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# SIMULTANEOUS POSITIVE AND NEGATIVE FREQUENCY-DEPENDENT SELECTION ON SPERM BINDIN, A GAMETE RECOGNITION PROTEIN IN THE SEA URCHIN *STRONGYLOCENTROTUS PURPURATUS*

**Don R. Levitan[1,2] and Andres Plata Stapper[1]**

[1]*Department of Biological Science, Florida State University, Tallahassee, Florida 32306*

[2]*E-mail: levitan@bio.fsu.edu*

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**Gamete-recognition proteins often, but not always, evolve rapidly. We explored how variation in sperm bindin influences reproductive success of the sea urchin *Strongylocentrotus purpuratus* during group spawning in the sea. Despite large variation in male and female abundance and neighbor distances, males with common genotypes had higher reproductive success than males with rare genotypes. However, males with a relatively uncommon proline-for-serine substitution were the most successful. Females also showed a fitness consequence of sperm-bindin genotype, suggesting linkage disequilibrium between the sperm-bindin locus and the egg receptor locus. Females with common genotypes had higher reproductive success than rare genotypes, but females with relatively uncommon insertions were most successful. Overall, these results suggest that rare male proteins are selected against, as supported by molecular evidence of purifying selection and probably caused by poor matches to the female receptor protein. Within the pool of moderately common to common alleles, however, individuals with less-common functional variants were favored and probably maintained by negative frequency-dependent selection. These results support the hypothesis that sperm availability and sexual conflict influence the evolution of gamete recognition systems in broadcast spawners and highlight the benefits of combining fitness measures with molecular signatures for estimation of patterns of selection.**

**KEY WORDS:** **Assortative mating, fertilization, positive selection, reinforcement selection, sexual conflict, sexual selection, sperm bindin, sperm competition, sperm limitation.**

Fertilization requires sufficient collisions of eggs with compatible sperm to allow for successful fusion. Too few collisions can prevent fertilization, whereas too many can result in polyspermy and developmental failure (Styan 1998; Franke et al. 2002; Levitan 2004; Levitan et al. 2007). Poorly matched gametes require higher numbers of collisions for successful fertilization or cannot fuse at all (Hagstrom 1956; Minor et al. 1991; Levitan 2002b). In the sea, sperm abundance and gamete compatibility interact to determine fertilization success. Higher levels of compatibility are needed for fertilization when collisions are rare, whereas lower levels of compatibility can prevent polyspermy when collisions are overabundant (Levitan and Ferrell 2006; Levitan et al. 2007). Although compatibility is generally considered as a mechanism influencing reproductive isolation between species or populations, the existence of variation in compatibility within populations is becoming more obvious (Zeh and Zeh 1996; Levitan 2002b; McCartney and Lessios 2002; Evans and Marshall 2005; Levitan and Ferrell 2006; Riginos et al. 2006; Styan et al. 2008).

Gamete-recognition proteins can mediate the likelihood of fertilization and determine the mating success of individuals within and across populations. In some taxa, these proteins show evidence of positive selection—more nonsynonymous substitutions than neutral expectations would predict (reviewed by Swanson and Vacquier 2002)—manifested as patterns of divergence across species (Swanson and Vacquier 1995; Biermann 1998; Hellberg et al. 2000; Zigler et al. 2005) and diversification within species (Metz and Palumbi 1996; Geyer and Palumbi 2003; Riginos et al. 2006; Moy et al. 2008). At first glance, this pattern seems odd; why should selection favor new proteins that probably cause mismatches between sperm and eggs?

Theory suggests that sexual selection and sexual conflict can drive this rapid evolution. When sperm are overabundant and polyspermy poses significant risk, then mutations that make eggs harder to fertilize might lower the effective concentration of sperm and allow the egg time to erect a successful block to polyspermy. A conflict over sperm–egg affinity therefore arises; females are selected for a lower affinity that reduces the risk of polyspermy whereas males are selected for a higher affinity that prevents outcompetition by other males. This conflict can lead to an "arms race" over affinities that results in divergence in recognition proteins within and among populations as sperm proteins chase the evolution of diversifying egg proteins (Gavrilets and Waxman 2002; Haygood 2004; M. Tomaiuolo and D. R. Levitan, unpubl. ms.).

Empirical tests of this idea are scant but revealing. A laboratory study indicated that males more similar to females at the sperm-bindin locus won in sperm competition over more dissimilar males (Palumbi 1999). This result suggests that the sperm-bindin genotype is informative in predicting fitness in females as well as males and that matching proteins can lead to higher fertilization rates. Laboratory (Levitan et al. 2007) and field (Levitan 2008) studies have also documented that, both within and across species, females that produce eggs that can be fertilized at lower sperm concentrations are more susceptible to polyspermy than females producing eggs requiring a higher concentration of sperm for fertilization. These results suggest not only that matching proteins might lead to higher fertilization rates but also that close matching might be costly to females under conditions of high sperm availability. Finally, a field study documented that males that matched females at the sperm-bindin locus had higher reproductive success than mismatched individuals at low levels of sperm availability but that, at high levels of sperm availability and polyspermy, mismatched individuals had higher success (Levitan and Ferrell 2006). These studies allow for a prediction about when and where gamete proteins should evolve rapidly. Demographic conditions that lead to high levels of sperm availability and the risk of polyspermy should result in an "arms race" and rapid evolution, whereas sperm-limited populations should experience purifying selection that will increase matching (Levitan and Ferrell 2006; M. Tomaiuolo and D. R. Levitan, unpubl. ms.).

These findings also point to the mystery of how female reproductive success can be predicted on the basis of the sperm-bindin locus, a protein seemingly not expressed in females (see, e.g., Palumbi 1999; Levitan and Ferrell 2006). A potential resolution of this issue is that assortative mating, driven by gamete affinities, leads to linkage disequilibrium between the sperm and egg recognition protein loci (Clark et al. 2009; M. Tomaiuolo and D. R. Levitan, unpubl. ms.).

In the work reported here, we examined the effects of sperm-bindin genotype on reproductive success in the sea urchin *Strongylocentrotus purpuratus* under natural conditions in the sea. Unlike the previously examined *Strongylocentrotus franciscanus,* which occurs at a range of population densities that can lead to conditions either of sperm limitation or of sperm oversaturation, *S. purpuratus* is more typically found at higher population densities and produces eggs that require higher sperm abundance to achieve fertilization and are resistant to both hybridization and polyspermy (Levitan 1993, 2002a,b, 2008; Levitan et al. 2007). The results suggest a complex pattern of both positive and negative frequency-dependent selection influencing the evolution of this protein.

## Methods

Field work was conducted in the springs of 2003–2005 in Barkley Sound in the Deer Island Group, British Columbia, Canada. In each of 15 independent spawning events, between 7 and 33 sea urchins were tagged, induced to spawn with an injection of 0.55 M KCl, and placed on their natural substratum over a range of population densities (1–270/$m^2$) that bracketed their natural range of densities (Levitan 2002a). Sea urchins were generally found in depressions or cracks in the rocky substratum, and after KCl injection they were returned to those locations. Generally within 1 min individuals began to spawn, and after 20–30 min of spawning, the positions of all individuals were mapped, and egg samples were collected approximately 15–20 cm above each female with a subtidal plankton pump. After eggs were collected, all males and females were collected, and tube-foot tissues samples were collected for genetic analysis. Water flow during these experiments was documented with an S4 current meter that recorded the depth and velocity in the E–W and N–S direction every 0.5 sec. These values were used to calculate the surge velocity (average of the absolute values of the 0.5 sec velocities) and advection (total straight line distance a parcel of water moved over the experimental time divided by the total time). The former measurement provides information on mixing and turbulence, and the latter provides information on the residence time of sperm over the spawning females (see Levitan 2008 for details of field experiment and study site).

Three hours after egg collection, at least 200 eggs were inspected for the presence of a raised fertilization envelope or later developmental stage. In 2004, when the bulk of the experiments were conducted (13 of the 15 trials), these inspected eggs were cultured for 2 days and inspected again for developmental success, which revealed the fraction of eggs developing normally as a measure of polyspermy. A sample of all collected eggs was cultured for 3 days without food, and 30–50 larvae from those cultures were individually frozen in 1 μl each of ultrapure filtered water.

For each spawning date, all adults and 20 larvae per female were genotyped with up to 12 microsatellite loci (7 loci from Cameron et al. 1999; 5 loci from Addison and Hart 2002) for determination of parentage of larvae and estimation of male and female reproductive success. Details of male and female reproductive success and patterns of sexual selection have been published previously (Levitan 2008). The relevant findings for the current study are that this species is often living at high densities, has high levels of female fertilization and high levels of male competition. This species exhibits polyspermy at higher sperm concentrations than congeners (Levitan et al. 2007) and polyspermy was rarely noted in this field experiment (Levitan 2008).

From the 15 spawning events in this field experiment, 11 were chosen that represented the five lowest and six highest spawning densities, and all adults were sequenced for the bindin locus AF077309 (Biermann 1998). This sampling scheme increased the chance of detecting interactions between spawning density, or sperm availability, and sperm-bindin genotype on reproductive success.

The sperm *bindin* gene codes for a protein 236 amino acids long translated from an mRNA of 708 bp. The variable locus AF077309 of *bindin* is composed of two exons separated by an intron 943 bp long. The first exon, which is 231 bp long and codes for a variable domain, was selected for genotyping in our study as it has in other studies (e.g., Debenham et al. 2000; Levitan and Ferrell 2006). This site has been suggested to play a role in species-specific interaction with glycoproteins (Lopez et al. 1993) such as the egg receptor for bindin protein (EBR1) for this species (Kamei and Glabe 2003). The second exon includes a highly conserved sequence followed by a variable repeat area. The conserved area is highly hydrophilic, suggesting a potential association to phospholipid bilayers, such as the egg matrix (Biermann 1998).

### SPERM-BINDIN GENOTYPING (DNA EXTRACTION, AMPLIFICATION, CLONING, AND SEQUENCING)

Tube feet from each male and female from the field experiment were stored in ethanol. Separate DNA extractions were performed on 5–7 tube feet from each individual, digested in a solution of CTAB and proteinase K incubated in a 65°C water bath for approximately 12 h. DNA extractions were performed with a SprintPrep DNA Purification kit (Agencourt Bioscience Corporation Beverly, MA), a magnetic-bead-based protocol. Extracts were stored at −20°C until ready for use in PCR reactions.

To obtain a clear sequence of our entire exon of interest, we designed gene-specific amplification and sequencing primers upstream from the start codon on the 5′ UTR region of the gene and downstream of the splice intron site. The UTR and intron sequences were obtained from the Sea Urchin Genome Sequencing Project by means of a blast search for the mature peptide sequence mRNA from the gene bank sequence NM_214518. The *bindin* gene is located in the scaffold 66,693 of the *S. purpuratus* genome project. We designed the following primers for amplification and sequencing respectively: PUB8 forward 5′CTTCATCTCGGGGCATTCTC3′ (91 bp upstream of start codon), PUB10 forward 5′CCGCAGTTTCTGACGATTCG3′ (44 bp upstream of start codon), PUB15 reverse 5′TTGGTGTGA CTACAGCGTGA3′ (146 bp downsteam of intron split site), and PUB16 reverse 5′ATGCCAGCCAAAGATACCAG3′ (240 bp downsteam of intron split site).

To decrease the possibility of genotyping errors due to PCR errors, we used a high-fidelity Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) with proofreading capabilities in all of our reactions. The PCR cocktail consisted of 12.95 μl double-distilled water, 2.5 μl 10× PCR buffer, 1.0 μl 2 mM MgCl, 2.5 μl 2 mM dNTPs, 0.15 μl Platinum HiFiTaq (Invitrogen), 1.2 μl 0.5 μM for PUB8 and PUB10, 1.2 μl μM for PUB15 and PUB16, 1.0 μl 10 μM bovine serum albumin, and 1.0 μl DNA (25 ng/μl). The PCR program was as follows: 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 56–59°C (depending on primers used), and 2 min at 72°C; and 7 min at 72°C. Sequencing reactions were performed on an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Initial sequence data determined whether individuals were homozygous or heterozygous at the target locus AF077309 with the sequencing primers PUB10 forward and PUB15 reverse.

All heterozygote individuals with more than one polymorphic site were subcloned into pCR2.1-TOPO vector from Invitrogen according to the manufacturer's protocol. The inserts from six recombinant clones for each sample were PCR amplified directly from the bacterial colonies with M13 forward and reverse primers, purified with magnetic beads, and sequenced on an Applied Biosystems 3100 Genetic Analyzer using T7 primers.

The obtained nucleotide sequences were initially analyzed and binned into distinct haplotypes, and individuals were assigned a diploid genotype by Sequencher 4.5 (Gene Codes, Ann Arbor, MI). Nucleotide sequences were aligned and analyzed with *MEGA* version 4 (Tamura et al. 2007). We assessed whether haplotypes deviated from neutral expectations using Tajima's D (Tajima

1989) and Fu and Li's D* (Fu and Li 1993) indices with DnaSP version 4.9 (Rozas et al. 2003).

# Results

## HAPLOTYPE AND ALLELIC DIVERSITY

A total of 40 haplotypes were noted among the 135 individuals (sequences deposited with GenBank, Accession numbers GU075625-GU075664). Of these, 31 were of 236 bp, the remaining nine had inserts of 3 (at site 41, one haplotype), 12 (at site 47, seven haplotypes), or 24 (at site 47, one haplotype) bp (Fig. 1A). These are all amino acid inserts and do not result in downstream coding changes. Of these 40 haplotypes, 17 had unique amino-acid sequences and are referred to as nonsynonymous alleles (Fig. 1). The three most common nonsynonymous alleles had frequencies of 0.58, 0.16, and 0.14, and the 10 rarest were only represented in the heterozygous form by single individuals at allele frequencies of 0.004 (Fig. 1). The nonsynonymous genotype frequencies did not differ from Hardy–Weinberg expectations (chi-square $P > 0.5$).

We reconstructed the phylogenetic history of sperm-bindin haplotypes using both parsimony (MP) and maximum likelihood (ML) with PAUP* (Swofford 2002). Parsimony applied equal weighting and maximum likelihood employed a HKY85 gamma model of evolution. Two divergent sequences from *S. droebachiensis* were used as outgroups. However, no well-supported tree was produced, with >300,000 equally parsimonious trees of 55 steps. When gaps were coded as a fifth character state, two more haplotype clades were recovered that corresponded to the two indels. All analyses recover haplotypes "3" and "17" together as the sister-clade to all other haplotypes. The key pattern was one of many haplotypes differing by only one or two substitutions and little hierarchical structure. Uncorrected genetic distance between the species (6.7–7.3%) well exceeded the maximum within each species (0.4% in *S. droebachiensis* and 2.5% in *S. purpuratus*).

Statistical parsimony networks were estimated with TCS (Clement et al. 2000). Gaps were not included as a fifth character state but were represented by three appended characters coding presence or absence of each of the three insertions respectively (Fig. 1B). The most common haplotype "1" appears to be ancestral, with many haplotypes, some with nonsynonymous changes, being connected by a single mutational step. In addition, three other groups of haplotypes form clusters; (1) one derived from common haplotype "4," with a single synonymous substitution from haplotype "1," (2) one derived from common haplotype "2" that differs from haplotype "1" by a single nonsynonymous substitution of proline for serine at amino acid site 61 and (3) one derived from common haplotype "11" that differs from haplotype "1" by amino acid insertions.

We examined the pattern of variation for the entire first exon of bindin. The unresolved tree structure, short sequence and degree of divergence of these data decrease the power and accuracy of tests of selection in PAML (e.g., BEB for site-specific selection—Yang et al. 2005, see Anisimova et al. 2001) and instead used tests designed for intraspecific comparisons. Tajima's D (Tajima 1989) and Fu and Li's D* (Fu and Li 1993) were

**A**

| Non-synonymous allele | Amino acid sequence | Frequency |
|---|---|---|
| A | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.579545 |
| K | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGPMGGP----PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.159091 |
| J | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGP--------PQFGALPPGQADTDFGSSSSSVDGGDTTISAR | 0.140152 |
| I | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGP--------PQFGPLPPGQADTDFGSSSSSVDGGDTTISAR | 0.030303 |
| P | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGGGPMGGL--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAK | 0.026515 |
| F | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGV-PMGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.022727 |
| H | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGL--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAK | 0.007576 |
| B | YVNAMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| C | YVNTMGYPQAMSPQMGDVNYGQPAQQGYGAQGMGGPVGGG-PMGGP--------PQFGALPPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| D | YVNTMGYPQAMSPQMGGVNYGQPVQQGYGAQGMGGPVGGG-PMGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| E | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGPQGMGGPVGGG-PMGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| G | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PLGGP--------PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| L | YVNTMGYPQAMSPQMGGVNYGQTAQQGYGAQGMGGPVGGG-PMGGPMGGP----PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| M | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGARGMGGPVGGG-PMGGPMGGP----PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| N | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PVGGPMGGP----PQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |
| O | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGPMGGP----PQFGALSPGQADTDFGSSSSSVDGGGTTISAR | 0.003788 |
| Q | YVNTMGYPQAMSPQMGGVNYGQPAQQGYGAQGMGGPVGGG-PMGGPMGGPMGGPPQFGALSPGQADTDFGSSSSSVDGGDTTISAR | 0.003788 |

**Figure 1.** ***Strongylocentrotus purpuratus*** **sperm bindin. (A) Amino acid sequences and frequencies. Asterisks indicate variable amino acid sites. The most common allele does not have the insert at site 41 or the variable-length insert at site 47. Asterisk with underline is site 61, the common serine replaced with the less common proline. (B) Haplotype network. Number indicates haplotype ID and letter indicates nonsynonymous allele ID, size indicates relative frequency. Red dots indicate nonsynonymous changes, black dots indicate synonymous changes, blue dots indicate indel changes. Haplotypes in yellow have the less common proline substitution at amino acid site 61, haplotypes in blue have amino acid insertions at site 47.**

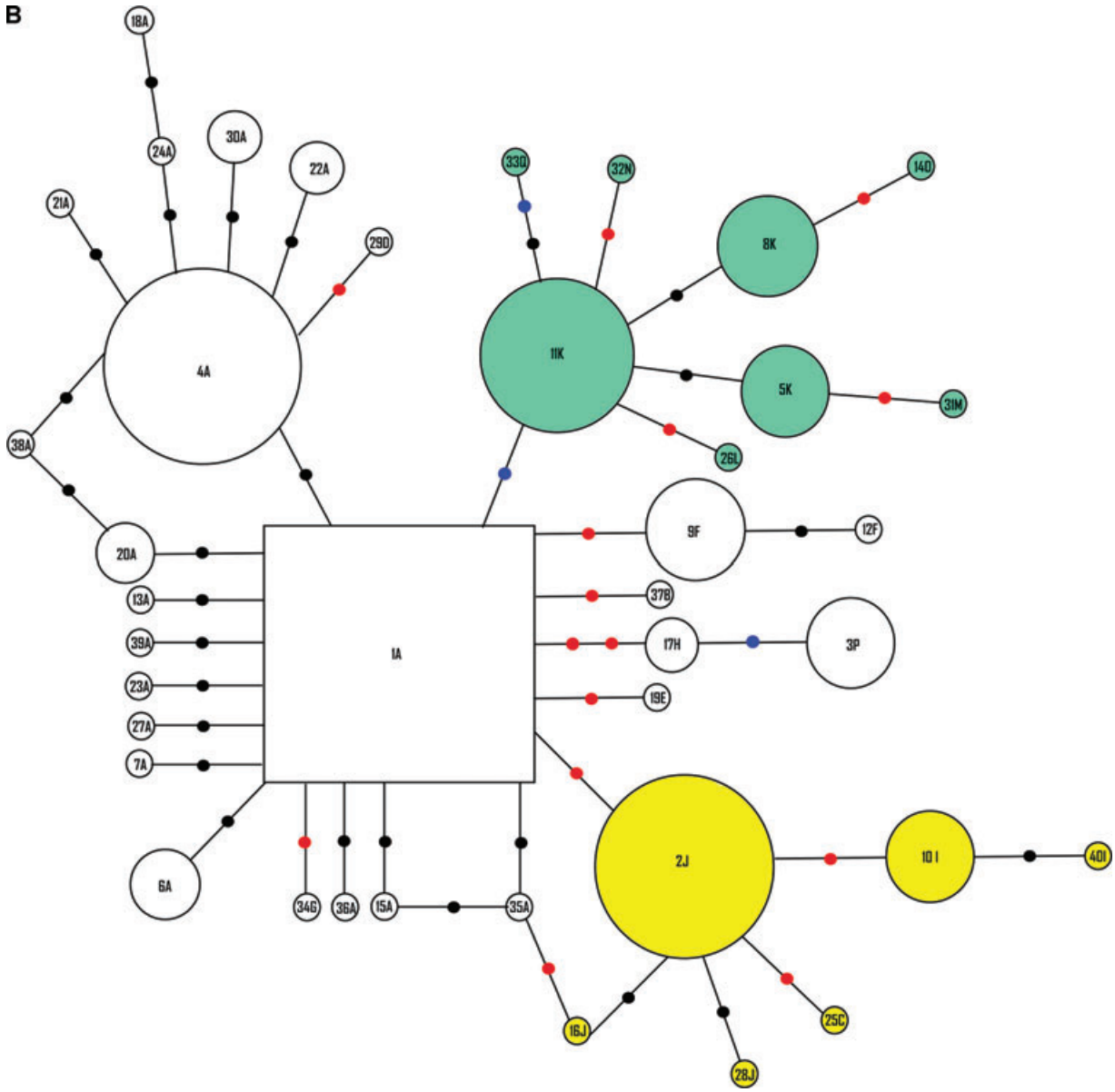


**Figure 1.** Continued.

employed to test for evidence of selection using DnaSP (Rozas et al. 2003). An excess of rare haplotypes generates negative values consistent with purifying selection, whereas an excess of common haplotypes generates positive values consistent with balancing selection (Nei and Kumar 2000). Both tests showed significant departure from neutrality and indicated an excess of rare variants with negative values of D ($-2.372$, $P < 0.01$) and D* ($-3.452$, $P < 0.02$), suggesting purifying selection (Table 1). We then examined a variety of sliding window scales (100, 50, 25 bps) and continued to find regions with a significant excess of rare variants (Fig. 2). The sliding window approach did identify two positive, but nonsignificant, regions (Fig. 2). The first region was associated with a point substitution resulting in a change from the common glycine to valine (amino acid site 40). The valine variant was noted in nonsynonymous allele F which comprises two haplotypes with a cumulative frequency of 0.02. The second region was associated with a point substitution resulting in a change from the more common serine to the less

**Table 1. Levels of polymorphism and tests of neutrality in exon 1 of the *Strongylocentrotus purpuratus* sperm-bindin locus. Number of haplotypes (*n*), number of sites (*L*) number of segregating sites (*S*), theta per site (Θ), and nucleotide diversity (π).**

| *n* | *L* | *S* | Θ | π | Tajima's D | Fu and Li's D* |
|---|---|---|---|---|---|---|
| 40 | 254 | 34 | 0.03648 | 0.01169 | −2.37167** | −3.45158* |

**$P<0.01$, *$P<0.02$.

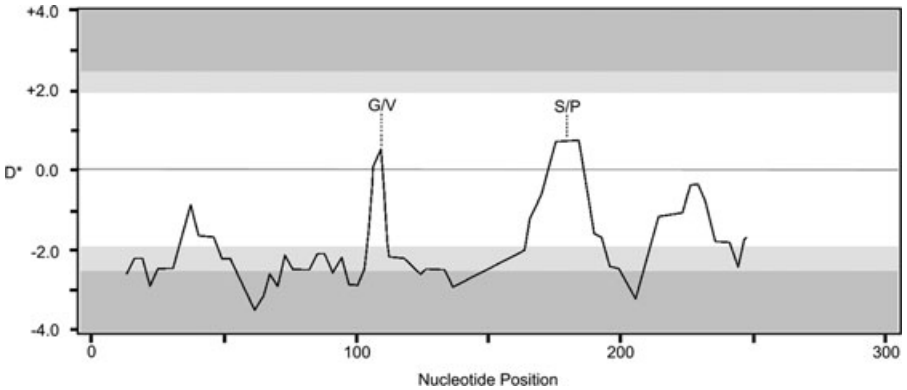


**Figure 2.** Values of Fu and Li's D* test for neutrality across the first exon of the *Strongylocentrotus purpuratus* sperm-bindin protein (Tajima's D test resulting in a similar pattern is not shown). A variety of window lengths (25, 50, and 100 bps) and step sizes (1–100) indicated regions with significant negative values and never significant positive values. This representative plot is based on a window size of 25 bps and a step size of 3. Lightly shaded regions indicate marginal significance ($0.10 > P > 0.05$), darkly shaded regions indicate significant departures from neutrality ($P < 0.05$). The two nonsignificantly positive peaks are identified as amino site 40 (valine for glycine) and site 61 (proline for serine).

common proline (amino acid site 61). The proline variant was noted in nonsynonymous alleles C, I, J, which comprise six haplotypes with a cumulative frequency of 0.17. This substitution is highlighted in the haplotype network (Fig. 1) and had important fitness consequences (see below).

## FITNESS CONSEQUENCES OF VARIATION IN BINDIN

We examined the fitness consequences of variation in this protein as a function of overall nonsynonymous genotype frequency (Fig. 1A) as well as the significance of two particular categories of functional differences (Table 2) noted in the haplotype network (Fig. 1B). The first functional category was based on two observed variable insertion sites located at positions 41 and 47. Sites 41 and 47 have been described as being absent in *S. purpuratus* but present in congeners, although only single individuals/species were reported in that study (Biermann 1998). The second category was based on a fairly common substitution of proline for serine at position 61 in this population. The substitution of proline for serine changes polarity and hydrophobicity of the protein at the site; such changes are known to influence protein structure and function (Gilles et al. 1986; Chen et al. 1992; Glenn and Novembre 2004; Kuiper et al. 2006; Jiao et al. 2008) and may influence the binding affinity of this sperm protein to its egg receptor. Although other specific amino-acid substitutions might influence reproductive success (e.g., site 40), they were not common enough to test independently and did not form large independent clusters in the haplotype network.

**Table 2.** All individuals were classified into bins based on functional characteristics observed in the sequences. One set of bins was based on a series of inserted amino acids at amino-acid sites 41 and 47. The second was based on the presence of a serine (a polar hydrophilic amino acid) or a proline (a nonpolar hydrophobic amino acid) at amino-acid site 61.

| Bin | Description | Frequency |
|---|---|---|
| Insertions | | |
| $X_0$ | No insertion | 0.796 |
| $X_1$ | Insertion at site 41 (G) | 0.030 |
| $X_2$ | Insertion at site 47 (MGGP) | 0.170 |
| $X_3$ | Insertion at site 47 (MGGPMGGP) | 0.004 |
| Proline/serine substitution | | |
| S | Serine at site 61 | 0.83 |
| P | Proline at site 61 | 0.17 |

## FITNESS CONSEQUENCES IN MALES

Male reproductive success was examined as a function of the covariates of male density (log transformed), the distance to the nearest female, the ratio between number of competing males and the number of female mates, water flow, and sperm-bindin nonsynonymous genotype frequency (log transformed). The two independent sets of bins of functional changes were treated as main effect in a general linear model (SAS).

Most variation in male success could be explained by factors associated with the absolute and relative amount of sperm a male could place next to females; male success increased with an increase in the ratio of mates to competitors, a decrease in the distance to the nearest female, and overall male density (Table 3). Male density ranged from less than 1 male/$m^2$ to over 140 males/$m^2$ (Fig. 3), the ratio of mates to competitors from 0.26 to 2.0, and the distance from a male to its nearest female from 2 to 127 cm.

In addition to the effects of the distribution and abundance of individuals that dictate sperm availability, sperm-bindin genotype influenced male success in two ways. Overall, males with

**Table 3.** General linear model of male reproductive success as a function of nonsynonymous genotype frequency (log transformed, GF), serine/proline substitution (and heterozygous, SP), ratio of mates to competitors (Ratio), male density (log transformed, Den), distance to nearest female (Fem), and interactions. Factors, such as the presence of insertions and interaction terms, with $P > 0.10$ were removed from the model.

| Source | DF | Type III SS | MS | F | $P>F$ |
|---|---|---|---|---|---|
| GF | 1 | 0.303 | 0.303 | 4.74 | 0.0326 |
| SP | 2 | 0.607 | 0.303 | 4.75 | 0.0113 |
| Ratio | 1 | 2.081 | 2.081 | 32.60 | <0.0001 |
| Den | 1 | 0.359 | 0.359 | 4.90 | 0.0298 |
| Fem | 1 | 2.124 | 2.124 | 33.27 | <0.0001 |
| GF × Den | 1 | 0.359 | 0.359 | 5.62 | 0.0203 |
| SP × Den | 2 | 0.360 | 0.180 | 2.82 | 0.0656 |
| GF × Den × Fem | 1 | 0.189 | 0.189 | 2.95 | 0.0897 |
| Error | 76 | 4.851 | 0.064 | | |
| Total | 86 | 10.503 | | | |

common sperm-bindin genotypes had higher reproductive success than males with rare genotypes (Fig. 4). Male density interacted significantly with genotype frequency; this positive relationship was more pronounced at lower male densities (Table 3). One functional set of changes significantly influenced male reproductive success. Males homozygous for a nonpolar proline substitution (amino acid site 61) had advantages over males homozygous or heterozygous for the polar serine substitution ($P < 0.05$). Individuals that were heterozygous at this site had intermediate success but were not significantly different from males homozygous for serine ($P = 0.15$, Fig. 4B). This functional substitution did not interact significantly with overall genotype frequency. The other functional category, the presence and type of insertions, was not a significant factor in male success.

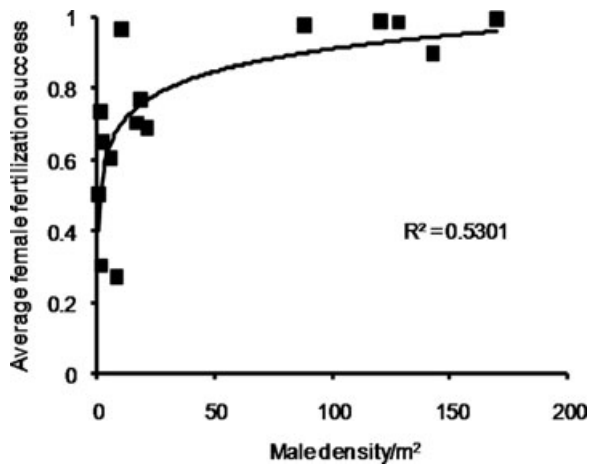


**Figure 3.** Average fertilization success of *Strongylocentrotus purpuratus* females in a spawning event as a function of the male density of that spawning event. Details of male and female density, nearest-neighbor distances, and water flow were reported by Levitan (2008).

## FITNESS EFFECTS IN FEMALES

Female reproductive success was examined as a function of the covariates of male density (log transformed), the average distance to all males, and sperm-bindin nonsynonymous genotype frequency (log transformed). The same two independent sets of bins of functional changes were treated as main effect in a general linear model (SAS; Table 4). Most variation in female success could be explained by the average distance to all males in a spawning event. Genotype frequency had a significant positive effect. Male density interacted marginally nonsignificantly with genotype frequency; the positive relationship was more pronounced at lower densities, because at higher densities females were saturated with sperm (Fig. 3).

In females, the signature of amino-acid insertions (Table 2) increased female reproductive success; less common and larger sequence insertions resulted in higher female reproductive success (Fig. 5). The most common insert genotype ($X_0X_0$, in 28 females) had no insertions and significantly lower fertilization success than the next most common insert genotype ($X_0X_2$, in with 15 females). The remaining insert genotypes were rare ($X_0X_1$, $X_0X_3$ and $X_2X_2$, in 3, 1, and 1 females respectively). The insertion at site 41 ($X_1$) did not differ significantly from individuals lacking insertions (pair-wise tests see Fig. 5); it appears that the larger set of inserts at site 46 ($X_2$ and $X_3$) have a greater effect on fertilization. Interestingly, the single $X_0X_3$ female had the highest reproductive success and the largest insertion (Fig. 5) even though she had the highest average distance to males (82 cm, compared to a mean of <45 cm for all other genotypes); this female produced eggs estimated to be highly fertilizable, given her success from a relatively isolated position in the spawning event. This functional change showed no significant interaction with overall genotype frequency. In females, the serine/proline substitution did not significantly influence reproductive success.

## PAIR-WISE REPRODUCTIVE SUCCESS

Because both male and female success could be predicted from their sperm-bindin genotype, we examined pair-wise reproductive success—the fraction of eggs fertilized by a particular male for a particular female within each group spawning event. A general linear model examined how male density (log transformed), pair-wise distance, male and female genotype frequency (log transformed), and main effects of the two functional categories (serine/proline and insertions) influenced pair-wise reproductive success (Table 5).

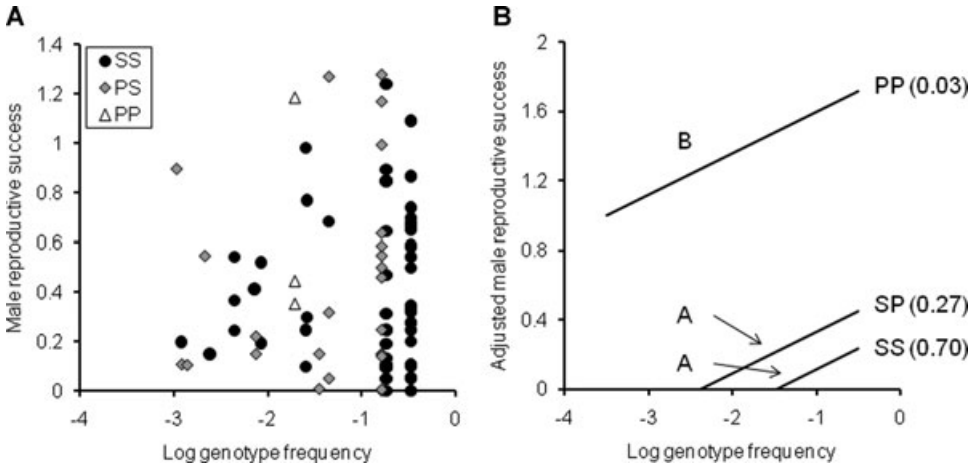


**Figure 4.** Male reproductive success as a function of sperm-bindin genotype frequency and serine (S), proline (P), or heterozygous (PS) condition at amino acid site 61. The SP substitution was treated as a main effect, and the slopes of these main effects were not significantly different (no main effect-by-genotype frequency interaction, Table 3). Raw data (A) and slope (B) of each SP condition adjusted by other factors in the ANCOVA (General Linear Model SAS). Letters on the left are least-square-mean estimates of pair-wise differences (genotypes that share a letter are not significantly different). SP identity and frequency are listed on right. Overall, common genotypes were more successful, but individuals with the less common proline substitution had higher success.

The results were similar, but more striking, compared to the patterns of total reproductive success of males and females (Table 5). Overall pair-wise success was significantly lower when both the male and female had rare genotypes (Fig. 6) but higher when the male was heterozygous and higher still when homozygous for the relatively uncommon proline substitution (Fig. 7A). Success was also higher when the female had the relatively uncommon amino-acid insertion (Fig. 7B). Significant interactions with density or distance were driven by decreases in slope as sperm reach saturation densities at higher male densities or short pair-wise distances (Fig. 3). The two functional categories (serine/proline and insertions) showed no significant interactions, nor did male and female genotype frequency or these functional categories and genotype frequencies (Table 5). Thus, in spite of the fact that rare genotypes had lower reproductive success, particularly when paired together (Fig. 6), the most successful pair-wise combinations involved males with the relatively uncommon proline substitution at site 61 (frequency of 17%) matched with females with the relatively uncommon insertion at site 46 (frequency of 17%—Fig. 7). These relatively uncommon (but not rare) protein variants have the highest reproductive success.

**Table 4.** General linear model of female reproductive success (arcsine transformed) as a function of average distance to all males (Ave), male density (log transformed, Den), nonsynonymous genotype frequency (log transformed, GF), insertion class (Insert), and interactions. Factors, such as the serine/proline substitution and interactions terms, with $P>0.10$ were removed from the model.

| Source | DF | Type III SS | MS | F | $P>$F |
|---|---|---|---|---|---|
| Ave | 1 | 0.687 | 0.687 | 14.77 | 0.0004 |
| Den | 1 | 0.002 | 0.002 | 0.04 | 0.8332 |
| GF | 1 | 0.337 | 0.337 | 7.23 | 0.0105 |
| Insert | 4 | 0.912 | 0.228 | 4.90 | 0.0027 |
| Den × GF | 1 | 0.187 | 0.187 | 4.03 | 0.0516 |
| Error | 39 | 1.815 | 0.047 | | |
| Total | 47 | 6.638 | | | |

## Discussion

Variation in the sperm-bindin protein of *S. purpuratus* influences patterns of fertilization in the sea. Despite ocean-driven turbulence and large differences in density, nearest-neighbor distances, and sex ratio, all of which influence local sperm concentration and fertilization rates (Levitan 2002a, 2008), males and females with common genotypes and with particular functional attributes produced more offspring than their counterparts. Below we first discuss the patterns of positive and negative frequency-dependent selection, then protein polymorphism and sperm availability and finally how assortative mating might explain why the sperm-bindin genotype predicts female reproductive success.

### FREQUENCY-DEPENDENT SELECTION

Common genotypes were more successful than rare genotypes. This is supported by (1) the molecular tests of sequence evolution indicating an excess of rare haplotypes and purifying selection (Table 1, Fig. 2), (2) the haplotype network indicating that rare haplotypes are clustered around the four common haplotypes and separated by only one mutational step, suggesting a balance between random mutations and selection around these ancestral

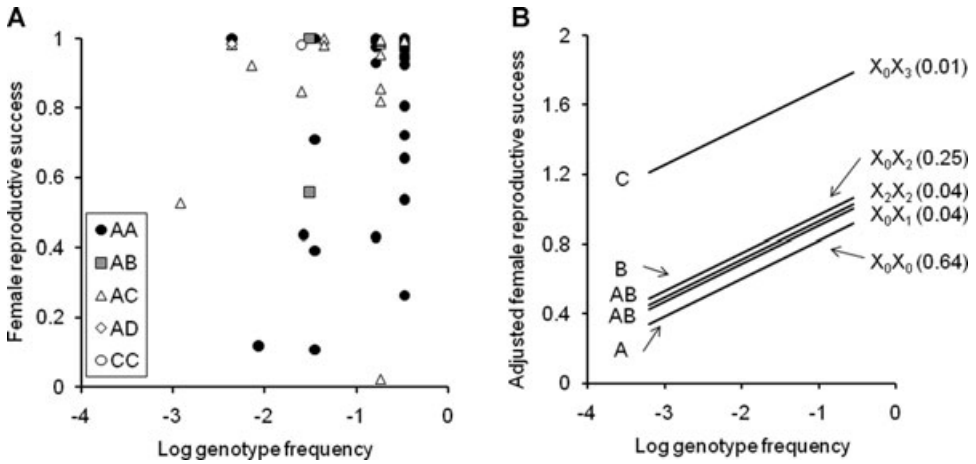


**Figure 5.** Female reproductive success as a function of overall sperm-bindin genotype frequency and the presence and condition (homozygous or heterozygous) of insertions (Table 2). Insertion condition was treated as a main effect, and the slopes of these main effects were not significantly different (no main effect-by-genotype frequency interaction. Table 4). Raw data (A) and slopes (B) of each Insertion condition adjusted by other factors in the ANCOVA (General Linear Model SAS). On panel "B" letters on the left are least square mean estimates of pair-wise differences (genotypes that share a letter are not significantly different). Insertion condition identity and frequency are listed on right. Insertion identities are described in Table 2. Overall, common genotypes were more successful, but the less common individuals carrying an insertion had higher success.

haplotypes (Fig. 1A), and (3) the field data indicating that rare genotypes have poor reproductive success (Fig. 4A, 5A and 6). Random mutations of the sperm-bindin protein likely have reduced gamete affinities with eggs and should have reduced fitness and be constantly swept away by purifying selection.

There are three relatively common nonsynonymous alleles at frequencies of 0.58 (Allele "A"), 0.16 ("J"), and 0.14 ("K"). The most common allele, "A," has serine at amino acid site 61 and no insertions. A less common allele, "J," has a proline substitution at amino acid site 61. No other amino acid variants were noted at this site, both the serine and proline forms of the protein had some reproductive success, but the less common proline variant had a twofold advantage over the more common serine variant (Fig. 4B, 7A). This less common allele appears to have high

**Table 5.** General linear model of pair-wise reproductive success as a function of pair-wise distance between individuals (Dist), male density (log transformed, Den), male and female nonsynonymous genotype frequency (log transformed, GF), serine/proline substitution in males (and heterozygous, SP), insertion in females (all insertions in Table 1 pooled as heterozygous or homozygous, Insert). Interactions terms with $P>0.10$ were removed from the model.

| Source | DF | Type III SS | MS | F | $P>$F |
|---|---|---|---|---|---|
| Dist | 1 | 0.244 | 0.244 | 20.96 | <0.0001 |
| Den | 1 | 0.556 | 0.556 | 47.74 | <0.0001 |
| Male GF | 1 | 0.135 | 0.135 | 11.64 | 0.0007 |
| Female GF | 1 | 0.004 | 0.004 | 0.33 | 0.5638 |
| Male SP | 2 | 0.181 | 0.090 | 7.76 | 0.0005 |
| Male GF × Dist | 1 | 0.058 | 0.058 | 4.99 | 0.0261 |
| Male GF × Den | 1 | 0.111 | 0.111 | 9.58 | 0.0021 |
| Female GF × Dist | 1 | 0.092 | 0.092 | 7.91 | 0.0052 |
| Female Insert | 2 | 0.138 | 0.069 | 5.94 | 0.0029 |
| Female Insert × Den | 2 | 0.096 | 0.048 | 4.11 | 0.0172 |
| Error | 381 | 4.436 | 0.012 | | |
| Total | 394 | 6.144 | | | |

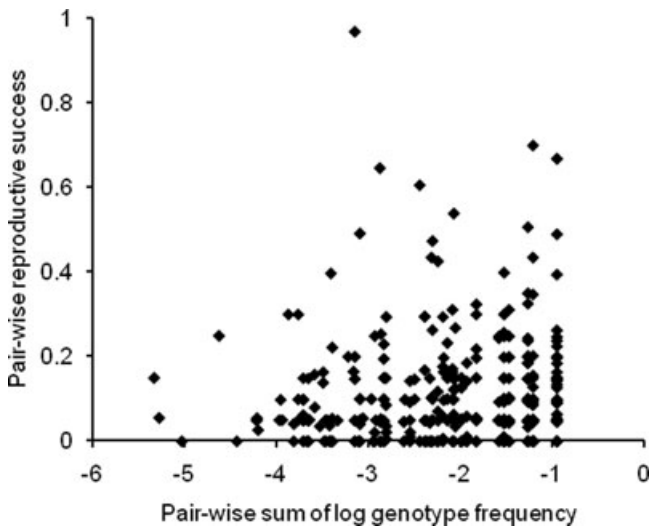


**Figure 6.** Pair-wise reproductive success and the sum of the genotypes of each pair. Pair-wise success is the fraction of eggs fertilized by a particular male. Increases in male and female genotype frequency independently (no interaction of male with female genotype frequency) influenced pair-wise success (Table 5), and these values are summed for visualization of their combined effect.

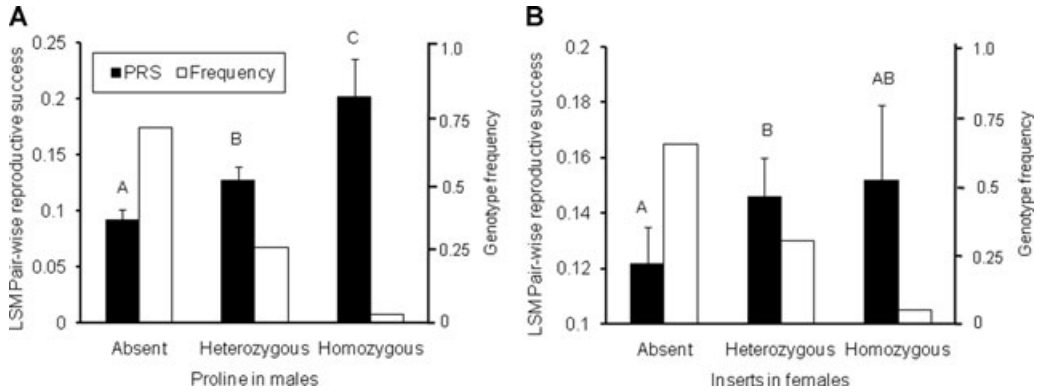


**Figure 7. Histograms showing influence of SP condition in males (A) and Insertion condition in females (B) (Table 2) on pair-wise reproductive success. Least square mean reproductive success and standard error are plotted in black bars with axis on left. Frequency of each condition plotted in white bars with axis on right. Letters are least square mean estimates of pair-wise differences (conditions that share letter on not significantly different). Reproductive success is negatively correlated with frequency in both panels.**

gamete affinities with at least some egg proteins and is maintained by frequency-dependent selection against the most common allele. This amino acid site (61) had the highest positive D and D* values (Fig. 2), and although these values were not significantly different from zero, they are consistent with the pattern of selection observed in the field experiment. Proline/serine substitutions have been noted to alter folding structure in other proteins (Gilles et al. 1986; Chen et al. 1992; Glenn and Novembre 2004; Kuiper et al. 2006; Jiao et al. 2008) and might influence the fit of the sperm-bindin protein to the egg receptor. Variants at other amino acid sites appear to reduce reproductive success and are, not surprisingly, rare.

The other less common allele, "K," has an insertion of four amino acids at site 46. Variation at this site did not influence reproductive success in males, but did significantly increase reproductive success in females by 25% (Fig. 5B, 7B). Although the sample size is not great for some of these genotypes, greater success seems related to greater insertion size and lower genotype frequency (Fig. 5B; Table 2). Biermann (1998) characterized *S. purpuratus* as not having any inserts at these sites, and this was the most common variant. Variation at the insertion site may be maintained by linkage disequilibrium with the egg receptor site as described in a section below and appear, indirectly, to be maintained by frequency-dependent selection. Although insertions and deletions are often overlooked in molecular studies of selection, positive selection has been noted at indel sites in sperm and seminal fluid proteins in primates (Podlaha and Zhang 2003), rodents (Podlaha et al. 2005) and flies (Schully and Hellberg 2006) suggesting these sites may play an important role in gamete recognition and sperm competition.

The finding of selection maintaining some, but not many protein variants is consistent with recent theory of how sexual conflict can generate a polymorphic population of gamete recognition alleles. When sperm compete, mutations in the sperm protein that decrease compatibility will be selected against, whereas those that increase compatibility will quickly sweep through the population (Gavrilets and Waxman 2002; Haygood 2004; M. Tomaiuolo and D. R. Levitan, unpubl. ms.). The result should be a pattern of excess rare alleles that form a balance between mutation and purifying selection. Superimposed on this landscape of selection against mutations that match poorly with egg receptors is the potential action of sexual conflict. At high sperm densities, where polyspermy poses a risk, selection will favor novel egg-surface proteins that are less compatible with the available sperm proteins; these lower-affinity eggs are more resistant to polyspermy (Levitan et al. 2007). Once these novel egg-protein alleles increase in frequency, specific mutations in the sperm protein that increase compatibility with these novel egg variants will have an advantage and will increase in frequency (Haygood 2004; M. Tomaiuolo and D. R. Levitan, unpubl. ms.). The equilibrium frequency of an allele is predicted by the balance between the affinity with its complement and the cost of polyspermy; gametes with lower affinity can be maintained at higher frequencies without suffering losses imposed by excess sperm (M. Tomaiuolo and D. R. Levitan, unpubl. ms.). This prediction matches the empirical result that the less common proline substitution in males and insertions in females had the highest reproductive success.

Together, these forces produce a pattern of positive frequency-dependent selection removing rare variants of sperm bindin that poorly match all available egg receptor proteins (selective sweeps of deleterious alleles; Figs. 4A, 5A, 6; Table 1) and negative frequency-dependent selection among the subset of variants that probably each match a variant egg receptor protein (Figs. 4B, 5B, 7).

The prediction that sexual conflict drives the negative frequency-dependent selection of these moderately common alleles hinges on one of two possible interpretations of the data. If this species is resistant to polyspermy because females produce

eggs that have a more efficient fast (electrical) or slow (cortical reaction) block to polyspermy, then this explanation is not satisfying. Under such conditions, females would be free from sexual conflict, as they would be unaffected by excess sperm. Theory under these conditions predicts no advantage for novel egg proteins and selection for the single most efficient sperm genotype (M. Tomaiuolo and D. R. Levitan, unpubl. ms.). This scenario cannot explain the variation or performance of these proteins noted in this study. However, if selection, driven by the risk of polyspermy, results in polymorphic egg and sperm proteins, then the match between theory and data is more compelling (Levitan and Ferrell 2006; Levitan et al. 2007; Moy et al. 2008). In this scenario, polymorphism reduces the effective concentration of highly compatibly sperm and reduces the risk of polyspermy.

The issue is not whether fast or slow blocks to polyspermy exist or are effective, it is the degree to which variation in resistance to polyspermy, among females (or species), can be attributed to variation in the effectiveness of these blocks or to gamete affinities. Laboratory tests of fertilization and polyspermy, within and among species, support this latter scenario (Levitan et al. 2007). Empirical data suggest that eggs that require fewer sperm for fertilization (higher affinity) are also more susceptible to polyspermy. The former scenario, based on better blocks, would predict that resistant eggs should have an increased range of sperm concentrations where fertilization is achieved, but polyspermy is resisted. This increased range was not apparent in these laboratory tests. Polyspermy in *S. purpuratus* is noted in the laboratory, but at higher sperm concentrations than seen in *S. franciscanus* or *S. droebachiensis*, two species that achieve fertilization at lower sperm (Levitan et al. 2007) and male (Levitan 2002a) densities.

### PROTEIN POLYMORPHISM AND SPERM AVAILABILITY

The pattern of greater success for males with common genotypes was also noted in a field study of *S. franciscanus*, a congener that lives under more variable levels of sperm availability (Levitan and Ferrell 2006). As in the current study, female success could also be predicted by their sperm-bindin genotype (Levitan and Ferrell 2006). A difference between the results of these studies is that in *S. franciscanus,* females with rare sperm-bindin genotypes were more successful. *Strongylocentrotus franciscanus* is generally found at lower densities, but is more susceptible to polyspermy at higher densities, than *S. purpuratus* (Levitan et al. 2007; Levitan 2008). If the demographic history of *S. franciscanus* was generally lower levels of sperm availability (Levitan 2002a), gametes with higher affinity would be favored and increase in frequency. When these individuals were experimentally tested at high levels of sperm availability, mismatched mates (common males and rare females) were favored because they avoided polyspermy (Levitan and Ferrell 2006).

Higher levels of sperm availability should also be correlated with increased allelic diversity, because adding protein variants decreases the effective concentration of compatible sperm types and reduces the risk of polyspermy (M. Tomaiuolo and D. R. Levitan, unpubl. ms.). Although allelic diversity can certainly be influenced by historic factors independent of those that influence sperm availability, the greater diversity of *S. purpuratus* (40 haplotypes, 17 nonsynonymous alleles) than of *S. franciscanus* (15 haplotypes and 7 nonsynonymous alleles; Levitan and Ferrell 2006) is the predicted outcome for higher levels of sperm availability.

### LINKAGE DISEQUILIBRIUM BETWEEN SPERM BINDIN AND THE EGG RECEPTOR

The sperm-bindin protein and the egg receptor protein (EBR1) are on different genomic scaffolds (Sodergren et al. 2006) and are not physically linked. However, linkage disequilibrium between the sperm and egg proteins is still predicted to form when sperm are overabundant and therefore compete (M. Tomaiuolo and D. R. Levitan, unpubl. ms.). This disequilibrium is formed by a balance of assortative mating, which places the matching alleles from these two proteins in the same offspring, and recombination, which disrupts this association. The degree of linkage disequilibrium is predicted to increase with the difference in compatibility between different matched proteins. Linkage disequilibrium is predicted to be strongest when different sets of matched sperm and egg proteins have very different levels of affinity; when gamete affinity differences are subtle, assortative mating is weak. Large intraspecific differences in affinity have been noted in both *S. purpuratus* and *S. franciscanus* in the laboratory (Levitan 2002b; Levitan et al. 2007). These intraspecific differences are also noted under field conditions (Levitan 1996, 2002a,b; Levitan and Ferrell 2006), so intraspecific differences in affinities are great enough not to be swamped by the random effects of water flow or which genotype is closest to a spawning individual in a particular spawning event. Although these population effects do explain much of the variation in individual reproductive success for a particular spawning event, individuals have been estimated to live for over 100 years (Ebert and Southon 2003). Because of such long lives and numerous spawning opportunities, the average effects of these gamete affinities will have large predictive effects on fitness. Evidence for this predicted linkage disequilibrium between egg and sperm recognition protein loci has recently been found in abalone (Clark et al. 2009).

## Conclusions

Gamete-recognition proteins can, but do not always, evolve rapidly (Swanson and Vacquier 2002). Reinforcement selection

against hybrids and sexual conflict over fertilization rates have been proposed as mechanisms that might promote the rapid evolution of these proteins (Swanson and Vacquier 2002). No consensus has so far been reached as to which of these or perhaps another mechanism might be more common (see, e.g., Styan et al. 2008). Some general patterns suggest reinforcement (Palumbi and Lessios 2005), and an explicit test has supported reproductive character displacement (Geyer and Palumbi 2003), a pattern consistent with, but not conclusive proof of, reinforcement selection (Howard 1993; Noor 1999). In many cases, however, reinforcement has been rejected, or less favored, than hypotheses in support of within-species processes (Levitan 2002b; Zigler and Lessios 2003; McCartney and Lessios 2004; Levitan and Ferrell 2006; Riginos et al. 2006; Lessios 2007; Slaughter et al. 2008; Turner and Hoekstra 2008). The current set of experiments indicates that variation in recognition proteins strongly influences patterns of within-species reproductive success, as have a previous field experiment (Levitan and Ferrell 2006) and a laboratory study (Palumbi 1999). Although these results do not rule out reinforcement, they do suggest that within-species processes can shape the evolution of gamete-recognition proteins.

Finally, our ability to untangle the complex set of positive and negative frequency-dependent selective forces outlined here indicates the power of combining tests of the signature of selection from molecular data with measurements of selection from field experiments. The sequence analysis indicating purifying selection provided important information that this protein is under selection and that certain variants are being swept away. However, the pattern of negative frequency-dependent selection, only hinted at in the analysis of molecular evolution, was uncovered by an analysis of the fitness consequences of specific point mutations and insertions. It was this field experiment that provided an explanation for why this protein can be maintained in a polymorphic state.

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