Research Article

# Sex-assortative shoaling in zebrafish (*Danio rerio*)

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*Abstract.* Shoals of fish are often composed of individuals similar in body size or other characteristics. This study tested for the assortment by sex (controlled for body size) in a species with a low degree of sexual dimorphism. Zebrafish (*Danio rerio*) shoaling was studied in the laboratory using four different experiments utilizing two different methodologies. In most of the experiments, females showed a statistically significant preference for shoaling with other females, but males typically showed no preference. In choice experiments zebrafish preferred swimming near a compartment containing another zebrafish compared to swimming near an empty compartment, but this preference was strongest for same-sex pairings, both female-female and male-male. In additional choice experiments, the results showed a preference among females (but not males) for a group of three same-sex fish over three opposite-sex fish. There was no consistent result when females were forced to choose between three females and six males, thus the preference for same-sex shoalmates may be balanced with a preference for larger shoals. The second methodology used freely-swimming groups of four fish. Females swam closer to females than to males, but males showed no preference. Few previous studies have demonstrated sex-assortative shoaling.

## Introduction

Many species of fish form loose aggregations of individuals called "shoals", whereas true schooling involves synchronization in terms of speed and direction (Pitcher, 1986). One of the most common adaptive explanations for shoaling and schooling is that movement of fish in a group creates confusion among their predators, making capture less likely (Landeau and Terborgh, 1986). However, when prey gather together, it is easy for a predator to focus on an individual that looks different from the rest, so predation has selected for similar phenotypes among conspecific prey and assortative shoaling: a preference for similar individuals (Theodorakis, 1989). Fish may assort in shoals based on many factors, including species, body length, color, presence of parasites, familiarity, and kinship (Krause et al., 2000; Peichel, 2004).

Fish may also be expected to assort by sex for several reasons in addition to possible antipredator benefits. For example, females may avoid harassment by males (Croft et al., 2006) and social groups may be less stable if they are composed of individuals with differing activity schedules (Conradt and Roper, 2000). Although many studies have examined assortative shoaling in fish, few have studied sex-assortative shoaling. Croft

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et al. (2006) demonstrated that Trinidadian guppies (*Poecilia reticulata*) separated by sex due to a combination of sex differences in predation risk and female avoidance of harassment by males (see also Griffiths and Magurran, 1998). However, Shohet and Watt (2004) reported that female Trinidadian guppies, when given a choice between water with olfactory cues from females or males, initially approached the female odor but eventually chose the male odor.

This series of experiments tests the effect of sex on the shoaling behavior of zebrafish (*Danio rerio*) in the laboratory. Zebrafish have been used as a model organism in many areas of biology for both biological and practical reasons. For example, embryonic development is easy to observe, their genetics are well known, and they are vertebrates that are easy to maintain in large numbers in a relatively small space. Despite the popularity of zebrafish as a model organism, little is known about the behavior and natural history of zebrafish in the wild (Engeszer et al., 2007). Until recently, few studies had documented the behavior of zebrafish in the laboratory, but behavioral studies have become more common (e.g., Pritchard et al., 2001; Delaney et al., 2002; Turnell et al., 2003; Engeszer et al., 2004; Ruhl and McRobert, 2005; Moretz et al., 2007). Zebrafish can discriminate among the sexes using visual cues alone (Turnell et al., 2003; Ruhl and McRobert, 2005), although pheromones also play a role in sex recognition (Bloom and Perlmutter, 1977) and males may rely more on olfaction than vision when identifying females (Turnell et al., 2003).

Ruhl and McRobert (2005) reported that male and female zebrafish preferred to swim near compartments containing a group of male or female zebrafish over empty compartments. Male subjects also showed a preference for a shoal containing females over one containing males. Female subjects showed no preference in that scenario. Neither males nor females showed a preference between a mixed-sex shoal and a single sex shoal. Experiments in our laboratory suggested that zebrafish may show sex-assortative shoaling (Lebron and Palestis, 2004; Kurta et al., 2007), contradicting the results of Ruhl and McRobert (2005). Here we expand on our work, which was published only in abstract form, and also report on additional experiments testing for positive assortment by sex.

## Materials and Methods

Adult zebrafish (*D. rerio*) were obtained from pet stores and maintained in various sized tanks. The tanks contained gravel and artificial plants to imitate realistic surroundings. The tanks were heated to approximately 24º C and aerated. The fish were fed tropical fish flakes daily, occasionally supplemented with brine shrimp.

In all experiments similar sized individuals were selected (within 0.5 cm), to control for size-assortative shoaling, although in previous experiments there was no significant effect of body size on shoaling in zebrafish (Kurta et al., 2007). All trials were performed in the afternoon to eliminate effects of mating behavior, as zebrafish spawn during the early morning (Laale, 1977; Westerfield, 2000). No individuals were tested more than once. Both male and female subjects in a trial were obtained from the same tank, to control for possible avoidance of strangers.

In each experiment behavior was quantified by instantaneous time sampling (Martin and Bateson, 1993), using two-minute intervals.

### Experiment 1

In this study the test tank was 70 cm long, 10 cm wide and 10 cm high. The tank was divided into three compartments, the middle compartment was 50 cm long, and the two end compartments were 10 cm long. The middle compartment was divided into five sections for observational purposes, by marking the outside of the test tank every 10 cm. The water in all compartments was maintained at the same level and contained gravel to simulate normal conditions in a tank. Two zebrafish were used for each trial – one test fish and one stimulus fish. There were a total of 33 trials. Eighteen of the trials used a female subject and 15 used a male subject. All four possible combinations of sexes were used (subject-stimulus: male-male, male-female, female-female, and female-male).

The test fish was placed in the middle compartment and the stimulus fish in a randomly selected end compartment. The fish were provided a habituation period of five minutes, then the test fish was observed for 20 minutes. Every 2 minutes, the position of the test fish (presence in one of the five 10 cm sections of the middle compartment) was recorded. For analysis, the proportion of time that the test fish spent in the 10 cm section nearest the stimulus fish was compared to the proportion of time in the 10 cm section nearest the empty compartment. The other 30 cm was considered as a neutral area. Data were arcsine-square root transformed, as is standard for proportions (Sokal and Rohlf, 1995).

**Experiment 2**

The test fish were either female (10 trials) or male (10 trials). The test fish had a choice between three males and three females using the tank described in Experiment 1. The subject was placed in the center of the middle compartment and the group of three female fish was placed in one of the end compartments and the male fish in the other end. The same protocol was used as in Experiment 1.

**Experiment 3**

The test fish had a choice among six males in one end compartment and three females in the other end compartment. There were a total of 10 trials and all test fish were female. Previous studies have shown that zebrafish prefer to shoal with larger groups (Pritchard et al., 2001; Ruhl and McRobert, 2005). This experiment was designed to determine if the female would prefer to shoal with a smaller group of the same sex or a larger group of the opposite sex. This experiment tested only females, based on the results of our earlier experiments and because Ruhl and McRobert (2005) reported a shoal size preference only among females. The same protocol was used as in Experiment 1.

**Experiment 4**

The apparatus used for this experiment was a 20 cm round bowl. The bowl was filled with water from the tanks to a depth of 2 to 3 cm. A web camera connected to a computer was placed above the bowl to record observations remotely. Tri-fold boards surrounding the bowl prevented the fish from seeing the experimenters or other external stimuli.

For each trial, two males and two females of similar sizes were placed in the test bowl and allowed to adjust to the new environment for 1 hour (to align with other studies in our laboratory on the effects of ethanol on behavior; Kurta and Palestis, in press). There were ten 12 minute trials. Digital photographs were taken every two minutes, with the first one at two minutes, giving a total of 6 pictures per trial.

The digital photos were analyzed using Image Tool software (http://ddsdx.uthscsa.edu/dig/itdesc.html). The distance between each fish, from head to head, was measured in pixels then converted to centimeters. Also the length of each fish was measured, to confirm that the fish within each trial were of similar body size.

Because 6 photos were taken per trial, for each trial there were 6 measures of male-male distance and 6 of female-female distance. The 6 distances within a sex were averaged to give one data point per trial per sex. However, each photo contained 4 measures of opposite-sex distance (male 1 to female 1, male 1 to female 2, male 2 to female 1, male 2 to female 2). The median opposite-sex distance was calculated for each photo, and then these medians were averaged across the 6 photos to again get a single measure of average distance for each trial. Medians were initially used rather than means, because of the possibility that one fish being distant from the shoal would have a disproportionate impact on the mean.

The experimental design allowed paired comparisons of same-sex versus opposite-sex inter-individual distances. Wilcoxon signed-ranks tests (the nonparametric version of the paired t-test) were performed using a Bonferroni-adjusted alpha level of $P = 0.025$ to avoid pseudoreplication, because the opposite-sex numbers were used in more than one comparison (male-male vs. opposite and female-female vs. opposite).

## Results

### Experiment 1

The first procedure confirmed that, using visual cues alone, zebrafish approach members of the same species in preference to an empty compartment, and also demonstrated that this preference is stronger in same-sex than in opposite-sex pairings. The test fish spent an average of 42% of their time near the stimulus fish and 14% of the time at the opposite end of the tank (paired $t_{32} = 4.82$, $P < 0.0001$). Test fish spent significantly more time near the stimulus fish than near the empty compartment in both same-sex ($t_{17} = 4.46$, $P < 0.0003$) and opposite-sex ($t_{14} = 2.57$, $P < 0.022$) pairings. However, there was significant variation among treatments (ANOVA $F_{3,28} = 4.13$, $P = 0.015$); time near the stimulus fish was significantly higher in same-sex than in opposite-sex pairings, for both females and males (Figure 1).

### Experiment 2

The second procedure also suggested that females prefer to shoal with other females, but no sexual preference was observed among males. When given a choice between a group of three females and a group of three males, female test fish spent 47% of their time near females and 26% near males (the remaining time was spent in the middle of the tank). This difference approaches statistical significance (paired $t_9 = 2.24$, $P = 0.052$). Males spent 34% of their time near females and 30% of their time near males ($t_9 = 0.41$, $P = 0.69$).

### Experiment 3

The third procedure demonstrated that females, given a choice between a group of females and a larger group of males, showed no significant sexual preference (paired $t_9 = 0.58$, $P = 0.57$). Overall, the stimulus fish spent 53% of their time near the three females, and 36% of their time near the six males. In 6 of 10 trials, the test fish switched from the female group to the larger group of males after approximately 10 minutes. The remaining 4 trials were evenly divided between test fish choosing the 3 females and choosing the 6 males.

### Experiment 4

The second methodology, which allowed for free swimming and both visual and olfactory cues, showed that, in a mixed-sex group, females shoaled closer to other females than to males, while males had no significant preference (Figure 2). There was a significant difference (Bonferroni-adjusted alpha = 0.025) between the female-female distance and opposite-sex distance (Wilcoxon signed-ranks test, $Z = 2.497$, $P = 0.0125$, n = 10) but not between the male-male and opposite sex distance ($Z = 0.255$, $P = 0.799$, n = 10).

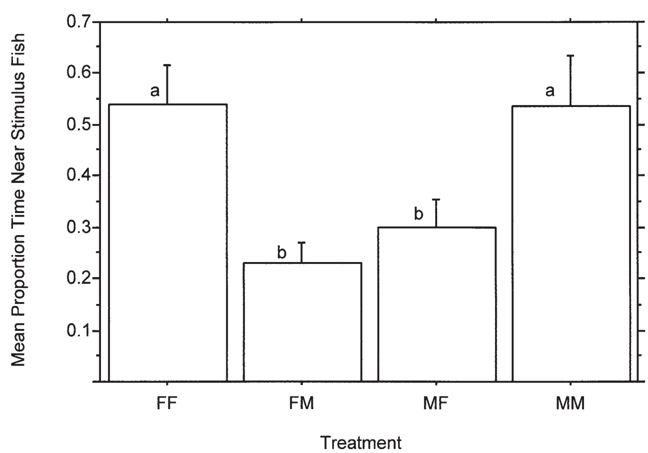


**Figure 1.** Proportion of time spent by test fish near stimulus fish in Experiment 1. The pairs of letters along the x-axis indicate the sex of test and stimulus fish, respectively (e.g., FM represents female test fish with male stimulus fish). Error bars are +/- SE. Letters above the bars indicate statistical significance (Fisher's PLSD after ANOVA, $P < 0.05$). Bars with different letters are significantly different.

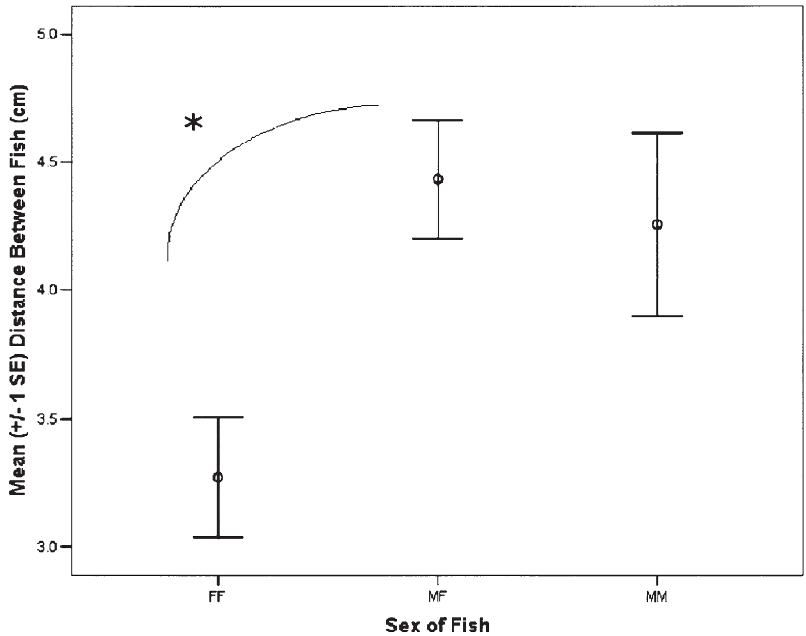


**Figure 2.** Mean distance between fish of each combination of sexes in a group of two females and two males in Experiment 4. Letters along the x-axis indicate which distances were measured: female-female, male-female, or male-male. Error bars are +/- SE. An asterisk indicates a statistically significant difference (Wilcoxon signed-ranks test with Bonferroni-adjusted alpha-level of $P < 0.025$).

## Discussion

These experiments demonstrate that female zebrafish prefer to shoal with other females over males. There have been few previous reports of sex-assortative shoaling, whereas assortment by other characteristics, such as body size is commonly reported (reviewed in Krause et al., 2000). Females that swim together may be less likely to be harassed by males (Croft et al., 2006) and groups of same-sex individuals may be more stable than mixed groups (Conradt and Roper, 2000). Another possible explanation for sex-assortative shoaling is that a female zebrafish within a group of mostly males would be more likely to be selected by a predator. To increase its survival via the confusion effect (Landeau and Terborgh, 1986; Theodorakis, 1989), a female fish should surround itself with other females. However, the degree of sexual dimorphism in zebrafish is small, and zebrafish live in streams with low visibility (Engeszer et al., 2007), so the antipredator benefits of assortative shoaling may be less important in zebrafish than in other species.

Of course, to allow reproduction females and males must come together. Others have shown opposite-sex zebrafish to approach each other (Delaney et al., 2002; Turnell et al., 2003; Ruhl and McRobert, 2005), but the experiments recorded in this paper were performed at a time of day (afternoon) when mating was unlikely. Ruhl and McRobert (2005) performed experiments very similar to ours, using a methodology like in Experiments 1 through 3, but they began testing in the morning. Other than time of day, it is difficult to explain why our results are so different from those of Ruhl and McRobert (2005).

Experiment 4 used the more realistic apparatus, a bowl, as fish could swim freely and interact as a group. The other three experiments used a tank that did not allow mixing of test fish and stimulus fish, or mixing of water, and thus provided only visual cues. The advantage of the tank is that a preference for one sex over another is easier to demonstrate, based on a choice for one end of the tank over the other. It is noteworthy that the results of these two very different experimental methodologies were similar.

It was unclear why sex-assortative shoaling was consistently present among females, while males showed a significant preference for males only in Experiment 1. Perhaps the trade-off between mating and survival differs between the sexes, due to differences in mating strategies and/or predation risk. For example, females of similar length to males would have larger girth and potentially could be preferred prey. Females may also be avoiding harassment by males, while males seek out females to mate. Interestingly, the females in Experiment 3 seemed to balance a preference for shoaling among other females with a preference for larger groups. The sex composition of zebrafish shoals in the wild is unknown, and field studies of zebrafish behavior are clearly needed.

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