Sex-Specific Spawning Behavior and Its Consequences in an External Fertilizer

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abstract: Identifying the target of sexual selection in externally fertilizing taxa has been problematic because species in these taxa often lack sexual dimorphism. However, these species often show sex differences in spawning behavior; males spawn before females. I investigated the consequences of spawning order and time intervals between male and female spawning in two field experiments. The first involved releasing one female sea urchin's eggs and one or two males' sperm in discrete puffs from syringes; the second involved inducing males to spawn at different intervals in situ within a population of spawning females. In both, fertilization success was measured as the fraction of eggs fertilized and the paternity share of each male. The results indicate that spawning after females imposes a cost on males but only during sperm competition. Further, the optimal interval between the initiations of male and female spawning depends on degree of sperm competition, distance between males and females, and water velocity. The results show that sex differences in spawning timing of marine invertebrates can be explained on the basis of the differential costs and benefits of spawning out of synchrony with the other sex and that the result of sexual selection on external fertilizers may be behavioral rather than morphological differentiation of the sexes.

Keywords: fertilization success, microsatellites, paternity analysis, reproductive synchrony, sea urchins, sexual dimorphism.

A fundamental issue in evolutionary biology is understanding the selection that produces intersexual differences in morphology and behavior (Darwin 1874; Andersson 1994). This issue is particularly compelling in taxa that release eggs and sperm into the sea for external fertilization (broadcast spawning) because these species often lack sexual dimorphism in adult traits. However, they do vary in a very simple way—the timing of gamete release (Levitan 1998*b*). This allows for an investigation of how mating behavior can influence mating success without the complications imposed by variation in adult morphological features, interactions within the female reproductive system, or post-mating (or pollination) investments that can all influence paternal and maternal success (Arnqvist and Rowe 1995; Havens and Delph 1996; Eberhard 1998). It also provides an avenue for exploring how the evolution of sexual dimorphism in adult traits may be related to the evolutionary transition to internal fertilization.

One of the most striking patterns among animals and in particular invertebrate taxa is that, generally, species that copulate or pseudocopulate exhibit sexual dimorphism whereas species that broadcast gametes do not (Strathmann 1990; Levitan 1998*b*). This pattern has been noted since at least the time of Darwin (1874), who suggested that this lack of sexual dimorphism was caused by an absence of sexual selection among such primitive animals. Recent evidence suggests that the potential for sexual selection can be high among broadcast-spawning invertebrates (Levitan 1998*b*, 2004; Franke et al. 2002). This apparent contradiction raises the issue of identifying the targets of sexual selection in broadcast-spawning taxa.

Two potential targets are gamete traits and spawning behavior. Previous work has shown that different egg and sperm trait values perform best under high and low levels of sperm availability (Levitan 1993, 1996, 1998*a*, 2002*a*; Marshall et al. 2000, 2002; Huchette et al. 2004) and that species typically experiencing different levels of sperm limitation show the trait values predicted to perform best under those conditions (Levitan 1993, 1998*a*, 2002*a*). In contrast, little information is available on the consequences of variation in spawning behavior in external fertilizers. The pattern of gamete release by adults influences sperm availability and the probability of gamete competition and is a likely target for sexual selection.

In external fertilizers, sexual selection may have produced differences in spawning behavior between the sexes. Thorson (1950) noted that, in 37 species of externally fertilizing marine invertebrates spread across seven phyla,

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males spawn before females. This pattern has been borne out by later observations; of 47 reports in which one sex spawned before the other, 44 indicated that males spawned first (Levitan 1998*b*). In some cases, males can initiate spawning hours before females (Hamel and Mercier 1996).

A similar pattern has been noted in externally fertilizing algae. Clifton (1997) observed broadcast spawning in 11 species across four genera of dioecious green algae, and in all 56 paired observations, males spawned before females. In these algal species, the average interval between male and female spawning times ranged from 2 to 20 min.

This potential outcome of sexual selection is similar to the sex differences in seasonal emergence times often noted in plants and animals (e.g., by Darwin 1874; Botterweg 1982; Fagerstrom and Wiklund 1982; Holzapfel and Bradshaw 2002). Male emergence time is viewed as a result of stabilizing sexual selection. Males that emerge too soon or too late relative to females and other males have lower reproductive success than males releasing at an intermediate optimal time. Although externally fertilizing adults may not emerge, their gametes do. However, it is not simple to intuit optimal spawning patterns or to estimate how priority effects will influence reproductive success in external fertilizers. Unlike copulating organisms, in which males can preempt mating by other males (Jivoff 2003), eggs may be difficult for single males to monopolize (Levitan 2004; D. R. Levitan, unpublished manuscript). Similarly, unlike plants and brooding invertebrates, which can slowly accumulate pollen (Alonso 2004) or sperm (Bishop 1998), eggs drift away from spawning events, and the window for fertilization can be very narrow (Levitan 2002*a*; but see Yund and Meidel 2003). Reproductive success of males and females as a function of the timing of spawning in externally fertilizers has not been directly measured.

I examined sex differences in the costs and benefits of spawning synchrony in the red sea urchin, *Strongylocentrotus franciscanus*, using microsatellite markers to determine paternity. Males spawn before females in this species (Levitan 2002*a*). In the study reported here, patterns of fertilization success under two types of field manipulations were examined. The first set of experiments was a tightly controlled study of gametes released from syringes into the ocean and recaptured under conditions with or without male competition. The second was a more natural manipulation in which sea urchins were induced to spawn in situ, and the pattern of reproductive success as a function of the temporal and spatial pattern of spawning was observed. The results suggest that the sexes differ in the consequences of spawning timing. The outcome of sexual selection in broadcast-spawning species may be manifested as adult behavioral rather than adult morphological differentiation.

Methods

Syringe Experiments

In the first set of experiments, sperm and eggs were released in discrete puffs over short time frames in a test of how male and female success varied as a function of exact timing and spatial configurations, with initial gamete concentrations held constant. The research was conducted in Barkley Sound, British Columbia, Canada, during the springs of 2001 and 2002. At the beginning of each experimental day, one female and two male sea urchins were injected with a 0.55 M solution of KCl, which induces spawning in the laboratory. The female sea urchin was inverted in filtered seawater, and 90 mL of eggs were collected and placed in a glass jar at a concentration of approximately 1×10^5 eggs/mL. Male sea urchins were kept upright and out of the water, and sperm were collected and placed on ice. For each male, six 0.1-mL aliquots of undiluted sperm were placed in separate 20-mL scintillation vials. Before sea urchins were returned to the sea, tube feet were collected for DNA analysis and placed in 95% ethanol.

Gametes were taken to a small cove on Dixon Island (48°07′N, 125°51′W) for an examination of how the spatial and temporal pattern of sperm and egg release influence male and female reproductive success in the presence and absence of sperm competition. All gametes were placed on ice during transportation to the field site and were used in field experiments within 3 h of spawning. The egg suspension was mixed with 20 mL of fluorescein dye (1 g/L of filtered seawater). Ten milliliters of this suspension was placed in each of nine 10-cm^3 syringes, and the syringes were capped and taken by a diver to the seabed at a depth of between 3 and 5 m. Sperm were kept undiluted until 1 min before use because sperm age effects become evident approximately 20 min after dilution in seawater (Levitan et al. 1991). Once the diver was situated on the bottom, the sperm for the first treatment were diluted with 9.9 mL of fluorescein dye. A 5-mL aliquot of this suspension was placed in a syringe, and the syringe (or syringes in treatments testing both males) was sent down to the diver. After the gametes from that treatment were released and recaptured (see below), the next set of sperm syringes were prepared and sent to the diver.

Sperm and eggs were released in nine patterns of spawning order in the presence or absence of sperm competition, that is, the release of sperm from more than one male (table 1). The nine treatments were subdivided into two experiments. The first tested for the influence of sex differences in the order of gamete release on male and female reproductive success with or without sperm competition. The second tested the influence of the delay between sperm and egg release on male reproductive success when eggs

Table 1: Patterns of sperm and egg release in the syringe experiments

	Time of release		
	0 s	20 s	40 s
Sperm release:			
Before egg release	Male 1 sperm	Eggs	…
After egg release	…	Eggs	Male 2 sperm
Before and after egg release	Male 1 sperm	Eggs	Male 2 sperm
Egg release:			
In center of sperm cloud	Male 1 sperm	…	Eggs
In center of sperm cloud	…	Male 2 sperm	Eggs
In center of sperm cloud	Male 1 sperm	Male 2 sperm	Eggs
1 m from center of sperm cloud	Male 1 sperm	…	Eggs
1 m from center of sperm cloud	…	Male 2 sperm	Eggs
1 m from center of sperm cloud	Male 1 sperm	Male 2 sperm	Eggs

Note: In the first set of trials, the eggs were released 20 s after the initiation of the trial. Sperm from males 1 and 2 were released at times 0 s and 40 s, either in independent trials (no competition) or in the same trials (in competition). In the second set of trials, eggs were released 40 s after the initiation of the trial. Sperm from males 1 and 2 were released at times 0 s and 20 s, either independently or in competition. These trails were repeated with eggs released in the center of the sperm cloud and eggs released 1 m from the center of the sperm cloud. All nine treatments from both sets of trials were conducted with gametes from the same female and two males for each replicate. The entire experiment was replicated 19 times on different days with different sea urchins.

were released either close to or more distant from the point of sperm release (again with or without sperm competition). In all trials sperm were released at a natural rate of 1×10^8 sperm/s (Levitan 1998*a*), and gametes were released at a height of approximately 0.5 m from the seafloor. In all nine treatments, eggs were recollected with a subtidal plankton pump (Levitan 1996, 1998*a*) 130 s after the first gametes were released into the water. After this interval, sperm are too dilute to influence fertilization further, but eggs are still concentrated enough to be visible as a wisp of dye and for collection of a reasonable sample (Levitan 1996). Immediately after the sample was collected, the intake of the pump was placed 5 m upcurrent from the sperm release point, and the sample and pump were rinsed for 1 min to remove excess sperm.

In the laboratory, after 3 h, at least 250 eggs were microscopically inspected for the presence of early development (raised fertilization envelope or early cleavage). In treatments in which sperm from both males were released, the embryos were cultured in 500-mL glass jars with filtered seawater without added food. Seawater in these jars was changed daily, and after 3 days, 50 larvae from each culture were individually frozen in 1 μL of nanopure water at -80°C (Levitan 2002*b*).

The adults and 20 larvae per treatment from these experiments were then subjected to analysis of microsatellite markers (for details of methods, see McCartney et al. 2004), and paternity was determined. I scored the alleles of six to nine loci for all potential adults and then chose (at least) the three most diagnostic loci for that particular set of parents to use on the larvae. Typically, three loci were enough both to identify (to exclude all but one set of parents) and to confirm (to have at least two diagnostic alleles present in both the larvae and adults) the parentage of larvae. If necessary, an additional set of three loci was used to resolve ambiguities. This entire set of trials was replicated 19 times with unique male and female sea urchins. Before statistical analysis, all percentage data were arcsine transformed.

In these experiments, I did not conduct the complementary treatment of releasing the eggs from two females, one before and one after sperm release, because eggs released later would be recollected in higher abundance than the eggs released earlier, and accounting for this bias would be difficult. Laboratory experiments have demonstrated, however, that the fundamental premise of this treatment, that higher egg number (or density) would decrease the likelihood that an individual egg would be fertilized, is not supported. An eightfold increase in egg density, over a range relevant to the current experiment, had no effect on the fraction of eggs fertilized in the laboratory (Levitan et al. 1991). To test this premise further, I conducted a small number of field trials intended to reveal whether the presence of a second female's eggs would decrease overall fertilization rate. Gametes were collected in the laboratory from two females and one male as described above, and then in the field sperm were released at time 0, and eggs from one female, a second female, or both females simultaneously were released in different treatments. Eggs were released 20, 40, or 60 s after sperm release

(three female treatments × three time intervals × three replicates).

In Situ Spawning Experiment

The second set of experiments more closely resembled natural sea urchin spawning events. Males and females were induced to spawn with an injection of KCl, in situ, at different times over a longer temporal scale. Individuals released an uncontrolled number of gametes in a plume rather than puff, and the spatial component was more variable as the sea urchins moved. In *Strongylocentrotus franciscanus*, eggs are released and reside on the aboral surface of the female until they are wafted up into the water column by water currents. In this experiment, eggs are collected in the water column as they drift away from the females but before they disperse and dilute to the point where capture becomes unfeasible (about 20 cm away).

The time eggs reside on the aboral surface of the females varies depending on flow conditions. Experiments in a laboratory flume suggest that fertilization occurs while the spawned eggs lay on aboral surface of female sea urchins and as they are advected off the females (Yund and Meidel 2003). These results indicate that at low flow conditions, eggs remain longer on females and are fertilized at a higher and faster rate compared to high flow conditions. In this experiment, eggs advect away naturally, so the differential time eggs spend on the aboral surface is incorporated into this assay. This method of inducing sea urchins to spawn in situ with KCl produces the same female fertilization rates as compared with estimates made during natural spawning events at those same spawning population densities (Levitan 2002*a*).

These experiments were conducted in the spring of 2003 at the mouth of Bamfield Inlet (48°50′N, 125°08′W) at a depth of 3–8 m within a natural population of *S. franciscanus* (see Levitan 2004 for a full site description). The densities and nearest-neighbor distances between spawning individuals in this experiment were well within the natural range of spawning individuals for this species (Levitan 2002*a*, 2004). Flow at this site has a directional component driven by incoming or outgoing tides but also has an oscillatory component caused by exposure to small to moderate size waves (usually less than 1 m; see fig. 1 for examples of the directional components of flow during these experiments).

At the beginning of the dive, a 1-m^2 PVC quadrat was placed on the seabed to establish the center of the experimental arena. An S4 current meter (InterOcean) was placed 5 m upcurrent from this location and set to record water velocity and direction at 0.5-s intervals 0.5 m from the bottom. Next, sea urchins were collected and placed in a location 10 m downstream from the quadrat. These sea urchins were induced to spawn with injections of KCl. Spawning males were kept downstream and placed into a large plastic tub. Between three and eight spawning females were tagged with numbered latex bands, brought upstream, and placed around and 1–2 m away from the PVC quadrat. After these females were in place, another group of sea urchins were injected within the PVC quadrat. When the first male was induced to spawn it was tagged and placed near the center of the quadrat. Five minutes later, additional sea urchins were injected until a second male was induced to spawn, tagged, and placed near the center of the quadrat. This process was continued until 20 min had elapsed (for a total of between three and five males, depending on the rapidity of spawning induction). Any females that were incidentally induced to spawn during this procedure were moved out of the experimental arena and ignored. Five minutes after the last male was induced to spawn, all individuals' spatial positions were mapped, and a sample of eggs was collected, with a subtidal plankton pump, in the water column directly above each spawning female (see Levitan 2002*a*, 2004, for similar method of egg collection). Eggs were collected for approximately 10 s, and the time interval between samplings of successive females was 2–3 min. At the end of the dive all tagged individuals were collected, and tube feet were collected and placed in 95% EtOH for microsatellite genotyping. This experiment was replicated nine times.

In this experiment, females were induced to spawn and placed in the spawning arena before males in order to reduce the diver influence of water (and gamete) mixing that would have taken place if divers had to swim through the sperm clouds in order to place the females and to avoid the sperm contamination of spawning additional males while trying to induce females (the parentage analysis confirms that egg contamination is extremely rare). The effect of inducing females first on the outcome of the experiment is likely to be minimal because the eggs released by this sea urchin species (in this habitat) are advected quickly away from the females (D. R. Levitan, unpublished data), and the eggs sampled were almost certainly spawned after the males were induced to spawn.

Larvae were examined, cultured, and collected as in the first set of experiments, and microsatellite markers of at least 20 larvae per female were analyzed, along with those of the adults. Overall, genotypes were determined for 34 males, 56 females, and 1,142 larvae across the nine replicate trials in this experiment. The fertilization success of a particular male-female pair was calculated as the product of the proportion of the female's eggs fertilized and the male's paternity share. Data were arcsine transformed and examined as a function of the distance between the male and female, the level of advection (distance the water mass moved over the current meter during the experimental

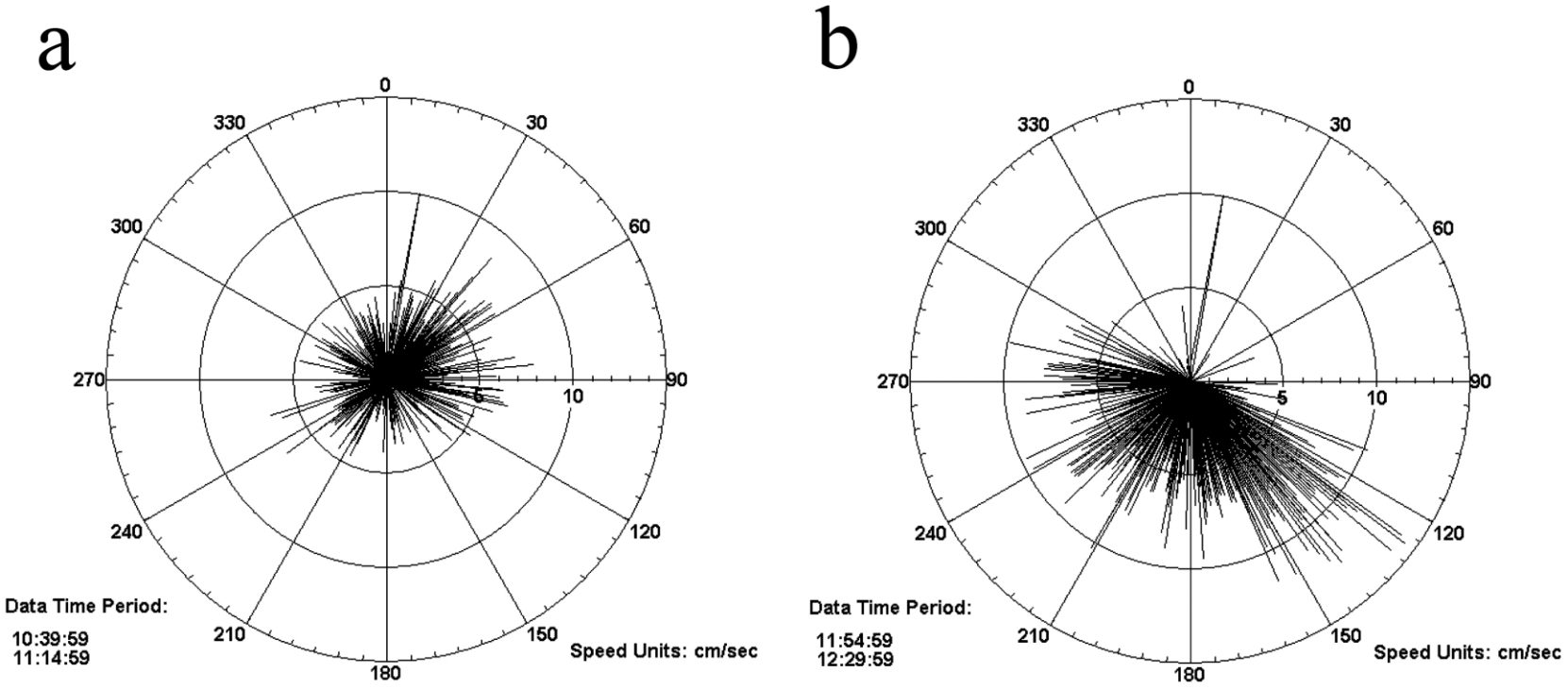


Figure 1: Directional components to water flow. Each line emanating from the center of the compass diagrams represents the direction and velocity of flow over a 0.5-s interval during the entire time period from inducing spawning in the male sea urchins to collecting the last egg sample (35 min). The average velocity is calculated as the mean of the absolute values of each vector (which gives an indication of the amount of water movement). The level of advection is the calculated as the sum of the vectors divided by the experimental time period (which gives the rate at which gametes move away from a sea urchin). March 13, 2003 (*a*), was the day with the least amount of flow with a mean velocity of 2.41 cm/s and advection of 0.67 cm/s in a direction of 40°. March 16, 2003 (*b*), was the day with the most amount of flow with a mean velocity of 5.11 cm/s and advection of 3.43 cm/s in a direction of 181°.

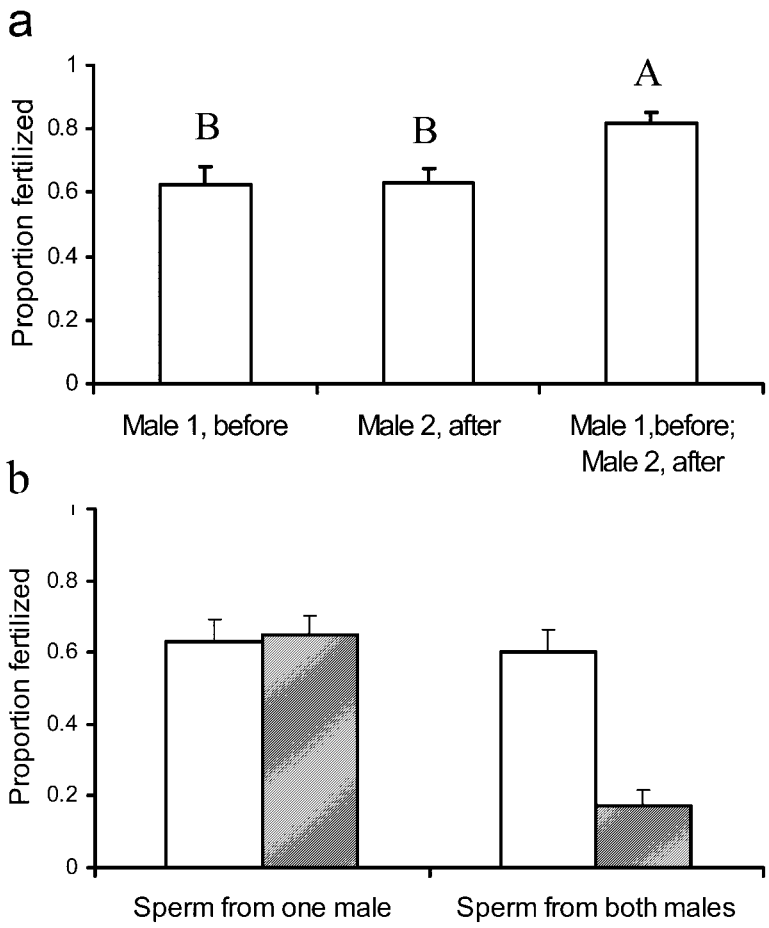


Figure 2: Reproductive success as a function of synchrony and sperm competition. *a*, Female reproductive success when sperm were released before, after, or both before and after egg release. The fraction of eggs fertilized was independent of spawning order but was higher when sperm from two males were released. Treatments with different letters were significantly different in multiple comparisons. *b*, Male reproductive success when sperm were released before (*white bars*) and after (*shaded bars*) egg release in the presence and absence of competing sperm. When sperm from only one male were released, order did not influence male reproductive success, but when sperm from two males were released, the sperm released before the eggs were released garnered an advantage. See table 2 for statistical analysis.

period), and the time interval during which the male was induced to spawn (early, 0–5 min; intermediate, 5–15 min; late, 15–20 min).

Results

Influence of Spawning Order and Sperm Competition on Male and Female Success

Sex differences in the order of spawning affected male but not female reproductive success. For females, the fraction of eggs fertilized was higher when sperm from both males were released into the water than when only one male's were released, but female reproductive success did not depend significantly on whether sperm were released before or after eggs (one-way ANOVA and Tukey pairwise comparison; fig. 2*a*).

For males, when sperm from only one male were released, the fraction of eggs fertilized was the same whether the sperm were released before or after the eggs, but when sperm from both males were released, one before and one after egg release (fig. 2*b*), the sperm from the second release fertilized far fewer eggs (17% rather than 65%). Results from a two-way ANOVA testing the effects of male timing (before or after the eggs) and competition (presence or absence of a competing male) on male reproductive success indicated a significant interaction. Independent tests of the effect of male timing on male reproductive success indicated a significant effect only in the presence of competition (table 2). Sperm from a male that spawns late in

Table 2: Two-way ANOVA testing the effects of male timing (early or late) and competition (present or absent) on male reproductive success (arcsine transformed)

Source	df	Type III SS	MS	*F*	*P*
Overall:					
Timing	1	.909	.909	12.99	.0006
Competition	1	1.388	1.388	19.38	.0001
Timing × competition	1	1.091	1.091	15.59	.0002
Error	72	5.039	.070		
Corrected total	75	8.428			
No competition:					
Timing	1	.004	.004	.06	.8045
Error	36	2.401	.067		
Corrected total	37	2.405			
Competition:					
Timing	1	1.997	1.997	27.24	.0001
Error	36	2.639	.073		
Corrected total	37	4.635			

Note: Because of a significant interaction, the effect of male timing was tested independently across the two competition treatments. Male timing was only significant in the presence of a competing male.

an event, after both males and females have spawned, may still encounter eggs, but those eggs may already have been fertilized by other males.

Influence of the Location of Gamete Release on Male and Female Success

Males seem to be subject to a trade-off between the advantage of early sperm release and the disadvantage of greater sperm dilution (fig. 3). When eggs were released in the center of the sperm cloud, sperm released in close synchrony (20-s treatment) with egg release fertilized similar percentages of eggs in the presence and the absence of competition from sperm released earlier (71% and 61% of fertilized eggs). The sperm released earlier (40-s treatment) were more diffuse by the time the eggs were released and therefore fertilized fewer eggs even in the absence of competing sperm (48%) and many fewer (16%) in competition with more synchronously released sperm. Sperm released early lost fertilization share to sperm released later (but still before egg release) when the eggs and sperm were released in the same central location.

In contrast, when the eggs were released 1 m from the sperm cloud, sperm released earlier fertilized more eggs (>20%) than did sperm released later and more synchronously with egg release (<5%), regardless of whether the two sets of sperm were in competition (fig. 3). Although the sperm released early were more dilute at the time of egg release, those sperm were dispersed over a greater

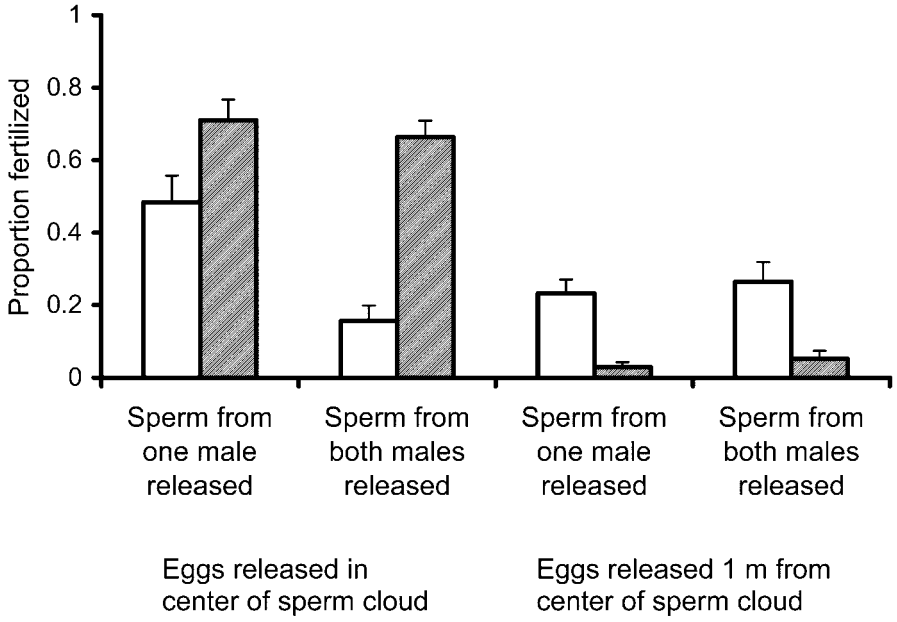


Figure 3: Male reproductive success when sperm were released 40 s (male 1, *white bars*) or 20 s (male 2, *shaded bars*) before eggs were released. Eggs were released either in the center of the sperm cloud or 1 m from the center of the sperm cloud. See table 3 for statistical analysis. Sperm released closer to the time of egg release showed higher success when eggs were released in the sperm cloud but lower success when the eggs were released away from the sperm cloud.

Table 3: Three-way ANOVA testing the effects of male timing (early or late), competition (present or absent), and position (egg release in the center or edge of sperm cloud) on male reproductive success (arcsine transformed)

Source	df	Type III SS	MS	*F*	*P*
Overall:					
Timing	1	.026	.026	.45	.5035
Competition	1	.195	.195	3.35	.0692
Position	1	6.490	6.490	111.83	.0001
Timing × competition	1	.217	.217	3.75	.0549
Timing × position	1	4.962	4.962	85.49	.0001
Competition × position	1	.682	.682	11.76	.0008
Three-way interaction	1	.241	.241	4.16	.0432
Error	143	8.300	.058		
Corrected total	150	21.038			
Edge:					
Timing	1	2.119	2.119	47.39	.0001
Competition	1	.074	.074	1.65	.2037
Timing × competition	1	.0003	.0003	.01	.9333
Error	71	3.175	.045		
Corrected total	74	5.359			
Center:					
Timing	1	2.874	2.874	40.37	.0001
Competition	1	.808	.808	11.36	.0012
Timing × competition	1	.461	.461	6.49	.0130
Error	72	5.125	.071		
Corrected total	75	9.269			
Center/no competition:					
Timing	1	.516	.516	5.65	.0228
Error	36	3.283	.091		
Corrected total	37	3.798			
Center/competition:					
Timing	1	2.820	2.820	55.12	.0001
Error	36	1.842	.051		
Corrected total	37	4.662			

Note: Because of a significant three-way interaction, the effect of male timing and competition was tested independently for egg release in the center or edge of the sperm cloud. In the edge treatment, the effect of male timing was significant but not the effect of male competition. In the center treatment, because of a significant two-way interaction, the effect of male timing was addressed independently in each competition treatment. The effect of male timing was significant in both independent tests.

spatial area and were more likely to come into contact with eggs released at a distance.

These results were confirmed by a three-way ANOVA testing the effects of male timing (early or late), competition (presence or absence), and position of egg release (center or edge) on male reproductive success. A significant three-way interaction prompted independent tests of the effects of male timing and competition on center and edge egg release points. In the edge egg release test, male timing had a significant effect, but competition did not. In the center egg release test, male timing and competition interacted significantly. In independent tests, the effect of male timing was significant in both competition treatments but more prominent in the presence of competition (table 3).

Female Competition Experiment

The small subset of trials that examined egg competition revealed no significant difference between the fertilization of eggs released with or without competition with a second female (ANCOVA; table 4). In this experiment, eggs from both females were released at the same time in the same place, and no effect of competition was noted. If females were separated in space, as they normally would be in the field, the chance would be even smaller that sperm taken up by the eggs of one female would diminish the likelihood of fertilization in a second female.

In Situ Spawning and the Effects of Temporal and Spatial Variation in Gamete Release

The fraction of eggs released by a particular female that was fertilized by a particular male depended on the distance between them, the temporal sequence of male spawning, and the speed of water movement. An ANCOVA testing the main effect of male timing (early, intermediate, or late), and the covariates of male-female distance and advection, found a significant effect of advection (fig. 4) and an interaction between male timing and male-female distance (arcsine-transformed data; table 5). Higher levels of advection removed sperm faster from the water column above the spawning females and resulted in lower levels of fertilization. Because of the significant interaction of male timing and male-female distance, fertilization success (arcsine transformed) was regressed on the effects of advection and male-female distance for each male time cat-

Table 4: ANCOVA testing the main effect on female fertilization success of releasing eggs from one female, a second female, or both females together at three intervals after release of sperm from a single male (egg competition experiment)

Source	df	Type III SS	MS	*F*	*P*
Female	2	390.73	195.37	.39	.684
Release time	1	3,611.69	3,611.69	7.15	.014
Error	22	11,119.52	505.43		
Corrected total	25	15,030.84			

Note: The covariate was the time interval (20, 40, or 60 s) between sperm and egg release. This experiment was replicated on 3 days with unique sea urchins. Eggs were not successfully recollected in one trial, so the total number of trials was 26 rather than 27. The effect of the covariate was significant (the shorter the interval, the higher the fertilization success), but the main effect of number and identity of females whose eggs were released was not significant.

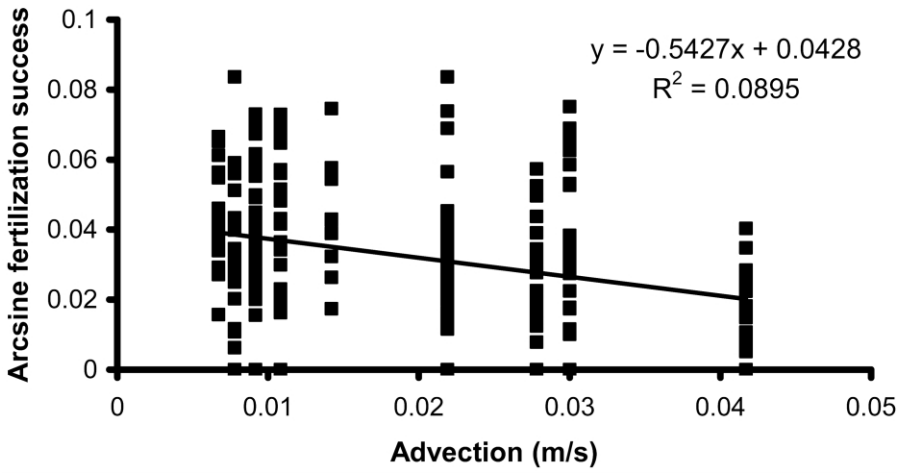


Figure 4: Fertilization success of a particular male-female pair as a function of the level of advection (straight line distance a particle of water moved divided by the length of the experimental time period) in the in situ spawning experiment. Increased water movement resulted in decreased fertilization success.

egory independently. In all three tests, increased advection significantly reduced fertilization. Early-spawning males were able to permeate the water more evenly with their sperm, eliminating any significant effect of male-female distance (fig. 5*a*). Intermediate-spawning males showed a nonsignificant trend of reduced fertilization success with increased male-female distance (fig. 5*b*). Late-spawning males showed a significant negative relationship between fertilization success and male-female distance (fig. 5*c*). Late-spawning males were able to fertilize fewer eggs released by distant females. Pairwise tests of least square means indicated that, overall, early-spawning males had higher levels of fertilization than did intermediate- ($P < .01$) or late-spawning ($P < .001$) males. Although the late-spawning males' reduced fertilization at greater distance is not surprising, it was not obvious that the earlier-spawning males would experience no distance effect.

Discussion

The Consequences of Spawning Behavior

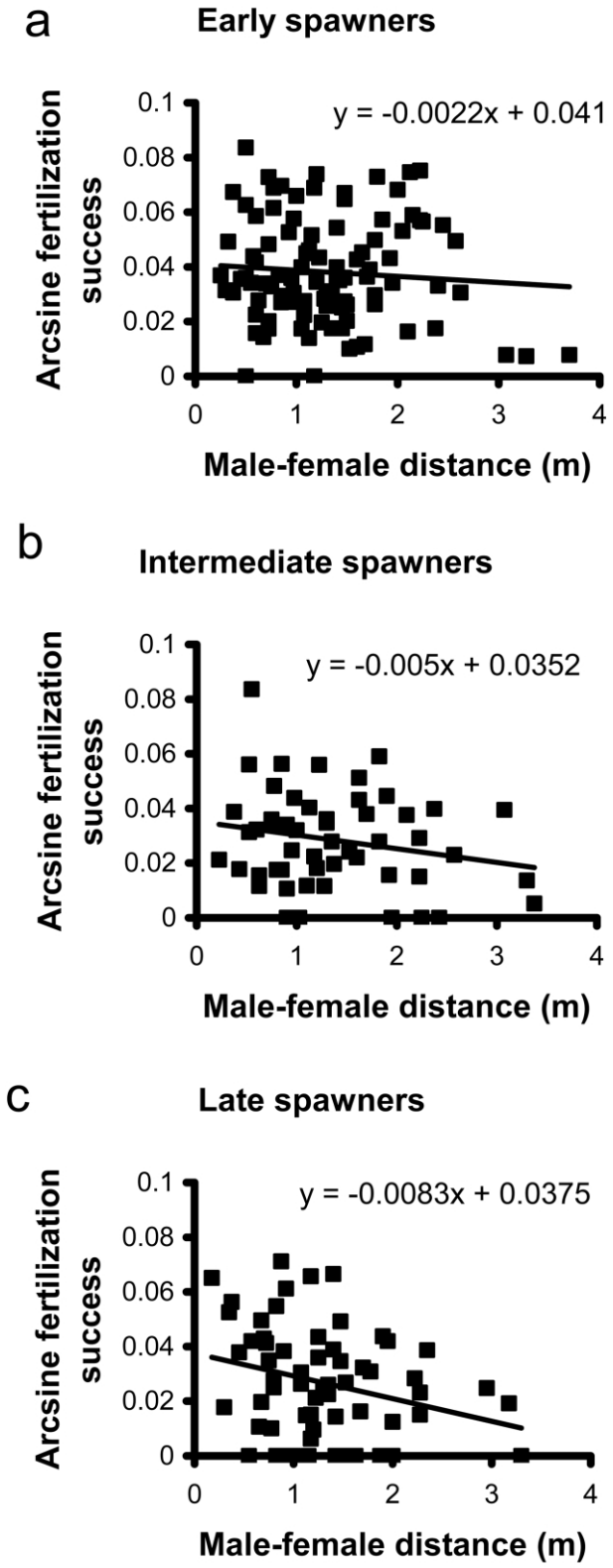
These results provide insight into the pattern of spawning timing of *Strongylocentrotus franciscanus* and perhaps more generally among externally fertilizing taxa. Results of the experiment in which gametes were released in puffs from syringes indicate that, in the absence of sperm competition, male reproductive success is independent of spawning order, but when more than one male is competing for fertilizations, the male with first access to the eggs garners a greater paternity share at the expense of the later-spawning male. Because spawning late is costly only to males, selection should favor males that spawn before females, explaining the male-first spawning observed in this species (Levitan 2002*a*) and in many other externally fertilizing marine invertebrates (Thorson 1950; Levitan 1998*b*).

The question of how early males should spawn is a more complicated problem, and the answer seems to depend on context. Some conditions might favor early sperm release, which would blanket a larger area with more diffuse sperm. The syringe experiments demonstrated an advantage to spawning early, when sperm clouds from different males did not completely overlap with egg clouds, because early-spawning males could cover a larger spatial area with sperm than could later-spawning males. However, when

Table 5: ANCOVA testing main effect of male timing (early-, intermediate-, or late-spawning male) on the fertilization success of a particular male-female pair (arcsine transformed) in the in situ spawning experiment

Source	df	Type III SS	MS	*F*	*P*
Male time	2	.001009	.000504	1.53	.218
Advection	1	.001468	.001468	4.46	.036
Distance	1	.000369	.000369	1.12	.291
Advection × time	2	.001797	.000899	2.73	.068
Distance × time	2	.002197	.001099	3.34	.038
Advection × distance × time	3	.001753	.000584	1.77	.153
Error	204	.067193	.000329		
Corrected total	215	.084575			

Note: The covariates were the level of advection and the distance between the male and the female. The effects of advection and of the interaction between distance and time were significant.



sperm from two males were directly competing within the same area, there was a cost to spawning too early, because the sperm were released in discrete puffs and the early release sperm were more dilute and at a disadvantage.

In the in situ spawning experiment, early-spawning males were still releasing sperm at the time of egg collection and were at an advantage regardless of competition. Early-spawning males gained higher average fertilization, more extensive spatial cover of fertilization, and far fewer cases of reproductive failure—only 3% of their pairwise matings produced zero fertilization, compared to 8% and 23% for intermediate- and late-spawning males.

Spawning too soon, however, would be disadvantageous. If early-spawning males slowed or ceased spawning before females completed egg release, the more concentrated sperm from later-spawning and nearby males would presumably gain the advantage. Clearly sperm advected away from spawning females before they release eggs would lose in competition to the sperm of later-spawning males.

The time interval between the initiations of male and female spawning should be correlated with the duration of sperm release by males; selection should favor males that spawn before and during egg release but stop in near synchrony with females. This seems to be the case in the species under study here (Levitan 2002*a*) and in an earlier study of a sea cucumber (Hamel and Mercier 1996). In *S. franciscanus,* males spawned for 1.5 h before females joined in, and 1 h later both stopped simultaneously (Levitan 2002*a*). In *Cucumaria frondosa*, males spawned for 4–5 h before females joined in, and 10–12 h later both stopped simultaneously (Hamel and Mercier 1996). Detailed observations from other taxa are needed to confirm this pattern.

The interesting trade-off, for a constant number of sperm, is how rapidly sperm should be released: explosively for a short time period before female spawning or more slowly over a longer period before female spawning. Models of individual sperm release have emphasized that increasing sperm release rate leads to greater reproductive success (Denny and Shibata 1989; Babcock et al. 1994; Levitan and Young 1995), yet individual males are often

Figure 5: Fertilization success of a particular male-female pair as a function of the distance between them for (*a*) early-spawning males, (*b*) intermediately spawning males, and (*c*) late-spawning males. Multiple regression testing the effects of advection and male-female distance on fertilization success indicated a significant effect of advection across all three male spawning times ($P < .05$). The effect of male-female distance was not significant for early spawners ($P > .5$), closer to significant for intermediate spawners ($P > .1$), and significant for late spawners ($P < .05$). For late spawners, greater distance between males and females resulted in lower levels of fertilization.

observed to release sperm slowly, for minutes to hours (e.g., by McEuen 1988; Levitan 2002*a*). These models considered only overlapping and synchronous sperm and egg plumes and not the benefits of bet hedging, in the form of slower sperm release over longer intervals. Slow release of sperm might be advantageous when competition is low or individuals are more widely spaced. Prolonged sperm release by nonaggregating sedentary benthic invertebrates, as noted above, is then not surprising.

A more explosive release, more synchronous with female spawning, would be advantageous under conditions of close male-female pairing and/or intense sperm competition. Where multiple males release overlapping plumes of sperm, males releasing sperm slowly would be outcompeted. A more explosive release of sperm would also be advantageous when rapid advection removes sperm from the spawning population before females release eggs. The much more explosive pattern of spawning noted in fish, which mate in very close proximity, often with competing males (reviewed in Petersen and Warner 1998), matches this prediction. Similarly, polychaetes that live in tight aggregations seem to release sperm in bursts rather than as constant plumes (Thomas 1994). Additional observations of the match between these predictions of spawning intensity as a function of male-female distance and the degree of sperm competition will be interesting.

An alternative hypothesis explaining why males spawn before females is that sperm are the cue for females to release eggs. Although sperm have been documented to act as a cue for egg release in some species, including sea urchins (Starr et al. 1990, 1992), the pattern is not universal, as eggs can trigger male spawning in other echinoderm taxa (Run et al. 1988). These cases suggest that males are not constrained to spawn first but do so in response to selection. The release and reception of spawning pheromones may be under sexual selection as the proximate mechanism generating sex differences in spawning to achieve an optimal time interval between male and female spawning.

The patterns noted here are analogous to patterns of emergence in plants and animals with internal fertilization. Sexual selection can act as a stabilizing force on optimal emergence (spawning) times that depends on the distribution of emergence (spawning) time of other males in the population (Iwasa et al. 1983; Parker and Courtney 1983; Thornhill and Alcock 1983; Zonneveld 1996; Holzapfel and Bradshaw 2002). The major difference between copulating and externally fertilizing taxa is that copulating males can guard mates and must choose among synchronously reproductive females (Shuster and Wade 2003). In contrast, species that release sperm (or pollen) cannot easily dominate matings with single females but can simultaneously mate with several females (Levitan 2004). These differences might result in a bet-hedging strategy that lowers the variance in male reproductive success (D. R. Levitan, unpublished manuscript) through early and protracted spawning that produces a temporally and spatially more homogenous sperm cover.

Constraints on the Experimental Design and the Diversity of Spawning Conditions

In the in situ spawning experiment, it is possible that some of the unfertilized eggs might have been fertilized if they had been left to drift until egg death. The magnitude of this cost is dependent on how long drifting eggs tend to remain near a spawning event where the sperm are most concentrated and have the highest likelihood of fertilization (Levitan 2002*a*; Yund and Meidel 2003). In locations where gametes are not advected away from the spawning event, fertilization may take place over a longer time interval dictated by oscillatory currents, tidal rhythms, or gamete longevity (Levitan 2002*a*; Yund and Meidel 2003; Marshall et al. 2004). Even in this study, where eggs rapidly dispersed, it is likely that some fraction of collected unfertilized eggs would have been fertilized had they been left in the water column. However, it is not clear how this potential artifact might bias the conclusion that earlier males have a larger spatial coverage of sperm and are more likely to fertilize more distant females compared to later-spawning males. While waiting additional time might have given later-spawning males additional time for their sperm to reach more distant females (and vice versa for eggs passing by more distant males), this would also be true of earlier-spawning males and sperm from these early-spawning males are still more likely to reach females first. The results from the syringe experiments suggest that this priority effect favors early-spawning males. This point is supported by the result that while water flow influenced reproductive success, there was no significant interaction between water flow and the timing of male spawning on reproductive success (table 5).

The Outcome of Sexual Selection in External Fertilizers and the Evolution of Sexual Dimorphism

Sexual selection, defined as the selection that arises from differences in mating success (Arnold 1994), may be an important force acting on external fertilizers (Levitan 1998*a*, 2002*a*, 2002*b*, 2004). The nature of sexual selection shifts as a function of sperm availability in external fertilizers (Levitan 2004). Under conditions of low sperm availability and sperm limitation, both males and females are under selection to enhance fertilization rate. As sperm availability increases, sexual selection becomes more asymmetrical as eggs become saturated with sperm, but males

still compete for fertilizations. At more extreme levels of sperm availability, sexual conflict may result, as males compete for the highest fertilization rate, while females might be under selection to decrease fertilization rate to avoid polyspermy (Franke et al. 2002; Levitan 2004).

The outcome of sexual selection in external fertilizers may be manifested in two forms: first, the evolutionary transition from isogamy to anisogamy (Parker 1984; Levitan 1996; Randerson and Hurst 2001; Bulmer et al. 2002) and variation in gamete traits under different conditions of sperm availability (Levitan 1993, 1998*b*, 2002*a*) and, second, the evolution of behavioral differentiation in the timing of spawning that reflects the different constraints on males and females.

The evolution of morphological differentiation in animals seems to be linked not directly to the intensity of sexual selection per se but rather to the initiation of tight behavioral interactions by adults. Interestingly, the rare exceptions of externally fertilizing marine invertebrates that show sexual dimorphism are pair spawners (Levitan 1998*b*). These include reduced male body size in pair-spawning brittle stars (Hendler 1991; Tominaga et al. 2004) and horseshoe crabs (Brockmann et al. 1994) and reduced male gonad size in pair-spawning sea stars (Run et al. 1988). These three examples are also among the rare cases in which eggs are released before sperm. Males of pair-spawning species may be able to wait for egg release and efficiently release sperm to garner the majority of fertilizations even in the presence of competing males (Brockmann et al. 1994). This pattern also appears to hold in marine fish, with sexual dimorphism being more pronounced in species that pair versus group spawn (Thresher 1984). Mate guarding, courtship, and territoriality are all social activities that select for sexual dimorphism in size, color, and specialized structures (Anderssen 1994). The continuum from group spawning through pair spawning, pseudocopulation, and copulation may represent a transition in the nature of sexual selection and a shift in what traits are the target of selection (Levitan 1998*b*, 2004).

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