

Sperm competition and the evolution of gametic compatibility in externally fertilizing taxa

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ABSTRACT: Proteins expressed on the surface of sperm and egg mediate gametic compatibility and these proteins can be subject to intense positive selection. In this review, we discuss what is known about the patterns of adaptive evolution of gamete recognition proteins (GRPs). We focus on species that broadcast eggs and sperm into the environment for external fertilization, as the ease of observing and manipulating gamete interactions has allowed for greater advances in the understanding of GRP evolution, uncomplicated by confounding behavioral and physiological components that offer alternative evolutionary targets in internal fertilizers. We discuss whether interspecific mechanisms, such as selection to avoid fertilization between species (reinforcement selection), or intraspecific mechanisms, such as selection to increase (or decrease) the affinity between eggs and sperm based on the intensity of sperm competition, may be responsible for the pattern of GRP evolution observed. Variation in these proteins appears to influence gametic compatibility; GRP divergence among species is a better predictor of hybrid fertilization than neutral genetic markers and GRP variation within species predicts reproductive success among individuals within a population. Evidence suggests that sperm competition may play a large role in the evolution of gametic compatibility.

Key words: sperm competition / gamete recognition protein / reinforcement / polyspermy / gamete compatibility

Introduction

Gametic compatibility can have important influences on the reproductive success of individuals within a species and the effectiveness of reproductive isolation across species boundaries (Clark *et al.*, 1999; Palumbi, 1999; Evans and Marshall, 2005; Vieira and Miller, 2006; Lessios, 2007). Examining the evolution of gametic compatibility in internal fertilizers has been challenging, as mating is a complex process involving both behavioral and physiological components that might confound studies of gamete interactions (Clark *et al.*, 2006; Vacquier and Swanson, 2011). In contrast, great strides have been made in species that broadcast eggs and sperm into the environment for external fertilization, because individuals release millions of gametes, multiple crosses are simple to generate and gamete interactions can be easily observed (Vacquier and Swanson, 2011). The added benefit of examining broadcast-spawning species is that this mode of reproduction is thought to be the ancestral mating strategy (Ruppert *et al.*, 2004) and thus provides a key insight into how gametes in general, and gametic compatibility in particular, have evolved.

The proteins mediating gametic compatibility, or gamete recognition proteins (GRPs), were first identified in sea urchins, a model broadcast-spawning taxon (Vacquier and Moy, 1977). The genes encoding GRPs have now been characterized in a suite of broadcast-spawning species, including snails, mussels, oysters, tunicates, sea urchins and sea stars (Gao *et al.*, 1986; Riginos and McDonald, 2003; Moy and Vacquier,

2008; Yamaguchi *et al.*, 2011; Hellberg *et al.*, 2012; Sunday and Hart, 2013). Despite the advances in identifying GRPs in many broadcast-spawning species, there have only been a few species in which both the male and female binding partners have been functionally identified (Swanson and Vacquier, 1997; Yamaguchi *et al.*, 2011; Vacquier, 2012). In internal fertilizers, identification of GRPs has lagged behind, although sperm receptors have been identified and several candidate sperm proteins that may act as a binding partner have been isolated in mammals (Gasper and Swanson, 2006; Turner and Hoekstra, 2008a; Vicens *et al.*, 2014). In insects, the focus has mainly been on reproductive proteins not directly involved in sperm–egg binding, but rather on proteins that are known to affect the female reproductive tract (Swanson *et al.*, 2001a, 2004; Panhuis *et al.*, 2006).

Interestingly, given their role in gametic compatibility, GRPs in some species are under positive selection (Swanson *et al.*, 2001b; Swanson and Vacquier, 2002; Moy *et al.*, 2008; Lessios, 2011; Pujolar and Pogson, 2011; Hellberg *et al.*, 2012), and for many of those species, GRP divergence has been found to be greater than non-reproductive protein divergence, indicating that rapid evolution is occurring (Metz *et al.*, 1998; Zigler *et al.*, 2005; Lessios and Zigler, 2012). Additionally, a relatively small number of non-synonymous mutations can have large effects on gametic compatibility; as few as 10 amino acid changes can lead to gamete incompatibility in sea urchins (Zigler *et al.*, 2005). Furthermore, high numbers of non-synonymous substitutions occur at protein-

binding sites in many of these species, implying that these substitutions may affect how the sperm and egg proteins interact (Lyon and Vacquier, 1999; Torgerson *et al.*, 2002; Clark *et al.*, 2007; Springer and Crespi, 2007; Hellberg *et al.*, 2012). Variation in these proteins appears to influence reproductive compatibility; protein divergence among species is a better predictor of hybrid fertilization than neutral genetic markers (Zigler *et al.*, 2005) and protein variation within species predicts reproductive success among individuals within a population (Palumbi, 1999; Levitan and Ferrell, 2006; Levitan and Stapper, 2010; Levitan, 2012).

Although there is ample evidence that reproductive compatibility (or incompatibility) can evolve fairly rapidly, there is no consensus on the evolutionary mechanisms that drive these patterns. In this review, we focus on what is known about the patterns of adaptive evolution of GRPs, and discuss whether interspecific mechanisms, such as selection to avoid fertilization between species (reinforcement selection), or intraspecific mechanisms, such as selection to increase (or decrease) the affinity between eggs and sperm based on the intensity of sperm competition, may be responsible for the patterns observed.

Predictions for adaptive evolution of GRPs and gametic compatibility

The idea that GRPs undergo adaptive evolution comes from the analysis of sequence data on these reproductive proteins in which divergence among species or diversification within species accumulates at a higher rate than predicted by neutral models of evolution. This is generally manifested as a higher ratio of non-synonymous nucleotide substitutions to synonymous substitutions than predicted by chance (reviewed in Palumbi, 1999). At first glance, this pattern of positive selection in either male or female proteins seem counterintuitive, because any mutation that causes an individual to have a unique GRP is likely to make that individual less compatible with his or her mates. Why should selection ever favor novel recognition variants? What are the circumstances in which a decrease in gametic compatibility is beneficial?

Preventing hybridization between species (reinforcement) or preventing egg death from polyspermy (sperm competition) could both lead to selection for decreased gametic compatibility (Swanson and Vacquier, 2002; Palumbi, 2009). In both of these scenarios, selection for novel GRPs on the egg surface that reduces deleterious fusions with sperm would be predicted. The circumstances for favoring novel mutations on the sperm surface are more complex. When novel egg proteins become frequent in the population, novel sperm proteins that match these new egg proteins would be favored (Swanson and Vacquier, 1998; Gavrillets *et al.*, 2001; Hayashi *et al.*, 2007), and when the risk of polyspermy is equally shared among males and females, i.e. when sperm competition among males is low, but within males is high, then novel mutations to sperm proteins can also be favored even in the absence of matching egg proteins (Tomaiuolo and Levitan, 2010).

Predictions for patterns of selection on gametic compatibility and novel GRPs due to reinforcement selection

Reinforcement is a selective force caused by the negative consequences of hybridization. Some species that overlap geographically may have very

little temporal separation in their spawning behavior, and exhibit only partial gametic incompatibility (Lessios, 2007). If fertilization between two such species (or populations) results in developmental failure, sterile offspring or offspring with reduced fitness, and the heterospecific fertilization prevents an otherwise successful conspecific fertilization event, then traits which prevent heterospecific fertilizations should be favored. Thus, under these conditions, reinforcement should select for decreased gamete compatibility between species. It is predicted that reinforcement selection should be strongest in sympatric populations, where species overlap, compared with allopatric populations, where no heterospecific sperm is present (Dobzhansky, 1940; Lessios, 2007). Novel GRPs expressed on the egg surface that decrease fusions with heterospecific sperm would be favored, while novel sperm GRPs that match the novel female would be favored as well. This would result in the pattern where GRPs exhibit a stronger signal of positive selection (sequence divergence between these species) in geographic areas where a sympatric species occurs (Dobzhansky, 1940; Vieira and Miller, 2006). Therefore, reinforcement selection is a potential explanation for seeing patterns of divergence in GRPs among species, and variation between allopatric and sympatric populations within a species. However, this selective scenario cannot explain persistent variation in GRPs within a population; reinforcement does not select for the maintenance of lower intraspecific sperm and egg affinities (Lessios, 2011).

Predictions for patterns of selection on gametic compatibility and novel GRPs due to sperm competition

Intraspecific variation in sperm availability may produce diverse patterns of GRP evolution (Fig. 1) as sexual selection is predicted to select for higher affinities between sperm and eggs when gamete collisions are rare and reduced affinities when polyspermy is a risk. Sperm availability can be modified by mate density, water flow and position in the spawning group (Pennington, 1985; Levitan *et al.*, 1992; Petersen *et al.*, 1992; Coma and Lasker, 1997; Franke *et al.*, 2002; Levitan, 2002). Species that do not aggregate, or that live in environments where sperm is rapidly diluted are more likely to experience sperm limitation, and traits that increase gametic compatibility would be favored (Levitan, 1993, 1998; Franke *et al.*, 2002). Under sperm limitation, selection may result in signatures of purifying selection in both the male and female GRPs, as any novel mutation that decreased gametic compatibility would not be favored in these species (Fig. 1A). Alternately, when sperm are saturating, the female protein may evolve neutrally, while the male protein is under positive selection to match any changes in the female protein if sperm competition is occurring.

When sperm are overly abundant, high levels of sperm competition can result in polyspermy and potentially sexual conflict. Sexual conflict is predicted to produce conflicting selection on affinities when male and female interests are not aligned due to increased sperm competition. High sperm concentrations can occur in those species that aggregate at high densities, live in environments where sperm can be easily concentrated, and/or have synchronized spawning behaviors in general (Franke *et al.*, 2002; Levitan, 2004; Levitan *et al.*, 2004). Species that are found in tidepools and low tidal zones can be routinely subjected to high levels of sperm competition, as sperm can become trapped in the rocky pools causing polyspermy rates as high as 62% (Franke *et al.*, 2002). Sperm competition and sexual conflict may result in GRP

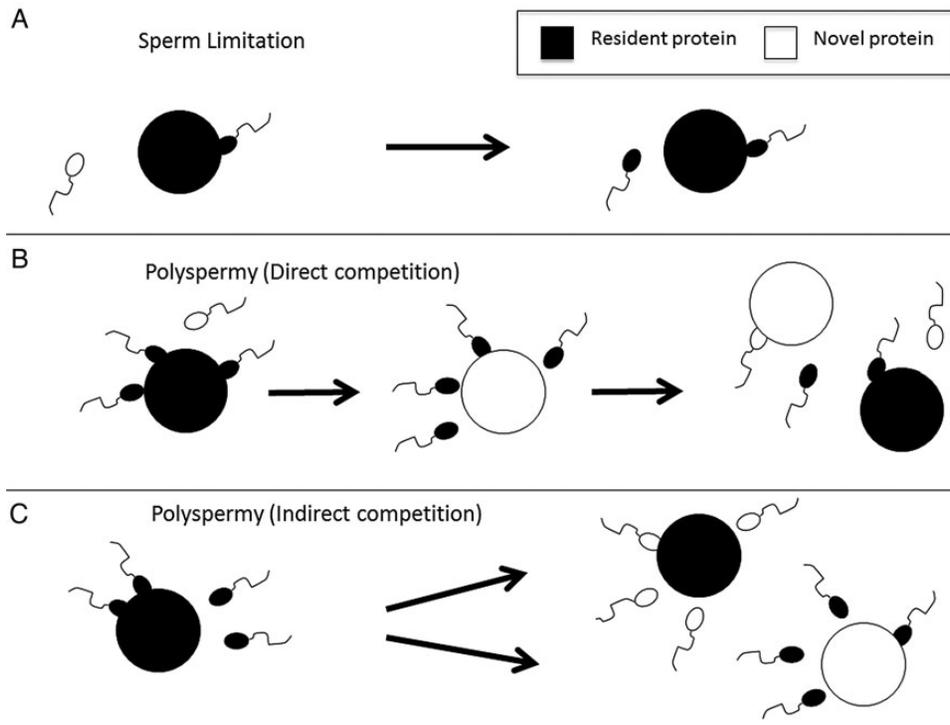


Figure 1 Cartoon of gamete recognition proteins (GRP) evolution as a function of patterns of sperm availability. Black symbols represent common resident protein and open symbols represent a novel mutation with lower gamete affinities. Arrows represent evolutionary transitions. **(A)** Under sperm-limited conditions, novel mutations that lower gamete compatibility in either eggs or sperm recognition proteins would be selected out of the population. **(B)** When sperm availability is high and there is a risk of developmental failure caused by multiple sperm fusions (polyspermy) and multiple males directly compete for the same eggs, then there is sexual conflict over gamete affinities. Females are selected to reduce compatibilities while males are always selected to produce high affinity gametes because they are directly competing for fertilizations. Under these conditions, a mutation to the egg surface proteins that lowers compatibility and the risk of polyspermy would be favored. Once these new egg proteins are common in the population, then a mutation to the sperm protein that matches this new egg receptor will be favored and can increase in frequency. **(C)** When sperm from only single males surround single eggs, and the risk of polyspermy is high, then a mutation to either the sperm or egg protein that lowers gamete compatibility and reduces the risk of polyspermy would be favored. In this scenario, sexual conflict has been reduced because in the absence of competing males, the cost of polyspermy is more equally shared by males and females. Under these conditions, selection only favors a decrease in compatibility and not necessarily well-matched sperm and egg proteins. Formal modeling can be found in Tomaiuolo and Levitan (2010).

diversification (Franke *et al.*, 2002; Levitan, 2004, 2008; Levitan and Ferrell, 2006; Levitan *et al.*, 2007). The rationale for this process is that when a mutation changes the affinity between sperm and eggs it lowers the rate of fertilization because either only a subset of sperm can fertilize the egg or because it takes sperm longer to fuse with eggs. These conditions that slow down the rate of sperm fusion provide more time for an egg to erect a successful block to polyspermy caused by secondary sperm fusions (Frank, 2000). This process of generating polymorphisms in GRPs in sperm and eggs can also lead to assortative mating between the best matching pairs of male and female GRPs. This assortative mating is predicted to generate linkage-disequilibrium between male and female GRP loci (Tomaiuolo and Levitan, 2010). Support for this notion has been found in both sea urchins (Palumbi, 1999; Levitan and Ferrell, 2006; Levitan and Stapper, 2010) and abalone (Clark *et al.*, 2009).

The path and conditions under which male and or female recognition proteins diversify hinge on whether sperm from single or multiple males compete for eggs (Tomaiuolo and Levitan, 2010). The two extreme conditions of sperm–egg interactions under polyspermic conditions

are when sperm from multiple males are well mixed and contact eggs near simultaneously, or when single eggs only face sperm from a single male before a block to polyspermy can be erected (Fig. 1B and C). When sperm from multiple males surround single eggs and males directly compete for fertilizations, then sexual conflict is predicted; females are being bombarded by too many sperm and are selected to avoid polyspermy by lowering gamete affinities. Males on the other hand are selected to produce sperm that bind more rapidly to eggs than their competitors. Although this might result in some degree of polyspermy in a batch of eggs, the male with the highest paternity share produces more offspring than his competitors; there is never an advantage to produce sperm that are out-competed by higher affinity sperm. This produces sexual conflict over fertilization rate. Females are selected to reduced gamete affinities, while males are selected to increase gamete affinities. Sexual conflict favors mutations to eggs that lower compatibility and once these novel egg proteins are common enough in the population, mutations to the sperm protein that match this new egg protein can also be favored (Fig. 1B). This conflict can produce GRP polymorphisms in first the egg and then the sperm (Gavrilets, 2000; Gavrilets and

Waxman, 2002; Haygood, 2004; Hayashi *et al.*, 2007; Tomaiuolo and Levitan, 2010). This process, under a restricted set of conditions, can also result in a polymorphism in the egg protein and a single sperm protein with intermediate compatibility with both egg proteins (Gavrilets and Waxman, 2002). This last scenario appears to be a transient state, as any asymmetries in the level of compatibility between the sperm and the two egg partners will lead to a polymorphic state in the GRPs in both sexes (Gavrilets and Waxman, 2002).

The other extreme in which polyspermy is a risk, is when eggs encounter sperm from only single males. Under this scenario sexual conflict is reduced because developmental failure caused by polyspermy reduces offspring production in both sexes. In such cases, mutations to either the sperm or egg protein that lower the risk of polyspermy would be favored (Fig. 1C). Depending on the degree of sperm availability and the compatibility of the novel GRPs, this can lead to the maintenance of GRP polymorphism through negative frequency-dependent selection or selection only favoring the lower compatibility GRPs male–female pairings. Thus, in the absence of among-male competition, mutations that produce matches between sperm and egg GRPs are not required, only mutations that lower gamete affinities and the probability of polyspermy (Tomaiuolo and Levitan, 2010). Most likely, these two extremes of sperm from multiple males arriving simultaneously or not represent ends in a continuum, and the distribution of these types of gamete encounters produce a complex pattern of selection on sperm and egg proteins (Tomaiuolo and Levitan, 2010).

Patterns of adaptive evolution in GRPs and gametic compatibility

Signatures of positive selection in GRPs among species

Tests for reinforcement as a mechanism for GRP divergence have typically been limited to comparing signatures of positive selection in GRPs among species, with the idea that species in sympatry, where hybrid fertilization is possible, will have stronger signals for positive selection. These studies have typically yielded conflicting results, with some species showing the expected strong signal of positive selection in sympatry and others showing equally high positive selection in allopatry (McCartney and Lessios, 2004; Clark *et al.*, 2007; Lessios, 2007, 2011; Slaughter *et al.*, 2008; Vacquier and Swanson, 2011). Perhaps the most comprehensive interspecific study on male GRP divergence looked for signatures of positive selection based on the presence of sympatric species in eight urchin genera (Palumbi and Lessios, 2005). As predicted, they did find a correlation between sympatry and GRP divergence, however, increased GRP divergence among species was also characterized by high intraspecific polymorphisms, suggesting that the divergence patterns seen may have been driven by intraspecific mechanisms (Palumbi and Lessios, 2005). Other studies in sea urchins have had somewhat less success in linking GRP divergence with sympatric species. One study comparing sea urchins from the genus *Echinometra* in the Pacific regions found that novel GRP variants were favored only in regions where species overlapped, however, other genera of urchins have shown no pattern of positive selection in regions of sympatry (Zigler and Lessios, 2004; Lessios *et al.*, 2012).

In other taxonomic groups, divergence in the male GRP in mussels is higher in populations with a sympatric species present than in allopatry,

while divergence between two species of tunicates follows no such pattern (Springer and Crespi, 2007; Nydam and Harrison, 2011). In abalones, divergence in GRPs appears to follow the typical pattern of reinforcement, with sympatric species exhibiting positive selection and allopatric species more prone to purifying selection, however, there are some species that exhibit a different pattern (Yang *et al.*, 2000; Clark *et al.*, 2007). For example, the female GRP of the abalone *Haliotis tuberculata* has recently undergone a gene duplication event and both copies appear to be under strong positive selection (Clark *et al.*, 2007). This species is also allopatric, so reinforcement is unlikely to be responsible for the positive selection observed on these female proteins (Clark *et al.*, 2007). Otherwise, female proteins in abalone generally have a high rate of divergence between species, but low intraspecific polymorphisms (Galindo *et al.*, 2002). In one of the few studies to date examining intra- and interspecific polymorphisms in female GRPs in mammals, no difference in selection pressures among populations was found in sympatry versus those found in allopatry, nor was there any evidence of novel GRP variants in the sympatric populations (Turner and Hoekstra, 2008b).

Maintenance of intraspecific polymorphisms in GRPs

As predicted for sexual conflict, species that tend to have strong signals of positive selection and maintain high intraspecific variation in male GRPs are generally those from intertidal areas or those who aggregate in high densities, for example mussels and some subtidal sea urchins, that would be more likely to experience polyspermic conditions (McCartney and Lessios, 2004; Riginos *et al.*, 2006; Levitan and Stapper, 2010; Hart *et al.*, 2012; Hellberg *et al.*, 2012; Sunday and Hart, 2013). Shifts in allele frequencies from one to two common GRPs on sperm were noted in a subtidal sea urchin over a 200-year period, likely associated with an increase in population density (Levitan, 2012). Older urchins conceived at lower population densities had a higher prevalence of the common GRP variant with a high affinity for available eggs, while younger urchins conceived at higher population densities have two equally frequent alleles with the emergence of a rare allele that is more resistant to polyspermy with the available eggs (Levitan, 2012).

Species that lack effective electrical blocks to polyspermy also exhibit high amounts of intraspecific GRP polymorphism. The oyster's electrical block to polyspermy can be slow to develop (Gould and Stephano, 2003; Vacquier and Swanson, 2011), and the male proteins are the most diverse GRPs discovered to date, generating polymorphisms through alternate splicing and recombination, as well as having a high non-synonymous substitution rate (Moy and Vacquier, 2008; Moy *et al.*, 2008). Little is known about the female protein, but it is predicted that male proteins are exhibiting this extreme diversity in response to female protein divergence if sexual conflict is occurring, or in parallel with female divergence under non-competitive conditions of polyspermy.

Few studies have been able to look at the patterns of male and female proteins simultaneously, mainly due to the large size and complexity of female proteins, which typically have many repeated regions further complicating haplotype reconstruction (Swanson and Vacquier, 1997; Hellberg *et al.*, 2012; Hart, 2013). However, the advent of next generation sequencing is making this process easier, and some of the patterns predicted by sexual selection and sexual conflict, such as positive

selection on the female protein and linkage-disequilibrium between male and female proteins, are slowly emerging (Clark *et al.*, 2009; Hellberg *et al.*, 2012; Sunday and Hart, 2013). In a species of abalone, the female protein appears to have diversified into two major clades, while the male protein has not; similar to one of the patterns predicted by sexual conflict (Swanson *et al.*, 2001c; Clark *et al.*, 2009). It remains to be seen whether the male protein has equal compatibility with both female proteins, as predicted by sexual conflict, or whether they are more compatible with only one of the versions. Correlated selection, with both male and female proteins exhibiting similar signatures of positive selection, has been found in some species that aggregate and may also signify that females are under selective pressures to diversify their protein to reduce polyspermy, while males are under selective pressures to increase compatibility with novel female proteins (Hellberg *et al.*, 2012; Sunday and Hart, 2013).

More tantalizing is the possibility that duplicate female genes might be acting to reduce polyspermy by offering false binding sites (Aagaard *et al.*, 2013). In abalone, a gene has been characterized that may be from a distant duplication event of the female GRP gene (Aagaard *et al.*, 2010, 2013). It produces proteins that have similar structural motifs and binding abilities as the currently recognized female sperm receptor, and also has a strong signature of positive selection (Aagaard *et al.*, 2010, 2013). The pattern of selection on these three genes is complex but intriguing. There appears to be a positive correlation in the rates of co-evolution between the male and female protein, as predicted if the female protein is under positive selection to reduce compatibility and male protein to increase it, but a negative correlation in the rates of co-evolution between male protein and the duplicate female protein (Aagaard *et al.*, 2013). This may be because the duplicate female protein acts as a decoy to decrease polyspermy (by binding up excess sperm) when compatibility between the male and female proteins is high, but not when the male protein is somewhat incompatible (Aagaard *et al.*, 2013). This would result in the pattern of positive selection observed, as positive selection would act on the duplicate gene to make it match the male protein when the male protein is not likely to be under positive selection, that is, when it has a high affinity with the original female protein. It would be interesting to see if this type of system, where a decoy female receptor is under positive selection to match the male protein, exists in other species.

Polymorphisms in GRPs and gametic compatibility

Studies examining differences in heterospecific gametic compatibility in individuals from sympatric and allopatric populations have rarely been carried out in conjunction with analyses of GRP polymorphisms (Zigler *et al.*, 2005; Lessios, 2007). Those that have, found a positive correlation between number of non-synonymous substitutions in GRPs and the rank order of gametic incompatibility; although this did not necessarily relate to species overlap, as the most divergent/incompatible species tended to be allopatric (McCartney and Lessios, 2004; Slaughter *et al.*, 2008). Thus, it appears that GRP divergence is a better predictor of heterospecific gametic incompatibility than overlaps in species ranges (McCartney and Lessios, 2004; Zigler *et al.*, 2005).

In mammals, comparisons of hetero- and conspecific crosses have shown that species tend to favor conspecific sperm (Lambert, 1984; Dean and Nachman, 2009). However, in some cases this advantage

for conspecific sperm disappears when crosses are performed *in vitro*, indicating that suppression of heterospecific sperm binding is mediated by other proteins present in either the female reproductive tract or in the seminal fluid for some species, but in others suppression does occur at the egg (Dean and Nachman, 2009). There is evidence of differing levels of divergence in GRPs among species (Clark *et al.*, 2006; Gasper and Swanson, 2006), and of differences in heterospecific gamete compatibility (Dean and Nachman, 2009), but tests that examine gametic compatibility based on GRP divergence are needed.

Among some non-compatible species, it has been noted that individuals can vary greatly in their ability to block heterospecific sperm, when exposed to sperm from a single heterospecific male (Harper and Hart, 2005; Slaughter *et al.*, 2008; Miranda *et al.*, 2010). Even when exposed to a mixture of heterospecific and conspecific sperm, some females show equivalent compatibilities to these, rather than exhibiting a preference for conspecific sperm (Harper and Hart, 2005; Miranda *et al.*, 2010). It is possible that heterospecific blocks are byproducts of selection for decreased intraspecific compatibility, as species that require low amounts of sperm for conspecific fertilization (i.e. are highly compatible) are also more likely to be fertilized by heterospecific sperm (Levitan, 2002; Fogarty *et al.*, 2012).

Within sea urchin species there is evidence that increased male GRP divergence is correlated with increased gametic compatibility within color morphs (Calderon *et al.*, 2010; Lopes and Ventura, 2012), and that matching GRPs tend to have higher compatibility than non-matching proteins (Palumbi, 1999; Levitan and Ferrell, 2006). However, the relationship between high compatibility and fertilization success appears to be dependent upon sperm concentration, as lower compatibility pairings are favored at higher sperm concentrations, as predicted under polyspermy conditions (Levitan and Ferrell, 2006). Although less is known about diversity in the female protein, the evidence that female as well as male reproductive success can be predicted by sperm recognition genotypes suggest that linkage-disequilibrium between sperm and egg loci can be generated by assortative mating (Levitan and Ferrell, 2006; Calderon *et al.*, 2010; Lopes and Ventura, 2012). Additionally, although there is no direct link between GRP identity and reproductive success and no known link between the genes encoding GRPs and those for color in sea urchins, the fact that GRP divergence is higher between light and dark color morphs, and those color morphs have higher reproductive success when mating within color morph than across color morphs, also indicates that assortative mating may be occurring. These patterns of assortative mating might potentially lead to sympatric speciation (Calderon *et al.*, 2010; Lopes and Ventura, 2012).

Conclusions and future directions

Polymorphisms in the genes encoding GRPs have been shown to affect individual reproductive performance in the laboratory (Palumbi, 1999) and under natural conditions in the sea (Levitan and Ferrell, 2006; Levitan and Stapper, 2010; Levitan, 2012). The amount of polymorphism maintained within species varies widely. Abalone and some sea urchin species possess relatively little variation, while oysters and other sea urchin species can be highly polymorphic (Metz *et al.*, 1998; Moy *et al.*, 2008; Lessios, 2011; Hart *et al.*, 2012). In internal fertilizers, little is known about the amount of variation present within species, as most studies have focused on identifying and documenting positive selection in candidate GRP genes (Swanson *et al.*, 2001a, b; Torgerson *et al.*, 2002; Gasper and Swanson, 2006;

Kelleher *et al.*, 2011), although a study that did compare inter- and intra-specific variation in female GRPs found a high amount of variation within species (Turner and Hoekstra, 2008b). In abalone and some sea urchins, high divergence in GRPs among species is related to the presence of sympatric species, while in other species it is not (Metz *et al.*, 1998; Palumbi and Lessios, 2005; Clark *et al.*, 2007; Geyer and Lessios, 2009). It is unclear if divergence in GRPs arose due to secondary contact between species that initially arose either in allopatry or sympatry, causing reinforcement to occur, or if sympatric species arose due to divergence in GRPs leading to a sympatric speciation event (Gavrilets and Waxman, 2002; Palumbi, 2009). On the other hand, the degree of intra-specific GRP polymorphism maintained within a population appears to be highly correlated to sperm availability and/or population density (Levitan and Stapper, 2010; Pujolar and Pogson, 2011; Levitan, 2012).

Distinguishing between selective forces requires additional information about the relationship between GRP alleles and gametic compatibility. Reinforcement predicts that species from sympatric populations would possess more divergent GRP alleles and be less likely to fuse with heterospecific sperm (Geyer and Palumbi, 2003). Current evidence is mixed, as some species are able to completely block heterospecific fertilizations, while others have individuals that allow conspecific and heterospecific fertilizations equally (McCartney and Lessios, 2004; Lessios, 2007; Miranda *et al.*, 2010). Testing gametic compatibilities based on GRP identity under differing sperm concentrations is the best way to determine if polymorphisms may be maintained through negative frequency-dependent selection mediated by sexual conflict, yet tests such as these have been restricted to a few species within a genus of sea urchins (Levitan and Ferrell, 2006; Levitan and Stapper, 2010; Levitan, 2012). Preliminary results suggest that rare allele advantage under polyspermy conditions may also be present in the tunicate *Gona intestinalis* (Kosman and Levitan, unpublished data), but more systems should be studied to determine the generality of these results.

More information about male and female co-evolution of proteins is needed to illuminate the evolutionary history of GRP divergence; currently our knowledge is restricted to a single species of abalone, although incomplete information regarding possible assortative mating exists for sea stars and the snail *Tegula* (Hellberg *et al.*, 2012; Hart, 2013). While patterns in male–female evolution cannot necessarily distinguish whether inter- or intraspecific processes are occurring, as both generally predict positive selection between male and female proteins, they can shed light on specific processes that may be occurring, for example whether balancing selection may be maintaining female polymorphisms, by trapping males between two alternate alleles, or whether a co-evolutionary chase is occurring (Gavrilets and Waxman, 2002; Civetta, 2003).

Fertilization is a complex process that involves several stages, each of which involves a different suite of proteins. Sperm from external fertilizers must often interact with a jelly coat, an egg coat and the egg membrane itself, each of which has a set of proteins and carbohydrates that can interact with sperm proteins (Moy *et al.*, 1996; Biermann *et al.*, 2004; Clark *et al.*, 2006; Turner and Hoekstra, 2008a, b). Internal fertilizers have an additional level of protein interactions, with proteins present in the seminal fluid and female reproductive tract, many of which are known to be positively selected and can play a role in gametic compatibility (Swanson *et al.*, 2001b, 2004; Clark *et al.*, 2006; Dean and Nachman, 2009; Vicens *et al.*, 2014). Information about other proteins involved in sperm–egg interactions may be needed to fully understand the evolutionary trajectories of the GRPs discussed

here, as they may offer alternate binding sites for excess sperm, as hypothesized in abalone, act as a filter by pre-selecting certain sperm types, or at the very least be under positive selection themselves, offering other loci upon which sexual selection can operate (Aagaard *et al.*, 2010; Pujolar and Pogson, 2011; Vacquier, 2012; Levitan, unpublished data).

In conclusion, examining the prevalence of polymorphism in genes encoding GRPs as well as the functional consequences of those polymorphisms on gametic compatibility in relation to ecological factors, such as sperm concentration, is necessary in understanding the forces that shaped the evolutionary history of these proteins.

Authors' roles

E.T.K. and D.R.L. are currently investigating the role of sperm availability on the evolution of GRPs in ascidians (E.T.K.) and sea urchins (D.R.L.). This review was equally conceived and outlined by both authors. Each author contributed sections of the manuscript that was initially compiled by E.T.K. and revised by D.R.L. The final version was approved by both authors.

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Conflict of interest

None declared.

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