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**Original Article**

# Hybridization, reinforcement selection, and sex-dependent reproductive character displacement of sperm and egg recognition proteins

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## Abstract

The establishment of reproductive isolation between species via gametic incompatibility initially requires within-species variation in reproductive compatibility. We investigate how within-species variation in sperm and egg recognition proteins, potentially generated via sexual conflict, influences reproductive isolation between two partially sympatric sea urchin species; the North American west coast *Mesocentrotus franciscanus* and the circumpolar *Strongylocentrotus droebachiensis*. Barriers to hybridization are stronger when eggs are given a choice of conspecific versus heterospecific sperm and the variation in hybridization among crosses can be explained by whether the sperm or egg protein variant is ancestral or derived. Derived proteins can be recognized as different and prevent hybridization. Examination of the allele frequencies of these proteins in *M. franciscanus* in and out of sympatry with *S. droebachiensis* along the west coast of North America reveals evidence of reinforcement selection and reproductive character displacement in eggs but not sperm, which likely reflects the differential cost of hybridization for males and females.

**Keywords:** conspecific sperm precedence, fertilization, gamete recognition proteins, hybridization, reinforcement selection, reproductive isolation

## Introduction

Regardless of whether reproductive isolation evolves allopatrically or sympatrically, mutations with isolating effects must arise and proliferate within a population such that the population can no longer reproduce with individuals from other populations. The proliferation of initially rare genotypes becomes particularly puzzling for traits associated with reproductive compatibility. How can a mutation, that by definition results in reduced compatibility with mates, have a fitness advantage over individuals that have high compatibility with those same mates (Levitan et al., 2019)? Taxa with external fertilization provide an excellent model for this question because of their ease of study and because they often exhibit wide variation in intraspecific gametic compatibility (Evans & Marshall, 2005; Hart et al., 2014; Levitan, 2002, 2012; Levitan & Ferrell, 2006; Levitan & Stapper, 2010; Levitan et al., 2019; Palumbi, 1999). One hypothesis that might explain the generation of intraspecific variation in gametic compatibility is that sexual conflict over fertilization rate is driven by the risk of polyspermy and selects for egg variants with reduced compatibility (Haygood, 2004; Kosman & Levitan, 2014; Tomaiuolo & Levitan, 2010). As the frequency of eggs with reduced compatibility increases, it provides an unexploited resource for mutant sperm that match this emerging egg variant and can produce a population with more than one matched compatibility group (Levitan et al., 2019).

Empirical support for this hypothesis was found in the sea urchin *Mesocentrotus franciscanus* (Levitan, 2012; Levitan et al, 2019). Increases in sea urchin abundance following the removal of sea otter predators during the late 1700's to early 1900's were associated with an increasing risk of polyspermy and a shift from one to two common nonsynonymous alleles in *M. franciscanus*, first at the egg (*EBR1*) and then by the sperm (*bindin*) recognition loci (Levitan et al., 2019). The historically common sperm and egg recognition alleles have high compatibility as do the more recently common sperm and egg recognition alleles, forming two compatibility groups. Although this process can generate variation in compatibility and compatibility groups, it might not be sufficient to explain the very low levels of compatibility (e.g., Levitan, 2002; Palumbi, 1994; Zigler et al., 2005) preventing or limiting hybridization across species.

Reinforcement is the selection on prezygotic traits to avoid producing low-fitness hybrids to minimize wasting gametic resources that could have been used to produce high-fitness conspecifics (Dobzhanksy, 1940; Howard 1999; Servedio & Noor, 2003). For external fertilizers, premating isolation (e.g., nonoverlapping gamete release) can reduce the probability of hybrid formation (Levitan et al, 2004), but sympatric species often spawn simultaneously in mixed aggregations (Harrison et al., 1984; Levitan, 2002; Levitan et al., 2004; Pearse et al., 1988) suggesting the importance of gametic incompatibilities

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in maintaining reproductive isolation. Reinforcement selection has been suggested as a mechanism for the rapid evolution of gamete recognition proteins (GRPs) and gametic incompatibility among often closely related species (Geyer & Palumbi, 2003; Swanson & Vacquier, 2002). Strong support for reinforcement is the presence of reproductive character displacement (RCD). RCD consists of reduced compatibility in heterospecific crosses in sympatry compared with allopatry because selection against producing low-fitness hybrids is only manifested in sympatry. Although evidence for RCD in gamete compatibility (Geyer & Palumbi, 2003) or GRP divergence (Yang et al., 2000) is noted in some species pairs, it is lacking in others (Geyer & Lessios, 2009; McCartney & Lessios, 2004; Nydam & Harrison, 2011) suggesting the possibility that within species, rather than among species, processes might drive the diversification of GRPs. However, a lack of support for RCD does not mean that hybrid production is not costly nor influence selection on gametic compatibility. There might be a more complicated interaction between within and among species processes and trade-offs between selection maximizing conspecific success and minimizing hybridization (Levitan, 2002).

An alternative means of examining how selection might influence reproductive isolation is to investigate variation in GRPs within a species and examine if the ancestral proteins shared across species have increased compatibility compared with the derived proteins unique to each species (Zigler et al., 2005). Zigler and colleagues' (2005) untested hypothesis was suggested based on patterns of asymmetry in compatibility between sperm and eggs in congeneric crosses with fixed differences in the echinoid sperm GRP (sperm *bindin*). A rigorous test of this hypothesis would be to select a species with intraspecific variation in GRPs that has both shared ancestral variants and novel derived variants with a sympatric species. The expectation is that novel proteins are recognized as being different by heterospecific gametes and lead to increased resistance to hybrid fertilization compared with the ancestral proteins shared by both species. These patterns also lead to specific *apriori* expectations for how allele frequencies of these proteins should shift in and out of allopatry via reinforcement selection. A final prediction is that if reinforcement selection is driven to increase conspecific zygote production, it should be stronger in egg proteins rather than sperm proteins, because (1) sperm greatly outnumber eggs and the vast majority of sperm fail to fertilize any egg and (2) eggs left unfertilized by heterospecific sperm are more likely to be available for conspecific fertilization than vice versa; sperm penetrating the jelly coat of eggs and failing to fertilize a heterospecific egg are less likely to detach and find and fertilize a conspecific egg.

This study focuses on the sea urchin *Mesocentrotus franciscanus* and its potential to hybridize with *Strongylocentrotus droebachiensis*. Both species are in the strongylocentrotid clade and diverged between 9 and 16 million years ago (Kober & Bernardi, 2013). *Mesocentrotus franciscanus* occurs along the west coast of North America, from Alaska, United States to Baja, Mexico. *Strongylocentrotus droebachiensis* is circumpolar and extends south on the west coast of North America into Oregon (Scheibling & Hatcher, 2001). Prior studies have indicated that these two species can produce F1 embryos with *S. droebachiensis* eggs being more susceptible to hybridization compared with *M. franciscanus* eggs (Levitan, 2002). The hybrids produced from *S. droebachiensis* eggs can metamorphose into juveniles, albeit at two orders of magnitude lower levels relative to that of the offspring of conspecific crosses (Levitan, 2002). These barriers appear to be effective as there is no evidence of introgression between these two species (Addison & Pogson, 2009; Glasenapp & Pogson, 2023).

Here, we explore the degree of hybrid fertilization between these species in no-choice and competitive choice assays to test for conspecific sperm precedence (CSP). We then examine if variation in hybrid fertilization depends on the GRP amino acid polymorphisms known to influence male (sperm *bindin*) and female (*EBR1*) fertilization success in *M. franciscanus* (Levitan, 2012; Levitan et al., 2019) and specifically test if derived protein variants are less likely to result in hybrid fertilization. Finally, the allele frequencies of these sperm and egg proteins in *M. franciscanus* are investigated along the west coast of North America in regions of sympatry (north) and allopatry (south) with the circumpolar *S. droebachiensis* to test for RCD.

## Background on sperm and egg recognition proteins

### Sperm bindin

During the process of fertilization, once the spermatozoon acrosomal process has punched through the jelly coat and contacted the vitelline layer, the sperm *bindin* and *EBR1* proteins have the opportunity to interact (Biermann et al., 2004). Sperm *bindin* was the first identified GRP (Vacquier & Moy, 1977). During the acrosomal reaction, *bindin* is released from the acrosomal vesicle of sperm and coats the surface of the acrosomal process. This protein is known to bind sperm to eggs and influences the fusion of the sperm and egg membranes (reviewed in Vacquier et al., 1995). All studied echinoids show a highly conserved core region surrounded by two variable flanking regions and *bindin* was likely present in the 250-million-year-old ancestor to all extant sea urchins (Zigler & Lessios, 2003). Some taxa show evidence of positive selection in at least one of these flanking regions, manifested as either divergence across taxa (Biermann, 1998) or intraspecific variation within species (Metz & Palumbi, 1996).

In *M. franciscanus*, in the first variable region (exon 1), there are two common amino acid polymorphisms, Arginine (R) or Glycine (G) at site 16 and Glycine (G) or Arginine (R) at site 38 (Table 1 and Supplemental Figure 2a, all amino site locations are based on Biermann, 1998 and match Zigler & Lessios, 2003 to facilitate species comparisons). The most common alleles are RG (sites 16 and 38, frequency ~0.6), GR (~0.3), and GG (~0.1). The RR allele is extremely rare (Levitan, 2012). Prior work focusing on intraspecific fertilization and compatibility has focused on the two most common alleles (RG and GR) which can be found in the homozygous state with sufficient frequency to use in laboratory crosses. Although less is known about the GG allele, it appears to have lower average levels of compatibility compared with the RG and GR alleles (Levitan, 2012). This work in the lab (Levitan, 2012; Levitan et al., 2019) and in the ocean (Levitan & Ferrell, 2006; Levitan, 2012) demonstrated the influence of these protein variants on reproductive success. In addition, the RG and GR alleles form compatibility groups with two alleles in a repeat in the egg protein, *EBR1* (see below). Less studied, but potentially important to an examination of interspecific fertilization is the GG allele. The GG state at these

**Table 1.** Summary of frequent intraspecific amino acid polymorphisms in sperm *bindin* and the four *EBR1* exons in *M. franciscanus* and *S. droebachiensis* found in sea urchins collected from Bamfield, British Columbia, Canada; full sequences in Supplemental Figure 2a and b. In all cases, the variable site in one species has a unique and a shared amino acid with the alternate species. Asterisks indicate the allele with the highest intraspecific outgroup probability (Haplotype networks and outgroup probabilities in Supplemental Figure 4a–g). Cells without asterisks indicate ambiguous probabilities (differences < 2%). Only the linked amino acids in *CUB7* (sites 49 and 51) in which the highest outgroup probabilities differed by ~3% and the phylogenomic signal was mixed, indicates an ambiguous ancestral state. Data for *S. purpuratus* (Kamei & Glabe, 2003; Pujolar & Pogson, 2011), *S. pallidus* (Pujolar & Pogson, 2011), *M. nudus* (Kober & Pogson, 2017), and *Pseudocentrotus subdepressus* (sister genera to *Mesocentrotus*, Kober & Pogson, 2017) presented for comparison (empty cells = no data).

| Species | bindin 16 | bindin 38 | TSP8 31 | CUB1 17 | CUB1 27 | TSP13 27 | CUB7 44 | CUB7 49 | CUB7 51 |
|---|---|---|---|---|---|---|---|---|---|
| *M. franciscanus* | R/G* | R/G* | S/G | D | S/P* | D/Y | S | G*/R | D/Y* |
| *M. nudus* | G | G | | | | | | | |
| *P. subdepressus* | G | G | | | | | | | |
| *S. droebachiensis* | G | G | G | D/G | P | D | S/P | R | D |
| *S. purpuratus* | G | G | G | A | P | D | S | G | G |
| *S. pallidus* | G | G | G | | | | | | |

amino acid sites is nearly ubiquitous in the echinoids (Zigler & Lessios, 2003 and Supplemental Figure 1) and specifically in this clade of strongylocentrotid sea urchins (Biermann, 1998 and Table 1). The sharing of the GG allele in *M. franciscanus* with *S. droebachiensis* (Table 1, Supplemental Figure 2A) provides the variance needed to test how a shared and potentially ancestral allele (GG) influences hybrid fertilization compared with unshared and potentially derived alleles (RG and GR).

### Egg Bindin Receptor (*EBR1*)

Kamei & Glabe (2003) characterized the *egg bindin receptor* (*EBR1*) in *S. purpuratus* and *M. franciscanus*. Inhibiting this protein decreases fertilization in a species-specific manner and beads coated with this protein result in sperm adhesion. *EBR1* has been sequenced in *S. purpuratus* (3,713 amino acids) and *M. franciscanus* (4,595 aa). In *M. franciscanus*, there is an ADAMTS-like domain followed by a series of 27 *TSP* (53–59 aa) and 20 *CUB* (104–123 aa) repeats. Most of these are tandem *TSP*/*CUB* repeats with the final 9 tandem repeats being near 100% identical to each other. Pujolar & Pogson (2011) examined one exon in this protein (*TSP8*) in four *Strongylocentrotus* spp. and noted interspecific positive selection at a single amino acid site, but overall, this region exhibited evidence of purifying selection. In *S. purpuratus*, there is evidence of purifying selection in 8 *EBR1* repeats and strong evidence of linkage-disequilibrium between sperm b*indin* and *EBR1* driven by assortative mating (Stapper et al., 2015). In *M. franciscanus*, an examination of 15 repeats (10 *TSP* and 5 *CUB*) found evidence for balancing selection in four repeats (*TSP8* site 31, *TSP13* site 27, *CUB1* site 27, and *CUB7* sites 49 and 51, Table 1) and little to no variation at other sites within these 4 repeats or in the remaining 11 repeats examined (details see Levitan et al., 2019 and Supplemental Figure 2B). These variable *EBR1* regions explained intraspecific variation in fertilization success, but only *TSP8* interacted with sperm *bindin* to produce two compatibility groups (Levitan et al., 2019). An S/G amino acid substitution at site 31 in *TSP8* interacts with the RG/GR genotypes at sites 16 and 38 in sperm *bindin* to form 2 compatibility groups. These 4 *EBR1* repeats lie within a 25,000 bp genomic region and the estimated distances between *TSP8* and the other 3 sites are 2,637, 17,460, and 21,793 bp for *CUB1*, *TSP13*, and *CUB7*, respectively (based on the *S. purpuratus* genome). Significant linkage disequilibrium was detected between *TSP8*, *CUB1*, and *TSP13*. The more distant *CUB7* was not statistically linked with *TSP8* or *CUB1* (Levitan et al., 2019).

## Methods

### Fertilization assays

Fertilization assays were conducted in the springs of 2017, 2018, and 2022 on the west coast of Vancouver Island, Canada at the Bamfield Marine Sciences Centre. All protocols were approved by their Animal Care Committee which complies with the Canadian Council on Animal Care. Sea urchins were collected from Barkley Sound on the west side of Vancouver Island and kept in flowing seawater tables for less than 1 week and fed kelp. One exception was a single collection of *S. droebachiensis* from the east side of Vancouver Island in the spring of 2022 to supplement sample sizes. The experiments were designed to examine no-choice fertilization success between *S. droebachiensis* and *M. franciscanus* males and females, reciprocally, and choice experiments consisting of *S. droebachiensis* eggs with a mixture of conspecific and heterospecific sperm. These experiments focused on choice experiments using *S. droebachiensis* eggs, because prior research has indicated high and variable levels of hybrid fertilization in this cross but reduced hybridization in the reciprocal cross (Levitan, 2002 and Results).

Two experimental designs were used, the first focused on variable ratios of sperm from males of the two species, while the second focused on using the most informative sperm ratio of the two species to increase the sample size of the less common sperm *bindin* alleles. Design one tested replicates of one male and female from *S. droebachiensis* and *M. franciscanus* in no-choice crosses and five choice crosses with varying ratios of sperm (0.9:0.1, 0.7:0.3, 0.5:0.5, 0.3:0.7, and 0.1:0.9) from the two males. These crosses were conducted under both sperm-saturating (10,000-fold dilution of dry sperm) and sperm-limited (1,000,000-fold dilution) conditions (Details of Methods in Supplemental, Supplemental Figure 3).

Design two was identical to design one, with the modification of only using the no-choice assays plus the 0.9:0.1 (*M. franciscanus*: *S. droebachiensis*) sperm ratio. This tested the ability of *M. franciscanus* sperm to fertilize *S. droebachiensis* eggs when they had the greatest numerical superiority. This reduced design allowed for testing an increased number of

replicates to get adequate sample sizes of the less frequent sperm *bindin* alleles.

Three hours after sperm and eggs were mixed, at least 200 eggs were inspected for the presence of a fertilization membrane or cleavage in each experimental vial. For the five sperm ratios (times two concentrations) containing sperm from both species, the eggs and zygotes from each vial were placed into a glass jar containing 500 mL of filtered seawater, and the embryos were allowed to develop for 3 days before being fixed in 95% EtOH for genetic analysis of paternity (species of male sire). Tube foot samples from each adult used in these crosses were collected and placed in 95% EtOH for genetic analysis of recognition proteins.

To determine if variation in paternal success at day 3 was due to gametic compatibility or early postzygotic survivorship prior to genotyping larvae, patterns of embryo survivorship were examined. In 2022, a subsample of zygotes or early cleavage embryos (mean = 50.3 zygotes, SE = 2.3) from the no-choice assays was hand-counted and placed into a 20-mL glass vial and allowed to develop for the 3-day period between fertilization and fixing larvae for genetic analysis. After 3 days, these larval cultures were counted for living pluteus larvae to test for evidence of differential survivorship among crosses (conspecific or heterospecific sperm) or sperm *bindin* genotype. The morphology of these larvae was not accessed, which might indicate their vitality; this was not an assay of larval fitness. This assay only tested if a bias existed at day 3 that might alter estimates of gametic compatibility.

### Genetic analysis

Adult tube foot DNA was extracted as in Levitan (2012), while larvae were extracted as in Levitan (2004). For hybrid determination of larvae, preliminary results indicated four primer pairs that differentially amplified regions of sperm (*bindin*) and egg (*EBR1*) GRPs that could be used to identify hybrid larvae (Supplemental Table 1 for primers). The conspecific (*S. droebachiensis*) and heterospecific (*M. franciscanus*) potential sires and at least 20 larvae from each experimental vial were examined for these identifying bands using gel electrophoresis.

To examine if GRP identity was associated with heterospecific fertilization success, the two polymorphic regions of sperm *bindin* (exon 1 and 2) and the four *EBR1* repeats (*TSP8*, *TSP13*, *CUB1*, and *CUB7*) that showed balancing selection in *M. franciscanus* (Levitan et al., 2019) were sequenced in both species (*M. franciscanus* primers—Levitan et al., 2019; *S. droebachiensis* primers—Supplemental Table 2). Heterozygote states were determined by the presence of two peaks in the sequencing chromatographs. In cases of ambiguity, the amplification product was re-sequenced in the opposite direction to confirm heterozygosity. The sequencing strategy was to sequence at least 20 individuals that were used in the experimental crosses ($N$ = 22–90) to detect amino acid variation at each locus. Polymorphisms were either at high frequency (minor allele > 0.2) or rare (<0.025). Only loci with high-frequency polymorphisms were sequenced in all individuals used in crosses and investigated for fertilization effects.

To provide additional insight into whether the alleles in *M. franciscanus* that are shared with *S. droebachiensis* are derived, intraspecific haplotype networks were constructed for *M. franciscanus* and S. droebachiensis loci with high-frequency polymorphisms using TCS 1.21 (Clement et al., 2000), which calculates the outgroup probabilities for each haplotype in the network (Posada and Crandall 2001).

### Geographic sampling of sperm bindin and EBR1 of *M. franciscanus* in and out of sympatry with *S. droebachiensis*

To investigate the evidence for RCD in the sperm and egg recognition proteins, samples of adult *M. franciscanus* individuals were collected from 14 sites along the west coast of North America from British Columbia to Southern California. Seven of these sites were north of the biogeographic break of Point Conception, CA, and within the species range of *S. droebachiensis* (Alaska through Oregon, USA—Scheibling & Hatcher, 2001). The remaining seven sites were in southern California (Supplemental Table 3). These individuals were sequenced for the sperm *bindin* exon 1 and the four *EBR1* loci (*TSP8*, *CUB1*, *TSP13*, and *CUB7*).

### Sequence variation of sperm *bindin* in strongylocentrotid sea urchins

Variation in the amino acid sequence of sperm *bindin* was obtained from the literature or GenBank. Sperm *bindin* sequences are available from 23 echinoid species throughout their 250 million-year history (Zigler & Lessios, 2003; Zigler et al., 2005). Detailed information was obtained within the strongylocentrotid clade (*Strongylocentrotus* and *Mesocentrotus*) in which samples sizes were large enough to detect evidence of intraspecific variation (Balakirev et al., 2008; Biermann, 1998; Kober & Pogson, 2017; Levitan, 2012; Levitan & Stapper, 2010; Levitan et al., 2019; Marks et. al., 2008; Pojular & Pogson, 2011). The phylogenetic relationship of the strongylocentrotid species was based on Biermann et al. (2003) and Kober & Bernardi (2013, 2017).

## Results

### Sequence variation in sperm *bindin* and *EBR1*

The first exon of sperm *bindin* in *M. franciscanus* revealed the two common amino acid polymorphisms noted to influence intraspecific compatibility (Levitan et al., 2019); sites 16 (Arginine [R] or Glycine [G]) and 38 (Glycine or Arginine) at frequencies of 0.51 (RG), 0.38 (GR), and 0.11 (GG) based on the 143 individuals used in these experiments (summary Table 1, full amino acid sequence Supplemental Figure 2A). In the subsample of 45 individuals sequenced for the second exon of sperm *bindin*, 88 of the 90 haploid sequences had the identical amino acid sequence as Biermann (1998); Supplemental Figure 2A.

In the *egg bindin receptor* (*EBR1*) of *M. franciscanus*, the four loci examined showed the same high-frequency polymorphisms as noted previously (Levitan et al., 2019); *TSP8* Serine (0.80)/Glycine (0.2) at site 31, *CUB1* Serine (0.59)/Proline (0.41) at site 27, *TSP13* Aspartic Acid (0.62)/Tyrosine (0.38) at site 27, and two linked sites in *CUB7* Glycine (0.63)/Arginine (0.37) at site 49 and Aspartic Acid (0.63)/Tyrosine (0.37) at site 51 Table 1, Supplemental Figure 2B.

In *S. droebachiensis*, in the first exon of sperm *bindin*, there was no variation in amino acids detected ($N$ = 36 individuals). At the two amino acid sites that were variable in *M. franciscanus*, *S. droebachiensis* was fixed at GG (sites 16 and 38, Table 1, Supplemental Figure 2A). In the second sperm *bindin* exon, there were 3 rare amino acid site polymorphisms found in single instances in the heterozygote form. There was also a rare indel noted in two instances (Supplemental Figure 2A).

The four EBR1 exons that showed balanced polymorphisms in *M. franciscanus* were sequenced in *S. droebachiensis*. In all

four of these exons, the amino acid site that was commonly variable in *M. franciscanus* was fixed in *S. droebachiensis* at one of the amino acids noted in *M. franciscanus* (Table 1, Supplemental Figure 2B). In *S. droebachiensis*, no amino acid variation was detected in *TSP8* ($N = 30$ individuals) or *TSP13* ($N = 22$). In *CUB1* ($N = 39$ individuals), there was one common amino acid substitution at site 17 of Glycine (0.22) for the common Aspartic Acid (0.78). This site was fixed at Aspartic Acid in *M. franciscanus*. In *CUB7*, there was one common amino acid substitution at site 44 of Phenylalanine (0.30) for the common Serine (0.70), which had a rare substitution in *M. franciscanus* (shared common Serine and unique rare Proline). In addition to these variable sites highlighted, in all these sperm and egg exons, there were numerous fixed differences between these species (Supplemental Figure 2A and B). It is noteworthy that in both sperm *bindin* and *EBR1*, in every case where there was a common amino acid variant within species, one variant was shared between species and one was unique to each species (Table 1). This pattern of variation allows for tests of how shared and unshared alleles influence the probability of hybrid fertilization.

A haplotype network analysis (TCS 1.21, Clement et al., 2000) was conducted at all exons that showed high-frequency polymorphisms (Table 1). This analysis included haplotype frequencies and estimated intraspecific outgroup probabilities (Supplemental Figure 4A–G). These probabilities were compared with the pattern of sharing between *M. franciscanus* and *S. droebachiensis* and when data were available, other species in this strongylocentrotid clade (Table 1). Because the network analysis incorporates allele frequencies and these frequencies have been documented to shift over decades (Levitan et al., 2019), the outgroup probabilities likely have some error. To address this possibility, whenever outgroup probabilities among haplotypes were near identical (within ~0.02), the phylogenetic signal of sharing alleles across taxa (Table 1) was used as the tie-breaker for estimating the ancestral state. The results indicate that in all but one case, the phylogenetic signal was either supported (*M. franciscanus* sperm *bindin* and *EBR1 CUB1*) or was not contradicted (near equal outgroup probabilities) by the network analysis (Table 1, Supplemental Figure 4). The *EBR1 CUB7* repeat in *M. franciscanus* was more complicated as it has two tightly linked amino acid polymorphisms (sites 49 and 51) with three combinations of association (G/D, R/Y, and G/Y ordered by frequency) that had outgroup probabilities within ~0.03, but the fourth possibility (R/D) was fixed in *S. droebachiensis* and G/G was fixed in *S. purpuratus* (Table 1); no conclusion on the ancestral state is supported. For all other variable loci, the alleles shared among taxa are designated as ancestral.

## No-choice fertilization assays

*Strongylocentrotus droebachiensis* eggs required a lower sperm concentration to achieve fertilization and were more susceptible to hybrid fertilization compared with *M. franciscanus* eggs (Figure 1). With conspecific sperm, *S. droebachiensis* eggs averaged 98% (high sperm concentration) and 76% (low sperm concentration) fertilization compared with 95% and 66% for *M. franciscanus* eggs. With heterospecific sperm, *S. droebachiensis* eggs averaged 55% (high sperm concentration) and 7% (low sperm concentration) compared with 13% and 1% for *M. franciscanus* eggs. An ANOVA examined fertilization (logit transformed proportions) with the main effects of the species of the egg donor, whether the cross was with conspecific or heterospecific sperm and whether the sperm concentration was high (saturated) or low (limited), plus all two and the one three-way interactions. The results revealed that all main effects were significant, as well as all interactions except between egg donor species and sperm concentration (Supplemental Table 4).

## Choice fertilization assays

In contrast to no-choice trials that demonstrated high levels of hybrid fertilization of *S. droebachensis* eggs, the presence of competing conspecific sperm eliminated hybridization in 75% of trials (Figure 2A), indicating strong average levels of CSP. The likelihood of hybridization increased with an increased ratio of *M. franciscanus* sperm (Figure 2B), but even at the most extreme ratios favoring *M. franciscanus* sperm (0.9:0.1), 47% of trials resulted in no detection of hybridization. The proportion of eggs hybridized (logit transformed) was examined with a mixed model (SAS, GLIMMIX) with a link-logit function, using the fixed effects of sperm concentration (limited or saturated), a covariate of the *S. droebachiensis*/*M. franciscanus* sperm proportion and a random effect of replicate. The results indicated a significant effect of *S. droebachiensis*/*M. franciscanus* sperm proportion ($F = 40.49$, $p < .0001$) but not sperm concentration ($F = 2.02$, $p = .156$, $N = 160$). Although the majority of eggs were unlikely to be hybridized in the presence of conspecific sperm, the degree of CSP varied among replicate crosses from absolute (no hybridization even under high heterospecific sperm ratios) to nonexistent (i.e., 50% heterospecific sperm resulted in 50% hybridization) (Figure 2B).

Variation in the degree of CSP was examined as a function of the presence or absence of the shared Glycine/Glycine (GG) allele (there were no GG homozygotes in these samples) in sperm *bindin* of the *M. franciscanus* male and the two variable *EBR1* exons (*CUB1*, *CUB7* in the shared and unique homozygous states and the heterozygote state) of the *S. droebachiensis* female. Only the most skewed sperm ratio treatment was used (0.9 *M. franciscanus*/0.1 *S. droebachiensis*) which had the greatest proportion of hybridization (Figure

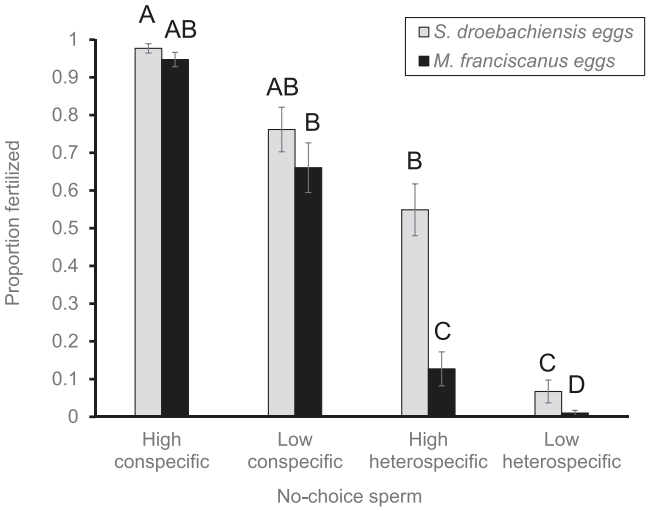


**Figure 1.** No-choice fertilization assays as a function of "High" or "Low" sperm concentration, conspecific or heterospecific sperm and species of egg (bars = SE). Statistical test on logit transformed data, proportions plotted. Treatments with different letters indicate Tukey-adjusted pairwise differences.

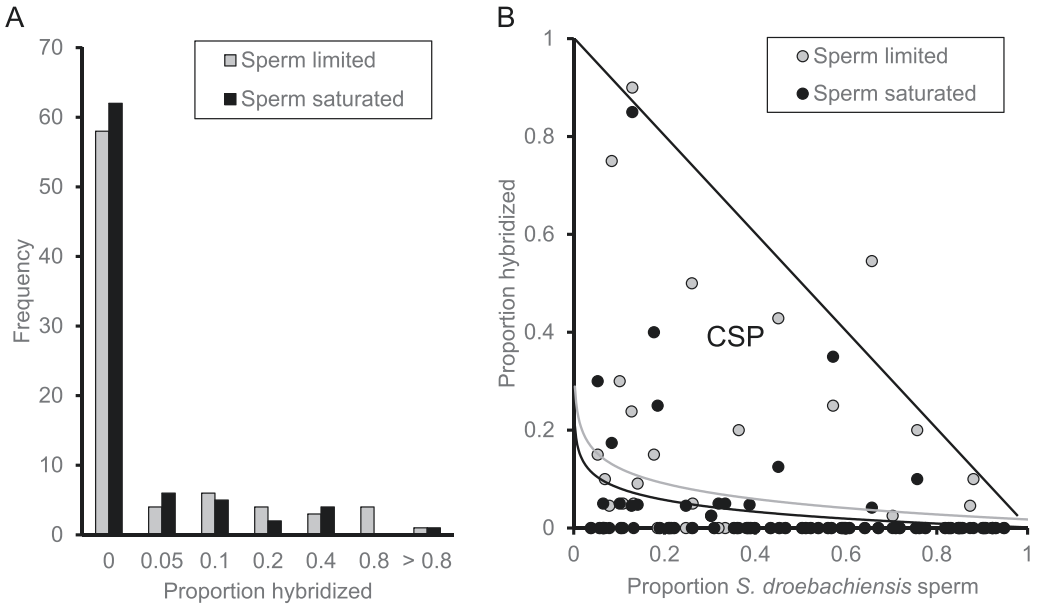


**Figure 2.** Hybridization of *S. droebachiensis* eggs in choice fertilization assays. (A) Distribution of the proportion of eggs hybridized under sperm-limited and sperm-saturated conditions. (B) Proportion of eggs hybridized as a function of the proportion of sperm suspension consisting of *S. droebachiensis* (conspecific) sperm. The black diagonal line indicates no evidence of conspecific sperm precedence (CSP, the proportion of hybrids is proportional to heterospecific sperm). The data below the line indicate CSP.

2B). This analysis made use of the second experimental design that only investigated this sperm ratio to increase sample size. The proportion of hybrids produced (logit transformed) was examined as a function of a main effects of sperm *bindin* and the two EBR1 exons. The results indicated a significant effect of the presence/absence of the GG allele in *M. franciscanus* males, but not the two *EBR1* exons in the *S. droebachiensis* females (Supplemental Table 5). When the GG allele was absent and only alleles unique to *M. franciscanus* (RG or GR) at sites 16 and 38 were present, hybridization averaged 3.6 % (SE = 5.7%). When the GG allele was present, always in the heterozygous form, hybridization increased to 20.0% (SE = 6.1%). The *M. franciscanus* GG allele is at a frequency of 0.09 in Barkley Sound and homozygous individuals are rare and not observed in this experiment. Although the two *EBR1* exons in *S. droebachiensis* were not significant in this model, in both *EBR1* exons, the trend was for the shared amino acid states to have higher rates of hybridization compared with the unique states (Figure 3). Analyses of variance on logit-transformed embryo survivorship found no evidence that either the species identity of the male ($F_{1,28}$ = 2.69, $p$ = .11) or the presence or absence of the ancestral GG allele in the *M. franciscanus* sire ($F_{1,12}$ = 0.002, $p$ = .96) influenced survivorship from the stage at which fertilization success was assayed to day 3 of development when larvae were fixed for genetic analysis.

## No-choice assays of *M. franciscanus* eggs as a function of *EBR1* genotype

Because hybrid fertilization is less likely in *M. franciscanus* eggs, the reciprocal choice experiments were not conducted; however, in each trial, *M. franciscanus* eggs were exposed to *S. droebachiensis* sperm in no-choice trials to further document the degree of reproductive isolation between these species. Low, but variable, hybridization was detected in these crosses (Figure 1). Variation in the hybridization of these eggs

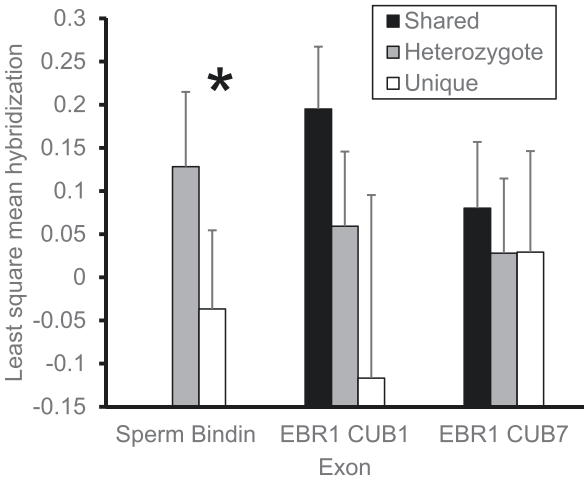


**Figure 3.** Hybridization *of S. droebachiensis* eggs in choice fertilization experiments as a function of *M. franciscanus* sperm *bindin* genotype (presence or absence of shared GG allele) and *S. droebachiensis EBR1 CUB1* and *CUB7* genotypes (shared and unique alleles). For these loci, the shared alleles are estimated to be ancestral (Table 1). Statistical analysis using logit transformed data, proportions plotted (bars = SE, asterisk, $p$ = .026).

was examined in light of variation in the four *M. franciscanus EBR1* repeats (Figure 4). An analysis of the likelihood of hybridization (logit transformed fertilization success) as a function of *M. franciscanus* dame *TSP8* site 31, *TSP13* site 27, *CUB1* site 27, and *CUB7* site 51 genotype (each as an independent factor) under sperm saturating conditions with sperm concentration and the polynomial of sperm concentration (to account for the effect of polyspermy) as covariates resulted in a significant effect of increasing hybridization with the shared *TSP8* site 31 Glycine allele (least square mean of 37% hybridization) compared with the unique Serine amino acid allele (10% hybridization, Supplemental Table 6). The

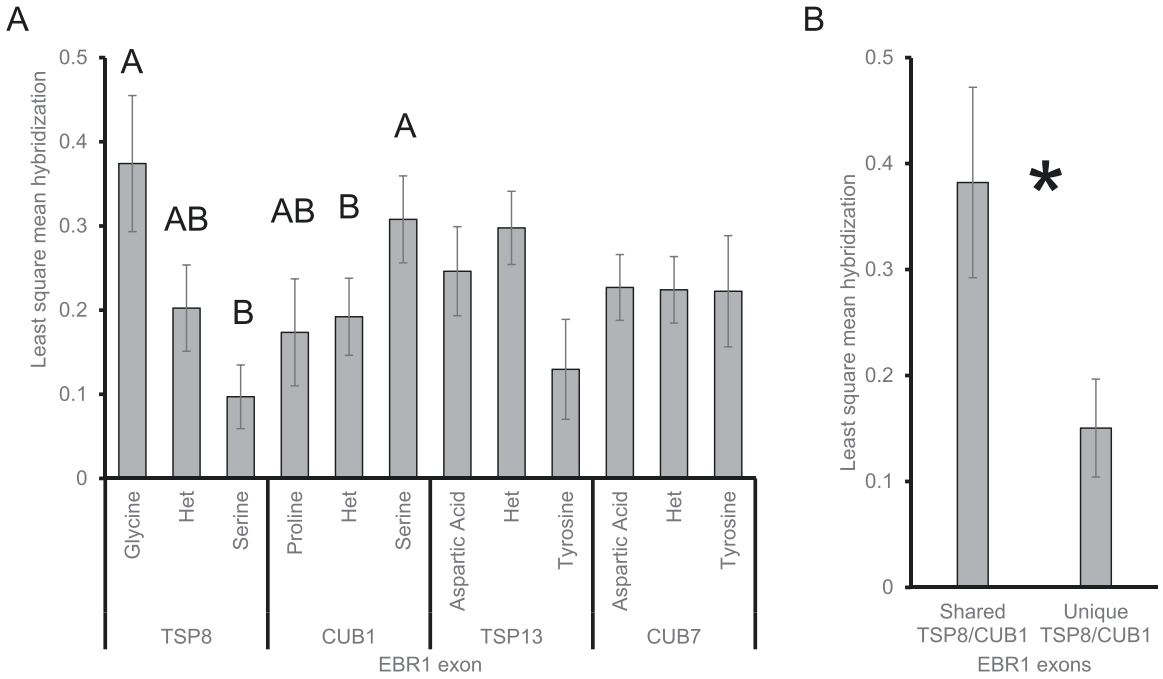


**Figure 4.** Hybridization of *M. franciscanus* eggs in no-choice fertilization experiments as a function of the four *EBR1* repeats that show evidence of balancing selection (*TSP8* site 31, *CUB1* site 79, *TSP13* site 79, and *CUB7* site 151). (A) Ordering of repeats represents ordering in the genome (Levitan et al., 2019). The shared variant is plotted on the left for each exon. For all loci, except *CUB7* (unresolved), shared alleles are estimated to be ancestral (Table 1). Treatments with different letters indicate Tukey-adjusted pairwise differences (pairwise tests conducted within each *EBR1* repeat and do not correspond to differences among repeats). (B) Secondary test reporting significant interaction of *TSP8* and *CUB1* indicating that shared genotypes were more likely to result in hybridization compared with individuals with unique amino acids. Statistical test on logit transformed data, proportions plotted (bars = SE, asterisk, $p = .03$).

only other *EBR1* locus to influence hybridization was *CUB1* site 27; however, a Tukey-adjusted pairwise test could not significantly distinguish the two homozygous states (Serine vs. Proline, Figure 4A). To further examine the influence of the two significant *EBR1* loci, the same statistical model was run with the addition of an interaction term between these two loci. The results indicated a significant interaction ($p = .031$, Supplemental Table 6) and the pairwise test of the shared Glycine (*TSP8*)/Proline (*CUB1*) state had a higher level of hybrid fertilization (least square mean 38%) compared with the unique Serine/Serine state (15%, $p = .0248$, Figure 4B).

## Shifts in GRP allele frequencies in and out of sympatry

GRP allele frequencies of *M. franciscanus* at seven locations in sympatry with *S. droebachiensis* (Oregon and North) and seven locations in allopatry (south of Point Conception, CA, Supplemental Table 3) were tested with ANCOVAs with region (allopatric versus sympatric) as the main effect and mean test diameter as the covariate (prior work has shown size/age class differences in allele frequencies at the Bamfield site—Levitan et al., 2019) independently for sperm *bindin* exon 1 and the four variable *EBR1* exons. Test diameter was not a significant factor for any exon but improved model fit and was retained in the analysis (Supplemental Table 7). There was no significant difference in the allele frequencies of the sperm *bindin* Glycine/Glycine (sites 16/38) allele in and out of sympatry. In *EBR1*, the only region with a significant shift was in the *TSP8* locus (higher frequency of the Glycine alleles at site 31 in allopatry); the shared Glycine allele that facilitates hybridization was more frequent in allopatry (Figure 5). A prior study conducted by Debenham et al. (2000) examined allele frequencies in sperm *bindin* in six locations along the west coast of North America (Alaska, USA to Baja, Mexico). It is worth noting that the frequencies they found for the sperm *bindin* GG allele were nearly identical to that reported here (0.091 vs. 0.092—sympatric and 0.118 vs. 0.114—allopatric for Debenham et al., 2000 vs. present study, respectively). Combining both data sets ($N = 10$ sites sympatric and $N = 10$ allopatric) revealed the same nonsignificant ($p = .23$) result; the GG sperm *bindin* allele did not vary between allopatric and sympatric sites.

## Protein variation in sperm *bindin* in the strongylocentrotid clade

In the first variable exon of sperm *bindin*, there are numerous fixed differences within the strongylocentrotid clade and throughout the echinoids (Biermann, 1998; Zigler & Lessios, 2003). At the variable sites within *M. franciscanus* (R or G at site 16 and G or R at site 38), the less frequent GG allele appears to be the fixed state in almost all echinoid taxa (Supplemental Figure 1), including the sister species *to M. franciscanus* (*M. nudus*, Kober & Pogson, 2017), and is likely the ancestral state (Table 1). The Arginine (R) variants at both these sites are derived and unique from the echinoids that have been sequenced.

Across the species range within *S. droebachiensis*, there are two frequent amino acid polymorphisms at sperm *bindin* sites 75 and 77 (Biermann, 1998; Marks et. al., 2008; Pojular & Pogson, 2011; Yund et al. unpublished GenBank sequences). One variant has Asparagine (N) at site 75 and Glutamic Acid (E) at site 77 (NE variant), and the other has Aspartic acid (D)

at site 75 and Glycine (G) at site 77 (DG variant). However, only the NE variant is found in geographic sampling in the Northwest and Northeast Pacific (Marks et al., 2008) and specifically in the current sampling along Vancouver Island ($N = 56$ individuals). The DG variant is noted across the North Atlantic (Marks et al., 2008). At these variable sites in *S. droebachiensis* (75 and 77), *M. franciscanus* is fixed at other amino acids (Alanine and Tyrosine, $N = 124$ individuals, Levitan, 2012).

## Discussion

The establishment of gametic incompatibility between two diverging populations requires the emergence of variation in compatibility between sperm and eggs and the establishment of compatibility groups that can lead to reproductive isolation (Hart et al., 2014; Levitan et al., 2019). Under conditions in which fertilization success limits reproduction, purifying selection should eliminate gametic variants that have reduced compatibility and result in a single most compatible set of sperm and eggs. When fertilization does not limit reproduction, purifying selection might be relaxed. When sperm are overabundant leading to polyspermy and developmental failure, theory (Haygood, 2004; Tomaiuolo & Levitan, 2010) and data (Levitan et al., 2019) support the hypothesis that diversifying selection would favor rare egg variants (and in noncompetitive scenarios sperm variants—Levitan, 2018) with reduced compatibility that allows blocks to polyspermy to become effective. Once variation in compatibility is established among eggs, it provides split resources for sperm that can select for matched compatibility and result in the establishment of compatibility groups. This pattern of intraspecific selection is expected to reduce compatibility to the level at which polyspermy has a reduced risk of causing developmental failure but is balanced by selection to avoid sperm limitation and fertilization failure. In contrast, mismatches in compatibility across species reduce hybridization and the cost of gamete wastage and should produce strong interspecific barriers. The effectiveness of these interspecific blocks is better predicted by divergence in GRPs compared with overall genetic similarity (Zigler et al., 2005). This prediction along with evidence that GRPs often (but not always) show evidence of positive selection (Swanson & Vacquier, 2002) suggests that selection rather than drift plays a prominent role in interspecific divergence in GRPs.

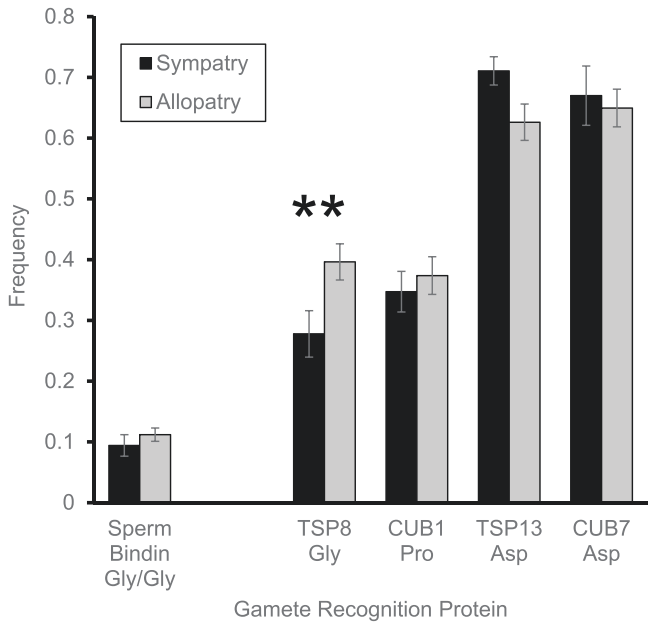


**Figure 5.** Frequency of sperm *bindin* GG allele and the polymorphisms in the four *EBR1* exons in *M. franciscanus* in sympatry ($N = 7$ sites) and allopatry ($N = 7$ sites) with *S. droebachiensis* (bars = SE). For sperm *bindin* and the four *EBR1* exons, the shared allele with *S. droebachiensis* is plotted (asterisks, $p = .0071$). For all loci, except *CUB7* (unresolved), shared alleles are estimated to be ancestral (Table 1).

To establish a link between how these within and among-species processes interact, we examined how the loci that influence compatibility within the strongylocentrid echinoid *M. franciscanus* affect compatibility with the sympatric strongylocentrotid *S. droebachiensis* that has been observed to spawn together in mixed aggregations (Levitan, 2002). Eggs of *S. droebachiensis* are more easily fertilized by both conspecific and *M. franciscanus* sperm in no-choice experiments compared with *M. franciscanus* eggs (Figure 1) as has been noted in a prior study (Levitan, 2002). The blocks to hybrid fertilization in crosses of *S. droebachiensis* eggs with *M. franciscanus* sperm strengthen considerably in the presence of conspecific sperm (Figure 2). Evidence for CSP was strong and in some replicates near absolute, even when conspecific sperm was greatly outnumbered (Figure 2B). This difference in hybridization based on the presence or absence of competing conspecific sperm points to these interspecific blocks being a gradient in the rate at which sperm collisions translate into fertilization events. Evidence that CSP is relevant in nature comes from field fertilization experiments in which both species were induced to spawn at their naturally co-occurring densities and *S. droebachiensis* eggs were less likely to be fertilized by heterospecific sperm than predicted by heterospecific male spawning density (Levitan, 2002). In that field study, CSP was not absolute and hybrid fertilization was noted, particularly when spawning conspecific males were at greater distances to spawning *S. droebachiensis* females compared with nearby high densities of spawning *M. franciscanus* males; under these conditions, conspecifics sperm was likely absent or at vanishingly small concentrations surrounding individual *S. droebachiensis* eggs. The relatively high levels of hybrid fertilization of *S. droebachiensis* eggs by *M. franciscanus* sperm in the no-choice experiments (Figure 1) suggest that interspecific variation in these recognition proteins are not different enough to prevent hybrid fertilization, given enough time and in the absence of competing sperm with higher affinities.

Although the average level of hybridization of *S. droebachiensis* eggs was low in the presence of competing sperm, there was high variation in the effectiveness of CSP in choice crosses. This variation was largely explained by the gamete recognition genotype of the individual *M. franciscanus* male. From the perspective of *S. droebachiensis* eggs, when exposed to *M. franciscanus* sperm with the shared GG sperm *bindin* allele, blocks to hybridization were weak, while hybridization was almost entirely blocked by conspecific sperm when the sperm only carried the derived and more common RG or GR alleles (Figure 3). *Strongylocentrotus droebachiensis* eggs have a lower affinity with the unshared and derived amino acid alleles in *M. franciscanus* and males with these alleles are outcompeted by conspecific sperm. The low average hybridization success of *M. franciscanus* sperm is a function

of the low frequency of the GG allele in this region (~9%). In general, the eggs of *M. franciscanus* are more resistant to hybrid fertilization with *S. droebachiensis*, but variable levels of hybridization were detected and related to their *EBR1* genotype. Eggs released from females with the ancestral and shared Glycine allele at site 31 in *TSP8* had significantly higher rates of hybridization compared with females with the derived Serine allele (Figure 4A). The only other *EBR1* repeat that demonstrated a significant effect was *CUB1*; however, in this repeat, the Tukey adjusted pairwise test failed to distinguish the two homozygous states (Proline vs. Serine). When the interaction of these two repeats was considered, the shared *TSP8*/*CUB1* variant was much more likely to result in hybrid fertilization compared with the unique variant (Figure 4B).

Regardless of the species of the sire and dam, variation in the likelihood of hybridization was influenced by whether the intraspecific sperm and egg variants in *M. franciscanus* were shared with *S. droebachiensis*. We did not detect variation in sperm *bindin* in *S. droebachiensis* and thus could not test for how variants might influence hybridization. We did detect variation in *EBR1* in *S. droebachiensis*, and similar to *M. franciscanus*, there was a trend for eggs with the derived alleles to be more likely to be hybridized. However, this trend was not significant and no firm conclusions can be drawn. Given this pattern of variation and it is influence on hybridization, it appears that ongoing reinforcement selection and RCD is most likely to be manifested in *M. franciscanus*.

### Reproductive character displacement

There is evidence for RCD in *M. franciscanus* at the egg locus (*TSP8*) that significantly influenced the likelihood of hybridization with *S. droebachiensis*. The unique protein variant that reduces hybridization was more frequent (41% vs. 26%) in sympatric locations compared with allopatric locations (Figure 5). The three other *EBR1* sampled repeats that vary in genomic distance from ~2,500 to 22,000 bp from *TSP8* did not significantly vary between sympatric and allopatric locations; any linkage-disequilibrium between *TSP8* and these other repeats was not strong enough to show a uniform shift in allele frequencies across sympatric and allopatric locations.

In sperm *bindin*, even though the unique protein variant increases the likelihood of fertilization, there was no significant shift in allele frequencies in and out of sympatry. The trend of the derived sperm protein being more frequent in allopatry was slight and nonsignificant (Figure 5). Even when the sample size was increased in the sperm *bindin* locus by including prior studies, the influence of location in or out of sympatry remained nonsignificant. The shift in allele frequencies in *TSP8* was evident despite studies that have found either no (Debenham et al., 2000—using the sperm *bindin* locus) or weak (Benham et al., 2012—using microsatellite markers) evidence for significant geographic population structure in *M. franciscanus* along this coastline. This result suggests that selection on *TSP8* to avoid hybrid fertilization might be strong enough to overcome periodic gene flow from sites in sympatry (Oregon and north) to sites sampled in allopatry (south of Point Conception). Studies of genetic structure and gene flow in species with planktonic dispersal have often found weak or equivocal evidence of barriers to gene flow along the west coast of North America (Dawson, 2001; Pelc et al., 2009). However, in some planktonically dispersing taxa, there is evidence of asymmetrical gene flow (north to south—Wares et al., 2001) and latitudinal gradients in allele frequencies (Sotka et al., 2004). These gradients have been attributed to selection overcoming weak or periodic gene flow associated with the oceanic currents and regions of upwelling that characterize this coastline (e.g. Sotka et al., 2004). A potential explanation for the shift in the egg protein in *M. franciscanus* in and out of sympatry compared with sperm *bindin* is that the cost of egg wastage via hybrid fertilization is likely to be much stronger than sperm wastage; selection is strong enough on the egg protein, but not the sperm protein to overcome the homogenizing effect of limited gene flow.

Interestingly, most studies that have searched for evidence for RCD in GRPs have focused on loci expressed in sperm and have found mixed results (Geyer & Lessios, 2009; Geyer & Palumbi, 2003; McCartney & Lessios, 2004; Nydam & Harrison, 2011; Yang et al., 2000). The equivocal result found across different species pairs could be due to unstudied genes being influential in this process, other selective forces driving GRP divergence, or that reinforcement selection is stronger on the less-tested egg, versus sperm, proteins.

Although reconstructing the exact pattern of protein evolution in these species is beyond the scope of this study, one hypothesis for the relation between within- and across-species processes is that sexual conflict, or perhaps more generally sexual selection, generates intraspecific variation that can then be acted upon by reinforcement selection. In *M. franciscanus*, there is evidence that sexual conflict arose from high sea urchin densities and the increased risk of developmental failure caused by polyspermy. This led to negative frequency-dependent selection on the egg protein for lower compatibility variants, and as these variants increased in frequency, they became a target for a matching sperm protein variant. This process generated the set of sperm-egg capability groups that formed the variation needed for the manifestation of reinforcement selection. In the present case, the emergence of the derived Serine (from Glycine) substitution in *TSP8 EBR1* provided a target for mutations for Arginine (at both sites 16 and 38 from Glycine) that formed new compatibility groups. This left the ancestral variants in both sperm and egg at lower frequencies, especially in sympatric regions where hybridization is costly.

In conclusion, protein variation that influences conspecific fertilization success also influences heterospecific fertilization success. From a phylogenetic perspective, derived proteins, generated by sexual conflict or perhaps other means, can be recognized as different by related species and provide a mechanism for CSP and reproductive isolation. Finally, patterns of RCD appear stronger in eggs than sperm perhaps reflecting differences in the cost of hybrid fertilization. These results suggest how within-species generation of variation might lead to across-species reproductive isolation.

## Supplementary material

Supplementary material is available online at *Evolution*.

## Data availability

Data uploaded to Dryad: DOI: 10.5061/dryad.vdncjsz5n

## Author contributions

D.R.L. designed all aspects of this study, conducted fertilization assays, analyzed all data, and wrote the first draft of this

manuscript. Y.H. conducted all the molecular benchwork, designed and tested PCR primers, sequenced all individuals, aligned sequences, scored polymorphisms for sequence variation, and scored larvae for paternity and contributed to the writing.

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## Conflict of interest

The authors declare no conflict of interest.

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