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The evolution of gametic compatibility and compatibility groups in the sea urchin *Mesocentrotus franciscanus*: An avenue for speciation in the sea

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The generation of reproductive incompatibility between groups requires a rare genotype with low compatibility to increase in frequency. We tested the hypothesis that sexual conflict driven by the risk of polyspermy can generate compatibility groups in gamete recognition proteins (GRPs) in the sea urchin *Mesocentrotus franciscanus*. We examined variation in the sperm (bindin) and egg (EBR1) GRPs, how this variation influences fertilization success and how allele frequencies shift in these GRPs over time. The EBR1 gene is a large, 4595 amino acid protein made up of 27 thrombospondin type 1 domain (TSP) and 20 C1s/C1r, uEGF and bone morphogenic protein subdomain (CUB) repeats. Two TSP and two CUB repeats each demonstrate two common non-synonymous haplotypes (alleles). Sperm bindin and one of these EBR1 repeats (TSP8) shift allele frequencies from one common to two common types over an approximate 200 year interval associated with the removal of predatory sea otters and rising sea urchin abundances; the egg receptor shifts first, followed by the sperm ligand. Laboratory crosses indicate that the historically common sperm and egg gamete recognition proteins have high compatibility as do the new common proteins, with mismatches having lower compatibility. This process of creating compatibility groups sets the stage for reproductive isolation and speciation.

KEY WORDS: **EBR1, fertilization, polyspermy, reproductive isolation, sexual conflict, sperm bindin.**

The biological species concept hinges on whether groups of organisms are reproductively isolated (Mayr 1963). For many plant and animal taxa, reproductive isolation is either a consequence of, or associated with, incompatibility between sperm and eggs (Palumbi 1994, Howard 1999). In order for gametic incompatibilities to evolve between groups, mutant genotypes with reduced compatibility must arise and proliferate within a group to the point where that group has reduced compatibly with other groups. The proliferation of gametes with reduced compatibility seems counterintuitive as these should lose in competition with more compatible mates and be selected against by purifying selection. There is broad evidence that mate choice can operate on gamete variants in a wide variety of internally and externally fertilizing taxa that include vertebrates, invertebrates, plants, and fungi (reviewed in Beekman et al. 2016; Kekelainen and Evans 2018), but what process generates this gametic variation? One mechanism that might drive selection for reduced compatibility and generate compatibility groups is the risk of polyspermy; developmental failure caused by more than one spermatozoon fusing with an egg (Gavrilets and Waxman 2002; Haygood 2004; Tomaiuolo and Levitan 2010). Under these circumstances of overabundant sperm, reduced compatibility slows the rate of fertilization and provides the time required for the egg to erect a successful block to polyspermy (Styan 1998; Franke et al. 2002; Levitan et al. 2007).

Theory has suggested that overabundant sperm and the risk of polyspermy can create polymorphisms in gamete recognition systems, which under some circumstances are predicted to lead to sympatric reproductive isolation (Gavrilets and Waxman 2002; Haygood 2004; Tomaiuolo and Levitan 2010). This can be caused

by sexual conflict in which males under sperm competition (competitive fertilization) are selected for a high fertilization rate and females are selected for a reduced fertilization rate to avoid polyspermy. Mutant egg genotypes with reduced compatibility are selected to increase in frequency and once these low compatible egg genotypes are frequent in the population, it provides an underexploited resource for a mutant sperm genotype with high compatibility to this increasingly common egg genotype. This process is predicted to results in balancing selection for two (or multiple) sets of sperm and egg variants maintained by negative frequency dependence (Tomaiuolo and Levitan 2010). A related scenario is conditions where sperm do not directly compete and individual eggs are typically surrounded by overabundant sperm from only one male (monogamous fertilization). Under these conditions sexual conflict is reduced and selection would favor reduced compatibility via mutations to the sperm or egg variant. Under monogamous fertilizations sperm with lower compatibility have high fitness because they typically are not in direct competition with other males and can fertilize eggs with a reduced likelihood of polyspermy (Tomaiuolo and Levitan 2010). Unlike the sexual conflict scenario that requires the novel sperm protein to be compatible with the newly established egg protein, the monogamous fertilization hypothesis only requires gametes to evolve to be less efficient at fertilization to avoid polyspermy; selection for wimpy sperm (Levitan 2018).

Testing these and other hypotheses on the evolution of compatibility has been problematic because fertilization involves a complex set of interactions involved with gamete release, attraction, collision, attachment, and fusion, each potentially mediated by different recognition systems (Levitan 1998; Evans and Sherman 2013). Here, we focus on gamete recognition proteins (GRPs) found on the surface of sperm and eggs, because they are relatively well described in a number of taxa (Swanson and Vacquier 2002) and would provide an important step in understanding the complex evolutionary dynamics of reproductive compatibility. There is good evidence for variation in GRPs within and across species and that GRPs often, but not always, evolve with a molecular signature of positive selection (Swanson and Vacquier 2002). These molecular signatures of selection have been used to test hypotheses of how sexual conflict, sperm competition, or reinforcement selection can drive GRP diversification in marine invertebrates, insects and mammals (reviewed in Turner and Hoekstra 2008). However, these studies generally do not measure intraspecific gamete affinities or demonstrate how selection on GRPs based on sperm availability can generate compatibility groups. Data is accumulating on the fitness consequences of intraspecific variation in sperm GRPs (Palumbi 1999, Levitan and Ferrell 2006; Levitan and Stapper 2010; Levitan 2012; Hart et al. 2014). However there is scant data on the functional significance of intraspecific variation in egg GRPs and how sperm and egg GRPs interact to influence fertilization success (but see Hart et al. 2014 for an example in sea stars).

Externally fertilizing taxa are a good model for examining the evolution of gametic compatibility because fertilization occurs without the added complication of adult control of sperm and egg encounters and the cryptic nature of internal fertilization (Evans and Sherman 2013). The sea urchin *Mesocentrotus* (formerly *Strongylocentrotus*) *franciscanus* provides a unique opportunity to address how sperm and egg GRPs evolve and influence reproductive success over a temporal gradient in sperm availability. Key attributes include experimental work demonstrating the relationship between sea urchin density, sperm limitation, and polyspermy (Levitan 2004), knowledge of the interacting sperm (Minor et al. 1991) and egg (Foltz et al. 1993; Kamei and Glabe 2003) GRPs, and a historic record of a species-wide shift in sperm availability (Estes et al. 2010, Levitan 2012). The GRPs located on the head of the sperm (sperm bindin) and the surface of the egg (EBR1 & 350-kDa) show species-specific adhesion to each other (Glabe and Vacquier 1978; Ohlendieck et al. 1993; Kamei and Glabe 2003) and appear to only be expressed in gametes and function during fertilization (Gao et al. 1986; Cameron et al., 1990; Kamei and Glabe 2003). These studies suggest little opportunity for post-zygotic selection on these fertilization-specific proteins. Thus, allele frequencies of old individuals provide insight into the allele frequencies present in the gametes that interacted at the moment of fertilization in times past (Levitan 2012).

This sea urchin species shows no evidence of senescence, has a predictable relationship between size and age, and has a life-span estimated to exceed 200 years (Ebert et al. 1999; Ebert 2008). Over this time interval, evidence indicates that this species has greatly increased in abundance following human exploitation of predatory sea otters from 1741 until the otters' near extinction in 1911 (Estes et al. 2010). Studies of the influence of sea otters on sea urchins suggest a shift of sea urchin densities from $>0.1/m^2$ in times past to current densities reaching tens per meter square (Estes and Duggins 1995; Rogers-Bennet et al. 1995; Levitan 2002; Watson and Estes 2011). We do not contend that sea urchin densities were uniformly low in times past and uniformly high at present, but that on average sperm availability has increased as sea urchin populations exploded following the removal of sea otter predators along the west coast of North America. This indicates an approximate 200 year transition from low densities, in which sperm limitation was more likely an important selective force on gamete evolution, to high densities, in which polyspermy is more likely to be an important selective force on gamete evolution, over the lifespan of the oldest individuals still present in the population (Levitan 2012).

The sperm bindin gene has a conserved core region surrounded by two variable flanking regions (Zigler and Lessios 2003). The first region is a 91 amino acid exon that in

Table 1. Primers used to generate EBR1 sequences.

	Primer	Sequence	Annealing temperature (°C)	Extension time
TSP1/TSP2 pt1	F	GGGCAGTTATGGTGATTGTC	59	3:00
	R*	TCCATCAATCTGGCACCAAC		
TSP4 pt2	F*	AAAGACCTGCCTCTTCACAG	61	3:00
	R	TCAACACACGACACTGAACG		
TSP8/TSP9 pt1	F	TTTCCTGTGGCAATGGTG	59	1:00
	R*	CCCCCAAAAATACCAAACAC		
TSP9 pt2/CUB1 pt1	F*	GGGACACTTTCGATTGATTG	57	3:30
	R	ACCAGGCCAGTGAAAATCAG		
CUB1 pt2/TSP10 pt1	F	CTTCACCTGGGTTTCTATAGGC	59	2:30
	R	GGCCAGTGAAAATCAGGATG		
sequencing primer	*	GCAAAGCCACAAACCCATTC		
CUB4 pt1 & pt2	F	TGATGAGTGTCCCGATGAAG	61	2:00
	R*	AAATGGTCGGAAGGTCTCAC		
TSP12	F*	AAGCCTTACTGTGGGAATGC	59	2:30
	R*	GAGCATGTACAGGGGGAAAG		
CUB5 pt1	F*	ACAACCCTTGAAGCCATGC	61	3:30
	R	AGCTGGCCTTTGGTCTCTAC		
CUB5 pt2/TSP13 pt1	F*	GCCAATCAAGTGAGAACTGC	61	2:30
	R	AGCTGGCCTTTGGTCTCTAC		
TSP13 pt2	F*	CCAATGAAAGGGCAAAGC	57	1:30
	R	TGCTGAAGATTCCCTCCTC		
CUB7 pt1	F	ACTGCCAAACGTCGATAAGC	61	3:00
	R	TTGGTCAAGAGAGAAGGATGG		
sequencing primer	*	TATGCCTGGTTCGTGGATAC		
CUB7 pt2/TSP15 pt1	F	CCACGACCAGAGAGTGTAATG	61	3:30
	R*	TTGGTCAAGAGAGAAGGATGG		
TSP15 pt2	F*	CCCAAGTTGTGTCCAAT	57	2:00
	R	AGACGCATTGCAGGTTCTC		
CUB8	F*	CGCTGGAAATTATGGAGCAG	57	4:00
	R*	GCAAGGCTGTATCTCTGAAGG		
TSP16	F*	ACGTAGCATGTGGTGATGAG	59	2:30
	R	GCCATACCAAACCAAAGATG		

*Primers used for sequencing.

M. franciscanus has polymorphisms correlated with fertilization success (Levitan and Ferrell 2006; Levitan 2012). We focus on the two most common alleles (non-synonymous haplotypes) in this region that make up approximately 90% of the allele frequencies in *M. franciscanus*. These two common alleles are distinguished by two non-synonymous point substitutions; the RG allele (Arginine at AA site 13 and Glycine at site 35, see Table 1 in Levitan 2012 for haplotypes and reference location) and the reciprocal GR allele. The GG allele is less common (~10% frequency) and the RR allele has a frequency <1% (details see Levitan 2012). Prior field work has demonstrated that males homozygous for the RG allele outperform males homozygous for the GR allele under sperm-limited conditions at low spawning densities, whereas the reciprocal is true under polyspermic conditions at high spawning densities (Levitan 2012). Without knowledge of the egg receptor for sperm bindin, it is not possible to distinguish if the lower compatibility of the GR allele is caused by it being less efficient at fertilization overall, or is highly compatible with only a rare egg receptor.

There appear to be two independent GRPs located on the surface of the egg; EBR1 (Kamei and Glabe 2013) and 350-kDa (variously referred to as hsp70, hsp110, and Obi1; Foltz et al. 1993; Hart and Foster 2013). Both proteins show species-specific adhesion to sperm bindin and might act independently or in concert to achieve fertilization (Vacquier 2012). Obi1 is the sea star homolog to 350-kDa and has been shown to interact with sperm bindin and correlate with fertilization success in a sea star (Hart et al. 2014). We chose to examine EBR1 because in sea

urchins this GRP in under positive selection (Pojular and Pogson 2011) and linkage disequilibrium exists between EBR1 and sperm bindin, generated by assortative fertilization (Stapper et al. 2015). The potential combinatory role of EBR1 with the 350-kDa protein remain unknown and beyond the scope of this study.

The Egg Bindin Receptor (EBR1) in *M. franciscanus* is composed of 4595 amino acids parsed into 47 repeat units, 27 TSP units and 20 CUB units, and shows a species-specific influence on fertilization and adhesion to the sperm bindin protein (Kamei and Glabe 2003). Within species variation in EBR1 has been noted in this species (Pujolar and Pogson 2011), another sea urchin, *Strongylocentrotus purpuratus* (Stapper et al. 2015), and a more distantly related sea star (Hart 2013). However no information is available on the functional significance of variation in this large and complicated protein. Here, we document intraspecific variation in sperm bindin and EBR1, how these two loci interact to influence fertilization success and how they have evolved over an approximate 200 year interval related to shifts in sperm availability.

Methods

COLLECTION SITE AND FIELD COLLECTIONS

All sea urchins were collected from Barkley Sound, British Columbia, Canada, from the shallow subtidal habitats (~3–15 m depth). Animals were kept in fresh flowing seawater and fed kelp at the Bamfield Marine Sciences Centre and returned to their collection site, generally within 2–5 days. Test diameters were taken on most individuals and tissue samples composed of tube feet were collected for each sea urchin and fixed in 95% EtOH. Data on genotype frequencies were generated via adults used in laboratory crosses described below, or from other field and laboratory experiments conducted since 2002. In addition, targeted searches for exceptionally large (>15 cm test diameter) and small (1–5 cm) sea urchins were added to more evenly distribute samples across size classes.

MOLECULAR ANALYSIS

DNA was extracted from tube feet samples as described in Levitan (2012). A 431-bp region of sperm bindin was amplified with primers developed by Debenham and colleagues (2000) and sequenced using an internal primer to generate the 273-bp variable region described by Minor and colleagues (1991). Details of the sperm bindin sequencing can be found in Levitan (2012). All sequences were aligned using Sequencher. We used the program PHASE 2.1 (Stephens and Smith 2001; Stephens and Scheet 2005) to determine the two haplotypes for each individual. If PHASE returned a probability of assignment <0.95 and if the uncertainty involved a non-synonymous substitution, we cloned the PCR product to resolve the haplotype. These sperm bindin sequences ($N = 542$ individuals) were used to determine the fitness consequence of sperm bindin identity in males for the fertilization assays, and in generating shifts in allele frequencies over size classes.

We examined variation within 15 of the 47 EBR1 repeats. This included 10 TSP repeats (TSP 1,2 [partial],4,8,9,10 [partial],12,13,15,16 [partial]) and five CUB repeats (CUB 1,4,5,7,8), representing 37% and 25% of the total number of TSP and CUB repeats, respectively, found in *M. franciscanus* by Kamei and Glabe (2003). The numbering system (e.g., TSP1, 2 . . .) refer to the order of these repeats as located in the *S. purpuratus* genome as described by Kamei and Glabe (2003). These repeats were chosen because we could develop unique primers based on the combination of the mRNA data (Kamei and Glabe 2003) and intron data available from the *Strongylocentrotus purpuratus* genome. Amplification protocols and primers can be found in Table 1. We sequenced 10–12 individuals for each of these regions and focused attention on those repeats that produced more than one common non-synonymous haplotype (common defined as a frequency >15%). Four of these repeats, TSP8, TSP13, CUB1, and CUB7, demonstrated common point substitutions that revealed heterozygote and both homozygote forms of the two most common non-synonymous haplotypes (Table 2). The remaining repeats were largely monomorphic or contained novel point substitutions only found in usually single instances in the heterozygote form (Table 3). We define alleles as haplotypes with unique non-synonymous amino acid sequences at each repeat. We sequenced all females in the fertilization assays for these four EBR1 repeats, plus additional individuals (total of 387 individuals) to bolster sample sizes for estimates of shifts in allele frequencies over size classes. All EBR1 sequences, within these four repeats, were binned into haplotypes using Phase 2.1 (Stephens and Smith 2001; Stephens and Scheet 2005). Linkage disequilibrium (LD) was estimated using the methods of Rogers and Huff (2009) for unphased data and tested for significance using Genepop (Rousset 2008) G test of association. Linkage disequilibrium within EBR1 was plotted as a function of genomic distance, using the *Strongylocentrotus purpuratus* genome and the available mRNA sequence (GenBank NM_214665.1).

USING TEST DIAMETER TO INFER SEA URCHIN AGE

The age of sea urchins was estimated based on research from Ebert and colleagues (1999), in which sea urchins were tagged with tetracycline and recollected after one year to estimate skeletal growth at 17 locations throughout the species range of *Mesocentrotus franciscanus*. This size-to-age estimate was later confirmed by radioisotope data that found the growth point at which nuclear bomb testing began in the 1950s (Ebert and Southon 2003). The size-to-age conversion was based on the average estimated age based on these 17 sites (see Levitan 2012 for details).

Table 2. Amino acid sequences of selected EBR1 repeats used in analysis.

Allele	Amino acid sequence	Freq.
	1 0 2 0 3 0 4 0 5 0 6 0 7 0	
A	Y T W V S E N F G E C S V T C G E G V R V R N V L C Y A I A S G S F Q P V E G S L C N P A L E P P S E E I C D L E E C G G V Y E A T P W S A	0.6702
B	. . . . . G	0.3150
C	. . . . . G . . . . . H	0.0040
D	. . . . . G . . . . . R	0.0013
E	. . . . . G . . . . F	0.0013
	+ + +	
	[TSP8] [TSP9-partial-1]	
A	L I D L E T N S I N S L C D E V D L P Y V Y E S T S S M L E V L F L T D A T V S R R G F S A T Y Q A V R A T A L D Y M T G E W E E	0.6250
B	. . . . . P	0.3132
C	. . . . . P . . . . . K	0.0471
D	. . . . . P . . . . . A	0.0059
E	. . . . . A . P	0.0029
F	. . . . . P . . . . . K	0.0015
G	. . . . . P . . . . G	0.0015
H	. . . . . P . . . . F	0.0015
I	. . . R . . . . . P	0.0015
	+ +	
	[CUB1-partial-2] [TSP10-partial-1]	
A	C S V T C G E G I E Y R D V E C R Q F S T G Q P F T D G F C R D Q R P A D S R P C S L P E C P	0.6963
B	. . . . . Y	0.2867
C	. . . . . R . . . Y	0.0071
D	. . . . . L	0.0028
E	. . . . . X	0.0014
F	. . . . . G	0.0014
G	. . . . . R	0.0014
H	. . . . . H . . . . . Y	0.0014
I	. . . . . Y . . . . . R	0.0014
	+ + + + +	
	[TSP13-partial-2]	
A	C D E T Y T R N N V S F E R D D F M D N E E C L Y N V T S L E G Q C V R I S V A W N L S P P P N G E D E C T G D S L K	0.6265
B	. . . . . R . Y	0.3348
C	. . . . . Y	0.0238
D	. . . . . E . . . . . R . Y	0.0060
E	. . . . . T . . . . . Y	0.0015
F	. . . . . Q	0.0015
G	. . . . . P . . . . R . Y	0.0015
H	. K . . . . . R . Y	0.0015
I	S . . . . . R . Y	0.0015
J	. . . . . R . N	0.0015
	+ + + + +	
	[CUB7-partial-1]	

\+ Syn substitution present at this site

Note: Letters indicate non-synonymous alleles. Frequency of each allele noted in last column. Brackets under each region indicate correspondence of sequence to repeats as identified by Kamei and Glabe (2003). "+" Symbols represent locations of synonymous substitutions.

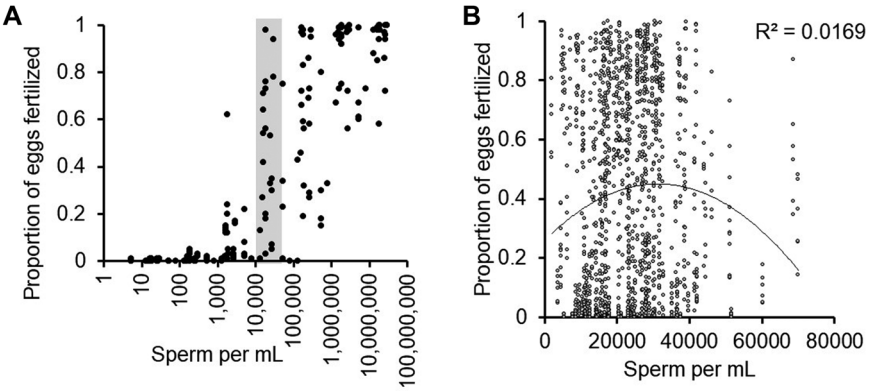

Figure 1. **(A) Preliminary fertilization assays over 10-fold serial dilutions used to determine optimal sperm concentration range for pairwise crosses in fertilization arrays. Gray bar indicates target range in which fertilization is highly variable and less influenced by polyspermy. (B) Fraction of eggs fertilized in the crosses used to determine patterns of compatibility. In this narrow range of sperm concentrations, most of the variation in fertilization is explained by factors other than sperm concentration.**

Table 3. Amino acid sequences of EBR1 repeats not used in analysis because of low levels of common polymorphisms. Letters indicate non-synonymous alleles. Frequency of each allele noted and sample size noted in last columns. Brackets under each region indicates correspondence of sequence to repeats as identified by Kamei and Glabe (2003). "+" Symbols represent locations of synonymous substitutions.

Allele	Amino acid sequence	Freq.	N
	10 20 30 40 50 60 70		
	* * * * * * *		
A	SSYQILSSQCSVTCGTGSETRVLACVDLEGNVVDDSFCTDERPPDVIECAPAPCPGVTYILFYDNYGE	0.8500	10
B	.....M.....	0.0500	
C	.....F.....	0.0500	
	.....I.....	0.0500	
	[TSP1] [TSP2-partial-1]		
A	CSVSCGSGVRTRSIFCLNEANQVVDDSFCAGLVRQTESESCNLASCTITYTYEAQPFPE	0.9500	10
B	.....P.....	0.0500	
	+		
	[TSP4-partial-2]		
A	CSVTCALGVQTREVSCVTRKGSGIVIAESDCSNMTRPSESRECYLDPCPLPYGCDQSYATEGSITYTLQS	0.9500	10
B	.P.....	0.0500	
	+ + + +		
	[TSP9-partial-2] [CUB1-partial-1		
	80 90 100 110		
	* * * *		
A (con't)	PGYPVGYPADLECSKILVAPEDNIIRITFTDLQLEEGCAYDAVR		
B(con't)	.....		
	CUB1-partial-1 (con't)]		
A	CDVRITEGNSKIEFPINDDYYLYNDECTLTITNENGCMNLFFISLDIDEGLGDTCYNDYLM	0.8636	11
B	...H.....	0.1364	
	[CUB4-partial-1]		
A	TFDPSNVYASKPYCGNAINTRSYQTLGNTVELTLRTEDADRFKSFEVFPSFG	1.0000	11
	[CUB4-partial-2]		
A	SCLTFAFVPGPWSE	1.0000	11
	[TSP12-partial-1]		
A	CSLSCDGGVRTRDVPCLNLLTRTPDLESMCEGSISYETMEECNTEECP	1.0000	11
	[TSP12-partial-2]		
A	VCSENVIRPGVIVSPGFSTDDRFLGPAYDEYDNNLNCLYNITNPISTECITVSFISFDLGQSSENCSDYV	1.0000	12
	+ +		
	[CUB5-partial-1]		
A	QITDTEGGEYALYCGLPENTTVPVFYSRSANVEVVLRTGEDEGNKGFEALINFRTCPEFGFFTGNFSE	0.9500	10
B	.....Y..	0.0500	
	+		
	[CUB5-partial-2] [TSP13-partial-1]		
A	IVDAAYPYRAASLCGRDDDQFISYGSTGLIYFTTDDEDRGGGFQIFTEFVDCPEYGFTASNFSE	0.9545	11
B	.....S.....	0.0455	
	+		
	[CUB7-partial-2] [TSP15 partial-1]		
A	CSASCGNSGFRTRDVLCTRLETGENVSRENCNETDTPSNYEPCNRQPCF	0.9545	11
B	.....H.....	0.0455	
	+ +		
	[TSP15-partial-2]		
A	DCNMFFADSGRFVSPEYPSDYPENLQCVYDFANQNNECWKISAIDFDLQDEENGQCRDRFS	0.95	10
B	.....I.....	0.05	
	[CUB8-partial-1]		
A	VEDVGFGGRREPYVACGDELSPVLSFSGTIRITFFTDDEYGGRGFDVLVISE	1	10
	[CUB8-partial-2]		
A	ACPVYGFLAGPYSEE	1	10
	[TSP16-partial- 1]		
A	CSATCGEGVVYRNVTCQDLMTREVVNDSLCSDLRPSEIQPCRQEPCP	1	10
	[TSP16-partial-2]		

\+ Syn sub present at this site

Note: Letters indicate non-synonymous alleles. Frequency of each allele noted and sample size noted in last columns. Brackets under each region indicates correspondence of sequence to repeats as identified by Kamei and Glabe (2003). "+" Symbols represent locations of synonymous substitutions.

DETERMINATION OF TARGET SPERM CONCENTRATION

The target sperm concentration chosen for the examination of the role of gamete recognition protein variation on fertilization success was based on a preliminary set of 27 independent crosses conducted in 1996 examining fertilization success over 10-fold serial dilutions. The goal was to use a narrow range of sperm concentrations near the inflection point where variation in fertilization success was high and largely independent of sperm concentration. This target was found at sperm concentrations from between 10,000 and 50,000 sperm/mL (Fig. 1A), which were achieved by diluting sperm via six serial 10-fold dilutions of released sperm. This dilution produced a narrow range of sperm concentrations, in which sperm concentration, in isolation of other factors, played a small role (1.7%) in explaining the large variance in fertilization success in the current experiments detailed below (Fig. 1B). In these fertilization assays, egg concentration, as a single factor, explained $<1\%$ of the variance in fertilization success.

COMPATIBILITY AS A FUNCTION OF SPERM AND EGG RECOGNITION PROTEINS

Fertilization assays each crossing a set of male and female sea urchins in all pairwise combinations were conducted in 2009, 2012, 2014–2017 (19 independent assays for a total of 1445 unique male–female crosses). Each assay was conducted in a day by injecting a set of sea urchins with 0.55 M KCl to induce spawning. Spawning individuals were sexed, with female being inverted and placed in filtered seawater to collect eggs and males being kept upright so that sperm could be collected with a Pasteur pipette and placed "dry" in an ice-cooled glass dish. Eggs were collected from each female and diluted to a concentration of $\sim$5000 eggs/mL (mean 4644/mL, SE = 115). An array of scintillation vials filled with 8 mL of filtered seawater was established (e.g., 100 vials in a 10 male by 10 female cross). To each row, 1 mL of the stock egg suspension was added from a female. Once eggs were situated, 0.01 mL of dry sperm was carefully placed into a vial containing 9.9 mL of seawater. The sperm would sit in an undisturbed clump on the bottom of the vial. Once sperm from all males were similarly situated at the top of each column, the sperm from the first male was swirled and put through three additional 10-fold dilutions to create the stock sperm suspension. One mL of this suspension was added to each experimental vial containing eggs (sixth 10-fold dilution) in that column (each male crossed with all females). Sperm were used within 2 min of dilution to minimize the aging effect of sperm following mixture with seawater; however the timing of each sperm dilution was recorded and used as a covariate to account for subtle effects of aging. As soon as sperm were added to each vial containing eggs, it was swirled three times by hand and left for 3 h before examination of 200 eggs for the presence of cleavage or a fertilization membrane. For each male's sperm, 1 mL of the third serial dilution was fixed with three drops of formaldehyde for eventual quantification of sperm concentration using a hemocytometer (eight replicate counts).

Data were analyzed in SAS using the GLIMMIX procedure with a beta-binomial distribution to eliminate over (or under) dispersion of data. The response variable was the square-root arcsine transformed proportion of fertilized eggs. The main effects were sperm bindin genotype (males with rare alleles were eliminated), the four EBR1 repeats, and interactions between each EBR1 repeat and sperm bindin. The covariates were sperm concentration, the polynomial of sperm concentration (to account for nonlinearity associated with sperm saturation and polyspermy), egg concentration, the order of sperm addition (to account for sperm age), and the interactions of sperm concentration $\times$ egg concentration (test for non-linearity with respect to sperm–egg ratio) and sperm order $\times$ sperm concentration (sperm age differently at different concentrations; Levitan et al. 1991; Levitan 2000). The array (block of males and female used per experiment) was used as a random effect. For the EBR1 repeats, TSP8, CUB1, and TSP13 were represented by their only common single point substitution (Table 2) in either of the homozygous conditions or heterozygous condition (e.g., A, G, or R for TSP8). For CUB7, because there were three common substitutions (see results), this repeat was represented by the combined SNP genotype (AAT, TAT, WAT, etc.).

THE RELATIONSHIP BETWEEN POLYSPERMY AND COMPATIBILITY

To confirm a relationship between a male's sperm bindin genotype ability to fertilize eggs and polyspermy, a serial sperm dilution experiment conducted in 2002 to specifically estimate rates of developmental failure caused by polyspermy was here reanalyzed by sequencing the males for their sperm bindin genotype. From these experiments, 39 crosses were identified with males having the RG/RG, RG/GR, or GR/GR sperm bindin genotype. Prior work has established the RG genotype has a higher affinity, on average, with available eggs (Levitan 2012, 2018, and see Results). This lab experiment documented the degree of developmental failure, defined as the reduction in normal development following peak fertilization as a function of increasing sperm concentration. This could be manifested as a failure to raise a fertilization membrane (after a peak of 100% of eggs raising membranes at lower sperm concentrations), broken or misshapen membranes, or uneven cleavage patterns. Gametes were obtained as noted above and fertilization was tested at three to eight 10-fold serial dilutions of sperm. Sperm concentration and the fraction of eggs noted to have polyspermy were both log transformed and analyzed with a GLM in SAS. Because polyspermy was not noted at sperm concentrations lower than 30,000 sperm/mL, these data were

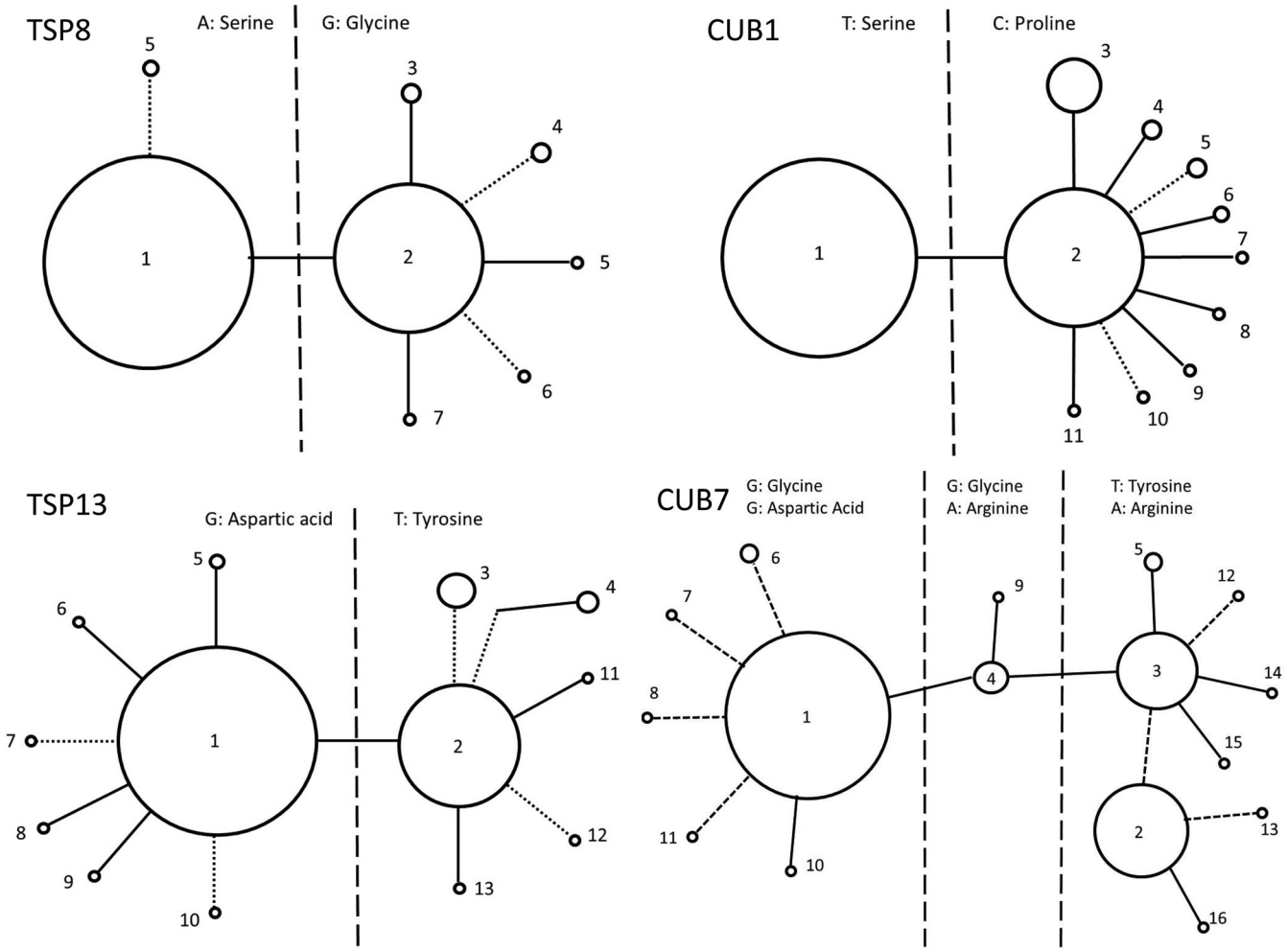

Figure 2. Haplotype networks of the four EBR1 regions examined. Circle size indicates haplotype frequency. Black connecting lines indicate nonsynonymous changes, dotted lines indicate synonymous changes. Dashed dividing lines substitutions that correspond with the common substitutions highlighted in the main text. In all cases, the "1" is the older common haplotype.

removed from the analysis that tested the degree of polyspermy as function of sperm bindin genotype and sperm concentration.

Results

The estimated distances between the SNPs on TSP8 and CUB1, TSP13, and CUB7 are 2,637, 17,460, and 21,793 bp, respectively. Haplotype networks for these four repeats were constructed using TCS 1.21 (Clement et al. 2000), which highlighted the common alleles in each region (Fig. 2). The four EBR1 exons demonstrated a negative relationship of Linkage Disequilibrium with the log of genomic distance (Fig. 3). Tests of independence noted significant LD at all but the two most distant repeat pairs (TSP8-CUB7 and CUB1-CUB7), which are estimated to be at least 19,000 bp apart on the genome.

The sperm bindin locus ($N = 542$ individuals) and the four variable EBR1 repeats ($N = 376$) were sequenced and individuals

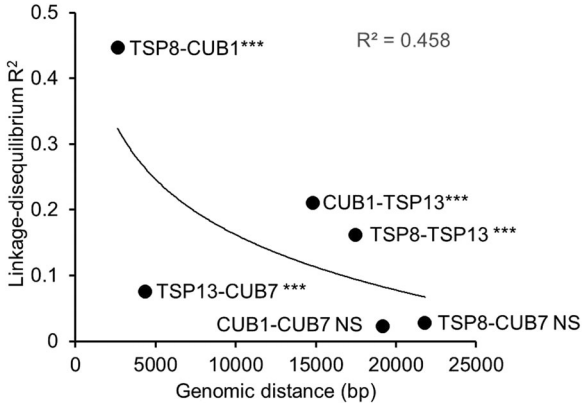

Figure 3. Linkage disequilibrium between EBR1 exons as a function of genomic distances (based on *Strongylocentrotus purpuratus* genome). The two EBR1 loci significantly influencing compatibility (TSP8 and CUB7) are unlinked. ***Indicates $P < 0.0001$.

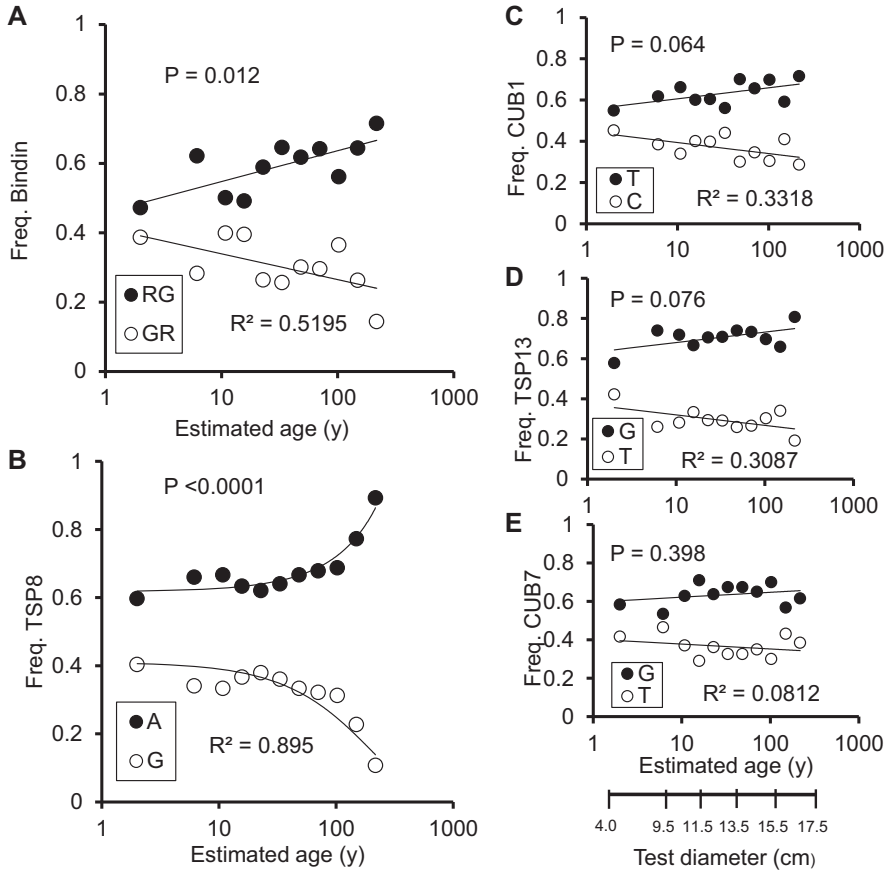

Figure 4. Shifts in allele frequencies in sperm and egg GRPs over size classes and estimated age. Sperm bindin (A), the most common non-synonymous point substitution in TSP8 (B), CUB1 (C), TSP13 (D), and CUB7 (E). The scale bar for the relationship between sea urchin size (Ebert et al. 1999, Levitan 2012). Allele frequencies tend to shift from one common allele in older individuals to two alleles being more equally common in younger individuals. These shifts are significant with the log of age classes in sperm bindin (A) and linearly with age classes in TSP8 (B).

binned into 11 size classes ranging from less than 7 cm to greater than 17 cm (number of alleles per size class = 70/62, 174/94, 108/66, 104/60, 114/58, 90/50, 60/48, 78/56, 66/64, 42/44, 28/28 for sperm bindin/EBR1). This is an estimated age range from <5 to >200 years old (Fig. 4). Sperm bindin allele frequencies shifted from a condition of one common and one rare allele in the largest, oldest individuals to two nearly equal allele frequencies in the smallest, youngest individuals (Fig. 4A). Variation in neutral microsatellite loci showed no pattern of change over time (Levitan 2012).

In EBR1, TSP8 revealed a larger and more rapid shift, compared with sperm bindin, from one to two common alleles over this size and age gradient (Fig. 4B). Two other repeats (CUB1 and TSP13) showed the same trend of transitioning from one to two common alleles but with marginal P-values ($P < 0.10$, Fig. 4C and D). The remaining repeat, CUB7 showed no trend over size and age (Fig. 4E). Only TSP8 showed a linear relation between estimated age and allele frequencies, the other regions were better represented by the log of estimated age; indicating the more rapid rise of the less common TSP8 allele over time, compared to either the sperm bindin or the other EBR1 repeats.

Laboratory crosses examining pairwise fertilization as a function of sperm bindin genotype in males and the four EBR1 exon genotypes in females, while including sperm concentration, sperm age, and egg concentration as covariates, did not detect a significant effect of TSP13 or CUB1, either as main effects or interacting with sperm bindin. A reduced model without these two nonsignificant EBR1 repeats found significant main effects of sperm bindin, TSP8, CUB7, and an interaction between TSP8 and sperm bindin (Table 4 and Figs. 5C and 6). In addition to these main effects, the covariates and interactions of sperm age and sperm concentration with sperm bindin were also significant (Table 4). The older common RG/RG sperm bindin genotype was most compatible with the older common A/A TSP8 genotype, while the newly common GR/GR genotype was most compatible with the newly common G/G TSP8 genotype (Fig. 5). To specifically test whether the two compatibility groups were significantly

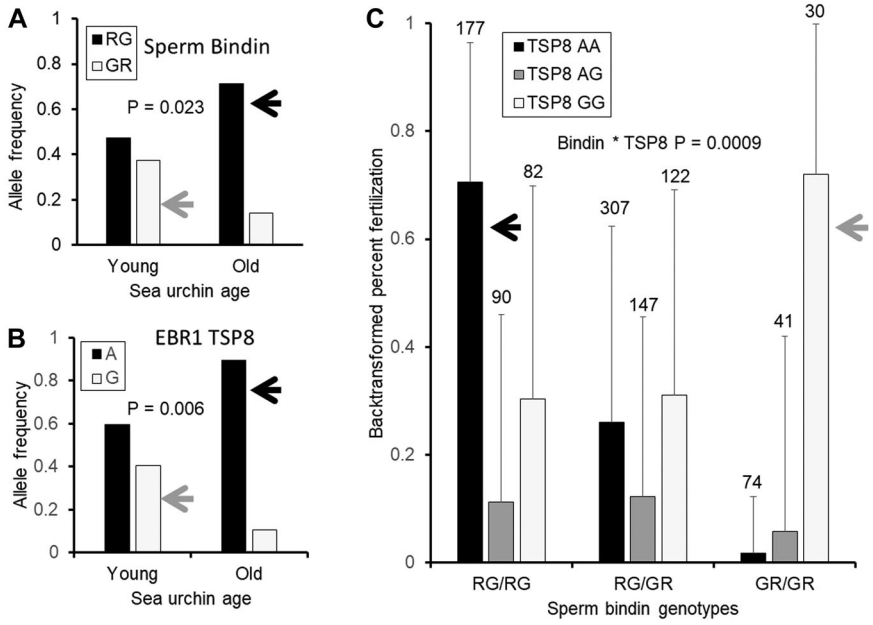

Figure 5. Allele frequencies of sperm bindin (A) and EBR1 TSP8 (B) in the smallest (Young) and largest (Old) size classes (Two tailed, Fisher exact test, sample sizes in text). (C) Back-transformed least square mean fertilization success based on sperm bindin in males and EBR1 TSP8 in females (standard error and sample sizes). There is a significant interaction ($P = 0.0009$; Table 4) between these loci; historically common sperm and egg proteins have high compatibility (black bars and arrows) as do recently common sperm and egg proteins (light bars and arrows). Mismatched and heterozygous individuals have lower compatibility compared to the two compatible groups ($P < 0.0001$, Table 5).

different from mismatched and crosses involving heterozygous individuals, a second model was explored, using the same covariates as noted above, but grouping these crosses into these three classes: the two compatible crosses (RG/RG × A/A and GR/GR × G/G) and a third group composed of all other crosses. This simplified model indicated no significant difference between the two compatible crosses, but both compatibility groups had higher fertilization compared to the group made up of the remaining crosses (Table 5).

As noted in prior work (Levitan 2012), the more common RG/RG genotype had higher overall success compared to heterozygotes or the less common GR/GR genotype (Fig. 6). The higher average success of the RG/RG sperm bindin genotype is caused by two factors: (1) it has high compatibility with the more common TSP8 A/A genotype and (2) it has relative high compatibility with mismatched TSP8. In contrast, the GR/GR sperm bindin genotype is highly compatible with the less common TSP8 G/G genotype and it has poor compatibility with mismatched TSP8 genotypes (Fig. 5C).

The second EBR1 repeat that significantly influences fertilization success (CUB7) does not strongly interact with sperm bindin ($P = 0.077$). The two EBR1 repeats that influenced fertilization (TSP8 and CUB7) are separated on the genome such that linkage disequilibrium between them could not be detected (Fig. 3).

The polyspermy experiments confirm that the, on average, more compatible RG genotype also is more likely to cause polyspermy at high sperm concentrations when crossed with a random sampling of females. The rank order of the likelihood of inducing polyspermy at high sperm concentrations was RG/RG, RG/GR, and GR/GR sperm bindin genotypes in males (Fig. 7).

Discussion

PATTERN AND PROPOSED PROCESS

The shift in allele frequencies in EBR1 and sperm bindin along with the fertilization assays are consistent with the hypothesis that polyspermy drives sexual conflict and balancing selection on gamete recognition proteins in this species; sperm limitation selects for purifying selection for high affinity gametes, whereas polyspermy selects for a novel egg GRP with lower compatibility to escape polyspermy, creating an unexploited resource for a novel sperm GRP that matches the novel egg GRP (Gavrilets and Waxman 2002; Haygood 2004; Tomaiuolo and Levitan 2010). This process can create distinct compatibility groups and provide an avenue for reproductive isolation and speciation.

The observed pattern is that the historically common sperm bindin RG allele and the historically common TSP8 A allele are highly compatible (Fig. 5). With progressively smaller and younger sea urchins, there is a rapid rise in the less common

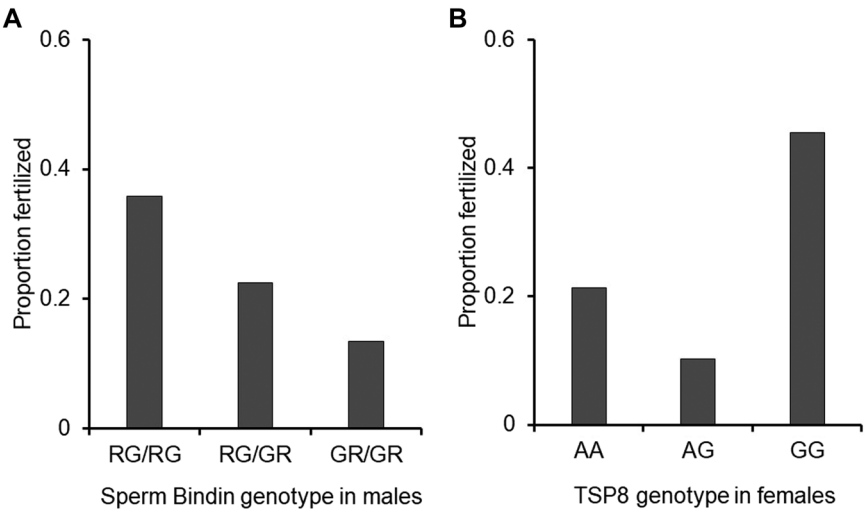

Figure 6. **Back-transformed least square mean fertilization success as a function of then main effects of sperm bindin and TSP8. As in prior work on sperm bindin (Levitan 2012, 2018), the average rank order of compatibility is RG/RG > RG/GR > GR/GR. In EBR1, TSP8, the less common GG genotype had, on average, higher success than the more common AA genotype.**

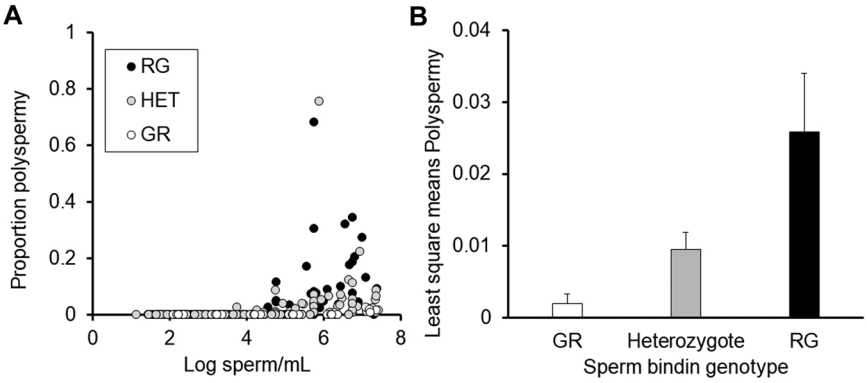

Figure 7. **The fraction of eggs suffering polyspermy as a function of sperm concentration for RG/RG, RG/GR, and GR/GR males. (A) Males and females were tested in 39 crosses using 10-fold serial dilutions of sperm. (B) Least square mean level of polyspermy.**

EBR1 TSP8 G allele (Fig. 4B), which is followed more slowly by a rise in the less common sperm bindin GR allele (Fig 4A). In the most recently conceived sea urchins, these newly common alleles are at near equal frequencies with the historically common alleles (Figs. 4A and B and 5A and B). These newly common sperm and egg recognition protein variants are also highly compatible with each other, but mismatches between these compatibility groups (RG/A and GR/G) are less compatible (Fig 5C).

A parsimonious explanation for the shift in allele frequencies, which matches these patterns of compatibility, is that prior to the extirpation of predatory sea otters, sea urchin densities were on average very low across the species range and sperm were more likely limiting, favoring the highly compatible sperm bindin RG and EBR1 TSP8 A allele. Because the sperm bindin RG allele has higher compatibility across all EBR1 TSP8 genotypes compared to the GR allele (Figs. 5C and 6A), it would be favored under sperm-limited conditions. As sea urchin densities increased following the removal of sea otter predators the risk of polyspermy increased and the common RG sperm bindin protein, which is more likely to cause polyspermy (Fig. 7), likely became increasingly deleterious to the highly compatible TSP8 A protein. Prior work has shown that at sperm concentrations where polyspermy become apparent, there is a positive relationship between sperm concentration and fertilization success in GR males and a negative relationship in RG males; evidence that GR reduce the risk of polyspermy at high sperm concentrations compared to RG sperm bindin proteins (Levitan 2012). Under these historic conditions in which the sperm bindin RG protein was common and polyspermy was a significant risk, the rarer, less compatible TSP8 G allele became favored and rapidly increased in frequency (Fig. 4B). The establishment of the newly common TSP8 G allele provided a resource for the highly compatible, but historically rare, sperm bindin GR allele (Fig. 5). This is reflected by the delayed, but equally large shift in sperm bindin allele frequencies

Table 4. GLIMMIX model testing fertilization (arcsine transformed) with a beta-binomial distribution. Main effects are sperm Bindin, EBR1 exon TSP8 and CUB1 with the covariates of sperm (sperm) and egg (egg) concentration, sperm age, and the polynomial of sperm concentration. The fertilization array (block of individuals) is a random factor, scale is the beta-binomial factor that adjusts for overdispersion (Chi-square/*df* = 1.0).

Parameter	Estimate	SE	
Array	1.8330	0.7562	
Scale	4.0034	0.2894	
Type III tests of fixed effects			
Effect	*df*	*F*-value	Pr > F
TSP8	2	3.55	0.0292
CUB7	9	2.20	0.0207
Bindin	2	8.03	0.0004
Sperm	1	65.59	<0.0001
CUB7	9	2.20	0.0207
Sperm	1	65.59	<0.0001
Egg	1	4.55	0.0333
sperm age	1	5.10	0.0243
Sperm × sperm age	1	6.75	0.0096
Sperm × egg	1	20.35	<0.0001
Polynomial sperm	1	20.46	<0.0001
Bindin × sperm	2	9.78	<0.0001
Bindin × TSP8	4	4.75	0.0009
Bindin × CUB7	16	1.55	0.0777

Table 5. GLIMMIX model testing fertilization (arcsine transformed) with a beta-binomial distribution on crosses categorized as the two compatibility groups (RG/RG AA and GR/GR GG) versus all other crosses (groups). Main effects are group (compatibility group) and EBR1 exon CUB7 with the covariates of sperm age, sperm concentration (sperm), egg concentration (egg), and the polynomial of sperm concentration. The fertilization array (block of individuals) is a random factor, scale is the beta-binomial factor that adjusts for overdispersion (Chi-square/*df* = 1.0). Multiple comparisons using Tukey method; raw P values and P values adjusted for multiple comparisons.

Parameter	Estimate	SE	
Array	1.7214	0.7068	
Scale	3.8483	0.2774	
Type III tests of fixed effects			
Effect	*df*	*F* value	Pr > F
Group	2	9.47	<0.0001
CUB7	9	2.39	0.0114
Sperm	1	58.57	<0.0001
Egg	1	7.55	0.0062
Sperm Age	1	3.49	0.0622
Sperm × sperm age	1	5.35	0.021
Sperm × egg	1	26.24	<0.0001
Polynomial of sperm	1	18.07	<0.0001
Group × sperm	2	5.41	0.0047
Comparison	*P*	Adjusted *P*	
RG-A versus GR-G	0.4869	0.7661	
RG-A versus other	0.0181	0.0474	
GR-G versus other	0.0007	0.0021	

(Fig. 4A). This process produced two distinct compatibility groups that reduced the effective sperm concentration (only half the sperm is highly compatible with an egg genotype). This established a balanced polymorphism maintained by negative frequency dependence; the more common protein suffers the higher cost of polyspermy (Tomaiuolo and Levitan 2010).

Although a variety of alternate hypotheses might explain the noted shifts in allele frequencies of sperm bindin and EBR1, the idea that it is driven by the risk of polyspermy is supported by (1) the evidence for how sea otter removal leads to increases in sea urchin abundance (Estes and Duggins 1995; Watson and Estes 2011); (2) the relationship between sea urchin abundance, sperm limitation, and polyspermy from field experiments conducted over a range of natural densities (Levitan 2004); (3) field experiments that demonstrate how variation in gamete recognition proteins influence patterns of fertilization in the sea (Levitan and Ferrell 2006; Levitan 2012, 2018), in the direction confirmed by laboratory experiments (Levitan 2012; Fig. 5C); (4) The relationship between gamete affinity and the likelihood of polyspermy of these alternate sperm bindin genotypes (Fig. 7); and that (5) the shift in allele frequencies matches the specific set of compatibilities of these protein variants (Fig. 5). These lines of evidence match the theoretical predictions for how these proteins should shift in allele frequencies with sperm availability and sexual conflict (Tomaiuolo and Levitan 2010).

An alternate hypothesis for the evolution of gamete recognition proteins is that egg proteins evolve neutrally and sperm proteins evolve to match these new egg receptors (Swanson et al. 2001). The hypothesis that egg proteins evolve neutrally suggests that egg GRP variants do not influence female success under natural ranges in sperm availability. This would be the case if sperm saturation is common, and blocks to polyspermy are strong enough to result in peak fertilization over the broad range of sperm concentrations experienced in nature. Studies investigating polyspermy demonstrate narrow peaks between sperm limitation and polyspermy, such that even under optimal sperm levels some eggs are limited by sperm and others are killed by sperm, due to stochastic variation in sperm–egg collisions (Styan 1998; Franke et al. 2002; Levitan 2004). A narrow fitness peak, coupled with the rapid and heterogeneous dilution of released sperm (Crimaldi 2012), suggests that eggs might regularly be under selection imposed by either too many or too few sperm (Levitan 2004).

We cannot rule out the potential effects of other environmental factors that might have shifted over this 200 year period (e.g., temperature, pH, nutrients), but it is not clear how other factors besides increasing sperm availability can explain the match between shifts in sperm and egg GRP allele frequencies and their patterns of compatibility.

THE EVOLUTION OF COMPATIBILITY GROUPS AND THE INITIATION OF REPRODUCTIVE ISOLATION

Incompatibility between groups of individuals, sympatrically or allopatrically, cannot evolve unless variation in compatibility arises within at least one group of individuals. The process predicted by theory (Gavrilets and Waxman 2002; Haygood 2004; Tomaiuolo and Levitan 2010) and reflected by the presented empirical data provides an illustration for how rare or mutant genotypes with low reproductive compatibility can proliferate and generate two compatibility groups. Once matched sets of compatibility groups are established, theory suggests several possible outcomes based on the degree of difference among sperm and egg compatibilities and the degree of polyspermy, that can lead to (1) a balanced polymorphism maintained by negative frequency-dependent selection, (2) a selective sweep by one or both sperm and egg variants, or (3) continued divergence and an avenue for sympatric speciation (Gavrilets and Waxman 2002; Haygood 2004; Tomaiuolo and Levitan 2010). There is no indication from the present data for a progression toward complete reproductive isolation, rather it appears that allele frequencies might be maintained as a balanced polymorphism. However, these various predicted outcomes might explain why in some taxa polymorphisms are maintained within species, while other taxa diverge with little within-species variation and a high degree of among-species incompatibility (Palumbi 1999; Swanson and Vacquier 2002; Kosman and Levitan 2014).

Fluctuations in adult population density caused by a variety of biotic and abiotic factors could result in purifying selection reducing protein diversity during times of low density and diversifying selection during times of high density. These processes might select for different combinations of matched proteins among isolated populations and reduce or eliminate the possibility of hybridization during secondary contact. Even in cases in which reinforcement selection against hybridization is detected in sympatry (Geyer and Palumbi 2003), generation of compatibility groups within a population provides the variation to produce eventual reproductive incompatibility between species. High and fluctuating densities would also enhance standing genetic variation such that populations could rapidly adapt to demographic challenges that create mismatches between reproductive traits and sperm availability. The rapid changes noted in this study were facilitated by the presence of low frequency variants.

Fertilization involves a cascade of interactions starting with synchrony in spawning, cues for aggregation, sperm chemotaxis to eggs, interactions between sperm and egg accessory structures, gamete surface recognition, and finally gamete fusion (Levitan 1998; Evans and Sherman 2013). Discrimination at all these stages might influence patterns of compatibility. At the level of GRPs, at least two independent proteins (EBR1 and 350-kDa) have been identified to interact with sperm bindin. Even within EBR1, other regions of this protein might have large effects on fertilization. Examining how all these recognition systems interact would be a fruitful area of research. Our finding of one repeat that influences fertilization and another that strongly interacts with sperm bindin to create two compatibility groups does not indicate that other aspects of fertilization or EBR1 are not critical to fertilization or compatibility. It does provide strong evidence for how compatibility can evolve. Related theory predicts how the risk of polyspermy can sympatrically generate temporal isolation in spawning times (Tomaiuolo et al. 2007). For many marine species (Palumbi 1994) and plants (Baack et al. 2015), gametic incompatibility and temporal differences in reproduction are predominant isolating mechanisms. Here, we provide evidence for how the risk of polyspermy can initiate reproductive barriers within an interbreeding population and provide a potential avenue for speciation.

AUTHOR CONTRIBUTIONS

D.R.L. was responsible for experimental design, fertilization assays, data analysis, and writing. R.B. and Y.H. designed and screened EBR1 primers, sequenced, analyzed sequences, and contributed to the writing.

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DATA AVAILABILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.c1t54v2

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