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Rapid evolution of reproductive proteins in abalone and *Drosophila*

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Observations from different taxa, including plants, protozoa, insects and mammals, indicate that proteins involved in reproduction evolve rapidly. Several models of adaptive evolution have been proposed to explain this phenomenon, such as sexual conflict, sexual selection, self versus non-self recognition and pathogen resistance. Here we discuss the potential role of sexual conflict in the rapid evolution of reproductive genes in two different animal systems, abalone (*Haliotis*) and *Drosophila*. In abalone, we reveal how specific interacting sperm–egg proteins were identified and discuss this identification in the light of models for rapid protein evolution and speciation. For *Drosophila*, we describe the genomic approaches taken to identify male accessory gland proteins and female reproductive tract proteins. Patterns of protein evolution from both abalone and *Drosophila* support the predicted patterns of rapid protein evolution driven by sexual conflict. We stress however that other selective pressures may contribute to the rapid evolution that is observed. We conclude that the key to distinguishing between sexual conflict and other mechanisms of protein evolution will be an integration of genetic, experimental and theoretical data.

Keywords: speciation; fertilization; adaptive evolution; sexual conflict; sexual selection

1. INTRODUCTION

Sequence data from diverse taxonomic groups reveal that reproductive genes are rapidly changing by adaptive evolution (Swanson & Vacquier 2002*a*,*b*). For example, comparisons of sequence data between *Drosophila* species show that male reproductive genes are evolving faster than non-reproductive proteins (Civetta & Singh 1995; Swanson *et al.* 2001*b*). Mammalian reproductive proteins involved in sperm–egg interactions are also evolving rapidly by adaptive evolution, as seen in a high per cent of divergence at the amino acid level between human and rodent (Makalowski & Boguski 1998; Swanson *et al.* 2001*c*, 2003). Other taxonomic groups that show this pattern are marine invertebrates. In abalone (*Haliotis*), turban snails (*Tegula*) and sea urchins (*Echinometra*), sperm proteins are extremely divergent between species due to adaptive evolution (Lee & Vacquier 1992; Metz *et al.* 1998*a*; Hellberg & Vacquier 1999; Hellberg *et al.* 2000; Yang *et al.* 2000).

The adaptive mechanism for rapid evolution of reproductive proteins has not been directly identified, but potential mechanisms include sexual selection, sexual conflict, immune defence and self versus non-self recognition (Swanson & Vacquier 2002*b*). Given that many reproductive proteins are involved in interactions (either directly or indirectly) with the opposite sex, insight into the evolutionary mechanisms can be gained by studying DNA sequence evolution of both male and female reproductive proteins. Here we will discuss the potential role of sexual conflict in the rapid evolution of reproductive genes in two systems, abalone and *Drosophila*, and discuss new data on female reproductive proteins in *Drosophila* (Swanson *et al.* 2004).

Sexual conflict is involved in any aspect of reproduction where evolutionary interests between males and females diverge (Parker 1979), such as the rate of mating and fertilization, the number of offspring produced from a given mating and the amount of parental investment in offspring (Haygood 2004). One way this occurs is when traits that evolve to benefit male fitness have a pleiotropic effect of causing female harm (Linder & Rice 2005). Females in turn will evolve traits to resist male harm (Rice 1998; Linder & Rice 2005). A potential evolutionary outcome of sexual conflict is a continual coevolutionary chase between the sexes where adaptations in one sex lead to counter-adaptations in the other sex, followed by counter–counter adaptations (Trivers 1972; Dawkins 1976; Parker 1979; Gowaty 1997; Rice & Holland 1997; Rice 1998; Arnqvist & Rowe *et al.* 2003; Chapman *et al.* 2003; Linder & Rice 2005). However, additional regimes distinct from continual coevolution are also predicted from models and simulations of sexual conflict (Gavrilets 2000*a*; Gavrilets & Waxman 2002). A prediction of sexually antagonistic coevolution is that it will facilitate rapid evolutionary change in the underlying male–female genes involved in the conflict, such as genes for sperm–egg proteins, reproductive tract proteins, mating behaviours and morphological traits (Rice 1998). This rapid change could generate divergence between isolated populations and possibly subsequent speciation (Parker & Partridge 1998; Rice 1998; Gavrilets 2000*b*; Gavrilets & Waxman 2002; Rowe *et al.* 2003).

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In the two systems presented here, abalone and *Drosophila*, there has been considerable focus on the role of sexual conflict in the evolution of their reproductive proteins (Swanson & Vacquier 2002*a*). In *Drosophila*, the main male reproductive proteins that have been studied are accessory gland proteins (Acps). Acps are predicted to mediate sexual conflict (Chapman *et al.* 1995, 2003). When Acps are ejaculated into females upon mating they have significant effects on female behaviour and physiology, including oogenesis, ovulation, remating rate and lifespan (Wolfner 1997). Several of these Acp effects are detrimental to female fitness, but beneficial to male fertilization success, potentially resulting in a sexually antagonistic coevolutionary race (Linder & Rice 2005). Consistent with this arms race are DNA analyses that reveal a twofold increase in *Acp* divergence relative to non-reproductive proteins (Swanson *et al.* 2001*b*), and the observation that several Acps studied in detail are rapidly evolving by positive selection (Tsaur & Wu 1997; Tsaur *et al.* 1998; Aguade 1999; Begun *et al.* 2000). What has been missing, until recently, is an analysis of the evolution of female reproductive proteins. To fully understand a process that facilitates coevolution between interacting proteins, we need information on both male and female protein evolution (Chapman *et al.* 2003). Here we will discuss exciting new research on female reproductive proteins (Swanson *et al.* 2004) and comment on what it can tell us about the role of sexual conflict in reproductive protein evolution.

In abalone (*Haliotis*), the potential for sexual conflict to promote rapid evolutionary change in reproductive proteins also exists. This gastropod mollusc is a free-spawning external fertilizer. The dynamics of free spawning sets up an environment for polyspermy (multiple sperm fertilizing a single egg) and sperm competition. The opportunity for polyspermy and sperm competition may be responsible for creating a conflict in fertilization rate between the sexes and can affect the evolution of sperm–egg proteins (Frank 2000). For instance, an increased fertilization rate may be beneficial to sperm if it increases the success in sperm competition. In females, however, where polyspermy can cause death to an egg, changes in the egg proteins that lower the fertilization rate may evolve (Rice & Holland 1997; Haygood 2004). This conflict may result in rapid coevolutionary changes in the sperm and egg proteins of abalone species. DNA analysis has revealed rapid divergence of the sperm acrosomal protein, lysin, which is necessary for egg penetration (Lewis *et al.* 1982; Lee & Vacquier 1992). The female receptor to the male protein has been identified and the evolution in this protein has been able to provide an explanation for the rapid adaptive evolution in lysin, which we discuss in detail below (Swanson & Vacquier 1997, 1998; Galindo *et al.* 2002, 2003).

2. COEVOLUTION OF ABALONE FERTILIZATION PROTEINS

Abalone are large marine molluscs of the genus *Haliotis*. Seven species co-exist in the Eastern Pacific Ocean, with overlapping breeding seasons and habitats (Vacquier & Lee 1993). For example, it is possible to collect *Haliotis rufescens* and *Haliotis corrugata* in the same location and both will have gravid gonads when examined in the laboratory. Since fertilization occurs externally in abalone, there may be an increased potential for hybridization. Investigations into the mechanism that maintains these groups as distinct species may provide clues into the process of speciation.

Although hybrids can be generated in laboratory crosses and are found in the wild at low frequency, the species are distinct in nature and exhibit species-specific fertilization. Sperm from *H. rufescens* fertilize *H. rufescens* eggs much more efficiently than sperm from *H. corrugata*. Species-specific fertilization could result from a variety of steps in the fertilization cascade (Vacquier 1998). First, there is chemotaxis of the sperm to the egg. In abalone, chemotaxis is species-specific with *H. rufescens* attracted to L-tryptophan released from eggs while *H. corrugata* sperm are not (Riffell *et al.* 2002). Once the sperm reach the egg, they encounter the egg vitelline envelope (VE). The VE is a tough, elevated, glycoproteineous envelope that surrounds the egg. Once the sperm contacts the VE, a signal transduction event causes the contents of the acrosome to be released. One of the proteins in the acrosome, lysin, dissolves a hole in the egg by a species-specific non-enzymatic mechanism. The sperm is able to then pass through the VE and fuse with the egg plasma membrane, an event hypothesized to be regulated by the sperm protein sp18 (Swanson & Vacquier 1995). Any or all of these steps could demonstrate species-specificity of sperm–egg interaction. We focus on the dissolution of egg VE by the sperm protein lysin, since it is such a well-characterized event.

Both lysin and egg VEs can be purified in large quantities, which allows for detailed biochemical assays. When lysin is mixed with egg VEs, the VEs are dissolved in a species-specific manner. For example, it takes approximately 7 μg of *H. rufescens* lysin to get 50% dissolution of a mixture of *H. rufescens* egg VEs. To dissolve the *H. rufescens* VEs with lysin from *H. corrugata*, it takes approximately 21 μg of lysin to achieve the same degree of dissolution (Vacquier & Lee 1993). It is important to note that in the heterospecific mixture of lysin and VE, there is complete dissolution, indicating species-specificity is not an all or none phenomenon. Through detailed biochemical analyses (Kresge *et al.* 2001), it was determined that lysin dissolves a hole in VE by competing for hydrogen bonds of tightly intertwined fibrous molecules in the VE. The fibrous molecules have the same dimensions as the purified *v*itelline *e*gg *r*eceptor for *l*ysin (VERL), which was identified through affinity chromatography and density gradient sedimentation assays (Swanson & Vacquier 1997). VERL is a giant glycoprotein of 1.5 million Daltons, with 50% of the mass being carbohydrate. Purified VERL and lysin interact with the same species-specificity as lysin mediated VE dissolution. Having now identified interacting sperm–egg proteins, we can study their coevolution to gain insights into the

molecular basis for the evolution of species-specific fertilization and potentially reproductive isolation (speciation).

The sperm protein lysin is extraordinarily divergent between closely related species (Lee & Vacquier 1992). A striking example of this rapid divergence is the observation that exons evolve up to 15 times faster than introns (Metz *et al*. 1998*b*). In order to determine if the rapid divergence is due to adaptive evolution or relaxed functional constraint, the rate of non-synonymous (amino acid altering; d_N) to synonymous (silent; d_S) substitutions per site was calculated. The neutral theory predicts that the d_N/d_S ratio should equal one (Yang & Bielawski 2000). For lysin, it was determined that the d_N/d_S ratio significantly exceeds one indicating a potential adaptive change in the amino acid sequence. Using methods to identify particular sites subjected to adaptive evolution, the N-terminal and C-terminal were predicted to contain a large number of sites subjected to positive Darwinian selection (Yang *et al*. 2000). By using site-directed mutagenesis to make chimeric lysins between *H. rufescens* and *H. corrugata*, it was demonstrated that these regions regulate species-specificity (Lyon & Vacquier 1999). This demonstrates that comparative analyses of adaptive evolution can provide insight into the functionally important regions of the proteins (figure 1), and further suggests that the proteins' adaptive evolution results in species-specific fertilization. The rapid adaptive evolution of the sperm protein begged the question of whether such extraordinary rates of evolution would be observed in the egg receptor, VERL.

When cloned, egg VERL was discovered to be an enormous, repetitive molecule containing 22 repeats of approximately 153 amino acids (Galindo *et al*. 2002). The repetitive nature was consistent with the previously determined stoichiometry of approximately 60 lysin molecules binding each VERL, indicating each VERL repeat may bind two lysin molecules. Phylogenetic analysis showed that repeats 3–22 were homogenized within the genome, with each repeat being more than 95% identical to other repeats 3–22 in the array (Swanson & Vacquier 1998). The homogenization is due to the random process of unequal crossing over and/or gene conversion acting upon repeats in the genome, and is often referred to as concerted evolution (Elder & Turner 1995). Statistical tests of neutrality showed that this region of the repeat array appears to be under slight purifying selection, with a d_N/d_S ratio not significantly different from one (Swanson & Vacquier 1998). Additional tests utilizing polymorphism data from the C-terminal repeats did not show any departure from equilibrium neutral expectations (Swanson *et al*. 2001*a*). The repetitive nature of the array suggested that the repeats might be redundant and subjected to relaxed purifying selection (Swanson & Vacquier 2002*a*). For example, if a mutation that was suboptimal for lysin–VERL binding arose in one VERL repeat, it may not be selected against since lysin could bind the remaining 21 repeats. By this mechanism, repeats that are suboptimal for lysin–VERL interaction could arise in the population. By chance, through the process of concerted evolution the repeat could be corrected back to wild-type or begin to spread through the repeat array. As the repeat becomes more prevalent, there would be selective pressure on lysin to adapt to the repeat type in order to efficiently compete with other sperm. This hypothesis suggests that lysin is adapting to a neutrally drifting egg receptor. It is reliant upon the idea that there are excess sperm in successful free-spawning events, so that eggs will eventually be fertilized. The adaptive pressure on the sperm is the competition with other sperm to be the first to fertilize the egg, thus the need to interact as efficiently as possible with the egg coat. This model's predictions are similar to predictions of other models of rapid reproductive protein evolution (e.g. sexual conflict and sexual selection), but differ in respect to the lack of adaptive evolution on the female locus.

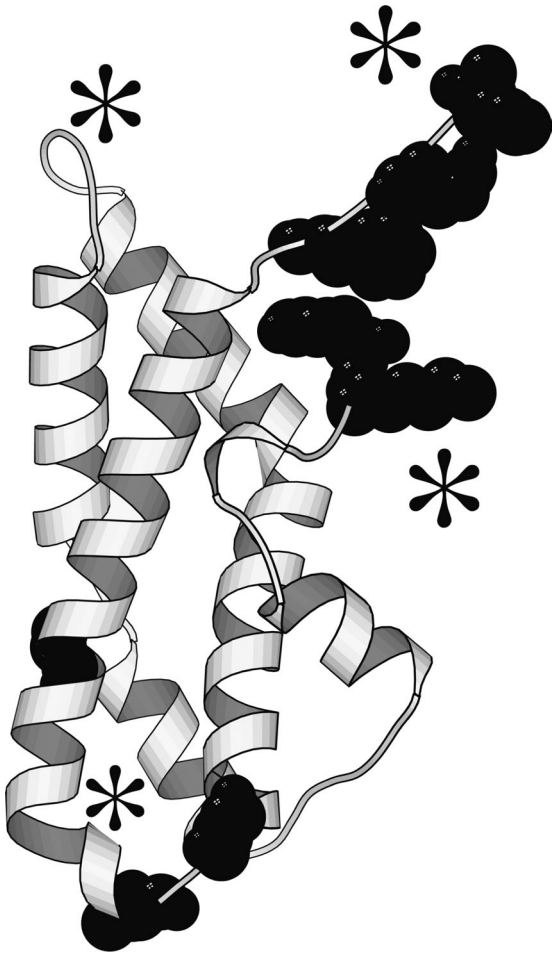

Figure 1. Three-dimensional structure of abalone sperm lysin showing sites predicted to be under adaptive evolution from an analysis of seven species. These sites correspond to regions experimentally demonstrated using site directed mutagenesis to regulate species-specificity (indicated by asterisks).

When the complete VERL gene was sequenced from *H. rufescens*, it became clear that the two N-terminal repeats were distinct from the main array of repeats 3–22. These repeats do not cluster indicating they are not subjected to homogenization through the process of concerted evolution (Galindo *et al*. 2002). To examine the evolutionary rates for this region of VERL, repeats one to three were PCR amplified and sequenced from eight abalone species. While the d_N/d_S ratio averaged across all sites for this region are less

than one, analysis for variation in the d_N/d_S ratio between sites revealed a subset of sites subjected to adaptive evolution with d_N/d_S ratios of 3.3 (Galindo *et al*. 2003). This is the first, and to date only, example of interacting sperm and egg recognition molecules where both components have been shown to be subjected to adaptive evolution as predicted by the sexual conflict theory.

The combined biochemical and evolutionary analyses have generated a hypothesis about the origin of species-specific fertilization in abalone, which could lead to reproductive isolation and speciation. There could be an initial interbreeding population that becomes geographically split, although theory also suggests the following process could occur in sympatry (Gavrilets & Waxman 2002). The egg VERL molecule is subjected to two forces: adaptive evolution of repeats one and two and drift due to the repetitive nature of repeats 3–22. In the different populations, the evolution of VERL could lead to different targets to which lysin must adapt. As the sperm and egg molecule co-evolve over time, species-specific fertilization may arise, and when the two populations come in contact there may be sufficient divergence to generate reproductive isolation.

In this scenario, speciation is the by-product of the coevolution of sperm–egg recognition molecules. The selective pressure driving the divergence of abalone reproductive genes remains unknown. One possibility is a sexual conflict hypothesis involving polyspermy. Polyspermy occurs if more than one sperm fuses with the egg. Eggs have evolved elaborate blocks to polyspermy, including rapid reversal of the egg membrane potential and slower blocks involving destroying the receptors for sperm on the egg surface. It is also possible that eggs are selected to slow down the fertilization process to regulate sperm entry in order to allow time to raise blocks to polyspermy. One method to regulate sperm entry may be to change the receptors for sperm on the egg surface, effectively slowing down the process. However, from the sperm perspective, they would be selected to get through the egg investments as quickly as possible in order to be the fertilizing sperm. This may particularly be the case in free-spawning invertebrates where there are sperm from multiple males competing to fertilize eggs.

If the rapid divergence between species of these reproductive molecules drove speciation, it should be possible to capture incipient speciation events by analyses of polymorphism levels within species at these loci. In an initial survey of 11 *H. corrugata* individuals, a dramatic level of polymorphism at the egg VERL locus was identified (Swanson *et al*. 2001*a*). The sequence of the last VERL repeat and portion of the non-repetitive region showed two very distinct clades, with six amino acid differences and an 11 amino acid indel separating the two clades. It was also striking to observe that all the amino acid differences occurred in the VERL repeat region (which is the presumptive functional unit binding lysin) and none occurred in the non-repetitive region. This observation suggests that we may be observing an incipient speciation event. Current work is aimed at increasing the sample size and genotyping sperm lysin and other non-reproductive loci, with the long-term goal of correlating levels of genetic diversity with different regimes predicted by simulations of sexual conflict (Gavrilets 2000*a*; Gavrilets & Waxman 2002).

The abalone system is a well-characterized system for studying the function and coevolution of reproductive genes. Although the selective pressure driving the divergence of sperm lysin and egg VERL remains a mystery, there are several striking observations that are consistent with mathematical and verbal models of sexual conflict. First, we observe rapid, adaptive evolution of both the male and female reproductive loci (Lee & Vacquier 1992; Galindo *et al*. 2003). Second, there is variation in the rate of lysin evolution between lineages. This observation is consistent with mathematical models suggesting rapid evolution can occur sporadically, with periods of absent or reduced positive selection (Gavrilets 2000*a*). Third, the polymorphism data show a split in the female genotype with two distinct alleles. This is highly reminiscent of the Buridan's ass regime suggested by Gavrilets (Gavrilets & Waxman 2002). In this regime of sexual conflict, females diverge into two types and effectively trap males, who are unable to adapt to both types simultaneously. Over time, the males also split resulting in a sympatric speciation event. Initial analyses of sperm lysin in this population only discovered one allele, but not all exons have been sequenced (Swanson *et al*. 2001*a*). Overall, there is surprisingly good fit between the experimental observations of adaptive evolution in abalone and mathematical models of sexual conflict.

3. IDENTIFICATION AND EVOLUTION OF *DROSOPHILA* REPRODUCTIVE PROTEINS

While the abalone is a fantastic system for biochemistry and comparative analyses, it lacks the power of genetics and experimental manipulation needed for robust tests of the sexual conflict theory. Therefore, we have also pursued studies of reproductive genes in *Drosophila* in order to rigorously test sexual conflict theory. The primary focus has been on the identification of reproductive genes in *Drosophila* and studies of their evolutionary rates. In particular, the focus is on identifying male (Swanson *et al*. 2001*b*) and female reproductive proteins (Swanson *et al*. 2004), with the long-term goal of isolating interacting partners. We briefly describe the approaches for identification of male Acps (Swanson *et al*. 2001*b*) and receptors in the female reproductive tract (Swanson *et al*. 2004).

The male accessory gland produces a majority of the *Drosophila* seminal fluid components, which are transferred along with sperm during mating (Wolfner 1997). The seminal fluid contains many Acps that have several effects on the mated female's physiology, sperm storage and sperm competition (Wolfner 1997). For example, the Acp sex peptide is known to reduce the female's propensity to remate (Soller *et al*. 1997); Acp26Aa is involved in induction of ovulation (Herndon & Wolfner 1995); and Acp36DE has been implicated in sperm storage (Neubaum & Wolfner 1999) and potentially sperm competition (Chapman *et al*. 2000). Additionally, the receipt of seminal fluid appears to reduce female lifespan (Chapman *et al*.

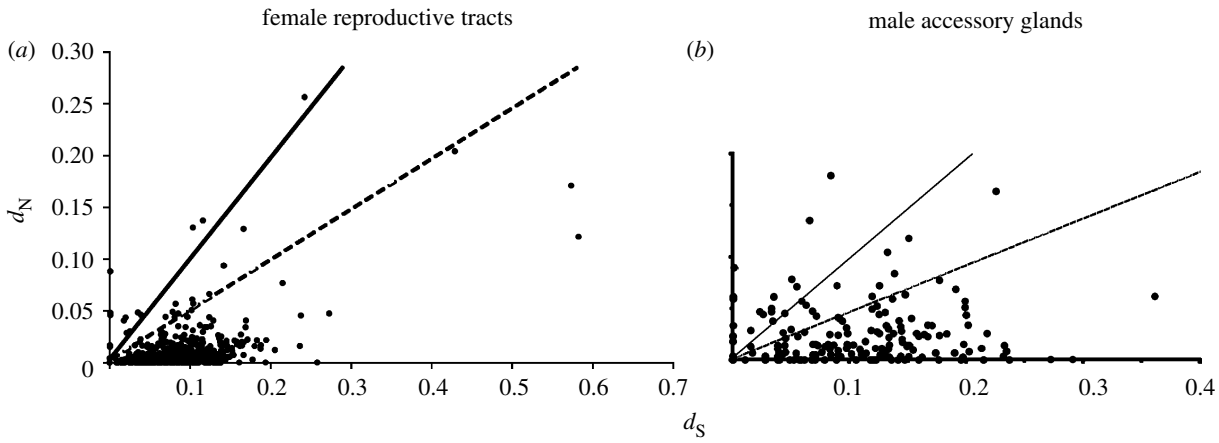

Figure 2. Evolutionary EST analyses from *Drosophila* (*a*) female reproductive tracts (Swanson *et al.* 2004) and (*b*) male accessory glands (Swanson *et al.* 2001*b*). For each coding region identified in the EST, d_N was plotted against d_S. Genes with a d_N/d_S ratio >1 (solid line) or 0.5 (dashed line) are prime candidates to have been recent targets of adaptive evolution. In both male accessory glands and female reproductive tracts, there are several genes that potentially have been subjected to adaptive evolution.

1995), potentially due to the transfer of the protease inhibitor Acp62F (Lung *et al.* 2002). These observations suggest that studies of Acps will be a particularly fertile area of research for studies of sexual conflict.

In order to obtain a genomic view of Acp function and evolution, a study was designed to identify the entire suite of *Acp* genes through analyses of expressed sequence tags (ESTs) (Swanson *et al.* 2001*b*). ESTs are small portions (approx. 200–500 bp) of an entire expressed gene that are useful for identifying unknown genes. Previously, 18 Acps had been identified through differential hybridization studies (DiBenedetto *et al.* 1987; Wolfner *et al.* 1997). Acp numbers were estimated to be approximately 100 through differential cDNA hybridization and two-dimensional protein electrophoresis. To identify the entire suite of Acps, a cDNA library was made from dissected accessory glands and subjected to differential hybridization (Swanson *et al.* 2001*b*). In this differential hybridization step, clones were hybridized to cDNA from both male and female flies. Clones that appeared to be enriched for male-biased expression were selected for sequencing and further study. An additional differential hybridization step was performed comparing hybridization of males with accessory gland to males with accessory glands ablated by expression of diphtheria toxin (DTA males; Kalb *et al.* 1993), this allowed a strict test of accessory gland specific expression as those ESTs that hybridize with wild-type males and not DTA males. Clones that hybridized to cDNA from wild-type males but not DTA males are by definition accessory gland specific. *Acps* were then identified as those ESTs that encoded proteins either containing signal sequences indicating secreted molecules or lost hybridization to cDNA from DTA male flies. By these criteria, 75 potential Acps were identified. By analyses of multiple hit rates, these Acps are estimated to represent 90% of the expressed Acps. Based upon the assumption that the genes will have equal expression, a Poisson distribution is expected for the number of times each gene is recovered in the clones (multiple hit rate). This expectation allows for an estimate of the total number of *Acp* genes expected (see Swanson *et al.* 2001*b* for details). The genes identified encode a variety of molecules with predicted functions consistent with activities known to exist in seminal fluid, such as proteases, protease inhibitors and lipases (Wolfner 1997). Although further refinement of this catalogue of Acps will be important, we are beginning to have a genomic level description of *Drosophila* male Acps.

While the EST screen was instrumental in the identification of male *Acps*, the rates of evolution for these genes is also of interest. Therefore, ESTs were generated from a cDNA library constructed from *Drosophila simulans* (Swanson *et al.* 2001*b*). *Drosophila simulans* is a close relative to *Drosophila melanogaster*, with approximately 11% divergence at synonymous sites (Bauer & Aquadro 1997). This allowed direct comparison of the cDNA coding sequences between the two species in order to calculate d_N/d_S ratios (figure 2). Overall, the Acps showed a twofold increase in d_N compared to non-Acps while d_S was not statistically different between the two categories. The twofold increase in d_N was consistent with previous protein electrophoresis studies (Civetta & Singh 1995); however, this observation by itself does not indicate adaptive evolution (Swanson *et al.* 2001*b*). Several candidates for recent targets of adaptive evolution were identified by virtue of having d_N/d_S ratios >1 (11%), but these need further investigation before it can be concluded that adaptive evolution drives their divergence.

As with abalone sperm lysin, the identification of substantial numbers of male Acps potentially being subjected to positive Darwinian selection begs the question of whether female receptors for the Acps will show similar rates of evolution. In order to initiate studies of the function and evolution of female receptors, an EST approach analogous to the male accessory gland EST project described previously was performed. A cDNA library was made from dissected female reproductive tracts (minus ovaries) and subjected to differential hybridization to enrich for genes with female-biased expression (Swanson *et al.* 2004). Candidate female reproductive genes were identified as

those containing either a signal sequence or transmembrane domains, indicating secreted proteins. By this approach, 169 putative female reproductive genes were identified. These putative receptors are now prime targets for future functional assays to determine if they interact with male Acps. It will be important to generate comparisons with other approaches, such as microarray experiments, to analyse for genes upregulated following mating (Lawniczak & Begun 2004; McGraw *et al.* 2004). Microarray methods have already provided insights into the identification of potential female receptors for Acps.

Like the male accessory gland, the female reproductive tract was an evolutionary EST screen with the ESTs derived from *D. simulans* and compared to the completed *D. melanogaster* genome (Swanson *et al.* 2004). From aligned coding sequences, the d_N/d_S ratio was calculated as a measure of selective pressure (figure 2). From comparison of published examples of adaptive evolution, it was proposed that genes with a d_N/d_S ratio >0.5 were good candidates to have been the target of recent adaptive evolution. When genes from the literature with an overall d_N/d_S ratio >0.5 were analysed by more powerful maximum-likelihood analyses incorporating variation in the d_N/d_S ratio between sites, more than 90% showed robust signatures of adaptive evolution (Swanson *et al.* 2004). This indicates a d_N/d_S ratio >0.5 is a good indicator for targets of recent adaptive evolution, but that additional studies are necessary prior to invoking adaptive evolution. From the female EST screen, 27 genes had a d_N/d_S ratio >0.5, indicating that they may have been the target of recent adaptive evolution. Eight genes were selected for in depth polymorphism and divergence analyses for signatures of adaptive evolution, and six were confirmed to have been the target of recent adaptive evolution (Swanson *et al.* 2004). Thus, as predicted by the sexual conflict theory, genes expressed in both the male and female reproductive tracts have been subjected to adaptive evolution (figure 2). Important future studies will be to identify interacting male–female reproductive molecules.

4. CONCLUSIONS AND FUTURE DIRECTIONS

We have discussed the recent molecular work on the evolution of male and female reproductive proteins in two different animal systems, abalone and *Drosophila*. The abalone system is the first to show that female–male interacting gametic proteins are rapidly evolving. The mechanism behind this rapid evolution remains unknown; however, we know that both proteins show signs of change by adaptive evolution (in partial regions of VERL). In *Drosophila*, we are making important strides toward identifying female reproductive proteins. Similar to male reproductive proteins, female proteins also are rapidly evolving by Darwinian selection. This molecular work is an important first step for investigating the coevolution of interacting male–female reproductive proteins. Patterns of protein evolution from both systems support the predicted patterns of rapid protein evolution driven by sexual conflict. We stress, however, that other selective pressures may contribute to the rapid evolution we observe in the reproductive proteins of abalone and *Drosophila*. For instance, models of sexual selection, pathogen resistance and self versus non-self recognition make similar predictions as sexual conflict (Chapman *et al.* 2003). The key to distinguishing between sexual conflict and other mechanisms will be an integration of genetic, experimental and theoretical data. An integrative approach will help provide a link between genotype and phenotype of male and female reproductive molecules. Correlations of genotype frequencies and predictions from theoretical models will also provide support for models of sexual conflict.

For genetic data we need to identify interacting male and female reproductive proteins (as has been done for abalone egg VERL and sperm lysin) from several taxonomic groups. This data will allow us to determine if female–male coevolution supports or contradicts models of sexual conflict. For example, in *Drosophila*, under predictions of sexual conflict, female receptors to male Acps involved in mediating costs to females should be rapidly evolving. Functional studies have identified that the male reproductive protein Acp62F is a candidate for influencing the cost of mating in females (Lung *et al.* 2002). When the female receptor to Acp62F is identified we will be able to determine if it shows signs of rapid evolution as would be predicted by sexual conflict.

Theoretical predictions about the rates and patterns of variability in reproductive genes can provide clues into the identification of new genes involved in reproduction. An example where prediction of variability was useful in the experimental identification of new reproductive genes is in plant species with self-incompatibility. Self-incompatibility in plants probably evolved to prevent the costs associated with inbreeding depression that can arise from self-fertilization. Self-incompatible genes are predicted to be subjected to adaptive evolution, have high levels of polymorphism and the male and female loci should be tightly linked (Nasrallah 2002). This pattern of molecular evolution has been characterized for self-incompatible loci in several plant genera, including Brassicaceae and Solanaceae (Schopfer *et al.* 1999; Richman & Kohn 2000). In Brassicaceae, combining the predicted molecular properties of these loci with experimental work has proven useful for identifying and characterizing the self-incompatible loci for both the male pollen and female stigma proteins (Kachroo *et al.* 2001).

For experimental tests of sexual conflict, it will be informative to include genetic analyses of reproductive genes. In many experimental tests, it is often indirectly assumed that genes, such as those encoding Acps in *Drosophila*, have responded during the experimental procedure. For example, in one of the original laboratory experiments demonstrating sexual conflict, *Drosophila* Acps became more toxic upon adaptation to a non-responding female genotype (Rice 1996). Now that several male Acps and potential female reproductive molecules have been identified, it will be relatively easy to genotype these loci during the course of experimental evolution studies and shed light on the role of sexual conflict in protein evolution.

With the emergence of data on the rapid evolution of reproductive genes from many different taxa, including

ciliate protozoa, green algae, fungi, plants, insects, marine invertebrates and mammals (see review in Swanson & Vacquier 2002*a*), we now have a good starting place for experimental and functional tests of reproductive genes which will be useful for identifying the adaptive mechanisms involved in rapid reproductive protein evolution. Continued integration of genetic, experimental and theoretical studies of sexual conflict will lead to a robust understanding of sexual conflict and the evolutionary implications.

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