

Males, but not females, contribute to sexual isolation between two sympatric species of *Gambusia*

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Abstract Reproductive isolation restricts genetic exchange between species. Various pre- and post-mating barriers, such as behavior, physiology and gametic incompatibility, have been shown to evolve in sympatry. In certain scenarios, isolation can be asymmetrical, where species differentially prefer conspecifics. We examined sexual isolation via conspecific mate preference between *Gambusia affinis* and *G. geiseri* in both sexes. To investigate male contribution to sexual isolation, we compared the number of mating attempts (gonopodial thrusts) directed at either a conspecific or a heterospecific female, in both species. We also examined sperm priming and expenditure in males in the presence of conspecific or heterospecific females. We then measured female preference for either a conspecific or heterospecific male, in both species. We found that males of both species preferred to mate with conspecific females, but showed no difference in sperm production or expenditure between conspecific and heterospecific females. Females of both species did not prefer conspecific over heterospecific males. Our results suggest that sexual isolation might be mediated by male mate choice in this system and not female choice, suggesting that there is asymmetrical reproductive isolation between the sexes in *G. affinis* and *G. geiseri*, but symmetrical species isolation.

Keywords *Gambusia* · Male mate choice · Sexual isolation · Sperm production

Introduction

Speciation results from the evolution of reproductive isolation between diverging populations. Various pre- and post-mating processes may play a role in the evolution of

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reproductive isolation, including differences in behavior, timing or location of reproduction, gametic incompatibilities, and reduced fitness of hybrid offspring (see Coyne and Orr 2004 for review). Sexual isolation results when there are divergent mate preferences for traits involved in mate choice between closely related taxa (Nosil et al. 2007). Conspecific mate choice has been shown to lead to and maintain reproductive isolation between species in diverse taxa (frogs: Reynolds and Fitzpatrick 2007; fish: Turner 1994; Alexander and Breden 2004; Rafferty and Boughman 2006; Kozak et al. 2009; *Drosophila*: Kaneshiro 1976, Kaneshiro and Boake 1987; Coyne and Orr 1989, 1997; Boake et al. 2000; butterflies: Friberg et al. 2008; salamanders: Arnold et al. 1993; birds: Sætre et al. 1997a, b; crickets: Jang et al. 2007). Sexual isolation can result from phenotypic differences between species that arise via adaptation to different ecological environments (Funk 1998; Jiggins et al. 2001; Rundle and Nosil 2005; Funk et al. 2006; Nosil et al. 2007), via direct selection on mate preferences (Servedio 2001), or via reinforcement (Dobzhansky 1937; Kirkpatrick 2001; Servedio 2004).

Asymmetrical reproductive isolation occurs when one species is more likely to mate with a heterospecific than individuals of the other species and has been demonstrated in *Drosophila* (Kaneshiro 1976), sticklebacks (Rafferty and Boughman 2006) and in salamanders (Arnold et al. 1996; Whiteman and Semlitsch 2005). Although the importance of asymmetries between species in reproductive isolation has been well studied, until recently few studies have examined the potential for asymmetries between the sexes within species in sexual isolation, especially in vertebrates (but see Shine et al. 2004; Sætre et al. 1997b; Kozak et al. 2009). In a theoretical study on sexual conflict over mating and speciation Parker and Partridge (1998) conclude that in conditions of incipient speciation or when previously diverged allopatric populations come into contact, females are expected to act as a force in favor of pre-mating isolation with males acting as a force against it.

As expected, many studies of sexual isolation have focused on examining female mate choice for conspecifics versus heterospecifics, as sexual isolation between sympatric species is thought to be driven by the sex that exhibits more choosiness in mate choice (Wirtz 1999; Kozak et al. 2009). Males of many species are considered to be more indiscriminate in mate choice than females, as they usually have lower costs associated with reproduction (Bateman 1948; Trivers 1972). However, there is increasing evidence that selection has favored male mate choice in many taxa. For example, males may be choosy if they receive direct benefits associated with the choice, such as more offspring from mating with more fecund females (Parker 1983; Olsson 1993; Verrell 1995; Monaghan et al. 1996; Jones et al. 2001; Bonduriansky 2001; Werner and Lotem 2003; Herdman et al. 2004). In addition, there could be pleiotropic effects of preference genes that result in the expression of mate choice in both sexes (Servedio and Lande 2006; Servedio 2007). The direct benefits of male mate choice may also vary, depending on the phenotype of the choosy individual (Basolo 2004). The existence of male mate choice could lead to sexual isolation between species if the female traits that males prefer differ between species (Hubbs and Delco 1960; Wiernasz 1995; Seehausen et al. 1999; Deering and Scriber 2002; Shine et al. 2004; Albert and Schluter 2004; Wong et al. 2005). Male mate choice may also arise as a result of considerable costs associated with sperm production (Dewsbury 1982; Nakatsuru and Kramer 1982; Shapiro et al. 1994; review in Wedell et al. 2002; Aspbury and Gabor 2004a, b). Physiological responses in the form of changes in gamete production and transfer may play a role in sexual isolation if males produce or transfer more sperm to conspecific females (Eady 2001; Aspbury and Gabor 2004a; Schlupp and Plath 2005; Reinhardt 2006; Robinson et al. 2008).

Here we present a study on sexual isolation between two sympatric species of live-bearing fish, the western mosquito fish, *Gambusia affinis* and the largespring mosquito fish, *Gambusia geiseri*. These two species are closely related (Lydeard et al. 1995), and they are sympatric in parts of Texas, including the San Marcos River, Hays County, Texas (Davis 1978). The natural western geographic limit of *G. affinis* passes through Texas, but it has also been said to have a “catholic ecology” by Hubbs and Delco (1960) as this species is widely distributed in the southeastern United States and throughout the world given that it has been largely introduced to many bodies of water for mosquito control (Krumholtz 1948; Lee 1980). *Gambusia geiseri*, on the other hand, are generally restricted to clear spring habitats in San Marcos and Comal springs, Texas (Hubbs and Springer 1957). Despite these habitat differences both species are syntopic in parts of the San Marcos River, Texas.

There is evidence for both female and male choice in *G. affinis*: female *G. affinis* prefer to associate with larger males (Hughes 1985) and males with larger gonopodia (Langerhans et al. 2005) and male *G. affinis* prefer larger females (Deaton 2008). Males in the genus *Gambusia*, including *G. affinis* and *G. geiseri* exhibit little pre-copulatory courtship behavior, but rather rely on coercive mating attempts (Pilastro et al. 1997; Plath et al. 2007; C. E. pers. obs.). In most poeciliids, females are only receptive for about 2 days out of 30 day cycles so most females are not receptive and males generally spend time searching for receptive females. Most poeciliids also store sperm and exhibit multiple paternity (Constantz 1989). Female *G. affinis* have specifically been shown to store sperm and exhibit multiple paternity (Robbins et al. 1987). There is, to date, no information on conspecific mating preferences of male or female *G. geiseri*. Hubbs and Delco (1960) found that male *G. affinis* ($n = 6$) prefer to mate with conspecific females over female *G. geiseri*. Male *G. geiseri* ($n = 3$) preferred to mate with conspecific females over female *G. affinis*. *Gambusia affinis* has been shown to hybridize with both *G. heterochir* (Hubbs 1957) and *G. holbrooki* (Scribner and Avise 1994). These three species, along with *G. geiseri* form a monophyletic group (Lydeard et al. 1995). It has yet to be shown, beyond anecdotal evidence (Hubbs and Delco 1960), whether or not *G. affinis* and *G. geiseri* can produce hybrid offspring. To examine sexual isolation in this system, we examined male mate choice, male sperm expenditure and production, female association preference, and the potential for hybridization in *G. affinis* and *G. geiseri*.

Materials and methods

Males and females of both *G. affinis* and *G. geiseri* were collected together in mixed sex and species shoals at the same locale in the San Marcos river (Hays County, Texas: 29.89 N, 97.93 W) in May–July 2007 and February 2008. Fish were maintained on a 14 h light:10 h dark cycle using UV lighting (40WCoralife Day-Max Aquarium daylight) and fed Ocean Star International Inc. Spirulina Flake mixed with Ocean Star International Inc. Freshwater Flake food twice daily and supplemented daily with live brine shrimp. All experimental data were collected during the mating season of each species (May–August 2007 and March 2008) between 0800 and 1,700 h. Because male mating behavior is affected by the stages of the female reproductive cycle (Park and Propper 2002), we used non-gravid females that were separated from males for at least 30 days (one reproductive cycle). All males were also separated from females for at least 7 days. All statistical analyses were done using JMP version 7 software (SAS Institute, Cary, North Carolina, USA).

Experiment 1: Male mate choice by *G. affinis* and *G. geiseri*

This experiment was designed to test the hypothesis that male *G. affinis* and *G. geiseri* exhibit conspecific mate preferences. At the beginning of each trial, individual male *G. affinis* ($n = 25$) or *G. geiseri* ($n = 25$) were placed in an acclimation chamber (a clear plastic 1 l cylinder) in the center of a 38 l aquarium ($54 \times 29 \times 33$ cm) that also housed a pair of size matched (within ± 1 mm standard length—SL) females (one heterospecific female and one conspecific female) (see Fig. 1a). After a 10 min acclimation period, the male was released and the fish were allowed to freely interact. After the male's first gonopodial thrust (thrusts), the number of thrusts directed at each female were counted for 10 min. We did not include males that did not thrust ($n = 1$ male *G. affinis* and $n = 2$ male *G. geiseri*) in the statistical analyses. To examine if there are male mating preferences, we used a paired *t*-test to compare the number of thrusts directed at females of the two species for both species of males. We then compared the strength of preference (SOP) for conspecifics (number of thrusts directed at conspecific females/total thrusts directed at both species) between male *G. affinis* and *G. geiseri* using an unpaired *t*-test. SOP data were arcsine transformed to meet assumptions of parametric analyses. We also tested whether or not the mean SOP was significantly greater than 0.5 (no preference) using a *Z*-test for each male species. Values of SOP greater than 0.5 indicate a conspecific preference, whereas values below 0.5 indicate a heterospecific preference. We examined the potential for species differences in mating effort by comparing the total number of thrusts by males of both species using an unpaired *t*-test.

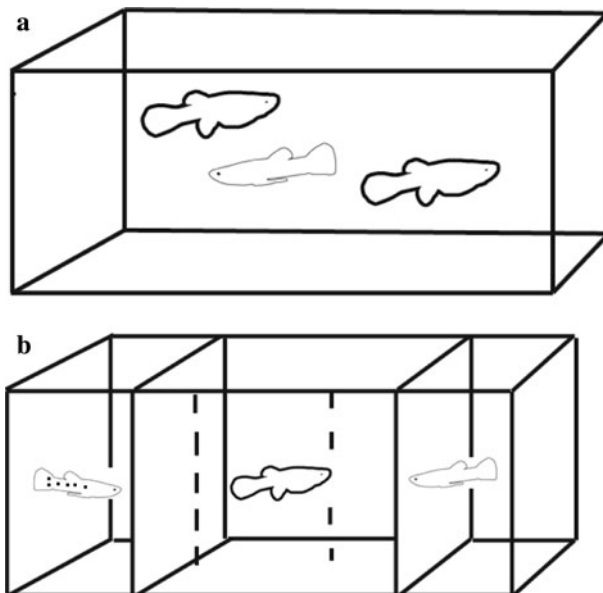


Fig. 1 Experimental setup for: **a** Experiment 1: male mate choice, **b** Experiment 3: female association preference. Dashed lines indicate preference zones. Experiment 2 was set-up like (a) but with one male and one female and a divider before mating was allowed. Drawings of females are darker than males, the spotted male is *Gambusia geiseri*

Experiment 2: Male sperm production and expenditure by *G. affinis* and *G. geiseri*

This experiment was designed to test the hypothesis that male *G. affinis* and *G. geiseri* exhibit conspecific mate preferences that are manifested by differential sperm priming and expenditure. On day 0 of the experiment, male SL was recorded and sperm was extracted from the focal males to establish the baseline sperm count from male *G. affinis* ($n = 50$) and *G. geiseri* ($n = 30$). Sperm extraction methods followed established protocols for poeciliid fishes (Aspbury and Gabor 2004a, b). The sperm cells collected from each male were placed into microcentrifuge tubes with 0.9% saline solution. Sperm cells were counted five times on an improved Hy-Lite Neubauer chamber hemocytometer under $400\times$ magnification. The total number of sperm cells was determined by multiplying the mean cell count by the sample's initial volume and dividing by the volume of the hemocytometer. All sperm samples were coded so that sperm counts were conducted blind to the experimental treatment (species of the female). By stripping available sperm from all males on day 0, subsequent sperm collections should only be affected by the treatment.

After sperm extraction, individual males were placed in separate aquaria with either a conspecific (one half of the males of both species) or a heterospecific female (one half of the males of both species). The male and female were separated by a Plexiglas divider that allowed for the potential passage of both chemical and visual cues of the female. After 3 days (with no tactile interactions between the two fish), the males were removed and sperm was stripped from each male. The extraction on day 0 (baseline) was subtracted from day 3 to determine the sperm priming response (Bozynski and Liley 2003). A positive number indicates a male increasing sperm production in the 3 days with the female stimulus, relative to the male's baseline. The male was returned to the testing aquarium for another 3 days with the same female to replenish sperm reserves (Aspbury and Gabor 2004a). On day 6 the divider was removed and the male and the female were allowed to interact for 10 min, during which time the total number of thrusts were recorded. After the mating trial, the males were removed from the aquarium, and sperm was again collected from all males. The day 6 sperm count is a measure of sperm availability post-mating (i.e., available sperm = the inverse of expenditure: Robinson et al. 2008). Males that did not have detectable sperm cells from samples collected on each day of sampling were excluded from analyses ($n = 2$ male *G. geiseri* and $n = 5$ male *G. affinis* were excluded on this basis).

To examine male mate preference for each species of male, we compared the total number of thrusts to conspecific and heterospecific females using a Wilcoxon signed rank test because no transformations of the thrust data resulted in meeting the assumptions of parametric analyses. To examine differential male sperm priming and expenditure for each male species we compared the total sperm cells primed, as well as the sperm cells remaining after mating for conspecific versus heterospecific females using unpaired *t*-tests.

Experiment 3: Female association preference for *G. affinis* and *G. geiseri*

This experiment was designed to test the hypothesis that female *G. affinis* and *G. geiseri* exhibit conspecific mate preferences. All trials were conducted in a 38 l aquarium ($54 \times 29 \times 33$ cm) divided into three compartments: a central compartment for the female (34 cm), and two outer compartments (10 cm) that each housed a heterospecific and a conspecific male. The outer compartments were separated from the center compartment (housing the female) with perforated plexiglas dividers, which allowed females to potentially assess both visual and chemical cues of the males. The middle 18 cm of the

central compartment was the no preference zone, and the 4 cm areas adjacent to each male side were the preference zones (see Fig. 1b). At the beginning of each trial one male *G. geiseri* and one male *G. affinis* were placed in the outer compartments (random placement of males in left or right compartment), and a female *G. affinis* ($n = 25$) or *G. geiseri* ($n = 25$) was placed in the center of the aquarium under an acclimation chamber. The males were size matched within ± 1 mm SL for all trials. After a 10 min acclimation period the female was released. When a female swam within 4 cm of either male side, timing was initiated. The amount of time the female spent in each preference zone was recorded for 10 min. To control for side bias, the males were switched between the end compartments after the first 10 min trial, and a second 10 min trial was initiated after another 10 min acclimation period. To compare association time with a heterospecific male and a conspecific male for each species, we compared the time that a focal individual spent on the right side of the tank within trials and between treatments using a paired *t*-test. This method of data analysis provides statistical independence of data for individual test fish (Gabor 1999). We also compared the SOP of both species for conspecifics (total association time with conspecific/total time associating with both males) between the two species using an unpaired *t*-test. SOP data were arcsine transformed to meet assumptions of parametric analyses. We also tested whether or not the mean SOP was significantly greater than 0.5 (no preference) using a Z-test for each female species. Values of SOP greater than 0.5 indicate a conspecific preference, whereas values below 0.5 indicate a heterospecific preference. We examined the potential for species differences in mating effort by comparing the total time associating with males by females of both species using an unpaired *t*-test.

Experiment 4: Hybridization

We examined the potential for hybridization since there were no studies to date (barring one uncertain example in Hubbs and Delco 1960) indicating whether hybridization between these species occurs. To examine this issue, we set up aquaria (18.93 l; $n = 10$) with heterospecific pairings and allowed them to interact for 30 days, during their mating season. Males and females were chosen at random (5 *G. affinis* males with 5 *G. geiseri* females and its reciprocal) and had been previously isolated for 30 days to control for any effects of female gravidity on male behavior (Park and Propper 2002). Fish were observed at least twice a day during morning and afternoon feedings.

Results

Experiment 1: Male mate choice by *G. affinis* and *G. geiseri*

Males of each species thrust significantly more towards conspecific females than towards heterospecific females (paired *t*-tests: *G. affinis*: $n = 24$, $t = -5.384$, $P < 0.0001$, Fig. 2a; *G. geiseri*: $n = 23$, $t = -3.070$, $P = 0.006$, Fig. 2b). Strength of preference for conspecific females did not differ between species (unpaired *t*-test: $t = 0.701$, $P = 0.487$; *G. affinis*: $n = 24$, \bar{X} SOP ± 1 SE = 0.785 ± 0.047 ; *G. geiseri*: $n = 23$, \bar{X} SOP ± 1 SE = 0.793 ± 0.048 ; Fig. 3b). For both species the SOP was significantly greater than 0.5 (*G. affinis*: Z-test: $n = 24$, $Z = 7.884$, $P < 0.0001$; *G. geiseri*: Z-test: $n = 23$, $Z = 5.083$, $P < 0.0001$; Fig. 3b). Male *G. affinis* and male *G. geiseri* did not differ in mating effort (unpaired *t*-test: $t = -1.870$, $P = 0.068$; *G. geiseri*: $n = 23$, \bar{X} total thrusts ± 1 SE = 20.174 ± 2.893 , *G. affinis*: $n = 24$, \bar{X} total thrusts ± 1 SE = 27.750 ± 2.832).

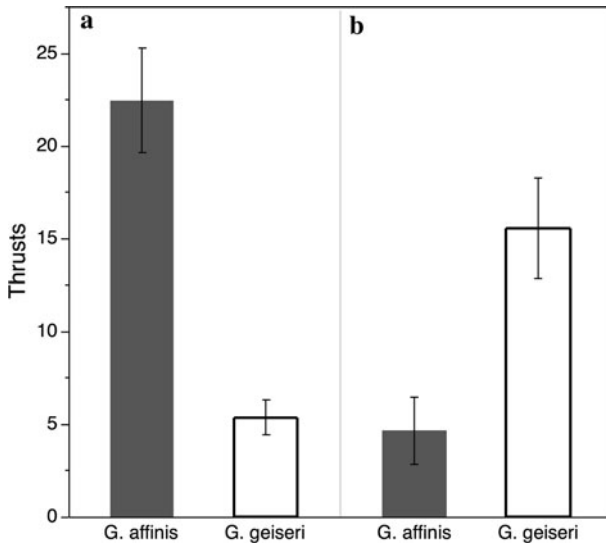


Fig. 2 Mean \pm SE male gonopodial thrusts (*thrusts*) directed at conspecific and heterospecific females by male **a** *Gambusia affinis*, and **b** *Gambusia geiseri*

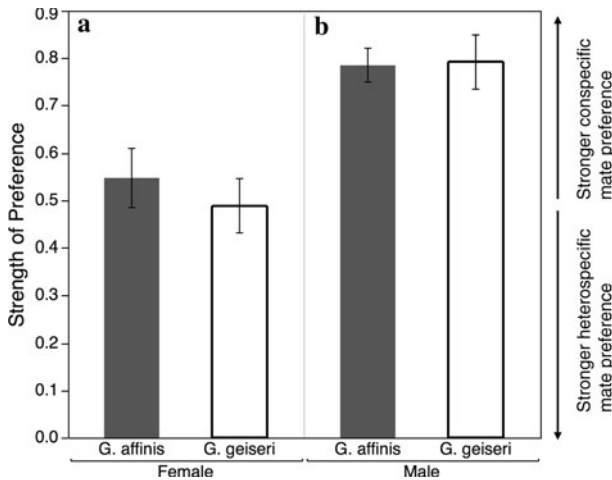


Fig. 3 Mean \pm SE strength of preference (*SOP*) for conspecifics by **a** Females and **b** Males for both *Gambusia affinis* (grey) and *G. geiseri* (white). For females *SOP* = the amount of time spent associating with a conspecific male/total time associating with both males. For males *SOP* = the number of gonopodial thrusts directed at conspecifics/total gonopodial thrusts to both females

Experiment 2: Male sperm production and expenditure by *G. affinis* and *G. geiseri*

Both species of males thrust significantly more towards conspecific females (Kruskal–Wallis tests: *G. affinis*: $n = 45$, $H = 5.61$, $df = 1$, $P = 0.018$; *G. geiseri*: $n = 28$, $H = 7.575$, $df = 1$, $P = 0.006$). Neither species of males differentially primed sperm

given a conspecific or heterospecific female stimulus (unpaired t -tests: *G. affinis*: $n = 45$, $t = 0.133$, $P = 0.895$; *G. geiseri*: $n = 28$, $t = 1.122$, $P = 0.276$). There was no significant difference between the female species treatments in sperm availability after mating (day 6) for either species (unpaired t -tests: *G. affinis*: $n = 45$, $t = -1.263$, $P = 0.215$; *G. geiseri*: $n = 28$, $t = -0.476$, $P = 0.638$). There were no significant correlations between thrusting, priming, or sperm available after mating (spearman rank correlations within treatments/species all $P > 0.05$).

Experiment 3: Female association preference for *G. affinis* and *G. geiseri*

Females of both species did not associate significantly more with conspecific or heterospecific males (paired t -tests: *G. affinis*: $n = 24$, $t = -0.021$, $P = 0.984$; *G. geiseri*: $n = 25$, $t = 0.219$, $P = 0.829$). Strength of preference for conspecific males did not differ between species (unpaired t -test: $t = 0.243$, $P = 0.810$; *G. affinis*: $n = 24$, \bar{X} SOP ± 1 SE = 0.547 ± 0.301 ; *G. geiseri*: $n = 25$, \bar{X} SOP ± 1 SE = 0.488 ± 0.287 ; Fig. 3a). For both species the SOP was not significantly greater than 0.5 (*G. affinis*: Z -test: $n = 24$, $Z = 0.765$, $P = 0.444$; *G. geiseri*: Z -test: $n = 25$, $Z = -0.209$, $P = 0.834$; Fig. 3a). Female *G. geiseri* spent significantly more time associating with males than female *G. affinis* (unpaired t -test: $t = 2.436$, $P = 0.019$; *G. affinis*: $n = 24$, \bar{X} total association time (s) ± 1 SE = 373.217 ± 44.305 ; *G. geiseri*: $n = 25$, \bar{X} total association time (s) ± 1 SE = 522.76 ± 42.496 ; Fig. 4).

Experiment 4: Hybridization

At the end of 30 days, no broods had been dropped in any of the aquaria, nor were any females gravid, suggesting that hybridization does not occur between these species.

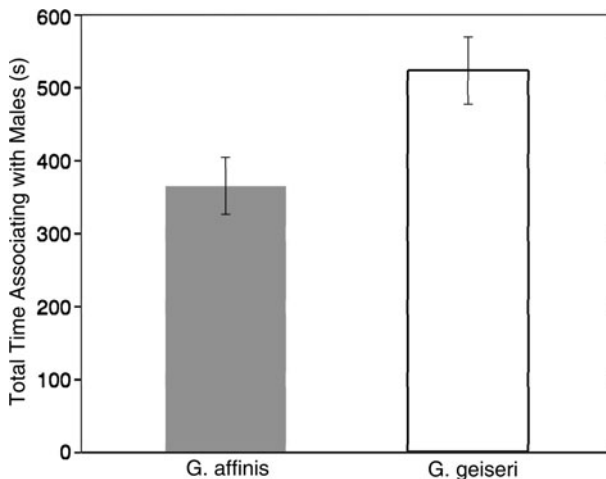


Fig. 4 Mean (s) \pm SE female association time with both males by female *Gambusia affinis*, and *Gambusia geiseri*

Discussion

When we examined sexual isolation in a syntopic population of *G. affinis* and *G. geiseri* we found that males of both species prefer to mate with conspecific females in both simultaneous and sequential mate choice trials. These results corroborate those found earlier by Hubbs and Delco (1960) but with more substantial sample sizes. Although males of both species exhibited strong mating preferences for conspecific females, males did not produce more sperm or expend more sperm when paired with conspecific females as has been found in other species of poeciliids (Aspbury and Gabor 2004a; Schlupp and Plath 2005; Robinson et al. 2008). Females of both species did not show evidence of conspecific mating preferences. Sexual isolation in this sympatric system of closely related species appears to be maintained as a result of male mate choice rather than female mate choice.

In many species of poeciliid fish, coercive mating is common (Farr 1989; Bisazza 1993; Bisazza et al. 2001), and overt female mate choice may play a limited role in the mating system of these species (Farr 1989; Bisazza and Marin 1995; Kolluru and Joyner 1997). For example, in the eastern mosquitofish, *G. holbrooki*, males do not exhibit courtship behaviors, and mating results only from coercive mating (McPeck 1992; Bisazza and Marin 1995). Male *G. affinis* and *G. geiseri* also show low levels of courtship behaviors (Plath et al. 2007, C. E. pers. obs.), especially compared to other poeciliid species with high levels of courtship behaviors (e.g., *Xiphophorus* spp. and some *Poecilia* spp.; Plath et al. 2007). Bisazza and Marin (1991) found no significant preference for large males by female *G. holbrooki*, and Taylor et al. (1996) found no female *G. holbrooki* preference for males with different body coloration. Despite a lack of evidence of pre-copulatory female choice in these species, the females may be able to bias paternity for conspecific or preferred male offspring after copulation (i.e., exhibit cryptic female choice, review in Eberhard 1996).

In our study, females may lack association preferences because males were size matched. In our population, male *G. affinis* have a significantly greater SL than do male *G. geiseri* (unpaired *t*-test: $t = 6.488$, $df = 73$, $P < 0.0001$; *G. affinis* mean SL (mm) \pm SE = 23.72 ± 0.29 ; *G. geiseri* mean SL (mm) \pm SE = 20.66 ± 0.37). However, there was no significant relationship between conspecific male size and female association time with conspecific males in both *G. affinis* and *G. geiseri* (Spearman ρ : *G. affinis* = 0.244, $P = 0.251$; *G. geiseri* = -0.076 , $P = 0.717$), suggesting that SL may not be a trait upon which females base mating decisions. However, there could be other size-related differences that were not examined in this study, such as body depth, total lateral projection area, or gonopodium length that are targets of female preferences. Furthermore, females may need access to more cues than visual and chemical cues that were allowed in this study. It is possible that tactile cues are needed for females to assess species differences or differences in male quality (review in Candolin 2003).

Differing sex ratios may influence mate choice since it directly affects the opportunity for interaction and therefore the potential to mate. It is possible that our lack of finding a female preference for conspecific males is that these females have a low opportunity to mate in natural populations (i.e., a female biased operational sex ratio (OSR)), and are therefore selected to be more permissive in mating preferences. However, for our studies *Gambusia* were collected from a small freshwater stream where we found both species in abundant numbers. During seine hauls and minnow trap collections, sex ratios, while not specifically examined, did not appear to favor one sex or species over another (C.E. pers obs).

Our results indicate that while mate choice is symmetrical relative to both species it is asymmetrical relative to the sexes of both species. Many studies on asymmetrical isolation have focused on female choice and have found that only one species over the other shows mate preference (Kaneshiro 1976; Watanabe and Kawanishi 1979; Kaneshiro 1980; Wasserman and Koepfer 1980; Arnold et al. 1996; Tiffin et al. 2001; Rafferty and Boughman 2006). However, several studies have examined the potential for sex differences in sexual isolation. Sætre et al. (1997b) found that females of two sympatric flycatcher species, *Ficedula hypoleuca* and *F. albicollis* preferred conspecifics whereas males of these two species showed no mate preferences. Similarly, Kozak et al. (2009) found that female sticklebacks (*Gasterosteus* spp.) recognized and preferred conspecific males, but males did not show conspecific mate preferences, despite showing evidence of species recognition. Additionally, theoretical models by Parker and Partridge (1998) predict females to be the force favoring premating isolation. Our results differ from these studies, as we found male sexual isolation, but no evidence of female sexual isolation. We are not aware of any other study that documents such a result.

Several different, yet not mutually exclusive, selective processes may account for the sexual isolation observed in *Gambusia*. First, species differences in morphology could have arisen in different ecological contexts. If these traits affect mate choice, then the observed isolation between *G. affinis* and *G. geiseri* could have evolved as a by-product of local adaptation (Rundle and Nosil 2005; Nosil et al. 2007). Langerhans et al. (2007) suggested that in *G. hubbsi*, sexual isolation has evolved as a by-product of divergent selection on morphology in populations that differ in predator regimes. In this system assortative mating for body shape results in higher levels of sexual isolation between populations of *G. hubbsi* that are more ecologically divergent. *Gambusia affinis* are more likely to inhabit high predation environments and *G. geiseri* are more likely to inhabit low predation sites (spring environments) (Davis 1978). Therefore our results could be a by-product of differences in species recognition traits that result from local adaptation to different ecological environments.

Sexual isolation could also have evolved as a result of direct selection on actual mate preferences of the males in this study (Servedio 2001). Differences between populations in the signal transmission characteristics of the habitats could lead to local adaptation. If there is a heritable basis for male mate preferences, direct selection on these alleles via increased reproductive success could lead to increased sexual isolation. This direct selection hypothesis, as well as the ecological by-product hypothesis, predicts that sexual isolation will be greater between more ecologically divergent populations. Therefore, it would be interesting to expand the current study to include allopatric population pairs of *G. affinis* and *G. geiseri* that differ in ecological variables, such as predator regime, or signal transmission characteristics.

Introduced species, such as *G. affinis*, can cause “genetic extinction” via hybridization and introgression with native species (Rhymer and Simberloff 1996). Although there is little information on the degree of hybridization between *G. affinis* and *G. geiseri* (Hubbs and Delco 1960; this study), *G. affinis* is known to hybridize with other *Gambusia* species, and hybridization has been implicated in the decline and extinction in native *Gambusia* populations where *G. affinis* has been introduced (e.g., *G. heterochir*: Hubbs 1957; Hubbs 1971; Hubbs et al. 2002; *G. amistadensis*: Edwards et al. 2002; *G. nobilis*: Hubbs and Springer 1957). Understanding the strength of mating preferences for conspecifics in both sexes could shed light on the potential for either the presence or absence of sexual isolation when closely related species co-occur. Interestingly, we found that males, but not females act as a force favoring pre-mating sexual isolation in this *Gambusia* population.

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