This is to certify that the

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Sexual Selection in
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Rebecca Claire Fuller

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Thomas Getty
Major professor

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SEXUAL SELECTION IN THE RAINBOW DARTER *ETHEOSTOMA CAERULEUM*

By

Rebecca Claire Fuller

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ABSTRACT

SEXUAL SELECTION IN THE RAINBOW DARTER, ETHEOSTOMA CAERULEUM

By

Rebecca Claire Fuller

Darters are a group of brightly colored, North American freshwater fishes that includes the genus Etheostoma, the most speciose fish genus in North America. Little is known about the roles of female choice, male competition or sperm competition in any species of Etheostoma. In this thesis, I examined female mating preferences, male competition, and sperm competition in the rainbow darter, Etheostoma caeruleum. I demonstrate the following:

1. Females exhibit measurable mating preferences.
2. Males display aggressive behavior when competing for females.
3. Large males are competitively dominant to small males.
4. Group spawnings where 2 or more males simultaneously spawn with a female are common.
5. Group spawnings are costly to dominant, primary males in terms of lost paternity.
6. Small males increase their sperm output when the potential for sperm competition is high.
7. Large males forego spawning opportunities when the potential for sperm competition is high.

In loving memory of Albert Greenleaf Fuller, Jr.

ACKNOWLEDGMENTS

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PREFACE

Darters are a group of North American freshwater fishes that includes the genus Etheostoma, the most speciose fish genus in North America. Despite the fact that males of most Etheostoma species bear conspicuous color patterns and/or presumably expensive ornaments, little is known about the dynamics of sexual selection in these species. Do females exhibit mate choice among males? Do males compete aggressively for females? Does sperm competition play a role in these mating systems? These questions are largely unanswered. To date, sexual selection has been investigated in only three darter species. Knapp & Sargent (1989) demonstrated that the egg mimic structures on the first dorsal fin of E. flabellare are preferred by females. Grant & Colgan (1983) provide some indirect evidence that E. nigrum females may prefer males that actively guard their nests. Pyron (1995), however, found no evidence for female mate choice in E. spectabile nor did he find evidence that male secondary sexual traits are involved in male/male competition.

This thesis centers on the spawning behaviors of the rainbow darter, Etheostoma caeruleum Storer. Prior to this thesis, the only studies of E. caeruleum were performed by Winn (1958 a, b) who documented the natural history of this species. E. caeruleum is a small bottom dwelling fish that inhabits shallow riffles in swift streams and gravel areas in clear lakes. The mating system is promiscuous, and there is no parental care (Winn 1958 a, b). The sexes are dimorphic in color; adult male color patterns contain brilliant blue and red hues whereas females and juveniles are a cryptic sand color (Page 1983, Page & Burr 1991). During the breeding season, males remain on riffles and guard small moving territories, while females dwell in quiet waters at the base of the riffle (Winn 1958 a, b). When a female is ready to spawn, she moves to the riffles and is immediately followed and defended by a male. The male attempts to keep competing males away by chasing and attacking them. During these activities, males may take on a temporary color pattern in which their entire body turns black. The female solicits spawns from the male by performing incomplete or complete nosedigs. In an incomplete nosedig, the female digs her nose into the gravel and quivers in a near vertical position. In a complete nose dig, after quivering, the female moves down and forward into the gravel so that her ventral half is buried in the substrate. The male can only spawn with a female after she has performed a nosedig and is buried in the gravel. The male then mounts the female, and the two fish vibrate rapidly during which time eggs and sperm are released (Winn 1958 a, b). Occasionally nearby males sneak in and release their sperm next to the pair of spawning fish (Breder & Rosen 1966).

In chapter 1, I examine the roles of female mate choice and male/male competition using three sequential mate choice trials followed by an observation period where the fish could interact. Specifically, I address the following questions: 1) Which behavioral variables are good measures of female mating preference in E. caeruleum? 2) Do females of E. caeruleum exert mating preferences for males from different populations? 3) Which male behaviors are used in the context of mating? 4) How do female choice and male interactions affect male mating success? This chapter provides information on how to measure female mating preferences and male competition. However, the link between female mate choice, male competition, and male spawning success was not clear. This is due in some part to the large amount of group spawning that occurred.

In Chapter 2, I measure the cost of group spawning to primary males by measuring the spawning success of secondary males relative to primary males. The paternity of offspring resulting from group spawnings where two males simultaneously mate with one female is assessed using males and females of known allomorphs. Knowing the spawning success of secondary males is important. If secondary males fertilize a negligible proportion of the eggs, then primary males should be willing to tolerate their presence.

In Chapter 3, I use E. caeruleum to test the predictions of a theoretical model. Theory considering the relationship between the number of competitors in a group spawning event and male sperm output indicates that males should ejaculate less sperm when sperm competition is higher than average and should ejaculate more sperm when sperm competition is lower than average (Parker et al. 1996). The reasoning behind this model is that males should reduce sperm output when sperm competition is high because males can wait for better mating opportunities in the future with fewer competing males. Similarly, males should take advantage of mating opportunities taking place under low sperm competition by increasing sperm output because such an opportunity may not arise again. In this chapter, I examine the effect of potential sperm competition and male size on male sperm output, male willingness to spawn, and a competitive behavior, adoption of black coloration. Specifically, I address the following questions: 1) Do males increase their sperm output under low sperm competition and decrease their sperm output under high sperm competition? 2) Are males less willing to mate with females under high sperm competition? 3) Is the adoption of black coloration used in male/male competition?

Finally, I end this thesis with a synthesis that integrates the results of the three chapters and points to directions for future research.

Chapter 1

Measuring female mate choice and male/male competition in rainbow darters

INTRODUCTION

Sexual selection is defined as the evolution of traits through variation in mating success (Andersson 1994). In most species with conventional sex roles, sexual selection occurs through male competition and/or female choice . Disentangling the two mechanisms can be problematic. Traditionally, female choice experiments have relied on physically separating two males and allowing the female to see, smell or hear the males (or male cues) in a choice arena. Some aspect of female behavior is then used to infer preference. The roles of mate choice and competition in darter mating systems (genus: Etheostoma) are poorly understood despite the fact that males of most species bear conspicuous color patterns and/or presumably expensive ornaments. Only three species have been investigated. Knapp & Sargent (1989) demonstrated in E. flabellare that males with egg mimic structures on their first dorsal fin are more likely to spawn than males that have had their egg-mimics removed. Whether this is due to competition or choice is unclear. Female E. flabellare tend to associate more with clay dummies that have had orange spots painted on their first dorsal fin. Grant & Colgan (1983) demonstrated in E. nigrum that males that actively guard their nests are visited more often by females and have more eggs in their nests. Using time as a measure of preference, Pyron (1995) found no evidence for female mate choice in E. spectabile nor did he find evidence that male secondary sexual traits are involved in male competition.

This study centers on the breeding behaviors of the rainbow darter, Etheostoma caeruleum Storer. The present study attempts to answer the following four questions: 1) Which behavioral variables are good measures of female mating preference? 2) Do females exhibit mating mating preferences for males from different populations with different sizes and color patterns? 3) Which male behaviors are used in the context of mating? 4) How do female choice and male interactions affect male mating success?

MATERIALS AND METHODS

Etheostoma caeruleum were collected with a kicknet between March and May 1995 at Seven Mile Creek, Kalamazoo County, and Prairieville Creek, Barry County, Michigan, USA. These streams occur in separate drainages of the Kalamazoo River, MI. Animals were returned to Kellogg Biological Station where they were housed. Females and males were kept in separate 40 l aquaria containing a gravel substrate. Animals were fed daily with live tubifex worms and chironomids.

Females were given a choice between males from two populations, Prairieville Creek and Seven Mile Creek. Males from these two populations were used because they appeared to differ in their color pattern and body size. Males from Seven Mile Creek appeared darker with more large patches of blue. Prairieville Creek males appeared lighter with more contrast between red and blue hues. Seven Mile Creek males were larger than Prairieville Creek males (Seven Mile Creek: $x = 52.18 \pm 1.663$ (SE) mm, Prairieville Creek: $x = 44.77 \pm 0.836$ (SE) mm, $T = 3.608$, $DF = 46$, $P = 0.001$). Similarly, Seven Mile Creek females were larger than Prairieville Creek females (Seven Mile Creek: $x = 50.125 \pm 0.972$ (SE) mm, Prairieville Creek: $x = 43.778 \pm 0.999$ (SE) mm, $T = 3.868$, $DF = 24$, $P = 0.001$).

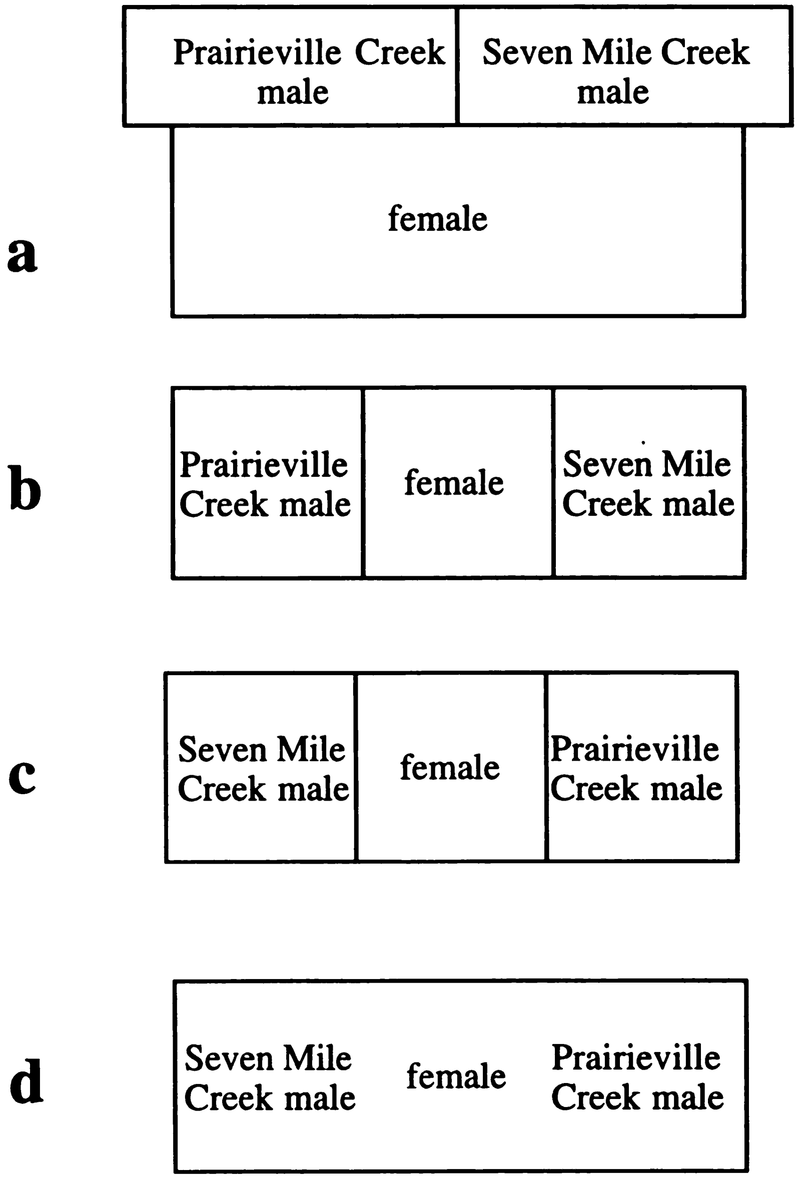
Three sequential mate choice trials were conducted on each group of fish (one Prairieville Creek male, one Seven Mile Creek male, and one female from one of the populations). In all three trials lighting was provided by a mixture of fluorescent and candescent spot lights. Due to a paucity of animals, three males from Seven Mile Creek were reused; two were each used in two replicates and one was used in three replicates. No males from Prairieville Creek were reused, and no females from either population were reused. Twenty-six replicates were conducted.

In trial 1, focal females were given visual access to the two males. In each replicate, a female was placed in a 40 l aquarium (50.8 cm x 26.4 cm x 31.75 cm) which abutted two 20 l aquaria each holding one male (Figure 1A). A piece of cardboard was placed between the aquaria holding the males to prevent visual access. Animals were allowed to interact for 30 minutes. In trial 2, the female's 40 l aquarium was divided into thirds with plexiglass barriers creating three separate compartments (Figure 1B). These barriers were not waterproof and allowed olfactory cues to pass. The female was placed in the central compartment, and males were transferred from the 20 l aquaria to the end compartments. Animals were allowed to interact for 30 minutes. In trial 3, the position of the males was switched to control for any side biases that females may have (Figure 1C). Again, the fish were allowed to interact for 30 minutes. The night prior to experimentation, the standard lengths of the animals were measured, and animals were placed in the aquaria for trial 1 (see below). Males were placed randomly in one of two 20 l aquaria. A visual barrier was placed between the female aquarium and the male aquaria to prevent visual access prior to the experiment.

Following the three mate choice trials, the fish were allowed to freely interact for 1.5 hours. If no spawnings took place during the 1.5 hour observation period, then the animals were left in the aquarium for 24 hours. These animals were monitored to see if they were in reproductive condition by videotaping their behaviors and checking aquaria for the presence of eggs. Each replicate was categorized as either having spawnings or no spawnings. The category spawnings included replicates where spawnings occurred during the 1.5 hour observation period and replicates where spawnings occurred during the subsequent 24 hours. The category no spawnings included replicates where no spawnings took place during either the 1.5 hour observation period or after the subsequent 24 hours.

In all three trials, behaviors were recorded with a video camera. Tapes were used to assess the number of nosedigs females performed in front of each male, the number of times females swam up vertically in the water column when positioned in front of each male, and the amount of time females spent in front of each male. Swims were only recorded if they were performed directly in front of one of the two compartments holding a male. The compartment containing the female was visually divided into two equal areas corresponding to the position of the two males. All of the female's time and all of her nosedigs were categorized as occurring in front of one of the two males. From these data, the number of nosedigs and the number of swims performed by the female were summed from all three trials yielding total number of nosedigs and total number of swims. Whether

Figure 1 Mate choice aquaria set-up. A: Trial 1. The female has visual access to both males. B: Trial 2. The female has both visual and olfactory access to both males. C: Trial 3. Identical aquarium set-up as in trial 2 only the positions of the males have been reversed. D: 1.5 hour observation period. Fish are allowed to interact freely for 1.5 hours.


Prairieville Creek male
Seven Mile Creek male
female
a
b
Prairieville Creek male
female
Seven Mile Creek male
c
Seven Mile Creek male
female
Prairieville Creek male
d
Seven Mile Creek male
female
Prairieville Creek male

each female behavior was performed primarily in the context of spawning was tested by comparing both the total number of nosedigs and total number of swims performed between replicates where spawnings did and did not take place.

In addition, nosedig, swim, and time scores were calculated for each trial. Nosedig scores were calculated as the proportion of nosedigs performed per trial to the Prairieville Creek male (number of nosedigs performed to Prairieville Creek male divided by the total number of nosedigs performed in that trial). Swim scores were calculated as the proportion of swims performed to the Prairieville Creek male in a trial. Time scores were calculated as the proportion of time each female spent in front of the Prairieville Creek. In addition, overall nosedig, swim, and time scores were calculated. These overall scores represent the proportion of nosedigs, swims, and time spent with each male over the course of the three trials. Scores above 0.5 indicate that females preferred the Prairieville Creek male, and scores below 0.5 indicate that females preferred the Seven Mile Creek male. The preferences of the females from the two populations were pooled providing no significant differences existed. A Kolmogorov-Smirnov Lilliefors test was used to discern whether the variables were normally distributed, and either a parametric one-sample t-test or a non-parametric Wilcoxon Signed Ranks test (depending on whether the assumptions of normality were met) was used to test whether female preferences differed statistically from 0.5, a null expectation of no choice.

During the 1.5 hour observation period following the three mate choice trials, the number of attacks, chases and guards performed by each male were recorded from direct observation. Guarding behavior occurs when one male is closest to the female and successfully prevents a competing male from coming between himself and the female. The number times each male was closest to the female when she performed a nosedig and the number of times each male spawned with the female were also recorded. In addition, the number of group spawnings where 2 males simultaneously spawn with a female and the number of spawnings in which each male mated as a primary male and as a secondary male were recorded. The primary male is defined as the male that begins spawning with the female. The secondary male is the male that sneaks in and releases sperm next to a pair of fish that have already begun spawning. From these data, the total number of attacks, chases, guards, and nosedigs were calculated as well as scores for each variable (proportion of the behaviors performed by or to the Prairieville Creek male; an attack score greater than 0.5 indicates that Prairieville Creek males performed more attacks than Seven Mile Creek males).

T-tests and Mann-Whitney U tests were performed on the total number of attacks, chases, and guards to determine whether these behaviors are performed primarily in the context of spawning. The correlation coefficients between spawning and nosedig scores, and attack, chase, guard, and preference scores were calculated to determine which behaviors were most closely related to spawning success. All probability tests are two-tailed and results are considered significant at $P < 0.05$. All analyses were performed with Systat (Wilkinson 1992).

RESULTS

Female Choice

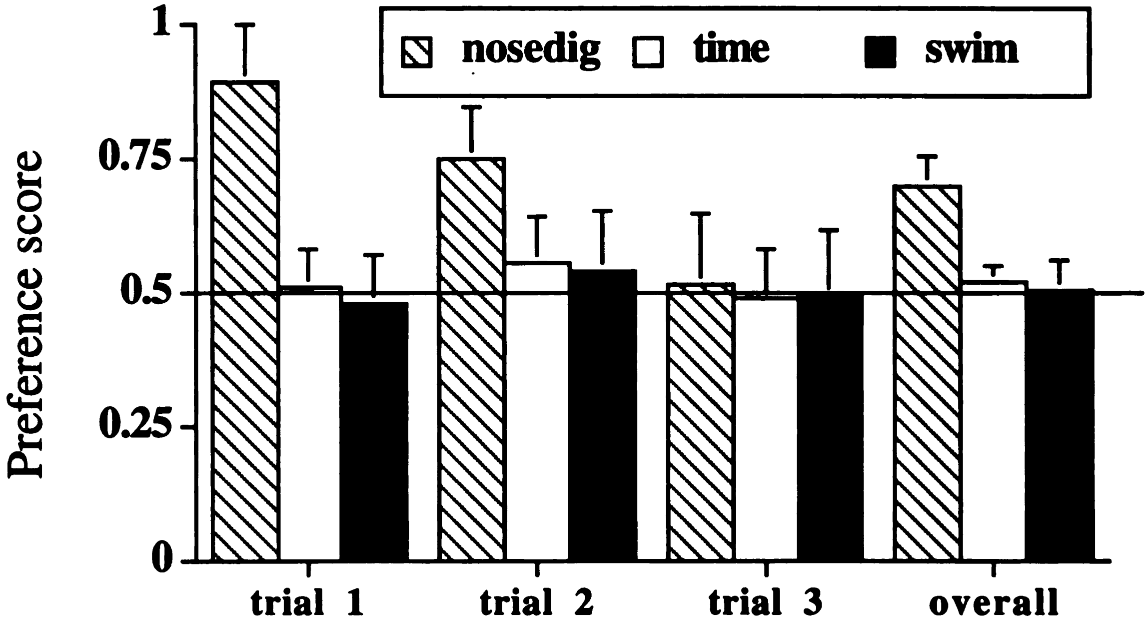
Of the 26 replicates, eleven contained spawnings during the 1.5 hour observation period, four contained spawnings during the subsequent 24 hours, and eleven contained no spawnings.

In order to discern which variables are appropriate measures of female mating preferences, I first compared the behavior of females that spawned with the behavior of females that did not spawn. Only females that spawned performed nosedigs (Mann-Whitney $U = 8.5$, $P = 0.000$, $n = 26$). In contrast, the number of swims performed by females did not differ in relation to whether or not the female spawned (Mann-Whitney $U = 52.00$, $P = 0.113$, $n=26$).

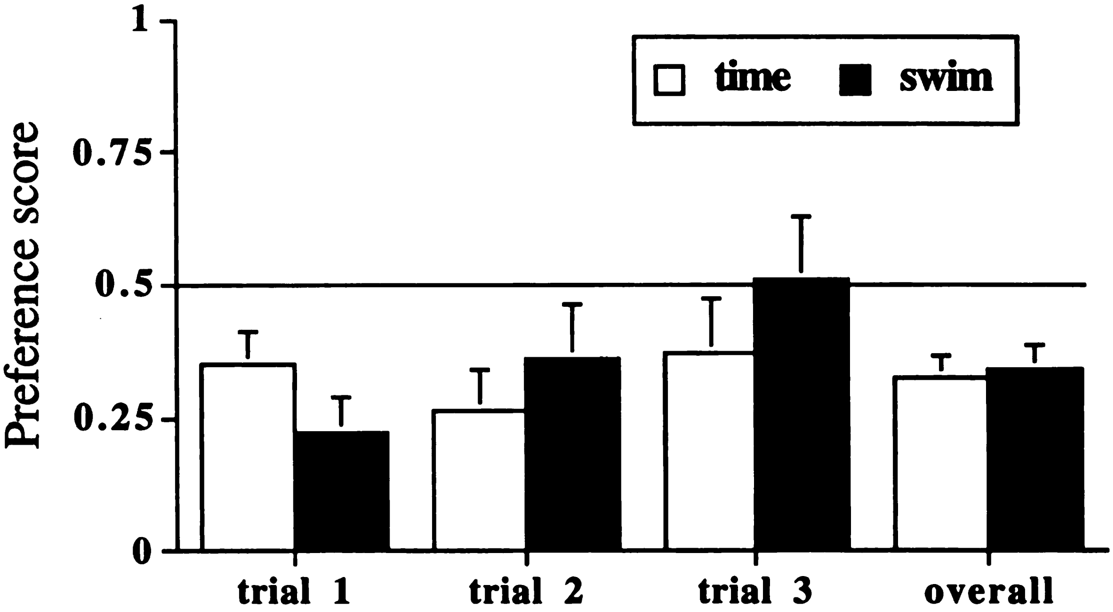
Female mating preferences among males were only detected when considering nosedig scores (Figure 2A). Females performed more nosedigs to Prairieville Creek males in trials 1 and 2 (trial 1: Wilcoxon Signed Ranks test $Z = -1.890$, $n=4$, $P = 0.059$, trial 2: One sample t-test $T = 2.632$, $DF = 9$, $P = 0.027$). The overall nosedig score also differed significantly from the null expectation of 0.5 (One sample t-test $T = 3.487$, $DF = 11$, $P = 0.005$). Nosedig scores did not differ significantly from 0.5 in trial 3 where the position of

Figure 2 Preference scores in trials 1, 2, 3, and the overall preference score using nosedigs, time, and swims as measures of preference. A: Preference scores in replicates where spawnings occurred. Sample sizes are as follows: nosedig scores trial 1 n = 4, trial 2 n = 10, trial 3 n=10, overall n = 12; time scores n=15 in all cases; swim scores trial 1 n=15, trial 2 n=14, trial 3 n=13, overall n=15. B: Preference scores for replicates where no spawnings occurred. Sample sizes for are as follows: time scores n=11 in all trials; swim scores trial 1 n = 11, trial 2 n = 11, trial 3 n = 9, overall n = 11. Means and standard errors are shown. Preference is detected when the preference scores differ from a null expectation of 0.5 denoted by the black line.

a


▨ nosedig
☐ time
■ swim
Preference score
1
0.75
0.5
0.25
0
trial 1
trial 2
trial 3
overall

b


☐ time
■ swim
Preference score
1
0.75
0.5
0.25
0
trial 1
trial 2
trial 3
overall

the males was switched (T = 0.109, DF = 9, P = 0.916). Sample sizes were less than fifteen because some females did not perform nosedigs in all of the trials.

Female mating preferences were not detected with time scores or swim scores (Figure 2A). Neither time scores nor the overall time score differed significantly from 0.5 in any of the trials ($P > 0.520$ in all cases). Swim scores in trial 1 differed among females in respect to their population of origin (T = 2.614, DF = 13, P = 0.021). Females from Prairieville Creek did not prefer males from either population on the basis of swim scores (x = 0.639 ± 0.091 (SE), T = 0.1476, DF = 8, P = 0.178). Females from Seven Mile Creek tended to prefer males from their own population when swim scores were considered (x = 0.246 ± 0.118 (SE), T = -2.151, DF = 5, P = 0.084). Females did not prefer males from either population in trials 2 and 3 on the basis of swim scores (trial 2: T = 0.367, DF = 13, P = 0.720, trial 3: Wilcoxon Signed Ranks Test Z = -0.457, n=13, P = 0.658). Overall swim scores did not differ significantly from 0.5 (Figure 2A, T = 0.091, DF = 14, P = 0.929).

Considering replicates in which no spawnings took place, a different pattern of female preference is obtained from time and swim scores. In trials where no spawnings took place, females spent more time and performed more swims in association with males from Seven Mile Creek (Figure 2B). Overall time scores and time scores in trials 1 and 2 indicated that females preferred Seven Mile Creek males (trial 1: T = -2.350, DF = 10, P = 0.041, trial 2: Wilcoxon Signed Ranks Test Z = 2.046, n=11, P = 0.041, Overall: T = -4.122, DF = 10, P = 0.002). Time scores in trial 3 differed among females in respect to their population of origin. In trial 3, Prairieville Creek females spent significantly more time with Seven Mile Creek males (x= 0.265 ± 0.088 (SE) T = -2.657, DF = 8, P = 0.018). A similar result was obtained with swim scores. Females performed more swims to Seven Mile Creek males in trial 1 (Wilcoxon Signed Ranks Test Z = 2.407, n=11, P = 0.016). The overall swim score also differed significantly from 0.5 (T = -3.361, DF = 10, P = 0.007).

In trial 3, reversing the position of the males had strong effects upon preference scores in replicates where spawnings occurred. All preference scores were negatively correlated between trials 2 and 3 (nosedig scores: R = -0.834, P = 0.010, N=8, time scores: $R = -0.608$, $P = 0.016$, n=15, swim scores: $R_S = -0.615$, n=13, $P < 0.05$). Conversely, in replicates where no spawnings occurred, reversing the position of the males had a less dramatic effect on female mating preferences. There were no statistically significant relationships between preferences scores in trials 2 and 3 (time scores: $R = -0.209$, $P = 0.537$, N=11; swim scores: $R_S = -0.147$, $P > 0.500$, N=11).

Male Mating Behaviors - 1.5 Hour Observation Period

Males behaved more aggressively within the context of mating. Males performed more attacks, chases, and guards in replicates where spawnings occurred (Figure 3 A-C, total attacks: Mann-Whitney $U = 24.5$, $P = 0.003$, n=26, DF = 1, total chases: Mann-Whitney $U = 22.5$, $P = 0.001$, n=26, DF = 1, total guards: Mann-Whitney $U = 13$, $P = 0.000$, n=26, DF = 1). As in the female choice experiment, females performed more nosedigs in replicates where spawnings took place (Mann-Whitney $U = 20$, $P = 0.001$, n=26, DF = 1).

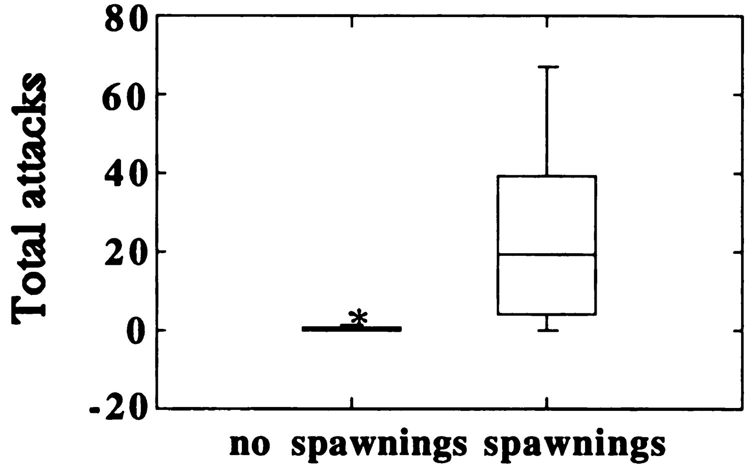
Group spawnings occur when a secondary male dashes in and releases his sperm next to a spawning pair of fish. Group spawnings occurred in 5 of the 11 replicates in which animals spawned during the 1.5 hour observation period. In 3 of the 5 replicates, both males spawned as the primary male at least once. In 4 of the 5 replicates, the smaller of the two males acted as the secondary male for the majority of group spawnings. The absolute difference in body size between the 2 males was smaller in replicates where group spawnings took place (Figure 4A, Mann-Whitney $U = 4$, $P = 0.043$, n=11, DF = 1). More chases and guards also took place in replicates where group spawnings took place (Figure 4B-C, total chases: Mann-Whitney $U = 26$, $P = 0.043$, n=11, DF = 1, total guards: Mann-Whitney $U = 27$, $P = 0.028$, n=11, DF = 1).

Aggression levels were higher in replicates where the two males were of similar size. Absolute size differences between the two males was inversely correlated with the total number of chases and attacks performed in replicates where spawnings occurred during the 1.5 hour observation period ($R_S = -0.721$, $P < 0.02$, n=11; $R_S = -0.676$, $P < 0.05$, n=11 respectively).

Males from the two populations did not differ in their tendencies to perform attacks,

Figure 3 Box plots of (A) total attacks, (B) total chases, and (C) total guards in replicates where spawnings took place (n=15) and in replicates where no spawnings took place (n=11). Box plots are used to represent the data. The middle line of the box corresponds to the median of the data. Outer edges of the box represent the first and third quartiles. Whiskers on the box represent 95% confidence limits. Dots falling outside of the whiskers represent outliers.

a


Total attacks
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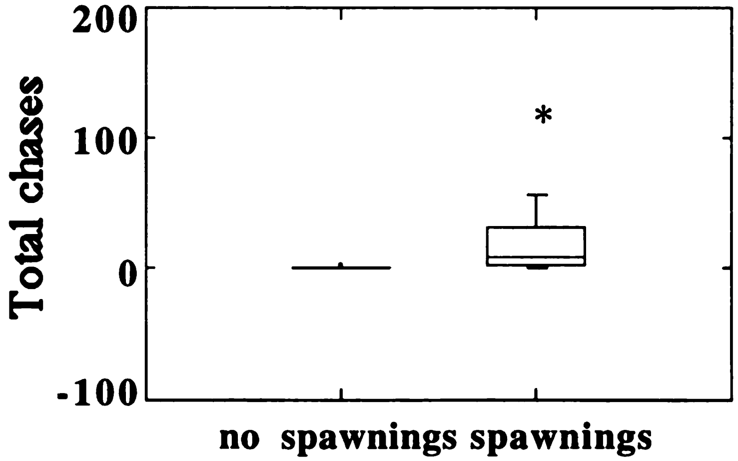
60
40
20
0
-20
*
no spawnings
spawnings

b


Total chases
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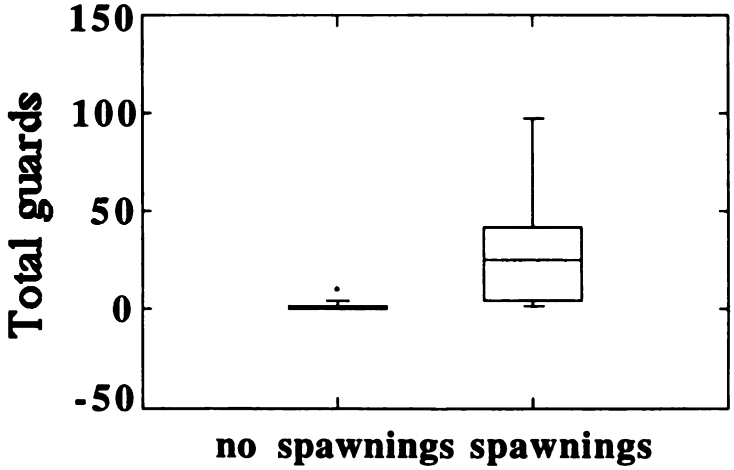
100
0
-100
*
no spawnings
spawnings

c


Total guards
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100
50
0
-50
no spawnings
spawnings

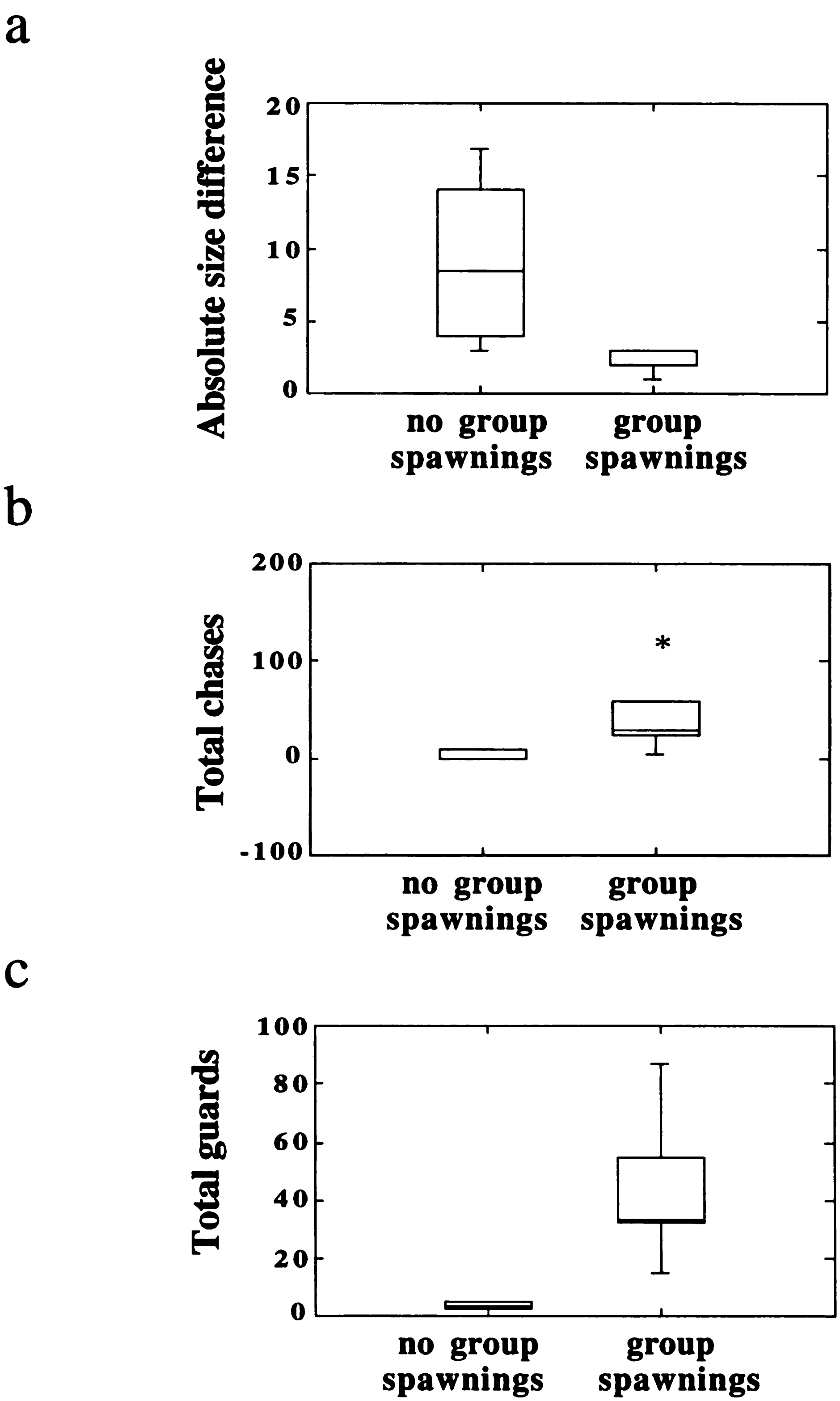

Figure 4 (A) Absolute size difference (mm) between the two males, (B) total number of chases, and (C) total number of guards in replicates where group spawnings took place (n=5) and in replicates where no group spawnings took place (n=6).

chases, guards, sneaky spawnings, nor in their ability to obtain spawnings (Wilcoxon Signed Ranks Test, $P > 0.50$ in all cases). Chase and attack scores were positively correlated ($R_S = 0.638$, $P < 0.05$, n=12). Chase and attack scores were not correlated with guard scores ($R_S = 0.199$, $R_S = 0.182$, $P > 0.500$, n=12). The larger of the two males was more likely to perform more aggressive behaviors. In each trial, the larger male of the two males was more likely to perform more chases (Sign Test $P = 0.039$) and tended to perform more attacks (Sign Test $P = 0.092$) than the smaller male. However, there was no relationship between male size and tendency to perform guards (Sign Test $P = 1.000$).

Does female mating preference or male competition affect male mating success?

The relationship between female mating preferences, aggressive male behaviors, male size, and spawning success is unresolved. There was no relationship between female mating preferences as measured in trials 1-3 and male spawning score or nosedig score in the 1.5 hour observation period. None of the overall preference scores (overall nosedig score, overall time score, overall swim score) correlated with spawning or nosedig scores in the 1.5 hour observation period ($P > 0.200$ all tests). Similarly, none of the preference scores from trials 1-3 correlated with spawning or nosedig scores ($P > 0.100$ in all tests). In the 1.5 hour observation period, guard scores were highly correlated with spawning and nosedig scores ($R_S = 0.880$, $P < 0.001$, n=11, $R = 0.790$, $P = 0.004$, n=11 respectively). In the 1.5 hour observation period, nosedig and spawning scores were highly correlated indicating that the male that was closest to the female when she performed a nosedig in the 1.5 hour observation period was most likely to spawn with her ($R_S = 0.915$, $P < 0.001$, n=11). Attack scores tended to correlate with nosedig scores ($R_S = 0.590$, $P < 0.10$, n=9) but were not correlated with spawning scores ($R_S = 0.368$, $P < 0.20$, n=9). Chase scores were not correlated with either nosedig scores or spawning scores ($R_S = 0.380$, $R_S = 0.154$, $P > 0.50$, respectively, n=8). In each trial, the larger male was not more likely to be closest to the female when she performed a nosedig (Sign Test $P = 0.388$), and was not more likely to spawn with the female (Sign Test $P = 1.00$).

DISCUSSION

Which behavioral variables are good measures of female mating preference?

Two main points emerge from the female choice experiments. First, nosedig behaviors are the most sensitive measures of female mating preference. Female nosedig behavior provides an appropriate measure of female mating preferences because the motivation for this behavior is unambiguous. Females want to mate. In nature, females only perform nosedigs prior to spawning (Winn 1958 a, b, Fuller personal observation). The results presented here support this finding, as nosedigs were only performed in trials where spawnings took place. Furthermore, the male that was closest to the female when she performed a nosedig in the 1.5 hour observation period was most likely to spawn with her.

The second point to emerge from this study is that the amount of time spent with a given male does not appear to be an accurate measure of female mating preference. Measures of female mating preferences based on time assume that females are constantly choosing among males, a potentially erroneous assumption. In contrast, not all females performed nosedigs in every trial. If the female did not perform a nosedig, then a preference score could not be calculated for her. The average time scores incorporate data from all three trials of all replicates where females spawned. However, females may not have been constantly choosing among males in each of these trials. The result of no female mating preferences may be inaccurate because mating preferences are inferred at times when no preferences exist.

In addition, it is interesting to note that time scores produce a different pattern of female preference in replicates where no spawnings occurred. Based on time scores, females preferred to associate with Seven Mile Creek males outside of breeding activities, while nosedig scores indicated that females preferred Prairieville Creek males in the context of mating. As a result of these two contrasting preferences, no significant female mating preferences were found on the basis of time scores. Again, this demonstrates the importance of knowing the motivation of the female when interpreting preferences scores;

otherwise, erroneous mating preferences could be inferred. For example, Pyron (1995) studied female mating preferences for bright versus dull males using time spent with each male as a measure of preference in E. spectabile. Based on time measures, he concluded that females do not have preferences for bright males. Although his analysis only considers animals that went on to spawn, it is still unclear whether females were exerting preferences at all times in his experiment especially as focal females tended to spend more time with control females than either of the two males. Since E. spectabile is in the same subgenus as E. caeruleum (Oligocephalus) and is very similar in morphology, color pattern, and behavior (Page 1983, Winn 1958 a, b), nosedigs may have been a better measure of female mating preferences in this species as well.

Measures of female mating preferences based on time have worked well in fish species where the reproductive state of the female is easily assessed. In poeciliids, females can store sperm and directly fertilize their eggs when they ovulate. As a result, these females are typically receptive to males both as virgins (when they have no sperm) and after giving birth (Basolo 1995, Endler & Houde 1995, Kodric-Brown 1985, Farr & Travis 1986). In other species, researchers have demonstrated that mating preferences based on time measures correlate with mating success and are therefore accurate measures of mating preferences (Berglund 1993, Forsgren 1992). However, when conducting mate choice experiments on previously unstudied organisms, it is imperative to either use a biologically relevant variable (i.e. a variable associated with reproduction) or demonstrate that time measures are associated with some aspect of reproductive success.

Swim scores appear not to be a reliable measure of female mating preferences in E. caeruleum. Although swim scores produced a similar pattern of association preferences in replicates where no spawnings occurred, the vertical swims performed by females are most likely an aquarium artifact. Like other darters, these E. caeruleum lacks a swim bladder and tends to make quick horizontal dashes on the bottom. E. caeruleum has rarely been observed swimming vertically into the upper water column (Fuller personal observation).

Finally, it is worth noting that reversing the position of males is not an effective control for side biases. Female mating preferences became somewhat linked to their original spatial location. Nosedig scores were negatively correlated between trials 2 and 3 which differed only in the location of the two males indicating that females did not follow the male they preferred in trial 2 to the other side of the aquaria in trial 3. This result cannot be accounted for by a preference for simply one side of the aquarium because Prairieville males were placed in the left and right aquaria at random. What can account for this behavior? Females may never have to follow males in the wild. Typically, females enter an area and are themselves followed and defended. Females may conceivably choose among males by performing nosedigs at locations that they associate with specific males. The practice of reversing the position of males to control for side biases has been used predominantly in studies of poeciliids (Kodric-Brown 1985) where males are very mobile and spawning is not restricted in location to a specific substrate. Hence, it is reasonable to expect that females should follow preferred males in poeciliids.

What accounts for female mating preferences?

The experiments on both species demonstrated female preference for particular males. In E. caeruleum females from both populations performed significantly more nosedigs in front of the Prairieville Creek males, a preference which is unexpected. Females were not preferring larger males, as Seven Mile Creek males were larger than Prairieville Creek males. Females must have relied on visual cues for mate choice because a preference was detected in trial 1 where no olfactory cues could be detected. Prairieville Creek males may have appeared more attractive because the experimental lighting conditions closely approximated lighting conditions of Prairieville Creek. The sensory environments of the two populations are different; Seven Mile Creek is deeper, contains more dissolved organic material, and has a darker substrate than Prairieville Creek (Fuller personal observation). Different sensory environments may result in the evolution of different male color patterns, and these color patterns may be perceived as more attractive when signaling takes place in their own environment (Endler 1992, 1993).

Which male behaviors are used in the context of mating?

Males of both species competed aggressively over spawning opportunities. During the observations of spawning dynamics for E. caeruleum, attacks, chases, and guards were performed primarily within the context of mating, and aggression levels were higher when males were of similar size and presumably competitive ability. Group spawnings occurred predominantly in replicates where males were of similar size. Group spawnings usually occur when one male is incapable of completely dominating another male. However, the fact that males spawning as secondary males were also willing to mate as primary males indicates that these males may be making the best of a bad situation (Dunbar 1982, Magnhagen 1992, 1994).

How do female choice and male interactions affect male mating success?

In this experiment, male guarding was important in determining mating success. Males that successfully guard females from competing males enjoy high spawning success. This simply raises the question what determines male guarding ability? Neither overt aggressive behavior (attacks and chases) nor male size was correlated with guard behavior or spawning success. Furthermore, female mating preferences were not related to male spawning success. The interactions between these phenomenon are most likely quite complex. The sample size in this study is undoubtedly too low to resolve these issues. However, similar results have been found in E. spectabile (Pyron 1995). Males that followed females and defended them from neighboring males were more likely to obtain spawnings (Pyron 1995). However, the relationship between male guarding ability and male size/color was not clear. The relationship between male size and spawning success may be obscured if female size is not taken into consideration (Verell 1991). Winn provides anecdotal evidence that size assortative mating may play a role in darters (1958a, but see Pyron 1996). Successful fertilization between large males and small females may be technically difficult to achieve. In addition, large males may choose not to exert energy and/or sperm in order to spawn with small females that release fewer eggs per spawning than larger females Winn also gives anecdotal evidence that large males may not always dominate in competition. "In all 11 of 15 cases an intermediate-sized male was dominant, the larger exhibiting no interest in breeding. If a dominant intermediate-sized male was challenged by a larger male, the larger fish usually became dominant in the tank . . ."

(Winn 1958 b). These observations indicate that the relationships between size, aggression, and spawning success are complex and in need of further study. When do males choose to compete for females? How do female mating preferences affect male mating success? Can female behavior affect the outcome of male competition? Which male characters are used in female choice and male competition? The answers to these questions will be important in assessing the relative importance of male competition and female choice in darter breeding systems.

Chapter 2

Costs of group spawning to primary males

INTRODUCTION

Sperm competition occurs when ejaculates of 2 or more males compete for fertilization of a set of eggs. It is a common phenomenon in fishes and can have important effects on the behavior and life history patterns in many animals (Breder & Rosen 1966, Halliday & Verrell 1984, Stockley et al. 1997, Stockley 1997). The rainbow darter, Etheostoma caeruleum, is a promiscuous species where sperm competition in group spawnings is common (Breder & Rosen 1966, Fuller & Porterfield in prep.). 'Secondary' males will often sneak in and release sperm next to a spawning pair. In one population, 80% of spawnings involve group spawnings where 2-5 males simultaneously spawn with a female (Fuller, unpublished data, Gull Lake, Kalamazoo Co., MI, USA). In comparison to the more well-known examples of alternative male strategies in salmon and bluegill (Gross 1982, 1984), there is no alternative male morphology associated with the secondary male strategy. All males will spawn as 'primary' guarding males when given the opportunity (Fuller & Porterfield in prep.). This observation indicates that the secondary male strategy represents a best of a bad job strategy (Dunbar 1982). However, behavioral observations indicate that group spawning may be quite costly to primary males. A primary male will often forego spawning opportunities with females if competing males are present indicating that secondary males may fertilize a significant amount of eggs.

This study addresses the following two questions: How costly is group spawning to primary males in terms of lost paternity? Do large males have an advantage over smaller males in spawning as primary males? The paternity of offspring resulting from group spawnings where two males simultaneously mate with one female is assessed using males and females of known allomorphs.

MATERIALS AND METHODS

E. caeruleum was collected from the upper Mill Pond Stream, 37th & G Streets, Kalamazoo county, MI, USA, and returned to Kellogg Biological Station, January 11 - February 2, 1997. Adults were prescreened for PGM-2 using a fin clip from their pelvic and caudal fins. Twelve sets of fish were established containing two males that were homozygous at alternate allomorphs (PGM-2: SS and FF). One male was given a mark so that the allomorph of each male was always evident. Marks were equally distributed among males of both allomorphs. Six sets of fish contained a female that was homozygous at one allomorph (SS), and six sets of fish contained a female homozygous at the alternate allomorph (FF). Each set of fish was placed in 20-liter aquaria with a rocky substrate, and housed in the laboratory until April 4. Standard length was measured at the beginning of the study. At the completion of this study, the allomorph of the adult fish was double checked. In one replicate, a male classified as homozygous was found to be heterozygous. This replicate was excluded from the paternity analysis.

Fish were fed twice daily with a diet of live chironomids and blackworms. Excess food items were always present in the rocks of the aquaria and ensured an adequate food supply. Lights were kept on a 14L:10D to mimic natural sunlight patterns during the breeding season. The thermostat in the laboratory was set at 14^{o}C, and water temperatures ranged between 10-15 ^{o} C for the majority of the breeding season. Two sets of fish died in the laboratory before spawning.

Fish were observed until they spawned. Each spawning was categorized as either a single spawning or a group spawning. For single spawns, the identity of the spawning male was recorded. For group spawns, the identities of the males spawning as primary and secondary males were recorded. The primary male was defined as the male that began the spawning and spawned on top of the female. The secondary male was defined as the male that sneaked in and quivered next to the spawning pair once they had already begun spawning.

After each spawning, eggs were obtained using a vacuum hose and the remaining water was returned to the aquarium. Darter sperm is effective at fertilizing eggs for approximately 20 seconds after it is released (Hubbs 1960). Occasionally, the fish spawned in the absence of an observer. Aquaria were periodically checked for eggs using a vacuum hose. When eggs were found, the fish were moved into a new aquarium with clean rocks.

Eggs were kept in plastic tubs and treated with methylene blue to prevent fungus infection. Water was replaced every 3-4 days. After hatching, fry were fed live brine shrimp. After 4 weeks, the fry were transferred to aquaria where they were fed brine shrimp 1-2 times a day. In late May, the fry were frozen (-80° C) in individual Eppendorf tubes containing 2 drops of grinding buffer. Paternity was determined using the electrophoresis methods described above.

The electrophoresis methods described here were adapted from Mather & Rusco (1992) and Hebert & Beaton (1989). Adult fin clips were obtained by clipping the posterior 1/3 of the pelvic fin and posterior 1/6 of the caudal fin. Juvenile fish were killed and placed directly into Eppendorf tubes. Tissues were placed in Eppendorf tubes containing 2 drops of grinding buffer (0.01 M tris-HCl, pH 8.0, with 1% Triton X-100) and two small ball bearings. The tissues were ground using an amalgamator which shook the Eppendorf tubes for 1 minute. The tissues were then centrifuged in an Eppendorf Centrifuge for 2 minutes at 14,000 RPM. The supernatant was decanted and used for electrophoresis. Liquid samples were loaded onto a Helena tray containing 12 individual holding wells. An applicator was used to transfer the liquid from the individual holding wells onto the cellulose acetate plates (Titan III, Helena Laboratories, Texas).

Electrophoresis was performed using cellulose acetate gels which had been soaked in buffer (50 mM Tris glycine (pH 8.5)) for at least 20 minutes. Gels were run at 200 V for 30 minutes at 4°C. Gels were stained for PGM-2 (EC 2.7.5.1) using the gel recipe developed by Hebert and Beaton (1989). Because the amount of protein contained on each gel was very small, a larger amount of enzyme (80 ul) was used for each stain. Gels were scored at PGM-2 as being either FF (fast-fast), SF (slow-fast) or SS (slow-slow).

The paternity of each male was estimated as the proportion of the clutch sired by each male. Clutches were only included in the paternity analysis if there were 4 or more fry. In two replicates, insufficient number of fry developed to allow analysis of paternity.

The mean paternity of each male was calculated for replicates from which multiple clutches were obtained.

RESULTS

The allomorph frequencies of prescreened adults did not differ from frequencies that would be expected if the population were in Hardy Weinberg equilibrium [OVERALL: FF (fast-fast) observed: 25, expected: 24.75; SF (slow-fast) observed: 51, expected: 49.5; SS (slow-slow) observed: 23, expected 24.75; MALES: FF observed: 15, expected 14.25; SF observed 28, expected 28.50; SS observed: 14, expected: 14.25; FEMALES FF observed: 10, expected 10.5; SF observed: 23, expected: 21; SS observed: 9, expected: 10.5].

Overall, large males spawning as primary males sired 51.3% of the offspring (Table 1). In three replicates, large male primary paternity differed from a null expectation of 0.5 (Table 1). In two of these replicates, the primary male sired more offspring than the secondary male. In one replicate, the secondary male tended to sire more offspring than the primary male. Large male primary paternity was not related to the magnitude of the size difference between the two males (Pearson correlation coefficient, R = -0.243, P = 0.599, n = 7). In two replicates, the smaller male spawned as the primary male on at least one occasion. In the second replicate, the secondary male spawned as a primary male two times. In one spawning he sired 100% of the offspring, and in the other spawning he sired 0% of the offspring. In the seventh replicate, the secondary male spawned as the primary male one time and sired 18% of the offspring.

Male size was important in determining competitive ability. In each pair, the larger of the two males was more likely to spawn singly with the female (paired T = 2.63, DF = 8, P = 0.030). In group spawns, the larger of the two males was more likely to spawn as the primary male (paired T = 3.53, DF = 8, P = 0.008). The difference in size between the two males tended to be correlated with the proportion of spawnings in which the larger male spawned as the primary male (R = 0.851, P = 0.004, n=9). Male allomorph was not related to competitive ability (paired T = 0.466, DF = 6, P = 0.658).

Rep	Female	L. Male	Size Diff	Number of Clutches	Total Fry Examined	L. Male Primary Pat. (SE)	Smaller Male as Primay?
	Allomorph						
1	SS	SS	16	5	81	42 (12)	no
2	SS	SS	1	3	15	11 (11)§	yes
3	SS	FF	5	1	10	60	no
4	SS	FF	4	4	21	83 (7)*	no
5	SS	FF	0	3	19	84 (8)*+	no
6	FF	SS	16	1	5	20	no
7	FF	SS	21	4	19	60 (14)	yes
					overall mean:	51.3 (0.108)	

Table 1 Replicates in which adequate numbers of offspring were obtained to assess the reproductive success of primary and secondary males. Replicate female allomorph, large male allomorph, absolute size difference between the two males (mm), number of clutches with 4 or more fry, total number of fry, large male primary paternity, and whether or not the smaller male every mated as a primary male is listed. + In this replicate there was no size difference between the two males. Since clutches were only obtained from spawnings where the FF male spawned in the primary position, that male's paternity is listed. This replicate is not included in the behavioral analyses. § indicates that the proportion of the clutch sired by the primary male differs from a null expectation of 0.5 at $P < 0.10$. * indicates that the proportion of the clutch sired by the primary male differs from a null expectation of 0.5 at $P < 0.05$.

DISCUSSION

Two points emerge from this study. First, group spawning is costly to primary males in terms of lost paternity. No primary male ever achieved 100% paternity . When groups spawns involve two males, each male has an equal probability of fertilizing the eggs. Does this mean that primary and secondary mating tactics provide males with equal fitness benefits? The answer is no. Males acting as primary males occasionally spawn singly with females. The advantage to primary males of mating singly with females (where they are assured 100% paternity) is large. Similar results have been found in other species. In a similar study, Foote et al. (1997) used electrophoresis to determine the relative spawning success of jacks (sneakers) relative to larger, older males in sockeye-salmon, Oncorhynchus nerka. They found that jack spawning success was variable and did not differ statistically from that of larger, older individuals. Thomaz et al. (1997) used minisatellite DNA markers to analyze paternity of parr males (sneakers) versus larger, older males in the Atlantic Salmon, Salmo salar. They found that individual parr fertilized up to 26% of the eggs.

The second point to emerge from this study is that male competitive ability is associated with male size. In paired contests, larger males spawn singly with females and spawn as primary males in group spawnings more often than smaller males. Furthermore, the ability of larger males to consistently spawn as primary males is associated with the difference in size between itself and its competitors. Large male mating advantage has been demonstrated in numerous mating systems (Andersson 1994). However, this is the first quantitative demonstration of large male mating advantage in darters. Previous studies provided only anecdotal information on large male mating advantage (Winn 1958, Distler 1972).

This study was somewhat artificial in that only two males could spawn with the female. In the field, one to five males may simultaneously spawn with the female. The manner in which additional males fertilize the female's clutch is unknown. Males mating closest to the female (i.e. on top and directly on each side) may fare best as their sperm is most likely released closer to the female's eggs. Secondary males located on the outside of the spawning group (i.e. with another secondary male between himself and the female) may not fertilize a proportion of the clutch that is commensurate with their relative sperm output. Use of more variable molecular markers (e.g. DNA finger printing, microsatellites) should resolve these issues.

Chapter 3

Sperm Competition Affects Male Sperm Output and Behavior

INTRODUCTION

Sperm competition, defined as competition between ejaculates of 2 or more males for fertilization of a set of eggs, is a common phenomenon and has important effects on the behavior and life history patterns in many animals (Eberhard 1996, Halliday & Verrell 1984, Stockley 1997). Many fishes engage in group spawnings which involve 2 or more males simultaneously spawning with one female (Breder & Rosen 1966, Stockley et al. 1997). Theory predicts that across populations the prevalence of sperm competition should be correlated with investment in sperm production (Parker et al. 1996). Comparative studies support this prediction, finding that sperm competition is correlated with investment in sperm production as measured by gonadosomatic index (gonad weight/body weight) (Stockley et al. 1997).

Within a species, the relationship between intensity of sperm competition and sperm output between spawning opportunities is not as straightforward. In many fishes, sperm production is costly, and therefore males should carefully allocate their sperm among mating opportunities (Dewsbury 1982, Nakatsuru & Kramer 1982, Shapiro et al. 1994). Models considering the relationship between the number of competitors in a group spawning event and male sperm output indicate that males should ejaculate less sperm when sperm competition is higher than average and should ejaculate more sperm when sperm competition is lower than average (Parker et al. 1996). The reasoning behind this model is that males should reduce sperm output when sperm competition is high because males can wait for better mating opportunities in the future with fewer competing males. Similarly, males should take advantage of mating opportunities taking place under low sperm competition by increasing sperm output because such an opportunity may not arise again.

In this chapter, I examine the effect of potential sperm competition and male size on male sperm output, male willingness to spawn, and a competitive behavior, adoption of black coloration, in the rainbow darter, Etheostoma caeruleum. Specifically, I address the following questions: 1) Do males increase their sperm output under low sperm competition and decrease their sperm output under high sperm competition? 2) Are males less willing to mate under high sperm competition? 3) Is the adoption of black coloration used in male/male competition?

MATERIALS AND METHODS

Fish were collected with a kicknet between January and April, 1997 at Mill Pond Outlet, Kalamazoo County, Michigan, USA. Animals were returned to Kellogg Biological Station where they were housed. The field season was extended by inducing fish to spawn early. Fish were brought into reproductive condition early by catching them prior to the onset of the breeding season, bringing them into the lab, and manipulating their water temperature and light ratios so as to mimic conditions during the breeding season (water temperature = 12° to 10 °C, day : night ratio 14 L : 10 D). The sex ratio of stock aquaria was roughly 1:1 with 2-3 males and 2-3 females in each aquarium. Animals were fed twice a day with live tubifex worms and frozen chironomid larvae. The first breeding activities were recorded on 97/02/14, and experiments began on 97/02/21.

Animals were maintained in mixed-sex stock aquaria so that the breeding stage of females could be monitored. Female E. caeruleum have a small window of time during which they can spawn. Once a female has ovulated her eggs, she has only a few days during which she is receptive to males (Fuller personal observation). After that time, she will drop the eggs into the gravel allowing them to go unfertilized (Fuller personal observation). Mixed-sex stock aquaria were kept in the same room where the experiment was conducted. When females were observed performing nosedigs or spawning, they were transferred to all-female holding aquaria and were used within 2 days.

To examine the effect of potential sperm competition on male sperm output and related guarding behaviors, I allowed a male and a female to spawn in one of four different treatments. One male and one female were allowed to spawn in the presence of either four males, one male, zero males, or one female. The one female treatment was used as a control to ensure that male responses were due to the presence of competing males as opposed to simply the presence of conspecifics.

Two aquaria set-ups were used in this experiment. In the first set-up, I divided 40 l aquaria (50.80 cm x 26.04 cm x 31.75 cm) into two equal sections (25.40 cm x 26.04 cm x 31.75 cm) using clear pieces of Plexiglas. Barriers were attached to the bottom and sides of the aquarium with silicone so that no sperm could pass under the barrier. Darter sperm is negatively buoyant (Fuller personal observation). A series of 3-5 mm holes were drilled in each barrier approximately 5 cm from the bottom so that olfactory cues could pass between the two compartments. This aquaria set-up was used for the one male, zero males, and one female treatments. For each trial, a male and female were placed in one section and the stimulus animal in the other section. In the second set-up, I divided 40 l aquaria into three sections - one large central section which held the focal animals (25.40 cm x 26.04 cm x 31.75 cm) and two smaller end sections each of which held two stimulus males (12.70 cm x 26.04 cm x 31.75 cm). This aquarium set-up was used for the 4 male treatment. The central section containing the focal animals was the same area as the sections holding focal animals in the first aquarium set-up. Again, clear plastic Plexiglas barriers were attached to the bottom and sides of aquaria with silicone. All barriers contained a series of 3-5 mm holes 5 cm from the bottom which allowed olfactory cues to pass between the sections. The bottom of all aquaria were lined with small-grain gravel. Following each trial, all water was removed from the aquarium in which the fish had spawned. The gravel in the section where fish spawned was removed from the aquaria and set aside for a period of at least 1 week. Prior to being reused, the gravel was rinsed with hot tap water.

For each trial, the focal male, female, and stimulus animals were placed in an aquarium at approximately the same time. If the female did not perform a nosedig within two hours, then the trial was canceled. Individuals were observed over the course of five spawnings. Occasionally, animals ceased to spawn part-way through the trial. In these cases, I observed the animals for 2-3 hours after the last spawning. If the female did not perform a nosedig during this time, then the trial was canceled. Following each trial, the standard length of focal and stimulus animals was measured to the nearest mm. Forty-nine trials were completed in total. Standard lengths of males and females did not differ significantly among treatments ($F_{3,45} = 0.469$, $P = 0.705$, $F_{3,45} = 0.128$, $P = 0.943$ respectively). Standard lengths of stimulus males did not differ between the one male and four male treatments ($T = 0.387$, $DF = 22$, $P = 0.702$).

Behavioral data was recorded over the course of the five spawnings. I recorded the number of complete nosedigs performed by the female prior to each spawning and whether or not the male turned black prior to each spawning. From these data, I calculated the mean number of missed opportunities to spawn and the black score. The number of missed opportunities to spawn is the number of complete nosedigs minus the one nosedig after which the male spawned with female. Each time the female performed a complete nosedig in which her body was buried in the gravel the male had an opportunity to spawn. Thus, when a female performed a nosedig and a male chose not to spawn, he missed an opportunity to spawn (because in the wild the female may have swam away and spawned with another male). The black score was calculated as the proportion of spawnings prior to which the male had turned black.

In this experiment, I measured male sperm output over a series of spawnings using the basic sperm collection techniques developed by Shapiro et al. (1994) modified here for darters. After each spawning, the sperm and eggs were removed from the aquarium by rapidly siphoning approximately 1300 ml of water from the aquaria into a bucket. During this process, I concentrated on siphoning primarily in the area where the fish had spawned. I then replaced fresh water in the aquarium. After the first, third, and fifth spawnings, I vigorously mixed the water in the bucket to suspend the sperm and took a 500 ml sample. Darter sperm is negatively buoyant. The sample was then treated with five drops of Rose Bengal dye and placed in a refrigerator. After 25 minutes, 25 ml of formalin was added to the solution to fix the stain. Following the completion of the experiment, the eggs were removed from the bucket and the remaining volume of water was measured to obtain a dilution factor.

At a later time, the sperm solution was processed. The sperm solution was first passed through a 35 μm mesh nylon filter to remove debris and then filtered through a 0.22

µm millipore filter using a vacuum pump. The filter was then dried on a hot plate for > 30 minutes. Finally, a portion of the filter was mounted on a slide with immersion oil. Using a compound microscope and an ocular grid, the number of sperm occurring in the grid was counted on 40 separate areas of the slide. By starting in the upper corner and working down and across the slide, the counts are presumed to be independent. Sperm estimates were then calculated with the following formula: sperm estimate = (# sperm counted/area counted)*(total area of millipore filter)*(volume of water remaining in bucket + volume of sample)/(volume of sample). For each male, the mean sperm output was calculated as the average of the estimated sperm outputs from the first, third, and fifth spawnings.

Eggs were retrieved from each spawning, placed in containers, treated with methylene blue to prevent fungus infection, and monitored for development. I measured fertilization success as the proportion of eggs that developed to the stage where they had pigmented eyes. The attainment of this developmental stage is a conservative, but reliable measure of fertilization success (Hubbs 1955).

All statistical tests were conduced using Systat statistical package. Nonparametric statistics are used when the underlying assumptions of parametric tests are violated. For all analyses of variance, Bartlett's test for homogeneity of variance was used to test for heteroscedasticity (Sokal & Rohlf 1995 p. 391). For analyses of covariance, the residuals from each regression were examined to ascertain whether they differed from normal using Kolmogorov-Smirnov Lilliefors test (Tessier personal communication). All probabilities are two-tailed.

Testing Sperm Collection Methods

Following Shapiro et al. (1994), I tested whether the above methods accurately estimated the amount of sperm released by males. Sperm solutions were created by hand stripping males and mixing sperm with water, formalin, and Rose Bengal dye to obtain a 5% formalin solution. For each trial, I used a pipette to deposit approximately two milliliters of sperm solution among the rocks on the bottom of an aquarium. I waited approximately 30 seconds, siphoned the sperm mixture out of the aquaria, and later estimated the total amount of sperm using the methods described above. I compared these values to a control treatment where an equivalent amount of sperm was released directly into a bucket.Sperm estimates for aquaria treatments were compared to control treatments using T-tests and linear regression. Twenty-one trials were run in total.

RESULTS

Sperm Collection Tests

The sperm collection method was relatively reliable. There were significant differences between the amount of sperm obtained from the aquaria and control treatments in two of the 21 trials. In one trial, the sperm estimate was greater in the aquaria treatment (T = 3.031, DF = 78, P = 0.003), whereas in the other trial the sperm estimate was greater for the control treatment (T = -2.089, DF = 78, P = 0.04). In the other 19 trials, there were no significant differences in the amount of sperm obtained between the aquarium and bucket controls ($P > 0.05$ in all tests). Overall, there was no significant difference in the amount of sperm obtained between the aquarium and bucket controls (T = -0.190, DF = 20, P = 0.851). Furthermore, the number of sperm collected in aquarium treatments correlated strongly with that obtained from bucket treatments (Figure 5). The slope of the line is approximately 1 ($b = 0.971X \pm 0.013$ SE).

Sperm Output and Male Behavior

Male sperm output did not differ significantly among treatments (Figure 6, Kruskal-Wallis Test Statistic = 4.317, P = 0.229). Pooling the four males and one male treatments as ‘competing males present’ and the one female and zero males treatments as ‘competing males absent’, males released larger amounts of sperm when mating in the presence of competing males (Mann-Whitney U = 202.00, P = 0.05).

The relationship between male size and sperm output differed among treatments (Table 2A, Figure 7). Male sperm output decreased with male size in the four male treatment (Figure 7A). This appears to be caused by small males increasing their sperm output relative to large males. In contrast, male sperm output increased slightly with male size in the one male treatment (Figure 7B). Male standard length had little relation to sperm

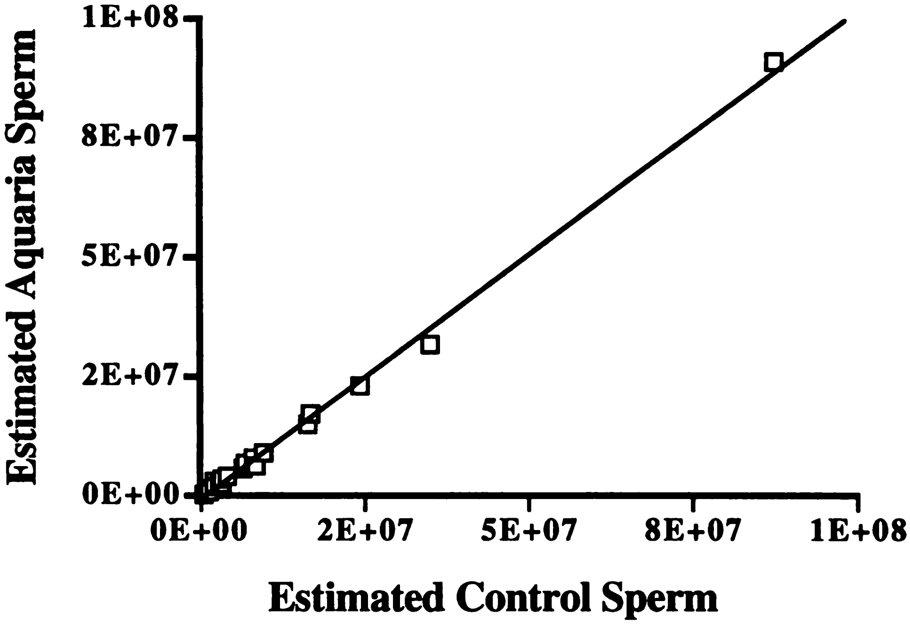

Figure 5 Relationship between estimated aquaria sperm and estimated control sperm. linear regression: Y = 0.971X + 296,982, where Y=the amount of sperm obtained from the aquarium treatment and X equals the amount of sperm obtained from the bucket treatment, F = 5434.80, P = 0.0001.

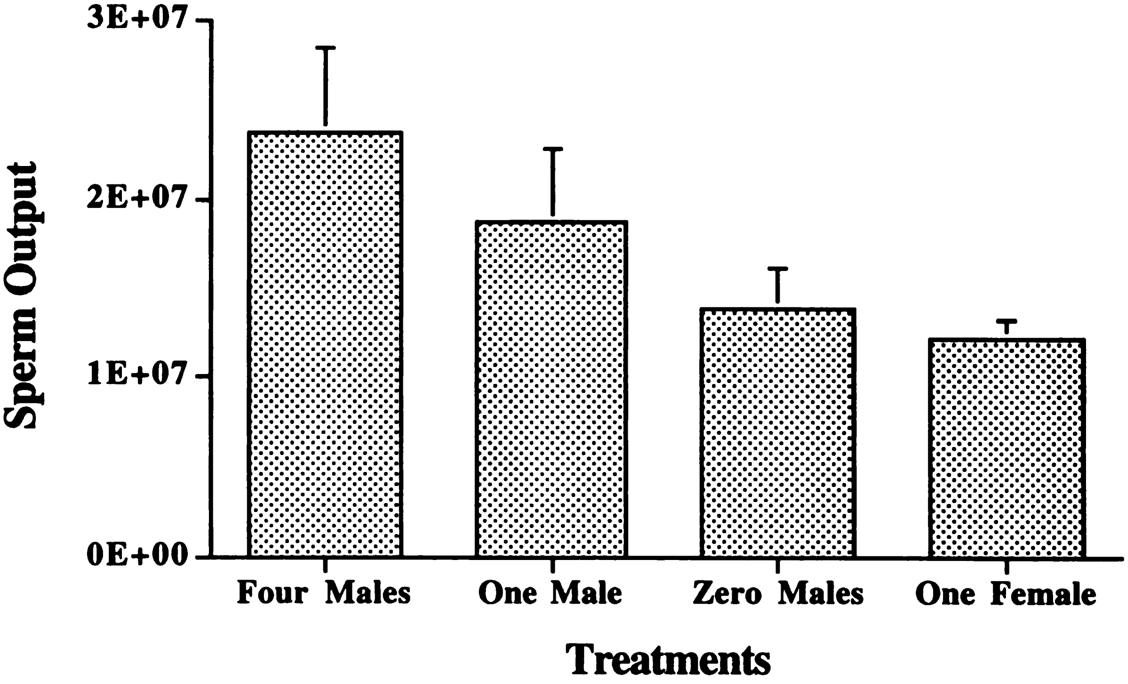

Figure 6 Sperm output in the four treatments. Means and standard errors are shown. Sample sizes were the following: four males n=14, one male n=10, zero males n=12, one female n=13.

A.

Dependent Variable = Sperm Output

Source	Sum-of Squares	DF	F	P
Male Size	.4226 E+14	1	0.345	0.560
Treatments	.1434 E+16	3	3.907	0.015
Treatments X Male Size	.1331 E+16	3	3.625	0.021
Error	.5017 E+16	41		

B.

Dependent Variable = Missed Opportunities to Spawn

Source	Sum-of -Squares	DF	F	P
Male Size	16.20	1	15.56	0.000
Treatments	8.08	3	2.59	0.066
Treatments X Male Size	10.50	3	3.36	0.028
Error	42.69	41		

C.

Dependent Variable = Black Score

Source	Sum-of-Squares	DF	F	P
Male size	0.168	1	3.51	0.068
Treatments	5.083	3	35.31	0.000
Error	2.111	44		

Table 2 Analyses of covariance on (A) sperm output, (B) missed opportunities to spawn, and (C) black score. The variable missed opportunities to spawn is measured as the number of opportunities males had to spawn prior to each spawning. The values are an average of the number of missed opportunities to spawn prior to each spawning over 5 spawnings. Black score is calculated as the proportion of spawnings prior to which the male had turned black.

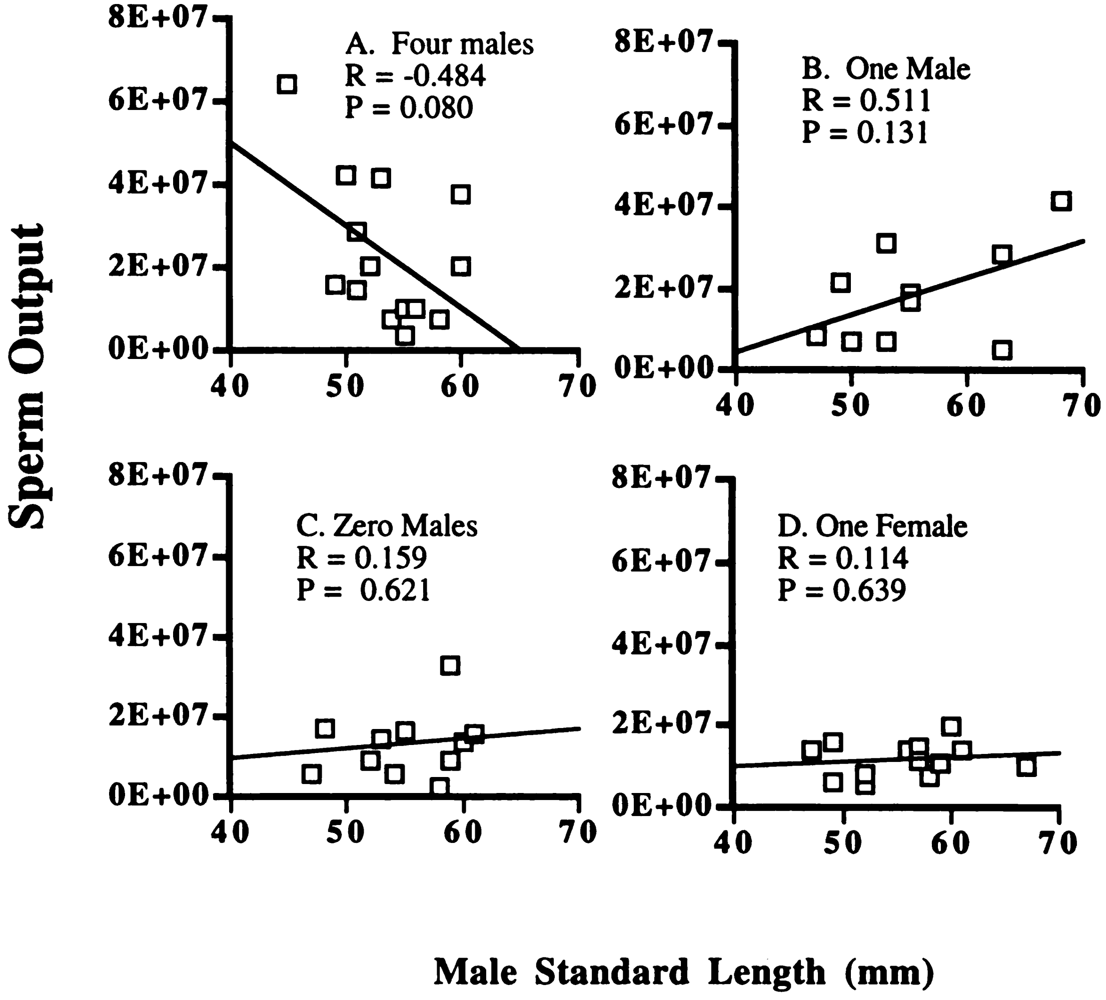

Figure 7 Relationship between sperm output (estimated sperm cell numbers) and male standard length (mm) among the four treatments (A-D).

output in female and zero male treatments.

The treatments had strong effects on the tendency of males to forego spawning opportunities (Figure 8, Kruskal-Wallis Test Statistic = 10.952, DF = 3, P = 0.012). To compare treatments, I used a nonparametric post-hoc test that corrects for multiple comparisons (Siegel & Castellan 1988, p. 213). Males in the four male treatment were significantly more likely to forego opportunities to spawn than males in the zero males or one female treatment (Figure 3, $Z_{crit.,\alpha=0.05} = 2.638$). There were no differences in the tendency of males to forego spawning opportunities between the four males and one male treatment, nor were there differences in the tendency of males to forego spawning opportunities between female, one male, and zero males treatments.

The relationship between male standard length and tendency to forego spawning opportunities varied significantly among treatments (Table 2B, Figure 9). Male standard length was positively correlated with missed opportunities to spawn in the four male treatment (Figure 9A). This pattern appears to be caused by large males foregoing more opportunities to spawn in the four males treatment.

Males were more likely to assume black coloration when in the presence of other males (Table 2C, Figure 10). After testing for homogeneity of slopes, analysis of covariance indicated that treatments had significant effects on the likelihood of males to turn black (Table 2C). Males in the four male treatment turned black more often than males in the one male, zero male, and female treatments (Tukey HSD Multiple Comparisons, P = 0.000 for all comparisons). Males in the one male treatment also turned black more often than males in the zero males or one female treatments (P = 0.025, P = 0.005 respectively). The likelihood of males to turn black did not differ significantly between zero males and one female treatments (P = 0.947, n = 14). Furthermore, the likelihood of males to turn black in the one female and zero males treatments did not differ significantly from zero (one sample T-tests T = 1.000, P = 0.337, T = 1.000, P = 0.339 respectively).

Having discerned the effects of treatments and male standard length on sperm output, black score, and number of missed opportunities to spawn, I examined the correlations between sperm output, black score, and missed opportunities to spawn in each of the four treatments. Bartlett chi-squared tests indicated that the overall correlation matrix

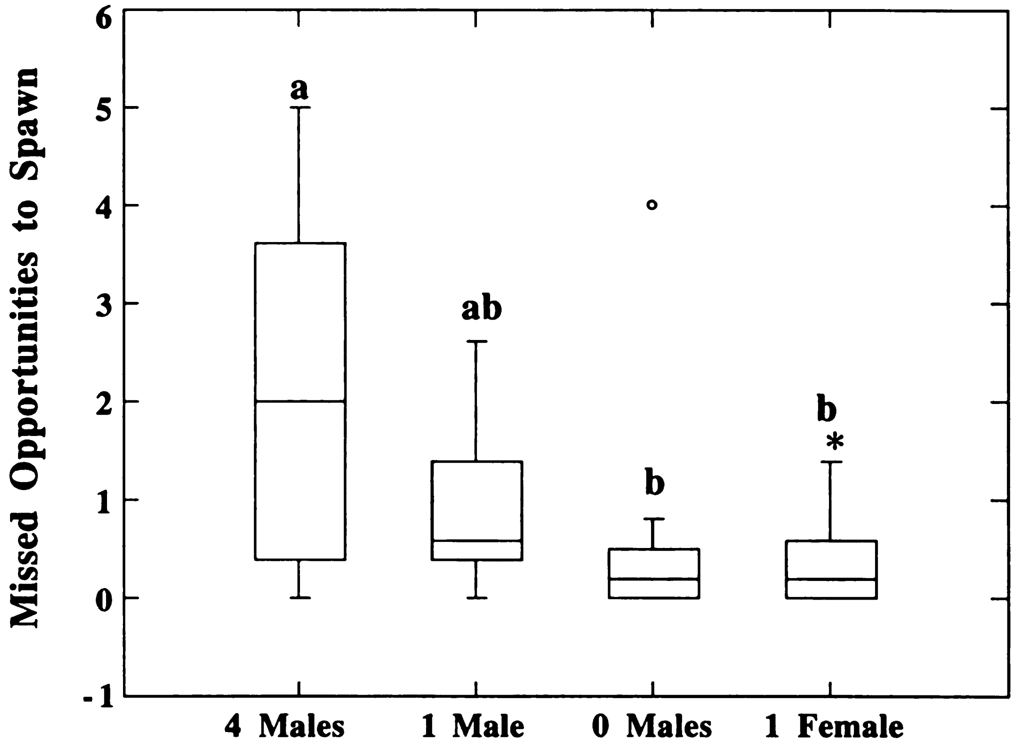

Figure 8 Box plots of missed opportunities to spawn in the four treatments. The middle line refers to the median of the data. The edges of the boxes refer to the first and third quartiles of the data. Whiskers mark ± 3 times the interquartile range from the median. Stars mark outside values, and circles mark far outside values. Sample sizes are as follows: four males n=14, one male n=10, zero males n=12, one female n=13.

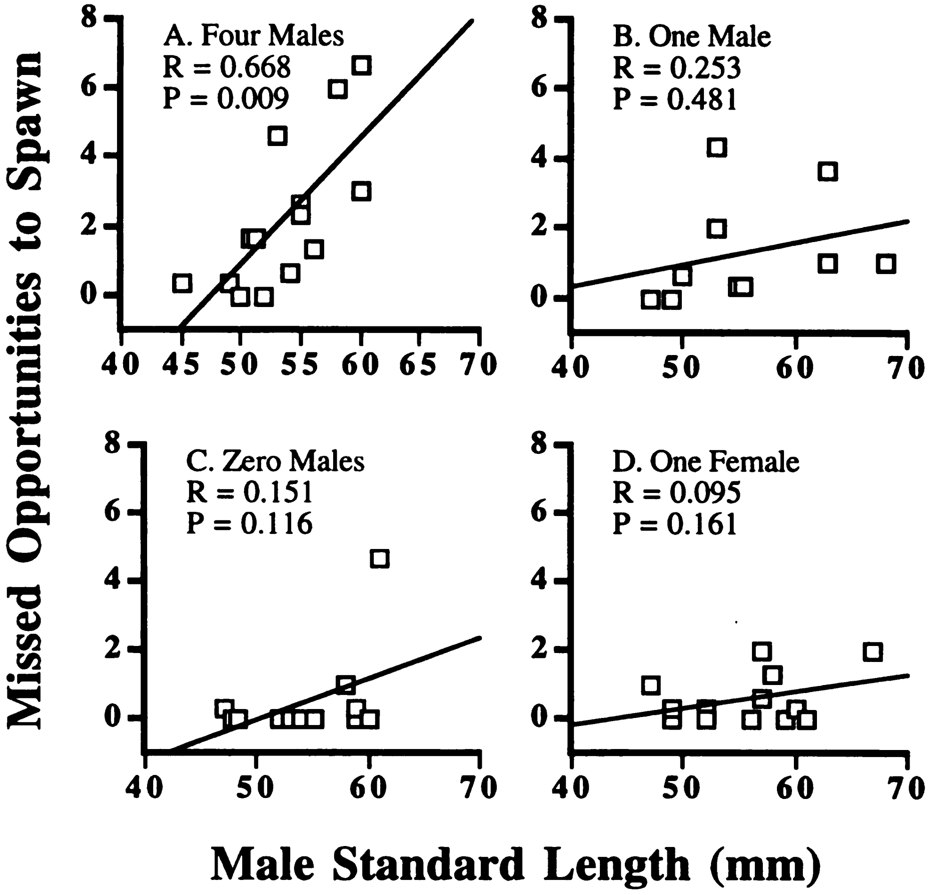

Figure 9 The relationship between missed opportunities to spawn and male standard length among the four treatments (A-D). The variable missed opportunities to spawn is measured as the number of opportunities males had to spawn prior to each spawning. The values are an average of the number of missed opportunities to spawn prior to each spawning over 5 spawnings. In the zero males treatment, an outlier resulted in a non-normal distribution of residuals from the individual regression. Excluding this data point from the analysis results in a normal distribution of residuals along the regression line, increases the overall fit of the model, and increases the significance of the interaction term.

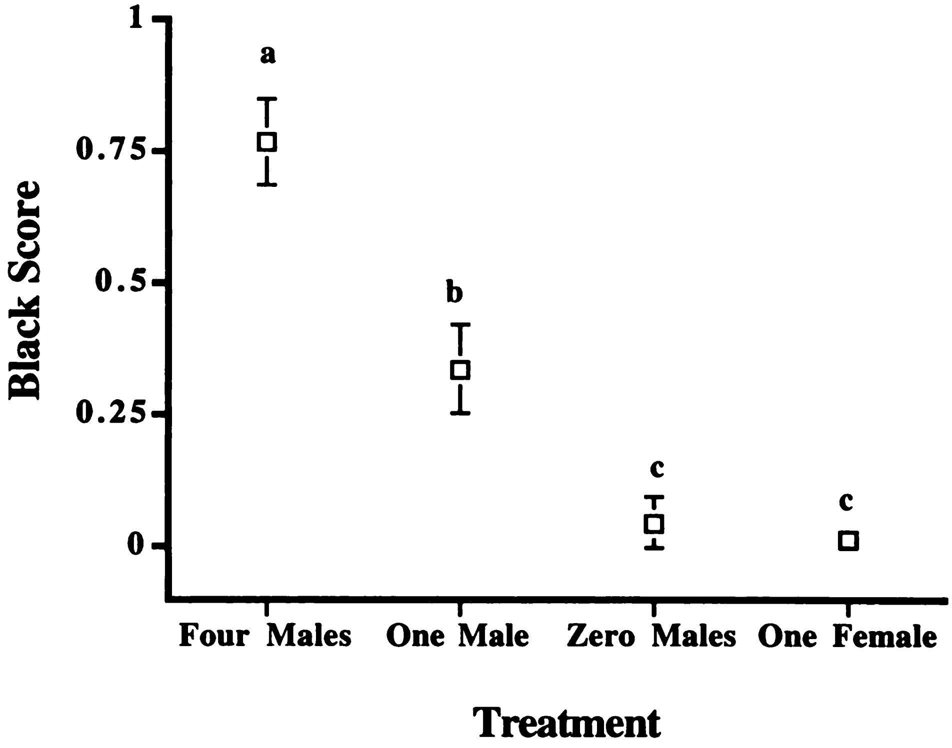

Figure 10 Black scores in the four treatments. Black score is calculated as the proportion of spawnings prior to which the male had turned black. Values range between 0-1. Means and standard errors are shown. Sample sizes are the following: 4 males n = 14, 1 male n = 10, 0 males n=12, 1 female n = 13.

accounted for a significant amount of variation only in the four male treatment (Bartlett X^2 = 8.043, DF = 3, P = 0.045). I therefore only considered correlations in the four male treatment. Black score and missed opportunities to spawn were positive correlated (R = 0.580, P = 0.030, N=14, Figure 11). Black score and sperm output tended to be negatively correlated (R = -0.464, P = 0.095, N=14). There was no relationship between missed opportunities to spawn and sperm output.

Effects of Female Size and Fertilization Success

To examine whether female size had effects on male behavior, I computed the correlation coefficient between female standard length and sperm output, missed spawning opportunities, and black score in each of the four treatments. Bartlett chi-squared tests indicated that the overall correlation matrix accounted for a significant amount of variation only in the four male treatment (Bartlett X^2 = 14.334, DF = 6, P = 0.026). A significant correlation existed between black score and female standard length (R = 0.620, P = 0.018, Figure 12). Female standard length and missed opportunities to spawn also tended to be positively correlated (R = 0.491, P = 0.075, n = 14).

Across all four treatments, there was no relationship between mean sperm output and mean fertilization success (R = -0.020, P = 0.896, range = 93.6% - 0.0%, mean = 43.4% ± 0.042 SE, n = 49, c.v. = 0.657). Female standard length correlated with the total number of eggs released over the five spawnings (R = 0.446, P = 0.001, N=49).

DISCUSSION

According to the model proposed by Parker et al. (1996), males should reduce their sperm output when under higher than average sperm competition and should increase their sperm output when under lower than average sperm competition. Applied to this experiment, males were predicted to reduce their sperm output in the four males treatment and increase their sperm output in the one male treatment. This prediction was not upheld.

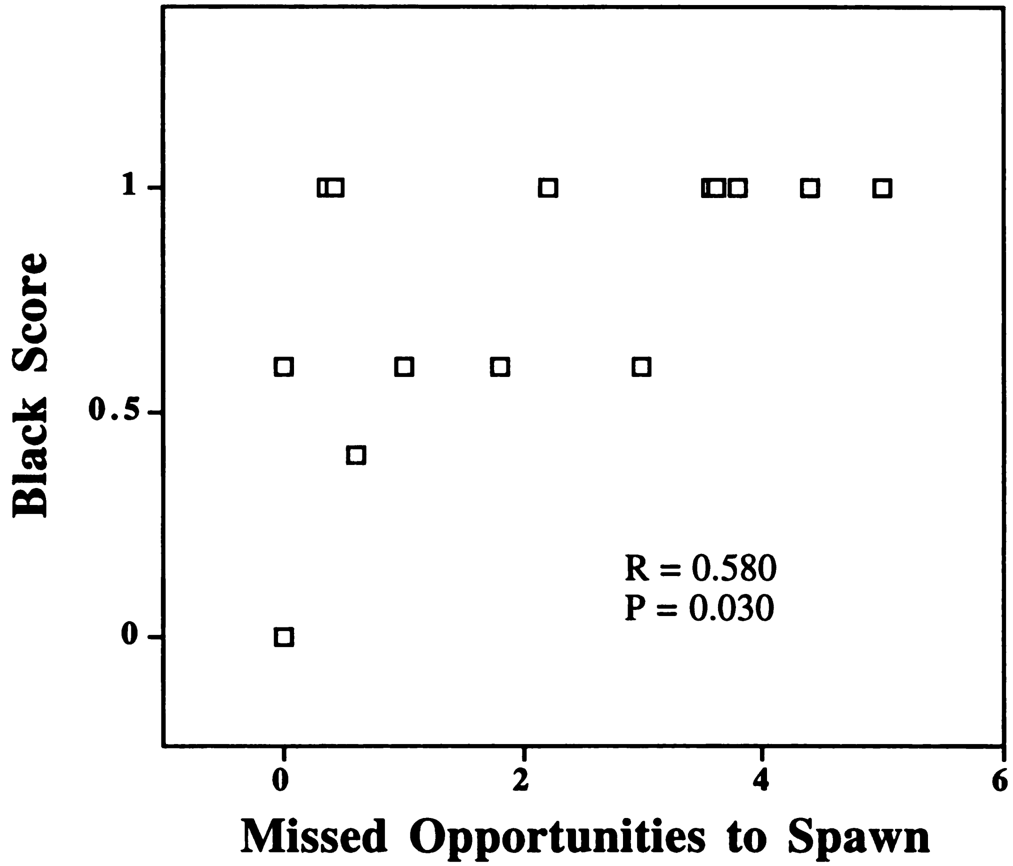

Figure 11 The relationship between black score and missed opportunities to spawn in the four male treatment. Black score is measured as the proportion of spawnings prior to which the male had turned black. Missed opportunities to spawn is measured as the the number of opportunities males had to spawn prior to each spawning. The values are an average of the number of missed opportunities to spawn prior to each spawning over 5 spawnings. Overlapping data points have been jittered for presentation.

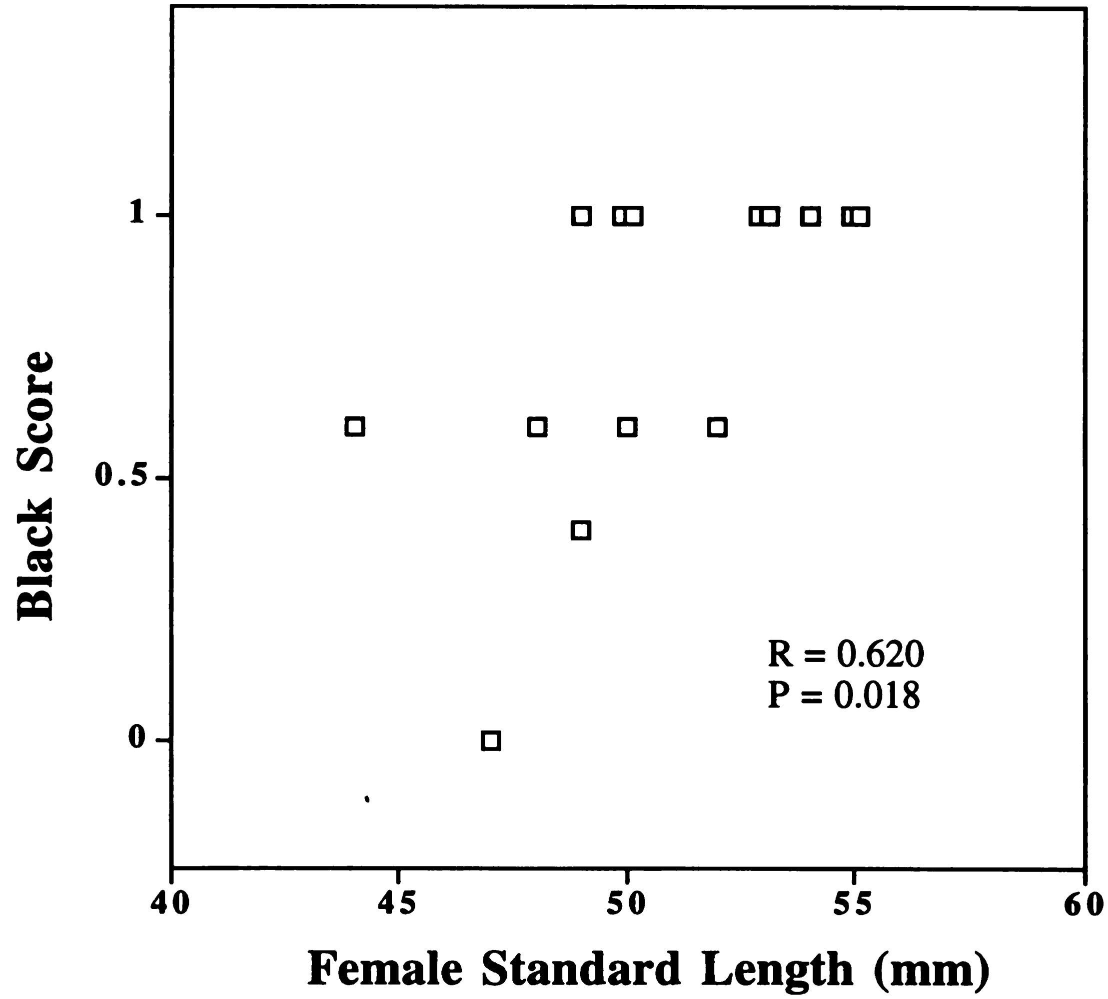

Figure 12 The relationship between black score and female standard length in the four male treatment. Overlapping data points have been jittered for presentation.

Other studies have provided empirical support for this model. Simmons and Kvarnemo (1997) demonstrated that when under a female biased operational sex ratio male bushcrickets decrease sperm numbers when mating with females that have a high probability of being multiply mated. In my study, males did respond to increased sperm competition by foregoing opportunities to spawn with females. This result is in accordance with the reasoning of Parker et al.'s model (1996) when considering a missed opportunity to spawn as zero sperm output. Guarding males most likely suffer decreases in reproductive success as the number of spawning males increases. Preliminary data indicate that in group spawns involving 2 males, each male fathers approximately 50% of the clutch (Fuller unpublished data). As more males engage in a group spawn, the reproductive success of the guarding male may decrease to a point where it is detrimental to even participate in the spawning. Similarly, Schwagmeyer & Parker (1990) showed in thirteen-lined ground squirrels that males will reject females that are apparently willing to mate due to the costs imposed by sperm competition.

This study also demonstrates size-dependent competitive strategies in males. Small males have high sperm output under high potential sperm competition relative to large males. Small males are less competitive than large males (Page 1983, Fuller unpublished data) which may affect their sperm output strategies. Theoretically, males that consistently spawn in disfavored roles should compensate by increasing ejaculate size (Parker 1990, Gage et al. 1995). The reasoning of Parker et al.'s (1996) model is that males should reduce sperm output when there are a greater than average number of males engaged in a groups spawning because males can wait for better spawning opportunities in the future with fewer competing males. However, uncompetitive males may not be capable of attaining better spawning opportunities and therefore consistently engage in group spawnings with more males.

The asymmetry in competitive ability between large and small males may be further exacerbated by their abilities to mate in favorable positions in groups spawns in which males spawning on top of the female and on each side of the female can release their sperm closer to the female's eggs than can males spawning further away. Distler (1972) provides anecdotal evidence for such spawning dynamics in E. cragini, another group spawning darter. In E. caeruleum, small males may be consistently engaging in group spawns with larger numbers of competitors and spawning in less favorable positions than large males. Their best strategy may be to release large numbers of sperm when spawning in favorable positions. In contrast, large males were particularly likely to forego spawning opportunities under high sperm competition. As large males dominate over small males in competition (Page 1983, Fuller unpublished data), they may be able to choose among spawning opportunities because they are more assured of their success in future contests. Hence, there may be variation not only in the number of males present at a group spawning but also in the position of the males relative to the female. If there is significant variation in the competitive abilities of males within a population, then it may be difficult to test model predictions based on population parameters.

The effect of spawning in disfavored versus favored roles has been documented to have similar effects on sperm output and competitive behaviors in other fish species. In blue headed wrasse, Thalassoma bifasciatum, large, territory holding, terminal phase males release less sperm per spawning and invest fewer resources into sperm production than do group spawning males (Shapiro et al. 1994, Warner et al. 1995). In the Atlantic salmon, Salmo salar, parr males (sneakers) produce greater numbers and volume of sperm per unit body weight than do anadromous males (Gage et al. 1995). Furthermore, parr sperm is more motile and longer lived than that of anadromous males indicating that males spawning in disfavored roles invest more in sperm production than do males spawning in favored roles. However, in contrast to T. bifasciatum and S. salar, male E. caeruleum do not adopt discrete mating strategies and instead exhibit a continuum of mating tactics that covary with size. All of the males used in this experiment were in typical nuptial coloration and spawned as guarding males. A similar continuous gradient in male reproductive tactics is found in guppies, Poecilia reticulata, where males that perform more sneaky mating attempts (and presumably mate in a disfavored role) produce more sperm than males that try to attract females predominantly through courtship (Matthews et al. 1997).

In this study, males turned black when in the presence of competing males indicating that this signal is used predominantly in intrasexual selection. Males rarely turned black in the zero males or one female treatments indicating that this signal is not used to transfer information to females. Such temporary color changes are common in many fish and appears to be linked to competitive and sexual behaviors (Demski 1992). In bluegill, Lepomis macrochirus, males develop a stereotyped banded color pattern when engaged in antagonistic behaviors (Stacey & Chiszar 1975). In E. caeruleum black coloration may serve as a warning signal to other males indicating escalating aggression levels. In P. reticulata, male display rates potentially serve as an indicator of male sperm supply (Matthews et al. 1997). This was not the case in E. caeruleum as sperm output tended to negatively correlated with black score. Whether other male nuptial colors correlate with sperm output is unknown.

Males were more likely to turn black when paired with large females in the four male treatment indicating that males are more likely to engage in active competition when guarding large females. Males may exert a type of cryptic choice among females by deciding whether or not to engage in competition. Male choice of females is not uncommon; males of many species exert direct preferences for large, fecund females (Sargent et al. 1986, reviewed in Turner 1993, although see Pyron 1996). In E. caeruleum, this behavior should be favored if it increases the male's probability of spawning with large females because large females release more eggs per spawning.

There was no relationship between mean sperm output and mean fertilization success. This lack of a relationship may be due to several factors. First, sample size may have been too small to detect such a relationship. Work on blue-headed wrasse found a significant relationship between fertilization success and sperm output only after collecting data on 1358 spawnings (Warner et al. 1995). Second, egg viability may rapidly decrease after females ovulate their eggs. If a female has been held without a male for too long, a large proportion of her eggs may be inviable even though she has not yet dropped them. Such a phenomenon has been demonstrated in other fish species (Bry 1981, Stacey 1984, Vincent 1994). Current research is investigating whether this phenomenon is present in E. caeruleum. Fortunately, this lack of a relationship between fertilization success and sperm output indicates that males were not adjusting their sperm output in relation to the perceived viability of females' eggs.

In conclusion, this study found that the predictions for sperm output made by

Parker et al.'s (1996) model were not upheld in E. caeruleum. However, males were more likely to forego copulation opportunities under high sperm competition which is in accordance with theory. This study also demonstrated size-dependent male sperm output and competitive behaviors. Small males have high sperm output under high potential sperm competition relative to large males. Small males may be competitively inferior and compensate for this disadvantage by investing in sperm production. In contrast, large males are more likely to forego spawning opportunities under high sperm competition. Large males may be better off waiting for future spawning opportunities when there is a lower potential for sperm competition. Finally, males were found to adopt black coloration only when spawning in the presence of other males. This signal does not indicate male sperm output. Instead, male black coloration may serve as a warning signal indicating increasing aggression levels.

SYNTHESIS

In many ways, this thesis raises more questions than it answers. This thesis demonstrates that female mating preferences exist and demonstrates how to measure them, but the degree to which preferences affect male mating success is unknown. Similarly, this thesis shows that male aggressive behavior is used in competition over females, but does not demonstrate a correlation between aggression and spawning success. Fortunately, these problems are tractable. Theory holds that male/male competition is a direct result of the operational sex ratio (proportion of males/females ready to mate at any moment in time) (Kvarnemo & Ahnesjö 1996). By manipulating the operational sex ratio and measuring the relationship between male size, color pattern, aggression, and spawning success, it should be possible to tease apart the roles of male/male competition and female choice.

This thesis also demonstrates that group spawning is common. Secondary males father a large proportion of the eggs. As a result, group spawning should reduce the variance in reproductive success among males. This may raise a problem for the evolution and maintenance of the male color pattern. Typically, we expect species with extravagant male color patterns to have a similarly extravagant variance in male mating success. What are the costs of the color pattern? To what degree does the color pattern and male spawning success vary among males? These questions need to be addressed.

Continued research on E. caeruleum must do two things: 1) complete a detailed study of individuals in a field setting; 2) develop molecular markers so that male mating success can be measured both in the field and in realistic experiments where group spawnings occur. Although these fish do not remain in breeding condition long enough to be the sole focus of a behavioral ecologist's research, they do provide a reliable peak of breeding behavior in April and would make a wonderful side-project for anyone so inclined. There are many opportunities for interesting research.

In this spirit, I close with the following words:

"These (darters) we found to be the most fascinating, vivacious, and individual of all river fishes." David Starr Jordan, *The Days of a Man*,1922.

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