Quantitative Analysis of Gametic Incompatibility Between Closely Related Species of Neotropical Sea Urchins

MICHAEL A. McCARTNEY1 AND H. A. LESSIOS

Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama

Abstract. Species of the sea urchin genus Echinometra found on the two coasts of Panamá are recently diverged and only partially isolated by incomplete barriers to interspecific fertilization. This study confirms previous work that revealed incompatibility between the eggs of the Atlantic E. lucunter and the sperm of the other two neotropical species, whereas eggs of its sympatric congener E. viridis and allopatric E. vanbrunti are largely compatible with heterospecific sperm. Here we quantify fertilization using a range of sperm dilutions. We demonstrate a much stronger block to cross-species fertilization of E. lucunter eggs than was previously shown at fixed sperm concentrations, and mild incompatibility of the other two species' eggs where previous crosses between species were not distinguishable from within-species controls. Additionally, we present evidence for intraspecific variation in egg receptivity towards heterospecific sperm. Our findings here again discount the "reinforcement model" as a viable explanation for the pattern of prezygotic isolation. Gamete incompatibility in these Echinometra has appeared recently-within the last 1.5 million years—but is weaker in sympatry than in allopatry. Accidents of history may help explain why incompatibility of eggs emerged in one species and not in others. Compensatory sexual selection on sperm in this species could follow, and promote divergence of proteins mediating spermegg recognition.

Introduction

How reproductive isolation evolves between species of marine invertebrates that shed their eggs and sperm into the

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sea is a matter of considerable interest (Palumbi, 1992, 1994). Apart from asynchrony of gamete release and possible chemical communication between individuals, courtship and mating behaviors that could isolate species are largely absent in free-spawning organisms. When spawning seasons of coexisting closely related species overlap (Lessios, 1985) or when more than one species participates in a mass spawning event (e.g., Babcock et al., 1986; McCuen, 1988; Pearse et al., 1988), incompatibility between gametes may be the most likely prezygotic reproductive barrier. Cross-species in vitro fertilizations are then an effective method for obtaining quantitative data on the strength of blocks to fertilization. Such data are crucial for judging the contribution of gamete incompatibility to the maintenance of reproductive isolation between free-spawning species, and may also provide clues to how fertilization barriers evolve.

Gamete compatibility between species has been studied in hydroids (Buss and Yund, 1989), corals (Knowlton et al., 1997; Miller and Babcock, 1997; Szmant et al., 1997), polychaetes (Pawlik, 1988; Marsden, 1992; Pernet, 1999), oysters (Banks et al., 1994), abalones (Leighton and Lewis, 1982), sea urchins (Branham, 1972; Summers and Hylander, 1975; Strathmann, 1981; Lessios and Cunningham, 1990; Minor et al., 1991; Palumbi and Metz, 1991; Metz et al., 1994, 1998a; Aslan and Uehara, 1997), and sea stars (Byrne and Anderson, 1994). Although complete reciprocal incompatibility between species is often observed and a complete lack of incompatibility is rare (Pernet, 1999), these are not the most common outcomes. Most fertilization barriers are "partial"-the percent of eggs fertilized by heterospecific sperm is not zero but is some fraction of the percent fertilized by sperm of conspecifics.

Often, incompatibility between gametes of recognized species is also asymmetric; percent fertilization of the eggs

¹ Current address: Department of Biological Sciences and Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin Moss Lane, Wilmington, NC 28409; E-mail: McCartneyM@uncwil.edu

of one species of a pair is lower than in the reciprocal cross (Vacquier *et al.*, 1995). Asymmetric incompatibility has been found between species of hydroids (Buss and Yund, 1989), polychaetes (Pawlik, 1988), oysters (Banks *et al.*, 1994), abalones (Leighton and Lewis, 1982), and is particularly common in sea urchins (Branham, 1972; Strathmann, 1981; Lessios and Cunningham, 1990; Minor *et al.*, 1991; Metz *et al.*, 1994; Aslan and Uehara, 1997). Why fertilization barriers should often be asymmetric and incomplete is not known and is worthy of further study.

The three species of Echinometra sea urchins found on the two coasts of the Americas provide an excellent case in which to examine partial, asymmetric gametic isolation. First, the phylogeny of these species and the timing of their speciation relative to the closure of the Isthmus are known (McCartney et al., 2000). Hence, we can determine whether fertilization barriers are stronger in species separated earlier than in species more recently split, and we can address whether the evolution of these barriers is accelerated in sympatric species compared to species living in different oceans. Echinometra vanbrunti ranges along the Pacific coast of America from the Gulf of California to Peru. Echinometra lucunter occurs from Bermuda south to Brazil and east to the Atlantic coast of tropical Africa. Echinometra viridis is restricted to the Caribbean, where it co-occurs with E. lucunter, and although E. viridis is usually found in deeper water, the spawning seasons of the two sympatric species overlap (Lessios, 1981b). E. viridis is morphologically most distinct from the other two species (Mortensen, 1928-1951; Lessios, 1981a), yet mitochondrial DNA (mtDNA) sequences show it to be a sister species to the sympatric E. lucunter, with the allopatric E. vanbrunti having split off earlier (McCartney et al., 2000). Thus, it is probable that closure of the Isthmus of Panama 3.1 million years ago (MYA) (Coates and Obando, 1996) split E. vanbrunti from the common ancestor of the two Atlantic species, whose mtDNA divergence places their speciation at ca. 1.5 MYA (McCartney et al., 2000).

An earlier study (Lessios and Cunningham, 1990) demonstrated asymmetric gamete compatibility of one species towards the other two. Both heterospecific crosses involving eggs of E. lucunter females showed a greatly lowered percent fertilization compared to homospecific controls. In contrast, fertilization in both of the reciprocal crosses between E. lucunter and the other two species, as well as in the E. viridis male \times E. vanbrunti female crosses, was no lower than in homospecific crosses. Fertilization in the E. van*brunti* male \times *E. viridis* female cross was slightly reduced relative to controls. Finally, incompatibility between the sympatric species was less than that between the allopatric E. lucunter and E. vanbrunti. This finding runs contrary to predictions from the "speciation by reinforcement" model (Dobzhansky, 1940; Butlin, 1989; Liou and Price, 1994). The model envisions that populations that have acquired a

degree of reproductive isolation in allopatry, will, when they become sympatric, develop pre-zygotic isolation to avoid gamete wastage in inferior hybrids. Reinforcement should therefore yield greater gamete incompatibility between *Echinometra* species that currently live in the same ocean than between those that have been allopatric for the last 3 MY.

Each of the above conclusions was reached in experiments that combined gametes at a single concentration. McClary (1992) suggested that a different pattern might have emerged if concentrations of sperm had been varied. Though there were reasons to consider this suggestion unlikely (Lessios and Cunningham, 1993), we here report a determination of percent fertilization at various sperm concentrations. We construct "fertilization curves" estimated using a series of sperm dilutions, then examine the degree to which the added sensitivity of these experiments permits a better understanding of the mechanisms by which sympatric and allopatric species have developed prezygotic isolation.

Materials and Methods

Gametic compatibility experiments

Sea urchins were collected from the Caribbean coast of Panamá near Portobelo (*Echinometra lucunter* and *E. viridis*), and from the Pacific coast of Panamá at Isla Taboguilla (*E. vanbrunti*) between August and October 1996, and again in June 1997. These are months during which all three species are reproductively active (Lessios, 1981b). Animals were induced to spawn by intracoelomic injection of 0.5 *M* KCl.

Females releasing eggs were inverted so that their gonopores were immersed in a 50-ml beaker partially filled with $0.22-\mu$ m-filtered seawater (FSW). Eggs were resuspended, poured through doubled gauze to trap debris, and then washed once by aspiration and replacement of the FSW. The eggs were settled in a volumetric centrifuge tube to estimate their volume, and their concentration was adjusted to 2 ml of eggs in 100 ml of FSW. Counts of eggs from three females per species indicated that 2% suspensions contained, on average, 58 eggs of *E. lucunter*, 50 eggs of *E. viridis*, and 104 eggs of *E. vanbrunti* per microliter. Eggs were stored at room temperature for no more than 6 h prior to their use.

Sperm was drawn directly off male gonopores after injection of KCl solution. Undiluted ("dry") sperm was stored in capped microcentrifuge tubes for less than 1 h at room temperature. Preliminary experiments indicated that dry sperm maintained full fertilizing potency for at least 1 h.

A polypropylene microplate (Whatman #7701-5200) with 96 wells of 2-ml capacity each was used to conduct the fertilization experiments. Each well was filled with 0.2 ml FSW. A starting suspension of sperm was prepared by diluting 5 μ l dry sperm in 25 ml FSW for homospecific

crosses and 5μ l of sperm into 2.5 ml FSW for crosses in which E. lucunter eggs were exposed to heterospecific sperm. These starting concentrations were chosen so that subsequent dilutions would bracket those necessary to produce the descending portion of the fertilization curves. Equal amounts (200 μ l each) of the starting suspension and FSW were mixed together, and 200 μ l of this diluted suspension was again transferred to 200 μ l FSW. This process was repeated to prepare 12 serial 2-fold dilutions. Egg suspension (200 μ l) was mixed into each well in succession, starting with the well containing the most concentrated sperm suspension. Each sperm dilution series was prepared from a fresh dilution of dry sperm. A portion of the original sperm suspension was saved for later spectrophotometric estimates of sperm concentration. The microplate was capped, then gently rotated on an orbital shaker at 30°C for 90 min. This amount of time was previously determined to ensure that all fertilized eggs had passed through at least two cleavage divisions. Incubations were stopped after 90 min by fixing with 400 μ l of 5.5% formaldehyde in FSW. In preliminary experiments, washing of the eggs to rid them of excess sperm had no effect on the yield of fertilized eggs at fixed sperm concentrations in homospecific crosses, so it was not considered necessary to remove the sperm during the experiments.

All animals spawned were used in fertilizations on a single day, then discarded. Each female was used in a single experiment involving crosses with conspecific males, and with males of either one or both of the other two Echinometra species. A total of 181 crosses on eight trial dates were performed. To score percent fertilization, a drop of fixed suspension was placed on a slide, and a haphazardly selected track through the suspension was scanned under a compound microscope. At least 100 eggs were scored as either cleaving or not cleaving. Cleavage was selected as a convenient index of fertilization in lieu of raised fertilization membranes because fertilization membranes are difficult to see in these species of Echinometra. Previous work (Lessios and Cunningham, 1990) has shown that although percent of cleaving eggs is in some crosses lower than percent of eggs with raised fertilization membranes, this reduction is slight and introduces no bias in comparisons between homospecific and heterospecific crosses.

Sperm concentration was quantified spectrophotometrically (Vacquier and Payne, 1973). First, a standard curve was constructed. Three males of *E. lucunter* and two males each of *E. vanbrunti* and *E. viridis* were spawned, and a dilution series consisting of 10 serial 2-fold dilutions in FSW was prepared for each male. Each sperm dilution was fixed in an equal volume of 5.5% formaldehyde in FSW, and its absorbance at 340 nm (A_{340}) was determined against a blank of 2.25% formaldehyde in FSW. We made triplicate hemacytometer counts of the number of spermatazoa in each dilution, then determined the correspondence between optical absorbance and sperm concentration using linear regression. Separate regressions for each species showed no consistent differences in slope, so a common curve with slope equal to the average was used. This standard curve was used to convert A_{340} readings taken from the males used in each of the experimental trials to numbers of sperm per microliter.

Data analyses

The nonlinear, sigmoid relation between number of sea urchin eggs fertilized and sperm concentration presents a problem for quantifying and statistically comparing gamete compatibility. One solution is to fit the data to an explicit nonlinear fertilization kinetics model (Vogel et al., 1982; Levitan, 1996, 1998). An alternative approach is to first linearize the response using an appropriate transformation. We preferred the latter approach, because it does not assume that cross-species fertilizations adhere to the kinetic model developed for crosses performed within species. This approach also permits the application of linear statistical models. We used a standard transformation that has been successfully applied to other sigmoid responses, such as toxicity. This is the logit transformation (Finney, 1964; Hewlett and Plackett, 1979). The proportion of eggs fertilized (P) was transformed to its logit as follows: logit (P) = $\ln (P/1 - P)$. Linear regression of logit (P) values was then performed on log-transformed sperm concentration for each of the 181 crosses.

As a measure of egg-sperm compatibility over the range of sperm concentrations tested, we calculated the F_{50} , or the sperm concentration at which 50% of the eggs were fertilized (see Levitan, 1996, 1998). F_{50} values were calculated from the linear regression by determining the sperm concentration at which logit (0.5) = 0 (Figs. 1B, 2B, 3B). To compare F_{50} values across experimental trials, we took two approaches, each of which relied on different assumptions. In the first approach, we treated each F_{50} estimate as a unique value, independent of all others. This would be true if gamete compatibility were an emergent property of a given egg-sperm combination and not reflective of sperm or egg "qualities" (*e.g.*, swimming speed, viability) that would influence multiple crosses.

For this analysis, we grouped the log F_{50} values calculated from individual crosses by the species of female used in the cross. We then compared the mean log F_{50} values among the three male species tested on each species of female. Results of a Kolmogorov-Smirnov test indicated that distributions of log F_{50} values were not significantly different from an ideal normal distribution with the same mean and variance. The distributions were then tested for heteroscedasticity across the three species of sperm donor by using Bartlett's test. In two of the three species of females, variances were not significantly different between species of male, and ANOVA was performed using log F_{50} as the response variable and species of male as the main effect. In the case of *E. lucunter* females, unequal variances were found (F = 7.590, P < 0.001), so the log F_{50} values were compared using Welch's (1951) approximate ANOVA. Multiple comparisons among mean log F_{50} values for each class of cross were conducted using the Games and Howell (1976) test at the 5% significance level. ANOVA and multiple comparisons were performed using StatView 5.0.1 (SAS Institute, 1999).

For the second approach, we assumed that percent fertilization of eggs of a given female by one male is not independent of percent fertilization of that female by a second male. In other words, we assumed that unknown qualities of the eggs of a female could influence their compatibility with any male tested; a similar argument applies to the sperm of a male used in more than one egg-sperm combination. To take such lack of independence into account, we analyzed the data using a randomized complete blocks design in ANOVA. Each of the three species of females was analyzed separately. It was also necessary to separately analyze each of three types of crossing designs to maintain a balanced statistical design. In all of the trials, eggs from a single female were mixed with sperm from a single male (no experiment involved mixtures of sperm of multiple males), but slightly different crossing designs were used to make efficient use of ripe animals available on a given day. In type I crosses, one female was tested with several conspecific males and several males of one of the other two Echinometra species. In type II crosses, one female was tested with several conspecific males and several males of the remaining *Echinometra* species. In type III crosses, one female was tested with a single male from each of the three species. The dependent variable was logtransformed F_{50} . Females were treated as blocks in the analysis, and species of male was treated as the main effect. Multiple comparisons among species of male were conducted using the Games and Howell (1976) procedure.

As a final method for comparing compatibility, we fit our data to the nonlinear sea urchin fertilization kinetics model developed by Vogel *et al.* (1982). Untransformed proportion of eggs fertilized (P) was fit to the following equation:

$$P = 1 - \exp\left(\frac{-\beta S_0}{\beta_0 E_0} \left(1 - e^{-\beta_0 E_0 \tau}\right)\right)$$

where S_0 = number of sperm/µl, E_0 = number of eggs/µl, τ = sperm/egg contact time (set to equal 90 min) and β and β_0 are parameters obtained from nonlinear regression of Pon S_0 (the ratio β/β_0 is roughly interpretable as the proportion of the egg surface area that is fertilizable (Vogel *et al.*, 1982). Because the eggs of the three species are different sizes, the number of eggs per microliter of seawater was estimated from counts made separately for each species. As measured by Lessios (1990), *E. viridis* has the largest eggs $(3.97 \times 10^5 \ \mu \text{m}^3 \text{ volume})$, *E. lucunter* has eggs intermediate in size $(3.40 \times 10^5 \ \mu \text{m}^3)$, and *E. vanbrunti* has the smallest eggs $(1.94 \times 10^5 \ \mu \text{m}^3)$. Values of β and β_0 were estimated separately for each of the 181 trials and are not given, but are available from the first author. Fits to this equation were performed using the Gauss-Newton least squares iterative method provided in JMP 2.0 (SAS Institute, 1989). The F_{50} value was calculated by solving the resulting nonlinear regression equation for S_0 at P = 0.5. Values of F_{50} obtained from fits to the Vogel *et al.* (1982) model were analyzed statistically in the same manner as were the log F_{50} values obtained from linear regression described above.

We found great variation among E. lucunter females in F_{50} values calculated from heterospecific crosses (Figs. 4 and 7, Appendix). To determine whether this was due to differences among females in egg "quality"-that is, whether it reflected a capacity to be fertilized by sperm of conspecific males-we performed the following analyses. We used the results from the 13 E. lucunter females that were each tested with one different male of each of the three species. We calculated the correlation (Kendall's τ) between the F_{50} values estimated for each of the possible pairwise comparisons among males: E. lucunter males with E. viridis males, E. lucunter males with E. vanbrunti males, and E. viridis males with E. vanbrunti males. Significant values of Kendall's τ in the former two comparisons would indicate that differences in fertilizability of eggs by heterospecific sperm arise due to differences in egg quality; significant values in the latter comparison would indicate that fertilizability of eggs by sperm of the allopatric species covaried with fertilizability by sperm of the sympatric species.

Results

Relation between sperm concentration and optical absorbance

The standard curve relating A_{340} to sperm concentration yielded a highly significant linear regression (F = 651.08; df = 1,46; P < 0.001; $R^2 = 0.935$). The regression equation ($f(x) = 1.84 \times 10^{-5} x + 0.014$), when solved for x at $A_{340} = 1$, produced an extinction factor of 5.35×10^4 sperm/ μ l per A_{340} unit. We used this value to convert optical density measurements to sperm concentrations in all of our fertilization experiments. Our calibration was very close to those published. Branham (1972) reported extinction factors of 5.37×10^4 for *E. mathei* and 4.94×10^4 for *E. oblonga*. Using methods similar to ours, Tyler *et al.* (1956) and Minor *et al.* (1991) estimated that two species of *Strongylocentrotus* and *Lytechinus variegatus* produced dry sperm with concentrations ranging between 2 and 9.5×10^7 produced dry sperm with a similar average concentration— 3.04×10^7 sperm/µl.

Fertilization curves

Fertilization of eggs in all homospecific and some heterospecific crosses rose sharply with increasing sperm concentration, producing characteristic sigmoid curves (Figs. 1, 2 and 3). Curve fitting to the fertilization kinetics model of Vogel et al. (1982) was generally good (Figs. 1A, 2A, 3A), as was the fit of the linear regressions on logit-transformed data (Figs. 1B, 2B, 3B). Crosses in which eggs of E. lucunter females were fertilized by sperm of either of the other two species produced curves that shifted greatly towards higher sperm concentrations (Fig. 1A, 1B). As Figure 1B shows, sperm of this E. viridis male needed to be more than 250 times more concentrated than that of E. lucunter to fertilize half the eggs of this E. lucunter female. Sperm of the *E. vanbrunti* male shown had to be about 2000 times more concentrated than that of E. lucunter to achieve the same effect. Other E. lucunter females showed even stronger incompatibilities towards heterospecific sperm (Appendix).

Incompatibility of eggs from the other two species with heterospecific sperm was more moderate, or lacking altogether. For example, to fertilize half the eggs of the *E. viridis* female shown in Figure 2B, *E. lucunter* sperm needed to be only three times more concentrated and *E. vanbrunti* sperm only about 25 times more concentrated than *E. viridis* sperm. Eggs of *E. vanbrunti* showed an even milder barrier to cross-species fertilization, or none at all. For example, the *E. vanbrunti* female shown in Figure 3 displayed no detectable gamete incompatibility towards the other two species. Fertilization of her eggs was actually highest when they were exposed to sperm of *E. viridis*, and fertilization by *E. lucunter* and by *E. vanbrunti* sperm was nearly indistinguishable.

Linear regression analysis

The logit-transformed fertilization data showed good fit to log sperm concentrations, with r^2 values from linear regression often exceeding 0.90 (Appendix). Of the 181 crosses analyzed, only 6 crosses did not yield significant regressions. In four of these cases, this occurred because several sperm dilutions were too dilute to yield detectable fertilization, resulting in a regression based on few values and in increased error in estimation. The two other cases involved highly incompatible crosses (between *E. vanbrunti* sperm and *E. lucunter* eggs) that showed poor dependence of percent fertilization on sperm concentration.

Comparison of F_{50} values

To compare the results obtained across multiple females, F_{50} values were estimated for all crosses. The F_{50} values

were then placed into nine groups representing the nine cross classes (with each class of cross defined as one species of male crossed with one species of female: Figs. 4, 5, and 6). Fertilization of *E. lucunter* eggs required considerably more heterospecific than conspecific sperm. The mean F_{50} values were 65 times higher for *E. viridis* sperm and over 1700 times higher for *E. vanbrunti* sperm than was the F_{50} value estimated for conspecific sperm crossed with *E. lucunter* eggs (Fig. 4, Table 1).

E. viridis females showed much more modest heterospecific incompatibility. Compared with conspecific sperm, just over twice as much *E. lucunter* sperm and about 18 times as much *E. vanbrunti* sperm fertilized half the *E. viridis* eggs (*Fig.* 5, Table 1). *E. vanbrunti* females were only slightly less fertilizable by heterospecific sperm (Fig. 6; Table 1). Just over 2.5 times as much *E. lucunter* as conspecific sperm fertilized half the eggs of this species. Only two *E. viridis* males were tested against *E. vanbrunti* eggs, but neither of them was incompatible.

Welch's ANOVA showed highly significant differences among male species tested with E. lucunter females (W =125.41, P < 0.001), and multiple comparisons showed significant differences between means in all three species of sperm donor (Games-Howell test). ANOVA also showed significant differences among male species tested on E. viridis females (F = 15.96, df = 2,43; P < 0.001). In this case, multiple comparisons revealed differences between E. viridis and E. vanbrunti males, but E. viridis and E. lucunter males were not distinguishable. ANOVA also showed marginally significant differences among species of sperm donor tested on *E. vanbrunti* females (F = 3.425; df = 2, 30; P = 0.046). In this case, significant differences were apparent between E. lucunter and E. vanbrunti males; the other two comparisons were not possible because there were only two (*E. viridis* male \times *E. vanbrunti* female) crosses.

A potential problem with the above analysis is that males and females were used in more than one fertilization experiment, so a randomized complete blocks analysis was used to allow for any non-independence. The results closely matched those from one-way ANOVA. Highly significant differences in fertilization of E. lucunter eggs were present between species of males, but these differences were less clear with eggs of *E. viridis* and *E. vanbrunti*. The seven *E*. lucunter females involved in crosses with multiple E. viridis and E. lucunter males had significantly fewer eggs fertilized by *E. viridis* than by *E. lucunter* sperm (F = 140.29, P < 140.29) 0.001). For the two E. lucunter females involved in crosses with multiple E. vanbrunti and E. lucunter males, significantly fewer eggs were fertilized by E. vanbrunti than by E. *lucunter* males (F = 142.0, P < 0.001). And for the 13 E. lucunter females, each crossed with a single male of each species, highly significant differences existed among species of males (F = 48.01, P < 0.001). Multiple comparisons



3 4 5

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Log (sperm per microliter)

-1



Figures 1–3. Representative results from *Echinometra* fertilization experiments performed at a series of sperm dilutions. Top panels (A) show the proportion of eggs fertilized (*P*) as a function of log sperm concentration (number of sperm/ μ l); curves are least-squares fits to the fertilization kinetics model of Vogel *et al.* (1982). Bottom panels (B) show logit (*P*) plotted against log sperm concentration; lines are from linear regression. Solid lines = *E. lucunter* (EL) males, dashed lines = *E. viridis* (EV) males, dotted lines = *E. vanbrunti* (EVB) males. The female involved in the crosses is indicated above the top panel. Data from other experiments are shown in the appendix.



Figures 4–6. Distributions of F_{50} values derived from linear regressions of logit-transformed data from *Echinometra* fertilization experiments. Bars indicate the number of crosses in which F_{50} values fell between the class limits labeled on the X-axis (note the logarithmic scale to the labels). Arrows mark the back-transformed value of the mean log F_{50} . The number of crosses in each cross category was 40 *E. lucunter* female × *E. lucunter* male, 40 *E. lucunter* female × *E. viridis* male, 22 *E. lucunter* female × *E. vanbrunti* male; 18 *E. viridis* female × *E. viridis* female × *E. viridis* female × *E. vanbrunti* male; 15 *E. vanbrunti* male; 15 *E. vanbrunti* male; 2 *E. viridis* male, and 16 *E. vanbrunti* female × *E. vanbrunti* male.

ranked compatibility of the males with E. lucunter eggs in the order E. lucunter > E. viridis = E. vanbrunti. In contrast, the five E. viridis females crossed with multiple males showed no significant difference between F_{50} values estimated for *E. lucunter* and *E. viridis* males (F = 1.329, P > 0.05). The three *E. viridis* crossed with a single male of each species showed significant differences between male species (F = 30.40, P < 0.01). But as in the one-way analysis, multiple comparisons discriminated between E. viridis and E. vanbrunti males, but not between E. viridis and E. lucunter males. Results with E. vanbrunti females were mixed: significantly fewer eggs were fertilized by E. lucunter than E. vanbrunti males in crosses with multiple males (F = 37.26, P < 0.001), whereas crosses with a single male of all three species revealed no differences among male-species (F = 0.919, P > 0.05).

Compatibility estimated using the fertilization kinetics model

Estimates of F_{50} from nonlinear fits to the fertilization kinetics model of Vogel *et al.* (1982) were highly correlated with the F_{50} values from linear regression (correlation between log F_{50} values = r = 0.970, P < 0.001). Differences between heterospecific and homospecific values tended to be smaller using the Vogel method, particularly in highly incompatible crosses (see Figs. 1, 2, and 3; Appendix). Mean F_{50} values calculated from the Vogel model estimated that 60 times as much *E. viridis* sperm and 735 times as much *E. vanbrunti* sperm as conspecific sperm was required to achieve 50% fertilization of *E. lucunter* sperm and about 12 times as much *E. vanbrunti* sperm



Figure 7. Correlation of F_{50} values from *Echinometra* fertilization experiments. Data shown are for 13 crosses of *E. lucunter* females, each of which was tested with a single male of each of the three species. Points are F_{50} values measured in the cross with a male of one species (ordinate) plotted against the F_{50} values measured in the cross with a male of the other species (abscissa).

fertilized half the *E. viridis* eggs. Just over twice as much *E. lucunter* as conspecific sperm fertilized half the eggs of *E. vanbrunti* (Table 2).

Correlation among F_{50} values

Females of *E. lucunter* differed by as much as 2 orders of magnitude in their receptivity towards heterospecific sperm (Fig. 4; Appendix). Differences in receptivity towards conspecific sperm do not explain these differences; that is, they do not arise from differences in gamete "quality." In 13 cases, fertilization trials combined the eggs of a single *E*.

lucunter female with one male of each of the three species. No correlation existed between the F_{50} value calculated (from linear regression) for the homospecific cross and for the cross with either E. viridis (Fig. 7A: Kendall's τ = -0.077, P > 0.05) or *E. vanbrunti* (Fig. 7B: $\tau = -0.154$, P > 0.05) males. Similarly, no correlation was found in the same comparisons for F_{50} values calculated using nonlinear regressions ($\tau = 0.154, P > 0.05$ for both comparisons). Significant, positive correlations would have indicated that females that required more heterospecific sperm for fertilization also required more conspecific sperm. This would implicate gamete quality differences as responsible, but none were apparent. In contrast, significant positive correlations were found for these same 13 trials between F_{50} values of E. viridis and those of E. vanbrunti males, when calculated using both linear (Fig. 7C: $\tau = 0.872$, P < 0.001) and nonlinear regression ($\tau = 0.795, P < 0.001$). These results show that females with eggs that were less discriminating towards sperm of one heterospecific were also less discriminating towards sperm of the other.

Discussion

Patterns of gametic incompatibility

The neotropical species of Echinometra each show partial cross-species gametic incompatibility, but to very different degrees. The eggs of E. lucunter are strongly incompatible with the sperm of its two most closely related species. Incompatibility can be defined as the ratio of the mean F_{50} values (the heterospecific value divided by the homospecific value: see Tables 1 and 2). With this approach, incompatibility between E. lucunter eggs and E. viridis sperm is estimated to range from 60-fold (using the F_{50} values estimated by fitting the Vogel et al. (1982) model) to 65-fold (using fits to linear regressions). Incompatibility between E. lucunter eggs and E. vanbrunti sperm ranges from > 700to > 1700-fold. This is a much stronger block than was found by Lessios and Cunningham (1990). If, from this previous study, incompatibility is defined as the inverse of the ratio of percent cleaving eggs in homospecific:heterospecific crosses, incompatibility of E. lucunter eggs with E. viridis sperm would be estimated at 5.2-fold, and with E. vanbrunti sperm only 11-fold. Hence, the present approach of using sperm dilution curves detects much greater quantitative differences in gamete compatibility, but the rankings among the species are the same.

Compatibility differences between homospecific and heterospecific gametes are much more moderate for eggs of the other two species. In the present study, we estimate compatibility of *E. viridis* eggs with *E. lucunter* sperm to be just 1.4–2.2 times lower, and with *E. vanbrunti* sperm 12–18 times lower than with conspecific sperm. *E. vanbrunti* eggs are only 2.2–2.6 times less compatible with *E. lucunter* than with conspecific sperm, and appear—from the two crosses

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Table 1

	v 50 0	1	<i>y 1</i>			
Female	Male	n	\bar{F}_{50}	F_{50} ratio	L_1	L_2
E. lucunter	E. lucunter	40	94	_	65	137
E. lucunter	E. viridis	22	6095	64.8	3359	11,059
E. lucunter	E. vanbrunti	40	162,554	1729	56,032	471,585
E. viridis	E. lucunter	18	136	2.19	85	216
E. viridis	E. viridis	23	62	_	39	100
E. viridis	E. vanbrunti	5	1143	18.4	737	1770
E. vanbrunti	E. lucunter	15	215	2.59	133	351
E. vanbrunti	E. viridis	2	81	0.98	NA	NA
E. vanbrunti	E. vanbrunti	16	83	—	52	132

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 F_{50} estimated by linear regression, as described in the text; n = number of crosses; $\bar{F}_{50} =$ mean F_{50} ; F_{50} ratio = F_{50} (heterospecific cross)/ F_{50} (homospecific cross); L_1 and $L_2 = 95\%$ confidence limits for \bar{F}_{50} ; means and confidence limits are back-transformed from their logs.

performed here and from the results of Lessios and Cunningham (1990)-to show no incompatibility with E. viridis sperm. So, although E. lucunter eggs have evolved a strong barrier to cross-species fertilization, weaker or undetectable barriers are present in eggs of the other two species. This result is in large part identical to that found in Lessios and Cunningham (1990), with the exception that the weak incompatibilities in E. viridis and E. vanbrunti were previously not detectable (the somewhat stronger incompatibility between E. viridis eggs and E. vanbrunti sperm was detected). Thus, testing sperm dilution series is a more sensitive method for identifying small fertility differences that are not apparent at single sperm concentrations. The question is whether unidirectional differences of small magnitude, such as those involving E. viridis and E. vanbrunti eggs, have any biological meaning. In the absence of any other isolating mechanism, it is unlikely that they alone could serve to keep two sympatric species from fusing.

A new approach to quantifying gametic incompatibility

In the present study, fitting by linear regression of logittransformed data was a more accurate method than was fitting to the Vogel *et al.* (1982) fertilization kinetics model. Fits to the latter model were poor in some heterospecific crosses, particularly those in which incompatibility was strong. This casts doubts on the general utility of the Vogel model for estimating gamete compatibility between species. Vogel *et al.* aimed their approach at homospecific fertilization and built their model from a kinetic study. When sperm and eggs of different species are involved, the probability of fertilization is likely to be affected by factors that their kinetic model cannot take into account, such as compatibility of gamete recognition molecules. As a more general alternative, loaded with fewer assumptions, we recommend the use of the linear regression approach adopted in our paper.

When using a single metric to compare fertilization curves, one must consider whether the chosen metric is biologically meaningful. For example, an F_{50} estimate obtained solely through extrapolation (in crosses in which 50% fertilization was never achieved) would be of questionable significance, and an alternative (say an F_{10} or F_{20} value) would be preferable. We chose to use F_{50} for the following reasons. First, it worked well for a majority of our

comparison of 1.50 values estimated from the nonlinear model of voget et al. (1992) for the time possible crosses of Lemnonical species									
Female	Male	п	\bar{F}_{50}	F_{50} ratio	L_1	L_2			
E. lucunter	E. lucunter	40	118	_	78	181			
E. lucunter	E. viridis	22	7030	59.5	4165	11,867			
E. lucunter	E. vanbrunti	40	86,696	735	54,465	138,000			
E. viridis	E. lucunter	18	153	1.45	90	261			
E. viridis	E. viridis	23	105	_	71	156			
E. viridis	E. vanbrunti	5	1288	12.3	458	3621			
E. vanbrunti	E. lucunter	15	160	2.28	102	253			
E. vanbrunti	E. viridis	2	55	0.78	NA	NA			
E. vanbrunti	E. vanbrunti	16	70		41	121			

Table 2

Comparison of F₅₀ values estimated from the nonlinear model of Vogel et al. (1982) for the nine possible crosses of Echinometra species

n = number of crosses; $\bar{F}_{50} =$ mean F_{50} ; F_{50} ratio = F_{50} (heterospecific cross)/ F_{50} (homospecific cross); L_1 and $L_2 = 95\%$ confidence limits for \bar{F}_{50} ; means and confidence limits are back-transformed from their logs.

crosses. In our data set, most (156 of 181 or 85%) of the crosses produced a maximum of \geq 50% fertilization of eggs, so extrapolation beyond the tested concentrations was not necessary. For the remaining 25 crosses in which maximum fertilization was less than 50% (maximum fertilization averaged 35% for these crosses), extrapolation of the curve was necessary to obtain an F_{50} . There was a difference in the fit of data to the regressions in these two groups of crosses. The r^2 value from linear regression was 0.917 for crosses in which \geq 50% was achieved, and r^2 equaled 0.847 for crosses from which F_{50} had to be extrapolated. Hence while there is a suggestion of a trend towards lower accuracy with these most incompatible crosses, extrapolation itself would not have created excessive additional error.

Our second reason for using F_{50} is that calculating F_{10} or F_{20} values from the fertilization curves would have introduced even greater inaccuracy. In the more compatible crosses, 10% or 20% fertilization was reached with extremely dilute sperm whose concentration is more difficult to estimate accurately and is less reproducible. Also, F_{10} and F_{20} values lie farther away from the bivariate mean of regression, which introduces additional error. Confidence intervals around F_{10} or F_{20} estimates (not shown) were wider than were those surrounding F_{50} for the great majority of our crosses.

The evolution of gamete incompatibility in sea urchins

The results from the present study indicate that incompatibility of sea urchin eggs does not steadily evolve at equal rates as species diverge. Based on mtDNA sequence comparisons, E. lucunter and E. viridis are sister species that separated about 1.5 MYA. This occurred after closure of the Isthmus of Panamá, which split E. vanbrunti from the E. lucunter/E. viridis ancestor some 3.1 MYA (McCartney et al., 2000). A scenario in which gametic incompatibility closely tracked time and genetic divergence would predict the following. First, we would expect eggs of E. viridis to show incompatibility with sperm of E. lucunter equal to that in the reciprocal cross. Second, eggs of E. vanbrunti would show the greatest block towards sperm of the other two species. Instead, the primary fertilization barrier in the eggs appears to have evolved in a single lineage leading to E. lucunter (see also Lessios and Cunningham (1990) and Lessios (1998)), during the last 1.5 million years (McCartney et al., 2000).

The barrier that eggs evolve to fertilization by heterospecific sperm does not appear to develop continuously as species diverge. Yet once such a barrier emerges, it does appear to strengthen with time. With *E. lucunter* eggs, *E. viridis* sperm is more compatible than is *E. vanbrunti* sperm, just as would be predicted from divergence estimates based on mtDNA. In contrast, if incompatibility were the product of reinforcement (Dobzhansky, 1937; Butlin, 1989; Liou and Price, 1994), it would be expected that selection against hybrids would lead to greater incompatibilities in the sympatric species pair than in the allopatric pair. *E. viridis* eggs are fertilized to a greater extent by *E. lucunter* than by *E. vanbrunti* sperm, again the ranking predicted by genetic distances, but not by the reinforcement model. Compatibility in other sea urchin species is due to the interaction of gamete recognition molecules on both eggs and sperm (*e.g.*, Minor *et al.*, 1991; Metz and Palumbi, 1996). Our data suggest that, in the American *Echinometra*, differences in sperm-egg recognition molecules do accumulate over time as species diverge.

Asymmetric gamete incompatibility like that observed here has often been found between species pairs of sea urchins and other free-spawning marine invertebrates. As Lessios and Cunningham (1990) and Palumbi (1994) point out, such cases are reminiscent of asymmetric behavioral isolation observed between closely related, allopatric Drosophila species (Kaneshiro, 1976, 1983). Why such a similar pattern should be shared between such different mechanisms for reproductive isolation is worthy of consideration. Borrowing from a hypothesis originally suggested by Muller (1942), Kaneshiro (1980) suggests that behavioral isolation evolves as a byproduct of disruptive or directional selection in allopatry, and its asymmetry is due to drift and founder effects in one but not both of the descendent populations. In an analogous fashion, asymmetric barriers to fertilization could emerge as an accident of history, such as a bottleneck in one of the two sister species, then become exaggerated as selection within the bottlenecked population promoted coevolutionary changes of sperm and eggs.

Asymmetric gametic compatibility between sympatric species, such as the one observed between E. lucunter and E. viridis, has been found several times. Putative morphospecies of Echinometra mathei that coexist in Okinawa show highly asymmetric gamete incompatibility (Aslan and Uehara, 1997). A high percentage of eggs of Strongylocentrotus droebachiensis are fertilized by sperm of its sympatric (but bathymetrically displaced) congener S. pallidus, but the reciprocal cross of S. pallidus eggs and S. droebachiensis sperm produces very low percent fertilization (Strathmann, 1981). Oyster species in the genus Crassostrea that co-occur in Japan show an asymmetric block to fertilization (Banks et al., 1994). Percent fertilization of eggs from white abalones (Haliotis sorenseni) by sperm of another California species, H. rufescens, is close to 100%, but is much lower in the reciprocal cross (Leighton and Lewis, 1982). In one case, asymmetric incompatibility exists between sympatric species that are distantly related. Close to 100% of the eggs of the Hawaiian sea urchin Colobocentrotus atratus can be fertilized by sperm of the sea urchins Echinometra mathei, Pseudoboletia indiana, and Tripneustes gratilla (the latter two of which are in a

different family). In contrast, the same concentration of *C*. *atratus* sperm fertilizes a much smaller percentage of eggs in the reciprocal crosses (Branham, 1972).

As in other cases where species are sympatric, it is tempting to propose an adaptive hypothesis to explain why E. lucunter should have "choosier" eggs than E. viridis. Lessios and Cunningham (1990) examined the hypothesis of natural selection against hybridization that differs in intensity between the two species. E. lucunter inhabits a narrow band from the water surface down to a depth of about 1 m. E. viridis individuals are often found within this zone as well, but they are more abundant in deeper coral reef, where E. lucunter is absent (McPherson, 1969; Lessios et al., 1984). Spawning seasons for the two species overlap (Lessios, 1981b, 1985), and neither species shows a lunar cycle (Lessios, 1991). Since sperm from one male can fertilize the eggs of many females, the infiltration of E. viridis into the E. lucunter zone may create a hazard to a large fraction of spawning E. lucunter females, and if hybrids are less viable, may strongly select for discrimination by its eggs. The zone of overlap, however, represents a small fraction of the E. viridis population, which would therefore be under much weaker selection to avoid hybridization. Under this hypothesis, discrimination against E. vanbrunti sperm would be a byproduct of discrimination against E. viridis sperm. However, as Lessios and Cunningham indicate, this "reinforcement" model predicting higher discrimination by E. lucunter eggs would also predict that E. lucunter eggs should protect themselves more effectively against sperm of the sympatric E. viridis than against sperm of the allopatric E. vanbrunti. This is not the case.

We found that different females of E. lucunter show great variation in their discrimination against heterospecific sperm. Recognition of heterospecifics that varies within species implies variation at compatibility loci in females. Such variation plays a crucial role in some models for the evolution of prezygotic isolation between species (Nei et al., 1983; Wu, 1985). These models invoke relaxed selection on females for compatibility with conspecific male variants and suggest that drift and mutational input may determine the fate of female compatibility alleles (Wu, 1985). An important prerequisite for these models is that female alleles are neutral with respect to their effect on female reproductive success. Our data are consistent with this, given that large differences in cross-species compatibility are not associated with fertility differences within species.

An incomplete barrier to fertilization of the strength described here could only partially account for the apparent lack of hybridization between sympatric *E. lucunter* and *E. viridis* in nature, shown by fixed differences in two isozymic loci (Lessios, 1979). It may be that habitat preferences work in concert with gametic incompatibility to severely lower the production of hybrid embryos; but a postzygotic isola-

tion mechanism not yet discovered is necessary to account for the complete lack of introgression between the sympatric species (Lessios and Cunningham, 1990).

Conclusion

Other studies have shown that fertilization barriers between free-spawning marine invertebrates can evolve rapidly (Palumbi and Metz, 1991; Metz et al., 1998b; Hellberg and Vacquier, 1999). Indeed, the gametic incompatibility between E. lucunter and E. viridis must have appeared within the last 1.5 million years (McCartney et al., 2000). However, the emergence of incompatibility must still represent an unlikely evolutionary event. In large part, the proteins mediating fertilization are subject to strong stabilizing selection, and many of them are greatly conserved (Vacquier, 1998). Hence, it is not surprising that gamete incompatibility in the neotropical Echinometra has not evolved in a continuous fashion, resulting in a complete barrier to genetic exchange. Rather the process has been lineage-specific and has produced asymmetric reproductive isolation. It may be that historical accidents such as population bottlenecks play an important role in the initial emergence of gamete incompatibility and explain why it does not always evolve. One likely scenario in the present case would be that accidental changes in gamete recognition molecules on eggs initiate the process. These changes could then drive compensatory sexual selection on cognate molecules in sperm and fuel their divergence. This scenario would predict greater divergence of proteins mediating sperm-egg recognition and binding in E. lucunter compared to its neotropical congeners.

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Appendix

Summary of fertilization data. n = number of sperm dilutions tested; $R^2 =$ coefficient of determination; F(reg) = F ratio testing significance of regression coefficient: ***P < 0.001, **P < 0.01, *P < 0.05, ns = not significant.

						Linear regression		Nonlinear
#	Date	Female	Male	n	R^2	F (reg)	F_{50} (linear)	(nonlinear)
1	8/13/96	EL1	EL1	10	0.957	178.1***	74	86
2	8/13/96	EL1	EL2	9	0.867	45.93***	16	32
3	8/13/96	EL1	EV1	12	0.922	118.2***	520	905
4	8/13/96	EL1	EV2	12	0.891	82.38***	675	1270
5	8/13/96	EL1	EV3	12	0.917	110.1***	967	1183
6	8/13/96	EL1	EV4	8	0.844	32.53***	2875	2461
7	8/13/96	EL2	EL1	10	0.870	53.80***	67	62
8	8/13/96	EL2	EL2	10	0.755	24.67***	19	40
9	8/13/96	EL2	EV1	12	0.890	80.49***	155	312
10	8/13/96	EL2	EV2	12	0.869	66.37***	159	237
11	8/13/96	EL2	EV3	12	0.705	24.01***	52	140
12	8/13/96	EL2	EV4	12	0.815	44.18***	320	305
13	8/13/96	EV1	EL1	10	0.975	310.5***	78	120
14	8/13/96	EV1	EL2	10	0.889	64.71***	114	151
15	8/13/96	EV1	EV1	9	0.823	32.59***	148	168
16	8/13/96	EV1	EV2	10	0.951	157.0***	105	133
17	8/13/96	EV1	EV3	10	0.875	55.91***	53	94
18	8/13/96	EV1	EV4	9	0.943	117.7***	30	48
19	8/20/96	EL3	EL3	11	0.972	317.8***	79	107
20	8/20/96	EL3	EL4	12	0.943	164.4***	64	119
21	8/20/96	EL3	EV5	5	0.983	177.6***	14764	17750
22	8/20/96	EL3	EV6	5	0.853	17.47*	8438	7968
23	8/20/96	EL3	EV7	12	0.883	75.26***	1901	3659
24	8/20/96	EL3	EV8	10	0.960	194.0***	7721	9646
25	8/20/96	EL4	EL3	11	0.891	73.68***	90	204
26	8/20/96	EL4	EV5	7	0.905	47.87***	19571	19336
27	8/20/96	EL4	EV6	12	0.897	87.47***	11395	11855
28	8/20/96	EL4	EV7	9	0.947	126.4***	8454	10138
29	8/20/96	EV2	EL3	7	0.734	13.80*	97	116
30	8/20/96	EV2	EL4	11	0.970	291.6***	181	313

SEA URCHIN GAMETIC INCOMPATIBILITY

Appendix (Continued)

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						Linear regression			
#	Date	Female	Male	п	R^2	F (reg)	F_{50} (linear)	Nonlinear regression F_{50} (nonlinear)	
31	8/20/96	EV2	EV5	12	0.935	146.1***	49	75	
32	8/20/96	EV2	EV6	11	0.875	63.13***	142	219	
33	8/20/96	EV2	EV7	8	0.983	360.4***	127	174	
34	8/20/96	EV2	EV8	12	0.973	365.2***	56	79	
35	8/29/96	EL5	EL5	7	0.817	22.32**	533	664	
36	8/29/96	EL5	EV9	12	0.909	100.3***	2231	2323	
37	8/29/96	EL5	EVB1	12	0.880	73.47***	5294	7181	
38	8/29/96	EL6	EL5	8	0.953	124.1***	137	143	
39	8/29/96	EL6	EV9	12	0.884	76.58***	283550	100147	
40	8/29/96	EL6	EVB1	10	0.898	71.14***	7530070	324044	
41	8/29/96	EL7	EL5	9	0.922	83.02***	184	159	
42	8/29/96	EL7	EV9	12	0.927	127.2***	1108	1301	
43	8/29/96	EL7	EVB1	12	0.957	223.7***	8437	11619	
44	8/29/96	EL8	EL5	8	0.885	46.33***	645	1030	
45	8/29/96	EL8	EV9	11	0.933	127.1***	174077	82327	
46	8/29/96	EL8	EVB1	6	0.613	6.358ns	315066738	513944	
47	8/29/96	EL9	EL5	10	0.908	79.11***	206	443	
48	8/29/96	EL9	EV9	8	0.956	132.7***	33471	40535	
49	8/29/96	EL9	EVB1	5	0.901	27.35*	105567	112169	
50	8/29/96	EV3	EL5	11	0.886	70.04***	217	200	
51	8/29/96	EV3	EV9	10	0.989	728.5***	64	107	
52	8/29/96	EV3	EVB1	8	0.931	80.96***	1409	1876	
53	8/29/96	EVB1	EL5	12	0.918	112.0***	67	76	
54	8/29/96	EVB1	EV9	12	0.909	100.4***	13	8	
55	8/29/96	EVB1	EVB1	10	0.902	73.85***	53	43	
56	9/3/96	EL10	EL6	9	0.971	235.5***	310	442	
57	9/3/96	EL10	EV10	10	0.709	19.56**	77923	51024	
58	9/3/96	EL10	EVB2	10	0.914	85.12***	163104	73180	
59	9/3/96	EL11	EL6	10	0.970	259.4***	387	430	
60	9/3/96	EL11	EV10	10	0.978	369.9***	3581	5822	
61	9/3/96	EL11	EVB2	9	0.941	113.1***	14156	22449	
62	9/3/96	EV4	EL6	9	0.919	80.2***	491	1090	
63	9/3/96	EV4	EV10	10	0.974	307.5***	142	222	
64	9/3/96	EV4	EVB2	7	0.967	147.4***	1424	2307	
65	9/3/96	EVB2	EL6	6	0.897	35.18**	373	278	
66	9/3/96	EVB2	EV10	6	0.798	15.82*	505	378	
67	9/3/96	EVB2	EVB2	6	0.974	155.8***	536	376	
68	9/10/96	EL12	EL7	1	0.977	396.0***	218	297	
69	9/10/96	EL12	EV11	11	0.982	510.7***	8503	12683	
70	9/10/96	EL12	EVB3	10	0.886	62.19***	77716	65298	
71	9/10/96	EL13	EL7	12	0.957	228.0***	154	233	
72	9/10/96	EL13	EV11	8	0.914	64.09***	43940	49352	
73	9/10/96	EL13	EVB3	5	0.816	13.33*	373349	212238	
74	9/10/96	EL14	EL7	12	0.964	275.2***	217	231	
75	9/10/96	EL14	EV11	12	0.905	95.31***	2442	2806	
76	9/10/96	EL14	EVB3	10	0.864	50.98***	10857	16817	
77	9/10/96	EL15	EL7	9	0.934	99.15***	291	323	
78	9/10/96	EL15	EV11	10	0.963	209.1***	24955	24195	
79	9/10/96	EL15	EVB3	7	0.960	120.3***	76890	64534	
80	9/10/96	EL16	EL7	8	0.975	234.8***	334	547	
81	9/10/96	EL16	EV11	5	0.973	108.9**	14813	19479	
82	9/10/96	EL16	EVB3	3	0.998	579.2***	54651	88914	
83	9/10/96	EL17	EL7	11	0.863	57.06***	196	115	
84	9/10/96	EL17	EV11	12	0.917	110.8***	3612	3512	
85	9/10/96	EL17	EVB3	10	0.938	122.7***	22039	29284	
86	9/10/96	EV5	EL7	10	0.898	70.59***	305	42	
87	9/10/96	EV5	EV11	10	0.953	165.59***	22	1227	
88	9/10/96	EV5	EVB3	10	0.871	54.05***	692	382	

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Appendix (Continued)

		Female		п		Linear regressio	n	
#	Date		Male		R^2	F (reg)	F_{50} (linear)	Nonlinear regression F_{50} (nonlinear)
89	9/10/96	EVB3	EL7	10	0.897	70.03***	1057	742
90	9/10/96	EVB3	EVB3	9	0.973	258.2***	608	504
91	9/19/96	EL18	EVB3	5	0.859	18.33*	159890	229668
92	9/19/96	EV6	EVB3	6	0.924	49.07**	2091	3764
93	9/19/96	EV7	EVB3	8	0.960	144.3***	671	857
94	9/19/96	EVB4	EVB3	11	0.960	220.7***	273	178
95	10/3/96	EL19	EL8	12	0.801	40.29***	146	191
96	10/3/96	EL19	EL9	5	0.953	61.45**	452	1568
97	10/3/96	EL19	EL10	5	0.973	108 1**	133	77
98	10/3/96	EL19	EL11	5	0.845	16.42*	720	3361
99	10/3/96	EL19	EVB4	7	0.925	62 00***	346555	223114
100	10/3/96	FI 19	EVB5	5	0.661	5 874ns	14271563	204606
101	10/3/96	FI 19	EVB5 EVB6	6	0.650	7 438*	427516	270235
102	10/3/96	FI 19	EVB0 EVB7	7	0.889	40.27**	128093	106011
102	10/3/96	EL19 EL 20	EVD, FIS	5	0.805	12 /3*	102	100011
103	10/3/96	EL20 EL20	ELO	5	0.830	14.60*	102	57
104	10/3/90	EL20 EL20	EL9 FL10	5	0.830	51 /1**	118	016
105	10/3/90	EL20 EL20	EL10 EL11	5	0.944	22 04**	473	51
100	10/3/90	EL20 EL20	EU11 EVD4	5	0.910	02.14***	76712	102441
107	10/3/90	EL20 EL20	EVB4	07	0.958	92.14***	/0/13	103441
108	10/3/96	EL20	EVBS	1	0.885	38.50**	14/808	112384
109	10/3/96	EL20	EVBO	2	0.888	24.00*	184934	145387
110	10/3/96	EL20	EVB/	8	0.932	83.13***	62560	84298
111	10/3/96	EVB5	EL8	8	0.921	69.86***	82	53
112	10/3/96	EVB5	EL9	9	0.961	1/6.23***	170	101
113	10/3/96	EVB5	EL10	8	0.916	65.96***	38	41
114	10/3/96	EVB5	ELII	8	0.936	89.13***	100	89
115	10/3/96	EVB5	EVB4	5	0.930	39.99**	37	30
116	10/3/96	EVB5	EVB5	6	0.972	140.79***	43	32
117	10/3/96	EVB5	EVB6	8	0.944	102.8***	41	30
118	10/3/96	EVB5	EVB7	8	0.952	121.1***	28	22
119	10/3/96	EVB6	EL8	8	0.927	77.11***	169	93
120	10/3/96	EVB6	EL9	10	0.977	345.1***	290	228
121	10/3/96	EVB6	EL10	9	0.931	94.96***	110	83
122	10/3/96	EVB6	EL11	10	0.966	229.6***	157	93
123	10/3/96	EVB6	EVB4	7	0.979	236.8***	39	31
124	10/3/96	EVB6	EVB5	7	0.850	28.46**	91	62
125	10/3/96	EVB6	EVB6	6	0.904	37.67**	73	64
126	10/3/96	EVB6	EVB7	6	0.978	179.3***	114	89
127	10/3/96	EVB7	EL8	8	0.954	126.8***	447	314
128	10/3/96	EVB7	EL9	10	0.908	79.69***	627	406
129	10/3/96	EVB7	EL10	9	0.962	178.0***	446	351
130	10/3/96	EVB7	EL11	9	0.969	220.4***	695	532
131	10/3/96	EVB7	EVB4	5	0.919	34.14**	49	46
132	10/3/96	EVB7	EVB5	6	0.943	66.61**	88	75
133	10/3/96	EVB7	EVB6	7	0.948	92.22***	100	72
134	10/3/96	EVB7	EVB7	7	0.892	41.37**	47	31
135	6/28/97	EL21	EL12	6	0.693	9.039*	70	60
136	6/28/97	EL21	EL13	4	0.944	34.25*	9	17
137	6/28/97	EL21	EL14	5	0.971	103.7**	51	55
138	6/28/97	EL21	EL15	5	0.914	32.26**	7	14
139	6/28/97	EL21	EV12	6	0.917	44.29**	4853	5885
140	6/28/97	EL21	EV13	7	0.874	34.85**	4187	5509
141	6/28/97	EL21	EV14	4	0.962	51.67*	25784	26663
142	6/28/97	EL21	EV15	8	0.971	205.4***	11555	16463
143	6/28/97	EL22	EL12	7	0.915	54.31***	40	33
144	6/28/97	EL22	EL13	5	0.933	41.79**	13	15
145	6/28/97	EL22	EL14	5	0.864	19.19*	39	22
146	6/28/97	EL22	EL15	3	0.999	1573*	74	82

SEA URCHIN GAMETIC INCOMPATIBILITY

Appendix (Continued)

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						Linear regression		
#	Date	Female	Male	п	R^2	F (reg)	F_{50} (linear)	Nonlinear regression F_{50} (nonlinear)
147	6/28/97	EL22	EV12	6	0.821	18.35*	13791	19123
148	6/28/97	EL22	EV13	5	0.823	14.03*	3340	3902
149	6/28/97	EL22	EV14	4	0.533	2.285ns	31424	47940
150	6/28/97	EL22	EV15	6	0.884	30.51**	32570	41095
151	6/28/97	EL23	EL12	7	0.969	157.6***	16	26
152	6/28/97	EL23	EL13	5	0.958	69.71**	10	10
153	6/28/97	EL23	EL14	5	0.880	22.03*	55	41
154	6/28/97	EL23	EL15	5	0.980	152.4***	57	67
155	6/28/97	EL23	EV12	8	0.948	109.5***	7275	8390
156	6/28/97	EL23	EV13	6	0.965	112.6***	2165	2272
157	6/28/97	EL23	EV14	6	0.903	37.61**	71798	41261
158	6/28/97	EL23	EV15	7	0.886	38.93**	38868	43075
159	6/28/97	EV8	EL13	4	0.822	9.261ns	141	144
160	6/28/97	EV8	EL14	4	0.965	56.31*	52	93
161	6/28/97	EV8	EL15	4	0.939	31.25*	154	97
162	6/28/97	EV8	EV12	7	0.989	488.4***	18	37
163	6/28/97	EV8	EV13	5	0.966	85.97**	36	76
164	6/28/97	EV8	EV14	4	0.989	193.6**	44	76
165	6/28/97	EV8	EV15	5	0.943	49.76**	266	395
166	6/28/97	EV9	EL12	5	0.972	107.2**	111	159
167	6/28/97	EV9	EL13	5	0.756	9.32*	170	119
168	6/28/97	EV9	EL14	4	0.985	132.9**	37	56
169	6/28/97	EV9	EL15	3	0.940	15.81ns	269	138
170	6/28/97	EV9	EV12	7	0.939	77.75***	2	5
171	6/28/97	EV9	EV13	5	0.900	27.03*	14	40
172	6/28/97	EV9	EV14	5	0.714	7.52ns	143	244
173	6/28/97	EV9	EV15	4	0.968	61.43*	229	213
174	6/28/97	EV10	EL12	5	0.977	129.3**	113	162
175	6/28/97	EV10	EL13	5	0.866	19.39*	27	23
176	6/28/97	EV10	EL14	4	0.701	4.71ns	41	21
177	6/28/97	EV10	EL15	3	0.907	9.840ns	1906	2705
178	6/28/97	EV10	EV12	7	0.895	42.83**	44	169
179	6/28/97	EV10	EV13	6	0.929	52.69**	48	111
180	6/28/97	EV10	EV14	3	0.973	36.14ns	150	334
181	6/28/97	EV10	EV15	4	0.902	18.49*	384	275