

ABSTRACT

Intrinsic Post-zygotic Isolation and Haldane's Rule in Lake Malawi Cichlids

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To assess the intrinsic fitness of hybrids and test for the action of Haldane's rule of two closely related Lake Malawi cichlid species, *Maylandia benetos* and *M.zebra*, fitness-related traits, sex ratios and sex-linked microsatellite loci were measured or genotyped in offspring from both conspecific and interspecific crosses. No differences were found between hybrid and non-hybrid offspring in fertilization, hatching and survival rate. There was no difference in sex ratio between the interspecific and conspecific crosses with *M.zebra* female. By contrast, males were absent in the interspecific cross with *M. benetos* female. The microsatellite data showed that *M. benetos* have an XY sex determination system. The gender of hybrids from both reciprocal crosses was determined by the allele they inherited from their *M. benetos* parental, suggesting a dominance effect of sex determination system in *M. benetos* to that in *M. zebra*.

Intrinsic Post-zygotic Isolation and Haldane's Rule in Lake Malawi Cichlids

by

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A Thesis

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TABLE OF CONTENTS

LIST OF FIGURES	v
LIST OF TABLES	vi
ACKNOWLEDGMENTS	vii
CHAPTER ONE: Introduction	1
The Role of Post-Zygotic Isolation in African Cichlid Speciation	1
Intrinsic Genetic Incompatibility: The Dobuzhansky-Muller Model	3
The Haldane's Rule	4
The Sex Determination System In African Cichlids	6
CHAPTER TWO: Methods and Materials	10
Fish and Cross Design	10
Fitness-Related Traits	13
Microsatellite Markers Selection And Genotyping	15
Data Analysis	17
CHAPTER THREE: Results	18
Fitness-Related Traits	18
Sex Genotypes And Determination Systems	23
CHAPTER FOUR: Discussion	26
CHAPTER FIVE: Conclusions	35
BIBIOGRAPHY	36

LIST OF FIGURES

Figure 1. Dobzhansky-Muller model from Wu & Ting, 2004	4
Figure 2. Four types of crosses	11
Figure 3. Incubating device	12
Figure 4. Identification of unfertilized eggs and dead embryos	14
Figure 5. Ovary vs. testis	15
Figure 6. Fitness-related traits between conspecific crosses	18
Figure 7. Fertilization rates between conspecific and interspecific crosses	19
Figure 8. Hatching rates between conspecific and interspecific crosses	20
Figure 9. Survival rates between conspecific and interspecific crosses	21
Figure 10. Sex ratios between cross types	22
Figure 11. Four possible scenarios of sex determination systems in <i>M. zebra</i> and sex determiners interaction	33

LIST OF TABLES

Table 1. Total survival at different stages in four types of crosses	15
Table 2. Sex-linked microsatellite markers	16
Table 3.1 Marker UNH2086	23
Table 3.2 Marker UNH2139	24
Table 3.3 Marker GM602	25

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CHAPTER ONE

Introduction

The Role of Post-Zygotic Isolation in African Cichlid Speciation

Understanding the mechanisms of speciation is a fundamental goal of evolutionary biology. As most models of speciation and maintenance of species boundaries require the development of reproductive isolation (Mayr, 1942; Jennings et al., 2011), the study of this process will provide important information into how species are formed. Reproductive isolation is traditionally divided into pre-zygotic and post-zygotic isolation. Pre-zygotic isolation refers to all processes that prevent fertilization and is influenced by ecological, molecular, structural, and behavioral factors (Coyne and Orr, 2004; Wiens, 2004; Perdeck, 1958; Vacquier, 1998). Post-zygotic isolation compromises all phenomena that reduce the fitness in hybrids (Coyne and Orr, 2004; Moyle et al., 2004). Post-zygotic isolation mainly results from intrinsic genetic incompatibilities (Dobzhansky, 1936; Muller, 1942; Turelli and Orr, 2000; Stelkens et al., 2009). Alternatively, it can result from extrinsic ecological or sexual selections if hybrids are ecologically or reproductively less fit than non-hybrids (Coyne and Orr, 2004; Naisbit et al., 2001).

Cichlids fishes in the Great Lakes of East Africa are the most diverse extant vertebrate radiation and are considered one of the most powerful model systems to study speciation (Kocher, 2004; Seehausen, 2006). Over 2,000 species of cichlids in these lakes have evolved in the past 10 million years (Kocher, 2004; Danley et al., 2012a). The

mechanisms underlying such an extraordinarily rapid radiation of African cichlids have been studied extensively. Cichlid speciation in Africa is thought to have been driven by pre-zygotic isolation (Kocher, 2004; Salzburger, 2009) as a result of the interplay of ecological and reproductive factors (van der Sluijs et al., 2008; Salzburger, 2009; Wagner et al., 2012). In addition, many of cichlid species are reproductively compatible in laboratory (Kocher, 2004) and post-mating isolation between closely related species is thought to be negligible (Stelkens et al., 2009). Consequently, fewer studies of post-zygotic isolation in cichlids have been performed.

While it has been broadly accepted that divergent ecological and sexual selections have played a very important role in cichlid speciation, several lines of evidence suggest the potential role of intrinsic genetic incompatibilities in cichlid speciation. Intrinsic genetic incompatibilities will gradually accumulate between allopatric populations and produce unfit hybrids given sufficient time. The high level of population structure across km scale distances in cichlids has likely created opportunities for micro-allopatric divergence of populations (van Oppen et al., 1997; Arnegard et al., 1999, Danley et al., 2000). A long history of lake level fluctuations further contributes to the geographic separation in cichlid populations (Danley et al., 2012a) and facilitates the accumulation of intrinsic genetic incompatibilities.

Intrinsic genetic incompatibilities might also arise when two species with different sex determining systems hybridize and produce intersex individuals (Ser et al., 2010). Multiple interacting loci have been identified which control sex determination in many species of cichlids (Seehausen et al., 1999; Ser et al., 2010; Parnell and Streelman, 2012; Kudo et al., 2015). The intense competition among the colorful cichlids drives the

spread of new color patterns, which might produce asymmetrical fitness effect on males and females. This may then create opportunities for the invasion of new sex determiners in linkage disequilibrium with genes controlling color patterns (Seehausen et al., 1999; Ser et al., 2010). Consequently, unfit hybrids might result from sex determination incompatibilities between species with different number of sex determiners or/and sex determining systems (XY and ZW sex determining systems).

The Dobzhansky-Muller Model

How does genetic incompatibilities evolve without populations passing through an adaptive valley (Wright, 1932)? In the simplest scenario, we consider one gene with only two alleles: A and a . The common ancestor has genotype aa . Any daughter species that evolves from aa to AA must experience the heterozygous state (Aa), which is inferior (Orr, 1997). How can speciation occurs without populations crossing such an adaptive valley? This fundamental problem was first unraveled by William Bateson (Bateson, 1909), and then independently solved by Dobzhansky (Dobzhansky, 1937) and elaborated by Muller later (Muller, 1942). Credit is usually given instead to Dobzhansky and Muller for their contributions (Orr, 1997). Their solution to the problem is known as the Dobzhansky-Muller Model. As explained by this model, alleles at different loci in two divergent populations may evolve independently. These mutated alleles, without being tested under foreign genomes, may cause genetic incompatibilities when brought to a different genetic background.

As an example, let us consider the simplest situation of the Dobzhansky-Muller model. The ancestor population has genotype $AABB$. When the population splits into two, A evolves into a in one population and produce genotype $aaBB$, and b evolves into B in

the other and produce genotype $AAbb$. Their hybrids will have genotype $AaBb$. Hybrids will be unfit if a and b are mutually incompatible. Since a and b are not simultaneously present in both pure species, the evolution of a - b incompatibility is possible (Fig. 1, Wu and Ting, 2004).

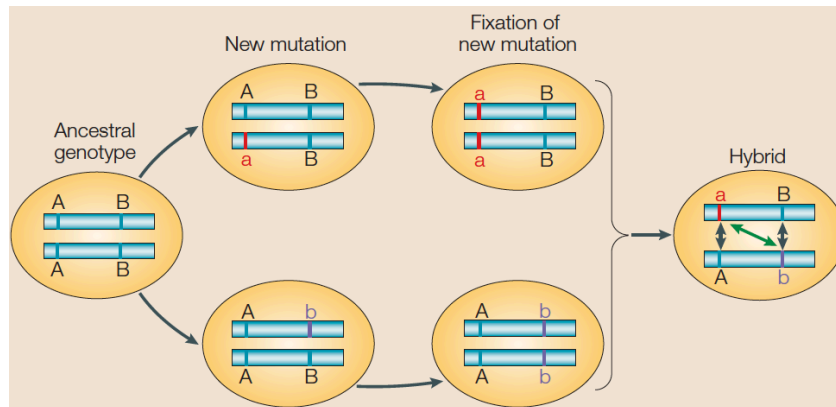


Figure 1. Dobzhansky-Muller model from Wu & Ting, 2004

The Haldane's Rule

One of the patterns characterizing post-zygotic isolation is Haldane's rule (Presgraves, 2002), which was first described by J.B.S Haldane as “When in the offspring of two different animals races one sex is absent, rare or sterile, that sex is the heterozygous/heterogametic sex” (Haldane, 1922). Haldane's rule has been observed in large number of animal groups, such as insects, birds, reptiles, mammals and bony fish (Laurie, 1997; Russell, 2003; Coyne and Orr, 2004; Presgraves, 2010), and has even been extended to plants with sex chromosomes (Brothers & Delph, 2010).

A large number of hypotheses have been proposed to explain Haldane's rule. These include genetic incompatibilities between X- and Y- linked genes or between Y and autosomal chromosomes (Muller, 1942; Pantazidis et al., 1993; Heikkinen and

Lumme, 1998), dosage compensation (Cline and Meyer, 1996), meiotic drive (Frank, 1991; Hurst and Pomiankowski, 1991), the Faster-X theory (Charlesworth et al, 1987), the Faster male theory (Wu and Davis, 1993; Wu et al., 1996), and the dominance theory (Muller, 1942). Unfortunately, none of these hypotheses applies unitarily to all cases (Coyne 1992), and most have been falsified (Orr, 1997). Only the faster male theory, the faster X theory and the dominance theory remain viable (Orr, 1997). The dominance theory is mostly applicable in Haldane's rule.

The dominance theory was initially proposed by Muller (1940) and originally known as the X-autosome imbalance theory (Muller, 1940; Muller, 1942; Muller and Pontecorvo, 1942). Muller's X-autosome imbalance hypothesis is based on the assumption that many species have a balance of genes in the X chromosome and the autosomes. While homogametic sex carries a haploid set of chromosomes from each species, the heterogametic sex lacks an X from one species. It is clear that hybrid males (assuming males are the heterogametic sex) will be more afflicted than hybrid females if detrimental interactions between X-linked genes and autosomal genes from the otherwise species are partially recessive in the hybrid genetic background. Muller's X-autosome imbalance theory was later renamed as the dominance theory to better describe its dominant/recessive nature (Wang, 2012).

The faster male theory (Wu and Davis, 1993; Wu et al., 1996) and faster X theory (Charlesworth et al, 1987) are two alternative hypotheses that explain Haldane's rule, both suffer some limitations. Wu and Davis (1993) suggest that hybrid male sterility evolves faster than hybrid female sterility due to sexual selection. While this hypothesis explains hybrid sterility of Haldane's rule well in male heterogametic taxa, it cannot

explain hybrid sterility in female heterogametic taxa in which hybrid females are preferentially afflicted. It does not apply to hybrid inviability neither, as there is no difference between hybrid male and female lethality (Orr, 1997). Charlesworth et al (1987) proposed the faster X explanation of Haldane's rule. They indicated that X-linked genes evolve more quickly due to enhancing selection on heterogametic-expressed genes. The faster X theory itself does not explain Haldane's rule. It relies on the dominance theory but exaggerates the effect of dominance (Orr, 1997).

While several proposed hypotheses have contributed to the explanation of Haldane's rule, all these theories must work within the confines of the Dobzhansky-Muller model (Orr, 1997). The phenomenon of Haldane's rule in hybrid offspring reflects intrinsic genetic incompatibilities between divergent parental species, which is thought to be less likely a mechanism influencing the diversification of African cichlids (Kocher, 2004; Stelkens et al., 2010). Demonstrating the existence of Haldane's rule will be strong evidence in support of the hypothesis that genetic incompatibilities may have contributed to the speciation of African cichlids.

The Sex Determination System In African Cichlids

Sex determination system plays a determinant role in Haldane's rule. Therefore, understanding the sex determination system is critical to examining Haldane's rule in divergent species. In African cichlids, the complexity of sex determination has largely hampered the test for Haldane's rule in this system. Published data indicates that sex chromosomes in cichlids of Lake Malawi are morphologically indistinct (Kornfield, 1984), and evidence shows that sex in tilapiine cichlids is environmentally and genetically determined (Römer & Beisenherz, 1996; Baroiller et al., 2009; Devlin and

Nagahama, 2002; Cnaani et al., 2008). Multiple sex determination systems exist in African cichlids (Albertson, 2002; Streelman et al., 2003, Ser et al., 2010; Parnell and Streelman, 2013). By applying sex-linked microsatellite markers to Lake Malawi cichlids, Ser et al. (2010) identified a male heterogametic (XY) system on linkage group 7 and a female heterogametic (ZW) system on linkage group 5 in different species. The ZW system showed epistatic dominance to the XY system when both are segregating within a family. Ser et al. (2010) also indicated that some species are segregating additional sex-determining loci. Similar results were found in a recent study (Parnell and Streelman, 2013). Parnell and Streelman (2013) used quantitative trait loci analyses and identified five sex-determining regions in a single hybrid cross between two Lake Malawi cichlid species. The five sex-determining regions interact epistatically to determine the sex of an individual. Their findings support the idea that linkage group 5 hosts a female heterogametic (ZW) locus. This ZW system is epistatically dominant to other sex systems identified, including two male heterogametic (XY and X'Y') loci on linkage group 7, because all WW genotypes were found to be females. However, the ZW and ZZ genotypes were not exclusively females and males, respectively.

Yet, few studies have focused on quantifying hybrid fitness in African cichlids. Sex ratios in most of these cases tended to be female biased and evidence consistent with Haldane's rule was found in hybrids between relatively distantly related Africa cichlids (Crapon de Caprona and Fritzsche, 1984). However, it is unknown which sex in the cichlid species studied by Crapon de Caprona and Fritzsche (1984) were heterogametic, thus leaving the test for Haldane's rule unresolved (van der Sluijs et al., 2008b). Recent studies on cichlids sex determination systems (Ser et al., 2010; Parnell and Streelman,

2013) provide a solution to testing for Haldane's rule in African cichlids. While it is clear that both the XY and ZW system exist in African cichlids and multiple sex determination systems are segregating in some species, many species have a pure or a primary sex determination system (Ser et al., 2010; Parnell and Streelman, 2013). For example, Ser et al (2010) observed that many species of *Metriaclima* (*Maylandia*) have a pure or primary XY system in which segregation of sex determinant alleles explains the sex of >90% of the individuals. The simplicity of the sex determination system in these species provides opportunity for testing for Haldane's rule and the effect of genetic incompatibilities on the speciation of African cichlids.

Maylandia zebra and *Maylandia benetos* are two closely related rock-dwelling species. While *M. zebra* is broadly distributed across the Lake, *M. benetos* is only found at Mazinzi Reef (Stauffer et al., 1997). On this reef, the two species share similar habitats and overlap with each other (Danley et al, 2012b). Hybridization between the two species occurs rarely in the lab as a result of strong assortative mating. Artificial hybridization in one direction of the cross in our lab produced only female offspring suggesting the potential of intrinsic post-zygotic isolation between these two closely related species. I made pure and hybrid crosses between *M.benetos* and *M.zebra* and then measured several fitness-related traits in the offspring, including fertilization rate, hatching rate, survival rate and sex ratio. I also used previously identified sex-linked microsatellite markers to identify sex-determining mechanisms in their hybrids. If genetic incompatibilities exist between these two species, I would expect to see one or more of these fitness-related traits being affected. In addition, if the post-zygotic isolation pattern is consistent with the

pattern of Haldane's rule, I would expect to see that the heterogametic sex are more afflicted than the homogametic sex.

CHAPTER TWO

Materials And Methods

Fish And Cross Design

M. zebra and *M. benetos* used in this study were from laboratory stocks. Both species were collected from Mazinzi Reef and have been maintained as laboratory stocks for at least 12 generations. Fish are kept at 26~28°C with a 12h L: 12h D light regime and fed with dry food daily. Hybridization between two species occurs rarely in the lab. Consequently, F1 offspring were produced via artificial fertilization for both interspecific crosses (female *M. zebra* × *M. benetos* & female *M. benetos* × *M. zebra*) and intraspecific species crosses (female *M. zebra* × *M. zebra* & female *M. benetos* × *M. benetos*) to control for the effects of artificial fertilization (Fig. 2). Eggs were collected by gently squeeze the abdomen of gravid females. Only mature eggs (no bloody tissues attached) were used in fertilization. Eggs from each single female were split into two even portions and placed on two 50mm diameter x 4mm deep petri dishes with water. After drying the body surface of males from each species with tissue papers, sperm was collected by squeezing males and directly placed onto the two petri dishes, respectively. Eggs and sperm were immediately mixed by sucking the mix back and forth with a dropper. After 5-10 min, the fertilized eggs were transferred to a special incubating device I designed. A total of 15 *M. benetos* females, 12 *M. zebra* females, 14 *M. benetos* males and 14 *M. zebra* males were used to produce 42 crosses of female *M. benetos* × *M. zebra* (BZ

cross), 42 female *M. benetos* × *M. benetos* (BB cross), 18 crosses of female *M. zebra* × *M. benetos* (ZB cross) and 18 crosses of female *M. zebra* × *M. zebra* (ZZ cross).

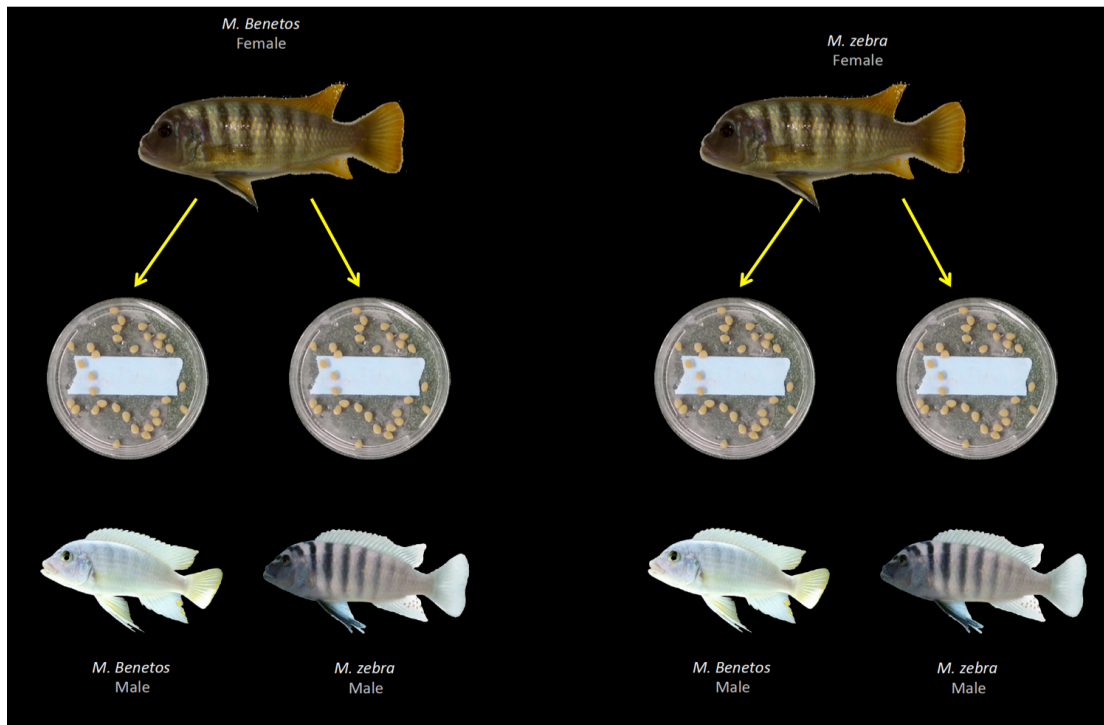


Figure 2. Four types of crosses

The incubating device consists of 6-8 50ml centrifuge tubes, a 10cm diameter cylinder with a plastic grid inside. The plastic grid divides the cylinder into upper and lower parts and acts as a platform to hold the tubes. The bottom of centrifuge tubes were cut off and the lid was replaced with a piece of mesh. The tubes then were put upside down on the plastic grid inside the cylinder. Fertilized eggs from different crosses were placed into different tubes. Air stones were held in the lower part of the cylinder. Bubbles produced from the air stones gently rock and fully aerate the eggs. One of the biggest advantages of this incubating device is that it can hold 6-8 crosses of eggs in a very small incubating area (within a 10cm diameter circle). It can efficiently control for the effect of variation in incubating environments between crosses.

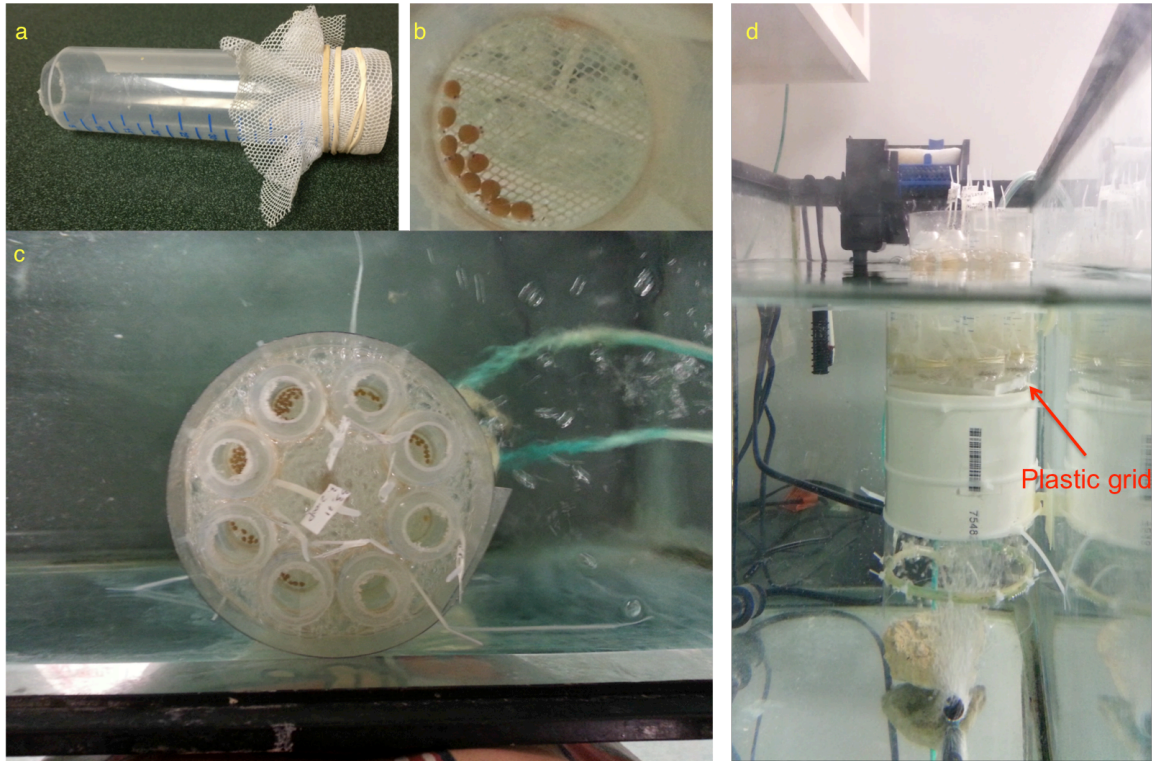


Figure 3. Incubating device. a. a single incubating tube; b. embryos in an incubating tube; c. 8 incubating tubes inside the incubating cylinder; d. the entire incubating system.

After 12 days hatching, the eggs hatched and fry became free swimming. Fry were then transferred into a $10 \times 9 \times 5 \text{ cm}^3$ cage. Each clutch was split into groups of 5 to standardize density. The remaining siblings were kept in a reserve cage and used to replace dead fish to keep the number of individual constant (van der Sluijs, 2008). At day 45, all fish from the same type of cross were transferred to a $231 \times 56 \times 28 \text{ cm}^3$ till adult. Fish were fed at constant per-fish-rate. Dead embryos and fish were removed and stored in 100% EtOH at -20°C for DNA extraction.

Fitness-Related Traits

Fertilization Rate

Fertilization rate was defined as the proportion of eggs successfully fertilized among the total number of eggs and calculated as the number of fertilized eggs/total number of eggs at day 3 post-fertilization (table 1). Fertilized eggs were distinguishable by color and texture at day 3 post-fertilization (Figure 3) (similar to 5 days post-fertilization in van der Sluijs, 2008; Stelkens et al., 2010).

Hatching Rate

Hatching rate was defined as the proportion of hatched eggs among the number of successfully fertilized eggs. Hatching rate was calculated as the number of developing embryos/total number of successfully fertilized eggs at day 12 post-fertilization (table 1). Dead embryos were white and had different texture from developing embryos (Figure 4). Heartbeat in developing embryos could be easily observed (Figure 4).

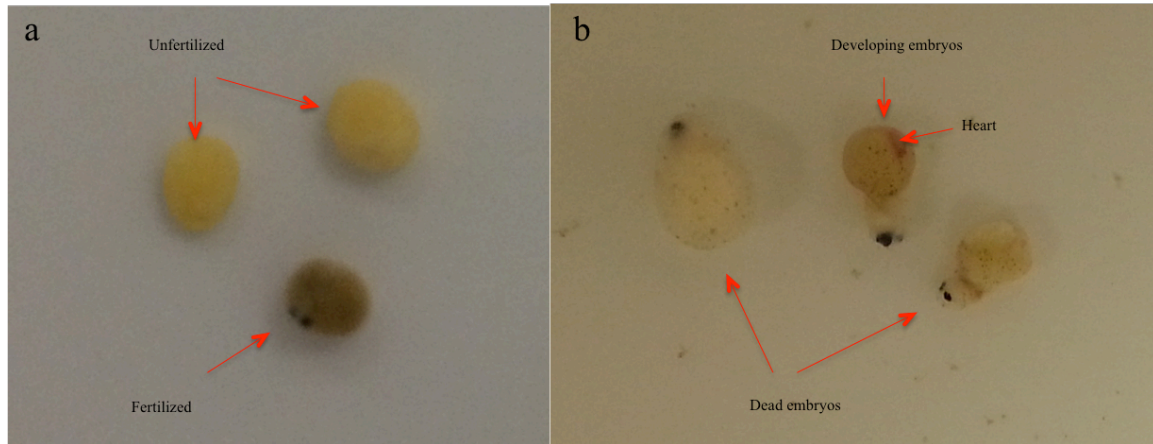


Figure 4. Identification of unfertilized eggs and dead embryos. a. Unfertilized eggs vs. fertilized eggs; b. Dead embryos vs. developing embryos.

Survival Rate

Survival rate was assessed at day 45 post-fertilization (table 1). Due to limited spaces, survival rate was only examined in 15 crosses of BZ, 15 crosses of BB, 8 crosses of ZB and 8 crosses of ZZ.

Sex Identification And Sex Ratio

All fish were sacrificed by lowering their body temperature in a freezer. Fish were then dissected and Gonads were examined to determine sex (Guerrero et al., 1974). All fish dissected were at least 8 months old. By the day all fish were dissected, there were 56, 36, 27, 19 individuals from crosses BZ, BB, ZB and ZZ, respectively. Ovary or testis could be clearly observed in all individuals (Figure 5).

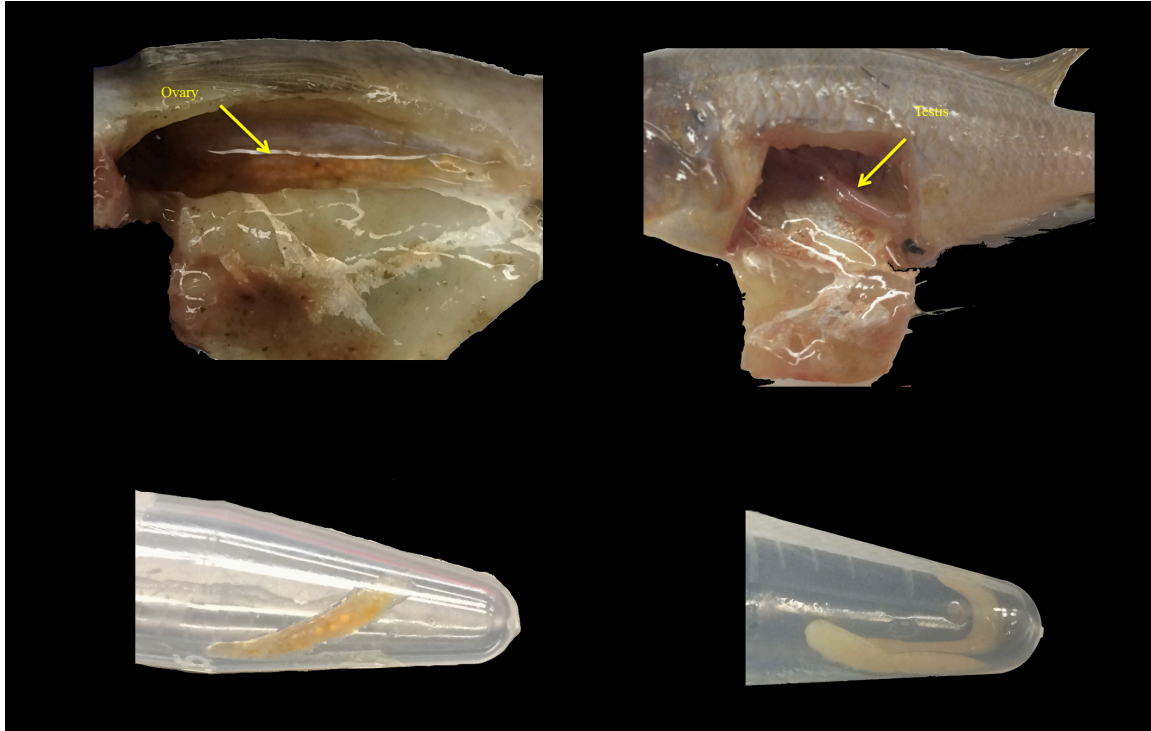


Figure 5. Ovary vs. testis

Sex ratio was defined as the proportion of males in a clutch and was calculated as the number of males/(females + males).

Table 1. Total survival at different stages in four types of crosses

Number of crosses	Cross type	Survival			
		Day 0	Day 3	Day 12	Day 45*
42	BB	987	595	396	94
42	BZ	982	544	386	108
18	ZZ	441	218	178	65
18	ZB	441	227	160	47

* denotes that the numbers of crosses for Day 45 are 15, 15, 8 and 8 in cross BB, BZ, ZZ and ZB, respectively.

Microsatellite Markers Selection And Genotyping

Microsatellite Markers Selection

The sequences for the 5 sex-linked RAD-tag SNP markers identified in Parnell et al. (2012) in NCBI (Table 2) were aligned to the Nile tilapia genome to find their locations on the genome. Microsatellite markers close to these locations were identified in the tilapia linkage map of Lee et al. (2005). By doing this, 5 microsatellite markers were obtained (Table 2). I also included the microsatellite marker UNH2139, which was identified to be linked to a ZW sex determining system on linkage group 5 (Ser et al., 2010).

Table 2. Sex-linked microsatellite markers

RAD-tag SNP ID	Linkage group	SNP rs number	Microsatellite markers
4384	3	rs267732498	GM150
9152	5	rs267732524	UNH884
27028	7	rs267732628	GM442
45045	7	rs267732730	UNH2086
11448	20	rs267732539	GM602
Ser et al (2010)			UNH2139

Sex Genotyping

PCR amplification of the 6 markers (table 2) was performed on genome DNA extracted from tissues from all individuals, including parents, dead and survived individuals by the day being dissected. A fluorescent labeling method was used to amplify these microsatellite markers (Schuelke, 2000). Three universal fluorescence labeled M13 (-21) primers were used and paired with 6 microsatellite marker primers. Each fluorescence labeled M13 (-21) was paired with two microsatellite marker primers.

Amplification products were then sent to Yale University for genotyping (<http://dna-analysis.research.yale.edu/>).

Data Analysis

Fertilization, hatching and survival rates were compared via paired t-test or Wilcoxon signed rank test between hybrid and non-hybrid crosses from the same maternal species, to determine if hybrid offspring were significantly less fit than non-hybrid offspring. The three traits were also compared via Welch two sample t test or Wilcoxon sum rank test between cross BB and cross ZZ, to determine if significant differences exist between offspring from two pure crosses. Whether parametric (t test) or non-parametric (Wilcoxon rank test) statistic tests were used depended on the normality of data. Normality was tested via Shapiro tests.

Person's chi-square tests were used to test if sex ratios were significantly different between two pure crosses and between hybrid and non-hybrid crosses from the same maternal species.

All microsatellite genotype calling was implemented in Geneious v8.1 (Drummond et al., 2011). Genotypes with haplotype frequencies less than 5% were removed from the data. Individuals from each of the four types of crosses were grouped according to gender by genotypes. Contingency tables were obtained by grouping individuals from each type of cross according to gender by presence or absence of a specific haplotype. Fisher's exact tests were applied to these contingency tables to determine if there were strong associations of markers with gender.

CHAPTER THREE

Results

Fitness-Related Traits

Comparisons between Conspecific Crosses

Fertilization, hatching and survival rates were compared between conspecific crosses. No significant effect (fertilization ratio: $W = 421$, $P = 0.493$; hatching ratio: $W = 267$, $P = 0.0871$; survival ratio: $t_{[21]} = -1.29$, $P = 0.217$) was found on any one of these three traits (Figure 6).

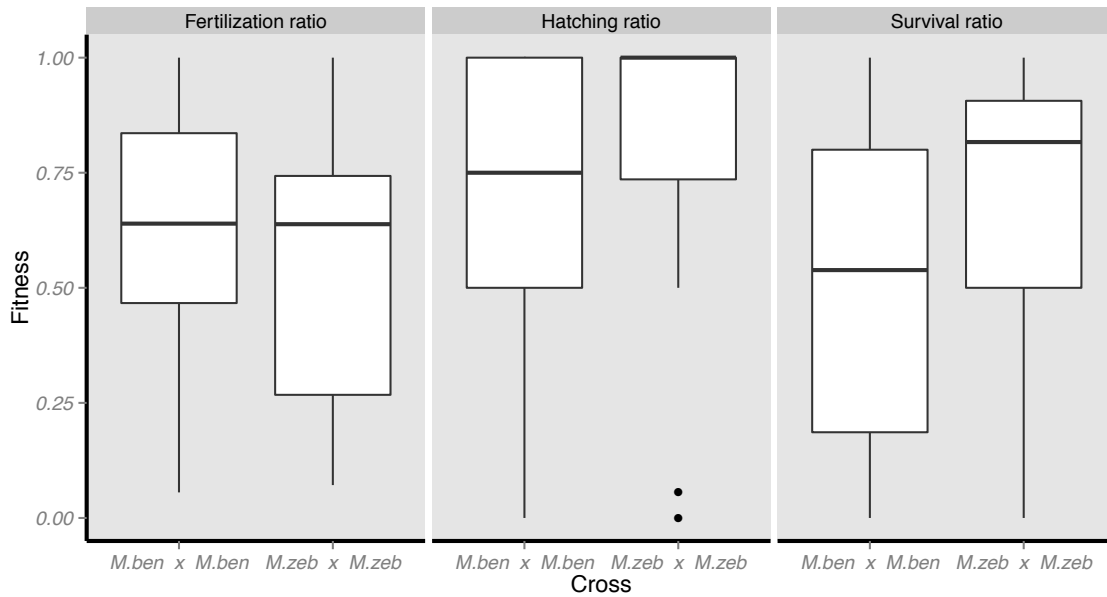


Figure 6. Fitness-related traits between conspecific crosses. Box-and-whisker plots for fertilization, hatching and survival rates.

Fertilization Rate

Fertilization rates were compared between BB and BZ crosses, and between ZZ and ZB crosses. No significant differences (Figure 7) were found in fertilization rates between BB and BZ ($V = 497$, $P = 0.245$) crosses, and between ZZ and ZB crosses ($t_{[18]} = -0.478$, $P = 0.638$).

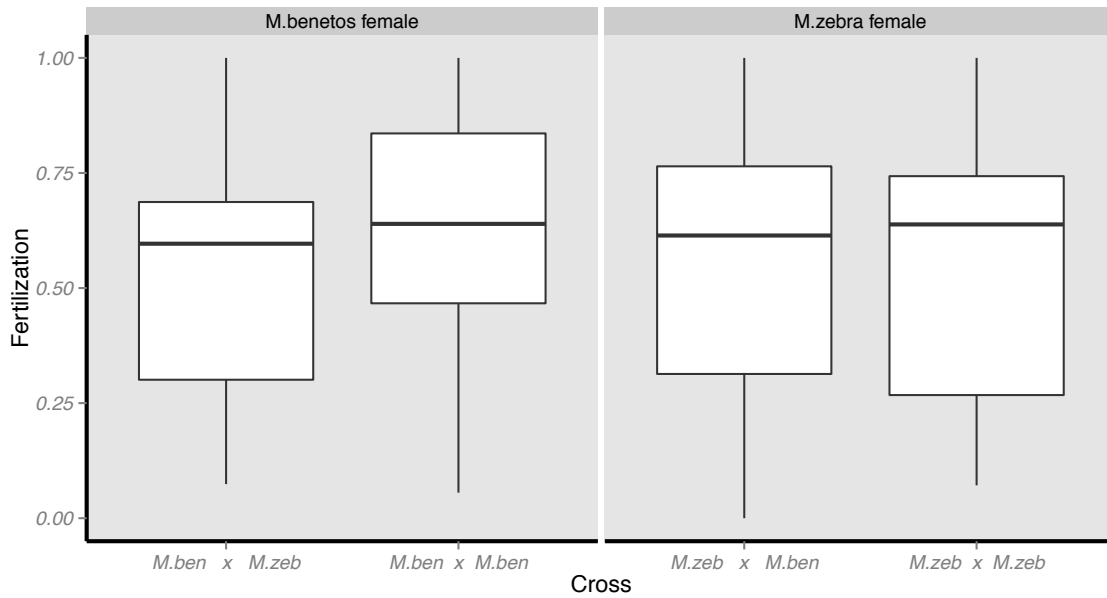


Figure 7. Fertilization rates between conspecific and interspecific crosses. Box-and-whisker plots for fertilization rate.

Hatching Rate

Hatching rates were compared between BB and BZ crosses, and between ZZ and ZB crosses. No significant differences (Figure 8) were found in hatching rates between BB and BZ ($V = 273.5$, $P = 0.906$) crosses, and between ZZ and ZB crosses ($V = 64$, $P = 0.208$).

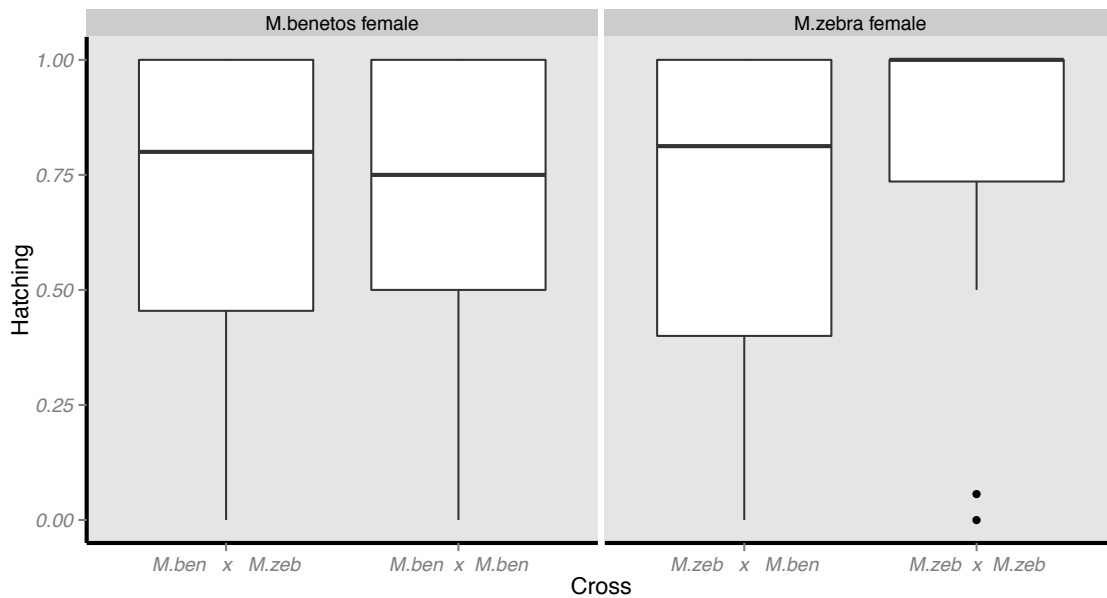


Figure 8. Hatching rates between conspecific and interspecific crosses. Box-and-whisker plots for hatching rate.

Survival Rate

Survival rates were compared between BB and BZ crosses, and between ZZ and ZB crosses. No significant differences (Figure 9) were found in survival rates between BB and BZ ($t_{[14]} = -0.771$, $P = 0.453$) crosses, and between ZZ and ZB crosses ($t_{[7]} = 1.61$, $P = 0.151$).

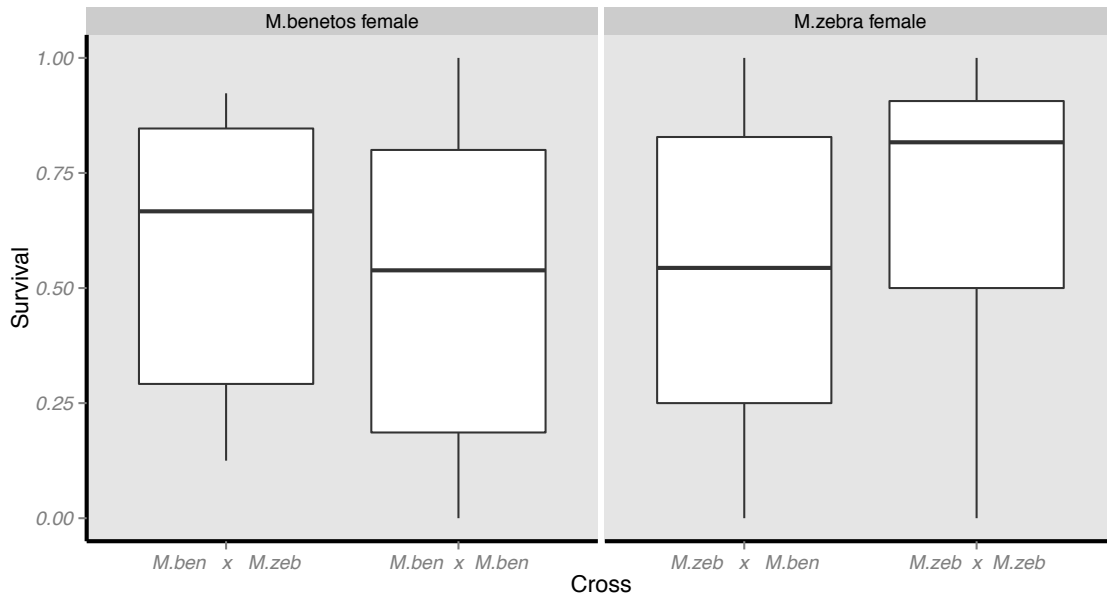


Figure 9. Survival rates between conspecific and interspecific crosses. Box-and-whisker plots for survival rate.

Sex Ratio

Sex ratios were slightly male biased but not significantly deviated from a 50:50 sex ratio in crosses BB (sex ratio = 0.556, $\chi^2 = 0.444$, $P = 0.505$), ZZ (sex ratio = 0.579, $\chi^2 = 0.474$, $P = 0.491$) and ZB (sex ratio = 0.592, $\chi^2 = 0.926$, $P = 0.356$). Males were absent in cross BZ (sex ratio = 0). Sex ratio in BZ was significantly deviated from the ratio 50:50 ($\chi^2 = 56$, $P < 0.0001$).

The sex ratio in the BZ interspecific cross significantly deviated from the conspecific cross BB (Fisher's exact test: $P < 0.0001$, Figure 10). However, there was no significant difference in sex ratio between the interspecific cross ZB and the conspecific cross ZZ (Fisher's exact test: $P = 1$, Figure 10).

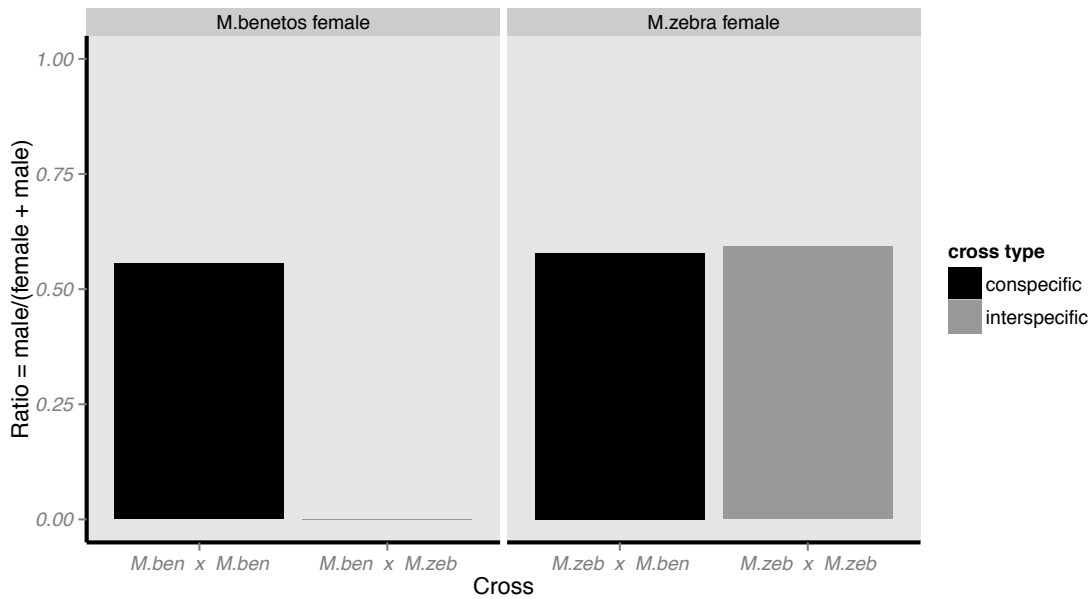


Figure 10. Sex ratios between cross types. The upper part is bar plots for number of different genders in crosses.

Sex Genotypes And Determination Systems

Among the 6 microsatellite markers, only marker UNH2086, UNH2139 and GM602 were successfully amplified. While no significant associations of marker UNH2139 (Table 3.2) and GM602 (Table 3.3) were detected with gender, a strong association was found between marker UNH2086 and the gender (Table 3.1).

Two alleles (170, 186) were obtained for marker UNH2086 in all *M. benetos* individuals, including parental *M. benetos* and the offspring from the BB cross. 24 out of 25 individuals with genotype 170/186 are males and 27 out of 28 individuals with genotype 186/186 are females. This is strong evidence for a male heterogametic (XY) sex determination system with haplotype 170 acting as a Y chromosome. The marker UNH2086 also has two alleles (188, 202) in *M. zebra*. The fisher's exact test result shows evidence for a female heterogametic (ZW) determination system: individuals with genotype 188/202 tend to be females, while individuals with genotype 202/202 tend to be males. However, the P value = 0.0496 was very close to the significance threshold 0.05. Out of 16 males, all but one from the ZB cross inherited the allele 170 from the *M. benetos* sire. Of the 11 females from the ZB cross, all but one inherited the 186 allele from the *M. benetos* sire. The *M. zebra* alleles show no effect on the gender of ZB hybrids, or its effect is completely masked by the *M. benetos* locus. This idea is further supported by the result from the cross BZ. All BZ hybrids were females and inherited the allele 186 from their *M. benetos* dam.

Table 3

Fisher's exact test for association of markers UNH2086 (table 3.1), UNH2139 (table 3.2), GM602 (table 3.3) with gender. Contingency tables are obtained by grouping individuals from the same cross type by gender x presence or absence of a haplotype. The '+' or '-' in front of a haplotype indicates genotypes with or without that haplotype. Bold letters are used for both haplotypes and corresponding p values if there is significant association. Males are absent in cross BZ; therefore, fisher's exact test is uninformative in cross BZ for all three markers.

Table 3.1 Marker UNH2086

UNH2086	Gender	Possible Haplotypes	Possible Genotypes				Fisher's Exact Test For Effect Of A Specific Haplotype On Gender							
			170/186	170/170	186/186		+170	-170		+186	-186			
BB	Female	170, 186	1	0	27		1	27	<0.0001	28	0	0.482		
	Male	170, 186	24	2	1		26	1		25	1			
			186/188	186/202										
BZ	Female	186, 188, 202	12	43					Not informative, male absent					
	Male	NA	NA	NA										
			202/202	188/202	188/188		+202	-202		+188	-188			
ZZ	Female	188, 202	6	10	2		16	2	0.214	12	6	0.0496		
	Male	188, 202	14	6	0		20	0		6	14			
			170/188	170/202	186/188	186/202	+170	-170		+186	-186		+188	-188
	Female	170, 186, 188, 202	1	0	2	8	1	10	<0.0001	10	1	<0.0001	2	9
	Male	170, 186, 188, 202	6	9	0	1	15	1		1	15		0	16
ZB							+202	-202						
	Female	170, 186, 188, 202					8	3	0.692					
	Male	170, 186, 188, 202					10	6						

Table 3.2 Marker UNH2139

UNH213 9	Gender	Possible haplotypes	Possible genotypes				Fisher's Exact Test For Effect Of A Specific Haplotype On Gender								
			210/241	210/210	241/241		+210	-210	P	+241	-241	P			
BB	Female	210, 241	23	0	6		23	6	0.524	29	0	0.453			
	Male	210, 241	15	1	7		16	7		23	1				
			210/241	210/246	241/246	241/241									
BZ	Female	210, 241, 246	1	18	32	1	Not informative, male absent								
	Male		0	0	0	0									
			240/246	246/246			+240	-240	P	+246	-246	P			
ZZ	Female	240, 246	1	16			1	16	1	17	0	1			
	Male	246	0	16			0	16		16	0				
			210/241	241/246			+210	-210	P	+241	-241	P	+246	-246	
ZB	Female	210, 241, 246	3	8			3	8	0.234	8	3	0.239	11	0	1
	Male	210, 241, 246	9	7			9	7		7	9		16	0	

Table 3.3 Marker GM602

GM602	Gender	Possible Haplotypes	Possible Genotypes					Fisher's Exact Test For Effect Of A Specific Haplotype On Gender								
			225/237	225/250	225/225	237/250	237/237	+225	-225	P	+237	-237	P	+250	-250	P
BB	Female	225, 237, 250	16	3	3	1	0	22	1	0.608	17	6	1	4	19	0.722
	Male	225, 237, 250	15	4	1	1	1	20	2		17	5		5	17	
BZ			225/209	225/221	225/240	237/209	237/221									
	Female	225, 209, 240, 237, 250, 221	5	9	6	1	16									
	Male	NA	0	0	0	0	0									
			237/240	250/221	221/221	250/240										
			5	6	1	2										
			0	0	0	0										
ZZ			209/240	221/240	221/237	221/221		+221	-221	P	+237	-237	P	+209	-209	P
	Female	221, 237	0	0	1	17		18	0	1	1	17	0.474	0	18	1
	Male	209, 240, 221	1	2	0	17		19	1		0	20		1	19	
								+240	-240	P						
	Female	221, 237						0	18	0.488						
	Male	209, 240, 221						2	18							
ZB			225/221	225/240	237/221	237/240	250/221	+225	-225	P	+221	-221	P	+237	-237	P
	Female	225, 221, 237, 240, 250	2	0	4	1	4	2	9	1	10	1	0.624	5	6	1
	Male	225, 221, 237, 240, 250	2	1	6	2	5	3	13		13	3		7	7	
								+240	-240	P	+250	-250	P			
	Female	225, 221, 237, 240, 250						1	10	0.624	4	7	1			
	Male	225, 221, 237, 240, 250						3	13		5	11				

CHAPTER FOUR

Discussion

Due to strong behavioral reproductive isolation between *M.benetos* and *M.zebra*, artificial fertilization was needed to produce the necessary hybrids. This method also allowed controlling for additional variables thought to influence fitness. In order to control for maternal effect (van der Sluijs et al. 2008), eggs from the same female were split into two even portions and fertilized with sperm from males of each species to create conspecific and interspecific crosses. To further control for the effect of incubating environments, all eggs were incubated in a newly designed incubating system (Figure 3). With this incubating system, paired conspecific and interspecific crosses could be reared in identical environments (within two 50ml centrifuge tubes neighbored with each other (Figure 3.c)). In addition, I measured the survival of offspring in a very early development stage (45 days post-fertilization). This was different from other studies of hybrid fitness of cichlids (van der Sluijs et al. 2008; Stelkens et al. 2010) in which survival was measured through to 6 months old or adult. Due to limited space and a large number of fish produced in this study, it was impossible to maintain all experimental fish individually. Many cichlid species are very aggressive and show different levels of aggression towards conspecific and interspecific species (Dijkstra et al., 2007; Dijkstra et al., 2011). While no work has been done on the direct influence of aggression on survival in cichlid species, it has been proved that there is an association between aggression rate and mortality in other fish species (Kaiser et al., 1995). As cichlid species grow, they

become increasingly aggressive. In this study, I was only interested in the reduction of fitness-related traits resulting from intrinsic genetic incompatibilities. To minimize the potential result bias, I only measured the survival of offspring over 45 days during their early development.

No significant differences were found between *M. benetos* and *M. zebra* and their hybrids in fitness related traits. The results are consistent with previous studies on African cichlids (van der Sluijs et al., 2008; Stelkens et al., 2010). In van der Sluijs et al. (2008), they investigated several similar fitness-related traits in a pair of Lake Victoria cichlid species and found a reduction of fitness in the first and second hybrid generations as a result of a significant maternal effect on fertility and survival. In this study, no maternal effect was found on any of the three fitness-related traits. In another study of reproductive incompatibilities in African cichlid system, Stelkens et al. (2010) found that, although post-mating isolation accumulates rapidly with divergence time, hybrid inviability between closely related species is negligible. Inviability resulting from fertilization failure and 14-day mortality were inconsequential for species pairs separated for up to 1.2/0.9/1.5 Ma (results from three different molecular clocks), whereas hatching mortality and 180-day mortality were independent of divergence time. In fact, their results suggested that hybrids between the most closely related species even experienced higher survival rates than conspecific crosses.

The absence of hybrid inviability in this study supports the idea that cichlid speciation has been driven by recent divergent ecological and sexual selection rather than by the accumulation of intrinsic genetic incompatibilities (Kornfield and Smith, 2000; Seehausen, 2000; Streelman and Danley, 2003; Kocher, 2004). While the estimated age

of Lake Malawi's species flock vary continues to be dated, most methods obtained an age less than 2.4 Ma (Sturmbauer et al., 2001; Genner et al., 2007; Koblmüller et al., 2008; Danely et al., 2012a). The rock-dwelling cichlid clade, including *M. benetos* and *M. zebra*, is thought to have emerged 0.313 Ma to 0.486 Ma. *M. benetos* is thought to have diverged from other *Maylandia* species ~100 Ka, shortly after the last refilling of Lake Malawi at around 120 Ka (Husemann et al., 2015). Thus the divergence time within *Maylandia* species and even the entire rock-dwelling cichlid clade is much shorter than 0.9 Ma, which is the minimum divergence time when reproductive incompatibilities become significant in African cichlids (Stelkens et al., 2010). To reach complete isolation, it requires 4.4/8.5/18.4 Ma, which is significantly longer than the age of Lake Malawi's species flock (Stelkens et al., 2010).

Although none of the three fitness-related traits demonstrated significant differences between con- and heterospecific crosses in this study, intrinsic genetic incompatibility might not be completely absent between this pair of cichlid species. An important limitation in this study is that only F1 hybrid inviability was inspected. It is reasonable that hybrid incompatibilities should be more likely to be observed after the F1 generation, since recombination of recessive alleles in higher hybrid generations would expose some of the Dobzhansky-Muller incompatibilities that have been masked in the F1 generation. In a recently published study, Stelkens et al. (2015) found that the fitness of F2 hybrids of African haplochromine cichlids was significantly compromised, with a reduction of 21% compared to F1 hybrids and 43% compared to the grandparental, non-hybrid crosses.

The sex ratios in cross BB, ZZ and ZB were slightly male skewed but not significantly different from 50:50. The results contradict what was observed in some other Lake Victoria and Malawi cichlids in which sex ratios were found to be overall female biased (Crapon de Caprona & Fritzsche, 1984; Seehausen et al., 1997; van der Sluijs et al., 2008; Ser et al., 2010). One possible explanation was that more females died before being sacrificed for sex identification. In this study, all individuals from the same type of cross were stocked in the same big tank after measurement of 45-day survival. Siblings showed a high level of aggression due to high stocking density. Females were generally less aggressive and smaller than males and thus may have received more attacks from siblings. Therefore, females potentially had a higher mortality than males by the day when they were sex-identified.

In contrast, males in cross BZ were completely absent. This pattern was also observed in a fraction of families from Stelkens et al. (2010). They also found one family in which female was absent. The finding that only one sex was present resembles the pattern of Haldane's rule in which hybrid sterility or inviability affects heterogametic sex preferentially. Although the sex determination system in BZ hybrids is not known, results from this study suggest that the Haldane's rule is unlikely to cause the absence of males in the case of the *M. benetos* and *M. zebra* pair. All experimentally proved hypotheses of Haldane's rule work within the confines of the Dobzhansky-Muller model and invariably assume that inviability is caused by intrinsic genetic incompatibilities (Orr, 1997). In the case of this study, no evidence of intrinsic inviability was observed. The results of non-reduced hybrid fitness combined with evidence from Stelkens et al. (2010) indicate that

Haldane's rule is probably not the cause of male absence in the hybrid offspring from two recently divergent species, *M. benetos* and *M. zebra*.

Results from the microsatellite analyses showed evidence that a male heterogametic (XY) sex determination system was segregating in *M. benetos*. Of the six microsatellite markers, only three were successfully amplified. Of these three, only alleles of the marker UNH2086 were significantly associated with the gender. All females from cross BB or parental *M. benetos* had genotype 186/186 (XX); and almost all males had genotype 186/170 (XY), except for two individuals in which a genotype 170/170 (YY) was found (Table 3.1). In contrast to *M. benetos*, *M. zebra* appears to have a ZW sex determination system linked to UNH2086. Individuals with genotype 188/202 (ZW) were more likely to be females and individuals with genotype 202/202 (ZZ) were more likely to be males (Table 3.1, $P = 0.0496$). It is worth noting, however, that the Fisher's exact test was very close to the significance threshold 0.05 and increasing or decreasing the sample size by only one would generate a non-significant P value. Additionally, the ZW sex determination system at linkage group 7 contradicts what was found in other similar studies (Ser et al., 2010; Parnell and Strelman, 2013). Both Ser et al. and Parnell and Strelman found that only a XY sex determination system, segregating on linkage group 7 in *M. zebra*. The result from Ser et al. (2010) also showed that while one of the two *M. zebra* families had an XY sex determination system based on UNH2086, the sex determination system of the other *M. zebra* family was unable to be determined based on exactly the same marker.

Microsatellite analyses on the two types of interspecific crosses, BZ and ZB, support the idea that the sex determining system in *M. benetos* has dominance effect to

the sex determination system in *M. zebra*. All individuals from the cross BZ inherited the allele 186 (the X chromosome) from their *M. benetos* mother and either the allele 188 or 202 from their *M. zebra* father. All these individuals were females. This pattern could result from either the dominance effect of *M. benetos*'s sex determination system or the death of all males (i.e. the Haldane's rule). It is possible, though improbable, that all male died during maturation since I was unable to identify the gender of dead embryos and fry. However, this seems unlikely given that the number of BZ surviving to adulthood was equivalent to those in the ZB and conspecific crosses. While further evidence is needed to confidently rule out the contribution of Haldane's rule in this case, the contribution from the dominance effect of sex determination system in *M. benetos* was confirmed by data from the reciprocal cross ZB. All ZB females inherited the X chromosome (the allele 186) and all males the Y chromosome (the allele 170) from their *M. benetos* father. It appears that the gender was exclusively determined by alleles they inherited from their *M. benetos* father.

Given that the Fisher's exact test produced a high sensitive P value for a ZW sex determination system in *M. zebra*, I proposed four scenarios to explain the observed data in which both XY and ZW sex determination system could occur in *M. zebra*. These four scenarios are (Figure 11): scenario 1) *M. zebra* has a ZW sex determination system at the same locus as XY in *M. benetos* and the sex determiner Y from *M. benetos* is dominant to the sex determiner W from *M. zebra*; scenario 2) *M. zebra* has an X'Y' sex determination system at the same locus as XY in *M. benetos* and the sex determiner X from *M. benetos* is dominant to the sex determiner Y' from *M. zebra*; scenario 3) *M. zebra* has a ZW sex determination system at different locus as XY in *M. benetos* and the sex determiner Y

from *M. benetos* is dominant to the sex determiner W from *M. zebra*; scenario 4) *M. zebra* has an X'Y' sex determination system at different locus as XY in *M. benetos* and the sex determiner X from *M. benetos* is dominant to the sex determiner Y' from *M. zebra*. Compared to scenario 1 and 3, scenario 2 and 4 are less likely since both scenarios require that a sex determiner Y (here it Y') be masked by an X. In scenario 1 and 3, the sex determiner Y from *M. benetos* is dominant to the sex determiner W from *M. zebra*. All these four scenarios suggest that sex determination system in *M. benetos* has dominance effect to the sex determination system in *M. zebra*. The dominance effect of one sex-determining locus to others was also found in other cichlid species (Ser et al. 2010; Parnell and Streelman, 2013). In both Ser et al. and Parnell and Streelman, the ZW sex determination system on linkage group 5 was dominant to the XY sex determination system on linkage group 7. To determine which scenario is true in this study, stronger sex-linked markers are needed.

		Parental genotypes	F1 genotypes and gender		Expected sex ratio (P value from Chi-square test on observed data)
Scenario 1 ZW sex determination system in <i>M. zebra</i> on the same locus as XY in <i>M. benetos</i>	Cross ZB	♀ <i>M. zebra</i> Z—W	Z—X ♀		♀ : ♂ = 1:1
			Z—Y ♂		
		♂ <i>M. benetos</i> X—Y	W—X ♀		
			W—Y ♂ If Y > W		
Scenario 2 X'Y' sex determination system in <i>M. zebra</i> on the same locus as XY in <i>M. benetos</i>	Cross ZB	♀ <i>M. zebra</i> X'—X'	X'—X ♀		♀ : ♂ = 1:1
			X'—Y ♂		
		♂ <i>M. benetos</i> X—Y			
	Cross BZ	♀ <i>M. benetos</i> X—X	X—X' ♀		All females
		♂ <i>M. zebra</i> Z—Z			
	Cross BZ	♀ <i>M. benetos</i> X—X	X—X' ♀		All females
		♂ <i>M. zebra</i> X'—Y'	X—Y' ♀ If X > Y'		

Figure 11. Four possible scenarios of sex determination systems in *M. zebra* and sex determiners interaction (continue).

	Parental genotypes	F1 genotypes and gender	Expected sex ratio (P value from Chi-square test on observed data)
Scenario 3 ZW sex determination system in <i>M. zebra</i> On different locus as XY in <i>M. benetos</i>	Cross ZB ♀ <i>M. zebra</i> $\begin{array}{c} Z \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} W \\ \text{---} \\ 0 \end{array}$ ♂ <i>M. benetos</i> $\begin{array}{c} 0 \\ \text{---} \\ X \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y \end{array}$	$\begin{array}{c} Z \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ X \end{array} \quad \text{♀}$ $\begin{array}{c} Z \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y \end{array} \quad \text{♂}$ $\begin{array}{c} W \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ X \end{array} \quad \text{♀}$ $\begin{array}{c} W \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y \end{array} \quad \text{♂ If } Y > W$	♀ : ♂ = 1:1
	Cross BZ ♀ <i>M. benetos</i> $\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} X \\ \text{---} \\ 0 \end{array}$ ♂ <i>M. zebra</i> $\begin{array}{c} 0 \\ \text{---} \\ Z \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ W \end{array}$	$\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Z \end{array} \quad \text{♀}$ $\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ W \end{array} \quad \text{♀}$	All females
Scenario 4 X'Y' sex determination system in <i>M. zebra</i> on different locus as XY in <i>benetos</i>	Cross ZB ♀ <i>M. zebra</i> $\begin{array}{c} X' \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} Y' \\ \text{---} \\ 0 \end{array}$ ♂ <i>M. benetos</i> $\begin{array}{c} 0 \\ \text{---} \\ X \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y \end{array}$	$\begin{array}{c} X' \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ X \end{array} \quad \text{♀}$ $\begin{array}{c} X' \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y \end{array} \quad \text{♂}$	♀ : ♂ = 1:1
	Cross BZ ♀ <i>M. benetos</i> $\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} X \\ \text{---} \\ 0 \end{array}$ ♂ <i>M. zebra</i> $\begin{array}{c} 0 \\ \text{---} \\ X' \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y' \end{array}$	$\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ X' \end{array} \quad \text{♀}$ $\begin{array}{c} X \\ \text{---} \\ 0 \end{array} \begin{array}{c} \text{---} \\ \text{---} \\ \text{---} \end{array} \begin{array}{c} 0 \\ \text{---} \\ Y' \end{array} \quad \text{♂}$	All females

Figure 12. Four possible scenarios of sex determination systems in *M. zebra* and sex determiners interaction (continue). '0' indicates a non-sex-determining allele.

CHAPTER FIVE

Conclusions

Hybrids suffered no reduction in viability based on three fitness related traits: fertilization rate, hatching rate and survival rate. Nonetheless, hybrids in one direction of the cross were all female, consistent with the genetic incompatibility of these species. This may support Haldane's rule, however additional data that elucidates the sex determining system on *M. zebra* is needed to make a conclusion concerning Haldane's rule in this cross. Nonetheless, the polygenic sex determination system in African cichlid species provides ample opportunities for post-zygotic isolation among cichlid species. Whether post-zygotic isolation drives speciation in this system or is merely a secondary outcome of the divergence process is unknown. Further research should focus on exploring the sex determination system of East African cichlids and its potential role in the speciation of recently diverged species, which to this point, have been largely ignored.

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