

ABSTRACT

Post-Zygotic Isolation and Haldane's Rule in Two Closely Related Lake Malawi Cichlid Species

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Cichlid fishes from the Great Lakes in East Africa experienced an extensive, rapid, and recent diversification resulting in over 2,000 species of fish over the course of 10 million years. Such extraordinary diversification is thought to have been driven by behavioral pre-zygotic isolation. However, limited research has been conducted evaluating the role of post-zygotic isolation in promoting the reproductive isolation of African Cichlid fishes. In order to test the hypothesis that post-zygotic isolation contributes to the reproductive isolation of two closely related Lake Malawi African Cichlid fishes, *Maylandia benetos* and *Maylandia zebra*, I artificially created conspecific and reciprocal hybrid crosses and examined the fitness of the first generation. Fertilization rate, hatching rate, survival rate, and sex ratios were analyzed and compared. I found no differences in fertilization rate, hatching rate, or survival rate. However, the interspecific cross with a *M. benetos* female produced no male offspring. The absence of males in the interspecific cross with a *M. benetos* female may provide support for the hypothesis that post-zygotic isolation contributes to the reproductive isolation of *M. benetos* and *M. zebra*.

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POST-ZYGOTIC ISOLATION AND HALDANE'S RULE IN TWO CLOSELY
RELATED LAKE MALAWI CICHLID SPECIES

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CHAPTER ONE

Background

Post-Zygotic Isolation

Understanding the mechanisms of speciation is a central concern of evolutionary biology (Thomas D Kocher, 2004). Speciation can be simply defined as the development of reproductive barriers between populations that allow the populations to maintain distinct phenotypic and genotypic characteristics, even when in close proximity (Seehausen et al., 2014). Key to speciation is the development of reproductive barriers among populations in isolation (Jennings, Mazzi, Ritchie, & Hoikkala, 2011; Seehausen et al., 2014). Reproductive isolation can be generally divided into two categories, pre-zygotic isolation and post-zygotic isolation. Pre-zygotic isolation includes factors that prevent fertilization, such as spatial isolation, behavioral isolation, temporal isolation, and mechanical isolation. Post-zygotic isolation describes factors that reduce hybrid fitness after successful fertilization, such as hybrid inviability, hybrid sterility, and hybrid breakdown.

Post-zygotic isolation can be further divided into extrinsic post-zygotic isolation and intrinsic post-zygotic isolation. Extrinsic post-zygotic isolation is dependent upon hybrids' interaction with their environment, potentially resulting in natural selection or sexual selection against the hybrid phenotype when the

hybrid phenotype is less fit than the conspecific phenotypes. (Naisbit, Jiggins, & Mallet, 2001; Ritchie, 2001; Seehausen et al., 2014). Intrinsic post-zygotic isolation is independent of the environment and is due to genetic incompatibilities between the parental genomes (Seehausen et al., 2014).

A common cause of intrinsic post-zygotic isolation is Dobzhansky-Muller incompatibilities. The Bateson-Dobzhansky-Muller Model (Dobzhansky-Muller Model) fundamentally relies upon negative epistatic interactions between conspecific genes, when they are mixed by sexual reproduction in the hybrid genome to, the evolution of reduced hybrid fitness and/or inviability. When species diverge from a common ancestor due to geographic isolation they accumulate changes in their genomes. These genetic changes from the ancestral state that differ between the conspecific genomes may have negative epistatic interactions with each other when brought together for the first time in the new background of the hybrid genome (Orr, 1997). The model proposes that these negative epistatic interactions produced by a novel combination of alleles result in reduced hybrid fitness, sterility, and/or inviability and, as a result, intrinsic post-zygotic isolation (Dobzhansky, 1937; Muller, 1942; Orr, 1997). Although the Bateson-Dobzhansky-Muller Model postulates a minimum of two interacting loci, it is likely that three or more loci in the hybrid genome contribute to a complex network of negative epistatic interactions that ultimately result in reduced hybrid fitness, sterility and/or inviability (Dobzhansky, 1936; Muller, 1942; Orr, 1997).

Gene duplications, transposition, and gene loss are genetic factors that have been demonstrated to contribute to the development of intrinsic post-zygotic

isolation (Bikard et al., 2009; Masly, Jones, Noor, Locke, & Orr, 2006; Ting et al., 2004). Additionally, intragenomic conflict may play a role in the development of Dobzhansky-Muller incompatibilities and thus contribute to intrinsic post-zygotic isolation (Crespi & Nosil, 2013). Intragenomic conflict is antagonistic interactions between DNA sequences within the same genome, in which elements distort their transmission rate or expression, interfere with the transmission of other alleles, or replicate disproportionately. The outcome of intragenomic conflict is the increase of allele frequency in the absence of positive effects on organism fitness (Crespi & Nosil, 2013). Meiotic drive, a form of intragenomic conflict, has been identified as a potential cause for the development of post-zygotic isolation in some taxa (Crespi & Nosil, 2013; Frank, 1991; Hurst & Pomiankowski, 1991). Meiotic drive involves alterations to meiosis that results the transmission of one allele or chromosome over another. These effects may be dampened by alleles that suppress this process. Post-zygotic isolation may result from meiotic drive when a meiotic drive element is present within a hybrid genome that lacks a suppressor (Crespi & Nosil, 2013; Presgraves, 2010).

Haldane's Rule

Haldane's rule is a characteristic pattern associated with intrinsic post-zygotic isolation. Haldane's rule states, "when in the F₁ offspring of two different animals races on sex is absent, rare, or sterile, that sex is the heterozygous/heterogametic sex" (Haldane, 1922). Since its description in 1922

Haldane's rule has been demonstrated in many different taxa (Coyne et al., 1997; Laurie, 1997; Orr, 1997; Russell, 2003). Additionally, Haldane's rule has been demonstrated both in taxa with an XY sex determination system in which the male is heterogametic and taxa with a ZW sex determination system in which the female is heterogametic (Orr, 1997). Haldane's rule is hypothesized to hold true for all organisms with sex chromosomes, which suggests that evolution by post-zygotic isolation occurs very similarly in very dissimilar lineages across a wide range of taxa (Orr, 1997).

Although Haldane's rule has been widely documented, there is not a single hypothetical mechanism that explains the pattern across all observed cases (Orr, 1997). Several hypotheses to explain Haldane's rule have been proposed. The majority of these hypothesis have been falsified; however, three remain as feasible explanations of the genetic causes of Haldane's rule: the dominance theory, the faster male theory, and the faster X theory (Orr, 1997). Each of these hypotheses operates within the confines of the Bateson-Dobzhansky-Muller Model.

The dominance theory is founded upon the assumption that at least some of the alleles causing Dobzhansky-Muller Incompatibilities, also called complementary alleles, are recessive in the context of the hybrid genome. If this is the case, X-linked recessive complementary alleles will be expressed in male hybrids, but not in female hybrids. This is due to the fact that female hybrids have a haploid set of autosomal and sex chromosomes from each lineage, while males are lacking one X chromosome. Thus, male hybrids are effected by all negative

epistatic interactions on the X chromosome, while female hybrids are only effected if the interactions are dominant in the context of the hybrid genome (Orr, 1997; Wu, 1996).

The faster male theory is based upon the assumption that genes effecting male sterility evolve faster than genes that effect female sterility (Orr, 1997; Wu & Davis, 1993). This could be due to several factors. Sexual selection could promote a more rapid evolution of genes expressed in males relative to genes expressed in females. If genes related to male reproduction evolve more rapidly, it stands to reason that more Dobzhansky-Muller Incompatibilities could form in these genes relative to genes related to female reproduction (Orr, 1997). The increased frequency of Dobzhansky-Muller Incompatibilities between genes associated with male reproduction would explain Haldane's Rule for taxa that have an XY sex determination system. However, the faster male theory cannot explain Haldane's rule for inviability or for taxa that have a ZW sex determination system (Orr, 1997).

The faster X theory is based upon the assumption that X-linked genes evolve more rapidly than autosomal genes (Charlesworth, Coyne, & Barton, 1987; Orr, 1997). As a result, populations in isolation will acquire more changes on X-linked genes than autosomal genes, increasing the likelihood of developing Dobzhansky-Muller Incompatibilities in the hybrid genome (Orr, 1997). Rapid evolution of X-linked genes alone does not explain the pattern observed in Haldane's rule. However, when combined with the dominance theory, the faster X theory enhances the dominance theory by postulating a greater effect of X-linked

negative interactions (Orr, 1997)

African Cichlid Fishes

Cichlid fishes from the Great Lakes in East Africa experienced an extensive, rapid, and recent diversification that produced over 2,000 species of fish over the course of 10 million years making them the most diverse family of vertebrates in existence (Danley et al., 2012; Kocher, 2004). Due to this extensive and rapid diversification, cichlid fishes provide a powerful model for the study of speciation. The haplochromine cichlids of Lake Malawi represent the largest monophyletic group of cichlid fishes, containing over 700 species (Turner et. al., 2001; Danley et al., 2012). Though the precise age of the cichlid species group in Lake Malawi is unknown, data suggests that the Malawian haplochromine cichlids descended from a single common ancestor seeded from Lake Tanganyika approximately one million years ago (Joyce et. al., 2011; Salzburger et. al., 2005).

Though reproductive isolation can result from both pre-zygotic isolation and post-zygotic isolation, it is largely believed that speciation of African cichlid fishes was driven primarily by pre-zygotic isolation (Kocher, 2004). A great deal of research has been done evaluating pre-zygotic isolation of African cichlid fishes; however, comparatively little research has been done evaluating the role of post-zygotic isolation in the speciation of African cichlid fishes. Post-zygotic

isolation has attracted relatively little attention because many species of cichlid fishes are reproductively compatible in the laboratory (Kocher, 2004).

However, it is possible that intrinsic post-zygotic isolation also plays an important factor in the evolution of African cichlid species. African cichlid species of Lake Malawi have experienced periods of geographic isolation due to water level fluctuations throughout their evolutionary history (Danley et al., 2012). Additionally, several species of rock-dwelling cichlids exhibit little gene flow between populations separated by minor kilometer distances (Danley, Markert, Arnegard, & Kocher, 2000). The limited gene flow between relatively close populations coupled with periods of geographic isolation due to fluctuations in water levels contributes to divergence of these species. Periods of geographic isolation due to fluctuations in lake levels allow for allopatric divergence between populations (Danley et al., 2012). Additionally, limited gene flow between populations undergoing strong environmental selection may produce divergence even given the occurrence of migration (Danley et al., 2000). Periods of divergence could allow for the gradual accumulation of genetic incompatibilities that produce reduced fitness in hybrid offspring. Furthermore, the diversity and rapid evolution of sex determination systems in African cichlid fishes also promotes speciation through post-zygotic isolation (Brawand et al., 2014; Ser, Roberts, & Kocher, 2010). Hybridization between species that have different sex determination systems can produce intersex hybrids that have reduced fitness, thus contributing to intrinsic post-zygotic isolation (Ser et al., 2010). Additionally, hybridization between species that have different numbers of sex determination

genes may result in incompatibilities between sex determination genes and hybrids with reduced fitness, also contributing to reproductive isolation (Seehausen, Lande, & Van Alphen, 1999).

Sex determination systems in African Cichlid Fishes

Sex determination systems in African Cichlid fishes are highly variable between species and often involve multiple alleles (Kudo et al., 2015; Parnell & Streelman, 2012; Seehausen et al., 1999; Ser et al., 2010). Sexual conflict has been hypothesized to play a large role in the variability and rapid evolution of sex determination systems in African Cichlid fishes (Parnell & Streelman, 2012; Roberts, Ser, & Kocher, 2009; Seehausen et al., 1999; Ser et al., 2010). Sexual conflict occurs when traits benefit one sex but are detrimental to the other. Many species of cichlid fishes are sexually dimorphic. Selective pressures such as male-male aggression and sexual selection can drive the evolution of novel male color patterns (Dijkstra & Groothuis, 2011; Ser et al., 2010). Bright color patterns enhance male reproductive success, but reduce female fitness by increasing the risk of predation (Roberts et al., 2009; Ser et al., 2010). Similarly cryptic color patterns enhance female fitness but reduce male fitness due to intense sexual selection (Roberts et al., 2009). This sexual conflict could be resolved through linking novel sex determination loci to loci regulating sex-specific color patterns. The evolution of novel sex determination systems in response to sexual conflict is proposed to be an important factor in the evolution of Lake Malawi African

Cichlid fishes (T D Kocher, 2004; Lande, Seehausen, & Alphen, 2001; Roberts et al., 2009; Seehausen et al., 1999; Ser et al., 2010).

Current research suggests that Lake Malawi African Cichlid fishes do not have morphologically distinct sex chromosomes (Kornfeild, 1984; Ser et al., 2010). However, Lake Malawi African Cichlid fishes have been demonstrated to have primarily genetic sexual determination systems, although it has not been negated that environmental factors may play a limited role in sex determination (Ser et al., 2010). Both XY and ZW sex determination systems are present in Lake Malawi African cichlid species (Parnell & Streelman, 2012; Roberts et al., 2009; Ser et al., 2010). Ser (2010) discovered an XY sex determination system on locus group 7 in twenty-one species of *Metriaclima*, a ZW sex determination system on locus group 5 in four species of *Metriaclima*, and found both XY and ZW systems segregating within *M. pyrsonotus*. Parnell (2013) confirmed presence of a ZW sex determination system on locus group 5 and a XY sex determination system on locus group 7 as well as two additional loci interacting epistatically to influence sex determination on chromosomes 3 and 20.

Speciation Through Genetic Conflict

Sex ratio is a selectable phenotype. Depending upon the environment, a sex ratio that deviates from the expected 1:1 male to female ratio can be advantageous to fitness or detrimental to fitness, and as a result can cause selective pressures (Kocher, 2004; Lande, Seehausen, & Alphen, 2001; Seehausen

et al., 1999; Wilkinson et al., 1998). Kocher (2004) and Lande (2001) each proposed models by which African Cichlid fishes may have speciated as a result of repeated cycles of genetic conflict between sex determiners over optimal sex ratios. In this model it is postulated that African Cichlid species during their evolutionary history went through fluctuating periods of small and large population sizes as they were exposed to new environments during lake level fluctuations. Different population sizes produce different selection pressures. In very small populations males experience intense competition for mates. As a result, proportionally fewer males than females successfully mate. Due to the limited mating success of males in a small population, it is advantageous to produce more female offspring than male offspring in this context. Thus, a dominant feminizing allele would be selected for in a small population. The emergence of a dominant feminizing allele would produce a female biased sex ratio, which would allow individuals carrying this allele to produce more offspring that would successfully mate. However, as the population grows and more females are available for mating, males suffer less intense competition and have greater mating success. As a result, it is no longer advantageous to maintain a female biased sex ratio and it is more advantageous to revert to an equally distributed sex ratio. Thus, a masculinizing allele would be selected for as the population grows. The emergence of a masculinizing allele would return the sex ratio to a 1:1 female to male sex ratio.

In the models proposed by Kocher (2004) and Lande (2001) a small population of cichlid fishes separated into a novel environment from the original

population due to lake level fluctuations could undergo rapid evolution in their sex determination system as a result of the fluctuating selective pressures on sex ratio as the population size changes. This model predicts the emergence of closely related species of fish with drastically different sex determination systems (Kocher, 2004; Lande, Seehausen, & Alphen, 2001; Seehausen et al., 1999).

Post-zygotic Isolation in African Cichlid Fishes

The complexity of the sex determination systems African Cichlid fishes has made analysis of Haldane's Rule, a hallmark of intrinsic post-zygotic isolation, difficult, due to the fact that it is challenging to determine definitively which sex is the heterogametic sex in several of these species. However, several studies have been conducted evaluating post-zygotic isolation in African Cichlid species.

Crapon de Caprona and Fritsch (1984) observed evidence of intrinsic post-zygotic isolation in bidirectional crosses of *Haplochromis burtoni* and *Haplochromis nubilus* as well as bidirectional crosses of *Haplochromis elegans* and *Haplochromis* "black lividus". Interestingly, the female *H. nubilus* x male *H. burtoni* cross produced lethal hybrids, while the reciprocal cross produced viable hybrids with 1:1 sex ratios in the F₁, F₂, and F₃, but female skewed sex ratios in back crosses. *H. elegans* and *H.* "black lividus" produced viable hybrids with female skewed sex ratios in each direction of the cross. Despite the observation of skewed sex ratios favoring the female sex in these crosses, it is unclear which sex

in these species is the heterogametic sex, so Haldane's rule could not be analyzed in these experiments.

Van Der Sluijs (2008) conducted a similar study on two recently diverged sister species of Lake Victorian cichlids, *Pundamilia pundamilia* and *Pundamilia nyererei*. Comparisons between the fecundity, fertility, egg size, survival, growth rate, and sex ratio in conspecific and reciprocal hybrid crosses of *P. pundamilia* and *P. nyererei* revealed no evidence of intrinsic post-zygotic isolation.

Stelkens (2010) utilized fitness data from different life stages in 15 different hybrid crosses in order to attempt to estimate the rate at which intrinsic post-zygotic isolation mechanisms develop in African Cichlid fishes. Using three different molecular clocks they estimated the relationship between genetic difference and the accumulation of genetic incompatibilities. The data indicate that intrinsic post-zygotic isolation is negligible in closely related species, but accumulates rapidly as time since isolation increases. Based on their data they estimate that complete hybrid inviability develops after 4.4, 8.5, or 18.4 million years, depending upon the molecular clock used.

The majority of studies evaluating post-zygotic isolation in African Cichlid fishes analyze only F₁; however, Stelkens (2015) conducted a study evaluating hybrid break down between F₁ and F₂ *Metriaclima estherae* and *Astatotilapia calliptera* hybrids. Comparisons of fertilization, hatching, and survival at day 180 revealed that F₂ considerably reduced fitness compared to both F₁ and conspecifics. This data is consistent with the theoretical prediction that incompatible alleles at different loci inherited from the parental species are

more harmful in the homozygous condition (which arises in F₂ and increasing hybrid generations) than they are in the heterozygous condition present in the F₁ (Muller 1942).

Additional studies evaluating sexual selection, a pre-zygotic isolating mechanism, in African Cichlid fishes have also provided evidence for extrinsic post-zygotic isolation in these populations by demonstrated reduced mating success of male hybrids with an intermediate phenotype (Seehausen et al., 1999; Stelkens et al., 2008).

In the context of previous research conducted evaluating post-zygotic isolation in African Cichlid species *Maylandia benetos* and *Maylandia zebra* present another promising system in which to study post-zygotic evolution. *M. benetos* and *M. zebra* are to closely related Lake Malawi rock-dwelling cichlids. While *M. benetos* is endemic to Mazinzi Reef, *M. zebra* is found distributed across Lake Malawi (Stauffer, Bowers, Kellogg, & Mckaye, 1997). In Mazinzi Reef, *M. benetos* and *M. zebra* share similar, overlapping habitats and a range of mating and aggressive behaviors (P. D. Danley, Husemann, & Chetta, 2012; Ding, Daugherty, Husemann, & Chen, 2014). *M. benetos* and *M. zebra* exhibit assortative mating, presumably based upon male color patterns, that prevents natural hybridization in the lab (Ding et al., 2014). However, viable hybrids can be produced artificially in the lab setting. I tested the hypothesis that post-zygotic isolation contributed to the reproductive isolation of *Maylandia benetos* and *Maylandia zebra* by artificially creating conspecific and reciprocal hybrid crosses and examining the fitness of the resulting offspring. Four fitness measures,

fertilization rate, hatching rate, survival rate, and sex ratios, were analyzed and compared between the four crosses. If genetic incompatibilities exist between *M. benetos* and *M. zebra* it is expected that there will be a significant difference between one or more of the four fitness measures in the hybrid offspring relative to the conspecific controls.

CHAPTER TWO

Methods and Materials

Fish

Maylandia benetos and *M. zebra* for the purpose of this study were chosen from laboratory stocks. *M. benetos* and *M. zebra* were collected from Mazinzi Reef in Lake Malawi in 2001 and have been outbred in the laboratory for approximately 12 generations. Fish were maintained in 28°C water with a pH of 8. Fish were kept on twelve-hour light and twelve hour dark cycles.

Creation of Conspecific and Interspecific Crosses

Due to the fact that *M. zebra* and *M. benetos* do not frequently mate naturally in the wild or in captivity, the crosses were artificially made in the lab setting. Females were carefully observed for the presence of an enlarged genital papilla, an indication of their readiness to lay eggs. Unfertilized eggs were extracted from gravid females by applying light pressure along the ventral side above the genital papilla. Reciprocal conspecific and interspecific crosses were created by collecting the unfertilized eggs from female *M. benetos* or *M. zebra* and mixing half of the eggs with sperm from a conspecific male and the other half

with sperm from an interspecific male in a petri dish with water (50 mm diameter x 4mm deep). Sperm from male fish was collected by first drying the male's abdomen with tissue paper and then applying light pressure to the abdomen to deposit semen into the petri dish. The eggs were allowed to fertilize in the petri dish for 5 minutes and eggs and sperm were mixed during this time by repeated pipetting with a bulb pipette. 15 *M. benetos* females, 12 *M. zebra* females, 14 *M. benetos* males, and 14 *M. zebra* males were used to generate 42 female *M. benetos* x male *M. benetos* crosses, 42 female *M. benetos* x male *M. zebra* crosses, 18 female *M. zebra* x male *M. zebra* crosses, and 18 female *M. zebra* x male *M. benetos* crosses (Figure 1).

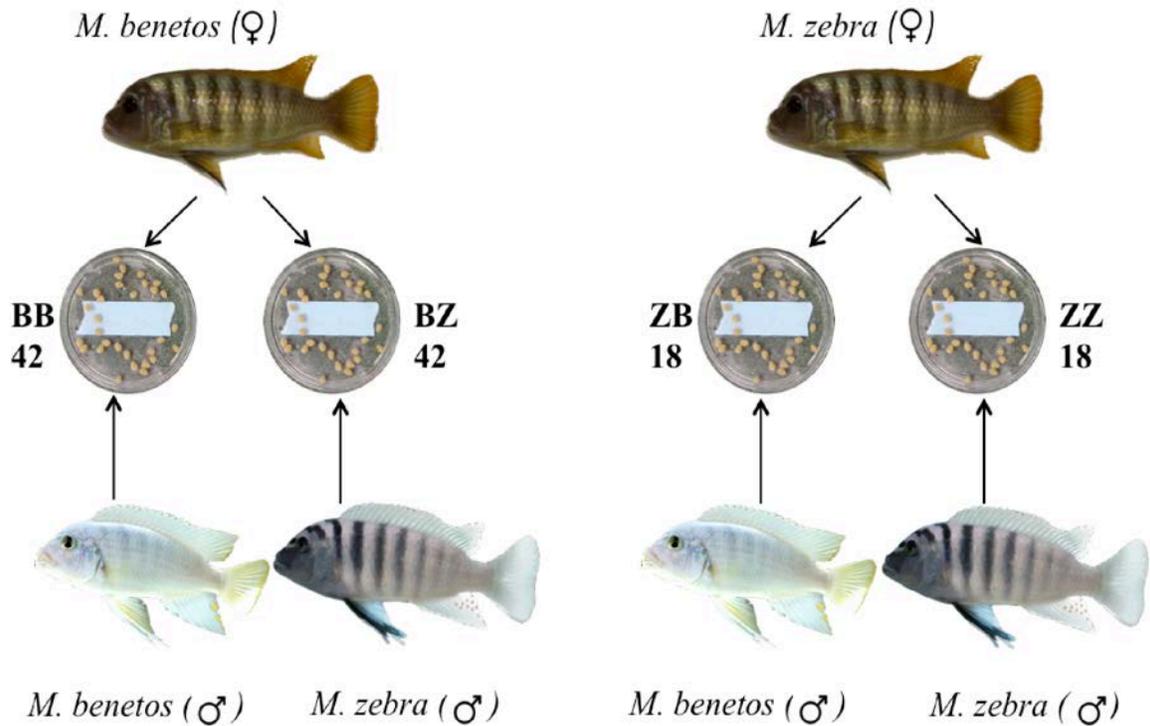


Figure 1: A diagram of the cross design. BB represents a conspecific *M. benetos* cross. BZ represents a female *M. benetos* crossed with a male *M. zebra*. ZB represents a female *M. zebra* crossed with a male *M. benetos*. ZZ represents a conspecific *M. zebra* cross.

Incubation and Maintenance

M. zebra and *M. benetos* are maternal mouth brooders and hold their offspring in their mouths for several weeks after laying. In order to mimic the conditions experienced by developing embryos and fry in the mother's mouth, we designed a novel incubation system (Figure 2).

Offspring were kept in the incubation system for twelve days following fertilization. At day twelve fry were transferred into a 10 x 9 x 5 cm³ container within the same central tank system. Offspring were maintained in small

enclosures until they reached 45 days old, at which time they were transferred to an open tank (231 x 56 x 28 cm³) with members of the same cross, where they were maintained until they reached sexual maturity. Fish were fed dry food at a constant per-fish rate daily. Dead eggs and dead fish were removed from the incubator/tank daily and stored in 100% ethanol at -20°C for DNA extraction.

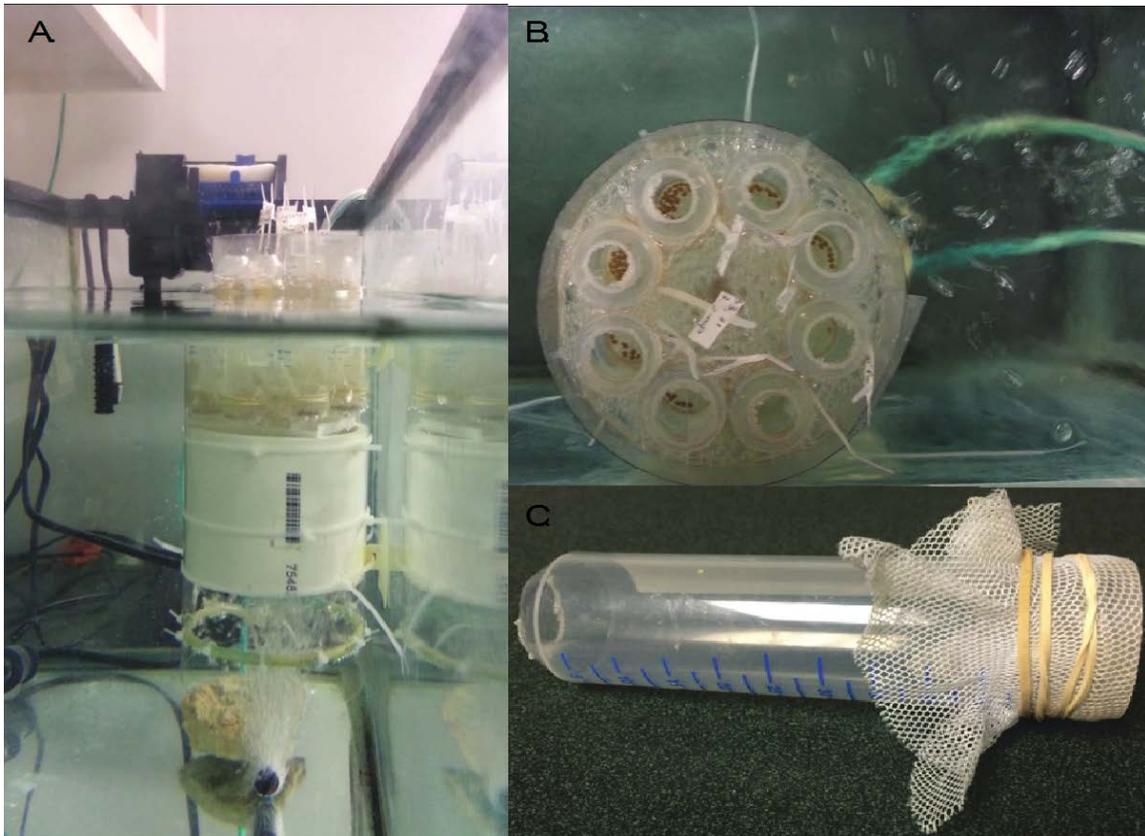


Figure 2: (A) The central compartment of the incubator is composed of PVC tubing and Plexiglas and is divided into two sections by a plastic egg crate in the center. In the bottom compartment an air stone connected to an air pump pumps air bubbles into the central chamber. In the top compartment (B) up to eight modified conical vials capped with a nonabrasive netting (C) rest on top of the plastic egg crate. Bubbles flow through the central compartment of the incubator and shuffle the eggs resting in the 50mL conical vials, maintaining constant, gentle movement.

Analysis Individual Fitness Characteristics

Individual fitness characteristics for the purpose of this investigation were defined as fertilization rate, hatching rate, and survival rate. The fertilization rate was visually assessed at day 3 after fertilization and defined as the number of eggs fertilized divided by the number eggs collected for fertilization. Fertilized eggs and unfertilized eggs can be visual distinguished by color and texture (Figure 3).



Figure 3: Fertilized eggs are physically distinguishable from unfertilized eggs. Fertilized eggs begin to develop eyespots at approximately day 3-4. Fertilized eggs are a dark golden brown, while unfertilized eggs are white or yellow. Unfertilized eggs begin to degrade around day 3.

Hatching rate was visually assessed at day 12 after fertilization, and was defined as the number of hatched individuals divided by the number of eggs fertilized. Fully hatched individuals no longer bore a yolk sac and were free

swimming. Dead embryos were easily distinguishable from live embryos and were removed daily (Figure 4).

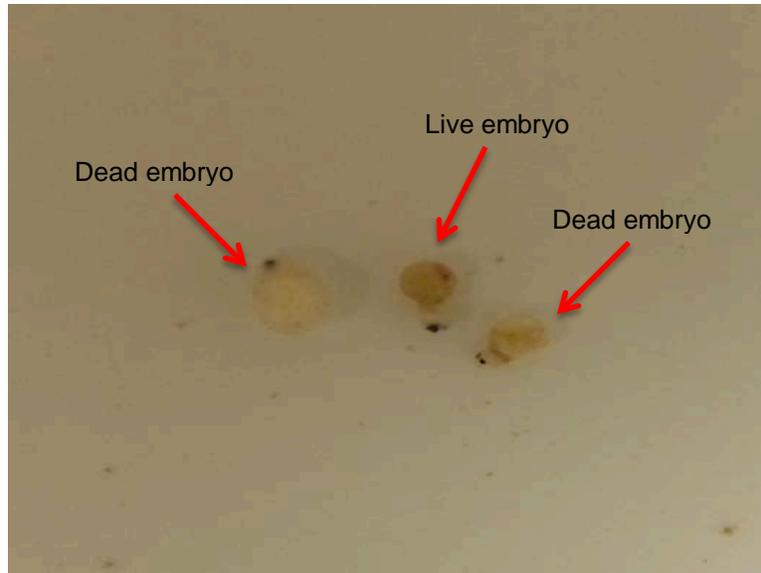


Figure 4: Fertilized embryos are physically distinguishable from unfertilized embryos. Fertilized embryos have a visible heartbeat. Unfertilized embryos turn white and begin to degrade.

Survival rate was recorded for 45 days and was defined as the number of surviving individuals divided by the number of hatched individuals.

In order to evaluate intrinsic post-zygotic isolation between the two species the fitness characteristics of the conspecific and interspecific crosses were compared using unpaired t-tests.

Sex Ratios

Sex ratio was utilized as a measure of population level fitness. Sex ratio was defined as the proportion of males. The sex of the fish was visually assessed by dissection and examination of the gonads (Figure 5). At approximately 8 months old the sexually mature fish were placed in the freezer, sacrificed, and the gonads were analyzed. Although histological methods exist for sexing juvenile fish (Guerrero & Shelton, 1974), individuals can be easily sexed by six months of age via dissection (Ser et. al., 2010).

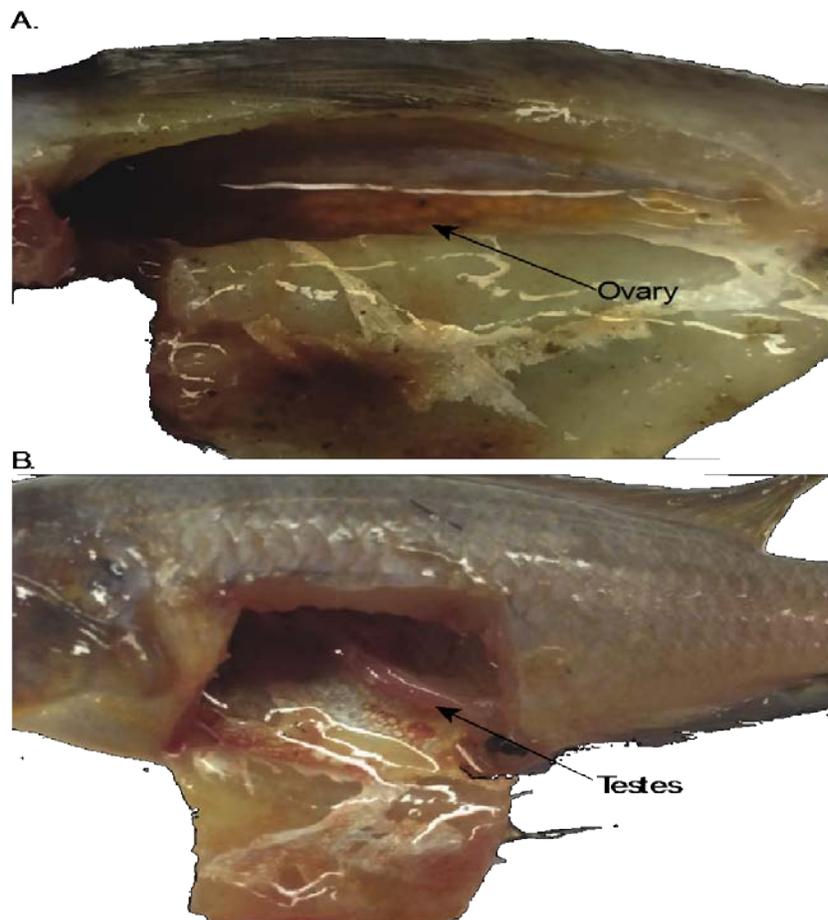


Figure 5: (A) Dissection of a female fish with labeled ovary (B) Dissection of a male fish with labeled testes.

CHAPTER THREE

Results

Fertilization Rate

Fertilization rate was defined as the number of eggs collected divided by the number of eggs fertilized by day three. The data indicate that there is no significant difference between fertilization rates among any of the crosses.

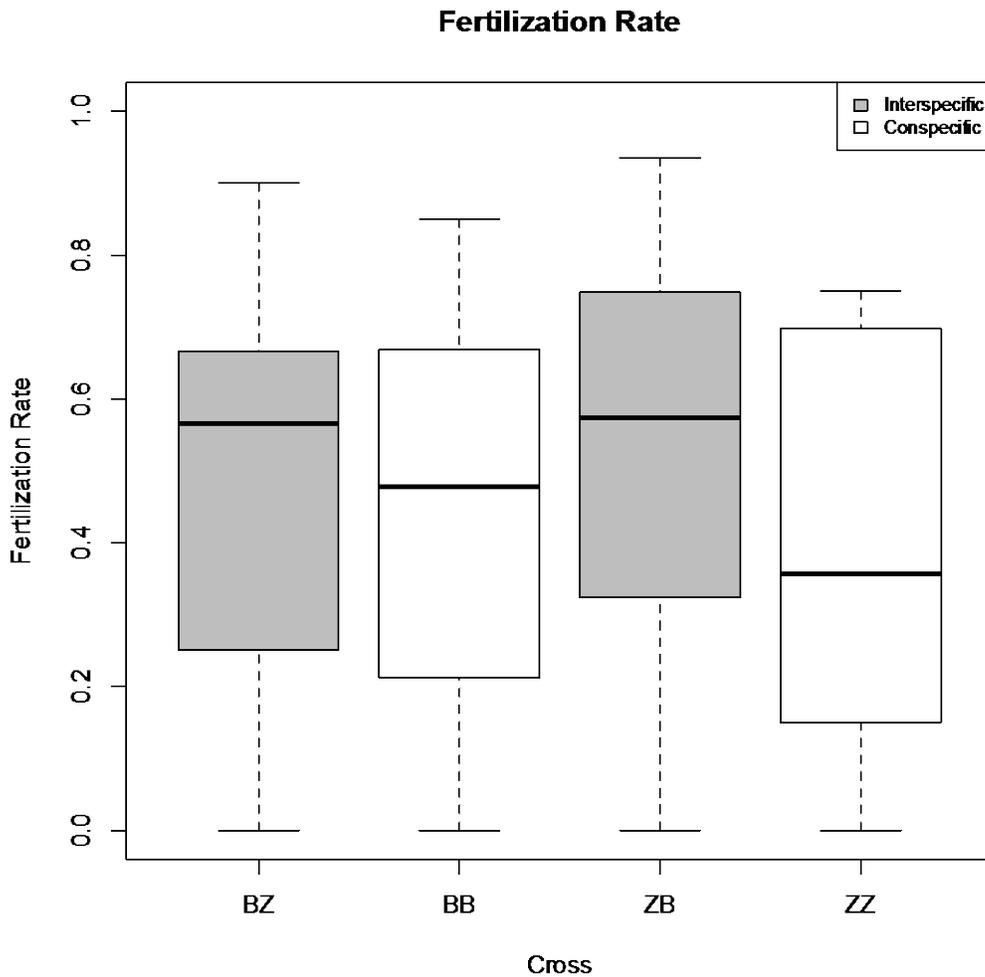


Figure 6: Fertilization Rate
22

Hatching Rate

Hatching rate was defined as the number of individuals that hatched divided by the total number of eggs fertilized. The data demonstrate no significant difference in hatching rate between any of the crosses (Table 1)

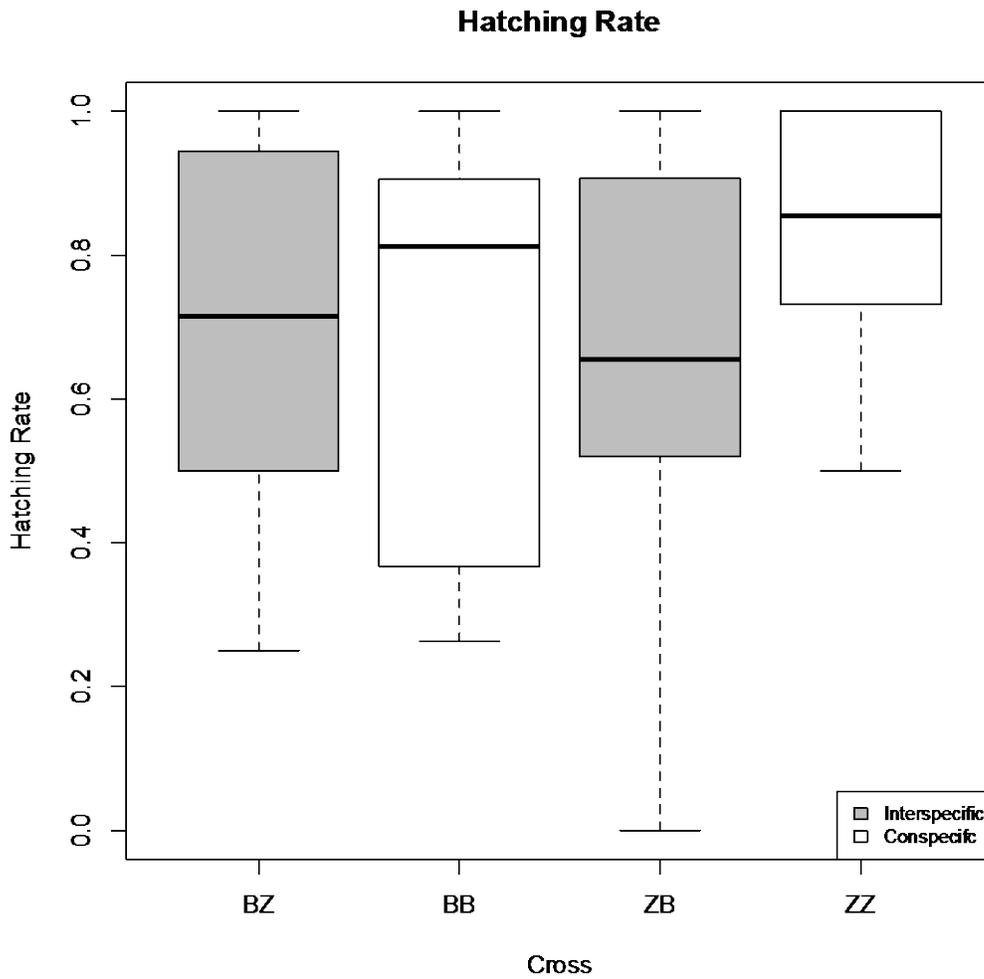


Figure 7: Hatching Rate

Survival Rate

The survival rate was measured at day forty-five. The data indicate that there was no significant difference in survival between any of the crosses (Table 1).

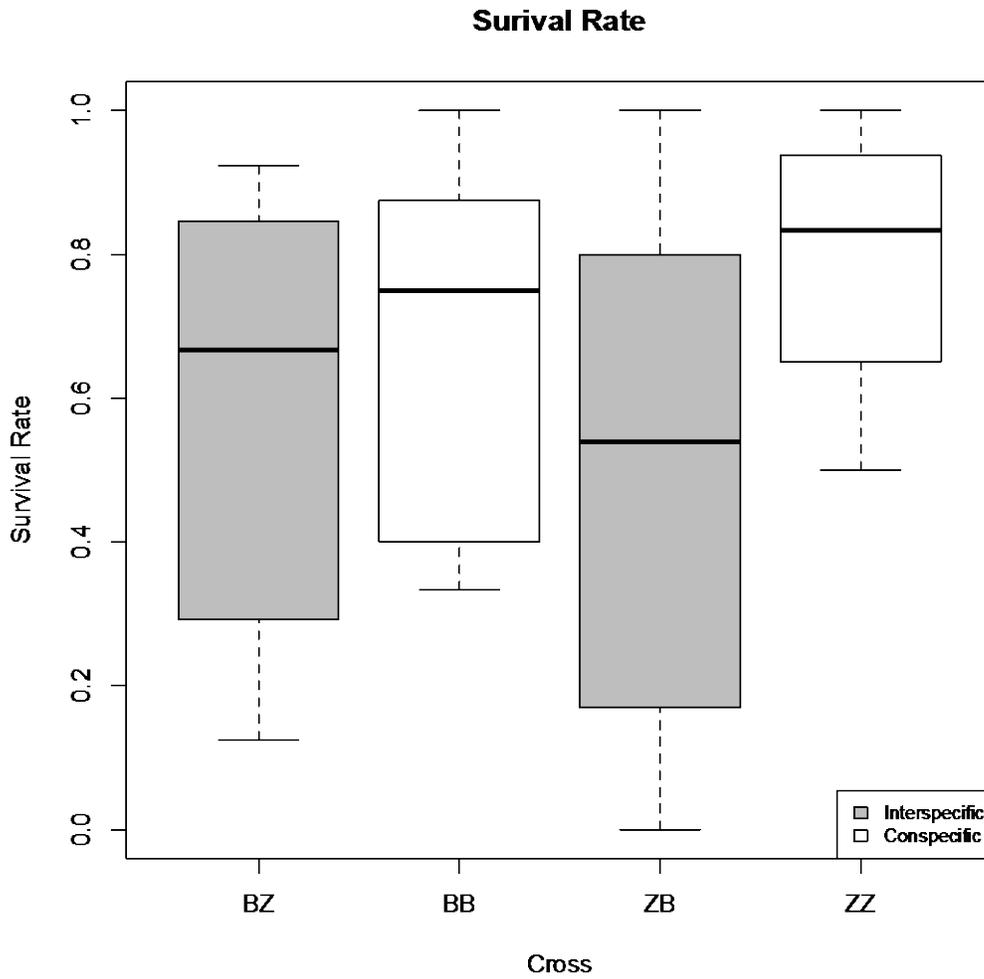


Figure 8: Fertilization Rate

Table 1:

Fitness Characteristics: Two Sample T-Test P-values			
	Fertilization Rate	Hatching Rate	Survival Rate
BB ~ BZ	0.4508	0.486	0.4063
BB ~ ZB	0.2679	0.9552	0.1836
BB ~ ZZ	0.1435	0.1153	0.1228
ZZ ~ BZ	0.335	0.257	0.4672
ZZ ~ ZB	0.7727	0.2769	0.07858

Sex Ratio

The sex ratios for the female *M. benetos* x male *M. benetos*, female *M. zebra* x male *M. zebra*, and female *M. zebra* x male *M. benetos* were all slightly skewed in favor of males, however; they did not deviate significantly from the expected 1:1 ratio of males to females (Table 2) Only the female *M. benetos* x male *M. zebra* cross deviated significantly from the expected 1:1 ratio. No males were produced in the female *M. benetos* x male *M. zebra* cross, resulting in a sex ratio of zero (Table 2).

The sex ratio in the female *M. benetos* x male *M. zebra* cross differed significantly from the sex ratio of the conspecific *M. benetos* cross (Fisher's exact test p-value = $8.753e^{-11}$). However, the female *M. zebra* x male *M. benetos* cross did not differ significantly from the conspecific *M. zebra* cross (Fisher's exact test

p-value = 1), nor did the conspecific crosses differ from one another (Fisher's exact test p-value = 1).

Table 2:

Sex Ratios			
Person's Chi-Squared Test			
Cross	Sex Ratio	χ^2	p-value
♀ <i>M. benetos</i> x ♂ <i>M. zebra</i>	0	56	7.247e ⁻¹⁴
♀ <i>M. benetos</i> x ♂ <i>M. benetos</i>	0.556	0.444	0.505
♀ <i>M. zebra</i> x ♂ <i>M. benetos</i>	0.593	0.926	0.336
♀ <i>M. zebra</i> x ♂ <i>M. zebra</i>	0.579	0.474	0.491

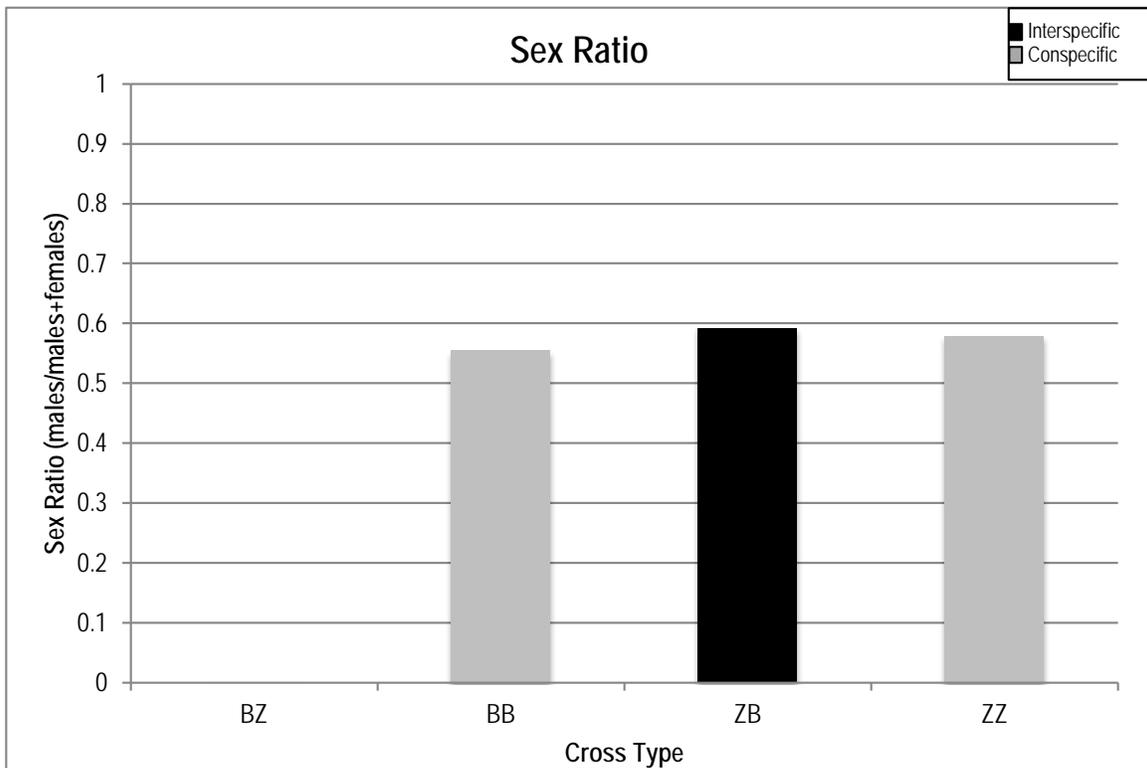


Figure 9: Sex Ratios

CHAPTER FOUR

Discussion

Few studies to date have tested intrinsic post-zygotic isolation in closely related species of African Cichlid fishes. In this study four fitness characteristics (fertilization rate, hatching rate, and survival rate, and sex ratios) were analyzed in conspecific and reciprocal hybrid crosses of *M. benetos* and *M. zebra*, in order to evaluate post-zygotic isolation between these closely related species of Lake Malawi African Cichlid fishes. I found no difference in fertilization rate, hatching rate, or survival rate between any of the four crosses tested. The absence of fitness disadvantages in the interspecific crosses compared to the conspecific crosses does not support the hypothesis that intrinsic post-zygotic isolation contributes to the reproductive isolation of *M. benetos* and *M. zebra*. However, the female *M. benetos* x male *M. zebra* cross produced no male offspring, resulting in a highly skewed sex ratio that differed significantly from the expected 1:1 sex ratio of males to females. The complete absence of males in the female *M. benetos* x male *M. zebra* cross may provide support for the hypothesis that post-zygotic isolation contributes to the reproductive isolation of *M. benetos* and *M. zebra*.

Due to strong assortative mating, *M. benetos* and *M. zebra* had to be artificially hybridized. The procedure used for hybridizing *M. benetos* and *M. zebra*, in which eggs were extracted from the female and half were fertilized with

sperm from *M. zebra* and half were fertilized with sperm from *M. benetos*, is unique in that it results in interspecific and conspecific half-siblings. This crossing procedure allows for the control of maternal effects, which have been demonstrated in studies of Lake Victorian cichlid fishes (Van Der Sluijs et. al., 2008). After fertilization, eggs were incubated in an incubation system designed for the purposes of this project (Figure 2), which controlled for incubation environment due to the fact that the paired conspecific and hybrid crosses could be incubated in the exact same environment. Contrary to previous studies, survival rate in this study was recorded at day 45, an early developmental stage, instead of day 180, at sexual maturity (Stelkens, Young, & Seehausen, 2010; Van Der Sluijs et al., 2008). Due to limited lab space and the large number of fish generated, it was not possible to house all of the fish individually. *M. benetos* and *M. zebra* display aggressive behavior in the natural environment and the laboratory setting (Danley, 2011). As the fish mature they become more aggressive, resulting in an increased frequency of death due to aggressive behavior. Therefore, in order to avoid biasing the survival rate results, survival rate was only measured until day 45.

The lack of significant differences between the fitness characteristics of hybrid and conspecific crosses noted in this study is consistent with previous studies on intrinsic post-zygotic isolation in African Cichlid fishes (Stelkens et. al., 2010; Van Der Sluijs et. al., 2008). Van der Sluijs (2008) studied fitness characteristics, including fecundity, fertility, egg size, survival at day 180, and growth rate in conspecific and reciprocal hybrid crosses of two closely related

Lake Victorian Cichlid fishes, *Pundamilia pundamilia* and *Pundamilia nyererei*.

Van der Sluijs (2008) found no significant difference in any of the fitness characteristics except survival rate between the crosses. The difference in survival rate was attributed to maternal effect and deemed unrelated to hybridization (Van Der Sluijs et al., 2008). Maternal effects were not observed in any of the hybrid or conspecific crosses of *M. benetos* and *M. zebra*.

Stelkens (2010) measured fertilization, hatching, survival rate at 14 days, and survival at day 180 in twenty six interspecific hybrid crosses between sixteen different species of haplochromine cichlids. Stelkens (2010) plotted hybrid inviabilities as a function of divergence time and concluded that intrinsic post-zygotic isolation between closely related species is negligible, but accumulates rapidly as divergence time increases. Stelkens (2010) projected that it requires a minimum of 4.4 million years divergence time for hybrid inviability to evolve. According to this estimate, *M. benetos* and *M. zebra* are too recently diverged to have evolved significant intrinsic hybrid inviabilities. Several different estimates date the emergence of the Lake Malawi cichlid population between 0.72 to 4.6 million years ago, with the best estimate of the emergence being approximately 1 million years ago (Danley et al., 2012; Genner et al., 2007; Koblmüller, Sefc, & Sturmbauer, 2008; Sturmbauer et. al., 2001). The rock-dwelling cichlid clade of Lake Malawi, which includes *M. benetos* and *M. zebra*, is estimated to have emerged 0.313 to 0.486 million years ago (Danley et al., 2012; Genner et al., 2007) *M. benetos* and *M. zebra* are estimated to have diverged from one another only 100,000 years ago (Huseman et. al., 2015). Based upon these estimated

emergence times, *M. benetos* and *M. zebra* diverged much more recently than 4.4 million years ago, Stelkens' earliest estimate of the amount of time after divergence necessary to develop complete separation by intrinsic post-zygotic isolation.

Although significant differences in fitness characteristics were not measured in the F₁ tested in this study, it is possible that hybrid inviabilities may arise in subsequent hybrid generations. Theory predicts that incompatible alleles at different loci inherited from the parental species are more harmful in the homozygous condition (which arises in F₂ and increasing hybrid generations) than in the heterozygous condition present in the F₁ (Muller 1942). Stelkens (2015) found that F₂ hybrids were 21% less fit than F₁ hybrids and 43% less fit than conspecific crosses. The current study on post-zygotic isolation between *M. benetos* and *M. zebra* is limited by the fact that only the F₁ generation was generated and analyzed for fitness characteristics. Future research analyzing F₂ and further generations of *M. benetos* and *M. zebra* hybrids could potentially uncover incompatibilities that were masked in the heterozygous state of the F₁.

Although analysis of fertilization rate, hatching, rate and survival rate does not support the hypothesis that post-zygotic isolation contributes to the reproductive isolation of *M. benetos* and *M. zebra*, the highly skewed sex ratio of the female *M. benetos* x male *M. zebra* cross may provide support for the hypothesis. The sex ratios of the conspecific *M. benetos*, conspecific *M. zebra*, and the female *M. zebra* x male *M. benetos* crosses were slightly skewed in favor of males; however, the sex ratios did not differ significantly from the expected 1:1

sex ratio for males to females. The presence of sex ratios skewed in the favor of males contradicts other studies of hybridization in African cichlid fishes, which predominately demonstrate female biased sex ratios (Crapon De Caprona & Frittsch, 1983; Seehausen, Lande, & Van Alphen, 1999; Stelkens et al., 2010; Van Der Sluijs et al., 2008). A possible explanation for the contradiction is that more females died prior to sex determination as a result of aggression. Females are smaller and less aggressive than males in both *M. benetos* and *M. zebra* populations. As a result, it is possible that females experienced more aggressive attacks from their siblings than males.

No males were produced in the female *M. benetos* x male *M. zebra* cross. The absence of males in a single cross is consistent with other studies in African cichlid fishes and other taxa (Orr, 1997; Seehausen et al., 1999; Stelkens et al., 2010). One potential explanation for the absence of males in one direction of the experimental cross is Haldane's rule. Haldane's Rule states that when one sex is absent or sterile in a hybrid cross it is the heterogametic sex (Haldane, 1922). It is not known definitively which sex is the heterogametic sex in these species or their hybrids. Ser (2010) revealed a putative XY sex determination system on locus group 7 of *M. benetos* and some families of *M. zebra*. However, Chen (2015 unpublished) in a similar study suggested the presence of a XY sex determination system on locus group 7 of *M. benetos*, but a ZW sex determination system on locus group 5 of *M. zebra*. Due to the ambiguity of the sex determination systems in these species, the presence of Haldane's Rule cannot be definitively supported or refuted as a result of this experiment.

There are other possible explanations for the absence of males in one direction of the experimental cross. It is possible that *M. benetos* has a novel dominant feminizing allele that causes the production of all females when crossed with *M. zebra* due to the absence of a masculinizing allele to rescue the male phenotype in the *M. zebra* genome. This phenomenon has been demonstrated in other studies on African Cichlid fishes (Seehausen et al., 1999). The absence of males in only one direction of the cross could also result from dominance of the sex determination system of *M. benetos* over that of *M. zebra*. Unpublished data by Ming Chen (2015) suggests that *M. benetos* does have an XY sex determination system on locus group 7 that has a dominant effect on a putative ZW sex determination system on locus group 5 in *M. zebra* (Chen, 2015 unpublished). The dominant effect of one sex determination system over another has been demonstrated in other studies on African Cichlid fishes (Parnell & Streelman, 2012; Ser et. al., 2010). However, previous studies have demonstrated that the ZW sex determination system on locus group five is semi-dominant over the XY sex determination system on locus group seven, contrary to the system suggested to be operating in *M. benetos* and *M. zebra* (Chen 2015 unpublished; Parnell & Streelman, 2012; Ser et. al., 2010).

The highly skewed sex ratio in the female *M. benetos* x male *M. zebra* cross may support the Speciation by Genetic Conflict Model proposed by Kocher (2004). *M. benetos* is endemic to Mazinzi Reef, while *M. zebra* is widely distributed across Lake Malawi (Stauffer, Bowers, Kellogg, & Mckaye, 1997). Due to its highly localized distribution, it is evident that the *M. benetos* population

has been maintained at a smaller size than the *M. zebra* population. It is highly likely that *M. benetos* experienced a population contraction to the degree that the *M. zebra* population has not experienced. Different population sizes result in different selection pressures. In very small populations selection favors a female biased sex ratio; however, as the population grows selection favors a 1:1 sex ratio (Kocher, 2004; Lande, Seehausen, & Alphen, 2001). A contraction in population size in the evolutionary history of *M. benetos* could have driven the fixation of a dominant feminizing allele according to the Speciation by Genetic Conflict Model (Kocher, 2004). Additionally in accordance with this model, as the population grew a masculinizing allele in the *M. benetos* population would be selected for in order to establish an advantageous 1:1 sex ratio. Thus, through this model it is possible that *M. benetos* underwent rapid evolution of novel sex determinant alleles. If the comparatively much larger *M. zebra* population did not experience a similar population contraction and similar selective pressures on sex ratio it would lack the feminizing allele and masculinizing allele present in *M. benetos*. As a result of differences in the sex determination systems of *M. benetos* and *M. zebra*, hybridization between *M. benetos* and *M. zebra* could produce the skewed sex ratios demonstrated in this study. The demonstrated incompatibilities between the sex determination systems of *M. benetos* and *M. zebra* could contribute to their reproductive isolation. A highly skewed sex ratio as demonstrated in the female *M. benetos* x male *M. zebra* cross may cause decreased fitness in a hybrid population and pose a considerable disadvantage to hybridization in each of the conspecific species. Selection pressures against mating that results in skewed sex

ratios in the progeny may encourage the evolution of sexual selection or enforced an already existent system of sexual selection, resulting in limited gene flow between populations with different sex determination systems (Seehausen et al., 1999). Selection pressures on sex ratio may have contributed to the evolution or reinforcement of pre-zygotic isolation between *M. benetos* and *M. zebra*.

In conclusion, no significant differences were found in fertilization rate, hatching rate, or survival rate between the hybrid crosses and the conspecific controls. However, the experimental cross between female *M. benetos* and male *M. zebra* produced no male offspring. This data suggests genetic incompatibilities between the sex determination systems of *M. benetos* and *M. zebra*. Despite the absence of males in one experimental cross, this data does not definitively support or refute Haldane's Rule, because the heterogametic sex is unknown. However, it is possible that genetic incompatibilities between the sex determination systems in *M. benetos* and *M. zebra* contributed to their divergence. The demonstration of genetic incompatibilities in the sex determination systems between *M. benetos* and *M. zebra* and the comparatively limited population size and distribution of *M. benetos* in Mazinzi Reef may provide experimental support for the Speciation by Genetic Conflict Model (Kocher, 2004).

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