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

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**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 sperm-egg recognition.

## Introduction

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

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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 × *E. vanbrunti* female crosses, was no lower than in homospecific crosses. Fertilization in the *E. vanbrunti* male × *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 $\mu$l dry sperm in 25 ml FSW for homospecific

crosses and 5μl 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 μl each) of the starting suspension and FSW were mixed together, and 200 μl of this diluted suspension was again transferred to 200 μl FSW. This process was repeated to prepare 12 serial 2-fold dilutions. Egg suspension (200 μl) 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 μl 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 spermatozoa 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 log-transformed $F_{50}$. 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:

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

where $S_0$ = number of sperm/$\mu$l, $E_0$ = number of eggs/$\mu$l, $\tau$ = sperm/egg contact time (set to equal 90 min) and $\beta$ and $\beta_0$ are parameters obtained from nonlinear regression of $P$ on $S_0$ (the ratio $\beta/\beta_0$ 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 ($3.97 \times 10^5$ $\mu$m$^3$ volume), *E. lucunter* has eggs intermediate in size ($3.40 \times 10^5$ $\mu$m$^3$), and *E. vanbrunti* has the smallest eggs ($1.94 \times 10^5$ $\mu$m$^3$). Values of $\beta$ and $\beta_0$ 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 $\tau$) 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 $\tau$ 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 $5.35 \times 10^4$ sperm/$\mu$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 $5.37 \times 10^4$ for *E. mathei* and $4.94 \times 10^4$ 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 $9.5 \times 10^7$ sperm/$\mu$l. The eight *Echinometra* males used in our study

produced dry sperm with a similar average concentration—$3.04 \times 10^7$ sperm/$\mu$l.

*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.

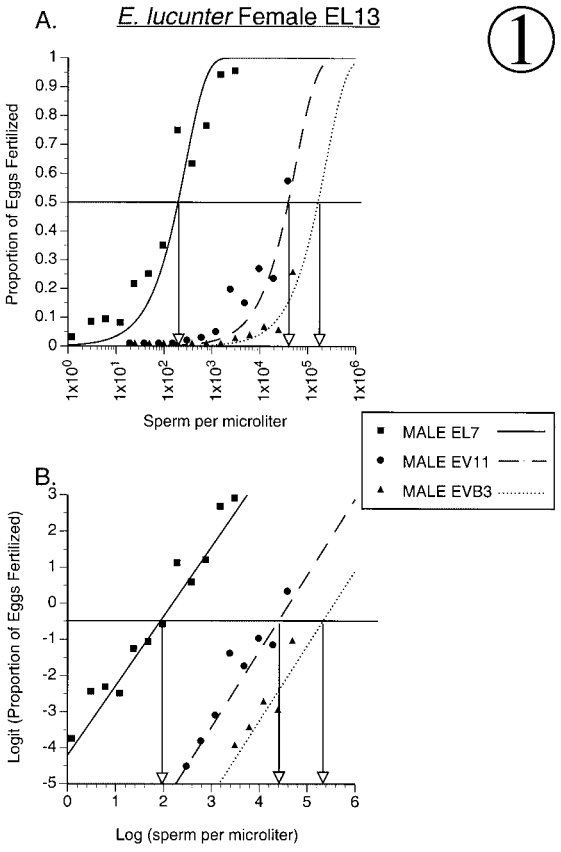
*Comparison of* $F_{50}$ *values*

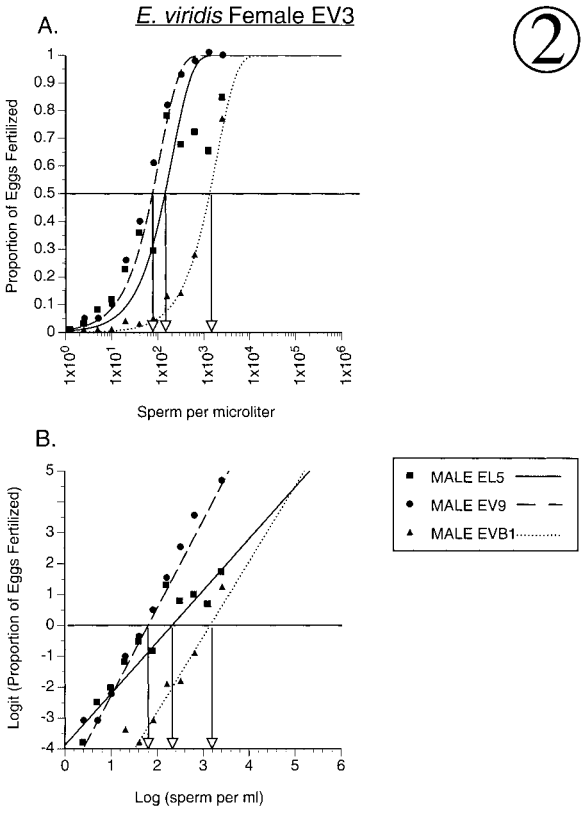
To compare the results obtained across multiple females, $F_{50}$ values were estimated for all crosses. The $F_{50}$ values were then placed into nine groups representing the nine cross classes (with each class of cross defined as one species of male crossed with one species of female: Figs. 4, 5, and 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).

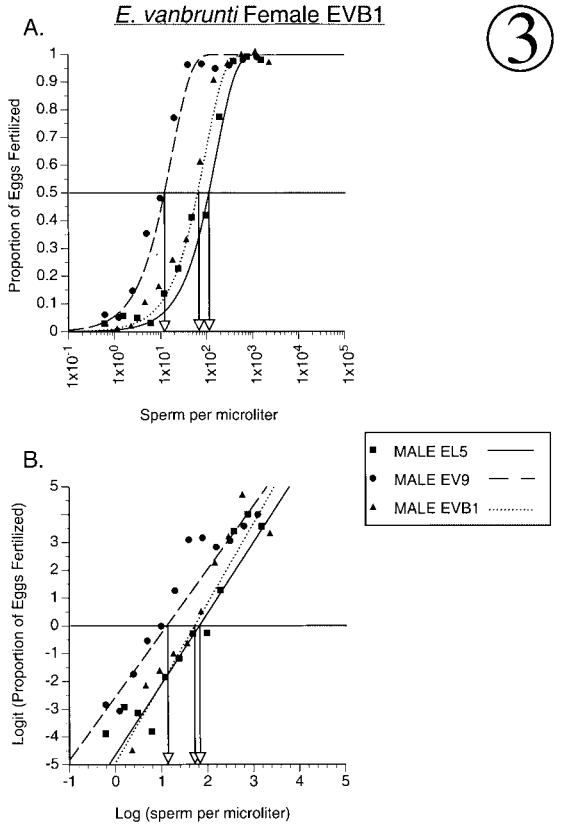
*E. viridis* females showed much more modest heterospecific incompatibility. Compared with conspecific sperm, just over twice as much *E. lucunter* sperm and about 18 times as much *E. vanbrunti* sperm fertilized half the *E. viridis* eggs (*Fig*. 5, Table 1). *E. vanbrunti* females were only slightly 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.

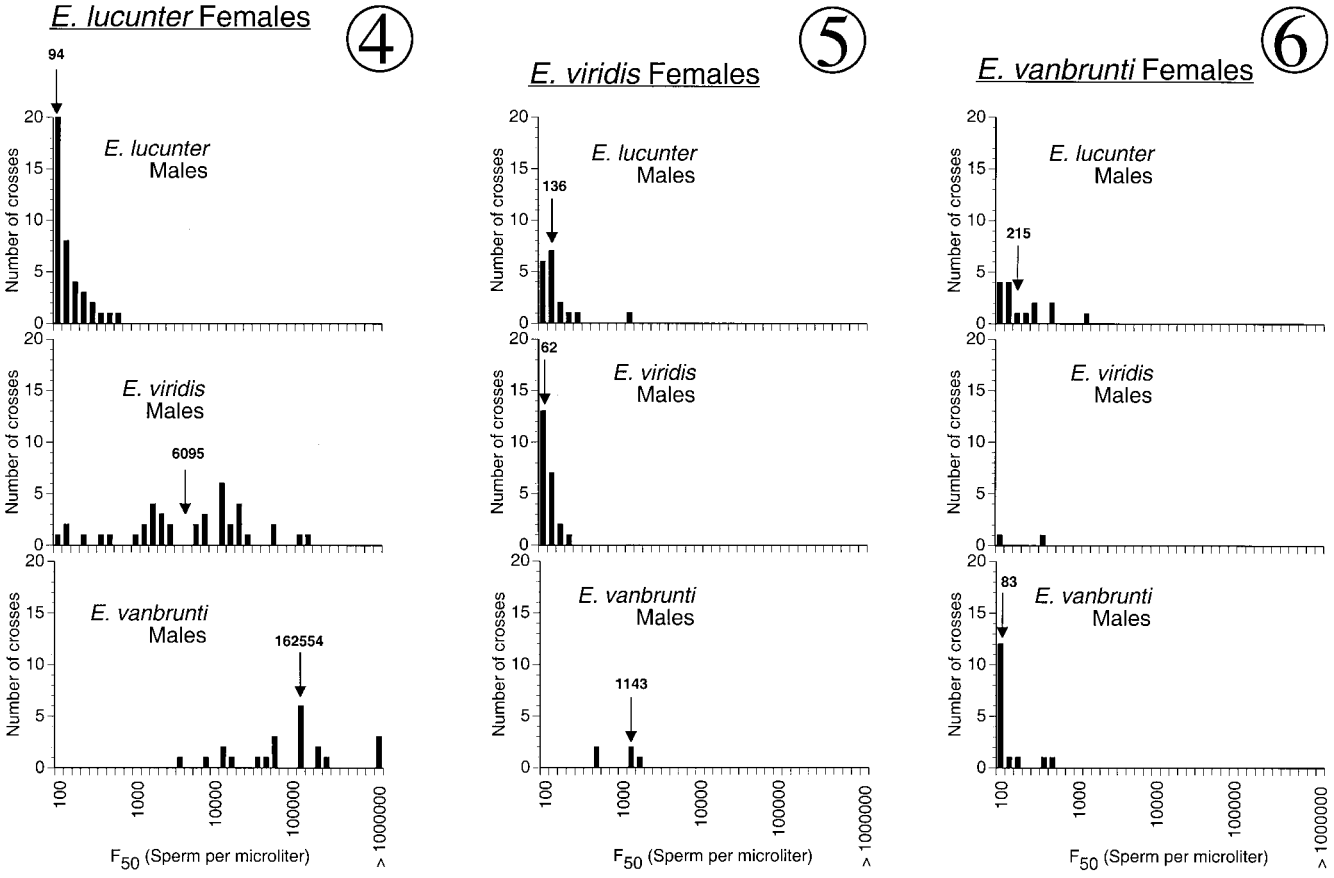
A potential problem with the above analysis is that males and females were used in more than one fertilization experiment, so a randomized complete blocks analysis was used to allow for any non-independence. The results closely matched those from one-way ANOVA. Highly significant differences in fertilization 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 ($F = 140.29$, $P < 0.001$). 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




**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/$\mu$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. lucunter* male, 23 *E. viridis* female × *E. viridis* male, 5 *E. viridis* female × *E. vanbrunti* male; 15 *E. vanbrunti* female × *E. lucunter* male, 2 *E. vanbrunti* female × *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* eggs. About 1.5 times as much *E. lucunter* sperm and about 12 times as much *E. vanbrunti* sperm

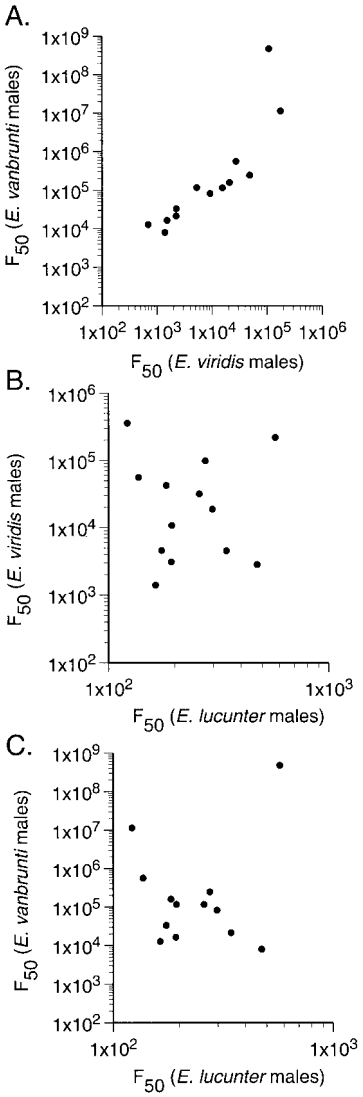



**Figure 7.** Correlation of $F_{50}$ values from *Echinometra* fertilization experiments. Data shown are for 13 crosses of *E. lucunter* females, each of which was tested with a single male of each of the three species. Points are $F_{50}$ values measured in the cross with a male of one species (ordinate) 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 $\tau = -0.077$, $P > 0.05$) 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 $>$ 700 to $>$ 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

**Table 1**

*Summary comparison of* $F_{50}$ *values among the nine possible crosses of* Echinometra *species.*

| 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 |

$F_{50}$ 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 logit-transformed 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

**Table 2**

*Comparison of* $F_{50}$ *values estimated from the nonlinear model of Vogel* et al. *(1982) for the nine possible crosses of* Echinometra *species*

| Female | Male | *n* | $\bar{F}_{50}$ | $F_{50}$ 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 |

$n$ = 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 ≥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 ≥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 isolation 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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## Literature Cited

**Aslan, L. M., and T. Uehara. 1997.** Hybridization and F1 backcrosses between two closely related tropical species of sea urchins (genus *Echinometra*) in Okinawa. *Invertebr. Reprod. Dev.* **31:** 319–324.

**Babcock, R. C., G. D. Bull, P. L. Harrison, A. J. Heyward, J. K. Oliver, C. C. Wallace, and B. L. Willis. 1986.** Synchronous spawnings of 105 scleractinian coral species on the Great Barrier Reef. *Mar. Biol.* **90:** 379–394.

**Banks, M. A., D. J. McGoldrick, W. Borgeson, and D. Hedgecock. 1994.** Gametic incompatibility and genetic divergence of Pacific and Kumamoto oysters, *Crassostrea gigas* and *C. sikamea. Mar. Biol.* **121:** 127–135.

**Branham, J. M. 1972.** Comparative fertility of gametes from six species of sea urchins. *Biol. Bull.* **142:** 385–396.

**Buss, L. W., and P. O. Yund. 1989.** A sibling species group of *Hydractinia* in the northeastern United States. *J. Mar. Biol. Assoc. UK* **69:** 857–874.

**Butlin, R. 1989.** Reinforcement of pre-mating isolation. Pp. 158–179 in *Speciation and its Consequences.* D. Otte and J. A. Endler, eds. Sinauer, Sunderland, MA.

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

**Coates, A. G., and J. A. Obando. 1996.** The geologic evolution of the Central American Isthmus. Pp. 21–56 in *Evolution and Environment in Tropical America,* J. B. C. Jackson, A. G. Coates, and A. Budd, eds. University of Chicago Press, Chicago.

**Dobzhansky, T. 1937.** *Genetics and the Origin of Species,* Columbia University Press, New York.

**Dobzhansky, T. 1940.** Speciation as a stage in evolutionary divergence. *Am. Nat.* **74:** 312–321.

**Finney, D. J. 1964.** *Statistical Method in Biological Assay,* Griffin, London.

**Games, P. A., and J. F. Howell. 1976.** Pairwise multiple comparison procedures with unequal *n*'s and/or variances: a Monte Carlo study. *J. Educ. Stat.* **1:** 113–125.

**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.

**Hewlett, P. S., and R. L. Plackett. 1979.** *The Interpretation of Quantal Responses in Biology.* University Park Press, Baltimore.

**Kaneshiro, K. 1976.** Ethological isolation and phylogeny in the planitibia subgroup of Hawaiian *Drosophila. Evolution* **30:** 740–745.

**Kaneshiro, K. 1980.** Sexual isolation, speciation, and the the direction of evolution. *Evolution* **34:** 437–444.

**Kaneshiro, K. Y. 1983.** Sexual selection and direction of evolution in the biosystematics of Hawaiian Drosophilidae. *Annu. Rev. Entomol.* **28:** 161–178.

**Knowlton, N., J. Maté, H. Guzmán, and R. Rowan. 1997.** Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panamá and Honduras). *Mar. Biol.* **127:** 705–711.

**Leighton, D. L., and C. A. Lewis. 1982.** Experimental hybridization in abalone. *Int. J. Invertebr. Reprod. Dev.* **5:** 273–282.

**Lessios, H. A. 1979.** Use of Panamanian sea urchins to test the molecular clock. *Nature* **280:** 599–601.

**Lessios, H. A. 1981a.** Divergence in allopatry: molecular and morphological differentiation between sea urchins separated by the Isthmus of Panama. *Evolution* 35: 618–634.

**Lessios, H. A. 1981b.** Reproductive periodicity of the echinoids *Diadema* and *Echinometra* on the two coasts of Panama. *J. Exp. Mar. Biol. Ecol.* **50:** 47–61.

**Lessios, H. A. 1985.** Annual reproductive periodicity in eight echinoid species on the Caribbean coast of Panama *in Echinodermata: Proceedings of the Fifth International Echinoderm Conference,* Galway. B. F. Keegan and B. D. O'Connor, eds. A. A. Balkema, Rotterdam.

**Lessios, H. A. 1990.** Adaptation and phylogeny as determinants of egg size in echinoderms from the two sides of the Isthmus of Panama. *Am. Nat.* **135:** 1–13.

**Lessios, H. A. 1991.** Presence and absence of monthly reproductive rhythms among eight Caribbean echinoids off the coast of Panama. *J. Exp. Mar. Biol. Ecol.* **153:** 27–47.

**Lessios, H. A. 1998.** The first stage of speciation as seen in organisms separated by the Isthmus of Panama. Pp. 186–201 in *Endless Forms: Species and Speciation.* D. Howard and S. Berlocher, eds. Oxford University Press, Oxford.

**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.

**Lessios, H. A., and C. W. Cunningham. 1993.** The evolution of gametic incompatibility in neotropical *Echinometra*: a reply to McClary. *Evolution* **47:** 1883–1885.

**Lessios, H. A., J. D. Cubit, D. R. Robertson, M. J. Shulman, M. R. Parker, S. D. Garrity, and S. C. Levings. 1984.** Mass mortality of *Diadema antillarum* on the Caribbean coast of Panama. *Coral Reefs* **3:** 173–182.

**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* **53:** 1043–1056.

**Liou, L. W., and T. D. Price. 1994.** Speciation by reinforcement of premating isolation. *Evolution* **48:** 1451–1459.

**Marsden, J. 1992.** Reproductive isolation in two forms of the serpulid polychaete, *Spirobranchus polycerus* (Schmarda) in Barbados. *Bull. Mar. Sci.* **51:** 14–18.

**McCartney, M. A., G. Keller, and H. A. Lessios. 2000.** Dispersal barriers in tropical oceans and speciation of Atlantic and eastern Pacific *Echinometra* sea urchins. *Mol. Ecol.* **9:** 1391–1400.

**McClary, D. 1992.** Gamete compatibility or incompatibility? A comment on Lessios and Cunningham (1990). *Evolution* **46:** 1579–1580.

**McCuen, F. S. 1988.** Spawning behaviors of northeast Pacific sea cucumbers (Holothuroidea: Echinodermata). *Mar. Biol.* **98:** 565–585.

**McPherson, B. F. 1969.** Studies on the biology of the tropical sea urchins *Echinometra lucunter* and *E. viridis. Bull. Mar. Sci.* **19:** 194–213.

**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. 1998a.** Mitochondrial DNA and bindin gene sequence evolution among allopatric species of the sea urchin genus *Arbacia. Mol. Biol. Evol.* **15:** 185–195.

**Metz, E. C., R. Robles-Sikisaka, and V. D. Vacquier. 1998b.** Nonsynonymous substitution in abalone sperm fertilization gene exceeds substitution in introns and mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **95:** 10676–10681.

**Miller, K., and R. Babcock. 1997.** Conflicting morphological and reproductive species boundaries in the coral genus *Platygyra. Biol. Bull.* **192:** 98–110.

**Minor, J. E., D. R. Fromson, R. J. Britten, and E. H. Davidson. 1991.** Comparison of the bindin proteins of *Strongylocentrotus franciscanus, S. purpuratus,* and *Lytechinus variegatus:* sequences involved in the species specificity of fertilization. *Mol. Biol. Evol.* **8:** 781–795.

**Mortensen, T. 1928–1951.** *A Monograph of the Echinoidea.* C. A. Reitzel, Copenhagen.

**Muller, H. J. 1942.** Isolating mechanisms, evolution and temperature. *Biological Symposia* **6:** 71–125.

**Nei, M., T. Maruyama, and C.-I. Wu. 1983.** Models of evolution of reproductive isolation. *Genetics* **103:** 557–579.

**Palumbi, S. R. 1992.** Marine speciation on a small planet. *Trends Ecol. Evol.* **7:** 114–118.

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

**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.

**Pawlik, J. 1988.** Larval settlement and metamorphosis of sabellariid polychaetes, with special reference to *Phragmatopoma lapidosa*, a reef-building species, and *Sabellaria floridensis*, a non-gregarious species. *Bull. Mar. Sci.* **43:** 41–60.

**Pearse, J. S., D. J. McClary, M. A. Sewell, W. C. Austin, A. Perez-Ruzafa, and M. Byrne. 1988.** Simultaneous spawning of six species of echinoderms in Barkley Sound, British Columbia. *Invertebr. Reprod. Dev.* **14:** 279–288.

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

**SAS Institute. 1989.** *JMP, Version 2.0.* Cary, NC.

**SAS Institute. 1999.** *StatView, Version 5.0.1.* Cary, NC.

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

**Summers, R. G., and B. L. Hylander. 1975.** Species-specificity of acrosome reaction and primary gamete binding in echinoids. *Exp. Cell Res.* **96:** 63–68.

**Szmant, A., E. Weil, M. Miller, and D. Colón. 1997.** Hybridization within the species complex of the scleractinian coral *Montastrea annularis. Mar. Biol.* **129:** 561–572.

**Tyler, A., A. Monroy, and C. B. Metz. 1956.** Fertilization of fertilized sea urchin eggs. *Biol. Bull.* **110:** 184–195.

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

**Vacquier, V. D., and J. E. Payne. 1973.** Methods for quantitating sea urchin sperm-egg binding. *Exp. Cell Res.* **82:** 227–235.

**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.

**Welch, B. L. 1951.** On the comparison of several mean values: an alternative approach. *Biometrika* **38:**330–336.

**Wu, C.-I. 1985.** A stochastic simulation study on speciation by sexual selection. *Evolution* **39:** 66–82.

## 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.

| # | Date | Female | Male | $n$ | Linear regression $R^2$ | Linear regression $F$ (reg) | Linear regression $F_{50}$ (linear) | Nonlinear regression $F_{50}$ (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 |

**Appendix** (*Continued*)

| # | Date | Female | Male | *n* | Linear regression $R^2$ | Linear regression *F* (reg) | Linear regression $F_{50}$ (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 |

**Appendix** (*Continued*)

| # | Date | Female | Male | *n* | Linear regression $R^2$ | Linear regression $F$ (reg) | Linear regression $F_{50}$ (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 | EL19 | EVB5 | 5 | 0.661 | 5.874ns | 14271563 | 204606 |
| 101 | 10/3/96 | EL19 | EVB6 | 6 | 0.650 | 7.438* | 427516 | 270235 |
| 102 | 10/3/96 | EL19 | EVB7 | 7 | 0.889 | 40.27** | 128093 | 106011 |
| 103 | 10/3/96 | EL20 | EL8 | 5 | 0.805 | 12.43* | 102 | 122 |
| 104 | 10/3/96 | EL20 | EL9 | 5 | 0.830 | 14.69* | 118 | 57 |
| 105 | 10/3/96 | EL20 | EL10 | 5 | 0.944 | 51.41** | 473 | 916 |
| 106 | 10/3/96 | EL20 | EL11 | 5 | 0.916 | 32.94** | 68 | 51 |
| 107 | 10/3/96 | EL20 | EVB4 | 6 | 0.958 | 92.14*** | 76713 | 103441 |
| 108 | 10/3/96 | EL20 | EVB5 | 7 | 0.885 | 38.50** | 147808 | 112384 |
| 109 | 10/3/96 | EL20 | EVB6 | 5 | 0.888 | 24.00* | 184934 | 145387 |
| 110 | 10/3/96 | EL20 | EVB7 | 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 | 176.23*** | 170 | 101 |
| 113 | 10/3/96 | EVB5 | EL10 | 8 | 0.916 | 65.96*** | 38 | 41 |
| 114 | 10/3/96 | EVB5 | EL11 | 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 |

**Appendix** (*Continued*)

| # | Date | Female | Male | *n* | Linear regression $R^2$ | Linear regression *F* (reg) | Linear regression $F_{50}$ (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 |