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Effect of Reinforcement Selection on Mate Selection Rates of *Gambusia affinis* and *Gambusia holbrooki*

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Effect of Reinforcement Selection on Mate Selection Rates of *Gambusia affinis* and
Gambusia holbrooki

by

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Abstract

This study was conducted in order to investigate the effects that reinforcement selection has on non-hybridized and hybridized populations of two separate species of *Gambusia* (*G. affinis* and *G. holbrooki).* In order to examine if organisms from a population that had been previously exposed to hybridization would choose mates so as to not produce species hybrid offspring, 29 trials consisting of 9 different mate preference combinations and 87 individuals were recorded and analyzed. Once mate preference was determined for each female used in each trial, the effect that reproductive isolation had on reinforcement in hybrid zones was analyzed. The results yielded evidence that contradicted and supported published literature discussed in this study. Major conclusions included the understanding that many more trials need to be conducted in order to accurately understand the effects of reinforcement selection on *Gambusia* spp. populations.

Dedication

I dedicate this to my mother and father for always believing in me.

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List of Abbreviations

Ff   Florida female

Fm   Florida male

Mf   Mississippi female

Mm   Mississippi male

Tf   Texas female

Tm   Texas male

MS   Mississippi

FL   Florida

TX   Texas

p-value   Calculated probability

R score   Preference score indicates the average location of the female within the tank during trial observations.

Introduction and Literature Review

A hybrid organism is the offspring of two divergent species that often has lower fitness than the parent species (Abbot et al., 2013; Kawakami *et al.*, 2012). Secondary contact occurs when two species, which were previously separated by a physical barrier, come together and have a portion of their range overlap (Durand *et al.*, 2009). Once secondary contact and inbreeding occur, the two species either mate amongst each other (hybridization), or they do not (complete reproductive isolation) (Kawakami *et al.*, 2012; Wilk and Horth, 2016; Wright, 2002). If the two species were able to mate and produce offspring, those offspring would be considered hybrids. Hybridizing populations can progress to a few outcomes: hybrid equilibrium, species fusion, reinforcement selection, and hybrid speciation. Reproductive isolation is an outcome of reinforcement, which occurs when the hybrid organism has lower fitness than that of both parent species, losing the ability to survive and reproduce through natural selection (Wilk and Horth, 2016; Mayfeild and Levine, 2010). Coexistence of similar species within a hybrid zone can also result in species fusion when two parent species produce hybrids, and then the gene pool of the species converges over time (Wright, 2002). Species fusion can occur within a hybrid zone when hybrid fitness is not less than the fitness of both parent species and when there is no reproductive isolation (Wright, 2002). When parent populations continually produce hybrids with reduced fitness, while also producing parent population offspring with normal fitness within a hybrid zone, then the environmental condition produced would be hybrid equilibrium (Wright, 2002). Hybrid equilibrium generally occurs when the rate of hybridization is low, or only occurring within a small range of overlap between large populations. Thus, two species with large ranges but a small

hybrid zone could produce many hybrids within the area of overlap, however, because the species have large ranges there would be no effect on the broader populations (Moore, 1977). Another result of similar species interbreeding within a hybrid zone is hybrid offspring becoming reproductively isolated from parent populations. In cases where hybrid offspring are no longer able to reproduce with individuals from parent populations, hybrids are isolated and continue to diverge, forming a new species in a process known as hybrid speciation (Wright, 2002). In some cases, hybrid speciation can occur within a single generation. This process is more common in plants, with an estimated 28% of plant species thought to be of hybrid origin (Whitham *et al.,* 1999).

Insect hybridization rates are lower than those of plants as exhibited by the North American fruit fly, also known as *Drosophila pseudoobscura* (Noor, 1995). Evidence suggests that natural selection against producing hybrids resulted in the reproductive isolation of *D. pseudoobscura* and *D. persimilis* (Noor, 1995). Mosquitofish are a common type of freshwater fish that are named for there affinity for eating mosquito larvae (Batty and Lim, 1999). They inhabit freshwater environments and can withstand varying salinities, which means they have a broad habitat range (Pyke, 2005). The two different species of mosquitofish used in this experiment are Eastern and Western mosquitofish. Eastern mosquitofish, also known as *Gambusia holbrooki,* are found in the southeastern United States along the Atlantic coast from Maryland to Florida and as far west as southeast Mississippi (Pyke, 2005). Western mosquitofish, or *Gambusia affinis,* are found in the western central United States and are found as far as south as the Gulf of Mexico and also in New Jersey (Pyke 2005). The range of overlap of these species is in southwestern Alabama and southeastern Mississippi (Pyke 2005).

The species *Gambusia affinis* and *Gambusia holbrooki* can produce hybrids with one another, as well as occur sympatrically (Scribner, 1993). They were classified together under the species *Gambusia affinis holbrooki* until they were divided into separate species (Wooten et al. 1988). *Gambusia holbrooki* and *Gambusia affinis* are sexually dimorphic organisms, meaning that males and females exhibit different physical characteristics (Batty and Lim, 1999). For example, the anal fin of males develops into the gonopodium, which functions to aid in the transfer of sperm during fertilization; females form a dark and round birth spot, located on the abdomen (Batty and Lim, 1999). Additionally, females can grow to lengths of 6 cm while males only reach 3.5 cm in length, which allows them to be easily distinguished from one another (McDowall, 2000). Females have a gestational period of three to four weeks. Immature *Gambusia spp.* male individuals reach sexual maturity at one month and females at six to seven weeks after birth (McDowall 2000).

Hybrid offspring from *G. affinis* and *G. holbrooki* are typically less fit than organisms from their parent species (Edmands, 2006). The fitness of two organisms can be measured and compared by assessing how many offspring each organism produces, thus it is understood that the hybrid offspring of *Gambusia* spp. do not produce as many offspring as nonhybrid individuals (Edmands, 2006). Individuals that are able to produce more viable offspring are considered most fit (Brown et al. 1993). Intrinsic processes such as inbreeding depression occur when genetic recombination causes gene complexes to change, which gives the gene complexes the potential to result in harmful mutations (Orr, 1996). Evolutionary theory would predict that reinforcement selection would result in lower rates of hybridization in sympatric populations (Scribner and Avise, 1994;

Brown et al. 1993). Because *Gambusia* spp. produce hybrid offspring with reduced fitness, evolutionary theory would predict there should be selection against hybridization in contact zones (Scribner and Avise, 1994; Brown et al. 1993).

A 1994 study conducted by Scribner and Avise showed that a virgin female *G. holbrooki* preferred males of its own species rather than mates from *G. affinis*. This study found that *G. holbrooki* and *G. affinis* hybridize when placed in contact with each other, but over time the population shifted toward a preference of producing pure *G. holbrooki* offspring rather than hybrids (Scribner and Avise, 1994). The results support the hypothesis that, because the hybrid offspring are less fit, selection should be against their breeding (Duvernell and Schaefer, 2013). Organisms from *G. holbrooki* exhibit different mate selection patterns than organisms from *G. affinis* (Wilk and Horth, 2016). Studies found that *G. holbrooki* were more likely to select mates from their species, while *G. affinis* does not show a mating preference among Gambusia species (Scribner and Avise, 1994). This can be concluded from a 2007 study conducted by Deaton, which showed *G. affinis* selecting mates randomly from both populations of *G. affinis* and populations of *G. holbrooki* (Deaton, 2007). Because both species used in this study can hybridize with each other with different mate preferences, they are ideal model organisms.

In this study, *G. affinis* and *G. holbrooki* parent species were collected from water sources in Texas, Florida, and Mississippi, encompassing a range overlap of the two species. In this study, a contact zone will refer to an area where both *G. affinis* and *G. holbrooki* are known to coexist, meaning that the populations had been exposed to hybridization. Populations outside of the contact zone refer to populations that had not been exposed to hybridization. The Texas, Mississippi, and Florida populations were

housed in separate tanks in the wet lab at The University of Southern Mississippi, Hattiesburg. It was important that the specimen be virginal, because evidence from previous studies suggests that experience is a factor in mate selection (Bisazza *et al*., 2011). Virginal males and females from each population collected were introduced to each other during observation, and the data obtained from these trials was used to determine if reinforcement selection that occurs within hybrid zones affects hybridization rates of *Gambusia* spp. that had not been previously exposed to the other. This study aims to test the hypothesis that reinforcement selection should alter mate choice in contact zone populations.

This study is important because it further explores the topic of reinforcement selection in addition to hybrid zone dynamics. Other studies that have been conducted have focused on different species or have not discussed the effects of reinforcement. One article investigated the dynamic of topminnows in contact zones (Durvernell and Schaefer, 2013). This study also analyzes hybrid zone dynamics but instead of using topminnows as the specimen of interest, we are using *Gambusia*. This study is unique because it involves two different species from the genus *Gambusia* and the resulting hybrid offspring.

Materials and Methods

Before this study, mature organisms were collected from within a contact zone in Simmons Bayou located in Ocean Springs, Mississippi. Organisms were also collected outside of contact zones in Galveston Bay, Texas and the Yellow River drainage system in Florida (Table 1). The organisms were housed in large holding tanks in the basement wet lab of Walker Science Building at The University of Southern Mississippi in

Hattiesburg, MS. Populations were fed freeze dried blood worms once a day and were kept on 16 h : 8 h light cycle at a constant temperature of approximately 24.4 °C. While in the holding tanks, mature males and females from the same population were able to breed and interact.

Table 1: Parent Population Location of Collection and Rate of Hybridization

Summarizes populations collected in each location and whether those locations were part of hybrid zones.

Species Collected	Location of Collection	Hybridization in Location of Collection
Gambusia affinis	Galveston Bay, Texas	Not within contact zone
Gambusia holbrooki	Yellow River Drainage, Florida	Not within contact zone
G. holbrooki and *G. affinis*	Simmons Bayou, Mississippi	Within contact zone

Populations were contained in the holding tanks for more than three weeks before gravid female collection. Females can store sperm, which enables them to produce offspring up to a year after sexual contact with a male. This means that gravid females could have been fertilized while in the holding tanks or before they were collected. Virgin offspring were obtained by procuring individual gravid females from each holding tank. Each gravid female was then housed within a rearing container inside separate 10-gallon aquarium tanks. The water was kept at 26 °C, and each 10-gallon tank contained a submersible aquarium heater, sponge filter, and an air pump. Each 10-gallon aquarium held one gravid female within a rearing chamber until the female gave birth to healthy offspring, in which case the female was returned to the holding tank containing the parent population and the virgin offspring were free to roam within the aquarium. Females have

a tendency to prey on their own young immediately following birth, which is why the females were removed from the aquarium closely following parturition during this study (Krumholz 1948). Offspring were fed newly hatched brine shrimp for three to four weeks until they were mature enough to eat freeze-dried blood worms. Offspring were separated into males and females when they were large enough to be reliably sexed, approximately four weeks after birth. They needed to be separated to eliminate previous sexual interaction as a variable in mate selection. These virginal specimens were counted and divided into six separate 15-gallon aquariums that corresponded to the gender and parent collection location of the offspring: Mississippi females (Mf), Mississippi males (Mm), Florida females (Ff), Florida males (Fm), Texas males (Tm) and Texas females (Tf).

The conductivity of each tank was kept between 400 and 1500 μS, measured using a YSI Model Professional Plus Conductivity Meter (Xylem).

Table 2: Individuals Available for Observations from Reared Groups

Sample size of virgin males and females from each population.

Tank Number	Origin Population and Sex	Tank Abbreviation	Number of Individuals
1	Mississippi Female	Mf	36
2	Mississippi Male	Mm	18
3	Florida Female	Ff	35
4	Florida Male	Fm	15
5	Texas Female	Tf	38
6	Texas Male	Tm	30

Underneath the 10-gallon observational aquarium tanks, lines (red lines in Figure 1) were drawn that separated the tank into three different compartments. The organisms were acclimated to the observational aquarium climate for 12-14 h before each trial was recorded. The aquariums were filled with water of appropriate salinity, and the experimental environment was kept on a constant 16 h : 8 h photoperiod and at a constant temperature of approximately 24.4 °C. The aquarium tanks were separated by opaque surfaces to ensure that the trials taking place in neighboring aquariums did not have an impact on the behavior of the observed specimen. GoPro Hero 3 waterproof cameras (GoPro) were positioned two feet above each tank and were used to record each trial. Numerical values that corresponded with the compartment in which the female was housed (bottom: 1, middle: 2, top: 3) were recorded every 15 s over a 15 min time period. Averages of those numerical values were used to determine the assumed mate preference for each female. The null hypothesis for this study states that the females would show no mate preference and exhibit an average preference score of 2.

Graphic Representation of Experimental Setup as Viewed from Above
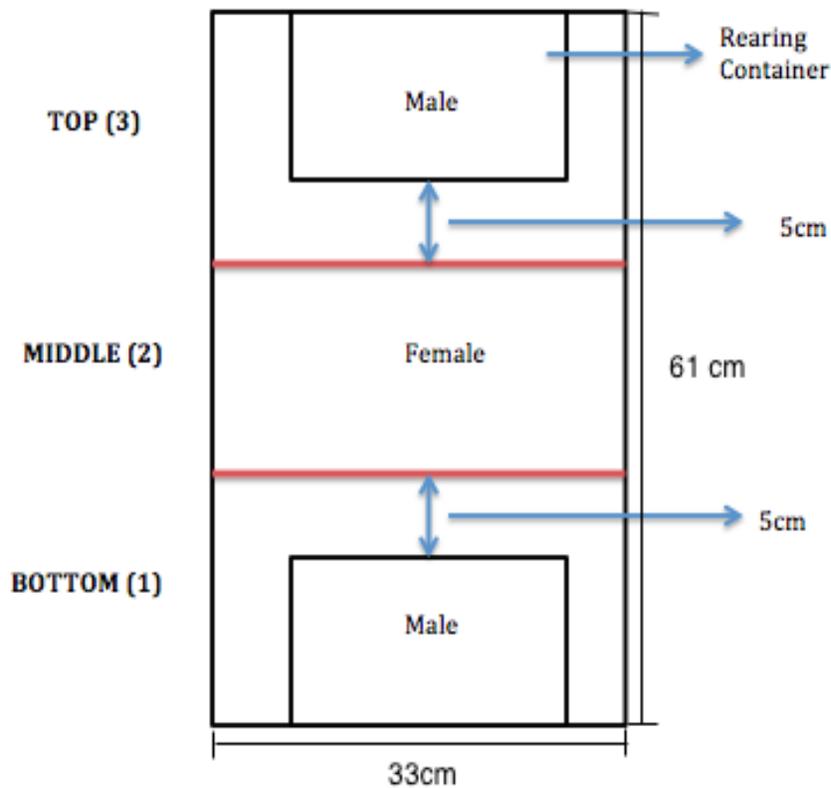
Figure 1: Illustration of the tank choice chamber set-up used for each trial. The numbers associated with the chambers of the 10-gallon tank were used to quantify mate preference for each female.

Nine different combinations of organisms were tested for mating preference during this study. The virginal specimens from each reared population were exposed to other virginal specimens from the other groups of bred organisms. Females from each group were introduced to two males from separate populations, and the position of the female within the tank was recorded in order to determine assumed mate preference.

Table 3: Mate Selection Trial Combinations and Frequencies

The trial combinations and frequency with which each combination was able to be

repeated

	Mississippi females	**Florida females**	**Texas females**
Texas and Mississipi males (n=4)	Tm-Mf-Mm	Tm-Tf-Mm	Tm-Ff-Mm
Texas and Florida males (n=3)	Tm-Mf-Fm	Tm-Tf-Fm	Tm-Ff-Fm
Mississippi and Florida males (n=2)	Mm-Mf-Fm	Mm-Tf-Fm	Mm-Ff-Fm

For each different mate selection trial combination (Table 3), the indicated

virginal male was contained within a floating transparent rearing box while the other

virginal male was captive in the rearing box at the opposite end of the tank as shown in

Figure 1. Each rearing container was transparent and had small openings that allowed

water to flow. Water flow was important because it made chemical interactions between

the males and female possible during acclimation and observations. Once both males

were secured within their respective containers, the female was free to swim within the

aquarium. After the 12-24 h acclimation period, the timed observation trials were

recorded for each repetition of the mate selection trial combinations. Table 2 represents

the orientation of each combination and the number of times each organism combination

was observed. The number of repeats for each trial was determined by the number of

virgin males and females that each population was able to produce, which is listed in

Table 2. The number of offspring was not uniform throughout the different groups, so an

unbalanced design was used in order to take advantage of all the individuals that were

available for observations.

Following the trials, the 81 specimens used during experimentation were

preserved in a formalin solution, which consisted of 10% formaldehyde and 90% water.

Each Mississippi female was individually preserved in 20 ml of the formalin solution

within separate 50-ml Falcon tubes following each trial. The remaining 72 organisms

were preserved together within a jar containing 50 ml of the formalin solution. The

species of each Mississippi female was determined after preservation of the organisms.

Results

A score of 2 would indicate no preference for either males. For preference to be

considered statistically significant, the p-value calculated from a one-sample t-test should

be less than 0.05.

During trials when *G. holbrooki* from the Mississippi (MS) population was

exposed to MS males and TX males the average preference (R) recorded was 2.51 with a

standard deviation of 0.41. A one-sample t-test with a 95% confidence interval was

performed, and it yielded a p-value of 0.17, which indicated that the preference exhibited

toward organisms from either population was not statistically significant. During trials

when females of the *G. holbrooki* MS population were exposed to FL males and TX

males the average preference could not be recorded. The *G. holbrooki* from the MS

population were not tested against Texas and Florida populations due to the small number

of trials and individuals available, which is addressed in the Discussion. Alternatively, the R score for the MS population *G. holbrooki* when exposed to organisms from trials with MS males and FL males was 2.36 with a standard deviation of 0.19. The p-value (0.73) calculated from a one sample t-test indicated that the preference observed was not significant.

During trials that tested the mate selection of *G. affinis* between MS and TX males the R score was 2.5. The R score indicated a preference for contact zone population organisms, though the preference cannot be distinguished as significant or insignificant because only one trial was observed. In trials that tested mate preference of *G. affinis* MS females between TX males and FL males the R score was 1.94 with a standard deviation of 0.32. The calculated p-value indicated that this preference was not significant.

The females from the Texas population of *G. affinis* did not show a preference for TX males when also exposed to MS males. The *G. affinis* females exhibited a R score of 1.59 with a standard deviation of 0.31. A one-sample t-test yielded a p value of 0.66, which indicated no preference for males from populations outside the contact zone was statistically insignificant. For each Texas female trial the average preference, which can be found in Table 5., was determined to be insignificant based on p values calculated for each organism combination shown in Table 4. The R score of Florida female *G. holbrooki* was 2.13 with a standard deviation of 0.35 when exposed to males from MS populations and TX populations a one-sample t-test was performed, and the p-value (0.41) was greater than 0.05, which indicated that the preference shown had no statistical significance. For each Florida female trial the observed preference, which can be located

in Table 5, was deemed insignificant after one sample t-tests were used to find p values that were consistently above 0.05 (Table 4.).

All results gained from this study were deemed statically insignificant based on the p values garnered from multiple one sample t-tests.

Table 4: Significance Of Mating Preference For Each Trial Combination

Results of one-sample t-tests testing for female mate choice by trial. Values below 0.05 would have indicated statistically significant mate preference. Values listed as 'N/A' indicate that information needed to perform a t-test was not available (addressed in the Discussion).

	Mississippi Females		**Florida Female**	**Texas females**
Trial combinations	*Gambusia holbrooki*	*Gambusia affinis*	*Gambusia holbrooki*	*Gambusia affinis*
Tm-Xf-Mm	0.17	N/A	.35	0.66
Tm-Xf-Fm	N/A	.62	.69	0.47
Mm-Xf-Fm	.73	N/A	.19	0.10

Table 5: Average Preference Observed (P)

Indicates the average preference score that was recorded for each organism combination.

	Mississippi Females		Florida Female	Texas females
Trial combinations	*Gambusia holbrooki*	*Gambusia affinis*	*Gambusia hobrooki*	*Gambusia affinis*
Tm-Xf-Mm	2.51 R	2.5 R	1.73 R	1.75 R
Tm-Xf-Fm	N/A	1.94 R	2.32 R	2.02 R
Mm-Xf-Fm	2.36 R	N/A	2.48 R	1.31 R

Discussion

This study aims to test the hypothesis that reinforcement selection should alter mate choice in contact zone populations. The null hypothesis for this study is that there will be no preference in mate choice for the population that had been previously exposed to hybridization.

The female Mississippi *G. holbrooki* individuals showed a statistically insignificant preference for Mississippi males every time they were placed in the same environment during observations. The R score for *G. holbrooki* was indicative of preference toward males from contact zone populations, but after a one-sample t-test was performed, it was determined that the preference was not significant (Table 4). These results do not support the hypothesis that reinforcement selection decreases the rate at which the species will hybridize for populations that are reared within contact zones. Although preference was observed during experimentation, it was not pronounced enough to be significant, which suggests that more trials need to be conducted in order to gain an accurate understanding of the effect reinforcement selection has on populations collected from contact zones. These preliminary results contradict the findings of Scribner and Avise, which state that, since interspecific hybrids produce less fit hybrid offspring, selection should be against the creation of hybrids (Scribner and Avise, 1994). Female *G. holbrooki* have been noted to prefer male *G. holbrooki* opposed to male *G. affinis* (Scribner and Avise, 1994*).* However, this study found that the females from the Florida population (*G. holbrooki*) preferred male *G. holbrooki* during some trials. Though this correlation was statistically insignificant, it is still important to recognize that this is inconsistent with observations from the Scribner and Avise study where *G. holbrooki* was

observed hybridizing upon contact with *G. affinis* (1994). The same study showed that over time the population began to favor *G. holbrooki* offspring over the production of hybrids (Scribner and Avise, 1994). Therefore, it can be surmised that, had the period of exposure been similar to the one used in the Scriber and Avise study, then the *G. holbrooki* females from the Florida population may have started to prefer males from the Florida population as well.

According to a study conducted in 2016, *G. affinis* were observed exhibiting different mating habits than *G. holbrooki* (Wilk and Horth, 2016). *Gambusia affinis* individuals were observed choosing mates randomly from both *G. affinis* and *G. holbrooki* (Deaton, 2007). The organisms from the Texas population were *G. affinis,* and they were observed preferring males from the Texas population five out of the six times they were exposed to one another. This observation contradicts the mating behavior seen by Deaton in 2007, suggesting that more trials are needed in order to accurately deduce the mating preference of the *G. affinis* females.

The experiment was designed as a mesocosm experiment, meaning that the trials and observations took place within a laboratory, in order to observe interactions between virginal *G. affinis* and *G. holbrooki*, a feat that would not be possible in their natural environment. However, because the specimens were not observed in their natural habitat, variables must be taken into account when their behavior and selection habits were recorded (Abbot *et al*., 2013). Although the mesocosm design of the experiment eliminated some major, otherwise uncontrollable, aspects of mate preference, it was not able to remove the possible exposure of males to the opposite sex. It is possible that a few of the organisms used in this study were not separated by sex as soon as they reached

maturity, which could have allowed for mating interactions. A problem encountered during this study was that the species of the Mississippi females were unknown before trial observations; this meant that there was not a way to determine which species were being used and with what frequency until after the females were preserved. This problem attributed to the lack of data available for some cells in Table 4 and Table 5.

In future studies, it would be beneficial to replicate each organism combination greater than ten times per combination. Increasing replication of trials allows for a more reliable set of data to be produced, because it decreases variability. An increase in replication would result in a smaller standard deviation, which would lead to more statistically valid results than were gained through this study. It would also be interesting to conduct this research by observing two females and a male, and seeing how the results compare to the results yielded from two males and a female. Although literature suggests that females are the determining organisms in mate selection (Scribner, 1993), it would still be interesting to investigate the mate selection based on male preference.

Conclusion

From the results gathered through this study, it can be deduced that more research needs to be conducted to gain a deeper understanding of the relationship between reproductive isolation and reinforcement. The interactions observed between *Gambusia* spp. during trial observations were not statistically significant. If this experiment were to be repeated in the future, it should include many more repetitions, which would not only decrease variability but would also allow for more female *G. affinis* to be observed interacting in trials in which males from contact zone and noncontact zone populations are present.

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