Reference: *Biol. Bull.* **209:** 113–126. (October 2005)

Gamete Compatibility and Sperm Competition Affect Paternity and Hybridization Between Sympatric *Asterias* Sea Stars

F. M. HARPER* AND M. W. HART[1]

Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada

Abstract. Gamete interactions may strongly influence speciation and hybridization in sympatric broadcast-spawning marine invertebrates. We examined the role of gamete compatibility in species integrity using cross-fertilization studies between sympatric *Asterias* sea stars from a secondary contact zone in the northwest Atlantic. In crosses between single males and single females, gametes of both species were compatible and produced viable, fertile hybrid offspring, but with considerable variation in the receptivity of eggs to heterospecific sperm. Differential compatibility of heterospecific gametes was detected in sperm competition studies in which we used a nuclear DNA marker to assign paternity to larval offspring. Several families showed conspecific sperm precedence in *A. forbesi* eggs, and one family showed competitive superiority of *A. forbesi* sperm fertilizing *A. rubens* eggs. Gametic interactions are an important component of prezygotic reproductive isolation in sympatric *Asterias*. The interaction between gametes of these closely related sea stars is consistent with the function of gamete recognition systems that are known to mediate fertilization success and speciation in other marine invertebrates.

Introduction

Understanding the mechanisms that reproductively isolate species is a fundamental component of studies of species formation. In marine invertebrates, differences in habitat selection, spawning synchrony (on diurnal and seasonal time scales), mate preference, and fertilization have been implicated in speciation events (review in Palumbi, 1994). Although adults of most broadcast-spawning marine invertebrates lack mating behaviors that contribute to reproductive isolation in copulating taxa such as insects, the interactions of gametes can limit heterospecific fertilization. Gametic incompatibility is considered particularly important in determining reproductive isolation for closely related broadcast-spawning species whose spawning periods overlap in sympatry (*e.g.,* Lessios and Cunningham, 1990; Palumbi and Metz, 1991; Levitan, 2002). Such incompatibility may arise as a consequence of neutral evolution and genetic drift in allopatry, by selection against the formation of low-fitness hybrids when populations become secondarily sympatric (reinforcement), or as a stochastic consequence of sexual selection (review in Pernet, 1999).

Two closely related broadcast-spawning species of *Asterias* sea stars are sympatric in the northwest Atlantic. *Asterias forbesi* (Desor) and *A. rubens* L. co-exist in the Gulf of Maine and along the eastern shore of Nova Scotia as the result of secondary contact following the retreat of the last Pleistocene glaciers (Worley and Franz, 1983; Wares, 2001). *A. forbesi* is endemic to North America, ranging from the eastern shore of Nova Scotia to the Gulf of Mexico (Clark and Downey, 1992). *A. rubens* has an amphi-Atlantic distribution (Tortonese, 1963), extending from the United Kingdom south to Portugal in the northeast Atlantic and from southern Labrador to North Carolina in the northwest Atlantic (Franz *et al.,* 1981; Clark and Downey, 1992). Phylogenetic and population genetic analyses suggest the initial vicariance of North Atlantic *Asterias* populations occurred about 3 Mya, followed by recent ($<$ 18,000 years) recolonization of North America by *A. rubens* (Wares, 2001).

Whether sympatric populations of *Asterias* spp. are reproductively isolated is unclear. Reports of specimens with intermediate morphologies (Clark, 1904; Perlmutter and

Received 14 March 2005; accepted 13 July 2005.

* To whom correspondence should be addressed, at Department of Biology, Rollins College, 1000 Holt Avenue-2743, Winter Park, FL 32789-4499. E-mail: fharper@rollins.edu

[1] Current address: Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada.

Nigrelli, 1960; Walker, 1973; Menge, 1986) and of hybridization in the laboratory (Ernst, 1967) have been interpreted as evidence of natural hybridization (Schopf and Murphy, 1973; Menge, 1986; Byrne and Anderson, 1994; Palumbi, 1994; Gardner, 1997). Clark and Downey (1992) state that *Asterias* hybrids are frequent but do not reach sexual maturity. In contrast, other studies find little evidence of intermediate phenotypes that might be hybrids (Worley and Franz, 1983; Wares, 2001; F. Harper, unpubl. data). The question then is how the phenotypic integrity of these two closely related species is maintained in a recently formed contact zone.

Temporal separation of spawning periods and spatial separation of adults with different temperature and habitat preferences are possible mechanisms of prezygotic reproductive isolation (Schopf and Murphy, 1973), but these may be incomplete barriers to hybridization in *Asterias*. Adults of these species are found in similar habitats, compete for the same resources (Menge, 1979), and are likely to spawn at the same time. For example, at one site in the contact zone (Bear Cove, NS), a male of *A. forbesi* was observed spawning within 30 cm of an individual of *A. rubens* in late July when both species were reproductively active (pers. obs.).

Differential thermal tolerance of gametes has also been suggested as a barrier to successful hybridization in broadcast-spawning marine invertebrates (McClary and Sewell, 2003), but it seems unlikely to prevent hybridization in sympatric *Asterias* populations. In New England and Atlantic Canada, peak spawning by *A. rubens* varies among years from April to July when ambient water temperatures are 6–15 °C (Smith, 1940; Franz *et al.,* 1981; Menge, 1986). *A. forbesi* is generally more tolerant of higher temperatures and spawns at about 15 °C in July and August (Boolootian, 1966; Franz *et al.,* 1981; Menge, 1986). In parts of the secondary contact zone such as the shallow subtidal area of the Gulf of Maine, surface sea temperatures vary from 10 to 20 °C between May and August (Yoder *et al.,* 2002) when the spawning seasons of the two species may coincide.

Gametic incompatibility has not been previously suggested as a prezygotic barrier to reproduction in *Asterias*. The gametes of the two species have been considered compatible on the basis of a single reference in Ernst (1967, p. 22): "the sperm of both species will fertilize the eggs of both." To quantitatively assess the role of gamete incompatibility in the dynamics of this secondary contact zone, we conducted a series of cross-fertilization studies. We performed crosses between single males and single females over a range of sperm concentrations and analyzed the data using both linear and nonlinear fertilization models. We also genotyped larvae to assign paternity to offspring produced in sperm competition studies involving crosses between a male from each species and a single female. Because fertilization was successful in both conspecific and heterospecific crosses, we further examined two potential postzygotic barriers to reproduction (hybrid inviability and sterility) by rearing hybrid offspring to sexual maturity and backcrossing them to field-collected adults.

Materials and Methods

Sampling of adults and collection of gametes

Mature specimens of *Asterias rubens* and *A. forbesi* were collected from Bear Cove, Nova Scotia (5–10 m depth) in July and August 2001 for cross-fertilization studies and in July 2003 for sperm competition and backcross studies. Both species contained ripe gonads during these months (pers. obs.). Animals were maintained in flow-through seawater at ambient temperature (14–17 °C) for up to one week and were fed mussels (*Mytilus* spp.) *ad libitum*. We used the diagnostic morphological characters described in Clark and Downey (1992) to assign animals to species.

Gametes were collected by dissecting gonadal tissue from ripe adults. A short incision (1–2 cm) was made along the dorsal margin of one side of the proximal part of an arm; about the same volume of gonad was removed from each animal. Testes were placed in 20 ml of 0.45-μm-filtered seawater (FSW), and ovaries were placed in 20 ml of 10^{-5} *M* 1-methyl adenine prepared in FSW. Sperm were extruded from the testes immediately, whereas eggs were incubated for 1 h to allow ovulation to be completed and oocytes to mature (Kanatani, 1979). Prior to use, sperm and eggs were separated from their respective gonads by decanting the gametes into clean beakers. Gamete collection and all experiments were performed at 12 °C.

Single-male cross-fertilization studies

Conspecific and heterospecific crosses were made for both *Asterias* species. For each heterospecific cross, the same female was used in a conspecific fertilization to serve as a positive control. Thus, each fertilization experiment consisted of two conspecific (FF, RR) and two heterospecific (FR, RF) matings among four adults in which the two-letter designation indicates the species of female and male parents (*e.g.,* FR = *A. forbesi* female × *A. rubens* male). We replicated these experiments five times, using new adults for each replicate (20 adult animals total). Replicates were conducted on different days to reduce potential cross-contamination.

Gametes of a single male and a single female were combined using specified dilutions of initial sperm and egg stocks. Actual gamete concentrations were subsequently determined from aliquots of sperm and egg stocks preserved in formalin-buffered seawater. Sperm concentrations were estimated from hemacytometer counts of a 10-fold dilution of the initial sperm stock, and eggs were counted using a dissecting microscope.

We used a broad range of sperm dilutions (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) to determine whether heterospecific

crosses required different sperm concentrations than conspecific crosses for successful fertilization in single-male/single-female matings. Fertilizations were conducted in sterile 25-ml petri dishes containing 8 ml of FSW and 1 ml of eggs pipetted from a well-mixed stock suspension. From the freshly prepared sperm suspensions, 1 ml of the appropriate sperm dilution was then pipetted over the eggs and mixed gently. Final egg concentrations were 76–296 eggs ml^{-1}, and final sperm concentrations were 10^{-3}–10^{3} sperm μl^{-1}.

We scored fertilization success using two criteria: presence of a fertilization envelope and cell cleavage. After 1 h, fertilization success was scored by examining at least the first 200 undamaged, haphazardly selected eggs on a dissecting microscope and counting the number with fertilization envelopes. Evidence of cell cleavage in intact eggs was scored after 3 h. In a pilot study, 3 h was sufficient time to allow all fertilized eggs to divide at least once. Both cleavage and fertilization envelopes were used as indices of fertilization to assess the potential for polyspermy at high sperm concentrations and to avoid overestimating effective fertilization success due to inclusion of polyspermic eggs (Styan, 1998).

To compare the effectiveness of fertilization across replicate crosses, we estimated the F_{50} value, the sperm concentration at which 50% of the eggs were successfully fertilized (Levitan, 1996, 1998). To determine the F_{50} for each replicate, data were fit to the nonlinear fertilization kinetics model developed by Vogel *et al.* (1982) for sea urchins. The untransformed proportion of eggs fertilized (P) was fit to the equation.

$$P = 1 - \exp((-\beta S_0)/(\beta_0 E_0)*(1 - e(-\beta_0 E_0 t))),$$

where S_0 = number of sperm per microliter, E_0 = number of eggs per microliter, t = sperm:egg contact time (in s), and β and β_0 are parameters obtained from nonlinear regression of P on S_0 (Vogel *et al.,* 1982). β is the rate constant of fertilization, based on sperm-egg contact and the fertilizability of the egg; and β_0 is the rate constant of sperm-egg contact, based on egg cross-sectional area and sperm swimming velocity (Levitan *et al.,* 1991). Both β and β_0 were estimated for each replicate using initial values of 3.8×10^{-6} and 3.3×10^{-4} $mm^3\ s^{-1}$, respectively (NLIN command in SAS ver. 8). Each F_{50} was then determined by solving the resulting nonlinear regression equation for S_0 at $P = 0.5$. F_{50} values were estimated for both indices of fertilization success.

Fertilization success was also analyzed using a logit transformation method that does not assume adherence to a fertilization kinetics model developed for intraspecific crosses (McCartney and Lessios, 2002). The proportion of eggs fertilized (P) was transformed as logit $P = \ln(P/1 - P)$. For each replicate, the logit P value was linearly regressed on the log-transformed sperm concentration. F_{50} values were estimated from the linear regression by determining the sperm concentration at which logit (0.5) = 0.

Values of F_{50} were initially analyzed using a one-way ANOVA with cross type (FF, FR, RR, RF) as the independent variable ($\alpha = 0.05$). Because the heterogeneity of variances violated the assumption of homoscedasticity required for ANOVA (Sokal and Rohlf, 1995), we then conducted a two-sample randomization test with 10,000 Monte Carlo simulations (Manly, 1991). F_{50} values for all heterospecific crosses (FR and RF) were combined, as were F_{50} values for all conspecific crosses (FF and RR), to compare the amount of sperm required to fertilize 50% of eggs in hybrid and nonhybrid crosses. Four separate analyses were conducted for F_{50} values based on counts of fertilization envelopes and counts of cleaved zygotes and estimated using the nonlinear fertilization model and linear regression.

Sperm competition studies

Sexually mature specimens of *A. forbesi* and *A. rubens* selected for the sperm competition studies were maintained in individual containers in a flow-through seawater system for up to 4 days and were fed mussels *ad libitum*. Males and females were selected for crosses such that paternity could be assigned to the offspring by using a single nuclear DNA marker from ethanol-preserved tube feet (described below).

Gametes were collected and stocks prepared as described above, except that gamete concentrations were determined before each experiment. Sperm concentrations were estimated from hemacytometer counts of the 10^{-1} sperm dilution, and eggs were counted using a dissecting microscope. Sperm from one conspecific and one heterospecific male were combined in glass beakers containing FSW such that the final sperm concentration for one male was high (200 sperm μl^{-1}) and the sperm concentration for the second male was low (20 sperm μl^{-1}). The sperm suspension was mixed immediately before the eggs were added. Egg concentrations were adjusted to 200 eggs ml^{-1}, which gave sperm-to-egg ratios of 1000:1 and 100:1, respectively, for the two males (final volume of sperm and egg suspension was 25 ml).

Eggs from each female were used in two separate sperm competition crosses. First, eggs were mixed simultaneously with conspecific sperm in high concentration and heterospecific sperm in low concentration. Second, eggs were mixed with the relative concentrations of conspecific and heterospecific sperm reversed. As positive controls for gamete quality and to ensure that all gametes involved in each experiment were capable of fertilization, separate crosses of eggs mixed with only conspecific sperm in high concentration were conducted. Only offspring from crosses in which both controls had greater than 90% fertilization success (based on the presence of fertilization envelopes after 1 h) were cultured and genotyped.

Larvae from each replicate experiment were transferred after 2 days into 250-ml containers with 125 ml FSW and 2 ml of a dense culture of *Isochrysis galbana*. Five days after initial fertilization, bipinnaria larvae were gently removed from the culture and preserved in 95% ethanol for genotyping.

Genomic DNA was obtained from a single preserved tube foot from each adult. The tube foot was rinsed twice in 100 μl of double-distilled, autoclaved water (ddH_2O), then placed in 40 μl of ddH_2O with 10 μg Proteinase K (Qiagen). The digestion was incubated for 60 min at 65 °C, then for 10 min at 85 °C. Between 0.75 and 1.5 μl of this digest was used as template for each PCR. Genomic DNA from larvae was similarly extracted: larvae were placed individually into wells in a 96-well plate, rinsed twice with ddH_2O, and digested in a volume of 10 μl of ddH_2O with 10 μg of Proteinase K. Each larval digest (0.75 μl) was used as template in the PCR.

Microsatellite markers were developed for *A. forbesi* and *A. rubens,* using the enrichment protocol of Hamilton *et al.* (1999; details in Harper, 2004). Few clones contained microsatellite sequences, and Southern blots of *Asterias* genomic DNA probed for microsatellite motifs (alongside blots of other animal genomes known to have many or few microsatellite loci) suggested that di- and tri-nucleotide microsatellite loci are rare (F. Harper, unpubl. results). Most candidate loci could not be reliably and consistently amplified from genomic DNA. Locus *Ar50* was a microsatellite with a $(GTT)_{15}$ repeat motif that amplified reliably and was polymorphic in both species (two alleles in *A. forbesi* and six alleles in *A. rubens;* one allele was shared by both species). Primer sequences for this locus were *Ar50-A:* 5′-AGCCCATGTCGGTCTTAG-3′ and *Ar50-B:* 5′-TTTGAAAGGCTCTAATGAG-3′. The 5′ end of *Ar50-A* was labeled with an IRD700 dye for visualization using a Li-Cor 4200 sequencer. Amplifications were performed in a 5-μl final volume containing 0.2 m*M* of each dNTP (MBI), 3.0 m*M* of $MgCl_2$ (MBI), 1.5 pmol of each primer, 1× polymerase buffer, and 0.1 units of *Tsg* Polymerase (Biobasic). The thermal cycling profile consisted of an initial denaturation at 95 °C for 5 min, followed by 36 cycles of 90 °C (30 s), 49 °C (90 s), 72 °C (60 s), and a final extension of 72 °C for 1 min. The amplified products were resolved in 6% (25 cm, 0.2 mm thick) denaturing polyacrylamide gels. Each larva was genotyped twice; adults were genotyped three to five times each. The paternity of each larva was then determined from the genotype combinations.

We collected paternity data (and inferred fertilization success) from a total of four matings (three using eggs of *A. forbesi* and one using eggs of *A. rubens*) under sperm competition conditions. Replication was limited by low fertilization rates in some positive controls and by the low polymorphism of the only co-dominant genetic marker that we could use to positively assign paternity in sperm competition conditions.

To compare the ratio of the number of offspring expected for each male if there was no preference for conspecific sperm (10:1 for high–low sperm concentrations) with the ratio of the number observed, we used a *G*-test for goodness of fit (also known as the log-likelihood ratio test, Sokal and Rohlf, 1995), corrected according to Williams (1976). Adjusted values of *G* were then compared with the χ^2 distribution to test for significant differences at $\alpha = 0.05$.

Rearing of F_1 offspring and backcross studies

Larvae produced from the conspecific and heterospecific crosses in the single-male cross-fertilization studies were raised in 4-1 glass jars containing 3 l of FSW (initial larval density 1–3 ml^{-1}). Cultures were stirred using a rotating paddle system (Strathmann, 1987) at a speed of 10 rpm. Larvae were fed a dense mixture of cultured algae (*Dunaliella, Isochrysis,* and *Rhodomonas*) every 2–3 days, at which time half the volume of FSW was removed by siphoning and replaced with fresh FSW. A biological film was allowed to grow on the inner surface of the glass jars.

After 8 weeks, brachiolaria larvae with well-developed juvenile rudiments were induced to settle and metamorphose by addition of small cultured mussels (*Mytilus edulis*) and fresh pieces of macroalgae (*Ulva* spp.) to the jars (L. Harris, University of New Hampshire, pers. comm.). Postsettlement juveniles were transferred to individual 250-ml containers preconditioned with the algal mixture to form a biological film. Small pieces of *Ulva* spp. provided additional substrate. Initially, juveniles were fed cultured *Mytilus edulis* spat; larger animals were fed field-collected mussels (*Mytilus* spp. and *Modiolus modiolus*). Filtered seawater was replaced every 2–3 days, and cultures were maintained at 12 °C.

After one year, each animal was visually examined for gonadal development by making a small incision along the dorsal margin of one side of the proximal part of an arm. Since gonads were not present in any individual, all animals were returned to culture. In their second year, juveniles were reared in individual containers in a flow-through tank at ambient seawater temperatures and fed mussels (*Mytilus* spp.) *ad libitum*. Unfortunately, a tank failure resulted in the subsequent loss of most of these juveniles. The two surviving animals, an FR hybrid and an RF hybrid, were reared until they reached sexual maturity at 2 years of age.

Backcross experiments were conducted with each of the surviving hybrids. Gametes were collected as described above and combined at concentrations of 200 eggs ml^{-1} and 200 sperm μl^{-1} in total volumes of 2 and 25 ml FSW at 12 °C. The FR hybrid was male and was crossed separately with an *A. forbesi* female and an *A. rubens* female. The RF hybrid was female and was crossed separately with an *A. forbesi* male and an *A. rubens* male. The development of embryos in the 2-ml containers was arrested after 2 h with 1–2 drops of formalin. Fertilization was scored by sampling

the first 200 undamaged eggs encountered and counting the number that had fertilization envelopes. After 48 h, larvae in the 25-ml containers were transferred to 125 ml of FSW and fed 2 ml of dense *Isochrysis* algae. Larvae were examined daily for evidence of feeding (pigmented stomachs) and subsequently preserved in ethanol after 6 days of development for future studies.

Results

Single-male cross-fertilization studies

Conspecific and heterospecific sperm successfully fertilized eggs of both *Asterias forbesi* and *A. rubens,* but the compatibility of the heterospecific gametes (measured as F_{50}) showed considerable quantitative variation. The proportion of fertilized eggs increased rapidly as sperm concentration increased. Fertilization success was low at sperm concentrations less than about 1 sperm μl^{-1} and was typically greater than 90% at concentrations above 100 sperm μl^{-1}, producing a sigmoid curve when fertilization was plotted against sperm concentration (Fig. 1).

To compare fertilization success among the conspecific and heterospecific crosses, F_{50} values were estimated from curves of the nonlinear fertilization model (Fig. 2) and linear regressions of logit-transformed fertilization data (Fig. 3). The model of Vogel *et al.* (1982) fit the fertilization data well for both indices of fertilization success (mean $R^2 = 0.986$ for fertilization envelopes and 0.901 for cell cleavage). The linear regressions of logit-transformed fertilization success scored on the presence of fertilization envelopes fit the data well (mean $R^2 = 0.884$), but regressions of fertilization success scored using cell cleavage did not (mean $R^2 = 0.526$). F_{50} concentrations estimated from both indices using the nonlinear curves were highly correlated ($r = 0.838$, $P < 0.001$), as were analyses of fertilization envelope data estimated using both methods ($r = 0.613$, $P < 0.005$).

In conspecific crosses, mean F_{50} sperm concentrations for *A. forbesi* ranged from 32 to 72 sperm μl^{-1} depending upon

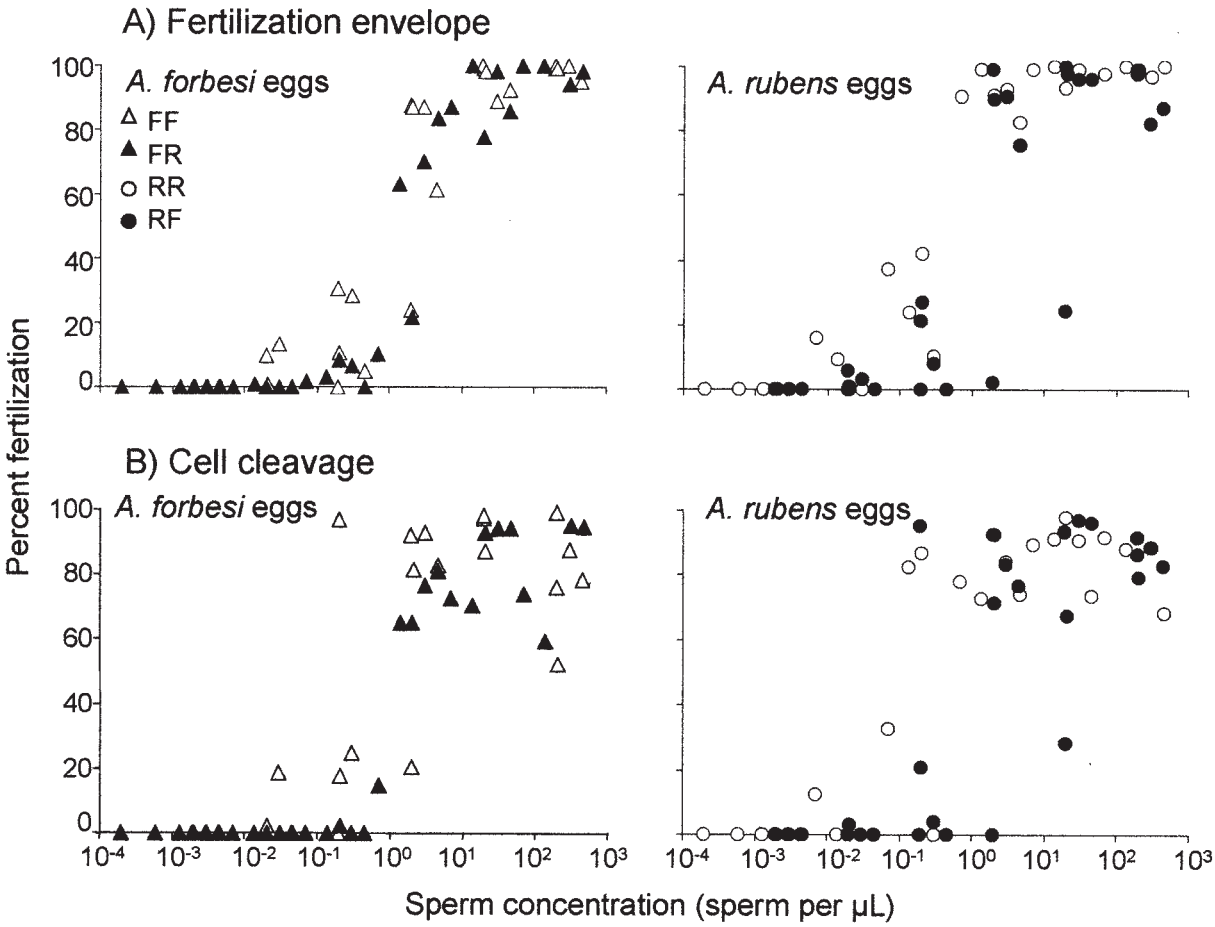

Figure 1. Results from conspecific and heterospecific cross-fertilization experiments across a range of sperm concentrations. Fertilization success scored on (A) presence of fertilization envelope after 1 h; (B) cell cleavage after 3 h. In the legend, F refers to *Asterias forbesi;* R refers to *A. rubens;* the female parent is indicated first, then the male parent. Five replicates for each cross FF, FR, RR, RF.

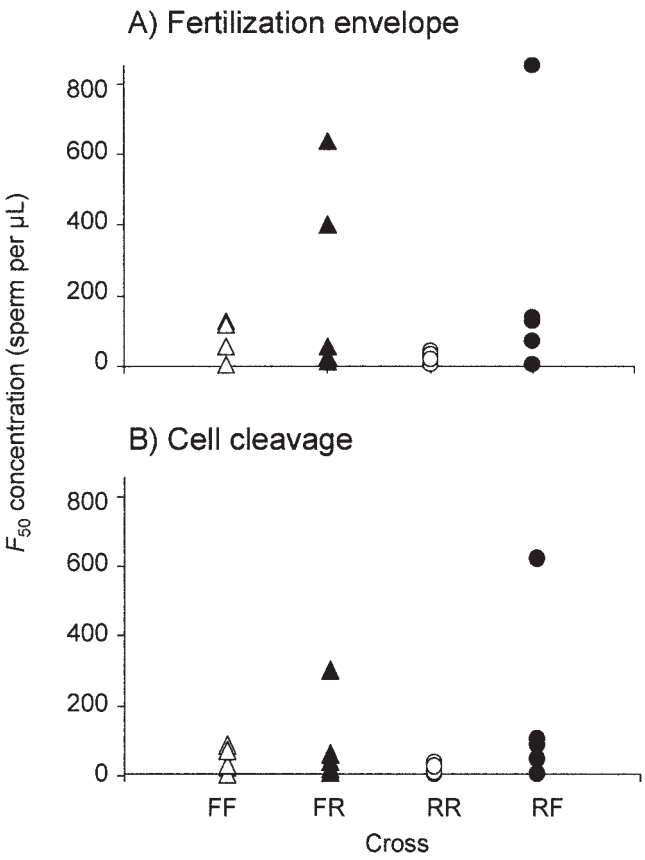

Figure 2. Sperm concentrations required to fertilize 50% of conspecific and heterospecific eggs estimated using a nonlinear fertilization kinetics model (Vogel *et al.,* 1982). Fertilization success scored on (A) presence of fertilization envelope after 1 h, (B) cell cleavage after 3 h. Five replicates for each cross FF, FR, RR, RF. Symbols as in Fig. 1.

the analytic method, while the conspecific *A. rubens* crosses varied from 16 to 25 sperm μl^{-1} (Table 1). In heterospecific crosses, *A. forbesi* eggs required 2 to 3 times more *A. rubens* sperm (measured as F_{50} values) for fertilization than with conspecific sperm, while *A. rubens* eggs required about 10 times more *A. forbesi* sperm than with conspecific sperm.

Some combinations of heterospecific gametes had F_{50} values comparable to those of conspecific crosses, but other combinations required an order of magnitude more heterospecific sperm for fertilization of 50% of the eggs. Females of both *A. rubens* and *A. forbesi* differed by an order of magnitude in their receptivity towards heterospecific sperm (Figs. 2, 3). The variance in the estimates of F_{50} for the heterospecific crosses was consistently much higher than the variance in the conspecific crosses (Table 1). As a result of this substantial variation among families within particular types of conspecific and heterospecific crosses, our quantitative tests did not indicate significantly different F_{50} values for conspecific *versus* heterospecific sperm fertilizing the eggs of either *A. forbesi* or *A. rubens* (in all two-sample randomization tests, estimated P values $>$ 0.05).

Both conspecific and heterospecific crosses showed evidence of polyspermy when sperm concentrations exceeded about 300 sperm μl^{-1} (Fig. 1). Although fertilization success did not decline at high sperm concentrations when scored from fertilization envelopes, fertilization decreased in most replicates when cell cleavage was scored. Cleavage was absent in some eggs with fertilization envelopes, and others showed irregular cleavage patterns. The F_{50} values estimated using the Vogel model were consistently lower when success was scored using cell cleavage than when fertilization envelopes were scored. As noted above, the fit of linear regressions to cell cleavage data was poor (R^2 = 0.526) Thus, in both the nonlinear fertilization model and the logit-transformation linear regression, the fits of the analyses to the data based on cleavage-stage embryos (and the inferred F_{50} values) may have been affected by decreased fertilization at high sperm concentrations.

Sperm competition studies

Of the 576 larvae collected, 455 were genotyped (79%). The remaining 21% yielded no amplifiable *Ar50* alleles from repeated PCR attempts, perhaps due to degradation of some larvae or impurities in the DNA extraction (Huvet *et*

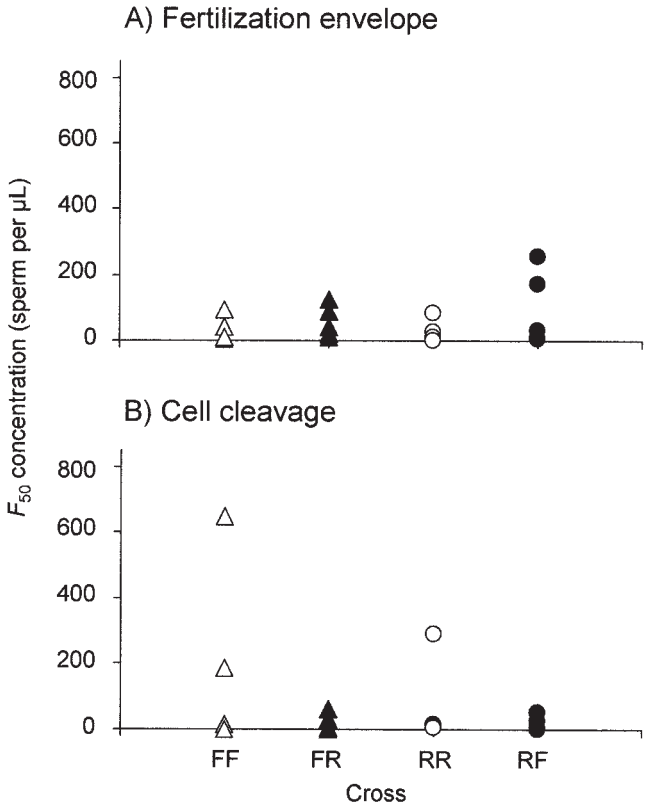

Figure 3. Sperm concentrations required to fertilize 50% of conspecific and heterospecific eggs estimated from linear regressions. Fertilization success scored on (A) presence of fertilization envelope after 1 h; (B) cell cleavage after 3 h.

Table 1

Comparison of mean F_{50} values (s.d.) among the conspecific and heterospecific crosses of Asterias *spp. calculated from nonlinear fertilization curves and linear regression*

Analysis	Index of fertilization success[1]	Female	Male	*n*	F_{50} (s.d.)	F_{50} ratio
Nonlinear fertilization curves	Fertilization envelope	*A. forbesi*	*A. forbesi*	5	71.50 (49.9)	
		A. forbesi	*A. rubens*	5	227.64 (279.5)	$\overline{3.2}$
		A. rubens	*A. rubens*	5	24.79 (14.5)	
		A. rubens	*A. forbesi*	5	238.13 (345.5)	$\overline{9.6}$
	Cell cleavage	*A. forbesi*	*A. forbesi*	5	39.85 (33.8)	
		A. forbesi	*A. rubens*	5	82.68 (120.9)	$\overline{2.1}$
		A. rubens	*A. rubens*	5	16.04 (12.5)	
		A. rubens	*A. forbesi*	5	168.25 (254.2)	$\overline{10.5}$
Linear regression[2]	Fertilization envelope	*A. forbesi*	*A. forbesi*	5	32.83 (36.25)	
		A. forbesi	*A. rubens*	5	58.15 (49.69)	$\overline{1.8}$
		A. rubens	*A. rubens*	5	24.40 (34.1)	
		A. rubens	*A. forbesi*	5	94.65 (112.6)	$\overline{3.9}$

[1] Fertilization data scored using two indices: presence of the fertilization envelope and cell cleavage.

[2] Due to the poor fit of the linear regressions to the fertilization data scored on the basis of cell cleavage (mean $R^2 = 0.526$), the mean F_{50} values for this analysis are not included above.

al., 2001). No nonparental alleles were observed in the larvae.

In the absence of sperm precedence and gametic incompatibility, paternity rates for conspecific and heterospecific males were expected to be proportional to sperm concentrations. The range of sperm concentrations (20–200 sperm μl^{-1}) included the typical conspecific F_{50} values (16–72 sperm μl^{-1}; Table 1) so that the relative fertilization success of the two males in competition was not expected to be significantly different from the 10:1 ratio of sperm concentrations.

Paternity rates among offspring from eggs of *A. forbesi* mixed with conspecific sperm in high concentration and heterospecific sperm in low concentration conformed to the expected 10:1 ratio in two out of three families (Table 2, Fig. 4). In the third family, all of the 63 offspring were sired by conspecific sperm, which suggests that abundant *A. forbesi* sperm were more effective than expected in competition with scarce *A. rubens* sperm. When the relative sperm concentrations were reversed, *A. forbesi* eggs mixed with high concentrations of heterospecific (and low concentrations of conspecific) sperm were significantly more likely to be fertilized by *A. forbesi* sperm in two families. In the third family, 89% of *A. forbesi* eggs were fertilized by *A. rubens* sperm, and this percentage was not significantly different from the expectation based on relative sperm concentrations. These results together suggest *A. forbesi* sperm precedence in fertilizing some clutches of *A. forbesi* eggs, in the presence of low or high concentrations of heterospecific sperm.

No evidence of conspecific preferential fertilization was detected in sperm competition studies using one clutch of *A. rubens* eggs. *A. forbesi* sperm at low concentration sired 82% of the offspring (significantly more than the expected 9%; Fig. 5). When the relative sperm concentrations were reversed (high heterospecific sperm concentration), most offspring again were sired by the same *A. forbesi* male, but the proportions were not significantly different from the expectation based on sperm concentration ($P > 0.05$, Table 2).

F_1 offspring and backcross studies

All conspecific and heterospecific crosses produced viable feeding larvae. There was high mortality in all culture jars, but larvae from at least one replicate of each conspecific and heterospecific cross were able to successfully settle and metamorphose. Larvae settled over an extended period from 8 to 13 weeks after fertilization.

Survival of F_1 juvenile offspring from both heterospecific and conspecific crosses decreased over time. After one year, 12 juveniles remained: 3 from each of the FF and RR crosses, 1 from the RF cross, and 5 from the FR cross. The animals ranged in size from 30 to 40 mm from the tip of the longest arm to the opposite interradius. Due to the small size of the animals, the species diagnostic characters (Clark and Downey, 1992) could not be reliably discerned.

Two hybrids survived to 2 years of age: one was a female RF hybrid; the other was a male FR hybrid. Both hybrids exhibited the orange madreporite and firm body typical of *A. forbesi,* and the long slender abactinal spines with a wreath of minor pedicellariae halfway up these spines typical of *A. rubens*. The only character that differed between the two was the major pedicellariae: the FR hybrid had the short, broad pedicellariae of *A. forbesi,* and the RF hybrid had pedicellariae of an intermediate character state, broad and pointed. Both animals produced viable gametes and were successfully backcrossed to field-collected adults of both species.

Table 2

Results of microsatellite DNA paternity analyses of 5-day-old bipinnaria larvae produced in crosses of eggs with combinations of conspecific and heterospecific sperm

Family	Cross	*n*	Observed paternity	Expected paternity	G_{adj}
F1	HF	63	$F = 63$	$10/11 \times 63 = 57.3$	11.8*
			$R = 0$	$1/11 \times 63 = 5.73$	
	HR	17	$R = 3$	$10/11 \times 17 = 15.45$	60.99*
			$F = 14$	$1/11 \times 17 = 1.55$	
F2	HF	74	$F = 67$	$10/11 \times 74 = 67.3$	0.04
			$R = 7$	$1/11 \times 74 = 6.73$	
	HR	66	$R = 42$	$10/11 \times 66 = 60$	36.3*
			$F = 24$	$1/11 \times 66 = 6$	
F3	HF	57	$F = 49$	$10/11 \times 57 = 51.8$	1.44
			$R = 8$	$1/11 \times 57 = 5.2$	
	HR	62	$R = 55$	$10/11 \times 62 = 56.4$	0.25
			$F = 7$	$1/11 \times 62 = 5.64$	
R1	HR	74	R = 13	$10/11 \times 74 = 67.3$	224.85*
			$F = 61$	$1/11 \times 74 = 6.72$	
	HF	49	$F = 45$	$10/11 \times 49 = 44.5$	0.16
			$R = 4$	$1/11 \times 49 = 4.45$	

Three families were constructed with *Asterias forbesi* females (F1–F3); one family was constructed with an *A. rubens* female (R1). HF refers to a 10:1 concentration of *A. forbesi* sperm to *A. rubens* sperm; HR refers to a 10:1 concentration of *A. rubens* sperm to *A. forbesi* sperm.

n is the number of larvae genotyped.

* = $P < 0.001$.

Under conditions that typically yielded 100% conspecific fertilization in studies reported here (egg concentrations of 200 eggs ml^{-1} and relatively high sperm concentrations of 200 sperm μl^{-1}), fertilization success for eggs of the hybrid female RF was 19% when backcrossed to a male *A. rubens* and 47% when backcrossed to a male *A. forbesi*. Similarly, the hybrid male FR fertilized 10% of eggs when backcrossed to a female *A. rubens* and 36% of eggs when backcrossed to a female *A. forbesi*. Although the sample of replicate backcrosses was small, in both cases gametes of F_1 hybrid offspring produced higher fertilization rates in backcrosses with *A. forbesi* than with *A. rubens*. All backcrosses produced viable, feeding bipinnaria larvae.

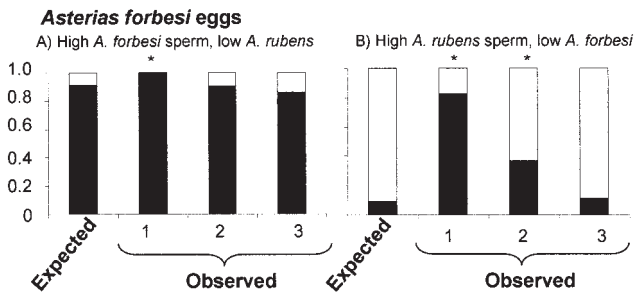

Figure 4. Paternity rates of *Asterias forbesi* eggs in sperm competition studies (based on inheritance of *Ar50* alleles). Offspring sired by *A. forbesi* are shaded in black and offspring sired by *A. rubens* are in white. (A) Crosses in which conspecific sperm are 10 times more abundant than heterospecific; and (B) crosses in which heterospecific sperm are 10 times more abundant than conspecific. Results for three families are shown. Expected rates are calculated on the basis of no preference for conspecific sperm. Crosses in which observed and expected frequencies were significantly different are indicated by * ($P < 0.001$).

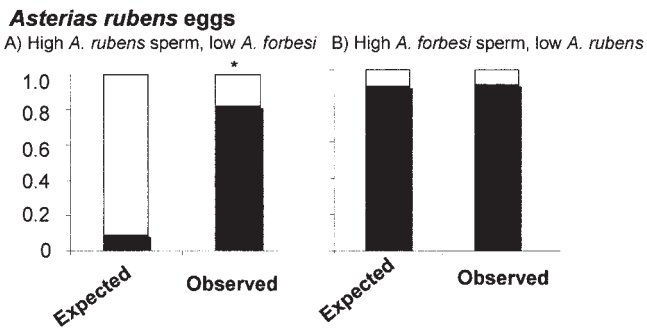

Figure 5. Paternity rates of *Asterias rubens* eggs in sperm competition studies (based on inheritance of *Ar50* alleles). Offspring sired by *A. forbesi* are shaded in black and offspring sired by *A. rubens* are in white. (A) Cross in which conspecific sperm are 10 times more abundant than heterospecific; and (B) cross in which heterospecific sperm are 10 times more abundant than conspecific. Expected rates are calculated on the basis of no preference for conspecific sperm. Crosses in which observed and expected frequencies were significantly different are indicated by *($P < 0.001$).

Discussion

Our results show that, in qualitative terms, *Asterias forbesi* and *A. rubens* have compatible gametes that can produce viable, fertile hybrid offspring without obvious pre- or post-zygotic barriers to hybridization. However, quantitative comparisons of fertilization kinetics in single-male heterospecific crosses and of paternity rates in two-male sperm competition experiments suggest that gametic interactions partly limit the success of heterospecific sperm and contribute to the reproductive isolation of sympatric populations of the two species.

Postzygotic barriers to hybridization

Hybrid inviability and sterility do not appear to be effective postzygotic barriers to hybridization in *A. rubens* and *A. forbesi*. At least some hybrid offspring produced from heterospecific crosses in both directions were viable and fertile, and were successfully backcrossed with parental species. Growth and development of the hybrids in culture was comparable to that recorded for *A. rubens* in the field (Nichols and Barker, 1984). In that study, juveniles in the northeast Atlantic reached an average diameter of 28.5 mm in the first year and became sexually mature in their second year at about 50 mm diameter. We did not measure hybrid fitness, which may be reduced relative to conspecific crosses under natural conditions. Although F_1 hybrids are viable and fertile, the fitness of these individuals relative to the offspring of conspecific parents is unknown, and the importance of hybrid dysfunction in limiting hybridization could not be determined. We were unable to compare the fertility of these two hybrid F_1 individuals to that of laboratory-reared nonhybrid offspring, and it is possible that the fertility of all offspring reared in the laboratory is low compared to that of adults collected from the field. In the absence of striking and obvious inviability or infertility of hybrids, we focused on possible prezygotic barriers produced by gamete incompatibility. However, if the mild gamete incompatibilities that we observed in this study have arisen through reinforcement (as a response to selection against low fitness of hybrid offspring), then more detailed studies of the growth and fecundity of hybrids might be warranted.

Single-male cross-fertilization studies

Examples of complete gamete incompatibility and asymmetric compatibility are common (review in Palumbi, 1994), but complete gamete compatibility is rare. In echinoderms, reciprocal gamete compatibility has been reported in laboratory crosses between congeneric sea urchins (*Echinometra:* Lessios and Cunningham, 1990; Rahman *et al.,* 2001; McCartney and Lessios, 2002; *Diadema:* Uehara *et al.,* 1990; *Pseudechinus:* McClary and Sewell, 2003; *Strongylocentrotus:* Levitan, 2002; *Arbacia:* Metz *et al.,* 1998) and a sea star (*Patiriella:* Byrne and Anderson, 1994). In our single-male cross-fertilization studies of sympatric *Asterias rubens* and *A. forbesi,* all combinations of gametes resulted in successful fertilization, but the compatibility of heterospecific gametes was highly variable. In some combinations, heterospecific gametes were reciprocally compatible: to achieve 50% fertilization of eggs, they required sperm concentrations similar to those needed by conspecific gametes. In contrast, other combinations of heterospecific gametes required an order of magnitude more sperm for successful fertilization.

Fertilization success in all conspecific and heterospecific crosses of *Asterias* spp. was affected by the sperm concentration, increasing rapidly from near zero at 0.1 sperm μl^{-1} to greater than 90% in most crosses when sperm concentrations were above 100 sperm μl^{-1}. The conspecific F_{50} values estimated for *Asterias* spp. were comparable to estimates for other echinoderm species (Table 3). Typically, sperm concentrations in the range of 10–100 sperm μl^{-1} were required to fertilize 50% of the conspecific eggs. Mean sperm concentrations required to fertilize 50% of eggs were about 3 times higher for heterospecific crosses with *A. forbesi* eggs and about 10 times higher with *A. rubens* eggs compared with conspecific crosses. Although hybrid crosses did not require significantly more sperm for fertilization, the results were confounded by considerable variation in the receptivity of the females of both *A. forbesi* and *A. rubens* to heterospecific sperm.

The variation in receptivity of *Asterias* spp. eggs to heterospecific sperm may be the result of intraspecific variation at compatibility loci. This suggestion has been proposed for both the sea urchin *Echinometra lucunter* (McCartney and Lessios, 2002) and the mussel *Mytilus edulis* (Rawson *et al.,* 2003). In a cross-fertilization study, females of *E. lucunter* varied by orders of magnitude in their discrimination against sperm from *E. vanbrunti* and *E. viridis*. Similarly, *M. edulis* eggs varied considerably in their receptivity to *M. trossulus* sperm. As was also the case in *Asterias,* however, females that required more heterospecific sperm did not also require more conspecific sperm, which would have indicated differences in gamete quality.

The high variance in heterospecific crosses may have also been the result of inter-individual variation in fertilizability. In a factorial experiment crossing multiple male and female sea urchins (*Heliocidaris erythrogramma*), Evans and Marshall (2005) observed male × female interactions influenced fertilization success. Their experiments crossing two females with the same four males revealed a strong significant female effect on fertilization success, as well as a significant male × female interaction. Heterospecific fertilization success in *Asterias* should be examined by conducting crosses with multiple conspecific and heterospecific males to determine whether the observed variation is the result of interactions between specific gametes or whether

Table 3

Some estimates of the concentration of sperm required to fertilize 50% of conspecific and heterospecific eggs (F_{50}) in echinoderm species

Maternal species	Conspecific F_{50} (sperm · μl^{-1})	Heterospecific F_{50} (sperm · μL^{-1})	F_{50} ratio	Model to estimate F_{50}	Reference
Strongylocentrotus droebachiensis	10^1	3.2×10^2 (average for *S. franciscanus* and *S. purpuratus*)	30	Vogel *et al.* (1982)	Levitan (2002)
S. franciscanus	10^2	2.5×10^6 (average for *S. droebachiensis* and *S. purpuratus*)	10^4		
S. purpuratus	10^2	2×10^7 (average for *S. franciscanus* and *S. droebachiensis*)	10^5		
Echinometra lucunter	94	7×10^3 (*E. viridis*)	100	Both linear regression & Vogel *et al.* (1982)	McCartney & Lessios (2002)
		1.6×10^5 (*E. vanbrunti*)	10^4		
E. viridis	62	1.1×10^3 (*E. vanbrunti*)	20		
		1.4×10^2 (*E. lucunter*)	30		
E. vanbrunti	83	2×10^2 (*E. lucunter*)	2		
		81 (*E. viridis*)	1		
Pseudechinus huttoni	50	30 (*P. albocinctus*)	0.6	Data modeled using 4-parameter logistic equation	McClary & Sewell (2003)
		30 (*P. novaezealandiae*)	0.6		
P. albocinctus	80	10 (*P. huttoni*)	0.25		
		10^3 (*P. novaezealandiae*)	10		
P. novaezealandiae	10^3	$> 10^5$ (*P. huttoni*)	100		
		$> 10^5$ (*P. albocinctus*)	100		
Asterias forbesi	72	228 (*A. rubens*)	3	Vogel *et al.* (1982)	This study
A. rubens	25	238 (*A. forbesi*)	10		

some females are more or less fertilizable regardless of the heterospecific male involved.

Many studies of heterospecific fertilization compare fertilization success at single sperm and egg concentrations (*e.g.,* Lessios and Cunningham, 1990; Uehara *et al.,* 1990; Byrne and Anderson, 1994). Construction of fertilization curves using serial sperm dilution permits a quantitative assessment of gamete compatibility and increases the sensitivity of the experiments (McCartney and Lessios, 2002). We analyzed fertilization data using both the nonlinear kinetics model of Vogel *et al.* (1982) and the linear regression method of McCartney and Lessios (2002). When fertilization success was scored using fertilization envelopes, the estimates of F_{50} from both analytic methods were highly correlated. In contrast, the linear regression analysis of the cell cleavage data did not fit the data well compared with the nonlinear model. Using an empirically based logit transformation to linearize the sigmoidal curve involves fewer assumptions than fitting the nonlinear fertilization model developed for intraspecific fertilization studies (McCartney and Lessios, 2002). However, the theoretically based Vogel *et al.* (1982) model is sufficiently parameter-rich to fit complex data from experiments that differ in several factors simultaneously (such as sperm and egg concentrations), even for heterospecific crosses in which the two species differ in some model parameters (Levitan, 2002).

Two indices of fertilization success, the presence of a raised fertilization envelope and cell cleavage, were measured to account for possible influences of polyspermy at high sperm concentrations on the estimated F_{50} values. Some studies have used both criteria to score fertilization (Vogel *et al.,* 1982; Levitan *et al.,* 1991; Levitan, 2002), whereas other studies have used only cell cleavage (McCartney and Lessios, 2002). In studies of corals, Oliver and Babcock (1992) scored only cleaving embryos and found decreased fertilization at high sperm concentrations, presumably the result of polyspermy. In the *Asterias* crosses, cell cleavage was absent in some eggs fertilized at sperm concentrations above 300 sperm μl^{-1}, and irregular patterns of cleavage were observed in other eggs that had raised fertilization envelopes. In a study of fertilization dynamics in the sea star *Acanthaster planci,* many eggs fertilized at concentrations of 100 sperm μl^{-1} showed abnormal divisions after 4–5 h and either did not complete gastrulation or did not develop into larvae (Benzie and Dixon, 1994). Further analyses should evaluate fertilization data using the

polyspermy-adjusted models developed by Styan (1998) to take into account multiple sperm-egg contacts.

Sperm competition studies

The gametes of *A. forbesi* and *A. rubens* have been reported to be reciprocally compatible (Ernst, 1967) and, indeed, some of our crosses between single males and single females supported this assertion. However, gametes of these two species were not reciprocally compatible when combined in our sperm competition studies. Although the replication of these experiments was low, our results suggest conspecific sperm preference in *A. forbesi*. In two cases, significantly more *A. forbesi* eggs were fertilized by conspecific sperm when *A. rubens* sperm were an order of magnitude more abundant in mixed suspension. In one replicate in which *A. forbesi* sperm were more abundant, no heterospecific fertilization of *A. forbesi* eggs was observed. However, *A. rubens* sperm were capable of fertilizing *A. forbesi* eggs, even when conspecific sperm were more abundant. Possible explanations for this partial species-specificity include differences in the gamete quality of the two species, and intraspecific variation in compatibility of sperm and egg recognition proteins (Palumbi, 1999; Levitan, 2002). It is not possible to distinguish among possible explanations of the partial species-specificity observed here for *A. forbesi*.

Although sperm of *A. forbesi* were competitively superior to those of *A. rubens* in fertilizing *A. forbesi* eggs, there was no evidence for conspecific fertilization precedence in *A. rubens* (in the single family we analyzed). We found, instead, that significantly more *A. rubens* eggs were fertilized by *A. forbesi* than by conspecific sperm. Differences in the quality of gamete characteristics such as sperm motility may account for this observation. Variation in sperm motility in multifactorial crosses of the oyster *Crassostrea gigas* has been suggested as an explanation for large variance in reproductive success (Boudry *et al.,* 2002). In addition, the condition of the male may have influenced the quality of the sperm: because this study was conducted in late July at the end of the spawning season for *A. rubens* in Nova Scotia, *A. rubens* sperm may have been of poorer quality than *A. forbesi* sperm. Replication of this result is clearly necessary.

Differential viability of hybrid offspring may have been a factor in this study, and it may be a significant source of error in all studies of sperm precedence that require growth and development of hybrid offspring before genotyping. Low hybrid viability could result in significantly greater observed conspecific paternity. While this explanation is plausible, the fact that some heterospecific paternity rates were in proportion to the sperm concentrations suggests that abortion of hybrid larvae was not extensive in the present study. Furthermore, our successful rearing of F_1 hybrid offspring through metamorphosis to sexual maturity indicates that at least some hybrids are fully viable and fertile.

Sperm competition can occur from the initial release of the sperm to the fusion of the sperm and egg membranes. Species-specific chemoattractants produced by eggs can play a role in gamete recognition by activating the motility of conspecific sperm and causing chemotaxis toward the egg (Miller, 1985a, 1997). Among echinoderms, specificity of sperm chemotaxis has been found mainly at the family level in holothurians and at the genus or species levels in ophiuroids (Miller, 1997). Within the asteroids, the families Asteriidae and Solasteridae have shown sperm attractant cross-reactivity between them, but not with any other asteroid families tested (Miller, 1985b). Since sperm chemotaxis is not known to be species-specific within asteroid families (Miller, 1985b), this mechanism is unlikely to have been responsible for the differential fertilization success of *A. forbesi* and *A. rubens* in this study.

Several gamete recognition systems have been identified and recognized for their roles in conspecific fertilization (reviews in Vacquier *et al.,* 1995; Vacquier, 1998; Swanson and Vacquier, 2002). In sea urchins, the sperm protein bindin is involved in species-specific sperm-egg attachment and sperm-egg fusion (Glabe and Vacquier, 1977; Glabe and Lennarz, 1979; Metz *et al.,* 1994). Barriers to cross-fertilization have been examined using bindin divergence; rapid divergence has been observed in some genera (*Echinometra:* Metz and Palumbi, 1996; *Strongylocentrotus:* Biermann, 1998; *Heliocardis:* Zigler *et al.,* 2003), but bindin divergence is much slower in other echinoid genera (*Arbacia:* Metz *et al.,* 1998; *Tripneustes:* Zigler and Lessios, 2003) In abalone (*Haliotus*) and teguline gastropods (*Tegula*), sperm release a soluble protein called lysin that allows the sperm to penetrate the egg envelope in a species-specific manner (Vacquier *et al.,* 1990; Shaw *et al.,* 1994; Hellberg and Vacquier, 1999). Abalone sperm also release a protein (sp18) that is thought to mediate fusion of sperm and egg (Swanson and Vacquier, 1995; Kresge *et al.,* 2001). A possible non-protein recognition system involving sulfated carbohydrates from egg jelly has recently been described in the urchins *Strongylocentrotus droebachiensis* and *S. pallidus* (Biermann *et al.,* 2004). Although a sperm-activating peptide released by sea star eggs (asterosap) and its protein receptor have been identified in *Asterias amurensis* (Nishigaki *et al.,* 2000; Matsumoto *et al.,* 2003), gamete recognition systems have not been explored in sea stars. Such sperm-egg molecular interactions could explain the conspecific sperm precedence demonstrated by *A. forbesi* in this study.

In a comparable study, Geyer and Palumbi (2005) reported conspecific sperm precedence in two *Echinometra* sea urchin species. As in *Asterias,* they found high gamete compatibility in single-male hybridizations (or no-choice experiments) but numerous examples of relatively higher conspecific fertilization in sperm competition. Pairs of hybridizing *Echinometra* individuals (23 in total) varied widely in inferred gamete compatibility under sperm com-

petition, and the authors tentatively concluded that conspecific sperm precedence and among-family variation in the strength of this precedence could be attributed to underlying genetic variation at the loci encoding bindin (expressed on sperm) or the bindin receptor (on eggs). We found among-family variation in conspecific sperm precedence in *Asterias* (though in fewer families), but we also found within-family variation associated with different conspecific and heterospecific sperm concentrations (Fig. 4, family 2; Fig. 5). We also suggest that such patterns could be explained by variation in gamete recognition loci and complex quantitative interactions between their gene products. Geyer and Palumbi (2005) suggested that conspecific sperm precedence might largely explain the rarity of *Echinometra* hybrids in sympatric populations. In contrast, we have found several examples of introgression between *Asterias* species (F. Harper, unpubl. data) that suggest incomplete reproductive isolation in spite of apparently strong conspecific sperm precedence in *A. forbesi.*

In several groups of congeneric echinoderm species in sympatry (*i.e.,* the sea stars *Acanthaster* and *Patiriella,* and the sea urchin *Strongylocentrotus*), genetic divergence and speciation is believed to have occurred without the evolution of gamete incompatibility (Lucas and Jones, 1976; Strathmann, 1981; Byrne and Anderson, 1994). However, these studies were not conducted using multiple males simultaneously (sperm competition; Parker, 1970). In a review of conspecific sperm precedence (the differential success of conspecific sperm in cases where conspecific and heterospecific males have contributed sperm to a female), Howard (1999) criticized single-male/single-female cross-fertilization studies as incapable of detecting differences between conspecific and heterospecific sperm in competition. Conspecific sperm and pollen precedence has been shown to be important in isolating closely related terrestrial taxa such as grasshoppers (Hewitt *et al.,* 1989; Bella *et al.,* 1992), beetles (Wade *et al.,* 1994), and ground crickets (Gregory and Howard, 1994). Indeed, the only reproductive barrier discovered to date in ground crickets is conspecific sperm precedence (Howard and Gregory, 1993; Gregory and Howard, 1994; Howard *et al.,* 1998; review in Howard, 1999). Although broadcast-spawning marine invertebrates cannot exhibit sperm precedence (*via* internal fertilization), gamete recognition and fertilization precedence could be mechanisms of reproductive isolation.

The need for gamete competition studies in marine invertebrates has been recognized (Grant *et al.,* 1998; McClary and Sewell, 2003), but as yet only a few studies have been conducted. In many cases, the offspring of conspecific and heterospecific fertilizations cannot be distinguished on the basis of their early developmental phenotypes. Molecular markers are needed to infer paternity from offspring genotypes (*e.g.,* Bierne *et al.,* 1998; Gerber *et al.,* 2000; Norris *et al.,* 2000). Conspecific fertilization precedence has been explored using microsatellites (in *Crassostrea* bivalves; Huvet *et al.,* 2001), intron-length polymorphisms (in *Mytilus* bivalves; Bierne *et al.,* 2002), and bindin coding sequences (in *Echinometra* sea urchins; Geyer and Palumbi, 2005). These studies suggest that some examples of heterospecific gametic compatibility are influenced by differences between conspecific and heterospecific sperm that are only detectable when heterospecific sperm compete with conspecific sires for fertilization of eggs. This general result suggests that many examples of apparent gamete compatibility between congeners (Table 3; see Geyer and Palumbi, 2005) should be revisited using sperm competition methods.

Acknowledgments

Thanks to J. Addison, A. Gillis, and the late D. Cook for advice and technical assistance in conducting the molecular biology portions of these studies, and to J. Addison, A. Gillis, and S. Watts for their help in collecting animals. We are grateful for thoughtful comments and statistical advice from C. Cunningham, R. Latta, and R. Scheibling. The comments of two anonymous reviewers improved this manuscript. We were supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canada Foundation for Innovation, by an NSERC postgraduate scholarship, by the Department of Biology at Dalhousie University, and by the Dr. Patrick Lett Fund.

Literature Cited

Bella, J. L., R. K. Butlin, C. Ferris, and G. M. Hewitt. 1992. Asymmetrical homogamy and unequal sex ratio from reciprocal mating-order crosses between *Chorthippus parallelus* subspecies. *Heredity* **68:** 345–352.

Benzie, J. A. H., and P. Dixon. 1994. The effects of sperm concentration, sperm:egg ratio, and gamete age on fertilization success in crown-of-thorns starfish (*Acanthaster planci*) in the laboratory. *Biol. Bull.* **186:** 139–152.

Biermann, C. H. 1998. The molecular evolution of sperm bindin in six species of sea urchins (Echinoidea: Strongylocentrotidae). *Mol. Biol. Evol.* **15:** 1761–1771.

Biermann, C. H., J. A. Marks, A.-C. E. S. Vilela-Silva, M. O. Castro, and P. A. S. Mourão. 2004. Carbohydrate-based species recognition in sea urchin fertilization: another avenue for speciation? *Evol. Dev.* **6:**353–361.

Bierne, N., S. Launey, Y. Naciri-Graven, and F. Bonhomme. 1998. Early effect of inbreeding as revealed by microsatellite analysis on *Ostrea edulis* larvae. *Genetics* **55:** 190–195.

Bierne, N., P. David, P. Boudry, and F. Bonhomme. 2002. Assortative fertilization and selection at larval stage in the mussels *Mytilus edulis* and *M. galloprovincialis. Evolution* **56:** 292–298.

Booloottian, R. A. 1966. Reproductive physiology. Pp. 561–614 in *Physiology of Echinodermata,* R.A. Booloottian, ed. Interscience, New York.

Boudry, P., B. Collet, F. Cornette, V. Hervouet, and F. Bonhomme. 2002. High variance in reproductive success of the Pacific oyster (*Crassostrea gigas,* Thunberg) revealed by microsatellite-based parentage analysis of multifactorial crosses. *Aquaculture* **204:** 283–296.

Byrne, M., and M. J. Anderson. 1994. Hybridization of sympatric *Patiriella* species (Echinodermata: Asteroidea) in New South Wales. *Evolution* **48:** 564–576.

Clark, H. L. 1904. The echinoderms of the Woods Hole region. *Bull. U.S. Fish Comm.* **22:** 547–576.

Clark, A. M., and M. E. Downey. 1992. *Starfishes of the Atlantic.* Chapman & Hall, New York.

Ernst, E. J. 1967. The distribution, ecology, environmental behavior and possible hybridization of the sea stars *Asterias forbesi* (Desor) and *Asterias vulgaris* Verrill in the subtidal zone of Long Island. Ph.D. dissertation, New York University. (University Microfilms #68-6060).

Evans, J. P., and D. J. Marshall. 2005. Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris erythrogramma. Evolution* **59:** 106–112.

Franz, D. R., E. K. Worley, and S. Merrill. 1981. Distribution patterns of common seastars of the Middle Atlantic continental shelf of the northwest Atlantic (Gulf of Maine to Cape Hatteras). *Biol. Bull.* **160:** 394–418.

Gardner, J. P. A. 1997. Hybridization in the sea. *Adv. Mar. Biol.* **31:** 1–78.

Gerber, S., S. Mariette, R. Streiff, C. Bodénès, and A. Kremer. 2000. Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. *Mol. Ecol.* **9:** 1037–1048.

Geyer, L. B., and S. R. Palumbi. 2005. Conspecific sperm precedence in two species of tropical sea urchins. *Evolution* **59:** 97–105.

Glabe, C. G., and W. J. Lennarz. 1979. Species-specific sperm adhesion in sea urchins. A quantitative investigation of bindin-mediated egg agglutination. *J. Cell. Biol.* **83:** 595–604.

Glabe, C. G., and V. D. Vacquier. 1977. Species specific agglutination of eggs by bindin isolated from sea urchin sperm. *Nature* **267:** 822–824.

Grant, C. M., S. H. Hooker, R. C. Babcock, and R. G. Creese. 1998. Synchronous spawning and reproductive incompatibility of two bivalve species: *Paphies subtriangulata* and *Paphies australis. Veliger* **41:** 148–156.

Gregory, P. G., and D. J. Howard. 1994. A postinsemination barrier to fertilization isolates two closely related ground crickets. *Evolution* **48:** 705–710.

Hamilton, M. B., E. L. Pincus, A. DiFiore, and R. C. Fleischer. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques* **27:** 500–507.

Harper, F. M. 2004. Sperm, spines, secondary contact and cytoplasmic introgression between sibling species of sea stars. Ph.D. dissertation, Dalhousie University, Nova Scotia, Canada.

Hellberg, M. E., and V. D. Vacquier. 1999. Rapid evolution of fertilization selectivity and lysin cDNA sequences in teguline gastropods. *Mol. Biol. Evol.* **16:** 839–848.

Hewitt, G. M., P. Mason, and R. A. Nichols. 1989. Sperm precedence and homogamy across a hybrid zone in the alpine grasshopper *Podisma pedestris. Heredity* **62:** 343–353.

Howard, D. J. 1999. Conspecific sperm and pollen precedence and speciation. *Annu. Rev. Ecol. Syst.* **30:** 109–132.

Howard, D. J., and P. G. Gregory. 1993. Postinsemination signaling systems and reinforcement. *Philos. Trans. R. Soc. Lond. B* **340:** 231–236.

Howard, D. J., P. G. Gregory, J. Chu, and M. L. Cain. 1998. Conspecific sperm precedence is an effective barrier to hybridization between closely related species. *Evolution* **52:** 511–516.

Huvet, A., K. Balabaud, N. Bierne, and P. Boudry. 2001. Microsatellite analysis of 6-hour-old embryos reveals no preferential intraspecific fertilization between cupped oysters *Crassostrea gigas* and *Crassostrea angulata. Mar. Biotechnol.* **3:** 448–453.

Kanatani, H. 1979. Hormones in echinoderms. Pp. 273–307 in *Hormones and Evolution,* Vol. 1, E.J.W. Barrington, ed. Academic Press, London.

Kresge, N., V. D. Vacquier, and C. D. Stout. 2001. Abalone lysin: the dissolving and evolving sperm protein. *Bioessays* **23:** 95–103.

Lessios, H. A., and C. W. Cunningham. 1990. Gametic incompatibility between species of the sea urchin *Echinometra* on the two sides of the Isthmus of Panama. *Evolution* **44:** 933–941.

Levitan, D. R. 1996. Effects of gamete traits on fertilization in the sea and the evolution of sexual dimorphism. *Nature* **382:** 153–155.

Levitan, D. R. 1998. Does Bateman's principle apply to broadcast-spawning organisms? Egg traits influence *in situ* fertilization rates among congeneric sea urchins. *Evolution* **52:** 1043–1056.

Levitan, D. R. 2002. The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. *Evolution* **56:** 1599–1609.

Levitan, D. R., M. A. Sewell, and F.-S. Chia. 1991. Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus:* interaction of gamete dilution, age, and contact time. *Biol. Bull.* **181:** 371–378.

Lucas, J. S., and M. M. Jones. 1976. Hybrid crown-of-thorns starfish (*Acanthaster planci* × *A. brevispinus*) reared to maturity in the laboratory. *Nature* **263:** 409–412.

Manly. B. F. J. 1991. *Randomization and Monte Carlo Methods in Biology.* Chapman and Hall, London.

Matsumoto, M., J. Solzin, A. Helbig, V. Hagen, S. Ueno, O. Kawase, Y. Maruyama, M. Ogiso, M. Godde, H. Minakata, U.B. Kaupp, M. Hoshi, and I. Weyand. 2003. A sperm-activating peptide controls a cGMP-signaling pathway in starfish sperm. *Dev. Biol.* **260:** 314–324.

McCartney, M. A., and H. A. Lessios. 2002. Quantitative analysis of gametic incompatibility between closely related species of neotropical sea urchins. *Biol. Bull.* **202:** 166–181.

McClary, D. J., and M. A. Sewell. 2003. Hybridization in the sea: gametic and developmental constraints on fertilization in sympatric species of *Pseudechinus* (Echinodermata: Echinoidea). *J. Exp. Mar. Biol. Ecol.* **284:** 51–70.

Menge, B. A. 1979. Coexistence between the seastars *Asterias vulgaris* and *A. forbesi* in a heterogeneous environment: a non-equilibrium explanation. *Oecologia* **41:** 245–272.

Menge, B. A. 1986. A preliminary study of the reproductive ecology of the seastars *Asterias vulgaris* and *A. forbesi* in New England. *Bull. Mar. Sci.* **39:** 467–476.

Metz, E. C., and S. R. Palumbi. 1996. Positive selection and sequence rearrangements generate extensive polymorphism in the gamete recognition protein bindin. *Mol. Biol. Evol.* **13:** 397–406.

Metz, E. C., R. E. Kane, H. Yanagimachi, and S. R. Palumbi. 1994. Fertilization between closely related sea urchins is blocked by incompatibilities during sperm-egg attachment and early stages of fusion. *Biol. Bull.* **187:** 23–34.

Metz, E. C., G. Gomez-Gutierrez, and V. D. Vacquier. 1998. Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia. Mol. Biol. Evol.* **15:** 185–195.

Miller, R. L. 1985a. Sperm chemo-orientation in the metazoa. Pp. 275–337 in *The Biology of Fertilization,* Vol. 2, C.B. Metz, Jr., and A. Monroy, eds. Academic Press, New York.

Miller, R. L. 1985b. Sperm chemotaxis in echinodermata: Asteroidea, Holothuroidea, Ophiuroidea. *J. Exp. Zool.* **234:** 383–414.

Miller, R. L. 1997. Specificity of sperm chemotaxis among Great Barrier Reef shallow-water holothurians and ophiuroids. *J. Exp. Zool.* **279:** 189–200.

Nichols, D., and M. F. Barker. 1984. Growth of juvenile *Asterias rubens* L. (Echinodermata: Asteroidea) on an intertidal reef in south-western Britain. *J. Exp. Mar. Biol. Ecol.* **78:** 157–165.

Nishigaki, T., K. Chiba, and M. Hoshi. 2000. A 130-kDa membrane protein of sperm flagella is the receptor for asterosaps, sperm-activating peptides of starfish *Asterias amurensis. Dev. Biol.* **219:** 154–162.

Norris, A. T., D. G. Bradley, and E. P. Cunningham. 2000. Parentage and relatedness determination in farmed Atlantic salmon (*Salmo salar*) using microsatellite markers. *Aquaculture* **182:** 73–83.

Oliver, J., and R. Babcock. 1992. Aspects of the fertilization ecology of

broadcast spawning corals: sperm dilution effects and *in situ* measurements of fertilization. *Biol. Bull.* **183:** 409–417.

Palumbi, S. R. 1994. Genetic divergence, reproductive isolation, and marine speciation. *Annu. Rev. Ecol. Syst.* **25:** 547–572.

Palumbi, S. R. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. *Proc. Nat. Acad. Sci. USA* **96:** 12632–12637.

Palumbi, S. R., and E. C. Metz. 1991. Strong reproductive isolation between closely related tropical sea urchins (genus *Echinometra*). *Mol. Biol. Evol.* **8:** 227–239.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. *Biol. Rev.* **45:** 525–567.

Perlmutter, A., and R. F. Nigrelli. 1960. A possible hybrid population of the starfish, *Asterias forbesi* (Desor), in western Long Island Sound. *Proc. Nat. Shellfish Assoc.* **51:** 93–96.

Pernet, B. 1999. Gamete interactions and genetic differentiation among three sympatric polychaetes. *Evolution* **53:** 435–446.

Rahman, M. A., T. Uehara, and J. S. Pearse. 2001. Hybrids of two closely related tropical sea urchins (Genus *Echinometra*): evidence against postzygotic isolating mechanisms. *Biol. Bull.* **200:** 97–106.

Rawson, P. D., C. Slaughter, and P. O. Yund. 2003. Patterns of gamete incompatibility between the blue mussels *Mytilus edulis* and *M. trossulus. Mar. Biol.* **143:** 317–325.

Schopf, T. J. M., and L. S. Murphy. 1973. Protein polymorphism of the hybridizing seastars *Asterias forbesi* and *Asterias vulgaris* and implications for their evolution. *Biol. Bull.* **145:** 589–597.

Shaw, A., Y.-H. Lee, D. Stout, and V. D. Vacquier. 1994. The species-specificity and structure of abalone sperm lysin. *Semin. Dev. Biol.* **5:** 209–215.

Smith, G. F. M. 1940. Factors limiting distribution and size in the starfish. *J. Fish. Res. Board Can.* **5:** 84–103.

Sokal, R. R., and F. J. Rohlf. 1995. *Biometry,* 3rd ed. W.H. Freeman, New York.

Strathmann, R. R. 1981. On barriers to hybridization between *Strongylocentrotus droebachiensis* (O.F. Muller) and *Strongylocentrotus pallidus* (G.O. Sars). *J. Exp. Mar. Biol. Ecol.* **55:** 39–47.

Strathmann, M. F. 1987. *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast.* University of Washington Press, Seattle.

Styan, C. A. 1998. Polyspermy, egg size, and the fertilization kinetics of free-spawning marine invertebrates. *Am. Nat.* **152:** 290–297.

Swanson, W. J., and V. D. Vacquier. 1995. Liposome fusion induced by a M(r) 18,000 protein localized to the acrosomal region of acrosome-reacted abalone spermatozoa. *Biochemistry* **34:** 14202–14208.

Swanson, W. J., and V. D. Vacquier. 2002. Reproductive protein evolution. *Annu. Rev. Ecol. Syst.* **33:** 161–179.

Tortonese, E. 1963. Differenziazione geografica e superspecie nel genere *Asterias* (Echinodermata). *Monit. Zool. Ital.* 70–71: 212–221.

Uehara, T., H. Asakura, and Y. Arakaki. 1990. Fertilization blockage and hybridization among species of sea urchins. Pp. 305–310 in *Advances in Invertebrate Reproduction,* M. Hoshi and O. Yamashita, eds. Elsevier, Amsterdam.

Vacquier, V. D. 1998. Evolution of gamete recognition proteins. *Science* **281:** 1995–1998.

Vacquier, V. D., K. R. Carner, and C. D. Stout. 1990. Species-specific sequences of abalone lysin, the sperm protein that creates a hole in the egg envelope. *Proc. Natl. Acad. Sci. USA* **87:** 5792–5796.

Vacquier, V. D., W. J. Swanson, and M. E. Hellberg. 1995. What have we learned about sea urchin sperm bindin? *Dev. Growth Differ.* **37:** 1–10.

Vogel, H., G. Czihak, P. Chang, and W. Wolf. 1982. Fertilization kinetics of sea urchin eggs. *Math. Biosci.* **58:** 189–216.

Wade, M. J., H. Patterson, N. W. Chang, and N. A. Johnson. 1994. Postcopulatory, prezygotic isolation in flour beetles. *Heredity* **72:** 163–167.

Walker, C. W. 1973. Morphology and histology of the reproductive system of *Asterias vulgaris.* Master's thesis, Cornell University, Ithaca, NY.

Wares, J. P. 2001. Biogeography of *Asterias:* north Atlantic climate change and speciation. *Biol. Bull.* **201:** 95–103.

Williams, D. A. 1976. Improved likelihood ratio tests for complete contingency tables. *Biometrika* **63:** 33–37.

Worley, E. K., and D. R. Franz. 1983. A comparative study of selected skeletal structures in the seastars *Asterias forbesi, A. vulgaris,* and *A. rubens* with a discussion of possible relationships. *Proc. Biol. Soc. Wash.* **96:** 524–547.

Yoder, J. A., S. E. Schollaert, and J. E. O'Reilly. 2002. Climatological phytoplankton chlorophyll and sea surface temperature patterns in continental shelf and slope water off the northeast US coast. *Limnol. Oceanogr.* **47:** 672–682.

Zigler, K. S., and H. A. Lessios. 2003. Evolution of bindin in the pantropical sea urchin *Tripneustes:* comparisons to bindin of other genera. *Mol. Biol. Evol.* **20:** 220–231.

Zigler, K. S., E. C. Raff, E. Popodi, R. A. Raff, and H. A. Lessios. 2003. Adaptive coevolution of bindin in the genus *Heliocidaris* is correlated with the shift to direct development. *Evolution* **57:** 2293–2302.