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THE RELATIONSHIP BETWEEN CONSPECIFIC FERTILIZATION SUCCESS AND REPRODUCTIVE ISOLATION AMONG THREE CONGENERIC SEA URCHINS

DON R. LEVITAN
Department of Biological Science, Florida State University, Tallahassee, Florida 32306-1100
E-mail: levitan@bio.fsu.edu

Abstract.—Few data are available on the effectiveness of reproductive isolating mechanisms in externally fertilizing taxa. I investigated patterns of conspecific and heterospecific fertilization among three coexisting sea urchin species, *Strongylocentrotus droebachiensis, S. franciscanus,* and *S. purpuratus*. In the laboratory, both among and within species, eggs from individual females whose eggs are more easily fertilized by conspecific sperm are also most susceptible to heterospecific fertilization. At one extreme, *S. droebachiensis* requires an order of magnitude fewer conspecific sperm to fertilize eggs than do the other two species and shows very little distinction between conspecific and heterospecific sperm in no choice experiments. *Strongylocentrotus franciscanus* has an intermediate susceptibility to fertilization by heterospecific sperm. At the other extreme, *S. purpuratus* rarely cross-fertilizes. Field observations indicate that *S. droebachiensis* is often surrounded by heterospecific sea urchins. Genetic analysis of larvae produced during heterospecific spawning events indicate that hybrids are generally produced if male conspecifics are more than 1 m from a spawning female *S. droebachiensis.* Laboratory cultures indicate that these hybrids suffer high mortality relative to conspecific larvae. Comparisons of reproductive success of *S. droebachiensis* during single-species and multispecies spawning events indicate that the benefits of producing easily fertilized eggs under conditions of sperm limitation may outweigh the costs of losing some offspring to hybrid fertilization. Patterns of variability in heterospecific fertilization are considered in light of three hypotheses: phylogenetic relatedness, reinforcement selection, and sexual selection.

Key words.—Echinoid, hybridization, reinforcement selection, reproductive isolation, sexual selection, sperm competition, sperm limitation.

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The reproductive isolation that leads to speciation is thought to evolve by chance in allopatry (Mayr 1963; Templeton 1981; Coyne and Orr 1989), through natural selection against hybridization in sympatry (Dobzhansky 1940; Liou and Price 1994; Noor 1999), or as a consequence of sexual selection driving mate choice within species (Fisher 1930; West-Eberhard 1983; Andersson 1994). Untangling these alternate hypotheses has proved to be difficult in taxa with internal fertilization, where adults, matings, and offspring can be closely monitored (Endler 1989; Noor 1999). In the large number of taxa with external fertilization, many of which release gametes during multispecies spawning events, these problems are greatly magnified (Harrison et al. 1984; Babcock et al. 1986; Pearse et al. 1988; Palumbi 1994; Knowlton et al. 1997). For example, on the Great Barrier Reef, Australia, more than 100 species release gametes during mass spawning events (Harrison et al. 1984; Babcock et al. 1986). How reproductive isolation is maintained, let alone initiated, during these kinds of multispecies spawning events is poorly understood.

For coexisting species that spawn simultaneously, gametic, rather than adult, interactions determine reproductive isolation. Variability among species in gamete compatibility may be caused by either genetic divergence independent of compatibility or by reinforcement selection against hybridization, but choosing between these alternatives has been difficult (Lessios and Cunningham 1990; Byrne and Anderson 1994). Variability of gamete-recognition proteins within species has at times been attributed to sperm competition and female choice (Metz and Palumbi 1996; Vacquier et al. 1997; Palumbi 1999), but patterns of variability have not been related to any relevant ecological parameter such as sperm availability.

At present a reasonable amount of information is available on heterospecific gamete compatibility (Hagström and Lönning 1967; R. Strathmann 1981; M. Strathmann 1987; Lessios and Cunningham 1990; Palumbi and Metz 1991; Knowlton et al. 1997), but none addresses the effectiveness of prezygotic barriers to external fertilization under natural conditions. As a result, discussions of the relative importance of alternate hypotheses on the evolution of reproductive isolation in these marine systems have been conducted without any information on natural levels of reproductive success (e.g., Lessios and Cunningham 1990; Palumbi and Metz 1991). Such information is critical for two reasons. First, the costs and benefits of complete or incomplete reproductive isolation cannot be quantified without measurements of the actual or potential for hybrid formation. Second, the suggestion that reproductive isolating mechanisms are enhanced by sperm competition and female choice hinges on the degree of sperm availability in nature. The emerging evidence from externally fertilizing species is that sperm are sometimes, but not always, limiting (Levitan 1998b, 2002; Yund 2000). In some cases gametic interactions take place where eggs are rarely fertilized, and in others high levels of female fertilization success and sperm competition may characterize gamete interactions. This continuum from sperm limitation to sperm competition may result in differing selective pressures on egg and spermatozoon traits (Levitan 1993, 1998a,b, 2002), which may have consequences for heterospecific fertilization and reproductive isolation.

Here I report on laboratory and field experiments that investigated patterns of gamete compatibility, reproductive success, and hybrid viability among three coexisting congeneric sea urchins. *Strongylocentrotus purpuratus, S. franciscanus,* and *S. droebachiensis* coexist along the northwest-

ern coast of North America. The first two coexist throughout their ranges from Alaska to the Baja California peninsula, whereas *S. droebachiensis* is circumpolar and only overlaps southward through the coast of Washington state. All three species spawn in the late winter and spring (Levitan 1998a) and have been observed to spawn simultaneously within the same aggregation (Levitan 2002).

My study demonstrated that these species, and females within each species, differ greatly in the susceptibility of eggs to fertilization by heterospecific sperm and that this variation is correlated with variation in conspecific fertilization rates. One species often produces hybrid larvae during multispecies spawning events. Survivorship of these larvae is much lower than that of conspecific offspring. The outcomes of these experiments are used to compare three alternative explanations for this variability in gametic compatibility: genetic distance, reinforcement selection, and a novel hypothesis that heterospecific fertilization is a by-product of selection on conspecific fertilization success.

Materials and Methods

Laboratory Crosses

Intra- and interspecific fertilization assays were conducted for the congeners *Strongylocentrotus purpuratus*, *S. franciscanus*, and *S. droebachiensis* during the spring of 1997. The spring is the peak spawning time for these species (Strathmann 1987). Sea urchins were collected from the Deer Island Group in Barkley Sound, British Columbia, Canada. On each experimental day, sea urchins from all three species were injected with 0.55 M KCl, which induced spawning. Sperm were collected off the aboral surface with a pipette and kept undiluted and on ice. Sperm of each species were diluted to form series of seven 10-fold dilutions. Separately for each species, eggs were collected from females that were inverted and placed in glass bowls filled with filtered seawater. Eggs were diluted to a stock concentration of 4000–6000 eggs/ml. One-milliliter aliquots of the egg suspensions were added to 63 scintillation vials (three egg-donor species × three sperm-donor species × seven sperm concentrations), each containing 8 ml of filtered seawater. A 1-ml aliquot of each sperm dilution (seven dilutions × three species) was added to each of three egg vials for each of the three species, bringing the final sperm dilution to 10^{-3} through 10^{-9} and all egg concentrations to 400–600 eggs/ml. Experimental vials were swirled for three rotations and then left in a shallow flowing-seawater table at the ambient seawater temperature of 12°C for 3 h. Because diluted sea urchin sperm can age rapidly (Levitan 1993), the interval between sperm dilution and addition was kept below 2 min. In these species and with these laboratory protocols, most fertilizations occur within the first few minutes of sperm addition. After 3 h, 100 eggs from each experimental vial were inspected for the presence of a fertilization membrane or further developmental stage. Sperm concentration was determined from eight replicate sperm counts with a hemocytometer at the 10^{-3} sperm dilution. Egg concentration was determined from three replicate egg counts of 0.1 ml from the stock egg suspension. On each experimental day, only one male and one female from each species were used, and the entire experiment was replicated on 12 different days.

An additional set of 17 conspecific and heterospecific laboratory crosses was conducted just for *S. droebachiensis* and *S. purpuratus*. These crosses were used to increase sample size for investigation of how variation in fertilization success within species was correlated with variation in heterospecific crosses. These assays were conducted in the same manner as described above.

Data from these fertilization assays were used in a fertilization-kinetics model to estimate the concentration of sperm needed to fertilize 50% of a female's eggs (F_{50}). The model (Vogel et al. 1982) predicts the proportion of eggs fertilized as a function of the concentration of eggs (E_0, eggs/μl) and sperm (S_0, sperm/μl), the sperm-egg contact time (t, sec), and two rate constants: the rate of sperm-egg collision (β_0, a product of the egg cross-sectional area in mm^2 and the sperm velocity in mm/sec) and the rate constant of fertilization (β, mm^3/sec) as follows:

$$\phi = 1 - \exp(-\beta S_0/\beta_0 E_0(1 - \exp(-\beta_0 E_0 t))) \qquad (1)$$

The values for β_0 and β were estimated by iteration according to the Marquart method of nonlinear regression (SAS, SAS Institute, Cary, NC), and then (1) was solved for S_0 with a φ of 0.5 (see Levitan et al. 1991; Levitan 1993) as the parameter F_{50}. I solved for the F_{50} values independently for each fertilization trial and log transformed for statistical analysis. A benefit of using the fertilization-kinetics model to solve for the F_{50}-value, rather than simply fitting a regression equation, is that variation in egg concentration can be accommodated. The result is a more precise estimate of the amount of sperm needed to fertilize a given concentration of eggs.

Larval Cultures

Larval cultures were established with eggs from *S. droebachiensis* and sperm from *S. droebachiensis*, *S. franciscanus*, and *S. purpuratus*. The eggs from six female urchins of each species were crossed with the sperm from one male of each species (18 crosses total). Cultures were established with 1 ml of concentrated eggs and one drop of dry sperm in 400 ml of filtered seawater. After 10 min the cultures were drained and rinsed twice with filtered seawater to remove excess sperm. Culture jars were placed in a flowing-seawater table at a 12°C. Water was changed twice daily in cultures for the first 3 days, and then the cultures were independently placed in 4-L glass jars, stirred continuously with a rack system of swinging paddles (Strathmann 1987). Water was changed and larvae were fed *Chronomonas salina* and *Isochrysis galbana* algae twice weekly. One month after initiation of the cultures, a plastic mesh screen that had acquired a natural biofilm was placed in each culture jar as a substrate for settlement.

After settlement, juveniles were transferred to plastic tubs with Nitex (Sefar Canada Inc., Scarborough Ontario) screen openings that allowed water flow and were maintained in a flowing-seawater table. The juveniles were initially fed the green alga *Ulva*, but as they grew the diet was gradually changed to the kelp *Macrocystis integrifolia*. When the sea urchins reached 1 year of age, they were moved to larger

containers with larger plastic mesh screening, which increased water flow. The sea urchins from each of the 18 crosses were maintained together in a separate container for a total of 18 independent containers.

Field Survey of Sea Urchins Surrounding Strongylocentrotus droebachiensis

I measured local densities of sea urchin species surrounding *S. droebachiensis* to estimate the likely conspecific and heterospecific spawning partners during spawning events. Every time an individual *S. droebachiensis* was located during four dives in Barkley Sound a 1-m^2 quadrat was centered on it. The numbers of sea urchins of all three species within the quadrat were recorded.

Field Experiment

In March 1998, I conducted a field experiment to determine the likelihood of hybridization in multispecies spawning events in the Deer Island Group in Barkley Sound, British Columbia (see map of site in Levitan 1998a). A 5 × 5 m square quadrat was haphazardly placed on the bottom in areas inhabited by both *S. franciscanus* and *S. droebachiensis*. Every urchin within the quadrat was picked up, injected with 0.55 M KCl, and replaced in its exact original location. Thirty minutes after the injections, the positions of all males and females of both species were mapped. Samples of eggs were collected in the water column directly above each spawning female with a subtidal pump connected to one of 12 filter chambers that retained eggs on a 30-μm mesh filter. After 5–10 sec of egg collection, I removed residual sperm by placing the intake hose at least 5 m from any spawning male and rinsing the sample for 1 min. After sample collection the pump was switched to the next filter and eggs from the next female were collected (for additional details see Levitan 1998a, 2002). Eggs from each female were incubated in the laboratory at ambient seawater temperature (12°C) for 3 h, and then at least 200 eggs were scored for the presence of a raised fertilization envelope or further developmental stage. Larvae were cultured in filtered seawater at 12°C for 3 days without food. Individual larvae were pipetted into the wells of depression slides, and the seawater was removed first with a Pasteur pipette and then by absorption with the edge of a Kimwipe tissue (Kimberg-Clarke, Roswell, GA). Each well was then filled with sterile, double-distilled H_20, and each larva, together with water totaling 1 μl, was pipetted into a 0.5-ml microcentrifuge tube, frozen, and stored at −80°C for later genetic analysis. This experiment was replicated four times.

Data from this experiment were compared to those from an identical field experiment, without the genetic component, that involved single-species spawning events (Levitan 2002). In this earlier experiment, all three species were independently (on different days and at different sites) induced to spawn at their natural densities. Additional trials were conducted with *S. purpuratus*, the species with the highest natural densities, at densities experimentally reduced to those observed for *S. droebachiensis* (Levitan 2002). The locations of these 64 experimental spawning events were spread haphazardly throughout the Deer Island Group and the southwestern corner of Barkley Sound. The sites were in rocky shallow subtidal environments (2–10 m) with varying degrees of macroalgal cover (e.g., *Macrocystis*, *Nereocystis*, *Pterygophora*, *Alaria*) and wave exposure (average surge velocity 2–25 cm/sec; for details see Levitan 2002).

Genetic Protocols

DNA extractions

Adults were dissected, and a sample of gonad tissue was preserved in 95% ethanol and stored at −20°C. Gonadal tissue (~250 μl) was macerated with a scalpel and placed in a 1.5-ml microcentrifuge tube. I added 500 μl of CTAB extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris [pH 8.0]), 5 μl of proteinase K (25-mg/ml stock solution) and incubated the samples at 65°C for 90 min. Samples were extracted twice with 500 μl of a 25:24:1 solution of phenol:chloriform:isoamyl alcohol and once with a 24:1 solution of chloriform:isoamyl alcohol. For each extraction step, reagents were added, and the sample was mixed thoroughly and spun for 18 min at 8000 rpm. The supernatant was then transferred for the next extraction. The DNA was precipitated with 1 ml of 95% ethanol, placed at −20°C for 20 min, and centrifuged for 20 min at 8000 rpm, and then the DNA pellet was rinsed twice in 70% ethanol. The sample was air dried and then resuspended in 50 μl solution of 10 mM Tris and 1 nM EDTA. The sample DNA was measured, and the final DNA concentration was adjusted to 5 ng/μl (Levitan and Grosberg 1993).

Larvae were extracted with a modified procedure. Individual larvae were extracted by addition of 6 μl of double-distilled H_20 and 8 μl of proteinase K (25 mg/ml) to the microcentrifuge tube containing the larva. A drop of mineral oil was added, and the sample was placed in a thermocycler (MJ 100; MJ Research Inc., Waltham, MA) for one cycle of 70°C for 20 min, 95°C for 5 min. It was then stored at 4°C. DNA was not measured and was used undiluted in the polymerase chain reaction (PCR; modified from S. Palumbi, pers. comm.).

Polymerase chain reactions and electrophoresis

Reaction mixtures contained 0.2 μl of a 5 unit/μl solution of Amplitaq (Applied Biosystems Foster city, CA) DNA polymerase; 0.625 μl each of 1 mM dATP, dCTP, dGTP, and dTTp; 1.25 μl of 10 mM $MgCl_2$, 2.5 μl of reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, and 15 mM $MgCl_2$); and sufficient sterile double-distilled H_20 to yield a total volume of 21 μl. To this mixture were added 3 μl of a RAPD primer (Operon Technologies, Alameda, CA) and 1 μl of the sample DNA, for a total volume of 25 μl. A drop of mineral oil was added before the tube was placed in the thermocycler. The thermocycler (MJ 100) profile started with one cycle of 2.5 min at 94°C, 1 min at 35°C, and 2 min at 72°C, followed by 44 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C. After all cycles were complete, the thermocycler remained at 4°C.

A 4-μl volume of Type 2 loading buffer (Maniatis et al. 1982) was added to the sample, and 10 μl was loaded onto

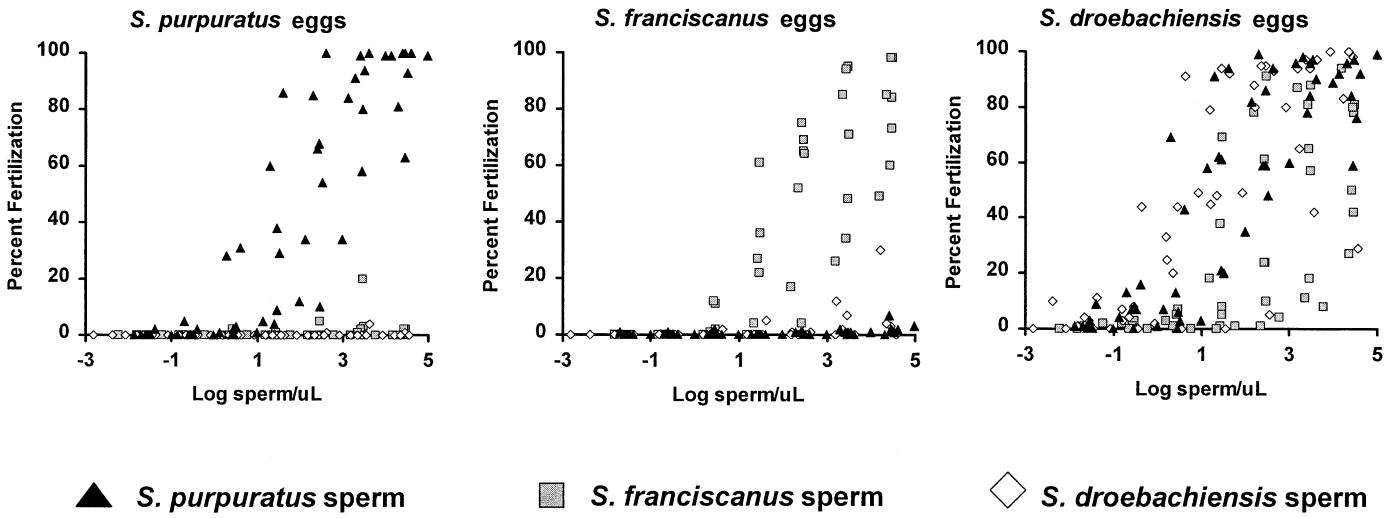

FIG. 1. Percentage of eggs fertilized, in the laboratory, as a function of sperm concentration of three congeners. Each panel represents the eggs from one species. Twelve replicate male-female pairs were used in each comparison (seven sperm dilutions within each replicate). Triangles, squares, and diamonds represent the sperm from *Strongylocentrotus purpuratus*, *S. franciscanus*, and *S. droebachiensis*, respectively.

a gel consisting of 0.6% Ultra-Pure agarose (Bio-Rad Laboratories, Hercules, CA) and 1.0% Synergel (Diversified Biotech, Boston, MA) in 0.5 TBE buffer (pH 8.0). Two DNA ladders were also loaded (123 bp and 1 kb), and the gel, immersed in 0.5 TBE buffer, was run at 100 V for 5 h. The gel was stained for 15 min in a bath of 2.5 mg/500 ml ethidium bromide, destained in water for 1 h, and photographed under UV light (Levitan and Grosberg 1993).

Primer selection

Initially six individuals each of *S. droebachiensis* and *S. franciscanus* were screened with 22 RAPD markers. The screening effort was concentrated on distinguishing between *S. droebachiensis* and *S. franciscanus* because under natural conditions *S. droebachiensis* eggs are most likely to be hybridized by *S. franciscanus* sperm, given the distribution of the three species (see Results section, Field Distribution of *Strongylocentrotus droebachiensis* in Relation to Other Species). Primer selection was based on two criteria: (1) that they revealed bands present in all individuals of one species but absent in the other species; and (2) that the bands were easy to score. These select primers were tested on 24 additional individuals from each species collected from three sites in Barkley Sound. These markers were also examined in larvae from conspecific and heterospecific crosses of these two species.

TABLE 1. Two-way analysis of variance of log F_{50}-values of heterospecific laboratory fertilization. The F_{50}-value is the concentration of sperm (per μl) needed to fertilize 50% of eggs at a concentration of 0.5/μl. The two factors were the species of the sperm donor and the species of the egg donor. Only heterospecific crosses were analyzed. Student-Neuman-Keuls pairwise comparisons indicate significant differences among all three egg donor species (mean log F_{50} = 7.3, 6.4, and 2.5 for *Strongylocentrotus purpuratus, S. franciscanus,* and *S. droebachiensis,* respectively).

Source	df	Type III SS	Mean square	*F*	*P*
Female	2	194.78	97.39	83.85	<0.0001
Male	2	10.91	5.46	4.70	0.0131
Female × male	1	3.38	3.38	2.91	0.0939
Error	55	63.88	1.16		
Total	60	345.74			

RESULTS

Overall, *S. purpuratus* produced eggs that were almost exclusively incompatible with heterospecific sperm, *S. franciscanus* eggs were largely incompatible with heterospecific sperm, and *S. droebachiensis* eggs were easily fertilized by sperm from all three species (Fig. 1). Fitting the results of these laboratory assays to a fertilization-kinetics model (eq. 1) provided an estimate of the concentration of sperm (number/μL) needed to fertilize 50% of eggs. On average, *S. purpuratus* eggs required approximately 2.0×10^7 heterospecific sperm per microliter to fertilize 50% of eggs, *S. franciscanus* eggs required 2.5×10^6, and *S. droebachiensis* eggs only 3.2×10^2 (Table 1). In fact, almost an order of magnitude fewer *S. purpuratus* sperm were needed to fertilize 50% of *S. droebachiensis* eggs than to fertilize eggs from its own species. The ease of heterospecific fertilization of *S. droebachiensis* eggs corresponded to the relative ease of conspecific fertilization in this species. *Strongylocentrotus droebachiensis* required an order of magnitude fewer conspecific sperm for 50% fertilization (10^1) than do the other two species (10^2). An ANOVA comparing conspecific crosses indicated a significant species effect (df species = 2, df error = 30, P = 0.0136); *S. droebachiensis* was significantly easier to fertilize than were the other two species (Duncan multiple comparison $P < 0.05$). In comparisons of heterospecific crosses, a two-way ANOVA with the species of the sperm and egg donor as main effects indicated significant main effects and no significant interaction (Table 1). Student-Neuman-Keuls multiple comparisons indicated significant differences among all

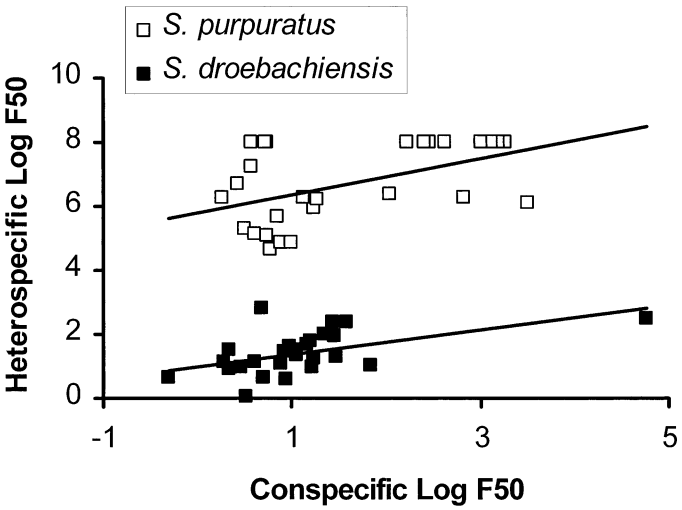

FIG. 2. The log amount of sperm needed to fertilize 50% of a female's eggs with heterospecific sperm as a function of the log amount of sperm needed to fertilize that female's eggs with conspecific sperm. Analysis of covariance indicates a significant main effect (species of egg donor, $P < 0.0001$) and covariate (log F_{50} with conspecific sperm, slope = 0.489, $P < 0.001$, R^2 of model = 0.91). Of 53 independent trials, four resulted in a maximum fertilization success of eggs less than 95% with conspecific sperm. When these trials were removed from the analysis, the results were similar ($P > 0.0001$ and 0.001 for the main effect and covariate; slope = 0.537; model $R^2 = 0.91$).

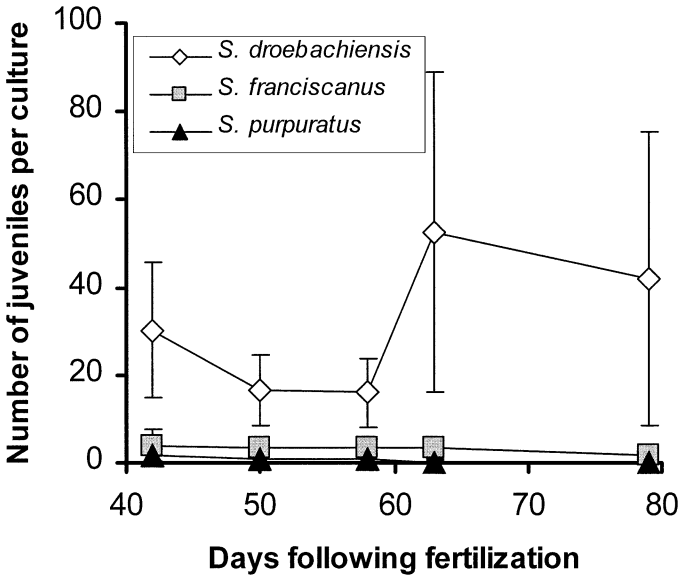

FIG. 3. Average number of juveniles produced from conspecific and heterospecific crosses plotted as a function of time from fertilization. Six independent cultures were established for each cross. All crosses used *Strongylocentrotus droebachiensis* eggs; symbols represent the species of the sperm donor. Bars represent standard errors.

pairwise comparisons of egg donors; ease of heterospecific fertilization was greatest for *S. droebachiensis* eggs, followed by *S. franciscanus* and *S. purpuratus* eggs (Table 1).

Comparisons within species indicated that, for *S. droebachiensis* and *S. purpuratus*, the two species at the extremes of the ability to cross-fertilize, females that produce eggs that are more easily fertilized with conspecific sperm were also more easily crossed with heterospecific sperm (Fig. 2). An ANCOVA indicated a significant main effect (species of egg donor, $P < 0.0001$) and covariate (log F_{50} using conspecific sperm, slope = 0.489, $P < 0.001$, R^2 of model = 0.91). In these fertilization trials, 49 of 53 independent trials resulted in greater than 95% of eggs fertilized with conspecific sperm at the higher sperm concentrations. When the four trials with lower levels of conspecific fertilization were removed from the analysis, the results were similar ($P > 0.0001$ and 0.001 for the main effect and covariate, respectively, with a slope of 0.537 and a model R^2 of 0.91). This result indicates that this variability in the ease of fertilization was not a result of using eggs that were not able to be fertilized. Both within and among these species, ease of conspecific fertilization was related to ease of heterospecific fertilization.

Larval Cultures

The fertilization success of the *S. droebachiensis* eggs in the cultures averaged 95%, 79%, and 85% for crosses with *S. droebachiensis*, *S. franciscanus*, and *S. purpuratus* sperm. All cultures produced abundant swimming pluteus larvae. Settlement was noted in all three treatments on day 42 and continued until day 63, at which point no swimming larvae were observed remaining in the cultures and no additional settlement was noted. At day 79, a Savage two-sample test (nonparametric) indicated a significantly greater abundance of conspecific juveniles ($n = 6$ cultures) than of heterospecific juveniles ($n = 12$ cultures, $P < 0.05$). More than an order of magnitude more juveniles were produced in conspecific than in heterospecific crosses, and no hybrid individuals produced with *S. purpuratus* sperm survived to day 79 (Fig. 3). Three years later, individuals from hybrid crosses involving *S. franciscanus* sperm and *S. droebachiensis* eggs, as well as pure *S. droebachiensis* crosses, still survived. Hybrid individuals produced with sperm from *S. franciscanus*, the species with the largest adult body size, were significantly larger than pure *S. droebachiensis* individuals (mean test diameter 29 mm and 21 mm; $n = 70$ individuals; ANOVA, $P < 0.05$).

Field Distribution of Strongylocentrotus droebachiensis in Relation to the Other Species

The distribution of *S. droebachiensis* in Barkley Sound is such that individuals are more likely to be surrounded by sea urchins of other species than by conspecifics. During four dives, 69 *S. droebachiensis* individuals were located in Barkley Sound. On average 20-fold more *S. franciscanus* and twofold more *S. purpuratus* individuals than additional *S. droebachiensis* individuals (Fig. 4) were present within the 1-m^2 quadrat. The sites were shallow (2–10 m), rocky subtidal habitats typical of Barkley Sound, dominated by crustose coralline algae with a fringe of macroalgae near the intertidal boundary.

Identification of Genetic Markers for the Field Experiment

Six individuals each of *S. droebachiensis* and *S. franciscanus* were screened with 22 RAPD markers. Four primers

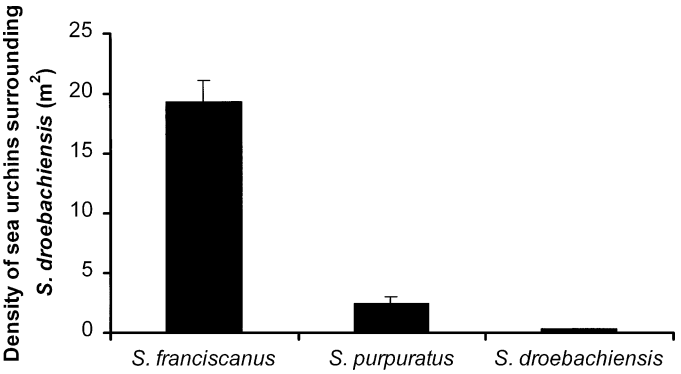

FIG. 4. Average number of sea urchins in 1-m^2 quadrats surrounding a focal *Strongylocentrotus droebachiensis* individual. Numbers for *S. droebachiensis* do not include the focal individual. Data collected from 69 quadrats.

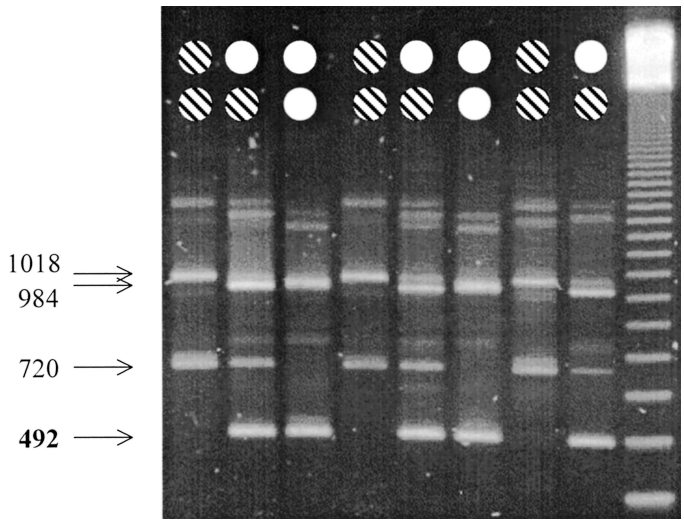

FIG. 5. Electrophoretic gel of RAPD PCR primer OP-AK-18 applied to three-day-old larvae from conspecific and heterospecific crosses. Hatched symbols are *Strongylocentrotus franciscanus* parents; open symbols are *S. droebachiensis* parents. Lanes with both symbols are larvae produced from a *S. franciscanus* male and a *S. droebachiensis* female. In these particular crosses, bands 1018 and 720 are diagnostic of *S. franciscanus*, and bands 984 and 492 are diagnostic of *S. droebachiensis*. Note that the hybrids show all four bands. In field-collected samples, bands 1018 and 984 were not scored because they were too similar in size, and band 720 was not completely diagnostic across all adults. Only band 492 (highlighted in bold) was used for field identification of larvae.

revealed six easily scored loci that were present in all individuals of one species but absent in the other species. These selected primers were tested on 24 additional individuals from each species collected from three sites in Barkley Sound. All six loci were found only in one species and ranged in frequency from 0.67 to 1.00 (Table 2). Two of these loci were monomorphic in one species, one locus for *S. franciscanus* and one locus for *S. droebachiensis*. All six markers were heritable as demonstrated in larvae from conspecific and heterospecific crosses of these two species (Fig. 5).

Field Experiment on Fertilization in the Presence and Absence of Heterospecific Spawning

Field experiments tested conspecific and heterospecific fertilization under natural demographic conditions. Hybrid status of offspring was determined by means of PCR RAPD genetic markers. On average, 30 larvae were scored for each spawning female (range = 18–42 larvae). Overall, the likelihood of hybridization increased with the distance to conspecific males (Fig. 6A) and decreased with distance to heterospecific males (Fig. 6B). Eggs from *S. droebachiensis* were almost exclusively fertilized by *S. franciscanus* sperm, unless a male *S. droebachiensis* was within 1 m of the female.

Comparisons between spawning events that included both *S. franciscanus* and *S. droebachiensis* and spawning events in which species spawned in isolation provide a measure of

TABLE 2. RAPD PCR loci used in parentage analysis of hybrid status. Primers were obtained from Operon Technologies (Alameda, CA). Diagnostic primers (and their sequences) were OP-AK-18 (5′-ACCCGGAAAC-3′), OP-AK-19 (5′-TCGCAGCGAG-3′), and OP-AK-20 (5′-TGATGGCGTC-3′). Primer identification, band size (kb), and band frequencies in each species are listed. Band frequencies are based on 30 individuals per species.

Primer	Band size	*Strongylocentrotus franciscanus*	*S. droebachiensis*
OP-AK-18	506	0	0.89
OP-AK-19	492	0	1.00
OP-AK-20	738	0	0.69
OP-AK-20	511	0	0.95
OP-AK-20	360	0.78	0
OP-AK-20	246	1.00	0

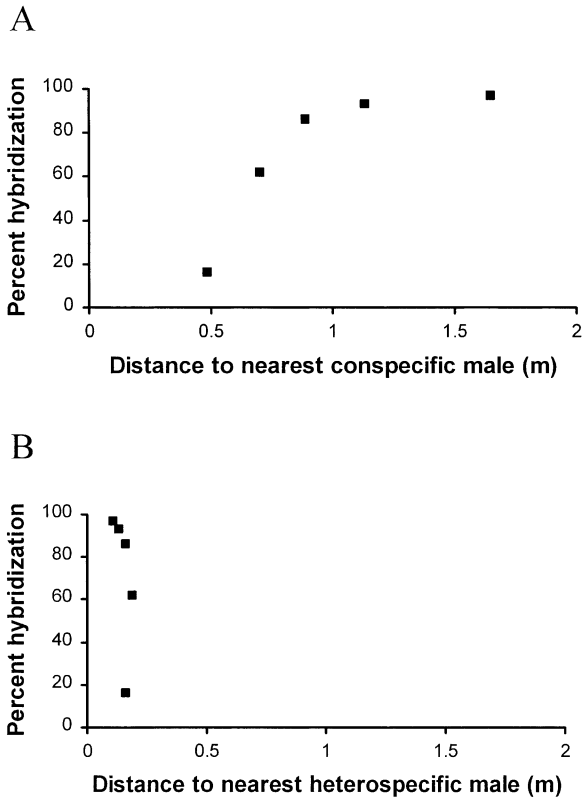

FIG. 6. Percentage of fertilized *Strongylocentrotus droebachiensis* eggs fertilized by *S. franciscanus* sperm as a function of the nearest (A) *S. droebachiensis* male and (B) *S. franciscanus* male.

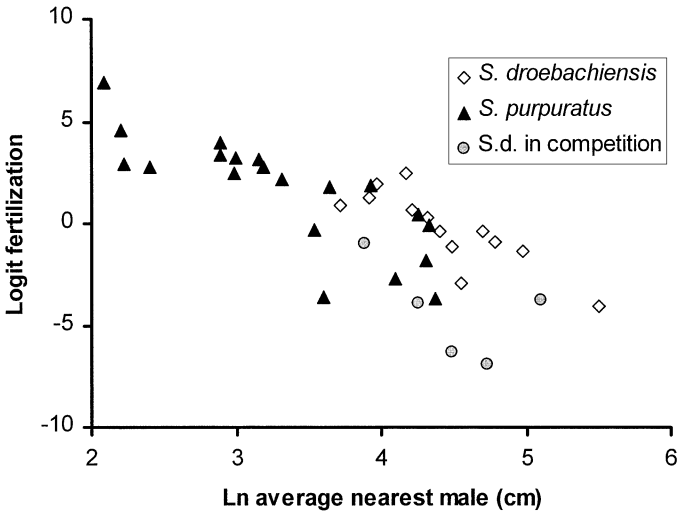

FIG. 7. Percentage of eggs fertilized by conspecific sperm as a function of the distance to the nearest conspecific male. Three experiments are compared: (1) *Strongylocentrotus droebachiensis* eggs fertilized when only *S. droebachiensis* individuals were spawning (open diamonds); (2) *S. droebachiensis* eggs fertilized by *S. droebachiensis* sperm when both *S. droebachiensis* and *S. franciscanus* were spawning (gray circles); (3) *S. purpuratus* eggs fertilized when only *S. purpuratus* individuals were spawning (solid triangles). The latter two treatments are significantly different from the first by ANCOVA (Table 3).

TABLE 3. Analysis of covariance for differences in female fertilization success as a function of the nearest conspecific male. Fertilization data are logit transformed; nearest-male distance is ln transformed. The three treatments of the main effect are: (1) *Strongylocentrotus droebachiensis* in single-species spawning events; (2) *S. droebachiensis* eggs fertilized by *S. droebachiensis* sperm in spawning events involving both *S. droebachiensis* and *S. franciscanus*; and (3) *S. purpuratus* in single-species spawning events (see Fig. 7). The covariate is the distance between the female urchin and the nearest conspecific male urchin. A preliminary test indicated no significant differences in the slopes of these three treatments ($P > 0.1$). Least-square means are the means adjusted by the average covariate value. Pairwise comparisons of these adjusted means are reported; means with the same letter are not significantly different ($P = 0.0001$ for significant differences between treatments 1 and 2; $P = 0.0021$ for significant differences between treatments 1 and 3; $P = 0.28$ for nonsignificant differences between treatments 2 and 3).

Source	df	Type III SS	MS	*F*	*P*
Main effect	2	68.08	34.04	15.02	<0.0001
Nearest male	1	87.14	87.14	38.44	<0.0001
Error	21	47.60	2.27		
Total	24	176.61			

Treatment	Least-square means	Pairwise groupings
1. *S. droebachiensis* spawning alone	−0.623	A
2. *S. droebachiensis* spawning with *S. franciscanus*	−4.557	B
3. *S. purpuratus* spawning alone	−3.487	B

the costs and benefits to *S. droebachiensis* of producing promiscuous eggs (Fig. 7). When only conspecific individuals spawned, female fertilization success decreased with the distance to spawning males. In these single-species spawning events, *S. droebachiensis* had significantly higher fertilization than did *S. purpuratus* at similar nearest-neighbor distances (Table 3). When *S. franciscanus* and *S. droebachiensis* spawned simultaneously, the percent of *S. droebachiensis* eggs fertilized by *S. droebachiensis* sperm was lower than during single-species spawning events but not significantly different from *S. purpuratus* fertilization success (Table 3). Together these results indicate three things. First, when *S. droebachiensis* spawns as a single species, it has higher fertilization success than do the other species at similar densities (Fig. 7; Levitan 2002). Second, when this species spawns in multispecies events at high density, it produces mostly conspecific offspring (Fig. 6A). Third, when this species spawns in multispecies events at lower densities, it loses a large proportion of reproductive success to heterospecific sperm but still produces as many conspecific offspring as the most hybridization-resistant species, *S. purpuratus*, at those low densities. Within the range of densities tested, the benefits of promiscuity outweigh the costs.

DISCUSSION

Reproductive Isolation among Three Strongylocentrotus Species

Although some differences in spawning times may exist and be unrecorded and some microhabitat differences in distribution exist, all three species can be found in mixed aggregations and have been observed to spawn simultaneously (Levitan 2002). Despite this opportunity for introgression, these three *Strongylocentrotus* species demonstrate good evidence of either pre- or postzygotic reproductive isolation. Eggs of *S. purpuratus* and *S. franciscanus* are resistant to hybrid fertilization from all three species in no-choice experiments. Even at the highest sperm concentrations, fertilization generally failed (Fig. 1). Often in choice experiments, the likelihood of heterospecific fertilization is further reduced as conspecific sperm outcompete the heterospecific sperm (Howard 1999). Given the generally high local abundance of these two species (Levitan 2002), eggs released by these females seem unlikely to be fertilized by heterospecific sperm.

In contrast, eggs of *S. droebachiensis* are very susceptible to heterospecific fertilization. On average, only an order of magnitude more heterospecific than conspecific sperm were needed to fertilize 50% of eggs in no-choice experiments. High levels of fertilization were noted with heterospecific sperm of both species at intermediate sperm concentrations. Even if choice experiments would reduce this estimate, the field experiments make clear that hybrid zygotes can often be produced as a result of the relative ease of heterospecific fertilization coupled with the high local abundance of *S. franciscanus* surrounding many *S. droebachiensis* in these locations.

Nevertheless, the hybrids produced by crosses involving *S. droebachiensis* eggs show much lower survivorship at the time of metamorphosis. More than an order of magnitude more hybrids than conspecifics perished under the benign conditions in the laboratory. The early life-history stages of planktonic marine invertebrates have notoriously high mortality rates in nature (Rumrill 1990). Under these harsher and more variable conditions, hybrids may be unlikely to survive

much past settlement. It remains unknown whether these hybrids produced in the laboratory can produce viable gametes. The evidence that introgression is weak, at best, is the presence of diagnostic genetic markers found in the present and other studies (e.g., Biermann 1998) and the well-defined morphological differences between gametes (Levitan 1993), larvae (Strathmann 1979; McEdward 1986), and adults (Kozloff 1973) of the three species.

The scattered populations on the outer coast of British Columbia could be sink populations produced by more concentrated populations elsewhere. These scattered individuals may produce few offspring because of hybrid fertilization from the much more abundant congeneric sea urchins and may not contribute many conspecific offspring. This restriction of offspring production might contribute to the southern range limit of this species of Washington state; adults survive on the outer coast of British Columbia but may not produce sufficient offspring to settle further south.

Variation in Gametic Compatibility and the Evolution of Reproductive Isolation

At least three hypotheses might explain the variation in heterospecific fertilization among these species. The first is that gametic compatibility is related to genetic similarity. Over time, selection (independent of heterospecific compatibility) or genetic drift results in increasing heterospecific incompatibility. The second is that gametic compatibility is related to the cost of hybridization. Reinforcement selection for incompatibility would be greatest for the species that coexist over their full range, and this selection would be lower in *S. droebachiensis* because of its smaller geographic overlap with the other two species. The third is a novel hypothesis that heterospecific gametic compatibility is a by-product of the ease of conspecific fertilization. Selection or genetic drift resulting in higher rates of conspecific fertilization results in eggs susceptible to hybrid fertilization. A potential selective mechanism driving differences in ease of fertilization is variation in sperm availability. The ability to have eggs fertilized at low sperm concentrations would be favored under conditions of low sperm availability, and the ability of eggs to be choosy and to avoid polyspermy would be favored under conditions of high sperm availability.

No data support the first hypothesis. Molecular phylogenetics suggests that *S. purpuratus* and *S. droebachiensis* are sister taxa that belong to a polytomy of four species (Biermann 1998) that diverged approximately 3.5 million years ago (Smith 1988). These two species are more closely related to species from two other genera than to *S. franciscanus*, which diverged as much as 20 million years earlier (Smith 1988). Eggs from *S. droebachiensis* are compatible with sperm from both its sister taxon, *S. purpuratus*, and the more distantly related *S. franciscanus*. Eggs from *S. purpuratus* are incompatible with sperm from both its sister taxon, *S. droebachiensis*, and the more distantly related *S. franciscanus*. Gametic compatibility is unrelated to phylogenetic relatedness.

Some support exists for the second hypothesis. Patterns of heterospecific gamete compatibility are generally consistent with reinforcement selection to prevent hybridization. Species with broad geographic overlap are incompatible, and species with minimal overlap are compatible. Because the geographic area where hybrid fertilization is likely is small compared with the nonoverlapping ranges, reinforcement selection is likely to be weakest in *S. droebachiensis*. A test for reinforcement selection between two species is higher mating discrimination in sympatry than in allopatry (Noor 1999). Although no data are available on fertilization of *S. droebachiensis* eggs from other circumpolar locations with *S. purpuratus* and *S. franciscanus* sperm, meaningfully greater promiscuity of *S. droebachiensis* eggs in allopatric comparisons is difficult to imagine. It is an open question whether promiscuity results because gene flow disrupts local selection against hybridization or because reinforcement selection is not operating. Mitochondrial DNA analysis of Atlantic and Pacific *S. droebachiensis* indicates small but significant genetic differences between the two populations (Palumbi and Wilson 1990). The authors suggest that occasional, but not continual, migration takes place between these regions.

Available data on a fourth species, *S. pallidus*, do not support the reinforcement-selection hypothesis. This species is circumpolar like *S. droebachiensis* but at slightly greater depths. Its sperm can fertilize *S. droebachiensis* eggs at moderate sperm concentrations, and hybrids can be formed (Hagström and Lönning 1967; Strathmann 1981). The reinforcement-selection hypothesis might still apply if the difference in depth distribution provided spatial isolation in spawning or if the two species differed in spawning time. No data on the fertilization ecology of *S. pallidus* are available.

The third hypothesis also has some support. A striking correlation between the amounts of conspecific and heterospecific sperm needed to fertilize eggs was evident both among (Fig. 1) and within (Fig. 2) species. Although species-specific recognition systems may play a large role in mediating among-species fertilization, the present study makes evident that some variance in the ability to cross-fertilize is influenced by within-species processes. The gamete traits correlated with variation in conspecific fertilization success in echinoids are: (1) the number of sperm-egg collisions (Farley and Levitan 2001), which is influenced by the target size of the egg (Levitan 1993, 1996, 1998a; Farley and Levitan 2001; Levitan and Irvine 2001; Podolsky 2001) and the velocity of the sperm (Levitan 2000a); and (2) the proportion of collisions that result in fertilization (Levitan 1993; Farley and Levitan 2001), which could be influenced by the proportion of the egg surface susceptible to fertilization (Vogel et al. 1982) and the compatibility of the sperm and egg recognition proteins (Palumbi 1999). Unless the barriers to hybrid fertilization are perfect, increasing the number or efficiency of sperm collisions could increase the likelihood of hybrid fertilization. In addition, any increase the range of acceptable sperm types could increase fertilization within and across species.

Among the three *Strongylocentrotus* species studied, *S. droebachiensis* has the largest eggs, the most receptive egg surfaces, and the slowest but longest-lived sperm and requires the least sperm to fertilize 50% of eggs. *Strongylocentrotus purpuratus* has the smallest eggs, less receptive egg surfaces, and the fastest but shortest-lived sperm and requires the most sperm to fertilize 50% of eggs. *Strongylocentrotus francis-*

canus has intermediate gamete traits and requires an intermediate amount of sperm for fertilization (Levitan 1993).

The gamete traits and performance of these species are correlated with patterns of fertilization and abundance in Barkley Sound. *Strongylocentrotus droebachiensis* is the rarest species at this study site (Levitan 1998a, 2002) and other sites along the west coast of North America (Kramer and Nordin 1978; Waddell et al. 1997). Its fertilization success averaged only 23% in Barkley Sound, because of its low abundance. *Strongylocentrotus franciscanus* is much more abundant than *S. droebachiensis* and covers extensive areas of subtidal environments (Schroeter 1978; Bureau 1996; Levitan 1998a, 2002). In Barkley Sound this species has intermediate fertilization success, averaging 65%, but ranges from near 0% to 100% of eggs fertilized dependent on the proximity and abundance of males (Levitan 2002). *Strongylocentrotus purpuratus* has a patchy distribution, but local densities are the highest of the three species (Schroeter 1978; Levitan 1998a, 2002); it averaged 95% fertilization in subtidal environments and typically 100% in tide pools (Levitan 2002). Interestingly, when the data are adjusted for differences among the three species in male distribution and abundance, the rank order of fertilization performance reverses. At the overall average densities for all species combined, *S. droebachiensis* has the highest level of fertilization, followed by *S. franciscanus* and *S. purpuratus* (Levitan 2002). Manipulation of *S. purpuratus* to the densities typical of *S. droebachiensis* resulted in fertilization success lower than that of *S. droebachiensis* (Levitan 2002; Fig. 7). Controlled experiments in both the laboratory and the field confirm that these differences in fertilization performance are caused by differences in gamete traits both among (Levitan 1998a) and within (Levitan 1996) species.

By either density-dependent selection or chance, the most sperm-limited species has gamete attributes (easily fertilized eggs, long-lived sperm) that lead to the best performance under conditions of sperm limitation, and the species living under conditions of sperm competition has gamete traits (small selective eggs and fast sperm) that suggest male competition and female choice. The strength of the argument that these differences in gamete performance are driven by density-dependent selection will depend on further studies that elucidate specieswide patterns of historic abundance and the amount of gene flow between populations that differ in density. It will be particularly interesting to compare gamete traits of *S. droebachiensis* in other circumpolar locations where densities can be higher than noted on the west coast of North America (e.g., Himmelman 1986).

Support for the hypothesis that gamete traits are influenced by density-dependent selection would suggest that density-dependent selection for optimizing intraspecific offspring production can influence variation in heterospecific fertilization and potentially reproductive isolation. The correlation between ease of heterospecific fertilization and the degree of sperm limitation noted in Barkley Sound for the three species is consistent with this hypothesis (Fig. 8). All species may occasionally experience sperm limitation and sperm competition, but depending on the average sperm availability, traits appropriate to sperm limitation may be selected more intensely than those appropriate to sperm competition or vice versa. As a consequence, species would be more or less susceptible to hybrid fertilization.

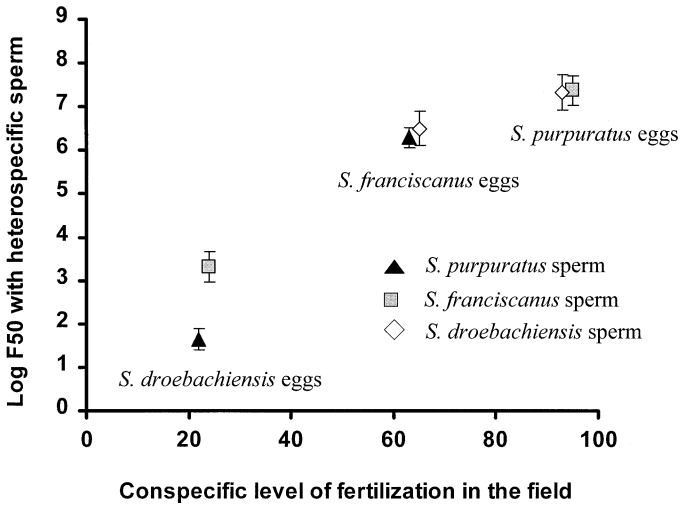

FIG. 8. Susceptibility to hybrid fertilization as a function of the average female fertilization success during conspecific spawning events in Barkley Sound, British Columbia (Levitan 2002). Symbols, as in Figure 1, represent the species of the sperm donor in laboratory fertilization assays. Bars represent standard errors.

The notion that sexual selection can result in positive selection on gamete-recognition proteins in some marine invertebrates (e.g., Metz and Palumbi 1996; Vacquier et al. 1997) and a strengthening of reproductive barriers (Gavrilets 2000) is consistent with this result. *Strongylocentrotus purpuratus* may often experience sperm competition, produces eggs more difficult to fertilize, and is the most resistant to hybrid fertilization. The novel point here is that, under conditions of sperm limitation, sexual selection can be just as intense but symmetrical across sexes, and both sexes are selected to maximize mating opportunities. Under these conditions, the selection produces more easily fertilized eggs and thus weakens isolating mechanisms. *Strongylocentrotus droebachiensis* may often experience sperm limitation, produces eggs that are easy to fertilize, and is the most susceptible to hybrid fertilization. The intermediate resistance to hybrid fertilization of *S. franciscanus* cannot be explained by reinforcement selection but can be explained by its intermediate level of conspecific sperm availability. The action of sexual selection on isolating mechanisms may therefore vary qualitatively in a density-dependent manner.

Although some scattered *S. droebachiensis* females may suffer drastic reductions in offspring production caused by hybrid fertilization, the overall benefits of producing promiscuous eggs may outweigh the costs. During low-density, single-species spawning events, *S. droebachiensis* females have much greater success than do the *S. purpuratus* females. During multispecies spawning events, although *S. droebachiensis* suffers a reduction in conspecific fertilization success, it still does as well as *S. purpuratus* at similar densities in producing conspecific offspring (Fig. 7). The cost of releasing promiscuous eggs during multispecies spawning events is no greater than that of releasing sperm-resistant eggs at low densities. For rare species, therefore, the benefits

of producing promiscuous eggs may outweigh the cost of hybrid fertilization.

The persistence of rare species often challenged by heterospecific sperm depends on pre- or postzygotic isolation, which prevents rampant introgression that might swamp the rarer species. Although prezygotic mechanisms may be more efficient, they may not always be selected for if constrained by conspecific fertilization efficiency.

This hypothesis and ones involving reinforcement selection are not mutually exclusive and may explain why heterospecific fertilization requires fewer sperm in *S. franciscanus* than in *S. purpuratus*, but in both these coexisting species, heterospecific fertilization is unlikely overall. Poorly defined reproductive isolation may exact a higher penalty in the two species that coexist and thus have increased selection for gametic incompatibility, but for *S. droebachiensis*, the benefits of promiscuous eggs may outweigh the costs of hybrid fertilization.

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Corresponding Editor: R. Burton