

CONSPECIFIC SPERM PRECEDENCE IN TWO SPECIES OF TROPICAL SEA URCHINS

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Abstract.—Conspecific sperm precedence occurs when females are exposed to sperm from males of multiple species, but preferentially use sperm of a conspecific. Conspecific sperm precedence and its mechanisms have been documented widely in terrestrial species, in which complex female behaviors or reproductive tract morphologies can allow many opportunities for female choice and sperm competition, however, the opportunity for conspecific sperm precedence in free spawning marine invertebrates has been largely ignored. Two sea urchin species, *Echinometra oblonga* and *E. sp. C*, have high levels of interspecific fertilization in no-choice lab crosses, but no natural hybrids have been found. We performed competitive fertilization assays to test for conspecific sperm precedence and found that eggs of both species showed a marked preference for conspecific sperm when fertilized with heterospecific sperm mixtures. Strong rejection of heterospecific sperm would not have been predicted from no-choice assays and helps explain the lack of natural hybrids. We also found significant variation in hybridization success among crosses. Conspecific sperm precedence in free spawning invertebrates shows that the simple surfaces of eggs and sperm provide ample opportunity for egg choice and sperm competition even in the absence of intricate behavior or complex reproductive morphologies.

Key words.—Conspecific sperm precedence, *Echinometra*, fertilization, hybrid, reproductive isolation, sea urchin.

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Darwin (1859) was one of the first to recognize the significance of conspecific sperm or pollen precedence in preventing hybridization between closely related species, yet the importance of this reproductive isolating mechanism has only recently come to prominence. Conspecific pollen or sperm precedence, or biased use of sperm from males of one species when a female is exposed to sperm from males of multiple species, is increasingly recognized as an important barrier to hybridization and gene flow between closely related species (Arnold et al. 1993; Price 1997; Markow 1997; Howard et al. 1998; Howard 1999). This type of postmating/prezygotic isolating mechanism can act as a cryptic barrier to fertilization between closely related species without obvious pre-mating reproductive isolating mechanisms, and signals the action of a broad suite of mechanisms that prevent fertilization after insemination or pollination.

Evidence for conspecific sperm precedence derives from experiments in which females have mated with more than one male (e.g. crickets [Howard and Gregory 1993; Howard et al. 2002], flour beetles [Robinson et al. 1994; Wade et al. 1995], *Drosophila* [Markow 1997; Price 1997; Price et al. 2000; Dixon et al. 2003]), or plant stigmas are colonized by more than one species' pollen (*Iris* [Arnold et al. 1993; Arnold 1997] and sunflowers [Riesberg et al. 1995]). These experiments indicate that postmating, prezygotic factors can have a strong effect on the number and type of offspring produced when females are multiply mated (Howard 1999; Bernasconi et al. 2004).

The mechanisms producing these effects are highly varied. In some cases, females affect fertilization by muscular control of sperm position within the reproductive tract (Fedina and Lewis 2004) or expulsion of sperm from the tract (Wagner et al. 2004). Other cases involve compatibility of genitalia or gamete morphology (Eberhard 1996). Males can impact fertilization or sperm competition after copulation via seminal fluid or other physiological interactions (Evans et al.

2003). Plants adjust fertilization profiles by differential pollen tube development (Carney et al. 1996; Arnold 1997). These varied mechanisms all rely on complex interactions within the female reproductive tract, which serves as a selective arena in which sexual selection and gender conflict can occur (Bernasconi et al. 2004).

For many species, especially free-spawning invertebrates, reproductive interactions occur in a smaller arena: the surfaces of gametes spawned into the water (Palumbi 1992). Without the complex behavioral and morphological structures that control fertilization in other taxa, it may seem that the opportunity for fertilization control is limited in these cases. However, recent studies of competitive mating in marine invertebrates (Yund and McCartney 1994; Palumbi 1999) have demonstrated that gamete interactions can act to produce biases in sperm usage among genotypes within species. To date only a few attempts have been made to examine these interactions in interspecific sperm use (Huvet et al. 2001; Levitan 2002; and Bierne et al. 2002).

Most studies of interspecific fertilization use no-choice experiments in which sperm from one species are exposed to eggs from another (Table 1). Among such studies, some have shown distinct barriers to interspecific fertilization (Palumbi and Metz 1991; Shaw et al. 1994; Hellberg and Vacquier 1999), which have suggested the evolution of reproductive isolation. However, other studies show only limited reproductive isolation (Table 1), which has been generally taken as evidence of potential hybridization in the wild. Recent work in terrestrial animals and plants suggests that no-choice experiments give a very limited picture of hybridization potential, and that experiments in which mating choices are available provide enhanced insight into evolutionary mechanisms of reproductive isolation (Howard 1999). The paucity of experiments using sperm mixtures in fertilization experiments with free spawning invertebrates may mask the contribution of conspecific sperm precedence to invertebrate evolution.

TABLE 1. Examples of no-choice fertilization studies used to assess hybridization potential and reproductive isolation in free-spawning invertebrates.

Taxon	% cross-fertilization rates	References
Polychaeta		
<i>Arctonoe</i>	80–100	Pernet 1999
<i>Phragmatopoma</i>	15–72	Pawlik 1988
<i>Sabellaria</i>	0–96	Pawlik 1988
<i>Spirobranchus</i>	0–17	Marsden 1992
Echinoidea		
<i>Arbacia</i>	100	Metz et al. 1998
<i>Alloccentrotus</i>	20–50	Moore 1959
<i>Echinometra</i>	5–100	Uehara et al. 1990; Lessios and Cunningham 1990; Palumbi and Metz 1991; Arakaki and Uehara 1991; Aslan and Uehara 1997; Rahman et al. 2000, 2001b; Fagonee et al. 2000; McCartney and Lessios 2002
<i>Lytechinus</i>	92–100	Durham et al. 1980; Cameron 1984
<i>Pseudoechinus</i>	40–100	McClary and Sewell 2003
<i>Strongylocentrotus</i>	0–98	Strathmann 1981
Asteroidea		
<i>Patiriella</i>	0–100	Byrne and Anderson 1994
Bivalvia		
<i>Crassostrea</i>	0–94	Galtsoff and Smith 1932; Davis 1950; Lyu and Allen 1999; Banks et al. 1994
<i>Mytilus</i>	0–80	Rawson et al. 2003
<i>Paphies</i>	0–60	Grant et al. 1998
Gastropoda		
<i>Haliotis</i>	16–96	Owen et al. 1971; Leighton and Lewis 1982; Shaw et al. 1994
<i>Tegula</i>	0–100 ¹	Hellberg and Vacquier 1999
Scleractinia		
<i>Acropora</i>	0–100	Willis et al. 1997; Hatta et al. 1999; Van Oppen et al. 2002
<i>Montipora</i>	9–48	Hodgson 1988
<i>Montastraea</i>	0–100	Knowlton et al. 1997; Szmant et al. 1997; Willis et al. 1997
<i>Platygyra</i>	0–100	Miller and Babcock 1997; Willis et al. 1997
Hydroidea		
<i>Hydractinia</i>	0–38	Buss and Yund 1989

¹ Given as vitelline envelope dissolution rates rather than fertilization.

Echinometra oblonga and *E. sp. C* are closely related tropical sea urchins that are interfertile in laboratory crosses. Uehara et al. (1990) demonstrated that sperm of both species could fertilize eggs of *E. oblonga* equally well. In the reciprocal cross (eggs of *E. sp. C*) there is some reduction in the ability of the heterospecific sperm to fertilize at low sperm concentrations. This high level of cross-fertilization is at odds with data showing differences between these species at the bindin locus. In Okinawa, Japan, *E. oblonga* and *E. sp. C* contain markedly different bindin gene sequences that show a signal of divergent selection (Geyer and Palumbi 2003). Other species pairs that have differences at this gamete attachment locus show strong fertilization barriers (Metz et al. 1994; Metz and Palumbi 1996). Species without strong bindin differences tend to be cross-fertile (Metz et al. 1998), so the facile fertilization between *E. sp. C* and *E. oblonga* despite bindin differentiation is unusual.

Echinometra oblonga and *E. sp. C* are part of a closely related complex of species recently described on the basis of morphological (Uehara 1990), reproductive (Uehara et al. 1990; Arakaki and Uehara 1991), and genetic evidence (Palumbi et al. 1997), although taxonomic names have not been assigned. The two species show slight differences in habitat preference in relation to water movement, but there are broad

areas of overlap where both species occur in mixed groups. Because spawning periods are not differentiated significantly between these species (Arakaki and Uehara 1991) the opportunity for hybridization should be high (Nishihira et al. 1991). Yet extensive sampling in field populations has revealed no natural hybrids between *E. oblonga* and *E. sp. C* (Palumbi et al. 1997; Geyer and Palumbi 2003). Postzygotic incompatibilities between the genomes may lead to very low survival among hybrid larvae and adults, though laboratory-raised hybrids appear to be viable and fertile (Uehara et al. 1990; Aslan and Uehara 1997; M. A. Rahman, pers. comm. 2000). A second hypothesis is that some form of premating isolation is in operation such that hybrid larvae are formed at a much lower rate than would be predicted given their high rate of cross-fertility. Such a limit to fertilization ability would be predicted by bindin comparisons. To test for these hidden biases, and to explore the potential for conspecific sperm precedence to operate in a very simple fertilization system, we conducted a series of competitive fertilization experiments between these two species.

MATERIALS AND METHODS

Specimens of *E. oblonga* and *E. sp. C* were collected from the reef on Sesoko-jima, Okinawa. They were transported to

the University of the Ryukyus, Nishihara, Okinawa, and maintained in closed seawater tanks until used in the experiment. Spawning was induced by injection with 1 ml 0.5 M KCl. Sperm were collected "dry" and stored at 4°C until ready for dilution. Dry stocks and dilutions were frozen and stored for later quantification. Eggs were collected in filtered seawater, rinsed three to four times to remove debris and then resuspended in filtered seawater to a concentration of approximately 1000 eggs/ml.

Fertilization Assays

Each adult was used in only one set of crosses. Dry sperm were serially diluted to 10^{-3} . For each fertilization, 200 μ l of eggs and 200 μ l of sperm were mixed for a final volume of 400 μ l. Equal volumes of diluted sperm from each species were mixed for competition crosses before addition to eggs. Each dilution of sperm was used for three fertilizations: one no-choice conspecific cross, one no-choice heterospecific cross, and one competitive cross using conspecific eggs. Simultaneously, a second set of fertilizations was performed with the male of the opposite species: the same male that was used in the competitive cross. All six possible crosses were done simultaneously from the same sperm dilutions to eliminate error due to differences in sperm concentration and age. Fertilization was allowed to proceed a minimum of 30 min before any eggs were transferred to culture or for counting. Because each male or female was only used in one set of crosses, each set of fertilization experiments is an independent datapoint.

Fertilization success was determined by counting the number of eggs that showed raised fertilization envelopes or cleavage at 100 \times magnification. All eggs showed high levels of fertilization (>85%) in homospecific fertilizations. Percent fertilization in no-choice heterospecific crosses was used to assay levels of potential hybridization in the absence of competition. Fertilized eggs from competition crosses were transferred to petri dishes with 40 ml of filtered seawater and allowed to develop without feeding at room temperature for two to three days. Individual four-armed plutei were harvested in 5 μ l of seawater and transferred to individual tubes. Five μ l of 3 mg/ml proteinase K was added to each, and larvae were incubated at 70°C for 15 minutes. The digested larvae were stored frozen until ready for polymerase chain reaction (PCR).

Sperm from each cross were quantified using DNA concentration as a proxy for relative sperm count. Frozen aliquots of sperm were thawed and vortexed to break up aggregations and resuspend sperm. A 150 μ l aliquot of each sperm dilution was incubated with 150 μ l of lysis buffer (Palumbi et al. 1991) and 15 μ l of 20 mg/ml proteinase K at 60°C for 2 h. Three hundred μ l of each sample was diluted to 2 ml with detection buffer (10mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 mM NaCl, 0.1 μ g/ml bisbenzimidazole; Carr and Shearer 1998). Fluorescence was detected on a Shimadzu RF-5301 PC spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD) with an excitation wavelength of 356 nm and a detection wavelength of 456 nm.

Genetic Characterization

Adult urchins were fixed in 30% ethanol and transported whole. DNA was extracted and PCR and sequencing was carried out as described in Geyer and Palumbi (2003). Cytochrome *C* oxidase subunit I (COI) was sequenced to confirm species identifications based on morphology. The first exon of *bindin* shows a fixed 30–36 bp difference in size between *E. oblonga* and *E. sp. C*. This region of *bindin* was amplified from each parent and larva. Amplification products were separated on 12% acrylamide gels, stained using SYBR Green (BioWhittaker Molecular Applications, Rockland, ME) and visualized with UV light. In each cross 45–55 larvae were scored for paternity. Amplifications from parents were included on each gel as controls.

Statistical Analysis

The percentage of hybrid larvae from each competitive cross was inferred from the *bindin* genotype. Raw values were arcsine transformed to calculate standard errors and variance for statistical tests. A two-tailed binomial test of average hybridization success was performed using 95% confidence intervals of the expected hybridization based on the average fertilization success in the no-choice crosses. The one-tailed binomial probability of each cross was calculated given an expected frequency of hybridization corrected for both the relative sperm concentration and the difference in fertilization success in no-choice experiments. The significance of each cross was evaluated using a sequential Bonferroni correction (Rice 1989). Sperm use probabilities were calculated according to Palumbi (1999) as $SR/(SR + 1)$, where $SR = (L1/L2) \times (S2/S1)$. *L1* and *L2* are the number of larvae sired by each male and *S1* and *S2* are the respective sperm concentrations.

RESULTS

No-Choice Crosses

Thirteen independent crosses with reciprocals were performed. Average fertilization in both conspecific crosses was greater than 99% (Fig. 1). In the heterospecific crosses, fertilization was also very high. Crosses using *E. sp. C* eggs and *E. oblonga* sperm averaged $95.4 \pm 5.7\%$ (mean \pm SE). This is not significantly different than the within-species fertilization rate for *E. sp. C*, although the variation is significantly greater (*F*-test, $P \ll 0.001$). Crosses using *E. oblonga* eggs and *E. sp. C* sperm achieved an average fertilization of $84.0 \pm 7.9\%$, which is significantly lower than within-species fertilization for *E. oblonga* (Wilcoxon rank sum, $P < 0.01$) and shows a much higher level of variation (*F*-test, $P \ll 0.001$; Fig. 1). This asymmetry in fertilization success is consistent with the fertilization behavior of these species reported by Uehara et al. (1990).

Choice Crosses

Twelve crosses with reciprocals were performed for a total of 24 competition experiments. In cross 7, no data were collected from *E. oblonga* eggs because cultures were contaminated and had to be discarded. Average hybridization using

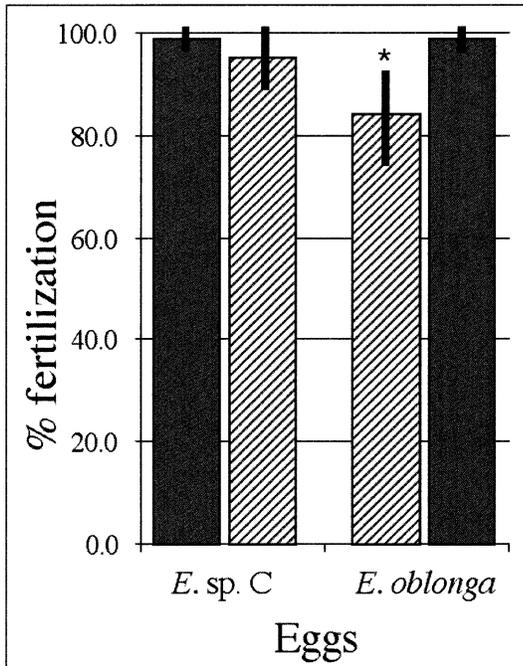


FIG. 1. Average fertilization in no-choice crosses in *Echinometra*. Solid bars indicate homospecific sperm; hatched bars indicate heterospecific sperm. Error bars are arcsine-transformed standard errors. $n = 13$ for each possible cross. The asterisk indicates the significant comparison (Wilcoxon rank sign $P < 0.01$).

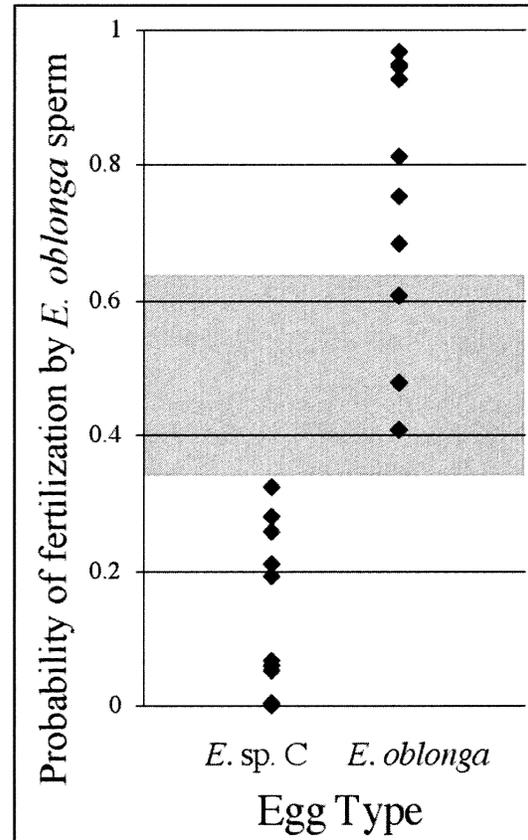


FIG. 2. Probability of fertilization by *Echinometra oblonga* sperm. Each point represents a separate experiment using mixed *E. oblonga* and *E. sp. C* sperm. Sample sizes: 11 crosses using *E. oblonga* eggs, 12 crosses using *E. sp. C* eggs. The gray area indicates 95% confidence intervals for detecting a difference from random fertilization given an average sample size of 50 larvae.

E. sp. C eggs was $12 \pm 5\%$ and using *E. oblonga* eggs averaged $26 \pm 7\%$. These averages are significantly lower than predicted given the results of the no-choice fertilizations (Table 2). However, there is considerable variability between individual crosses (Fig. 2). Individual tests for biased sperm usage (Table 3) reveal that 11 of 12 crosses with *E. sp. C* eggs showed significant deviations from random sperm usage. In addition, the distribution of hybridization rates appears to be distinctly bimodal, with 7 of 12 crosses having less than 8% hybrids and the remaining 5 crosses averaging 25% hybrids. This distribution is significantly non-normal ($P = 0.025$, Shapiro-Wilk W test). Only 7 of 11 crosses with *E. oblonga* eggs show a significant bias. There is significantly more variation in hybridization success with *E. oblonga* eggs than with *E. sp. C* eggs (Conover squared rank test, $P < 0.2$), and normality cannot be rejected ($P = 0.21$). Both species show significantly lower levels of hybridization than predicted by no-choice fertilization, and in addition the asymmetry in hybridization rates is in the opposite direction than that predicted from fertilization success.

Natural Hybrids

Thirty-four specimens of *E. oblonga* and 36 specimens of *E. sp. C* were collected for these experiments. Of these, a single specimen had an anomalous genotype. This specimen (OK216) was a female that conformed to the brown morph of *E. sp. C* and had a matching CO1 haplotype, but was heterozygous at the *bindin* locus for alleles 2 and 3a. These alleles were previously described (Geyer and Palumbi 2003) and are characterized by multiple amino acid differences and two insertion/deletion mutations. In 73 samples of *E. sp. C* surveyed to date, clade 2 alleles were diagnostic. Clade 3a and b alleles were diagnostic among 58 *E. oblonga* samples in the West Pacific (Geyer and Palumbi 2003). This is the

TABLE 2. Average hybridization in choice crosses using a two-tailed binomial test of hybridization based on expectations from no-choice fertilization success. Hybridization probability calculated as $H/(H + 1)$, where H is the proportion of hybrids observed, corrected for differences in average sperm concentration.

Female species	% hybrids	SE	Hybrid probability	Expected	95% confidence limits
<i>Echinometra sp. C</i>	12.2	5.0	0.140	0.490	0.344–0.627
<i>E. oblonga</i>	25.8	7.0	0.162	0.460	0.320–0.609

TABLE 3. Binomial probabilities per cross. This probability is calculated with an expected number of hybrids in each cross that is corrected for both relative sperm concentration and relative fertilization success in the absence of competition. The significance is evaluated with a sequential Bonferroni correction for multiple tests (Rice 1989). A single asterisk indicates a significant result. A double asterisk indicates a highly significant result, where P is an order of magnitude below α .

Egg type	Cross no.	No. of larvae	% hybrids	Binomial P
<i>Echinometra</i> sp. C	1	46	21.7	$1.071 \times 10^{-4**}$
	3	54	22.2	$2.411 \times 10^{-4**}$
	4	52	0.5	$3.576 \times 10^{-23**}$
	5	5	0.0	$1.553 \times 10^{-3*}$
	6	48	8.3	5.707×10^{-2}
	7	35	0.7	$4.843 \times 10^{-21**}$
	8	54	5.6	$3.572 \times 10^{-19**}$
	9 ¹	51	23.5	$7.320 \times 10^{-4**}$
	10	56	1.7	$4.742 \times 10^{-39**}$
	11	50	30.0	$1.723 \times 10^{-7**}$
	12	52	26.9	$1.623 \times 10^{-3*}$
	13	48	6.3	$5.175 \times 10^{-12**}$
	<i>E. oblonga</i>	1	45	6.7
3		48	4.2	$5.635 \times 10^{-14**}$
4		55	5.5	$4.848 \times 10^{-8**}$
5		55	12.7	$4.907 \times 10^{-3*}$
6		50	4.0	$4.129 \times 10^{-68**}$
8		43	27.9	6.465×10^{-2}
9		48	64.6	4.602×10^{-2}
10		54	38.8	$2.980 \times 10^{-4**}$
11		54	48.2	1.083×10^{-2}
12		47	27.7	$2.398 \times 10^{-4**}$
13		48	37.5	4.763×10^{-2}

¹ Because the female in this cross was a heterozygote, the number of hybrids is estimated as the number of clade 3 (*E. oblonga*) homozygotes plus one half the heterozygotes.

first instance of hybridization in the field that has been documented between these two species. Despite its hybrid origin, its eggs perform as normal *E. sp. C* eggs in crosses (cross 9). If the bindin recognition system is responsible for these interactions, this would indicate that this individual is most likely not an F_1 hybrid since a heterozygote at the bindin receptor would show reduced fertilization success. However, without additional diagnostic loci for these species and a better understanding of the controls of fertilization in eggs, we cannot speculate further.

DISCUSSION

Our data demonstrate strong, reciprocal, conspecific sperm precedence between *Echinometra oblonga* and *E. sp. C* in a very simple fertilization system. Despite very high levels of interspecific fertilization in no-choice crosses, hybrid formation was significantly reduced in choice crosses. Because average fertilization between *E. oblonga* females and *E. sp. C* males was 84%, in the absence of sperm precedence we would expect approximately 42% hybrids from the choice crosses (Table 2). The observed proportion of 26% is significantly lower than this expectation. Likewise, hybridization of *E. sp. C* eggs in choice experiments was 12% instead of the 49% expected if no sperm precedence was present. On average, there appears to be a strong bias toward use of conspecific sperm in both species. These data suggest that competition among males and an egg's choice of sperm may play a significant role in reducing the incidence of hybrids in these species.

Mechanistically, sperm precedence in free-spawning invertebrates operates on simple cellular and genetic systems.

In sea urchins, fertilization involves only the interaction of egg and sperm after they are shed into the water. Sperm are activated in the presence of conspecific eggs, penetrate a jelly coat surrounding the egg, attach to the vitelline envelope, and fuse with the egg membrane (Palumbi 1992). Metz et al. (1994) demonstrated that the specificity of fertilization between *E. oblonga* and *E. mathaei* is controlled by the interaction of the sperm protein bindin with a receptor on the egg surface. Other urchins show differentiation in activation among species (Biermann 1998). In Okinawa, *E. oblonga* and *E. sp. C* are characterized by very different bindin alleles (Geyer and Palumbi 2003) that show significant amino acid and length differences, which can explain the strong cryptic discrimination between these species. Unfortunately, relatively little is known about the egg receptor for bindin (Kamei and Glabe 2003), which must also have a strong influence on fertilization success. Variation at the bindin receptor could explain a portion of the variance in hybridization success seen in this study.

In terrestrial systems, the importance of conspecific sperm precedence is associated with opportunity for eggs to be fertilized by more than one male after multiple matings, or long exposure of flowers to pollen from diverse sources (Howard 1999; Bernasconi et al. 2004). In free-spawning invertebrates, conspecific sperm precedence likewise is ecologically and evolutionarily important only if eggs are exposed to sperm from multiple males before fertilization. Conditions for multispecies fertilization may be stringent. Because eggs tend to be fertilizable only for short periods (e.g., Rahman et al. 2001a), and sperm are diluted quickly in seawater (Denny and Shibata 1989), successful fertilization in the sea requires

proximity of spawning adults in time and space (reviewed in Levitan and Petersen 1995; Yund 2000; Levitan 2002). When these conditions are not met, many eggs may go unfertilized (Yund 2000), potentially reducing the opportunity for hybridization (Levitan et al. 2004).

Conditions for exposure of eggs to sperm of multiple species may be met in shallow water habitats where multiple congeners coexist at high densities and spawn as a consequence of the same environmental cues. For instance, Levitan (2004) demonstrated that in field populations of the temperate sea urchin *Strongylocentrotus franciscanus*, an average of 42% of a female's eggs were fertilized by multiple, nonfocal males, indicating that sperm mixing is likely when sperm is not a limiting factor. Sympatric *Echinometra* species tend to live in dense stands, with population densities as high as 100 individuals \times m⁻² (McClanahan and Muthiga 2001), indicating that relatively high sperm concentrations may be experienced in the field. Isolated individuals and low densities are most likely to be found on the periphery of habitats, areas in which the habitat preferences of the different *Echinometra* species are likely to be more pronounced (Nishihira et al. 1991), and exposure to heterospecific males is probably rare. Other species groups in which mass spawnings have been observed include reef-building corals (Miller and Babcock 1997; Van Oppen et al. 2002), sponges, polychaetes, and brittlestars (Hagman and Vize 2003). These observations suggest that multiple species spawning in shallow habitats may be common; however, Levitan et al. (2004) show in a carefully observed coral mass spawning that subtle differences in timing can reduce hybridization potential substantially. Thus, the ecology of fertilization in the sea is an intrinsic part of understanding the evolution of marine mating systems.

Sympatric *E. oblonga* and *E. sp. C* in Okinawa probably have ample opportunity for hybridization because most individuals are within a meter of potential interspecific mates, and both species spawn in response to storm cues. The sperm precedence we document here reduces hybridization potential, but does not eliminate it. Given our fertilization results, a hybridization rate greater than 5–10% would be expected. Additional postzygotic factors may yet be discovered that can explain the comparative rarity of hybrids.

Abundant variation among individuals shows that conspecific sperm precedence is not an absolute barrier to hybridization. *Echinometra* sp. C eggs show an approximately bimodal capacity to use *E. oblonga* sperm. In 7 of 12 crosses, eggs show strong rejection of *E. oblonga* sperm, with hybridization rates of less than 8%. By contrast, 5 of 12 crosses show approximately 25% hybridization, although this is still significantly lower than expected with no sperm precedence (expected 50%). This bimodality suggests that there may be at least two major egg phenotypes in *E. sp. C*; however, our sample sizes do not allow us to test this distribution effectively. Similarly, individual tests of hybridization success reveal that a small fraction of *E. oblonga* females do not significantly discriminate between males of different species. Mechanisms that explain this failure of the hybridization barrier remain unexplored to date.

A number of authors have suggested that conspecific sperm precedence observed through competitive crosses may be the initial step in the evolution of reproductive isolation between

closely related species (Price 1997; Howard 1999; Bernasconi et al. 2004). By contrast, marine studies have tended to use data from no-choice crosses to examine potential for reproductive isolation because such crosses are vastly simpler. Although fertilization potential from no-choice crosses is probably a good indicator of reproductive isolation when fertilization success is low (e.g., Table 1; *Echinometra*, Palumbi and Metz 1991; *Spirobranchus*, Marsden 1992; *Allocentrotus*, Moore 1959; *Montipora*, Hodgson 1988; *Hydractinia*, Buss and Yund 1989), noncompetitive fertilization assays can miss more subtle aspects of prezygotic reproductive isolation when barriers to fertilization are not absolute. The high levels of conspecific sperm precedence seen here despite high levels of cross-fertilization indicate that caution should be taken when trying to make assessments of hybridization potential from fertilization data. For example, Pernet (1999) found small but consistent gamete incompatibilities in no-choice crosses of species of the polychaete worm *Arctone* similar to the levels we see between *E. oblonga* and *E. sp. C* (Table 1), yet concluded that gamete incompatibilities are absent. As in *Echinometra*, these species are sympatric but hybrids are rare or absent.

Surprisingly few studies have attempted to look at assortative fertilization in free-spawning animals. Levitan (2002) showed that the likelihood of hybrid formation in field fertilizations of *Strongylocentrotus droebachiensis* was highly dependent on the arrangement of congeneric urchins in space but did not attempt to perform choice experiments to assess sperm competition. Huvet et al. (2001) found an excess of hybrids and possible heterosis in competitive fertilizations between *Crassostrea gigas* and *C. angulata*; however, the species status of these taxa is questionable (Menzel 1974). Bierne et al. (2002) examined assortative fertilization between two species of mussels, *Mytilus edulis* and *M. galloprovincialis*, that form natural hybrids in the field. They found fewer hybrids than expected from completely random mating. There are no data on gamete incompatibility currently available for these species, however, Rawson et al. (2003) found significant reduction in fertilization between *M. edulis* and a third species, *M. trossulus*, indicating that gamete incompatibility may have a role in reducing hybrid formation among these species.

Studies of mating behavior and sexual selection have traditionally focused on organisms with complex premating behaviors such as color recognition (Seehausen et al. 1998), sexual selection on size (Fleming 1996), mating calls (Brown 1999; Ptacek 2000), and female choice of extreme male traits (Andersson and Iwasa 1996). Mate choice experiments have long been the standard tool for understanding mating behavior in organisms with a strong ethological component to reproductive isolation. Only recently have studies of postcopulatory, prefertilization reproductive isolation begun to use this technique to assess sperm precedence in animals (Howard 1999). Although the mating system of free-spawning organisms is comparatively simple, our data demonstrate that interactions at the level of cell-cell interfaces may provide ample opportunity for complex mating-system dynamics. Female choice or sperm competition, if possible among gametes in sea urchins, may also be possible in taxa with internal fertilization. This opens the possibility that sperm prece-

dence, female choice, gender conflict, and sexual selection could be played out on the surfaces of gametes in insects, mammals, and birds (Evans et al. 2003). The tendency in the literature of sperm precedence has been to emphasize interactions between seminal fluids and female reproductive tracts in such taxa, yet sperm-egg contact and penetration is a universal aspect of fertilization in sexual systems. Although the precise mechanism of egg-sperm attachment is not homologous between phyla, as a general mechanism, gamete-level assortative fertilization may turn out to be an important mode of reproductive isolation in many taxa.

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