.

Panel A-D: Testes of the M1, M2, M3 and M4 stages. Panel E-H: Transforming gonads of the T1, T2, T3 and T4 stages. Panel I-L: Ovaries of the F1, F2, F3 and F4 stages. Abbreviations: ao – atretic oocytes; ca – cortical alveolus oocytes; g – gonia; po – perinucleolar oocytes; r.spz – residual spermatozoa; sc – spermatogonia; spz – spermatozoa; st – spermatids; vi – vitellogenic oocytes.

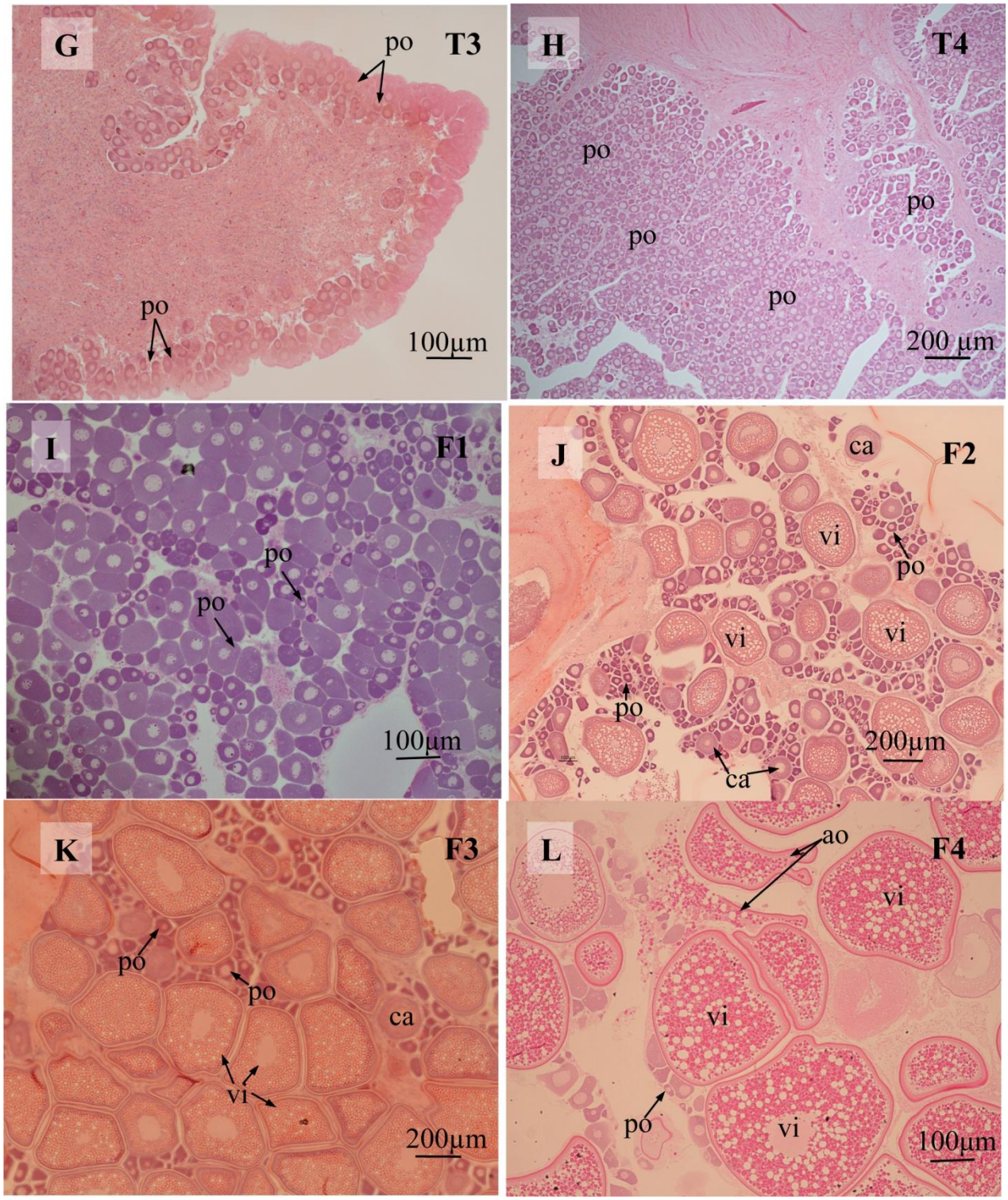

Figure 10 (cont'd). Stages of gonad maturation collected from adult Asian seabass based on the classification by Guiguen *et al.* 1994.

Table 3. Classification of gonads collected from juvenile Asian seabass between 4.5-9 months post hatching (mph).

S/N	Age (mph)	No. collected	Average Weight (g)	Gonadal Maturation Index (number of seabass)
1	4.5	11	65±12	Undifferentiated (11)
2	7.5	6	155±18	M1 (1); M2 (5)
3	8-9	10	368±63	M1 (2); M2 (1); M3 (7)

At 4.5 mph, the gonads were undifferentiated in appearance and contained no visible testicular or ovarian tissues except for the presence of germ cells at the gonia stage (**Figure 11**). However, at 7.5 mph, all the gonads collected were either M1 or M2 stage testes with M3 stage (spermiating) testes appearing at 8-9 months old seabass. In addition, these M3 stage testes or 'juvenile testes' were very small in physical size in comparison to the adult M3 stage testes (**Figure 12**).

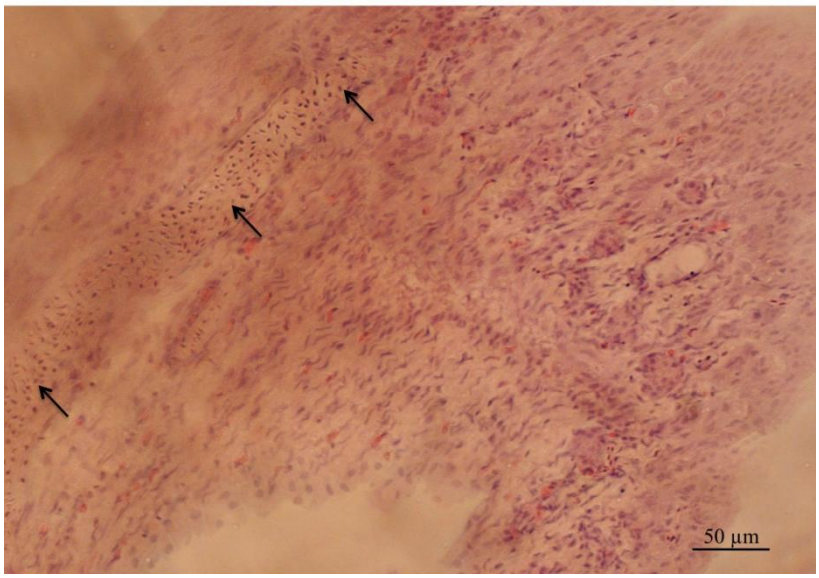

Figure 11. Undifferentiated Asian seabass gonad with no testicular or ovarian tissues.
The gonad was collected from a 4.5 mph juvenile seabass weighing about 69 g. The arrows indicate gonial cells.

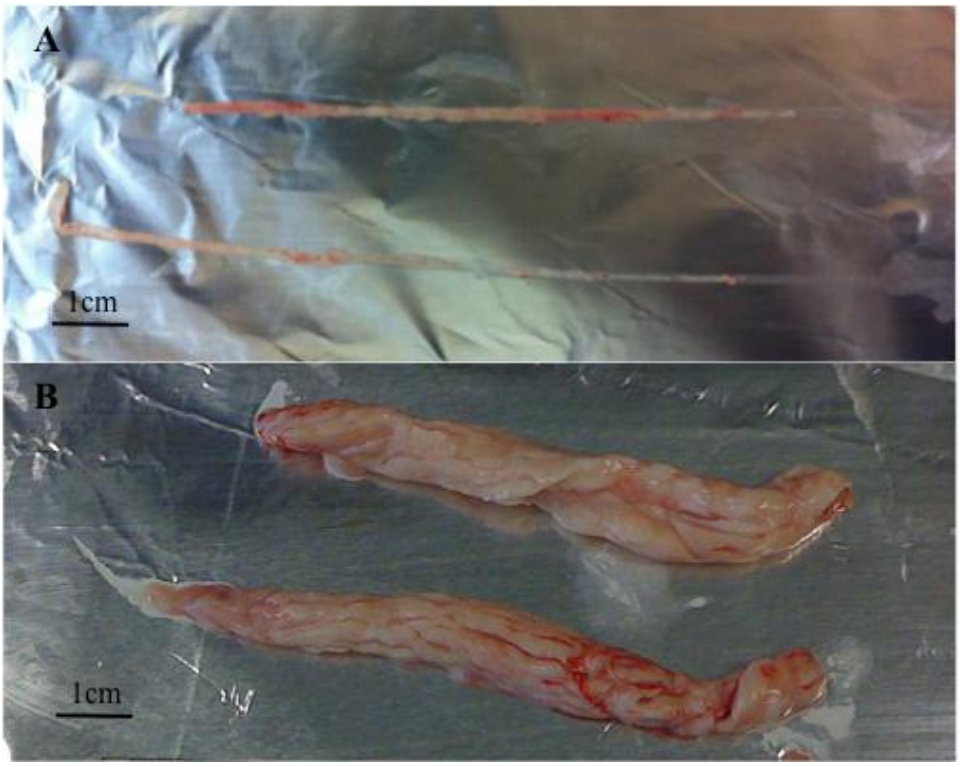

Figure 12. Physical appearance of M3 stage testes collected from a 9 mph juvenile seabass (A) and an adult seabass (B).
Despite having similar histological structure, the physical size of the juvenile testis from a 9 mph seabass weighing about 400 g is very small compared to a fully matured testis from an over 2 years old seabass weighing 3.3 kg.

Among the specimen in the collection of Asian seabass testes histology, one of the adult testes was confirmed to have primary (or perinucleolar) oocytes within the testicular tissues. This phenomenon was also found in one out of the 16 juveniles that were 7.5-9 mph of age (**Figure 13**). However, the rest of juvenile or adult testes had no primary oocytes residing within them.

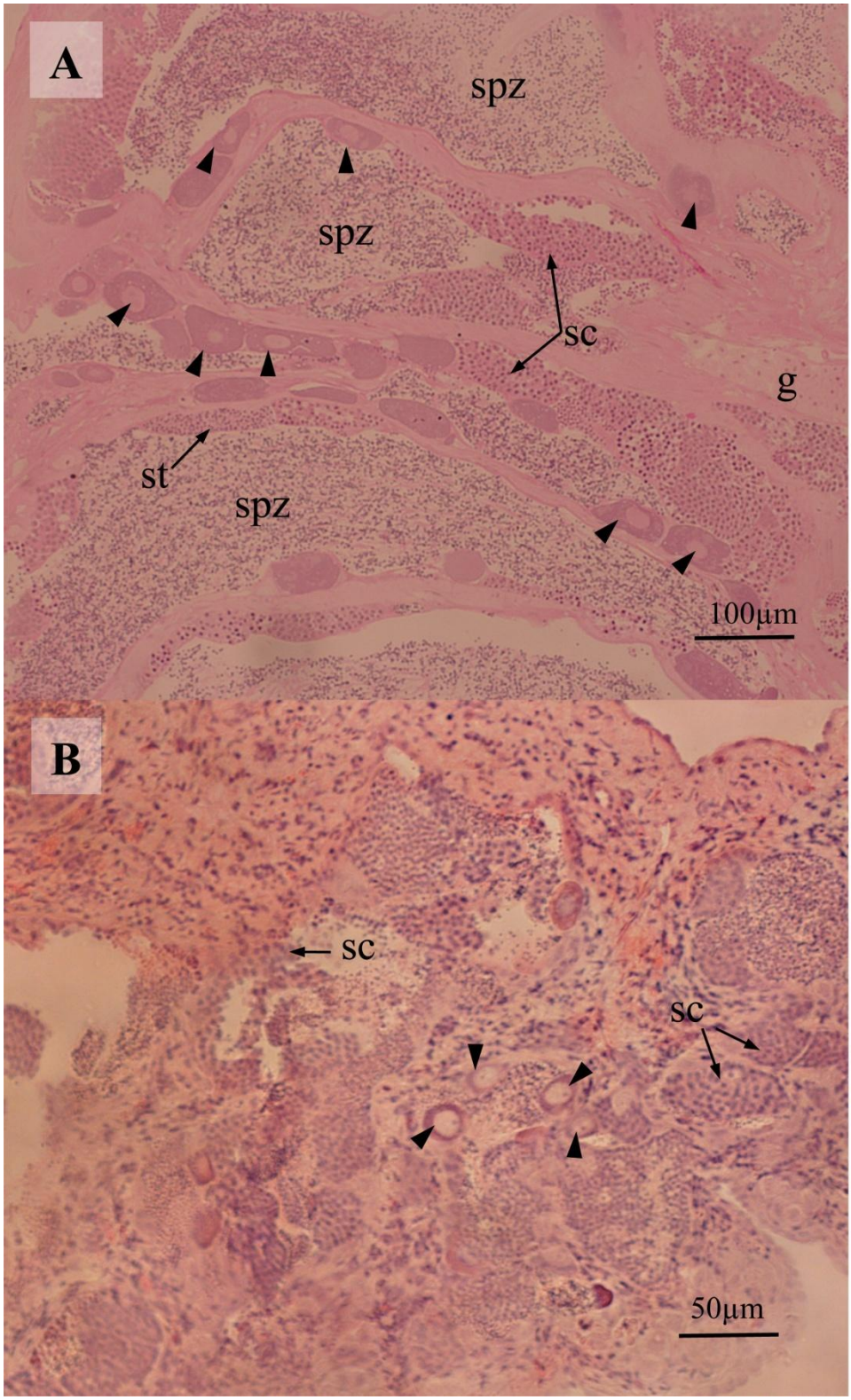

Figure 13. The presence of primary oocytes in adult (A) and juvenile (B) testes. (A) Perinucleolar oocytes could be detected from the testis of an adult male seabass. (B) One of the gonads from 7.5 months old Asian seabass investigated also contained primary oocytes in its M2 stage testis. Abbreviations: sc – spermatocytes; spz – spermatozoa; st – spermatids. Arrowheads indicate primary oocytes.

The analysis of the Asian seabass gonads was also extended to broodstocks in order to detect for the presence of primary females and terminal males as described previously on Asian seabass reared in Papua New Guinea and Northern Australia (Moore 1979, Davis 1982). This time, the Asian seabass were not sacrificed, but sexed through cannulation or massaging of abdomen to detect for presence of milt or oocytes.

A very small proportion of females (2%) could be identified among a batch of 1.8 years old Asian seabass. This was at an age when only 31% were milting when induced with GnRH while no oocytes or sperm could be detected from the rest (**Figure 14**). In a separate stock of 2.5 years old seabass, the percentage of females and milting males detected both rose to 9% and 53%, respectively. This result confirmed the presence of primary females that either went through the male phase early and quickly or entirely skipped through the male phase.

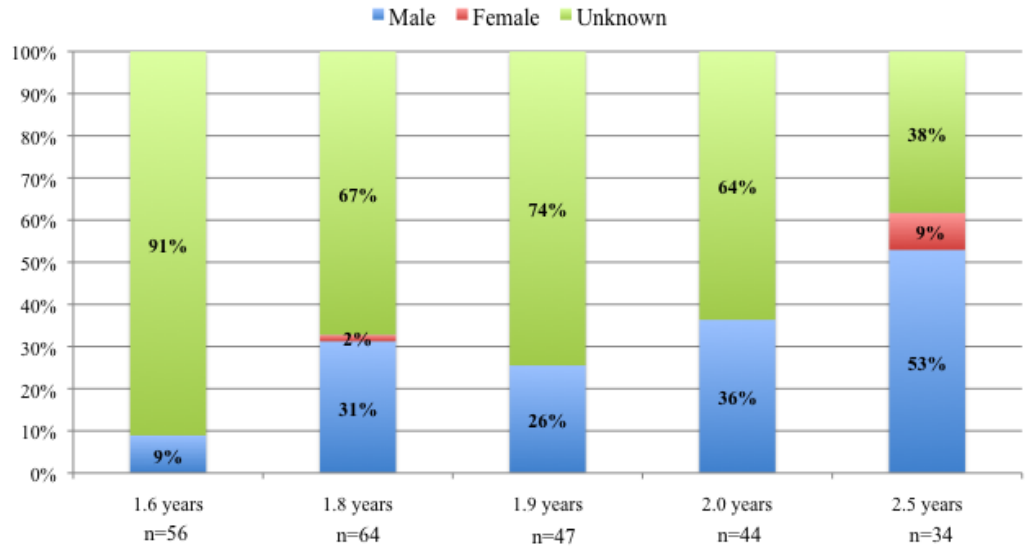

Figure 14. Female Asian seabass could be found as early as 1.8 years of age.
Five different batches of Asian seabass of various ages were sexed by checking for the presence of gametes (sperm or oocyte). One out of 64 seabass (2%) at 1.8 years old was a female while three females could be found from among 34 seabass of 2.5 years of age (9%). The proportion of milting males could also be observed to increase with age from 9% at 1.6 years old to 26-36% at 1.8-2.0 years old and 53% at 2.5 years old.

Similarly, old males could be found among a stock of 16 Asian seabass aged 13 years that were still spawning regularly upon GnRH induction and producing viable larvae (Table 4). In 2013, there were 18 spawning days and hatched larvae could be obtained from the majority of these spawning events. This indicated the presence of functional male seabass among those in the spawning nucleus, hence providing a strong indication for the presence of terminal males that do not change sex.

Table 4. Frequency of spawning occasions in a group of 13 years old Asian seabass.

S/N	Date of spawning	No. of eggs spawned (million)	No. used for culture (million)	No. of hatched larvae (million)	Hatching rate (%)	Type of spawning
1	22-Jan-2013	4.5	3	2.7	88%	Induced
2	23-Jan-2013	3.5	2	1.4	70%	Induced
3	20-Feb-2013	Not available	1.8	1.3	73%	Induced
4	21-Feb-2013	N.A.	1.5	1.2	80%	Induced
5	28-Mar-2013	2.7	2	1.7	85%	Induced
6	29-Mar-2013	2.0	1.2	1.0	83%	Induced
7	30-Mar-2013	2.0	1.9	1.6	84%	Induced
8	24-Apr-2013	3.3	2.6	2.0	77%	Induced
9	25-Apr-2013	3.0	2.5	2.1	84%	Induced
10	26-Apr-2013	0.01	Not collected	N.A	N.A.	Induced
11	26-Jun-2013	6.5	5	3.9	78%	Induced
12	27-Jun-2013	5.0	4.8	4.0	83%	Induced
13	31-Jul-2013	8.0	5	4.1	82%	Induced
14	1-Aug-2013	2.5	1.7	1.3	76%	Induced
15	18-Sep-2013	2.0	Not collected	-	-	Natural
16	20-Sep-2013	1.8	Not collected	-	-	Natural
17	27-Nov-2013	Not available	1.1	0.8	73%	Natural
18	28-Nov-2013	Not available	1.1	0.7	64%	Natural

3.3 Identification of Nile tilapia orthologous proteins in the Asian seabass transcriptome

Due to the lack of sufficient number of Asian seabass EST sequences available from NCBI at the start of this study for the species, next generation sequencing technology was used to generate the Asian seabass transcriptome. A total of 363,369 transcript contigs were assembled from the transcriptome sequencing efforts. To estimate the completeness of the transcriptome sequencing, Blastx was carried out against the Nile tilapia RefSeq protein database containing 26,675 sequences (downloaded Oct 2013). Based on the criteria of e-value < 1e-6 and aligned length > 65 amino acids, a total of 22,497 (84.3%) Nile tilapia proteins were found to have significant hits against the Asian seabass transcriptome. Hence, by using the Nile tilapia protein database as a proxy, the Asian seabass transcriptome was estimated to be about 84% complete. The information from the sequenced transcriptome was subsequently used to generate primers for a mid-throughput qPCR array and a high throughput expression microarray.

3.4 Gene expression analysis revealed differences in the sexual maturation stages of testes and ovaries

To analyze the differences between and within groups of adult Asian seabass testis and ovary samples at the various sexual maturation stages, a 48.48 qPCR array based on the BioMark HD system (Fluidigm Corporation) was generated. This qPCR array was used to analyze the expression patterns of 37 sex-related genes in 30 adult Asian seabass testes and ovaries (Table 5). Orthologs of the selected genes had known reproductive functions in mammals and/or other vertebrate species. Eight genes (*18 s*, *bactin*, *ef1a*, *gapdh*, *rpl8*, *tuba*, *catd* and *ubq*) were also included as internal control

genes of which *rpl8*, *ef1a* and *ubq* had the highest gene expression stability based on GeNorm analysis (Vandesompele *et al.* 2002) and were used as reference genes.

Table 5. Type of gonad samples used for the 48.48 qPCR array.

Sex type	Index	Gonadal maturation stage	Number of samples
Female	F1	Pre-vitellogenesis	4
	F3	Vitellogenesis	7
	F4	Atretic	6
Male	M1	Testis gonia	3
	M2	Spermatogenesis	2
	M3	Spermiation	6
	M4	Post-spawning	2
		Total number of samples	**30**

3.4.1 Sexually dimorphic expression of genes between seabass testes and ovaries

Of the 37 genes chosen (not including the eight reference genes), 86% (32/37) showed sexually dimorphic expression (p-value <0.05 and fold-change ≥1.5 or ≤-1.5) when ovaries of F3 and F4 stages were compared against testes of M3 and M4 stages (Table 6). The expression of 25 of these genes was enhanced in testes, with 13 genes showing more than 10-fold up-regulation when compared with ovaries. In contrast, seven genes were found to have ovary-enhanced expression, of which *zp2* showed the highest up-regulation (7.1-fold) in ovaries compared with testes.

3.4.2 The expression profiles of 36 genes were sufficient to distinguish between male and female gonads types

All the genes that were tested, except *foxl2* and the reference genes, showed significant differences in their expression levels across the various gonad types in a one-way ANOVA analysis (p-value < 0.05). The expression patterns of these 36 genes were subsequently used to generate a hierarchical clustering map (**Figure 15**).

Table 6. Genes that were analyzed between Asian seabass testes (M3 and M4) and ovaries (F3 and F4) and classified according to functions and pathways.

Gene symbol	Accession	Fold-change (Ovary vs. Testis)	p-value
Known sex-related			
sox9	KF444460	−51.8	4.95E-04
wt1	KF444464	−25.0	0.024
amh	GAMU01071817	−19.7	9.49E-10
nr5a2	KF444453	−15.2	0.007
dmrt1	KF444450	−7.8	0.000
nr0b1	KF444458	−7.6	0.013
Germ cell markers			
odf3	GAMU01119126	−2628.1	2.64E-13
sycp3l	GAML01036838	−25.8	1.16E-06
tdrd7	GAML01004579	−15.4	1.37E-08
piwil1	GAML01007253	−10.6	4.26E-10
sept6	KF444459	−7.5	2.27E-10
vasa	KF444462	−3.2	1.18E-04
tdrd1	GAML01005267	−2.2	0.002
sept7	GAML01001618	−1.5	0.026
zp2	KF444465	7.1	0.003
Steroidogenesis			
cyp11c1	KF444447	−377.8	1.56E-15
hsd3b	KF444455	−42.1	0.038
hsd11b2	KF444456	−12.8	0.025
cyp19a1	AY684256.1	−5.5	1.47E-04
cyp17a1	KF444448	−5.0	0.005
hsd17b1	KF444457	−4.2	0.786 (N.S.)*
Wnt signaling			
foxl2	KF444454	−4.9	0.434 (N.S.)
axin1	KF444443	−3.1	0.242 (N.S.)
ctnnbip1	KF444446	1.5	0.072 (N.S.)
ck2a	KF444444	2.0	0.042
ctnnb1	KF444445	3.5	0.001
dvl2	KF444451	4.5	0.001
Retinoic acid signaling			
cyp26b1	KF444449	−36.3	5.61E-06
stra6	GAML01004693	1.3	0.079 (N.S.)
cyp26a1	GAML01005182	1.8	0.030
NF-κB-related			
nfkb2	GAMU01013914	−3.1	2.74E-04
nkap	GAML01003947	−3.0	0.001
Apoptosis			
tp53	KF444461	2.0	0.020
Others			
esr1	KF444452	−25.1	6.68E-05
ar	KF444442	−4.9	2.29E-06
psap	GAML01003564	−2.1	0.001
vtgr	KF444463	2.9	0.009

*N.S. - Not significant (fold-change < 1.5 or > -1.5 or p-value ≥ 0.05)

Published in Ravi and Jiang et al. 2014, Table 2.

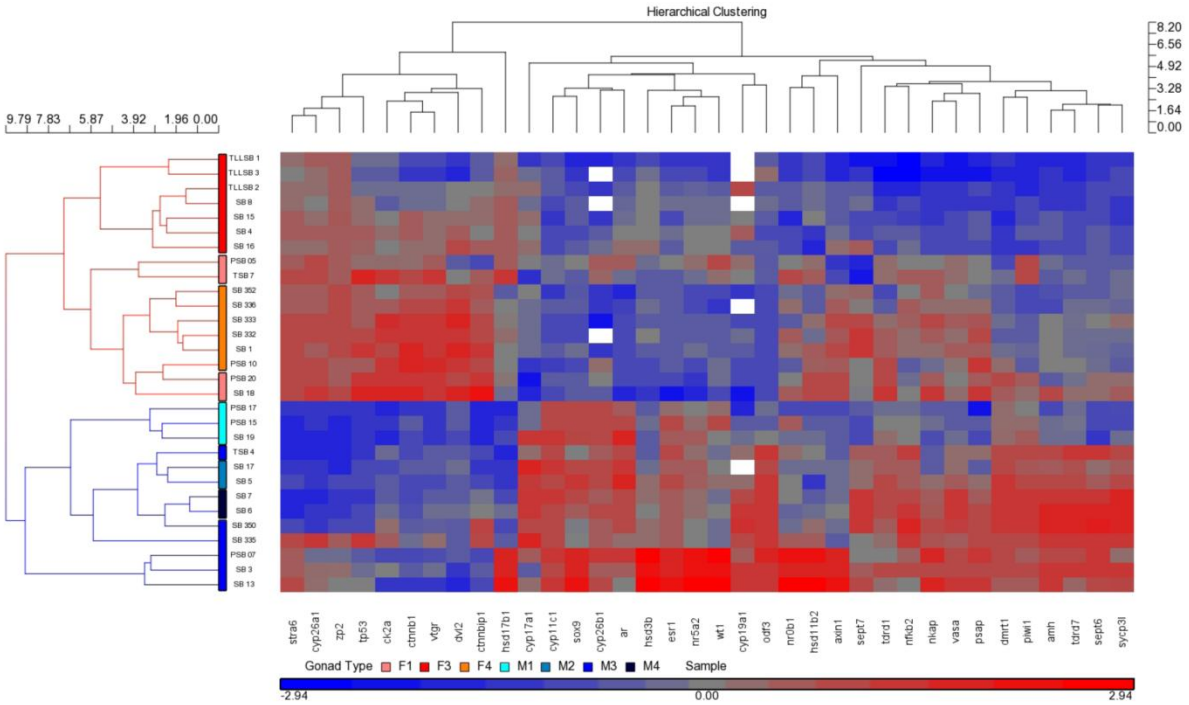

Figure 15. Male and female Asian seabass gonads could be clearly distinguished based on the expression of 36 genes with sex-related function.
The expression profiles of 36 sex-related genes with p-value (gonad type) < 0.05 under a one-way ANOVA analysis were used to generate the hierarchical clustering map. Male and female gonads were clustered into two different clades. Within the female gonad clade (top section), F3 and F4 ovaries were further divided into two sub-clades, which indicated that their gene expression profiles were different. Red boxes indicate high expression, whereas blue boxes indicate low expression. White boxes within the clustering map indicate missing values for a particular sample and gene.
Published in Ravi and Jiang et al. 2014, Figure 1.

In the hierarchical clustering map, the ovarian and testicular samples were clustered into two separate primary clades and within the ovary clade, the F3 ovaries were clustered into their own sub-clade. This indicated that the gene expression profile of F3 ovaries was different from those of ovaries from other stages. In particular, while F3 and F4 ovaries differed only in the presence of atresia in the oocytes of the F4 but not F3 ovaries, 70% of the tested genes (26/37) were differentially expressed between them.

The genes that were up-regulated in the F4 ovaries included germ cell markers, such as *vasa*, *piwil1*, *tdrd1* and *sycp3l*; testis-enhanced genes such as *dmrt1*, *amh*, *nr0b1*; Wnt signaling member genes such as *axin1*, *dvl2*, *ctnnb1*, *ck2a* and *ctnnbip1*; apoptosis-related genes such as *tp53* and genes involved in retinoic acid pathway (*cyp26a1* and *stra6*) (see appendices Table A 3).

3.4.3 Female-like expression levels of amh and germ cell markers in M1 testes

Similarly, in the hierarchical clustering map, within the testes clade, the inactive M1 testes were clustered in their own sub-clade. M1 testes contained predominantly gonia and they were inactive and not capable of spawning. Gene expression analysis showed that these M1 testes have female-like expression levels of the testis-enhanced germ cell markers, *odf3*, *sycp3l*, *septin6* and *tdrd7* and also *amh* (**Figure 16**).

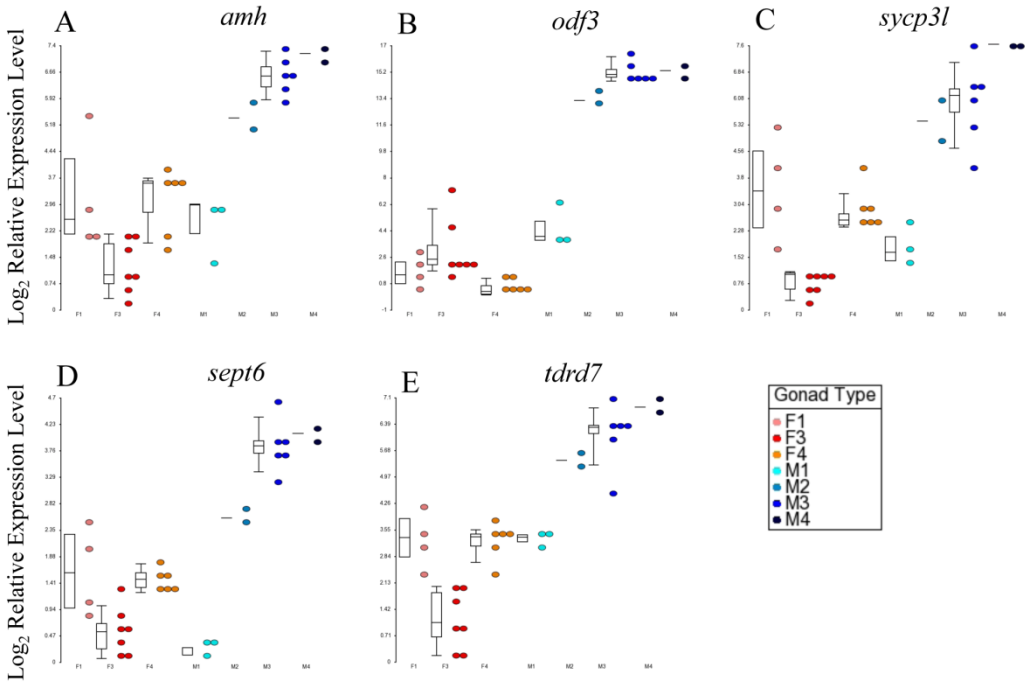

Figure 16. M1 testes showed female-like expression levels of *amh* and male germ cell markers.
Dot plots overlaid with box plots of the relative $\log_2$ gene expression values are shown. The M1 testes are labeled with cyan dots.
Published in Ravi and Jiang et al. 2014, Figure 2.

3.4.4 Increased variation of testicular zp2 expression as a consequence of the presence of primary oocytes in some Asian seabass testes

Zona pellucida glycoproteins, comprising of Zp1, Zp2 and Zp3 in mammals, are found on the extracellular matrix of oocytes and serve as receptors for binding of sperm (Wassarman 1999, Howes *et al.* 2001). Zp orthologs were found in fish oocytes (Hamazaki *et al.* 1989). As expected, in the Asian seabass, *zp2*, an oocyte marker, exhibited a 7.1-fold up-regulation in the ovaries compared to testes. However, while *zp2* expression showed consistently high expression values across all the female gonads, there was a high variation among the male gonads, with some M3 testes showing increased *zp2* transcript levels compared to the other male gonads (**Figure 17**). The high *zp2* expression variation may be related to the presence of primary oocytes that could be found in the histological sections of at least one M3 testis.

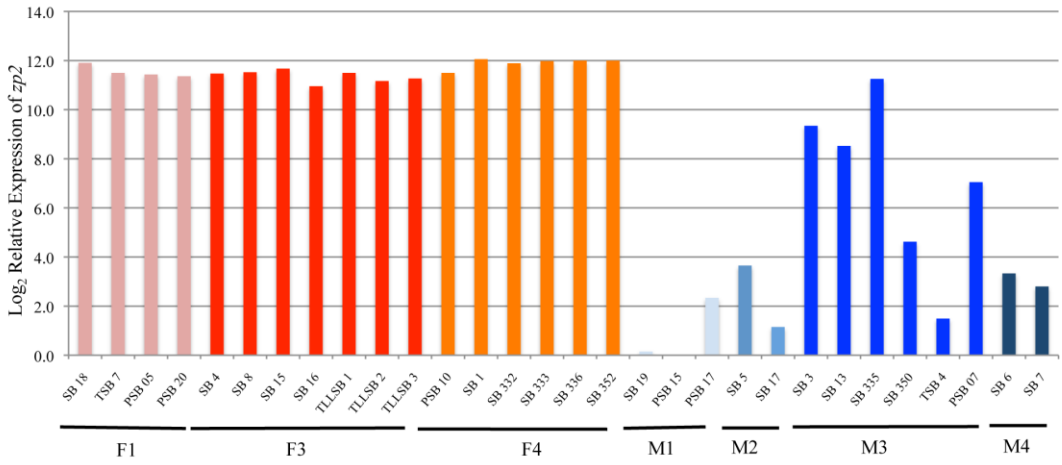

Figure 17. The expression of oocyte marker *zp2* showed a wide variation and an increased level in some M3-type testes.
The relative $\log_2$ gene expression values of *zp2* are shown. Female gonads (F1, F3 & F4; pink, red & orange) are on the left, whereas male gonads (M1-M4; light to dark blue) are on the right.
Published in Ravi and Jiang et al. 2014, Figure 3.

3.4.5 Sexually dimorphic expression of cyp11c1 and esr1 is independent from the maturation status of gonads

In addition, among the studied genes, the expression of *cyp11c1* and *esr1* were found to be distinctly sexually dimorphic between the gonads of the male and female sex regardless of the maturation stage (**Figure 18**). Both genes showed testis-enhanced expression, even at the inactive M1 and M2 stages, while their expression levels were uniformly low across the F1, F3 and F4 ovaries.

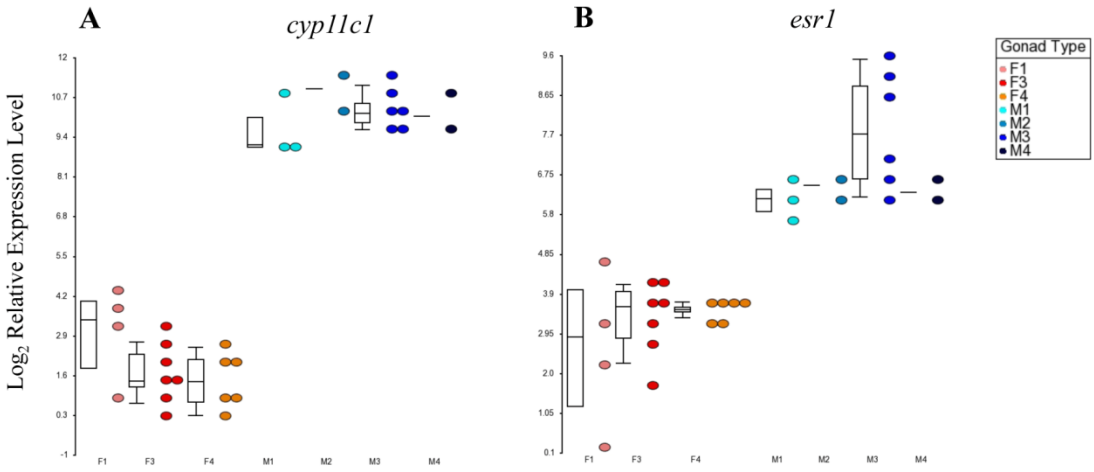

Figure 18. The expressions of *cyp11c1* and *esr1* were high across all testes types. At the same time, the expressions of the two genes were low in the ovaries regardless of their sexual maturation stage. Dot plots that are overlaid with box plots of the relative $\log_2$ gene expression values are shown.
Published in Ravi and Jiang et al. 2014, Figure 5.

3.5 Microarray analysis revealed a similarity in transcriptomic profiles between the undifferentiated and early transforming gonads

To investigate how the gonadal transcriptome profile changes across the various developmental stages, a microarray analysis of six key gonadal developmental stages that ranged across the entire developmental phases of the Asian seabass sexual development was carried out (Table 7).

Table 7. Types of gonad samples used for the microarray analysis.

S/N	Type of gonads	No. used	Age
1	Undifferentiated	4	4.5 months
2	†Juvenile testis (M3-stage)	3	8-9 months
3	¶Adult Testis (M3-stage)	4	5 years
4	Early Transforming (T1-T2-stage)	3	>2 years*
5	Late Transforming (T3-T4-stage)	4	>2 years*
6	Ovary (F3-stage)	4	5 years

†All testes obtained from juvenile seabass (<1 year of age) were labeled as juvenile testes.
¶All testes obtained from adult seabass (5 years of age) were labeled as adult testes.
*Age was estimated based on the weight of the Asian seabass.

As the analysis of the adult gonads using a mid-throughput qPCR array showed that there existed a distinction in transcriptomic profile between the F3 and F4 stage ovaries, only F3 stage ovaries were used in this microarray analysis. Similarly, only juvenile and adult testes that were at the M3 stage were used to minimize potential variability.

The microarray, comprising of 60,080 probes, revealed a large number of transcripts that were differentially expressed when transiting from one gonad type to the other in sequential order of development (Table 8).

Table 8. Number of differentially expressed transcripts (DETs) between two gonad types that are in sequential order of development

S/N	†Comparison	*Up-regulated	*Down-regulated	Total
1	Juvenile Testis vs. Undifferentiated	4,935	3,857	8,792
2	Adult Testis vs. Juvenile Testis	293	543	836
3	Early Transforming vs. Adult Testis	5,462	5,922	11,384
4	Late Transforming vs. Early Transforming	6,250	4,703	10,953
5	Ovary vs. Late Transforming	1,805	1,167	2,972
6	Ovary vs. Adult Testis	9,328	8,395	17,723
7	Early Transforming vs. Undifferentiated	783	1,043	1,862

*Significant change in expression when p-value FDR <0.01 and fold-change ≥1.5 or ≤-1.5.
†The comparisons between ovary and adult testis and early transforming and undifferentiated gonads were also included.

Principal component analysis (PCA) of the overall transcriptome profiles of the gonads showed a bifurcation in the clustering pattern with the undifferentiated gonads at roughly the centre and the testes and ovaries at two opposite ends (**Figure 19**). The juvenile testes and the late transforming gonads clustered closed to their succeeding gonad types, the adult testes and ovaries respectively, while the early transforming gonads were clustered between the undifferentiated and late transforming gonads.

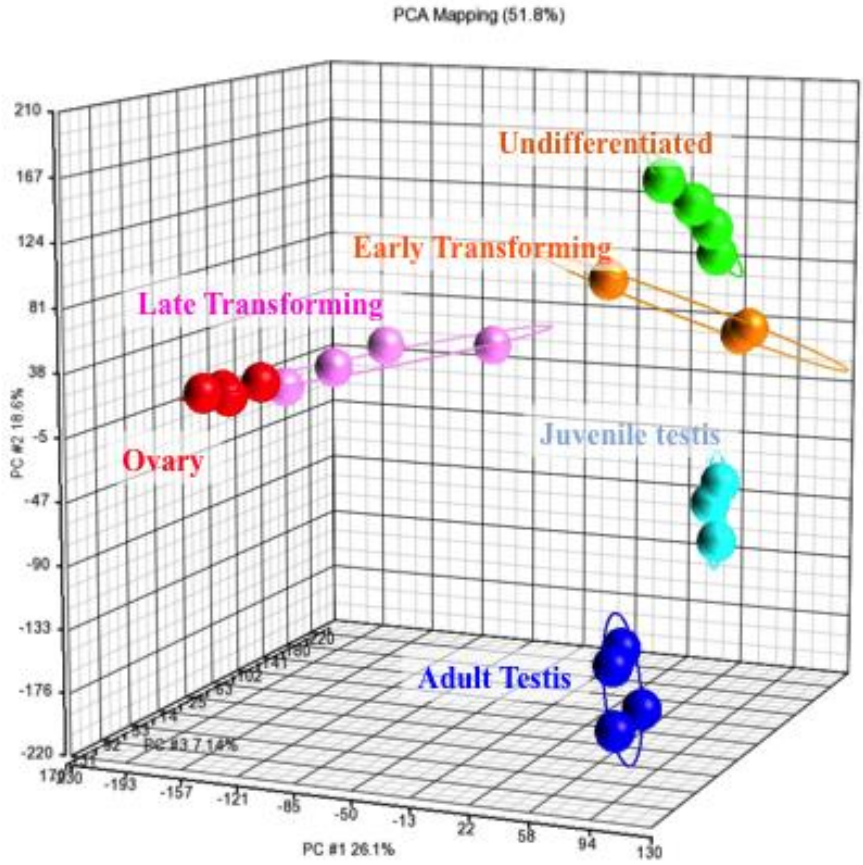

Figure 19. The gonadal transcriptome profiles formed a V-shape clustering pattern with undifferentiated gonads at the base and ovaries and testes at the two ends.
A bifurcation could be observed with the undifferentiated gonads at roughly the centre and the testes and ovaries at two opposite ends. The late transforming gonads clustered closed to the ovary while the early transforming gonads clustered between the late transforming and undifferentiated gonads. The juvenile testes clustered slightly away from the adult testes. Each individual sphere represented the overall transcriptomic profile of a gonad sample with the color indicative of the type of gonad. Green – undifferentiated; cyan – juvenile testis; blue – adult testis; orange – early transforming; pink – late transforming; red – ovaries.

A hierarchical clustering map using the expression values of 14,007 probes that detected significant differences across the six gonad types in a one-way ANOVA analysis (p-value with FDR < 0.0001) was subsequently generated to further visualize the differences and similarities between the gonad types (**Figure 20**).

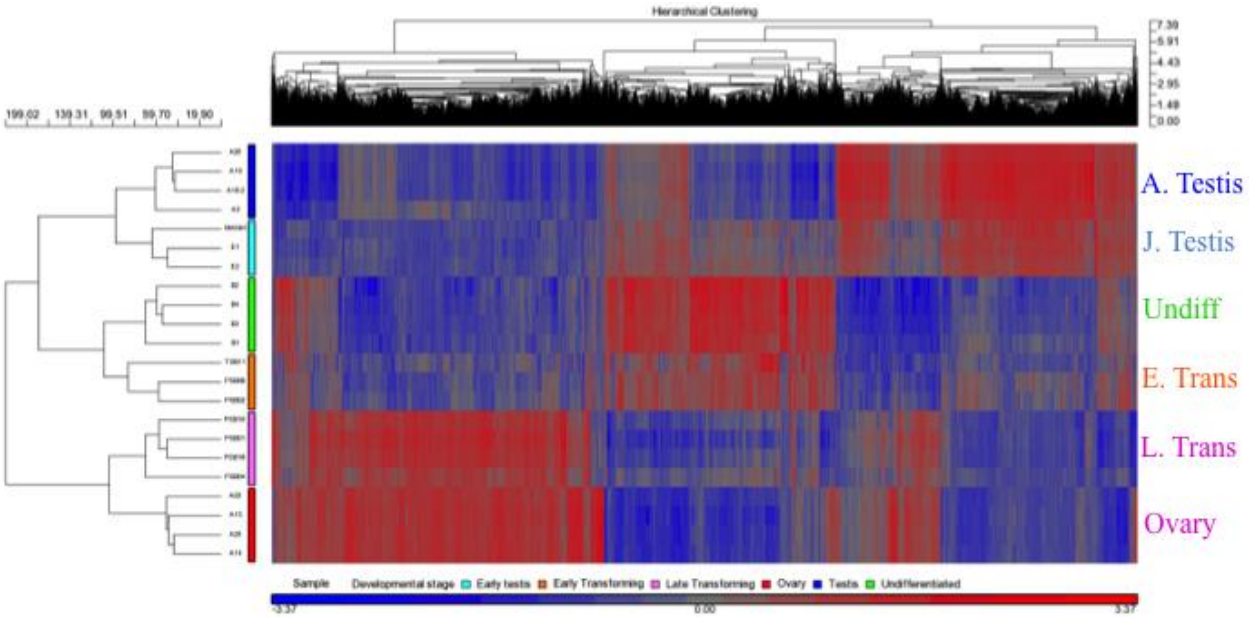

Figure 20. The undifferentiated and early transforming gonads were clustered in the same secondary clade under the hierarchical clustering map.
Total RNA from 22 gonads at six different sexual maturation stages were hybridized onto three custom-made Agilent 8x60K expression microarray slides. The hierarchical clustering map bifurcated into two primary clades with the ovary (F3 stage) and late transforming gonads (T3-T4 stage) forming one primary clade and the other four gonad types forming the other clade. The co-clustering of the undifferentiated gonads in the same primary clade as the testes showed that the undifferentiated gonads had a more similar transcriptome profile to the testes than to the ovaries. The hierarchical clustering map was generated using 14,007 probes that showed p-value with FDR (gonad type) < 0.0001 in a one-way ANOVA analysis. Abbreviations: A.Testis – Adult Testis; J.Testis – Juvenile Testis; Undiff – Undifferentiated; E.Trans – Early Transforming; L.Trans – Late Transforming.

The hierarchical clustering map separated the six gonad types into two major groups, with the ovaries and late transforming gonads forming one primary clade and the other four gonad types forming the other clade. Surprisingly, despite the huge differences between the age of fish from which the undifferentiated (4.5 mpf) and

early transforming (> two years old) gonads were collected from, these two gonad types clustered together in the same secondary clade (**Figure 20**). This indicated that the transcriptomes of the undifferentiated and early transforming gonads were more similar to each other than to the other gonad types and they had about 1,862 DETs between them, in contrast to the other comparisons that typically showed a much higher number of DETs with the exception of the 'juvenile testis vs. testis' comparison that showed only 836 DETs in Table 8.

In addition, the co-clustering of the undifferentiated gonads with the testis (rather than into their own primary clade) showed that the transcriptomes of the undifferentiated gonads were more similar to that of the testes than to the ovaries. Also, the juvenile testes and the adult testes, which have similar histological structures, expectedly clustered together but in different sub-clades, reflecting the slight differences between the juvenile and adult M3-stage testes (< 1000 DETs) (Table 8).

3.6 Real-time qPCR verified the involvement of genes and pathways during Asian seabass gonad transformation as shown by microarray analysis

In order to verify the results from the microarray experiment, the 48.48 qPCR array described previously was expanded into a 96.96 qPCR array, that allowed for the parallel analysis of up to 96 genes and 32 samples (in triplicates). For the validation, additional genes were shortlisted from the microarray results and where possible, gonad samples that were not used previously for the microarray were utilized (Table 9). A total of 88 genes (not including six control genes:*18 s*, *bactin*, *ef1a*, *gapdh*, *rpl8* and *ubq*) and 27 gonadal samples (an additional 63% of new samples) were used. In this experiment, GeNorm analysis (Vandesompele *et al.* 2002)

showed that *rpl8*, *ubq* and *bactin* had the highest gene expression stability and were used as reference genes.

Using the seven comparisons shown in Table 8, a total of 330 out of 612 data points (54%) from the microarray were validated (*i.e.* real-time qPCR results showing same direction of fold-change or absence of significant fold-change as shown by the microarray results) (Table A 4). Despite having only 54% of the data points validated, a PCA plot created using the qPCR results (**Figure 21**) showed a similar clustering of the six gonad types as seen in the PCA plot created using the microarray data shown earlier (**Figure 19**). A subset of the validated genes that showed differential expression during the gonad transformation process is listed in Table 10 (see appendices Table A 4 for full results).

Table 9. Number of samples used for the real-time qPCR validation.

S/N	Type of gonads	Used previously for microarray	New samples	Total no. of samples
1	Undifferentiated	1	4	5
2	Juvenile testis (M3-stage)	3	2	5
3	Adult Testis (M3-stage)	0	4	4
4	Early Transforming (T1-T2-stage)	2	4	6
5	Late Transforming (T3-T4-stage)	3	0	3
6	Ovary (F3-stage)	1	3	4
	Total			**27**

Table 10. Genes that were differentially expressed during the gonad transformation process as verified by both microarray and real-time qPCR results.

*Gene Info		Early Transforming vs. Testis		Late vs. Early Transforming	
S/N	***Gene Sym***	**p-value**	**fold-change**	**p-value**	**fold-change**
Male germ cell-related					
1	*sept6*	0.000	-17.59	-	-
2	*odf3*	0.004	-44.32	-	-
3	*sycp1*	0.000	-40.36	-	-
4	*tekt1*	0.000	-44.26	0.000	-61.85
Female germ cell-related					
5	*zp3*	-	-	0.000	450.56
6	*rttn*	-	-	0.000	20.48
Apoptosis and protein degradation					
7	*tp53*	-	-	0.000	3.58
8	*bfar*	0.000	-10.59	-	-
9	*sh3rf1*	-	-	0.016	4.27
10	*ctsk*	0.043	2.15	-	-
Known sex genes					
11	*dmrt1*	0.000	-6.56	-	-
12	*sox9*	-	-	0.000	-8.47
13	*gsdf1*	0.008	3.20	0.003	-8.76
Hormone-related					
14	*ar*	-	-	0.000	-6.83
15	*esr2*	-	-	0.000	-5.25
16	*pr*	-	-	0.007	-4.22
Wnt signaling					
17	*wnt3*	-	-	0.002	7.86
18	*tnks*	-	-	0.000	-6.83
19	*fzd1*	-	-	0.000	29.12
20	*psen1*	0.001	-3.61	-	-
21	*fzd8*	-	-	0.000	-4.21
22	*ck2a*	-	-	0.001	5.04
23	*wnt16*	-	-	0.002	29.13
Notch signaling					
24	*jag1b*	-	-	0.000	8.40
25	*dlc*	-	-	0.010	-2.80
Hedgehog signaling					
26	*shh*	-	-	0.006	-4.75
27	*gli1*	0.029	-9.72	-	-
NF-κB signaling					
28	*rtkn1*	-	-	0.045	-1.85
29	*rtkn2*	0.000	-21.26	-	-
30	*ikbe*	0.002	1.82	-	-
Retinoic acid signaling					
31	*cyp26a1*	-	-	0.000	129.21
32	*cyp26b1*	-	-	0.010	-4.99
33	*stra6*	-	-	0.000	379.29
34	*rdh3*	-	-	0.001	-8.06
TGF-ß-Smad Signaling					
35	*acvr1*	-	-	0.035	-1.84
36	*smad4*	-	-	0.000	-2.15
37	*bmp1*	0.000	-43.33	0.002	3.10
38	*gdf9*	-	-	0.000	86.21
39	*bmp2*	-	-	0.000	-10.19
Immune-related					
40	*c6*	0.000	23.64	0.011	-17.31

Table 10. Genes that were differentially expressed during the gonad transformation process as verified by both microarray and real-time qPCR results (continued).

***Gene Info**		**Early Transforming vs. Testis**		**Late vs. Early Transforming**	
S/N	***Gene Sym***	**p-value**	**fold-change**	**p-value**	**fold-change**
41	*c7*	-	-	0.000	-17.04
42	*peli1*	0.000	-10.98	0.011	-5.09
Neuron-related					
43	*sema4e*	0.000	-160.94	-	-
44	*unc5a*	0.043	-8.87	-	-
45	*npb*	0.002	-13.59	0.009	6.25

*These genes were classified into signaling pathways and functions. The p-value and fold-change in this table were taken from the real-time qPCR results. Empty cells indicate that results from real-time qPCR and microarray do not agree or do not have significant fold-change.

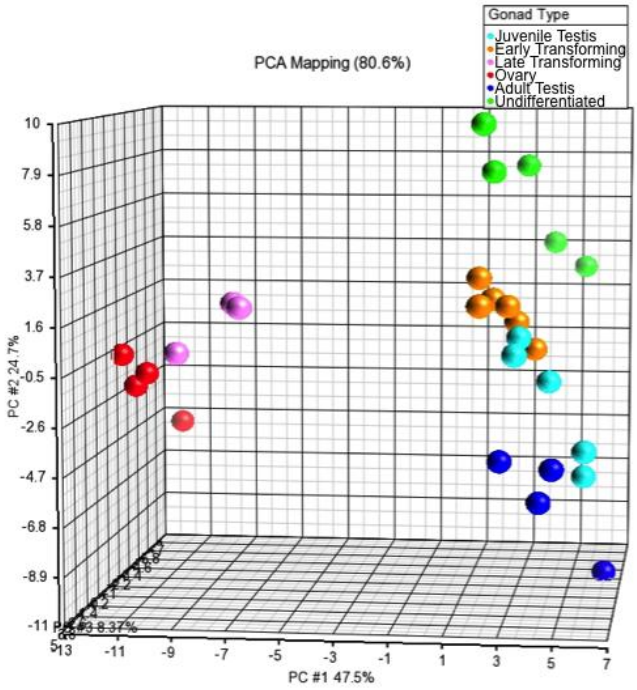

Figure 21. PCA plot drawn using data from the real-time qPCR showed a similar clustering pattern to that produced from the microarray experiment.
Gene expression values from 88 genes could generate a clustering profile similar to that generated using over 60,000 expression data from a microarray. Green – undifferentiated; cyan – juvenile testis; blue – adult testis; orange – early transforming; pink – late transforming; red – ovaries.

3.7 No widespread difference was found between the transcriptomic profiles of adult Asian seabass male and female whole brains

To understand the potential role of the brain in Asian seabass sex change, four brains from adult males with M3 stage testes and four brains from adult females with F3 stage ovaries were tested for transcriptomic differences using the same microarray. The results found only three DETs (all significantly higher in the female brains with p-value FDR <0.05 and fold-change ≥1.5). Of these three DETs, two were genes with unknown function while the third DET was the mitochondrial gene, *cox1* that showed 13.5-fold higher expression in female brain.

3.8 Two GnRH isoforms were identified in the transcriptome of Asian seabass

Gonadotropin-releasing hormone (GnRH; also called as Luteinizing-hormone-releasing hormone or LHRH) is a ten amino acid peptide produced by the brain and regulating the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Lovejoy *et al.* 1992). There have been up to nine forms of GnRH described to date and more than one forms can exist within a teleost (Gothilf *et al.* 1996). To determine the GnRH isoforms present within the Asian seabass brains, GnRH mRNA sequences from the existing NCBI database were BLAST searched against the Asian seabass transcriptome consisting of 363,369 assembled contigs. Although 'salmon GnRH' was readily detected, 'chicken GnRH-II' previously described in Asian seabass (Tan *et al.* 2008) could not be found from the transcriptome data. Nevertheless, based on both results, there are currently at least two forms of GnRH found in Asian seabass (**Figure 22**).

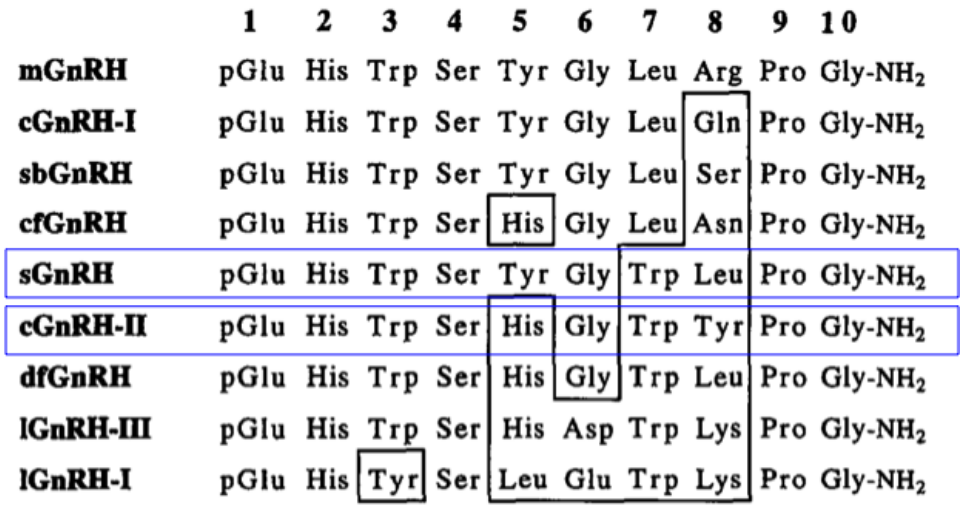

Figure 22. The transcriptome of Asian seabass showed the presence of at least two isoforms of GnRH, the salmon and chicken-II.
Nine forms of GnRH core peptide are known to exist. These GnRH were named according to the species that the GnRH isoforms were first isolated from. Asian seabass transcriptome data revealed the presence of salmon GnRH isoform, while chicken-II GnRH was isolated in a previous study by Tan et al., 2008 (boxed). The GnRH isoforms from descending order are: mammalian, chicken-I, seabream, catfish, salmon, chicken-II, dogfish, lamprey-III and lamprey-I. Figure modified from Gothilf *et al.* (1996).

3.9 GnRH induction resulted in sexually dimorphic increase in mucus 11-KT production

As part of the efforts to create a non-invasive method of sexing Asian seabass and to devise ways to promote early sexual maturity in this species with long generation time, the effects of GnRH, a popular hormone used in the aquaculture industry, were analyzed.

Mucus samples were collected from Asian seabass ranging from two mph of age to adults (>two years old) immediately prior to and a day after induction with a synthetic GnRH analog, LHRH. The samples were analyzed for 11-KT levels. The results showed that Asian seabass older than nine mph of age responded to GnRH induction with an increase in their mucus 11-KT production (**Figure 23**). This corresponded with the age at which juvenile testis could be detected in Asian seabass.

On the other hand, two months old seabass did not respond to the GnRH induction most likely because of their undifferentiated gonads (since at 4.5 mph seabass had undifferentiated gonads).

In addition, the spike in 11-KT production was the highest in adult male seabass (7.91-fold), followed by 1.8 years old seabass (5.64-fold), 9 months old seabass (2.17-fold) and lastly adult female seabass (1.52-fold) (Table 11). Results described in the previous section (Section 3.2) showed that all Asian seabass individuals at 9 mph and 1.8 years of age showed the presence of juvenile testes rather than undifferentiated gonads or ovaries. This indicated that testes resulted in a higher spike in 11-KT production in response to GnRH than ovaries. In addition, the gradual increase in magnitude of 11-KT spike between the 9 mph, 1.8 years of age and adult males may be due to the increase of testis size during that period.

Furthermore, while adult male seabass had higher mucus 11-KT levels compared to adult female seabass on average prior to GnRH induction, there were still some females with higher mucus 11-KT than some males (**Figure 23**, panel A). However, after the GnRH induction, due to the greater spike of 11-KT levels in males than in females, the average difference between the two sexes in their mucus 11-KT levels increased from 2.04-fold (p-value: 0.010) to 10.62-fold (p-value: 2.3e-6). In addition, none of the induced males showed lower mucus 11-KT level than the highest value in induced females (**Figure 23**, panel B).

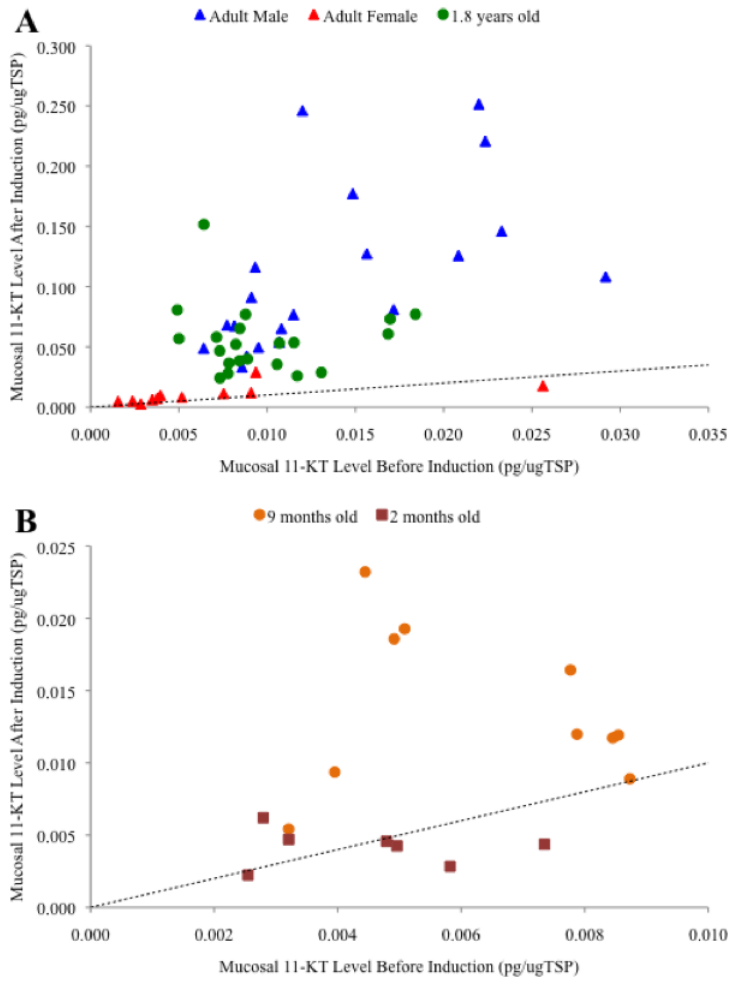

Figure 23. GnRH induction resulted in increased mucus 11-KT levels except among juveniles at two months of age.
Every single point indicates the readings from an individual seabass before and after GnRH induction. (A) In general, adult male seabass responded with the greatest increase in mucus 11-KT production compared to the 1.8 years old seabass which is in turn higher then the adult females. (B) Nine months old seabass, but not the two months old ones, also responded to the GnRH induction. The dotted line (y=x) indicates parity in mucus 11-KT level before and after induction.

Table 11. Change in mucus 11-KT levels of Asian seabass due to GnRH induction.

After vs Before (GnRH induction)	***P-value**	**Fold-Change**
Adult Male	1.0E-06	7.91
Adult Female	0.049	1.52
1.8 years old	2.4E-07	5.64
9 months old	0.002	2.17
2 months old	0.360	0.93

*P-value is obtained from a one-tailed paired Student's T-Test

3.10 Long term treatment of GnRH can promote development of testis

Three groups of eight months old Asian seabass (20 individuals per group; average weight 284±72 g) were implanted with GnRH and various combinations of domperidone (Dom; an inhibitor of dopamine) and 17α-methyltestosterone (17MT; a synthetic male steroid) pellets and reared over a period of three months. A fourth group of seabass (18 individuals) implanted with blank implants served as controls. These seabass were checked for presence of milt in the second and third month of implantation and in the third month, some seabass from each group were sacrificed for gonadal histology.

The hormone implantation experiment showed that GnRH alone cause a consistent increase in number of milting males (**Figure 24**), which corresponded to this group having more individuals with M3 stage testis among those sacrificed (**Figure 25**). However, the addition of both domperidone as well as domperidone+17MT resulted in a decreased number of milting males or seabass with M3 stage testis compared to the GnRH group. In addition, with 17MT, there was even an adverse effect on testis development compared to the control as there were more M1 stage testes and less M3 stage testes compared to the control.

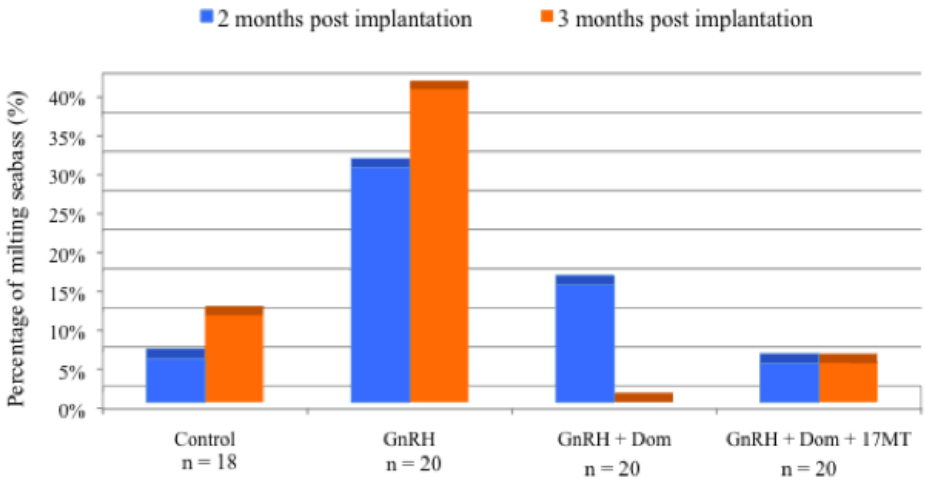

Figure 24. GnRH implants, but not their combination with other compounds, resulted in consistent increase in percentage of milting juveniles over that shown by the control group.

During the second and third month of implantation, all juvenile seabass were stripped to check for presence of milt. The group with GnRH implants had the largest proportion of juveniles that were milting by the second month of implantation.

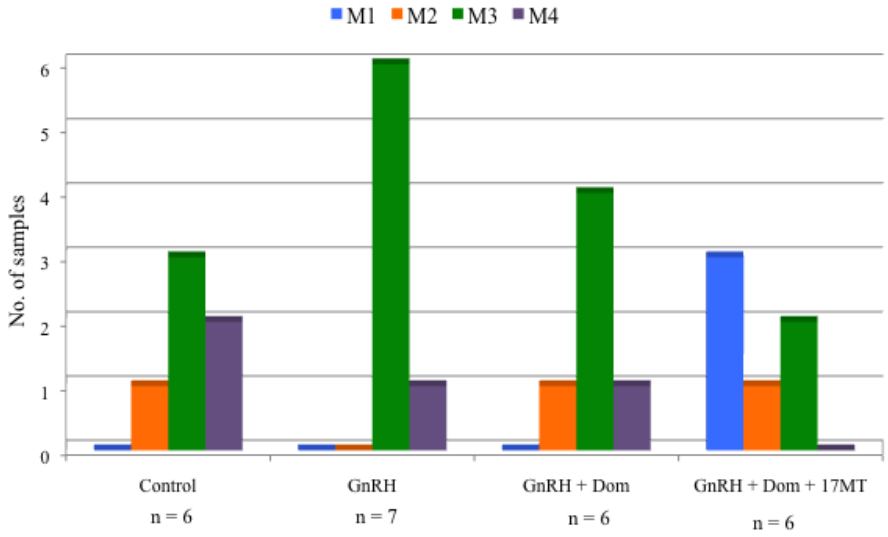

Figure 25. GnRH implants resulted in most M3 stage testis among sacrificed individuals.

Six to seven juvenile seabass were randomly sacrificed from each group for histological analysis of the gonad development at three months post implantation. Six out of seven juveniles sacrificed from the GnRH implant group had M3 stage testis while the other groups had relatively less individuals with M3 stage testis.

3.11 Zebrafish 'juvenile ovary-to-testis' type gonad transformation involves the differential expression of several Wnt signaling genes

Previously, our laboratory had carried out microarray analysis of transforming zebrafish male gonads that suggested the roles of several genes involved in zebrafish gonad transformation, including members of the Wnt signaling pathway (Sreenivasan. *et al.* 2008 poster presentation).

In this study, 57 genes from the microarray were shortlisted for validation by real-time qPCR on a separate set of gonadal samples collected at 35 dpf. Of these 57 genes, 44 were differentially expressed genes and 13 were non-differentially expressed genes on the microarray. Among the 57 genes, 41 were validated as they had the direction of differential expressions or non-differential expressions similarly indicated by real-time qPCR (Table A 5). A subset of the results is shown in Table 12.

Table 12. Differentially expressed genes between juvenile ovotestes (JOT) and juvenile ovaries (JO).

Gene Symbol	**Accession**	***Microarray**	***qPCR**
		Fold-change (JOT / JO)	**Fold-change (JOT / JO)**
Genes with known sex-related functions			
cyp11c1	NM_001080204.1	12.1	69.9
star	NM_131663.1	3.6	36.7
esr2b	NM_174862.3	4.5	9.6
ts1	EF554575.2	1.9	3.7
dmrt1	NM_205628.1	2.6	2.9
sycp3	NM_001040350.1	2.0	2.4
zp2	NM_131330.1	-5.1	-5.8
zp3	NM_131331.1	-8.2	-7.4
Genes involved in Wnt Signaling			
dkk3	NM_001089545.1	2.4	5.1
psen1	NM_131024.1	-1.9	-1.6
ctnnbip1	NM_131594.1	-1.7	-1.7

*Conditions for differential expression: microarray: ≥1.5-fold difference and p-value <0.01; real-time qPCR: ≥1.5-fold difference and p-value <0.05.
Published in Sreenivasan and Jiang et al. 2014, Table 2.

3.12 Functional analysis of the role of Wnt signaling in zebrafish gonad transformation

Three Wnt signaling member genes, *dkk3*, *psen1* and *ctnnbip1* were shown to be differentially expressed (Table 12), indicating that Wnt signaling may be involved in zebrafish 'juvenile ovary-to-testis' type gonad transformation process. Similarly, several Wnt signaling member genes were also found to have differential expression during Asian seabass gonadal transformation (Table 10). Hence, the zebrafish was used for functional validation of the role of Wnt signaling in gonad differentiation.

3.12.1 No detection of dGFP expression in Tg(TOP:GFP) zebrafish gonads

To visualize the activity of canonical Wnt signaling within the zebrafish gonad, the transgenic zebrafish strain, *Tg(TOP:GFP)w25*, was utilized. The TOPdGFP construct expresses a destabilized GFP (dGFP) under the control of ß-catenin responsive promoter (Lef) (Dorsky *et al.* 2002). This facilitates the visualization of regions where canonical Wnt signaling is active.

From 1-3 dpf, intense GFP expression could be visualized directly under the fluorescence microscope in the head region of the developing embryos. However, no GFP expression could be observed directly from the gonadal regions in both adults and developing juveniles (30-40 dpf). Hence, immunohistochemistry was carried out against dGFP using 2 dpf zebrafish heads as positive controls. The immunohistochemistry results confirmed that dGFP expression could be detected in the 2 dpf heads, but not in the developing gonads at 35 dpf and 45 dpf (**Figure 26**).

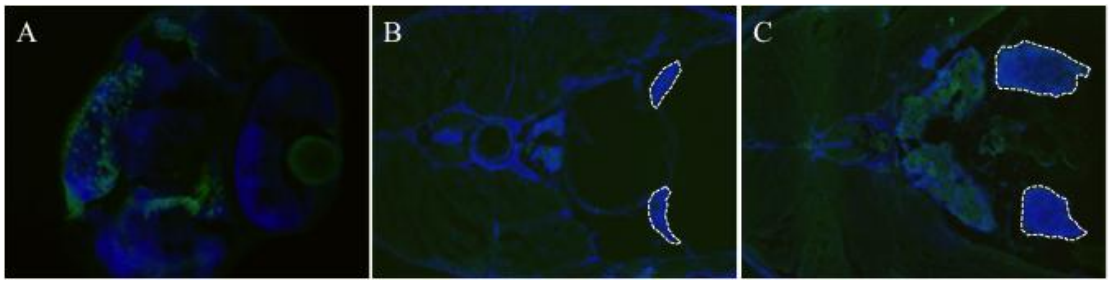

Figure 26. dGFP could not be detected in the gonads of *Tg(TOP:GFP)* zebrafish. Immunohistochemical analysis showed that destabilized GFP was highly expressed in individual neurons of the head region at 2 dpf (A), but could not be detected in the gonad regions of 35 dpf (B) and 45 dpf (C) transgenic zebrafish. The dGFP was labeled using chicken polyclonal anti-GFP antibody and visualized using green fluorescent Alexa Fluor 488-labelled goat anti-chicken IgG antibody. DNA was labeled with DAPI. The gonads are outlined with white dotted lines. Note that the green fluorescence was deliberately over-exposed in panels B & C.

3.12.2 Chemical treatments did not consistently change sex ratio, but down-regulated the expression of cyp19a1a

In order to provide functional evidence for the involvement of canonical Wnt signaling in zebrafish gonad differentiation, a small molecule inhibitor of Wnt signaling, IWR-1-endo was utilized. IWR-1-endo promotes ß-catenin destruction by stabilizing Axin, hence causing the down-regulation of the Wnt/ß-catenin pathway (Chen *et al.* 2009). The treatment of IWR-1-endo from 15-30 dpf interfered with fin development in all treated groups and down-regulated *cyp19a1a* expression by 2.02-fold (p-value = 0.04) in one group that was sacrificed at 30 dpf, after 15 days of IWR-1-endo treatment. However, while there was increased proportion of males in 3 out of 7 groups, there was no consistent male bias as a consequence of inhibitor treatment (Table 13).

Table 13. Effect of IWR1-endo treatment on family sex ratios.

S/N	Family	Treatment Period	% Male			*N	
			DMSO	ENDO	Difference	DMSO	ENDO
1	vas(+/+) 1C	15-39 dpf	71%	70%	-1%	38	30
2	vas(+/+) 1C	15-31 dpf	23%	55%	32%	52	22
3	vas(+/+) 12D	15-30 dpf	32%	42%	10%	62	151
4	vas(+/+) 12D	15-39 dpf	31%	31%	0%	72	42
5	vas(+/+) 3D	15-30 dpf	11%	38%	27%	74	157
6	vas(+/+) 3D	15-39 dpf	27%	17%	-10%	30	35
7	vas(+/+) 2D	15-40 dpf	61%	61%	0%	31	44

*N refers to number of individuals which were sexed. More individuals were allocated to the IWR1-endo treatment group due to high mortality. The fish were sexed at 4 months or older based on Egfp expression. Significant change in sex ratio (>10%) was observed in three out of seven experiments only (S/N 2, 3, 5). Furthermore, the change in sex ratio could not be replicated even within the same family.
Published in Sreenivasan and Jiang et al. 2014, Supplemental Table S4.

3.12.3 Transgenic inhibition of canonical Wnt signaling pathway promoted testis formation in zebrafish

Subsequently, the heat-inducible *Tg(hsp70l:dkk1b-GFP)w32* zebrafish line (from here onwards dkk) was used to promote ubiquitous expression of the Wnt antagonist, *dkk1b* (Stoick-Cooper *et al.* 2007). The Dkk1b protein is known to inhibit canonical Wnt signaling by binding to the LRP5/6 co-receptors, thus preventing downstream signaling (Mao *et al.* 2001). Transgenic dkk zebrafish at 44 dpf were heat-shocked for two hours at 39ºC and were sacrificed four hours later for gonadal immunohistochemistry against *dkk1b-GFP*. The result confirmed that the transgene was expressed in the somatic cells of both the juvenile ovary and testis, but not in the germ cells following the heat shock treatment (**Figure 27**).

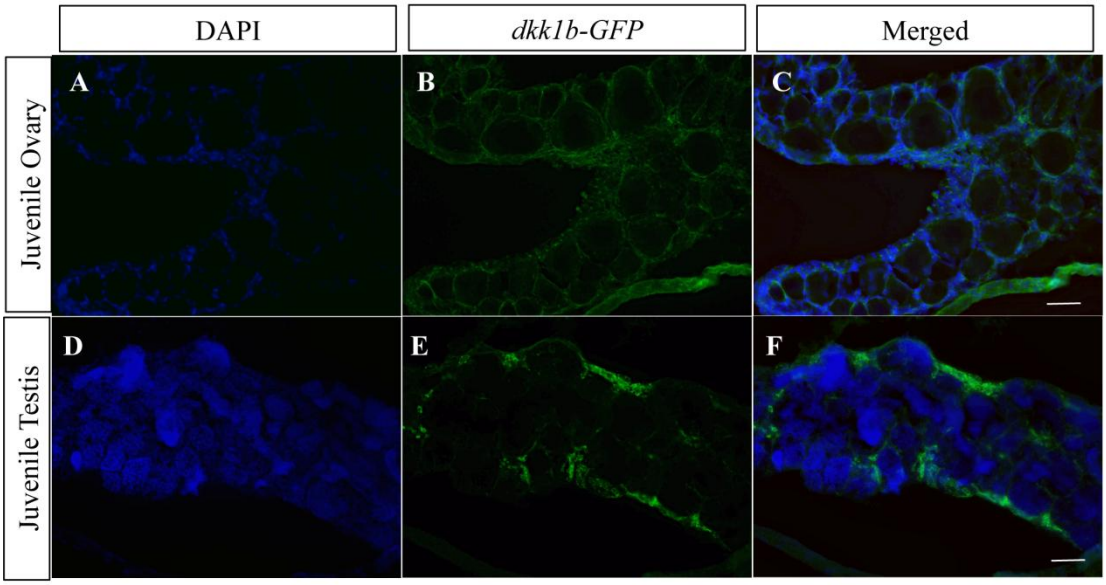

Figure 27. GFP was highly expressed in the somatic cells of both the ovary and testis of 44 dpf *Tg(hsp70l:dkk1b-GFP)w32* zebrafish at four hours post heat shock treatment.
Immunohistochemical analysis showed that GFP was highly expressed in the somatic, but not germ cells of both the ovary and testis. DNA was labeled with DAPI (A & D). The GFP was labeled using chicken polyclonal anti-GFP antibody and visualized using green fluorescent Alexa Fluor 488-labelled goat anti-chicken IgG antibody (B & E). The green and blue fluorescence are merged in C & F. Scale bar is 100 μm.
Published in Sreenivasan and Jiang et al. 2014, Supplemental Figure S3.

Hemizygous dkk-transgenic zebrafish, *Tg(dkk)/-*, were then crossed to wild-type partners in order to obtain F_1 progeny containing both wild-type and transgenic *Tg(dkk)/-* offspring. This would generate equal number of wild-type and *Tg(dkk)/-* offspring for the experiment. To induce *dkk1b* expression, F_1 progeny (both transgenic and wild-type) were incubated daily for two hours at 39°C from 20-60 dpf, including and well beyond the period when zebrafish gonad differentiation is known to occur normally (Wang *et al.* 2007b).

Tg(dkk)/- F_1 individuals could be easily distinguished from their wild-type siblings via the Egfp expression after heat-shock or by partial deformation of their caudal and dorsal fins. The effect on the caudal and dorsal fin remained permanent even after the termination of heat-shock treatment (**Figure 28**).

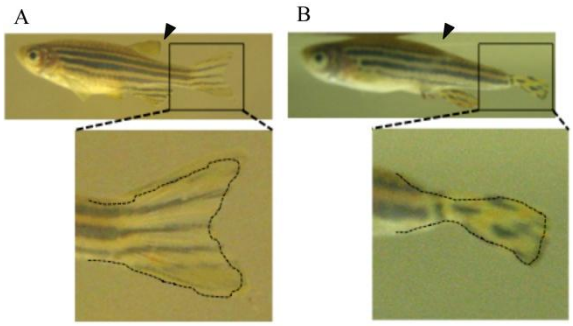

Figure 28. Induced expression of *dkk1b* resulted in deformation of the caudal fin and disappearance of the dorsal fin.
(A) A typical adult wild-type F_1 progeny with intact dorsal and caudal fins. (B) Deformed caudal fin and absent dorsal fin of a typical adult *Tg(dkk)/-* F_1 progeny heat-shocked on a daily basis from 20-60 dpf . Inserts show the enlarged caudal fins, whereas the arrowheads point to the dorsal fins.
Published in Sreenivasan and Jiang et al. 2014, Supplemental Figure S4.

The heat-shock treatment resulted in significant increase of the proportion of males in the *Tg(dkk)/-* F_1 progeny compared to their wild-type full siblings (**Figure 29**). There was also more mortality in the *Tg(dkk)/-* F_1 progeny since equal numbers of *Tg(dkk)/-* and wild-type offspring were expected. However, even by hypothetically increasing the number of transgenic *Tg(dkk)/-* with females such that *n* would be equal for both *Tg(dkk)/-* and wild-type F_1, two of the families (A1 and A2) would still show male-biased sex ratios. Hence, it is unlikely that the increase in female-to-male sex reversal was due exclusively or even primarily to the increase in mortality. This indicates that Wnt signaling plays a role in regulating zebrafish gonad differentiation.

On the other hand, there were no significant morphological differences between the gonads of heat-shocked *Tg(dkk)/-* individuals and heat-shocked wild-type individuals at 3.5 months post fertilization (**Figure 30**). Furthermore, motile sperm could be observed under the microscope in the testes of both heat-shocked groups. This indicated that the over-expression of *dkk1b* during the treatment period did not affect the subsequent functionality of the ovaries and testes of the adults.

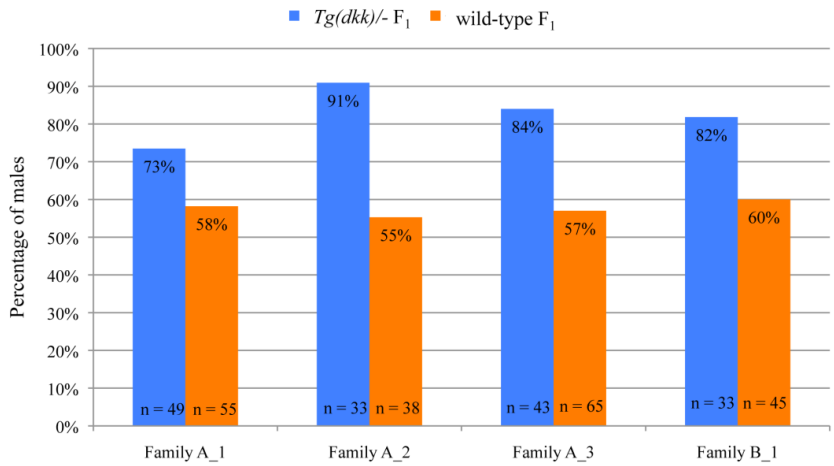

Figure 29. Heat-induced expression of *dkk1b* resulted in significant increase of zebrafish males.

A mixture of *Tg(dkk)/-* individuals and their non-transgenic siblings were heat-shocked at 39°C for two hours on a daily basis between 20 and 60 dpf. At 100-120 dpf, the fish were sexed by dissection and gonads were observed under wet-mount microscopy to confirm sex. Within each family, the heat-shocked transgenic progenies always had a higher percentage of males (15-36% higher) compared to their wild-type full siblings. Three batches from different spawnings were tested from Family A with consistent male-bias seen in the transgenic progenies following heat-shock, while a second family (Family B; one clutch) also showed the same result. A paired t-test with one-tailed distribution showed that the difference between transgenics and controls is significant with p-value = 0.005.

Published in Sreenivasan and Jiang et al. 2014, Figure 3.

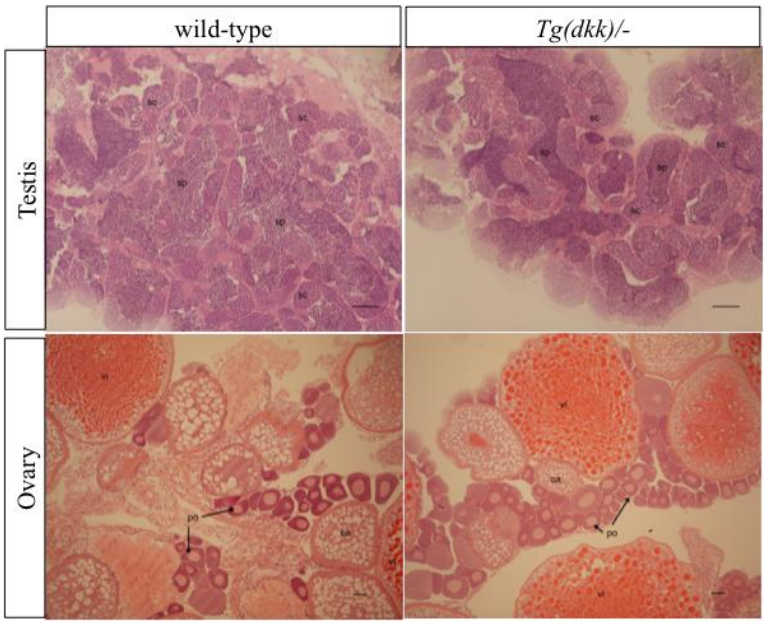

Figure 30. The gonadal histology of wild type (left) and *Tg(dkk)/-* (right) zebrafish adults was unaffected following the heat shock.

Abbreviations: ca - cortical alveolus; po - perinucleolar oocyte; sc – spermatocyte; sp - spermatozoa; vi – vitellogenic oocyte. Scale bar = 50 μm.

Published in Sreenivasan and Jiang et al. 2014, Supplemental Figure S5.

3.12.4 Heat shock-based activation of dkk1b resulted in responsive down-regulation of cyp19a1a

To identify the genes that were affected by the transgenic inhibition of Wnt signaling, the expression level of 42 genes were quantified by real-time qPCR in wild-type and transgenic siblings from one family after 20 days of heat-shock treatment at 36 dpf (see appendices Table A 6 for the complete results). Both the wild-type and transgenic sibling groups comprised of a mix of JO and JOT individuals as they could not be distinguished. Overall, *cyp19a1a* expression was significantly down-regulated by 5.75-fold in the transgenic individuals compared to wild-types (see subset of results in Table 14).

Members of the Wnt signaling pathway, *wnt4a* and *lef1*, also showed significantly down-regulated expression in the heat-shocked *Tg(dkk)/-* offspring (Table 14). Additionally, *lef1* is known to be a downstream target of canonical Wnt signaling and has shown up-regulated expression by Wnt/ß-catenin signaling in HEK293 cells and in human colon cancers (Hovanes *et al.* 2001, Filali *et al.* 2002). While Wnt/ß-catenin signaling has been shown to inhibit *sox9b* expression in regenerating zebrafish fins, the down-regulation of *sox9b* in the heat-shocked *Tg(dkk)/-* offspring was expected since *sox9b* expression is also found in the oocytes (Day *et al.* 2005, Rodriguez-Mari *et al.* 2005). The down-regulation of *inhbb*, however appeared contradictory as *Inhbb* is inhibited by Wnt/ß-catenin signaling in mouse ovaries (Yao *et al.* 2006, Liu *et al.* 2010). However, zebrafish has three inhibin-ß paralogs: *inhbaa*, *inhbab* and *inhbb*. The regulation of all three paralogs is not fully understood and there is currently no study that would show the regulation of *inhbb* by the Wnt pathway in zebrafish.

Table 14. Differentially expressed genes between individuals with transgenic *dkk1b* over-expression and controls.

Gene Symbol	Accession no.	p-value	Fold-change
Up-regulated genes			
dkk1b	NM_131003.1	0.000	323.7
esr2b	NM_174862.3	0.003	1.4
Down-regulated genes			
cyp19a1a	NM_131154.2	0.006	-5.8
lef1	NM_131426.1	0.008	-2.0
sox9b	NM_131644.1	0.000	-1.6
inhbb	NM_131068.2	0.048	-1.5
wnt4a	NM_001040387.1	0.023	-1.3

Published in Sreenivasan and Jiang et al. 2014, Table 3.

Besides the reduced expression of *cyp19a1a* in the gonads of the transgenic individuals caused by the heat-induced over-expression of *dkk1b*, there was also a decreased variation in the expression of *cyp19a1a* among the transgenics (range: 0.015-0.190) compared to their wild-type siblings (range: 0.040-1.000) (**Figure 31**). This indicates that even the gonads of those transgenics that would eventually be able to resist the 'pressure to transform' would have reduced *cyp19a1a* levels compared to their wild-type siblings due to the inhibition by *dkk1b*.

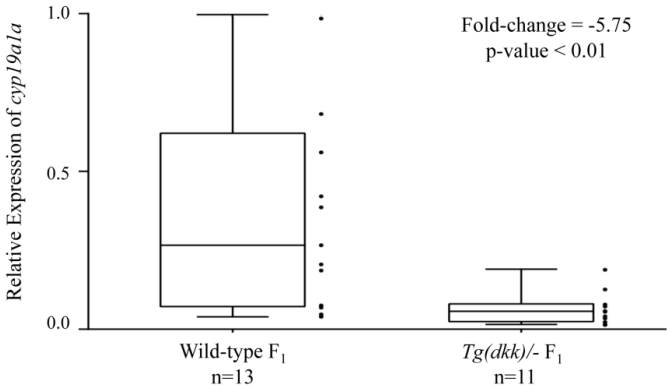

Figure 31. The relative expression of *cyp19a1a* was significantly decreased in heat-shocked transgenics, compared to their control siblings (also heat-treated). Altogether, 13 wild-type F_1 and 11 *Tg(dkk)/-* F_1 originating from the same family were tested. These F_1 individuals had undergone the daily heat-shock treatment for 16 days and were sacrificed at 36 dpf for gene expression study. Real time qPCR was performed on trunks segments. The wild-type F_1 samples showed a greater variation in *cyp19a1a* expression compared to that of their *Tg(dkk)/-* siblings.
Published in Sreenivasan and Jiang et al. 2014, Figure 4.

To investigate the responsiveness of *cyp19a1a* down-regulation to the transgene expression, a group of 35 dpf transgenic dkk zebrafish (which have not been heat-shocked before) were heat-shocked and random individuals were sacrificed at four and eight hours post treatment for analysis. As early as four hours post-treatment, *dkk1b* was up-regulated by over 700-fold compared to transgenic dkk zebrafish without any heat shock (control). There was also a corresponding decrease in *cyp19a1a* expression by 7.2-fold (**Figure 32**). Then, at 8 hours post-treatment, the magnitude of *dkk1b* up-regulation compared to control was reduced to 338-fold (from over 700-fold) and the magnitude of *cyp19a1a* down-regulation was also similarly reduced to 4.7-fold (from 7.2-fold).

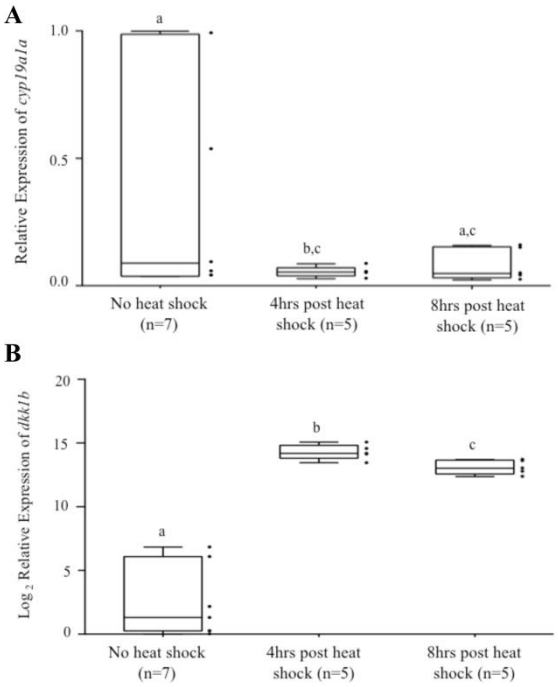

Figure 32. The down-regulation of *cyp19a1a* expression (A) was responsive to the heat activation of *dkk1b* (B) in 35 dpf dkk transgenic zebrafish.
As early as 4 hours post treatment, *cyp19a1a* expression was significantly down-regulated by 7.2-fold while the up-regulation of *dkk1b* expression was over 700-fold. At 8 hours post treatment, the magnitude of *dkk1b* up-regulation compared to control was reduced (338-fold up-regulation) and the magnitude of *cyp19a1a* down-regulation was also similarly reduced (4.7-fold down-regulation). Real time qPCR was performed on eviscerated trunks and *rpl13* and *eef1a1l1* were used as reference genes. a,b,c indicate p-value < 0.05.
Published in Sreenivasan and Jiang et al. 2014, Figure 5

4 Discussion

4.1 New insights into the reproductive life cycle of the Asian seabass

In this study, the results from the collection and analysis of the Asian seabass gonads showed an agreement with the existing literature on several aspects of the sexual development of the species. They have also extended our current knowledge on the juvenile and transforming gonads, in particular the molecular pathways involved in the sex change process.

4.1.1 Mandatory juvenile testis stage

Juvenile testis could be found in Asian seabass juveniles as early as 8 months post-hatching of age and these juvenile testes typically ranged from the M1 to M3 maturation stages. This finding is in unison with a recent study on Asian seabass reared in intensive freshwater recirculation system that also found the presence of juvenile testes in 9 months old juveniles (Szentes *et al.* 2012). In addition, no ovary-like gonads among juveniles of those ages had been reported by us or in that study. However, even though the M3 stage juvenile testes contained spermatozoa, the testes were too small to be functional in nature. In both studies, the juvenile testes were found at around the same age despite one batch being reared in seawater (our study) and the other in freshwater.

In contrast, among adults, a study on wild Asian seabass in Papua New Guinea reported that mature gonads (testes and ovaries) were only found from individuals caught from coastal waters where water salinity was high (Moore 1982). This may be related to the life history of the Asian seabass, a catadromous species that migrates

from inland waters of low salinity to coastal waters of high salinity for spawning (Moore 1982). Hence, while water salinity or other aspects of the environment could be important in triggering gonad maturation and/ or even sex change in adults, the development of the juvenile testis in juvenile seabass appears to be independent from the environment.

Furthermore, the transcriptomic profiles of the undifferentiated gonads were more similar to that of the testes than the ovaries (**Figure 20**), suggesting that differentiation into a juvenile testis is a 'shorter and more direct' route, requiring fewer changes to gene expression patterns that a differentiation into an ovary requires. However, admittedly, it could also be argued that while the undifferentiated gonads presented no testicular tissues histologically, the gonads could already be in the process of testis differentiation at the time of sample collection (4.5 mph). This would hence explain the similarity of the transcriptome of an undifferentiated gonad to that of a testis. Even so, the exclusive presence of testes only in juvenile seabass strongly indicates that the differentiation into testis by the undifferentiated gonads is mandatory.

The collection of Asian seabass gonads also supported existing data in the literature on the presence of primary females and terminal males (Moore 1979, Davis 1982). However, as with the other studies, based on the results from this set of experiments, it could not be determined whether the primary females skipped the male phase entirely or transited through it very quickly. Given that the juvenile testis is a mandatory stage, it is possible that the ovaries of primary females were differentiated from the juvenile testes.

4.1.2 'Oocytes-in-testis' as a possible sign of sex change

Primary oocytes were also found residing in the testicular tissue of some of the young and adult Asian seabass individuals (**Figure 13**). Primary oocytes in adult testis had been previously described (Guiguen *et al.* 1994) but this is the first time they have been detected in the testis of a juvenile seabass. However, primary oocytes could only be found in a subset of our Asian seabass testis collection, suggesting that not all testes have primary oocytes residing within them. This observation was also reflected in the wide variation in expression levels of the oocyte marker, *zp2*, among the adult testes (**Figure 17**).

In mammals, the Sertoli and Leydig cells of the testis are suggested to be derived from the same bipotential somatic precursors of the genital ridge as those of the ovarian granulosa and theca cells, respectively, due to them having parallel functions in the two gonads (Svingen and Koopman 2013). In mammalian XY gonads, Sertoli cells are the first to be specified and differentiated from the genital ridge due to the expression of *Sry* which leads to the feed forward expression of *Sox9* while the absence of *Sry* expression results in default expression of Rspo1/Wnt/ß-catenin signaling that leads to granulosa cells differentiation (Ungewitter and Yao 2013, Sekido and Lovell-Badge 2008). The Sertoli cells subsequently serve as master regulators driving the differentiation of the androgen-producing Leydig cells and the spermatogonia of the mammalian testis (Svingen and Koopman 2013). On the other hand, in the XX gonad, following the differentiation of the granulosa cells, theca cells appear and together with the somatic cells, surround the oocytes to form the follicles (Ungewitter and Yao 2013). Similar to the Leydig cells of the testis, the theca cell produce androgens that are transported to the granulosa cells where the androgens are converted to estrogens and progesterone (Ungewitter and Yao 2013).

Hence, it can be hypothesized that during sex change in the Asian seabass, the Sertoli and Leydig cells trans-differentiate into the granulosa and theca cells of the ovary, respectively. However, unlike in the mammalian system, germs cells can play a key role in deciding the sexual fate of the gonad in several fish species, possibly explaining the presence of primary oocytes in the Asian seabass testis. In the zebrafish, the presence of germ cell is required for the development of the ovary while in the stickleback, it is shown that higher PGC numbers can lead to ovarian differentiation (Siegfried and Nusslein-Volhard 2008, Slanchev *et al.* 2005, Lewis *et al.* 2008).

Primary oocytes were also found in the testes of the protandrous black porgy before sex change. They were surrounded by Sertoli cells, indicating that the oocytes were able to maintain their existence in the testicular environment (Wu and Chang 2013a, Wu and Chang 2013b). Results obtained with the black porgy had also suggested that ectopically-induced primary oocytes could lead to the trans-differentiation of adjacent Sertoli cells to follicle-like cells (Wu and Chang 2013a).

Similarly, in the opposite direction of sex change, during regression of ovary in the protogynous wrasse (*H. trimaculatus*), few somatic cells were found to undergo apoptosis and instead, those surrounding the degenerating oocytes underwent proliferation, incorporating experimentally supplied BrdU which were subsequently found in the Sertoli cells of the transformed testis (Nozu *et al.* 2013). In Nile tilapia, during Fadrozole-induced female-to-male sex change, ovarian somatic cells residing near putative germline stem cells were also found to trans-differentiate into the male somatic cells to create the micro-steroidal environment required for the subsequent trans-differentiation and proliferation of the spermatogonia (Sun *et al.* 2014).

While the studies cited from the wrasse and tilapia involved female-to-male sex change, these evidences, together with those from the black porgy, do suggest that during sex change, initial pools of somatic cells of one sex type originated through trans-differentiation from existing somatic cells and this trans-differentiation was likely to be induced by the earliest oogonia or spermatogonia. The earliest oogonia or spermatogonia were in turn likely to arise from undifferentiated germ cells. Hence, it could be speculated that the presence of primary oocytes in Asian seabass testis could be an early indication of sex change and oocytes in the juvenile testes may be suggestive of a primary female.

4.2 qPCR-based analysis of a limited set of sex-related genes is a powerful method to uncover new molecular insights

In this study, a custom-designed 48.48 qPCR array, containing 37 candidate sex genes and 8 reference genes, was developed for the analysis of Asian seabass reproduction. These genes have been carefully selected according to their functions and pathways that have been shown earlier to be involved in reproduction in teleosts and other vertebrate species. The results from the qPCR array had provided several insights into the Asian seabass gonad development.

4.2.1 Complexity and variability of gonad development in Asian seabass

It was possible to divide the gonads into the male and female sexes based on just the expression patterns of 36 genes. The hierarchical clustering map generated using such a limited set of transcriptomic data (**Figure 15**) also uncovered the differences in individual gene expression among testes/ovaries across and within different sexual maturation stages.

In particular, the F3 and F4 ovaries were found to have distinctly different transcriptomic profiles. The key histological feature that separates the F3 and F4 ovaries was the presence of atretic oocytes in the latter, but not in the former (**Figure 10**). Apoptosis regulates the death of the oocytes and expectedly, the pro-apoptotic gene *tp53* (Amaral *et al.* 2010) was found to be up-regulated (2.3-fold) in the F4 ovaries compared to the F3 ovaries. In addition, F4 ovaries also showed higher expression of two RA-responsive genes, *cyp26a1* (2.7-fold) and *stra6* (3.5-fold), indicating a higher level of RA in the F4 ovaries (Taneja *et al.* 1995, Li *et al.* 2012). While RA is known to regulate multiple physiological processes, one of them involves the activation of apoptosis as applied in the treatment of tumors (Das *et al.* 2014). Recently, the human ortholog of *stra6* was also found to have a role in p53-induced apoptosis in human cancer cells (Carrera *et al.* 2013). However, whether higher RA levels indeed lead to increased apoptosis in the ovaries needs to be confirmed as RA can also enhance survival and proliferation depending on the cell type (Noy 2010). In addition, the up-regulation of 24 genes and down-regulation of only three genes in F4 ovaries compared to F3 ovaries suggest that the former may be more transcriptomically active than the latter.

This result showed the complexity and variability in the process of gonad development in the Asian seabass. Studies on gonad differentiation in the zebrafish had also reported such a trend (Wang *et al.* 2007b, Orban *et al.* 2009). The individual variability of gonad development thus implies that more biological samples would be needed in order to obtain a more representative result for any sex-related experiment. In this sense, the use of a 48.48-type qPCR array would prove useful as it allows the affordable, parallel analysis of only the important genes in several individuals (up to

13) in parallel. The platform could also be expanded into a 96.96 array to analyze even more genes and samples and this had been utilized in later parts of the study.

4.2.2 Identification of genes with expression characteristic of specific gonad types

One of the molecular insights obtained was that the M1 testes were found to have much lower expression levels of the testis-enhanced germ cell markers, *odf3*, *sycp3l*, *sept6* and *tdrd7* and the pro-male *amh* than the other three testicular stages (**Figure 16**). Homologs of *odf3* and *sept6* are known to be components of the cytoskeletal structure of the mammalian sperm tail (Petersen *et al.* 1999, Estey *et al.* 2011), the protein product of *Sycp3l* is a structural component of the synaptonemal complex in the mouse sperm (Yuan *et al.* 2000) and *Tdrd7* is associated with germinal granules in murine male germ cells (Hosokawa *et al.* 2007). Expectedly, these genes showed higher expression in the M3-M4 stage testes compared to the F3-F4 stage ovaries (Table 6). Their low expression in the M1 stage testis is hence likely to be a direct consequence of the lack of the spermatids and spermatozoa. In the case of *amh*, a member of the transforming growth factor-ß family known to promote mammalian testis differentiation through the regression and apoptosis of the Mullerian duct (Allard *et al.* 2000), it had been shown in the black porgy that active testes have higher expression levels of *amh* than inactive ones (Wu 2010). These evidences together may indicate that a higher level of *amh* is required to initiate spermatogenesis.

A second insight was that the consistently testis-enhanced expression of *cyp11c1* and *esr1* (regardless of maturation stage) could be suitable markers for the testes. Both genes showed testis-enhanced expression from M1 to M4 stages, but

remained low in the F1, F3 and F4 ovaries. This indicated the importance of the two genes in maintaining the male sex of the gonad.

Cyp11c1 (11ß-hydroxylase) is an important steroidogenic gene involved in the conversion of testosterone to estrogen and 11ß-hydroxytestosterone, an androgen and its expression is generally high in testis and low in ovary (Baroiller *et al.* 1999). Androgen levels are generally higher in males as they are required for stimulating male sexual differentiation, male behavior and spermatogenesis (reviewed by Borg, 1994 and Nagahama 1994). This explained why *cyp11c1* expression was high in the various stages of the testis compared to the ovaries.

On the other hand, estrogen receptors are intracellular receptors. When bound to estrogens, they form homo- or heterodimers and translocate to the nucleus where they bind to the estrogen response elements (ERE) of DNA to activate gene expression (Nelson and Habibi 2013). Estrogen receptors are well-known for their function in livers where they activate vitellogenesis in presence of estrogens (Nelson and Habibi 2013). However, given the complexity of estrogen signaling, the roles of the estrogen receptor subtypes in the gonads are less well understood and could be found localized in the somatic and/or germ cells depending on the species.

While the up-regulation of *esr1* in testis may seems counter-intuitive given the importance of estrogens in ovary development, estrogen receptors (ER) had been suggested to have a role in the control of testicular function as both *ERa* and *ERb* are expressed in the human testis (Cavaco *et al.* 2009). A higher testicular expression level of *esr1* compared to that of the ovary was also observed in the medaka, sea bream and Atlantic cod and Esr1 has been suggested to be required for spermatogenesis and Leydig cell maintenance (Socorro *et al.* 2000, Chakraborty *et al.* 2011, Nagasawa *et al.* 2014).

4.3 Sex change in Asian seabass involved apoptosis and de-differentiation of testis before gradual progression of ovarian differentiation

From the microarray analysis, the early transforming gonads were found to have a similar transcriptomic profile as the undifferentiated ones, suggesting that the testis had to degenerate and return to a near undifferentiated state before ovarian differentiation. The differences in expression profile between the early transforming and undifferentiated gonads could be partly attributed to the presence of residual spermatocytes and spermatozoa found in the former, but not in the latter. Indeed, the real-time qPCR results showed that the expressions of male germ cells markers, *odf3*, *sycp1* and *tekt1* were significantly up-regulated in the early transforming gonads compared to the undifferentiated ones (see appendices Table A 4).

Histological observation of the transforming gonads showed that the disappearance of spermatocytes, spermatids and the spermatogonia occurred in T1-T2 transforming gonads before the gradually increasing abundance of primary oocytes in late T3-T4 transforming gonads (**Figure 10**). This indicated that the male germ cells were cleared while the female germ cells appeared gradually. Similarly, the microarray results and real-time qPCR validation confirmed that male germ cell markers [*sept6* (Kissel *et al.* 2005, Estey *et al.* 2011), *odf3* (Petersen *et al.* 1999), *sycp1* (de Vries *et al.* 2005) and *tekt1* (Xu *et al.* 2001)] were down-regulated first during early transformation before the up-regulation of female germ cell markers [*zp3* (Hamazaki et al. 1989) and *rttn* (Faisst et al. 2002)] during the late transformation stages (Table 10).

Clearing of the germ cells of one sex to make way for those of the other was achieved through apoptosis. This had been shown in several species during gonad transformation, including the zebrafish (juvenile ovary-to-testis transformation)

(Uchida *et al.* 2002) and rice field eel (*Monopterus albus*) (female-to-male sex change) (He *et al.* 2010). In support of the role of apoptosis in removing germ cells during gonad transformation in the Asian seabass, the anti-apoptotic gene *bfar* (Roth *et al.* 0000, Stegh *et al.* 2002) was found to be down-regulated during early transformation while the pro-apoptotic gene *tp53* (Amaral *et al.* 2010) was up-regulated during late transformation. Expression of the gene encoding Sh3rf1, an apoptosis-promoting scaffold protein for the JNK pathway (Xu *et al.* 2003, Xu *et al.* 2006), was also up-regulated during late transformation in Asian seabass. In addition, the lysosomal protease, *ctsk* (Guha and Padh 2008), was also up-regulated during gonad transformation, indicating the increased degradation of proteins.

4.4 Gene expression changes during sex change reinforce the notion of conservation of sex differentiation

The potential role of several genes and pathways in gonad differentiation during the transformation process was uncovered from the microarray and real-time qPCR results (Table 10). This was achieved through identifying genes with changes in expression patterns during sex change and this link was further strengthened when their involvement in gonad differentiation had been suggested or confirmed in other species. However, signaling networks are known to be complex with cross talks, redundancies and pleiotropic effects. Hence, the confirmation of the involvement of each pathway or gene during gonad transformation would require functional experiments in future, either in the Asian seabass itself or in other easily manipulated model species such as the zebrafish.

4.4.1 Down-regulation of the expression of pro-male genes during sex change

Among genes with known roles in sex differentiation, the expression of pro-male *dmrt1* was down-regulated during the early transforming phase while that of *sox9* was down-regulated at the late transforming phase. Both *dmrt1* and *sox9* were also shown to have higher expression in the adult testis than in the ovary in Asian seabass (Table 6) and in zebrafish (Guo *et al.* 2005, Rodriguez-Mari *et al.* 2005). Specifically, *dmrt1* was shown to be expressed in the zebrafish male germ cells (spermatogonia, spermatocytes, and spermatids) (Guo *et al.* 2005), while *sox9a* was shown to be expressed in the zebrafish male somatic cells (Rodriguez-Mari *et al.* 2005). Both *dmrt1* and *sox9* are known transcription factors. In mammals, *Dmrt1* has been shown to be required for spermatogonia proliferation by inhibiting meiosis and promoting mitosis (Matson *et al.*, 2010), while *Sox9* is required for the specification of Sertoli cells (Ungewitter and Yao, 2013).

Based on these data, what we had observed in the Asian seabass could possibly suggest that the degeneration of the male germ cells occurred before the trans-differentiation of the male somatic cells. However, in the black porgy, *dmrt1* was shown to be expressed only in the Sertoli cells and not the germ cells (Wu *et al.* 2012, Wu and Chang 2013b). Thus, an *in-situ* hybridization of *dmrt1* and *sox9* localization would be required to confirm the relationship between *dmrt1* and *sox9* in the Asian seabass.

Interestingly, the expression of pro-male gene, *gsdf1* was unexpectedly up-regulated during early sex change before undergoing a bigger magnitude of down-regulation during late sex change (Table 10). This result suggests that in addition to its conserved role as a pro-male gene, *gsdf1* may have other roles not related to testis differentiation during early sex change. This finding parallels that made in the

rainbow trout where *gsdf* was found to be expressed in both the ovarian granulosa and testicular Sertoli cells, suggesting multiple functions of the trout ortholog (Sawatari *et al.* 2007). Hence, the larger down-regulation of *gsdf1* in late sex change could be attributed to the disappearance of Sertoli cells. In addition, it was shown that the *gsdf* gene is localized in a syntenic chromosomal region well conserved in vertebrates and the surrounding cluster of genes were found to be preferentially expressed in medaka and zebrafish ovaries, specifically in the primary oocytes (Gautier *et al.* 2011). Hence, this makes *gsdf* an interesting candidate gene for future studies, given that the ortholog of this gene seems to be missing from the genome of mammals and birds (Gautier *et al.* 2011) and it has also become the sex determining gene of two teleost species (Table 1).

4.4.2 The role of cyp19a1, cyp11c1 and hsd17b1 in Asian seabass reproduction

Androgens and estrogens are generally known to be the "male" and "female" sex hormones, respectively, as androgens function in males to promote testis differentiation, male behavior and spermatogenesis (reviewed by Borg 1994 and Nagahama 1994), while estrogens are known to promote ovarian differentiation, vitellogenesis in the liver and oocyte growth in females (Nelson and Habibi 2013, Nagahama 1994). Hence, androgens levels are expected to be high in males and low in females. Similarly, *cyp11c1* (11ß-hydroxylase), involved in the production of 11ß-hydroxytestosterone from testosterone, and c*yp19a1*, involved in the production of E2 from testosterone (Baroiller *et al.* 1999), are expected to have testis-enhanced and ovary-enhanced expression respectively.

However, there was no significant change in the expression of these two genes during gonad transformation that could be shown conclusively by both microarray

and/or qPCR. Furthermore, qPCR analysis of the Asian seabass gonads showed that *cyp19a1* was up-regulated in the testes by 5.5-fold compared to the ovaries (Table 6). This is contrary to the zebrafish, where higher expression levels of *cyp19a1a* were detected in the ovaries than in the testes (Rodriguez-Mari *et al.* 2005, Wang and Orban 2007). Through biochemical analysis of Asian seabass gonads, it was found that E2 levels were typically higher in all stages of ovaries (F1-F4) compared to all stages of the testes (M1-M4) (Guiguen *et al.* 1993). However both qPCR and microarray results showed, as per expectation, that *cyp11c1* expression was highly up-regulated by over 300-fold (qPCR result; Table 6) in the Asian seabass testes (M3-M4) compared to the ovaries (F3-F4). This is concurrent with the expression pattern seen in the zebrafish (Wang and Orban 2007).

Given that both *cyp19a1* and *cyp11c1* act on the same precursor, testosterone, this indicate that estrogens level may still be low relative to androgens in the adult males and vice-versa for the adult females despite the higher aromatase expression in the testis. Indeed, a previous biochemical analysis of Asian seabass plasma and gonadal steroid levels showed that although male Asian seabass had higher gonadal 11ß-hydroxyandrostenedione (another product of Cyp11c1) and plasma 11-KT compared to female seabass, the plasma and gonadal E2 levels were higher in the females than the males (Guiguen *et al.* 1993). This showed that despite the higher expression of aromatase transcripts in Asian seabass testes, the actual estrogen levels were still higher in the females.

In addition, there are known extra-gonadal sites of steroid production. For example, blood and liver have been shown to be alternate sites of androgen production in the rainbow trout (Schulz, 1986). In Asian seabass males, it had been shown that while 11-KT was the major androgen in the plasma, 11ß-

hydroxyandrostenedione was instead the major steroid in the gonad, suggesting a possible extra-gonadal site of 11-KT production (Guiguen *et al.* 1993). Similarly, in the same study, plasma E2 levels seemed to be higher in females with F3 and F4 stage gonads compared to the transforming individual although gonadal E2 levels were significantly higher in T1-T4 transforming gonads compared to F1-F4 ovaries (Guiguen *et al.* 1993). This may hence, also hint at possible extra-gonadal sites of E2 production.

The higher *cyp19a1* expression in Asian seabass testis also indicated that E2 could have important roles to play in the Asian seabass testis. In support of this, the estrogen receptor, *esr1*, also showed have higher expression levels in the Asian seabass testes compared to the ovaries (**Figure 18**). In mammals, estrogens have been shown to play a physiological role in the regulation of spermatogenesis as aromatase and estrogen receptors could be localized to the rat's Leydig and germ cells within the testis (Carreau *et al.* 2003). In aromatase knock out mice, males are initially fertile, but become infertile after 5 months of age due to failure in spermatogenesis (phytoestrogens in diet was suggested to result in the slow onset of infertility) (Robertson *et al.* 1999) Similarly, aromatase could also be detected in the Sertoli, Leydig and germ cells of the rainbow trout testes (Kotula-Balak *et al.* 2008). However, the exact function of estrogens in regulating spermatogenesis is still unknown.

Nevertheless, the unexpected high testicular and low ovarian expression of *cyp19a1* in the Asian seabass is worth further investigation in the future. In the case of *cyp11c1*, the failure to detect significant decrease in expression during Asian seabass gonad transformation may be due to the variability among the transforming gonads and the gradual down-regulation of the gene extending throughout the period of sex

change, hence resulting in non-significant p-values and weak down-regulation magnitude.

During early gonadal transformation, the gene with one of the highest magnitude of up-regulation shown by the microarray was the *hsd17b1* gene (252-fold up-regulation), a steroidogenic gene involved in the interconversion of adrenostrone to 11-KT; and estrone to E2 (Baroiller *et al.* 1999). In a study based on recovery of metabolites from incubation of radioactive androstenedione on Asian seabass gonads, it was shown that Hsd17b1 activity predominate in the T1 transforming gonads (Guiguen *et al.* 1995). However, the subsequent real-time qPCR did not manage to validate the microarray results, despite them being concurrent with what had been shown on the enzymatic activity level.

4.4.3 Activation of Wnt/ß-catenin signaling pathway during sex change

The real-time qPCR results also validated the differential expression of member genes of several pathways that may be required for ovarian differentiation. Wnt signaling has been known to be involved in teleost ovarian differentiation (Wu and Chang 2009, Nicol and Guiguen 2011, Nicol *et al.* 2013). Similarly, there was a differential expression of several Wnt signaling member genes during Asian seabass gonad transformation. In particular, the activators of the Wnt signaling pathway, *wnt3, wnt16* (Filali *et al.* 2002), *fzd1* (Gao and Chen 2010) and *ck2a* (Song *et al.* 2003, Gao and Wang 2006) were up-regulated during late transformation, while one of the negative regulators, *psen1* (Soriano *et al.* 2001, Xia *et al.* 2001, Kang *et al.* 2002, MacDonald *et al.* 2009), was down-regulated during early transformation. These results strongly suggest that Wnt signaling is activated during late gonad transformation in Asian seabass to drive ovarian differentiation.

4.4.4 Implications for other genes and signaling pathways during sex change

In mammals, Notch signaling pathway is known to be involved in ovary development, while hedgehog signaling plays a role in both ovary and testis development (Johnson *et al.* 2001, Wijgerde *et al.* 2005, Nguyen *et al.* 2009, Barsoum and Yao 2011). *JAG1* of Notch signaling was found to be expressed exclusively in the mammalian oocytes (Johnson *et al.* 2001) and in the Asian seabass, *jag1b* expression was found to be up-regulated during late sex change while the expression of *dlc*, the Notch ligand (Bray 2006), was down-regulated (Table 10). These results thus indicate the involvement of Notch signaling in Asian seabass gonad transformation. With regards to Hedgehog signaling, *sonic hedgehog* and its target gene, *gli1* (Katoh and Katoh 2006) were down-regulated during the transformation process, hence also implicating the pathway's involvement.

Members of the *NF-κB* and retinoic acid (RA) signaling pathways were also differentially regulated during Asian seabass gonad transformation (Table 10). Moreover, some of these genes were found to have pro- and anti-apoptotic consequences in other species, leading to the speculation that these pathways were (in)activated during the transformation to possibly drive apoptosis. NF-κB signaling is involved in numerous physiological processes including apoptosis (Gerondakis *et al.* 2006). In particular, *rtkn2* was down-regulated by about 21-fold during early gonad transformation and rhotekin (rtkn)-mediated activation of NF-κB had been shown to induce resistance to apoptosis in human cancers and lymphocytes (Liu *et al.* 2004, Collier *et al.* 2009). The inhibitor of NF-κB, IκBε (Verma *et al.* 1995, Jacobs and Harrison 1998), was also up-regulated, albeit to a smaller extent, during early sex change.

The qPCR results also showed a very high up-regulation of the expression of *cyp26a1* (129-fold) and *stra6* (379-fold), two RA-responsive genes during late gonad transformation (Taneja *et al.* 1995, Li *et al.* 2012). These results hence indicate that RA level was high during late gonad transformation. *Cyp26a1* was also shown to have higher expression in the Asian seabass ovary compared to the testis (Table 6) and its product is known to convert RA into non-active forms (Thatcher and Isoherranen 2009). Recently, the human ortholog of *stra6* was found to have a role in p53-induced apoptosis in human cancer cells (Carrera *et al.* 2013).

Genes that belong to the TGF-ß-Smad signaling pathway such as *smad4*, *bmp1*, *bmp2* and *gdf9*, were also found to be differentially expressed during gonad transformation. In particular, *Gdf9* was expressed in mammalian follicles (Fitzpatrick *et al.* 1998), zebrafish follicles (Liu and Ge 2007) and in male and female germ cells of the ricefield eel (*Monopterus albus*) (He *et al.* 2012). Gdf9 is a member of the transforming growth factor-beta superfamily, a secreted signaling factor found to be produced in mammalian oocytes to regulate the functions of surrounding granulose and theca cells to promote follicle development (Juengel *et al.* 2004). *Gdf9* was also down-regulated during ovary-to-testis sex reversal in rainbow trout (Baron *et al.* 2008). Concurring with the ovarian role of *gdf9* in other species, it was found to be highly up-regulated during Asian seabass gonad transformation.

Interestingly, several genes with immune-related functions were, such as complement components *c6* and *c7*, were also found to be differentially expressed during transformation (Table 10). Although complement factors were known to be secreted by human granulosa cells to promote oocyte development and maturation (Yoo *et al.* 2013), both *c6* and *c7* were down-regulated during late sex change in the Asian seabass, suggesting a different role. Peli1 is an E3 ubiquitin ligase with roles in

immunity, specifically toll-like receptor signaling and interlukin-1 receptor signaling (Schauvliege *et al.* 2007, Chang *et al.* 2009). The expression of *peli1* was also found to be significantly down-regulated during both early and late transformation phases in the Asian seabass. It is not known how immunity could be related to the sex change, but reproductive-immune interactions were shown to exist across vertebrates and one aspect is the regulation of various immune processes by the sex steroids (reviewed by Lutton and Callard, 2006). Immune cells such as macrophages and leukocytes could also be found in teleost gonads, including the gilthead seabream where it was found that several of the immune-related genes showed fluctuations in expression across the reproductive cycle (Chaves-Pozo *et al.* 2008). Hence, it is possible that the changes in the expression of these genes could be related to alteration of the immune process cause by changes in sex steroids levels.

Several genes with neuron-related function were also found to be differentially expressed in the gonad during the sex change process. The expression of *sema4e* was highly down-regulated (161-fold) during early transformation and its product, a transmembrane protein has been shown to function as a repulsive guidance molecule for the directional growth of axons in zebrafish (Xiao *et al.* 2003). Similarly, the netrin receptor, Unc5a, also functions to repulse axons in *Drosophila* (Keleman and Dickson 2001) and the expression of its ortholog was also down-regulated during early gonad transformation in Asian seabass. These results hence suggest that there may be an increased innervation in the gonad during the transformation process. Another neuron-related gene, *npb* was also down-regulated during early transformation in the gonad before a subsequent, modest up-regulation during late transformation, suggesting a potential role for this neuropeptide in the gonad. This neuropeptide is a known ligand for mammalian G-protein coupled receptors found in

the brain (Tanaka *et al*. 2003) and has been suggested to be involved in the regulation of several physiological functions such as feeding behavior (Hondo *et al.* 2008).

4.5 Sexual differences in brain may be mild, localized and possibly transient

In this study, microarray analysis showed that there was an absence of widespread differences between the transcriptomes of whole Asian seabass adult brains of the two sexes. The brain is an important organ in relation to sex as it has a critical role in sexual development through the control of the endocrine system via the BPG axis (Zohar *et al.* 2010). In the Senegalese sole (*Solea senegalensis*), *kiss2* and *kiss2r*, the genes involved in the BPG axis, showed differential fluctuations of their expression patterns between the brains of the sexes during a reproductive cycle (Mechaly *et al.* 2012).

In addition, the brain may have a role to play during sex change. For example, in the bidirectional hermaphroditic bluebanded goby (*Lythrypnus dalli*), it was found that female brains had higher levels of E2 than male brains and during social induction of protogynous sex change, testosterone and 11-KT levels changed in the brain (Lorenzi *et al.* 2012). As a result, there had been numerous studies in detailing the sexual dimorphism in gene expression in teleost brains (Santos *et al.* 2008, Sreenivasan *et al.* 2008, Taboada *et al.* 2012). In mammals and birds, there are also gene expression differences between the male and female brains (Yang *et al.* 2006, Naurin *et al.* 2011, Trabzuni *et al.* 2013). Studies using specimens like the gynandromorphic zebra finch (*Taeniopygia guttata*), chicken embryos and agonadal mouse have shown that such sexual differences in mammals and birds are caused not just by gonadal hormones, but they might also develop autonomously from the sex chromosomes that the cells carry (Agate *et al.* 2003, Scholz *et al.* 2006, Büdefeld *et*

al. 2008). While not all teleosts may carry sex chromosomes, the teleost brain is also known to be a target of sex steroids (Diotel *et al.* 2011).

Hence, the detection of only three transcripts that showed sexually dimorphic expression in the Asian seabass whole brain was unexpected. This is especially since several large-scale studies had already shown that gene expression patterns differed substantially between the sexes in mammals, birds and other teleosts. However, many of these studies also showed contrasting results in the number of genes reported, ranging from tens to over thousands of genes (Table 15). Our detailed meta-analysis revealed that these differences in the number of genes reported are in part due to the differences in statistical criteria used. For example, in mouse, microarray analysis detected widespread differences of over 600 genes with significant differential expression ($p<0.01$), but when a fold-change of greater than two was considered, the number of genes dropped drastically to only six (Yang *et al.* 2006).

Similarly, a microarray analysis of the brains of mature and immature Atlantic salmon could identify over 400 clones with differential expression, but all showed limited changes only (most much lesser than two-fold) (Guiry *et al.* 2010). Hence, it seems that the analysis of the brain expression pattern could be compounded by the small fold-differences between the sexes. It is likely that some genes may have only differences in expression in certain parts of the brain and the use of whole brain may further dilute the differences. Besides the spatial factor, the differences may also be temporal and hence the use of adult brains may not necessary capture the differences. In a study on the rainbow trout, it was found that the brain aromatase, estrogen receptors genes and *cyp11a1* showed sexually dimorphic expression only when the gonads were still morphologically undifferentiated and the differences in expression disappeared thereafter (Vizziano-Cantonnet *et al.* 2011).

Hence, the failure to detect widespread transcriptomic differences between the whole brains of the two sexes of Asian seabass (in this case defined as p-value FDR < 0.05 and fold-change ≥ 1.5 or ≤ -1.5) suggests that any existing differences in expression are likely to be mild, localized and possibly transient. This finding suggests that future studies on brain sexual differences will be required to investigate different portions of the brains separately and at stages other than adult.

Table 15. Summary of gene expression differences detected in brains of teleost, mammals and birds.

S/N	Organism	Organ/ Method	No of differentially expressed genes	Statistical criteria	Reference
1	Zebrafish	Whole brain/ Microarray	1,641	p<0.01; fold-change > 1.5	Sreenivasan *et al.* 2008
2	Zebrafish	Whole brain/ Microarray	42	p<0.05 (Benjamini and Hochberg multiple testing correction); fold-change > 1.2	Santos *et al.* 2008
3	Turbot (*Scophthalmus maximus*)	Whole brain/ cDNA-AFLP	No difference	Not applicable	Taboada *et al.* 2012
4	Human	Separate brain regions/ Microarray	2.6% of all expressed genes (448/17,501)	p (FDR) < 0.01; no fold-change consideration	Trabzuni *et al.* 2013
5	Mouse	Whole brain/ Microarray	612 (>1 fold) 34 (>1.2 fold) 6 (>2 fold) 3 (>3 fold)	p< 0.01	Yang *et al.* 2006
6	Zebra finch (*Taeniopygia guttata*)	Telencephalon / Microarray	417 genes	*P* with FDR set at 3.9%; no fold change consideration	Naurin *et al.* 2011
7	Common whitethroat (*Sylvia communis*)	Whole brain/ Microarray	299 genes	*P* with FDR set at 3.5%; no fold change consideration	Naurin *et al.* 2011

4.6 Potential existence of long-term temporal distribution of GnRH isoforms in Asian seabass

In this study, sexually mature Asian seabass brains from both sexes were used for deep sequencing of the Asian seabass transcriptome and only salmon GnRH was detected from the transcriptome data. However, in another study utilizing juvenile seabass brains (age or size not mentioned) for the generation of a brain EST library, only chicken GnRH-II was found (Tan *et al.*, 2008). Given that deep sequencing was used in this study, the failure to detect chicken GnRH-II isoform from the transcriptome data is likely due to the absence or very low expression of the transcript.

One possible explanation for the absence of chicken GnRH-II in the transcriptome data is that besides having a spatial distribution of GnRH isoforms (Chen and Fernald 2008), it is possible that there is a temporal distribution which extends not just in the short-term across the spawning cycle (Gothilf *et al.* 1996) but also in the long-term across the developmental stage of the fish. It would be interesting in the future to further analyze when the switch in use of GnRH isoform occurs and whether it coincides with any specific stage of the sexual development.

4.7 Positive effect of GnRH on mucus 11-KT level

In this study, the sexually dimorphic effect of GnRH on mucus 11-KT levels has been demonstrated to be a potential non-invasive method of sexing the Asian seabass.

In aquaculture, GnRH and their analogs are commonly used to promote ovulation, spermiation and induce spawning of broodstock individuals of several species (Zohar and Mylonas 2001), including induced spawning in Asian seabass

(Almendras *et al.* 1988). GnRH is produced from the brain and worked via inducing the release of gonadotropins (FSH and LH) from the pituitary which subsequently stimulate the gonads to release sex steroids and progestagens to promote gametogenesis and spermiation/ ovulation respectively (Mylonas *et al.* 2010).

In teleosts, the main and most potent androgen is 11-KT, while E2 is one of the main female hormones (Hishida and Kawamoto 1970, Baroiller *et al.* 1999). It was previously shown that the levels of 11-KT in the surface mucus of koi carp (*Cyprinus carpio*) corresponded with those in blood serum and muscle (Schultz *et al.* 2005). In addition, it was also shown in male striped bass (*Morone saxatilis*) that plasma 11-KT spiked significantly at seven days after treatment of sustained release GnRH with corresponding increased production of milt (Mylonas *et al.* 1997).

The results from this study have shown that Asian seabass can respond to GnRH induction with a spike in mucus 11-KT depending on the gonadal maturation stage. Immature seabass with undifferentiated gonads were unresponsive, while males responded with greater 11-KT spike than females. Older males with presumably larger testes and hence greater capacity to produce 11-KT, responded with greater spikes. This result also indicated that juvenile seabass at 9 months of age with juvenile testes had a functional pituitary with GnRH receptors and capable of producing gonadotropins. On the other hand, juveniles with undifferentiated gonads failed to respond either due to an un-functional pituitary (lack of receptors or inability to produce gonadotropins) or un-functional gonad (lack of gonadotropin receptors or inability to produce steroids) or both.

In this study, mucus 11-KT could be detected from both sexes of the Asian seabass and interestingly, several females had higher mucus 11-KT levels than some males, indicating a potential anomaly (**Figure 23**).

This is because androgens are known for their roles in stimulating male sexual differentiation, male behavior and spermatogenesis, and are hence expected to be detected at higher levels in males (reviewed by Borg, 1994). It is known that generally the Leydig cells of the testes are the major sites of androgen production (Devlin and Nagahama 2002). Similarly, the ovaries can also produce androgens including 11-KT. Cyp11c1 (11ß-hydroxylase), involved in the indirect production of 11-KT from T, could be detected via immunohistology in the theca cells of the ovary of the protandrous anemonefish (*Amphiprion clarkii*) (Miura *et al.* 2008). Transcripts of *cyp11c1* could also be detected in the ovary of the Malabar grouper (*E. malabaricus*) and *cyp11c1* was further suggested to be involved in the female-to-male sex change of the species (Murata *et al.* 2010). In the Japanese eel (*Anguilla japonica*), the ovary was found to be able to produce 11-KT *in vitro* (Matsubara *et al.* 2003).

As such, it is not unusual to detect 11-KT in the mucus or blood serum of female fish. In the review by Borg (1994), 11-KT was summarized to be found in the serum of females from several fish species, although its level was generally lower in them compared to that of the males. In another study, 11-KT was also detected in the blood serum of females from several fish species across various families, including the *Acipenseridae*, *Salmonidae*, *Cyprinidae* and those from the *Periformes* order (Lokman *et al.* 2002).

Androgens, while primarily male hormones, do also have female-related roles. In the rainbow trout and European eel, androgens can stimulate the production of vitellogenesis in the liver (Peyon *et al.* 1996, Mori *et al.* 1998). In two species of eels (*Anguilla japonica* and *A. dieffenbachia*), treatment of 11-KT can result in increased oocytes size, suggesting a role of 11-KT in oogenesis (Matsubara *et al.* 2003). Hence it is possible for some females to have higher levels of 11-KT compared to some

males, although the exact role of 11-KT in female Asian seabass is unknown. In addition, some males may have lower levels of 11-KT as they may not be undergoing spermatogenesis. Nevertheless, on average, male Asian seabass had higher mucus 11-KT compared to females (2.04-fold before GnRH induction; 10.62-fold after induction).

4.8 Positive effect of GnRH on testis development

In this study, the long-term treatment with GnRH alone can promote the development of M3-stage testis in juvenile seabass. In several species, GnRH solely or in combination with testosterone and dopamine inhibitors have been used to promote sexual maturation to varying degree of success in the Atlantic salmon (*Salmo salar*), striped bass, European eel (*Anguilla anguilla*) and honeycomb grouper (Henry *et al.* 1998, Hassin *et al.* 2000, Vidal *et al.* 2004, Kanemaru *et al.* 2012). Based on these studies, GnRH, domperidone and 17MT was tested for their effects on promoting early sexual maturation in the Asian seabass.

However, the use of domperidone and/or 17MT with GnRH did not promote the development of testis beyond the level shown by GnRH implants alone. The addition of both domperidone as well as domperidone+17MT resulted in a decreased number of milting males or seabass with M3 stage testis compared to the GnRH group. Moreover, with the combined treatment (domperidone+17MT), there was even an adverse effect on testis development compared to the control as there were more M1 stage testes and less M3 and M4 stage testes compared to the control.

Dopamine is known to inhibit GnRH-stimulated release of gonadotropins from the pituitary (Levavi-Sivan *et al.* 2010). In cultured carps, it is known that GnRH must be used in conjunction with dopamine antagonist in order to successfully induce

ovulation (Peter *et al.* 1988). However, in the Asian seabass, GnRH alone is sufficient to induce ovulation (Almendras *et al.* 1988). Hence, this inhibition mechanism regulated by dopamine may not be significant in the Asian seabass, at least in the final stages of gametogenesis. In the European eel and striped mullet (*Mugil cephalus*), dopamine was shown to be involved in the inhibition of puberty although in other species such as the salmonids and striped bass (*Morone saxatilis*), there seemed to be no or low dopamine involved in the process (reviewed by Dufour *et al.* 2010). These studies thus showed that the inhibitory effect of dopamine is not ubiquitous in all fish species and the results in this study have shown that dopamine inhibition of GnRH is insignificant in Asian seabass.

17MT, on the other hand, is a synthetic steroid with known masculinizing and growth-promoting effects on teleosts (Baker *et al.* 1988, Kuwaye *et al.* 1993). The adverse effect of 17MT on testis development compared to the control group suggested that the use of 17MT in this experiment resulted in an inhibitory role in the progression of testis maturation and spermatogenesis. It has been shown that testosterone can inhibit 11-KT-induced spermatogenesis in African catfish (*Clarias gariepinus*) (Cavaco *et al.* 2001). In addition, in fathead minnow (*Pimephales promelas*), 17MT was found to be converted by aromatase into 17alpha-methylestradiol (17ME2) a metabolite that could bind similarly to the estrogen receptor albeit with lesser affinity (Hornung *et al.* 2004). Hence, it is possible that 17MT may exert its inhibitory effect by either inhibiting spermatogenesis or through the estrogenic effect of its metabolites *in vivo* especially when used in non-optimal doses resulting in the opposite effect in the sensitive regulation of the neuroendocrine system.

4.9 Gonad differentiation in zebrafish is regulated by the canonical Wnt signaling pathway

The role of Wnt/ß-catenin signaling in mammalian sex determination and differentiation is well known and gene expression studies on other teleosts such as the black porgy and rainbow trout have also implicated Wnt signaling in teleost gonad differentiation (see introduction).

Previous work by our laboratory on the microarray analysis of the zebrafish gonad transformation process had implicated the involvement of Wnt signaling. In this study, three members of the Wnt/beta-catenin signaling pathway (*ctnnbip1*, *psen1* and *dkk3*) were verified to be differentially expressed by real-time qPCR (Table 12). While the set of Wnt signaling member genes that were differentially expressed in the zebrafish 'juvenile ovary-to-testis' transformation were different from those shown during Asian seabass testis-to-ovary transformation, these evidences do suggest a potential role of Wnt signaling in gonad differentiation. Hence, the zebrafish was used to functionally validate the role of Wnt signaling in gonad development.

However, Wnt/ß-catenin signaling could not be visualized to be active in the gonads of *Tg(TOP:dGFP)w25* zebrafish (**Figure 26**). A possible explanation is that the TOPdGFP construct may not be sufficiently expressed for it to be detected in the gonads. Functionally, it has been shown in rainbow trout that the use of the Wnt signaling chemical inhibitor, IWR-1-endo, could result in temporary down-regulation of the expression of *fst* and c*yp19a1a* in the differentiating ovary, but the adult sex ratios of the treated individuals were not reported (Nicol *et al.* 2013). Similarly, our initial attempt to inhibit Wnt signaling using IWR-1-endo on zebrafish also resulted in the down-regulation of *cyp19a1a* (*fsta* was not tested). However, there were no consistently male-biased sex ratios caused by these treatments. In our case, it was not

possible to further increase the concentration of IWR-1 beyond 2 μM or to extend the treatment beyond 40 dpf as both would result in excessive mortality. Thus, a reason for the inconclusive result could be attributed to the insufficient repression of Wnt/ß-catenin signaling and/or the potential reversibility of the transformation process during the gonad differentiation period.

Henceforth, a transgenic system generated earlier in the Moon laboratory (Stoick-Cooper *et al.* 2007) was used that allowed for the repression of Wnt/ß-catenin signaling from 20 dpf until 60 dpf without incurring significant mortalities. While the process of gonad transformation could have already been initiated at 20 dpf, this treatment period would allow the investigation of the role of Wnt signaling in regulating zebrafish gonad transformation (Wang *et al.* 2007b). Through the use of *Tg(hsp70l:dkk1b-GFP)w32* zebrafish, heat-induced inhibition of canonical Wnt signaling during the gonad differentiation period resulted in male-biased sex ratios. The transgenic inhibition of Wnt signaling during this period also down-regulated *cyp19a1a* expression significantly. In addition, *lef1*, a Wnt signaling target gene was also down-regulated.

Given the ubiquitous expression of the *dkk1b-GFP* transgene, there is a possibility that male-biased sex ratios could be due to the effect of *dkk1b-GFP* on other tissues involved in reproduction (*e.g.* brain), causing the down-regulation of *cyp19a1a* as a consequence. Nevertheless, *cyp19a1a* down-regulation was shown to be responsive to changes in levels of *dkk1b-GFP* transgene expression and reduced expression of the gonadal aromatase gene could be detected after the very first heat shock. Immunohistochemical analysis has also confirmed that *dkk1b-GFP* was expressed in the somatic cells of the developing gonads upon heat shock which coincided with the known localization of *cyp19a1a* (Wang and Orban 2007). In

addition, through computational prediction using PROMO (open web-based software), LEF-1 and TCF-4 binding sites could be found at the upstream region of zebrafish *cyp19a1a* (Messeguer *et al.* 2002, Farré *et al.* 2003). The proximal region of mammalian *CYP19A1* could also be amplified from DNA obtained from chromatin immunoprecipitation (ChIP) assays performed using ß-catenin antibodies (Parakh *et al.* 2006). This indicates that *cyp19a1a* is a direct target of canonical Wnt signaling. Furthermore, although chemical inhibition of the canonical Wnt pathway using IWR-1 did not conclusively change the sex ratios in all treated groups, *cyp19a1a* was down-regulated by 2.02-fold. In another study on the rainbow trout, the inhibition of Wnt pathway for 5 days using IWR-1 during ovarian differentiation similarly resulted in the down-regulation of *cyp19a1a* (Nicol *et al.* 2013).

Taken together, these evidences strongly suggest that the decreased *cyp19a1a* expression is largely a direct consequence of the inhibition of Wnt signaling resulting from *dkk1b-GFP* transgene expression. Hence, it is likely that the male-biased sex ratios are due to the decreased *cyp19a1a* expression.

Using the zebrafish as a model, Wnt/ß-catenin signaling was shown to be involved in teleost gonad differentiation. This is the first functional proof from a teleost species that manipulation of the pathway during that period resulted in altered sex ratios that lasted to adulthood. The inhibition of canonical Wnt signaling also resulted in partial deformation of the caudal and dorsal fins. This observation hence supports existing studies that Wnt signaling is required not only for vertebrate fin regeneration, but for fin development as well (Yokoi *et al.* 2003, Neto *et al.* 2012).

However, in the Asian seabass, while the gene expression analysis strongly suggested that Wnt signaling was being activated during late gonad transformation, there was no significant up-regulation of *cyp19a1*. Moreover, *cyp19a1* was further

shown to have higher expression in the testis than in the ovary. Hence, this indicates that Wnt signaling may also work to promote ovarian differentiation via different means.

4.10 A model of Asian seabass sexual development

In summary, differentiated testes could be found in juvenile Asian seabass as young as 8-9 months of age. Given that undifferentiated gonads have a transcriptomic profile more similar to the testis than to the ovary, the differentiation into juvenile testes by the undifferentiated gonad seems to be mandatory. Furthermore, oocytes-in-testis may be an early indication of sex change and oocytes in the juvenile testes may be suggestive of a primary female. The microarray analysis of the gonads at various stages of Asian seabass reproductive cycle also reveals that the gonadal transcriptomic profile undergoes a de-differentiation before ovary differentiation occurs. In addition, several genes and pathways possibly involved in apoptosis were found to be differentially expressed during the transformation phase. Evidences from the gene expression changes also strongly indicate the activation of the pro-female Wnt signaling pathway during late transformation.

qPCR analysis of the M1-M4 testes and F1-F4 ovaries have also shown that *esr1* and *cyp11c1* expression remain high in all testis stages and low in all ovary stages while *amh* is possibly required to initiate spermatogenesis as its expression is low in M1 testes. Additionally, Asian seabass can respond to GnRH induction with a spike in mucus 11-KT depending on the gonadal maturation stage and long-term treatment of GnRHa alone can promote the development of M3-stage testis in juvenile seabass. With these data, a model of the Asian seabass sexual development is shown in **Figure 33**.

4.11 Working hypothesis for the molecular processes involved in gonad differentiation

In this study, the analysis of the testis-to-ovary transformation in the Asian seabass has shown that pro-male genes such as *dmrt1*, *sox9* and *gsdf* had to be down-regulated while the pro-female canonical Wnt signaling pathway had to be up-regulated during the process. These pro-male and pro-female genes had already been shown in several other species to be required for testicular and ovarian differentiation respectively. Hence, it can be inferred that the differentiation into an ovary (be it from an undifferentiated gonad or from a testis) involved the down-regulation of pro-male genes and the up-regulation of pro-female genes.

The same is true for a differentiation into a testis, but the direction of change for the expression of pro-male and pro-female genes is reversed, as shown by female-to-male sex reversal experiments carried out in the Nile tilapia (Sun *et al.* 2014) and rainbow trout (Baron *et al.* 2008). The difference between a direct differentiation into the testis or ovary from the bipotential gonad and the transformation into the testis or ovary from the precursor sex is the involvement of apoptosis that is required to clear the existing differentiated germ cells in the latter process. Another difference is that during gonad transformation, the somatic cells of the new sex may develop through trans-differentiation of existing pools of sex-differentiated somatic cells. This is unlike the case of a direct differentiation into a testis or ovary where the somatic cells develop from an undifferentiated state. Hence, despite the additional involvement of apoptosis and possible difference in the origins of the somatic cells, the differentiation of the bipotential gonad into the testis or ovary in gonochoristic species and the gonadal transformation of the testis or ovary in sequential hermaphrodites essentially involved the same processes, albeit in the appropriate direction.

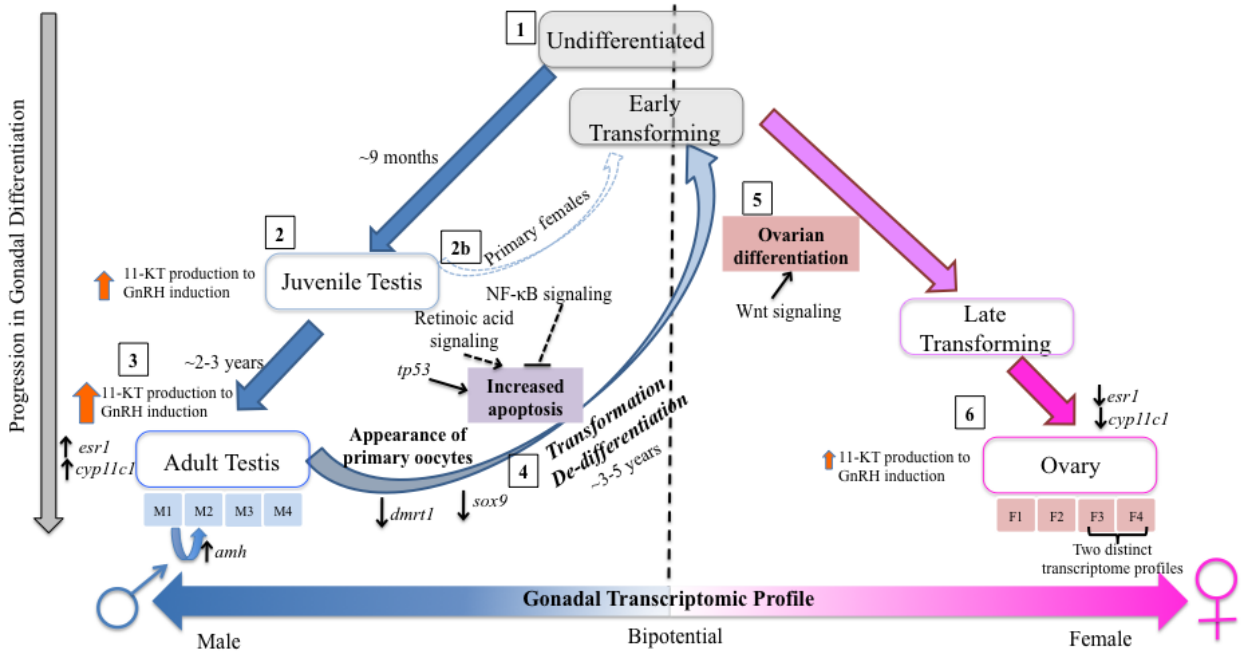

Figure 33. Model of Asian seabass sexual development shows return of early transforming gonad to near-undifferentiated transcriptomic state.
[1] The undifferentiated gonad is shifted to the left of the bipotential line as its transcriptomic profile is more similar to the testis than the ovary. [2] The differentiation to an juvenile testis occurs at around 9 months of age and may be mandatory. At this point of development, the Asian seabass is able to respond to GnRH induction with moderate increase in 11-KT production. [2b] Primary females may transform after this juvenile testis phase while most individuals transform only after the adult testis phase at 3-5 years of age. [3] In the adults, the testes are able to respond to GnRH induction with the greatest increase in 11-KT production. Across the sexual maturation stages of M1-M4, *esr1* and *cyp11c1* expression are elevated compared to the ovaries. In addition, increase in *amh* expression may be required to initiate spermatogenesis. [4] The earliest sign of transformation possibly involves the appearance of primary oocytes within the testis. During transformation, pro-male genes *dmrt1* and *sox9* are down-regulated. Apoptosis is activated to clear the male germ cells, regulated by *tp53* and possibly retinoic acid signaling and NF-κB signaling. The early transforming gonad thus assumes a near-undifferentiated transcriptomic state with the disappearance of testicular tissues. [5] Subsequently, ovarian differentiation from the early transforming gonad involves the up-regulation of canonical Wnt signaling pathway. [6] The adult ovaries are also able to respond to GnRH induction, but with the lowest increase in 11-KT production compared to the adult and juvenile testes. Distinct transcriptomic profiles were also observed between the F3 and F4 stage ovaries.

However, it is important to note that gonad differentiation or transformation is a much more complicated process. First, in several gonochoristic teleosts, the number of primordial germ cells (PGCs) may play a role in determining the sex of the fish.

The presence of germ cells is required for the development of the zebrafish ovary and an empty somatic gonadal shell forms in knock-down morphants injected with anti-*dnd* morpholino (Slanchev *et al.* 2005, Siegfried and Nusslein-Volhard 2008). In the medaka and stickleback, higher PGC numbers can lead to ovarian differentiation (Satoh 1974, Lewis *et al.* 2008). It is not known how PGCs can regulate gonad differentiation in these species and PGCs may not have such roles, if any, in sequential hermaphrodites.

Secondly, genes or pathways may have different functions during different developmental period. For example, while Wnt signaling has been shown, in this study and several other studies, to be involved in ovarian differentiation, Wnt signaling can also function to affect early germ cell development. In mouse, Wnt4 has been shown to maintain female germ cell survival by inhibiting *Inhbb* expression via β-catenin in the somatic cells (Liu *et al.* 2010). Aberrant activation of Wnt/β-catenin signaling in mammalian PGCs is also deleterious for their normal development (Kimura *et al.* 2006). Another example is NF-κB signaling which has been shown to play a role in mammalian spermatogenesis through the activation of the testis-enriched *LRWD1* gene (Teng *et al.* 2012), while activation of NF-κB signaling has been shown to result in increased survival of oocytes that can result in female-biased sex ratios in zebrafish (Pradhan *et al.* 2012). The pro-male *gsdf* is another gene that has been shown to have possible non-testicular role in Asian seabass given its up-regulation during early sex change before a down-regulation during late sex change.

Lastly, there are also exceptions as not all sex genes have the same function or effect in every species. For example, the supposedly pro-female *foxl2* showed up-regulation instead of the expected reduced expression in the protogynous wrasse during ovary-to-testis transformation (Kobayashi *et al.* 2010). Similarly, while

microarray analysis indicated that *foxl2* was up-regulated (183-fold) during early gonad transformation in Asian seabass, the subsequent qPCR verification did not manage to show any significant down-regulation. In addition, the gonadal aromatase, *cyp19a1*, showed higher expression in the Asian seabass testis compared to the ovary. It was recently found that in some cichlid lineages, both the ovarian and brain aromatases can have testicular function and as a result the sex steroid pathway has been suggested to be not as conserved among teleosts (Böhne *et al.* 2013).

Despite the complexity of gonad development described above, a generalized working hypothesis has been set up to explain the gene expression changes observed during the analysis of gonad development in vertebrates (**Figure 34**).

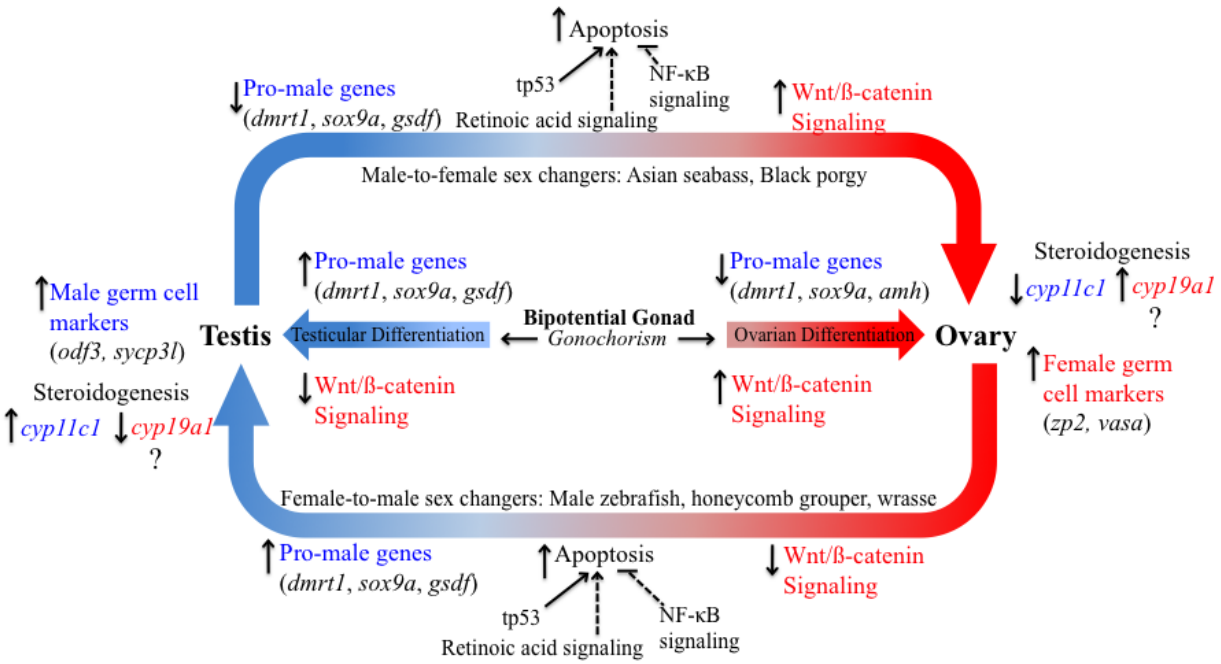

Figure 34. Simplified working hypothesis for the processes involved in gonad differentiation.

The differentiation of the bipotential gonad into the testis or ovary in gonochoristic species and the transformation of the testis or ovary in sequential hermaphrodites involve a largely overlapping set of processes, albeit in the appropriate direction. During testicular differentiation, pro-male genes such as *dmrt1*, *sox9a* and *gsdf* must be up-regulated for testis differentiation while the pro-female Wnt/ß-catenin pathway must be down-regulated. The opposite is true for ovarian differentiation. Likewise, during testis-to-ovary transformation, pro-male genes must be down-regulated and pro-female genes must be up-regulated. In addition, apoptosis needs to be activated in order to clear the existing male germ cells. Similarly, during ovary-to-testis transformation, pro-female and pro-male genes must be down-regulated and up-regulated, respectively. However, unlike the pro-male and pro-female genes whose direction of change had to be reversed, apoptosis is activated in the transforming ovary (as it is in the transforming testis) in order to clear the female germ cells. The differentiation into testis will result in high expression levels of male germ cell markers such as *odf3* and *sycp3l*, while the differentiation into ovary results in high expression of female germ cell markers, such as *zp2* and *vasa*. These changes are likely to be consequences of the gonad differentiation process. Beside the germ cell markers, the expression of the pro-male steroidogenic gene, *cyp11c1* is high in the testes and low in the ovaries. However, while the gonadal aromatase is shown in the zebrafish to be highly expressed in the ovary and not in the testis, the same is not true for the Asian seabass.

5 Concluding remarks and future directions

The study of gonad development in Asian seabass will always be a challenging area, as the sexual maturation period of the species is long (a two years long period for a male and even longer for a female) and even the earliest developments in the testis take place at 7.5 months of age. Furthermore, it is currently not possible to predict when would a particular individual transform naturally. The trigger for sex change in the species is still unknown, hence eliminating the option of experimental induction of the process for the purpose of research. Thus, compared to the black porgy where sex change is more predictable with 50% of the males changing into females during the third year of their lives (Wu and Chang 2013b), the study of Asian seabass gonad transformation is currently confined to the random collection of gonads from adults and analysis of the transformants found during this process. In addition, the Asian seabass is relatively large-sized (more than two kg of weight beyond two years of age), thus requiring greater resources in keeping the fish for any experiments.

Hence, to facilitate the future study of sex change in the Asian seabass, there is a need to identify the trigger for sex change. The environment could be a potential trigger and one of the candidate abiotic factors is the change in water salinity (from low to high). It was previously shown that only Asian seabass caught from coastal waters where water salinity was high had ripe testes and ovaries, as opposed to those caught from inland waters where water salinity was low (Moore 1982). On the other hand, a freshwater-based aquaculture facility in Hungary reported the absence of females among the captive broodstock (K. Szentes, personal communication). The potential requirement for high salinity waters for gonad maturation and sex change

may be related to the life history of the catadromous species which return to the sea for spawning (Moore 1982). The understanding of the trigger for sex change would also greatly facilitate the selective breeding of the species by promoting early sex change in males, thus shortening the intergeneration waiting period.

Our laboratory is also currently on its way to complete the sequencing of the Asian seabass genome. The completion of the Asian seabass genome, that of the first true hermaphrodite teleost, will provide the opportunity to further unravel the genetic regulation of sex differentiation. In this study, the microarrays and qPCR arrays generated for the study of Asian seabass have only provided one aspect of the genetic regulation of sex differentiation *i.e.* relative mRNA levels.

However, in the past few decades, several 'non-central dogma' mechanisms have been discovered to have important roles in regulating the expression of genes. This includes DNA methylation that can result in gene silencing (Newell-Price *et al.* 2000). Recently, the whole methylome of the tongue sole was sequenced and it was shown that DNA methylation is involved in the temperature-induced sex reversal of the species and which could be inherited (Shao *et al.* 2014). Earlier, it was already shown that the promoter region of *cyp19a1a* was methylated in response to heat treatment in the European seabass, resulting in female-to-male sex reversal (Navarro-Martin *et al.* 2011). Hence, it will be fruitful to similarly analyze the methylomes of the male and female Asian seabass and compare them with that of the early sex changers. This way, if methylation is indeed involved in natural sex change of Asian seabass, we may be able to find the key gene(s) required for initiating sex change.

Besides searching through the methylome, it will also be useful to search through the sequenced genome for possible duplication of the key sex genes such as *dmrt1*. This is because most of the candidate master sex genes found to-date in teleosts are

duplicated copies of known sex genes such as *dmrt1*, *amh* and *gsdf*. While the Asian seabass is a hermaphrodite and as such it is unlikely to have a single "sex determining gene", the existence of duplicated copies of key genes that play a role in the sex change as switches for the primary and secondary sex determination (see **Figure 1**) cannot be ruled out.

In this study, the zebrafish was used as a model to functionally validate the role of Wnt signaling in promoting ovarian development. This would be a very difficult task in the Asian seabass, given their large physical sizes, late sexual maturation period and the lack of transgenic lines. In zebrafish, the earliest sign of gonad differentiation is at 10-14 dpf with the juvenile-ovary-to-testis transformation starting at around 20 dpf (review: Orban *et al.* 2009). Hence, while the development of the reproductive organs is a relatively late process compared to other organs such as fin, brain and liver, their development in the zebrafish is still relatively early compared to the Asian seabass. Also, given the conservation of genes involved in gonad differentiation, the zebrafish could be further utilized to serve as a model for the study of gonad differentiation, for example in validating the role of *gsdf* and Notch and Hedgehog signaling in gonad development.

The knowledge obtained in this thesis and tools developed during the process will hopefully set the groundwork for future experiments that can improve the sex control of this species, either in early sexual maturation or sex change and initiate studies into other biologically important complex traits of the species such as disease resistance.

Bibliography

Papers published

- *Ravi, P., ***J. Jiang**, W. Liew and L. Orban (2014). "Small-scale transcriptomics reveals differences among gonadal stages in Asian seabass (*Lates calcarifer*)." *Reprod Biol Endocrinol* **12**(1): 5.
- *Sreenivasan, R., ***J. Jiang**, X. Wang, R. Bartfai, H. Y. Kwan, A. Christoffels and L. Orban (2013). "Gonad differentiation in zebrafish is regulated by the canonical wnt signaling pathway." *Biol Reprod* **90**(2):45,1-10
- ***Jiang, J**., *M. Masato, C. Chan, S. Y. Ngoh, W. Liew, J. M. Saju, K. S. Ng, F. S. Wong, Y. S. Lee, S. F. Chang and L. Orbán (2014) "Differential transcriptomic response in the spleen and head kidney following vaccination and infection of Asian seabass with *Streptococcus iniae*." *PLoS One **9**(7): e99128.*

*Equal contribution.

Manuscript in preparation

- **Jiang, J**., A. Soo, B. Liang, W. Liew and L. Orbán (2014) "Male-to-female sex change in Asian seabass (*Lates calcarifer*) involves return to near-undifferentiated transcriptomic state in the transforming gonads", in preparation

References

Agate, R., W. Grisham, J. Wade, S. Mann, J. Wingfield, C. Schanen, A. Palotie and A. Arnold (2003). "Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch." *Proc Natl Acad Sci U S A* **100**: 4873 - 4878.

Alam, M. A., Y. Kobayashi, R. Horiguchi, T. Hirai and M. Nakamura (2008). "Molecular cloning and quantitative expression of sexually dimorphic markers *Dmrt1* and *Foxl2* during female-to-male sex change in *Epinephelus merra*." *Gen Comp Endocrinol* **157**(1): 75-85.

Alhazzaa, R., A. R. Bridle, P. D. Nichols and C. G. Carter (2011). "Replacing dietary fish oil with Echium oil enriched barramundi with C18 PUFA rather than long-chain PUFA." *Aquaculture* **312**(1–4): 162-171.

Allard, S., P. Adin, L. Gouedard, N. di Clemente, N. Josso, M. C. Orgebin-Crist, J. Y. Picard and F. Xavier (2000). "Molecular mechanisms of hormone-mediated Mullerian duct regression: involvement of ß-catenin." *Development* **127**(15): 3349-3360.

Almendras, J. M., C. Duenas, J. Nacario, N. M. Sherwood and L. W. Crim (1988). "Sustained hormone release. III. Use of gonadotropin releasing hormone analogues to induce multiple spawnings in sea bass, *Lates calcarifer*." *Aquaculture* **74**(1–2): 97-111.

Amaral, J. D., J. M. Xavier, C. J. Steer and C. M. Rodrigues (2010). "The role of p53 in apoptosis." *Discov Med* **9**(45): 145-152.

Anderson, J. L., A. Rodriguez Mari, I. Braasch, A. Amores, P. Hohenlohe, P. Batzel and J. H. Postlethwait (2012). "Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics." *PLoS One* **7**(7): e40701.

Artavanis-Tsakonas, S., M. D. Rand and R. J. Lake (1999). "Notch signaling: cell fate control and signal integration in development." *Science* **284**(5415): 770-776.

Baker, I. J., I. I. Solar and E. M. Donaldson (1988). "Masculinization of chinook salmon (*Oncorhynchus tshawytscha*) by immersion treatments using 17α-methyltestosterone around the time of hatching." *Aquaculture* **72**(3–4): 359-367.

Baroiller, J. F., Y. Guiguen and A. Fostier (1999). "Endocrine and environmental aspects of sex differentiation in fish." *Cell Mol Life Sci* **55**(6-7): 910-931.

Baron, D., R. Houlgatte, A. Fostier and Y. Guiguen (2008). "Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens." *Gen Comp Endocrinol* **156**(2): 369-378.

Barske, L. A. and B. Capel (2008). "Blurring the edges in vertebrate sex determination." *Curr Opin Genet Dev* **18**(6): 499-505.

Barsoum, I. and H. H. C. Yao (2011). "Redundant and differential roles of transcription factors *Gli1* and *Gli2* in the development of mouse fetal leydig cells." *Biol Reprod* **84**(5): 894-899.

Bedell, V. M., Y. Wang, J. M. Campbell, T. L. Poshusta, C. G. Starker, R. G. Krug Ii, W. Tan, S. G. Penheiter, A. C. Ma, A. Y. H. Leung, S. C. Fahrenkrug, D. F. Carlson, D. F. Voytas, K. J. Clark, J. J. Essner and S. C. Ekker (2012). "In vivo genome editing using a high-efficiency TALEN system." *Nature* **491**(7422): 114-118.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the false discovery rate: a practical and powerful approach to multiple testing." *J R Statist Soc B* **57**(1): 289-300.

Biran, J., O. Palevitch, S. Ben-Dor and B. Levavi-Sivan (2012). "Neurokinin Bs and neurokinin B receptors in zebrafish - potential role in controlling fish reproduction." *Proc Natl Acad Sci U S A* **109**(26): 10269-10274.

Böhne, A., C. Heule, N. Boileau and W. Salzburger (2013). "Expression and sequence evolution of aromatase *cyp19a1* and other sexual development genes in East African cichlid fishes." *Mol Biol Evol* **30**(10): 2268-2285

Borg, B. (1994). "Androgens in teleost fishes." *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **109**(3): 219-245.

Boulanger, L., M. Pannetier, L. Gall, A. Allais-Bonnet, M. Elzaiat, D. Le Bourhis, N. Daniel, C. Richard, C. Cotinot, Norbert B. Ghyselinck and E. Pailhoux (2014). "*FOXL2* Is a Female Sex-Determining Gene in the Goat." *Curr Biol* **24**(4): 404-408.

Bradley, K. M., J. P. Breyer, D. B. Melville, K. W. Broman, E. W. Knapik and J. R. Smith (2011). "An SNP-based linkage map for zebrafish reveals sex determination loci." *G3 (Bethesda)* **1**(1): 3-9.

Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." *Nat Rev Mol Cell Biol* **7**(9): 678-689.

Büdefeld, T., N. Grgurevic, S. A. Tobet and G. Majdic (2008). "Sex differences in brain developing in the presence or absence of gonads." *Dev Neurobiol* **68**(7): 981-995.

Carrera, S., S. Cuadrado-Castano, J. Samuel, G. D. D. Jones, E. Villar, S. W. Lee and S. Macip (2013). "*Stra6*, a retinoic acid-responsive gene, participates in p53-induced apoptosis after DNA damage." *Cell Death Differ* **20**(7): 910-919.

Cavaco, J. E., J. Bogerd, H. Goos and R. W. Schulz (2001). "Testosterone Inhibits 11-Ketotestosterone-Induced Spermatogenesis in African Catfish (*Clarias gariepinus*)." *Biol Reprod* **65**(6): 1807-1812.

Cavaco, J. E., J. G. Lambert, R. W. Schulz and H. J. T. Goos (1997). "Pubertal development of male African catfish, *Clarias gariepinus*. *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids." *Fish Physiol Biochem* **16**(2): 129-138.

Cavaco, J. E., S. S. Laurentino, A. Barros, M. Sousa and S. Socorro (2009). "Estrogen receptors alpha and beta in human testis: both isoforms are expressed." *Syst Biol Reprod Med* **55**(4): 137-144.

Carreau, S., S. Lambard, C. Delalande, I. Denis-Galeraud, B. Bilinska and S. Bourguiba (2003). "Aromatase expression and role of estrogens in male gonad : a review." *Reprod Biol Endocrinol* **1**(1): 35.

Chakraborty, T., Y. Shibata, L.-Y. Zhou, Y. Katsu, T. Iguchi and Y. Nagahama (2011). "Differential expression of three estrogen receptor subtype mRNAs in gonads and liver from embryos to adults of the medaka, *Oryzias latipes*." *Mol Cell Endocrinol* **333**(1): 47-54.

Chaves-Pozo, E., S. Liarte, L. Fernández-Alacid, E. Abellán, J. Meseguer, V. Mulero and A. García-Ayala (2008). "Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata L.*)." *Mol Immunol* **45**(10): 2998-3011.

Chang, M., W. Jin and S.-C. Sun (2009). "Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production." *Nat Immunol* **10**(10): 1089-1095.

Chen, B., M. E. Dodge, W. Tang, J. Lu, Z. Ma, C. W. Fan, S. Wei, W. Hao, J. Kilgore, N. S. Williams, M. G. Roth, J. F. Amatruda, C. Chen and L. Lum (2009). "Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer." *Nat Chem Biol* **5**(2): 100-107.

Chen, C. C. and R. D. Fernald (2008). "GnRH and GnRH receptors: distribution, function and evolution." *J Fish Biol* **73**(5): 1099-1120.

Chenoweth, S. F., J. M. Hughes, C. P. Keenan and S. Lavery (1998). "When oceans meet: a teleost shows secondary intergradation at an Indian–Pacific interface." *Proc R Soc B* **265**(1394): 415-420.

Childs, A. J., G. Cowan, H. L. Kinnell, R. A. Anderson and P. T. K. Saunders (2011). "Retinoic acid signalling and the control of meiotic entry in the human fetal gonad." *PLoS One* **6**(6): e20249.

Christoffels, A., E. G. Koh, J. M. Chia, S. Brenner, S. Aparicio and B. Venkatesh (2004). "Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes." *Mol Biol Evol* **21**(6): 1146-1151.

Cnaani, A. (2013). "The tilapias' chromosomes influencing sex determination." *Cytogenet Genome Res* **141**(2-3): 195-205.

Collier, F. M., A. Loving, A. J. Baker, J. McLeod, K. Walder and M. A. Kirkland (2009). "RTKN2 induces NF-κB dependent resistance to intrinsic apoptosis in HEK cells and regulates BCL-2 genes in human $CD4^+$ lymphocytes." *J Cell Death* **2**: 9-23.

Crespo, B., A. Gómez, M. J. Mazón, M. Carrillo and S. Zanuy (2013). "Isolation and characterization of *Ff1* and *Gsdf* family genes in European sea bass and identification of early gonadal markers of precocious puberty in males." *Gen Comp Endocrinol* **191**(0): 155-167.

Cutting, A., J. Chue and C. A. Smith (2013). "Just how conserved is vertebrate sex determination?" *Dev Dyn* **242**(4): 380-387.

Das, B. C., P. Thapa, R. Karki, S. Das, S. Mahapatra, T.-C. Liu, I. Torregroza, D. P. Wallace, S. Kambhampati, P. Van Veldhuizen, A. Verma, S. K. Ray and T. Evans (2014). "Retinoic acid signaling pathways in development and diseases." *Bioorg Med Chem* **22**(2): 673-683.

Davis, T. (1982). "Maturity and sexuality in Barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria." *Mar Freshwat Res* **33**(3): 529-545.

Day, T. F., X. Guo, L. Garrett-Beal and Y. Yang (2005). "Wnt/ß-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis." *Dev Cell* **8**(5): 739-750.

De Mitcheson, Y. S. and M. Liu (2008). "Functional hermaphroditism in teleosts." *Fish Fish* **9**(1): 1-43.

De Santa Barbara, P., N. Bonneaud, B. Boizet, M. Desclozeaux, B. Moniot, P. Sudbeck, G. Scherer, F. Poulat and P. Berta (1998). "Direct interaction of SRY-related protein SOX9 and Steroidogenic Factor 1 regulates transcription of the human Anti-Müllerian Hormone gene." *Mol Cell Biol* **18**(11): 6653-6665.

de Vries, F. A., E. de Boer, M. van den Bosch, W. M. Baarends, M. Ooms, L. Yuan, J. G. Liu, A. A. van Zeeland, C. Heyting and A. Pastink (2005). "Mouse *Sycp1* functions in synaptonemal complex assembly, meiotic recombination, and XY body formation." *Genes Dev* **19**(11): 1376-1389.

Deeming, D. C., M. W. J. Ferguson, U. Mittwoch, U. Wolf, M. Dorizzi, P. Zaborski and H. Sharma (1988). "Environmental regulation of sex determination in reptiles." *Philos Trans R Soc Lond B Biol Sci* **322**(1208): 19-39.

DeFalco, T. and B. Capel (2009). "Gonad morphogenesis in vertebrates: divergent means to a convergent end." *Annu Rev Cell Dev Bio* **25**(1): 457-482.

Delfino, F. and W. H. Walker (1998). "Stage-specific nuclear expression of NF-κB in mammalian testis." *Mol Endocrinol* **12**(11): 1696-1707.

Deshpande, G., L. Swanhart, P. Chiang and P. Schedl (2001). "Hedgehog signaling in germ cell migration." *Cell* **106**(6): 759-769.

Devlin, R. H. and Y. Nagahama (2002). "Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences." *Aquaculture* **208**(3–4): 191-364.

Dhert, P., P. Lavens, M. Duray and P. Sorgeloos (1990). "Improved larval survival at metamorphosis of Asian seabass (*Lates calcarifer*) using ω3-HUFA-enriched live food." *Aquaculture* **90**(1): 63-74.

Dhert, P., P. Lavens and P. Sorgeloos (1992). "State of the art of Asian seabass *Lates calcarifer* larviculture." *J World Aquacult Soc* **23**(4): 317-329.

Diotel, N., J.-L. Do-Rego, I. Anglade, C. Vaillant, E. Pellegrini, H. Vaudry and O. Kah (2011). "The brain of teleost fish, a source and a target of sexual steroids." *Front Neurosci* **5**. doi: 10.3389/fnins.2011.00137

Ditewig, A. C. and H. H.-C. Yao (2005). "Organogenesis of the ovary: a comparative review on vertebrate ovary formation." *Organogenesis* **2**(2): 36-41.

Dorsky, R. I., L. C. Sheldahl and R. T. Moon (2002). "A transgenic Lef1/β-Catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development." *Dev Biol* **241**(2): 229-237.

Dufour, S., M. E. Sebert, F. A. Weltzien, K. Rousseau and C. Pasqualini (2010). "Neuroendocrine control by dopamine of teleost reproduction." *J Fish Biol* **76**(1): 129-160.

Eggers, S. and A. Sinclair (2012). "Mammalian sex determination—insights from humans and mice." *Chromosome Res* **20**(1): 215-238.

El-Greisy, Z. A. and A. E. El-Gamal (2012). "Monosex production of tilapia, *Oreochromis niloticus* using different doses of 17α-methyltestosterone with respect to the degree of sex stability after one year of treatment." *Egypt J Aquat Res* **38**(1): 59-66.

Estey, M. P., M. S. Kim and W. S. Trimble (2011). "Septins." *Curr Biol* **21**(10): R384-R387.

Faisst, A. M., G. Alvarez-Bolado, D. Treichel and P. Gruss (2002). "Rotatin is a novel gene required for axial rotation and left–right specification in mouse embryos." *Mech Develop* **113**(1): 15-28.

Farré, D., R. Roset, M. Huerta, J. E. Adsuara, L. Roselló, M. M. Albà and X. Messeguer (2003). "Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN." *Nucleic Acids Res* **31**(13): 3651-3653.

Filali, M., N. Cheng, D. Abbott, V. Leontiev and J. F. Engelhardt (2002). "Wnt-3A/β-Catenin signaling induces transcription from the LEF-1 promoter." *J Biol Chem* **277**(36): 33398-33410.

Fitzpatrick, S. L., D. M. Sindoni, P. J. Shughrue, M. V. Lane, I. J. Merchenthaler and D. E. Frail (1998). "Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues." *Endocrinology* **139**(5): 2571-2578.

Fleming, N. I., K. C. Knower, K. A. Lazarus, P. J. Fuller, E. R. Simpson and C. D. Clyne (2010). "Aromatase Is a direct target of FOXL2: C134W in granulosa cell tumors via a single highly conserved binding site in the ovarian specific promoter." *PLoS One* **5**(12): e14389.

Gao, C. and Y. G. Chen (2010). "Dishevelled: The hub of Wnt signaling." *Cell Signal* **22**(5): 717-727.

Gao, Y. and H.Y. Wang (2006). "Casein Kinase 2 Is activated and essential for Wnt/β-Catenin Signaling." *J Biol Chem* **281**(27): 18394-18400.

Garcia-Ortiz, J., E. Pelosi, S. Omari, T. Nedorezov, Y. Piao, J. Karmazin, M. Uda, A. Cao, S. Cole, A. Forabosco, D. Schlessinger and C. Ottolenghi (2009). "*Foxl2* functions in sex determination and histogenesis throughout mouse ovary development." *BMC Dev Biol* **9**(1): 36.

Gautier, A., F. Le Gac and J.-J. Lareyre (2011). "The *gsdf* gene locus harbors evolutionary conserved and clustered genes preferentially expressed in fish previtellogenic oocytes." *Gene* **472**(1–2): 7-17.

Gerondakis, S., R. Grumont, R. Gugasyan, L. Wong, I. Isomura, W. Ho and A. Banerjee (2006). "Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models." *Oncogene* **25**(51): 6781-6799.

Gibson-Kueh S, Crumlish M, Ferguson HW (2004) "A novel 'skinny pot-belly' disease in Asian seabass fry, *Lates calcarifer* (Bloch)". *J Fish Dis* **27**: 731-735.

Gjerde, B. (2005) "Prediction of Breeding Values." *Selection and Breeding Programs in Aquaculture.* T. Gjedrem, Springer Netherlands: 197-231.

Gothilf, Y., J. A. Muñoz-Cueto, C. A. Sagrillo, M. Selmanoff, T. T. Chen, O. Kah, A. Elizur and Y. Zohar (1996). "Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization." *Biol Reprod* **55**(3): 636-645.

Guha, S. and H. Padh (2008). "Cathepsins: fundamental effectors of endolysosomal proteolysis." *Indian J Biochem Biophys* **45**(2): 75-90.

Guiguen, Y., B. Jalabert, E. Thouard and A. Fostier (1993). "Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass, *Lates calcarifer*." *Gen Comp Endocrinol* 92(3): 327-338.

Guiguen, Y., J.-F. Baroiller, M.-J. Ricordel, K. Iseki, O. M. McMeel, S. A. M. Martin and A. Fostier (1999). "Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*)." *Mol Reprod Dev* **54**(2): 154-162.

Guiguen, Y., C. Cauty, A. Fostier, J. Fuchs and B. Jalabert (1994). "Reproductive cycle and sex inversion of the seabass, *Lates calcarifer*, reared in sea cages in

French Polynesia: histological and morphometric description." *Environ Biol Fish* **39**(3): 231-247.

Guiguen, Y., B. Jalabert, A. Benett and A. Fostier (1995). "Gonadal in vitro androstenedione metabolism and changes in some plasma and gonadal steroid hormones during sex inversion of the protandrous sea bass, *Lates calcarifer*." *Gen Comp Endocrinol* **100**(1): 106-118.

Guiry, A., D. Flynn, S. Hubert, A. O'Keeffe, O. LeProvost, S. White, P. Forde, P. Davoren, B. Houeix, T. Smith, D. Cotter, N. Wilkins and M. Cairns (2010). "Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (*Salmo salar*)." *BMC Genomics* **11**(1): 211.

Guo, Y., H. Cheng, X. Huang, S. Gao, H. Yu and R. Zhou (2005). "Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *Dmrt1*." *Biochem Biophys Res Commun* **330**(3): 950-957.

Hacker, A., B. Capel, P. Goodfellow and R. Lovell-Badge (1995). "Expression of *Sry*, the mouse sex determining gene." *Development* **121**(6): 1603-1614.

Hamazaki, T. S., Y. Nagahama, I. Iuchi and K. Yamagami (1989). "A glycoprotein from the liver constitutes the inner layer of the egg envelope (zona pellucida interna) of the fish, *Oryzias latipes*." *Dev Biol* **133**(1): 101-110.

Hassin, S., M. Claire, H. Holland and Y. Zohar (2000). "Early maturity in the male striped bass, *Morone saxatilis*: Follicle-stimulating hormone and luteinizing hormone gene expression and their regulation by gonadotropin-releasing hormone analogue and testosterone." *Biol Reprod* **63**(6): 1691-1697.

Hattori, R. S., J. I. Fernandino, A. Kishii, H. Kimura, T. Kinno, M. Oura, G. M. Somoza, M. Yokota, C. A. Strussmann and S. Watanabe (2009). "Cortisol-induced masculinization: does thermal stress affect gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination?" *PLoS One* **4**(8): e6548.

Hattori, R. S., Y. Murai, M. Oura, S. Masuda, S. K. Majhi, T. Sakamoto, J. I. Fernandino, G. M. Somoza, M. Yokota and C. A. Strüssmann (2012). "A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination." *Proc Natl Acad Sci U S A* **109**(8): 2955-2959.

Hayashi, Y., H. Kobira, T. Yamaguchi, E. Shiraishi, T. Yazawa, T. Hirai, Y. Kamei and T. Kitano (2010). "High temperature causes masculinization of genetically female medaka by elevation of cortisol." *Mol Reprod Dev* **77**(8): 679-686.

Hayden, M. S. and Ghosh, S. (2008). "Shared principles in NF-κB signaling." *Cell* **132**(3): 344-362.

He, Y., X. Shang, J. Sun, L. Zhang, W. Zhao, Y. Tian, H. Cheng and R. Zhou (2010). "Gonadal apoptosis during sex reversal of the rice field eel: implications for an evolutionarily conserved role of the molecular chaperone heat shock protein 10." *J Exp Zool B Mol Dev Evol* **314B**(4): 257-266.

He, Z., Y. Wu, J. Xie, T. Wang, L. Zhang and W. Zhang (2012). "Growth differentiation factor 9 (Gdf9) was localized in the female as well as male germ cells in a protogynous hermaphroditic teleost fish, ricefield eel *Monopterus albus*." *Gen Comp Endocrinol* **178**(2): 355-362.

Henry, J. C., E. McLean, I. Mayer and E. M. Donaldson (1998). "Induction of precocious maturation in masculinized Atlantic salmon by treatment with sustained-release LHRHa and testosterone." *Aquacult Int* **6**(4): 261-268.

Hishida, T.-O. (1965). "Accumulation of estrone-16-C14 and diethylstilbestrol-(monoethyl-1-C14) in larval gonads of the medaka *Oryzias latipes*, and determination of the minimum dosage of estrogen for sex reversal." *Gen Comp Endocrinol* **5**(2): 137-144.

Hishida, T.-O. and N. Kawamoto (1970). "Androgenic and male-inducing effects of 11-ketotestosterone on a teleost, the medaka (*Oryzias latipes*)." *J Exp Zool* **173**(3): 279-283.

Hornung, M. W., K. M. Jensen, J. J. Korte, M. D. Kahl, E. J. Durhan, J. S. Denny, T. R. Henry and G. T. Ankley (2004). "Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17alpha-methyltestosterone: conversion of 17alpha-methyltestosterone to 17alpha-methylestradiol." *Aquat Toxicol* **66**(1): 15-23.

Hondo, M., M. Ishii and T. Sakurai (2008). "The NPB/NPW neuropeptide system and its role in regulating energy homeostasis, pain, and emotion." *Results Probl Cell Differ* **46**: 239-256.

Hong, W. and Q. Zhang (2003). "Review of captive bred species and fry production of marine fish in China." *Aquaculture* **227**(1–4): 305-318.

Horiguchi, R., R. Nozu, T. Hirai, Y. Kobayashi, Y. Nagahama and M. Nakamura (2013). "Characterization of gonadal soma-derived factor expression during sex change in the protogynous wrasse, *Halichoeres trimaculatus*." *Dev Dyn* **242**(4): 388-399.

Hosokawa, M., M. Shoji, K. Kitamura, T. Tanaka, T. Noce, S. Chuma and N. Nakatsuji (2007). "Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: Domain composition, intracellular localization, and function in male germ cells in mice." *Dev Biol* **301**(1): 38-52.

Hovanes, K., T. W. H. Li, J. E. Munguia, T. Truong, T. Milovanovic, J. Lawrence Marsh, R. F. Holcombe and M. L. Waterman (2001). "ß-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer." *Nat Genet* **28**(1): 53-57.

Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, L. Matthews *et al.* (2013). "The zebrafish reference genome sequence and its relationship to the human genome." *Nature* advance online publication doi:10.1038/nature12111

Howes, E., J. C. Pascall, W. Engel and R. Jones (2001). "Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization." *J Cell Sci* **114**(22): 4127-4136.

Hsiao, C. D. and H. J. Tsai (2003). "Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism in vivo." *Dev Biol* **262**(2): 313-323.

Ijiri, S., H. Kaneko, T. Kobayashi, D.-S. Wang, F. Sakai, B. Paul-Prasanth, M. Nakamura and Y. Nagahama (2008). "Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*." *Biol Reprod* **78**(2): 333-341.

Ingham, P. W. and McMahon, A. P. (2001). "Hedgehog signaling in animal development: paradigms and principles." *Genes Dev* **15**(23): 3059-3087.

Jacobs, M. D. and S. C. Harrison (1998). "Structure of an IκBα/NF-κB Complex." *Cell* **95**(6): 749-758.

Jaillon, O., J.-M. Aury, F. Brunet, J.-L. Petit, N. Stange-Thomann, E. Mauceli, L. Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, *et al.* (2004). "Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype." *Nature* **431**(7011): 946-957.

Johnson, J., T. Espinoza, R. W. McGaughey, A. Rawls and J. Wilson-Rawls (2001). "Notch pathway genes are expressed in mammalian ovarian follicles." *Mech Dev* **109**(2): 355-361.

Jolliffe, I. T. (1988). "Principal Component Analysis." Springer, New York.

Jordan, B. K., M. Mohammed, S. T. Ching, E. Delot, X. N. Chen, P. Dewing, A. Swain, P. N. Rao, B. R. Elejalde and E. Vilain (2001). "Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans." *Am J Hum Genet* **68**(5): 1102-1109.

Juengel, J. L., K. J. Bodensteiner, D. A. Heath, N. L. Hudson, C. L. Moeller, P. Smith, S. M. Galloway, G. H. Davis, H. R. Sawyer and K. P. McNatty (2004). "Physiology of GDF9 and BMP15 signalling molecules." *Anim Reprod Sci* **82–83**(0): 447-460.

Kamiya, T., W. Kai, S. Tasumi, A. Oka, T. Matsunaga, N. Mizuno, M. Fujita, H. Suetake, S. Suzuki, S. Hosoya, S. Tohari, S. Brenner, T. Miyadai, B. Venkatesh, Y. Suzuki and K. Kikuchi (2012). "A trans-species missense SNP in *Amhr2* is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (Fugu)." *PLoS Genet* **8**(7): e1002798.

Kanemaru, T., M. Nakamura, R. Murata, K. Kuroki, H. Horie, K. Uchida, B. Senthilkumaran and H. Kagawa (2012). "Induction of sexual maturation of the female honeycomb grouper, *Epinephelus merra*, in the non-breeding season by modulating environmental factors with GnRH analogue implantation." *Aquaculture* **358–359**(0): 85-91.

Kang, D. E., S. Soriano, X. Xia, C. G. Eberhart, B. De Strooper, H. Zheng and E. H. Koo (2002). "Presenilin couples the paired phosphorylation of ß-Catenin independent of Axin: Implications for ß-Catenin activation in tumorigenesis." *Cell* **110**(6): 751-762.

Katersky, R. S. and C. G. Carter (2009). "Growth and protein synthesis of barramundi, *Lates calcarifer*, fed lupin as a partial protein replacement." *Comp Biochem Physiol A Mol Integr Physiol* **152**(4): 513-517.

Katoh, Y. and M. Katoh (2006). "Hedgehog signaling pathway and gastrointestinal stem cell signaling network (review)." *Int J Mol Med* **18**(6): 1019-1023.

Keenan, C. (1994). "Recent evolution of population structure in Australian barramundi, *Lates calcarifer* (Bloch): An example of isolation by distance in one dimension." *Mar Freshwat Res* **45**(7): 1123-1148.

Keleman, K. and B. J. Dickson (2001). "Short- and long-range repulsion by the *Drosophila* Unc5 netrin receptor." *Neuron* **32**(4): 605-617.

Kettleborough, R. N. W., E. M. Busch-Nentwich, S. A. Harvey, C. M. Dooley, E. de Bruijn, F. van Eeden, I. Sealy, R. J. White, C. Herd, I. J. Nijman, F. Fenyes, S.

Mehroke, C. Scahill, R. Gibbons, N. Wali, S. Carruthers, A. Hall, J. Yen, E. Cuppen and D. L. Stemple (2013). "A systematic genome-wide analysis of zebrafish protein-coding gene function." *Nature* **496**(7446): 494-497.

Kimura, T., T. Nakamura, K. Murayama, H. Umehara, N. Yamano, S. Watanabe, M. M. Taketo and T. Nakano (2006). "The stabilization of ß-catenin leads to impaired primordial germ cell development via aberrant cell cycle progression." *Dev Biol* **300**(2): 545-553.

Kissel, H., M.-M. Georgescu, S. Larisch, K. Manova, G. R. Hunnicutt and H. Steller (2005). "The Sept4 Septin locus Is required for sperm terminal differentiation in mice." *Dev Cell* **8**(3): 353-364.

Kitano, T., K. Takamune, Y. Nagahama and S.-I. Abe (2000). "Aromatase inhibitor and 17α-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*)." *Mol Reprod Dev* **56**(1): 1-5.

Kobayashi, Y., R. Horiguchi, R. Nozu and M. Nakamura (2010). "Expression and localization of forkhead transcriptional factor 2 (*Foxl2*) in the gonads of protogynous wrasse, *Halichoeres trimaculatus*." *Biol Sex Differ* **1**(1): 3.

Kobayashi, Y., Y. Nagahama and M. Nakamura (2013). "Diversity and plasticity of sex determination and differentiation in fishes." *Sex Dev* **7**(1-3): 115-125.

Kobayashi, Y., M. Nakamura, T. Sunobe, T. Usami, T. Kobayashi, H. Manabe, B. Paul-Prasanth, N. Suzuki and Y. Nagahama (2009). "Sex change in the gobiid fish is mediated through rapid switching of gonadotropin receptors from ovarian to testicular portion or vice versa." *Endocrinology* **150**(3): 1503-1511.

Kotula-Balak, M., R. Zielińska, J. Glogowski, R. K. Kowalski, B. Sarosiek and B. Bilińska (2008). "Aromatase expression in testes of XY, YY, and XX rainbow trout (*Oncorhynchus mykiss*)." *Comp Biochem Physiol A Mol Integr Physiol* **149**(2): 188-196.

Krueger, W. and K. Oliveira (1999). "Evidence for environmental sex determination in the American eel, *Anguilla rostrata*." *Environ Biol Fish* **55**(4): 381-389.

Kuwaye, T. T., D. K. Okimoto, S. K. Shimoda, R. D. Howerton, H.-R. Lin, P. K. T. Pang and E. G. Grau (1993). "Effect of 17α-methyltestosterone on the growth of the euryhaline tilapia, *Oreochromis mossambicus*, in fresh water and in sea water." *Aquaculture* **113**(1–2): 137-152.

Lau, Y.-F. C. and Y. Li (2009). "The human and mouse sex-determining SRY genes repress the Rspol/β-catenin signaling." *J Genet Genomics* **36**(4): 193-202.

Lei, N., K. I. Hornbaker, D. A. Rice, T. Karpova, V. A. Agbor and L. L. Heckert (2007). "Sex-specific differences in mouse DMRT1 expression are both cell type- and stage-dependent during gonad development." *Biol Reprod* **77**(3): 466-475.

Leu, M.-Y. (1997). "Natural spawning and mass larviculture of black porgy *Acanthopagrus schlegeli* in captivity in taiwan." *J World Aquacult Soc* **28**(2): 180-187.

Levavi-Sivan, B., J. Bogerd, E. L. Mañanós, A. Gómez and J. J. Lareyre (2010). "Perspectives on fish gonadotropins and their receptors." *Gen Comp Endocrinol* **165**(3): 412-437.

Lewis, Z. R., M. C. McClellan, J. H. Postlethwait, W. A. Cresko and R. H. Kaplan (2008). "Female-specific increase in primordial germ cells marks sex differentiation in threespine stickleback (*Gasterosteus aculeatus*)." *J Morphol* **269**(8): 909-921.

Li, J., P. Hu, K. Li and Q. Zhao (2012). "Identification and characterization of a novel retinoic acid response element in zebrafish *cyp26a1* promoter." *Anat Rec (Hoboken)* **295**(2): 268-277.

Li, M.-H., H.-H. Yang, M.-R. Li, Y.-L. Sun, X.-L. Jiang, Q.-P. Xie, T.-R. Wang, H.-J. Shi, L.-N. Sun, L.-Y. Zhou and D.-S. Wang (2013). "Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs." *Endocrinology* **154**(12): 4814-4825.

Liew, W. C., R. Bartfai, Z. Lim, R. Sreenivasan, K. R. Siegfried and L. Orban (2012). "Polygenic sex determination system in zebrafish." *PLoS One* **7**(4): e34397.

Liu, C.-A., M.-J. Wang, C.-W. Chi, C.-W. Wu and J.-Y. Chen (2004). "Rho/Rhotekin-mediated NF-κB activation confers resistance to apoptosis." *Oncogene* **23**(54): 8731-8742.

Liu, C. F., K. Parker and H. C. Yao (2010). "WNT4/ß-Catenin pathway maintains female germ cell survival by inhibiting Activin bB in the mouse fetal ovary." *PLoS One* **5**(4): e10382.

Liu, L. and W. Ge (2007). "Growth Differentiation Factor 9 and its spatiotemporal expression and regulation in the zebrafish ovary." *Biol Reprod* **76**(2): 294-302.

Lokman, P.M., Harris, B., Kusakabe, M., Kime, D.E., Schulz, R.W., Adachi, S. and Young, G. (2002). "11-oxygenated androgens in female teleosts: prevalence, abundance, and life history implications." *Gen. Comp. Endocrinol.* **129**: 1–12.

Lorenzi, V., R. L. Earley and M. S. Grober (2012). "Differential responses of brain, gonad and muscle steroid levels to changes in social status and sex in a sequential and bidirectional hermaphroditic fish." *PLoS One* **7**(12): e51158.

Lovejoy, D. A., Fischer, W. H., Ngamvongchon, S., Craig, A. G., Nahorniak, C. S., Peter, R. E., Rivier, J. E. and Sherwood, N. M. (1992). "Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution." *Proc Natl Acad Sci U S A* **89**(14): 6373-6377.

Lutton, B. and I. Callard (2006). "Evolution of reproductive–immune interactions." *Integr Comp Biol* **46**(6): 1060-1071.

Maack, G. and H. Segner (2003). "Morphological development of the gonads in zebrafish." *J Fish Biol* **62**(4): 895-906.

MacDonald, B. T., K. Tamai and X. He (2009). "Wnt/ß-catenin signaling: components, mechanisms, and diseases." *Dev Cell* **17**(1): 9-26.

Mana, R. R. and G. Kawamura (2002). "A comparative study on morphological differences in the olfactory system of red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) from wild and cultured stocks." *Aquaculture* **209**(1–4): 285-306.

Mao, B., W. Wu, Y. Li, D. Hoppe, P. Stannek, A. Glinka and C. Niehrs (2001). "LDL-receptor-related protein 6 is a receptor for Dickkopf proteins." *Nature* **411**(6835): 321-325.

Marchand, O., M. Govoroun, H. D'Cotta, O. McMeel, J.-J. Lareyre, A. Bernot, V. Laudet and Y. Guiguen (2000). "DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*." *Biochim Biophys Acta* **1493**(1–2): 180-187.

Matson, C. K., M. W. Murphy, M. D. Griswold, S. Yoshida, V. J. Bardwell and D. Zarkower (2010). "The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells." *Dev Cell* **19**(4): 612-624.

Matson, C. K., M. W. Murphy, A. L. Sarver, M. D. Griswold, V. J. Bardwell and D. Zarkower (2011). "DMRT1 prevents female reprogramming in the postnatal mammalian testis." *Nature* **476**(7358): 101-104.

Matsubara, M., P. M. Lokman, A. Senaha, Y. Kazeto, S. Ijiri, A. Kambegawa, T. Hirai, G. Young, T. Todo, S. Adachi and K. Yamauchi (2003). "Synthesis and possible function of 11-ketotestosterone during oogenesis in eel (*Anguilla spp.*)." *Fish Physiol Biochem* **28**(1-4): 353-354.

Matsuda, M., Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, C. E. Morrey, N. Shibata, S. Asakawa, N. Shimizu, H. Hori, S. Hamaguchi and M. Sakaizumi (2002). "DMY is a Y-specific DM-domain gene required for male development in the medaka fish." *Nature* **417**(6888): 559-563.

McElreavey, K., E. Vilain, N. Abbas, I. Herskowitz and M. Fellous (1993). "A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development." *Proc Natl Acad Sci U S A* **90**(8): 3368-3372.

Mechaly, A. S., J. Viñas and F. Piferrer (2012). "Sex-specific changes in the expression of kisspeptin, kisspeptin receptor, gonadotropins and gonadotropin receptors in the Senegalese sole (*Solea senegalensis*) during a full reproductive cycle." *Comp Biochem Physiol A Mol Integr Physiol* **162**(4): 364-371.

Meng, X., M. B. Noyes, L. J. Zhu, N. D. Lawson and S. A. Wolfe (2008). "Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases." *Nat Biotech* **26**(6): 695-701.

Messeguer, X., R. Escudero, D. Farré, O. Núñez, J. Martínez and M. M. Albà (2002). "PROMO: detection of known transcription regulatory elements using species-tailored searches." *Bioinformatics* **18**(2): 333-334.

Meyer, A. and M. Schartl (1999). "Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions." *Curr Opin Cell Biol* **11**(6): 699-704.

Miura, S., R. Horiguchi and M. Nakamura (2008). "Immunohistochemical evidence for 11beta-hydroxylase (P45011beta) and androgen production in the gonad during sex differentiation and in adults in the protandrous anemonefish *Amphiprion clarkii*." *Zoolog Sci* **25**(2): 212-219.

Moore, R. (1979). "Natural sex inversion in the giant perch (*Lates calcarifer*)." *Mar Freshwat Res* **30**(6): 803-813.

Moore, R. (1982). "Spawning and early life history of burramundi, *Lates calcarifer* (Bloch), in Papua New Guinea." *Mar Freshwat Res* **33**(4): 647-661.

Mori, T., H. Matsumoto and H. Yokota (1998). "Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes." *J Steroid Biochem Mol Biol* **67**(2): 133-141.

Morrish, B. and A. Sinclair (2002). "Vertebrate sex determination: many means to an end." *Reproduction* **124**(4): 447-457.

Murata, R., H. Karimata, Y. Kobayashi, R. Horiguchi, K. Kishimoto, M. Kimura, T. Kobayashi, K. Soyano and M. Nakamura (2011). "Differentiation of steroid-producing cells during ovarian differentiation in the protogynous Malabar grouper, *Epinephelus malabaricus*." *Int J Dev Biol* **55**(6): 619-625.

Mylonas, C. C., A. Fostier and S. Zanuy (2010). "Broodstock management and hormonal manipulations of fish reproduction." *Gen Comp Endocrinol* **165**(3): 516-534.

Mylonas, C. C., A. P. Scott, E. L. Vermeirssen and Y. Zohar (1997). "Changes in plasma gonadotropin II and sex steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems." *Biol Reprod* **57**(3): 669-675.

Myosho, T., H. Otake, H. Masuyama, M. Matsuda, Y. Kuroki, A. Fujiyama, K. Naruse, S. Hamaguchi and M. Sakaizumi (2012). "Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*." *Genetics* **191**(1): 163-170.

Nagahama, Y. (1994). "Endocrine regulation of gametogenesis in fish." *Int J Dev Biol* **38**(2): 217-229.

Nagasawa, K., C. Presslauer, L. Kirtiklis, I. Babiak and J. M. O. Fernandes (2014). "Sexually dimorphic transcription of estrogen receptors in cod gonads throughout a reproductive cycle." *J Mol Endocrinol* **52**(3): 357-371.

Nasevicius, A. and S. C. Ekker (2000). "Effective targeted gene 'knockdown' in zebrafish." *Nat Genet* **26**(2): 216-220.

Naurin, S., B. Hansson, D. Hasselquist, Y.-H. Kim and S. Bensch (2011). "The sex-biased brain: sexual dimorphism in gene expression in two species of songbirds." *BMC Genomics* **12**(1): 37.

Navarro-Martin, L., J. Vinas, L. Ribas, N. Diaz, A. Gutierrez, L. Di Croce and F. Piferrer (2011). "DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass." *PLoS Genet* **7**(12): e1002447.

Nelson, E. R. and H. R. Habibi (2013). "Estrogen receptor function and regulation in fish and other vertebrates." *Gen Comp Endocrinol* **192**: 15-24.

Nelson, J. S. (1994). "Fishes of the world." 3rd edn. *John Wiley and Sons, Inc. New York*

Neto, A., N. Mercader and J. L. Gomez-Skarmeta (2012). "The *Osr1* and *Osr2* genes act in the pronephric anlage downstream of retinoic acid signaling and upstream of *Wnt2b* to maintain pectoral fin development." *Development* **139**(2): 301-311.

Newell-Price, J., A. J. L. Clark and P. King (2000). "DNA methylation and silencing of gene expression." *Trends Endocrinol Metab* **11**(4): 142-148.

Nguyen, N. T., D. P. Lin, S. Y. Yen, J. K. Tseng, J. F. Chuang, B. Y. Chen, T. A. Lin, H. H. Chang and J. C. Ju (2009). "Sonic hedgehog promotes porcine oocyte maturation and early embryo development." *Reprod Fertil Dev* **21**(6): 805-815.

Nicol, B. and Y. Guiguen (2011). "Expression profiling of Wnt signaling genes during gonadal differentiation and gametogenesis in rainbow trout." *Sex Dev* **5**(6): 318-329.

Nicol, B., A. Yano, E. Jouanno, A. Guerin, A. Fostier and Y. Guiguen (2013). "Follistatin Is an early player in rainbow trout ovarian differentiation and is both colocalized with aromatase and regulated by the Wnt pathway." *Sex Dev* **7**(5):267-76.

Norfatimah, M. Y., M. N. Siti Azizah, A. S. Othman, I. Patimah and A. F. J. Jamsari (2009). "Genetic variation of *Lates calcarifer* in Peninsular Malaysia based on the cytochrome b gene." *Aquaculture Res* **40**(15): 1742-1749.

Noy, N. (2010). "Between Death and Survival: Retinoic Acid in Regulation of Apoptosis." *Annu Rev Nutr* **30**(1): 201-217.

Nozu, R., R. Horiguchi, R. Murata, Y. Kobayashi and M. Nakamura (2013). "Survival of ovarian somatic cells during sex change in the protogynous wrasse, *Halichoeres trimaculatus*." *Fish Physiol Biochem* 39(1): 47-51.

Ogawa, S., P. N. Ramadasan, M. Goschorska, A. Anantharajah, K. We Ng and I. S. Parhar (2012). "Cloning and expression of tachykinins and their association with kisspeptins in the brains of zebrafish." *J Comp Neurol* **520**(13): 2991-3012.

Onichtchouk, D., K. Aduroja, H.-G. Belting, L. Gnügge and W. Driever (2003). "Transgene driving GFP expression from the promoter of the zona pellucida gene *zpc* is expressed in oocytes and provides an early marker for gonad differentiation in zebrafish." *Dev Dyn* **228**(3): 393-404.

Orban, L., R. Sreenivasan and P. E. Olsson (2009). "Long and winding roads: testis differentiation in zebrafish." *Mol Cell Endocrinol* **312**(1-2): 35-41.

Ospina-Álvarez, N. and F. Piferrer (2008). "Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change." *PLoS One* **3**(7): e2837.

Ottolenghi, C., E. Pelosi, J. Tran, M. Colombino, E. Douglass, T. Nedorezov, A. Cao, A. Forabosco and D. Schlessinger (2007). "Loss of *Wnt4* and *Foxl2* leads to female-to-male sex reversal extending to germ cells." *Hum Mol Genet* **16**(23): 2795-2804.

Pannetier, M., S. Fabre, F. Batista, A. Kocer, L. Renault, G. Jolivet, B. Mandon-Pepin, C. Cotinot, R. Veitia and E. Pailhoux (2006). "FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development." *J Mol Endocrinol* **36**(3): 399-413.

Parakh, T. N., J. A. Hernandez, J. C. Grammer, J. Weck, M. Hunzicker-Dunn, A. J. Zeleznik and J. H. Nilson (2006). "Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires ß-catenin." *Proc Natl Acad Sci U S A* **103**(33): 12435-12440.

Patowary, A., R. Purkanti, M. Singh, R. Chauhan, A. R. Singh, M. Swarnkar, N. Singh, V. Pandey, C. Torroja, M. D. Clark, J. P. Kocher, K. J. Clark, D. L. Stemple, E. W. Klee, S. C. Ekker, V. Scaria and S. Sivasubbu (2013). "A sequence-based variation map of zebrafish." *Zebrafish* **10**(1): 15-20.

Payne, A. H. and D. B. Hales (2004). "Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones." *Endocr Rev* **25**(6): 947-970.

Pender, P. J. and R. K. Griffin (1996). "Habitat history of barramundi *Lates calcarifer* in a North Australian river system based on barium and strontium levels in scales." *T Am Fish Soc* **125**(5): 679-689.

Peter, R. E., H.-R. Lin and G. Van Der Kraak (1988). "Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine antagonists." *Aquaculture* **74**(1–2): 1-10.

Petersen, C., L. Füzesi and S. Hoyer-Fender (1999). "Outer dense fibre proteins from human sperm tail: molecular cloning and expression analyses of two cDNA transcripts encoding proteins of ~70 kDa." *Mol Hum Reprod* **5**(7): 627-635.

Pethiyagoda, R. and A. C. Gill (2012). "Description of two new species of sea bass (Teleostei: Latidae: Lates) from Myanmar and Sri Lanka." *Zootaxa* **3314**: 1-16.

Peyon, P., S. Baloche and E. Burzawa-Gérard (1997). "Investigation into the possible role of androgens in the induction of hepatic vitellogenesis in the European eel:in vivo andin vitro studies." *Fish Physiol Biochem* **16**(2): 107-118.

Pierce, C. (2006). The next big fish. *The Boston Globe Magazine* 26 Nov 2006.

Pradhan, A., H. Khalaf, S. A. Ochsner, R. Sreenivasan, J. Koskinen, M. Karlsson, J. Karlsson, N. J. McKenna, L. Orbán and P.-E. Olsson (2012). "Activation of NF-κB protein prevents the transition from juvenile ovary to testis and promotes ovarian development in zebrafish." *J Biol Chem* **287**(45): 37926-37938.

Raymond, C. S., M. W. Murphy, M. G. O'Sullivan, V. J. Bardwell and D. Zarkower (2000). "Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation." *Genes Dev* **14**(20): 2587-2595.

Renault, A. D., S. Ricardo, P. S. Kunwar, A. Santos, M. Starz-Gaiano, J. A. Stein and R. Lehmann (2009). "Hedgehog does not guide migrating *Drosophila* germ cells." *Dev Biol* **328**(2): 355-362.

Ribeiro, F. F. and J. G. Qin (2013). "Modelling size-dependent cannibalism in barramundi *Lates calcarifer*: cannibalistic polyphenism and its implication to aquaculture." *PLoS One* **8**(12): e82488.

Robertson, K. M., L. O'Donnell, M. E. E. Jones, S. J. Meachem, W. C. Boon, C. R. Fisher, K. H. Graves, R. I. McLachlan and E. R. Simpson (1999). "Impairment of spermatogenesis in mice lacking a functional aromatase (*cyp 19*) gene." *Proc Natl Acad Sci U S A* **96**(14): 7986-7991.

Rodriguez-Mari, A., C. Canestro, R. A. Bremiller, A. Nguyen-Johnson, K. Asakawa, K. Kawakami and J. H. Postlethwait (2010). "Sex reversal in zebrafish *fancl* mutants is caused by Tp53-mediated germ cell apoptosis." *PLoS Genet* **6**(7): e1001034.

Rodriguez-Mari, A., Y. L. Yan, R. A. Bremiller, C. Wilson, C. Canestro and J. H. Postlethwait (2005). "Characterization and expression pattern of zebrafish Anti-Mullerian hormone (*Amh*) relative to *sox9a*, *sox9b*, and *cyp19a1a*, during gonad development." *Gene Expr Patterns* **5**(5): 655-667.

Rondeau, E., A. Messmer, D. Sanderson, S. Jantzen, K. von Schalburg, D. Minkley, J. Leong, G. Macdonald, A. Davidsen, W. Parker, R. Mazzola, B. Campbell and B. Koop (2013). "Genomics of sablefish (*Anoplopoma fimbria*): expressed

genes, mitochondrial phylogeny, linkage map and identification of a putative sex gene." *BMC Genomics* **14**(1): 452.

Roth, W., P. Kermer, M. Krajewska, K. Welsh, S. Davis, S. Krajewski and J. C. Reed (0000). "Bifunctional apoptosis inhibitor (BAR) protects neurons from diverse cell death pathways." *Cell Death Differ* **10**(10): 1178-1187.

Rouiller-Fabre, V., S. Carmona, R. A. Merhi, R. Cate, R. Habert and B. Vigier (1998). "Effect of Anti-mullerian hormone on Sertoli and leydig cell functions in fetal and immature rats." *Endocrinology* **139**(3): 1213-1220.

Santos, E. M., P. Kille, V. L. Workman, G. C. Paull and C. R. Tyler (2008). "Sexually dimorphic gene expression in the brains of mature zebrafish." *Comp Biochem Physiol A Mol Integr Physiol* **149**(3): 314-324.

Satoh, N. (1974). "An ultrastructural study of sex differentiation in the teleost *Oryzias latipes*." *J Embryol Exp Morphol* **32**(1): 195-215.

Sawatari, E., S. Shikina, T. Takeuchi and G. Yoshizaki (2007). "A novel transforming growth factor-β superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*)." *Dev Biol* **301**(1): 266-275.

Schauvliege, R., S. Janssens and R. Beyaert (2007). "Pellino Proteins: novel players in TLR and IL-1R signalling." *J Cell Mol Med* **11**(3): 453-461.

Scherer, G. (2008). The molecular genetic jigsaw puzzle of vertebrate sex determination and its missing pieces. The Genetics and Biology of Sex Determination, *John Wiley & Sons, Ltd*: 225-239.

Scholz, B., K. Kultima, A. Mattsson, J. Axelsson, B. Brunstrom, K. Halldin, M. Stigson and L. Dencker (2006). "Sex-dependent gene expression in early brain development of chicken embryos." *BMC Neurosci* **7**(1): 12.

Schultz, D. R., N. Perez, C. K. Tan, A. J. Mendez, T. R. Capo, D. Snodgrass, E. D. Prince and J. E. Serafy (2005). "Concurrent levels of 11-ketotestosterone in fish surface mucus, muscle tissue and blood." *J Appl Ichthyol* **21**(5): 394-398.

Schulz, R. (1986). "In vitro metabolism of steroid hormones in the liver and in blood cells of male rainbow trout (*Salmo gairdneri Richardson*)." *Gen Comp Endocrinol* **64**(2): 312-319.

Sekido, R. and R. Lovell-Badge (2008). "Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer." *Nature* **453**(7197): 930-934.

Shao, C., Q. Li, S. Chen, P. Zhang, J. Lian, Q. Hu, B. Sun, L. Jin, S. Liu, Z. Wang, H. Zhao, Z. Jin, Z. Liang, Y. Li, Q. Zheng, Y. Zhang, J. Wang and G. Zhang (2014). "Epigenetic modification and inheritance in sexual reversal of fish." *Genome Res*. doi:10.1101/gr.162172.113. In press.

Shibata, Y., Paul-Prasanth, B., Suzuki, A., Usami, T., Nakamoto, M., Matsuda, M. and Nagahama, Y. (2010). "Expression of gonadal soma derived factor (GSDF) is spatially and temporally correlated with early testicular differentiation in medaka." *Gene Expr Patterns* **10**(6): 283-289.

Siegfried, K. R. and C. Nusslein-Volhard (2008). "Germ line control of female sex determination in zebrafish." *Dev Biol* **324**(2): 277-287.

Slanchev, K., J. Stebler, G. de la Cueva-Mendez and E. Raz (2005). "Development without germ cells: the role of the germ line in zebrafish sex differentiation." *Proc Natl Acad Sci U S A* **102**(11): 4074-4079.

Smith, C. A., K. N. Roeszler, T. Ohnesorg, D. M. Cummins, P. G. Farlie, T. J. Doran and A. H. Sinclair (2009). "The avian Z-linked gene DMRT1 is required for male sex determination in the chicken." *Nature* **461**(7261): 267-271.

Socorro, S., D. Power, P. Olsson and A. Canario (2000). "Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution." *J Endocrinol* **166**(2): 293-306.

Song, D. H., I. Dominguez, J. Mizuno, M. Kaut, S. C. Mohr and D. C. Seldin (2003). "CK2 phosphorylation of the armadillo repeat region of β-Catenin potentiates Wnt signaling." *J Biol Chem* **278**(26): 24018-24025.

Soriano, S., D. E. Kang, M. Fu, R. Pestell, N. Chevallier, H. Zheng and E. H. Koo (2001). "Presenilin 1 negatively regulates β-Catenin/T Cell Factor/Lymphoid Enhancer Factor-1 signaling independently of β-Amyloid precursor protein and notch processing." *J Cell Biol* **152**(4): 785-794.

Sreenivasan, R., M. Cai, R. Bartfai, X. Wang, A. Christoffels and L. Orban (2008). "Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain." *PLoS One* **3**(3): e1791.

Sreenivasan, R., Wang X.G., Bartfai R., Kwan H.Y., Christoffels A., Orban L. (2008) "Global expression profiling in zebrafish reveals genes with potential roles in sexual differentiation." *41st Annual Meeting of the Society for the Study of Reproduction, May 27-30, 2008, Kailua-Kona, Hawaii, USA. Biol Reprod* **78**: 269.

Stegh, A. H., B. C. Barnhart, J. Volkland, A. Algeciras-Schimnich, N. Ke, J. C. Reed and M. E. Peter (2002). "Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein." *J Biol Chem* **277**(6): 4351-4360.

Stoick-Cooper, C. L., G. Weidinger, K. J. Riehle, C. Hubbert, M. B. Major, N. Fausto and R. T. Moon (2007). "Distinct Wnt signaling pathways have opposing roles in appendage regeneration." *Development* **134**(3): 479-489.

Sun, L.-N., X.-L. Jiang, Q.-P. Xie, J. Yuan, B.-F. Huang, W.-J. Tao, L.-Y. Zhou, Y. Nagahama and D.-S. Wang (2014). "Transdifferentiation of differentiated ovary into functional testis by long term treatment of aromatase inhibitor in Nile tilapia." *Endocrinology* **0**(0): en.2013-1959.

Suster, M., H. Kikuta, A. Urasaki, K. Asakawa and K. Kawakami (2009). Transgenesis in Zebrafish with the Tol2 Transposon System. *Methods Mol Biol* **561:** 41-63.

Svingen, T. and P. Koopman (2013). "Building the mammalian testis: origins, differentiation, and assembly of the component cell populations." *Genes Dev* **27**(22): 2409-2426.

Szentes, K., E. Mészáros, T. Szabó, B. Csorbai, G. Borbély, G. Bernáth, B. Urbányi and Á. Horváth (2012). "Gonad development and gametogenesis in the Asian sea bass (*Lates calcarifer*) grown in an intensive aquaculture system." *J Appl Ichthyol* **28**(6): 883-885.

Taboada, X., D. Robledo, L. Del Palacio, A. Rodeiro, A. Felip, P. Martínez and A. Viñas (2012). "Comparative expression analysis in mature gonads, liver and brain of turbot (*Scophthalmus maximus*) by cDNA-AFLPS." *Gene* **492**(1): 250-261.

Takahasi, H. (1977). "Juvenile Hermaphroditism in the Zebrafish, *Brachydanio rerio*." *Bull. Fac. Fish. Hokkaido Univ* **28**: 57-65.

Takehana, Y., K. Naruse, S. Hamaguchi and M. Sakaizumi (2007). "Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena*." *Chromosoma* **116**(5): 463-470.

Tan, S.-L., A. Mohd-Adnan, N. Y. Mohd-Yusof, M. R. J. Forstner and K.-L. Wan (2008). "Identification and analysis of a prepro-chicken gonadotropin releasing hormone II (preprocGnRH-II) precursor in the Asian seabass, *Lates calcarifer*,

based on an EST-based assessment of its brain transcriptome." *Gene* **411**(1–2): 77-86.

Tanaka, H., T. Yoshida, N. Miyamoto, T. Motoike, H. Kurosu, K. Shibata, A. Yamanaka, S. C. Williams, J. A. Richardson, N. Tsujino, M. G. Garry, M. R. Lerner, D. S. King, B. F. O'Dowd, T. Sakurai and M. Yanagisawa (2003). "Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8." *Proc Natl Acad Sci U S A* **100**(10): 6251-6256.

Taneja, R., P. Bouillet, J. F. Boylan, M. P. Gaub, B. Roy, L. J. Gudas and P. Chambon (1995). "Reexpression of retinoic acid receptor (RAR) gamma or overexpression of RAR alpha or RAR beta in RAR gamma-null F9 cells reveals a partial functional redundancy between the three RAR types." *Proc Natl Acad Sci U S A* **92**(17): 7854-7858.

Tang, H., J. Brennan, J. Karl, Y. Hamada, L. Raetzman and B. Capel (2008). "Notch signaling maintains Leydig progenitor cells in the mouse testis." *Development* **135**(22): 3745-3753.

Taylor, J. S., I. Braasch, T. Frickey, A. Meyer and Y. Van de Peer (2003). "Genome duplication, a trait shared by 22,000 species of ray-finned fish." *Genome Res* **13**(3): 382-390.

Teng, Y.-N., P.-J. Chuang and Y.-W. Liu (2012). "Nuclear Factor-κB (NF-κB) regulates the expression of human testis-enriched Leucine-Rich Repeats and WD Repeat Domain Containing 1 (LRWD1) gene." *Int J Mol Sci* **14**(1): 625-639.

Thatcher, J. E. and N. Isoherranen (2009). "The role of CYP26 enzymes in retinoic acid clearance." *Expert Opin Drug Metab Toxicol* **5**(8): 875-886.

Trabzuni, D., A. Ramasamy, S. Imran, R. Walker, C. Smith, M. E. Weale, J. Hardy, M. Ryten and C. North American Brain Expression (2013). "Widespread sex differences in gene expression and splicing in the adult human brain." *Nat Commun* **4**.

Uchida, D., M. Yamashita, T. Kitano and T. Iguchi (2002). "Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish." *J Exp Biol* **205**(Pt 6): 711-718.

Ungewitter, E. K. and H. H. Yao (2013). "How to make a gonad: cellular mechanisms governing formation of the testes and ovaries." *Sex Dev* **7**(1-3): 7-20.

Vainio, S., M. Heikkila, A. Kispert, N. Chin and A. P. McMahon (1999). "Female development in mammals is regulated by Wnt-4 signalling." *Nature* **397**(6718): 405-409.

Valenzuela, N. (2008). "Sexual development and the evolution of sex determination." *Sex Dev* **2**(2): 64-72.

Vandeputte, M., M. Dupont-Nivet, H. Chavanne and B. Chatain (2007). "A polygenic hypothesis for sex determination in the European sea bass *Dicentrarchus labrax*." *Genetics* **176**(2): 1049-1057.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." *Genome Biol* **3**(7): RESEARCH0034.

Veitia, R. A. (2010). "FOXL2 versus SOX9: A lifelong "battle of the sexes"." *BioEssays* **32**(5): 375-380.

Verma, I. M., J. K. Stevenson, E. M. Schwarz, D. Van Antwerp and S. Miyamoto (1995). "Rel/NF-κB/I-κB family: intimate tales of association and dissociation." *Genes Dev* **9**(22): 2723-2735.

Vidal, B., C. Pasqualini, N. Le Belle, M. C. H. Holland, M. Sbaihi, P. Vernier, Y. Zohar and S. Dufour (2004). "Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: a neuroendocrine lock for the onset of puberty." *Biol Reprod* **71**(5): 1491-1500.

Vigier, B., M. G. Forest, B. Eychenne, J. Bézard, O. Garrigou, P. Robel and N. Josso (1989). "Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries." *Proc Natl Acad Sci U S A* **86**(10): 3684-3688.

Vizziano-Cantonnet, D., I. Anglade, E. Pellegrini, M.-M. Gueguen, A. Fostier, Y. Guiguen and O. Kah (2011). "Sexual dimorphism in the brain aromatase expression and activity, and in the central expression of other steroidogenic enzymes during the period of sex differentiation in monosex rainbow trout populations." *Gen Comp Endocrinol* **170**(2): 346-355.

Volff, J. N. (2004). "Genome evolution and biodiversity in teleost fish." *Heredity* **94**(3): 280-294.

von Hofsten, J. and P. E. Olsson (2005). "Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes." *Reprod Biol Endocrinol* **3**: 63.

Wallis, M. C., P. D. Waters and J. A. M. Graves (2008). "Sex determination in mammals — before and after the evolution of SRY." *Cell Mol Life Sci* **65**(20): 3182-3195.

Wang, C., Z. Bai, X. He, G. Lin, J. Xia, F. Sun, L. Lo, F. Feng, Z. Zhu and G. Yue (2011). "A high-resolution linkage map for comparative genome analysis and QTL fine mapping in Asian seabass, *Lates calcarifer*." *BMC Genomics* **12**(1): 174.

Wang, D.-S., T. Kobayashi, L.-Y. Zhou, B. Paul-Prasanth, S. Ijiri, F. Sakai, K. Okubo, K.-i. Morohashi and Y. Nagahama (2007). "*Foxl2* up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding protein/Steroidogenic Factor 1." *Mol Endocrinol* **21**(3): 712-725.

Wang, X. G., R. Bartfai, I. Sleptsova-Freidrich and L. Orban (2007). "The timing and extent of 'juvenile ovary' phase are highly variable during zebrafish testis differentiation." *J Fish Biol* **70**: 33-44.

Wang, X. G. and L. Orban (2007). "Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males." *Dev Dyn* **236**(5): 1329-1338.

Ward, R. D., B. H. Holmes and G. K. Yearsley (2008). "DNA barcoding reveals a likely second species of Asian sea bass (barramundi) (*Lates calcarifer*)." *J Fish Biol* **72**(2): 458-463.

Wassarman, P. M. (1999). "Mammalian Fertilization: Molecular Aspects of Gamete Adhesion, Exocytosis, and Fusion." *Cell* **96**(2): 175-183.

Westerfield, M. (2000). "The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*)." 4th ed., *Univ. of Oregon Press, Eugene*.

Wilkins, A. S. (1995). "Moving up the hierarchy: A hypothesis on the evolution of a genetic sex determination pathway." *BioEssays* **17**(1): 71-77.

Wijgerde, M., M. Ooms, J. W. Hoogerbrugge and J. A. Grootegoed (2005). "Hedgehog signaling in mouse ovary: Indian hedgehog and desert hedgehog from granulosa cells induce target gene expression in developing theca cells." *Endocrinology* **146**(8): 3558-3566.

Wittenrich, M. L. and P. L. Munday (2005). "Bi-directional sex change in coral reef fishes from the family Pseudochromidae: an experimental evaluation." *Zoolog Sci* **22**(7): 797-803.

Wu, G.-C. and C.-F. Chang (2013a). "Oocytes survive in the testis by altering the soma fate from male to female in the protandrous black porgy, *Acanthopagrus schlegeli*." *Biol Reprod* **88**(1): 19, 11-10.

Wu, G.-C. and C.-F. Chang (2013b). "The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegeli*." *Fish Physiol Biochem* **39**(1): 33-38.

Wu, G.-C., P.-C. Chiu, C.-J. Lin, Y.-S. Lyu, D.-S. Lan and C.-F. Chang (2012). "Testicular *dmrt1* is involved in the sexual fate of the ovotestis in the protandrous black porgy." *Biol Reprod* **86**(2): 41, 41-11.

Wu, G.-C., S. Tomy and C.-F. Chang (2008). "The expression of *nr0b1* and *nr5a4* during gonad development and sex change in protandrous black porgy fish, *Acanthopagrus schlegeli*." *Biol Reprod* **78**(2): 200-210.

Wu, G. C. (2010). "The expression of *amh* and *amhr2* is associated with the development of gonadal tissue and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*." *Biol Reprod* **83**(3): 443-453.

Wu, G. C. and C. F. Chang (2009). "*wnt4* Is associated with the development of ovarian tissue in the protandrous black Porgy, *Acanthopagrus schlegeli*." *Biol Reprod* **81**(6): 1073-1082.

Xia, J., G. Lin, X. He, B. Yunping, P. Liu, F. Liu, F. Sun, R. Tu and G. Yue (2014). "Mapping quantitative trait loci for omega-3 fatty acids in asian seabass." *Mar Biotechnol* **16**(1): 1-9.

Xia, J. H., F. Feng, G. Lin, C. M. Wang and G. H. Yue (2010). "A first generation bac-based physical map of the Asian seabass (*Lates calcarifer*)." *PLoS One* **5**(8): e11974.

Xia, J. H., G. Lin, X. He, P. Liu, F. Liu, F. Sun, R. Tu and G. H. Yue (2013). "Whole genome scanning and association mapping identified a significant association between growth and a SNP in the IFABP-a gene of the Asian seabass." *BMC Genomics* **14**(1): 295.

Xia, J. H., P. Liu, F. Liu, G. Lin, F. Sun, R. Tu and G. H. Yue (2013). "Analysis of stress-responsive transcriptome in the intestine of Asian seabass (*Lates calcarifer*) using RNA-Seq." *DNA Res* **20**(5): 449-460.

Xia, X., S. Qian, S. Soriano, Y. Wu, A. M. Fletcher, X.-J. Wang, E. H. Koo, X. Wu and H. Zheng (2001). "Loss of Presenilin 1 is associated with enhanced β-

catenin signaling and skin tumorigenesis." *Proc Natl Acad Sci U S A* **98**(19): 10863-10868.

Xiao, T., W. Shoji, W. Zhou, F. Su and J. Y. Kuwada (2003). "Transmembrane Sema4E guides branchiomotor axons to their targets in zebrafish." *J Neurosci* **23**(10): 4190-4198.

Xu, M., Z. Zhou, C. Cheng, W. Zhao, R. Tang, Y. Huang, W. Wang, J. Xu, L. Zeng, Y. Xie and Y. Mao (2001). "Cloning and characterization of a novel human TEKTIN1 gene." *Int J Biochem Cell Biol* **33**(12): 1172-1182.

Xu, Z., N. V. Kukekov and L. A. Greene (2003). "POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis." *EMBO J* **22**(2): 252-261.

Xu, Z., A. Sproul, W. Wang, N. Kukekov and L. A. Greene (2006). "Siah1 Interacts with the Scaffold Protein POSH to Promote JNK Activation and Apoptosis." *J Biol Chem* **281**(1): 303-312.

Yamaguchi, T., N. Yoshinaga, T. Yazawa, K. Gen and T. Kitano (2010). "Cortisol is involved in temperature-dependent sex determination in the Japanese flounder." *Endocrinology* **151**(8): 3900-3908.

Yang, X., E. E. Schadt, S. Wang, H. Wang, A. P. Arnold, L. Ingram-Drake, T. A. Drake and A. J. Lusis (2006). "Tissue-specific expression and regulation of sexually dimorphic genes in mice." *Genome Res* **16**(8): 995-1004.

Yano, A., R. Guyomard, B. Nicol, E. Jouanno, E. Quillet, C. Klopp, C. Cabau, O. Bouchez, A. Fostier and Y. Guiguen (2012). "An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*." *Curr Biol* **22**(15): 1423-1428.

Yao, H. H., J. Aardema and K. Holthusen (2006). "Sexually dimorphic regulation of inhibin beta B in establishing gonadal vasculature in mice." *Biol Reprod* **74**(5): 978-983.

Yao, H. H.-C., W. Whoriskey and B. Capel (2002). "Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis." *Genes Dev* **16**(11): 1433-1440.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T. Madden (2012). "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction." *BMC Bioinformatics* **13**(1): 134.

Yokoi, H., A. Nishimatsu, K. Ozato and K. Yoda (2003). "Cloning and embryonic expression of six wnt genes in the medaka (*Oryzias latipes*) with special reference to expression of *wnt5a* in the pectoral fin buds." *Dev Growth Differ* **45**(1): 51-61.

Yoo, S. W., T. Bolbot, A. Koulova, R. Sneeringer, K. Humm, Y. Dagon and A. Usheva (2013). "Complement factors are secreted in human follicular fluid by granulosa cells and are possible oocyte maturation factors." *J Obstet Gynaecol Res* **39**(2): 522-527.

Yoshimoto, S. and M. Ito (2011). "A ZZ/ZW-type sex determination in *Xenopus laevis*." *FEBS J* **278**(7): 1020-1026.

Yuan, L., J.-G. Liu, J. Zhao, E. Brundell, B. Daneholt and C. Höög (2000). "The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility." *Mol Cell* **5**(1): 73-83.

Yue, G. H., Z. Y. Zhu, L. C. Lo, C. M. Wang, G. Lin, F. Feng, H. Y. Pang, J. Li, P. Gong, H. M. Liu, J. Tan, R. Chou, H. Lim and L. Orban (2009). "Genetic variation and population structure of Asian seabass (*Lates calcarifer*) in the Asia-Pacific region." *Aquaculture* **293**(1–2): 22-28.

Zerbino, D. R. and E. Birney (2008). "Velvet: Algorithms for de novo short read assembly using de Bruijn graphs." *Genome Res* **18**(5): 821-829.

Zhang, C.-P., J.-L. Yang, J. Zhang, L. Li, L. Huang, S.-Y. Ji, Z.-Y. Hu, F. Gao and Y.-X. Liu (2011). "Notch signaling is involved in ovarian follicle development by regulating granulosa cell proliferation." *Endocrinology* **152**(6): 2437-2447.

Zhang, Y., F. Li, D. Sun, J. Liu, N. Liu and Q. Yu (2011). "Molecular analysis shows differential expression of R-spondin1 in zebrafish (*Danio rerio*) gonads." *Mol Biol Rep* **38**(1): 275-282.

Zhou, L., T. Charkraborty, X. Yu, L. Wu, G. Liu, S. Mohapatra, D. Wang and Y. Nagahama (2012). "R-spondins are involved in the ovarian differentiation in a teleost, medaka (*Oryzias latipes*)." *BMC Dev Biol* **12**(1): 36.

Zhu, Z. Y., C. M. Wang, L. C. Lo, G. Lin, F. Feng, J. Tan, R. Chou, H. S. Lim, L. Orban and G. H. Yue (2010). "A standard panel of microsatellites for Asian seabass (*Lates calcarifer*)." *Anim Genet* **41**(2): 208-212.

Zohar, Y., J. A. Muñoz-Cueto, A. Elizur and O. Kah (2010). "Neuroendocrinology of reproduction in teleost fish." *Gen Comp Endocrinol* **165**(3): 438-455.

Zohar, Y. and C. C. Mylonas (2001). "Endocrine manipulations of spawning in cultured fish: from hormones to genes." *Aquaculture* **197**(1–4): 99-136.

Table A 1. Gene symbols, gene names, accession numbers and primer sequences used for Asian seabass.

S/N	*Gene Symbol*	*Gene Name*	Accession Number	Forward Primer	Reverse Primer
1	*18S*	*18S ribosomal RNA*	GQ507431.1	GGTAGTAGCGACGGGCGGTG	GAGGCGTGGGTAACCCGCTG
2	*acvr1*	*activin A receptor, type I*	GAQL01018032	TCTCCCCCACATTTGATGCT	GGTGTTTAGACGCATCCACC
3	*acvr2b*	*activin receptor Iib*	GAQL01332399	AGGAGCTCAGGTGAAGGATG	AGCAGCACTACCTCAGACAG
4	*amh*	*anti-mullerian hormone*	GAMU01071817	CCGGCACTTGAGCATCTTGACTCC	CCAAGTACAGCACACACTGCATGC
5	*ar*	*androgen receptor*	KF444442	ACTCAGCTGCCACCCTACTC	CAGGGCAAACACCATCACC
6	*axin1*	*axin1*	KF444443	GAGGGCAAAGCTGGCCAAGG	GATGCGGCCTTCCCACACAG
7	*bactin*	*beta-actin*	GU188683.1	GGCCTCTGGGCAACGGAACC	TGGGTACCGCTGCCTCCTCC
8	*bfar*	*bifunctional apoptosis regulator*	GAQL01302998	GCCGCTTTGTATTCCCAGAG	GACGAAGAGCTGCTAAAGCC
9	*bmp1*	*bone morphogenetic protein 1*	GAQL01283156	ACTCCGTAACCCTTCTCAGC	GCCTTAAAGCCGAGGTCAAG
10	*bmp2*	*bone morphogenetic protein 2*	GAQL01015224	CGGCTTAAAGAGTCCTGCAC	CCCCTAACAACAGCTCCAGA
11	*btg1*	*B-cell translocation gene 1*	GAQL01134858	TGCATTCGCATCAACCACAA	ATGCGGTAGGACACCTCAAA
12	*c6*	*complement component C6*	GAQL01003411	GTTGATGCAGCGTCCGTTAT	CCTGTTACCCCTCCACAGAG
13	*c7*	*complement component C7*	GAQL01304558	AGTCGTCACAGGACGTGAAT	GGGTGTTTGTCAGCTTGGAG
14	*casp1*	*caspase 1*	GAQL01021430	ATCAGGGGTGCAGGAAAGAA	GATGTCCAACAGTCTGCGTC
15	*catD*	*cathepsin D*	EU143237.1	GCGGCTTTAGCTCTGAGCAGC	TGAGTCGGTCAACTCGCGCC
16	*ck2a*	*casein kinase 2 alpha*	KF444444	AATAGCGGGAGGCCACCCTG	GCACAGGGATGTGAAGCCCC
17	*comt*	*catechol-O-methyltransferase*	GAQL01025469	TGCCCAAGCAATGACTTGAC	TTGGAGCTGGGAACCTACTG
18	*ctnnb1*	*catenin beta 1*	KF444445	ATGCCTCCAGCTTCTACAATG	TATGGCAACCAGGAAAGCAA
19	*ctnnbip1*	*catenin beta interacting protein 1*	KF444446	GCCCCGGGGAAGTCTCCTGAAG	AGCTGGCTCATCTGACTGTGGACC
20	*ctsk*	*cathepsin K*	GAQL01015896	CGATCACGGTTCATTGGGAC	GATGAACCACCTGGGAGACA
21	*ctsz*	*cathepsin Z*	GAQL01254737	AGCGAGGTTCAGGAGTTCAT	CAAGTTGCTACCAGCCAGTG
22	*cul1*	*cullin 1*	GAQL01125635	ATCACACTCTCGTCTGACCC	TTTTACACCCAGCAGTGGGA
23	*cyp11c1*	*cytochrome P450, family 11, subfamily C, polypeptide 1*	KF444447	CACCGCTGTCGTGTCGACCC	ACACCGGGGTTCTGGGCCAG
24	*cyp17a1*	*cytochrome P450, family 17, subfamily A, polypeptide 1*	KF444448	AACGCAGTGCTGAGAATAAC	CTGGATACACCTCTGCACC
25	*cyp19a1*	*cytochrome P450 aromatase*	AY684256.1	TGACTACGGTGCAGCGCAGC	CCTGACAGGTCCGGGCTTGC
26	*cyp26a1*	*cytochrome P450, family 26, subfamily A, polypeptide 1*	GAML01005182	AGCTGCTCCAAGGACTTGAT	GACAGACTGAGGCAGGAACT
27	*cyp26b1*	*cytochrome P450, family 26, subfamily b, polypeptide 1*	KF444449	GCGCCCGTCACTCGGATCAG	GCGAGACCTGCCACTGGCTC
28	*dlc*	*deltaC*	GAQL01326070	TCGTCGATGTTGGTCTCACA	AGTGTTTGGACCTCGGTGAT
29	*diablo*	*diablo, IAP-binding mitochondrial protein*	GAQL01268499	CATGTTCCGCTCCTGATGTG	TCTGGCAGGTGATCATTGGT
30	*dmrt1*	*doublesex and mab-3 related transcription factor 1*	KF444450	CTCTCTGCCTCTCGGCTATC	CATCCAAAGGCCAGAAACCC
31	*dvl2*	*dishevelled, dsh homolog 2*	KF444451	TTCGCGGCGGTCCTGGAAAC	GGTCAGGGACCGCATGTGGC
32	*ef1a*	*elongation factor 1-alpha*	GQ507427.1	GGCATCCAGAGCTTCCAGCAGTG	CGCCACTGTTGCCTTTGTCCC
33	*epha4*	*eph receptor A4*	GAQL01333781	ATTCCTGGGGAGGGCTTTAC	ACGAAGTGACACCAGCTACA
34	*ephb3*	*eph receptor B3*	GAQL01004769	GGCTGTCGGACATTACACAC	ACATTCCCAGAGAGTGGGTG
35	*ephb4*	*eph receptor B4*	GAQL01010987	CAGCTGGCTGTGATGATTCC	AAAGACGCTAAAGGGAGGCT
36	*esr1*	*estrogen receptor 1*	KF444452	TGGCCCAGGCGATCATGTGG	CTGCTCCAGGGTGCTGAGCC
37	*esr2*	*estrogen receptor 2*	GAQL01027449	CTGTGAATCCAGGGACCACT	AGATCCTGGACACACACCTG

Table A 1. Gene symbols, gene names, accession numbers and primer sequences used for Asian seabass. (continued)

S/N	*Gene Symbol*	*Gene Name*	Accession Number	Forward Primer	Reverse Primer
38	*fgfr1a*	*fibroblast growth factor receptor 1a*	GAQL01295010	GACATCGCATGGAGAAACCC	CTGTTGGAAAGTGGGTCTGC
39	*foxl2*	*forkhead box L2*	KF444454	CTGGGGAGCGCCATGCTCTG	CAACCGCCCACCCCGATGTC
40	*fsta*	*follistatin a*	GAQL01268786	TTCCTCATCCTCCTCCTGGT	GCGCTGTTGTGGTCCATATT
41	*fzd1*	*frizzled homolog 1*	GAQL01343711	GCCACAAAACTCAGGAGGAC	TGTAGACGGAGCAGAGGAAC
42	*fzd8*	*frizzled homolog 8*	GAQL01271658	GGATCCTGAACAGGGACACA	CAGAACCTGGACAATCTGCG
43	*gadph*	*glyceraldehyde-3-phosphate dehydrogenase*	GQ507430.1	GGCACCACCCTTCAAGTGAGCAG	GAGAGGGACCCCGCCAACATC
44	*gdf9*	*growth differentiation factor 9*	GAQL01307614	GTAGTGGGAGGGGATACACG	AGTACAACCCCAGGTACTGC
45	*gli1*	*GLI-Kruppel family member 1*	GAQL01025385	ATGGGGGTTTCTGACCTCTG	ACTCGGACAATCCCAGTTGT
46	*gsdf1*	*gonadal soma derived factor 1*	GAQL01288594	ACCATGGTACCTGTGATGGG	GGCGATTGCATTTGTCTTGC
47	*hsd11b2*	*11-beta-hydroxysteroid dehydrogenase type 2*	KF444456	TGGAGATGCCGAGCTGTCGC	TCGCAGCAGTGGCAGGAAGC
48	*hsd17b1*	*17-beta-hydroxysteroid dehydrogenase type 1*	KF444457	TGCCGGTGACCAGAATGCGG	GATGGGGCCACTGGAGCTGC
49	*hsd3b*	*3 beta-hydroxysteroid dehydrogenase*	KF444455	TGATGGGACCAAACCCCAGGG	TGGGCCAGCAGGGTTCTCTG
50	*igf1*	*insulin-like growth factor 1*	GAQL01220206	AACCAGGCTATGGCTCCAAT	TGCCTTAGTCTTGGGAGGTG
51	*igf2*	*insulin-like growth factor 2*	GAQL01005606	ATAGTTGTCCGTGGTGAGCA	CAGGCGGAGAAGATCAAAGC
52	*ikba*	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*	GAQL01007286	TGGTGGAGAAGCTCCTGAAG	ACACGATGGAGGTAAGGTGG
53	*ikbe*	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon*	GAQL01342786	ACGTGATGTCATGCAGTTCG	TTCACTTGGCTGCACTGAAC
54	*il13ra2*	*interleukin 13 receptor, alpha 2*	GAQL01286502	ACGCTCCCATTGTACTTCCA	ATCCAAAACCACGTGAAGCC
55	*inhbb*	*inhibin, beta B*	GAQL01354340	AGCAGCTTGAAGTAGAGCCA	GACATCCAAGTCCAGCCTCT
56	*insr*	*insulin receptor*	GAQL01155747	TCTCCATGTTGTCCAGGTCC	CACCCATCTTTCCAGGAGGT
57	*jag1b*	*jagged 1b*	GAQL01021133	GGGAGCACATGCAACGATAG	CGATACAAACGACTGCAGGG
58	*junb*	*jun B proto-oncogene*	GAQL01202237	TAGCTGGATGCGGAAGAGAG	GAGTTACGACGTGTTCACCG
59	*nanos1*	*nanos homolog 1*	GAQL01279018	TACGAGGGCGAATCTCTGTC	TACGATCTCAAGGAGCGCAT
60	*nfil3*	*nuclear factor, interleukin 3 regulated*	GAQL01307699	GATCCCTGCTGGAAGAACCT	TGAAATTTGGCCTGGTGAGC
61	*nfkb2*	*NF-kappa-B 2*	GAMU01013914	GCTGGGAGGCGTTCGGTGAC	CTGTGCTGTGGTACGGCGGC
62	*nkap*	*NF-kappa-B-activating protein*	GAML01003947	GCGCTGTTGCCAGGTGAAGG	TGCGATCTCCTCGCTGGTGAG
63	*nodal*	*nodal homolog*	GAQL01221532	CATTTTTGTTGGAGCGCAGC	CTACCGCTGTGAGGGATCTT
64	*nog*	*noggin*	GAQL01225420	CTTGTGCTTCTTGCCGAACT	GGACAAATACACCGGGAACG
65	*nova1*	*neuro-oncological ventral antigen 1*	GAQL01012356	CCAAAATGGCACCAACCAGA	ACAGGGGCTTCCAATGGTTA
66	*npb*	*neuropeptide B*	GAQL01282305	GGGATCACGTTGTTACCTGC	CTGTCCGGTATCAGGAGGTC
67	*nr0b1*	*nuclear receptor subfamily 0 group B member 1*	KF444458	GCTCGTGCTGGGACTCGCTC	CGCTCTGCCTGTCGGGCAAA
68	*nr5a2*	*nuclear receptor subfamily 5 group A member 2*	KF444453	CCCAATACCAATACACAGCCTTC	GACACTGAGGGGTACATGTCTGG
69	*odf3*	*outer dense fiber of sperm tails 3*	GAMU01119126	GCCCGCTCGGGTGATGTTGG	GCACTGCCTGGACTCACAGGTG
70	*peli1*	*pellino homolog 1*	GAQL01328021	GAAGGCCAGAGTGTTGAAGC	ACTCATCGACCTCTGTGGTG
71	*piwil1*	*piwi-like 1*	GAML01007253	TGCTGGGGAGGACCACCACC	GGTCCACTTGGGATCCGCATCC
72	*pr*	*progesterone receptor*	GAQL01031563	ATGCAGTCATTTCGTCCAGC	ATGGAGTCTTGACCTGTGGG
73	*psap*	*prosaposin*	GAML01003564	CCCTGGCAAAGGCTCAGGCC	GGGAGCCTCCTGCTTGGGAC
74	*psen1*	*presenilin 1*	GAQL01019997	TCTCCACCAGGATCCTCAGA	AAGTACCTGCCAGAGTGGAC
75	*rdh3*	*retinol dehydrogenase 3*	GAQL01318752	TAATCAAGAAGGCCAGGGGG	ACACCGTACTTGGAGACAGG

Table A 1. Gene symbols, gene names, accession numbers and primer sequences used for Asian seabass. (continued)

S/N	*Gene Symbol*	*Gene Name*	Accession Number	Forward Primer	Reverse Primer
76	*rpl8*	*ribosomal protein L8*	GQ507429.1	ACCTTGCGTCCAGCAGGTGC	AACTGCTGGCCACGTGTCCG
77	*rprma*	*reprimo, TP53 dependent G2 arrest mediator candidate a*	GAQL01184498	GGTCTCCTGTCCGTTACCAA	GATAGCCGTCATGTGCGTTT
78	*rtkn1*	*rhotekin 1*	GAQL01352899	ACATCTTCTTGCCGGTGGTA	CAGCCTCACTGTATGACCCA
79	*rtkn2*	*rhotekin 2*	GAQL01025139	GCGAAGGGTTGATGATGGAC	TAAAGTGCAGCCGACTCTCA
80	*rttn*	*rotatin*	GAQL01105479	AAGCTCCCATACAGCGAGTT	ACCCTTCACAACCTCTGCTT
81	*rxfp3*	*relaxin/insulin-like family peptide receptor 3*	GAQL01019782	ATCCCAGTTGACCACGTTGA	CACCATCGTTGTCCTCTCCT
82	*sema4e*	*semaphorin 4e*	GAML01028495	GGAGGAAAGCTGGAGGAAGT	GATTTGATGGTGAGGACGGC
83	*sept6*	*septin 6*	KF444459	ACAGTGAGTTTGAGGCGCAC	CTGTGTTGGTGAGACGGGAC
84	*sept7*	*septin7*	GAML01001618	TCGAGCAGCAGAAACTGGACGC	AGGGCCACAACAGACGTGAGC
85	*sh3rf1*	*SH3 domain containing ring finger 1*	GAQL01304276	TTGCATGCTGTGCTCCATTT	TCGCTCTTGACAGGGTGAAT
86	*shh*	*sonic hedgehog*	GAQL01006243	TGGACTTGACTGGCGAAGAT	CGGAGGCTCTTCTACGTGAT
87	*smad4*	*MAD homolog 4*	GAQL01008285	CAGACAAACTGATGGCAGGG	AGGTGTTTGACTTGCGTCAG
88	*smo*	*smoothened homolog*	GAQL01017046	CCAAAAGCGAGGAACCCAAA	ATTCGGGGTGTCATGACCTT
89	*sox9*	*SRY-box containing gene 9*	KF444460	GCGTTCATGGGTCTTTTGACG	CGGTGTCCCAGGTGTTGAAG
90	*spry1*	*sprouty homolog 1*	GAQL01018294	CCATGCACAGGAAACGAGAG	CCACTGCTCCAATGACGATG
91	*stat3*	*signal transduction and activation of transcription 3*	GAQL01301147	TCATTCCACAGTGCCAGGAT	ACTCTGGCTGTCAGATCACC
92	*stra6*	*stimulated by retinoic acid gene 6 homolog*	GAML01004693	GTGGACTGGTCAGGCTCGGC	ACCCATCCCTGCTGGGCTCC
93	*sycp1*	*synaptonemal complex protein 1*	GAQL01102493	TTGTTTGAAGCTCTGCCACC	AGCCTTCGTATTCGAGCAGA
94	*sycp3l*	*synaptonemal complex protein 3 like*	GAML01036838	CGTTCGTCCAGGGAGCGCAG	GAGACTTGCGGACGGTGGCC
95	*tac1*	*tachykinin 1*	GAQL01003257	AGATGACCGTTTTTAGCGGC	TGGCAAATGCACAGATCACC
96	*tcf7*	*transcription factor 7 (T-cell specific, HMG-box)*	GAQL01300917	AGGTGGAATCCTTGGTCCTG	ATGTCAAAAGAGCAGCAGGC
97	*tdrd1*	*tudor domain containing 1*	GAML01005267	GCTCTGTGATGTGCCAGGCAG	AGGCTGGAGGCATCACCATGC
98	*tdrd7*	*tudor domain containing 7*	GAML01004579	ACTGCCAGCTACCCTCCAGAGG	AGCGGGCTAGACAGAACTTGCC
99	*tekt1*	*tektin 1*	GAQL01036461	ATCTCCAAGTGGCCATCACA	AAGGTCTCAGGCGACGTAAA
100	*tnfa*	*tumor necrosis factor a (TNF superfamily, member 2)*	GAQL01232152	TGAGGGGCGTGAGGTATTTT	CTCAGACCGGCCTCTACTTC
101	*tnfrsf10b*	*tumor necrosis factor receptor superfamily, member 10b*	GAQL01140952	TTTTGTGCTCCTGACCAAGC	TTTCTTGCACTCCGTGTTGG
102	*tnfrsf1a*	*tumor necrosis factor receptor superfamily, member 1a*	GAQL01309610	GCACAAACAACACAAGCACC	GAACCCTGCAAGAGTTGTGG
103	*tnks*	*tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase*	GAQL01035852	GCGTTGATGCCGATCTCTTT	GCTCGACATGACCATCAACC
104	*tp53*	*tumor protein p53*	KF444461	TGAGCCCTCTGGCTGCCCTC	ATGTGGCGGAAGTGGTCCGC
105	*tuba*	*alpha tublin 1*	EU136175.1	CCGCAAACTGGCTGATCAGTGC	AGCCAGAGCCAGTTCCTCCCC
106	*ubq*	*ubiquitin*	GQ507428.1	CTTGTCGCAGTTGTATTTCTGG	GCACACTGTCTGACTACAACATCC
107	*unc5a*	*unc-5 homolog A*	GAQL01308363	AATCAAGTGGTCGTCCTGGT	TTGTGAAGAACAAGCCGGTG
108	*vasa*	*vasa homolog*	KF444462	TGGCCCCAACCAGGGAGCTC	AACCACCGGACGCACACACG
109	*vtgr*	*vitellogenin receptor*	KF444463	GTGCATCCCTGCCAGCTGGG	TGGACGAACACTTCGCCGGC
110	*wnt16*	*wingless-type MMTV integration site family, member 16*	GAQL01017753	AAACACCGTGACAACGACAG	CGCTGATCACCATGAACCAG
111	*wnt3*	*wingless-type MMTV integration site family, member 3*	GAQL01088979	CACCACCTGGTCCTTGTTTG	ATGTGGTCGAGGATGGTCTG
112	*wt1*	*wilms' tumor 1*	KF444464	GGTGCCCAGCATCGCTCCAG	TGCACCCTGGGTAGGCACAC
113	*zp2*	*zona pellucida glycoprotein 2*	KF444465	GCTGGGCCACCTCTACCCCA	GAGCCGTCCACAGGCACCAC
114	*zp3*	*zona pellucida glycoprotein 3*	GAQL01279491	AGGTGCGACCTTTTCAAACC	TGGGCCTTTTATCAGCTCCT

Table A 2. Gene symbols, gene names, accession numbers and primer sequences used for zebrafish.

S/N	Gene Symbol	Gene Name	Accession	Forward Primer	Reverse Primer
1	*18s*	18s ribosomal RNA	BX296557	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATCA
2	*actb1*	actin, beta 1	NM_131031.1	GGCACGAGAGATCTTCACTCCCC	GGGAAAACAGCACGAGGGGC
3	*ar*	androgen receptor	NM_001083123.1	GAATGACCCTGGGAGCCCGC	CTCCGACGGGCCCTGAACTG
4	*anxa1a*	annexin A1a	NM_181758.1	ACCAGCAAGCCACTAGCAAGCC	TGTGCCAGCACCCTTCATGGC
5	*amh*	anti-Mullerian hormone	NM_001007779.1	ACAACCCGAAGGTCAACCCGC	GTGGCATGTTGGTCAGTTGGCTG
6	*axin1*	axin 1	NM_131503	ACCTGCTGACGACATGGAGAGG	AATGCTCCCGTAAGGGCCCC
7	*btg4*	B-cell translocation gene 4	NM_198121.1	GGAGAGGTGTCATGCCGGTATGG	CCTACGAGAGAACTCGCCGTCTC
8	*csnk1g1*	casein kinase 1, gamma 1	NM_001008635	GCGGGTAGCGGGATATCACCAC	CCGCCAGCACATTCCACGATG
9	*ck2a1*	casein kinase 2 alpha 1	NM_131252.1	TGCAAAGGTGCTGGGTACAGAGG	CACCTCTTCCGAGAGTGCCTGC
10	*casp9*	caspase 9	NM_001007404.2	CACATGGCACTGAGGCAAGCC	GGGCTTGCCCTGAAGAGAAGGAC
11	*ctnnb1*	catenin, beta 1	NM_131059.2	ACGGATTGTCGCCATTATTCGCG	CCAGCTCCATCAAGTCAGACTGGG
12	*ctnnbip1*	catenin, beta interacting protein 1	NM_131594.1	CTGTCGGGATGTGACCCCGG	CTCCTGACGCACCGCTCTCC
13	*ctssb.1*	cathepsin S, b.1	NM_001024409.2	CACCACCTCCTGTTCAGACGACG	ACACGGCAAACAGCAAGCTCC
14	*cldnd*	claudin d	NM_180964.2	TGCCCGAAGAACGAGGGTCG	CCAGAAAGTCGGTGCGTCACAC
15	*col1a1b*	collagen, type I, alpha 1b	NM_201478.1	ACGGTCATGTGCGACGAGGTG	CCAGCAGGACCCACGGTATCAAC
16	*cyc1*	cytochrome c-1	NM_001037393.1	AGCTGCACCCGCCGACATAC	GATATCCACGACGAACGCTGGC
17	*cyp11c1*	cytochrome P450, family 11, subfamily C, polypeptide 1	NM_001080204.1	CCTCGGGCCCATATACAGAGA	CGTCCCGTTCTTGAGGAAGA
18	*cyp19a1a*	cytochrome P450, family 19, subfamily A, polypeptide 1a	NM_131154.2	GATATTTGCTCAGAGCCATGGA	GCTCTGGCCAGCTAAAACACT
19	*cyp19a1b*	cytochrome P450, family 19, subfamily A, polypeptide 1b	NM_131642.1	AAAGAGTTACTAATAAAGATCCACCGGTAT	TCCACAAGCTTTCCCATTTCA
20	*cyp26b1*	cytochrome P450, family 26, subfamily b, polypeptide 1	NM_212666.1	GCAAGCTGCCCATGCCCAAG	GCATGGAAACCTGCACCCTGAAAG
21	*cyp26a1*	cytochrome P450, subfamily XXVIA, polypeptide 1	NM_131146.2	GACGAGCAAGAACTGGTGGAAGC	ATTGCGTGCCCTCAAACCCCTG
22	*dnd*	dead end	NM_212795.1	TCGTGGAAGCTTTTCGGAACCGG	TGTCCTCGACGCGCTTGGAC
23	*dkk1b*	dickkopf 1b	NM_131003.1	AGAGTTCGTGTCCATCGCCCATG	CCCTCCAGACCTTTCAGCATCTGG
24	*dkk3*	dickkopf homolog 3	NM_001089545.1	GCATGTGGCTCACGGACAAACC	TTCTCCATCTGATGCACAGCCTCC
25	*dvl3a*	dishevelled, dsh homolog 3a	NM_131757.1	TGCCCATCCCTGCCGAAAGG	TGACCACCCCAAAGTCATCGTCC
26	*dmnt1*	DNA (cytosine-5-)-methyltransferase 1	NM_131189.1	GGCTTCCCAGACACCTACCGC	TGACCTCCAGGCCAATGGTTTCG
27	*dnmt3*	DNA (cytosine-5-)-methyltransferase 3	NM_131386.1	CGACTCCCTACGTGATAGCGACC	CCATAACCACCACCGTCCGTCC
28	*dmrt1*	doublesex and mab-3 related transcription factor 1	NM_205628.1	TGCCCAGGTGGCGTTACGG	CGGGTGATGGCGGTCCTGAG
29	*esr2a*	estrogen receptor 2a	NM_180966.2	TGAAATGTGGGTTGCGGCGAG	TTCCCACTGAGAGGACGCGG
30	*esr2b*	estrogen receptor 2b	NM_174862.3	CACGTTCACACAGCGCCTGG	GCTCGCGGAGGGATTCAAGC
31	*eef1a1l1*	eukaryotic translation elongation factor 1 alpha 1	NM_131263.1	GGAGGCTGCCAACTTCAACGC	GGCGATGTGAGCAGTGTGGC
32	*fancl*	Fanconi anemia, complementation group L (fancl)	NM_212982.1	ACACTCCAGCTGAAGGCAGAGG	CCAAGGTGCTCTGAGAGGTCCAC
33	*fgf20a*	fibroblast growth factor 20a	NM_001037103.1	CTGGTGGGCAGCTTCTCTCACG	TCATCAGCGCACCAGAGTCCC
34	*fgf20b*	fibroblast growth factor 20b	NM_001039172.1	CGGGACGTTTTTGCAGAGCCTG	ATCTGCGGAGGTGCTCCGTG
35	*fsta*	follistatin a	NM_131037.3	ATGCATCAAGGCCAAGTCATGCG	GCGCCCGCGACTCATCTTTG
36	*foxl2*	forkhead box L2	NM_001045252.1	AAACACTGGGAAGGTTTGCGTGC	TTTGTCCGGCCCCTTCTCTGG
37	*gtf3ab*	general transcription factor IIIA, b	NM_001089544.2	TACTGCAGACACACGGGGCTG	ACCCGCTGACTGAACACAGGTAAG
38	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	NM_001115114.1	GTGTCCCCACCCCCAATGTCTC	TGGACACAACCTGGTGCTCCG
39	*hsf5*	heat shock transcription factor 5	FJ969446.1	GGCAGCTCAACCTCTACGG	TCAGCCTCTTCAGATGGACC
40	*hsd11b2*	hydroxysteroid 11-beta dehydrogenase 2	NM_212720.1	CAGCCCTTCAGGTGAGCATCCC	CCCCATGGATCCAGCTCGTGAC
41	*inhbb*	inhibin, beta B	NM_131068.2	GCCCACAACGAGGTGCAAGAAG	ACAGCCACAGATTTGCCTGCAAC
42	*lgals3bpb*	lectin, galactoside-binding, soluble, 3 binding protein b	NM_212873.1	GACATCATGTTGCTCCTGTGGCC	GCTGCTTTGGTCGGTCAAACAAGG
43	*lef1*	lymphocyte enhancer binding factor 1	NM_131426.1	CGAGGGAGACCCGCACAAGG	GGGACTGTCTAGCTGCGTCGTG
44	*mitfa*	microphthalmia-associated transcription factor a	NM_130923.1	CTGCCGGCCGTCAAAAGGG	CCAGGGCTCTGACTTCTGCTTCTAC
45	*mid1ip1*	MID1 interacting protein 1	NM_213439.1	AATCCAGCTTTGCCATGATGCAGC	GTTGGCTGCGGCGATGAACC

Table A 2. Gene symbols, gene names, accession numbers and primer sequences used for zebrafish. (continued)

S/N	Gene Symbol	Gene Name	Accession	Forward Primer	Reverse Primer
46	*nanos3*	nanos homolog 3	NM_131878.1	GCAAGAAATGCAATCTGACG	GTGTACACGGCCTCAGTCTC
47	*nkap*	NFKB activating protein	NM_001003414.1	GTGCCGCCATGGCAGAGTTC	TACGCCGATGTCTGCTTCCACTC
48	*odf3b*	outer dense fiber of sperm tails 3B	NM_199958.1	AAACTCCAGGTCCAGCTGCGTAC	ACCGCTGAAGGAGACGTTTGGG
49	*piwil1*	piwi-like 1	NM_183338.1	ATCTCAAGAGGCTGCAAAGC	CACCTCTCTCCCCGATCTTC
50	*pou5f1*	POU domain, class 5, transcription factor 1	NM_131112.1	GGAAGGCACAGTCCGTTCTGC	CGCACTACATCTCTCTCCAGGCC
51	*psen1*	presenilin 1	NM_131024.1	TGGCGGACAGTGCTGAAACCAG	GTGGGAGCCATCGCAACTACCTG
52	*pomca*	proopiomelanocortin a	NM_181438.3	GAAAACGCCCGCTGTCGAGAC	AGGAGGTCGATTTGCTCCGGC
53	*ppp4ca*	protein phosphatase 4 (formerly X), catalytic subunit a	NM_001110414.1	GTGTGCACGGTGGTCTTTCTCC	CCCAGCCTGTGGTGTCTTCTGG
54	*pycard*	PYD and CARD domain containing	NM_131495.2	GCGTGTTCACATCAAAAGACGCG	AGCACCTTTGCTTTCTGATTGCCC
55	*rspo1*	R-spondin homolog	NM_001002352.1	AGGCCTGTACTCGCATAGAGGGC	GGCCCCATGGACTCCACTCAC
56	*rxrgb*	retinoid X receptor, gamma b	NM_001002345.1	CGCCTCCAGGACAAGACCTGAC	TGACGGACGGATGACCCACC
57	*rbp1b*	retinol binding protein 1b	NM_212895.2	AGAAAGGCGAGGTTGAAGGCCG	TCCCCCTGCTCTCAGTTCCAAATG
58	*rbp2a*	retinol binding protein 2a	NM_153004.1	TGCCAGCCGATTTCAACGGC	GGCGAAGTCGATGTCCAAAGCC
59	*rbp4*	retinol binding protein 4	NM_130920.1	GCCAAGAAAGACCCTGTTGGGC	GCGCACATCTCCCAGTTGTTGAG
60	*rdh10b*	retinol dehydrogenase 10b	NM_201331.1	TTGAAGCCCTCCACCGGGTC	GTCTCCCACCTCACTGCGAACC
61	*rdh14a*	retinol dehydrogenase 14a	NM_001006031.1	GTCCGCGTGTTCTGCGAAGG	AGCAAACTGCATCTCGAAGCCG
62	*rpl13*	ribosomal protein L13	NM_198143.1	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
63	*setd8b*	SET domain containing (lysine methyltransferase) 8b	NM_001100089.1	TCTGCCAAAAGGAGACCTGCAGAC	TGTCCTTGAGCTGTCGTGGGC
64	*CO352993*	similar to differentially regulated trout protein 1	CO352993	AGACGTGTTTTGGAAGCTTCGACG	AGGAACGGATGGAGGCGGAAC
65	*sox9a*	SRY-box containing gene 9a	NM_131643.1	ACACTCAGGCCAGTCCCAGGG	GAGGACGGGCCTCTCGTTTCAG
66	*sox9b*	SRY-box containing gene 9b	NM_131644.1	AGTTCGACCAGTACCTGCCTCCG	TCGCGAGTGGTTTGTGCATCCAG
67	*star*	steroidogenic acute regulatory protein	NM_131663.1	TGAACAAGCTCTCCGGACCTGG	CAATTGGACTGCTGAGCAAGGAGC
68	*stra6*	stimulated by retinoic acid gene 6 homolog	NM_001045312.1	TGGAGGGTCGTCATCACGGC	GGGAGTAGCAGCGGTATCCTGG
69	*sycp3*	synaptonemal complex protein 3	NM_001040350.1	AGCGGATCTGACGAAGACACGAG	ATGTCCGCACCAAATCTTTCCAGC
70	*tbp*	TATA box binding protein	NM_200096.1	ATGTTTTGCGCGCTCCCTGC	AGCAGTGGTTCAGGGCTTCCC
71	*trdmt1*	tRNA aspartic acid methyltransferase 1	NM_001018143.1	GCAGGGAGATGTTGCCGACCC	TGAAGCAAAGCATCCCTTGCAGC
72	*ts1*	ts 1	EF554575.2	GGAGAGCAGTGAGACGACATCACC	ACCCAGATGTATCGGCCCCATG
73	*tdrd7*	tudor domain containing 7	NM_001099343.1	TCGCATAACGTCAAACCTCGCTTG	TCTGGAGGAGGGCAGGTCGG
74	*tp53*	tumor protein p53	NM_131327.1	TTTACCCTGCAGGTGAGGGGC	TCTGAGGCAGGCACCACATCAC
75	*vasa*	vasa homolog	NM_131057.1	TCCCTGGTCAAAGTCCTTTCAGGGG	CGCTCTTGAAGGATCCTCCCTTCCG
76	*vtg5*	vitellogenin 5	NM_001025189.1	AAGGCCCTGCCAACTGACCG	TGGGTCCAGTAACCATCGGTATTGC
77	*wt1a*	wilms tumor 1a	NM_131046.1	ATGAGAGCGACCCCAGCACAC	ACTCTCCGCACATCCTGAAGGC
78	*wnt4a*	wingless-type MMTV integration site family, member 4a	NM_001040387.1	ACGGAGTCAGTCCAGAAGGTTTCC	ACTCCGTTCCCTGATGTCCACG
79	*zp2*	zona pellucida glycoprotein 2	NM_131330.1	CCCACAGTCTCTGTCTCCTGTGC	TTCCTTGCGCAACTCTGCTCAC
80	*zp3*	zona pellucida glycoprotein 3	NM_131331.1	AATCATGGGTGCTTTGTGGATGCC	GCCTCCAACTGGAAACGGAGC
81	*zgc:55413*	#N/A	CO350518	TCCAGTGTCTTTCTGGCCCCG	CCCCATCATTTGGTCCGCCAATC
82	*zgc:195154*	#N/A	CO351481	ATCTCGAGGAGGTGACCAGCTTTG	ATGATGAACCCGCCTGCTTCTCC
83	*zgc:194314*	#N/A	CO352680	TGAGCTCATCCCTTTCGCCAAGG	CAAATGCGCCGCCCACCAG
84	*zgc:194626*	#N/A	NM_001128777.1	CTAGCCTGCTGCCTGCTGTTTC	TCTGCACAGAGGTTGAGGGTGTG
85	*CCL-C11a*	chemokine CCL-C11a	AB331767.1	CGGAGGATGCAACATTCCCGC	TCGCCTGGACCCAGTCACTTC

Table A 3. The list of differentially expressed genes between Asian seabass F3 and F4 ovaries

S/N	Gene symbol	Fold-change (F4 vs F3)	p-value	Remarks
1	*vasa*	5.2	3.47E-05	
2	*nkap*	4.5	6.38E-06	
3	*axin1*	4.3	0.001	
4	*tdrd1*	4.2	0.002	
5	*sycp3l*	4.2	6.06E-05	
6	*dmrt1*	3.8	0.001	
7	*psap*	3.8	1.32E-04	
8	*vtgr*	3.8	3.94E-04	
9	*tdrd7*	3.7	2.34E-04	
10	*amh*	3.6	0.003	
11	*stra6*	3.5	3.26E-04	
12	*dvl2*	3.4	0.005	
13	*nfkb2*	3.2	0.003	
14	*ctnnb1*	3.2	0.001	
15	*ck2a*	2.9	0.002	
16	*nr0b1*	2.8	0.004	
17	*hsd11b2*	2.7	0.023	
18	*cyp26a1*	2.7	0.001	
19	*sept7*	2.4	0.004	
20	*tp53*	2.3	0.002	
21	*piwil1*	1.9	0.013	
22	*sept6*	1.9	3.67E-04	
23	*ctnnbip1*	1.9	0.001	
24	*hsd17b1*	-2.0	0.007	
25	*cyp19a1*	-4.5	0.050	
26	*odf3*	-20.7	0.014	
27	*zp2*	1.4	0.001	N.S.
28	*foxl2*	-2.1	0.057	N.S.
29	*hsd3b*	-2.0	0.065	N.S.
30	*ar*	-2.2	0.066	N.S.
31	*sox9*	1.5	0.116	N.S.
32	*nr5a2*	-1.4	0.368	N.S.
33	*cyp17a1*	-1.6	0.389	N.S.
34	*wt1*	-1.5	0.447	N.S.
35	*esr1*	1.0	0.544	N.S.
36	*cyp11c1*	-1.1	0.651	N.S.
37	*cyp26b1*	2.5	0.741	N.S.

N.S. - Not significant (fold-change < 1.5 or p-value > 0.05)

Table A 4. Real-time qPCR validation of microarray results.

Gene Info		Juvenile Testis vs Undifferentiated							Adult Testis vs Juvenile Testis						
		real-time qPCR			Microarray			Validated?	real-time qPCR			Microarray			Validated?
S/N	*Gene Sym*	p-value	fold-change	Direction	p-value	fold-change	Direction		p-value	fold-change	Direction	p-value	fold-change	Direction	
Known sex genes															
1	*wt1a*	0.024	-3.40	DOWN	0.009	-4.63	DOWN	YES	0.929	1.76	N.S.	0.038	-4.65	N.S.	YES
2	*foxl2*	0.056	13.24	N.S.	0.851	1.21	N.S.	YES	0.181	3.45	N.S.	0.415	-2.41	N.S.	YES
3	*dmrt1*	0.031	2.93	UP	0.184	1.91	N.S.	NO	0.004	2.25	UP	0.969	1.05	N.S.	NO
4	*amh*	0.007	25.13	UP	0.202	-3.85	N.S.	NO	0.008	5.73	UP	0.042	-16.53	N.S.	NO
5	*sox9*	0.679	-1.23	N.S.	0.004	-6.34	DOWN	NO	0.583	1.50	N.S.	0.093	-3.62	N.S.	YES
Steroidogenic/ hormone-related															
6	*hsd17b1*	0.020	54.44	UP	0.027	22.43	N.S.	NO	0.360	1.81	N.S.	0.084	-21.39	N.S.	YES
7	*fsta*	0.069	16.10	N.S.	0.134	-2.64	N.S.	YES	0.325	3.26	N.S.	0.397	-2.11	N.S.	YES
8	*ar*	0.489	1.07	N.S.	0.152	-2.29	N.S.	YES	0.097	-1.65	N.S.	0.006	-10.99	DOWN	NO
9	*comt*	0.047	1.50	UP	0.443	-1.40	N.S.	NO	0.104	1.63	N.S.	0.020	-3.76	N.S.	YES
10	*esr2*	0.759	-1.01	N.S.	0.026	-2.25	N.S.	YES	0.026	-1.56	DOWN	0.007	-4.17	DOWN	YES
11	*esr1*	0.665	1.04	N.S.	0.639	-1.52	N.S.	YES	0.130	1.59	N.S.	0.701	-1.63	DOWN	NO
12	*pr*	0.656	-1.27	N.S.	0.037	-3.13	N.S.	YES	0.767	1.09	N.S.	0.069	-3.55	N.S.	YES
13	*cyp11c1*	0.660	-1.07	N.S.	0.297	-4.21	N.S.	YES	0.676	-1.24	N.S.	0.694	-2.38	N.S.	YES
Wnt Signaling															
14	*ctnnb1*	0.648	-1.94	N.S.	0.000	-3.11	DOWN	NO	0.498	1.20	N.S.	0.001	-3.77	DOWN	NO
15	*wnt3*	0.009	14.79	UP	0.003	3.68	UP	YES	0.418	-1.21	N.S.	0.156	-2.07	N.S.	YES
16	*tnks*	0.165	1.48	N.S.	0.593	1.41	N.S.	YES	0.032	1.78	UP	0.635	1.52	N.S.	NO
17	*dvl2*	0.585	1.00	N.S.	0.019	-2.01	N.S.	YES	0.163	1.43	N.S.	0.493	-1.33	N.S.	YES
18	*fzd1*	0.426	8.79	N.S.	0.026	-6.85	N.S.	YES	0.166	3.62	N.S.	0.864	1.33	N.S.	YES
19	*tcf7*	0.000	1555.38	UP	0.000	1329.18	UP	YES	0.018	2.88	UP	0.879	1.53	N.S.	NO
20	*psen1*	0.152	1.55	N.S.	0.023	3.84	N.S.	YES	0.008	2.28	UP	0.187	2.66	N.S.	NO
21	*fzd8*	0.014	15.59	UP	0.083	1.78	N.S.	NO	0.467	2.61	N.S.	0.009	-3.56	DOWN	NO
22	*ck2a*	0.008	2.59	UP	0.425	-1.24	N.S.	NO	0.001	2.28	UP	0.118	-1.68	N.S.	NO
23	*wnt16*	0.001	4.36	UP	0.221	2.17	N.S.	NO	0.122	1.77	N.S.	0.831	1.29	N.S.	YES
Retinoic acid															
24	*cyp26a1*	0.001	10.88	UP	0.679	1.71	N.S.	NO	0.051	4.92	N.S.	0.918	1.28	N.S.	YES
25	*cyp26b1*	0.973	-1.77	N.S.	0.903	-1.17	N.S.	YES	0.153	1.48	N.S.	0.138	-5.96	N.S.	YES
26	*stra6*	0.037	58.27	UP	0.899	1.26	N.S.	NO	0.041	5.36	UP	0.446	4.21	N.S.	NO
27	*rdh3*	0.714	-1.07	N.S.	0.007	-3.88	DOWN	NO	0.610	1.19	N.S.	0.003	-9.10	DOWN	NO
NF-Kappa B															
28	*rtkn1*	0.066	1.86	N.S.	0.153	-1.83	N.S.	YES	0.667	1.12	N.S.	0.216	-1.97	N.S.	YES
29	*rtkn2*	0.002	15.87	UP	0.000	101.78	UP	YES	0.001	4.65	UP	0.621	1.84	N.S.	NO
30	*ikba*	0.440	1.13	N.S.	0.011	-3.21	N.S.	YES	0.316	1.26	N.S.	0.812	-1.21	N.S.	YES
31	*ikbe*	0.313	1.32	N.S.	0.061	-2.71	N.S.	YES	0.499	-1.21	N.S.	0.240	-2.25	N.S.	YES
Notch															
32	*jag1b*	0.963	-1.32	N.S.	0.115	-2.17	N.S.	YES	0.935	-1.03	N.S.	0.085	-2.98	N.S.	YES
33	*deltaC*	0.332	2.47	N.S.	0.000	-4.14	DOWN	NO	0.315	3.30	N.S.	0.293	-1.59	N.S.	YES
Hedgehog															
34	*smo*	0.648	-1.44	N.S.	0.021	-3.86	N.S.	YES	0.400	-1.20	N.S.	0.024	-5.75	N.S.	YES
35	*shh*	0.073	14.84	N.S.	0.007	2.95	UP	NO	0.291	2.68	N.S.	0.103	-2.19	N.S.	YES
36	*gli1*	0.219	3.32	N.S.	0.649	1.28	N.S.	YES	0.148	3.00	N.S.	0.010	5.72	N.S.	YES
TGF-b/ Smad Signaling															
37	*inhbb*	0.425	-2.13	N.S.	0.003	-13.86	DOWN	NO	0.605	1.92	N.S.	0.079	-6.31	N.S.	YES
38	*acvr1*	0.028	3.50	UP	0.065	-1.88	N.S.	NO	0.585	2.21	N.S.	0.008	-3.79	DOWN	NO
39	*acvr2b*	0.072	2.08	N.S.	0.530	-1.40	N.S.	YES	0.611	1.06	N.S.	0.011	-5.98	N.S.	YES
40	*btg1*	0.044	-2.01	DOWN	0.000	-4.49	DOWN	YES	0.028	2.55	UP	0.962	1.03	N.S.	NO
41	*nog*	0.677	-1.13	N.S.	0.003	-16.13	DOWN	NO	0.656	2.59	N.S.	0.118	-5.64	N.S.	YES
42	*smad4*	0.002	2.35	UP	0.264	-1.33	N.S.	NO	0.968	-1.02	N.S.	0.044	-1.96	N.S.	YES
43	*nodal*	0.034	9.87	UP	0.196	3.00	N.S.	NO	0.325	2.22	N.S.	0.023	-13.56	N.S.	YES
44	*bmp1*	0.003	38.92	UP	0.000	44.07	UP	YES	0.049	3.47	UP	0.714	1.43	N.S.	NO
45	*gdf9*	N.A.	N.A.	N.A.	0.365	2.21	N.S.	N.A.	0.695	1.94	N.S.	0.704	1.69	N.S.	YES
46	*bmp2*	0.002	12.99	UP	0.189	2.24	N.S.	NO	0.695	2.03	N.S.	0.004	-15.27	DOWN	NO
FGF Signaling															
47	*spry1*	0.855	-1.05	N.S.	0.000	-9.84	DOWN	NO	0.960	1.46	N.S.	0.168	-2.02	N.S.	YES
48	*fgfr1a*	0.009	4.49	UP	0.295	1.29	N.S.	NO	0.071	-1.90	N.S.	0.008	-2.55	DOWN	NO
Tumour necrosis factor (TNF)															
49	*tnfa*	0.081	6.30	N.S.	0.702	-1.36	N.S.	YES	0.518	4.13	N.S.	0.336	-2.43	N.S.	YES
50	*tnfrsf10b*	0.786	-1.31	N.S.	0.000	-6.37	DOWN	NO	0.540	1.36	N.S.	0.033	-2.91	N.S.	YES
51	*tnfrsf1a*	0.931	-1.12	N.S.	0.019	-3.68	N.S.	YES	0.318	-1.22	N.S.	0.143	-2.76	N.S.	YES
Jak-Stat signaling															
52	*junb*	0.642	1.06	N.S.	0.007	-4.65	DOWN	NO	0.083	1.70	N.S.	0.645	-1.47	N.S.	YES
53	*stat3*	0.764	-1.23	N.S.	0.103	-2.19	N.S.	YES	0.904	-1.00	N.S.	0.052	-3.37	N.S.	YES
Insulin signaling															
54	*igf1*	0.029	-4.69	DOWN	0.001	-9.10	DOWN	YES	0.205	1.94	N.S.	0.143	2.99	N.S.	YES
55	*rxfp3*	0.388	-1.92	N.S.	0.001	-29.12	DOWN	NO	0.784	2.42	N.S.	0.030	-14.57	N.S.	YES
56	*igf2*	0.167	-1.86	N.S.	0.001	-10.87	DOWN	NO	0.659	1.65	N.S.	0.151	-3.22	N.S.	YES
57	*insr*	0.889	1.12	N.S.	0.466	-1.41	N.S.	YES	0.374	1.38	N.S.	0.007	-5.77	DOWN	NO
Eph receptors and ephrins															
58	*epha4*	0.005	35.56	UP	0.875	-1.25	N.S.	NO	0.142	3.49	N.S.	0.749	1.78	N.S.	YES
59	*ephb4*	0.016	4.92	UP	0.204	-1.65	N.S.	NO	0.545	2.16	N.S.	0.013	-4.00	N.S.	YES
60	*ephb3*	0.036	2.54	UP	0.310	1.58	N.S.	NO	0.006	3.39	UP	0.027	3.71	N.S.	NO
Apoptosis															
61	*tp53*	0.411	1.17	N.S.	0.082	-2.09	N.S.	YES	0.482	1.22	N.S.	0.712	-1.30	N.S.	YES
62	*casp1*	0.044	2.81	UP	0.051	-2.77	N.S.	NO	0.845	2.01	N.S.	0.307	-2.02	N.S.	YES
63	*diablo*	0.000	3.80	UP	0.715	-1.16	N.S.	NO	0.534	-1.14	N.S.	0.821	-1.16	N.S.	YES
64	*bfar*	0.000	20.59	UP	0.000	132.13	UP	YES	0.001	3.18	UP	0.340	2.16	N.S.	NO
65	*rprma*	0.005	23.92	UP	0.111	3.10	N.S.	NO	0.488	2.65	N.S.	0.027	-8.20	N.S.	YES
Protein degradation															
66	*ctsz*	N.A.	N.A.	N.A.	0.618	1.42	N.S.	N.A.	0.019	44.86	UP	0.146	3.17	N.S.	NO
67	*ctsk*	0.467	-1.59	N.S.	0.032	-9.20	N.S.	YES	0.056	-2.72	N.S.	0.411	-3.16	N.S.	YES
68	*sh3rf1*	0.012	7.16	UP	0.851	-1.09	N.S.	NO	0.444	2.68	N.S.	0.010	-3.76	N.S.	YES
69	*cul1*	0.003	5.01	UP	0.020	2.00	N.S.	NO	0.003	3.41	UP	0.110	1.81	N.S.	NO
70	*peli1*	0.094	2.68	N.S.	0.066	3.53	N.S.	YES	0.011	3.36	UP	0.158	3.46	N.S.	NO
Female germ cell-related (germ cell markers)															
71	*nanos1*	0.373	-1.69	N.S.	0.000	-15.19	DOWN	NO	0.906	1.82	N.S.	0.007	-11.78	DOWN	NO
72	*zp3*	0.057	41.72	N.S.	0.294	2.73	N.S.	YES	0.076	4.06	N.S.	0.523	2.37	N.S.	YES
73	*piwil1*	0.045	7.83	UP	0.736	-1.74	N.S.	NO	0.046	1.54	UP	0.182	10.44	N.S.	NO
74	*rttn*	0.001	9.25	UP	0.013	4.52	N.S.	NO	0.040	1.51	UP	0.933	-1.11	N.S.	NO
Male germ cell-related															
75	*sept6*	0.004	5.17	UP	0.003	3.10	UP	YES	0.001	4.17	UP	0.089	2.13	N.S.	NO
76	*odf3*	0.000	5926.92	UP	0.000	3112.33	UP	YES	0.010	2.67	UP	0.963	1.24	N.S.	NO
77	*sycp1*	0.005	213.38	UP	0.001	37.71	UP	YES	0.006	4.24	UP	0.867	1.35	N.S.	NO
78	*tekt1*	0.000	266.68	UP	0.000	513.93	UP	YES	0.018	2.28	UP	0.860	-1.53	N.S.	NO
Neurons, neuropeptides															
79	*sema4e*	0.000	301.25	UP	0.000	272.06	UP	YES	0.009	4.44	UP	0.966	1.11	N.S.	NO
80	*tac1*	0.005	2.47	UP	0.533	-1.83	N.S.	NO	0.293	-1.29	N.S.	0.353	-3.02	N.S.	YES
81	*unc5a*	0.015	5.80	UP	0.069	4.27	N.S.	NO	0.257	2.63	N.S.	0.235	3.42	N.S.	YES
82	*npb*	0.000	9.80	UP	0.465	1.63	N.S.	NO	0.011	6.46	UP	0.071	4.25	N.S.	NO
Immune-related															
83	*il13ra2*	0.009	4.98	UP	0.481	2.02	N.S.	NO	0.003	-4.12	DOWN	0.085	-7.78	N.S.	NO
84	*c6*	0.556	1.08	N.S.	0.204	-3.44	N.S.	YES	0.021	-10.03	DOWN	0.006	-54.28	DOWN	YES
85	*c7*	0.188	2.86	N.S.	0.852	1.32	N.S.	YES	0.279	-2.12	N.S.	0.022	-36.31	N.S.	YES
86	*nfil3*	0.172	1.66	N.S.	0.185	-1.61	N.S.	YES	0.512	1.78	N.S.	0.165	-1.90	N.S.	YES
Others															
87	*nova1*	0.418	2.14	N.S.	0.002	-7.10	DOWN	NO	0.347	2.94	N.S.	0.006	-9.48	DOWN	NO
88	*gsdf1*	0.011	3.91	UP	0.512	1.96	N.S.	NO	0.001	-5.91	DOWN	0.006	-47.23	DOWN	YES

Table A 4. Real-time qPCR validation of microarray results. (continued)

Gene Info		Early Transforming vs Adult Testis							Late vs Early Transforming						
		real-time qPCR			Microarray			Validated?	real-time qPCR			Microarray			Validated?
S/N	*Gene Sym*	p-value	fold-change	Direction	p-value	fold-change	Direction		p-value	fold-change	Direction	p-value	fold-change	Direction	
Known sex genes															
1	*wt1a*	0.485	-2.55	N.S.	0.000	14.11	UP	NO	0.106	-1.81	N.S.	0.000	-14.35	DOWN	NO
2	*foxl2*	0.363	-3.01	N.S.	0.000	182.59	UP	NO	0.123	1.55	N.S.	0.936	-1.11	N.S.	YES
3	*dmrt1*	0.000	-6.56	DOWN	0.001	-6.28	DOWN	YES	0.000	-54.03	DOWN	0.007	-3.77	DOWN	YES
4	*amh*	0.000	-17.95	DOWN	0.021	10.96	N.S.	NO	0.434	-1.46	N.S.	0.008	-16.68	DOWN	NO
5	*sox9*	0.181	-2.05	N.S.	0.005	5.50	UP	NO	0.000	-8.47	DOWN	0.002	-7.36	DOWN	YES
Steroidogenic/ hormone-related															
6	*hsd17b1*	0.565	-1.73	N.S.	0.000	251.64	UP	NO	0.046	1.90	UP	0.928	-1.21	N.S.	NO
7	*fsta*	0.092	-19.70	N.S.	0.003	7.32	UP	NO	0.004	-6.78	DOWN	0.026	-4.10	N.S.	NO
8	*ar*	0.217	1.47	N.S.	0.000	13.18	UP	NO	0.000	-6.83	DOWN	0.001	-8.04	DOWN	YES
9	*comt*	0.040	-1.99	DOWN	0.000	8.31	UP	NO	0.062	-1.91	N.S.	0.000	-6.52	DOWN	NO
10	*esr2*	0.226	1.24	N.S.	0.000	4.80	UP	NO	0.000	-5.25	DOWN	0.000	-19.72	DOWN	YES
11	*esr1*	0.009	-2.50	DOWN	0.931	1.13	N.S.	NO	0.000	-7.32	DOWN	0.143	-3.00	N.S.	NO
12	*pr*	0.300	-1.35	N.S.	0.001	7.71	UP	NO	0.007	-4.22	DOWN	0.001	-7.65	DOWN	YES
13	*cyp11c1*	0.028	-4.71	DOWN	0.327	-3.85	N.S.	NO	0.000	-38.64	DOWN	0.148	-6.56	N.S.	NO
Wnt Signaling															
14	*ctnnb1*	0.258	-1.34	N.S.	0.000	7.14	UP	NO	0.006	2.31	UP	0.016	-1.73	N.S.	NO
15	*wnt3*	0.058	1.43	N.S.	0.001	4.82	UP	NO	0.002	7.86	UP	0.000	6.33	UP	YES
16	*tnks*	0.005	-2.49	DOWN	0.054	-2.89	N.S.	NO	0.000	-6.83	DOWN	0.006	-5.13	DOWN	YES
17	*dvl2*	0.060	-1.66	N.S.	0.003	2.43	UP	NO	0.069	1.32	N.S.	0.006	2.27	UP	NO
18	*fzd1*	0.008	-77.39	DOWN	0.923	-1.16	N.S.	NO	0.000	29.12	UP	0.000	83.05	UP	YES
19	*tcf7*	0.001	-44.70	DOWN	0.001	-125.62	DOWN	YES	0.094	-13.57	N.S.	0.245	-4.92	N.S.	YES
20	*psen1*	0.001	-3.61	DOWN	0.001	-7.84	DOWN	YES	0.013	-1.76	DOWN	0.066	2.77	N.S.	NO
21	*fzd8*	0.157	-10.00	N.S.	0.000	6.79	UP	NO	0.000	-4.21	DOWN	0.002	-2.96	DOWN	YES
22	*ck2a*	0.000	-3.95	DOWN	0.154	1.43	N.S.	NO	0.001	5.04	UP	0.000	11.26	UP	YES
23	*wnt16*	0.026	-2.57	DOWN	0.991	-1.02	N.S.	NO	0.002	29.13	UP	0.000	16.21	UP	YES
Retinoic acid															
24	*cyp26a1*	0.062	-5.04	N.S.	0.231	3.78	N.S.	YES	0.000	129.21	UP	0.001	70.55	UP	YES
25	*cyp26b1*	0.342	1.46	N.S.	0.003	19.40	UP	NO	0.010	-4.99	DOWN	0.026	-7.77	DOWN	YES
26	*stra6*	0.001	-75.31	DOWN	0.736	-1.79	N.S.	NO	0.000	379.29	UP	0.001	236.96	UP	YES
27	*rdh3*	0.923	-1.72	N.S.	0.000	22.39	UP	NO	0.001	-8.06	DOWN	0.000	-15.84	DOWN	YES
NF-Kappa B															
28	*rtkn1*	0.122	-1.47	N.S.	0.004	3.50	UP	NO	0.045	-1.85	DOWN	0.001	-5.47	DOWN	YES
29	*rtkn2*	0.000	-21.26	DOWN	0.000	-80.74	DOWN	YES	0.435	1.26	N.S.	0.001	17.26	UP	NO
30	*ikba*	0.855	-1.05	N.S.	0.001	4.67	UP	NO	0.030	3.10	UP	0.867	1.11	N.S.	NO
31	*ikbe*	0.002	1.82	UP	0.000	13.27	UP	YES	0.044	1.99	UP	0.177	-2.00	N.S.	NO
Notch															
32	*jag1b*	0.776	-1.14	N.S.	0.002	5.21	UP	NO	0.000	8.40	UP	0.009	3.70	UP	YES
33	*deltaC*	0.082	-11.98	N.S.	0.001	3.34	UP	NO	0.010	-2.80	DOWN	0.001	-3.72	DOWN	YES
Hedgehog															
34	*smo*	0.188	1.46	N.S.	0.000	16.17	UP	NO	0.001	-3.43	DOWN	0.011	-4.37	N.S.	NO
35	*shh*	0.085	-11.84	N.S.	0.000	4.77	UP	NO	0.006	-4.75	DOWN	0.000	-7.35	DOWN	YES
36	*gli1*	0.029	-9.72	DOWN	0.000	-13.53	DOWN	YES	0.069	-5.98	N.S.	0.969	-1.03	N.S.	YES
TGF-b/ Smad Signaling															
37	*inhbb*	0.298	-2.80	N.S.	0.000	48.75	UP	NO	0.774	-1.28	N.S.	0.043	-4.94	N.S.	YES
38	*acvr1*	0.186	-5.15	N.S.	0.000	7.63	UP	NO	0.035	-1.84	DOWN	0.004	-2.77	DOWN	YES
39	*acvr2b*	0.501	1.06	N.S.	0.000	11.55	UP	NO	0.882	1.04	N.S.	0.429	-1.49	N.S.	YES
40	*btg1*	0.306	-1.43	N.S.	0.000	4.05	UP	NO	0.001	1.95	UP	0.398	-1.24	N.S.	NO
41	*nog*	0.940	-1.48	N.S.	0.000	65.35	UP	NO	0.454	-2.28	N.S.	0.897	-1.18	N.S.	YES
42	*smad4*	0.629	1.05	N.S.	0.000	2.77	UP	NO	0.000	-2.15	DOWN	0.000	-2.94	DOWN	YES
43	*nodal*	0.058	-7.80	N.S.	0.004	12.48	UP	NO	0.005	-10.20	DOWN	0.030	-5.97	N.S.	NO
44	*bmp1*	0.000	-43.33	DOWN	0.000	-71.48	DOWN	YES	0.002	3.10	UP	0.003	6.61	UP	YES
45	*gdf9*	0.048	-10.26	DOWN	0.345	2.26	N.S.	NO	0.000	86.21	UP	0.000	34.83	UP	YES
46	*bmp2*	0.263	-5.06	N.S.	0.000	31.59	UP	NO	0.000	-10.19	DOWN	0.000	-14.73	DOWN	YES
FGF Signaling															
47	*spry1*	0.413	-2.27	N.S.	0.000	11.02	UP	NO	0.188	1.54	N.S.	0.273	-1.53	N.S.	YES
48	*fgfr1a*	0.111	1.54	N.S.	0.000	3.37	UP	NO	0.007	8.63	UP	0.000	4.98	UP	YES
Tumour necrosis factor (TNF)															
49	*tnfa*	0.641	-4.80	N.S.	0.001	11.25	UP	NO	0.080	-3.96	N.S.	0.231	-2.24	N.S.	YES
50	*tnfrsf10b*	0.294	-1.54	N.S.	0.000	14.24	UP	NO	0.006	6.83	UP	0.001	3.75	UP	YES
51	*tnfrsf1a*	0.323	1.33	N.S.	0.001	6.98	UP	NO	0.010	-4.68	DOWN	0.001	-6.90	DOWN	YES
Jak-Stat signaling															
52	*junb*	0.756	1.01	N.S.	0.000	12.05	UP	NO	0.032	2.05	UP	0.114	-2.24	N.S.	NO
53	*stat3*	0.102	-1.36	N.S.	0.003	4.61	UP	NO	0.001	-3.00	DOWN	0.005	-4.12	DOWN	YES
Insulin signaling															
54	*igf1*	0.001	-16.09	DOWN	0.001	-9.03	DOWN	YES	0.037	-2.23	DOWN	0.165	-2.18	N.S.	NO
55	*rxfp3*	0.249	-5.82	N.S.	0.000	53.46	UP	NO	0.563	-1.96	N.S.	0.553	-1.81	N.S.	YES
56	*igf2*	0.372	-2.14	N.S.	0.001	12.29	UP	NO	0.136	-2.57	DOWN	0.096	-2.75	N.S.	NO
57	*insr*	0.049	-2.47	DOWN	0.009	3.18	UP	NO	0.000	-7.69	DOWN	0.000	-9.03	DOWN	YES
Eph receptors and ephrins															
58	*epha4*	0.019	-21.06	DOWN	0.314	-2.94	N.S.	NO	0.000	-5.96	DOWN	0.000	85.93	UP	NO
59	*ephb4*	0.144	-5.34	N.S.	0.000	7.00	UP	NO	0.012	-1.90	DOWN	0.002	-3.83	DOWN	YES
60	*ephb3*	0.002	-4.22	DOWN	0.000	-10.54	DOWN	YES	0.015	-3.98	DOWN	0.016	2.89	N.S.	NO
Apoptosis															
61	*tp53*	0.101	-1.60	N.S.	0.002	4.01	UP	NO	0.000	3.58	UP	0.001	5.22	UP	YES
62	*casp1*	0.547	-3.07	N.S.	0.001	7.37	UP	NO	0.080	-4.53	N.S.	0.003	-5.01	DOWN	NO
63	*diablo*	0.685	1.05	N.S.	0.003	3.09	UP	NO	0.282	1.50	N.S.	0.009	-2.55	DOWN	NO
64	*bfar*	0.000	-10.59	DOWN	0.000	-93.37	DOWN	YES	0.184	-1.54	N.S.	0.896	1.12	N.S.	YES
65	*rprma*	0.125	-10.87	N.S.	0.004	8.46	UP	NO	0.000	-13.04	DOWN	0.004	-8.69	DOWN	NO
Protein degradation															
66	*ctsz*	0.017	-25.45	DOWN	0.262	-2.00	N.S.	NO	0.000	865.35	DOWN	0.000	52.83	UP	NO
67	*ctsk*	0.043	2.15	UP	0.008	15.72	UP	YES	0.232	-2.35	N.S.	0.073	-5.77	N.S.	YES
68	*sh3rf1*	0.100	-9.51	N.S.	0.000	5.99	UP	NO	0.016	4.27	UP	0.000	4.59	UP	YES
69	*cul1*	0.000	-15.51	DOWN	0.000	-6.85	DOWN	YES	0.000	1.75	UP	0.010	2.12	N.S.	NO
70	*peli1*	0.000	-10.98	DOWN	0.002	-9.05	DOWN	YES	0.011	-5.09	DOWN	0.005	-7.18	DOWN	YES
Female germ cell-related (germ cell markers)															
71	*nanos1*	0.426	-2.85	N.S.	0.000	54.76	UP	NO	0.000	-5.10	DOWN	0.001	-9.72	DOWN	YES
72	*zp3*	0.001	-69.45	DOWN	0.899	-1.22	N.S.	NO	0.000	450.56	UP	0.000	134.11	UP	YES
73	*piwil1*	0.003	-2.38	DOWN	0.942	-1.21	N.S.	NO	0.716	1.11	N.S.	0.001	156.58	UP	NO
74	*rttn*	0.001	-2.96	DOWN	0.505	-1.53	N.S.	NO	0.000	20.48	UP	0.000	12.73	UP	YES
Male germ cell-related															
75	*sept6*	0.000	-17.59	DOWN	0.000	-5.94	DOWN	YES	0.742	1.01	N.S.	0.033	-2.05	N.S.	YES
76	*odf3*	0.004	-44.32	DOWN	0.007	-202.08	DOWN	YES	0.007	-543.67	DOWN	0.067	-29.14	N.S.	NO
77	*sycp1*	0.000	-40.36	DOWN	0.007	-11.97	DOWN	YES	0.001	-18.91	DOWN	0.018	-8.19	N.S.	NO
78	*tekt1*	0.000	-44.26	DOWN	0.002	-56.70	DOWN	YES	0.000	-61.85	DOWN	0.002	-63.23	DOWN	YES
Neurons, neuropeptides															
79	*sema4e*	0.000	-160.94	DOWN	0.000	-124.02	DOWN	YES	0.013	-6.35	DOWN	0.106	-4.58	N.S.	NO
80	*tac1*	0.015	-2.10	DOWN	0.475	1.97	N.S.	NO	0.000	-9.32	DOWN	0.001	-24.54	DOWN	YES
81	*unc5a*	0.043	-8.87	DOWN	0.000	-40.82	DOWN	YES	0.664	1.49	N.S.	0.803	-1.30	N.S.	YES
82	*npb*	0.002	-13.59	DOWN	0.003	-6.98	DOWN	YES	0.009	6.25	UP	0.001	9.67	UP	YES
Immune-related															
83	*il13ra2*	0.011	2.62	UP	0.002	20.97	UP	YES	0.013	-5.50	DOWN	0.004	-16.39	DOWN	YES
84	*c6*	0.000	23.64	UP	0.000	679.95	UP	YES	0.011	-17.31	DOWN	0.001	-48.21	DOWN	YES
85	*c7*	0.069	2.96	N.S.	0.000	209.14	UP	NO	0.000	-17.04	DOWN	0.002	-42.14	DOWN	YES
86	*nfil3*	0.092	-3.87	N.S.	0.000	4.64	UP	NO	0.003	-2.70	DOWN	0.001	-3.67	DOWN	YES
Others															
87	*nova1*	0.136	-8.17	N.S.	0.000	33.48	UP	NO	0.000	-6.73	DOWN	0.001	-8.80	DOWN	YES
88	*gsdf1*	0.008	3.20	UP	0.002	21.83	UP	YES	0.003	-8.76	DOWN	0.029	-7.53	DOWN	YES

Table A 4. Real-time qPCR validation of microarray results. (continued)

Gene Info		Ovary vs Late Transforming							Ovary vs Adult Testis						
		real-time qPCR			Microarray			Validated?	real-time qPCR			Microarray			Validated?
S/N	*Gene Sym*	p-value	fold-change	Direction	p-value	fold-change	Direction		p-value	fold-change	Direction	p-value	fold-change	Direction	
Known sex genes															
1	*wt1a*	0.020	-1.81	DOWN	0.033	-3.52	N.S.	NO	0.067	-8.34	N.S.	0.010	-3.58	N.S.	YES
2	*foxl2*	0.676	1.07	N.S.	0.580	-1.68	N.S.	YES	0.908	-1.82	N.S.	0.000	98.29	UP	NO
3	*dmrt1*	0.077	-1.85	N.S.	0.421	-1.55	N.S.	YES	0.000	-655.69	DOWN	0.000	-36.73	DOWN	YES
4	*amh*	0.314	2.52	N.S.	0.361	-2.89	N.S.	YES	0.002	-10.44	DOWN	0.098	-4.39	N.S.	NO
5	*sox9*	0.189	-1.47	N.S.	0.040	-3.60	N.S.	YES	0.000	-25.42	DOWN	0.004	-4.82	DOWN	YES
Steroidogenic/ hormone-related															
6	*hsd17b1*	0.123	-1.22	N.S.	0.904	-1.32	N.S.	YES	0.470	-1.11	N.S.	0.000	157.39	UP	NO
7	*fsta*	0.012	-3.06	DOWN	0.817	1.24	N.S.	NO	0.007	-409.03	DOWN	0.150	2.22	N.S.	NO
8	*ar*	0.232	1.47	N.S.	0.711	-1.32	N.S.	YES	0.009	-3.18	DOWN	0.697	1.24	N.S.	NO
9	*comt*	0.012	-3.78	DOWN	0.001	-5.82	DOWN	YES	0.000	-14.35	DOWN	0.000	-4.56	DOWN	YES
10	*esr2*	0.688	1.06	N.S.	0.004	-3.25	DOWN	NO	0.000	-4.02	DOWN	0.000	-13.33	DOWN	YES
11	*esr1*	0.244	1.79	N.S.	0.221	-2.77	N.S.	YES	0.001	-10.22	DOWN	0.005	-7.33	DOWN	YES
12	*pr*	0.111	-1.90	N.S.	0.009	-4.68	DOWN	NO	0.000	-10.80	DOWN	0.002	-4.65	DOWN	YES
13	*cyp11c1*	0.382	-1.51	N.S.	0.890	-1.35	N.S.	YES	0.001	-275.12	DOWN	0.004	-34.04	DOWN	YES
Wnt Signaling															
14	*ctnnb1*	0.038	3.15	UP	0.038	1.65	N.S.	NO	0.007	5.45	UP	0.000	6.82	UP	YES
15	*wnt3*	0.183	1.37	N.S.	0.940	-1.06	N.S.	YES	0.000	15.36	UP	0.000	28.95	UP	YES
16	*tnks*	0.024	4.28	UP	0.179	2.25	N.S.	NO	0.010	-3.97	DOWN	0.001	-6.60	DOWN	YES
17	*dvl2*	0.015	5.13	UP	0.989	1.01	N.S.	NO	0.017	4.07	UP	0.000	5.56	UP	YES
18	*fzd1*	0.006	-2.22	DOWN	0.227	-3.00	N.S.	NO	0.306	-5.89	N.S.	0.000	23.94	UP	NO
19	*tcf7*	0.006	-1.49	N.S.	0.860	-1.46	N.S.	YES	0.000	-901.26	DOWN	0.000	-902.42	DOWN	YES
20	*psen1*	0.008	6.14	UP	0.968	-1.05	N.S.	NO	0.681	-1.03	N.S.	0.030	-2.96	N.S.	YES
21	*fzd8*	0.000	-4.31	DOWN	0.001	-3.80	DOWN	YES	0.010	-181.63	DOWN	0.078	-1.66	N.S.	NO
22	*ck2a*	0.008	2.01	UP	0.008	-2.12	DOWN	NO	0.001	2.56	UP	0.000	7.60	UP	YES
23	*wnt16*	0.138	1.63	N.S.	0.702	-1.36	N.S.	YES	0.000	18.50	UP	0.000	11.66	UP	YES
Retinoic acid															
24	*cyp26a1*	0.117	-1.37	N.S.	0.387	-2.95	N.S.	YES	0.006	18.77	UP	0.000	90.54	UP	YES
25	*cyp26b1*	0.079	-4.68	N.S.	0.092	-5.21	N.S.	YES	0.030	-15.99	DOWN	0.370	-2.09	N.S.	NO
26	*stra6*	0.172	1.57	N.S.	0.924	1.25	N.S.	YES	0.016	7.92	UP	0.000	165.75	UP	YES
27	*rdh3*	0.734	1.36	N.S.	0.288	-1.72	N.S.	YES	0.040	-10.21	DOWN	0.656	-1.22	N.S.	NO
NF-Kappa B															
28	*rtkn1*	0.003	7.90	UP	0.000	8.61	UP	YES	0.035	2.91	UP	0.000	5.51	UP	YES
29	*rtkn2*	0.011	4.67	N.S.	0.469	-1.93	N.S.	YES	0.009	-3.62	DOWN	0.003	-9.05	DOWN	YES
30	*ikba*	0.749	-1.16	N.S.	0.011	-3.38	DOWN	NO	0.005	2.55	UP	0.258	1.54	N.S.	NO
31	*ikbe*	0.042	2.14	UP	0.870	1.14	N.S.	NO	0.000	7.80	UP	0.000	7.58	UP	YES
Notch															
32	*jag1b*	0.097	2.96	N.S.	0.223	1.90	N.S.	YES	0.001	21.87	UP	0.000	36.60	UP	YES
33	*deltaC*	0.425	-1.23	N.S.	0.006	-2.82	DOWN	NO	0.015	-41.13	DOWN	0.001	-3.13	DOWN	YES
Hedgehog															
34	*smo*	0.024	-2.41	DOWN	0.007	-5.54	DOWN	YES	0.002	-5.66	DOWN	0.424	-1.50	N.S.	NO
35	*shh*	0.007	-10.00	DOWN	0.982	1.02	N.S.	NO	0.004	-563.19	DOWN	0.195	-1.52	N.S.	NO
36	*gli1*	0.544	1.44	N.S.	0.696	1.27	N.S.	YES	0.002	-40.44	DOWN	0.000	-11.00	DOWN	YES
TGF-b/ Smad Signaling															
37	*inhbb*	0.171	-1.39	N.S.	0.125	-3.71	N.S.	YES	0.110	-5.00	N.S.	0.161	2.66	UP	NO
38	*acvr1*	0.000	6.56	UP	0.007	2.83	UP	YES	0.749	-1.45	N.S.	0.000	7.78	UP	NO
39	*acvr2b*	0.020	5.50	UP	0.014	3.64	N.S.	NO	0.020	6.08	UP	0.000	28.30	UP	YES
40	*btg1*	0.915	1.08	N.S.	0.003	-2.45	DOWN	NO	0.224	1.47	N.S.	0.191	1.33	N.S.	YES
41	*nog*	0.004	-1.84	DOWN	0.269	-2.81	N.S.	NO	0.288	-6.24	N.S.	0.001	19.75	UP	NO
42	*smad4*	0.000	4.37	UP	0.002	2.55	UP	YES	0.003	2.14	UP	0.001	2.40	UP	YES
43	*nodal*	0.508	-1.26	N.S.	0.597	-1.71	N.S.	YES	0.003	-100.36	DOWN	0.816	1.22	N.S.	NO
44	*bmp1*	0.029	2.14	UP	0.801	1.24	N.S.	NO	0.006	-6.53	DOWN	0.000	-8.75	DOWN	YES
45	*gdf9*	0.289	1.50	N.S.	0.851	1.27	N.S.	YES	0.016	12.63	UP	0.000	99.72	UP	YES
46	*bmp2*	0.016	-2.65	DOWN	0.500	-1.61	N.S.	NO	0.008	-136.59	DOWN	0.613	1.33	N.S.	NO
FGF Signaling															
47	*spry1*	0.008	5.64	UP	0.030	2.48	N.S.	NO	0.033	3.82	UP	0.000	17.81	UP	YES
48	*fgfr1a*	0.066	2.11	N.S.	0.678	-1.13	N.S.	YES	0.000	28.07	UP	0.000	14.79	UP	YES
Tumour necrosis factor (TNF)															
49	*tnfa*	0.051	-8.39	N.S.	0.003	-11.07	DOWN	NO	0.022	-159.74	DOWN	0.180	-2.21	N.S.	NO
50	*tnfrsf10b*	0.040	3.32	UP	0.019	2.57	N.S.	NO	0.002	14.67	UP	0.000	136.95	UP	YES
51	*tnfrsf1a*	0.108	-1.98	N.S.	0.023	-3.69	N.S.	YES	0.000	-6.97	DOWN	0.008	-3.64	DOWN	YES
Jak-Stat signaling															
52	*junb*	0.056	1.87	N.S.	0.494	1.52	N.S.	YES	0.002	3.89	UP	0.000	8.18	UP	YES
53	*stat3*	0.012	2.18	UP	0.788	1.20	N.S.	NO	0.016	-1.87	DOWN	0.497	1.34	N.S.	NO
Insulin signaling															
54	*igf1*	0.005	3.28	UP	0.248	2.06	N.S.	NO	0.004	-10.92	DOWN	0.000	-9.58	DOWN	YES
55	*rxfp3*	0.318	1.34	N.S.	0.624	-1.74	N.S.	YES	0.248	-8.48	N.S.	0.001	16.96	UP	NO
56	*igf2*	0.086	-4.00	N.S.	0.002	-10.48	DOWN	NO	0.003	-21.96	DOWN	0.115	-2.35	N.S.	NO
57	*insr*	0.006	6.18	UP	0.315	1.62	N.S.	NO	0.024	-3.08	DOWN	0.132	-1.75	N.S.	NO
Eph receptors and ephrins															
58	*epha4*	0.635	1.50	N.S.	0.479	-2.36	N.S.	YES	0.002	-83.93	DOWN	0.008	12.35	UP	NO
59	*ephb4*	0.002	4.99	UP	0.008	3.14	UP	YES	0.743	-2.03	N.S.	0.000	5.74	UP	NO
60	*ephb3*	0.068	-4.86	N.S.	0.779	1.19	N.S.	YES	0.007	-81.78	DOWN	0.005	-3.08	DOWN	YES
Apoptosis															
61	*tp53*	0.032	1.54	UP	0.432	1.46	N.S.	NO	0.003	3.45	UP	0.000	30.54	UP	YES
62	*casp1*	0.248	-2.08	N.S.	0.120	-2.34	N.S.	YES	0.028	-28.93	DOWN	0.308	-1.59	N.S.	NO
63	*diablo*	0.142	1.66	N.S.	0.280	1.51	N.S.	YES	0.000	2.61	UP	0.046	1.83	UP	YES
64	*bfar*	0.167	1.58	N.S.	0.761	-1.29	N.S.	YES	0.000	-10.26	DOWN	0.000	-106.81	DOWN	YES
65	*rprma*	0.004	-2.11	DOWN	0.847	1.23	N.S.	NO	0.007	-299.30	DOWN	0.801	1.20	N.S.	NO
Protein degradation															
66	*ctsz*	0.062	2.75	N.S.	0.426	1.75	N.S.	YES	0.005	93.49	UP	0.000	46.01	UP	YES
67	*ctsk*	0.403	-2.68	N.S.	0.319	-3.03	N.S.	YES	0.017	-2.93	DOWN	0.922	-1.11	N.S.	NO
68	*sh3rf1*	0.053	2.02	N.S.	0.284	-1.52	N.S.	YES	0.441	-1.10	N.S.	0.000	18.13	UP	NO
69	*cul1*	0.115	2.03	N.S.	0.990	1.01	N.S.	YES	0.006	-4.35	DOWN	0.000	-3.21	DOWN	YES
70	*peli1*	0.840	-1.02	N.S.	0.616	1.52	N.S.	YES	0.001	-57.22	DOWN	0.000	-42.65	DOWN	YES
Female germ cell-related (germ cell markers)															
71	*nanos1*	0.006	-4.05	DOWN	0.013	-5.22	N.S.	NO	0.010	-58.94	DOWN	0.906	1.08	N.S.	NO
72	*zp3*	0.460	1.24	N.S.	0.767	-1.47	N.S.	YES	0.017	8.04	UP	0.000	75.20	UP	YES
73	*piwil1*	0.055	-2.30	N.S.	0.840	1.51	N.S.	YES	0.000	-4.91	DOWN	0.000	195.62	UP	NO
74	*rttn*	0.011	-2.26	DOWN	0.030	-3.83	N.S.	NO	0.001	3.07	UP	0.119	2.18	N.S.	NO
Male germ cell-related															
75	*sept6*	0.049	2.18	UP	0.883	1.08	N.S.	NO	0.001	-7.98	DOWN	0.000	-11.24	DOWN	YES
76	*odf3*	0.074	-1.79	N.S.	0.917	-1.40	N.S.	YES	0.000	-43144.62	DOWN	0.000	-8261.54	DOWN	YES
77	*sycp1*	0.006	-8.54	DOWN	0.817	-1.35	N.S.	NO	0.000	-6518.73	DOWN	0.000	-132.22	DOWN	YES
78	*tekt1*	0.013	-3.45	DOWN	0.337	-3.65	N.S.	NO	0.000	-9453.02	DOWN	0.000	-13103.50	DOWN	YES
Neurons, neuropeptides															
79	*sema4e*	0.737	1.19	N.S.	0.616	-1.82	N.S.	YES	0.000	-859.66	DOWN	0.000	-1036.05	DOWN	YES
80	*tac1*	0.002	-3.76	DOWN	0.165	-3.66	N.S.	NO	0.000	-73.56	DOWN	0.000	-45.55	DOWN	YES
81	*unc5a*	0.408	1.36	N.S.	0.177	3.13	N.S.	YES	0.129	-4.36	N.S.	0.000	-16.95	DOWN	NO
82	*npb*	0.416	-1.59	N.S.	0.064	-3.32	N.S.	YES	0.073	-3.45	N.S.	0.098	-2.40	N.S.	YES
Immune-related															
83	*il13ra2*	0.011	-6.43	DOWN	0.452	-2.20	N.S.	NO	0.000	-13.49	DOWN	0.516	-1.72	N.S.	NO
84	*c6*	0.007	-24.09	DOWN	0.005	-21.77	DOWN	YES	0.003	-17.64	DOWN	0.632	-1.54	N.S.	NO
85	*c7*	0.001	-11.64	DOWN	0.009	-26.88	DOWN	YES	0.004	-67.02	DOWN	0.085	-5.42	N.S.	NO
86	*nfil3*	0.000	7.89	UP	0.001	4.75	DOWN	NO	0.992	-1.32	N.S.	0.000	6.01	UP	NO
Others															
87	*nova1*	0.027	-2.27	N.S.	0.003	-6.75	DOWN	NO	0.006	-124.79	DOWN	0.217	-1.78	N.S.	NO
88	*gsdf1*	0.002	-8.90	DOWN	0.003	-26.66	DOWN	YES	0.000	-24.34	DOWN	0.009	-9.20	DOWN	YES

Table A 4. Real-time qPCR validation of microarray results. (continued)

Gene Info		E. Transforming vs Undifferentiated						
		real-time qPCR			Microarray			‡Validated?
S/N	*Gene Sym*	p-value	fold-change	*Direction	p-value	fold-change	†Direction	
Known sex genes								
1	*wt1a*	0.006	-4.94	DOWN	0.569	-1.52	N.S.	NO
2	*foxl2*	0.001	15.15	UP	0.000	91.75	UP	YES
3	*dmrt1*	0.669	1.01	N.S.	0.043	-3.14	N.S.	YES
4	*amh*	0.023	8.02	UP	0.144	-5.81	N.S.	NO
5	*sox9*	0.734	-1.68	N.S.	0.040	-4.17	N.S.	YES
Steroidogenic/ hormone-related								
6	*hsd17b1*	0.000	57.15	UP	0.003	263.83	UP	YES
7	*fsta*	0.007	2.66	UP	0.798	1.31	N.S.	NO
8	*ar*	0.687	-1.05	N.S.	0.352	-1.91	N.S.	YES
9	*comt*	0.632	1.23	N.S.	0.360	1.58	N.S.	YES
10	*esr2*	0.571	-1.27	N.S.	0.101	-1.95	N.S.	YES
11	*esr1*	0.540	-1.51	N.S.	0.413	-2.19	N.S.	YES
12	*pr*	0.288	-1.56	N.S.	0.622	-1.44	N.S.	YES
13	*cyp11c1*	0.001	-6.23	DOWN	0.024	-38.54	N.S.	NO
Wnt Signaling								
14	*ctnnb1*	0.505	-2.17	N.S.	0.059	-1.64	N.S.	YES
15	*wnt3*	0.013	17.42	UP	0.001	8.59	UP	YES
16	*tnks*	0.495	1.06	N.S.	0.722	-1.35	N.S.	YES
17	*dvl2*	0.939	-1.16	N.S.	0.865	-1.10	N.S.	YES
18	*fzd1*	0.028	-2.43	DOWN	0.067	-5.95	N.S.	NO
19	*tcf7*	0.003	100.08	UP	0.082	16.22	N.S.	NO
20	*psen1*	0.577	-1.02	N.S.	0.772	1.30	N.S.	YES
21	*fzd8*	0.006	4.07	UP	0.005	3.40	UP	YES
22	*ck2a*	0.092	1.50	N.S.	0.206	-1.46	N.S.	YES
23	*wnt16*	0.003	3.00	UP	0.161	2.74	N.S.	NO
Retinoic acid								
24	*cyp26a1*	0.004	10.63	UP	0.099	8.29	N.S.	NO
25	*cyp26b1*	0.353	1.22	N.S.	0.364	2.79	N.S.	YES
26	*stra6*	0.016	4.15	UP	0.536	2.97	N.S.	NO
27	*rdh3*	0.075	-1.54	DOWN	0.436	-1.57	N.S.	NO
NF-Kappa B								
28	*rtkn1*	0.175	1.42	N.S.	0.986	-1.03	N.S.	YES
29	*rtkn2*	0.038	3.47	UP	0.388	2.31	N.S.	NO
30	*ikba*	0.205	1.35	N.S.	0.800	1.20	N.S.	YES
31	*ikbe*	0.017	2.00	UP	0.205	2.18	N.S.	NO
Notch								
32	*jag1b*	0.780	-1.54	N.S.	0.782	-1.25	N.S.	YES
33	*deltaC*	0.778	-1.47	N.S.	0.078	-1.98	N.S.	YES
Hedgehog								
34	*smo*	0.990	-1.18	N.S.	0.707	-1.37	N.S.	YES
35	*shh*	0.055	3.36	N.S.	0.001	6.43	UP	NO
36	*gli1*	0.783	1.03	N.S.	0.268	-1.85	N.S.	YES
TGF-b/ Smad Signaling								
37	*inhbb*	0.176	-3.11	N.S.	0.596	-1.79	N.S.	YES
38	*acvr1*	0.137	1.50	N.S.	0.936	1.07	N.S.	YES
39	*acvr2b*	0.049	2.34	UP	0.629	1.38	N.S.	NO
40	*btg1*	0.835	-1.13	N.S.	0.881	-1.08	N.S.	YES
41	*nog*	0.701	1.55	N.S.	0.822	-1.39	N.S.	YES
42	*smad4*	0.002	2.42	UP	0.918	1.06	N.S.	NO
43	*nodal*	0.139	2.81	N.S.	0.312	2.76	N.S.	YES
44	*bmp1*	0.020	3.11	UP	0.927	-1.13	N.S.	NO
45	*gdf9*	N.A.	N.A.	N.A.	0.032	8.44	N.S.	
46	*bmp2*	0.003	5.21	UP	0.033	4.63	N.S.	NO
FGF Signaling								
47	*spry1*	0.265	-1.63	N.S.	0.199	-1.80	N.S.	YES
48	*fgfr1a*	0.016	3.64	UP	0.052	1.70	N.S.	NO
Tumour necrosis factor (TNF)								
49	*tnfa*	0.000	5.42	UP	0.123	3.39	N.S.	NO
50	*tnfrsf10b*	0.470	-1.48	N.S.	0.609	-1.30	N.S.	YES
51	*tnfrsf1a*	0.981	-1.03	N.S.	0.609	-1.46	N.S.	YES
Jak-Stat signaling								
52	*junb*	0.174	1.82	N.S.	0.378	1.77	N.S.	YES
53	*stat3*	0.178	-1.68	N.S.	0.430	-1.60	N.S.	YES
Insulin signaling								
54	*igf1*	0.000	-38.91	DOWN	0.000	-27.51	DOWN	YES
55	*rxfp3*	0.040	-4.63	DOWN	0.052	-7.94	N.S.	NO
56	*igf2*	0.055	-2.41	N.S.	0.151	-2.84	N.S.	YES
57	*insr*	0.368	-1.61	N.S.	0.062	-2.55	N.S.	YES
Eph receptors and ephrins								
58	*epha4*	0.006	5.89	UP	0.619	-2.05	N.S.	NO
59	*ephb4*	0.086	1.99	N.S.	0.963	1.06	N.S.	YES
60	*ephb3*	0.067	2.04	N.S.	0.257	-1.80	N.S.	YES
Apoptosis								
61	*tp53*	0.764	-1.12	N.S.	0.456	1.48	N.S.	YES
62	*casp1*	0.040	1.84	UP	0.723	1.32	N.S.	NO
63	*diablo*	0.000	3.47	UP	0.044	2.29	N.S.	NO
64	*bfar*	0.004	6.19	UP	0.106	3.06	N.S.	NO
65	*rprma*	0.000	5.82	UP	0.154	3.20	N.S.	NO
Protein degradation								
66	*ctsz*	N.A.	N.A.	N.A.	0.266	2.25	N.S.	N.A.
67	*ctsk*	0.248	-2.01	DOWN	0.673	-1.85	N.S.	NO
68	*sh3rf1*	0.043	2.01	UP	0.388	1.46	N.S.	NO
69	*cul1*	0.440	1.10	N.S.	0.056	-1.89	N.S.	YES
70	*peli1*	0.782	-1.22	N.S.	0.786	1.35	N.S.	YES
Female germ cell-related (germ cell markers)								
71	*nanos1*	0.074	-2.66	N.S.	0.091	-3.27	N.S.	YES
72	*zp3*	0.160	2.44	N.S.	0.119	5.31	N.S.	YES
73	*piwil1*	0.070	5.07	N.S.	0.325	4.98	N.S.	YES
74	*rttn*	0.003	4.72	UP	0.144	2.66	N.S.	NO
Male germ cell-related								
75	*sept6*	0.365	1.23	N.S.	0.868	1.11	N.S.	YES
76	*odf3*	0.009	357.69	UP	0.180	19.14	N.S.	NO
77	*sycp1*	0.021	22.41	UP	0.159	4.24	N.S.	NO
78	*tekt1*	0.006	13.73	UP	0.211	5.92	N.S.	NO
Neurons, neuropeptides								
79	*sema4e*	0.007	8.31	UP	0.472	2.43	N.S.	YES
80	*tac1*	0.787	-1.10	N.S.	0.330	-2.80	N.S.	NO
81	*unc5a*	0.090	1.72	N.S.	0.274	-2.80	N.S.	NO
82	*npb*	0.009	4.66	UP	0.996	-1.01	N.S.	NO
Immune-related								
83	*il13ra2*	0.024	3.17	UP	0.109	5.44	N.S.	NO
84	*c6*	0.064	2.54	N.S.	0.254	3.64	N.S.	YES
85	*c7*	0.001	3.99	UP	0.123	7.61	N.S.	NO
86	*nfil3*	0.602	-1.31	N.S.	0.329	1.52	N.S.	YES
Others								
87	*nova1*	0.861	-1.30	N.S.	0.273	-2.01	N.S.	YES
88	*gsdf1*	0.066	2.12	N.S.	0.975	-1.10	N.S.	YES

*Significant change when p-value < 0.05 and fold-change ≥ 1.5 or ≤ -1.5).
†Significant change when p-value FDR < 0.01 and fold-change ≥ 1.5 or ≤ -1.5).
‡Microarray results were validated when real-time qPCR results showed the same direction of significant change.
N.S. - Not significant; N.A. - Data not available.

Table A 5. Gene expression patterns of 57 genes between juvenile ovotestis (JOT) and juvenile ovary (JO) as tested by microarray and real-time RT-PCR.

S/N	Gene Symbol	Accession no.	*Microarray		**Real-Time RT-PCR			Validation
			Fold Change (JOT/JO)	Result	Fold Change (JOT/JO)	p-value	Result	
1	*CO352993*	CO352993	39.6	Up in JOT	188.0	0.005	Up in JOT	Yes
2	*cyp11c1*	NM_001080204.1	12.1	Up in JOT	69.9	0.006	Up in JOT	Yes
3	*lgals3bpb*	NM_212873.1	9.6	Up in JOT	6.5	0.038	Up in JOT	Yes
4	*chemokine CCL-C11a*	AB331767.1	8.4	Up in JOT	28.4	0.000	Up in JOT	Yes
5	*col1a1b*	NM_201478.1	8.2	Up in JOT	33.8	0.000	Up in JOT	Yes
6	*zgc:195154*	CO351481	8.0	Up in JOT	9.5	0.001	Up in JOT	Yes
7	*rbp2a*	NM_153004.1	7.8	Up in JOT	8.2	0.004	Up in JOT	Yes
8	*zgc:194626*	NM_001128777.1	6.6	Up in JOT	41.8	0.000	Up in JOT	Yes
9	*zgc:194314*	CO352680	6.2	Up in JOT	12.1	0.000	Up in JOT	Yes
10	*anxa1a*	NM_181758.1	6.1	Up in JOT	12.6	0.000	Up in JOT	Yes
11	*esr2b*	NM_174862.3	4.5	Up in JOT	9.6	0.000	Up in JOT	Yes
12	*rbp4*	NM_130920.1	3.9	Up in JOT	14.4	0.004	Up in JOT	Yes
13	*star*	NM_131663.1	3.6	Up in JOT	36.7	0.044	Up in JOT	Yes
14	*pycard*	NM_131495.2	2.9	Up in JOT	16.1	0.003	Up in JOT	Yes
15	*hsd11b2*	NM_212720.1	2.9	Up in JOT	4.4	0.015	Up in JOT	Yes
16	*dmrt1*	NM_205628.1	2.6	Up in JOT	2.9	0.016	Up in JOT	Yes
17	*csnk1g1*	NM_001008635	2.5	Up in JOT	-1.6	0.006	Down in JOT	No
18	*dkk3*	NM_001089545.1	2.4	Up in JOT	5.1	0.000	Up in JOT	Yes
19	*sycp3*	NM_001040350.1	2.0	Up in JOT	2.4	0.011	Up in JOT	Yes
20	*ck2a1*	NM_131252.1	1.9	Up in JOT	-1.5	0.049	Down in JOT	No
21	*ts1*	EF554575.2	1.9	Up in JOT	3.7	0.009	Up in JOT	Yes
22	*dnmt3*	NM_131386.1	1.7	Up in JOT	-4.4	0.000	Down in JOT	No
23	*ctnnb1*	NM_131059.2	1.7	Up in JOT	1.3	0.099	N.S.	No
24	*rxrgb*	NM_001002345.1	1.6	Up in JOT	-1.2	0.391	N.S.	No
25	*zp3*	NM_131331.1	-8.2	Down in JOT	-7.4	0.014	Down in JOT	Yes
26	*mid1ip1*	NM_213439.1	-5.9	Down in JOT	-6.9	0.001	Down in JOT	Yes
27	*gtf3ab*	NM_001089544.2	-5.4	Down in JOT	-6.4	0.003	Down in JOT	Yes
28	*ctssb.1*	NM_001024409.2	-5.4	Down in JOT	-7.4	0.004	Down in JOT	Yes
29	*zgc:55413*	CO350518	-5.3	Down in JOT	-6.4	0.003	Down in JOT	Yes

Table A 5. Gene expression patterns of 57 genes between juvenile ovotestis (JOT) and juvenile ovary (JO) as tested by microarray and real-time RT-PCR. (continued)

S/N	Gene Symbol	Accession no.	*Microarray		**Real-Time RT-PCR			Validation
			Fold Change (JOT/JO)	Result	Fold Change (JOT/JO)	p-value	Result	
30	*zp2*	NM_131330.1	-5.1	Down in JOT	-5.8	0.015	Down in JOT	Yes
31	*cyc1*	NM_001037393.1	-5.0	Down in JOT	-1.4	0.008	N.S.	No
32	*cldnd*	NM_180964.2	-4.3	Down in JOT	-7.5	0.007	Down in JOT	Yes
33	*btg4*	NM_198121.1	-4.3	Down in JOT	-10.7	0.016	Down in JOT	Yes
34	*dmnt1*	NM_131189.1	-3.3	Down in JOT	-3.0	0.162	N.S.	No
35	*setd8b*	NM_001100089.1	-3.2	Down in JOT	-6.4	0.000	Down in JOT	Yes
36	*rdh10b*	NM_201331.1	-3.0	Down in JOT	-7.2	0.002	Down in JOT	Yes
37	*rdh14a*	NM_001006031.1	-2.4	Down in JOT	-3.7	0.000	Down in JOT	Yes
38	*trdmt1*	NM_001018143.1	-2.4	Down in JOT	-2.3	0.002	Down in JOT	Yes
39	*cyp19a1a*	NM_131154.2	-2.3	Down in JOT	-1.4	0.162	N.S.	No
40	*rbp1b*	NM_212895.2	-2.3	Down in JOT	-5.3	0.004	Down in JOT	Yes
41	*psen1*	NM_131024.1	-1.9	Down in JOT	-1.6	0.004	Down in JOT	Yes
42	*ppp4ca*	NM_001110414.1	-1.9	Down in JOT	-4.6	0.000	Down in JOT	Yes
43	*ctnnbip1*	NM_131594.1	-1.7	Down in JOT	-1.7	0.004	Down in JOT	Yes
44	*tdrd7*	NM_001099343.1	-1.7	Down in JOT	24.4	0.865	N.S.	No
45	*amh*	NM_001007779.1	1.5	N.S.	213.5	0.008	Up in JOT	No
46	*vtg5*	NM_001025189.1	1.4	N.S.	1.2	0.928	N.S.	Yes
47	*nkap*	NM_001003414.1	1.3	N.S.	-1.4	0.004	N.S.	Yes
48	*esr2a*	NM_180966.2	1.1	N.S.	3.7	0.000	Up in JOT	No
49	*hsf5*	FJ969446.1	1.1	N.S.	3.0	0.016	Up in JOT	No
50	*vasa*	NM_131057.1	1.1	N.S.	1.1	0.757	N.S.	Yes
51	*tp53*	NM_131327.1	1.0	N.S.	1.0	0.885	N.S.	Yes
52	*pomca*	NM_181438.3	-1.1	N.S.	2.0	0.174	N.S.	Yes
53	*pou5f1*	NM_131112.1	-1.1	N.S.	-12.8	0.000	Down in JOT	No
54	*nanos3*	NM_131878.1	-1.2	N.S.	-7.7	0.003	Down in JOT	No
55	*axin1*	NM_131503	-1.2	N.S.	-3.8	0.001	Down in JOT	No
56	*dnd*	NM_212795.1	-1.3	N.S.	-2.3	0.002	Down in JOT	No
57	*piwil1*	NM_183338.1	-1.3	N.S.	1.1	0.819	N.S.	Yes

*Microarray experiment was carried out using six JO samples (three from 32 dpf and three from 35 dpf) and 17 JOT samples (four from 32 dpf, four from 34 dpf, three from 35 dpf and six from 36 dpf). Conditions for differential expression: microarray: >=1.5-fold difference; p-value <0.01.

**Real-time RT-PCR experiment was carried out using 5 JO and 5 JOT samples collected from 35 dpf zebrafish (different batch of samples from that collected for microarray experiment). Conditions for differential expression: real-time RT-PCR: >=1.5-fold difference; p-value < 0.05.

Table A 6. List of genes that were tested for differential expression between heat-shocked transgenic and heat-shocked wild-type full-sib progenies.

S/N	Gene Symbol	Accession no.	p-value	Fold-change (transgenic vs wild-type)	Significance (p-value < 0.05 and fold-change >= 1.3)
1	*dkk1b*	NM_131003.1	0.000	323.7	Up-regulated
2	*esr2b*	NM_174862.3	0.003	1.4	Up-regulated
3	*cyp19a1a*	NM_131154.2	0.006	-5.8	Down-regulated
4	*lef1*	NM_131426.1	0.008	-2.0	Down-regulated
5	*sox9b*	NM_131644.1	0.000	-1.6	Down-regulated
6	*inhbb*	NM_131068.2	0.048	-1.5	Down-regulated
7	*wnt4a*	NM_001040387.1	0.023	-1.3	Down-regulated
8	*foxl2*	NM_001045252.1	0.276	-1.3	Not significant
9	*axin1*	NM_131503	0.182	-1.3	Not significant
10	*fgf20a*	NM_001037103.1	0.245	-1.3	Not significant
11	*cyp19a1b*	NM_131642.1	0.383	-1.2	Not significant
12	*fsta*	NM_131037.3	0.015	-1.2	Not significant
13	*stra6*	NM_001045312.1	0.001	-1.2	Not significant
14	*casp9*	NM_001007404.2	0.018	-1.2	Not significant
15	*cyp26b1*	NM_131146.2	0.242	-1.2	Not significant
16	*ctnnb1*	NM_131059.2	0.179	-1.1	Not significant
17	*dvl3a*	NM_131757.1	0.189	-1.1	Not significant
18	*fgf20b*	NM_001039172.1	0.287	-1.1	Not significant
19	*dkk3*	NM_001089545.1	0.358	-1.1	Not significant
20	*ctnnbip1*	NM_131594.1	0.409	-1.1	Not significant
21	*star*	NM_131663.1	0.611	-1.1	Not significant
22	*rspo1*	NM_001002352.1	0.357	-1.1	Not significant
23	*psen1*	NM_131024.1	0.435	-1.1	Not significant
24	*ppp4ca*	NM_001110414.1	0.255	-1.1	Not significant
25	*sox9a*	NM_131643.1	0.688	-1.1	Not significant
26	*hsf5*	FJ969446.1	0.677	1.0	Not significant
27	*cyp11c1*	NM_001080204.1	0.803	1.0	Not significant
28	*wt1a*	NM_131046.1	0.632	1.0	Not significant
29	*ts1*	EF554575.2	0.729	1.0	Not significant
30	*ck2a1*	NM_131252.1	0.605	1.0	Not significant
31	*fancl*	NM_212982.1	0.814	1.0	Not significant
32	*mitfa*	NM_130923.1	0.842	1.0	Not significant
33	*ar*	NM_001083123.1	0.848	1.0	Not significant
34	*dmrt1*	NM_205628.1	0.657	1.0	Not significant
35	*csnk1g1*	NM_001008635	0.565	1.1	Not significant
36	*odf3b*	NM_199958.1	0.535	1.1	Not significant
37	*pou5f1*	NM_131112.1	0.703	1.1	Not significant
38	*tdrd7*	NM_001099343.1	0.394	1.3	Not significant
39	*piwil1*	NM_183338.1	0.464	1.4	Not significant
40	*amh*	NM_001007779.1	0.193	1.5	Not significant
41	*cyp26a1*	NM_131146.2	0.251	1.6	Not significant
42	*zp2*	NM_131330.1	0.293	7.7	Not significant