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Costs of Group Spawning to Guarding Males in the Rainbow Darter, *Etheostoma caeruleum*

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The rainbow darter (*Etheostoma caeruleum*) is a promiscuous fish with a moderate rate of group spawning and sperm competition (1–5 males may simultaneously mate with one female). In any given spawning event, males may spawn as either a guarder or sneaker. Theory indicates that the manner in which individual males behave in sperm competition depends on the expected fitness they receive when spawning in either the guarder or sneaker role. This study uses allozyme markers to compare the fertilization success of males spawning as guarders and sneakers for a common set of eggs. Two points emerge from this study. First, group spawning is costly to guarders in terms of lost paternity. Males spawning as guarders did not achieve 100% paternity. When group spawns involve two males, each male has roughly an equal probability of fertilizing the eggs. Second, male competitive ability is associated with male size. In paired contests, larger males spawn singly with females and spawn as guarding males in group spawnings more often than smaller males. The benefits to competitively superior males may come from single spawns with females where they are assured 100% paternity.

SPERM competition occurs when ejaculates of two 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 and Rosen, 1966; Stockley et al., 1997; Petersen and Warner, 1998). *Etheostoma caeruleum* is a small bottom-dwelling fish that inhabits shallow riffles in swift streams and gravel areas in clear lakes (Winn, 1958; Page, 1983). *Etheostoma caeruleum* is a promiscuous species where sperm competition resulting from group spawnings is common (Breder and Rosen, 1966; R. C. Fuller and J. C. Porterfield, unpubl. data). Females spawn multiple times (approximately 39 times) over the spawning season (Fuller, 1998a). The male attempts to keep competing males away by chasing and attacking them. In one population, 80% of spawnings involve group spawnings where 2–5 males simultaneously spawn with a female (unpubl. data, Gull Lake, Kalamazoo Co., MI). Theory indicates that the manner in which individual males behave depends on the expected fitness gains they receive when spawning in either the guarder or sneaker role (Parker et al., 1996; Parker, 1998).

Individual male *E. caeruleum* may spawn as a guarder or a sneaker depending on context. In contrast to the more well-known examples of alternative male strategies in salmon (*Oncorhynchus kisutch*) and bluegill (*Lepomis macrochirus*; Gross, 1982, 1984), there is no alternative male morphology or life-history pattern associated with the sneaker tactic. All males spawn as guarders when given the opportunity (Fuller, 1998b; e.g., when competitively dominant or the only male present). This observation indicates that the sneaker tactic represents a best of a bad job strategy (Dunbar, 1982). However, behavioral observations also indicate that group spawning may be quite costly to guarding males. A guarding male will often forego spawning opportunities with females if competing males are present (Fuller, 1998b), indicating that sneaker males may fertilize a significant proportion of eggs.

This study uses allozyme markers to compare the fertilization success of males spawning as guarders and sneakers for a common set of eggs and addresses two questions. How costly is group spawning to guarding males in terms of lost paternity? Do large males have a competitive advantage over smaller males? I report the first measurements of guarder and sneaker fertilization success in percid fishes.

MATERIALS AND METHODS

Etheostoma caeruleum was collected from the upper Mill Pond Stream, 37th and G Streets,

Kalamazoo County, Michigan, and returned to Kellogg Biological Station, 11 January to 2 February 1997. Adults were prescreened for phosphoglucomutase [PGM-2 (EC 5.4.2.2)] using a fin clip. Twelve sets of fish were established, each containing two males that were homozygous at alternate allomorphs (PGM-2: SS and FF). One male in each pair was given a subcutaneous mark above the pectoral fin using an elastomer fluorescent dye so that the allomorph of each male was always evident. Marks were equally distributed among males of both allomorphs. Six sets also contained a female that was homozygous at one allomorph (SS), and six sets contained a female homozygous at the alternate allomorph (FF). Each set of fish was placed in a 20-liter aquarium with a rocky substrate and housed in the laboratory until 4 April. Standard length (SL) was measured (± 0.5 mm) with a ruler at the beginning of the study.

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 14:10 L:D to mimic natural sunlight patterns during the breeding season. The thermostat in the laboratory was set at 14 C, and water temperatures ranged between 10 and 15 C for the majority of the breeding season.

Fish were observed until they spawned. Once females appeared gravid, an observer spent the majority of the day (0800–2100 h) in the aquaria room. Prior to spawning, females perform nosedigs in the rocks, which makes a rattling sound. By listening and observing fish, the observer could monitor the reproductive status of the fish. Occasionally, 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.

Each spawning was categorized as either a single spawning or a group spawning. A single spawning involves one male spawning with one female. A group spawning involves two males spawning with one female. For single spawns, the identity of the spawning male was recorded. For group spawns, the identities of the males spawning as the guarder and sneaker were recorded. The guarding male was defined as the male that began the spawning and spawned on top of the female. The sneaker male was defined as the male that sneaked in and quivered next to the spawning pair once they had 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 sec after it is released (Hubbs, 1960); thus, sperm remaining in the aquarium do not affect future spawnings. 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 four 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 two drops of grinding buffer. Paternity was determined using electrophoresis.

The electrophoresis methods described here were adapted from Mather and Rusco (1992) and Hebert and Beaton (1989). When prescreening the adults, fin tissues were obtained by clipping the posterior third of the pelvic fin and posterior sixth of the caudal fin. The body of juvenile fish was used as a tissue sample. Tissues were placed in Eppendorf tubes containing two 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 min. The tubes were then centrifuged in an Eppendorf Centrifuge for 2 min 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 that had been soaked in buffer [50 mM Tris glycine (pH 8.5)] for at least 20 min. Gels were run at 200 V for 30 min at 4 C. Gels were stained for phosphoglucomutase PGM-2 (EC 5.4.2.2) using the gel recipe developed by Hebert and Beaton (1989). Enzyme names and numbers follow International Union of Biochemistry, Nomenclature Committee, (1984). Because the amount of protein contained on each gel was very small, a larger amount of enzyme (80 µl) 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. The mean paternity of each male from group spawnings was calculated for replicates from which multiple clutches were obtained.

A total of 607 eggs were obtained from group spawnings. Four hundred eleven fry were still alive by 31 March 1997. Some small clutches (<

TABLE 1. GUARDER PATERNITY ESTIMATES FROM GROUP SPAWNS FOR LARGE AND SMALL MALES. * indicates that the proportion of the clutch sired by the guarder differs from 50% at $P < 0.05$. § indicates that the proportion of the clutch sired by the guarder differs from 50% at $P < 0.10$. In replicate 6, there is no difference in standard length between the two males. The paternity of the FF male is listed because he spawned as the guarder.

Rep	Female morph (SL)	Large male morph (SL)	Number of clutches (total fry) large males spawned as guarder	Large male guarder paternity % (SE)	Small male SL	Number of clutches (total fry) small males spawned as guarder	Small male guarder paternity % (SE)
1	SS (63)	SS (59)	4 (58)	46 (6)	43	0	—
2	SS (51)	SS (61)	3 (15)	11 (11)§	60	2 (11)	50 (50)
4	SS (60)	FF (61)	1 (10)	60	56	0	—
5	SS (51)	FF (60)	2 (12)	76 (4)	56	2 (10)	8 (8)
6	SS (57)	FF (59)	3 (19)	84 (8)*	59	0	—
7	SS (58)	SS (63)	2 (11)	28 (12)	40	0	—
8	SS (62)	SS (61)	4 (18)	71 (13)	40	1 (11)	18
				Mean = 53.7			*Mean* = 25.3

4) were discarded if they were from replicates with many other large clutches. Two hundred eighty fry were frozen for analysis. This included a few fry that were found dead in their aquarium. Forty cellulose acetate gels were run. Six gels (38 fry) failed due to technical difficulties (i.e., old enzyme solutions, agarose gel too cool, etc.). The bands from an additional 43 fry were unreadable. This was mainly a result of trying to analyze fry that had been found dead in their aquaria. Some unreadable bands were also due to fry that were too small. One hundred ninety-nine fry were scored for their PGM-2 allomorph. However, 24 of these fry were excluded from the analysis presented here because they came from clutches that were too small (< 4).

Twelve replicates were originally established. Two sets of fish died. At the completion of this study, the allomorphs of the adult fish were double checked. Three replicates were used for behavioral analysis only. In one replicate, a male classified as homozygous was found to be heterozygous. In two other replicates, clutches were too small (< 4) and were excluded from the paternity analysis. Clutches were only included in the paternity analysis if there were four or more fry. In one replicate, the two males were the same size and thus excluded from the behavioral analysis but included in the paternity analysis. Seven and nine replicates were used in the paternity and behavioral analysis, respectively.

RESULTS

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

Overall, large males spawning as guarders sired on average 53.7% of the offspring resulting from group spawns and ranged between 11% and 84% (Table 1). In replicate 6, guarder paternity (84%) was significantly higher than expected (50%, each male sires half the offspring). In replicate 2, guarder paternity (11%) was lower than expected (50%). Large male guarding paternity was not related to the magnitude of the size difference between the two males (Pearson correlation coefficient, $r = -0.175$, $P = 0.707$, n = 7). In three replicates, the smaller male spawned as guarders on at least one occasion. In the second replicate, the smaller male spawned as a guarder two times. In one spawning, he sired 100% of the offspring (seven fry examined), and in the other spawning he sired 0% of the offspring (four fry examined). In the fifth replicate, the smaller male spawned as a guarder two times. In one spawning, he sired 0% of the offspring (four fry examined), and in the other spawning he sired 17% of the offspring (six fry examined). In the eighth replicate, the smaller male spawned as the guarding male one time and sired 18% of the offspring (11 fry examined). Similar means were obtained when clutches with fewer than four eggs were included in the analysis although the variance surrounding the means increased due to a lack of precision.

Male size was important in determining competitive ability. In each pair, the larger of the

TABLE 2. LARGE MALE MATING ADVANTAGE IN SINGLE AND GROUP SPAWNS.

Rep	Female SL	Large Male SL	Small Male SL	Large male single spawns	Small male single spawns	Large male as guarder	Small male as guarder	Clutch size (SE)
1	63	59	43	0	0	7	0	13.6 (4.9)
2	51	61	60	2	0	3	7	5.5 (1.3)
3	60	60	58	1	1	9	7	3.6 (1.7)
4	60	61	56	0	1	4	1	6.7 (3.3)
5	51	60	56	4	3	12	4	6.1 (2.7)
7	58	63	40	2	0	7	0	7.9 (2.9)
8	62	61	40	4	0	21	1	6.3 (1.3)
10	59	61	58	0	0	2	2	2.0 (1.7)
11	57	58	56.5	2	0	15	2	6.0 (1.7)

two males tended to be more likely to spawn singly with the female (Table 2, paired $t = 2.17$, df = 8, $P = 0.062$). In group spawns, the larger of the two males was more likely to spawn as the guarder (Table 2, paired $t = 2.60$, df = 8, $P = 0.032$). The difference in size between the two males was correlated with the proportion of spawnings in which the larger male spawned as the guarder ($r = 0.736$, $P = 0.024$, n = 9). Male allomorph was not related to spawning success (single spawns: paired $t = -1.137$, df = 9, $P = 0.285$; guarding in group spawns: paired $t = -0.794$, df = 9, $P = 0.448$; these two t-tests include replicate 6 where the two males were the same standard length).

DISCUSSION

Two points emerge from this study. First, group spawning is costly to guarding males in terms of lost paternity. Males spawning as guarders did not achieve 100% paternity. When group spawns involve two males, each male has roughly an equal probability of fertilizing the eggs. Does this mean that guarder and sneaker mating tactics provide males with equal fitness benefits? The answer is no. Males acting as guarder males occasionally spawn singly with females where they are assured 100% paternity. Similar results have been found in other species. 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. As in this study, larger males can be the sole attendants of females (Foote and Larkin, 1988). In contrast to this study, 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*, and found that individual parr fertilized a smaller proportion of the eggs than larger, older males.

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 guarding males in group spawnings more often than smaller males. Furthermore, the ability of larger males to consistently spawn as guarding males is positively 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. In the orangethroat darter, *Etheostoma spectabile*, large males are more likely to engage in aggressive behavior than small males, but increased aggression does not lead to increased spawning success (Pyron, 1995). Other studies have 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 spawn with a female. The relative fertilization success of additional males is unknown. Males mating closest to the female (i.e., on top and directly on each side) may fare best because their sperm is most likely released closer to the female's eggs. Sneaker males located on the outside of the spawning group (i.e., with another sneaker male between himself and the female) may not fertilize a proportion of the clutch that is commensurate with their relative sperm output. Relative fertilization success of males in larger spawning groups may be measured using more variable molecular markers (e.g., DNA finger printing, microsatellites).

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