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The Study of DMRT1 in Zebrafish and How it Impacts Sex Determination

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THE STUDY OF *DMRT1* IN ZEBRAFISH AND HOW IT IMPACTS SEX DETERMINATION

A Thesis Presented

by

RAYMOND POIRIER

Submitted to the Office of graduate studies,
University of Massachusetts Boston,
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2024

Biology Program

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THE STUDY OF DMRT1 IN ZEBRAFISH AND HOW IT IMPACTS SEX DETERMINATION

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ABSTRACT

THE STUDY OF DMRT1 IN ZEBRAFISH AND HOW IT IMPACTS SEX DETERMINATION

May 2024

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The *dmrt1* gene is common amongst most animals and functions to determine or maintain male sex during development. Similarly, in zebrafish *dmrt1* is important for male sex determination and maintaining proper testis morphology. This gene is expressed in two different cell types of the testis in zebrafish, germ cells and Sertoli cells. While we know where this gene is expressed and what its role is, it is not known if it is sufficient to drive male fate. If so, then in which cells is it sufficient to drive male fate in the testis? I aimed to answer this question by analyzing two different *dmrt1* transgenic lines that express *dmrt1* specifically in either the germ cells or the Sertoli cells. We then analyzed sex ratios of the transgenic fish compared to the wild-type siblings. We found that *dmrt1* overexpression in both cell types leads to an overwhelming male bias in the population as compared to wild type fish. We also found that *dmrt1* overexpression exclusively in the germ cells led to a higher frequency of males in comparison to the wild type fish. Meanwhile, *dmrt1* overexpression exclusively in the Sertoli cell led to no change in the number of males

compared to wild type fish. I then wanted to see if the *dmrt1* gene was sufficient to rescue male fate in *dmrt1* mutant zebrafish. After testing out the individual transgenic lines against mutant *dmrt1* fish, we found that neither transgene by themselves could rescue the male sex determination defects in the progeny of mutant fish. Combining all of these data together, we see that *dmrt1* overexpression in the germ cells is sufficient to drive male sex determination. However, *dmrt1* expression from each of our transgenic lines is not sufficient to drive male sex determination, under *dmrt1* mutated conditions in the zebrafish testis.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Kellee Siegfried, who has guided me throughout my journey in graduate school. I have gained so much knowledge through her and have learned so many skills from her that I am forever grateful for the last few years I got to be in her lab. I am also thankful for Dr. Linda Huang who has helped me become a better teacher and has taught me many useful lessons. And, I am thankful for Dr. Labib Rouhana who has always been a charm to present in front of during current literature seminar every Friday.

I would like to take the time to thank the present and former graduate students in Kellee's lab including Kazi Islam, Jess MacNeil, Kavita Venkataramani and Travis Churchill. They were so welcoming and were always there whenever I needed help in lab. I would also like to thank my former undergraduate Andrew Karam for helping me to complete some of the work in this thesis. Without his services, I wouldn't have been able to finish some of the work in here. And finally, I would like to thank my closest friends and family for giving me the love and support I needed to persevere and finish my thesis work.

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INTRODUCTION

1.1 Sex Determination

As a broad definition, sex determination is a system that causes some members of a species to be one sex and the rest to become another (Zarkower, 2001). This mechanism can fall into two separate categories for how sex can be determined. The first way is by environmental factors including temperature, population density and social factors that determine what sex an organism will become. The second way is genetically, when sex is determined from the genetic makeup of an organism. It usually involves sex chromosomes where one of the sexes is the heterogametic sex with two different sex chromosomes like XY males for mammals and ZW females for birds (Gamble and Zarkower et al, 2012). However, sex chromosomes do not have to be involved to still have genetic sex determination. Polygenic sex determination is when multiple genes throughout the genome determine sex with the absences of sex chromosomes (Bulmer and Bull, 1982). The difference between these two types of genetic sex determination mechanisms can be found by looking for genetic linkage with sex. If a single chromosome is linked to a certain sex, like the Y chromosome for males or the W chromosome for females, then you have chromosomal sex determination (Liew et al, 2012). This type of sex determination leads to the progeny population having a 50:50 chance of being male or female based on the inheritance of the sex chromosome (Liew et al, 2012). However, if you have polygenic sex determination, then no chromosome is linked to a certain sex but instead certain expression of genes or alleles in the genome are linked to one sex or the other. Regardless, many organisms are not exclusive to having one way to determine sex as some organisms can have multiple genetic and/or environmental sex determination properties, like reptiles, fish, and amphibians (Gamble and Zarkower et al, 2012). For example, the fish Nile Tilapia determine sex through a XX/XY chromosomal sex determination system with the male being the heterogametic sex (Müller-Belecke and

Hörstgen-Schwark, 1995). However, changes in temperature can override the XX/XY system in that a prolonged period of time at high temperatures during development can result in XX individuals developing as males (Baroiller et al, 1995).

Zebrafish are another example of an organism that can have multiple sex determination systems. Domesticated zebrafish, like most laboratory lines, determine sex genetically with no sex chromosomes present (Liew et al, 2012). The domesticated zebrafish show a wide range of sex ratios. Some clutches of offspring were overwhelmingly male while some were overwhelmingly female and everywhere in between (Liew et al, 2012). In addition, selective breeding experiments were able to get an almost 100% male or female populations (Liew et al, 2012). This shows that no single chromosome was linked to sex and, because sex can be selected for, shows that genetic factors are at play when it comes to sex being determined (Liew et al, 2012). Six different locations in the zebrafish genome have been identified as loci with sex determining genes. Chromosome 3 and 5 have been linked to male sex determination, with chr 5 containing the *doublesex and mab-3 related transcription factor-1* (*dmrt1*) gene, and chromosomes 2, 11 and 16 have been linked to female sex determination (Luzio et al, 2015; Bradley et al, 2011; Anderson et al, 2012; Howe et al, 2013). Within these loci *dmrt1* is known to have a role in male sex determination (Webster et al. 2017), other genes known to have roles in sex determination or sex differentiation include the *antimullerian hormone* (*amh)* and *androgen receptor (ar)* genes, which are known to be involved in male sex determination, while genes like *wnt4a* and *foxL2a/foxL2b* are known to be involved in female sex determination (Kossack and Draper, 2019).

In the wild, zebrafish consist of ZZ/ZW sex chromosomes. Studies have shown that chromosome 4 in wild zebrafish is the sex chromosome (Wilson et al, 2014). Meanwhile, chromosome 4 in domesticated zebrafish does not possess the properties for being a sex chromosome, confirming that polygenic sex determination is only present in domesticated zebrafish and not those in the wild (Wilson et al, 2014).

Environmental factors can also have an effect on zebrafish sex determination. However, this is only observed under extreme conditions. For instance, high temperature resulted in male biased sex ratios, hypoxic conditions favored male development, and a high

rearing/population density of fish led to more male sex being determined (Uchida et al, 2004; Abozaid et al, 2011; Shang et al, 2007; Lawerence et al, 2007; Liew et al, 2012).

1.2 The dmrt1 Gene

For this study, *dmrt1* from the doublesex and Mab-3 (DM) domain family will be the gene of focus. The DM domain is encoded within a family of genes that code for transcriptional regulators which commonly act in the gonad (Matson and Zarkower, 2012). The *dmrt1* gene plays a role in male sex determination and/or differentiation. It can also maintain proper testis morphology and repress ovary production once sex has been determined during development. However, this varies from organism to organism. The *dmrt1* gene was first discovered when the genes *doublesex (dsx)* in *Drosophila* and *mab-abnormal-3(mab3)* in *C. elegans* were compared and shown to have common DM domains (Raymond et al, 1998). With the *dsx* gene being a prominent sex determination gene and the *mab3* gene being prominent in male sex differentiation in their respective organisms, it showed that these two genes have some kind of role in sexual development. Orthologs of these genes, with DM domains, were later found in other organisms with the first of these discovered being named *dmrt1* (Raymond, et al 1998). This gene is usually found in the autosomes but in some organisms it can reside on the sex chromosomes and have a primary role in male sex determination. For instance, in mammals the *SRY* gene on the Y chromosome is the prominent male sex determining gene, which means *dmrt1* is not the primary male sex determining gene. However, once male sex is determined *dmrt1* becomes active in the proper formation of testis while repressing oogenesis specific genes from being active. This was shown in mice, when *dmrt1* was knocked out, males were still able to be born with some testis formation. However, later on the gonad had abnormal differentiation, developing more female characteristics (Raymond, 2000). Further experiments would later explain this in detail, showing that *dmrt1* is functioning to repress the ovarian granulosa cell forming gene *Foxl2*, to make sure Sertoli cells in the testis are maintained (Matson et al, 2011). Overall, this shows that while *dmrt1* is not necessary for male sex determination in mammals, it is important to maintain proper testis morphology and function. In nonmammals, *dmrt1* is occasionally found within the sex chromosome. In Medaka (a kind of teleost fish) the *dmrt1*

paralog (*DMY*) acts as the primary male sex determining gene on the Y chromosome (Matsuda et al, 2007; Nanda et al, 2002). Medaka also have an autosomal copy of the *dmrt1* gene which plays a role in testis development post male sex determination like in mammals (Matson and Zarkower, 2012; Masuyama et al, 2011). Plus, in birds with ZZ/ZW sex chromosomes, the number of copies of the *dmrt1* gene on the Z chromosome can determine testes fate (Ayers et al, 2013). The males need two copies of the Z chromosome in order to possess enough *dmrt1* expression to develop testes. However, if only one copy of the Z chromosome is present, then not enough *dmrt1* is expressed leading to genes on the W chromosome to overwhelm *dmrt1* to promote ovary development in the embryo (Matson and Zarkower, 2012).

Dmrt1 is primarily expressed in two cell types in the gonad of vertebrate animals, the germ cells and the Sertoli cells (which surround the areas where the germ cells reside). In mice, *dmrt1* expression in both cell types is required for normal postnatal development to occur. However, *dmrt1* in the Sertoli cells has the additional task of helping the germ cells survive as when the gene is knocked out in the Sertoli cells, germ cells are not able to go through meiosis and started dying off (Kim et al, 2007). Overall, in both the Sertoli and germ cells *dmrt1* is heavily involved in male development and/or the male sex determination and this applies to zebrafish as well.

1.3 Previous Work on dmrt1 in Zebrafish

In previous work, it was found that zebrafish have three different splice variants of the *dmrt1* gene (Guo et al, 2005). All three variants showed expression in the testis, more so than in the ovary, with a 267 bp splice variant showing the more significant expression compared to the other two (Guo et al, 2005). Currently there are three additional protein coding splice variants predicted in the current genome assembly, GRCz11 (www.ensembl.org). The roles and specific expression of these splice variants has not been explored.

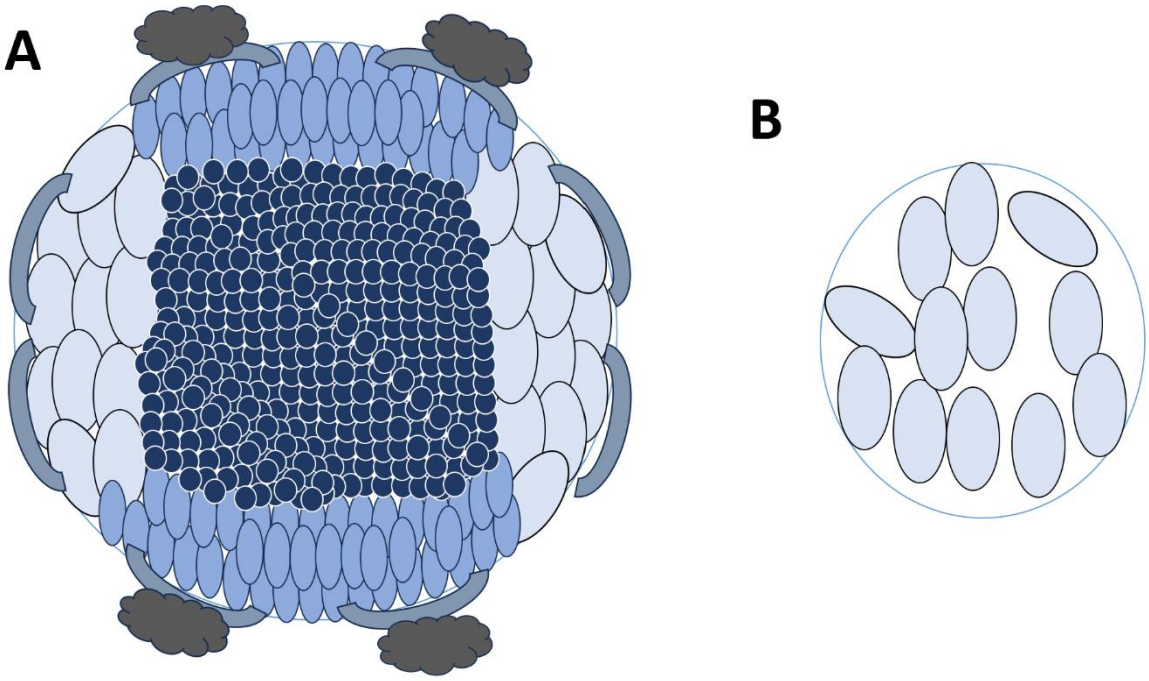
Overall, like with most animals, *dmrt1* promotes male development in zebrafish. This was shown in the sex ratio analysis of zebrafish with loss of function mutation in this gene. Because domesticated zebrafish have polygenic sex determination and variable sex ratios,

sex determination defects are assayed by comparing sex ratios of the mutants to the wild types within the same population. The mutant *dmrt1* populations skewed very heavily female, around 40% more females, as compared to the wild-type population (Webster et al, 2017). The heterozygous populations had similar sex ratios to the wild-type populations, which showed that only complete loss of *dmrt1* function leads to the female biases (Webster et al, 2017). These data shows that *dmrt1* has an impact on male sex determination.

On top of this, *dmrt1* is required for normal spermatogenesis and normal testis development to occur. This was shown with histology of mutant testes, which I have drawn graphic representation of in figure 1.1 with accompanying figure legend. A wild-type zebrafish testis has germ cells undergoing spermatogenesis inside the testis tubules. The spermatogonia are the mitotic proliferating population, the spermatocytes are in meiosis, and mature sperm are spermatozoa. (figure 1.1A). The cysts of spermatogonia and spermatocytes are supported by Sertoli cells and are arranged around a lumen which contains the spermatozoa (Siegfried and Draper, 2020). The Leydig cells are outside of the tubules and produce steroid hormones (figure 1.1A). However, in *dmrt1* mutant testis a small number of spermatogonia are often present but no spermatozoa were seen (figure 1.1B) (Webster et al, 2017). The testis tubules are also very disorganized and are much smaller than the wild-type testis (figure 1.1B) (Webster et al, 2017). This shows that without *dmrt1* function, the testis is very disorganized and males are infertile due to no presence of spermatozoa.

Dmrt1 may also help to repress ovary development. In *dmrt1* mutants, expression of some female-expressed genes were increased and repression of oocyte development was defective (Webster et al, 2017). This was shown experimentally when qPCR was done, measuring the level of expression of the *foxl2* gene in the ovaries and testes of *dmrt1* mutant and wild-type zebrafish. The results show that there was a 41-fold increase in the expression of *foxl2* in the mutant testes compared to the wild-type testes (Webster et al, 2017). The *foxl2* gene codes for a transcription factor that functions in the granulosa cells of ovaries (Kossack and Draper, 2019). So, with this expression increasing in the *dmrt1* mutant testis, it shows that the *dmrt1* gene is necessary to prevent expression of ovarian development genes. In addition to showing increased expression of ovarian genes, *dmrt1* mutants also have defects

in repressing ovarian development. During development all zebrafish start off with early oocytes in their gonad prior to sex differentiation. However, at around 20-30dpf sex differences start to occur and by 90dpf zebrafish are sexually mature (Leerberg et al, 2017). The *dmrt1* gene is important for activation of apoptosis in these early oocytes to form the testis. When *dmrt1* is mutated fewer apoptotic germ cells are seen and the early oocytes stay within the gonad in zebrafish. This results in most fish becoming female (Webster et al, 2017). Overall we know generally what the gene *dmrt1* does in zebrafish but the full extent of this gene's function is still shrouded in mystery.



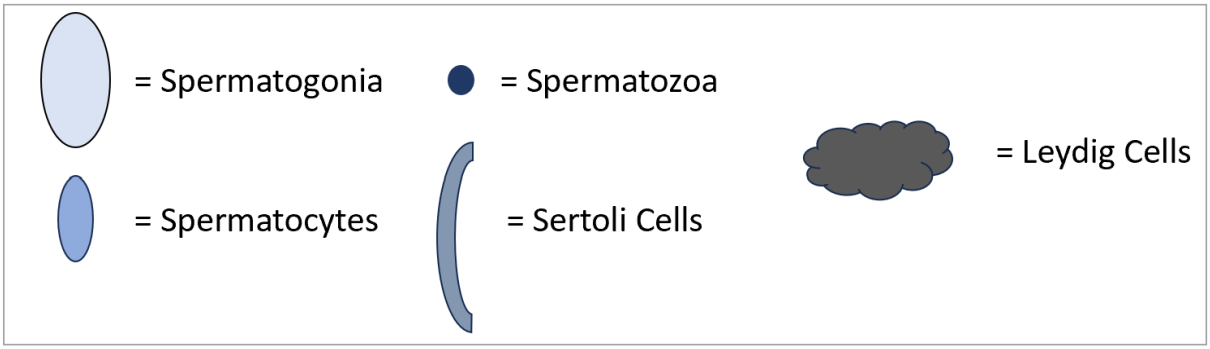


Figure 1.1. Testis Drawings. A) Testis drawing of a wild-type zebrafish testis tubule. This drawing shows the inside of a single testis tubule, in which there many within the testis organ. B) Testis drawing of a *dmrt1* mutant zebrafish testis. The key beneath the images shows what each of the shapes inside represents.

1.4 The Relationship Between dmrt1 and dmrt2a

Based on previous data, it has been suggested that *dmrt1* and *dmrt2a* cooperate during early development in zebrafish (Steinfeld et al, 2021). There are five different genes in the DM domain family in zebrafish. These genes are *dmrt1, dmrt2a, dmrt2b, dmrt3,* and *dmrt5*. *Dmrt2a* is expressed in the muscles as well as the testis in adult zebrafish (Zhou et al, 2008). During development, *dmrt2a* is expressed in developing somites (Meng et al, 1999). Morpholino knockdowns experiments of *dmrt2a* led to disruptions in left-right symmetry during segmentation and fast muscle differentiation (Lu et al, 2017; Saúde et al, 2005). In previous studies, it has been shown that when the *dmrt2a* gene is mutated the fish die between 7 and 12 days post fertilization (dpf) (Steinfeld et al, 2021). However, when *dmrt2a* mutants were double mutant with *dmrt1* some survived. When a line of *dmrt1;dmrt2a* double heterozygous fish were crossed with each other, around 10% of the progeny zebrafish were double mutant as adults (Steinfeld et al, 2021). While this was still lower than the expected number of fish based on Mendelian genetics (around 25% of the total population), it showed that the mutated *dmrt1* gene might have been able to rescue lethality in some mutant *dmrt2a* fish. However, there could also be some background modifiers that caused these *dmrt2a* mutants to survive with no involvement of *dmrt1*.

1.5 Aims of this Research

The previous research described above is what inspired my thesis work, which is centered around these aims: 1) *How effective can dmrt1 be at driving male fate within zebrafish?* 2) *In which cell type does dmrt1 expression function to drive male fate?* And 3) *What is causing the rescue of dmrt1;dmrt2a double mutant fish from lethality?* We know that when *dmrt1* function is completely gone from zebrafish we get and overwhelming number of female zebrafish in the progeny and the few males have a very disorganized testis with no mature sperm cells leading to them being infertile. I want to extend beyond this knowledge and test if the *dmrt1* gene is sufficient for male fate in zebrafish. This was done first by overexpressing this gene to see if it will increase males in the population. Then, I tested overexpression of this gene in each of the two cell types where it is normally expressed, the germ cells and the Sertoli cells, to see which cell type *dmrt1* can be overexpressed in to drive male fate. I also wanted to see, if reintroducing expression of this gene in either the germ cell or Sertoli cell in *dmrt1* mutant zebrafish could lead to any rescue in the female biased sex ratio of the mutants. Finally, I wanted to see if introducing a *dmrt1* mutation into the *dmrt2a* mutant line could lead to the survival of any *dmrt2a* mutant fish. My goal is to know the limitations or lack thereof for the *dmrt1* gene and to get a clearer image of how this gene impacts sex determination in zebrafish.

Chapter 2

METHODS

2.1 Ethics Statement

All experiments and procedures done on the zebrafish were approved by the University of Massachusetts Institutional Animal Use and Care Committee (IACUC).

2.2 Creating the dmrt1 cDNA Transgenes

In order for *dmrt1* to be overexpressed in the zebrafish, two constructs were made and transgenic lines were established for each. The first transgene is called *umb14 Tg[ziwi:dmrt1-V5]*. This transgene consists of the *zebrafish piwi-like 1* (*ziwi*) promoter connected to a cDNA transcript copy ENSDART00000124637.4 of the *dmrt1* gene, followed by a V5 tag and then ending with the *dmrt1* 3' UTR. The ziwi promoter specifically expresses in the germ cells, which allows for this transgene to be only expressed in the germ cells (Leu and Draper, 2010). The *dmrt1* 3' UTR was used at the end of this transgene because the regulation of gene expression in germ cells is often controlled by regulatory elements in the 3' UTR. The second transgene is called *umb15 Tg[gsdf:dmrt1-V5]*. This transgene consists of the *gonadal soma derived factor* (*gsdf*) promoter connected to the same cDNA transcript copy of the *dmrt1* gene from the germ cell transgene. The *dmrt1* cDNA is followed by a V5 tag and then ending with an SV40_polyA sequence. The gsdf promoter specifically expresses in the Sertoli cells of the testes and the granulosa cells of the ovary (Gautier et al, 2011). Each construct had *Tol2* transposon sequences that were used to generate transgenic zebrafish and γ-crystallin:GFP, which expresses in the eye lens, to identify transgenic fish. This work was done by Jocelyn Steinfeld who made the *gsdf:dmrt1* transgenic line and Jess McNeil who made the *ziwi:dmrt1* transgenic line. I will refer to these two transgenic lines as GC:*dmrt*1

and SC:*dmrt*1 to represent the germ cell-expressed transgene and Sertoli cell-expressed transgene, respectively.

2.3 RT-qPCR

To perform RT-qPCR, I collected GC*:dmrt1* transgene testes, SC*:dmrt1* transgene testes and wild type testes at 4-5 months post fertilization (mpf). I placed 3-4 testes per sample into tubes of Trizol reagent and homogenized them. I then isolated the RNA from the homogenized tissue following the manufactures protocol. I then precipitated the RNA with isopropanol and washed them after in cold 70% ethanol. Afterwards I measured the RNA concentration using the Nano Drop for each sample. Then, using superscript IV reverse transcriptase, the RNA was turned into cDNA and then diluted 1:10. While the cDNA was being diluted, I created a master mix that included a Thermo Fisher PowerUp SYBR green master mix (that included Taq), primers (either targeting *dmrt1* or the housekeeping gene *rpl13a*) and water. I then added 9ul of master mix and 1ul of diluted cDNA or 1ul of water for negative controls into the wells of a qPCR plate. Each sample was replicated three times with each biological replicate having three additional technical replicates. The data was collected on a Bio-Rad qPCR machine as C_t values. Using Microsoft Excel I compared the *dmrt1* expression levels of transgenic and wild-type testes as log fold changes. The statistical analysis was done on Graph Pad Prism. Primers are listed in table 2.1.

2.4 Genotyping

DNA was extracted from the tails of adult zebrafish using the HOTSHOT method. Briefly, fins were digested in 100 ul of NaOH at 90ºC for 20 minutes then neutralized by adding 10 ul of 1M Tris-HCl at pH 8 (Meeker et al, 2007). Then, 1 ul of DNA was added to each PCR reaction. Once the PCR was complete, how we looked at the samples depended on which kind of allele we wanted to detect. To look at the GC*:dmrt1* or SC*:dmrt1* transgenes we ran the finished PCR product on a 1.5% agarose gel at 120 v for 50 minutes. If we saw the presence of a band at 293 base pairs (bp) for the GC:*dmrt1* transgenes or 164 bp for the SC:*dmrt1* transgene, then the sample had that specific transgene present. To assay for the *dmrt1*^{uc27} mutant allele we ran an enzyme digest on the PCR product using 0.5 µl of the

MluCl enzyme at 37ºC for an hour. Afterwards, we ran the samples on a 6% TBE-acrylamide gel at 130 v for 50 minutes. If one band was present at 242 bp, then the sample would contain the loss of function mutation. If three bands at 242 bp, 145 bp and 97 bp along with a heteroduplex band above the 242 bp band were present, the sample would be heterozygous for the *dmrt1*^{uc27} mutant allele. If only two bands at 145 bp and 97 bp were present, then the sample was wild type. To assay for the *dmrt1*^{umb19} mutant allele we ran an enzyme digest on the PCR product using 0.5 µl of the Mspl enzyme at 37ºC for an hour. Afterwards, the samples were run on an 6% TBE-acrylamide gel with the same parameters as the gel used for the *dmrt1*^{uc27} allele. If only three bands were present at 125 bp, 42 bp and 25 bp, then the sample had the *dmrt1*^{umb19} mutation. If four bands were present at 25 bp, 42 bp, 125 bp and 167 bp along with a heteroduplex band above the 167 bp band, then the sample would be heterozygous for the *dmrt1*^{umb19} mutation. If only two bands were present at 167 bp and 25 bp, then the sample would be wild type. To identify the *dmrt2a*^{umb12} allele we ran an enzyme digest on the PCR product using 0.5 µl of the Bsli enzyme at 37ºC for an hour. The samples were then run on a 6% TBE-acrylamide gel. If one band is present at 146 bp, then the sample has the mutation. If three bands are present at 146 bp, 110 bp and 35 bp along with a heteroduplex band above the 146 bp band, then the sample would be heterozygous for the *dmrt2a*^{umb12} allele. If only two bands are present at 110 bp and 35 bp, then the sample is wild type. Primers are listed in table 2.1.

2.5 Sex Ratio Analysis

We identified the sex of each fish during the fin clipping process before DNA extraction. This was done by observing the color of their anal fin. If the fin has an orange color then the fish is male, but if the fin is yellow then it is female. Once the genotyping was done we matched the genotype with the specific fish to see what its sex was.

2.6 Histology

Zebrafish torsos were fixed in Bouin's fixative overnight on a shaker at room temperature. The torso samples were then washed in 70% ethanol 3 times for 30 minutes.

Then in 100% ethanol (3x for 30 mins), washed once in half ethanol half citrisolv, washed in 100% citirisolv (3x for 30 mins) and then filled with half citrisolv half paraffin and stored in a paraffin 59ºC incubator overnight. The paraffin was then changed twice a day for the next two days while in the incubator. The samples were then embedded into paraffin molds. Using a microtome, torsos were sectioned into 5µm sections and placed on a slide warmer overnight. The sections were stained by the following protocol:, washed twice in 100% citrisolv for 5 mins each, washed twice in 100% ethanol for 5 mins, washed twice in 95% ethanol for 5 mins, washed three times in DI water for 5 mins, stained in Modified Harris Hematoxylin for 8 minutes, rinsed in tap water for roughly 5 seconds, then quickly dipped in acid alcohol until the hematoxylin turned blue (roughly 30 seconds), the sections were rinsed again in tap water for roughly 5 seconds, then quickly dipped in ammonia water for roughly 10 seconds, they were quickly rinsed in tap water, then stained with eosin for one minute, the samples were then directly washed in 95% ethanol for 3 minutes, washed three times in 100% ethanol for 5 mins, washed in 100% citrisolv for 5 mins and finally Permount and a coverslip are placed on the slides and dried overnight at room temperature (Siegfried and Steinfeld, 2021). Sections were then imaged using a brightfield microscope.

2.7 Generation of dmrt1^{umb19} Mutants

Two guide RNA's (gRNAs) targeting *dmrt1* were designed using chopchop (https://chopchop.cbu.uib.no/) and crRNAs were synthesized by IDT. The target region of the genome was 3'-GGCTTCGTGTCACCGCTGAAGGG-5' (gRNA 1) and the other was 3'-AAGCGTTTGTGGCCCTTCAGCGG-5' (gRNA 2). Each crRNA was hybridized with tracr RNA to create the guide (gRNAs), following the manufactures protocol. 1 ul of each gRNA was combined with Cas9 mRNA and phenol red and injected into 1-cell embryos. To check that mutations were generated, we collected some of the injected and non-injected embryos and ran a PCR reaction to amplify the targeted region of *dmrt1*. We used the primers KS753 and KS754 to detect the mutated embryos (Table 2.1). We saw multiple sized bands on the gel indicating multiple random mutations. The injected fish were raised and were crossed to generate F1 embryos. Clutches in which mutants were detected were raised and genotyped as adults using fin tissue. We then subcloned DNA from fish with mutations using the pGEM-T

Easy cloning method (Promega). We then sent the plasmid DNA to EtonBio for Sanger sequencing. We used Blast to compare sequenced *dmrt1*^{umb19} gene with the wild-type *dmrt1* gene sequence to determine the sequence of the mutation.

Table 2.1. Primer Chart

Primer Name	Sequence (5'-3')	Targeted Gene	Use	Reference
JM117	ACACCCTTTACCAGTCCCAAGT	*Umb14 Tg[ziwi:dmrt1-V5]*	GC:*dmrt1* transgene genotyping	Steinfeld et al, 2021
JM118	TGGACAGCGACCCGTTAGTC	*Umb14 Tg[ziwi:dmrt1-V5]*	GC:*dmrt1* transgene genotyping	Steinfeld et al, 2021
JS135	GCTGCAGTCCGAGCTCCTCC	*Umb15 Tg[gsdf;dmrt1-V5]*	SC:*dmrt1* transgene genotyping	Steinfeld et al, 2021
JS136	AGCCGTGGTTTCTGCAGCGA	*Umb15 Tg[gsdf;dmrt1-V5]*	SC:*dmrt1* transgene genotyping	Steinfeld et al, 2021
KW1	GCCATCACCATTAGCTGTCA	$dmrt1^{uc27}$	genotyping	Webster et al, 2017
JS29	ATGGCCTTGTCGAGGATGTC	$dmrt1^{uc27}$	genotyping	Steinfeld et al, 2021
KS753	GTCGCTGTCCATCAGGAAAC	$dmrt1^{umb19}$	genotyping	Siegfried, unpublished
KS754	GGCCTTGTCGAGGATGTCTA	$dmrt1^{umb19}$	genotyping	Siegfried, unpublished
JS137	CGGAGAGCGATGATCAGGCGG	$dmrt2a^{umb12}$	genotyping	Steinfeld et al, 2021
JS138	TGGTTCCTACATCGGGCGCA	$dmrt2a^{umb12}$	genotyping	Steinfeld et al, 2021
JS180	AGCTCAGGACCGCCATCAC	*dmrt1*	RT-qPCR	Lin et al, 2017
JS181	GGAGGCGTCCACCATTAGGT	*dmrt1*	RT-qPCR	Lin et al, 2017
KW46	TCTGGAGGACTGTAAGAGGTATGC	*rpl13a*	RT-qPCR	Tang et al, 2007
KW47	AGACGCACAATCTTGAGAGCAG	*rpl13a*	RT-qPCR	Tang et al, 2007

Chapter 3

RESULTS

I contributed to every figure in this section. I had some help from other people who also contributed to this work and they are Jess MacNeil, who contributed to figures 3.2, 3.4 and 3.7. Andrew Karam, who contributed to figures 3.3, 3.5 and 3.6. Chistopher Wood, who contributed to figures 3.3 and 3.5. And Andrew Cogliano, who contributed to figure 3.2.

3.1 Transgenes are Validated

Before we could start any of our experiments, we needed validate our transgenes to make sure they expressed as expected. The two transgenes we are using are the germ cell *ziwi:dmrt1-V5* transgene and the Sertoli cell *gsdf:dmrt1-V5* transgene. Throughout the rest of this paper, I will refer to them as the GC*:dmrt1* (*ziwi*) and the SC*:dmrt1* (*gsdf*) transgene for simplicity. Previous V5 immunofluorescent work in our lab has shown that these transgenes are expressing in the correct areas, with the GC:*dmrt1* transgene expressing in the germ cells and the SC:*dmrt1* transgene expressing in the Sertoli cells (Jess MacNeil, unpublished). So, while we know that our *dmrt1* transgenes are expressing in the correct areas, we do not know if these transgenes are providing additional *dmrt1* expression to those cell types. To test for this, we did an RT-qPCR experiment in which we dissected out GC:*dmrt1* transgenic testes from one population, SC:*dmrt1* transgenic testes from another population and wild type testes from both populations. RNA was extracted and converted to cDNA via reverse transcriptase. We then preformed RT-qPCR on three replicates for each genotype for both *dmrt1* and our housekeeping gene *rpl13a*. Looking at the log fold change for each genotype, we saw that both the GC:*dmrt1* and the SC:*dmrt1* transgenes had a significantly higher level of *dmrt1* expression than their wild-type siblings (Figure 3.1). This shows that our transgenes are in fact increasing the level of *dmrt1* expression in the areas of the testis where we want them to express.

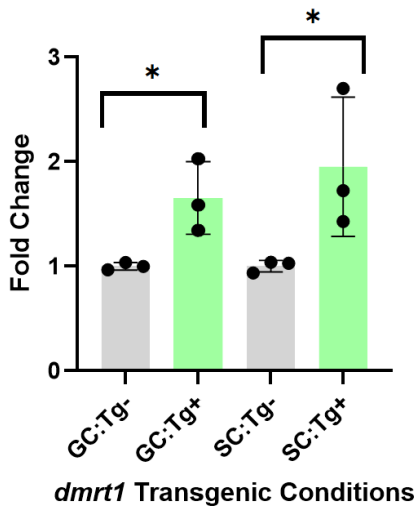


Figure 3.1. Level of *dmrt1* expression increases when transgenes are present. This is the fold change graph representing our RT-qPCR data. The signal for all genotypes were normalized to *rpl13a* expression. The grey bars represent the *dmrt1* expression of the wild-type testis from each population. The green bars represent the *dmrt1* expression for each transgenic testis populations. The black points represent the replicates for each. The error bars represent the standard deviation for each population. The significance is marked with the asterisk. The germ cell population had a p-value of 0.0159 and the Sertoli cell population had a p-value of 0.0300. The p-values were calculated using a two-tailed T-test.

3.2 Overexpression of dmrt1 in Both Germ Cells and Sertoli Cells Leads to More Males

To ask if overexpressing *dmrt1* expression is sufficient to drive male fate, we overexpressed *dmrt1* in both the germ cells and Sertoli cells. From previous research, we knew that *dmrt1* loss of function leads to an overwhelming female biased sex ratio, indicating that *dmrt1* has an important role in male sex determination (Webster et al, 2017). With this, we wanted to see if overexpressing *dmrt1* is sufficient to promote more males in the population. To do this, we compared the sex ratios of fish carrying the GC:*dmrt1* and the SC:*dmrt1* transgenes to siblings with neither transgene. We crossed fish that were double heterozygous for both transgenes with wild-type fish. The expected progeny would result in double transgene, single transgene for either transgene and wild-type fish (Figure 3.2A). We observed that when both transgenes were present, the fish were significantly more likely to become male then if they possessed neither transgene (Figure 3.2b). This shows that when

dmrt1 is overexpressed in the gonad, male sex is determined more often than under wild-type levels of *dmrt1* expression. Therefore, *dmrt1* overexpression is sufficient to drive male sex determination in zebrafish.

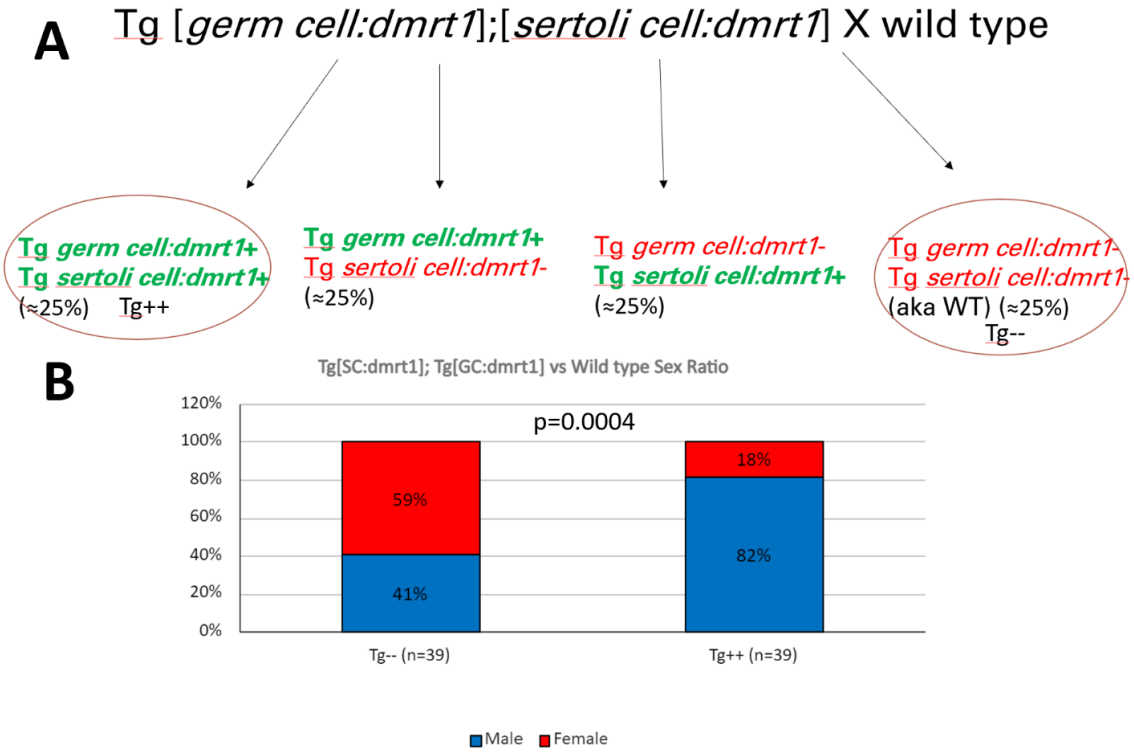


Figure 3.2. A higher percentage of males develop when *dmrt1* is overexpressed in the gonads. A) Diagram of the crosses set up for the sex ratio analysis. In the parent crosses, one parent had both the GC:*dmrt1* and SC:*dmrt1* transgenes and the other parent was wild type. The progeny then consisted of four distinct genotypes. The green lettering represents the presence of the transgene. The red lettering represents the absence of the transgene. The circled genotypes are what were counted for the sex ratio analysis. B) The sex ratio chart for the progeny that resulted from the cross in part A. The left side is the sex ratio for the transgene negative/wild-type progeny and the right side is the sex ratio for the double transgene positive progeny. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=78 (n=39 for each genotype). P-value was calculated using a Fisher's exact test. Data for this chart was also contributed by Jess MacNeil and Christopher Wood.

3.3 Overexpression of dmrt1 in Sertoli Cells is Not Sufficient for Male Sex Determination

After finding that overexpression of *dmrt1* is sufficient for male sex determination in zebrafish, we wanted to test if expression in one cell type could promote male fate. Since we know that germ cells and Sertoli cells are the only cells in the gonad where *dmrt1* is expressed those were the two cell types we tested. We first tested if Sertoli cell overexpression of *dmrt1* could drive male fate. To test this, we set up a cross, in which both parents had one or two copies of the SC:*dmrt1* transgene. This would lead to the progeny consisting of fish that either had no copies of the transgene or at least one copy of the SC:*dmrt1* transgene (Figure 3.3A). We found that when the SC:*dmrt1* transgene was present, there was no significant increase in the number of males compared to the wild-type sibling population (Figure 3.3B). This result shows that when *dmrt1* was overexpressed only in the Sertoli cells, there was no increase in the percentage of males. In other words, *dmrt1* overexpression in Sertoli cells is not sufficient to drive male fate in sex determination. Alternatively, expression of *dmrt1* from this transgene is either not at sufficient levels to drive male fate or different and/or additional isoforms are required.

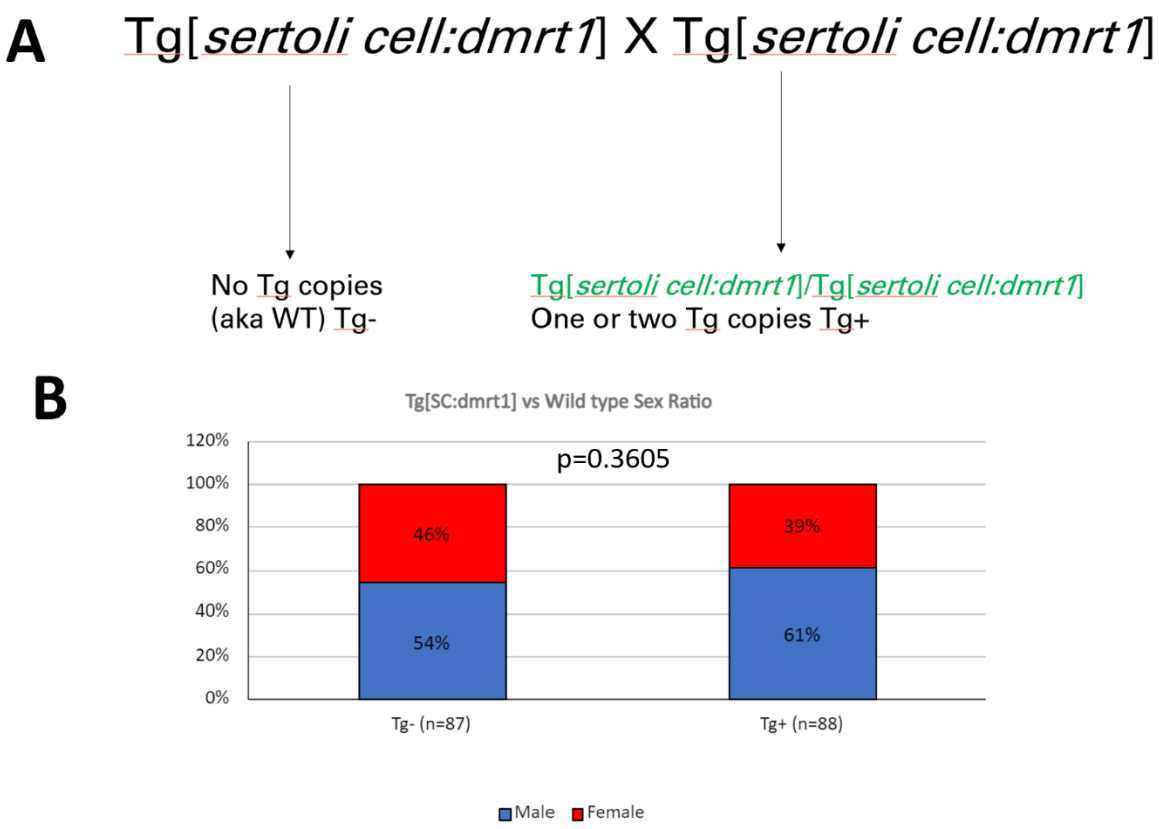


Figure 3.3. The sex ratio was similar to wild types when *dmrt1* was overexpressed in the Sertoli cells of the gonad. A) Diagram of crosses set up for the sex ratio analysis. In the parent crosses, both parents had at least one copy of the SC:*dmrt1* transgene. The progeny would then consist of three distinct genotypes. The green lettering represents the presence of one or two copies of the transgene. B) The sex ratio chart for the progeny that resulted from the crosses in part A. The left side is the sex ratio for the wild-type progeny and the right side is the sex ratio for the progeny with one or two copies of the transgene. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=175 (n=87/88 for each genotype). P-value was calculated using Fisher's exact test. Data for this chart was also contributed by Christopher Wood and Andrew Karam.

3.4 Overexpression of dmrt1 in Germ Cells is Sufficient for Male Sex Determination

After seeing no change in the number of male zebrafish when *dmrt1* was overexpressed only in the Sertoli cells, we next tested to see how the sex ratios would be affected by *dmrt1* overexpression in the germ cells only. To do this we set up a cross in which both parents had

one or two copies of the GC:*dmrt1* transgene. This cross resulted in progeny that had at least one copy of the GC:*dmrt1* transgene or were wild-type (Figure 3.4A). We observed a significantly higher percentage of male zebrafish when the GC:*dmrt1* transgene was present compared to wild type (Figure 3.4B). This result shows that when *dmrt1* is overexpressed in only the germ cells, it is sufficient to promote male fate during sex determination.

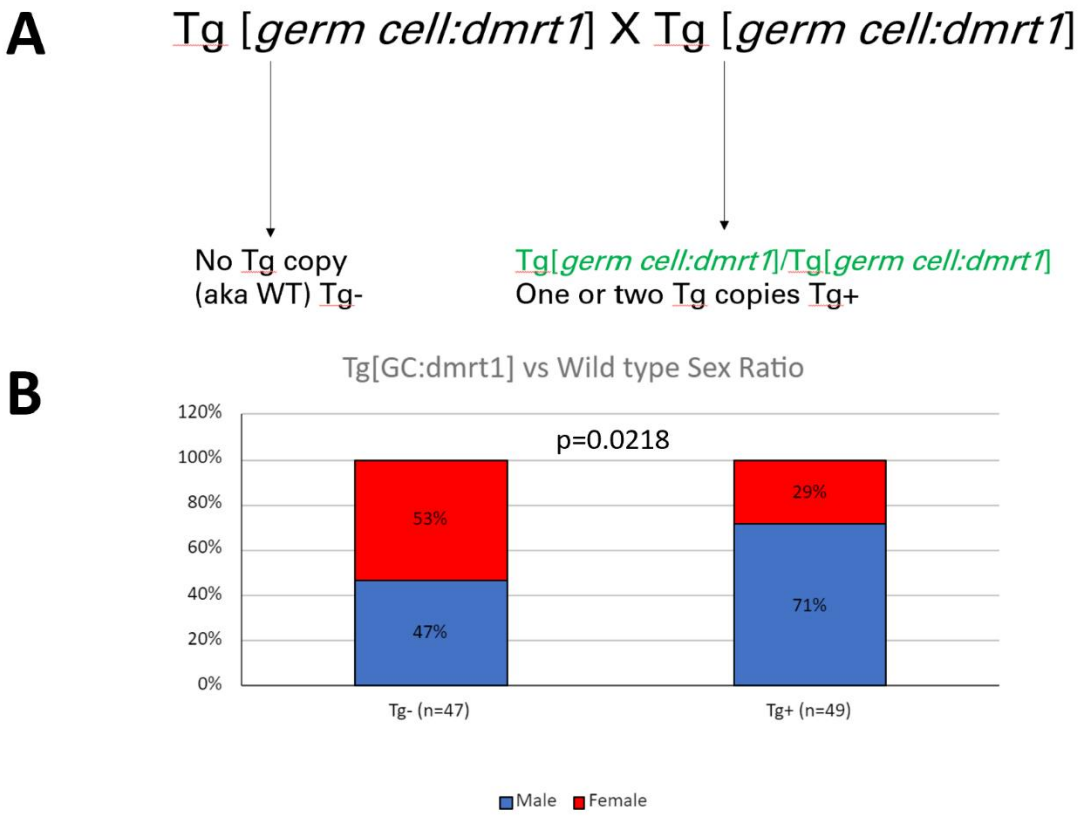


Figure 3.4. The percentage of males increased when *dmrt1* was overexpressed in the germ cells. A) Diagram of crosses set up for the sex ratio analysis. In the parent crosses, both parents would have at least a copy of the GC:*dmrt1* transgene. The progeny would then consist of three distinct genotypes. The green lettering represents the presence of one or two copies of the transgene. B) The sex ratio chart for the progeny that resulted from the cross in part A. The left side is the sex ratio for the wildtype progeny and the right side is the sex ratio for the progeny with one or two copies of the transgene. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=96 (n=47/49 for each genotype). P-value was calculated using Fisher's exact test. Data for this chart was also contributed by Jess MacNeil.

3.5 Sertoli Cell dmrt1 Expression Does Not Rescue the dmrt1 Mutant Phenotype

We next wanted to ask if *dmrt1* expressed in either the germ cells or Sertoli cells can rescue male sex determination in *dmrt1* mutant zebrafish. The *dmrt1* loss of function mutant zebrafish had a significant decrease in the percentage of male zebrafish compared to the wild-type siblings (Webster et al, 2017). To ask if *dmrt1* function was necessary in only Sertoli cells or germ cells for male sex determination, we tested if either the SC:*dmrt1* or GC:*dmrt1* transgenes could rescue the female biased sex ratio in the *dmrt1* mutants.

We first asked if the SC:*dmrt1* transgene could rescue the female biased sex ratios in *dmrt1* mutants. We set up a cross in which the female was homozygous mutant for *dmrt1* and the male was heterozygous for *dmrt1* (since male *dmrt1* mutants are sterile). One of the parents also had one copy of the SC:*dmrt1* transgene. This resulted in progeny in which the zebrafish could have either the SC:*dmrt1* transgene and be heterozygous for *dmrt1*, have the SC:*dmrt1* transgene and be mutant for *dmrt1*, be heterozygous for *dmrt1* with no transgene, or be mutant for *dmrt1* with no transgene (Figure 3.5A). When comparing the *dmrt1* mutant fish with and without the SC:*dmrt1* transgene, we saw no significant change in the percentage of males (Figure 3.5C). To confirm that these *dmrt1* mutants displayed the female biased sex ratios, we compared the *dmrt1* heterozygous fish with and without the SC:*dmrt1* transgene in a subset of our crosses. In these fish, we saw that each population was entirely male (Figure 3.5B). It has been shown that *dmrt1* heterozygous fish have similar sex ratios to their wild-type siblings (Webster et al, 2017). As expected, the *dmrt1* mutant population was female biased relative to the heterozygotes when the transgene was absent. These data tells us that Sertoli cell *dmrt1* expression via the SC:*dmrt1* transgene could not rescue female sex ratio bias in mutant *dmrt1* zebrafish, which shows that germ cell *dmrt1* expression might not be necessary for male sex determination since these fish have no functional *dmrt1* expressed in their germ cells. It is also possible that *dmrt1* is required in both Sertoli and germ cells for male sex determination. Additionally, additional splice variants or improper *dmrt1* expression from the transgene could cause the sex ratio to not be rescued in *dmrt1* mutants.

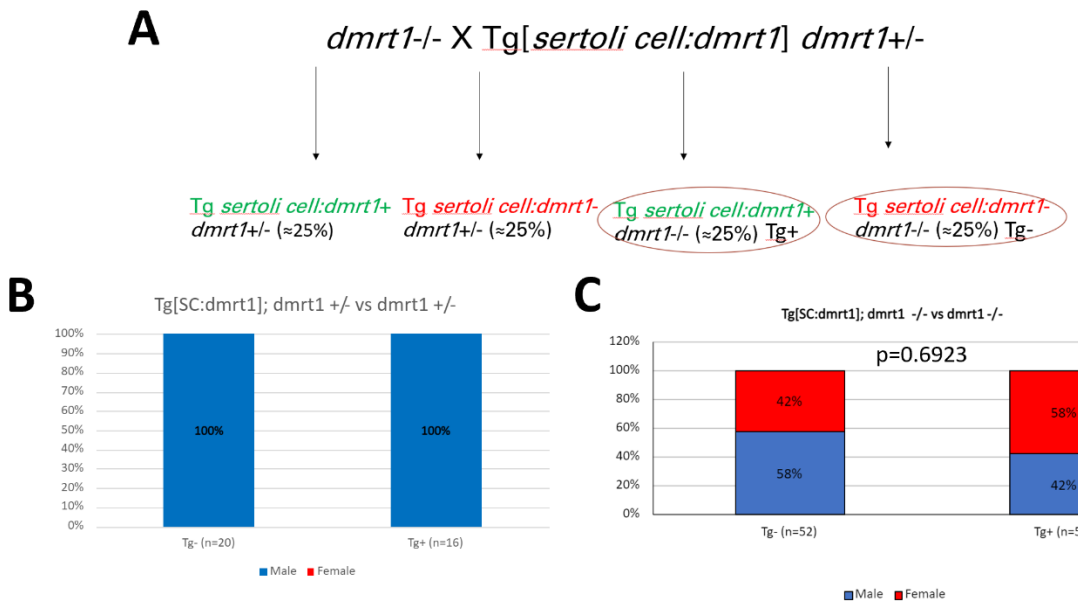


Figure 3.5. The ratio of males to females was not rescued when *dmrt1* was expressed exclusively in the Sertoli cells of *dmrt1* mutants. A) Diagram of the crosses set up for the sex ratio analysis. In the parent crosses, the female was mutant for *dmrt1* and the male was heterozygous. One of the parents had the SC:*dmrt1* transgene. The progeny then consisted of four distinct genotypes. The green lettering represents the presence of the transgene. The red lettering represents the absence of the transgene. The two circles represent the genotypes of interest for panel C. B) The sex ratio chart from the progeny that resulted from the heterozygous fish found in the cross in part A. The left side is the sex ratio for the non-transgene heterozygous fish and the right side is the sex ratio for the transgene positive heterozygous fish. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=36 (n=20/16 for each genotype). C) The sex ratio chart from the progeny that resulted from the mutants found in the cross in part A. The left side is the sex ratio for the non-transgene mutant fish and the right side is the sex ratio for the transgene positive mutant fish. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=104 (n=52 for each genotype). P-value was calculated using Fisher's exact test. Data for these charts were also contributed by Christopher Wood and Andrew Karam.

To test if expressing *dmrt1* exclusively in the Sertoli cells could rescue testis morphology in *dmrt1* mutants, we analyzed *dmrt1* mutant testes with and without the SC:*dmrt1* by histology. In wild-type conditions, a zebrafish testis has well organized testes tubules with spermatogonia, spermatocytes and mature spermatozoa present (Figure 3.6A). However, in zebrafish *dmrt1* loss of function mutants, they have a disorganized testis and lack mature

sperm cells (Figure 3.6B) (Webster et al 2017). To test if the SC:*dmrt1* transgene could rescue any of these mutant testes defects, we compared the testes of 10 months post fertilization (mpf) adult *dmrt1* mutant fish with and without the SC:*dmrt1* transgene. We found that in both groups the testes looked similar to each other, with no improvements found in the transgenic fish (Figures 3.6B & 3.6C). Both groups had small testes, were disorganized and had no mature sperm cells present (Figures 3.6B & 3.6C). However, both mutant genotypes had some spermatogonia present or no germ cells present (Figures 3.6B and 3.6C). This shows that *dmrt1* expression in the Sertoli cells via the SC:*dmrt1* transgene is not sufficient to rescue testes defects in the *dmrt1* mutant zebrafish.

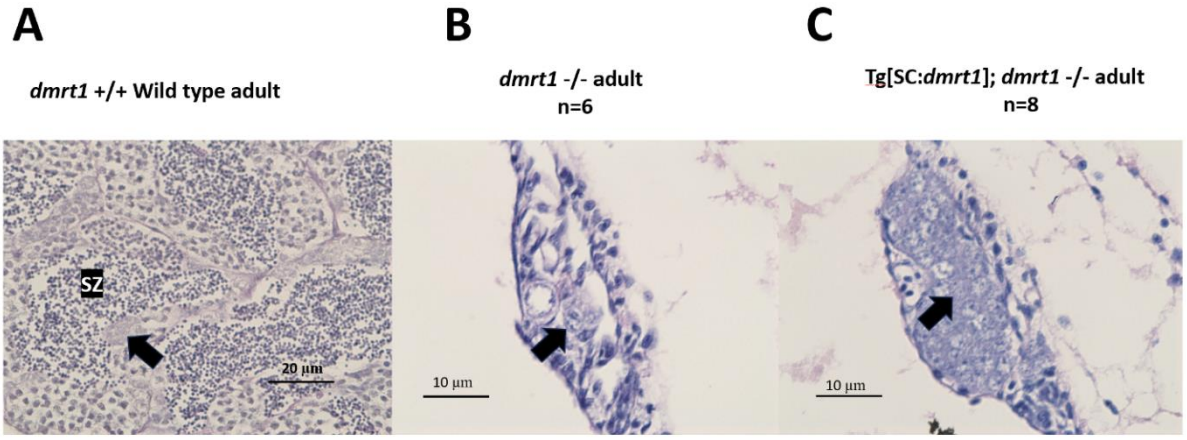


Figure 3.6. Testes of *dmrt1* mutant zebrafish are similar with or without the SC:*dmrt1* transgene. A) Histology image of *dmrt1* wild-type zebrafish testis. Section was stained using Hematoxylin and Eosin (H&E). B) Histology image of *dmrt1* mutant zebrafish testis without the SC:*dmrt1* transgene. Sections were stained using H&E. Total n=6. C) Histology image of *dmrt1* mutant zebrafish testis with the SC:*dmrt1* transgene. Sections were stained using H&E. Total n=8. The black arrow shows the spermatogonia and the 'SZ' labels the spermatozoa. Images were taken with the help of Andrew Karam.

3.6 Germ Cell dmrt1 Expression Does not Rescue the dmrt1 Mutant Phenotype

We next tested for *dmrt1* germ cell expression to see if that can rescue male fate in mutant zebrafish. To test this we set up a cross in which the female was a *dmrt1* homozygous mutant and the male was heterozygous for *dmrt1*. One of the parents also had a copy of the GC:*dmrt1* transgene. This resulted in progeny in which the zebrafish could have either the

GC:*dmrt1* transgene and be heterozygous for *dmrt1*, have the GC:*dmrt1* transgene and be mutant for *dmrt1*, be heterozygous for *dmrt1* with no transgene, or be mutant for *dmrt1* with no transgene (Figure 3.7A). When we compared the heterozygotes for *dmrt1*, with and without the transgene, we saw that both populations were predominantly male and both had similar ratios of males to females (Figure 3.7B). This shows what our wild-type genotype would look like in this population since wild-type and *dmrt1* heterozygous have similar sex ratios (Webster et al, 2017). Also, when comparing our heterozygous *dmrt1* sex ratio to the mutant *dmrt1* sex ratio, without our transgene, it shows the female sex bias for our *dmrt1* mutant population, as expected (Figures 3.7B & 3.7C). We then wanted to compare *dmrt1* mutant sex ratios, with and without the GC:*dmrt1* transgene to see if *dmrt1* expression exclusively in the germ cells could rescue this mutant phenotype. When we looked at the *dmrt1* mutant populations we saw that both the transgenic and the non-transgenic fish had similar sex ratios (Figure 3.7C). This shows that when *dmrt1* is expressed in the germ cells of *dmrt1* mutant fish, it cannot rescue male sex determination. It also shows that Sertoli cell *dmrt1* expression may not be necessary for male sex determination or that expression in both cell types is required. However, like with the Sertoli cell transgene, transgenic dmrt1 expression in the germline, or expression of this splice variant alone, may be insufficient to rescue the biased sex ratio observed in dmrt1 mutants.

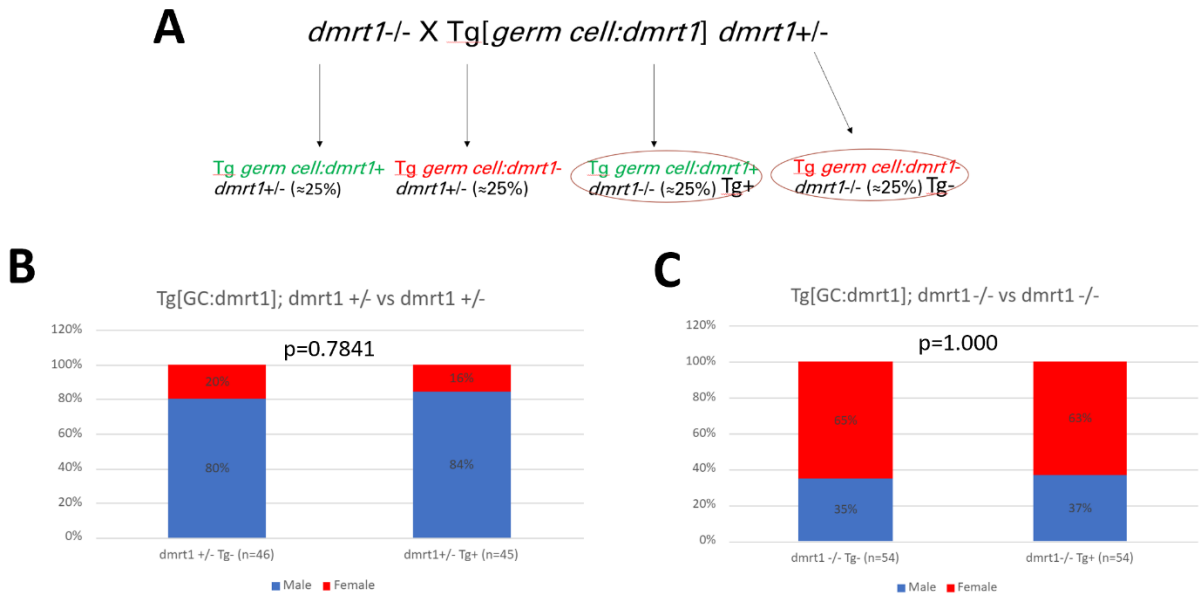


Figure 3.7. The female biased sex ratio was not rescued when *dmrt1* was expressed exclusively in the germ cells of *dmrt1* mutants. A) Diagram of the crosses set up for the sex ratio analysis. In the parent crosses, *dmrt1* mutant females were crossed to heterozygous males. One of the parents also had the GC:*dmrt1* transgene. The progeny then consisted of four distinct genotypes. The green lettering represents the presence of the transgene. The red lettering represents the absence of the transgene. The two circles represent the genotypes of interest for part C. B) The sex ratio chart from the progeny that resulted from the heterozygous fish found in the cross in part A. The left side is the sex ratio for the non-transgene heterozygous fish and the right side is the sex ratio for the transgene positive heterozygous fish. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=91 (n=46/45 for each genotype). P-value was calculated using a Fisher's exact test. C) The sex ratio chart from the *dmrt1* mutant progeny that resulted from the cross in part A. The left side is the sex ratio for the non-transgene mutant fish and the right side is the sex ratio for the transgene positive mutant fish. Blue areas are the percentages of males and the red areas are the percentages of females. Total n=108 (n=54 for each genotype). P-value was calculated using Fisher's exact test. Data for these charts were also contributed by Jess MacNeil and Andrew Cogliano.

3.7 The Larval Lethality of dmrt2a Mutants Cannot Be Rescued by Mutations in dmrt1

We next moved our attention to another gene in the *dmrt* family that is also involved in zebrafish development, which is the *dmrt2a* gene. Previous research has shown that predicted loss of function mutations in *dmrt2a* caused larval lethality. For example, the $dmrt2a^{umb12}$ allele, which is tightly linked to wild-type *dmrt1*, causes larval lethality (Steinfeld et al 2021). However, when a similar loss of function mutant allele $dmrt2a^{umb11}$, was double mutant for loss of function $dmrt1^{uc27}$, there was a 40% survival rate (Steinfeld et al, 2021). Because *dmrt1* and *dmrt2a* are tightly linked genes, the double mutant fish were generated by creating new mutations of one gene on the mutated chromosome of the other gene (e.g. the $dmrt1^{uc27}$ $dmrt2a^{umb11}$ mutant chromosome was made by generating the $dmrt2a^{umb11}$ mutation in fish carrying the $dmrt1^{uc27}$ mutation). We wanted to see if loss of *dmrt1* function is the cause of the *dmrt2a* mutant fish surviving in the double mutants or if it is another genetic modifier in the background that is causing this. To distinguish these possibilities, we first generated a new *dmrt1* mutation in the $dmrt2a^{umb12}$ mutant line using CRISPR/Cas9 genome editing. We tested the new *dmrt1* mutation for linkage to $dmrt2a^{umb12}$ by crossing the double heterozygotes with wild-type zebrafish and testing if progeny inherited both mutations or if the two mutations segregated away from each other. When we saw that the new *dmrt1* mutation was always present when our progeny fish were heterozygous for $dmrt2a^{umb12}$, we knew that these mutations were linked. We then sequenced the *dmrt1* gene in double heterozygous fish and saw one 4bp deletion, which we named $dmrt1^{umb19}$ (Figure 3.8A). We then looked at the amino acid sequence that was generated from these $dmrt1^{umb19}$ mutants and saw that the 4bp deletion causes a frameshift leading to a shorter DM domain sequence (Figure 3.8B). The $dmrt1^{umb19}$ mutation leads to truncation of the DM domain similar to the loss of function $dmrt1^{uc27}$ mutation and is therefore predicted to be a loss of function allele (Figure 3.8B). To test if the $dmrt1^{umb19}$ mutation can rescue $dmrt2a^{umb12}$ lethality, we incrossed fish that were heterozygous for $dmrt2a^{umb12}$ and $dmrt1^{umb19}$ and raised the progeny to adulthood. We found no surviving double mutant zebrafish showing that mutating the *dmrt1* gene does not contribute to the survival of *dmrt2a* mutant zebrafish and some other

background modifier is causing a small percentage of these mutant fish to survive in the *dmrt2a*^{umb12} line (Figure 3.9).

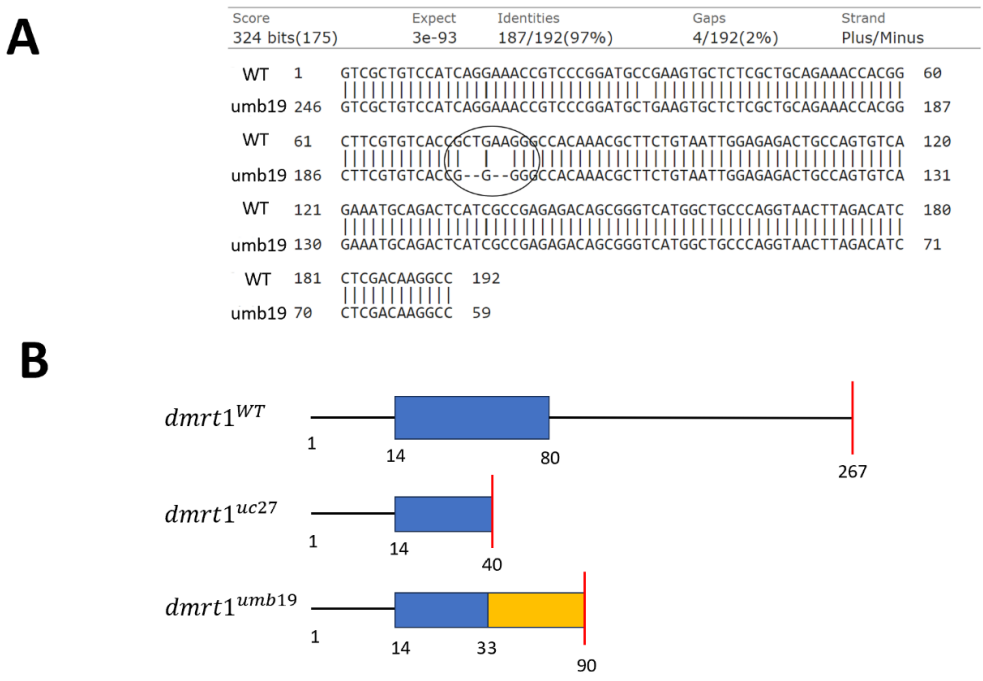


Figure 3.8. The *dmrt1*^{umb19} Mutation. A) Sequence alignment of the *dmrt1* wild-type and the *dmrt1*^{umb19} sequence. The top sequence represents the wild-type *dmrt1* sequence and the bottom sequence represents the *dmrt1*^{umb19} mutant sequence. The circle highlights the 4bp deletion found in the mutant sequence. Sequences were aligned using Blast. B) Diagram of the predicted proteins generated from the *dmrt1* gene. The top row is the wild-type protein, the middle row is the loss of function *dmrt1*^{uc27} mutant protein and the bottom row is the frameshift *dmrt1*^{umb19} mutant protein. The numbers represent the number of amino acids in each protein. The blue box represents the DM domain of the *dmrt1* protein. The orange box represents the frameshifted protein sequence. The red line represents the stop codon.

$\frac{dmrt1^{+};dmrt2a^{+}}{dmrt1^{umb19};dmrt2a^{umb12}} X \frac{dmrt1^{+};dmrt2a^{+}}{dmrt1^{umb19};dmrt2a^{umb12}}$		
Genotypes	**Expected**	**Observed**
$\frac{dmrt1^{+};dmrt2a^{+}}{dmrt1^{+};dmrt2a^{+}}$	30	27
$\frac{dmrt1^{+};dmrt2a^{+}}{dmrt1^{umb19};dmrt2a^{umb12}}$	60	73
$\frac{dmrt1^{umb19};dmrt2a^{umb12}}{dmrt1^{umb19};dmrt2a^{umb12}}$	10	0
Total	100	100

Figure 3.9. The *dmrt1^{umb19};dmrt2a^{umb12}* mutants were homozygous lethal. The progeny that resulted from a double heterozygous incross of *dmrt1^{umb19};dmrt2a^{umb12}*. Since these genes are linked, only three genotypes would be possible. The expected numbers were calculated based on previous data using different alleles showing that 10% of the population were double mutants as adults. The observed numbers were what was actually counted. The Chi-Square value was 13.117 with two degrees of freedom resulting in a p-value equal to 0.0014.

Chapter 4

DISCUSSION

4.1 dmrt1 Overexpression is Sufficient for Male Sex Determination

Using sex ratio analysis, we saw that when we overexpressed zebrafish *dmrt1* in both the germ cells and Sertoli cells using the GC:*dmrt1* and the SC:*dmrt1* transgenes they had a significantly higher percentage of males compared to the wild-type siblings, by about 40% (Figure 3.2B). We know from previous research that when *dmrt1* was mutated in zebrafish, the number of males decreased significantly compared to the wild-type siblings, by around 50% (Webster et al, 2017). These experiments show that *dmrt1* is important for male sex to be determined in zebrafish. However, it is not required, as some males were present in the mutant experiment and some females were present in the double transgene overexpression experiment (Webster et al, 2017; Figure 3.2B). Other genes, such as *androgen receptor* (*ar*) could act in male sex determination in the absence of *dmrt1* expression. The *ar* gene is a candidate gene for this because it has been shown to be expressed in testes when *dmrt1* is mutated and when that gene is mutated itself, the sex ratio results in a decrease of male fate (Crowder et al, 2018; Yu et al, 2018). So, this gene could act independently of *dmrt1* to have a small percentage of males be determined when the *dmrt1* gene is absent. However, without the *dmrt1* gene, the odds of a fish becoming a male is extremely low. Likewise, if the *dmrt1* gene is overexpressed, the odds that a fish will become a male is greatly increased. This proves that *dmrt1* is sufficient to drive male fate during sex determination. *Dmrt1* could also be considered a candidate to be a primary male sex determining gene in zebrafish, as this gene is located on chromosome 5 which has been shown to be a sex determination locus in zebrafish (Bradley et al, 2011).

4.2 Germ Cell Overexpression of dmrt1 is Sufficient for Male Sex Determination

Using sex ratio analysis, we saw that overexpression of *dmrt1* in germ cells from the GC:*dmrt1* transgene, there was a significant increase in the percentage of males compared to their wild-type siblings by about 20% (Figure 3.4B). We also saw that when *dmrt1* was overexpressed in the Sertoli cells from the SC:*dmrt1* transgene, there was no significant change in the percentage of males compared to their wild-type siblings (Figure 3.3B). This shows that germ cell overexpression of *dmrt1* likely has a bigger impact in male sex determination of zebrafish than Sertoli cell overexpression of *dmrt1*. However, the percentage of additional males is lower when germ cell *dmrt1* is overexpressed in germ cells compared to when it is overexpressed in both cell types (Figure 3.2B; Figure 3.4B). These data show that the expression of Sertoli cell *dmrt1* does have some involvement in male sex determination. In other animals, like mice, Sertoli cells *dmrt1* expression is vital to the survival and differentiation of germ cells in spermatogenesis as well as the maintenance of male-specific Sertoli cell fate (Kim et al, 2007; Matson et al, 2011). So, while overexpression of Sertoli cell *dmrt1* expression only using our transgenic line is not sufficient to drive male fate, there still could be involvement of Sertoli cell *dmrt1* expression in male sex determination. Nevertheless, *dmrt1* overexpression in the germ cells is sufficient to drive male fate in zebrafish during sex determination and likely has a bigger effect on male sex determination in zebrafish.

4.3 Germ Cell or Sertoli Cell Expression of dmrt1 is Not Sufficient to Rescue Male Sex Determination in dmrt1 Mutants

Through sex ratio analysis, we saw that when mutant *dmrt1* fish expressed *dmrt1* exclusively in the germ cells from the GC:*dmrt1* or exclusively in the Sertoli cells from the SC:*dmrt1* transgene, male sex determination defects could not be rescued (Figure 3.5C; Figure 3.7C). We also saw that SC:*dmrt1* transgene, could not rescue the testis morphology defects and these looked more like the mutant testis phenotype then the wild-type testis phenotype (Figure 3.6). Previous research has shown that when *dmrt1* is mutated, the sex ratio is female biased and the testes of the few mutant *dmrt1* males were disorganized with either only a few spermatogonia present or no germ cells present (Webster et al, 2017). It is

not too surprising that the SC:*dmrt1* transgene was not able to rescue *dmrt1* mutant female biased sex ratios or testis morphology since the overexpression of that transgene was not sufficient to drive male fate during sex determination. So, the SC:*dmrt1* transgene itself might not be strong enough to make up for the lack of *dmrt1* expression in the mutants. However, with the germ cell *dmrt1* overexpression being significant to drive male fate, it is a bit surprising that the germ cell *dmrt1* expression was not sufficient to rescue male fate in the mutant sex ratio. Some explanations for this could be that the GC:*dmrt1* transgene on its own is just not strong enough, like the SC:*dmrt1* transgene, to replace the lost expression of *dmrt1* in the mutants. The GC:*dmrt1* transgene with endogenous expression of *dmrt1* was able to significantly promote male fate, but without that endogenous expression, it was not able to rescue mutant female sex ratio bias. Another explanation could be that we are using a splice variant or transcript of *dmrt1* in both transgenes that is not producing the right protein isoforms needed for full function of the gene. We used *dmrt1* cDNA transcript ENSDART00000124637.4 for both of the transgenes, but there are up to 9 different cDNA transcripts of *dmrt1*, with 6 of them, including the one we used, code for a protein sequence (www.ensembl.org). One or multiple of these splice variants could contain proteins with different functions that could be needed for full function of the *dmrt1* gene. Regardless, the transgenes that we used individually are not sufficient to rescue male sex determination in *dmrt1* mutant zebrafish.

4.4 Mutations Disrupting dmrt1 Function Do Not Cause Survival of Mutant dmrt2a Fish

Using double mutant analysis, we saw that a predicted *dmrt1* loss of function mutation could not rescue lethality of *dmrt2a* mutants (Figure 3.9). From previous research we knew that when the *dmrt2a* gene is mutated in zebrafish, it resulted in lethality between 7 and 12 dpf (Steinfeld et al, 2021). However, when a *dmrt2a* mutation was generated in the *dmrt1* loss of function mutation (*dmrt1*^{uc27}) line, a small percentage of zebrafish were able to survive to adulthood when double mutant for *dmrt1* (Steinfeld et al, 2021). Though, when we generated a *dmrt1* mutation in the lethal *dmrt2a* line, no double mutants survived to adulthood (Figure 3.9). This shows that there is some background modifier(s) that is causing the survival of *dmrt2a* mutant fish in the line carrying the *dmrt1*^{uc27} allele. This makes sense

because in another paper that was not focused on studying the *dmrt1* gene, they too had mutant *dmrt2a* zebrafish survive in all of their mutant lines (Pinto et al, 2018). This shows that *dmrt2a* mutants can survive in some lines but not in others, regardless of loss of function of the *dmrt1* gene. Something other than *dmrt1* mutations is contributing to survival of *dmrt2a* mutant zebrafish. It remains unknown what is causing this survival but we can rule out that a mutation to the *dmrt1* gene is the cause of it.

4.5 Conclusion

Overall, we found that *dmrt1* overexpression is sufficient to drive male sex determination in zebrafish. The overexpression of *dmrt1* in germ cells is sufficient to drive male fate but, overexpression of *dmrt1* in Sertoli cells is not sufficient to drive male fate using the transgenic lines in this study. We also found that *dmrt1* expression in both the germ cells and Sertoli cells individually cannot rescue male sex in mutant *dmrt1* fish using the same transgenes. Finally, we found that mutations in the *dmrt1* gene do not cause the survival of *dmrt2a* mutant zebrafish. This project shows how effective *dmrt1* can be at promoting male sex determination. We know from previous studies that it is involved in sex determination and we know that it is important for proper testis morphology (Webster et al, 2017). However, with this project, we were able to see how adding more *dmrt1* expression can improve the chances of male sex being determined. This gene can drastically increase the number of males in a population based on expression levels.

Future experiments would help to see how much expression of *dmrt1* would it take to rescue female bias in *dmrt1* mutant sex ratios. Clearly, the expression of the transgenes by themselves could not rescue male fate. So, maybe more transgene expression or a different/additional splice variant/cDNA transcript could help to push male fate back into *dmrt1* mutant fish. However, despite no rescue in the *dmrt1* mutants, we see that *dmrt1* is sufficient to drive male sex determination which helps to improve the knowledge of zebrafish sex determination and the *dmrt1* gene as a whole. The gene *dmrt1* is important for male sex determination and from what was gathered here, this gene could be considered a candidate as a primary sex determination gene in zebrafish.

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