

Use of microsatellite paternity analysis to determine male mating success in the western mosquitofish, *Gambusia affinis*

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Summary

I tested the effects of male body size on male mating behavior and reproductive success in the western mosquitofish, *Gambusia affinis*. In two separate behavioral experiments (male–male competition and no-competition), I tested the predictions that (1) larger males out-compete smaller males for mates and (2) small males increase their number of mating attempts in the absence of a larger competitor. I estimated male mating success both indirectly (via behavioral experiments) and directly (using microsatellite DNA to assign parentage) and compared the two measures. Results from behavior experiments showed that, when in direct competition, large males were more aggressive and attempted more copulations than small males. In addition, paternity analyses illustrated that large males sired more offspring. I found no significant correlations between male body size (and other correlated traits) and mating success in a male–male competition study, suggesting that relative male size influences male mating behavior but absolute male size does not. When competition was removed, small males mated at equal rates to larger males. Finally, indirect estimates of male reproductive success explained about 67% of the variation in parentage by males, suggesting that indirect measures of mating are good predictors of actual male reproductive success in mosquitofish.

Keywords: male size, mating success, competition, coercive mating, mosquitofish, microsatellite paternity analysis.

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Introduction

Many factors are known to influence male mating behaviors, and ultimately, reproductive success, including dominance/aggression (Gozlan et al., 2003), body size (Kissner et al., 2005), parasites (Wiehn et al., 1997) and body condition (Kodric-Brown, 1989). Male body size has been shown to be an important component in both intrasexual competition and intersexual mate choice (Andersson, 1994), and is generally accepted as one of the most fundamental predictors of male reproductive success (Perrin, 1998; Wikelski, 2005). However, male size might also indirectly affect fitness via correlative associations with other traits (e.g., dominance status, aggression and condition; Haley et al., 1994; Teder, 2005; Candolin, 2005; Fisher et al., 2006). For example, larger males often have a competitive advantage over smaller males because they are more aggressive (Riesch et al., 2006) and, thus, more likely to court females (Morris, 1991; Savalli & Fox, 1999, but see Friedl & Klump, 2005). As a result, smaller males in some species often use alternative reproductive tactics such as sneaky or coercive (forced) matings, rather than the traditional courtship behaviors often exhibited by larger and/or dominant males (Bisazza & Marin, 1995; Gross, 1996; Pilastro et al., 1997). For example, in bluegill sunfish, small males often adopt a cuckolding strategy where they sneak into the larger (parental), guarding male's nest and fertilize eggs (Gross, 1991).

The livebearing fishes in the family Poeciliidae show considerable variation in male size (Snelson, 1989), making them good candidates for studies of body size effects on mating success. For example, large sailfin molly males display an enlarged, brightly colored dorsal fin to attract females, while smaller males, lacking the sail-like fin, coerce females (Riesch et al., 2006). Also, in the related guppy, larger, more colorful males court females, while smaller males use sneaky behaviors to obtain copulations (Houde, 1997). Traditionally, these alternative mating strategies by small males were viewed as less effective (Pilastro et al., 1997); however, alternative strategies recently have been suggested as important mechanisms for the maintenance of small male body size relative to females in species with extreme size polymorphism (Bisazza & Pilastro, 1997). In moquitofish and other livebearers, for example, the coercive strategy of small males gives them a mating advantage, possibly due to smaller males having an easier time maneuvering around and underneath the female in order to insert the intromittent organ for sperm transfer (Bisazza & Pilastro, 1997; Bisazza et al., 2000).

The livebearing mosquitofishes (Genus *Gambusia*) have pronounced male size polymorphism (Bisazza & Marin, 1991, 1995; Zulian et al., 1995; Campton & Gall, 1988) where males have determinant growth (Snelson, 1989), maturing between 11 and 24 mm standard length (SL; Campton & Gall, 1988). The mosquitofish mating system is largely male driven, based on male coercion with less courting of females than other livebearers (Bisazza & Pilastro, 1997). Moreover, there is little reported evidence for female choice (Bisazza & Marin, 1991, 1995; but see Hughes, 1985; McPeck, 1992; Gould et al., 1999; Bisazza et al., 2001), suggesting that intrasexual competition may be an important mechanism for the maintenance of male size variation. Little is known regarding the genetic basis for male size polymorphism in mosquitofish. In the related swordtails, male size has been linked to variation in the pituitary locus on the Y-chromosome (commonly referred to as the 'P gene'; Zimmerer & Kallman, 1989; Ryan et al., 1992). A genetic link to male body size has also been shown in other livebearing fishes (Erbelding-Denk et al., 1994). Therefore, most studies on mating behavior mosquitofish and other livebearers assume some heritable component to male body size (Bisazza & Pilastro, 1997; Pilastro et al., 1997; Riesch et al., 2006). However, there also is a known social component to male size at maturity (Campton & Gall, 1988; Snelson, 1989), further complicating the causal mechanisms of size of maturity in male mosquitofish. Thus, the exact determinants of male body size in mosquitofish are largely unexplored and remain unknown.

Hughes (1985) tested the effects of male body size in the western mosquitofish and found that large males outcompete smaller males for matings when in direct competition. He estimated male mating success using an indirect, behavioral approach and showed that large males are more aggressive, and small males rely more on forced copulations than larger males. In this study, I used both behavioral methods (as in Hughes, 1985) and molecular tools to address the effects of body size (and other correlated traits) on male reproductive success in the western mosquitofish. I hypothesized that body size influences overall mating success of male western mosquitofish, *G. affinis*. In addition, based on results by Hughes (1985) and personal observations, I predicted that large males would have higher reproductive success (number of offspring sired) than small males when in direct competition, presumably because they are more aggressive (as shown in the western mosquitofish; Hughes, 1985; and other livebearers; Bisazza & Marin,

1991; Bisazza et al., 2000) and, thus, better competitors for mates. Further, Hughes (1985) suggested that small males may use alternative mating strategies (e.g., 'sneaky' or coercive mating tactics rather than courtship behaviors) to increase their reproductive success when in competition with large males. Thus, I predicted that small males should gain at least a portion of matings and sire some offspring, since small males do persist in natural populations of mosquitofish.

For comparison, I also assessed male mating behavior in a noncompetitive situation. I predicted that small males would mate at rates equal to larger males when there is no larger competitor present.

Finally, I statistically compared indirect (via behavioral observations) and direct estimates of mating success (actual number of offspring sired). I assessed the relationship between the two measures of male reproductive success in order to determine the predictive value (if any) of male mating behaviors on actual male mating success.

Materials and methods

In July 2005, I collected male mosquitofish from a small pond in Norman, OK, USA. Fish were returned to a greenhouse laboratory and acclimated in two 340-l community tanks for two weeks. Because I could not collect enough small males from the field, I also reared male offspring in the laboratory from pregnant females collected from the same pond in May 2005. In a separate study, I found no significant changes in male mating behaviors between captive reared males and wild caught males, regardless of male size (R.D., unpubl. data). All fish were fed commercial flake food daily.

Experiment I: male competition and paternity

Indirect measures of male mating success (behavioral observations)

To measure male mating behavior in a competitive setting, I visually sorted all males into two non-overlapping size groups (small and large). Males in the 'small male' group ranged in size from 11–18.5 mm SL and averaged 17.0 ± 2.38 mm SL, while males in the 'large male group' ranged from 19–26.5 mm SL and averaged 23.18 ± 1.2 mm SL (measured post-experiment). Males in the 'large male group' were significantly larger than males assigned

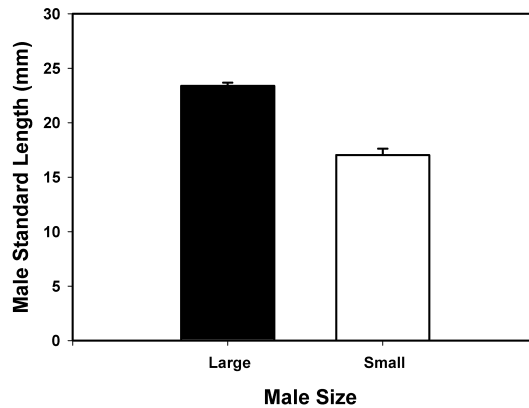


Figure 1. Comparison of males in large versus small size class for the competition experiment (two-sample t -test, $t = -9.8$, $df = 26$, $p < 0.0001$). Error bars represent one standard error.

to the 'small male group' (two-sample t -test, $t = -9.8$, $df = 26$, $p < 0.001$; Figure 1). The sizes of males used in this experiment ranged well within the natural size variation in the study population. I placed males from each size group together in 10-l plastic boxes for one week prior to experiments.

I matched females for size (within 2–3 mm SL) and other phenotypic characteristics such as pigmentation, girth, and size of gravid spot (a dark pigment spot which appears in the abdominal region when females are gravid, Snelson, 1989) to minimize the effects of female phenotypes on male mating behaviors. Phenotypically matched females were placed in a large 340-l holding tank for one week prior to experiments. I also used virgin females because female mosquitofish can store sperm for up to several months (Constantz, 1989).

I used a free-swimming 'choice' design (Houde, 1997) to estimate male mating success (number of mating attempts). Behavioral observations were conducted 05 August 2005 through 08 August 2005 and were made in early morning (between 0700 and 0930) or near dusk (1730 and 2030), as mosquitofish mating activity peaks during these times (C. Hubbs, personal communication). Previous studies have shown no difference in male mating between early morning and evening (R.D., unpubl. data); therefore, data from morning and evening samples were pooled.

For each behavioral trial ($N = 27$), I randomly selected one male from each of the two previously assigned size groups, and randomly selected one

female from the female stock tank. Selecting males randomly from each size group allowed for natural variation in size differences between males. I allowed fish to acclimate for 10 min in 20-l experimental aquaria prior to data collection. All aquaria were lined with a standardized amount of gravel (approximately 2 cm deep), emptied and rinsed thoroughly between trials.

Following the acclimation period, I conducted 5-min focal observations on each male. I randomly selected the order of observations for each male prior to each behavioral trial. I recorded the number of copulation attempts by each male (measured as the number of times the male thrust his gonopodium toward the female's genital pore), and all aggressive behaviors toward the other male. Common male aggressive behaviors included chasing or lunging toward the other male, nipping, back arch and gonopodial display (Krotzer, 1990; Houde, 1997). Aggression was quantified as the sum of the recorded aggressive behaviors (see Hughes, 1985).

Direct measures of male mating success (paternity analysis)

Following behavioral trials, each experimental group (small and large male plus female; $N = 27$) was placed in a 3.7-l plastic box with a mesh cover for several weeks to ensure fertilization of embryos and maintain a more natural, competitive scenario between males. Fish were fed commercial flake food once daily (to excess) and checked for neonates 2–3 times daily. Females near parturition (those with extremely large gravid spots and a high width to length ratio) were isolated in breeding traps to prevent cannibalism (Hubbs, 1991). Once females started to give birth, males were removed from the box and placed in a separate container to prevent predation. After females completed parturition, neonates were collected and preserved in ethanol for DNA extractions. At 4 weeks into the experiment, only four females had given birth to live neonates while the remainder gave birth to dead neonates, aborted their broods, or remained gravid. This may have occurred because I was using virgin females, which typically have lower fecundity than non-virgin females and abortion of neonates is not uncommon (R.D., personal observation). Therefore, on 10 September 2005, I killed females by stunning them in ice water and immediately preserving them in ethanol for DNA analyses. I also stunned the males in ice water, quickly clipped a portion of the caudal fin for genetic analyses, and immediately preserved the remainder of the specimen in 5% formalin.

I dissected fertilized embryos from each female, and extracted DNA using Chelex (R) 100 resin (modified from Burkhart et al., 2002) from all neonates and embryos per female, females (using a portion of the musculature tissue at the caudal peduncle), and potential fathers (using ethanol-preserved fin clips). Specifically, ethanol preserved specimens were added to 210 μl of 6% Chelex solution (1000 μl 1 M Tris, 20 μl 0.5 M EDTA and 6 g Chelex resin), heated for 20 min at 60°C, 20 min at 100°C, centrifuged for 10 s and held at 4°C.

Nineteen of the 27 females had developing broods, most of which were late stage embryos (stage 4 to stage 6; based on Meffe, 1985). I did not process the eight non-gravid females for genetic analyses. Of the nineteen females processed for genetic analyses, I was able to determine paternity of all offspring with high levels of certainty (94–100%) from nine females. The remaining ten females did not have offspring with enough genetic variation to accurately assign parentage.

After conducting a preliminary experiment to test for variation in microsatellite loci (using 7 microsatellite primers developed for *G. affinis* by Spencer et al., 1999 and three developed by Zane et al., 1999), I used the two most variable loci for my population (Gaf < mu > 4 and Gaf < mu > 5 from Spencer et al., 1999) to assess paternity (Table 1). Assigning parentage allows for a direct measure of mating success by estimating the number of offspring sired by each male. I used polymerase chain reaction (PCR; Applied Biosystems 9700 Thermocycler) with fluorescent-labeled primers to amplify microsatellite markers for paternity analyses. I optimized annealing temperatures and PCR reagent concentrations for each primer pair to reliably amplify the expected microsatellite fragment (see also Table 1; modi-

Table 1. Summary of loci (from Spencer et al., 1999) and PCR conditions used for paternity analyses.

Locus	Repeat	Size (bp)	Annealing temperature (°C)	MgCl ₂ (μl)	DMSO (μl)
Gaf < mu > 4	[CT] ₂₇	218 (171–225)	60–54 (TD)	0.8	0.2
Gaf < mu > 5	[GA] ₇ A ₂ [GA] ₁₁	264 (243–291)	48	0.8	0.1

TD, touchdown PCR (see Hecker & Roux, 1996).

fied from Spencer et al., 1999). Each PCR reaction for both loci was carried out in a total volume mixture of 10 μ l, using 1.0 μ l Promega *Taq* buffer, 0.2 μ l *Taq* polymerase, 0.8 μ l dNTPs, 0.8 μ l MgCl₂, 0.33 μ l of each primer (forward labeled and reverse) and 2 μ l DNA. For $Gaf < \mu > 4$, 0.1 μ l DMSO and 4.44 μ l dd H₂O were added to the total volume, while 0.2 μ l DMSO and 4.34 μ l dd H₂O were used for $Gaf < \mu > 5$. I used Touchdown PCR (Hecker & Roux, 1996) to amplify $Gaf < \mu > 4$, with cycling parameters of 1 min at 94°C for 1 cycle, 15 s at 60°C for 5 cycles, 15 s at 58°C for 5 cycles, 15 s at 56°C for 2 cycles, 15 s at 54°C for 20 cycles and 1 min at 72°C for 1 cycle. For $Gaf < \mu > 5$, I used standard PCR with cycling parameters of 1 min at 94°C (1 cycle), then 10 s at 94°C, 15 s at 48°C and 15 s at 72°C, for a total of 40 cycles, then 1 min at 72°C for 1 cycle.

Genetic analyses were conducted in the Systematics Laboratory at the Oklahoma Biological Survey and OU Department of Zoology Multi-User Molecular Laboratory using Applied Biosystems 3130XL Genetic Analyzer to generate microsatellites. Genotypes for each individual were determined with the aid of GeneMapper version 3.7 (Applied Biosystems).

Other male measurements

I measured male standard length and gonopodium length to the nearest 0.5 mm. Males were dissected, testes and liver removed, and dried along with soma for 10 days at 40°C. To assess body condition, soma and livers were weighed to the nearest 0.001 g, rinsed six times overnight (or longer) in petroleum ether to extract soluble nonstructural fats (Heulett et al., 1995; Trexler, 1997), dried overnight at 40°C and reweighed. Body condition was quantified as the standardized residual from least squares linear regression of mass of somatic fat/liver fat (calculated as pre-extraction mass minus post-extraction mass) on pre-extraction mass (Marsh-Matthews et al., 2005, Marsh-Matthews & Deaton, 2006).

Experiment II: male mating in a non-competitive situation

To test for effects of size on male mating behavior when male–male competition was removed, I followed the same methods as described for Experiment I, except I tested one male (randomly chosen from a group of males ranging in size from 15.0–26.5 mm SL) with one non-virgin female using a ‘forced

choice' (or 'no choice'; see Shackleton et al., 2005), free-swimming experimental design (Houde, 1997). This design has the advantage of testing male mating behaviors while eliminating the confounding effects of male–male interactions, but perhaps the disadvantage of being a less realistic mating scenario for mosquitofish reproductive behaviors, since livebearers are shoaling fish (Houde, 1997). Following the experiment, I euthanized males by stunning them in ice water, followed by preservation in 5% formalin. Males were then measured to the nearest 0.05 mm SL. I separated males into size classes post-behavioral trials for statistical analyses. Males in the small size class ranged from 15–20.5 mm SL and averaged 17.25 ± 1.59 mm SL, while males in the large size class ranged from 20.5–26.5 mm SL, averaging 22.8 ± 1.91 mm SL. Male sizes tested in this experiment ranged well within the natural size variation of the study population, and was comparable to those sizes used in the male–male competition experiment, with the exception of very small males (from 11–14.5 mm SL) which were not available.

Statistical approach

I tested for normality of data using the Kolmogorov–Smirnov test (Sokal & Rohlf, 1997). All data were normally distributed with the exception of male aggressive behaviors. Therefore, I performed both non-parametric and parametric statistics to test for differences between pairs in both experiments (competition and no competition). Results from nonparametric (Wilcoxon Signed-Rank test) and its parametric counterpart (paired *t*-test) yielded nearly identical results. Therefore, I present results from parametric statistics in this report. Because of a priori expectations based on the study by Hughes (1985), I report one-tailed results for Experiment I (male–male competition). Two-tailed tests are reported for all other analyses.

Because of the non-independence of data in the competition study (due to two males per replicate), I used the difference in number of gonopodial thrusts between the two males for the dependent variable (male mating attempts) and independent variables (male body size, testes mass, gonopodial length and condition). In addition, because male body size and other male characteristics measured were highly correlated (Pearson correlation, $r \geq 0.67$, $p < 0.01$, $N = 18$), I used residuals of the regressions of each male trait on male body size (SL) as independent variables (Brown & Prescott, 1999). Therefore, male body size (SL), residuals of the regression of gonopodial length on SL (GL_SL), somatic body condition on SL

(SBC_SL), liver body condition on SL (LBC_SL), and testes mass on SL (TM_SL) were predictor variables for male mating attempts (measured as the number of gonopodial thrusts).

I used linear regression to compare indirect (number of mating attempts) and direct (proportion of offspring sired) measures of male mating success, using one randomly selected male from each pair to eliminate the confounding effects of non-independence of males in each trial. All statistical analyses were performed in SPSS version 13.0.

Results

Experiment I: male competition and paternity

On average, large males were three times as aggressive (paired *t*-test, one-tailed, $t = -3.89$, $df = 26$, $p < 0.001$; Figure 2a), and attempted twice as many matings (paired *t*-test, one-tailed, $t = -2.17$, $df = 26$, $p = 0.02$; Figure 2b). The difference in male body size between males in each competitive dyad (large male SL minus small male SL) did not predict number of mating attempts, aggression, or number of offspring sired by males in either size class. Further, additional male characteristics measured (testes mass, gonopodial length and condition) did not affect male mating.

Of the nine broods for which paternity was assigned with high levels of certainty (see Table 2), eight of the nine broods showed mixed parentage, and only one brood was sired by a single male (the larger male; Table 3). In all of the mixed broods, the large males sired about four times the number of the offspring as small males (see Table 3). Within the nine broods for which paternity was assigned, large males attempted about three times the number of matings as small males (paired *t*-test, one-tailed, $t = 2.3$, $df = 8$, $p < 0.01$, Figure 3a) and sired twice the number of offspring as small males (paired *t*-test, one-tailed $t = 3.35$, $df = 8$, $p < 0.01$, Figure 3b).

The number of mating attempts by males explained 67% of the variation in the proportion of offspring sired ($R^2 = 0.668$, $N = 9$, $p = 0.007$; Figure 4).

Experiment II: male mating in a non-competitive situation

There was no significant difference between number of mating attempts between large and small males (two-sample *t*-test, $t = -0.051$, $df = 18$,

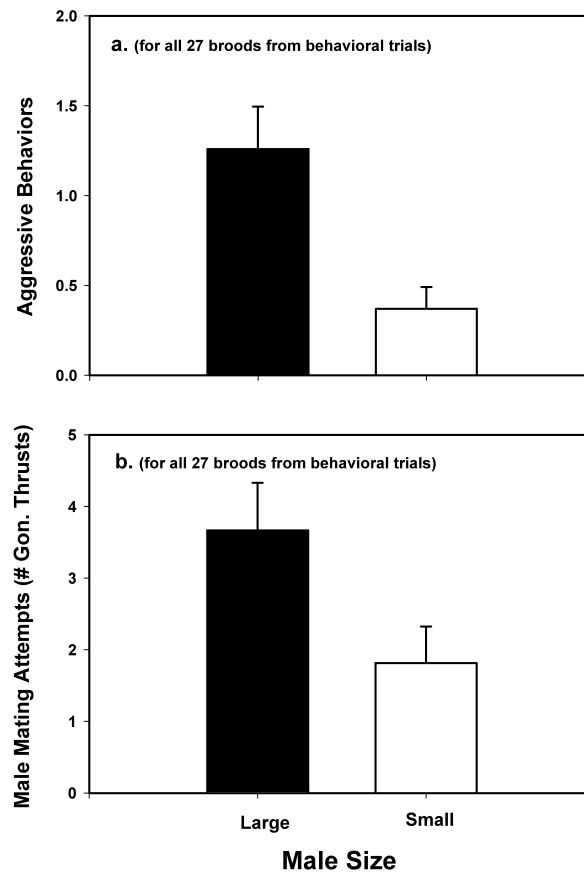


Figure 2. Comparison of mean number of aggressive behaviors (a) between large and small males (paired t -test, $t = -3.89$, $df = 26$, one-tailed, $p < 0.001$) and mean number of mating attempts (b, measured as number of gonopodial thrusts) between large and small males in the male–male competition experiment (paired t -test, $t = -222.17$, $df = 26$, one-tailed $p = 0.02$). Error bars represent one standard error.

$p > 0.05$), averaging 3.1 versus 3.0 mating attempts, respectively. Further, the number of male mating attempts was not predicted by male size ($R^2 = 0.01$, $N = 41$, $p > 0.05$).

Discussion

I tested the hypothesis that male body size influences overall reproductive success in the western mosquitofish, *G. affinis*, by measuring male mating

Table 2. Maternal and paternal genotypes, and offspring alleles for the nine broods for which paternity was assigned with high levels of certainty (at least 94% of the brood) in the male–male competition experiment. Genotypes presented are from the microsatellite locus used to assign paternity for that brood (also shown). The number of offspring scored per brood (# scored) divided by the total number of offspring in that brood (total), yielding a percent of the brood that was assigned paternity (% brood scored) is also presented.

Brood No.	Maternal genotype	Paternal genotypes (large/small)	Offspring alleles	Locus	No. scored/total (% brood scored)
2	231/231	231/231(lg) 233/233(sm)	231 233	<i>Gaf4</i>	16/17 (94)
4	189/227	189/197(lg) 233/233(sm)	189 197 227 233	<i>Gaf4</i>	8/8 (100)
5	276/276	259/276(lg) 257/257(sm)	257 259 276	<i>Gaf5</i>	24/25 (96)
7	189/231	197/233(lg) 231/231(sm)	189 197 231 233	<i>Gaf4</i>	19/19 (100)
8	189/197	189/197(lg) 231/231(sm)	189 197 231	<i>Gaf4</i>	28/29 (97)
13	197/231	197/233(lg) 231/231(sm)	197 231 233	<i>Gaf4</i>	15/15 (100)
17	231/233	189/231(lg) 197/233(sm)	189 231 233	<i>Gaf4</i>	18/18 (100)
19	257/276	259/259(lg) 276/276(sm)	257 259 276	<i>Gaf5</i>	20/20 (100)
26	259/276	276/276(lg) 259/259(sm)	257 259 276	<i>Gaf5</i>	13/13 (100)

Table 3. Comparison of male mating success (number of offspring sired) from male–male competition experiment. Brood number (female), male size (measured as standard length (SL) to the nearest 0.5 mm, number of offspring sired by each male (large vs. small) and proportion of offspring sired by large male are shown. Only those broods in which paternity for at least 94% of all offspring could be assigned are reported.

Brood No. (Female)	Male size (mm SL. Lg/Sm)	Offspring sired by Lg/offspring sired by Sm (%)	Approx. proportion sired by large male
2	24.5/12.0	12(0.75)/4(0.25)	3/4
4	22.0/17.0	5(0.63)/3(0.37)	2/3
5	23.0/14.5	15(0.63)/9(0.37)	2/3
7	25.0/19.5	11(0.58)/8(0.42)	2/3
8	22.0/18.0	23(0.82)/5(0.18)	4/5
13	22.0/20.0	9(0.6)/6(0.4)	2/3
17	22.0/20.0	16(0.875)/2(0.125)	7.8
19	23.5/15.0	10(0.5)/10(0.5)	1/2
26	24.0/16.0	13(1.0)/0(0)	1.0

success both indirectly (via behavioral sampling of male mating attempts, see also Hughes, 1985) and directly (using microsatellite paternity analysis). I tested this hypothesis in both a competitive and non-competitive situation. In the male competition experiment, my results supported the prediction that large males are more competitive than smaller males for mates. These findings were not surprising, considering that Hughes (1985) also reported similar results of *G. affinis*. I also found that larger males attempted more copulations than smaller males, and were more aggressive than small males. Similar results have been reported for the western mosquitofish (Hughes, 1985) as well as many other livebearing fishes (Houde, 1997), including the closely related eastern mosquitofish, *G. holbrooki* (Bisazza & Marin, 1995).

In this study, I examined the degree to which large males out-compete smaller males in a male–male competition experiment. Although I predicted that large males would have a competitive advantage (presumably because they are more aggressive; see Hughes, 1985), I also predicted that small males would acquire some matings, even in direct competition with larger males. Further, small males persist in most populations of *G. affinis*; therefore, it was expected that large males would not entirely out-compete small males for mates. I tested this prediction using microsatellite DNA to determine the actual number of offspring sired by males. Smaller males attempted

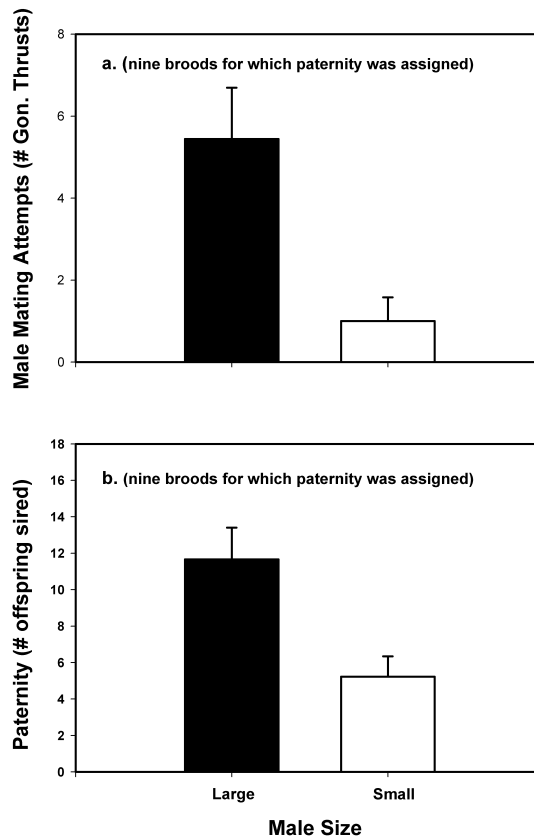


Figure 3. Comparison of mean number of mating attempts (a) measured as number of gonopodial thrusts between large and small males (paired t -test, $t = 2.3$, $df = 8$, one-tailed, $p < 0.01$) and paternity (b) measured as mean number of offspring sired between large and small males (paired t -test, $t = 3.35$, $df = 8$, one-tailed, $p = 0.005$) in the male–male competition experiment for the nine broods for which I was able to assign parentage. Error bars represent one standard error.

about a third the number of matings and sired almost half of the number of offspring as larger males. In all but one brood, small males fathered at least a quarter of the offspring as the larger male. My results show that even though large males have a competitive advantage over small males, they do not completely exclude smaller males from mating with females. Hughes (1985) also showed a large male competitive advantage in *G. affinis*, but only when presented with non-virgin, male-deprived females. In this study, I used virgin females, which were also male deprived, and did not compare male mat-

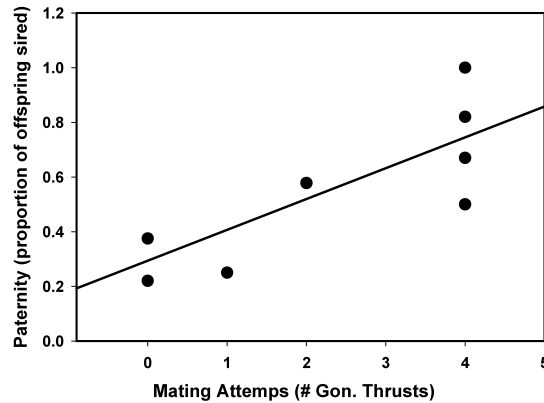


Figure 4. Male mating success (measured as the number of offspring sired) as a function of number of mating attempts measured as the number of gonopodial thrusts (GT); $MMS = 0.112GT + 0.029$, $R^2 = 0.67$, $N = 9$, $p < 0.01$.

ing behavior between virgin and non-virgin females. Because female mosquitofish store sperm (Constantz, 1989), it was necessary to use virgins to accurately determine reproductive success using genetic analyses.

In a non-competitive situation, Hughes (1985) found that small males were more likely to use forced inseminations, while large males were more likely to court, and suggested size correlated mating differences by males. It is possible that small males use alternative reproductive tactics, such as sneaky copulations to gain matings during competitive and/or non-competitive situations, which has been suggested for other livebearing fishes (Houde, 1997). However, this has not been directly tested for *G. affinis*. In the non-competition experiment, I found no difference between the number of mating attempts of large and small males, nor was male size a significant predictor of male mating attempts. These results differ somewhat from those found in the one-sided livebearer, *Jenynsia multidentata*, where small males mated at higher frequencies than large males when no larger competitors were present (Bisazza et al., 2000). I found no evidence for a small male mating advantage in *G. affinis* in this study, but further tests are needed to draw definitive conclusions.

In many species, male body size can influence mating success either directly (Savalli & Fox, 1999, Scharl et al., 1993), or indirectly (Wikelski, 2005). In this study, I used a multiple regression to test for predictive effects of male size and other correlated traits that may also influence mat-

ing success. Variation in male body size (or any other trait correlated with body size) did not predict male mating behavior. However, large males attempt more matings and obtain higher reproductive success than small males when in a competitive situation. In addition to Hughes (1985) for *G. affinis*, Scharl et al. (1993) showed a large male mating advantage in another live-bearing fish, *Limia perugiae*, where large males obtained all matings in small groups when competing with smaller males. However, when group size was increased, the large male advantage disappeared and small males actually obtained all of the matings. The authors concluded that in a group setting, large males are spending their time and energy maintaining their status in the dominance hierarchy, while small males can mate. Not much evidence of dominance hierarchies has been reported for *Gambusia*; thus, it remains uncertain whether the large male mating advantage found here would break down in the presence of several males, albeit possible.

Many studies on mating behavior in livebearing fishes assume that indirect measures of mating success accurately predict number of offspring sired (McPeck, 1992; Pilastro et al., 1997; Bisazza et al., 2000, 2001). However, the extent to which indirect measures can be used to predict actual mating success (using paternity measures) has only been tested in one livebearing fish, *L. perugiae* (Scharl et al., 1993), a species with strong female choice for colorful males. Based on nine families tested here, the minimum number of mating attempts by males accounted for a significant amount of variation in parentage (67%), despite the low sample size. The sample size for this study was compromised for several reasons: individuals in the study population showed little genetic variation at several microsatellite loci, which inhibited parentage assignments for several broods; and, surprisingly, several females prematurely aborted their broods or gave birth to dead neonates and, thus, could not be included in genetic analyses. For the outlined reasons, I was able to resolve parentage for all offspring from only nine of the 27 total broods. This population has gone through several bottlenecks, which likely caused the decreases in genetic variation seen in this study. However, the premature abortion of broods by females is unlikely due to the small amount of genetic variation, or to stressful laboratory conditions, because many females from this study population have been reared under identical laboratory conditions and had multiple healthy broods over the reproductive season. I believe these results stem from using virgin females, as they tend

to have lower reproductive success than non-virgins (R.D., personal observation). However, this idea needs to be further investigated to draw definitive conclusions.

Importantly, in this study, male mating behavior (number of copulation attempts) explained a large amount of variation in male reproductive success (paternity). This suggests that indirect measures of mating success are strongly predictive of actual fertilization success in mosquitofish. This pattern may also be likely in other species of mosquitofish exhibiting a coercive mating system. I believe these results have strong implications for behavioral studies of livebearing fishes (especially the mosquitofishes), as they show that indirect measures of reproductive success can be used to predict the actual number of offspring sired by males. These findings are important because molecular and genetic techniques are expensive and time consuming. Thus, if indirect measures of mating success can be used as a surrogate measure for actual reproductive success, then researchers can save time and money by conducting behavioral studies to predict male reproductive success.

Finally, most researchers studying mosquitofishes also assume that multiple paternity occurs in natural populations. Multiple paternity in a wild population of the closely related eastern mosquitofish (*G. holbrooki*) has been reported by Zane et al. (1999), and Green and Brown (1991) reported mixed paternity in the western mosquitofish using electrophoresis techniques. This study supports the assumption that multiple paternity in mosquitofishes is common by confirming that mixed paternity occurs in the laboratory when multiple males compete for matings. This, coupled with the verification that male mating success can be predicted by indirect, behavioral studies, provides valuable information for behavioral ecologists studying the mating system of livebearing fishes, especially those exhibiting a coercive mating system.

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