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Tests of Reproductive Isolation Between the Fishes Fundulus heteroclitus and F. grandis

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Tests of Reproductive Isolation Between the Fishes *Fundulus heteroclitus* and *F. grandis*

by

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"So long, and thanks for all the fish."

- Douglas Adams

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ABSTRACT

The closely related killifishes *Fundulus heteroclitus* and *F. grandis* hybridize in a small region where their ranges overlap in coastal northeastern Florida. Hybrids of these species are rare in frequency within the contact zone, suggesting the presence of relatively strong reproductive isolation between these species. The objective of this study was to elucidate barriers to reproduction between *F. heteroclitus* and *F. grandis* in the lab, as well as to quantify the relative strengths and contributions of various isolating barriers. Pre-zygotic (mating and fertilization) and post-zygotic (hatching) barriers were investigated by performing a variety of choice and no-choice laboratory mating experiments. The results revealed that under no-choice conditions, barriers to mating had the biggest influence on hybrid production in *F. grandis*, whereas hatching barriers contributed to the majority of reproductive isolation in *F. heteroclitus*. However, under choice conditions pre-zygotic barriers had the greatest influence on both species' ability to produce hybrids. The total relative reproductive isolation that was observed in females of each species was stronger in *F. heteroclitus* than in *F. grandis* overall, and was nearly complete in *F. heteroclitus* females under choice conditions while moderate in *F. grandis* females. These results reveal an asymmetry in the potential gene flow between these two species, with *F. grandis* being more likely to hybridize than *F. heteroclitus* in the absence of environmental influences.

INTRODUCTION

Speciation is the evolutionary process by which new genetically distinct species are formed. Species can be defined (under the biological species concept) as genetically distinct populations that have the ability to interbreed, but remain isolated genetically by barriers that prevent successful reproduction between them (Mayr 1940; Mayr 2000). These barriers can occur at different times during sexual reproduction and are generally recognized as acting pre-zygotically by preventing heterospecific mating and/or fertilization events, or acting post-zygotically by decreasing hybrid fitness either through a decrease in survival or fertility (Dobzhansky 1937; Mayr 1942; Coyne and Orr 1998, 2004). It is important to note that multiple isolating mechanisms fall under the category of prezygotic isolation, such as spatial, temporal, behavioral, and gametic isolation, and previous research has indicated that multiple isolating mechanisms can contribute to the total reproductive isolation that exists between species (Coyne and Orr 1998, 2004; Schluter 2001; Ramsey et al. 2003; Martin and Willis 2007; Matsubyashi and Katakura 2009). Additionally, studies of the relative strengths of reproductive barriers have suggested that total reproductive isolation between species does not tend to occur by one strong barrier acting to block successful reproduction, but that multiple isolating mechanisms of moderate strength typically act in concert to prevent gene flow between species (Coyne and Orr 2004; Matsubyashi and Katakura 2009).

A key question in evolutionary biology is the sequence in which these barriers to reproduction evolve (Coyne and Orr 1989, 1998, 2004; Mendelson 2003; Mendelson et al. 2007). Previous work has hypothesized that the existence of post-zygotic barriers induces the formation of pre-zygotic barriers in order to prevent the production of unfit hybrid offspring (Dobzhansky 1937), yet empirical studies have found that pre-zygotic isolation evolves before

post-zygotic isolation in some animal taxa (Coyne and Orr 1989; Mendelson 2003). In spite of this evidence, further research is needed in order to have a more complete assessment of the sequence in which isolating mechanisms tend to evolve between taxa.

When barriers to reproduction between species are incomplete, hybrid zones often form in areas where the species overlap in range (Barton and Hewitt 1985). Hybrid zones serve as ideal locations to study evolutionary processes as they occur in real-time, where speciation can be observed in its early stages since reproductive isolation has not yet gone to completion. This allows for the opportunity to identify specific reproductive barriers that may exist between species within the hybrid zone. Additionally, the study of hybrid zones allows for the observation of initial barriers to reproduction, which is of importance since the specific barriers that initially caused reproductive isolation cannot be distinguished from those that evolved after reproductive isolation was complete in completely isolated taxa.

The size and stability of a hybrid zone is maintained by the dispersal of parental genotypes into the zone of sympatry, as well as by the strength and form of selection on hybrid genotypes (Barton and Hewitt 1985; Hilbish et al. 2012). Reproductive isolation can be driven by factors that are both dependent of the hybrid zone environment (exogenous selection against hybrids) and independent of the environment within the hybrid zone (endogenous selection against hybrids). For example, in the mosaic hybrid zone model the parental taxa specialize in different habitats, and hybrids have lower fitness in those habitats resulting in exogenous selection working against the hybrid genotypes (Harrison 1986). Hybrid superiority may also occur where the parental genotypes, which are usually most successful in their individual habitats, have a lower fitness than hybrids when in an intermediate habitat (Hewitt 1988). Conversely, in the tension-zone hybrid zone model endogenous selection against hybrids results

from intrinsic genetic incompatibilities, and hybrid fitness is not influenced by environmental factors within the hybrid zone (Barton and Hewitt 1985).

The closely related killifishes *Fundulus heteroclitus* and *F. grandis* are known to inhabit the southeastern United States, and have an area of range overlap in northeastern Florida centered near Flagler Beach (Gonzalez et al. 2009). Some evidence of hybridization was found by Gonzalez et al. (2009) but hybrids were relatively uncommon, suggesting relatively strong reproductive isolation between the two species. Specifically, a lack of F_1 hybrids has been observed within the Flagler Beach hybrid zone and suggests that relatively strong reproductive barriers may exist between *F. heteroclitus* and *F. grandis*, but F_2 and backcrossed individuals have also been observed and are suggestive of some degree of hybrid fertility (Gonzalez et al. 2009; unpublished data). The hybrid zone between *F. heteroclitus* and *F. grandis* provides an excellent opportunity to observe reproductive barriers between two taxa that are not completely isolated from one another. Study of the isolation between these species under laboratory conditions will allow for the assessment of isolating mechanisms that act independently of the environment, and may provide evidence for the importance of environmental influence on the speciation of these fishes in situations where barriers observed in the laboratory cannot account for patterns observed in nature.

The Flagler Beach hybrid zone lies in an ecotone consisting of tidal saltmarsh that has been infiltrated with mangroves, most likely as the result of recent changes in global climate (Dale et al. 2013). It has been hypothesized that the distribution of *F. heteroclitus* and *F. grandis* may be determined by the utilization of different habitats (Case and Taper 2000; Galleher et al. 2010), as *F. heteroclitus* is known to utilize marsh areas that are dominated by grasses such as *Spartina spp.* (Able and Hagan 2003), and *F. grandis* are known to utilize mangrove-dominated

habitats (Ellis and Bell 2004). Since it is known that such environmental variability exists within the hybrid zone and could potentially influence the reproductive isolation between these species under natural conditions, it is important to achieve a better understanding of the degree of reproductive isolation that exists between *F. heteroclitus* and *F. grandis* that is independent of environmental influence.

Fundulids have a pivotal role as mesoconsumers in the trophic dynamics of salt marshes, functioning as both a key predator of small invertebrates and as an important prey source for many predator species (Darnell 1961; Kneib 1986), many of which have commercial importance. In order to determine whether increased range overlap will increase hybridization, and whether a change in the amount of hybridization will affect the population dynamics, we need to have a better understanding of the reproductive barriers which exist between *F. heteroclitus* and *F. grandis* and whether endogenous or exogenous selection is acting on the hybrid genotypes of these two fish species. Fishes belonging to the genus *Fundulus* have been used as laboratory subjects for decades, and since their genetics, embryology and physiology are well documented (Kneib 1986), they can serve as model organisms for studying speciation. Additionally, the ease of laboratory spawning and rearing of *Fundulus spp.*, as well as the robustness of their embryos and relatively short generation time make them ideal subjects for the study of evolution.

During the course of this study, barriers to reproduction between *F. heteroclitus* and *F. grandis* were investigated by analyzing the prevalence or lack of gene flow between species in a variety of laboratory experiments which focused on two questions: 1) How strong is pre-zygotic isolation between *F. heteroclitus* and *F. grandis* and their hybrids? 2) How strong is post-zygotic isolation among *F. heteroclitus* and *F. grandis* and their hybrids? If behavioral, mechanical and/or gametic isolation are driving forces of speciation in *F. heteroclitus* and/or *F. grandis*, then

pre-zygotic barriers should be relatively strong between species. Sexual dichromatism in the form of male nuptial coloration has been observed in *F. heteroclitus* (Jordan and Evermann 1900; Newman 1907) and this may result in the behavioral isolation between these species. These fishes exhibit external fertilization that is initiated by clasping of the dorsal and anal fins of a spawning pair (Newman 1907; Foster 1967; Able and Hata 1984), making mechanical isolation a potential reproductive barrier. Additionally, since these fishes exhibit external fertilization, gametic isolation may also play a role in their speciation and could result from the failure of gamete recognition/binding during heterospecific spawning events (Palumbi 1998), or due to conspecific sperm precedence when females spawn successively with heterospecific and conspecific males in a localized area. However, post-zygotic isolation may also be involved in the speciation of *F. heteroclitus* and/or *F. grandis*, and if so, then hatching success of hybrid offspring should be reduced compared to parental genotypes as seen in the laboratory.

This study aims to assess the relative strengths of pre- and post-zygotic reproductive barriers between these two species in the absence of environmental factors to see if endogenous barriers can account for the amount of hybridization observed in previous genetic studies. Weak or non-existent barriers to hybrid formation in the laboratory would instead provide evidence for the existence of exogenous reproductive barriers between *F. heteroclitus* and *F. grandis* in the Flagler Beach hybrid zone.

MATERIALS AND METHODS

All animal collection, care, and use during this study was approved by the University of North Florida's Institutional Animal Care and Use Committee (protocol IA#13-016).

Specimen Collection and Husbandry

Adult fishes were obtained from sites that have been previously identified as containing relatively pure populations of either *F. heteroclitus* or *F. grandis* (Gonzalez et al. 2009). *Fundulus heteroclitus* were collected from a marsh in the Guana Tolomato Matanzas National Estuarine Research Reserve (GTMNERR) near Ponte Vedra Beach, Florida, and *F. grandis* were collected from two coastal sites near Cedar Key, Florida. Approximately 200 individuals of each species were collected using standard minnow traps baited with commercial dog food. All fishes were transported to and housed in a wet lab at the University of North Florida in 37.8 L aquaria and were separated by species, and by sex. All fish underwent a minimum of a two-week acclimation period before being utilized in experimental crosses. Specimens were exposed daily to indirect natural sunlight of seasonally variable length through windows in the laboratory. Fishes were held at room temperature (22 ± 2°C), at a salinity of 26.0 ± 0.1‰ and were fed once daily; adults and juveniles were fed a rotating diet of earthworm and beef heart flakes/pellets and standard aquaculture pellet feed, and fry were fed live *Artemia* nauplii. Husbandry and culture parameters chosen for this study were based upon working knowledge of the husbandry and culture of other egg-scattering subtropical and tropical fish species.

Breeding Experiments

Water conditions in spawning aquaria were maintained at a salinity of 26.0 ± 0.1‰ and a temperature of 27.2 ± 1.1°C to induce spawning. It has previously been reported that gonadosomal indices of *F. heteroclitus* are highest among both males and females during the

nighttime hours compared to during the day (Taylor et al. 1979), and therefore fishes used for this study were bred under nighttime conditions to facilitate spawning; no direct light was provided during trials except when eggs were collected. Fishes used for each trial were haphazardly selected, and pairs were randomly put into 14 L spawning aquaria each containing two spawning brushes to provide the fishes with a substrate for oviposition.

Fishes were kept in culture year-round, and trials took place in 2014 from June through August, and in 2015 from January through March and August through September. Since *F. heteroclitus* and *F. grandis* are known to be semilunar spawners (Taylor et al. 1979; Hsiao and Meier 1986, 1989; Hsiao et al. 1996), trials were held during full and new moon phases. Each trial lasted for three days, and tanks were checked twice daily (morning and evening) during trials for the presence of eggs. If eggs were present, they were collected, counted, assessed for fertilization by visually identifying the absence of a nucleus and/or the occurrence of early cleavage, and placed in a small measuring cup containing approximately 60 ml of 26.0 ‰ saltwater and stored at a temperature of 27.2 ± 1.1°C; unfertilized eggs were discarded. Fertilized eggs received daily 100% water changes, and were assessed daily for mortality as noted by yellow or white cloudy eggs, and/or hatching. At the end of each trial, each individual adult was measured (TL), recorded, and returned to broodstock tanks, separated by species. Individuals that were used once for no-choice trials may have been utilized for one choice trial as well, however no individuals were re-used for the same trial type during the course of this study.

No-Choice Breeding Trials

No-choice breeding trials (one male and one female) were performed to assess both pre- and post-zygotic isolation between *F. heteroclitus* and *F. grandis*. On each trial date no-choice crosses were performed using a randomized block design with no less than two replicates of each

cross, and including all possible combinations of sex and species, including *F. heteroclitus* x *F. heteroclitus* (H♂xH♀), *F. heteroclitus* x *F. grandis* (H♂xG♀), *F. grandis* x *F. heteroclitus* (G♂xH♀), and *F. grandis* x *F. grandis* (G♂xG♀). Each cross was replicated 25 times, for a total of 100 crosses performed.

Pre-zygotic isolation was measured by evaluating both mating and fertilization success rates among all crosses. Mating success was assessed in three ways: 1) the proportion of successful mating attempts, 2) differences in mean number of eggs produced, and 3) time to first successful mating. The proportion of successful mating attempts was measured as the number of replicates that result in the production of eggs out of the total number of replicates for that cross. Comparisons of mating success rates among crosses were made using Fisher's exact tests of independence. The total number of eggs laid by each female (clutch size) was averaged across all replicates for each cross and means were compared among crosses. Time to first successful mating was estimated by the time that eggs were first found on the spawning substrate for each replicate and compared among crosses. Fertilization success in no-choice experiments was determined as the number of eggs fertilized in each replicate divided by the total number of eggs laid by the female, and the mean proportion of fertilized eggs was also compared among crosses. All measurements of clutch size, timing of mating, and fertilization success were tested for statistical significance among crosses using independent-samples Kruskal-Wallis tests. Mean total lengths of males and females used for no-choice trials were tested for statistical significance among crosses using independent-samples Mann-Whitney U tests.

Post-zygotic isolation was measured by assessing the hatching success rates of embryos produced by each cross type. The probability of hatching was measured as the number of fertilized eggs that hatched relative to the total number of fertilized eggs that were produced by

each replicate. Time to first successful hatching was estimated by the time that larvae were first observed for each replicate that produced fertilized eggs, and was compared among crosses. Measurements of hatching success and timing to first hatching were tested for statistical significance among crosses using independent-samples Kruskal-Wallis tests. To test if timing of hatching had any effect on hatching success, mean percent hatched and mean time to hatching were both compared using Quade's rank analysis of covariance among crosses from each trial that produced a clutch of eggs.

Once hybrids that were produced by the no-choice crosses were successfully reared to reproductive size (approximately 15 months), hybrid fertility and mating success were tested by performing no-choice backcrosses with fishes of each parental species. A total of 24 F_1 hybrid backcrosses were performed. Even though hybrids were successfully produced in both directions, only H♂xG♀ hybrids were used for backcrosses due to the fact that only a few of the G♂xH♀ hybrids reached reproductive size by the time the backcross trials were took place; F_2 crosses were not performed because of the low number of hybrids were produced in general, but also due to the disproportionate number of H♂xG♀ to G♂xH♀ hybrids. Backcrosses were performed with hybrids and *F. heteroclitus* and *F. grandis* in both directions, and included non-sibling HG♂xH♀, HG♂xG♀, H♂xHG♀, and G♂xHG♀ combinations. Each cross was replicated 6 times, for a total of 24 crosses performed. The mean total lengths of males and females used for backcrosses were tested for statistical significance among crosses using independent-samples Mann-Whitney U tests. Pre-zygotic and post-zygotic isolation was assessed in the same manner as all other no-choice trials.

Choice Breeding Trials

Choice breeding trials (one female and two males) were performed to elucidate female mate choice as well as possible post-mating/pre-fertilization isolation (i.e.: conspecific sperm precedence) that may exist between *F. heteroclitus* and *F. grandis*. Courtship and spawning behaviors have been well documented in *F. heteroclitus* and *F. grandis*, and previous works have described spawning events as when the dorsal and anal fins of a male clasp tightly with those of a female, then holding their bodies an s-shaped posture they vibrate and release their gametes simultaneously (Newman 1907; Foster 1967; Able and Hata 1984). *Fundulus heteroclitus* and *F. grandis* exhibit external fertilization, and this clasping behavior prevents multiple males from fertilizing a female's eggs during a single spawning event.

Choice crosses involved a female of one of the species and one male of each species. Each combination was replicated 10 times. Eggs were collected, counted, assessed for fertilization, and allowed to develop for a maximum of three days to ensure the presence of enough tissue for DNA extraction. In general, embryo failure occurred later than three days post-fertilization, and post-zygotic complications were unlikely to have influenced the results. Fertilized eggs received daily 100% water changes, and failed/deceased embryos (white/yellow cloudy eggs) were removed daily. Three days post-fertilization, embryonic development was arrested by placing the embryos in 70% ethanol, preserving them for genotypic analysis to determine paternity.

Due to financial and time constraints it was impossible to genotype all of the preserved embryos. For small clutches made up of 40 or less embryos the entire clutch was used for genotypic analysis, however for larger clutches containing more than 40 embryos a subsample of 40 embryos was used for genotyping. Total DNA was isolated from preserved whole embryos by

using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA) protocol for spin-column extraction of DNA from whole tissue. Isolated DNA was amplified via polymerase chain reaction (PCR) for the nuclear *recombination activating gene 1* (*RAG1*) locus or the nuclear *glycosyltransferase gene* (*GYLT*) locus using forward and reverse primers (*RAG1* forward: 5'-CAG AGC GAA ATG CAA TGA AA-3'; *RAG1* reverse: 5'-CCC GAT TTC ATC CTG AAA GA-3'; *GYLT* forward: 5'-TAG CCC AGG AGT TCC AAA TG-3'; *GYLT* reverse: 5'-GCT GGC TTA CTC TTC ATG CC-3'). PCR products were verified by electrophoresing them through a 1% agarose gel at 110V for 45 minutes. Once verified, PCR products underwent restriction enzyme digestion using *Mlu*I for *RAG1* or *Eco47III* for *GYLT* according to the manufacturer's protocols. PCR products of *F. heteroclitus* are diagnostically cut by *Mlu*I and *Eco47*III while PCR products of *F. grandis* remain uncut. Digested PCR products were electrophoresed through a 2.5% agarose gel at 125V for 1.25 hours, and the samples were then genotyped by assessing for differences in restriction fragment length polymorphisms (RFLPs).

Once paternity had been determined, pre-zygotic isolation was assessed by measuring differences in the proportion of successful mating attempts, and the proportion of eggs fertilized. These measurements were performed to assess each male used for each cross. For example, a cross in which only one of the two males successfully sired offspring was deemed a successful mating for that male, and an unsuccessful mating for the other male. A cross in which eggs were fertilized by both males was considered successful for each. Differences in the proportion of successful mating events for heterospecific and conspecific males in each cross was compared using Fisher's exact tests of independence. Differences in the mean proportion of eggs fertilized by conspecific and heterospecific males was compared using independent-samples Mann-Whitney U tests for each cross type. Mean total lengths of males and females used in choice

trials were also tested for statistical significance among crosses using independent-samples Kruskal-Wallis tests. Statistical significance was set at $\alpha = 0.05$ for all analyses.

Calculations of Relative Reproductive Isolation

The relative strengths of pre- and post-zygotic reproductive barriers between *F. heteroclitus* and *F. grandis* were calculated by following the methods outlined by Sobel and Chen (2014). This model results in values ranging from -1 (only heterospecific matings are successful) to 1 (only conspecific matings are successful) in a linear manner. Under this model, the amount of reproductive isolation (RI) was recognized as being directly proportional to the probability of gene flow (x) between the two species and was calculated as the following:

$$RI = 1 - 2x$$

This model estimated the probability of gene flow (x) between species as the proportion of heterospecific (H) mating events relative to all mating events involving both heterospecifics and conspecifics (C), and was calculated as:

$$x = \left(\frac{H}{H + C}\right)$$

The calculation used to estimate relative reproductive isolation for mating, fertilization, and hatching barriers tested during this study was performed by combining the two equations above into the following:

$$RI = 1 - 2\left(\frac{H}{H + C}\right)$$

Calculations of relative reproductive isolation were performed for all three barriers under choice and no-choice conditions. Although mating success was measured three ways during this study, only frequency of mating was used to assess the relative strength of mating isolation since it

represented a direct measurement of mating preference, and because the other measurements did not reveal any statistical significance. Hatching frequency data was only obtained from the no-choice trials since choice embryos were arrested during development for genotypic analysis, therefore hatching frequencies that were observed during no-choice trials were applied to the calculations of hatching isolation under both no-choice and choice conditions.

The absolute contributions (AC) of each reproductive barrier and the total relative strength of reproductive isolation (RI_{Total}) for *F. heteroclitus* and *F. grandis* were calculated as the combination of relative pre- and post-zygotic isolation for each species under no-choice and choice conditions. Since individual isolating mechanisms occur during different times during the life history of an organism, the sequence in which the barriers occur must be taken into consideration; a specific reproductive barrier can only reduce gene flow that has not already been reduced by previously acting barriers. The calculations for the absolute contributions of for each reproductive barrier used here have taken into account the amount of gene flow reduction that has already occurred as each successive barrier was measured. Estimations of the AC for each barrier and RI_{Total} for each species were made using the following formulas as outlined by Ramsey et al. (2003):

$$AC_{Mating} = RI_{Mating}$$

$$AC_{Fertilization} = RI_{Fertilization} * \left(1 - AC_{Mating}\right)$$

$$AC_{Hatching} = RI_{Hatching} * \left(1 - \left(AC_{Mating} + AC_{Fertilization}\right)\right)$$

$$RI_{Total} = \sum AC$$

The relative contribution (RC) of each reproductive barrier was also calculated for each species under no-choice and choice conditions using the following formula adapted from Ramsey et al. (2003):

$$RC = \frac{AC}{RI_{Total}}$$

RESULTS

No-Choice Experiments

Out of the 100 crosses attempted, 47 successfully produced eggs, with a total of 1,482 eggs that were laid of which 1,032 were fertilized. No significant differences were observed in the mean total lengths of males and females used for each cross type (H♂xH♀: $U = 68.500$, $z = -1.831$, $p = 0.067$; H♂xG♀: $U = 71.500$, $z = -1.702$, $p = 0.089$; G♂xH♀: $U = 99.500$, $z = -0.540$, $p = 0.595$; G♂xG♀: $U = 100.000$, $z = -0.519$, $p = 0.624$). There were no significant differences in mean clutch size among crosses ($H = 2.772$, $df = 3$, $p = 0.428$; Fig. 1). The large amount of variance observed in the mean clutch size of G♂xH♀ crosses was due to a single replicate that produced more than 450 eggs; when the replicate was removed from analysis, there was no change in statistical significance ($H = 4.504$, $df = 3$, $p = 0.212$). Frequency of mating between cross types was nearly significantly different ($\chi^2 = 5.842$, $df = 2$, $p = 0.053$) with a trend toward lower mating frequency in heterospecific crosses than conspecific crosses. Pairwise comparisons among the four types of crosses revealed a significant difference in mating frequency only between H♂xH♀ and H♂xG♀ crosses ($\chi^2 = 6.190$, $df = 2$, $p = 0.045$; H♂xH♀ & G♂xH♀: $\chi^2 = 4.844$, $df = 2$, $p = 0.089$; H♂xH♀ & G♂xG♀: $\chi^2 = 0.513$, $df = 2$, $p = 0.773$; H♂xG♀ & G♂xH♀: $\chi^2 = 0.000$, $df = 2$, $p = 1.000$; G♂xG♀ & H♂xG♀: $\chi^2 = 4.873$, $df = 2$, $p = 0.087$; G♂xG♀ & G♂xH♀: $\chi^2 = 3.676$, $df = 2$, $p = 0.159$). Since the no-choice trials were performed during both new and full moon phases, differences in moon phase could have potentially affected mating success, however no statistical differences in mating frequency were observed between the different moon phases ($\chi^2 = 0.258$, $df = 1$, $p = 0.689$). When the two types of conspecific crosses were pooled and compared to the pooled heterospecific crosses, conspecifics mated significantly

more frequently than heterospecifics ($\chi^2 = 9.972$, $df = 2$, $p = 0.007$; Fig. 2). There was no significant difference in the timing of first mating among crosses ($H = 6.631$, $df = 3$, $p = 0.085$; Fig. 3).

Mean percent fertilization was significantly different among crosses ($H = 8.834$, $df = 3$, $p = 0.032$; Fig. 4), with higher fertilization frequencies observed in both heterospecific crosses than was seen among conspecific crosses. G♂xG♀ crosses tended to have the lowest fertilization success, with significantly lower fertilization success than either H♂xG♀ crosses ($U = 25.000$, $z = -2.260$, $p = 0.024$) or G♂xH♀ crosses ($U = 31.000$, $z = -2.260$, $p = 0.026$), and a nearly significant reduction compared to H♂xH♀ crosses ($U = 70.000$, $z = -1.895$, $p = 0.058$). All other pairwise comparisons were not significantly different.

The average proportion of eggs that successfully hatched varied significantly among all crosses ($H = 12.771$, $df = 3$, $p = 0.005$; Fig. 5). Pairwise comparisons revealed that the difference was due only to the lower hatching success of G♂xH♀ crosses compared to all others (vs H♂xH♀: $p = 0.008$; vs H♂xG♀: $p = 0.011$; vs G♂xG♀: $p = 0.002$). In fact, G♂xH♀ hybrid embryos were about three times less likely to hatch than any other cross. Average time to first hatching was not significantly different among cross types ($H = 6.674$, $df = 3$, $p = 0.083$; Fig. 6). The potential influence of the timing of hatching on hatching frequency was considered, and the variability in hatching frequency due to timing of mating was removed using rank analysis of covariance. This test indicated that there was a still a significant relationship in mean hatching frequency between crosses when adjusting for mean time to first hatching across all cross types ($F_{(3, 38)} = 3.732$, $p = 0.020$), and the pooled data show that in general as time to hatching increased, hatching success decreased (Fig 7). Post-hoc pairwise comparisons of mean hatching frequency by cross type once the covariate of mean time to first hatching was removed revealed

a significant difference between the mean hatching frequencies of G♂xH♀ and G♂xG♀ crosses only (p = 0.015), which was what was also observed when timing of mating was not considered.

No-choice Hybrid Backcrosses

Only 2 of the 24 backcrosses successfully produced eggs, and there was no statistically significant difference in mating success among crosses (χ^2 = 2.182, df = 3, p = 0.536). No significant differences were observed in the mean total lengths of males and females used for each backcross type (HG♂xH♀: U = 14.500, z = -0.565, p = 0.589; H♂xHG♀: U = 14.500, z = -0.565, p = 0.589; G♂xGH♀: U = 9.500, z = -1.363, p = 0.180; HG♂xG♀: U = 16.500, z = -0.241, p = 0.818). A total of 20 eggs were laid, all of which were fertilized. One HG♂xH♀ cross produced 19 eggs, while one HG♂xG♀ cross produced one egg. Pairwise comparisons of mating success between the successful backcrosses and the parental no-choice crosses indicated that the frequencies of successful mating observed in each of the successful backcrosses was significantly lower than each conspecific no-choice cross (HG♂xH♀ & H♂xH♀: χ^2 = 10.117; df = 1, p = 0.002; HG♂xH♀ & G♂xG♀: χ^2 = 7.643, df = 1, p = 0.011; HG♂xG♀ & H♂xH♀: χ^2 = 10.117; df = 1, p = 0.002; HG♂xG♀ & G♂xG♀: χ^2 = 7.643, df = 1, p = 0.011), but was not significantly different than each of the heterospecific no-choice crosses (HG♂xH♀ & H♂xG♀: χ^2 = 2.467; df = 1, p = 0.220; HG♂xH♀ & G♂xH♀: χ^2 = 3.147 , df = 1, p = 0.119; HG♂xG♀ & H♂xG♀: χ^2 = 2.467; df = 1, p = 0.220; HG♂xG♀ & G♂xH♀: χ^2 = 3.147 , df = 1, p = 0.119). The hatching frequency for the clutch produced by the HG♂xH♀ cross was 0.21, and the single embryo produced by the HG♂xG♀ cross also hatched successfully (Table 1). No statistical analyses to assess differences in mean fertilization and hatching frequency among crosses were performed since there was only one pair from each cross that successfully produced eggs, and therefore mean values could not be obtained.

Choice Breeding Experiments

Of the 20 choice crosses attempted, 15 produced eggs, for a total of 1,042 eggs of which 1,011 were fertilized. 13 of the 15 clutches produced contained fertilized eggs, and clutch size varied from 1 to 205 eggs. No significant differences were observed in the mean total lengths of males and females used for each cross type (H♀ choice: $H = 2.271$, $df = 2$, $p = 0.321$; G♀ choice: $H = 1.947$, $df = 2$, $p = 0.378$). Of the clutches that were produced, a total of 10 clutches were genotyped to determine paternity; two clutches that consisted of only one fertilized egg each were lost during the course of handling for collection or preservation, and were not included in the genotypic analyses. No significant difference in mating frequency was found between conspecifics and heterospecifics for H♀ choice crosses ($\chi^2 = 6.000$, $df = 1$, $p = 0.061$; Fig. 8), or for G♀ choice crosses where mating frequencies between heterospecifics and conspecifics was found to be equal. Even though the average frequencies of successful matings were observed to be highest in conspecifics relative to heterospecifics, when mating success frequencies from conspecific crosses were pooled and compared to the pooled mating success from heterospecific crosses, the difference in mating success was not statistically significant ($\chi^2 = 1.667$, $df = 1$, $p = 0.333$). Within H♀ choice crosses, *F. heteroclitus* males sired significantly more embryos than *F. grandis* males ($U = 0.000$, $z = -2.989$, $p = 0.002$; Fig 9). Conspecifics also tended to have a higher fertilization success rate than heterospecifics in choice trials involving G♀, but the difference was marginally insignificant ($U = 1.000$, $z = -2.021$, $p = 0.057$).

Relative Strengths and Contributions of Reproductive Isolation

No-choice breeding trials were performed to identify the presence of pre-zygotic and post-zygotic reproductive isolation between *F. heteroclitus* and *F. grandis*, as well as to provide baseline measurements of the strengths of reproductive isolation between these species in the

absence of mate competition and environmental influences. No-choice conditions revealed mating isolation, no fertilization barrier, and asymmetrical hatching isolation between females of *F. heteroclitus* and *F. grandis* (Table 2). Under no-choice conditions, mating isolation accounted for nearly all of the total relative reproductive isolation in *F. grandis* females ($RI_{mating\ G♀}$ = 0.914-1.401), however post-zygotic isolation accounted for the majority of reproductive isolation in *F. heteroclitus* females ($RI_{post\text{-}zygotic\ H♀}$ = 0.793). A slight negative value for relative fertilization isolation was obtained for both species under no-choice conditions, and this caused the value for the relative contribution of mating isolation to be greater than a frequency of 1 in *F. grandis*. Therefore, the range of the relative contribution of mating isolation in *F. grandis* presented here represents the values obtained when fertilization isolation is excluded (low value) and included (high value) from total relative reproductive isolation seen under no-choice conditions. Total relative reproductive isolation under no-choice conditions was found to be considerably weaker in *F. grandis* females ($RI_{Total\ No\text{-}choice\ G♀}$ = 0.208) than for *F. heteroclitus* females ($RI_{Total\ No\text{-}choice\ H♀}$ = 0.613) which showed an approximate three-fold greater degree of isolation.

Under choice conditions, pre-zygotic barriers were stronger than post-zygotic barriers in both *F heteroclitus* and *F. grandis* females (Table 2). Fertilization isolation was found to be moderate in strength in *F. grandis* females ($RI_{Fertilization\ G♀}$ = 0.412), and it accounted for nearly all of the total relative reproductive isolation in that species ($RC_{Fertilization\ G♀}$ = 0.948). On the other hand, mating isolation contributed to a large proportion of the total reproductive isolation seen in *F. heteroclitus* females ($RC_{mating\ H♀}$ = 0.508), and when combined with fertilization and post-zygotic isolation lead to nearly complete reproductive isolation in *F. heteroclitus* females under choice conditions ($RI_{Total\ Choice\ H♀}$ = 0.984). Conversely, no mating barrier was seen under choice conditions in *F. grandis* females, and the total relative reproductive isolation in *F. grandis*

females was found to be less than half of that observed in *F. heteroclitus* females ($RI_{Total\ Choice\ G♀}$ = 0.437). Hatching isolation had little effect on females of either species, and contributed to less than 1% of the total reproductive isolation seen in *F. heteroclitus* and *F. grandis* under choice conditions.

DISCUSSION

Total reproductive isolation between *F. heteroclitus* and *F. grandis* was found to be asymmetrical and incomplete in the laboratory, with a greater total relative strength of reproductive isolation in *F. heteroclitus* females than in *F. grandis* females under both choice and no-choice conditions (Table 2). Total reproductive isolation for both species was observed to be stronger under choice conditions versus no-choice conditions, and most of the individual isolating barriers exhibited the same trend. Overall, conspecifics mated more frequently than heterospecifics (Fig. 1), yet hybrids were successfully produced in both directions during no-choice trials and at least some hybrids are capable of backcrossing in both directions. The strong mating and fertilization barriers seen in both species under choice conditions suggest that pre-zygotic isolation likely has the largest influence on their rate of hybridization within the area of range overlap between the two species.

Gonzalez et al. (2009) reported that measurements of allele frequencies of a single strongly differentiated nuclear locus had indicated that the population within the *F. heteroclitus* and *F. grandis* hybrid zone near Flagler Beach, Florida was not in Hardy-Weinberg equilibrium, and that there was a deficit of heterozygous (putative hybrid) genotypes at that site. Additionally, unpublished data utilizing three nuclear loci showed a bimodal hybrid index with primarily pure *F. heteroclitus* and *F. grandis* genotypes and low frequencies of F_1 and later generation hybrid genotypes. Bimodal hybrid zones that are driven by pre-zygotic barriers are known to be associated with strong assortative mating and/or fertilization (Jiggins and Mallet 2000). The results of the current study revealed that pre-zygotic barriers make up a majority of the reproductive isolation between *F. heteroclitus* and *F. grandis* when environmental variability is

absent, and suggests that the genetic structure of the Flagler Beach hybrid zone may be largely driven by barriers which prevent hybrid zygote formation between these two species.

Mating isolation was moderately strong in both species under no-choice conditions, and accounted for most of the total reproductive isolation that was observed in *F. grandis* females and nearly half of the total reproductive isolation observed in *F. heteroclitus* females (Table 2). *Fundulus heteroclitus* females exhibited even stronger mating isolation during the choice trials, whereas no mating preference was detected in *F. grandis* females under choice conditions. Since the breeding trials were carried out under laboratory conditions, the mating isolation that was observed between *F. heteroclitus* and *F. grandis* during this study could not have resulted from spatial isolation due to differences in habitat utilization. Temporal differences in the semilunar spawning cycles of *F. heteroclitus* and *F. grandis* under laboratory conditions have been previously reported (Hsiao and Meier 1989), however during the present study both species successfully produced offspring during each trial conducted and any mating isolation observed cannot be due to temporal isolation between the two species.

The mating isolation that was observed between *F. heteroclitus* and *F. grandis* could possibly be the result of differences in behaviors or morphological cues exhibited by each species during courtship. Differences in behavioral or morphological traits can affect how attractive an individual is to members of the opposite sex, and variation in female preferences for specific male traits can lead to differential mating success between species (Sargent et al. 1998; Coyne and Orr 2004). Differences in male body size have been known to influence female mate choice in other Cyprinodontiform fishes with larger males having a mating advantage over smaller males (Reynolds and Gross 1992). On average, *F. grandis* males were observed to be larger than *F. heteroclitus* males, however this was controlled for in the present study by

haphazardly pairing males and females by size and comparing the measurements of all individuals used for each trial. No significant differences were detected in the mean total lengths of individuals used for each cross in all breeding trials attempted, and therefore differences in male body size cannot account for the differences in mating success observed during the present study.

The ability of individuals to recognize conspecific mates has been identified as an important force behind the evolution of behavioral isolation between co-existing species and is dependent on the ability of individuals to broadcast and/or receive a sensory signal (Endler 1992; Coyne and Orr 2004). The eyes of killifishes are well-developed (Foster 1967), and *Fundulus spp.* have been documented as being heavily reliant on visual displays during courtship (Newman 1907; Foster 1967; Able and Hata 1984). However, previous research on the courtship behaviors displayed by members of the *F. heteroclitus-F. grandis* complex have revealed very few differences between the two species, and all of the mating behaviors that were observed were present at some level in both species during conspecific matings (Able and Hata 1984). The current study did not collect ethological data, and whether or not those behaviors are displayed in a similar fashion during heterospecific matings of *F. heteroclitus* and *F. grandis* individuals remains unknown.

Sexual dichromatism is present in killifishes, and is well documented in *F. heteroclitus* (Foster 1967), with females being generally silver in coloration and males exhibiting bright nuptial colors (Jordan and Evermann 1900; Newman 1907). During this study, *Fundulus heteroclitus* males exhibited breeding coloration in the laboratory that was dark blue on all surfaces of the body, except ventrally between and/or on the pectoral and anal fins where a yellow-orange coloration was often observed (Fig. 10). The observed male nuptial coloration of

F. grandis in the laboratory was a yellow-gold body and fins with a dark blue coloration only on and/or near the operculum, as well as the presence of eye bars. Assortative mating based on color has been identified in many fish species and is considered to have been instrumental in the speciation of some species of sympatric fishes (Seehausen et al. 1997; Seehausen and van Alphen 1998; Salzburger et al. 2006; Elmer et al. 2009). Since dramatic differences in male nuptial coloration exist between of *F. heteroclitus* and *F. grandis*, species recognition and discrimination against heterospecific individuals may have played a role in female mate selection during the choice breeding trials. The presence of pigmented photoreceptors in the eye that can detect wavelengths within the visible spectrum have been documented in *F. heteroclitus* (Flamarique and Harosi 2000), and may be responsible for discernment of mates in the present study, however the potential impacts that visual cues may have on mate selection within and between *F. heteroclitus* and *F. grandis* has yet to be determined.

Female mate choice based on visual cues has been shown in other fish species to be affected by biotic and abiotic factors that can affect signal perception such as visual acuity and turbidity (Seehausen et al. 1997; Engstrom-Ost and Candolin 2006; Fuller and Noa, 2010). Previous studies of the effects of light attenuation on mate choice in fishes from anthropogenically-induced eutrophic systems have shown decreased mating frequencies due to the inability of females to identify mates in turbid versus clear waters (Engstrom-Ost and Candolin 2006), as well as increased hybridization due to the inability of females to recognize visual signals from conspecific males (Seehausen et al. 1997). While water clarity was not an issue in the controlled environments used for current study, breeding trials were conducted in the dark and this may have impacted the fishes' ability to see during the trials.

Data from the current study revealed high frequencies of assortative mating especially under choice conditions, and due to the aforementioned high reliance on visual cues during courtship it could be suggested that females were still able to identify conspecific males during breeding trials using vision. However, studies involving other teleosts have suggested the importance of non-visual sensory signals during conspecific courtship such as acoustic, chemical, and mechanosensory cues (Sargent et al. 1998; Plath et al. 2004; Plenderleith et al. 2005; Blais et al. 2009; Smith and Staaden 2009), and it is possible that one or more sensory cues may have played a role in the patterns of mating success that were observed during the present study. Further investigation is needed to identify which sensory signals are involved in mate selection in *F. heteroclitus* and *F. grandis*, and whether differences in mating success between species can be attributed to differences in the sensory cues between the two species.

No fertilization barrier was detected between *F. heteroclitus* and *F. grandis* under no-choice conditions (Table 2), and the overall trend observed was that heterospecific fertilization occurred more frequently than conspecific fertilization (Fig. 4). The opposite was seen in the choice trials where conspecific fertilizations occurred more frequently (Fig. 9). In fact, fertilization isolation played a large role in the total reproductive isolation observed in females of both species under choice conditions, especially in *F. grandis* (Table 2). The lack of heterospecific fertilization success that was seen under choice conditions could be due to a higher proportion of successful mating events by conspecifics versus heterospecifics, or may be due to some form of gametic isolation that resulted in conspecific sperm precedence. Sperm competition between species, also known as conspecific sperm precedence (CSP), has been documented in several animal taxa (Howard et al. 1998; Fricke and Arnqvist, 2003; Geyer and Palumbi 2005; Ludlow and Magurran 2006; Martín-Coello et al. 2009; Matsubayashi and

Katakura 2009), and assessments of the strength of CSP between species requires measurements of fertilization success as well as sperm concentrations (Mendelson et al. 2007). Conspecific sperm precedence could not be directly assessed by the present study since sperm concentrations were not quantified. That said, there are at least two observations from the present study that suggest the conspecific advantage in fertilization success is not due to CSP. First, these species are known to exhibit clasping behavior prior to the release of gametes (Newman 1907; Foster 1967; Able and Hata 1984), so it is unlikely that eggs would be exposed to sperm from multiple males simultaneously. Second, in non-competitive situations, there was no fertilization barrier and actually a trend toward a heterospecific advantage. Therefore, the existence of CSP between *F. heteroclitus* and *F. grandis* within the Flagler Beach hybrid zone appears to be highly unlikely, making the conspecific fertilization advantage observed here most likely due to conspecific males dominating the number of mating events during each trial.

Even though gametic isolation was not observed in this study it is possible that results would be different in the field. Salinity-based differences in fertilization success rates have been documented in *F. heteroclitus*, and previous research has indicated that individuals that spawned in salinities that are representative of their native habitat have higher fertilization success rates than individuals spawned in salinities that are atypical of where they are found in nature (Able and Palmer 1988). The salinity that both species were held and spawned under during this study was approximately ± 2.0‰ of the salinities recorded from the sites where each species was collected, and therefore salinity should not have had a marked effect on fertilization success in either species. However, even though environmental conditions were controlled during this study and were similar to those observed when the fishes were collected, the spawning conditions

chosen may not have represented the optimal fertilization conditions for *F. heteroclitus* or *F. grandis*.

Strong hatching barriers were observed in *F. heteroclitus* females during no-choice trials but not in *F. grandis* females (Table 2); H♂xG♀ hybrids successfully hatched >3.5 times more frequently than G♂xH♀ hybrids (Fig. 5). However, due to the previous action of pre-zygotic barriers, the absolute contribution of hatching isolation was only 1.5 percent of the total reproductive isolation seen in *F. heteroclitus* females and 2.2 percent of total reproductive isolation in *F. grandis* females under choice conditions, and made the smallest contribution to total reproductive isolation. Differential survival of hybrid embryos from reciprocal *F. grandis* x *F. similis* and *F. heteroclitus* x *F. majalis* crosses have been documented, and was suspected to be due to differences in egg size (Newman 1908; Hubbs and Drewry 1959). Differences in egg size were observed between *F. heteroclitus* and *F. grandis*, and it appeared that the eggs of *F. heteroclitus* were smaller than those of *F. grandis*, however egg size was not measured during the present study. The low contribution of hatching isolation to total reproductive isolation under choice conditions is most likely the result of the strong mating and fertilization barriers that were observed prior to hatching. This suggests that post-zygotic isolation may not play a large role in the speciation of *F. heteroclitus* and *F. grandis* in the absence of environmental influence. However, previous studies of *F. heteroclitus* have linked variability in hatching success to abiotic factors such as temperature, and salinity (Tay and Garside 1975; Tingaud-Sequeira et al. 2009). Ultimately, more research is needed in order to identify any potential influence that the environment may have on hatching success of hybrid offspring produced by *F. heteroclitus* and *F. grandis*, as well as any effects on post-zygotic isolation between these two species in general.

Since so few G♂xH♀ hybrids were successfully produced during the no-choice trials, no F_1 hybrid crosses were performed and only H♂xG♀ hybrids were used for backcrosses to wild-caught *F. heteroclitus* and *F. grandis* individuals. However, few backcrosses could be performed during this study due to time constraints and the difference in the sizes of the lab-cultured hybrids from the no-choice trials and the wild-caught *F. heteroclitus* and *F. grandis*. Even though the hybrids were reared in the laboratory for approximately 15 months, they tended to be smaller in size on average than the wild-caught individuals that were used for the backcross trials. These differences were not statistically significant though. Of the trials performed, two male H♂xG♀ hybrids successfully produced offspring during the no choice backcross trials, one with a *F. heteroclitus* female and the other with a *F. grandis* female (Table 1). This suggests that at least some male H♂xG♀ hybrids are fertile and capable of reproducing with either of the parental species. Whether female H♂xG♀ hybrids are fertile cannot be ascertained from the current study, but differences in fertility between hybrids of different sexes is relatively common in nature (Orr 1987; Zeng and Singh 1993; Laurie 1997; Nasbit et al. 2002; Martin and Willis 2010).

In situations where the two sexes show differences in fertility, the heterogametic sex is almost universally the sex that shows elevated infertility in situations where there is asymmetrical post-zygotic isolation (Haldane's Rule) (Haldane 1922). The present study did not measure mortality of hybrids post-hatching and the sample sizes of the backcrosses were not sufficient enough to say whether there is a difference in fertility between the sexes of *F. heteroclitus/F. grandis* hybrids. Therefore, there is no way to directly test whether Haldane's Rule is observed in this group. Furthermore, there is no evidence of heterogametic sex determination in either *F. heteroclitus* or *F. grandis,* even though the karyotypes of both species

have been investigated and evidence for heterogametic males have been documented for *F. parvipinnus* and *F. diaphanus* (Chen and Ruddle 1970; Chen 1971; Kornfield 1981; Mank et al. 2006). It is also possible that some of the hybrids used in the backcross trials may have not had enough time to reach sexual maturity before the backcross trials were performed. Evidence of second generation and beyond hybrid individuals from the Flagler Beach hybrid zone population also suggests some degree of fertility in *F. heteroclitus-F. grandis* hybrids (unpublished data).

The backcross trials also indicated that hybrids had a reduced mating success compared to that of conspecific crosses involving each of the parental species, but not to that of heterospecific crosses. This suggests that hybrids may be pre-zygotically isolated from both parental species. Previous research involving reproductive isolation in the *F. notatus* species complex, showed a much different pattern in which a breakdown in prezygotic isolation in F_2 hybrid and parental backcrosses was observed relative to parental crosses (Vigueira et al. 2007). During the present study, an intermediate phenotype of male nuptial coloration was observed rarely and inconsistently in hybrids, but may have been a factor in the reduced mating success that was observed during the hybrid backcross trials relative to the no-choice trials. Further investigation needs to be done in order to determine the extent to which hybrids of *F. heteroclitus* and *F. grandis* are fertile and their relative mating success.

Previous work by Gonzalez et al. (2009) suggested that *F. grandis* alleles were introgressing into *F. heteroclitus* populations within the Flagler Beach hybrid zone, and measurements of cytonuclear disequilibrium suggested that matings between these species occurs in both directions yet most of the hybrids reported in that study had mitochondrial haplotypes of *F. heteroclitus*. The current study indicated the opposite, that *F. grandis* females were more likely to hybridize than *F. heteroclitus* females, and hybrid offspring of *F. grandis*

females were more likely to hatch and survive to reproductive age than hybrid offspring of *F. heteroclitus* females. The discrepancy between the directionality of hybridization predicted by Gonzalez et al. (2009) and that which was observed during the current study is likely due to low sample size in the former study but may also suggest that the patterns observed in the present laboratory study are modified by environmental influences in the field. Additional work on the genetic structure of the hybrid zone should be able to address this contradiction.

The present study concluded that reproductive barriers exist between *F. heteroclitus* and *F. grandis* in the absence of environmental influence, and that total reproductive isolation is relatively strong in *F. heteroclitus* females while moderate in strength in *F. grandis* females. Additionally, pre-zygotic barriers appeared to be the most developed of the barriers observed under choice conditions. Previous research on reproductive isolation in other *Fundulus spp.* has revealed strong pre-zygotic and weak post-zygotic isolation during heterospecific crosses between species who overlap in some part of their ranges (Vigueira et al. 2008), which was also what was observed during the present study. The results of the present research suggest that intrinsic reproductive isolation may be driving the nature of the Flagler Beach hybrid zone. While this laboratory study has provided valuable insight into the nature of reproductive isolation between *F. heteroclitus* and *F. grandis*, the importance of environmentally-dependent isolating mechanisms between these species remains unknown. The potential influence of extrinsic barriers to successful reproduction between *F. heteroclitus* and *F. grandis* needs to be investigated further, as these barriers may also play a significant role in the total reproductive isolation between these two species within the Flagler Beach hybrid zone.

WORKS CITED

Able K, Hagan S (2003) Impact of Common Reed, *Phragmites australis*, on Essential Fish Habitat: Influence on Reproduction, Embryological Development, and Larval Abundance of Mummichog (*Fundulus heteroclitus*). Estuaries 26:40–50

Able K, Hata D (1984) Reproductive Behavior in the *Fundulus heteroclitus-F. grandis* Complex. Copeia 1984:820–825

Able K, Palmer R (1988) Salinity Effects on Fertilization Success and Larval Mortality of *Fundulus heteroclitus*. Copeia 1988:345–350

Barton N, Hewitt G (1985) Analysis of Hybrid Zones. Annu Rev Ecol Syst 16:113–148

Blais J, Plenderleith M, Rico C, Taylor MI, Seehausen O, Oosterhout C van, Turner GF (2009) Assortative Mating Among Lake Malawi Cichlid Fish Populations is Not Simply Predictable from Male Nuptial Colour. BMC Evol Biol 9

Case T, Taper M (2000) Interspecific Competition, Environmental Gradients, Gene Flow, and the Coevolution of Species' Borders. Am Nat 155:583–605

Chen T (1971) A Comparative Chromosome Study of Twenty Killifish Species of the Genus *Fundulus* (Teleostei: Cyprinodontidae). Chromosoma 453:436–453

Chen TR, Ruddle FH (1970) A Chromosome Study of Four Species and a Hybrid of the Killifish Genus *Fundulus* (Cyprinodontidae). Chromosoma 29:255–267

Coyne JA, Orr HA (1989) Patterns of Speciation in *Drosophila*. Evolution 43:362–381

Coyne JA, Orr HA (1998) The Evolutionary Genetics of Speciation. Philos Trans R Soc Lond B Biol Sci 353:287–305

Coyne JA, Orr AH (2004) Speciation. Sinauer Associates, Inc., Sunderland, Massachusetts

Dale P, Eslami-Andargoli L, Knight J (2013) The Impact of Encroachment of Mangroves into Saltmarshes on Saltwater Mosquito Habitats. J Vector Ecol 38:330–8

Darnell R (1961) Trophic Spectrum of an Estuarine Community, Based on Studies of Lake Pontchartrain, Louisiana. Ecology 42:553–568

Dobzhansky TG (1937) Genetics and the Origin of Species. No. 11. Columbia University Press, New York, New York

Ellis W, Bell S (2004) Conditional Use of Mangrove Habitats by Fishes: Depth as a Cue to Avoid Predators. Estuaries 27:966–976

Elmer KR, Lehtonen TK, Meyer A (2009) Color Assortative Mating Contributes to Sympatric Divergence of Neotropical Cichlid Fish. Evolution 63:2750–2757

Endler JA (1992) Signals, Signal Conditions, and the Direction of Evolution. Am Nat 139:S125–S153

Engstrom-Ost J, Candolin U (2006) Human-Induced Water Turbidity Alters Selection on Sexual Displays in Sticklebacks. Behav Ecol 18:393–398

Flamarique I, Hárosi F (2000) Photoreceptors, Visual Pigments, and Ellipsosomes in the Killifish, *Fundulus heteroclitus*: A Microspectrophotometric and Histological Study. Vis Neurosci 17:403–420

Foster NR (1967) Trends in the Evolution of Reproductive Behavior in Killifishes. In: Proceedings of the International Conference on Tropical Oceanography. p 549–566

Fricke C, Arnqvist G (2004) Conspecific Sperm Precedence in Flour Beetles. Anim Behav 67:729–732

Fuller RC, Noa LA (2010) Female Mating Preferences, Lighting Environment, and a Test of the Sensory Bias Hypothesis in the Bluefin Killifish. Anim Behav 80:23–35

Galleher SN, Gilg MR, Smith KJ (2010) Comparison of Larval Thermal Maxima Between *Fundulus heteroclitus* and *F. grandis*. Fish Physiol Biochem 36:731–40

Geyer LB, Palumbi SR (2005) Conspecific Sperm Precedence in Two Species of Tropical Sea Urchins. Evolution 59:97–105

Gonzalez I, Levin M, Jermanus S, Watson B, Gilg MR (2009) Genetic Assessment of Species Ranges in *Fundulus heteroclitus* and *F. grandis* in Northeastern Florida Salt Marshes. Southeast Nat 8:227–243

Haldane JB (1922) Sex Ratio and Unisexual Sterility in Hybrid Animals. J Genet 12:101–109

Harrison R (1986) Pattern and Process in a Narrow Hybrid Zone. Heredity 56:337–349

Hewitt GM (1988) Hybrid Zones-Natural Laboratories for Evolutionary Studies. Trends Ecol Evol 3:158–67

Hilbish TJ, Lima FP, Brannock PM, Fly EK, Rognstad RL, Wethey DS (2012) Change and Stasis in Marine Hybrid Zones in Response to Climate Warming. J Biogeogr 39:676–687

Howard D, Gregory P, Chu J, Cain M (1998) Conspecific Sperm Precedence is an Effective Barrier to Hybridization Between Closely Related Species. Evolution 52:511–516

Hsiao SM, Limesand SW, Wallace RA (1996) Semilunar Follicular Cycle of an Intertidal Fish: The *Fundulus* Model. Biol Reprod 54:809–18

Hsiao SM, Meier AH (1986) Spawning Cycles of the Gulf Killifish, *Fundulus grandis*, in Closed Circulation Systems. J Exp Zool 240:105–112

Hsiao SM, Meier AH (1989) Comparison of Semilunar Cycles of Spawning Activity in *Fundulus grandis* and *F. heteroclitus* Held Under Constant Laboratory Conditions. J Exp Zool 252:213–218

Hubbs C, Drewry GE (1959) Survival of F1 Hybrids Between Cyprinodont Fishes, With a Discussion of the Correlation Between Hybridization and Phylogenetic Relationship. Inst Mar Sci 6:81–91

Jiggins CD, Mallet J (2000) Bimodal Hybrid Zones and Speciation. Trends Ecol Evol 15:250–255

Jordan D, Evermann B (1898) The Fishes of North and Middle America: A Descriptive Catalogue of the Species of Fish-Like Vertebrates Found in the Waters of North America, North of The Isthmus of Panama. No. 47. US Government Printing Office, Washington, District of Columbia

Kneib R (1986) The Role of *Fundulus heteroclitus* in Salt Marsh Trophic Dynamics. Am Zool 26:259–269

Kornfield I (1981) Distribution of Constitutive Heterochromatin and the Evolution of Sex Chromosomes in *Fundulus*. Copeia 1981:916–918

Laurie CC (1997) The Weaker Sex is Heterogametic: 75 Years of Haldane's Rule. Genetics 147:937–951

Ludlow AM, Magurran AE (2006) Gametic Isolation in Guppies (*Poecilia reticulata*). Proc R Soc London Biol Sci 273:2477–82

Mank JE, Hall DW, Kirkpatrick M, Avise JC (2006) Sex Chromosomes and Male Ornaments: a Comparative Evaluation in Ray-Finned Fishes. Proc R Soc London Biol Sci 273:233–236

Martin NH, Willis JH (2007) Ecological Divergence Associated with Mating System Causes Nearly Complete Reproductive Isolation Between Sympatric *Mimulus* Species. Evolution 61:68–82

Martin NH, Willis JH (2010) Geographical Variation in Postzygotic Isolation and Its Genetic Basis Within and Between Two *Mimulus* Species. Philos Trans R Soc Lond B Biol Sci 365:2469–78

Martín-Coello J, Benavent-Corai J, Roldan ERS, Gomendio M (2009) Sperm Competition Promotes Asymmetries in Reproductive Barriers Between Closely Related Species. Evolution 63:613–23

Matsubayashi KW, Katakura H (2009) Contribution of Multiple Isolating Barriers to Reproductive Isolation Between a Pair of Phytophagous Ladybird Beetles. Evolution 63:2563–80

Mayr E (1940) Speciation Phenomena in Birds. Am Nat 74:249–278

Mayr E (1942) Systematics and The Origin of Species, From The Viewpoint of a Zoologist. Harvard University Press, Cambridge, Massachusetts

Mayr E (2000) The Biological Species Concept. In: Wheeler QD, Meier R (eds) Species Concepts and Phylogenetic Theory: A Debate. Columbia University Press, New York, New York, p 17–29

Mendelson TC (2003) Sexual Isolation Evolves Faster Than Hybrid Inviability in a Diverse and Sexually Dimorphic Genus of Fish (Percidae: *Etheostoma*). Evolution 57:317–27

Mendelson TC, Imhoff VE, Venditti JJ (2007) The Accumulation of Reproductive Barriers During Speciation: Postmating Barriers in Two Behaviorally Isolated Species of Darters (Percidae: *Etheostoma*). Evolution 61:2596–2606

Naisbit RE, Jiggins CD, Linares M, Salazar C, Mallet J (2002) Hybrid Sterility, Haldane's Rule and Speciation in *Heliconius cydno* and *H. melpomene*. Genetics 161:1517–1526

Newman H (1907) Spawning Behavior and Sexual Dimorphism in *Fundulus heteroclitus* and Allied Fish. Biol Bull 12:314–348

Newman HH (1908) The Process of Heredity as Exhibited by the Development of *Fundulus* Hybrids. Dev Genes Evol 26:502–503

Orr HA (1987) Genetics of Male and Female Sterility in Hybrids of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 116:555–563

Palumbi SR (1998) Species Formation and the Evolution of Gamete Recognition Loci. In: Howard DJ, Berlocher SH (eds) Endless Forms: Species and Speciation. Oxford University Press, New York, New York, p 271–278

Plath M, Parzefall J, Korner KE, Schlupp I (2004) Sexual Selection in Darkness? Female Mating Preferences in Surface- and Cave-Dwelling Atlantic Mollies, *Poecilia mexicana* (Poeciliidae, Teleostei). Behav Ecol Sociobiol 55:596–601

Plenderleith M, Oosterhout C van, Robinson RL, Turner GF (2005) Female Preference for Conspecific Males Based on Olfactory Cues in a Lake Malawi Cichlid Fish. Biol Lett 1:411–414

Ramsey J, Bradshaw HD, Schemske DW (2003) Components of Reproductive Isolation Between the Monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534

Reynolds J, Gross M (1992) Female Mate Preference Enhances Offspring Growth and Reproduction in a Fish, *Poecilia reticulata*. Proc R Soc London Biol Sci 250:57–62

Salzburger W, Niederstätter H, Brandstätter A, Berger B, Parson W, Snoeks J, Sturmbauer C (2006) Colour-assortative Mating Among Populations of *Tropheus moorii*, a Cichlid Fish from Lake Tanganyika, East Africa. Proc R Soc London Biol Sci 273:257–66

Sargent RC, Rush VN, Wisenden BD, Yan HY (1998) Courtship and Mate Choice in Fishes: Integrating Behavioral and Sensory Ecology. Am Zool 38:82–96

Schluter D (2001) Ecology and the Origin of Species. Trends Ecol Evol 16:372–380

Seehausen O, van Alphen J, Witte F (1997) Cichlid Fish Diversity Threatened by Eutrophication That Curbs Sexual Selection. Science 277:1808–1811

Seehausen O, van Alphen J (1998) The Effect of Male Coloration on Female Mate Choice in Closely Related Lake Victoria Cichlids (*Haplochromis nyererei* Complex). Behav Ecol Sociobiol 42:1–8

Smith AR, Staaden MJ van (2009) The Association of Visual and Acoustic Courtship Behaviors in African Cichlid Fishes. Mar Freshw Behav Physiol 42:211–216

Sobel JM, Chen GF (2014) Unification of Methods for Estimating the Strength of Reproductive Isolation. Evolution 68:1511–1522

Tay KL, Garside ET (1975) Some Embryogenic Responses of Mummichog, *Fundulus heteroclitus* (L.) (Cyprinodontidae), to Continuous Incubation in Various Combinations of Temperature and Salinity. Can J Zool 53:920–933

Taylor M, Leach G, DiMichele L (1979) Lunar Spawning Cycle in the Mummichog, *Fundulus heteroclitus* (Pisces: Cyprinodontidae). Copeia 1979:291–297

Tingaud-Sequeira A, Zapater C, Chauvigné F, Otero D, Cerdà J (2009) Adaptive Plasticity of Killifish (*Fundulus heteroclitus*) Embryos: Dehydration-Stimulated Development and Differential Aquaporin-3 Expression. Am J Physiol Regul Integr Comp Physiol 296:R1041–52

Vigueira PA, Schaefer JF, Duvernell DD, Kreiser BR (2007) Tests of Reproductive Isolation Among Species in the *Fundulus notatus* (Cyprinodontiformes: Fundulidae) Species Complex. Evol Ecol 22:55–70

Zeng LW, Singh RS (1993) The Genetic Basis of Haldane's Rule and the Nature of Asymmetric Hybrid Male Sterility Among *Drosophila simulans, Drosophila mauritiana* and *Drosophila sechellia*. Genetics 134:251–260

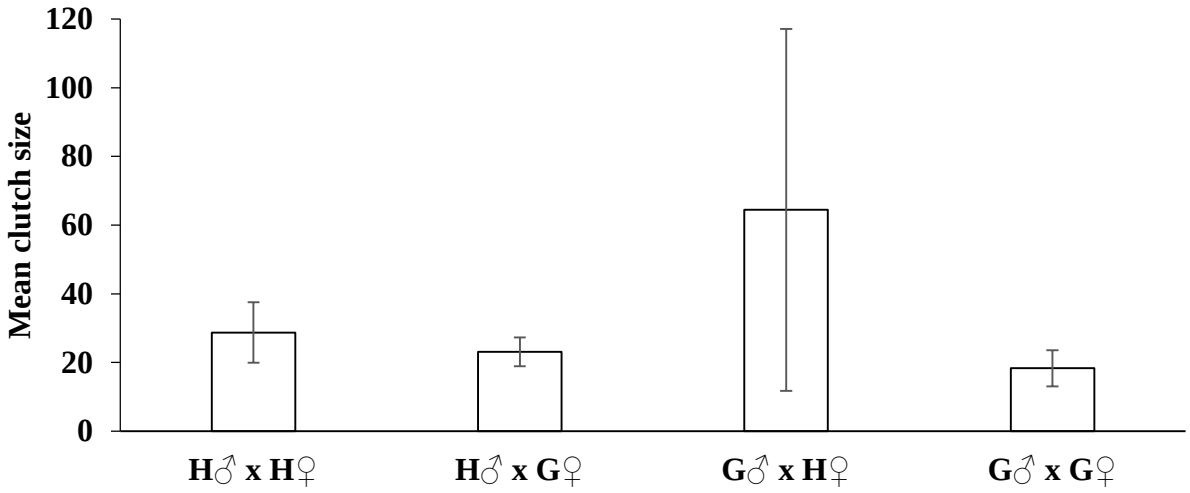

Figure 1. Mean number of eggs per clutch for each cross type that produced eggs during the course of the no-choice trials. Error bars represent standard error for each cross type.

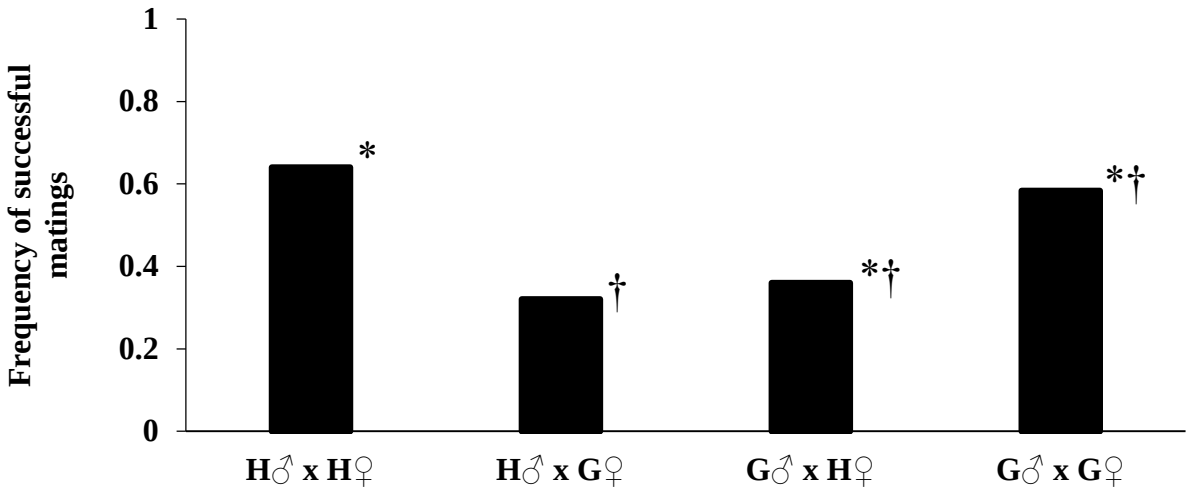

Figure 2. Proportion of no-choice breeding trials that successfully produced at least one egg for each different type of cross. Asterisks and daggers represent statistical significance between cross types.

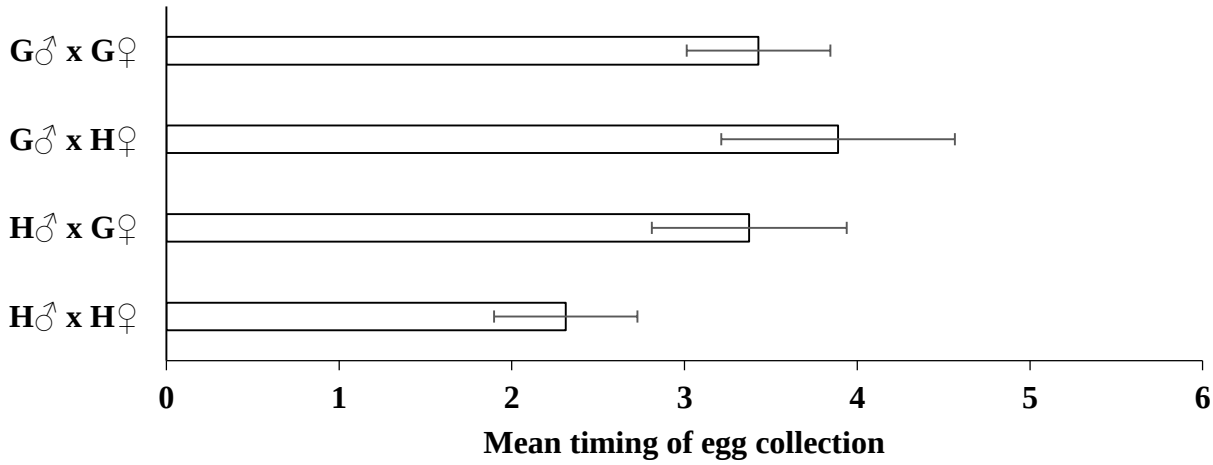

Figure 3. Average number of observations made (±se) before first successful mating for each cross type that produced eggs during no-choice breeding trials. Tanks were checked twice daily for the presence of eggs over a three-day period, resulting in a total of six observations during each trial.

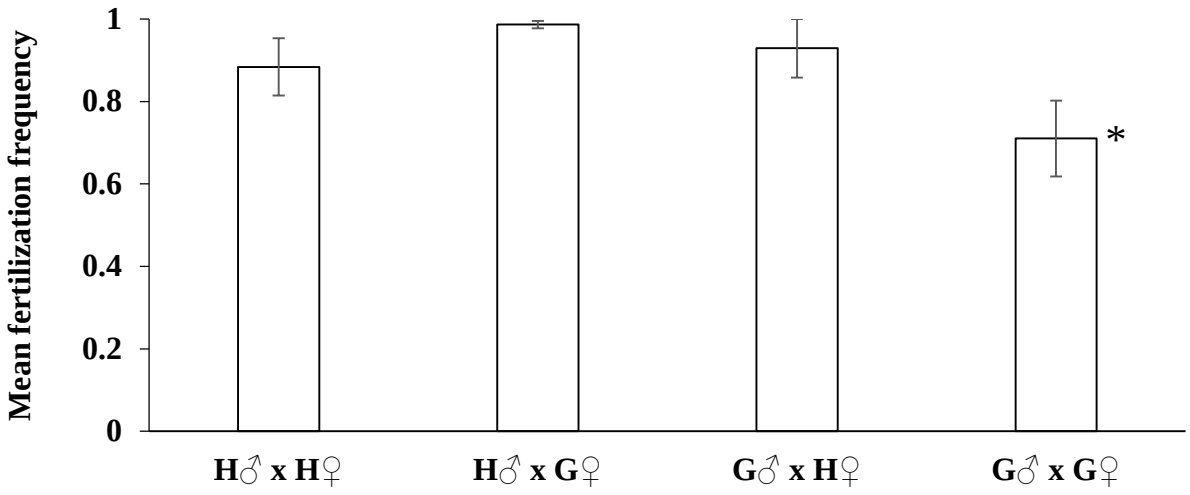

Figure 4. Mean (±se) proportion of eggs that were successfully fertilized for each cross type during the course of the no-choice breeding trials. Significant differences are denoted by asterisks.

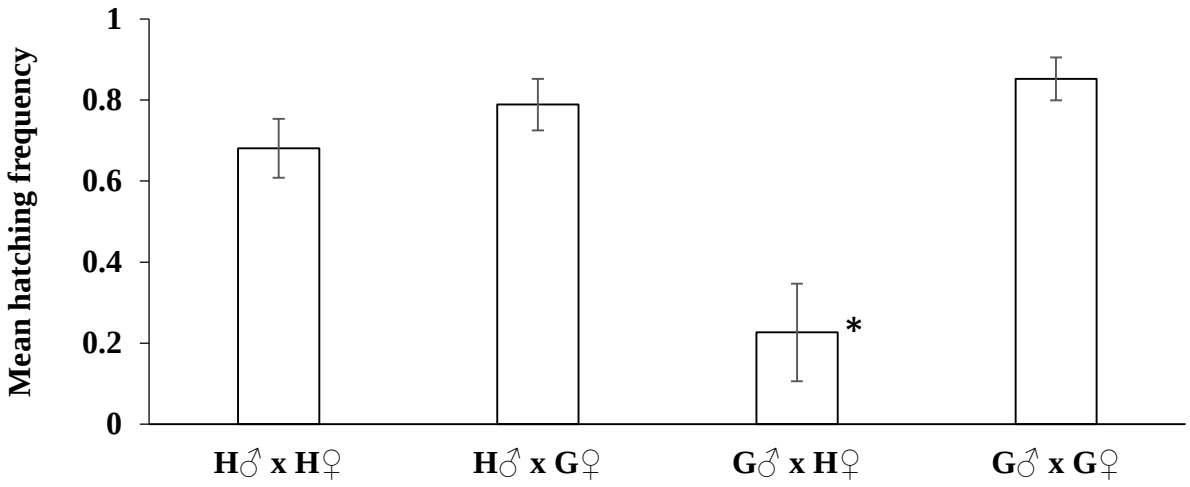

Figure 5. Mean (±se) proportion of embryos that successfully hatched during no-choice trials. Significant differences are denoted by asterisks.

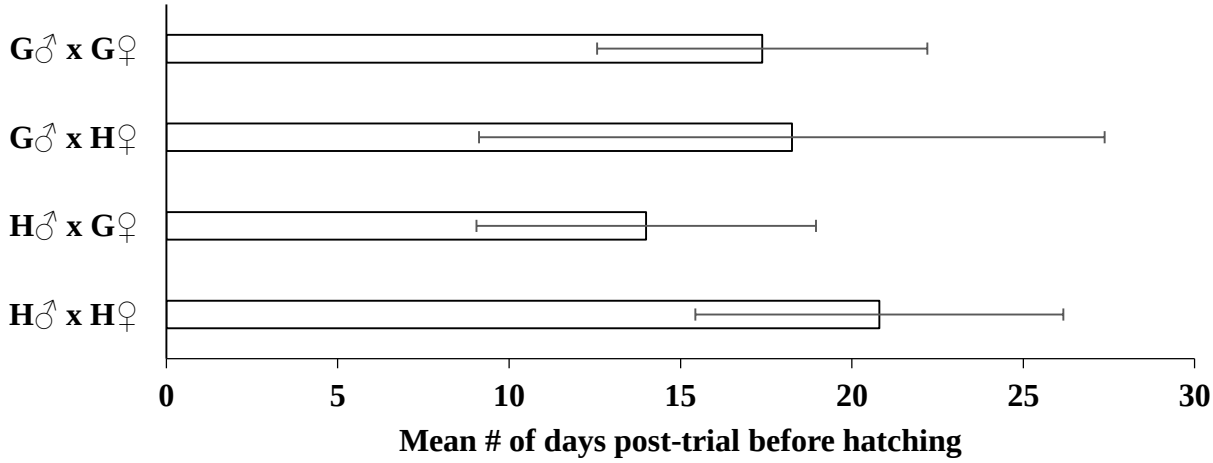

Figure 6. Average time to first hatching for each cross type as seen in crosses that produced viable fertilized eggs during no-choice breeding trials. Error bars represent standard error.

Table 1. Results from backcross breeding trials.

Cross	n	# of successful matings	Clutch size	# of eggs fertilized	# of embryos hatched
HG♂ x H♀	6	1	19	19	4
H♂ x HG♀	6	0			
HG♂ x G♀	6	1	1	1	1
G♂ x HG♀	6	0			

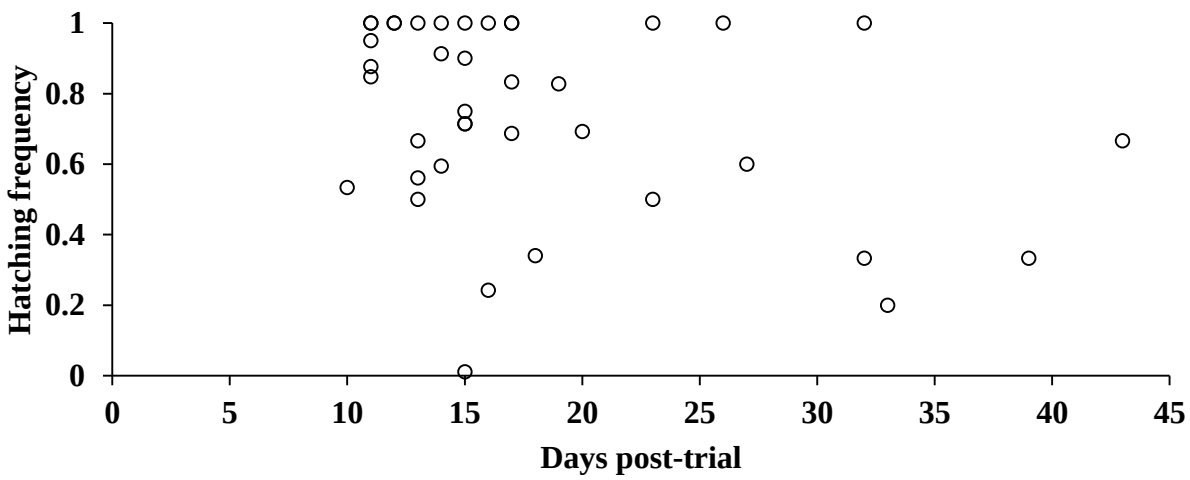

Figure 7. Association between hatching frequency and timing of hatching.

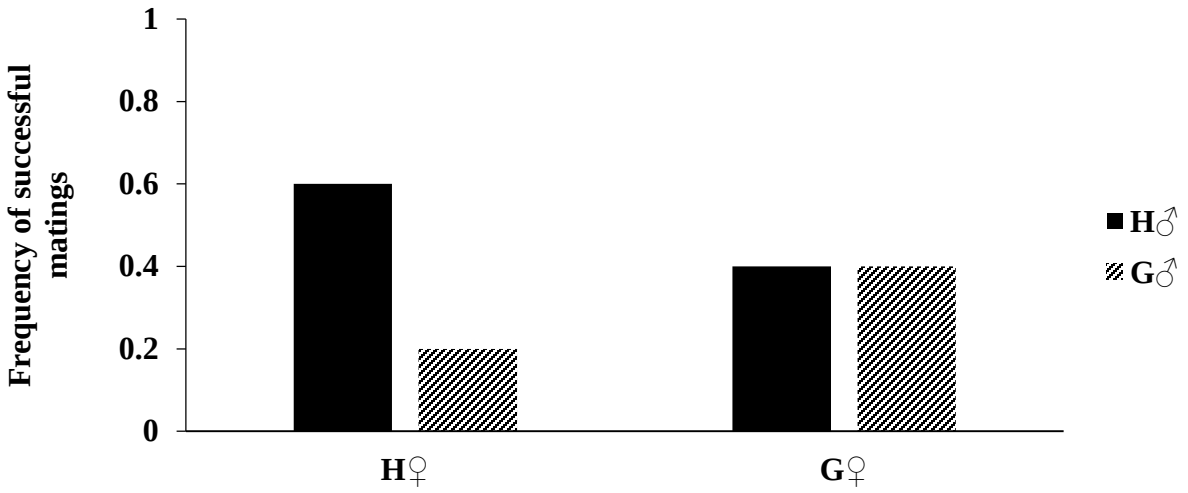

Figure 8. The proportion of choice breeding trials that successfully produced eggs that were sired by either a conspecific or heterospecific male.

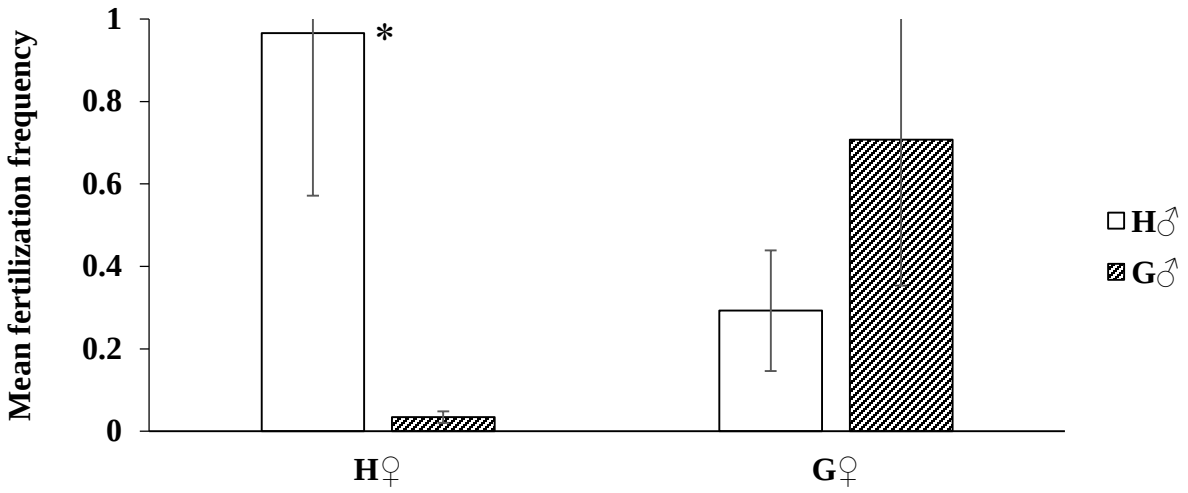

Figure 9. Mean (±se) proportion of eggs fertilized by conspecific or heterospecific males during choice breeding trials. Significant differences are denoted by asterisks

Table 2. Relative strengths and contributions of pre-zygotic and post-zygotic reproductive isolation between *F. heteroclitus* (*F. h*) and *F. grandis* (*F. g*) as seen under no-choice and choice laboratory breeding conditions. Post-zygotic isolation was measured using the embryos that were successfully produced under no-choice conditions, and the values obtained were used to calculate total reproductive isolation under both no-choice and choice conditions. The daggered values represent the range of the relative contribution of mating pre-zygotic isolation when fertilization isolation is included (high value) and excluded (low value) from total relative reproductive isolation in *F. grandis* under no-choice conditions.

	Relative RI Strength				**Relative RI Contribution**			
	No-Choice		**Choice**		**No-Choice**		**Choice**	
Isolating Barrier	*F. h*	*F. g*	*F. h*	*F. g*	*F. h*	*F. g*	*F. h*	*F. g*
Pre-zygotic								
Mating	0.280	0.291	0.500	0.000	0.443	0.914-1.401†	0.508	0.000
Fertilization	-0.025	-0.163	0.938	0.415	-0.028	-0.554	0.476	0.948
Post-zygotic								
Hatching	0.501	0.038	0.501	0.038	0.793	0.153	0.016	0.052
Total Relative Reproductive Isolation	0.615	0.208	0.984	0.437				

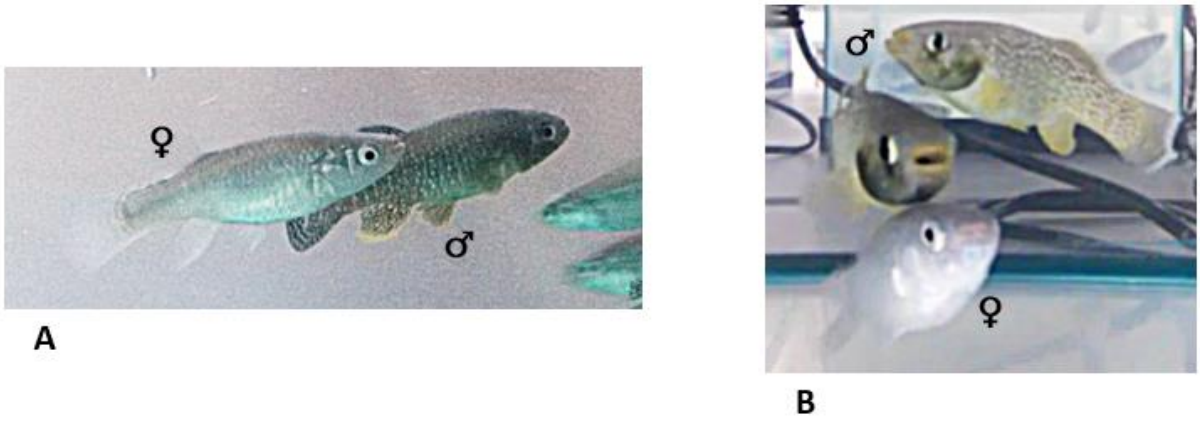

Figure 10. Differences in male nuptial coloration observed in *F. heteroclitus* (A) and *F. grandis* (B).

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