Measuring Fertilization Success of Broadcast-Spawning Marine Invertebrates Within Seagrass Meadows

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Abstract. Increasing current velocity has been negatively correlated with the fertilization success of marine broadcast-spawning invertebrates. Seagrass has been shown to affect seawater hydrodynamics by slowing the movement of water. In this study we aimed to tease apart the relationship between fertilization success in sea urchins inside and outside of seagrass beds in St. Joseph Bay, Florida. Fluorescein dye diffusion, as a proxy for gamete diffusion, indicated higher rates of diffusion in sand habitats outside of seagrass beds. We quantified the proportion of eggs that remained on a female compared to being advected off a female over a 2-min interval in and out of grass beds. More eggs were collected inside of seagrass beds than over sand habitats, suggesting increased residence time of gametes within the beds. We induced sea urchins to spawn in experimental arrays in and out of grass beds and measured the fertilization success of eggs released from females and captured in the water column with a plankton pump. The fertilization success of eggs was significantly higher in grass beds. We concluded that seagrasses have the potential to mitigate gamete diffusion and increase the reproductive success of broadcast-spawning species that spawn in them.

Introduction

Broadcast-spawning, the release of sperm and eggs into the environment, is a common reproductive strategy in benthic marine organisms (Giese and Kanatani, 1987; Levitan, 1998a). It is commonly accepted that the fertilization success of broadcast-spawners is largely dependent on the time that sperm and eggs remain mixed together at concen-

trations sufficient to allow for fertilization (Pennington, 1985; Babcock *et al.*, 1994; Levitan, 1995). Gamete concentration (Denny, 1988; Denny and Shibata, 1989) and dispersal (Benzie *et al.*, 1994) are in turn affected by hydrodynamic regimes. Most studies on variation in fertilization have focused on examining how the distribution and abundance of mates (Pennington, 1985; Yund, 1990; Levitan, 1991; Levitan and Young, 1995) or water flow (Pennington, 1985; Levitan *et al.*, 1992; Petersen *et al.*, 1992; Levitan, 2002) influence fertilization success. Studies on water flow generally examine temporal variation in flow (Pennington, 1985; Levitan *et al.*, 1992) or flow along a gradient of wave exposure (Levitan, 2002). Fewer studies have examined how features of benthic habitats, such as submerged vegetation, may influence fertilization through changes induced in flow (but see Wahle and Peckham, 1999).

Underwater plant canopies have been shown to affect flow structure in a variety of ways depending on the ratio between canopy height and water depth (Nepf and Vivoni, 2000). Submerged vegetation can mitigate low-frequency turbulence while generating high-frequency turbulence (Anderson and Charters, 1982; Ackerman and Okubo, 1993; Koch and Gust, 1993) and can both increase and decrease the total ambient turbulence (Eckman *et al.*, 1989; Gambi *et al.*, 1990; Nepf, 1999). Although vegetation can have complex effects on water mixing, there is general agreement that it slows advection (Fonseca *et al.*, 1982; Gambi *et al.*, 1990; Ackerman and Okubo, 1993; Worcester, 1995; Koch and Gust, 1999; Verduin and Backhaus, 2000; Abdelrhman, 2003; Zong and Nepf, 2010). Reduced advection increases fertilization success in a variety of species (Pennington, 1985; Levitan *et al.*, 1992; Petersen *et al.*, 1992; Levitan, 1995, 2002). Reduced advection increases fertilization by increasing the residence times of gametes near the point of

release within spawning aggregations (Levitan, 2002). Given the effects of vegetation on water flow and water flow on fertilization, here we directly examine how vegetation influences water flow, gamete dispersal, and fertilization.

Models have been made of the contribution of different habitats to the average population-scale zygote production (Meidel and Scheibling, 2001) and to the rate of fertilization as eggs drift in the water column (Lauzon-Guay and Scheibling, 2007). These models hypothesized that fertilization for sea urchins was lowest in habitats where sea urchins were spatially dispersed in kelp beds, highest in grazing fronts (on edge of kelp beds), and intermediate in transitional barrens (no kelp). These models did not, however, account for different flow rates within each habitat or for a possible shift in sea urchin distribution during spawning events and thereby may have underestimated the total zygote production in kelp beds.

In this study we examine patterns of water flow, egg dispersal, and fertilization success in and out of seagrass beds in St. Joseph Bay, Florida. Seagrass beds in St. Joseph Bay offer a mosaic of distinct large and small seagrass beds within open unvegetated sandy areas. Many broadcast-spawning species inhabit seagrass meadows, including echinoderms, molluscs, cnidarians, and ascidians (Munguia, 2007). These species are important economic and ecological resources, yet anthropogenic impacts have contributed to seagrass meadows becoming one of the most threatened marine habitats (Green and Short, 2003; Lotze et al., 2006; Orth et al., 2006). This general worldwide decline is primarily due to estuarine eutrophication and suspended sediment loads caused by human population growth along coastlines (Orth and Moore, 1988; Fonseca et al., 1998). Humans also cause smaller scale disturbances within seagrass meadows from boat propellers, vessel groundings, and human trampling, all of which fragment continuous seagrass beds into seagrass patches (Sargent et al., 1995; Eckrich and Holmquist, 2000; Bell et al., 2001; Uhrin and Holmquist, 2003). In this study we investigated the effect of habitat, specifically seagrass meadows, on fertilization success in the sea urchin Lytechinus variegatus (Lamarck, 1816). Sea urchins have been used extensively in studies of the fertilization success of marine broadcast-spawners (Pennington, 1985; Levitan, 1991, 2002; Levitan et al., 1992; Wahle and Peckham, 1999) because gamete release is easily induced with potassium chloride injections.

Materials and Methods

Dye blob experiment

Seagrass in St. Joseph Bay, Florida, is dominated by *Thalassia testudinum* and is found in distinct patches or islands of various sizes (ranging within our study from 42 to 550 m^2) in an open sandy matrix. To identify whether seagrass mitigates particle diffusion, we calculated rates of fluorescein dye diffusion inside and outside of grass beds by using video recordings of blobs of fluorescein dye in one two-dimensional vertical plane. The pattern of seagrass beds enabled us to pair our dye releases: for any given seagrass bed we recorded the release of a dye blob in the center of the bed (vegetated) and then immediately afterward recorded a release 3 m from the edge of the same grass bed in the sand (unvegetated). We chose seagrass beds that were isolated from other grass beds by at least 10 m. We released dye in six paired locations during 3 sequential days. Diffusion coefficients were calculated by tracing the outline of dye blobs within six frames of video through time and standardized with an in-frame scale. Each tracing was converted to x-y coordinates. Then, using a principal components analysis, we calculated the eigenvalues associated with each tracing for the two major directions, σ_a^2 and σ_b^2, giving us the variance along these two axes. We calculated the diffusion coefficients K_a and K_b (note that units are m^2/s) by calculating linear regressions for plots of σ_a^2 and σ_b^2 versus time and multiplying the slope of the regression by 1/2 (Koehl et al., 1993).

Egg dispersal experiment

When female sea urchins spawn, eggs can sometimes remain on the surface of the female before they are eventually advected away (Minchin, 1992; Levitan, 2002; Himmelman et al., 2008). This temporary retention of eggs on the female can potentially increase fertilization in two ways. First, stationary eggs can sample sperm as they drift past, essentially acting as a sperm filter, unlike eggs floating within a parcel of water (Yund and Meidel, 2003). Second, eggs retained within the area where spawning is occurring are more likely to be exposed to a high sperm concentration, because sperm concentration quickly decreases with distance from the source of sperm release (e.g., Pennington, 1985; Yund, 1990; Levitan, 1991). We estimated the degree to which eggs were advected away from females rather than remaining on the aboral surface of the females when spawning in either sand or seagrass environments.

Eggs were collected with a plankton pump (Styan, 1997; Levitan, 1998b, 2002) placed downstream from the experiments and consisting of a 3-m hose running inline into one of nine filter chambers that catches eggs against 32-μm Nitex mesh. The water (and sperm) exits the filter chamber and then passes through a battery-operated bilge pump that produces the suction. The 3-m hose provides separation between the sampling location and the downstream location of the exiting sperm. After an egg sample has been collected, the first filter chamber is closed and the next one opened for the next replicate or trial.

For each replicate, one sea urchin was induced to spawn via a 1-ml injection of 0.55 mol l^{-1} KCl. If the sea urchin was female and spawned eggs, the released eggs were

washed off the female and the female was immediately placed on the seafloor either in the center of a seagrass bed or 3-m away from the edge of that bed (paired as previously). Each female was tested for both released and retained eggs in a particular habitat (sand or seagrass). Each trial took about 2 min, and in each replicate the order of treatments (retained or released) was alternated. These females spawned for at least 10 min so that conducting the pair of trials sequentially with a single sea urchin was possible, and no difference in the amount of eggs released was detected on the basis of the order of treatments (see Results). To quantify the number of eggs retained on the surface of the female, the sea urchin was allowed to spawn for 2 min and then the hose of the plankton pump was used for 10 s to clean the surface of the female of eggs. To quantify the number of eggs released from the female, the hose from the plankton pump was placed 15 cm downstream of the female and eggs were collected over the full 2-min spawning period. The 2-min period was a trade-off between a reasonable fraction of the full spawning period (around 10 min) and the ability to pair both treatments without a decrease in spawning intensity. Although some eggs are likely to escape the pump using this method, it provides a relative measure of the fraction of eggs retained *versus* released in a particular habitat. A total of eight females were used over four paired seagrass and sand locations.

To quantify the number of eggs from each trial, a subsample of eggs from each filter from both the retained and dispersed treatments was collected and placed in an acrylic plastic block with a 3×3-mm groove holding a 1.4-ml volume. All the eggs in the block were counted, and from this we multiplied the count by 71.43 to calculate the total number of eggs in the 100 ml sample.

Fertilization experiment

We examined fertilization success within paired habitats both inside and outside of 25 seagrass beds, using the same seagrass meadow as in the dye blob experiments and the egg retention experiments. Gamete release was induced with a 1-ml injection of 0.55 mol l^{-1} KCl. Each sea urchin was then placed on its aboral surface on a white plastic board until it began releasing gametes. Sea urchins were induced separately and sorted by sex. Spawning equipment was washed with fresh water after each individual to eliminate pre-experimental fertilization. The current direction was determined by a fluorescein dye release.

Each experimental array consisted of three male sea urchins located 40 cm upstream of three female sea urchins. The spawning males were tethered to the seafloor with a 0.5-cm-wide rubber band attached to a 4-in galvanized nail that was inserted into the substrate. After the experimental sea urchins were all in place and allowed to spawn for 1

min, we collected the eggs as they advected off of the females. The hose of the plankton pump was placed 15 cm downstream of each female, and the eggs were collected for 20 s before the hose was moved to the next female. Eggs from all three females in a trial were pooled into one filter chamber. After egg collection, the hose was placed at least 2 m upstream of the sperm source to pump seawater for 60 s to rinse sperm from the hose and filter. Multiple males were used to decrease variability in the amount of sperm released by the males, and multiple females were used in order to collect enough eggs from the water column. Twenty-five paired (inside and outside of each seagrass bed) replicates using independent sea urchins were conducted.

On shore, eggs were washed out of each filter into plastic vials with fresh seawater and transported to the laboratory. A subsample of 100 eggs were counted with a light microscope 3 h after the fertilization event and categorized as either fertilized or not fertilized. Eggs were considered fertilized if a fertilization envelope or cell cleavage was observed.

Along with data on fertilization, four attributes of each seagrass bed were recorded: (1) area of the seagrass bed, (2) the depth of water at each experimental array, (3) the average lengths of 80 randomly selected seagrass blades (calculated by an average length of all blades from substrate to tip), and (4) the average density of seagrass blades within 20 randomly placed 10×10-cm quadrats.

During our experiment we searched the seagrass meadow for spawning aggregations of urchins in order to describe their spatial distribution during natural spawning events. We were specifically interested in whether sea urchins aggregated within seagrass beds during spawning events.

Statistical analysis

A paired Student's *t*-test was used to test whether diffusion coefficients (separately for K_a and K_b for each location treatment) varied by location treatment. An ANOVA was used to test the number of eggs collected with three main effects: when eggs were collected (during *vs.* after 2 min); habitat (seagrass *vs.* sand); and treatment order (temporal sequence of egg collection trial). Fertilization data were transformed into a proportion of eggs fertilized. A block ANOVA was then used to examine fertilization success in and out of seagrass blocked by seagrass bed. A correlation analysis was used to determine the relationship of fertilization within each location treatment against all data on the environmental characteristics of the site independently.

Results

Dye blob experiment

Fluorescein dye diffused significantly faster in the unvegetated sand habitat than in the grass habitat. Mean diffusion
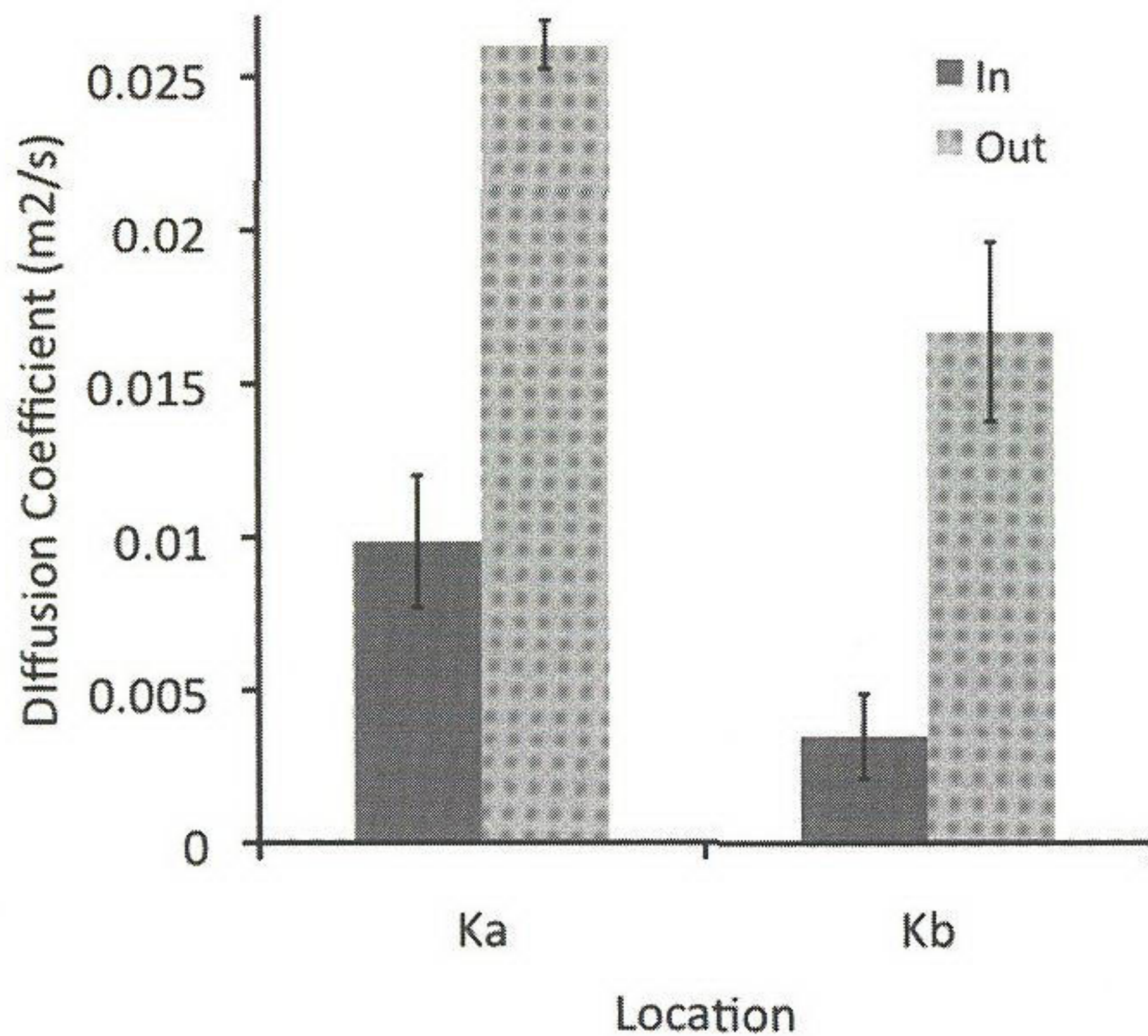
Figure 1. The mean and standard error of the diffusion constants K_a and K_b, for locations, either inside seagrass beds (dark grey) or outside seagrass beds (light grey).

Figure 2. The mean and standard error of the total number of eggs captured on the aboral surface after 2 min of spawning (After Spawning; light grey) or captured in the water column during 2 min of spawning (During Spawning; dark grey) from a single spawning sea urchin, either *inside or outside* seagrass beds.

coefficients for K_a and K_b inside of grass beds were 0.00985 m²/s and 0.00350 m²/s and outside of grass beds were 0.0260 m²/s and 0.0167 m²/s. The expansion coefficients for the two major axes of the dye blob, K_a and K_b, exhibited large differences between the sand and grass habitats ($P = 0.0003$ and $P = 0.008$; Fig. 1). These data suggest that, under flow conditions typical during the spawning season in St. Joseph Bay, seagrasses likely act as a buffer to tidal and wind-driven currents, which influence water flow at these sites and as a result reduce the diffusion of dye particles.

Egg dispersal experiment

Significantly more eggs (3.1-fold) were captured from both the aboral surface and the water column from females spawning within seagrass beds than from females spawning over unvegetated areas (Fig. 2, Table 1). In addition, significantly more eggs (5.5-fold) were collected in the water column while the female was spawning during the 2-min interval than were collected off the aboral surface of the female after the 2-min interval, in both seagrass and sand habitats. The large difference in the number of eggs captured from the water column between the grass and sand locations suggests that in the sand the majority of eggs escaped capture by the pump. Under the assumption that the majority of eggs in the seagrass were captured from the water column, we can use that value as the denominator for how many eggs were released on average by these sea urchins during the 2-min interval. This calculation suggests that 15% of spawned eggs were retained on the aboral surface of females in seagrass habitats and only 7% of eggs
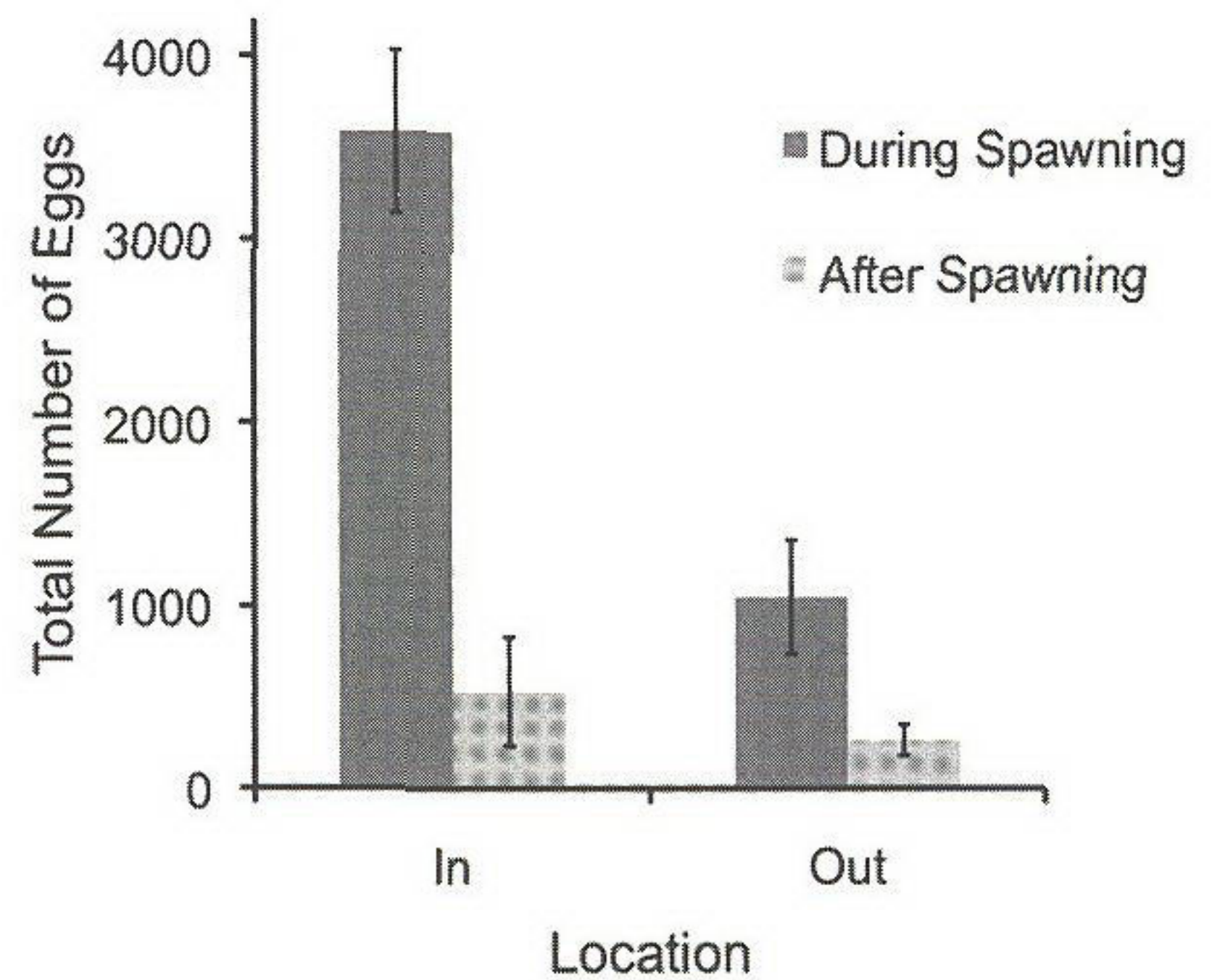
were retained on the aboral surface of females in the sand habitat. This estimate is likely an underestimate as some eggs also likely escaped the pump while it was collecting eggs in the water column in the seagrass habitats. However, regardless of how many eggs were released *versus* recaptured in the water column (the denominator used), the data indicate that twice as many eggs remained on the aboral surface of the sea urchins in the seagrass as in the sand habitats.

Fertilization experiment

A higher proportion of eggs were fertilized within grass habitats than in sand habitats (Fig. 3). The block ANOVA revealed a significant habitat effect ($P = 0.017$) and block

Table 1

Statistical results of an ANOVA testing three factors against average total number of eggs dispersed away from or residing against a female across a 2-min interval: (1) treatment, either eggs dispersed or retained from the female sea urchins aboral surface, (2) location, inside of or outside of seagrass beds, and (3) the order the treatments were conducted with each female, either first or second

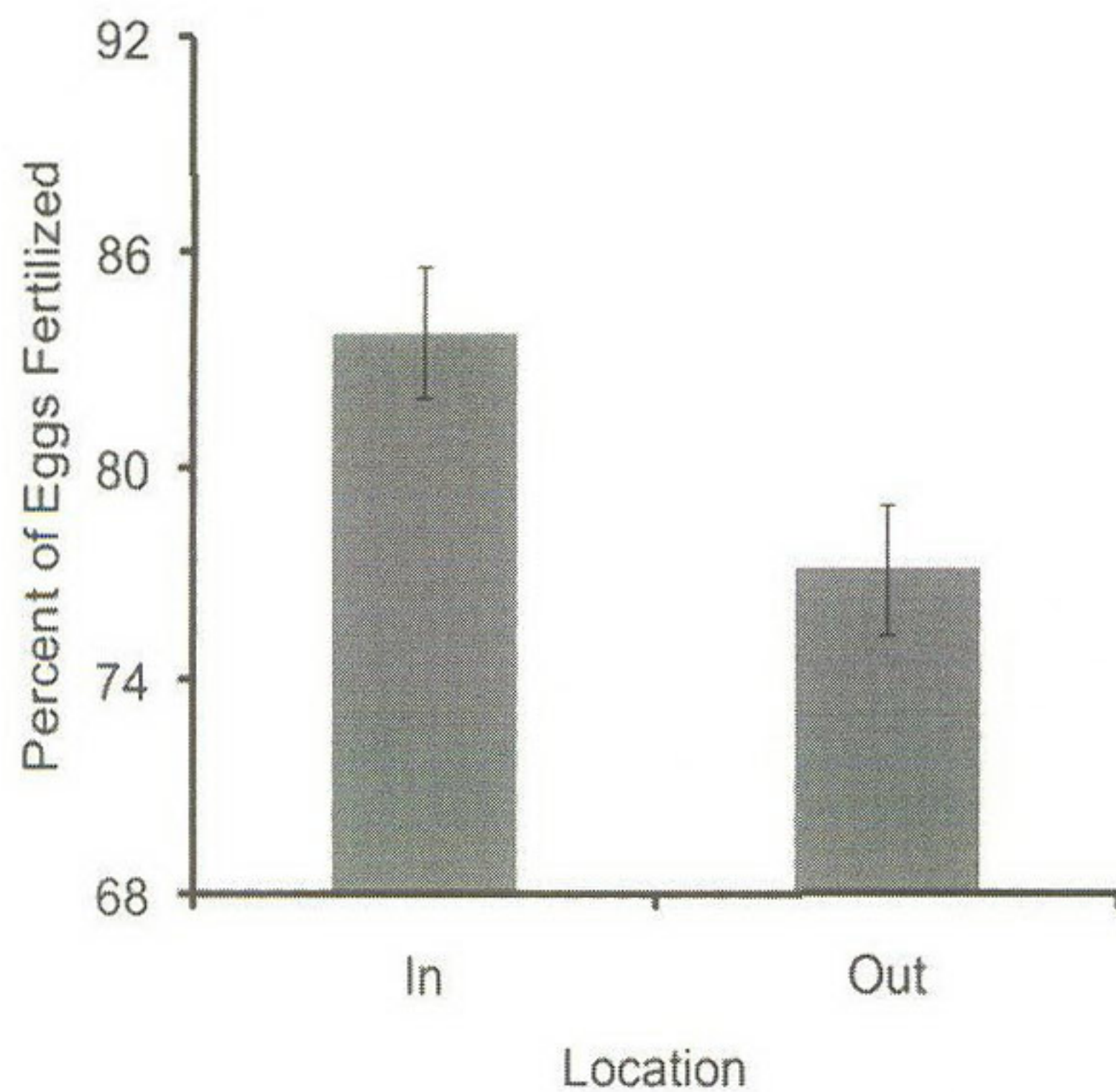| Source	Type III SS	Mean Square	F	P
Treatment	2916.0	2916.0	26.37	0.0003
Location	1560.3	1560.3	14.11	0.0032
Treatment Order	25.0	25.0	0.23	0.6438
Treatment × Location	1024.0	1024.0	9.26	0.0112

Degrees of freedom = 1 for all tests.

Figure 3.   The mean and least square mean estimates of the standard error of the proportion of *Lytechinus variegatus* eggs fertilized inside or outside seagrass beds. Data collected with a plankton pump from free-drifting eggs in the water column. The scale is reduced to highlight differences among treatments.

effect ($P = 0.04$). Fertilization success in seagrass was on average 6.6% higher than in sand habitats.

Fertilization success was not correlated with any of the environmental parameters measured. Seagrass beds varied in size from 42 to 550 m². The water was significantly deeper outside of the grass beds than inside them ($P = 0.002$): depth inside of beds was 0.65–1.5 m, and depth outside was 0.75–1.8 m. Average seagrass blade length in each bed ranged from 0.016 to 0.025 m, and average blade density ranged from 1600 to 3000 blades m⁻².

Observed natural spawning event

A single aggregated spawning event was witnessed by one of us (T.N.S) during our experiment on 13 June 2009 (6 days after full moon). About 250 sea urchins (*L. variegatus*) formed an aggregation approximately 2 m in diameter centered 0.75 m from the edge of a 17 × 6-m seagrass bed. Sea urchins were observed moving from all directions toward the aggregation. Within the aggregation sea urchins were extremely dense and piled two high in some places. No gametes were sampled, but water above the aggregation was cloudy. Many pinfish and other small fish were feeding heavily in the cloudy water above the aggregation. The next day sea urchins at this location were no longer aggregated. On two other occasions, single males were observed (by D.R.L) to spawn in isolation, within seagrass beds (17 September 2009 and 9 September 2010), a day prior and a day after the new moon, respectively.

Discussion

Seagrasses are disappearing across their range, yet we have little knowledge of the effects of seagrass declines on the diverse communities found within them (Huges *et al.,* 2009). In this study, we have shown that seagrasses can affect broadcast-spawning events by facilitating increased fertilization success. It is commonly accepted that the fertilization success of broadcast-spawners depends largely on sperm concentrations and residence times (Pennington, 1985; Babcock *et al.,* 1994; Levitan, 1995). Gamete concentration (Denny, 1988; Denny and Shibata, 1989) and dispersal (Benzie *et al.,* 1994) are in turn affected by hydrodynamic regimes. Worcester (1995) conducted dye diffusion experiments nearly identical to those of this study, reporting decreased advection within eelgrass beds with low shoot density in low currents. Similarly, our dye blob experiment showed that seagrass beds significantly slow the horizontal diffusion of particles in the water column. Levitan and Young (1995) hypothesized that the diffusion of fluorescein dye and sperm may be equal at moderate flow conditions, while Benzie *et al.* (1994) examined sperm dispersal in the asteroid *Acanthaster planci* and reported that sperm diffused as plumes of neutrally buoyant particles. These studies suggest that dye diffusion is a reasonable proxy for sperm diffusion. Although viscosity differences between spawned materials and dye and between the spawned materials of different species may influence diffusion rates (Thomas, 1994), higher rates of dye diffusion should correlate with higher rates of gamete diffusion, such that sperm and eggs should diffuse at higher rates in unvegetated habitats.

For both treatments in our egg dispersal experiment we captured significantly more eggs from females placed inside of seagrass beds than from females placed in unvegetated habitats. Assuming that the sea urchins tested in seagrass and unvegetated habitats spawned the same numbers of eggs, the most parsimonious explanation for this result is that more eggs escaped the plankton pump outside of seagrass beds than inside them. In addition, more eggs were retained on females in the seagrass compared to unvegetated habitats. Both results suggest that flow conditions in seagrass habitats decrease the dispersal of eggs away from females and from the location of spawning, a pattern that is likely to increase the number of encounters with sperm. In accordance with our dye release results, these data suggest that seagrass beds have decreased water movement, which in turn increases the residence times of both sperm and eggs near the point of gamete release.

Our *in situ* fertilization experiment showed that females had a higher fraction of eggs fertilized when spawning in seagrass beds than in unvegetated habitats. This assay integrates the fertilization success that accumulated while the eggs remained on the aboral surface and then as they were

advected away into the water column, before collection. Although some fertilization might have occurred had the eggs been allowed to drift for longer periods, eggs adrift in the water column are exposed to additional dispersal and move into ever-decreasing concentrations of sperm.

The fertilization assays coupled with the dye diffusion study and egg dispersal experiment suggest that reduced water movement in vegetated areas increases the collision frequency between sperm and eggs. This is likely accomplished by decreasing the rates of gamete diffusion; as eggs and sperm are spawned in seagrass beds they have time to accumulate within the spawning aggregation where gamete concentrations are the highest, before diffusing away and becoming too dilute to have a high probability of fertilization.

Our results show that vegetation affects particle diffusion rates as well as sea urchin fertilization success. Models that calculate the contribution of different habitats to sea urchin zygote production (Meidel and Scheibling, 2001; Lauzon-Guay and Scheibling, 2007) should thus consider how the characteristics of the habitat influence water flow and particle diffusion, and therefore fertilization rates. The fertilization rates of sea urchins within vegetated habitats would likely increase for models that include the effect of vegetation on reducing current velocity and particle diffusion. Kelp has been shown to affect current flow over rocky substratum (Eckman et al., 1989) and increase the fertilization success of eggs retained within them (Wahle and Peckham, 1999).

The aforementioned models also assume that the spatial distribution of sea urchins during spawning events is equivalent to their non-spawning distributions. The fact that one of us observed urchins converging on a spawning aggregation in a grass bed from both unvegetated and grassy habitats but found the aggregation to have dissipated by the next day suggests that this assumption is false. Thus the models may underestimate densities within benthic structures such as seagrasses, kelp beds, or reef structures during spawning events. To more accurately predict the contribution of different habitats to sea urchin zygote production, models must account not only for the effect of benthic structure (kelp beds) on current velocity and diffusion coefficients, but also for shifts in the spatial distribution of sea urchins during spawning events.

What we do not address in this study is the likelihood of predation on gametes and zygotes in and out of vegetated areas. Vegetation can provide refuges for predators (Stoner, 1979; Coen et al., 1981; but see Mattila et al., 2008) and fertilization gains associated with reduced flow in vegetation might be offset by increased predation in these habitats. This potential trade-off would be a fruitful area of research.

Summary

We hypothesized that seagrass beds modify water flow, thereby increasing the fertilization success of the sea urchin *Lytechinus variegatus*. Our results confirmed this hypothesis, suggesting that seagrasses can mitigate gamete diffusion during broadcast-spawning events, thus possibly increasing the fitness of those that spawn within them. Our results are supported both by our fluorescein dye release trials, showing that particles diffuse significantly more rapidly outside as opposed to inside seagrass beds, and by our egg dispersal experiment, showing that egg dispersal was mitigated by seagrass. The differences in fertilization success between locations were not large (6.6%), yet the flow conditions of St. Joseph Bay are minimal and mainly wind-driven. We have shown that benthic structure can affect fertilization success even in minimal flow conditions, and we predict that these effects are likely to be more pronounced in high-energy wave-swept shores and should be further explored.

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