SEX DETERMINATION IN ZEBRAFISH: GENETICS OF SEX AND *WNT4A*

by

SAMANTHA KAITLIN HIGH

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Student: Samantha Kaitlin High

Title: Sex Determination in Zebrafish: Genetics of Sex and *wnt4a*

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Phil Washbourne	Chairperson
John Postlethwait	Advisor
Judith Eisen	Core Member
Patrick Phillips	Core Member
Mike Harms	Institutional Representative

and

Scott L. Pratt	Dean of the Graduate School

Original approval signatures are on file with the University of Oregon Graduate School.

Degree awarded June 2016

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DISSERTATION ABSTRACT

Samantha Kaitlin High

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Effective reproduction is essential for species survival. Sexual reproduction depends upon functional gonads and reproductive ducts. Zebrafish (*Danio rerio*) is a popular model organism, but the genetic basis of zebrafish sex determination, gonad development, and reproductive tract development are not fully understood, and understanding this basis could inform about the evolutionary conservation of these genes and the use of zebrafish to investigate and treat reproductive diseases. In chapter I, I give a overview of sex determination systems, gonad development, and reproductive duct development in mammals and fish, and ask how sex is determined and how reproductive ducts develop in zebrafish. In chapter II, I used genome wide association studies (GWAS) to investigate if the genetic basis of sex determination in a variety of zebrafish strains -- two 'wild-type' strains cultured for about 30 years in the lab, and four 'natural' strains, wild-type strains isolated directly or recently from nature in India -- and identified a sex-associated region on zebrafish chromosome 4 in natural zebrafish strains that was lacking in the lab strains. In chapter III, I investigated whether or not *wnt4a* is important for zebrafish ovarian development, and found that *wnt4a* is expressed in the early bipotential gonad and that loss of *wnt4a* results in male-biased sex ratios, indicating that

wnt4a is important for zebrafish ovarian development. In chapter IV, I investigated whether or not *wnt4a* is important for male reproductive duct development, and found that loss of *wnt4a* slows the formation of male reproductive ducts and prevents the male fused ducti deferens from connecting to the genital orifice in zebrafish males. Results further showed that *wnt4a* is expressed in tissue around the site where this connection should occur before and after the connection is formed, revealing a novel *wnt4a* phenotype in zebrafish that hasn't been seen thus far, and indicating that *wnt4a* is necessary for proper male reproductive duct development in zebrafish.

This dissertation contains both published and unpublished co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Samantha Kaitlin High

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon
Oregon State University, Corvallis, Oregon

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2016, University of Oregon
Bachelor of Science, Biology, 2009, Oregon State University

AREAS OF SPECIAL INTEREST:

Molecular biology and development
Biology of gonad development and sex determination across species

PROFESSIONAL EXPERIENCE:

GTF, Department of Biology, University of Oregon, Eugene, 2010-2011

GRANTS, AWARDS, AND HONORS:

Developmental Training Grant (T32HD007348), 2015 to present

PUBLICATIONS:

Wilson C, **High SK**, McCluskey BM, Amores A, Yan YL, Titus TA, Anderson JL, Batzel P, Carvan MJ 3rd, Schartl M., Postlethwait J. (2014) Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. Genetics. 198(3): 1291-1308

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CHAPTER I

INTRODUCTION

Question

In sexual reproduction, each species is generally divided between two reproductive groups, male and female, which both have unique and complementary reproductive features. Generally, fully functional males and females require functional gonads and reproductive tracts. Without functional gonads, gametes would fail to be produced, and without a functional reproductive tract, the gametes produced in the gonad wouldn't be transported away from the gonad for proper fertilization and development. For the survival of many species it is imperative to understand the way gonads and reproductive tracts normally develop so we can recognize and potentially correct failures in this development to ensure species survival. To gain an understanding of these processes, we must first ask: what are the genetic mechanisms that control the proper development of fully functional male and female gonads and reproductive tracts?

Sex Determination

Genetic vs. environmental sex determination

Sex determination is the mechanism that specifies whether an embryo develops into a male or female. This process is extremely variable across species. Vertebrate gonads generally differentiate into ovaries or testes through environmental or genetic sex determination.

Environmental sex determination is controlled by a variety of environmental factors, the most prevalent of which is temperature. The Barbour's map turtle (*Graptemys barbouri*) and the pond slider turtle (*Trachemys scripta*) have temperature regulated environmental sex determination, where males develop at lower temperatures than females (Ewert et al. 1994). Environmental sex determination is just one way different sexes can develop; the other, and more prevalent, major sex determination pathway is genetic sex determination.

Genetic sex determination usually involves sexually dimorphic chromosomes that contain a master switch gene that triggers either male or female development (Bachtrog et al. 2014). In this form of sex determination, also called chromosomal sex determination, a network of genes, often found across various autosomes, are usually activated in either the presence or absence of the master switch gene found on one of the sex chromosomes (Bachtrog et al. 2014). The two main chromosomal sex determination systems involve either the XX/XY or the ZZ/ZW sex chromosomes (Bachtrog et al. 2014).

Mice (*Mus musculus*), humans (*Homo sapiens*), and fruit flies (*Drosophila melanogaster*) have XX/XY sex determination (Bachtrog et al. 2014; Eggers et al. 2014; She et al. 2014). These species have one X and one Y sex chromosome. Females are homogametic (have two X sex chromosomes) and males are heterogametic (have one X and one Y sex chromosome) (Barske et al. 2008; Kashimada et al. 2010; Bachtrog et al. 2014; Eggers et al. 2014; She et al. 2014). Even though these species utilize the same sex chromosome system, the genetic basis of sex determination in mammals (*M. musculus* and *H. sapiens*) and fruit flies (*D. melanogaster*) differs, because different master switch

genes with different mechanisms are used (*Sry*, a dominant Y-linked gene in mammals and *Sxl*, a dosage-sensitive X-linked gene in fruit flies) (Bachtrog et al. 2014). The difference in which master switch genes are utilized by these species is not the only difference.

In addition to different genetic bases for sex determination, taxa differ in dosage compensation, differences in gene expression that compensate for the extra copies of genes found on the second X chromosome in females. In mammals, one X chromosome is inactivated (X-inactivation) in females so that the expression of X-linked genes is the same in males and females (Sharma and Meister 2016). In flies, X-linked genes are up-regulated in the male to match the expression in females (Sharma and Meister 2016). The different master switch genes and different dosage compensation strategies show the complexity that arose in the evolution of this chromosomal sex determination system.

The XX/XY sex determination system is not the only chromosomal sex determination system that has evolved. Other chromosomal sex determination systems, such as ZZ/ZW sex determination, have also evolved and have some similarities to XX/XY sex determination. Avian species, such as chicken (*Gallus gallus domesticus*), have ZZ/ZW sex determination. These species have one Z and one W sex chromosome, males are homogametic (have two Z sex chromosomes) and females are heterogametic (have one Z and one W sex chromosomes) (Ser et al. 2009; Ezaz et al. 2006). In these species, the exact genetic mechanism of sex determination remains unclear. Under one hypothesis, females have a master switch gene on the W chromosome, similar to the male master switch gene found on the Y chromosome in mammals and fruit flies (Ayers et al. 2013). Under an alternative hypothesis, sex in ZW species is determined via dosage,

where males would have a higher expression of genes found on the Z chromosome than females, which is seen when looking at the gene *DMRT1*, found on the Z chromosome, which shows greater expression in males than females (Ayers et al. 2013).

Although XX/XY and ZZ/ZW chromosomal sex determination systems are found in many species, variations on these chromosomal sex determination systems also exist. Many other species, such as the duck billed platypus (*Ornithorhynchus anatinus*), roundworm (*Caenorhabditis elegans*), Eri silkworm (*Samia cynthia ricini*), and Shinju silkworm (*Samia cynthia* subsp. indet.), have many variations on the XX/XY and ZZ/ZW chromosomal sex determination systems. The duck-billed platypus (*Ornithorhynchus anatinus*), for example, has five X sex chromosomes and five Y sex chromosomes, where females have two sets of the five X chromosomes ($X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$) and males have one set of the five X sex chromosomes and one set of the five Y sex chromosomes ($X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$) (Grützner et al. 2004). The roundworm (*Caenorhabditis elegans*) has one X sex chromosome but lacks the Y sex chromosome, where hermaphrodites have a set of X chromosomes (XX) and males have only one X chromosome (X0) (Sharma and Meister 2016). The Eri silkworm (*Samia cynthia ricini*) has one Z sex chromosome but lacks the W sex chromosome, where females have only one Z chromosome (Z0) and males have a pair of Z chromosomes (ZZ) (Yoshido et al. 2013). The Shinju silkworm (*Samia cynthia* subsp. indet.) has one W sex chromosome and two Z sex chromosomes, where females have one W chromosome and one combination of the two Z chromosomes (WZ_1Z_2) and males have two sets of the two Z chromosomes ($Z_1Z_1Z_2Z_2$) (Yoshido et al. 2013).

In some species, sex determination is not driven by sex chromosomes and instead is polygenic. Some species that lack sexually dimorphic chromosomes still have polygenic sex determination, such as the harpacticoid copepod (*Tigriopus californicus*) and the European sea bass (*Dicentrarchus labrax*); genes across a variety of autosomes control sex determination (Alexander et al. 2015; Palaiokostas et al. 2015). In both the copepod and European sea bass, multiple sex-associated regions were identified across multiple chromosomes (although the sex-associated regions were not the same in both species), indicating that these species have polygenic sex determination, but the genes involved in sex determination have yet to be identified in both of these species (Alexander et al. 2015; Palaiokostas et al. 2015).

In summary, various sex determination systems are complicated and extremely variable across species. Understanding the mechanisms behind how these sex determination systems work is important for understanding the evolution of sex determination systems as well as for recognizing and potentially correcting failures in these systems to ensure species survival. The complexity of sex determination mechanisms can be further understood by looking at one of the better-studied sex determination mechanisms, mammalian sex determination.

Mammalian gonad development and sex determination

Some of the best-understood mechanisms behind sex determination and gonad development are in mammals. Mammalian sex is initially determined by the presence or absence of the Y sex chromosome, but the overall sex determination mechanism is actually more complicated and involves multiple genes (see Figure 1.1). Early in mammalian sex development, both XX and XY individuals develop bipotential gonads

that are capable of becoming ovaries or testes (Kashimada et al. 2010). Initially primordial germ cells, which are the precursors to germ cells, migrate into the gonadal ridge, which is where the bipotential gonad will start developing (Albrecht et al. 2001; Kim et al. 2006). The bipotential gonad requires multiple transcription factors to develop properly, including Emx2, Wt1, Lhx9, Nr5a1, and Cbx2 (Eggers et al. 2014). Mice independently lacking either Emx2, Wt1, Lhx9, or Nr5a1 fail to develop gonads, indicating that these proteins are critical for proper bipotential gonad formation (Eggers et al. 2014). Mice lacking Cbx2 have impaired gonad development, indicating that this protein is also important for proper bipotential gonad development (Eggers et al. 2014).

Mammalian XY-containing bipotential gonads develop into testes. *Sry,* the master switch for mammalian sex determination found on the Y chromosome, is necessary and sufficient to cause the supporting cells in the gonad to become Sertoli cells and to initiate testis development (Kashimada et al. 2010; DiNapoli et al. 2008; Eggers et al. 2014). Sry induces the expression of *Sox9* in Sertoli cell precursors both directly and by acting cooperatively with Sf1, a protein encoded by Nr5a1, which up-regulates *Sox9* (Kashimada et al. 2010; DiNapoli et al. 2008; Sekido et al. 2008). Sox9 causes the development of Sertoli cells and the development of testis (Kashimada et al. 2010; Dinapoli et al. 2008; Sekido et al. 2008), by initiating *Fgf9* (fibroblast growth factor – 9) expression, which in turn up-regulates *Sox9* expression in a positive feedback loop (She et al. 2014). Mouse gonads that contain XY chromosomes but lack *Fgf9*, a growth factor expressed in the bipotential gonad, through a mutation do not develop Sertoli cells and show male-to-female sex reversal (Kim et al. 2006; She et al. 2014). *Sry* expression is

nevertheless initiated normally in these *Fgf9*-deficient gonads, but *Sox9* expression is quickly silenced and the cells of these gonads start to express genes from the female

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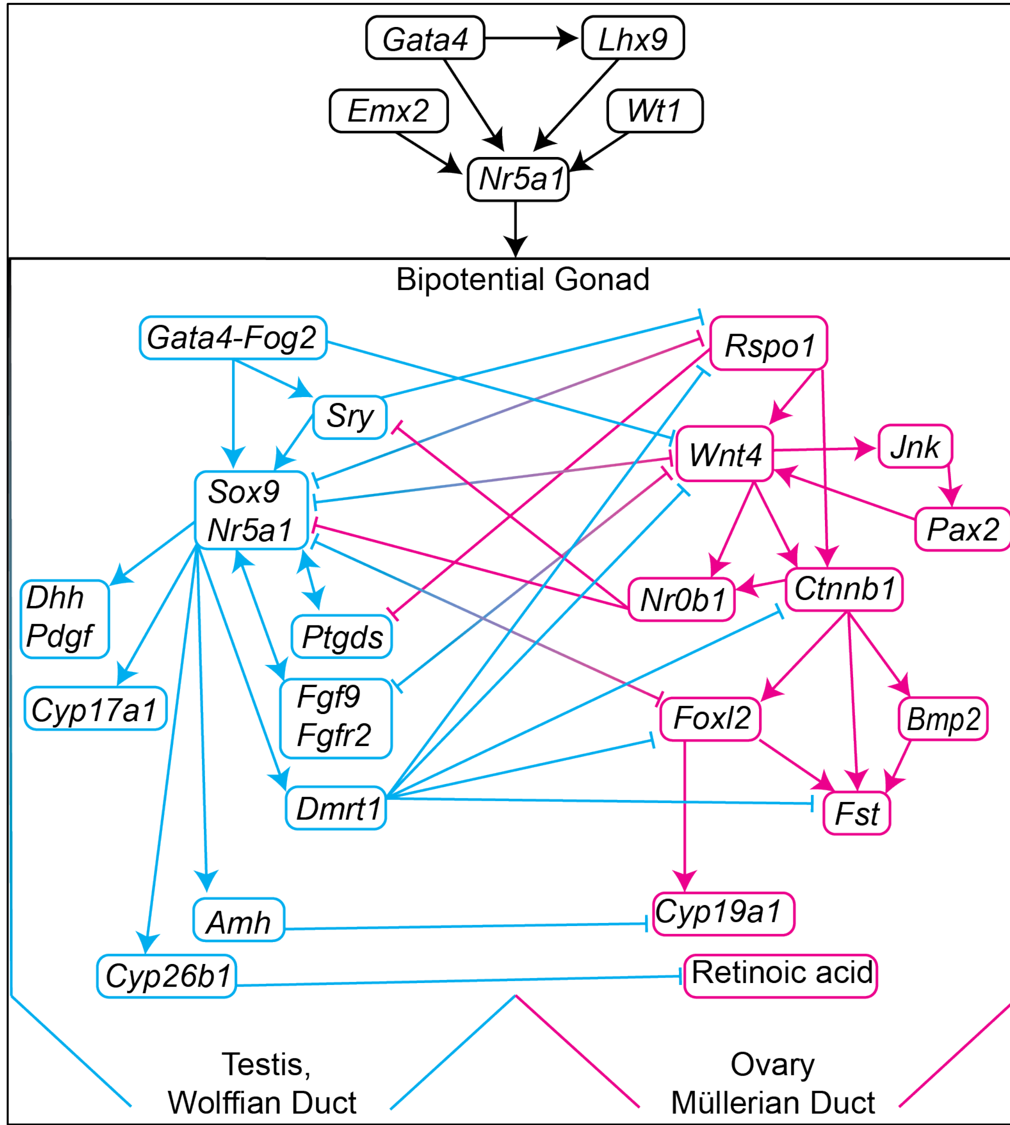
Figure 1.1 (next page) – Mammalian sex determination pathway. Initial bipotential gonad development requires multiple genes. Together *Emx2*, *Gata4*, *Lhx9* (expression is induced by *Gata4*), and *Wt1* promote the expression of *Nr5a1* leading to proper bipotential gonad formation through genital ridge formation and coelomic epithelium thickening (She and Yang 2014). In the male sex determination pathway, the *Gata4-Fog2* pathway is essential for the correct initiation and up-regulation of *Sry*, the mammalian master switch gene found on the Y chromosome in males. The *Gata4-Fog2* pathway in conjunction with *Sry* initiates *Sox9* and *Sf1* expression. *Sox9* up-regulates *Fgf9* expression and Fgf9, through Fgfr2, establishes a positive feedback loop with *Sox9* maintaining high *Sox9* expression levels (Kim et al. 2006). *Sox9* also up-regulates *Ptgds* and Ptgds signaling promotes Sox9 nuclear translocation, facilitating Sertoli cell differentiation. The Sertoli cells express *Dhh* and secrete Pdgf leading to Leydig cell differentiation. *Sox9* and *Nr5a1* activate *Cyp17a1* and *Cyp26b1*, which are required for steroidogenesis in Sertoli cells (Park et al. 2010; Kashimada et al. 2011). *Sox9* and *Nr5a1* up-regulate *Amh* expression, which initiates Müllerian duct regression. Testis development is also promoted through the inhibition of genes in the female sex determination pathway. *Gata4* inhibits initial *Wnt4* expression, then in the presence of *Sry*, *sox9* inhibits *Rspo1* and *Foxl2* expression and both *Sox9* and *Fgf9* inhibit *Wnt4* expression, and *Dmrt1* inhibits *Rspo1*, *Wnt4*, *Ctnnb1*, *Fst*, and *Foxl2* expression, preventing ovarian development and through doing so, promoting testis development (Herpin and Shartl 2015; Matson et al. 2011). *Cyp26b1* also inhibits retinoic acid expression preventing the initiation of meiosis in germ cells and leading to a failure in ovarian development. In the female sex determination pathway, *Rspo1* activates *Wnt4* expression in the somatic cells of the gonad, and together they activate the canonical Wnt/β-catenin pathway through *Ctnnb1* (β-catenin) activation (Chassot et al. 2008). The canonical Wnt/β-catenin pathway activates *Foxl2* and *Bmp2* expression, which then activate *Fst* expression, promoting folliculogenesis in the ovary (Kashimada et al. 2011). *Foxl2* also up-regulates *Cyp19* (aromatase) expression, which regulates estrogen biosynthesis in the developing ovary (Kashimada et al. 2011). *Wnt4* also activates *Jnk, which* up-regulates *Pax2*, which initiates Müllerian duct formation, and elongation (Bernard and Harley 2007; Prunskaite-Hyyryläinen et al. 2016; Dressler 2011). Ovary development is also promoted through the inhibition of male sex determination pathway genes. *Nr0b1*, which is up-regulated by *Wnt4* and the canonical Wnt/β-catenin pathway, inhibits *Sry*, *Sox9*, and *Nr5a1* expression (Tanaka and Nishinakamura 2014; Mizusaki et al. 2003; Kousta et al. 2010). *Rspo1* inhibits both *Sox9* and *Ptgds* expression and *Wnt4* inhibits *Sox9* and *Fgf9* expression, preventing Sertoli cell development and testis formation. *Foxl2* inhibits *Sox9* expression, which inhibits *Cyp26b1* expression and increases retinoic acid levels (Kashimada et al. 2011).



pathway, including *Wnt4* (Kim et al. 2006; She et al. 2014). These results show that F*gf9* is essential for the maintenance but not the initiation of *Sox9* expression, and is required to maintain testis development and suppress ovary development (Kim et al. 2006; She et al. 2014).

In most cases XX-containing bipotential gonads develop into ovaries. *Wnt4* (wingless-type MMTV integration site family, member 4), a gene expressed in the somatic cells of the bipotential gonad, is important for ovary development and inhibits

testis development (Kim et al. 2006; She et al. 2014). Chromosomally XX gonads in mouse that lack Wnt4 function up-regulate of *Fgf9* and *Sox9* and show partial female-to-male sex reversal (DiNapoli et al. 2008; She et al. 2014). This finding suggests that *Wnt4* is important for the female sex determination pathway and suppresses testis development (DiNapoli et al. 2008; She et al. 2014). Mutations in *Wnt4* can also lead to a variety of reproductive diseases that affect ovary development in humans, such as SERKAL syndrome and Polycystic ovary syndrome (Mandel et al. 2008, Canto et al. 2006). SERKAL syndrome results in the development of testes in XX humans, indicating female-to-male sex reversal (Mandel et al. 2008). Polycystic ovary syndrome can, but does not always, result in polycystic ovaries (ovaries with multiple small follicular cysts), chronic anovulation (absence of ovulation), oligomenorrhea (light or infrequent menstruation), amenorrhea (absence of menstruation), and hyperandrogenism (excess of androgens) (Canto et al. 2006).

Overall these findings suggest that the balance between *Fgf9* and *Wnt4* (also called the *FGF9/WNT4* see-saw hypothesis) is important for mammalian sex determination (DiNapoli et al. 2008, Kim et al. 2006, Kashimada et al. 2010), and that defects in *Wnt4* can cause reproductive diseases in mammalian females.

The ability to understand reproductive diseases and to discover effective treatments would be beneficial for human health. Reproductive deficits are not just localized to defects in gonad development, but also affect the rest of the reproductive system, the reproductive ducts. Investigating how reproductive ducts form in mammals can provide a greater understanding for how reproductive ducts may form in other

species, as well as give us a way to understand and treat diseases that may affect all parts of the overall reproductive system.

Mammalian reproductive duct development

In mammals, male reproductive ducts develop from Wolffian ducts and female reproductive ducts develop from Müllerian ducts. Precursors of the Wolffian and Müllerian ducts initially develop in both male and female fetuses (Staak et al. 2003). If the animal continues to develop as a female, the Wolffian ducts regress and the Müllerian ducts differentiate into the oviduct, uterine horns, cervical canal, and upper vagina (Staak et al. 2003; McLennan and Pankhurst 2015). If the animal continues to develop as a male, the Müllerian ducts regress, which is initiated by Amh (AntiMüllerian Hormone, a peptide hormone produced and secreted by the Sertoli cells of the testes), and induced by testosterone, the Wolffian ducts differentiate into epidiymides, vas deferens, and seminal vesicles (Staak et al. 2003; McLennan and Pankhurst 2015). In the adult male, the seminiferous tubules produce gametes, which travel through the rete testis, efferent ducts, epididymis, and vas deferens (Staak et al. 2003). Many genes are involved in reproductive duct development, including *Wnt4*, a signaling molecule that is necessary for proper ovary development in mammals.

Wnt4 and mammalian reproductive duct development

In mice, *Wnt4* was found to be important for the development of the male reproductive system (Jeays-ward et al. 2004). *Wnt4-/-* XY mice have fewer and more disorganized testicular cords, which eventually become seminiferous tubules, prior to birth (Jeays-ward et al. 2004). *Wnt4-/-* adult XY mice have normal (indistinguishable

from wild type) testes, reproductive ducts, and sperm production (Vainio et al. 1999), but *Wnt4-/-* male mice do have malformed testicular cords prior to birth, which indicates that this defect must be compensated for at birth (Jeays-Ward et al. 2004, Satoh 1991). *Wnt4-/-* XX mice have deformed Müllerian ducts, which persist after birth, indicating that *Wnt4* is important for proper Müllerian duct formation (She et al. 2014).

Mammals provide a great model to understand sex determination, gonad development, and reproductive duct development, but looking at just mammalian model organisms doesn't allow us to fully understand how these mechanisms evolved, and if other organisms conserve these mechanisms. To better understand the evolution of the mechanisms behind duct development, we can look at whether or not these mechanisms are conserved in teleosts.

Fish development

Sex determination in fish

Teleosts have a variety of sex determination systems. Some teleost species, such as the Indian rice fish (*O. dancena*), have XX/XY sex determination (Takehana et al. 2007), other rice fish, such as *O. hubbsi*, have ZZ/ZW sex determination (Takehana et al. 2007), some, such as some Lake Malawi cichlids, have polygenic sex determination (Ser et al. 2010) and others, such as poeciliid fish (*Poeciliopsis sphenops*), have environmental sex determination (Barón et al. 2002). The platyfish (*Xiphophorus maculatus*) has X, W, and Y sex chromosomes; females can have either two X sex chromosomes, or one W and one X sex chromosome, or one W and one Y sex chromosome, and males can have either one X and one Y sex chromosome or two Y sex

chromosomes (Shartl 2004). Even though sex can be determined using a variety of factors, the sex determination mechanisms are poorly understood in some model organisms, such as zebrafish (*Danio rerio*), and studying sex determination in zebrafish could either inform us of the variations in sex determination mechanisms utilized across teleost species.

Reproductive duct development in medaka (*Oryzias latipes*)

In one teleost, medaka (*O. latipes*), the male and female reproductive ducts are not formed from Müllerian or Wolffian ducts and they do not develop in both sexes as seen in mammals, and instead develop independently in males and females. The reproductive ducts in both medaka males and females initially form as a mass of somatic cells (cell mass) near the posterior end of either the testis or ovary around 20 to 30 days post hatching (dph), and the reproductive ducts are fully formed by 50 to 90 dph in males or 80 to 90 dph in females (Suzuki and Shibata 2004).

In medaka (*O. latipes*) males, reproductive ducts consist of the efferent ducts and sperm duct (Suzuki and Shibata 2004). The primordial reproductive duct develops from the posterior end of the testis as a mass of somatic cells (cell mass) around 20 to 30 days post hatching (dph) (Suzuki and Shibata 2004). The mass begins to elongate posteriorly as a rod-like structure that finally forms a lumen. The rod differentiates into the efferent duct, which is positioned along the testes, and sperm ducts, which continue from the posterior end of the efferent ducts (Suzuki and Shibata 2004). Male reproductive ducts are fully formed, with the connection of the sperm duct with the urethra, by 50-90 dph (Suzuki and Shibata 2004). In the adult medaka, gametes travel from the testes to the

efferent ducts, then to the sperm duct, and finally through the connection to the urethra and into the external environment.

In medaka females, reproductive ducts consist of the ovarian cavity and oviduct. As in medaka males, at around 20 to 30 dph a mass of somatic cells (cell mass) form at the posterior end of the ovary, but unlike medaka males, cell layers extend from both the ventral and dorsal sides of the posterior end of the ovary and connect with the cell mass to form eventually the primordial reproductive duct of the female; this primordial reproductive duct elongates posteriorly as a rod-like structure, that does not yet contain a lumen (Suzuki and Shibata 2004). The ovarian cavity, located along the dorsal side of the ovary, differentiates from the cell layers that initially extended from the dorsal and ventral sides of the ovary by 30-50 dph, and the oviduct forms from the rod-like posteriorly projecting cell mass, which forms a lumen by 30-50 dph (Suzuki and Shibata 2004). By 40-60 dph, the ovarian cavity and oviduct have connected, and the oviduct has continued to develop posteriorly (Suzuki and Shibata 2004). By 80 to 90 dph the oviduct, which up to this point continued developing as a tube-like structure containing a lumen, has finally fully connected and opened to the exterior of the fish (Suzuki and Shibata 2004). In the adult female medaka, gametes travel from the ovary to the ovarian cavity and then through the oviduct and into the external environment.

Medaka is just one species among the 25,000 species in the subclass of teleosts. Teleosts consist of a wide range of different species, and the way reproductive ducts are organized and formed could vary across species. Investigating the reproductive duct development of other teleost species, such as zebrafish (*D. rerio*), could either inform us of the variations in reproductive duct development or confirm that reproductive ducts

develop similarly in multiple teleost species. To effectively compare reproductive duct and gonad development in teleosts to other vertebrates, such as mice or humans, we need to understand genome duplication events that occurred throughout evolution.

Genome duplication events

The subclass *Teleostei*, or teleosts, consists of many bony fishes, including medaka (*O. latipes*), three-spined Stickleback (*Gasterosteus aculeatus*), and Japanese black porgy (*Acanthopagrus schlegeli*). Teleosts diverged from basal ray-finned fish approximately 320-350 million years ago (mya) (Glasauer and Neuhauss 2014), and ray-finned fish diverged around 384-402 million years ago (Near et al. 2012). Prior to the teleost divergence, three whole genome duplications occurred (Amores et al. 1999; Dehal and Boore 2005; Pascual-Anaya et al. 2013; Glasauer and Neuhauss 2014). Ideally one whole genome duplication results in a two daughter genes called paralogs (Force et al. 1999; He and Zhang 2005), two whole genome duplications result in four paralogs, and three whole genome duplications result in eight paralogs. Initially these paralogs are identical, but eventually develop neofunctionalization (one paralog retains ancestral function while the other acquires new function), subfunctionalization (both paralogs retain some ancestral function, but the functions retained are different in each paralog), subneofunctionalization (both paralogs retain some ancestral function as well as develop new function) or non-functionalization (the function of one paralog is lost) (Force et al. 1999; He and Zhang 2005; Glasauer and Neuhauss 2014).

Two rounds of whole genome duplication occurred early in vertebrate evolution (Dehal and Boore 2005; Pascual-Anaya et al. 2013; Glasauer and Neuhauss 2014). The evidence for these duplication events can be found in genes that follow the one-to-four

rule, meaning species that evolved before the genome duplication have only one copy of the ancestral gene and species that evolved after two rounds of genome duplication have four copies of that same ancestral gene (Dehal and Boore 2005). Evidence of this one-to-four rule, and thus for these two whole genome duplication events, can be found when looking at *Hox* clusters, specifically comparing the number of *Hox* clusters of cephalochordates, which diverged before the first two rounds of whole genome duplication, to tetrapods, which diverged after the first two rounds of whole genome duplication (Pascual-Anaya et al. 2013). Cephalochordata, one of the two main subgroups of Chordata, is the sister group to the other subgroup, the Olfactores, and tetrapods are a super class of the vertebrates belonging to the Olfactores subgroup (Pascual-Anaya et al. 2013), which makes the cephalochordates a valuable outgroup for *Hox* cluster number comparison. Cephalochordates contain only a single *Hox* cluster, whereas tetrapods contain 4 *Hox* clusters, which indicate that two rounds of genome duplication occurred before around the divergence of vertebrates (Pascual-Anaya et al. 2013). Even though evidence exists for two rounds of whole genome duplication, this hypothesis is complicated by the fact that many genes do not follow the one-to-four rule (Dehal and Boore 2005), but this could result from the non-functionalization of some paralogs (Amores et al. 1998; He and Zhang 2005; Glasauer and Neuhauss 2014), meaning that some of the paralogs were lost, and therefore some species only have one, two, or three copies of a gene instead of the expected four.

After the first two rounds of whole genome duplication and prior to the divergence of teleosts, a third teleost specific whole genome duplication occurred (Amores et al. 1998; Glasauer and Neuhauss 2014). Following the same logic seen in the

one-to-four rule (Dehal and Boore 2005), this third genome duplication should result in eight copies of a single gene in cephalochordates. Again evidence for this can be found in *Hox* clusters, specifically looking at the European eels (*Anguilla anguilla*), Japanese eels (*A. japonica*), and zebrafish (*D. rerio*). European and Japanese eels have the full set of eight *Hox* clusters (Glasauer and Neuhauss 2014) which is as expected if there was a third whole genome duplication event. Again, just as with the two rounds of whole genome duplication hypothesis, this hypothesis is complicated by the fact that most teleosts do not have eight *Hox* clusters, but again this could be explained by the non-functionalization of paralogs (Glasauer and Neuhauss 2014). Zebrafish (*D.* rerio) are teleosts that have seven instead of eight *Hox* clusters, which probably resulted from the non-functionalization of one of the paralogs (Amores et al. 1998; Glasauer and Neuhauss 2014). Zebrafish, as a teleost that diverged following the teleost genome duplication, make a great model to understand the evolution of gene function, because the resulting paralogs undergo a variety of modifications resulting in new functions, alternate functions, or no function at all (He and Zhang 2005; Glasauer and Neuhauss 2014), as described earlier.

Zebrafish development

Zebrafish as a model organism

Zebrafish are currently widely used as model organism in laboratory research. Zebrafish was first discovered and described in 1822 (Hamilton, 1822). Zebrafish are tropical freshwater fish found across India, Pakistan, Bangladesh, Myanmar, and Nepal (Lawrence 2007). Zebrafish live in a variety of freshwater habitats ranging from clear

rivers to murky swamps and fast moving streams to still rice paddies (Engeszer et al. 2007). In these wild habitats, zebrafish breed year round, hatch around 4 to 7 days post fertilization (dpf) and reach maturity 2 to 4 months post fertilization (or as early as 57 days post fertilization under laboratory conditions) (Lawrence et al. 2012).

Zebrafish became a popular aquarium fish before becoming a popular model organism for laboratory research 1960s (Grunwald and Eisen 2002; Schartl 2014). George Streisinger, who used zebrafish to study vertebrate embryonic development, was the first to recognize the utility of zebrafish as a genetic model organism (Grunwald and Eisen 2002). Streisinger recognized several advantages of zebrafish for laboratory genetic analyses. Firstly, zebrafish breed year round and produce hundreds of progeny. Secondly, zebrafish males fertilize eggs externally, and these gametes can be harvested at a single cell stage and utilized for genetic manipulation. Thirdly, zebrafish embryos are transparent and internal development can be easily viewed and characterized (Grunwald and Eisen 2002). Zebrafish research has continued to expand in the fifty years since its first use as a model organism. Tens of thousands of mutations have already been generated and described in zebrafish, which resulted in its popularity as a model organism used for biomedical research, especially in areas concerning treatments for human diseases (Grunwald and Eisen 2002; Schartl 2014). Zebrafish could provide a means to model and discover treatments for reproductive diseases. To facilitate the use of zebrafish as a model, we need to know more about zebrafish gonad development and sex determination.

Zebrafish gonad development

Like mammals, early zebrafish gonad development starts with primordial germ cells, which group together and are surrounded by somatic cells (Maack et al. 2003). The gonads are undifferentiated and still bipotential until about 20 dpf (Takahashi 1977, Maack et al. 2003). By approximately 20 dpf, the gonad in all individuals has female and supporting cells and can be considered a juvenile ovary (Takahashi 1977, Maack et al. 2003). By about 35 dpf, the gonads have transitioned into sexually determined, but still immature, gonads (Takahashi 1977, Maack et al. 2003). If the animal will eventually become male, then the gonad transitions into a testis by oocyte apoptosis (Maack et al. 2003; Takahashi 1977; Uchida et al. 2002). If the animal will eventually become female, then the oocytes enter meiosis (Maack et al. 2003; Rodriguez-Mari et al. 2005; Siegfried and Nüsslein-Volhard et al. 2008). The overall zebrafish genetic sex determination pathway is still unknown.

Zebrafish sex determination

It was previously thought that zebrafish did not have sex chromosomes (Krovel and Olsen 2004; Postlethwait et al. 2000; Amores and Postlethwait 1999), although new data suggest that zebrafish have chromosomes associated with sex determination, and that this sex determination might be polygenic (Anderson et al. 2012; Bradley et al. 2011; Howe et al. 2013). A zebrafish cross using the AB and Nadia zebrafish strains, in which a Nadia female was crossed with an AB male showed a sex-associated locus on zebrafish chromosome 4 (Chr4) where as the reciprocal mating (female-AB-by-male-Nadia) showed sex-associated loci on both Chr4 and Chr3 (Anderson et al. 2012). Another

zebrafish cross utilizing the AB and India zebrafish strains, in which an AB female was crossed with an India male showed sex-linked loci on Chr5 and Chr16 but none on Chr3 or Chr4 (Bradley et al. 2011). An F_2 map constructed from a gynogenetic doubled-haploid TU (Tuebingen) female and a gynogenetic doubled-haploid AB male identified a unique sex-linked region on Chr16 that does not overlap with the one observed in the AB-by-India zebrafish cross (Bradley et al. 2011; Howe et al. 2013). These studies indicate that zebrafish might have chromosomal regions associated with sex determination, but that these sex-associated regions may vary among strains. Repeated matings of zebrafish pairs from AB, TU, and Toh strains produce consistent sex ratios, but different pairs can give quite different sex ratios, which suggests that zebrafish has polygenic sex determination that might also vary among zebrafish strains (Liew et al. 2012). Overall, these results indicate that zebrafish utilize a polygenic sex-determination mechanism (Liew and Orban 2014), and that different zebrafish strains utilize different genetic mechanisms to determine sex. Therefore, we must test the hypothesis that the genetic basis of sex determination varies across different zebrafish strains.

In addition to having sex-associated regions that vary between strains, zebrafish may have a variety of genes that are also involved in sex determination. Zebrafish have multiple genes that are orthologous to the genes found in the mammalian sex determination pathway, and the expression of many of these genes was previously investigated (Figure 1.2). One particular gene that hasn't been thoroughly investigated in zebrafish, *wnt4a*, is orthologous to mammalian *WNT4*, which is required for ovary development in mice (DiNapoli et al. 2008, Mandel et al. 2008). Thus, we must test the

hypothesis, that *wnt4a,* like mammalian *Wnt4,* is important for zebrafish ovarian development.

Zebrafish sex determination can be affected by a variety of factors. There could be strain specific effects, or specific genes that affect gonad development. The gonad is only one part of the fully functional reproductive system. Functional reproductive ducts

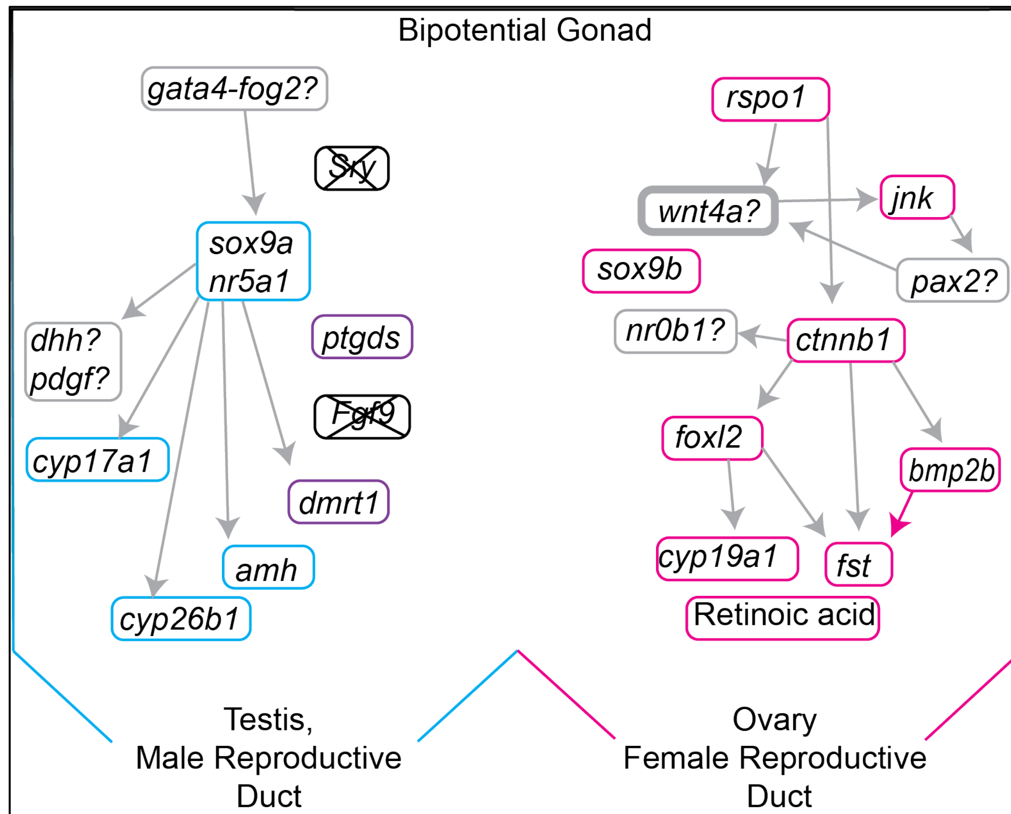


Figure 1.2 – Potential zebrafish sex determination pathway. Based on the mammalian sex determination pathway and zebrafish gene expression. Genes expressed in the developing testis: *sox9a* and *amh* (Rodriguez-Mari et al 2005; Siegfried and Nüsslein-Volhard 2008). Genes expressed in the adult testis: *cyp17a1a*, *cyp26b1,* and *nr5a1* (Hinfray et al. 2011; Von Hofsten et al. 2005). Genes expressed in both the developing testis and ovary: *dmrt1* and *ptgds* (Guo et al. 2005; Jorgensen et al. 2010). Genes expressed in the developing ovary: s*ox9b*, *cyp19a1a*, and *foxl2* (Rodriguez-Mari et al 2005; Siegfried and Nüsslein-Volhard 2008). Genes expressed in the adult ovary: *ctnnb1*, *rspo1*, *fst*, *bmp2b*, *jnk* (Zhang et al. 2010; Pradhan and Olsson 2014; Wu et al. 200; Li et al. 2013; Xiao et al. 2013). Zebrafish lack orthologs to mammalian *Sry* and *Fgf9*. Other genes expression is still being elucidated.

are also needed for a fully functional reproductive system. The zebrafish reproductive system may develop similarly to mammals or other teleosts. So far I have discussed zebrafish gonad development, but what about zebrafish reproductive duct development?

The zebrafish reproductive tract

In zebrafish, the adult male reproductive tract differs from that of mammals because, like other teleosts, zebrafish do not form Müllerian or Wolffian ducts like mammals and zebrafish males and females do not initially form two sets of ducts as seen mammals. Instead the adult zebrafish male reproductive tract is more similar to the reproductive tract in medaka. The adult zebrafish male reproductive system is made up of the testes, ducti deferens, fused ducti deferens, and a genital orifice (Menke et al. 2011). The ducti deferens and fused ducti deferens of the adult zebrafish are similar to the sperm duct in medaka, but how do zebrafish reproductive ducts develop, and what genes are involved in this development? It is possible that a lot of factors contribute to zebrafish reproductive duct development. In mammals *Wnt4* up-regulates *Jnk*, which then up-regulates *Pax2* initiating Müllerian duct formation and elongation, and *Wnt4* is necessary for Müllerian duct formation in mammals (Mericskay et al. 2004; Kobayashi et al. 2011; Prunskaite-Hyyryläinen 2016). Loss of *Wnt4* function in mice also causes disorganized testicular cords, the most anterior part of the reproductive duct system, in the embryo; this is later corrected at birth (Jeays-Ward et al. 2004). Both the development of the male zebrafish reproductive tract and whether *wnt4a* is involved are currently unknown. Even though zebrafish reproductive ducts are structured differently than mammalian reproductive ducts, we must test the hypothesis that *wnt4a* is important for male reproductive duct development.

Chapters

Chapter II, which was previously published with co-authors, uses genome wide association studies (GWAS) to investigate the question of the genetic basis of sex determination in a variety of zebrafish strains, two 'wild-type' strains cultured for about 30 years in the lab, and four 'natural' strains isolated directly or recently from nature in India. Surprisingly, results identified a sex-associated region on zebrafish chromosome 4 in natural zebrafish strains that was lacking in the lab strains. These results indicate that the zebrafish strains currently used to investigate sex determination are strains that lack the sex-associated region, and therefore, these studies should be revisited using natural zebrafish strains that retain the natural genetic sex determinant. Even so, all strains, including the laboratory strains lacking the sex-associated region, make males and females indicating that all strains might share the same sex-determination mechanism that is downstream from the natural genetic sex determinants contained in the sex-associated region in wild-type strains, meaning that laboratory strains are still useful for identifying conserved genes in the zebrafish sex determination pathway.

Chapter III, which is unpublished but also involves co-authors, investigates the question of whether *wnt4a* is important for zebrafish ovarian development. In this study, we found that *wnt4a* is expressed in the early bipotential gonad and that loss of *wnt4a* results in male-biased sex ratios, indicating that *wnt4a* is important for zebrafish ovarian development. These results are similar to what was found in mammals, indicating that Wnt4a has a conserved function in the zebrafish gonad. Also, zebrafish lacking Wnt4a function have a phenotype similar to some SERKAL syndrome patients, who had full

female-to-male sex reversal, and could provide insight and a means for treatment discovery for SERKAL syndrome.

Chapter IV, which is unpublished with co-authors, investigates the question of whether *wnt4a* is important for male reproductive duct development. We found that loss of *wnt4a* slows the formation of male reproductive ducts and prevents the male fused ducti deferens from connecting to the genital orifice in zebrafish males and that *wnt4a* is expressed in tissue around the site where this connection should occur before and after the connection is formed. These results reveal a novel *wnt4a* phenotype in zebrafish that hasn't been seen thus far, and indicate that *wnt4a* is necessary for proper male reproductive duct development in zebrafish.

CHAPTER II

WILD SEX IN ZEBRAFISH: LOSS OF THE NATURAL SEX DETERMINANT IN DOMESTICATED STRAINS

This work was previously published as: Wilson C, **High SK**, McCluskey BM, Amores A, Yan YL, Titus TA, Anderson JL, Batzel P, Carvan MJ 3rd, Schartl M., Postlethwait J. (2014) Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. Genetics. 198(3): 1291-1308.

I worked with C. Wilson on the zebrafish portion of this paper. I helped collect, dissect, and sex zebrafish from the EkkWill, Cooch Behar, Wild India Kolkata, and Tuebingen strains, and extract and prep genomic DNA from these strains for RAD-tag genotyping. C Wilson analyzed the majority of the data. I helped analyze and interpret part of the data, specifically I helped find genes within the sex-associated region. I also helped design and test multiple sex-genotyping primers, including the Nadia sex-genotyping primers that worked successfully. I helped C Wilson write the initial rough draft of the paper, and critiqued the final draft of the paper. BM McCluskey worked on the phylogenic reconstruction. A Amores mapped the sex-associated RAD-tags to Chr4. YL Yan and JL Anderson collected Nadia and AB fish and prepped the libraries. TA Titus performed the Medaka library prep. P Batzel helped with statistical analysis. MJ Caravan supplied the EkkWill strain. M Schartl provided the Medaka.

Introduction

Considering the fundamental importance of sex for species propagation, it is surprising that primary sex-determining mechanisms are not strongly conserved among

animal taxa (Bull 1983; Charlesworth 1996; Ming et al. 2011; Bachtrog et al. 2014). Closely related species or even populations of the same species can have different sex-determining mechanisms (Takehana et al. 2007; Ross et al. 2009; Kobayashi et al. 2013; Heule et al. 2014; Larney et al. 2014). Zebrafish (*Danio rerio*) is a popular model for studies of vertebrate development, behavior, physiology, evolution, disease, and human health (Mills et al. 2007; Seth et al. 2013; Braasch et al. 2014; Ota and Kawahara 2014; Wilkinson et al. 2014), but researchers struggle with highly variable and distorted sex ratios, and investigations into the genetic nature of zebrafish sex determination are conflicting. To help understand these issues, we conducted a population genomic study of sex determination in multiple zebrafish strains.

Zebrafish exhibit a juvenile ovary phase in which the gonad contains meiotic oocytes in all individuals: in some juveniles, oocytes survive and the individual becomes a female, but in others, oocytes die from about 19 to 27 days post fertilization and the fish becomes a male (Takahashi 1977; Uchida et al. 2002; Wang et al. 2007; Rodriguez-Mari et al. 2010). In the absence of germ cells (Slanchev et al. 2005; Siegfried and Nusslein-Volhard 2008) and in mutants in which oocytes undergo apoptosis (Rodríguez-Marí et al. 2005, 2010; Rodríguez-Marí and Postlethwait 2011), gonads develop as testes and individuals become males. Remarkably, mutants that produce oocytes early and reproduce as females can transform into fertile males after oocyte depletion, showing that oocytes are necessary both for primary sex determination and for maintenance of female phenotype in adult zebrafish (Dranow et al. 2013).

As expected from the hypothesis that oocyte death is a major feature of zebrafish sex determination, harsh environmental conditions tend to shift sex ratios in favor of

males; such factors include gamma rays, hypoxia, high density, high temperature, altered thermocycles, and poor nutrition (Walker-Durchanek 1980; Shang et al. 2006; Lawrence et al. 2008; Abozaid et al. 2011, 2012; Liew et al. 2012; Villamizar et al. 2012). Zebrafish does not, however, have a typical environmental sex-determination (ESD) mechanism like some sauropsids for which temperature is a cue (Charnier 1966; Lang and Andrews 1994; Merchant-Larios and Diaz-Hernandez 2013; Mork et al. 2014). It is more probable that zebrafish is like medaka (*Oryzias latipes*) in having a genetic sex-determination mechanism that is sensitive to environmental conditions (Hattori et al. 2007; Sato et al. 2005; Selim et al. 2009; Hayashi et al. 2010).

Zebrafish not only lacks classic ESD, but most stocks investigated do not have cytogenetically detectable chromosomal sex determination (Schreeb et al. 1993; Pijnacker and Ferwerda 1995; Daga et al. 1996; Gornung et al. 1997; Amores and Postlethwait 1999; Gornung et al. 2000; Sola and Gornung 2001; Traut and Winking 2001; Phillips et al. 2006). In contrast, the only investigation of zebrafish taken directly from nature in India concluded that zebrafish females are the heterogametic sex (Sharma et al. 1998). The disagreement in cytogenetic results suggests that different zebrafish strains may have different karyotypic bases for sex determination.

Not only is the chromosomal nature of zebrafish sex unresolved, but its genetic sex-determination (GSD) mechanism remains elusive. Repeated matings of zebrafish pairs from AB, TU (Tuebingen), and Toh strains produce consistent sex ratios, but different pairs can give quite different sex ratios (Liew et al. 2012). This result is expected if zebrafish has a genetic basis for sex determination with polygenic control that differs among strains (Liew et al. 2012). Furthermore, recent studies of sex genetics in

different zebrafish strains identified sex-associated loci, confirming a genetic component to sex determination, but different studies identified different sex-associated loci (Orban et al. 2009; Siegfried 2010; Tong et al. 2010; Bradley et al. 2011; Anderson et al. 2012; Liew et al. 2012; Howe et al. 2013; Liew and Orban 2014). A cross between a female of the NA (Nadia) natural strain by a male of the AB laboratory strain identified a single sex-linked locus on zebrafish chromosome 4 (Chr4) while the reciprocal mating (female-AB-by-male-NA) showed sex-associated loci on both Chr4 and Chr3 (Anderson et al. 2012). In contrast, analysis of a female-AB-by-male-IN (India) cross-identified sex-linked loci on Chr5 and Chr16 but none on Chr3 or Chr4 (Bradley et al. 2011). An F_2 map constructed from a gynogenetic doubled-haploid TU female and a gynogenetic doubled-haploid AB male (i.e., both parents had only female-derived chromosomes) identified a sex-linked region on Chr16 that does not overlap with the one observed in the AB-by-IN mating (Bradley et al. 2011; Howe et al. 2013). Together, these results have been interpreted to support a polygenic sex-determination mechanism (Liew and Orban 2014), but they also support the hypothesis that different zebrafish strains utilize different genetic mechanisms to determine sex.

Mapping crosses like those cited above that mate two zebrafish strains that differ in their major genetic sex-determining mechanisms might give difficult to interpret or even spurious results due to epistatic interactions between loci. Furthermore, because environmental factors can influence zebrafish sex ratios, an individual's phenotypic sex may not match its genotypic sex. Thus, a traditional F_2 mapping cross may not identify a major sex-determining locus if one of the P_0 or F_1 individuals is by chance sex reversed.

In addition, brother-by-sister matings to make F_2 families and gynogenesis protocols lead to inbreeding, which results in strongly male-biased sex ratios (Brown et al. 2012a).

To help resolve the confusing state of zebrafish sex genetics, we conducted a genome-wide association study (GWAS) based on a population genomic analysis of RAD-tags (Baird et al. 2008) to identify SNPs found differentially in males or females (the "RAD-sex" method). This approach analyzes genotypes without regard to parentage and can identify loci of major effect (Atwell et al. 2010). To identify sex-specific SNPs in RAD-tags, we utilized Stacks, a program that infers genotypes from short-read sequences (Catchen et al. 2011, 2013). We investigated six zebrafish strains, including domesticated strains made lethal free for mutagenesis (AB and TU), natural strains cultured for a few years in the laboratory without deliberate genetic manipulations [EKW (EkkWill) and WIK (Wild India Kolkata)], and strains acquired directly from the wild in India [NA and CB (Cooch Behar)].

Analysis of >25,000 SNPs in each of these six strains identified alleles differentially associated with sex phenotype. In all four natural strains, results identified a single sex-associated locus at the end of the long (right) arm of Chr4 (Chr4R), a locus previously identified as *sar4* (*sex-associated region Chr4*) (Anderson et al. 2012). Results showed that the distribution of *sar4* alleles in populations and their inheritance patterns in crosses were consistent with natural zebrafish populations having a WZ/ZZ sex-determination system, as previously suggested (Tong et al. 2010), and are in accord with the reported WZ/ZZ karyotype inferred for a zebrafish population taken directly from the wild (Sharma et al. 1998). Surprisingly, our experiments failed to detect any sex-linked loci in either AB or TU, two strains domesticated for mutagenesis experiments

and sequenced for the zebrafish reference genome. Domestication led either to the evolution of new methods of sex determination during recent decades of selection by zebrafish researchers or to the unveiling of preexisting minor genetic sex-determining mechanisms.

Materials and Methods

Fish strains

Zebrafish (*D. rerio*) were raised in the University of Oregon Zebrafish Research Facility under standard conditions (Westerfield 2007). Strains included the following:

1. AB (ZFIN ID: ZDB-GENO-960809-7), originating from a mating of strain A and strain B purchased at two different times from a pet shop in Albany, Oregon, in the late 1970s and screened for making large numbers of lethal-free embryos by *in vitro* fertilization and subsequently bottlenecked through 21 gynogenetic half-tetrad individuals produced by early pressure treatment to establish the current AB strain (Walker-Durchanek 1980; Streisinger et al. 1981; Chakrabarti et al. 1983; C. Walker, personal communication)
2. TU (Tuebingen, ZFIN ID: ZDB-GENO-990623-3), originating from a German pet store and selected to be lethal free ca. 1990 from multiple single pair crosses (Mullins et al. 1994)
3. NA (Nadia, ZFIN ID: ZDB-GENO-030115-2, Anderson et al. 2012), the eighth generation of animals taken from nature in Nadia, India, in 2000
4. WIK (Wild India Kolkata, ZFIN ID: ZDB-GENO-010531-2), which “derives from a wild catch in India” (Rauch et al. 1997), presumably Kolkata, about 140 km south

of Nadia, originating from a single pair mating

5. EKW (EkkWill, ZFIN ID: ZDB-GENO-990520-2), zebrafish of unknown origin maintained for many years in large populations at EkkWill Waterlife Resources (Ruskin, FL), which has supplied fish to the pet store trade since 1962 (http://www.ekkwill.com/aboutekkwill. html) and obtained from M. Carvan (University of Wisconsin—Milwaukee) (Loucks and Carvan 2004)
6. CB (Cooch Behar), a new strain derived from fish collected in 2012 from Cooch Behar, India, $500 km north of Nadia, and purchased from Eugene Research Aquatics, LLC.

Cooch Behar individuals taken directly from the wild gave small clutches and showed greatly reduced sex dimorphism. Dissection of CB fish taken directly from nature revealed 28 females, 4 hermaphrodites that contained both ovary and testis tissue, 1 fish with translucent tissue at the location expected for gonads, and 8 males (20% males), suggesting disrupted sex development by stress, endocrine-disrupting substances during development, or differential survival during acquisition of these animals. Group crosses of adults from each strain generated populations of fish from which we arbitrarily selected individuals for RAD-sex analysis. AB fish (UO stock no. S22191) came from stocks that the University of Oregon Zebrafish Research Facility maintains for shared use involving 10 crosses, each with 2 males and 2 females. Our TU population (S23232) came from *in vitro* fertilization of eggs from 4 females mixed with sperm pooled from 12 males. NA fish (S23847) derived from a natural cross of an unknown number males and females. WIK fish came from two different generations, one (S23069) a natural cross of 1 female by 2 males and the other (S24746) derived from multiple natural crosses. EKW

fish were maintained in three tanks of 60 unsexed fish that were bred *en masse* by natural matings. CB individuals were from natural matings of the first generation offspring of 28 females and 8 males captured in India. The sex of each animal was determined by microscopic observation of dissected gonads. Individuals of undetermined sex were excluded from analysis.

A population of dwarf danio (*D. nigrofasciatus*), a close relative of zebrafish (Mayden et al. 2007; Tang et al. 2010), was raised in the University of Oregon fish facility under standard zebrafish culture conditions (Westerfield 2007). A population of medaka (*O. latipes*) from the Carbio strain (WLC#2674) was raised at the University of Würzburg according to standard laboratory practices (http://shigen.lab.nig.ac.jp/medaka/medakabook/index.php)(Kirchen and West 1976). Male and female medaka were selected at random from the standing aquarium population, which is maintained by natural matings of ~50 males and 50 females each generation. The phenotypic sex of medaka was first determined from secondary sex characteristics (shape of dorsal and anal fins, spines on male anal fin rays) and confirmed by macroscopic inspection of dissected gonads. The genotypic sex of medaka was identified from the presence or absence of the *dmrt1bY* gene by PCR from fin clip DNA essentially as described (Nanda et al. 2003) using allele-specific primers: DMT1k (59 CAA CTT TGT CCA AAC TCT GA 39) and DMT1l (59 AAC TAA TTC ATC CCC ATT CC 39) at an annealing temperature of 56C.

<u>RAD-tag genotyping</u>

Genomic DNA was isolated from caudal fin clips and muscle with a Qiagen DNeasy blood and tissue kit. DNA was digested with high-fidelity *SbfI* restriction

enzyme (New England Biolabs, no. R3642S). Barcode adapters five or six nucleotides long were ligated to each sample. Restriction-site associated DNA (RAD) libraries were prepared as described (Baird et al. 2008; Amores et al. 2011; Anderson et al. 2012) and were sequenced on an Illumina HiSequation 2000 or 2500 using 100-nucleotide single-end reads. We used *Stacks* software (http://creskolab.uoregon.edu/stacks/) to organize reads into loci and to identify polymorphisms (Catchen et al. 2011, 2013). We RAD-sequenced 20 males and 20 females from AB, 24 males and 24 females from TU, 25 males and 25 females from NA, 21 males and 37 females from EKW, 39 males and 28 females from WIK (of which 34 males and 27 females were retained for analysis), and 49 males and 28 females from CB. Illumina sequences were quality filtered with the *process_radtags* program of *Stacks* (Catchen et al. 2011) and then aligned to the zebrafish genome (v. Zv9)(Howe et al. 2013) using *GSNAP* (Wu and Nacu 2010), allowing nine mismatches. Medaka sequences were aligned to the *O. latipes* genome (v. MEDAKA1) (Kasahara et al. 2007). Sequences that aligned to multiple sites in reference genomes were discarded. Genotypes were called from aligned reads using the *refmap.pl Stacks* pipeline, requiring a minimum stack depth of 10 (2m 10). Reads that did not align to the genome were analyzed with the *denovo_map.pl Stacks* pipeline using the following parameters: a minimum stack depth of 10 (–m 10), up to three differences when merging stacks into loci (–M 3), and up to two differences between loci when building the catalog (–n 2).

Zebrafish linkage group numbers assigned based on genetic length (Postlethwait et al. 1994; Johnson et al. 1996) are given the name “chromosome” in the Zv9 version of the zebrafish reference genome; here we abbreviate “linkage group” as Chr according to

zebrafish nomenclature conventions (https://
wiki.zfin.org/display/general/zfin+zebrafish+nomenclature+ guidelines). Cytogeneticists numbered zebrafish chromosomes based on physical length (Pijnacker and Ferwerda 1995; Daga et al. 1996; Gornung et al. 1997, 2000; Amores and Postlethwait 1999; Sola and Gornung 2001), so zebrafish genetic linkage group number and cytogenetic chromosome number are generally not the same. We assigned genetic linkage groups to physical chromosomes by fluorescent *in situ* hybridization (Phillips et al. 2006), which showed that, for example, cytogenetic chromosome 3 is linkage group 4, called chromosome 4 in the reference genome and Chr4 in this work.

Sequences for zebrafish, medaka, and dwarf danio RAD-tags were archived under accession no. SRP044635 and mitochondrial sequences under KM196113–KM196120.

Statistical analysis

Haplotypes were exported from the Stacks web interface requiring a minimum stack depth of three reads, and a blacklist of overmerged tags was generated with a custom python script. The *Stacks ref_map* pipeline identifies RAD-tags based on position in the genome. Over-merged tags arise when different regions of the genome have highly similar sequences, so that genomically separate RAD-tags align to the same location, resulting in a stack of RAD-tags with a biologically impossible number of alleles. We defined overmerged tags as those that had more than two alleles in more than one fish, and excluded these tags from further analysis.

Polymorphic RAD-tags were exported from Stacks in genomic format using *Stacks' populations* program (Catchen et al. 2013). RAD-tags were required to be present in 75% of the individuals of each sex, and blacklisted tags were excluded. For each

polymorphic SNP, the program SNPstats1 (http://webpages.uidaho.edu/hohenlohe/software.html) (Hohenlohe et al. 2010) calculated a G-test statistic comparing genotypes in males and females. False discovery rate (FDR) was calculated using the Qvalue R package (Storey and Tibshirani 2003) according to the method of Benjamini and Hochberg (1995). Markers that were significantly associated with sex after the first round of analysis were then inspected by hand. An arbitrarily selected subset of five individuals was evaluated in the *Stacks* web interface for all loci significantly associated with sex to ensure that *Stacks* had called genotypes correctly. If an error was identified in any of these five individuals, then all fish in the panel were checked at that locus and corrected by hand; for example, if three or more reads of an undersequenced allele were present in a stack, the genotype was corrected to a heterozygote in the genomic output file and statistics were recalculated.

Four WIK males were excluded from analysis because they had numerous genotypes throughout the genome that were not present in the vast majority of other fish from the population, suggesting migrants. One male and one female WIK were excluded because they had numerous alleles that were undersequenced and uncalled by Stacks, likely due to barcode contamination.

Mapping unassembled scaffolds

Despite the high quality of the zebrafish reference genome (Howe et al. 2013), many scaffolds remain unassembled, especially in areas rich in repeats, such as the heterochromatic and late-replicating right arm of zebrafish cytogenetic chromosome 3 (genetic linkage group 4, Ensembl Chr4) (Pijnacker and Ferwerda 1995; Daga et al. 1996; Amores and Postlethwait 1999; Traut and Winking 2001; Phillips et al. 2006;

Anderson et al. 2012; Howe et al. 2013). Some RAD-tags that were strongly associated with sex reside on scaffolds that are either unassembled or are on Chr14 in Zv9. To test for misassembly, we used a previously published data set of *SbfI*-based RAD-tags mapped on the zebrafish HS (heat shock) meiotic recombination mapping panel (Kelly et al. 2000; Postlethwait et al. 2000; Woods et al. 2000, 2005; Catchen et al. 2011). Filtered raw reads from RAD-tagging the HS panel were aligned to the Zv9 assembly with GSNAP, allowing five mismatches. Because these gynogenetic fish were homozygous at all loci, we relaxed *Stacks* parameters and required a minimum stack depth of two reads (2m 2) to call genotypes. RAD-tags that aligned to Chr4 and Chr14 in the assembled genome and to all unassembled contigs and scaffolds were used to generate meiotic linkage maps for Chr4 and Chr14.

Markers were initially grouped in JoinMap 4.1 using the Independence LOD parameter under population grouping with a minimum LOD value of 8.0. Subsequent grouping was performed at a minimum LOD value of 6.0. Marker ordering was performed using the maximum-likelihood algorithm in JoinMap 4.1 with default parameters. The expected recombination count feature in JoinMap4.1 was used to identify individuals with a higher than expected number of recombination events and visual inspection of marker order was performed. When needed, marker order was optimized manually after visual inspection of the colorized graphical genotypes in JoinMap 4.1. If moving a marker or group of markers reduced the total number of recombination events, the marker was manually moved to the new position. This analysis resulted in the positioning of many unplaced contigs and scaffolds across the entire Zv9 reference genome.

Nadia sex-genotyping primers

A pair of primers (NA_sx.F_5-CCGGCCCTCAAGGACCGAAA-3 and NA_sx.R_5-GGTTGCTCAAGTGTTGGTGAGA-3) was designed within the sequence of a RAD-tag that aligned to Chr14:37,865,815–37,865,909 and included a sex-specific indel in the NA strain. GoTaq Flexi DNA polymerase (Promega, M8298) was used to amplify the product with the following PCR protocol: denaturation at 94C for 6 min, 40 cycles of 94C denaturation, 55C annealing, and 72C extension followed by a final extension step of 72C for 10 min.

Phylogenetic reconstruction

To infer the history of zebrafish strains, we sampled RAD-tag sequences from 10 females and 10 males arbitrarily selected from each strain and identified 9,442 RAD-tag loci that were present in all 120 samples. To root the tree, we included orthologous RAD-tags from 1 male and 1 female *D. nigrofasciatus* for the 4,765 RAD-tags for which orthology could be inferred across species. RAD-tag loci were considered orthologous across strains and species if they mapped to the same unique location in the zebrafish reference genome Zv9. Tags with more than one best mapping location were excluded. We aligned sequences from each RAD-tag locus using Muscle (v. 3.8.31) with default parameters. Aligned loci were concatenated into a single 887.5-kb alignment. Using this alignment, we inferred phylogenetic trees and performed bootstrap replicates with RAxML (v. 7.2.8) using maximum parsimony and maximum likelihood under a GTR+I+Γ model with *D. nigrofasciatus* as the outgroup (Stamatakis 2014).

For zebrafish strains, we assembled sequences of the mitochondrial *cytochrome-b* gene from Illumina reads arising from small amounts of contaminating mitochondrial DNA. All reads used for RAD-tag analysis in *Stacks* have 6 bases left by the *SbfI* enzyme digestion (TGCAGG); reads without this sequence are from contaminating nuclear or mitochondrial genomic DNA. Reads with a correct barcode, but lacking a TGCAGG motif were identified with the *process_radtags* program from *Stacks* (Catchen et al. 2011) and aligned to the zebrafish genome using *GSNAP* (February 20, 2014, release) (Wu and Nacu 2010).

Results

Verifying the RAD-sex method: Medaka

To verify that a RAD-tag-based population genomics approach would identify a major sex-determination locus if one exists, we first tested a species in which the sex chromosome and major sex-determination locus has already been identified. Although Japanese medaka has a strong XY sex-determination system based on the sex-determining gene *dmrt1bY* located on chromosome Ola1 (*O. latipes* chromosome 1) (Matsuda et al. 2002; Nanda et al. 2002; Kondo et al. 2006, 2009), environmental temperature can override the system and cause genotypic females to develop as phenotypic males (Sato et al. 2005; Hattori et al. 2007; Selim et al. 2009; Hayashi et al. 2010). We analyzed 21,909 RAD-tags (Table 1) from 30 female and 31 male medaka and conducted a G-test for genotypes that are significantly associated with male or female sex at 36,115 SNPs. Although the medaka Y chromosome is fully assembled, including *dmrt1bY* in the middle of Ola1 (NCBI accession nos. AP006150– AP006153 (Kondo et

al. 2006)), the male-specific region is not assembled well in the reference genome sequence (http://www.ensembl.org/Oryzias_latipes/Info/Index) and *dmrt1bY* itself is on the unassembled scaffold1535, which contains no *SbfI* site and hence no RAD-tags, thus excluding *dmrt1bY* from our analyses. Nevertheless, results revealed SNPs between 14.3 and 32.5 Mb on Ola1 that were strongly associated with sex (Figure 2.1A). Only six sex-linked RAD-tags failed to align to the reference genome, none of which were as strongly associated with sex as the best hits within the genome. In the medaka reference genome, a region of 18.2 Mb around the position of the sex-determining locus *dmrt1bY* contained polymorphisms that are highly associated with sex, and 3.5 Mb of this region showed 100% correlation with the genotypic sex that had been determined previously by PCR, consistent with recombination suppression over the region showing strongly sex-specific RAD-tags. PCR genotyping and RAD- sex analysis both agreed that 2 of 31 medaka individuals with a male phenotype had a female genotype, showing that despite occasional sex reversal, the RAD-sex method is robust enough to identify sex-linked markers. Sex-linked SNPs were also identified in single RAD-tags on Ola3, Ola13, and Ola17. These SNPs are in linkage disequilibrium with the sex-linked SNPs on Ola1 (Figure 2.1, B and C), a result expected if these parts of Ola3, Ola13, and Ola17 were misassembled in the medaka reference genome. These experiments show that a RAD-tag-based GWAS study is an effective method for identifying a major sex-determining region despite occasional sex reversal.

Identifying a major zebrafish sex-determination locus

To identify loci linked to sex phenotype in zebrafish, we initially analyzed *SbfI*-associated RAD-tags (Table 2.1) from the widely used AB and TU strains. We tested 20

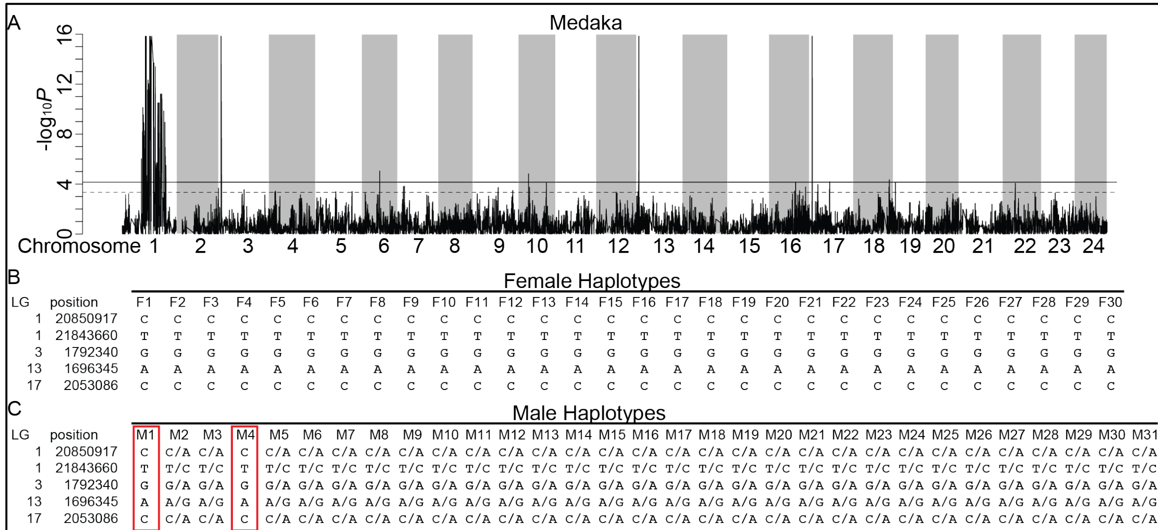


Figure 2.1 - RAD-sex for the medaka (*O. latipes*) assembled genome sequence. (A) The -log10P of a G-test of genotypes associated with male or female phenotype plotted against position in the 24 medaka linkage groups, with odd-numbered linkage groups having a white background and even-numbered linkage groups having a gray background. The solid horizontal line represents a q-value of 0.01, and the dashed line represents a q-value of 0.05. The analysis identified a broad peak of sex-associated SNPs on chromosome 1, (Ola1) the medaka sex chromosome. Isolated single SNPs on chromosomes 3, 13, and 17 were also highly linked to sex. (B) Medaka females were homozygous at SNPs strongly linked to sex, as expected from an XX karyotype, on Ola1 (only two of which are shown), and Ola3, Ola13, and Ola17. (C) Most medaka males were heterozygous at SNPs strongly linked to sex, as expected from an XY sex-determination system. Phenotypic males M1 and M4 had a female genotype. SNPs on Ola3, Ola13, and Ola17 were in linkage disequilibrium with sex-linked SNPs on Ola1, a result that would occur if these regions are on Ola1 in the fish genome but have been misassembled in the medaka reference genome sequence.

males and 20 females of the AB strain and 24 males and 24 females of the TU strain and compared results to 25 males and 25 females from NA, a natural strain from India. We analyzed 31,002 SNPs in AB and 40,391 SNPs in TU but found no markers that were significantly linked to sex (Figure 2.2, A and B). In contrast, analysis of 26,291 SNPs in NA fish revealed several sharp peaks of association with phenotypic sex. Sex-linked SNPs in NA were located at the right end of Chr4 between position 61.1 and 62.1 Mb, in

the middle of Chr14 between 37.87 and 37.90 Mb, and on the "Not Assembled" contigs NA482 and NA683 (Figure 2.2C).

Puzzled by the differences between AB and TU fish vs. NA fish, we analyzed two additional commonly used wild-type strains: WIK and EKW. We analyzed 39,256 polymorphic sites in 34 male and 27 female WIK fish and identified sex-associated SNPs located between 61.4 and 62.1 Mb at the end of Chr4, between 37.87 and 37.90 Mb on Chr14, and on the unassembled contigs NA482 and NA683 as found for the NA strain; in addition, sex-associated SNPs for WIK appeared on contig NA851, scaffold3519, and scaffold3545 (Figure 2.2D). Analysis of 49,182 SNPs in 21 male and 37 female EKW fish again identified sex-associated SNPs on Chr4 between 61.1 and 61.2 Mb, and the unassembled fragments NA851 and scaffold3519, both of which were also identified in the WIK RAD-sex results (Figure 2.2E). No locus on Chr14 was associated with sex in EKW.

To verify the discrepancy between domesticated and natural stocks, we obtained zebrafish directly from a natural population in Cooch Behar in India. We bred wild-caught individuals in the lab to establish the CB strain and used some of the offspring of wild-caught individuals for RAD-sex analysis. We analyzed 94,497 polymorphic sites in 49 males and 28 females (this strain showed much higher levels of heterozygosity than any of the other stocks examined) and identified sex-linked SNPs on Chr4:60.6 Mb and on scaffold3519, which was also found to harbor sex-linked SNPs in WIK (Figure 2.2F).

DNA for the zebrafish reference genome sequence initially came from several thousand TU embryos (Howe et al. 2013), some of which would have become males and others females, despite our finding that TU had no loci strongly linked to sex phenotype.

Table 2.1 Number of RAD-tags analyzed for each population tested												
	Total no. RAD-tags in population			Tags present in at least 75% of Individuals								
				Total no. RAD-tags			Polymorphic RAD-tags			SNPs analyzed		
Population	Denovo	Refmap	Total	Denovo	Refmap	Total	Denovo	Refmap	Total	Denovo	Refmap	Total
Medaka												
Carbio	8054	65849	73903	2802	51309	54111	1034	20875	21909	1588	34527	36115
Zebrafish												
AB	6455	54389	60844	1789	33542	35331	440	15099	15539	709	30293	31002
Tuebingen	6408	80284	86692	1983	46844	48827	469	19535	20004	770	39621	31002
Nadia	12020	44314	56334	1430	20705	22135	536	11828	12364	977	25314	26291
WIK	15792	138963	154755	3223	44481	47704	751	19143	19894	1202	38054	39256
EkkWill	14440	119701	134141	2303	40218	42521	772	22396	23168	1328	47854	49182
Cooch Behar	21712	75353	97065	1335	33149	34484	1161	28494	29655	3158	91339	94497

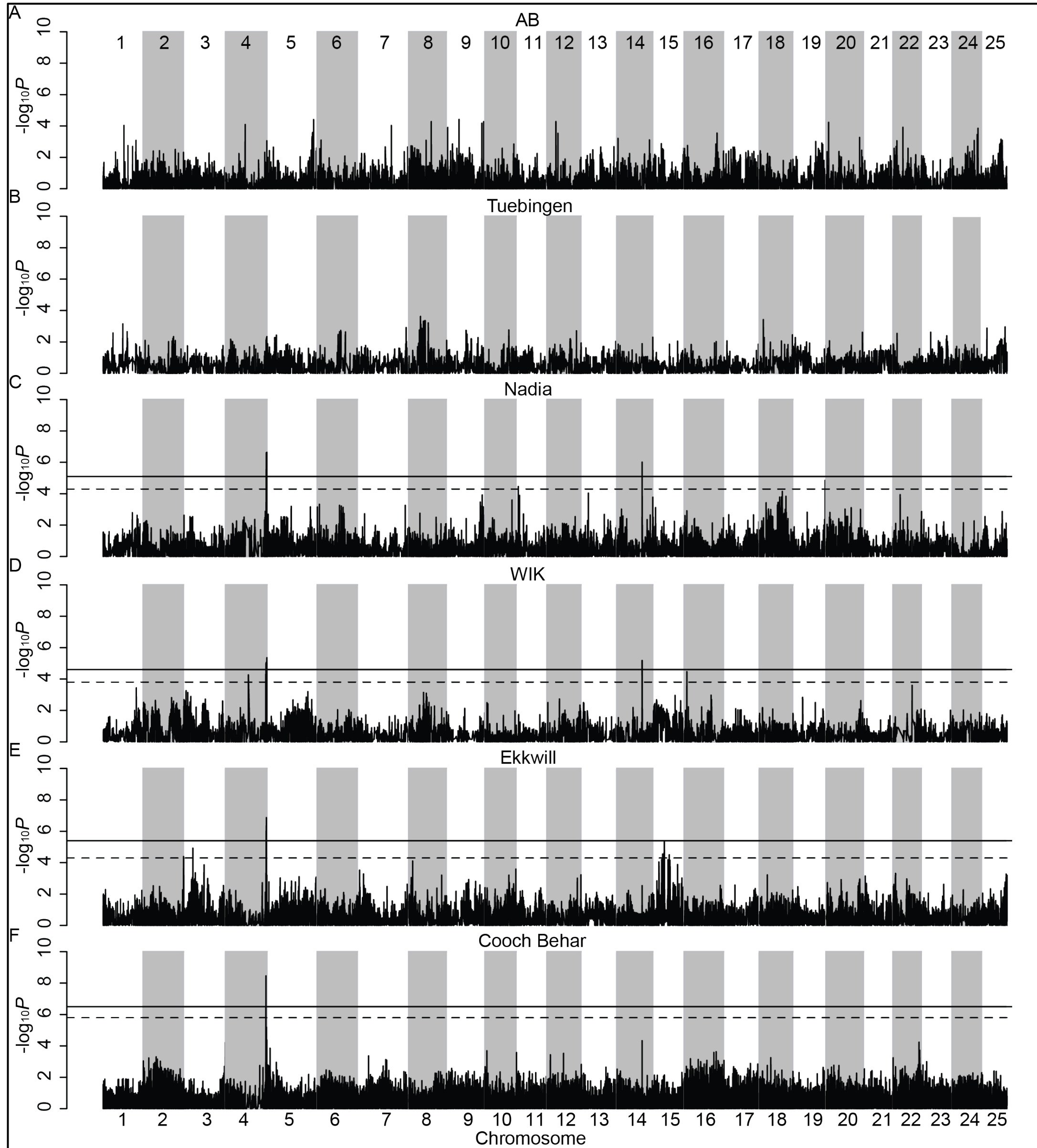


Figure 2.2 - RAD-sex results for zebrafish strains show plots of the -$\log_{10}$P of a G-test of genotypes associated with male or female phenotype in zebrafish plotted against the 25 linkage groups of the assembled zebrafish genome Zv9, with odd-numbered linkage groups having a white background and even-numbered linkage groups having a gray background. Solid lines represent a q-value of 0.01, and dashed lines represent a q-value of 0.05. No SNPs were significantly associated with sex in (A) AB or (B) Tuebingen strains. In contrast SNPs significantly associated with sex were identified on Chr4 and Chr14 in (C) Nadia and (D) WIK, while only loci on Chr4 were associated with sex in (E) EkkWill and (F) Cooch Behar. In addition to the assembled genome, sex-associated SNPs appeared on unassembled contigs NA482 and NA683 for WIK and NA, NA851 for WIK and EKW, scaffold3519 for WIK, EKW, and CB, and scaffold3545 for WIK.

If the reference genome lacked one of the sex chromosomes, then many sex-linked RAD-tags from wild stocks would fail to align to it. Analysis, however, showed that only one sex-linked RAD-tag in NA, one in EKW, and four in WIK failed to align to the zebrafish reference genome. SNPs in all nonaligning sex-linked RAD-tags were less strongly associated with sex than those that actually aligned to assembled or unassembled portions of the Zv9 reference genome. This result suggests that the assembly does not lack substantial amounts of any sex chromosome or that the major sex determinant is in a genomic region or unassembled contig with no *SbfI* sites, as is true for medaka.

Sex-linked unassembled scaffolds map to the sex-associated region at the right tip of Chr4R

While all four natural populations we studied had a strong sex-associated region near the right telomere of Chr4, several strains showed sex-associated loci at other locations, including a small portion of Chr14 and a number of unassembled contigs and scaffolds. Suspecting that the Chr14 locus and unassembled contigs and scaffolds belong on Chr4R, we utilized a previously published data set of RAD-tags generated from the zebrafish HS meiotic mapping panel (Kelly et al. 2000; Postlethwait et al. 2000; Woods et al. 2000; Woods et al. 2005; Catchen et al. 2011) to identify the position of these loci on a genetic map and hence to correct possible misassembly of these regions in Zv9. Results showed that nonassembled contigs NA683 and NA851 and scaffold3519, which are linked to sex in multiple natural populations, all localized to the end of Chr4 near other sex-linked tags in the meiotic mapping panel (Figure 2.3). Sex-associated fragments NA482 and scaffold3545 lacked any RAD-tags that were polymorphic in the meiotic mapping panel and so could not be mapped. Two sex-associated RAD-tags that had

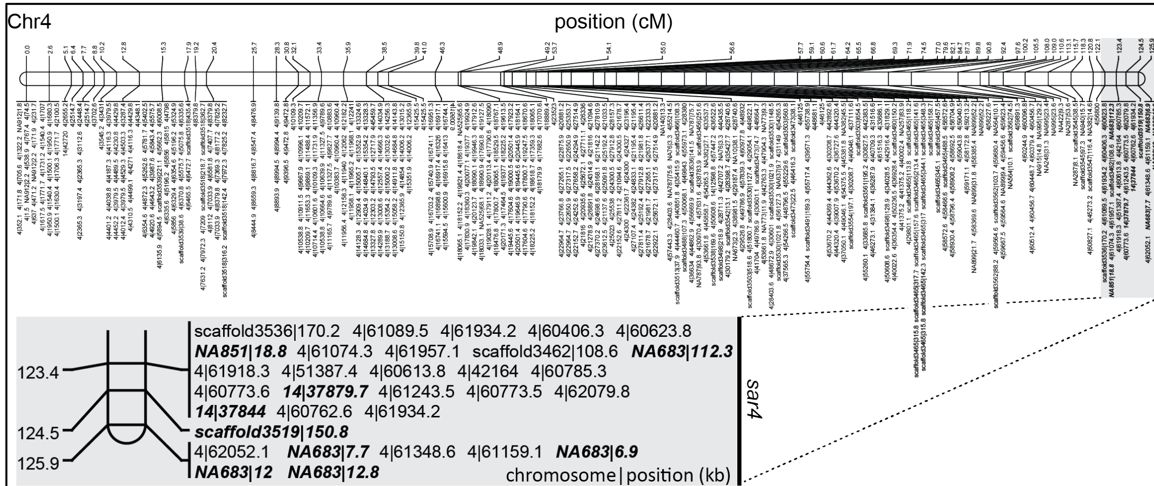


Figure 2.3 - All sex-associated RAD-tags map to Chr4. Sex-associated SNPs were mapped on the HS meiotic mapping cross panel (Kelly et al. 2000; Postlethwait et al. 2000; Woods et al. 2000; Catchen et al. 2013). The *sar4* region is indicated by shading. RAD-tags that aligned to Chr14 at 37.8 Mb and to the unassembled scaffolds NA683, NA851, scaffold3519, scaffold3462, and scaffold3536 (indicated in boldface italic type) all mapped to the distal tip of Chr4R on the HS panel.

aligned to Chr14 at positions Chr14:37,844,151 and Chr14:37,879,718 also mapped to Chr4R near other sex-linked RAD-tag polymorphisms. The finding that all mappable sex-linked RAD-tags in four natural strains (NA, WIK, EKW, and CB) occupy a single 1.5-Mb region at the right tip of Chr4 supports the conclusion that Chr4 represents a sex chromosome in natural populations of zebrafish. The failure to detect any sex-linked loci in AB and TU in these analyses suggests that a wild sex determinant was lost or greatly modified in the domestication of zebrafish for laboratory work and that other mechanisms have since taken the place of the natural wild genetic sex-determination system.

Wild zebrafish populations have a ZW/ZZ sex chromosome system

To determine whether natural zebrafish utilize an XY or ZW sex-determination system, we scrutinized the SNP with the strongest statistical support for linkage to sex phenotype in each of the four natural populations. In NA, the SNP most strongly

associated with sex was an A . T (nucleotide A in the reference genome vs. T in NA) polymorphism on the unassembled contig NA683 at nucleotide position 12,119 with support of $-\log_{10}P = 6.8$. All 15 fish with the homozygous T/T genotype were males, while 77% (23/30) of A/T heterozygotes developed as females. No individual developed as a female that did not have at least one A allele at this SNP. Only one individual was homozygous A/A, and it, rather surprisingly, developed as a male (Figure 2.4A). An A/A male could result from sex reversal or from recombination events that separated the sex-linked RAD-tag locus from the causative sex-determination locus. The rather small size of the zebrafish sex-associated region, about 1.5 Mb (60.6–62.1 Mb when considering all four natural strains and including all SNPs with a q-value ,0.01) compared to the large region we detected in medaka (14.3–32.5 Mb, or 18.2 Mb using the same q-value) suggests that recombination suppression is stronger around the medaka sex locus than around the zebrafish sex locus.

These data for NA show that: (1) the A allele of this SNP in NA is linked to a dominant factor that is necessary but not sufficient for development of a female phenotype; (2) the NA strain has a heterozygous female/homozygous male (e.g., ZW female/ZZ male) sex-determination system; (3) some A/T and A/A “genetic females” are sex reversed to a male phenotype due to the effects of the environment or to segregating minor genetic modifiers that our protocol could not detect; and (4) the small number of individuals with an A/A genotype could be due to the infrequent mating of a normal heterozygous A/T genotypic female to a sex-reversed heterozygous genotypic A/T male, that the homozygous A genotype is semi lethal, or that these genotypes come from a recombination event between the RAD-tag and the functional sex locus.

This pattern was repeated in the other natural populations. In WIK, an A . C polymorphism at Chr4:62,060,103 showed the strongest statistical support for sex linkage ($-\log_{10}P = 5.4$). All 16 homozygous C/C WIK fish were male, but 64% (18/28) of A/C heterozygotes and 60% (9/15) of A/A homozygotes were females (Figure 2.4B). In EKW, an A . T polymorphism at scaffold3519:177,330 was statistically most strongly linked to sex ($-\log_{10}P = 12.5$).

All 18 A/A homozygotes developed as males, while 94% (32/34) of A/T heterozygotes and all four T/T homozygotes became females (Figure 2.4C). In CB, a C . T polymorphism at Chr4:60,623,846 was statistically most strongly linked to sex ($-\log_{10}P = 8.5$). All 24 homozygous T/T fish were male, while 52% (17/33) of heterozygous T/C individuals and all seven homozygous C/C fish were female (Figure 2.4D). While the CB result could be interpreted as supporting a balancing sex-determination system, with T/T fish developing as males, C/C fish developing as females, and heterozygotes developing as either sex, a more parsimonious explanation that mirrors other native strains is that the C allele is linked to a locus that is necessary but not sufficient for female sex development and that homozygous C/C fish come from the mating of a normal T/C female to a sex-reversed phenotypic T/C male. Taken together, data from all four wild stocks provide strong support for a female-heterogametic sex-determination system.

Segregation in a mapping cross supports a ZW/ZZ sex chromosome system in wild zebrafish

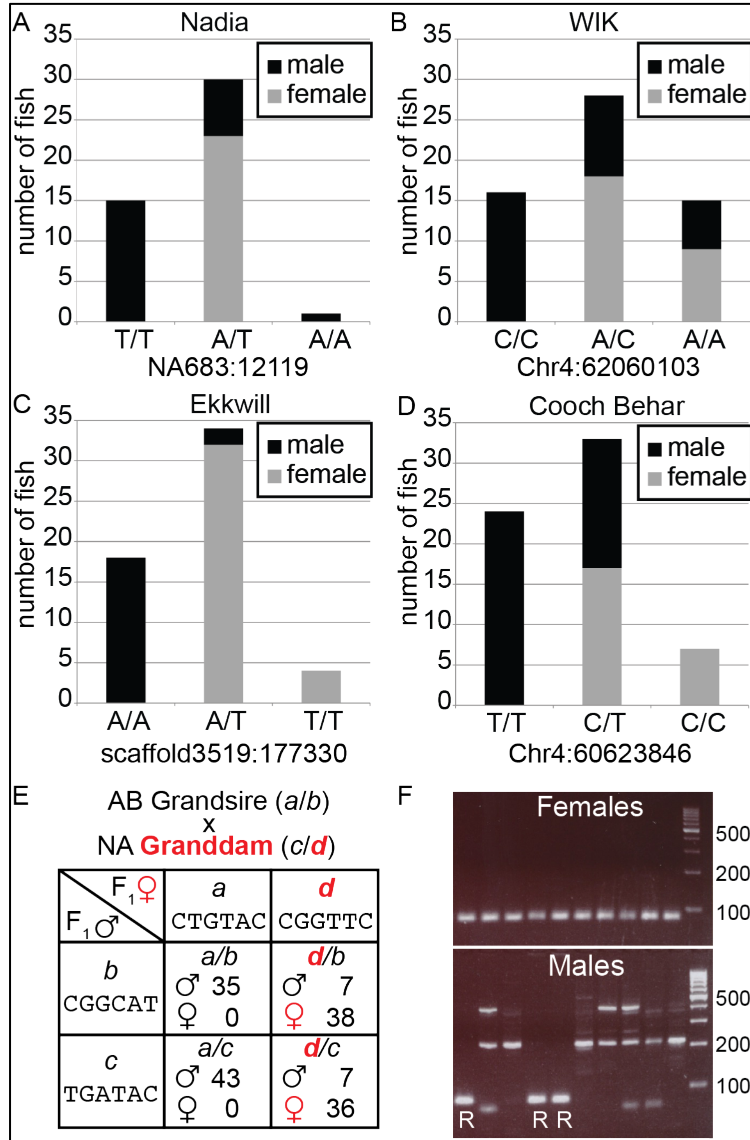
Reanalysis of a female-NA-by-male-AB F_2 mapping panel (Anderson et al. 2012) supports female heterogamety. For RAD-tag 32204 (Anderson et al. 2012), all 78 F_2 individuals that inherited the grandsire's *a* allele became males, but a is not a male-specifying allele because it was inherited through the F_1 female. The grandsire's *b* allele and the granddam's *c* allele were about equally likely to be found in males and females in the F_2 (52 and 58% males, respectively), so they are unrelated to sex differentiation. In contrast, 84% (74/ 88) of F_2 fish with the granddam's *d* allele, inherited through the F_1 female, themselves became females and no fish without the *d* allele became a female (Figure 2.4E). These results show that a sequence linked to the *d* allele is necessary but not sufficient for female development in this female-NA- by-male-AB cross, independent of the other allele, consistent with a dominant, environmentally sensitive, female-determining locus at *sar4* and female heterogamety.

Genotyping sex in NA

One of the RAD-tags (4086) that was tightly linked to sex in NA ($-\log_{10}P = 6.0$) contained a 12-bp indel with the insertion present in the female-linked allele. Although this RAD-tag aligned uniquely to Chr14:37,865,815–37,865,909 in Zv9, it mapped to the right tip of Chr4 on the HS meiotic mapping panel (Figure 2.3). Within the RAD-tag, we designed a forward primer containing four female-specific SNPs and a reverse primer with one female-specific SNP that amplify a single 85-bp band in NA females and a 73-bp band or a non-specific banding pattern in NA males (Figure 2.4F). We tested all 50

individuals from our NA population genomic analyses, of which *Stacks* had genotyped 24 phenotypic females and 22 phenotypic males at this locus. PCR verified the genetic sex determined by RAD-tag analysis for all individuals, including those experiencing female-to-male sex reversal. These primers also accurately identified the sex of individuals that *Stacks* did not genotype at this locus (due to insufficient read depth) but were genotyped for sex at nearby SNPs.

Figure 2.4 (on next page) - Genotypes for SNPs linked to sex phenotype with the highest statistical significance for four natural populations of zebrafish. (A) Nadia SNP at NA683:12,119 (nonassembled contig:nucleotide position), A . T, $-\log_{10}P = 6.8$. All T/T fish were male; 77% of A/T fish were female; the only A/A fish was male. (B) WIK SNP Chr4:62,060,103, A . C, $-\log_{10}P = 5.4$. All C/C fish were male; 64% of A/C and 60% of A/A fish were females. (C) EkkWill SNP at scaffold3519:177,330, A . T, $-\log_{10}P = 12.5$. All A/A fish were male; 94% of A/T and all T/T fish were females. (D) Cooch Behar SNP Chr4:60,623,846, C . T, $-\log_{10}P = 8.5$. All T/T fish were male; 52% of T/C and all seven C/C fish became female. In each of the four populations, all individuals homozygous for the “male allele” (T in NA, C in WIK, A in EKW, and T in CB) developed as males, heterozygotes (A/T in NA, C/A in WIK, T/A in EKW, and C/T in CB) developed mostly as females, and homozygotes for the “female” allele (A in NA, A in WIK, T in EKW, and C in CB) were rare and usually female. In each strain, fish with the homozygous “male allele” all became males and no individuals without the “non-male allele” (the “female allele”) developed into a female. Individuals homozygous for the “female allele” were obtained much less frequently than expected from random mating, which would be expected either if there were few matings between a male and a female both of which had at least one “female” allele or if homozygotes for the “female allele” were less likely to survive. These patterns would be expected of a WZ female/ZZ male sex-determination system with some female genotypes sex reversing to become males. (E) Analysis of the AB 3 NA F2 sex mapping cross (Anderson et al. 2012). RAD tag 32204, for example, which aligns to Chr4:61,934,186–61,934,280, has allele d that is present in the granddam, the F1 female, and all F2 females as expected if it resides on the W of a WZ/ZZ sex-determination system. (F) Sex-genotyping primers for NA. In the NA strain, the “female allele” in RAD-tag 4086 (which aligned to Chr14:37,865,815–37,865,909 in Zv9 but mapped to the right tip of Chr4 on the HS meiotic mapping panel, Figure 3) has a 12-nt deletion relative to the reference sequence. Primers with the sequence of the female allele in this RAD-tag gave an 85-bp fragment in females but produced an alternative amplification pattern in males. Sex-reversed individuals (as determined by this and other sex- linked RAD-tags) are indicated with an R.



After verifying primers on the RAD-sex population, we tested other NA fish. Of eight phenotypic females tested, all had the female genotype and of eight phenotypic males tested, seven had the male genotype; the other phenotypic male had a female genotype, consistent with occasional female-to-male sex reversal observed in other natural stocks. These results show that this primer pair can identify NA fish that will definitely become males or that have a high probability of becoming females. These sex-genotyping primers

will be useful for identifying genetic sex long before phenotypic sex becomes evident, which will facilitate the study of developmental mechanisms.

Phylogenomics of sex determinant loss

To determine whether our four natural strains represent separate accessions from the wild and to understand their historical relationships to the two strains that lack the Chr4R sex determinant, we conducted a phylogenetic analysis using RAD-sex sequence data. We collected RAD-tag genomic data from dwarf danio (*D. nigrofasciatus*), one of the closest extant relatives of zebrafish, for use as outgroup (Mayden et al. 2007; Tang et al. 2010). Because *D. nigrofasciatus* is native to the Sittang basin in Myanmar, which is far outside the range of *D. rerio*, the two species are not sympatric (Engeszer et al. 2007; Whiteley et al. 2011). Analysis utilized 888 kb of sequence from 9442 RAD-tags present in all six *D. rerio* strains, including 58,282 variable positions of which 51,444 SNPs were parsimony informative.

Analysis of the maximum-likelihood tree (Figure 2.5) and the maximum-parsimony tree provided several conclusions:

1. Maximum-likelihood and maximum-parsimony both gave strong support for the same phylogenetic relationships among strains. In contrast, relationships between individuals within a strain varied between the two approaches and across maximum-likelihood bootstrap replicates.
2. Analyses consistently recovered the same relationships among the three terminal taxa [EKW (AB,TU)] across bootstrap replicates under a GTR+I+G model. Because AB and TU occupied a monophyletic clade that was sister to EKW, phylogenomics does not resolve the question of whether the lack of the Chr4R

sex-determination locus in AB and TU is due to two independent events during their separate and independent routes to domestication in Oregon and Germany or to a single event that occurred either in nature or in the pet trade before the divergence of these two populations.

3. Recent extractions from East India (NA, WIK, CB) were genetically distinct from the [EKW (AB,TU))]clade, although relationships among NA, WIK, and CB varied across bootstrap replicates, as evidenced by poor support of the respective internal nodes (41 and 43%, Figure 2.5). The inability to resolve relationships among these strains even with a large amount of available sequence data are due to variation in the placement of the root of the tree. The length of the branch between zebrafish and dwarf danio is orders of magnitude longer than the length of the internal nodes separating strains because dwarf danio lacks zebrafish-specific RAD-tags. Unfortunately, outgroups closer to zebrafish are as yet unknown.
4. CB appears to have two subclades, suggesting population substructure within that isolate. In addition, CB harbors more genetic variation than the other strains as evidenced by the depth of the root of that strain and analysis of heterozygosity (Figure 2.5). This result is expected for the offspring of individuals taken directly from nature.
5. Males and females do not group separately within strains, showing that standing genetic variation in the bulk of the genome masks any sex-specific differences in the phylogenetic analyses. In sum, phylogenomics showed that each of the six strains used is a distinct population, that the most recently accessed natural strains

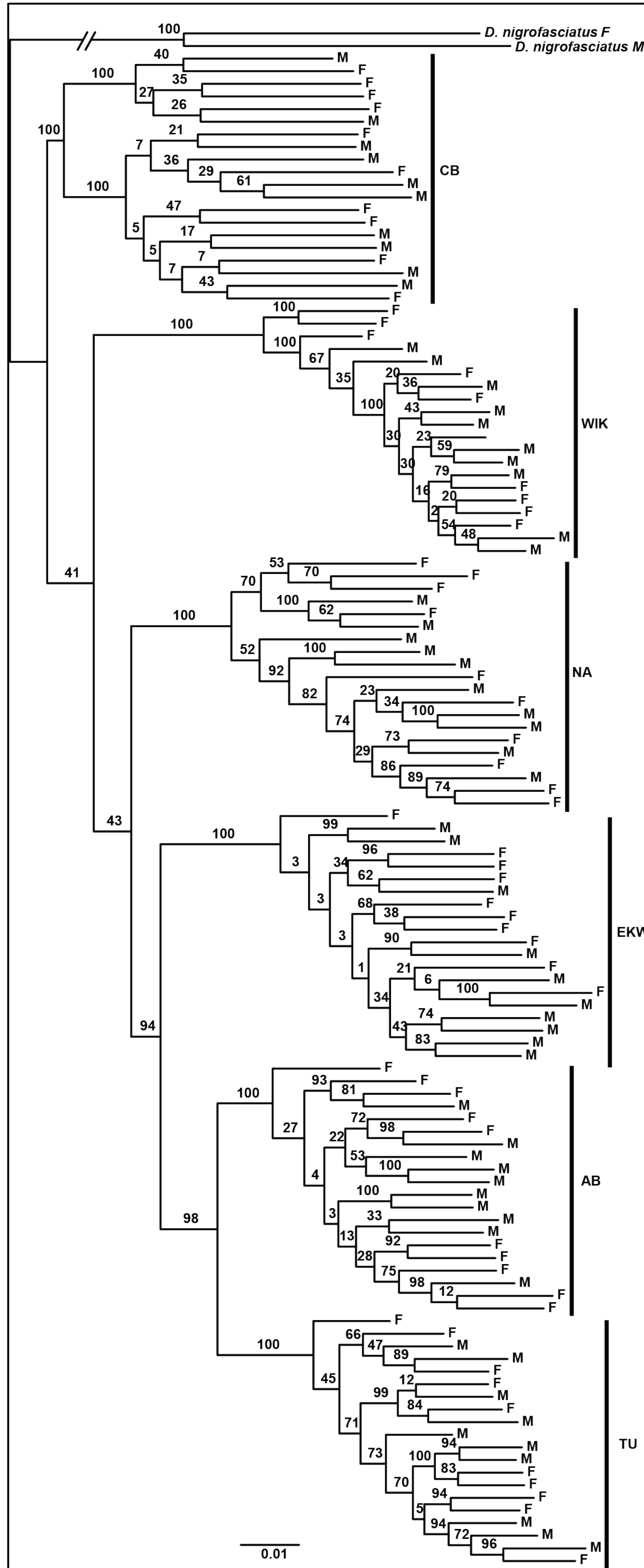


Figure 2.5 - Maximum-likelihood phylogeny of six *D. rerio* strains. The phenotypic sex of each individual is abbreviated as F (female) or M (male). Branch labels reflect bootstrap support. Each zebrafish strain represents a distinct clade.

lie basal in the tree, and that the domesticated strains AB and TU are rather closely related among the strains we tested.

To determine whether our strains cover the broad diversity of zebrafish natural variation, we investigated cytb, encoded by the mitochondrial genome. Zebrafish populations collected widely across India fall into three major clades: haplogroup 1 in Northern India and Eastern and Western Nepal, haplogroup 2 in Bangladesh and Southern India, and haplogroup 3 in Central Nepal (Whiteley et al. 2011). Infrequent mitochondrial genome contamination in RAD-tag libraries allowed us to reconstruct the cytb locus and assign populations to mitochondrial clades (accession nos. KM196113–KM196120). Results identified 14 SNPs that were shared across all our strains and all fish of haplogroup 1 but were absent from all haplogroups 2 and 3, showing that NA, WIK, EKW, and CB all derived from haplogroup 1 (Whiteley et al. 2011). Among the six strains we used and the 16 populations in the Whiteley et al. (2011) study, only AB appeared in both studies, and in both cases was in haplogroup 1. We conclude that our samples represent a single branch of the broad diversity of mitochondrial lineages across the zebrafish species.

Discussion

A major genetic sex determinant is linked to the distal tip of Chr4R in natural zebrafish populations RAD-sex analysis identified a 1.5-Mb interval containing a major genetic sex determinant near the telomere of zebrafish Chr4R in two natural laboratory strains (WIK and EKW) and in two recent accessions from the wild (NA and CB). These four natural strains have not been extensively manipulated for domestication and were derived from natural populations in India (NA, WIK, and CB) or are of unknown, but

likely unmanipulated, origin (EKW). Results also detected sex-linked loci within a small portion of Chr14 and on several unassembled contigs and scaffolds, as might be expected if zebrafish sex phenotype is polygenic (Tong et al. 2010; Bradley et al. 2011; Anderson et al. 2012; Liew et al. 2012; Howe et al. 2013; Liew and Orban 2014). To determine whether these several sex-linked sequences represented a polygenic sex-determination system or whether they were due to errors in the genome assembly, we mapped the unassembled and Chr14 sex-linked loci on a meiotic mapping panel. Because results showed that all sex-linked sequences map to the right tip of Chr4 in Zv9, we conclude that natural wild zebrafish have a single major sex-determining region within a short segment of Chr4R, *sar4*.

In natural zebrafish populations, Chr4 is likely a sex chromosome

The discovery of a strongly sex-linked locus only in Chr4R in natural zebrafish raises the question of whether Chr4 is a sex chromosome. Genetically defined Chr4 (Postlethwait et al. 1994; Johnson et al. 1996) corresponds to cytogenetic chromosome 3 (Phillips et al. 2006), the right arm of which is the only arm that is late replicating and heterochromatic (Pijnacker and Ferwerda 1995; Gornung et al. 1997; Amores and Postlethwait 1999; Gornung et al. 2000; Sola and Gornung 2001; Traut and Winking 2001; Phillips et al. 2006). Chr4R is impoverished in protein-coding genes, contains most of the genome's 5S–RNA genes, is enriched in satellite repeats, and has high GC content (Anderson et al. 2012; Howe et al. 2013); these properties are shared with sex chromosomes in other species (Peichel et al. 2004; Charlesworth et al. 2005). Finding sex-linked markers only on the sole chromosome arm with cytogenetic properties of sex

chromosomes is consistent with the hypothesis that Chr4 is a sex chromosome in zebrafish.

Females are the heterogametic sex in natural zebrafish populations

The conclusion that Chr4 is a sex chromosome raises the question of whether zebrafish has a female-XX/male-XY, a female-WZ/male-ZZ, or other type of chromosomal sex mechanism. Our population genomics and meiotic mapping both support the conclusion from testosterone-treated AB 3 WIK individuals (Tong et al. 2010) that females are the heterogametic sex (WZ) in natural populations of zebrafish. For the SNP that is statistically associated most strongly to sex phenotype in each natural population: (1) all individuals that were homozygous for one allele (e.g., *m/m*), which could be on the Z chromosome, developed as males; (2) most individuals that were heterozygous (e.g., *m/f*), which could represent a WZ karyotype, became females; and (3) fish that were homozygous for the allele that is not homozygous in most males (e.g., *f/f* fish, presumably WW) usually developed as females and were fewer than expected by random mating and equal viability. These fish would occur from the mating of a genetic (WZ) female with a, sex-reversed (WZ) phenotypic male. And (4) although some fish with a female genotype (*f/m*) developed as males, no individual with a male genotype (*m/m*) became a female. This result suggests that (5), an allele (*f*) is necessary but not sufficient to make a female phenotype, or the Z version of the native Chr4 has a dosage-sensitive locus for which two doses guarantees male development and one dose favors, but does not assure, female development.

The dominant-female-allele hypothesis and the two-dose-male–one-dose-female hypothesis have both been described in vertebrates. A W-linked, dominantly acting

truncated copy of *DMRT1* triggers ovary development in the frog *Xenopus laevis*, likely acting as a dominant-negative inhibitor of the normal *DMRT1* gene (Yoshimoto et al. 2008; Yoshimoto et al. 2010; Yoshimoto and Ito 2011). *DMRT1* is also involved in sex determination in birds, but the agent is a Z-linked copy that affects sex development by a dosage-sensitive mechanism (Smith et al. 2009). The Z, but not the W, in half-smooth tongue sole has a functional copy of *dmrt1*, consistent with the dosage-sensitive hypothesis (Chen et al. 2014). A variant of dmrt1 is also the major Y-linked sex determinant of Japanese medaka *O. latipes* (Matsuda et al. 2002; Nanda et al. 2002; Kondo et al. 2006, 2009). In some species of *Oryzias*, variants of different genes in the sex-determination pathway, including *gsdf* and *sox3*, are at the top of the sex-determination hierarchy (Takehana et al. 2007, 2014; Kondo et al. 2009; Myosho et al. 2012; Kikuchi and Hamaguchi 2013), while in other species of fish, other genes are at the top of the hierarchy, including a variant of *irf9* in trout and *amhr* in fugu (Kamiya et al. 2012; Yano et al. 2012). The molecular genetic nature of the zebrafish sex determinant is as yet unknown. About 80% of Chr4R genes have no apparent human ortholog and are highly duplicated, with, for example, 109 genes encoding NOD-like receptors and zinc finger proteins (Howe et al. 2013), and as discussed below, the strains from which the Zv9 zebrafish reference genome was derived lack or have a greatly modified Chr4-linked sex-determination system, making it likely that the reference genome does not contain the normal wild sex determinant.

The only karyotypes known to involve zebrafish collected directly from nature (Mansar Lake, Jammu) tentatively support the WW/WZ chromosomal sex system (Sharma et al. 1998). The relevance of the Mansar Lake fish to our populations, however,

is unknown. Mansar Lake is 2000 km west of Nadia, Kolkata, and Cooch Behar and the phylogenetic relationship of its zebrafish to our populations is unknown. Furthermore, the Z chromosome in Mansar Lake fish was cytogenetically much larger than the W chromosome (Sharma et al. 1998). Assuming that *SbfI* sites are distributed with approximately the same density across Z and W chromosomes, a large proportion of sex-linked RAD-tags should be homozygous in ZZ males and heterozygous in WZ females. In our experiments, however, only a small fraction of Chr4-linked RAD-tags fit these criteria, suggesting that our Indian populations, while maintaining a WZ/ZZ system, might have a different sex chromosome karyotype than the one suggested by published analyses of the Mansar Lake population.

<u>Domesticated zebrafish strains lack a single strong sex-linked locus</u>

In contrast to natural strains, RAD-sex failed to detect any sex-linked loci in AB and TU, which is surprising given that published studies involving these strains identified sex-biasing loci. For the SATmap study, we made fully homozygous (doubled haploid) gynogenetic AB and TU fish, some of which became males and others females despite all fish having only female-derived chromosomes (Howe et al. 2013). Crossing a fully homozygous female TU fish to a fully homozygous male AB fish produced a clutch of genetically identical F_1 heterozygotes, some of which became males and others females (Howe et al. 2013), showing that genetic differences are not essential for zebrafish sex determination. Apparently, in the absence of genetic differences, other forces influence zebrafish sex determination; for example, stochastic differences might cause some eggs to have less yolk than others or environmental differences might arise if late hatching

larvae have less access to food; both situations would lead to poorer nutrition, slower growth, and a greater likelihood of developing as a male (Lawrence et al. 2008).

To produce the F_2 SATmap population, a heterozygous AB/TU F_1 male was crossed to his genetically identical sister (Howe et al. 2013). Analysis identified a single significant peak linked to sex at Chr16:19 Mb–23 Mb. Homozygotes for the granddam (TU) allele were 70% likely to become female but homozygotes for the grandsire (AB) allele were only 26% likely to become female, while heterozygotes were about equally likely to be male or female (Howe et al. 2013). This result shows that a locus on Chr16 has female-favoring allele(s) in TU and/or male-favoring alleles in AB. This result does not address the question of whether sex-determination alleles are polymorphic within each strain, just that alleles differ between strains. For example, AB fish might have partially deleterious alleles at this locus so that AB homozygotes have fewer primordial germ cells, are slower developing, or are less successful at obtaining nutrition and thus more likely to become males relative to individuals homozygous for TU alleles (Lawrence et al. 2008; Siegfried and Nusslein-Volhard 2008; Rodriguez-Mari et al. 2010; Rodríguez-Marí and Postlethwait 2011; Dranow et al. 2013). In contrast, RAD-sex detects sex-linked polymorphisms segregating within a strain, not between strains. If the Chr16 sex-biasing factor that varies between AB and TU is not polymorphic within AB or within TU, our protocol would not find it. Furthermore, the failure to find *sar4* in the SAT-map experiments supports our finding that AB and TU lack *sar4*.

Modification of the Chr4R sex determinant in domesticated strains

AB and TU both experienced selection to remove preexisting mutations for mutagenesis experiments (Walker-Durchanek 1980; Streisinger et al. 1981; Mullins et al.

1994). C. Walker produced AB from 21 females derived by half-tetrad gynogenesis (Streisinger et al. 1981), which produces offspring that are homozygous except for regions distal to a recombination event; thus, half-tetrad gynogenesis might or might not eliminate heterozygosity at the distally located *sar4* locus. Because homozygosity can result in male bias due to loss of fitness (Brown et al. 2012a,b), some half-tetrad animals might sex reverse to male development despite heterozygosity at *sar4*. The mating of a WW (or ZZ) gynogenetic female (or male) to a sex-reversed WW (or ZZ) gynogenetic male (or female) might result in the loss of the Z (or W) chromosome making a homozygous WW (or ZZ) strain. Sex-biasing alleles or environmental factors could cause some WW (or ZZ) individuals to become males (or females), allowing the strain to propagate. Researchers might select for any preexisting male- or female-biasing alleles as they set up mating pairs; eventually a new genetic sex-determining mechanism might evolve in the domesticated strain, perhaps similar to the rapid evolution of new sex chromosomes in cichlids (Roberts et al. 2009; Ser et al. 2010; Parnell and Streelman 2013).

TU originated from a pet store in Germany about 1990. Mullins et al. (1994) used multiple single-pair matings to make a lethal-free strain without gynogenesis and with a conscious effort to maintain genetic variation. A hypothesis is that the major wild male or female sex determinant was linked to lethal or deleterious alleles, leading to the loss of the sex determinant along with lethal allele loss. In the absence of male or female alleles of the major sex determinant, occasional sex reversal would allow for stock maintenance, and researchers would strongly select for alleles favoring the development of both sexes.

If this hypothesis were true, then the evolution of new sex determinants in TU provides a unique opportunity to study the evolution of new sex-determining mechanisms.

Despite the absence of a strong sex-linked locus on Chr4, AB and TU may have retained some components of the wild sex-determination mechanism:

1. A female-AB-by-male-IN cross (MGH cross) identified sex-linked loci at Chr5:44.5–46.6 Mb and Chr16:13–17 Mb that together account for just 16% of the variance with regard to sex (Knapik et al. 1996, 1998; Bradley et al. 2011). If the AB parental female lacked a strong female sex determinant as in our AB fish, then the MGH cross might miss *sar4* even if IN females, which were not involved in the cross, do possess the natural wild sex determinant.
2. Our female-AB-by-male-NA cross identified sex-linked loci in addition to *sar4*, specifically, one on Chr3, although the reciprocal cross identified only *sar4* (Anderson et al.2012).
3. As discussed above, the female-TU-by-male-AB SATmap cross identified sex-linked loci on Chr16, but in a different location than in the MGH cross (Howe et al. 2013).

The identification of autosomal sex-associated loci (Chr3, Chr5, Chr16) could represent either the unmasking of weak sex determinants given the loss of the *sar4*, or the rapid evolution of new sex-determination systems after the divergence of EKW, which has the Chr4R sex-determination system, from the common ancestor of AB and TU.

<u>Female-to-male sex reversal</u>

What causes male genotypes to develop only as males but female genotypes to sometimes develop as sex-reversed phenotypes? Answers likely lie in environmental

factors and background genetic features that affect the strength of a meiotic oocyte-derived pro-female signal that inhibits oocyte apoptosis, probably by maintaining aromatase production (Slanchev et al. 2005; Houwing et al. 2007; Wang et al. 2007; Siegfried and Nusslein-Volhard 2008; Rodriguez-Mari et al. 2010, 2011; Rodríguez-Marí and Postlethwait 2011; Pradhan et al. 2012; Dranow et al. 2013). In general, harsh conditions, including high density and poor nutrition, tend to promote male development (Walker-Durchanek 1980; Pelegri and Schulte-Merker 1999; Shang et al. 2006; Lawrence et al. 2008; Abozaid et al. 2011, 2012; Liew et al. 2012; Villamizar et al. 2012). These harsh factors may act to decrease the pro-female signal by depressing the pool of meiotic oocytes, either by inhibiting primary germ-cell proliferation or entry into meiosis or by promoting oocyte apoptosis. Sex reversals can happen even in species with a strong genetic sex determinant. In medaka, high temperature and hypoxia can cause female-to-male sex reversal, accompanied by increased cortisol and depressed aromatase (Sato et al. 2005; Hattori et al. 2007; Selim et al. 2009; Kitano et al. 2012; Cheung et al. 2014). It remains to be tested whether stressful conditions cause sex reversal in zebrafish by a similar mechanism.

In addition to environmental factors, background genetic factors might decrease the strength of *sar4* activity in domesticated stocks. Although our experiments showed that WIK has a strong sex determinant, inbreeding, which results in homozygosis of partially deleterious alleles, biases WIK fish toward male development (Brown et al. 2012a). Especially likely would be an interaction of background genotype with environmental factors that could override the influence of *sar4* on phenotypic sex.

Population diversity and the modification of sex-determining mechanisms in domesticated strains

Did AB and TU lose *sar4* independently or was this feature present in the last common ancestor of the two strains? Phylogenomic analyses showed that AB and TU occupy a monophyletic sister clade to EKW, suggesting that AB and TU may have been derived from EKW-related fish—AB from a pet store in Oregon and TU from a pet store in Germany. By parsimony, these results do not rule out the possibility that the lack of sex-linked loci in Chr4R was a shared trait derived from the last common ancestor of AB and TU. Nevertheless, due to their independent domestication in Eugene and Tuebingen, and the presence of *sar4* in EKW, we suspect that the loss of a strong sex determinant occurred independently during the separate domestication of the two lineages, a conclusion that is not incompatible with the phylogenomic data.

Analysis of the mitochondrial genome-encoded cytb gene showed that our strains all derived from mitochondrial haplogroup 1, suggesting that a broader understanding of the genetics of sex determination across the full diversity of zebrafish will require investigations of haplogroups 2 and 3 (populations in Nepal and Southern India, respectively), which diverged from haplogroup 1 about 3 million years ago (Whiteley et al. 2011). Investigations of Danio species closely related to zebrafish will also add to our understanding, although the *sar4* sex-determination locus may not be widely conserved with other danios. The closest lineage to zebrafish (Cypriniformes; Cyprinidae; Danio) that has a genome-wide analysis of conserved syntenies is the gudgeon (Cypriniformes; Cyprinidae; Gnathopogon) (Kakioka et al. 2013), whose lineage separated from the zebrafish lineage about 117 million years ago (Saitoh et al. 2011). Analyses showed

strong conservation of syntenies among all zebrafish chromosomes except Chr4R (Kakioka et al. 2013).

Conclusions

Results presented here show that zebrafish in nature has a strong sex determinant linked to the right tip of Chr4, the only chromosome arm with cytogenetic features frequently found in sex chromosomes; that the determinant is necessary but not sufficient for female development; and that in natural populations, females are WZ and males are ZZ. In contrast, domesticated strains cleaned of background mutations for mutagenesis experiments lack or have greatly weakened versions of the Chr4 sex-determination system.

These conclusions have several important implications:

1. Because domesticated strains make males and females (often with widely fluctuating sex ratios) even without the full complement of natural genetic sex determinants, these strains apparently have a functional sex-determining mechanism, perhaps due to polygenic sex determination unmasked by the evolutionary strengthening of weak sex-ratio modifiers under the heavy hand of "unnatural selection" wielded by zebrafish researchers, or the unmasking of latent but preexisting environmental sex-determination mechanisms.
2. Some studies investigating the biology of zebrafish sex determination using TU and AB fish should be revisited using sex-genotyped animals from a strain that possesses the natural genetic sex determinant. 3. Although mechanisms downstream of *sar4* are likely to be the same in all zebrafish stocks, future work that includes studies of natural strains containing the wild sex determinants would

provide richer understanding.

4. The zebrafish genome sequence, which was derived mainly from TU with input from AB and substantial correction using the SATmap, is unlikely to contain strong alleles of both the male and the female alternatives of the major natural sex-determining gene.
5. We need to concentrate efforts to identify the molecular genetic basis of wild sex in zebrafish.

Bridge

In the GWAS of Chapter II, we found a sex-associated region on linkage group 4 in natural zebrafish strains. This sex-associated region could contain the master switch for gonad development in zebrafish, but a question remains: How does that gene work, what is its down-stream pathway, to regulate male vs. female development? In other species, a number of genes regulate sex development and one of these in mammals is *Wnt4*. In mammalian sex determination, the master switch for male gonad development is SRY, and the presence of SRY initiates a genetic cascade that results in the animal developing as a male. In the absence of mammalian SRY, mammalian WNT4 both inhibits the male sex determination pathway and initiates the female sex determination pathway causing the individual to develop as a female. Zebrafish have multiple genes that are orthologous to genes in the mammalian sex determination pathway. One particular gene, *wnt4a*, is orthologous to mammalian *Wnt4*. In chapter III, I investigated the hypothesis that *wnt4a,* like mammalian *Wnt4,* is important for zebrafish ovarian development.

CHAPTER III

ROLE OF WNT4A IN ZEBRAFISH GONAD DEVELOPMENT

The research described in this chapter was performed by **High SK**, Yan YL, BreMiller R, and Postlethwait J. I designed and performed the sex ratio (*wnt4a-/-* compared to *wnt4a+/+* siblings), and chemical treatment experiments. I analyzed the data from all of the experiments and wrote the initial drafts of the paper. Yan YL, BreMiller R helped me design and perform the *in situ* hybridization experiments.

Introduction

Without effective reproduction, species would face extinction. In sexual reproduction species are generally divided between two reproductive groups, male and female, which develop due to the process of sex determination. The mechanisms that regulate this process vary across species. Vertebrate gonads generally differentiate into ovaries or testes through genetic or environmental sex determination. Mammals, which have the best studied sex determination system, have an XY/XX chromosomal mechanism in which females are homogametic (XX) and males are heterogametic (XY) (Barske et al. 2008; Kashimada et al. 2010; She et al. 2014; Eggers et al. 2014). Early in mammalian sex development, both XX and XY individuals develop bipotential gonads that are capable of becoming either ovaries or testes (Kashimada et al. 2010). Initially primordial germ cells migrate into the gonadal ridge where the bipotential gonad will start developing (Albrecht et al. 2001; Kim et al. 2006). In mammals, the presence or absence of the Y chromosome leads to the development of either a testis (presence of the Y chromosome), found in males, or an ovary (absence of the Y chromosome), found in

females. In XY mammalian gonads, *Sry,* the master switch for mammalian sex determination found on the Y chromosome, activates the male sex determination pathway and the previously bipotential gonad develops into a testis. In XX mammalian gonads, which lack the Y chromosome and *Sry*, the absence of *Sry* allows activation of the female sex determination pathway and the previously bipotential gonad develops into an ovary. The male and female sex determination pathways contain a variety of sex-associated genes, some of which are highly conserved among other species and some of which are not. Master switch genes, such as *Sry* in mammals, are not typically conserved among other species and actually vary quite a bit. For example, different species in the genus *Oryzias* can have different master sex determination genes, $gsdf^{Y}$ in *O. luzonensis* (Myosho et al. 2012), *sox3* in *O. dancena* (Takehana et al. 2014), and *dmy/dmrt1bY* in *O. latipes* and *O. curvinotus* (Matsuda et al. 2002; Nanda et al. 2002; Heule et al. 2014). Even though these master switch genes can vary among fish species, most genes downstream of these master switch genes are actually quite conserved. Evidence for conservation is found in the roles of *Sox9*, *Wt1*, *Nr5a1*, *Foxl2*, *Cyp19a1a*, and *Dmrt1*, which act in the sex determination pathway and are conserved from fish to mammals (Shartl 2004; Trukhina et al. 2013). One gene, *Wnt4* (wingless-type MMTV integration site family, member 4), which is primarily involved in ovary development, is also evolutionarily conserved in birds, reptiles, amphibians, some fish, and mammals. In chickens (*Gallus gallus domesticus), Wnt4* mRNA is expressed in both male and female gonads prior to sex differentiation and becomes specifically expressed in just the developing oocytes of chicken ovaries after sex differentiation (Smith et al. 2008; Chassot et al 2014). In the red-eared slider turtle (*Trachemys scripta*), *Wnt4* transcription

is up-regulated in females during ovarian development, down-regulated in males during testes development, up-regulated in males treated with estrogen during gonad development, and down-regulated in females treated with aromatase inhibitor during gonad development, suggesting that *Wnt4* transcription is controlled by estrogen signaling in turtles (Mork and Capel 2013; Chassot et al 2014). In wrinkled frogs (*Rana rugosa*), *Wnt4* expression is maintained in a non-sexually dimorphic manner until the gonad differentiates into a testis or an ovary (Oshima et al. 2005; Chassot et al 2014). In the protandrous Black Porgy (*Acanthopagrus schlegeli*), which initially contain a bisexual gonad (both testicular and ovarian tissue are present) and live as functional males during the first two years of spawning seasons and can then undergo male-to-female sex change during their third year of life, *wnt4* expression increases during this transition from male-to-female and during late ovarian growth (Wu and Chang 2009; Chassot et al 2014). In humans (*Homo sapiens*) and mice (*Mus musculus*), *Wnt4* is expressed in the somatic cells of both the fetal and adult ovary (Jaaskelainen et al. 2010; Chassot et al 2014). These findings indicate that *Wnt4* is important for general gonad development in some amphibians and ovarian development in some birds, reptiles, fish, and mammals. Knowing that *Wnt4* is conserved among species raises the question: How does *Wnt4* regulate gonad development in mammals?

In most cases XX-containing mammalian bipotential gonads develop into ovaries. *Wnt4*, which encodes the Wnt4 protein and is expressed in somatic cells of the bipotential gonad, is important for ovary development and inhibits testis development in mammals (Kim et al. 2006; She et al. 2014). In developing ovaries in XX mammals, first Wnt4 activates the canonical Wnt/β-catenin pathway in nearby cells by binding to Frizzled

receptors and LRP co-receptors; this binding leads to the stabilization of Ctnnb1 (β-catenin), which normally promotes ovary development and can cause male-to-female sex reversal in XY males, and finally to the transcription of sex-associated target genes, such as *Fst*, which is involved in folliculogenesis within the ovary (Maatouk et al. 2008; Chassot et al. 2012; Niehrs 2012; Chassot et al. 2014; Eggers et al. 2014). In mammalian gonads lacking Wnt4 function, germ cells proliferate normally during early development, but germ cells undergo apoptosis around 16.5 days post coitum (dpc), suggesting that *Wnt4* helps ovary development through the prevention of germ cell apoptosis (Yao et al. 2004; Chassot et al. 2014). XX gonads lacking Wnt4 function also have increased expression of the testis markers *Fgf9* and *Sox9*. *Sox9* causes the development of Sertoli cells, supporting cells that help with sperm production, and the development of testis (Kashimada et al. 2010; Dinapoli et al. 2008; Sekido et al. 2008), by initiating *Fgf9* (fibroblast growth factor – 9) expression, which in turn up-regulates *Sox9* expression in a positive feedback loop (She et al. 2014). *Fgf9* and *Wnt4* also have an antagonistic relationship, in which higher expression levels of one inhibit the expression of the other (Kim et al. 2006; Eggers et al. 2014). Because of this mechanism, increased expression of *Fgf9* and *Sox9* in XX gonads lacking Wnt4 function leads to the masculinization of the gonad by 14.5 dpc and the development of Sertoli cells by birth (Kim et al. 2006; Chassot et al. 2014). This result suggests that *Wnt4* is important for ovary development by suppressing testis development (Kim et al. 2006; DiNapoli et al. 2008; She et al. 2014; Chassot et al. 2014).

Rspo1, a signaling protein, also activates the canonical Wnt/β-catenin pathway, by binding to the Lgr4, Lgr5, and Lgr6 co-receptors, which then stimulate canonical Wnt

signaling (Chassot et al. 2014). Rspo1 also binds to Znrf3 and Rnf43, negative-feedback regulators that ubiquinate lysines in frizzled receptors, to prevent the degradation of Wnt receptors and allow for Wnt4 to activate the canonical Wnt signaling pathway (de Lau et al. 2014; Chassot et al. 2014). Gonads lacking Rspo1 function have some germ cells that do not proliferate normally and a masculinized gonad that eventually develops Sertoli cells by birth, suggesting that *Rspo1* is also important for the development of the ovary. Rspo1 apparently supports ovary development by allowing germ cell proliferation and suppressing masculinization (Auguste et al. 2011; Chassot et al. 2012; Chassot et al. 2014). Gonads lacking both Wnt4 and Rspo1 function show full female-to-male sex reversal, suggesting that both proteins are necessary for proper ovary development and thus act in at least partially distinct pathways (Auguste et al. 2011; Chassot et al. 2012; Maatouk et al. 2013; She et al 2014; Chassot et al. 2014).

Mutations in *Wnt4* can also lead to a variety of reproductive diseases that effect ovary development in humans, such as SERKAL (female SEx reversal and dysgenesis of Kidneys, Adrenals, and Lungs) and Polycystic ovary syndrome (Mandel et al. 2008, Canto et al. 2006). SERKAL syndrome results in the development of testes in XX humans and abnormal Müllerian duct formation in some humans, indicating female-to-male sex reversal (Mandel et al. 2008). Polycystic ovary syndrome usually results in ovaries with multiple small immature follicles, chronic anovulation (absence of ovulation), oligomenorrhea (light or infrequent menstruation) amenorrhea (absence of menstruation), and hyperandrogenism (excess of androgens) (Canto et al. 2006). The ability to study reproductive diseases and discover effective treatments would be beneficial for human health. One model organism that can be used to study reproductive

diseases, and investigate the evolution of genes associated with sex is zebrafish (*Danio rerio*).

Zebrafish is a popular model organism used for developmental and genetic studies but its mechanism of sex determination is still unknown. Zebrafish seems to have both an environmental and genetic contribution to sex determination but contributions of both are as yet unclear. To investigate the possibility of environmental sex determination in zebrafish, the effect of temperature, density, and other factors were studied. One study raised zebrafish at different temperatures and found that increasing temperatures resulted in oocyte apoptosis and a male bias (Uchida et al. 2004). To investigate the effect of rearing densities on sex ratios, zebrafish were raised in tanks containing either higher or lower densities of fish, and the clutches reared at higher densities showed a male bias and those reared at lower densities had a female bias (Lawrence 2007; Liew et al. 2012). Another study set up repeated matings under different environmental conditions and found extremely variable sex ratios across families, ranging from 4.8% to 97.3% males, but when they used the same breeding pair for repeated matings, they found similar sex ratios among the resulting offspring, indicating that parental genotypes affected zebrafish sex ratios (Liew et al. 2012). These findings indicate that zebrafish sex determination is partially environmental.

Because zebrafish sex determination is also not purely environmental, what is the genetic mechanism of sex determination in zebrafish? It was previously thought that zebrafish did not have sex chromosomes (Krovel and Olsen 2004; Postlethwait et al. 2000; Amores and Postlethwait 1999), although new data from our lab shows that zebrafish have chromosomes containing regions associated with sex determination

(Anderson et al. 2012, Wilson et al. 2014). In our GWAS study, we found a sex-associated region on linkage group 4 in natural zebrafish strains, which likely contains the master switch for gonad development in zebrafish and indicates that zebrafish sex determination is likely regulated genetically in natural populations.

Zebrafish have multiple genes orthologous to the mammalian sex determination pathway. One particular gene, *wnt4a*, is orthologous to mammalian *Wnt4.* While the *Wnt4/Fgf9* seesaw regulates ovary vs. testis development in mammals, zebrafish lacks an *Fgf9* ortholog (Itoh and Konishi 2007) so the seesaw either doesn't work in zebrafish or *Fgf9* paralogs might be playing a similar role to *Fgf9* in zebrafish (Katoh and Katoh 2005). In either event, *wnt4* might play somewhat different roles in zebrafish. I hypothesize that *wnt4a* functions and is expressed similarly to mammalian *Wnt4* during ovary development, with the exception of *Fgf9* inhibition. The hypothesis that *wnt4a* is expressed similar to mammalian *Wnt4*, predicts that *wnt4a* should be in the somatic cells of the bipotential gonad (Jaaskelainen et al. 2010). The hypothesis that Wnt4a functions similar to mammalian Wnt4, excepting *Fgf9* inhibition, predicts that loss of Wnt4a function should produce female-to-male sex reversal, as seen in mice and humans lacking Wnt4 function (DiNapoli et al. 2008; She et al. 2014; Chassot et al. 2014). Previous studies showed that the number of germ cells in the gonad influence zebrafish gonad development, where the germ-line is required for ovary development and zebrafish lacking germ cells develop testes (Siegfried and Nüsslein-Volhard et al. 2008). The number of germ cells can also affect mammalian ovary development (Choi and Rajkovic 2006; Siegfried and Nüsslein-Volhard et al. 2008) although mammalian Wnt4 does not affect the proliferation of germ cells, it does prevent germ cell apoptosis (Yao et al. 2004;

Chassot et al. 2014). The hypothesis that zebrafish Wnt4a functions similarly to mammalian Wnt4, predicts that loss of Wnt4a function would have no effect on the number of germ cells in the gonad (Yao et al. 2004; Chassot et al. 2014), but the alternative hypothesis that zebrafish Wnt4a functions differently than mammalian Wnt4 by increasing the number of germ cells in the gonad promoting female development, predicts that loss of Wnt4a function would reduce germ cell number (Siegfried and Nüsslein-Volhard et al. 2008). The hypothesis that zebrafish Wnt4a functions similarly to mammalian Wnt4, predicts up-regulation of the canonical Wnt signaling pathway, as seen in mammalian gonad development (Maatouk et al. 2008; Chassot et al. 2012; Niehrs 2012; Chassot et al. 2014; Eggers et al. 2014).

In this study we show that zebrafish Wnt4a functions similarly to mammalian Wnt4, excepting *Fgf9* inhibition, and that Wnt4a function is necessary for proper ovary development. To investigate if *wnt4a* is expressed in the same cells and at the same time during gonad development in zebrafish as in mammals, I utilized *in situ* hybridization and found that *wnt4a* is expressed in the somatic cells of the gonad during the bipotential gonadal stage, similar to mammalian *Wnt4*. To investigate if Wnt4a function has an effect on proper ovary development, I utilized the $wnt4a^{fh294/+}$ mutant line [a loss-of-function mutation (Choe et al. 2013) developed by the Moens lab (et al. 2009)], to determine if Wnt4a function loss produces female-to-male sex reversal as seen in mammals. I found female-to-male sex reversal in *wnt4a* mutants, as expected. To investigate if loss of Wnt4a function results in the reduction of germ cell number, I again utilized the $wnt4a^{fh294/+}$ mutant line, and found that loss of Wnt4a function did not result in germ cell number reduction, indicating that Wnt4a functions similarly to mammalian WNT4 by not

reducing the number of germ cells present in the gonad. To investigate if the canonical Wnt signaling pathway is involved in zebrafish ovary development, I used a Wnt signaling pathway agonist to up-regulate Wnt signaling. The preliminary results did not show any effect of Wnt signaling up-regulation on zebrafish gonad development, but it is possible that a sufficient amount of Wnt Agonist II did not actually stimulate Wnt signaling in these experiments and so I will need to look at the expression of downstream canonical Wnt signaling target genes in these animals to determine if target gene expression increased in treated animals. Overall, these findings indicate that *wnt4a* is necessary for proper ovary development in zebrafish.

Significance

Reproductive diseases, such as SERKAL syndrome and Polycystic ovary syndrome, are increasing problems for human health (Dumesic et al. 2007). Understanding the mechanism of sex determination in zebrafish could lead to more uses of this species as a model organism to investigate reproductive diseases. If *wnt4a,* like mammalian *Wnt4*, is important for zebrafish ovarian development, then zebrafish could be used as a model for discovering drugs that can help treat human syndromes, such as SERKAL syndrome, caused by the loss of WNT4 function.

Material and Methods

Fish Stocks

Fish stocks used were AB wild type line, *vasa::EGFP* transgenic line (Krovel et al. 2002), and *wnt4a*$^{fh294/+}$ mutant line (Moens et al. 2009). The *vasa::EGFP* transgenic

line and *wnt4a*$^{fh294/+}$ mutant line were developed in an AB background. The *wnt4a*$^{fh294/+}$ mutant line was created by treating adult AB zebrafish males with ENU and identifying sequence changes in the *wnt4* gene (Moens et al. 2009). The resulting *wnt4a* mutation is a point mutation that creates a premature stop codon at amino acid 307 (Moens et al. 2009). Fish were maintained as previously described by Westerfield (2007).

Genotyping

The primers used for genotyping the *wnt4a*$^{fh294/+}$ mutant line were Wnt4a(C307*)F1 (5-GTCAAGACCTGCTGGAAAGC-3) and Wnt4a(C307*)R1 (5-CTTGCGACACTGTTTGCATT-3) (Moens et al. 2009). The PCR conditions were: 1'94°C; 35 cycles of: 30"94°C, 30"60°C, 1'30"72°C; followed by 15°C until program is ended. Following the *wnt4a*$^{fh294/+}$ genotyping PCR, the amplicons were digested with DdeI at 37°C overnight (Moens et al. 2009). Sizes of bands after DdeI digest: 384bp (WT), 270bp + 114bp (mutant).

Immunohistochemistry and Histology

Animals were collected at multiple stages of zebrafish gonad development. Animals were then euthanized, fixed, and cryosectioned as previously described (Rodriguez-Mari et al. 2005). The probe for *vasa* was created as previously described (Yoon et al. 1997). The probe for *wnt4a* was created using the following primers: Wnt4a+589 (5-CCGCAACCGCCGATGGAACT-3) and Wnt4a-1207 (5-ACCGGGCGTTCTGGGGTCAT-3). Both *vasa* and *wnt4a* cDNA were cloned using the TOPO vector and used to synthesize DIG-labeled probes. For the *in situ* hybridization experiment four 10 dpf, five 12 dpf, four 18 dpf, three 25 dpf, three 35 dpf male, and two

35 dpf female zebrafish were used. For hematoxylin and eosin staining, animals were euthanized and fixed in bouin's solution for more than 48 hours, then were washed multiple times in 70% ethanol and paraffin sectioned. For primordial germ cell counting 13 wild-type and 12 *wnt4a*$^{fh294/+}$ mutant zebrafish were used. Imaging was done using a Leica compound microscope.

Chemical Treatments

Wnt Agonist II (SKL2001, Calbiochem) was used to up-regulate Wnt signaling in *vas::EGFP* individuals from 5 days post fertilization (dpf) to 12 dpf, which is prior to zebrafish gonad differentiation. Wnt Agonist II was dissolved in DMSO to make a 100 mM stock solution. An appropriate amount of stock solution was added to fish water to obtain treatment concentrations of 5uM, 10uM, 15uM, and 30uM. Equal volumes of DMSO were added to each treatment and control group. Fish water was changed twice daily. Sex ratios were measured at 43 dpf, when females and males can be distinguished by *vas::EGFP* transgene fluorescence, where females had distinguishably more transgene fluorescence than males (Tzung et al. 2015; Krovel et al. 2004).

Phylogenetic Tree

Phylogenetic tree was constructed as previously described (Vilella et al. 2009) using the gene tree tool found on ensemble.org.

Ethics

Zebrafish were handled in accordance with good animal practices and animal work was approved by the University of Oregon Institutional Animal Care and Use

Committee (Animal Welfare Assurance Number A-3009-01, IACUC protocol #14-08RA)

Results

wnt4a, an ortholog of mammalian *Wnt4*

Human has a single *wnt4* gene, whereas zebrafish has two *wnt4* genes, *wnt4a* and *wnt4b* (Garriock 2007). To discover if zebrafish *wnt4a* was orthologous to mammalian *Wnt4,* I generated a gene tree using the gene tree tool found on ensembl.org (Figure 3.1). This analysis shows two Wnt4 clades, one that includes zebrafish *wnt4a*, spotted gar *wnt4a*, coelacanth *WNT4*, mouse *Wnt4*, and human *WNT4*, and another that includes zebrafish *wnt4b*, spotted gar *wnt4b*, coelacanth *wnt4b*, but no human or mouse *wnt4b*, indicating that *wnt4a* and not *wnt4b* is orthologous to mammalian *Wnt4.*

wnt4a expression during zebrafish gonad development

If *wnt4a* plays a role in gonad determination in zebrafish like it does in mouse, then it should be expressed before sex differentiation, specifically during the bipotential gonadal stage. Utilizing *in situ* hybridization to histological sections, I studied the expression of *wnt4a* throughout a zebrafish developmental time series covering various stages of gonad development, from 10 days post fertilization (dpf), when the gonad is considered to be bipotential (Uchida et al. 2002; Rodriguez-Mari et al. 2005) through 35 dpf, when the gonads are sexually determined (Uchida et al. 2002) (Figure 3.2). In order to determine if *wnt4a* was expressed in either the somatic cells or the primordial germ cells of the gonad, I compared *wnt4a* to *vasa*, which is expressed in the primordial germ

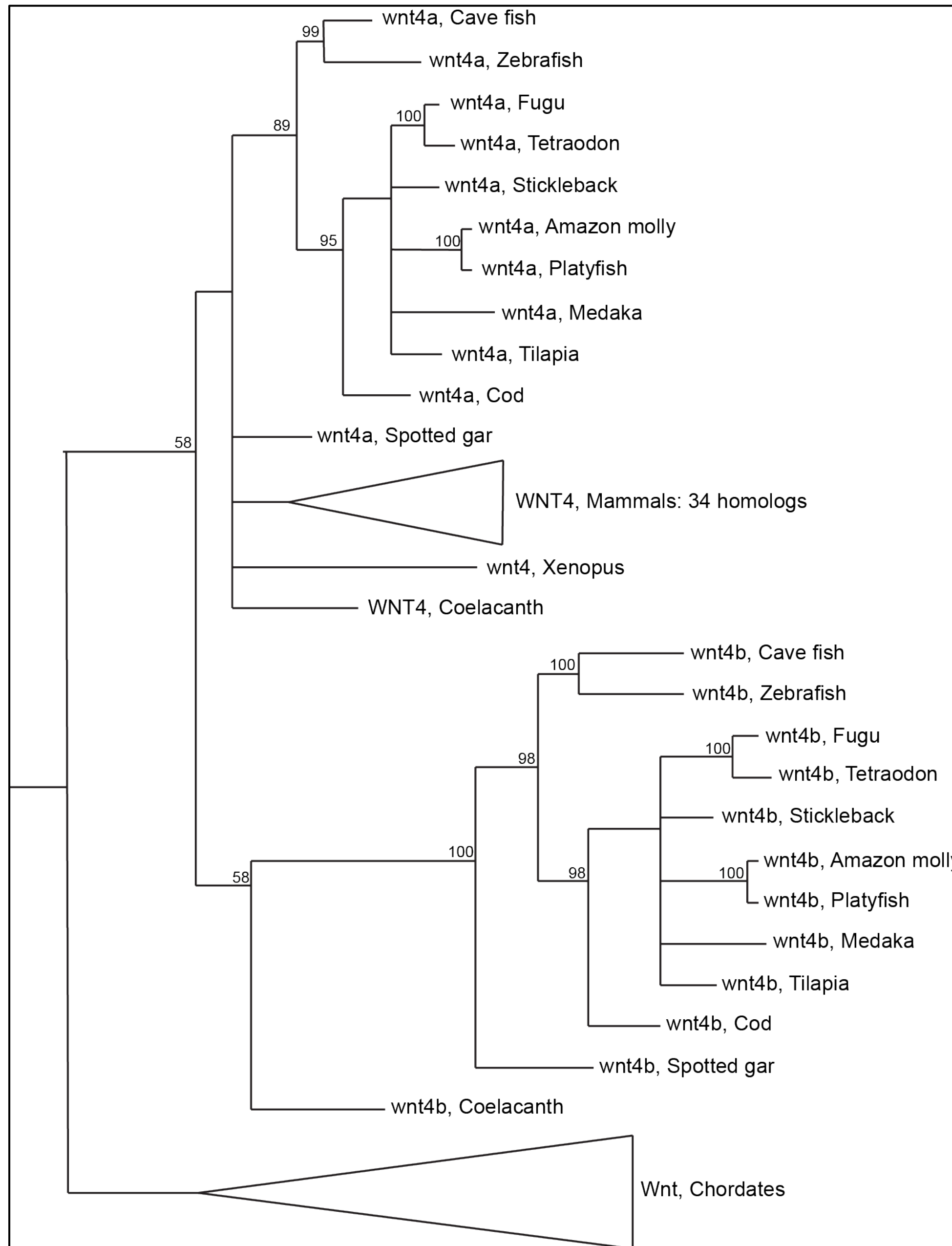


Figure 3.1 - Phylogenetic analysis of *wnt4* genes. Two *wnt4* clades are present, once containing zebrafish *wnt4a* and mammalian *Wnt4*, and the other containing zebrafish *wnt4b* but no orthologous mammalian gene. Created using the gene tree tool found on ensemble.org.

cells (Braat et al. 1999), using adjacent sections. Evidence from *in situ* hybridization experiments revealed *wnt4a* expression in the somatic cells of 12 dpf zebrafish gonads (Figure 3.2), showing that *wnt4a* is expressed during the bipotential gonadal stage in

zebrafish. The expression pattern was similar to what was seen in mammals, where *Wnt4* is expressed in the somatic cells during the bipotential gonadal stage (Jaaskelainen et al. 2010). I conclude that zebrafish *wnt4a* expression is appropriate for playing a similar role in gonad determination as seen in mouse.

Effect of Wnt4a loss on female gonad development

If Wnt4a is important for ovary development by suppressing testis development like it is in mice, then I would expect to see female-to-male sex reversal when Wnt4a function is lost. To test this I used the $wnt4a^{fh294}$ mutant line (Moens et al. 2009), which has a point mutation that creates a premature stop codon at amino acid 307 truncating 45 amino acids at the C-terminus of Wnt4a. This mutation results in the absence of 10 conserved cysteines that are necessary for the proper folding of Wnt4a (Miller 2002), and are needed to properly bind to Frizzled receptors (Janda et al. 2012) (Figure 3.3). The loss-of-function of this allele was also previously confirmed with a *wnt4a* morpholino (Choe et al. 2013).

To test if Wnt4a is important for ovary development in mice I utilized the $wnt4a^{fh294/+}$ line and genotyped the offspring of heterozygous $wnt4a^{fh294/+}$ fish, which enabled the examination of sex ratios for three genotypes (*wnt4a*+/+, *wnt4a*+/-, and *wnt4a*-/-) to determine if the *wnt4a*-/- fish had a significantly different sex ratio than of their wild type (*wnt4a*+/+) and heterozygous (*wnt4a*+/-) siblings. Figure 3.4 shows combined data from six different *wnt4a*+/- in-crosses. First I investigated whether or not the *wnt4a* mutation was lethal, by comparing the number of individuals that were homozygous wild type, heterozygous, or homozygous mutant. If the *wnt4a* mutation is viable then I would expect to see a population comprised of 25% homozygous wild-type

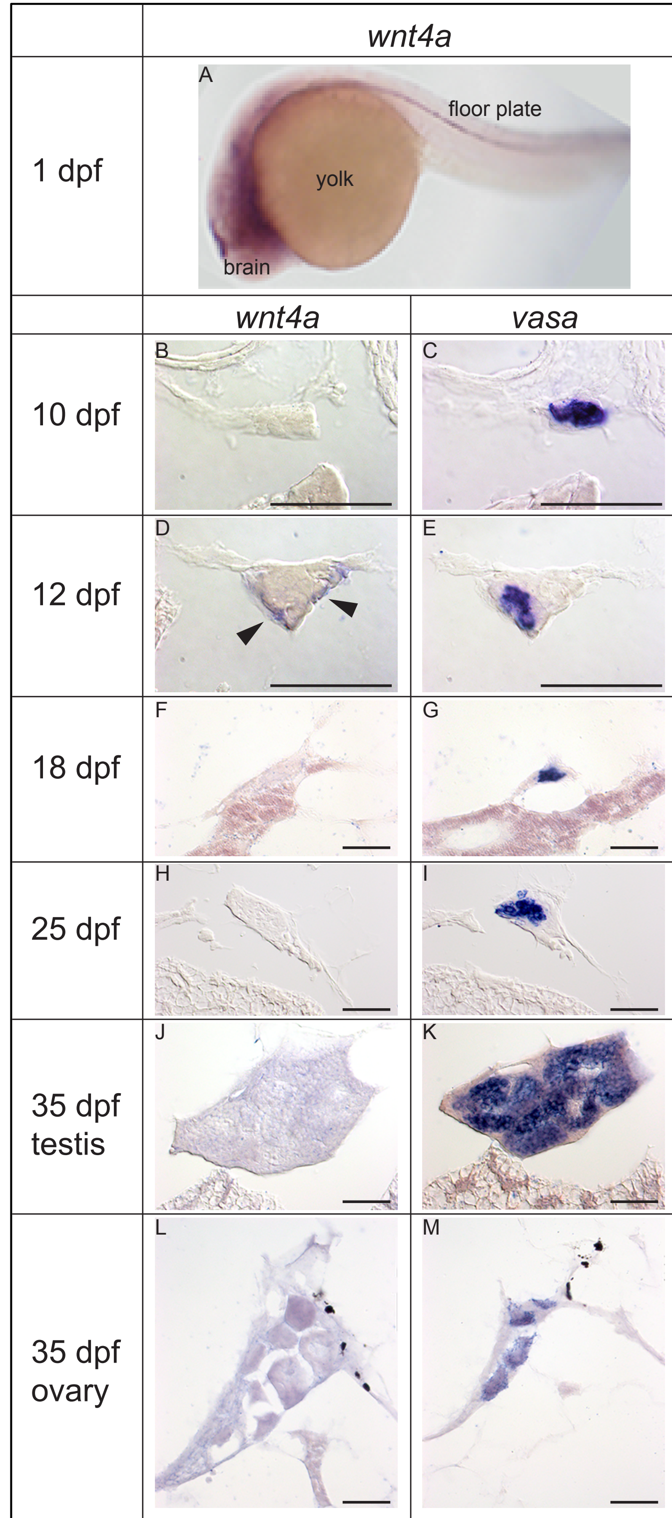


Figure 3.2 - Expression of *wnt4a* and *vasa* across zebrafish gonad development. (A) *wnt4a* expression seen in floor plate and brain at 1 dpf. (B) No *wnt4a* expression in gonad at 10 days post fertilization (dpf) (D) *wnt4a* expression in the somatic cells of the 12 dpf gonad (black arrows) (F, H, J, L) No *wnt4a* expression in gonad from 18 – 35 dpf. (C, E, G, I, K, M) *vasa* expression in PGCs of gonads at various ages. Scale bar = 50µm.

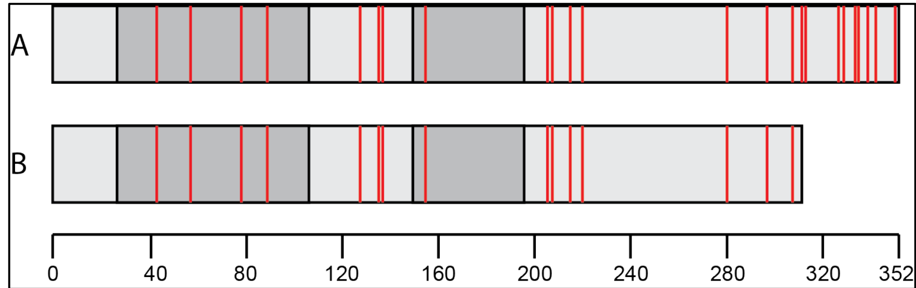


Figure 3.3 – Wnt4a protein structure. (A) shows Wnt4a protein with 5 exons (alternating light and dark gray) and 24 conserved cysteines, which are necessary to form the disulfide bonds necessary for proper protein folding, and this cysteine rich region binds to the Frizzled receptors, as evidenced in other studies (Janda et al. 2012). (B) shows the mutant Wnt4a protein resulting from a premature stop codon at amino acid 307. This protein lacks ten conserved cysteines necessary for proper protein folding at the C-terminus.

individuals, 50% heterozygous individuals, and 25% homozygous mutant individuals. If the *wnt4a* mutation is lethal, then I would expect to see a population comprised of one third wild-type individuals and two thirds heterozygous individuals. Among the 612 individuals scored (Figure 3.4), 26% were wild type (162 individuals), 50% were heterozygous (307 individuals), and 24% were mutant (143 individuals), and this ratio was not significantly different than expected (p-value = 0.55, chi-square test), indicating that this *wnt4a* mutation is not lethal.

Next I investigated whether or not proper Wnt4a function requires one or two wild-type *wnt4a* alleles by comparing sex ratios in *wnt4a*+/+ and *wnt4a*+/- fish to see if the mutant allele was dominant (both wild-type alleles are required for proper Wnt4a function) or recessive (only one wild-type allele is required for proper Wnt4a function). If the *wnt4a* mutant allele were dominant, then I would expect the *wnt4a*+/+ and *wnt4a*+/- sex ratios to be significantly different. Alternatively, if the *wnt4a* mutant allele were recessive, then I would expect the *wnt4a*+/+ and *wnt4a*+/- sex ratios to be similar. The *wnt4a*+/+ population was 65% male (105 males and 57 females, SD +/-16.6%), and the

wnt4a+/- population was 61% male (187 males and 120 females, SD +/-12.7%). These results revealed no significant difference between the sex ratios of these two populations (Figure 3.4, p-value = 0.33, chi-square test), indicating that the *wnt4a* mutant allele is recessive with respect to sex phenotype.

To learn whether *wnt4* is important for sex phenotype, I examined sex ratios in homozygous mutants. The *wnt4a-/-* population was 98.6% male (141 males and 2 females, SD +/-1.8%), showing that the *wnt4a-/-* population has a significant male bias (Figure 3.4). These results showed that the *wnt4a-/-* population had significantly greater fraction of males than their wild-type siblings (p = 3.63E-17, chi-square test) and significantly greater proportion of males than their heterozygous siblings (p = 3.00E-20, chi-square test) (Figure 3.4). These results support the conclusion that loss of Wnt4a activity causes significant female-to-male sex reversal.

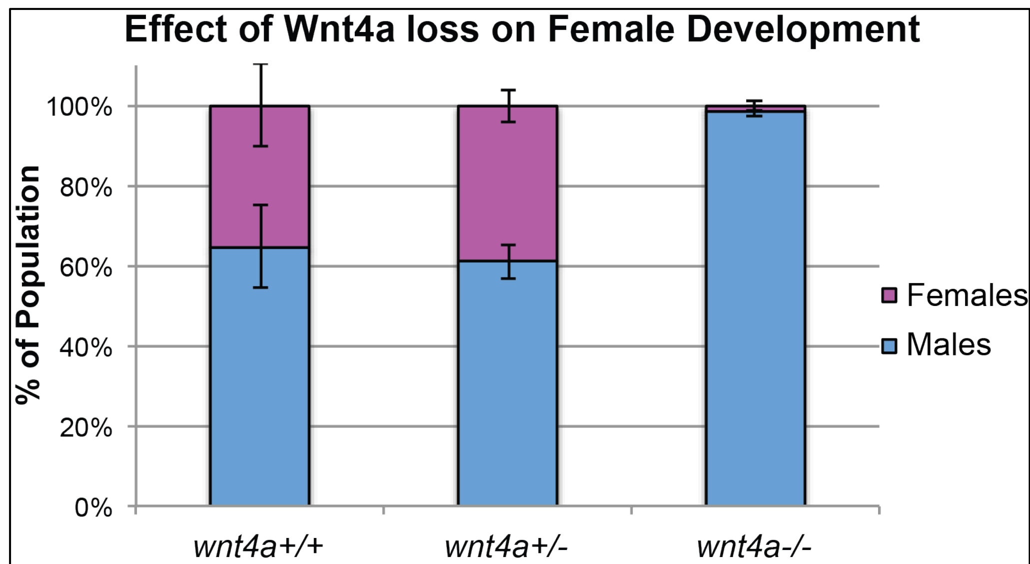


Figure 3.4 - Loss of *wnt4a* causes female-to-male sex reversal. Percent of each sex for *wnt4a+/+*, *wnt4a+/-*, and *wnt4a-/-* siblings resulting from six heterozygous in-crosses.

Effect of Wnt4a loss on primordial germ cell number

The number of germ cells in the gonad is a predictor of sex phenotype in zebrafish and other teleosts, and manipulations that reduce the number of germ cells bias sex phenotype toward males (Siegfried and Nüsslein-Volhard et al. 2008; Wang et al. 2007; Tzung et al. 2015; Lewis et al. 2008; Herpin et al. 2007). The hypothesis that Wnt4a functions in zebrafish similarly to WNT4 in mammals predicts that loss of Wnt4a function would have no effect on germ cell number (Yao et al. 2004; Chassot et al. 2014). Alternatively, if Wnt4a plays a different role in zebrafish, then germ cell numbers might be reduced in mutant fish. To test these hypotheses, I examined the number of primordial germ cells and stage I meiotic oocytes at 18 dpf in each genotype. All *wnt4a-/-* zebrafish and their wild-type siblings (homozygous and heterozygous siblings) produced primordial germ cells and more wild-type siblings produced a greater number of PGCs than *wnt4a-/-* zebrafish, but the difference between these two frequency distributions was not significant (p-value = 0.99, two-sample Kolmogorov-Smirnov test) (Figure 3.5). The difference between the mean number of PGCs in wild-type (mean = 640+/-273) and *wnt4a-/-* (mean = 578+/-326) was also not significant (p=value = 0.60, two-sample t-test). It was possible that the one wild-type individual with an abnormally low PGC count (88 PGCs) was an outlier that was skewing our results, but upon the removal of this individual from the data set, the difference between the mean number of PGCs in the wild-type population (mean = 683+/-231) and *wnt4a-/-* (mean = 578+/-326) was still not significantly different (p=value = 0.37, two-sample t-test). Only 4 of 12 (33.3%) *wnt4a-/-* individuals produced stage-I meiotic oocytes, whereas 9 out of 14 (64.3%) of their wild-type siblings produced stage-I meiotic oocytes, but the difference

between these two frequency distributions was not significant (p-value = 0.89, two-sample Kolmogorov-Smirnov test). The difference between the mean number of stage-I meiotic oocytes in wild type (mean = 95+/-126) and *wnt4a-/-* (mean = 58+/-119) was also not significant (p=value = 0.45, two-sample t-test). Even though data showed a difference between the numbers of individuals that produced stage I meiotic oocytes in *wnt4a-/-* zebrafish and their wild type siblings, this difference was not significant.

Up-regulation of canonical Wnt signaling during female gonad development

The hypothesis that Wnt4a in zebrafish functions like Wnt4 in mammals predicts that up-regulating the canonical Wnt signaling pathway in developing fish would skew sex ratios towards females, as seen in mammalian gonad development (Maatouk et al. 2008; Chassot et al. 2012; Niehrs 2012; Chassot et al. 2014; Eggers et al. 2014). To test this prediction, I used the *vasa::EGFP* transgenic line, which allows identification of males and females through differences in transgene fluorescence, and Wnt Agonist II, a compound that stimulates Wnt signaling by disrupting the interaction between Axin and β-Catenin and preventing the proteasomal degradation of β-Catenin, which in turn up-regulates Wnt signaling through β-Catenin targeted transcription (Gwak et al. 2012). The first Wnt Agonist II experiment had two duplicates of three different experimental groups (six fish tanks consisting of 25 fish each) and two duplicates of one control group (two tanks consisting of 25 fish each). Experimental groups had either 5 μM, 10 μM, or 15 μM of Wnt Agonist II dissolved in DMSO added to the tank water, and control groups had the same amount of DMSO as the experimental group added to the tank water but no Wnt Agonist II. To analyze the results I combined both replicates of each group. Results showed a slightly female-biased sex ratio (21 females, 9 males, 30% males) in the

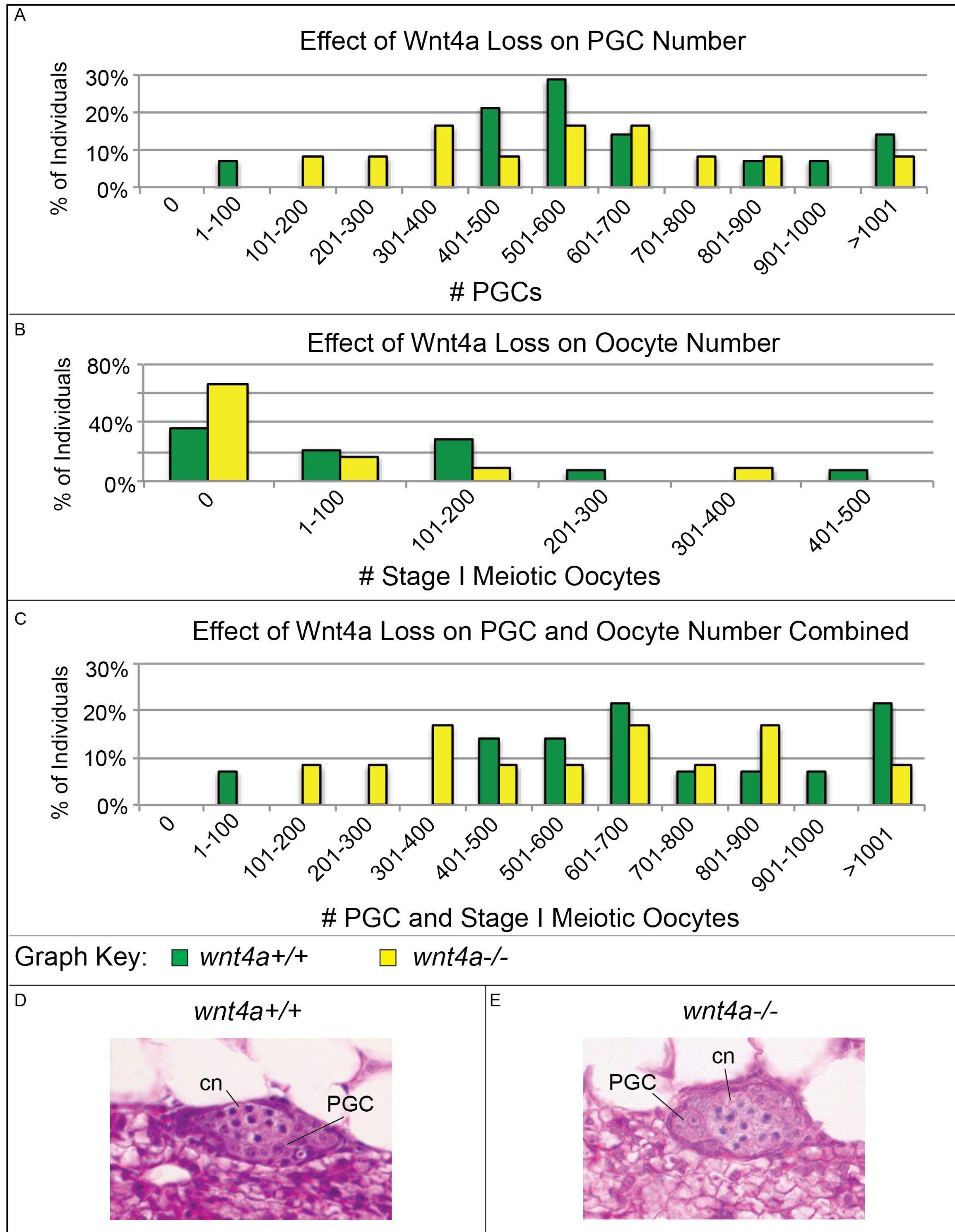


Figure 3.5 – Effect of Wnt4a loss on PGC and stage I meiotic oocyte number. (A) Effect of Wnt4a loss on PGC number (B) Effect of Wnt4a loss on PGC stage I meiotic oocyte number (C) Combined data from A and B. (D, E) Representative examples of *wnt4a+/+* and *wnt4a-/-* gonads containing PGC and stage I meiotic oocytes (cn).

experimental group that received the highest concentration (15 μM) of Wnt Agonist II; this sex ratio was significantly different from the control group's sex ratio (17 females, 25 males, 60% males) (p-value = 0.0008, chi-square test) (Figure 3.6A). Results showed no significant differences in the sex ratios between the 5 μM treatment group (19 females, 22 males, 54% males) and the control group sex ratio (p-value = 0.44, chi-square test) and no significant difference between the 10 μM treatment group (18 female, 20 male, 53% male) and control group sex ratio (p-value = 0.30, chi-square test) (Figure 3.6A). This first Wnt Agonist II experiment suggested that up-regulating Wnt signaling with a 15 μM concentration of Wnt Agonist II significantly affects female gonad development. If this result were to be confirmed, then it would support the prediction that gain of Wnt signaling function should produce male-to-female sex reversal. The 15 μM concentration of Wnt Agonist II did not result in full male-to-female sex reversal, in which all of the fish would develop as female.

To confirm the previous result and to test if a higher concentration of Wnt Agonist II would result in greater male-to-female sex reversal, I repeated the 15 μM concentration and introduced a 30 μM concentration of Wnt Agonist II. The second preliminary Wnt Agonist II experiment had two duplicates of two different experimental groups (four tanks consisting of 25 fish each) and two duplicates of one control group experiment (two tanks consisting of 25 fish each). The experimental groups received either 15 μM or 30 μM of Wnt Agonist II dissolved in DMSO, and control groups received the same amount of DMSO as the experimental group but no Wnt Agonist II. Results showed no significant differences in sex ratios between the 15 μM treatment group (17 females, 13 males; 43% males) and the control group sex ratio (16 females, 19

males, 54% males) (p-value = 0.23, chi-square test) and no significant difference between the 30 μM treatment group sex ratio (12 females, 19 males; 61% males) and the control group sex ratio (p-value = 0.43, chi-square test) (Figure 3.6B). This second preliminary experiment gave no evidence to support the conclusion that Wnt signaling significantly affected zebrafish sex ratios.

To confirm whether treating zebrafish with 15 μM of Wnt Agonist II had a significant effect on zebrafish sex ratios, I repeated the 15 μM concentration treatment in a third experiment (Figure 3.6C). The third Wnt Agonist II experiment used two duplicates of one experimental group and two duplicates of one control group. The experimental group received 15 μM of Wnt Agonist II dissolved in DMSO, and the control group received only the same amount of DMSO as the experimental group but no Wnt Agonist II. Results showed no significant difference between the 15 μM treatment group sex ratio (25 females, 22 males, 47% males) and the control group sex ratio (18 females, 18 males; 50% males) (p-value = 0.95, chi-square test) (Figure 3.6C). This result provided no evidence to support the conclusion that Wnt signaling significantly affected zebrafish sex ratios.

These results would be expected if Wnt signaling is not involved in zebrafish sex determination. It is possible, however, that Wnt signaling is involved in both the female (Wnt4a) and male (Wnt3a, Wnt10b) sex determination pathways, as proposed by some mammalian studies (Dong et al. 2015), and the treatment stimulated signaling by male and female specific Wnts equally. It is also possible that a sufficient amount of Wnt Agonist II did not actually stimulate Wnt signaling in these experiments, for example, the compound might not have entered treated zebrafish or it got in but did not stimulate Wnt

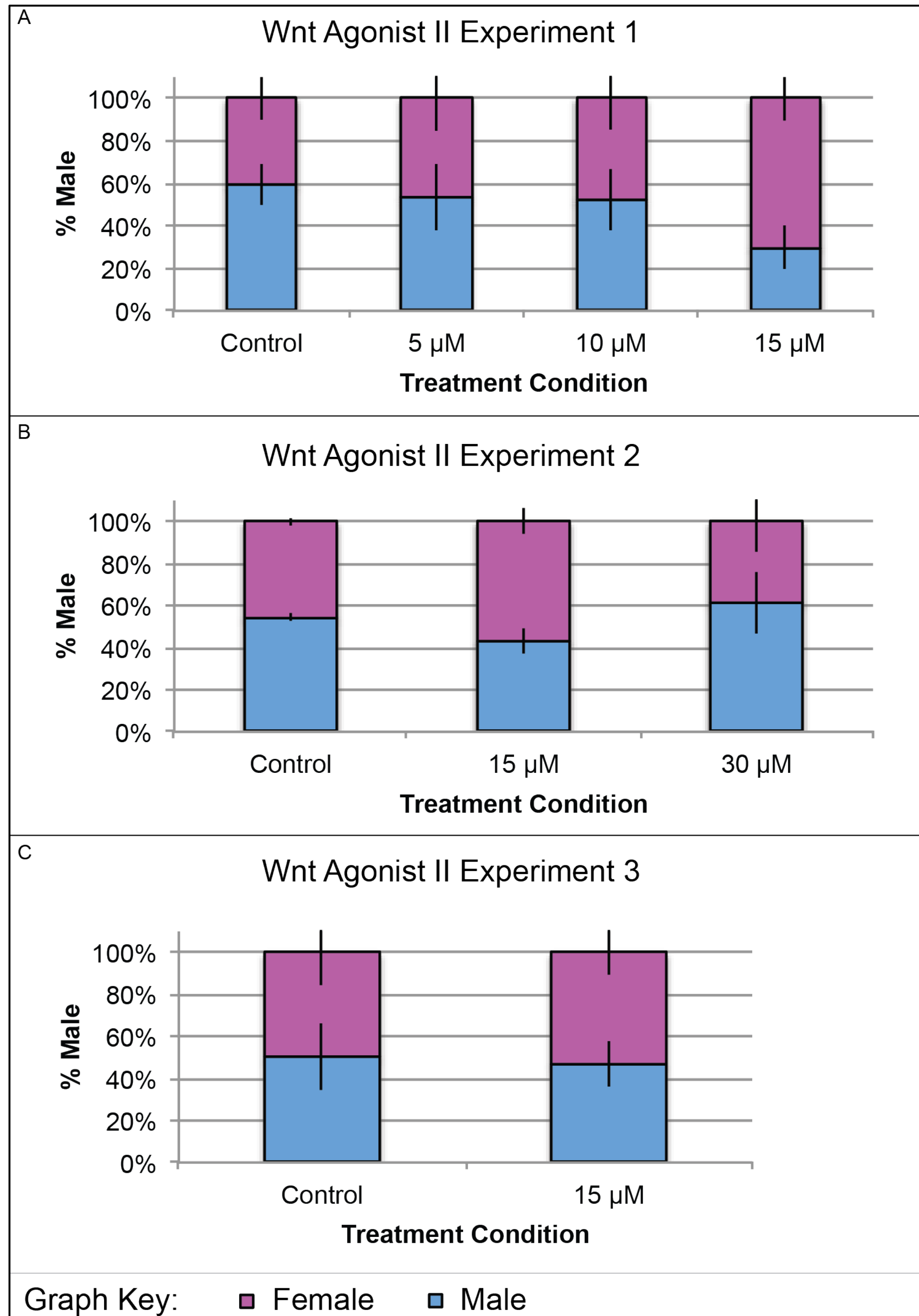


Figure 3.6 – Effect of Wnt4a loss on female gonad development. (A) Results from experiment one. (B) Results from experiment two. (C) Results from experiment three.

signaling due to its inability to interact with zebrafish Wnt pathway proteins. It is also possible that Wnt4a functions non-canonically, a process that Wnt Agonist II should not disrupt.

Discussion

Even though zebrafish (*Danio* rerio) are a popular model organism, the mechanism of zebrafish sex determination is still unknown. Multiple genes have been found to affect zebrafish gonad development, such as *amh*, *dmrt1, sox9a*, *sox9b*, and *cyp19a1a*, but the complete mechanism of zebrafish sex determination is still elusive (Rodríguez-Marí et al. 2005; Liang et al. 2015). In mammalian gonad development, *Wnt4* is expressed in the bipotential gonad to promote ovary development and inhibit testis development (She et al. 2014). Wnt4, secreted by somatic cells in mouse, activates the canonical Wnt/β-catenin pathway in nearby germ cells (Chassot et al. 2014), preventing germ cell apoptosis (Chassot et al. 2014), and inhibiting *Fgf9* and *Sox9* expression, which cause the masculinization of the gonad by initiating Sertoli cell development (Kashimada et al. 2010; Dinapoli et al. 2008; Sekido et al. 2008; She et al. 2014).

In zebrafish, we found *wnt4a* expression occurs at the time and place consistent with a functional role in gonad development. The *wnt4* gene was expressed during the early zebrafish bipotential gonadal stage (12 dpf) specifically in the somatic cells surrounding the primordial germ cells. This finding puts the expression of *wnt4a* in zebrafish, like mammals, in cells and at times that would be expected if *wnt4a* is important in zebrafish ovary development. The expression of *wnt4a* was absent at earlier stages (10 dpf) and *wnt4a* expression disappeared later in the bipotential gonadal stage (18 dpf) similar to the expression of mammalian *Wnt4,* as expected if *wnt4a* played a similar role in gonad determination as seen in mammals.

In mice, W*nt4* is important for the female sex determination pathway and suppresses testis development (She et al. 2014, Chassot et al. 2014). In mice,

chromosomally XX gonads lacking WNT4 function show a partial female-to-male sex reversal; they no longer develop a complete ovary, and instead develop an ovotestis containing both ovarian and testicular tissue (DiNapoli et al. 2008). In humans with SERKAL syndrome, chromosomally XX gonads lacking WNT4 function no longer develop as a complete ovary, and instead develop either an ovotestis that contains both ovarian and testicular tissue, or a full testis, full female-to-male sex reversal (Mandel et al. 2008). These findings led us to predict that loss of Wnt4a function should result in female-to-male sex reversal in zebrafish. When comparing the sex ratios of *wnt4a-/-* mutants to the sex ratios of *wnt4a+/-* and *wnt4a+/+* siblings, however, I found that 99% of *wnt4a-/-* fish became males compared to just 65% of homozygous wild types. All except two homozygous *wnt4a* mutants developed testes, and none developed an ovotestes, instead all of the 20 males developed testes. This finding supports the conclusion that Wnt4a function is required to prevent female-to-male sex reversal in AB strain zebrafish. The full female or full male phenotypes shown by *wnt4a* mutant zebrafish are different than the partial female-to-male sex reversal seen in mice (Vainio et al. 1999), but similar to the full female-to-male sex reversal phenotypes seen in some SERKAL syndrome patients (Mandel et al. 2008).

The expression of *wnt4a* in the bipotential zebrafish gonad and the male-biased sex ratios seen in *wnt4a-/-* zebrafish indicate that Wnt4a function is necessary for zebrafish ovary development, but what exactly does *wnt4a* do? Previous studies showed that the number of germ cells in the gonad influences gonad development in fish, because the germ-line is required for ovary development and fish lacking germ cells develop testes (Siegfried and Nüsslein-Volhard et al. 2008; Wang et al. 2007; Tzung et al. 2015;

Lewis et al. 2008; Herpin et al. 2007). In both threespine stickleback and medaka, fish with a greater number of primordial germ cells are more likely to develop as females and fish with fewer primordial germ cells are more likely to develop as males (Lewis et al. 2008; Herpin et al. 2007). The number of germ cells in the gonad is also important for zebrafish gonad development; zebrafish with a greater number of primordial germ cells tend to develop as females and those with fewer develop as males (Siegfried and Nüsslein-Volhard et al. 2008; Wang et al. 2007; Tzung et al. 2015). One hypothesis is that Wnt4a possesses different functions in zebrafish than mammals and acts to increase the number of primordial germ cells in the bipotential gonad, whereas the absence of Wnt4a activity hinders ovary development by leading to a decrease in the number of primordial germ cells in the bipotential gonad. If zebrafish Wnt4a functions by increasing the number of germ cells in the bipotential gonad promoting female development, then we might observe a reduction of germ cell number in *wnt4a-/-* zebrafish as compared to their wild-type siblings. In mammals, Wnt4 does not lead to an increase in the number of germ cells in the developing gonad, but does prevent germ cell apoptosis (Liu et al. 2010; Chassot et al. 2014), which leads to our hypothesis that zebrafish Wnt4a functions like mammalian Wnt4. If zebrafish Wnt4a functions like mammalian WNT4, then loss of Wnt4a function would have no effect on the number of germ cells in the gonad. A comparison of *wnt4a-/-* zebrafish to their wild-type siblings revealed no significant difference between genotypes in the number of primordial germ cells in the gonad, indicating that *wnt4a* does not control primordial germ cell development. We also observed that some *wnt4a-/-* individuals produced stage I meiotic oocytes, indicating that these individuals still initially develop a juvenile ovary. All AB zebrafish develop

oocytes before the gonads differentiate into either an ovary or testes (Uchida et al. 2002). In males, oocyte apoptosis occurs and the gonad becomes a testis, but in females, the oocytes mature and gonad becomes an ovary (Uchida et al. 2002). It is possible that *wnt4a* is important in preventing oocyte apoptosis, and that hypothesis could explain why zebrafish develop predominately as males when Wnt4a function is lost. To test this hypothesis we could preform TUNEL or anti-active Caspase 3 assays to detect apoptotic cells or compare apoptosis-related gene transcript levels using RNA-seq.

In the majority of mammalian studies, W*nt4* was found promote ovary development by stimulating the canonical Wnt signaling pathway (Eggers and Sinclair 2012), although another study using cell culture instead of *Wnt4* mutants indicated that Wnt4 promotes ovary development by stimulating non-canonical Wnt signaling (Bernard et al. 2008). Our fluorescent studies treating zebrafish larvae and juveniles with an agonist to canonical Wnt signaling did not detect any effect of increasing Wnt Agonist II concentrations on zebrafish sex ratios. One reason for this finding could be that insufficient Wnt Agonist II entered the bodies of treatment group zebrafish, which remains to be investigated by looking at the upregulation of target genes in canonical Wnt signaling. These results could indicate that Wnt signaling is not involved in zebrafish sex determination, or that Wnt signaling is involved in both the female (Wnt4a) and male (Wnt3a, Wnt10b) sex determination pathways, as proposed by some mammalian studies (Dong et al. 2015), and the treatment stimulated signaling by Wnt4a, Wnt3a, and Wnt10b equally. It is also possible that Wnt4a functions non-canonically, as seen in the previous cell culture study (Bernard et al. 2008), which would mean that targeting the canonical Wnt pathway might not have an effect. To further test if the canonical Wnt signaling

pathway is involved in the female zebrafish sex determination pathway, future experiments could compare the expression of Wnt signaling target genes between *wnt4a-/-* zebrafish and their wild type siblings through RNA-seq analysis.

Overall we found evidence that *wnt4a* is important for ovary development in zebrafish, because *wnt4a* is expressed early on in zebrafish gonad development and the lack of Wnt4a function creates a significantly male biased population. Our findings indicate that Wnt4a functions similarly to mammalian Wnt4. Zebrafish *wnt4a* is expressed in the somatic cells of the bipotential gonad, similar to mammalian *Wnt4*. Zebrafish lacking Wnt4a function experience full female-to-male sex reversal similar to some phenotypes seen in human SERKAL syndrome patients, in which XX individuals developed complete testes, but differ from phenotypes seen in *Wnt4-/-* mice, in which XX individuals developed ovotestes instead of complete testes, indicating that Wnt4a function is important for proper ovary development in zebrafish, similar to mammals. Loss of Wnt4a function, while tending to reduce germ cell number, does not achieve statistical significance, indicating that Wnt4a functions similar to mammalian Wnt4. In mice gonads lacking Wnt4 function, germ cells proliferate normally during early development, but germ cells undergo apoptosis around 16.5 days post coitum (dpc), suggesting that *Wnt4* helps ovary development through the prevention of germ cell apoptosis (Yao et al. 2004; Chassot et al. 2014). In mice gonads lacking Rspo1 function, some germ cells do not proliferate normally and form a masculinized gonad that eventually develops Sertoli cells by birth, suggesting that *Rspo1* is also important for the development of the ovary by allowing germ cell proliferation and suppressing masculinization (Auguste et al. 2011; Chassot et al. 2012; Chassot et al. 2014). In mice

gonads lacking both Wnt4 and Rspo1 function, XX mice show full female-to-male sex reversal, suggesting that both proteins are necessary for proper ovary development and thus act in at least partially different pathways (Auguste et al. 2011; Chassot et al. 2012; Maatouk et al. 2013; She et al 2014; Chassot et al. 2014). It is possible that zebrafish *wnt4a* and *rspo1* act together to promote ovary development, which is the focus of future studies. These data together support the hypothesis that *wnt4a,* like mammalian *Wnt4,* is important for zebrafish ovarian development.

Bridge

In this study we show that zebrafish Wnt4a functions like mammalian Wnt4 in the context of sex determination and is necessary for proper ovary development. We found that *wnt4a* is expressed in the somatic cells of the gonad during the bipotential gonadal stage, similar to mammalian *Wnt4*. We found female-to-male sex reversal when Wnt4a function was lost, similar to mammalian phenotypes. We found that loss of Wnt4a function does not reduce germ cell number in mammals. Our experiments failed to show that a compound that purportedly activates Wnt signaling promotes ovary development in zebrafish, although these results still need to be investigated further. Knowing that zebrafish Wnt4a functions similarly to mammalian Wnt4, and that loss of Wnt4 function can cause defects in both Müllerian and Wolffian duct formation in mammals (Morcel et al. 2007; Pai and Shakir 2013), we can then investigate whether or not *wnt4a* is required for proper male reproductive duct development and fertility.

CHAPTER IV

ROLE OF WNT4A IN ZEBRAFISH MALE REPRODUCTIVE DUCT DEVELOPMENT

The research described in this chapter was performed by **High SK**, Yan YL, BreMiller R, and Postlethwait J. I designed and performed all of the fertility experiments. I analyzed the data from all of the experiments and wrote the initial drafts of the paper. Yan YL, BreMiller R helped me design and perform the *in situ* hybridization and H&E experiments.

Introduction

In sexual reproduction, each species is generally divided between two reproductive groups, male and female, which both have unique and complementary reproductive features. Generally, fully functional males and females require functional reproductive tracts. Without a functional reproductive tract, gametes -- and in the case of mammals, even embryos -- would not be able to exit the body for fertilization. To gain an understanding of reproductive tract development, we review first mammalian reproductive tract development, which is the best understood, and then study how reproductive tract development differs in other species, such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*).

In mammals, two reproductive tracts, the Wolffian ducts, which will develop into the male reproductive tract, and Müllerian ducts, which will develop into the female reproductive tract, develop in both male and female fetuses. In mice of both sexes, the Wolffian ducts form before the Müllerian ducts, around embryonic day (E) 8.5, elongate

caudally from the gonad and connect to the cloaca, the posterior opening to the exterior of the animal, at E10.5 (Chiga et al. 2014). Müllerian ducts start forming around E11.5, elongate caudally using the Wolffian ducts as a guide, and connect to the urogenital sinus, the ventral part of the cloaca, at E13.5 (Chiga et al. 2014). If the animal continues to develop as a female, the Wolffian ducts regress prior to birth due to low amounts of testosterone, and the Müllerian ducts differentiate into the oviduct, uterine horns, cervical canal, and upper vagina (Staak et al. 2003; McLennan and Pankhurst 2015). If the animal continues to develop as a male, the Müllerian ducts regress, which is initiated by Amh (AntiMüllerian Hormone, a peptide hormone produced and secreted by the Sertoli cells of the testes), prior to birth, and induced by testosterone, the Wolffian ducts differentiate into epidiymides, vas deferens, and seminal vesicles (Staak et al. 2003; Chiga et al. 2014; McLennan and Pankhurst 2015). In the adult male, the seminiferous tubules of the testis produce gametes, which travel through the rete testis, efferent ducts, epididymis, and vas deferens (Staak et al. 2003). Proper Müllerian duct, and in some cases Wolffian duct, formation depends on *Wnt4* (Morcel et al. 2007; Pai and Shakir 2013)

Three Wnt (Wingless-type MMTV integration site family) genes, *Wnt4*, *Wnt5a*, and *Wnt7a*, are involved in Müllerian duct formation; *Wnt4* is critical for its initiation and extension and *Wnt5a* and *Wnt7a* are important for its differentiation (Mericskay et al. 2004; Kobayashi et al. 2011; Prunskaite-Hyyryläinen et al. 2016). Müllerian ducts that started developing fail to elongate once Wnt4 function is blocked after initial duct development, and overexpressing *Wnt4* promoted cell migration (Prunskaite-Hyyryläinen et al 2016), indicating that *Wnt4* is necessary for Müllerian duct elongation. *Wnt4-/-* XX mice have masculinized Müllerian ducts; the Müllerian ducts are more coiled and look

more similar to the male Wolffian duct epidiymal region than to the wild type Müllerian duct, indicating that *Wnt4* is important for proper Müllerian duct formation (Vainio et al. 1999; She et al. 2014).

Müllerian ducts fail to form in genetically female humans with Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome. MRKH is a complex syndrome and the exact genetic cause has been under some debate (Pizzo et al 2013). MRKH might be caused by mutations in multiple genes, such as *Wt1*, *Pax2*, *Amh*, and *Wnt4* (Pizzo et al 2013). A mutation in Wnt4 resulting from an L12P (amino acid 12 has changed from a Leucine to a Proline) substitution was found in all 28 female MRKH patients in one study (Philibert et al. 2008; Pizzo et al. 2013), indicating that *Wnt4* is important for proper Müllerian duct formation in humans as well as mice. Interestingly, MRKH syndrome has also been found in two genetically male patients who had female siblings that also had MRKH syndrome, and results in underdeveloped Wolffian ducts and infertility, suggesting that Wnt4 may play a role in male reproductive duct development as well, although no genetic tests were performed on these patients (Zlotogora 1995; Wellesley and Slaney 1995; Morcel et al. 2007; Pai and Shakir 2013). In SERKAL syndrome, which is also caused by a mutation in *Wnt4*, XX patients develop testes, which differs from MRKH patients who develop ovaries, and have abnormally formed Müllerian ducts, similar to some MRKH patients (Mandel et al. 2008; Philibert et al. 2008; Pizzo et al. 2013). The effect of SERKAL syndrome on Wolffian duct development has not been studied. *Wnt4* is primarily involved in mammalian Müllerian duct formation; it also might play a roll in male reproductive duct development.

In mice, *Wnt4* is important for development of the male reproductive system (Jeays-ward et al. 2004). Prior to birth, *Wnt4-/-* XY mice have fewer and more disorganized testicular cords, which eventually become seminiferous tubules (Jeays-ward et al. 2004). After birth, however, *Wnt4-/-* adult XY mice have normal (indistinguishable from wild type) testes, reproductive ducts, and sperm production (Vainio et al. 1999). Evidently the malformed testicular cords found in *Wnt4-/-* fetal male mice are rescued by birth (Jeays-Ward et al. 2004, Satoh 1991).

Mammals provide a great model to understand reproductive duct development, but looking at just one group of vertebrates doesn't allow us to fully understand the evolution of mechanisms controlling reproductive duct development, and if they are conserved. To better understand evolution of the mechanisms behind duct development, we examined whether these mechanisms are conserved in teleosts. Because *Wnt4* is involved in mammalian reproductive duct development, we decided to examine the roles of *wnt4* in zebrafish development.

Unlike mammals, teleosts do not develop Müllerian or Wolffian ducts (Lasala et al. 2004; Rodríguez-Marí et al 2005; Lee et al. 2011). Instead, teleost reproductive ducts are derived from other tissues besides Müllerian or Wolffian ducts and the male and female reproductive ducts do not develop simultaneously (Lee et al. 2011). In general, male teleost reproductive ducts develop as an extension from the testis and female teleost reproductive ducts develop as an extension from the ovary (Suzuki and Shibata 2004; Lee et al. 2011).

The literature disagrees on the terminology for male teleost reproductive ducts (Lee et al. 2011). Male salmonid and cyprinid species are described as having

seminiferous tubules, testicular efferent ducts, and testicular main ducts (Lahnsteiner et al. 1993; Lahnsteiner et al. 1994), whereas male medaka (*Oryzias latipes*) is described as having efferent ducts and sperm ducts (Suzuki and Shibata 2004), and male zebrafish are described as having ducti deferens, fused ducti deferens, and genital orifice (Menke et al. 2011). Despite disagreement over male reproductive duct terminology, the general structures of male reproductive ducts are shared among teleosts.

In medaka, the male and female reproductive ducts do not develop in both sexes as in mammals, and instead develop independently in males and females. The reproductive ducts in both medaka males and females initially form as a mass of somatic cells (cell mass) near the posterior end of either the testis or ovary around 20 to 30 days post hatching (dph), and the reproductive ducts are fully formed by 50 to 90 dph in males or 80 to 90 dph in females (Suzuki and Shibata 2004).

In medaka (*Oryzias latipes*) males, reproductive ducts consist of the efferent duct and sperm duct (Suzuki and Shibata 2004). The primordial reproductive duct develops from the posterior end of the testis as a mass of somatic cells around 20 to 30 dph (Suzuki and Shibata 2004). The mass begins to elongate posteriorly as a rod-like structure that finally forms a lumen. The rod differentiates into the efferent duct, which is positioned along the testes, and sperm ducts, which continue from the posterior end of the efferent ducts (Suzuki and Shibata 2004). Male reproductive ducts are fully formed, with the connection of the sperm duct with the urethra by 50-90 dph (Suzuki and Shibata 2004). In adult male medaka, gametes travel from the testes to the efferent ducts, then to the sperm duct, and finally through the connection to the urethra and into the external environment.

In medaka females, reproductive ducts consist of the ovarian cavity and oviduct. As in medaka males, at around 20 to 30 dph a mass of somatic cells form at the posterior end of the ovary, but unlike medaka males, cell layers extend from both the ventral and dorsal sides of the posterior end of the ovary and connect with the cell mass eventually to form the primordial reproductive duct of the female; this primordial reproductive duct elongates posteriorly as a rod-like structure, that does not yet contain a lumen (Suzuki and Shibata 2004). The ovarian cavity, located along the dorsal side of the ovary, differentiates from the cell layers that initially extended from the dorsal and ventral sides of the ovary by 30-50 dph, and the oviduct forms from the rod-like posteriorly projecting cell mass, which forms a lumen by 30-50 dph (Suzuki and Shibata 2004). By 40-60 dph, the ovarian cavity and oviduct have connected, and the oviduct has continued to develop posteriorly (Suzuki and Shibata 2004). By 80 to 90 dph the oviduct, which up to this point continued developing as a tube-like structure containing a lumen, has finally fully connected and opened to the exterior of the fish (Suzuki and Shibata 2004). In the adult female medaka, gametes travel from the ovary, to the ovarian cavity, and then through the oviduct and into the external environment.

Medaka is just one species among the 25,000 species in the subclass of teleosts. Teleosts consist of a wide range of different species, and the way reproductive ducts are organized and formed could vary across species. Investigating the reproductive duct development of other teleost species, such as zebrafish, could either inform us of the variations in reproductive duct development or confirm that reproductive ducts develop similarly in multiple teleost species. To effectively compare reproductive duct and gonad

development in teleosts to other vertebrates, such as mice or humans, we need to understand genome duplication events that occurred throughout evolution.

Zebrafish is a popular model organism, but much is yet unknown about the development of their reproductive tract. In fully developed adult zebrafish, the male reproductive ducts are found to be similar to those seen in medaka. The adult zebrafish male reproductive system is made up of the testes, ducti deferens, fused ducti deferens, and genital orifice (Menke et al. 2011). The development of the male zebrafish reproductive tract and which genes are involved in reproductive tract development are as yet unknown. A working hypothesis is that *wnt4a*, like mammalian *Wnt4,* is involved in zebrafish reproductive duct development. Although zebrafish male reproductive ducts, unlike mammalian male reproductive ducts, do not develop from a Wolffian duct like structure, we hypothesize that *wnt4a*, retains a function similar function to mammalian *Wnt4*, and is important for zebrafish male reproductive duct development.

Material and Methods

Fish Stocks

Fish stocks used were AB wild type and the $wnt4a^{fh294/+}$ mutant line (Moens et al. 2009), which was developed in an AB background. The resulting *wnt4a* point mutation creates a premature stop codon at amino acid 307, which disrupts the Wnt4 domain that binds to Frizzled receptors (Moens et al. 2009). Fish were maintained as previously described by Westerfield (2007).

Genotyping

The primers used for genotyping the *wnt4a*$^{fh294/+}$ mutant line were Wnt4a(C307*)F1 (5-GTCAAGACCTGCTGGAAAGC-3) and Wnt4a(C307*)R1 (5-CTTGCGACACTGTTTGCATT-3) (Moens 2009). The PCR conditions were: 1'94°C; 35 cycles of: 30"94°C, 30"60°C, 1'30"72°C; followed by 15°C until program is ended. Following the *wnt4a*$^{fh294/+}$ genotyping PCR, the amplicons were digested with DdeI at 37°C overnight (Moens 2009). Sizes of bands after DdeI digest: 384bp (WT), 270bp + 114bp (mutant).

Immunohistochemistry and Histology

Animals were collected at multiple stages of zebrafish male reproductive duct development. Animals were then euthanized, fixed, and cryosectioned as previously described (Rodriguez-Mari et al. 2005). The probe for *wnt4a* was created using the following primers: Wnt4a+589 (5-CCGCAACCGCCGATGGAACT-3) and Wnt4a-1207 (5-ACCGGGCGTTCTGGGGTCAT-3). The *wnt4a* cDNA was cloned using the TOPO vector and used to synthesize DIG-labeled probes. For the *in situ* hybridization experiment two 25 dpf, two 35 dpf male, and two 55 dpf male zebrafish were used. For hematoxylin and eosin staining, animals were euthanized and fixed in bouin's solution for more than 48 hours, then were washed multiple times in 70% ethanol and paraffin sectioned. Imaging was done using a Leica compound microscope.

Fertility Experiments

Zebrafish from the *wnt4a*$^{fh294/+}$ mutant line were used for fertility experiments. For natural mating experiments, multiple pairwise crosses were set up using *wnt4a-/-* males and *wnt4a+/+* female siblings. For *in vitro* fertilizations, testes were extracted from euthanized *wnt4a-/-* males, macerated and added to eggs obtained from *wnt4a+/+* female siblings. These procedures were done as previously described by Westerfield (2007).

Ethics

Zebrafish were handled in accordance with good animal practices and animal work was approved by the University of Oregon Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A-3009-01, IACUC protocol #14-08RA)

Results

Effect of Wnt4a function loss on sperm production

Because loss of Wnt4 function in adult male mice did not affect sperm production (Vainio et al. 1999), we did not expect loss of Wnt4a function to affect sperm production in zebrafish. To test this prediction, seven *wnt4a-/-* zebrafish males and five *wnt4a+/+* male siblings were cross-sectioned and H&E stained. In such sections, sperm are detected as dark purple areas (Figure 4.1). Sections showed that all *wnt4a-/-* males and *wnt4a+/+* male siblings contained sperm in their testes (Figure 4.1), and that there was no

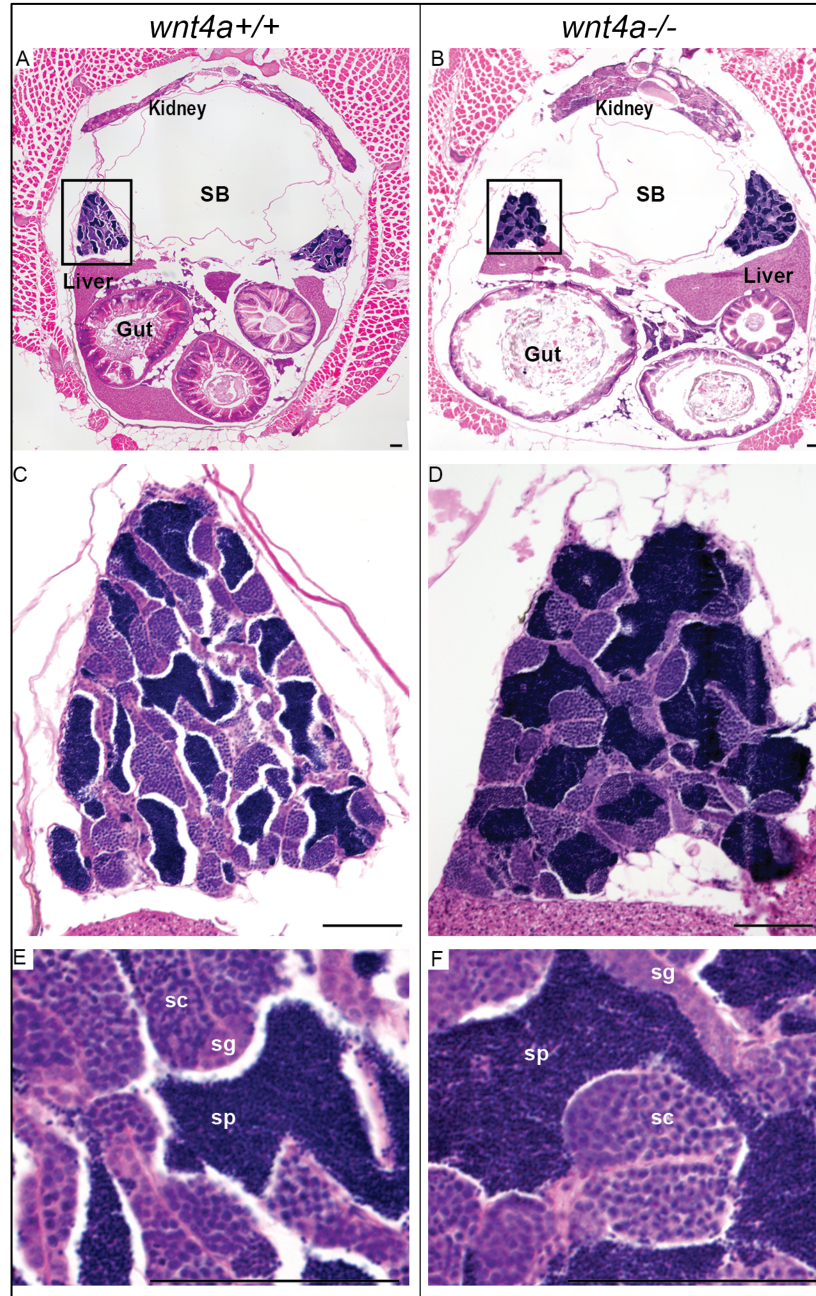


Figure 4.1 - Cross sections of wild types and *wnt4a* mutants. (A) Whole body, Wild type male. (B) Whole body, *wnt4a-/-* male. (C) Right testis of wild type. (D) Right testis of mutant. (E) Close-up of wild type testis tubules. (F) Close-up of mutant tubules. (SB = swim bladder, sc = spermatocytes. sg = spermatogonia. sp = spermatids) Scale bar = 100µm.

difference in the sperm area (gonad area that consists of sperm) between *wnt4a-/-* males and *wnt4a+/+* male siblings (p-value = 0.318, two sample t-test). These data show that loss of Wnt4a function does not block sperm production because sperm is clearly present in both *wnt4a-/-* zebrafish and their wild-type siblings.

Effect of Wnt4a function loss on fertility

Histology showed that mutant males lacking normal Wnt4a function produce sperm. We next wondered how the loss of Wnt4a affects zebrafish fertility. In the first experiment we compared the capability of *wnt4a-/-* males and their wild-type siblings (*wnt4a+/+*) to yield viable offspring in natural matings. Nine *wnt4a-/-* males and nine *wnt4a+/+* male siblings were individually crossed to 2-3 wild-type females. The *wnt4a-/-* males exhibited similar mating behavior as their wild-type siblings. Results showed that 69.2% of the offspring from the *wnt4a+/+* male siblings were viable and 0% of the offspring from all of the *wnt4a-/-* crosses were viable (Figure 4.2). We conclude that *wnt4* mutant males are sterile.

Several possible mechanisms could explain why mutant males, which have plenty of mature sperm in their testes, are sterile due to a defect in sperm release, sperm motility, ability to penetrate the micropile, or defective male pronucleus. To determine if *wnt4a-/-* sperm can function once outside the testis, we stimulated sperm release by gently squeezing along the sides of the *wnt4a-/-* males and *wnt4a+/+* male siblings (Westerfield 2007), and activated the sperm while viewing with a microscope. We tested 16 *wnt4a-/-* males and 21 *wnt4a+/+* male siblings. 76.1% (16/21) of the *wnt4a+/+* male siblings released sperm and 0% (0/16) of the *wnt4a-/-*

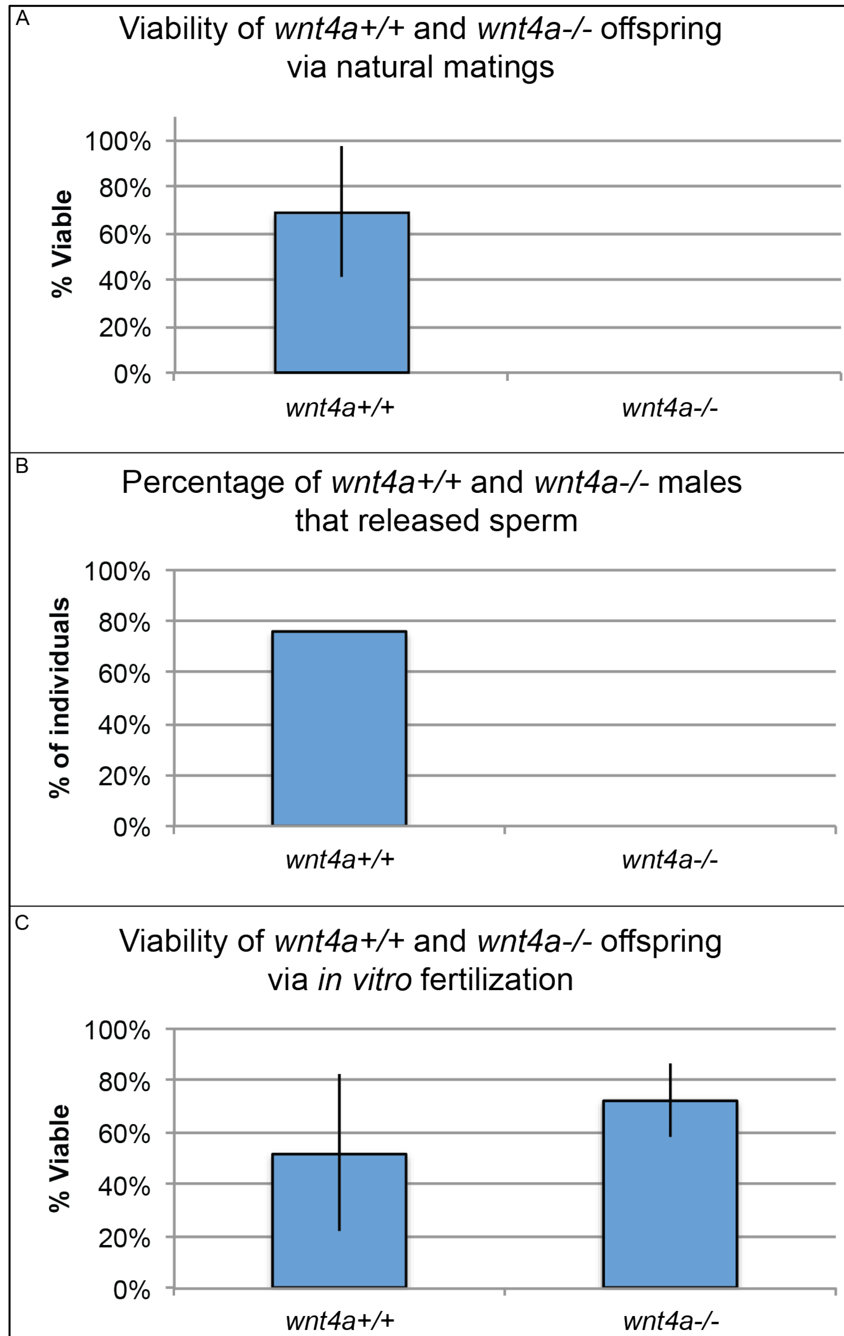


Figure 4.2 - Testing *wnt4a* mutant male fertility. (A) Viability of *wnt4a-/-* offspring obtained via natural matings compared to viability of offspring from their *wnt4a+/+* siblings obtained via natural matings. (B) Percentage of *wnt4a+/+* and *wnt4a-/-* males that released sperm upon stimulation. (C) Viability of *wnt4a-/-* offspring obtained *in vitro* fertilization following testes dissection and maceration compared to the viability of the offspring from their *wnt4a+/+* siblings obtained by *in vitro* fertilization following testes removal and maceration.

males released any fluid (p-value = 1.09E-12, chi-square test; Figure 4.2). This result suggests that *wnt4a-/-* males are not able to release sperm.

To directly test the ability of *wnt4a-/-* sperm to fertilize eggs, we preformed *in vitro* fertilization by dissecting testes from *wnt4a-/-* males and their wild-type siblings, macerating the testes, and exposing the resulting fluid to eggs laid by *wnt4a+/+* AB females. We tested five *wnt4a-/-* males and three *wnt4a+/+* male siblings, and looked at the viability of the offspring. Results showed that 52.2% (283 +/- 26.5) of the offspring of *wnt4a+/+* male siblings were viable and 72.7% (405 +/- 66.7) of the offspring from the *wnt4a-/-* males were viable (Figure 4.2). This result shows that the sperm produced by *wnt4a-/-* males is functional, but that sperm are unable to exit the body. Results also raise the possibility that *wnt4a* mutants have a defect in the male reproductive ducts.

<u>Effect of Wnt4a function loss on adult reproductive ducts</u>

The hypothesis that Wnt4a function is important for the development of zebrafish reproductive ducts predicts that *wnt4a-/-* male zebrafish should develop malformed reproductive ducts. To test this prediction, I examined reproductive ducts of five *wnt4a-/-* males and four *wnt4a+/+* male siblings (diagram shown in Figure 4.4A). All four *wnt4a+/+* male siblings had normal ducti deferens, fused ducti deferens, and genital orifices (Figure 4.3A-D). In contrast, of the five *wnt4a-/-* males, one did not form any posterior ducti deferens or fused ducti deferens (figure not shown), two had malformed ducti deferens and malformed fused ducti deferens that contained sperm (Figures 4.3E-F), and two had malformed ducti deferens and malformed fused ducti deferens that had cellular tissue covering the ducts which prevented sperm travel (Figures 4.3G-H). All *wnt4a-/-* males had malformed ducti deferens and malformed fused ducti

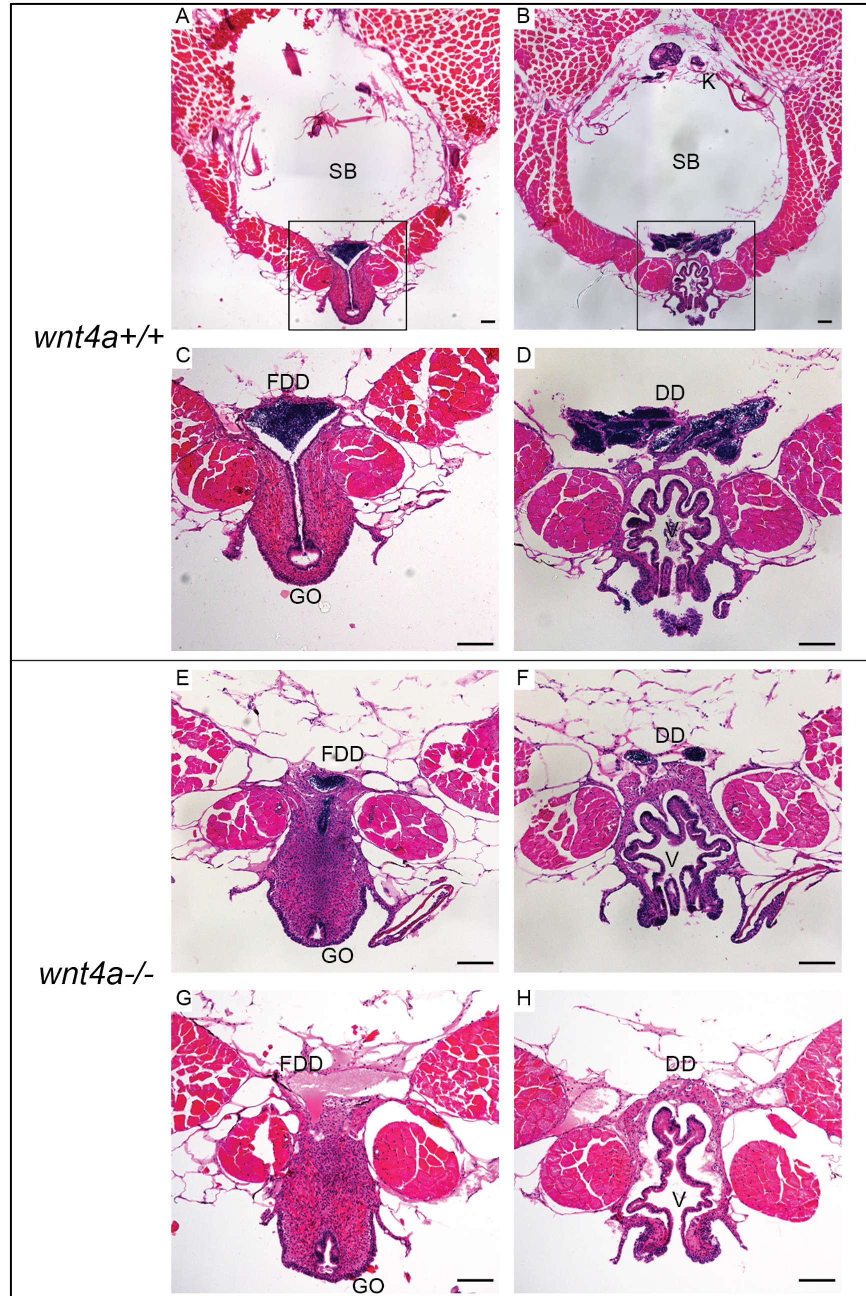


Figure 4.3. Duct morphology in *wnt4a* mutant and wild type males. A, B - Cross sections of a representative *wnt4a*+/+ male is shown at different anterior-posterior levels. The level of section in A shows the fused ducti deferens and genital orifice, and the level of section in B shows the ducti deferens and vent. C - Cross section of a *wnt4a*+/+ male showing fused ducti deferens and genital orifice. D - Cross section of a *wnt4a*+/+ male showing ducti deferens and vent. E, G - Cross sections of two *wnt4a*-/- male fused ducti deferens and genital orifice phenotypes. F, H – Cross sections of two *wnt4a*-/- male ducti deferens and vent phenotypes. (SB = swim bladder. FDD = fused ducti deferens. GO = genital orifice. DD = ducti deferens. V = vent) Scale bar = 100μm.

deferens (Figures 4.3E-H), and all *wnt4a-/-* males tested failed to connect the fused ducti deferens to the genital orifices (Figures 4.3E, 4.3G). This result indicates that loss of Wnt4a function causes defects in male reproductive duct formation, and thus Wnt4a function is necessary for the formation of zebrafish male reproductive ducts.

Effect of Wnt4a function loss on reproductive duct development

Results presented above show that loss of Wnt4a function causes a failure in the connection of the reproductive ducts to the genital orifice preventing sperm release in male zebrafish, but neither the timeline of normal zebrafish reproductive duct development nor the first deviant phenotype resulting from Wnt4a loss is currently known. To discover when and how male reproductive ducts develop, I collected *wnt4a+/+*, *wnt4a+/-*, and *wnt4a-/-* individuals at 25, 35, 45, and 55 dpf. These fish were cross-sectioned, H&E stained, and analyzed in serial sections to determine the schedule of defects that occur in *wn4a* mutant ducts. Animals were scored for duct position (from the posterior end of the testes to the posterior end of the genital orifice). Figure 4.4A shows a diagram of adult reproductive ducts. Duct extremities were measured and are represented as a percentage in Figure 4.4B. In this figure, *wnt4a+/+* and *wnt4a+/-* are combined and treated as wild type.

At 25 dpf, both the wild-type and *wnt4a-/-* male reproductive ducts had already started to develop as a cell mass, starting from the posterior testes and terminating well before the genital orifice. Development of the combined wild-type reproductive ducts and *wnt4a-/-* reproductive ducts was indistinguishable (Figure 4.4B, p-value = 0.064, ns, two-sample t-test).

At 35 dpf, wild-type reproductive ducts had grown significantly further than the *wnt4a-/-* reproductive ducts (Figure 4.4B, p-value = 0.001, two-sample t-test). The reproductive duct lumen had started to form from the posterior region of the testis posteriorly toward the genital orifice with the cell mass continuing more posteriorly toward the genital orifice in wild types, but not in their *wnt4a-/-* siblings. The *wnt4a-/-* reproductive duct still consisted only of a cell mass, and had not developed a lumen.

At 45 dpf, wild-type reproductive ducts were still significantly more developed than *wnt4a-/-* reproductive ducts (Figure 4.4B, p-value = 0.015, two-sample t-test). The reproductive duct lumen had developed further posteriorly in the wild-type individuals, and had started to form in *wnt4a-/-* mutants.

At 55 dpf, wild-type reproductive ducts were fully developed and functional, whereas *wnt4a-/-* reproductive ducts had not yet reached the position where the connection between the fused ducti deferens and genital orifice will occur (p-value = 0.001, two-sample t-test). In wild-type adult males (243 – 304 dpf), reproductive ducts remained fully developed but *wnt4a-/-* males were still missing the connection between the fused ducti deferens and genital orifice. Although this difference was no longer statistically significant (Figure 4.4B, p = 0.063, ns, two-sample t-test), this result is still significant in a biological sense, as none of the *wnt4a-/-* males have fully functional reproductive ducts and therefore can't release sperm to reproduce. In elderly wild-type male zebrafish (531 dpf), reproductive ducts were still normal but *wnt4a-/-* males were still missing the connection between the fused ducti deferens and genital orifice (Figure 4.4B, p = 0.246, ns, two-sample t-test).

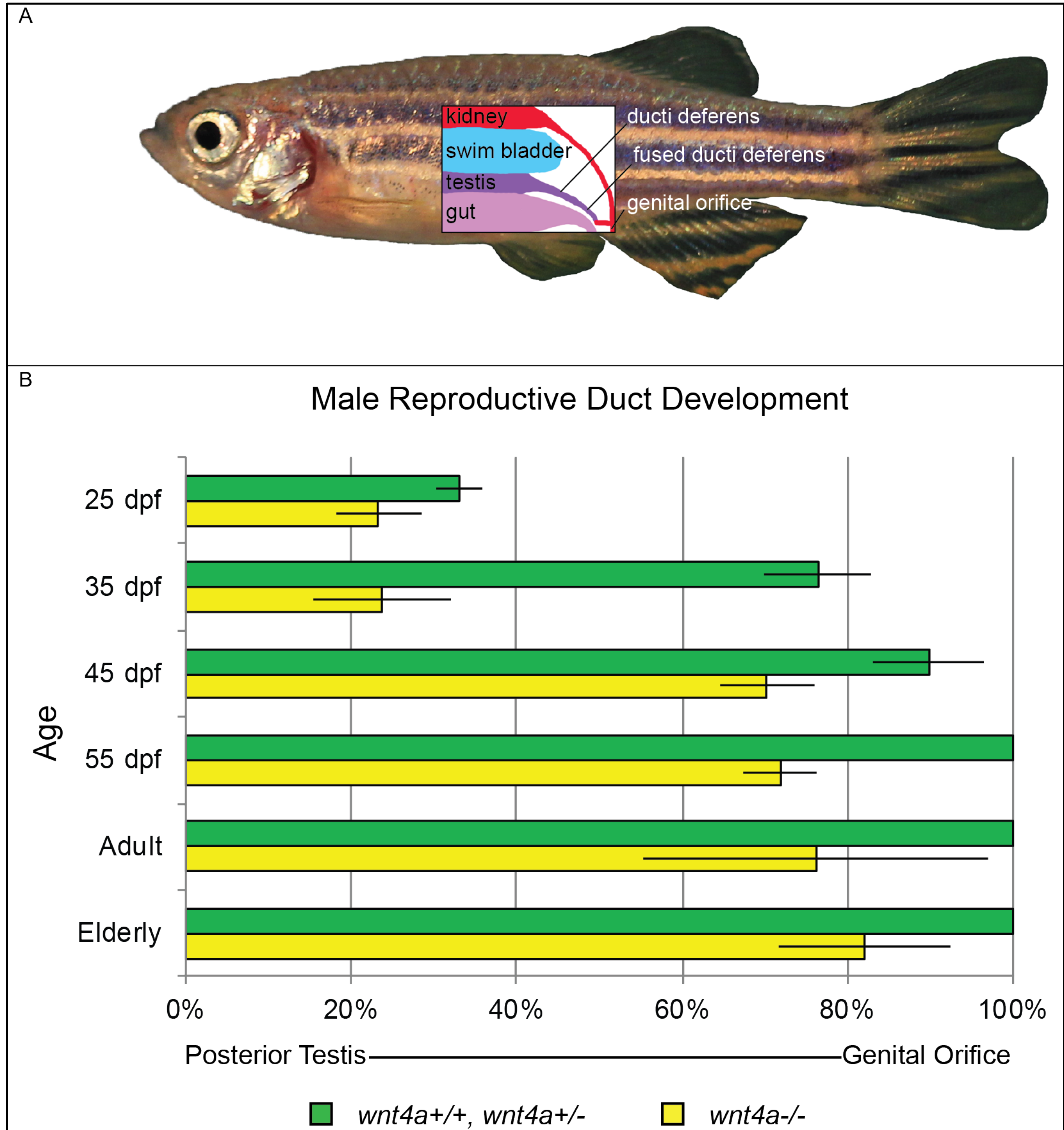


Figure 4.4 Growth of the zebrafish male reproductive duct over time. (A) Schematic showing the testis, ducti deferens, fused ducti deferens, and genital orifice. (B) A timeline (25 dpf to 531dpf) comparing heterozygotes plus wild types to *wnt4a-/-* mutants for growth of the reproductive duct from the posterior testis to the genital orifice.

These results show that male reproductive ducts were already present at 25 dpf, and wild-type individuals had fully formed ducts by 55 dpf. The time point with the greatest difference between the two genotypes was at 35 dpf, indicating that loss of Wnt4a function may cause an initial delay in reproductive duct development as well as

prevent the connection between the fused ducti deferens and genital orifice from forming. Elderly *wnt4a-/-* zebrafish still did not have fully formed reproductive ducts, indicating that reproductive duct development never fully recovered from the initial developmental delay.

wnt4a expression in developing zebrafish reproductive ducts

Results from the time-course experiment showed that normal growth of the male reproductive duct depends on *wnt4a* activity. Under one hypothesis, the developing duct would express *wnt4a* and secrete the Wnt4a protein, which initiates signals in nearby cells that reciprocally signal to the duct to move toward them. A competing hypothesis is that the tissues around the genital orifice express *wnt4a* and secrete the Wnt4a protein, creating a gradient (or stimulating the local secretion of a different signal that creates a gradient) toward which the duct grows. To test these hypotheses, we used *in situ* hybridization on tissue sections to examine *wnt4a* expression in the zebrafish reproductive ducts and surrounding areas at 25, 35, and 55 dpf. Results showed no expression along the duct at 25 dpf (Figure 4.5A), but showed faint expression along the duct at 35 dpf (Figure 4.5 E and F). At 25 and 35 dpf, *wnt4a* expression was seen in the most posterior region of the reproductive duct tract starting around the vent and continuing posteriorly to where the connection between the genital orifice and fused ducti deferens will eventually develop (Figure 4.5 C, D, G, H). This expression precedes the arrival of the ducts to this region. At 55 dpf, after the reproductive ducts have fully developed, *wnt4a* expression persisted in the posterior region and is seen anterior to the connection between the fused ducti deferens and genital orifice around the connection as well (Figure 4.5 I, J, K). The lack of *wnt4a* expression

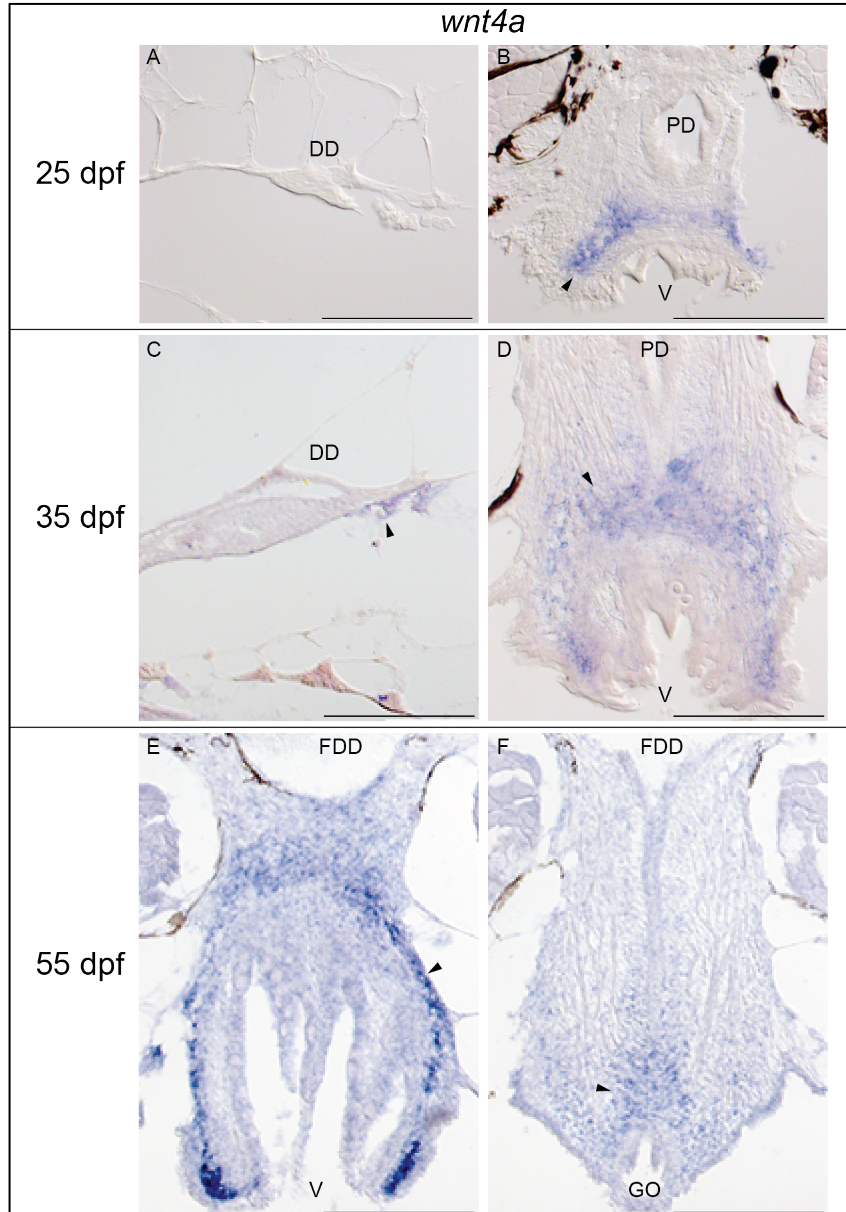


Figure 4.5 Expression of *wnt4a* during reproductive duct development in zebrafish AB strain males. A - 25 dpf ducti deferens, no expression. B - *wnt4a* expression (black arrow) dorsal to posterior vent, just anterior to eventual fused ducti deferens and genital orifice connection at 25 dpf. C - *wnt4a* expression (black arrow) in ducti deferens at 35 dpf. D - *wnt4a* expression (black arrow) dorsal to posterior vent, just anterior to eventual fused ducti deferens and genital orifice connection at 35 dpf. E - *wnt4a* expression (black arrow) dorsal to posterior vent, just anterior to the fused ducti deferens and genital orifice connection at 55 dpf. F - *wnt4a* expression (black arrow) dorsal to genital orifice at 55 dpf. (PD = pronephric duct. FDD = fused ducti deferens. GO = genital orifice. DD = ducti deferens. V = vent) Scale bar = 100μm.

around the most posterior end at 25 and 35 dpf indicates that our first hypothesis, that the developing duct would express *wnt4a* and secrete the Wnt4a protein, which initiates signals in nearby cells that reciprocally signal to the duct to move toward them, is incorrect. But the *wnt4a* expression seen anterior to the eventual fused ducti deferens and genital orifice connection does support the hypothesis that the tissues around the genital orifice express *wnt4a* and secrete the Wnt4a protein, creating a gradient (or stimulating the local secretion of a different signal that creates a gradient) toward which the duct grows.

Discussion

This first temporal analysis of zebrafish male reproductive duct development shows that in wild-type fish the ductus deferens initially extends as a rod-like structure, which does not yet contain a lumen and forms a cell mass located at the posterior end of the testes by 25 dpf. The rod-like structure then forms a lumen by 35 dpf, and continues extending posteriorly until finally developing into the fully formed ducti deferens and fused ducti deferens and connecting to the genital orifice to form a fully functional reproductive tract that can release sperm by 55 dpf.

The development of the zebrafish reproductive ducts is similar to that in medaka. In medaka at 20-30 dph, the male reproductive duct also starts to form from a cell mass located at the posterior end of the testes and this initial reproductive duct resembles a rod-like structure that does not yet contain a lumen (Suzuki and Shibata 2004), which is very similar to what we see in AB zebrafish males at 25 dpf. During medaka male reproductive duct development, the rod-like structure elongates posteriorly, develops a lumen, and differentiates into the sperm ducts, which is similar to what we see in

zebrafish. The medaka sperm duct connects to the urethra to form a fully functional reproductive tract similar to the zebrafish fused ducti deferens connecting to the genital orifice. These results confirm that the reproductive ducts develop similarly in at least two teleost species, zebrafish and medaka.

Zebrafish male reproductive duct development differs from mammalian reproductive duct development. In mammals, two reproductive tracts, the Wolffian ducts, which differentiate into the epidiymides, vas deferens, and seminal vesicles, and the Müllerian ducts, which differentiate into the oviduct, uterine horns, cervical canal, and upper vagina, initially develop in both male and female fetuses and if the individual starts developing as a male the Müllerian ducts will regress, whereas if the individual starts developing as a female the Wolffian ducts regress (Staak et al. 2003; Chiga et al. 2014; McLennan and Pankhurst 2015). Zebrafish, like other teleosts, do not develop Wolffian or Müllerian ducts (Lasala et al. 2004; Rodríguez-Marí et al 2005; Lee et al. 2011). Instead, zebrafish males only develop one reproductive duct and the ductus deferens initially develops as an extension from the testis. To further understand variations in reproductive duct development, zebrafish and mammalian reproductive duct development could be compared to threespine stickleback and spotted gar reproductive duct development.

Even though zebrafish and mammalian male reproductive duct development is different, it is possible that some of the genes involved in mammalian reproductive duct development are involved in zebrafish reproductive duct development. *Wnt4* is important for both ovary development and Müllerian and Wolffian duct development in mammals, as seen in humans with Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome who had

either underdeveloped Müllerian ducts in females or underdeveloped Wolffian ducts in males (Zlotogora 1995; Wellesley and Slaney 1995; Morcel et al. 2007; Pai and Shakir 2013). I previously investigated the effect of zebrafish *wnt4a*, which is orthologous to mammalian *Wnt4*, in ovary development and found that the majority of *wnt4a-/-* zebrafish developed as males (see Chapter III). Upon setting up natural matings with *wnt4a-/-* male zebrafish and wild-type AB female zebrafish, no offspring were ever produced even though the *wnt4a-/-* males exhibited normal mating behavior comparable to their wild-type male siblings and the wild-type AB females laid eggs, which was the first indication that loss of Wnt4a function affects male reproductive duct development. I was unable to study the effect of Wnt4a function loss on female reproductive duct development, because there were not enough female *wnt4a-/-* mutants to study (see Chapter III).

How was zebrafish *wnt4a* affecting male reproductive duct development? In contrast to wild types, *wnt4a-/-* zebrafish never fully form functional reproductive ducts, and although the reproductive ducts grow towards the vent, these ducts grow more slowly than wild types and never make a connection between the fused ducti deferens and genital orifice. At first, it looks like the reproductive ducts of *wnt4a-/-* zebrafish might just be developmentally delayed, as they seem to continue to develop, just at a slower pace. Homozygous *wnt4a-/-* male zebrafish past their reproductive prime at nearly two years post fertilization had still not fully formed their reproductive ducts, whereas their wild-type siblings still have functional ducts. This indicates that *wnt4a* is necessary for the formation of fully functional reproductive ducts in zebrafish.

Zebrafish *wnt4a* is necessary for functional male reproductive duct formation, but what exactly does *wnt4a* do? It is possible that *wnt4a* is expressed in the developing duct and initiates signals in nearby cells that reciprocally signal to the duct to move toward them or *wnt4a* might be expressed in the tissues around the genital orifice which secrete Wnt4a protein creating a gradient towards which the duct grows. At 25 and 35 dpf, the strongest *wnt4a* mRNA expression is seen in the most posterior region of the reproductive duct tract, before the reproductive ducts have fully extended to this region. Expression of *wnt4a* persists after the reproductive ducts have fully formed at 55 dpf. These data indicate that *wnt4a* is acting as a "come hither" signal for the ducts, guiding the development of the ducts toward the genital orifice. To test this hypothesis, future experiments could determine if receptors for Wnt4a signaling, such as LRP5/6 or Frizzled receptors, are present at the posterior tip of the developing ducts.

The *wnt4a* mRNA expression around the fused ducti deferens and genital orifice connection prior to and after full reproductive duct development, and the absence of this connection in *wnt4a-/-* males indicates that *wnt4a* is necessary for the formation of this connection. The slower formation of the male reproductive ducts suggests that *wnt4a* is also necessary for the normal speed of duct elongation. Our findings show novel functions for Wnt4a in zebrafish reproductive duct development. It is possible that Wnt4a is functioning similarly to Wnt4 in mammals, as evidenced by the infertility and underdeveloped Wolffian ducts seen in some males with Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome, which can be caused by a mutation in *Wnt4*, although zebrafish male reproductive ducts, unlike mammalian male reproductive ducts, do not develop from a Wolffian duct like structure. Overall, these data together support the

hypothesis that *wnt4a* activity plays an evolutionarily conserved role in male reproductive duct development across vertebrates.

CHAPTER V

CONCLUSION

Zebrafish is a widely used model organism in laboratory research, but the genetic components of zebrafish sex determination, gonad development, and reproductive tract development are not fully understood. Understanding the genetic components involved in zebrafish sex determination, gonad development, and reproductive tract development could help us understand which genes might be evolutionarily conserved and possibly lead to more uses of this species as a model organism to investigate reproductive diseases.

Throughout this dissertation I described some of the genetic components involved in zebrafish sex determination, gonad development, and reproductive tract development. In chapter II, I used genome wide association studies (GWAS) to investigate if the genetic basis of sex determination in a variety of zebrafish strains, two 'wild-type' strains cultured for about 30 years in the lab, and four 'natural' strains, wild-type strains isolated directly or recently from nature in India. Surprisingly, results identified a sex-associated region on zebrafish chromosome 4 in natural zebrafish strains that was lacking in the lab strains. These results indicate that the zebrafish strains currently used to investigate sex determination are the strains that lack the sex-associated region, and therefore, these studies should be revisited using natural zebrafish strains that retain the natural genetic sex determinant. Even so, all strains, including the laboratory strains lacking the sex-associated region, make males and females indicating that all strains might share the same sex-determination mechanism that is downstream from the natural genetic sex determinants contained in the sex-associated region in wild-type strains, meaning that

laboratory strains are still useful for identifying conserved genes in the zebrafish sex determination pathway.

In chapter III, I investigated whether or not *wnt4a* is important for zebrafish ovarian development. In this study, I found that *wnt4a* is expressed in the early bipotential gonad and that loss of *wnt4a* results in male-biased sex ratios, indicating that *wnt4a* is important for zebrafish ovarian development. These results are similar to what was found in mammals, indicating that Wnt4a has a conserved function in the zebrafish gonad. Also, zebrafish lacking Wnt4a function have a phenotype similar to some SERKAL syndrome patients, who had full female-to-male sex reversal, and could provide insight and a means for treatment discovery for SERKAL syndrome. These results support the hypothesis that zebrafish Wnt4a functions like mammalian Wnt4.

In chapter IV, I investigated whether or not *wnt4a* is important for male reproductive duct development. I found that loss of *wnt4a* slows the formation of male reproductive ducts and prevents the male fused ducti deferens from connecting to the genital orifice in zebrafish males and that *wnt4a* is expressed in tissue around the site where this connection should occur before and after the connection is formed. These results reveal a novel *wnt4a* phenotype in zebrafish that hasn't been seen thus far, and indicate that *wnt4a* is necessary for proper male reproductive duct development in zebrafish. These results support the hypothesis that *wnt4a* activity plays an evolutionarily conserved role in male reproductive duct development across vertebrates.

Overall the data presented in this thesis show that zebrafish in nature have a strong sex determinant linked to Chr4 which is most likely the master switch gene of sex determination in zebrafish, and that zebrafish *wnt4a*, a gene that is downstream in the sex

determination pathway, is important for zebrafish ovary development and necessary for zebrafish male reproductive duct development. The *wnt4a* phenotypes seen in these zebrafish could provide insight and a means for treatment discovery for SERKAL syndrome, which effects both ovary and Müllerian duct development in humans, and MRKH syndrome, which effects Müllerian duct and Wolffian duct development in humans.

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