Reproductive ecology of *Fundulus heteroclitus* and *Fundulus diaphanus* in a New England watershed

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The killifish, *Fundulus heteroclitus*, has been studied intensively, yet little is known about its reproductive behavior in the field. The majority of what is known about its reproductive biology has been done using individuals from populations south of Cape Cod, often with the southern subspecies, and most of the work has been done in the laboratory. During 2003, we studied the reproductive biology of the northern subspecies of *F. heteroclitus* in a salt marsh habitat (Northeast Creek) and of a congener, *F. diaphanus*, in a nearby freshwater lake (Lakewood). Specifically, we examined the mating systems of these species, factors affecting time and intensity of spawning, and salinity effects on sperm motility and fertilization ability.

Daily patterns of spawning activity were assessed in the field by observing areas from the shoreline in Northeast Creek and snorkeling in Lakewood. From the edge of the creek, *F. heteroclitus* could be seen spawning daily on a rocky substrate during most (20 of 22) high tides and was never seen spawning at other times in the tidal cycle. *Fundulus diaphanus* could be seen spawning throughout the day in shallow (< 2m) water at Lakewood. The spawning intensity of *F. heteroclitus* was measured as the number of spawning events counted during the most active 30 minutes of observation. For both species, number of males per spawning event was recorded, as were time of day and salinity, and time relative to high tide for *F. heteroclitus*. To further assess tidal influences on the reproductive biology of *F. heteroclitus*, we checked the fecundity of 50 females caught using a small seine before high tide during different phases of the lunar cycle. Both percent of gravid females and number of eggs per female were recorded as measures of reproductive activity within this species.

To evaluate the effect of salinity on sperm motility, we qualitatively examined milt from 54 *F. heteroclitus* and 33 *F. diaphanus*. For each trial, milt was pipetted from a male directly to two microscope slides containing water with a salinity of 10 parts per thousand (ppt) (control) and water of one of the salinity trials (1, 2, 4, 5, 6, 8, 15, 20, 25, 27, 30 ppt for *F. heteroclitus* and 0, 0.5, 2, 10, 15, 20 ppt for *F. diaphanus*). Artificial seawater was used for all salinity trials. Initial motility was evaluated in a scale of 0-5 (5 indicating greatest motility) and all runs in which sperm in the control slide scored less than 4 were discarded. Duration of sperm motility was recorded when the percentage of sperm showing forward movement was estimated as 50%, 5%, and 0%. Each of these three estimates of motility showed similar trends, and only the results for time to 0% motility are reported here.

To measure the effects of salinity on fertilization success, eggs were stripped into a petri dish containing 10ml of water (of one of the trial salinities) and simultaneously mixed with fresh sperm from 2-3 males. Fertilization success was evaluated at 1, 2, 4, 10, 30 ppt for *F. heteroclitus* ($n = 30$) and at 0.5, 4, 10, 15, 20, 25, 30 ppt for *F. diaphanus* ($n = 37$). A sample of the sperm-containing water was taken shortly after to evaluate sperm concentration and to verify that sperm concentrations stayed roughly equal between trials. Eggs were considered successfully fertilized if they developed into clearly discernable embryos (Stage 12 of Armstrong and Child[1]). Once at this stage, all embryos we continued to follow through development successfully hatched.

We observed natural spawning in the field in both species. Spawning intensity in *F. heteroclitus* showed a steady decline from the earliest spawning observations in mid-June to the last spawning in the end of July (r = -.49, p = .02). *Fundulus heteroclitus* spawned over the entire range of salinities recorded during the breeding season (11.7 to 32.7 ppt) and during all phases of the lunar cycle. Salinity had no apparent effect on spawning intensity of *F. heteroclitus* (p = .63 in multiple regression with effect of date removed). There was no consistent difference in intensity of spawning between the lower day high tides and the higher night high tides (paired t-test p = .31, n = 10 pairs). In Lakewood, salinity was always < 1ppt. *Fundulus diaphanus* was observed spawning during most daylight hours, with a peak of spawning in the early afternoon.

In contrast to previous aquarium studies[4] we found that the predominant mode of spawning in *F. heteroclitus* was group spawning, with an estimated 3 males per spawn and approximately 80% of spawns containing more than one male (n > 500 spawns). In contrast, we observed much lower levels of sperm competition in *F. diaphanus*, with pair spawns accounting for over 90% of the observed spawns in this lower density species (n > 60 spawns). These differences are reflected in differences in allocation to testis in the two species. *Fundulus heteroclitus* had a significantly higher gonosomatic index (GSI; testis was 7.6% of body weight) compared with *F. diaphanus* (less than 2%). There was a slight decrease in GSI with individual size in male *F. heteroclitus* GSI (r = -.28, n = 51, p = .049).

Sperm from *F. heteroclitus* remained motile for extended periods of time at a broad range of salinities (Figure 1). Lowered motility was observed at both ends of the salinity spectrum, as found elsewhere[5]. Lowered motility at low salinities is also true for *F. diaphanus*. However, above 10 ppt the two species diverge, with *F. heteroclitus* sperm showing sperm motility across a wide range of salinity while *F. diaphanus* sperm have substantially reduced motility at salinities > 10 ppt (Figure 1, Mann-Whitney U tests, 10, 15, 20 ppt *F.h.*> *F.d.*; 2 ppt *F.h.* = *F.d.*).

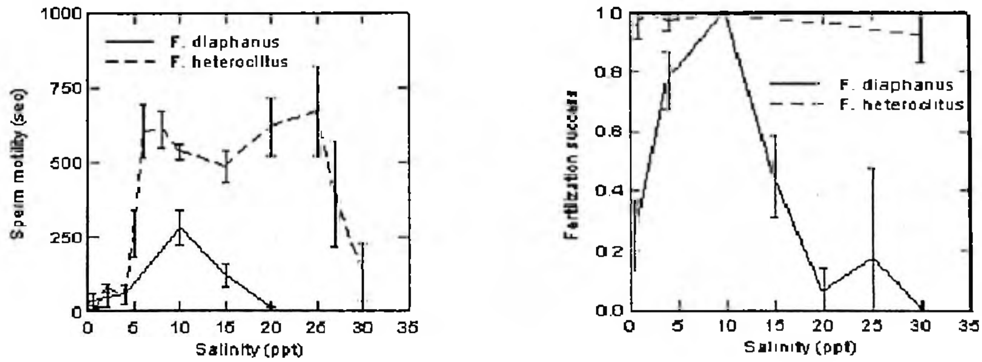

Figure 1. Sperm motility and fertilization success in *F. heteroclitus* and *F. diaphanus*. Error bars are ± 1 SE. Sperm motility is time to no motility, and fertilization success is the percentage of eggs successfully developing.

Similarly, fertilization success of *F. heteroclitus* was very high over a wide range of salinities. Despite minor falls in fertilization success at extreme salinities (Figure 1), over 99% of eggs were successfully fertilized in the other salinities. *Fundulus diaphanus* exhibited a different pattern. The optimum salinity was also 10 ppt (86.7% success), yet fertilization at low salinities was remarkably lower than that of *F. heteroclitus* (Figure 1). Fertilization did not occur in *F. diaphanus* in trials above 10ppt, as expected for a freshwater species. Fertilization success was less sensitive to salinity than sperm motility. Despite reduced motility at extreme salinities, fertilization success remained at or near 100% at all natural salinities for *F. heteroclitus* (Figure 1).

These results emphasize the differences in the reproductive biology of the closely-related *F. heteroclitus* and *F. diaphanus*. The abundant estuarine species, *F. heteroclitus*, spawns in groups at high tides, but does not exhibit a marked semi-lunar periodicity in its timing of reproduction as reported for the southern subspecies[2, 6]. The freshwater *F. diaphanus* exhibited less sperm competition, with large males defending territories, and spawned over a broader period during the day. In most fish species studied to date, sperm motility is activated upon spawning by a change in the relative osmolality of the external environment compared to the male reproductive tract[3]. These cues work in opposite directions for freshwater and marine species. In *F. heteroclitus*, the same unidirectional processes do not appear to be occurring, as sperm were active over the wide range of hypoosmotic and hyperosmotic conditions. Understanding the mechanisms that allow for this high salinity tolerance in reproductive environment in *F. heteroclitus* may be very important in understanding the success of this abundant estuarine fish.

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