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Sperm competition affects male behaviour and sperm output in the rainbow darter

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Rainbow darters, *Etheostoma caeruleum*, are promiscuous fish with moderate rates of group spawning (between one and five males may simultaneously mate with one female). In this study, I examined male sperm output and male willingness to spawn under different levels of sperm competition intensity. One male and one female were allowed to spawn in an aquarium where they had visual and olfactory access to one of four treatments: four males, one male, zero males, or one female. Theory predicts that males should reduce sperm output when there are more than the average number of males at a group spawning (four-male treatment) and should increase sperm output when there are fewer than average males at a group spawning (one-male treatment). Mean sperm output did not differ among treatments. However, males released more sperm when spawning in the presence of competing males (four-male and one-male treatments pooled) than when spawning in the absence of competing males (zero-male and one-female treatments pooled). Males were also most likely to forego spawning opportunities when sperm competition intensity was high. Furthermore, male willingness to spawn was size dependent. Large males were more likely to forego spawning opportunities under high sperm competition intensity. Large males may be better off waiting for future spawning opportunities when there is a lower potential for sperm competition intensity.

Keywords: alternative mating strategies; *Etheostoma caeruleum*; group spawning; sneaky mating behaviour; willingness to spawn

1. INTRODUCTION

Sperm competition, defined as competition between ejaculates of two or more males for fertilization of a set of eggs (Parker 1970), is a common phenomenon and has important effects on the behaviour and life-history patterns in many animals (Smith 1984; Eberhard 1996; Stockley 1997; Birkhead & Møller 1998). Many fish engage in group spawnings, which involve two or more males simultaneously spawning with one female (Breder & Rosen 1966; Stockley *et al.* 1997; Petersen & Warner 1998). Theory predicts that across populations the risk of sperm competition should be correlated with investment in sperm production (Parker *et al.* 1996). Comparative studies support this prediction, finding that sperm competition risk is correlated with investment in sperm production as measured by the gonadosomatic index (gonad mass/body mass) (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 fish, 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 intensity is higher than average and should ejaculate more sperm when sperm competition intensity is lower than average (Parker *et al.* 1996). The reasoning behind this model is that males should reduce sperm output when sperm competition intensity is high because they can wait for better mating opportunities in the future with fewer competitors. Similarly, males should take advantage of mating opportunities taking place under low sperm competition intensity by increasing sperm output because such an opportunity may not arise again.

In this paper, I examine the effect of sperm competition intensity and male size on male sperm output and male willingness to spawn in the rainbow darter, *Etheostoma caeruleum*. Specifically, I address the following questions. (i) Do males increase their sperm output under low sperm competition intensity and decrease their sperm output under high sperm competition intensity? (ii) Are males less willing to mate under high sperm competition intensity?

(a) *Natural history of* Etheostoma caeruleum

Etheostoma caeruleum Storer is a small bottom-dwelling fish that inhabits shallow riffles in swift streams and gravel areas in clear lakes (Page 1983). The mating system is promiscuous, and there is no parental care. During the breeding season, males remain on riffles and guard small moving territories, while females dwell in

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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. 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 nosedig, 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 complete 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*). However, if competing males are present, the guarding male will often opt to chase and fight nearby males, leaving the female buried in the gravel. Occasionally, nearby males sneak in and release their sperm next to the pair of spawning fish. In one *E. caeruleum* population, 80% of the observed spawnings involved group spawning, in which two to five males mated simultaneously with one female (R. Fuller, unpublished data, Gull Lake, Kalamazoo Co., MI, USA).

2. 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 before the onset of the breeding season, bringing them into the laboratory, and manipulating their water temperature and light ratio so as to mimic conditions during the breeding season (water temperature = 10–12 °C, day : night ratio 14L:10D). The sex ratio of stock aquaria was roughly 1:1, with two to three males and two to three 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 14 February 1997, and experiments began on 21 February 1997.

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, she has only a few days during which she is receptive to males (R. Fuller, personal observation). After that time, she will drop the eggs into the gravel and allow them to be left unfertilized (R. Fuller, personal observation). Mixed-sex stock aquaria were kept in the 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 two days.

To examine the effect of sperm competition intensity on male sperm output and related guarding behaviours, 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 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. A similar control with four females was not used owing to time constraints.

Two aquarium set-ups were used in this experiment. In the first, I divided a 40-litre aquaria (50.80 cm × 26.04 cm × 31.75 cm) into two equal sections (25.40 cm × 26.04 cm × 31.75 cm) by using clear pieces of Plexiglas. Barriers were attached to the bottom and sides of the aquarium with silicone. A series of holes 3–5 mm in diameter were drilled in each barrier approximately 5 cm from the bottom so that olfactory cues could pass between the two compartments. This aquarium set-up was used for the one-male, zero-male 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 a 40-litre aquaria into three sections: one large central section, which held the focal animals (25.40 cm × 26.04 cm × 31.75 cm) and two smaller end sections, each of which held two stimulus males (12.70 cm × 26.04 cm × 31.75 cm). This aquarium set-up was used for the four-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 Plexiglas barriers were attached to the bottom and sides of aquaria with silicone so that no sperm could pass under the barrier. Darter sperm is negatively buoyant (R. Fuller, personal observation). As before all barriers contained a series of holes, 3–5 mm in diameter and 5 cm from the bottom, which allowed olfactory cues to pass between the sections. The bottoms of all aquaria were lined with small-grain gravel. After 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 one week. Before 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 the trial was cancelled. 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 h after the last spawning. If the female did not perform a nosedig during this time the trial was cancelled. After each trial, the body length of focal and stimulus animals was measured to the nearest millimetre. All body lengths reported in the paper are standard lengths. All males used in this experiment were in breeding coloration. Forty-nine trials were completed in total. Body 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). Body lengths of stimulus males did not differ between the one-male and four-male treatments ($t = 0.387$, d.f. = 22, $p = 0.702$).

Behavioural data were recorded over the course of the five spawnings. I recorded the number of complete nosedigs performed by the female before each spawning. From these data, I calculated the mean number of missed opportunities to spawn. (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 might have swum away and spawned with another male).

In this experiment, I measured male sperm output over a series of spawnings by means of the basic sperm collection techniques developed by Shapiro *et al.* (1994) and 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 aquarium into a bucket. A separate bucket was used for each spawning. During this process, I concentrated on

siphoning primarily in the area where the fish had spawned. I then added fresh water into 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. The sample was then treated with five drops of Rose Bengal dye and placed in a refrigerator. After 25 min, 25 ml of formalin was added to the solution to fix the stain. After 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 by using a vacuum pump. The filter was then dried on a hot plate for more than 30 min. Finally, a portion of the filter was mounted on a slide with immersion oil. Using a compound microscope, the number of sperm occurring within an ocular grid was counted on 40 separate areas of the slide. Slides were examined systematically such that no area was counted twice. The counts were presumed to be independent. Sperm estimates were then calculated with the following formula: sperm estimate = (no. of 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).

(a) *Testing the sperm collection method*

Following the methods of Shapiro *et al.* (1994), I tested whether the sperm collection 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 2 ml of sperm solution among the rocks on the bottom of an aquarium. I waited approximately 30 s, siphoned the sperm mixture out of the aquariuma, and later estimated the total amount of sperm by the methods described above. I compared these values with a control treatment in which an equivalent amount of sperm was released directly into a bucket. Sperm estimates for aquarium treatments were compared with control treatments by using *t*-tests and linear regression. Twenty-one trials were run in total.

I conducted a second test to see whether any residual sperm was left in the aquaria after the trials and whether this varies among treatments. I measured the residual sperm remaining in the aquarium after the last spawning for a subset ($n=16$) of the experimental trials. From these data, I calculated the residual sperm, the proportion of residual sperm relative to the sperm output of the last spawning for that trial (residual sperm/(residual sperm + last sperm output)), and the proportion of residual sperm relative to the estimated cumulative sperm output for that trial (residual sperm/(residual sperm + (mean sperm output × number of spawns))). I then compared these variables among treatments.

All statistical tests were conducted with the SYSTAT statistical package (Wilkinson 1992). Non-parametric statistics were used when the underlying assumptions of parametric tests were 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, by using the Kolmogorov–Smirnov Lilliefors test (Tessier, personal communication). All probabilities are two-tailed.

3. RESULTS

(a) *Sperm collection tests, residual sperm, and stimulus males*

The sperm collection method was relatively reliable. There were significant differences between the amount of sperm obtained from the aquaria and control treatments in 2 out of the 21 trials. In one trial, the sperm estimate was greater in the aquarium treatment ($t=3.031$, d.f. $=78$, $p=0.003$), whereas in the other trial the sperm estimate was greater for the control treatment ($t=-2.089$, d.f. $=78$, $p=0.04$). 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 ($t=-0.190$, d.f. $=20$, $p=0.851$). Furthermore, the number of sperm collected in aquarium treatments correlated strongly with that obtained from bucket treatments (linear regression: $Y=0.971X+296\,982$, where Y is the amount of sperm obtained from the aquarium treatment and X is the amount of sperm obtained from the bucket treatment, $F_{1,20}=5434.80$, $p<0.001$). The slope of the line is approximately one ($b=0.971X \pm 0.013$ s.e.).

Absolute residual sperm in the aquaria was $2.20\times10^6 \pm 5.02\times10^5$ (s.e.) sperm ($n=16$) and did not differ among treatments (Kruskal–Wallis test statistic $=4.400$, d.f. $=3$, $p=0.222$). The average proportion of residual sperm relative to the sperm output of the last spawning was 0.149 ± 0.028 (s.e.) and did not differ among treatments (Kruskal–Wallis test statistic $=3.624$, d.f. $=3$, $p=0.305$). The average proportion of residual sperm relative to the estimated cumulative sperm output was 0.023 ± 0.003 (s.e.) and did not differ among treatments ($F_{3,12}=1.545$, $p=0.254$). If all the residual sperm resulted from the last spawning, then the sperm collection methods missed 15% of the sperm on average. If the residual sperm accumulated equally from all the spawnings, then the collection methods missed 2.3% of the sperm on average. Regardless, the sperm collection method should not have biased the results qualitatively because the proportion of residual sperm left in the aquaria did not differ among the treatments.

Stimulus males were presumably not releasing sperm, with one exception. In one trial of the four-male treatment, a stimulus male exhibited the quivering behaviour typical of a spawning male. The water was removed and sperm was measured (6.53×10^5 sperm). Removal of this trial does not qualitatively affect the results. In all other trials, no stimulus male exhibited quivering behaviour and presumably none released sperm.

(b) *Sperm output and male behaviour*

Male sperm output did not differ significantly among treatments (Kruskal–Wallis test statistic $=4.317$, $p=0.229$).

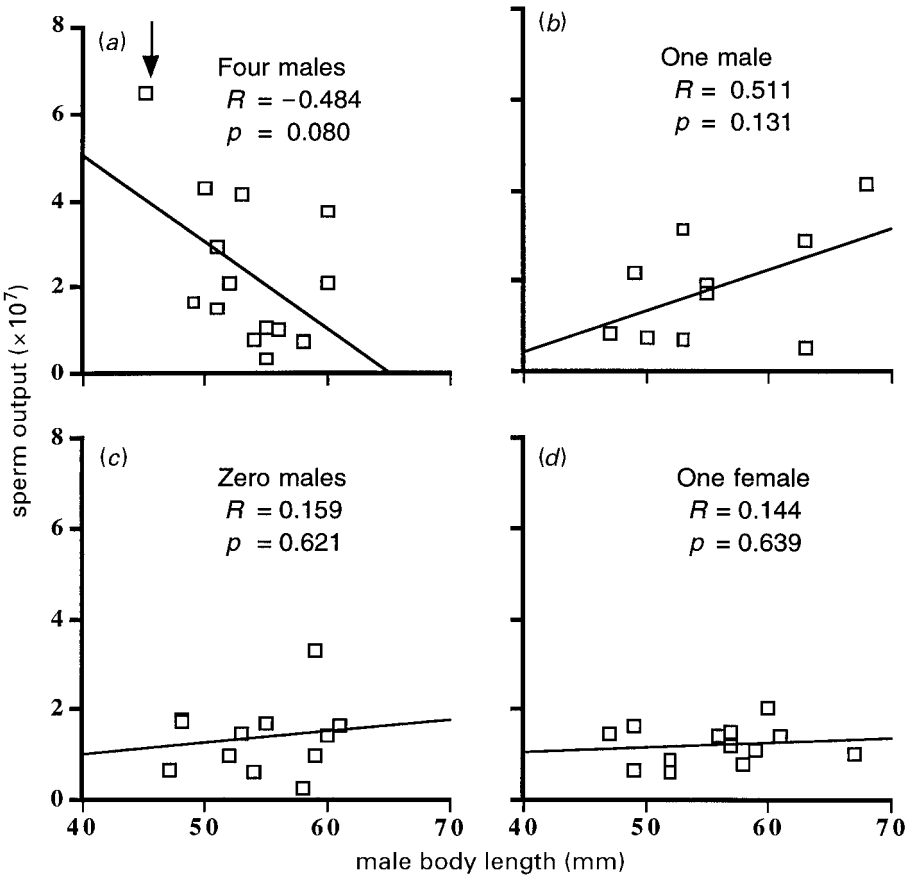

Figure 1. Relation between sperm output (estimated numbers of sperm cells) and male body length (mm) among the four treatments. Linear regression coefficients (R) and probability values (p) are given for each treatment. In the four-male treatment (*a*), one data point with somewhat high leverage (0.378) is indicated with an arrow.

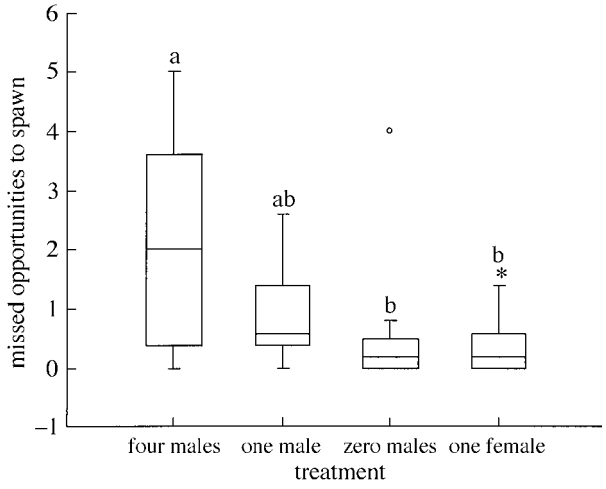

Figure 2. Box plots of missed opportunities to spawn in the four treatments. Letters denote statistically significant differences. The middle line refers to the median of the data. The edges of the boxes refer to the first and third quartiles of the data. Bars, ±3 times the interquartile range from the median; asterisk, outside values; circle, far-outside values. Sample sizes are as follows: four males, $n=14$; one male, $n=10$; zero males, $n=12$; one female, $n=13$.

If the four-male and one-male treatments were pooled as 'competing males present' and the one-female and zero-male treatments as 'competing males absent', males released larger amounts of sperm when spawning in the presence of competing males (competing males present: mean $=2.167\times10^7\pm3.18\times10^6$ (s.e.) sperm, $n=24$; competing males absent: mean $=1.29\times10^7\pm1.25\times10^6$ (s.e.) sperm, $n=25$; Mann–Whitney U-test $=202.00$, $p=0.05$).

The relation between male size and sperm output differed among treatments (figure 1, treatment × male body length: $F_{3,41}=3.625$, $p=0.021$). However, this result is dependent on one data point with somewhat high leverage (leverage $=0.378$, figure 1*a*). Removal of this point renders the interaction between male body size and treatment non-significant ($F_{3,40}=0.989$, $p=0.408$) and weakens the relationship between sperm output and male body size in the four-male treatment ($R=-0.159$, $p=0.605$). However, analysis of the residuals from the linear regression does not indicate that this point is an outlier. Furthermore, there was nothing unusual about this trial to warrant its deletion. Although not robust, this analysis indicates that small males increase their sperm output compared with large males in the four-male treatment (figure 1*a*). In contrast, male sperm output increased slightly with male size in the one-male treatment (figure 1*b*). Male body length had little relation to sperm output in the one-female and zero-male treatments.

The treatments had strong effects on the tendency of males to forego spawning opportunities (figure 2, Kruskal–Wallis test statistic $=10.952$, d.f. $=3$, $p=0.012$). To compare treatments, I used a non-parametric post hoc test that corrected 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-male or one-female treatments (figure 2, $Z_{\mathrm{crit.},\alpha=0.05}=2.638$, correcting for multiple comparisons). There were no differences in the tendency of males to forego spawning opportunities between the four-male and one-male treatments, nor were there differences in the tendency of males to forego spawning opportunities between female, one-male, and zero-male treatments.

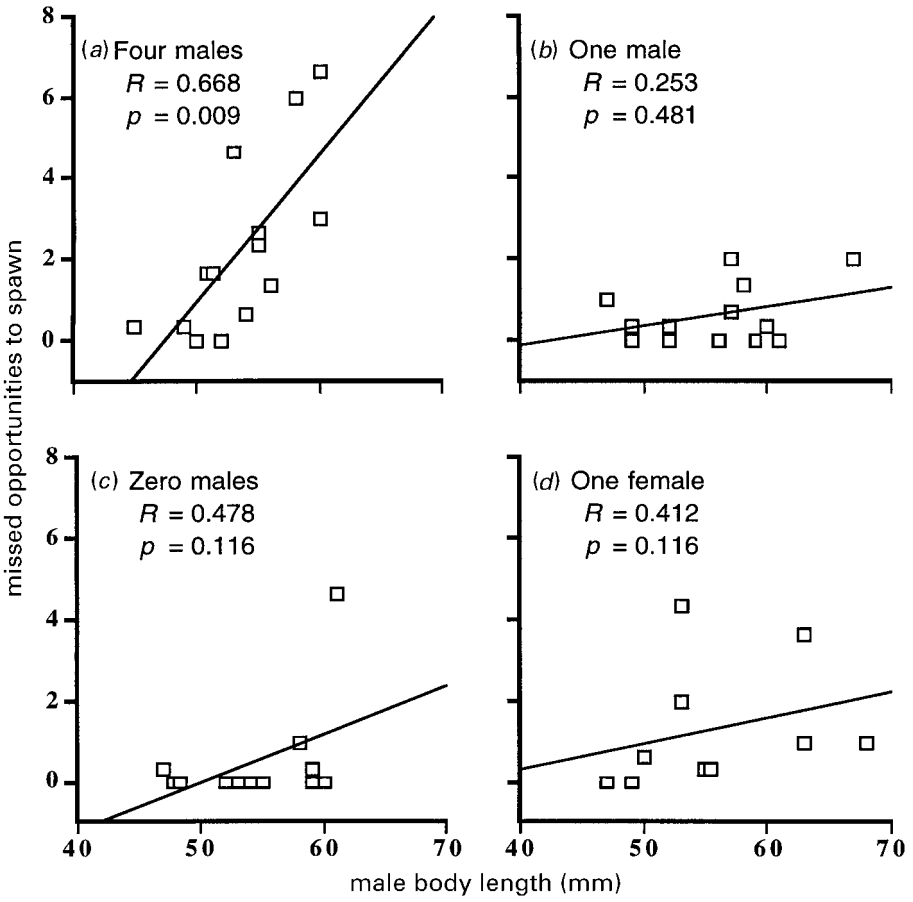

Figure 3. The relation between missed opportunities to spawn and male body length among the four treatments. The variable 'missed opportunities to spawn' is measured as the number of opportunities males had to spawn before each spawning. The values are an average of the number of missed opportunities to spawn before each spawning over five spawnings. Linear regression coefficients (R) and probability values (p) are listed for each treatment. In the zero-male 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.

The relation between male body length and tendency to forego spawning opportunities varied significantly among treatments (figure 3, treatment × male body length: $F_{3,41}=3.360$, $p=0.028$). Male body length was positively correlated with missed opportunities to spawn in the four-male treatment (figure 3*a*). This pattern appears to be caused by larger males foregoing more opportunities to spawn than smaller males in the four-male treatment.

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 s.e., $n=49$, coefficient of variation=0.657). Fertilization success varied significantly among females (Kruskal–Wallis test statistic=158.072, d.f.=47, $p<0.001$, one case deleted owing to lack of data). Within replicates, there was no relation between sperm output and fertilization success ($F_{1,79}=0.0024$, $p=0.9888$, five cases deleted owing to lack of variance). Female body length correlated with the total number of eggs released over the five spawnings ($r=0.446$, $p=0.001$, $n=49$).

4. DISCUSSION

According to the model proposed by Parker *et al.* (1996), males should reduce their sperm output when under higher than average sperm competition intensity and should increase their sperm output when under lower than average sperm competition intensity. If this model is applied to this experiment, males were predicted to reduce their sperm output in the four-male treatment and to increase their sperm output in the one-male treatment. This prediction was not upheld. Although males released more sperm when competing males were present, sperm output did not differ between high and low sperm competition intensity. Other studies have provided empirical support for this model. Simmons & Kvarnemo (1997) demonstrated that, when under a female-biased operational sex ratio, male bush crickets 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, is in accordance with the reasoning of the model of Parker *et al.* (1996). Guarding males most probably suffer decreases in reproductive success as the number of spawning males increases. Preliminary data indicate that in group spawns involving two males, each male fathers approximately 50% of the clutch (R. 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, owing to the costs imposed by sperm competition.

Parker *et al.* (1996, 1997) distinguish between sperm competition risk and sperm competition intensity. The term 'sperm competition risk' applies to scenarios where sperm competition is rare and the maximum number of competitors is two. The term 'sperm competition intensity' refers to the scenario where sperm competition is common and there are on average two or more competing ejaculates. A key assumption of this

experiment is that the sex-ratio treatment effectively manipulated the perceived level of sperm competition. This experiment assumes that male *E. caeruleum* use the number of males in close proximity as a cue to the level of sperm competition intensity. Males may instead use the number of males actually participating in the group spawn as their cue for the amount of sperm to release. However, if this were the case, then we would expect to find no treatment effect on any response variable. This was not the case. Males of different sizes responded differently to the sex-ratio treatment. Another possibility is that males did not perceive all of the stimulus males as being equally likely to participate in a group spawn. If so, the one-male and four-male treatments may represent lower levels of sperm competition intensity than indicated by the sex ratio.

This study also demonstrates size-dependent male behavioural strategies in response to sperm competition intensity. Large males were particularly likely to forego spawning opportunities under high sperm competition. As large males dominate over small males in competition (Page 1983; R. Fuller, unpublished data), they may be able to choose among spawning opportunities because they are more assured of their success in future contests. In contrast, the data at this time suggest that small males may increase sperm output under high potential sperm competition intensity relative to large males. Theoretically, males that consistently spawn in disfavoured roles should compensate by increasing ejaculate size (Parker 1990; Gage *et al.* 1995). In *E. caeruleum*, small males are less likely to spawn singly with females and are less likely to spawn as the defending, primary male when a larger male is present (R. Fuller, unpublished data). Small males may be competitively inferior and compensate for this disadvantage by releasing more sperm under high sperm competition intensity. Similar phenomena have been documented in the blue-headed wrasse (*Thalassoma bifasciatum*). Territory-holding, terminal-phase males release less sperm per spawning and invest fewer resources in sperm production than do group-spawning males (Shapiro *et al.* 1994; Warner *et al.* 1995). A comparative study of continuously breeding mammals also found that small males invest proportionately more in sperm production than large males (Stockley & Purvis 1993).

There was no relation 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 relation. Work on the 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. 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 (R. Fuller, unpublished data). Such a phenomenon has been demonstrated in other fish species (Bry 1981; Stacey 1984; Vincent 1994).

In conclusion, this study found that the predictions for sperm output made by the model of Parker *et al.* (1996) were not upheld in *E. caeruleum*. Sperm output did not differ between high and low sperm competition intensity. However, males were more likely to forego spawning opportunities under high sperm competition; this result is in accordance with theory. Males did release more sperm when competing males were present (four-male and one-male treatments pooled) than when competing males were absent (zero-male and one-female treatments pooled). This study also demonstrated size-dependent male behavioural strategies. Large males were more likely to forego spawning opportunities under high sperm competition intensity. Large males may be better off waiting for future spawning opportunities when there is a lower potential for sperm competition.

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