Causes and consequences of the evolution of reproductive proteins

LESLIE M. TURNER* and HOPI E. HOEKSTRA

Department of Organismic and Evolutionary Biology and The Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA

ABSTRACT **Proteins involved in reproduction often evolve rapidly, raising the possibility that changes in these proteins contribute to reproductive isolation between species. We review the evidence for rapid and adaptive change in reproductive proteins in animals, focusing on studies in recently diverged vertebrates. We identify common patterns and point out promising directions for future research. In particular, we highlight the ways that integrating the different but complementary approaches of evolutionary and developmental biology will provide new insights into fertilization processes.**

KEY WORDS: *evo-devo, gamete, positive selection, reproductive isolation, fertilization proteins, speciation*

Introduction

While the burgeoning field of "evo-devo" has formally united the study of evolution and development, the historical directions and approaches of these two fields have been distinct. A primary goal of evolutionary biology is to explain how genetic variation arises and is maintained in nature. While variation initially arises within populations, it is the variation between diverging populations that fuels speciation, and ultimately biological diversity. In contrast, developmental biology is concerned with the mechanisms by which an individual organism takes shape, processes that are generally highly conserved not only within species but often among widely divergent taxa. The study of fertilization, however, unites these two fields: traits associated with fertilization, a first and critical step in development common to all sexually reproducing organisms, are often remarkably variable between species and sometimes even within species. Consequently, fertilization is a process that is of common interest to both evolutionary and developmental biologists.

One of the most striking patterns observed in natural populations of animals is the rapid evolution of fertilization proteins. Although some of the earliest and most dramatic examples of rapid change in reproductive proteins were identified through studies of fertilization by developmental biologists (e.g., Lee and Vacquier, 1992), this pattern and its potential consequences have been embraced by evolutionary biologists. This is, in part, because rapid evolution of reproductive proteins can make an important contribution to reproductive isolation between diverging taxa, and under-

standing reproductive isolation is the key to understanding speciation (Coyne and Orr, 2004). While rapid evolution and positive selection of reproductive proteins have been seen in many groups of animals and plants (Singh and Kulathinal, 2000; Swanson and Vacquier, 2002b; Clark *et al.*, 2006), in most cases, the functional consequences of amino acid changes on fertilization potential are unknown. Tools used by developmental biologists provide the means to determine the precise effects of amino acid mutations on protein interactions, egg-sperm binding and fertilization success. Conversely, evolutionary analyses can provide new insights into the mechanics of fertilization. For example, sequence analyses can identify rapidly evolving protein regions that may be important in critical interactions during fertilization.

The earliest evidence for rapid evolution of reproductive proteins came from studies of abalones and sea urchins (reviewed in Kresge *et al.*, 2001; Zigler *et al.*, 2005; see article by Zigler in this issue). Additional work on reproductive protein evolution has since accumulated in diverse taxa. Evolutionary sequence analyses in insects (primarily *Drosophila*) and vertebrates have shown adaptive evolution of functionally diverse reproductive proteins. The molecular-evolution approaches commonly applied to detect adaptive evolution in vertebrates are most statistically powerful when widely divergent sequences are compared; therefore studies of vertebrate reproductive proteins within groups of closely related

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***Address correspondence to:** Leslie M. Turner. Department of Evolutionary Genetics, Max Planck Institute for Evolutionary Biology, August Thienemann Str. 2, 24306 Ploen, Germany. Fax: +49 45022-763287. e-mail: turner@evolbio.mpg.de

TABLE 1

STUDIES OF REPRODUCTIVE PROTEIN EVOLUTION IN CLOSELY RELATED SPECIES

Taxon	Protein	Function	Result	Reference
Marine invertebrates				
sea urchins (*Echinometra*)	bindin (S)	egg vitelline-envelope (VE) binding	HP, BS	(Lessios and Cunningham, 1990; McCartney and Lessios, 2004)
sea urchins (*Echinometra*)	bindin (S)	VE binding	R, P, HP, RE, SD, GI	(Metz *et al.*, 1994; Metz and Palumbi, 1996; Palumbi, 1999; Geyer and Palumbi, 2003; Geyer and Palumbi, 2005)
sea urchins (*Strongylocentrotus*)	bindin (S)	VE binding	R, P, GI, HP, DD, SD	(Biermann, 1998; Levitan, 2002; Levitan and Ferrell, 2006; Levitan *et al.*, 2007)
sea urchins (*Heliocidaris*)	bindin (S)	VE binding	R, P, GI	(Zigler *et al.*, 2003)
sea urchins (*Strongylocentrotus*)	EBR1 (E)	sperm binding	R, L, M	(Kamei and Glabe, 2003)
sea urchins (*Strongylocentrotus*)	suREJ1, suREJ2, suREJ3 (S)	egg-jelly binding, unknown, acrosome reaction signal transduction?	R, P	(Mah *et al.*, 2005)
abalones (*Haliotis*)	lysin (S)	VE dissolution	R, P, GI, M, MA	(Lee and Vacquier, 1992; Lee *et al.*, 1995; Lyon and Vacquier, 1999; Yang *et al.*, 2000; Yang and Swanson, 2002)
abalones (*Haliotis*)	sp18 (S)	sperm-egg cell membrane fusion?	R, P	(Swanson and Vacquier, 1995)
abalones (*Haliotis*)	VERL (E)	lysin-binding	R, P	(Swanson and Vacquier, 1998; Galindo *et al.*, 2003)
turban snails (*Tegula*)	lysin (S)	VE dissolution	R, P, M	(Hellberg and Vacquier, 1999)
turban snails (*Tegula*)	TMAP (S)	unknown	R, P, L	(Hellberg *et al.*, 2000)
mussels (*Mytilus*)	M7 lysin (S)	VE dissolution	R, P, BA[1], RE[2]	(Riginos and McDonald, 2003; Riginos *et al.*, 2006)[1] (Springer and Crespi, 2007)[2]
Insects				
fruit flies (12 species from *melanogaster* and *virilis* species groups)	unidentified proteins from ovary and testis (M,F)	unknown	R	(Civetta and Singh, 1995)
D. melanogaster and *D. simulans*	Acps (M)	sperm competition, sperm storage, stimulates ovulation in females, stimulates female immune system, decreased longevity in females	R, P, MA	(reviewed in Panhuis *et al.*, 2006; Ram and Wolfner, 2007)
D. pseudoobscura	Acp26Aa (aka ovulin) (M)	stimulates ovulation in females	R, P	(Wagstaff and Begun, 2005a)
D. pseudoobscura	Acps (M)	sperm competition, sperm storage, stimulates ovulation in females, stimulates female immune system, decreased longevity in females	R, P, L	(Schully and Hellberg, 2006)
D. mojavensis and *D. arizonae*	female reproductive tract proteins (F)	proteases, unknown	R, D, P	(Kelleher *et al.*, 2007)
field crickets (*Gryllus*)	seminal proteins (M)	unknown	R, P	(Andres *et al.*, 2006)
ground crickets and field crickets (*Allonemobius, Gryllus*)	seminal proteins (M)	unknown	R, P	(Braswell *et al.*, 2006)
Rodents				
mice (*Mus*), rats (*Rattus*)	RHOX5 (aka PEM, M,F)	transcription factor, regulates testis and ovary genes - affects sperm production, sperm motility, fertility	R, P	(Sutton and Wilkinson, 1997; MacLean *et al.*, 2005)
mice (*Mus*)	ZP3 (E)	zona pellucida (ZP) structure, sperm binding	R, P	(Jansa *et al.*, 2003)
mice (*Mus*), murine rodents	CATSPER1 (S)	sperm motility	R, P, L	
Australasian murine rodents	ZP3 (E)	sperm binding	R, P	(Swann *et al.*, 2007)
deer mice (*Peromyscus*)	ZP2, ZP3 (E)	ZP structure, sperm binding	R, P	(Turner and Hoekstra, 2006)
pinyon mouse (I, *Peromyscus truei*) and Osgood's mouse (*P. gratus*)	ZP3 (E)	ZP structure, sperm binding	HP, BS	(Turner and Hoekstra, 2008) (Podlaha *et al.*, 2005)
deer mice (*Peromyscus*)	LRRC50 (M); GM1276 (M); ACR (S); DDC8 (M); GSG1 (M)	protein binding; receptor activity, signal transduction; ZP binding, dispersal of acrosomal contents; unknown; unknown	R, P	(Turner *et al.*, in press)
Primates				
humans and great apes	PRM1 (S), PRM2 (S), TNP2 (M)	DNA packaging in sperm	R, P	(Wyckoff *et al.*, 2000)
great apes	SEMG1, SEMG2 (M)	copulatory plug formation	R, L	(Jensen-Seaman and Li, 2003)
humans (I, *Homo sapiens*), chimpanzees (I, *Pan troglodytes*), gorillas (I, *Gorilla gorilla*)	SEMG1 (M)	copulatory plug formation	R, P	(Kingan *et al.*, 2003)
humans (I), primates	zonadhesin (S)	ZP binding	R, P, BS	(Gasper and Swanson, 2006)
humans (I), primates	PKDREJ (S)	ZP binding?	R, P, BS	(Hamm *et al.*, 2007)
humans (I), primates	ESX1 (M)	spermatogenesis	R, P	(Wang and Zhang, 2007)
humans (I), chimpanzee	NYD-SP12 (M)	spermatogenesis	R, P, HP	(Zhang *et al.*, 2007)

Taxon - common and scientific taxon names are given, (I) indicates intraspecific comparison. *Protein* – location of protein is given in parentheses (E = egg; S = sperm; F = female, other or unknown; M = male, other or unknown). *Function* – question marks indicate evidence for function is preliminary or suggestive. *Result* – R = rapid evolution, P = positive selection, D = gene duplication, L = length variation, BS = balancing selection, RE = reinforcement, DD = density-dependent selection, HP = high polymorphism, SD = sperm discrimination by females, GI = gametic incompatibility (low interspecific fertilization rates and/or conspecific sperm precedence), M = molecular evidence (*in vitro* functional assays), MA = mutational analysis (site-directed mutagenesis, chimeric proteins, and/or deletions); superscripts indicate conflicting results and number corresponds to reference superscript. See also Herlyn and Zischler, 2008.

species, though important in assessing the contribution of these proteins to speciation, have been lacking until recently.

Here we focus on recent advances in understanding reproductive protein evolution within and between closely related species. We first give a brief overview of the selective forces that may cause rapid reproductive protein evolution and discuss the potential contribution of reproductive proteins to speciation. We then review patterns of change observed in reproductive proteins across animals. Finally, focusing on mammals, we synthesize recent results, identify some common patterns, and suggest future directions. Throughout this review, we concentrate on the ways in which evolutionary and developmental approaches are complementary and how their integration is essential to a comprehensive understanding of fertilization.

The causes of rapid reproductive protein evolution

An obvious first question is *why* reproductive proteins evolve rapidly. Many evolutionary forces have been implicated, including pathogen resistance, sperm competition, cryptic female choice, sexual conflict, reinforcement, and avoidance of heterospecific fertilization. We describe these forces only briefly as they are explained more fully in previous reviews (Howard, 1999; Swanson and Vacquier, 2002a; Clark *et al.*, 2006).

Selection can act on reproductive proteins at several points during fertilization. To achieve fertilization, sperm and eggs must come in contact with foreign molecules (the gametes of the opposite sex); this, along with necessary exposure to a novel environment (e.g., sperm traveling through the female reproductive tract) makes gametes vulnerable to attack from microbial pathogens. Egg and sperm proteins may thus be selected to evade and resist these attacks independently of any evolutionary forces promoting fertilization success. Selection can also act on gamete interactions themselves. First, selection may arise from interactions between conspecific individuals. In polyandrous species, in which a female mates with multiple males, females may discriminate between sperm from different males, exerting "cryptic choice". In addition, intrasexual competition between the sperm of different males to fertilize the egg can cause strong selection on sperm to rapidly penetrate and fertilize the egg. Females, however, may prefer a lower optimal rate of fertilization than males to reduce the risk of polyspermy. This difference between sexes in optimal fertilization rates can yield sexual conflict at the gamete level. This conflict may result in maintenance of polymorphism or an endless coevolutionary arms race between egg and sperm proteins (Palumbi, 1999; Gavrilets, 2000). Second, selection may arise from heterospecific interactions. If gametes from different species meet and hybrids have reduced fitness, selection will favor changes in protein interactions that reduce heterospecific fertilization, as this is a waste of reproductive effort. The various types of selection that can act on reproductive proteins are difficult to disentangle and may operate simultaneously in a single species (Coyne and Orr, 2004).

The consequences of rapid reproductive protein evolution

The second question, on which we focus here, is: what are the consequences of reproductive protein evolution – do changes in

reproductive proteins really matter for speciation?

In other words, do changes in the amino acid sequences of reproductive proteins contribute to the initial stages of reproductive isolation between diverging populations? This question is difficult to answer, and requires combining studies of variation in nature with functional molecular assays in the laboratory.

The critical element is one of timing; *when* reproductive proteins change relative to other factors promoting divergence (e.g., ecological specialization) determines whether reproductive genes are important in incipient speciation. Specifically, we want to know if changes in reproductive proteins *cause* speciation, or if they simply accumulate after species have already diverged. Figure 1 depicts a simple model of speciation in which gametic incompatibility arises as a result of rapid evolution of gamete interaction proteins in two physically separated populations. In contrast, if reproductive protein divergence occurs after populations are completely reproductively isolated due to other mechanisms (e.g. mate choice, habitat preference, timing), it has no consequences for fertilization potential.

While many studies have begun to shed light on either the
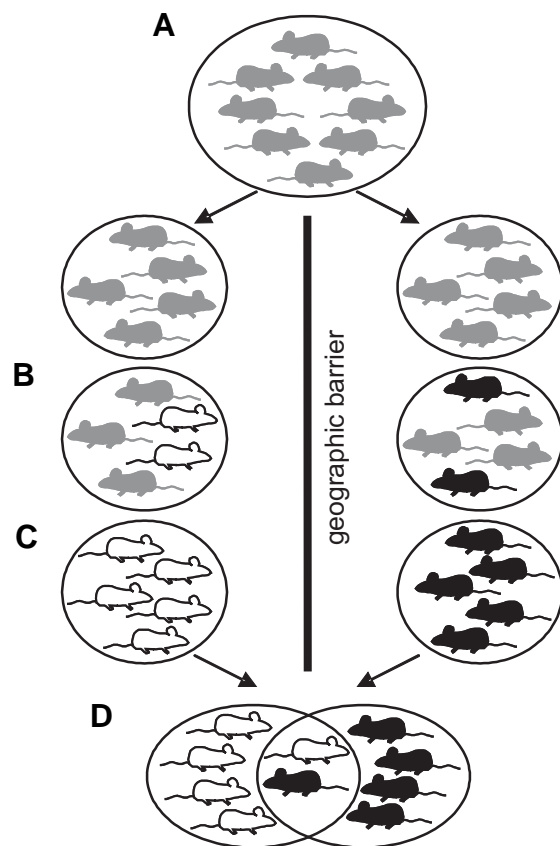
Fig. 1. A simple model of reproductive isolation arising from gametic incompatibility. (A) *A single population splits into two by a geographic barrier.* **(B)** *A new sperm gene allele arises in one population (indicated in black) and a new egg gene allele arises in the other (indicated in white).* **(C)** *The new alleles replace the old alleles in each population.* **(D)** *The populations come into contact. When individuals from different populations mate, eggs and sperm are no longer compatible. This gametic incompatibility results in reproductive isolation between the populations (indicated by a solid line around each).*

evolutionary patterns of reproductive proteins or on the molecular mechanisms of fertilization, there are no systems in which the complete story has been told. We highlight below some of the most thorough studies, emphasizing how evolutionary and developmental approaches can inform one another to illuminate the causes and consequences of rapid reproductive protein evolution.

Marine invertebrates

The earliest studies of fertilization protein evolution involved marine invertebrates. These species have long been used as models for fertilization research because their gametes are copious and easily collected (Kresge *et al.*, 2001). In addition, because fertilization occurs externally — gametes are simply released into the water — fertilization success is primarily mediated by gamete proteins. In contrast, internal fertilization involves complex interactions between many proteins present in the sperm and semen of males and the egg and reproductive tract of females. Consequently, selection can act on many different proteins and at many different times, even before mating, to control which individuals exchange gametes (e.g., selection on behaviors to avoid heterospecific fertilization or to choose the best mate). Thus, gamete proteins in externally fertilizing species are obvious targets of selection because they mediate a large portion of the fertilization process compared to internally fertilizing species. In fact, rapid evolution and positive selection of fertilization proteins have been documented in several taxa of marine invertebrates including abalones, sea urchins, turban snails, and mussels (Table 1). Here, we highlight studies in abalones and sea urchins in which the molecular function of gamete-interaction proteins and their potential role in reproductive isolation are particularly well understood.

Gamete interaction in abalones

The interaction of the gamete proteins lysin and VERL in abalones (genus *Haliotis*) is arguably the best characterized fertilization process in animals (reviewed in Kresge *et al.*, 2001; Panhuis *et al.*, 2006). The abalone fertilization process was first studied by developmental biologists in the lab. To fertilize an egg, an abalone sperm must pass through a layer of egg jelly until it reaches the vitelline envelope (VE). Contact with the VE stimulates release of the contents of the acrosome, which disassociates fibers of the VE and allows the sperm to pass through to the egg cytoplasmic membrane (Fig. 2A,B). Isolation of the contents of the acrosome revealed two major protein components. One of these was named "lysin" because of its ability to non-enzymatically dissolve, or lyse, the VE (Lewis *et al.*, 1982). The egg protein that physically interacts with lysin was later identified and named VERL (vitelline envelope receptor for lysin; Swanson and Vacquier, 1997). VERL is a large glycoprotein composed of 22 tandem repeats, each of which binds a lysin dimer (Fig. 2E, Swanson and Vacquier, 1997; Galindo *et al.*, 2002). Binding of lysin to VERL causes the molecules of VERL to disentangle, creating a hole in the VE through which the sperm can swim.

In abalones, egg-sperm binding appears species-specific. *In vitro* hybridization experiments have shown that optimal sperm concentrations are ten times higher and maximal fertilization rates much lower for heterospecific than for conspecific sperm (Leighton and Lewis, 1982). Moreover, dissolution of the VE by purified lysin and, to a lesser extent, *in vitro* binding of lysin and VERL, show the same species specificity as does egg-sperm binding (Swanson and Vacquier, 1997; Lyon and Vacquier, 1999), demonstrating that the interaction of these two proteins is the species-specific step of fertilization in abalones.

Comparison of amino acid sequences between closely related abalone species revealed that lysin evolves remarkably rapidly. Lysin is one of the rare molecules in which a strong signal of positive selection is observed: the amino acid substitution rate exceeds the synonymous substitution rate ($d_N/d_S > 1$; Lee and Vacquier, 1992; Lee *et al.*, 1995; Nunney and Schuenzel, 2006). In fact, lysin is one of the most rapidly evolving proteins ever seen (Metz *et al.*, 1998b). However, only the first two repeats of VERL are subject to positive selection (Fig. 2E), suggesting this part of the protein could be coevolving with lysin (Galindo *et al.*, 2003). [In contrast, repeats 3-22 of VERL evolve neutrally; concerted evolution probably homogenizes most of these repeat sequences within a given species (Swanson and Vacquier 1998).] One possible scenario is that coevolution results from conflict over fertilization rate; VERL evolves to decrease sperm-binding rates and avoid polyspermy, while lysin is selected to increase binding speed in response, leading to an ongoing "coevolutionary chase" (Galindo *et al.*, 2003). Why then does diversifying selection act on repeats 1-2 but not 3-22? One explanation is that lysin could have different binding affinities for different repeated regions of VERL. We know species specificity is strongest as VE dissolution is initiated (Swanson and Vacquier, 1997), so perhaps lysin binds in a species-specific manner to repeats 1 and 2, causing VERL molecules to begin to unravel, and then bind with less specificity to repeats 3-22 (Fig. 2F; Galindo *et al.*, 2003).

Evolutionary analysis provided hints about which regions of lysin interact with VERL. Amino acid divergence varies between different regions of lysin – some are conserved between species while others are highly variable. The divergent regions are strong candidates for mediating species specificity of binding. Molecular analyses were used to test this hypothesis; variable regions of lysin from red abalone and pink abalone were exchanged via site-directed mutagenesis to produce chimeric proteins (Fig. 2C; Lyon and Vacquier, 1999). Differences in the ability of these chimeric proteins to dissolve VEs from the two abalone species showed that the N- and C-termini and one internal region are major determinants of species specificity (Fig. 2D). The crystal structure of lysin from two species of abalone has been determined, allowing visualization of the relative positions of these regions; the N- and C-termini are adjacent to each other on one side of the folded protein, suggesting that these two domains constitute the major site where lysin binds to VERL (Shaw *et al.*, 1993; Shaw *et al.*, 1995; Kresge *et al.*, 2000a,b). This series of evolutionary and molecular studies clearly have shown that gamete proteins have the potential to play a prominent role in maintaining species boundaries in abalones.

Bindin evolution in sea urchins

Studies in sea urchins provide further insight into the evolutionary consequences of gametic interactions in external fertilizers. The egg-binding protein bindin has received the most attention,
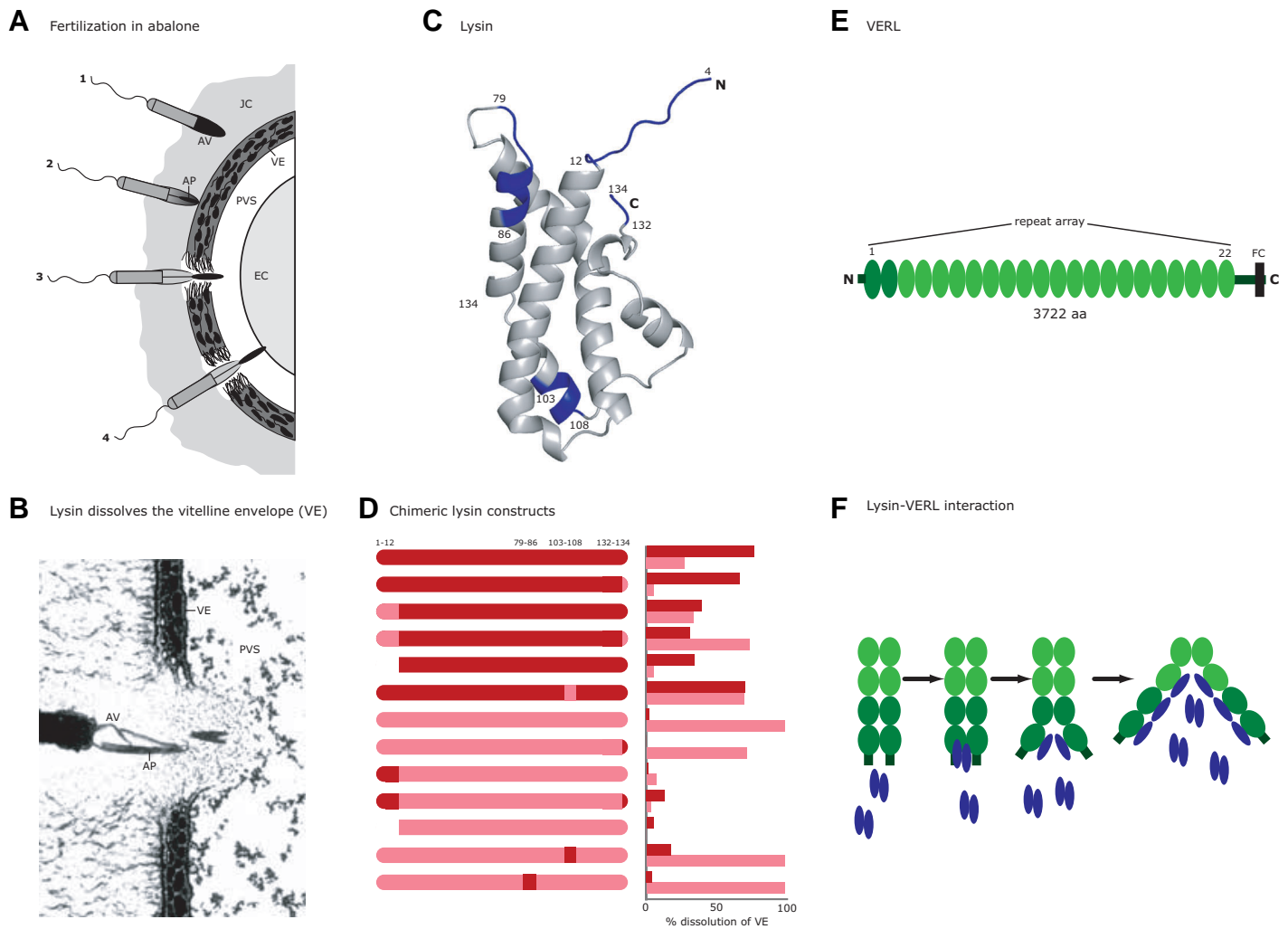
Fig. 2. Interaction of lysin and "vitelline envelope receptor for lysin" (VERL) during abalone fertilization. (A) *Steps of fertilization in abalone (adapted from Kresge* et al., *2001). (1) The sperm penetrates the egg jelly coat (JC). (2) The sperm comes into contact with the egg vitelline envelope (VE), which stimulates release of the contents of the acrosomal vesicle (AV) including lysin and elongation of the acrosomal process (AP). Lysin dissolves the VE, causing a hole to form. (3) The sperm passes through the hole in the VE into the perivitelline space (PVS). (4) The membrane of the acrosomal process fuses with the egg plasma membrane and the sperm enters the egg cytoplasm (EC).* **(B)** *Thin section transmission electron micrograph of a sperm dissolving a hole in the VE (21,400X, used with permission from Lewis* et al., *1982).* **(C)** *Structure of red abalone lysin; regions interchanged between species to produce chimeric proteins are in blue (adapted from Lyon and Vacquier, 1999).* **(D)** *Chimeric lysin constructs tested by Lyon and Vacquier (1999). Graph shows the percent dissolution of red (red bars) and pink (pink bars) abalone VEs by 20 μg of protein after four minutes (adapted from Lyon and Vacquier, 1999).* **(E)** *VERL structure (adapted from Galindo* et al., *2002); repeats subject to positive selection are in dark green, repeats evolving neutrally by concerted evolution are in light green; FC, furin cleavage site.* **(F)** *A model of the molecular interaction between lysin (blue) and VERL (green) (adapted from Kresge* et al., *2001). A lysin dimer binds to a VERL repeat, disrupting hydrogen bonds between VERL molecules and causing lysin to monomerize. As the molecules separate, more repeats are accessible to lysin and the process continues, unraveling the VERL molecules. Binding of lysin to the first two repeats of VERL is predicted to be more species-specific than binding to repeats 3-22 (Galindo* et al., *2003).*

having been studied within and between several sea urchin species representing diverse genera (reviewed in Zigler *et al.*, 2005; see article by Zigler in this issue). Like abalone lysin, bindin diverges rapidly between species and shows strong evidence for positive selection in many (Zigler *et al.*, 2005) but not all genera (e.g. Metz *et al.*, 1998a; Zigler and Lessios, 2003, 2004). Comparative analyses suggest that amino acid changes in bindin may play an important role in reproductive isolation in sea urchins; divergence at nonsynonymous sites in bindin correlates with gametic incompatibility, while divergence at synonymous sites and in a mitochondrial gene does not (Zigler *et al.*, 2005). Detailed

intraspecific analyses and geographic studies of allelic variation suggest that in some, but not all species, divergence was promoted by selection against hybridization (reinforcement; Geyer and Palumbi, 2003; McCartney and Lessios, 2004). However, evolutionary forces are likely to be complex; comparisons of reproductive success among individuals in one natural population of sea urchins (*Strongylocentrotus franciscanus*) showed that success of a particular bindin genotype depends on sex, genotype frequency, and sperm density (Levitan and Ferrell, 2006). Such studies have focused on the causes of rapid bindin divergence, but the detailed functional analyses necessary to molecu-

larly characterize the precise consequences of amino acid change on fertilization potential are awaited.

So far, research on the evolution of egg-sperm binding in sea urchins has focused solely on bindin. The egg receptor for bindin (EBR1) recently has been identified and sequenced in two species (Kamei and Glabe, 2003), and as in lysin and VERL, evolutionary analyses of bindin and EBR1 may help characterize their molecular interaction. What is perhaps most exciting is the opportunity to compare male-female pairs with known genotypes (at both sperm protein and egg receptor genes), which may help identify the precise molecular causes of variation in fertilization success in nature.

Insects

While gamete proteins have been the focus of study in marine invertebrates, egg-sperm binding proteins have yet to be identified in insects. Perhaps the best-studied group of reproductive proteins comprises the proteins produced by the accessory glands of male *Drosophila* (Acps). Here we give just a brief overview of Acp evolution, as this has been reviewed previously (Swanson and Vacquier, 2002b; Panhuis *et al.*, 2006; Ram and Wolfner, 2007).

More than 100 Acps have been identified in *D. melanogaster* (Ram and Wolfner, 2007). Acps influence female behavior and physiology, affecting storage and use of sperm, oviposition rates, and remating rates. While most of these effects increase the reproductive success of males, they are harmful to females, decreasing their longevity (Ram and Wolfner, 2007). Consequently, sexual conflict may be an important force driving Acp evolution (reviewed in Panhuis *et al.*, 2006; Ram and Wolfner, 2007). In fact, Acp genes evolve twice as fast as non-Acp genes in comparisons of *D. melanogaster* with its sibling species *D. simulans* (Swanson *et al.*, 2001a), and many of these Acp genes show a signature of positive selection (Ram and Wolfner, 2007). Comparison of *D. mojavensis* and *D. arizonae* suggests that Acps evolve even faster among these more promiscuous species (Wagstaff and Begun, 2005b).

Recently, female proteins that may interact with male Acps have been identified (Lawniczak and Begun, 2004; Swanson *et* *al.*, 2004; Kelleher *et al.*, 2007). Many of these candidate female proteins also show evidence for positive selection (Swanson *et al.*, 2004; Panhuis and Swanson, 2006; Lawniczak and Begun, 2007) and, as with Acps, evolve faster in more promiscuous groups (Kelleher *et al.*, 2007).

Evolutionary-genomic approaches have identified many rapidly evolving male and female proteins that probably interact and coevolve. For several Acps, knockout experiments have characterized their effects on female fitness (Ram and Wolfner, 2007). As a next step, evolutionary data can guide molecular experiments to identify and characterize Acp-female protein interaction pairs and their role in fertilization. For example, *in vitro* assays may be used to test the interaction between Acps and proteases present in the female reproductive tract (Lawniczak and Begun, 2004; Swanson *et al.*, 2004; Kelleher *et al.*, 2007); these experiments could test the specific hypothesis that female proteases degrade male Acps.

Male reproductive proteins have also been identified in honeybees, mosquitoes and crickets (Andres *et al.*, 2006; Braswell *et al.*, 2006; Collins *et al.*, 2006; Davies and Chapman, 2006; Dottorini *et al.*, 2007). In crickets there is evidence for positive selection on male seminal proteins, suggesting that adaptive evolution of these proteins may be common although additional taxa must be surveyed. Knowledge of the identity and evolution of other reproductive proteins, such as egg-sperm interaction proteins, is lacking in insects. Although recent large-scale studies using proteomic (Dorus *et al.*, 2006) and comparative genomic (Haerty *et al.,* 2007) approaches have begun to identify additional rapidly evolving male reproductive proteins in *Drosophila*. While these studies represent a first step, functional studies of these proteins are required to understand their contribution, if any, to reproductive isolation.

Vertebrates

Research on vertebrates shows that adaptive evolution of gamete proteins is not limited to externally fertilized taxa. Among vertebrates, knowledge of reproductive protein evolution is limited primarily to mammals (but see Berlin and Smith, 2005), but has been broad in scope, including proteins involved in many
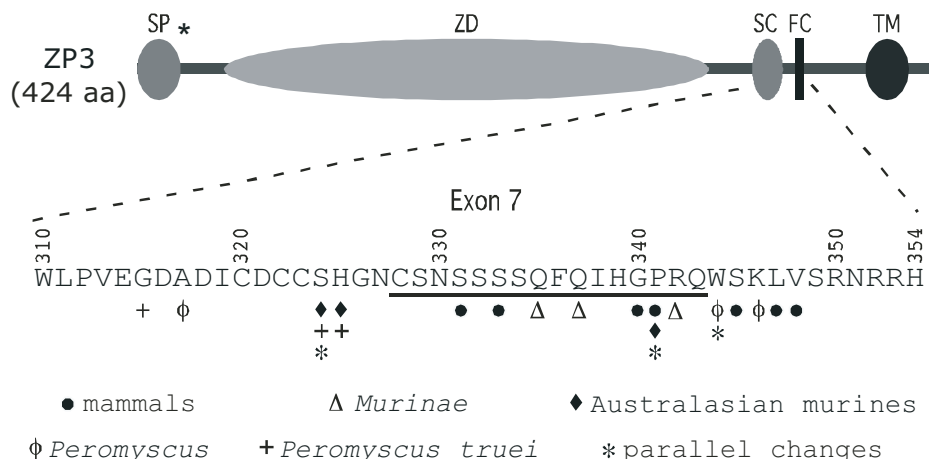
Fig. 3. Adaptive evolution of ZP3 in mammals. *The* Mus musculus *amino acid sequence for exon 7 is shown; the sperm-combining region (following Kinloch* et al., *1995) is underlined. Sites subject to positive selection in studies of diverse mammals (Swanson* et al., *2001b),* Mus *(Jansa* et al., *2003), Australasian rodents (Swann* et al., *2007), and* Peromyscus *(Turner and Hoekstra, 2006), as well as variable sites in* Peromyscus truei *(Turner and Hoekstra,* 2008*) are indicated. Sites with parallel amino acid substitutions are highlighted. The structure of ZP3 is shown above; SP, signal peptide; ZD, zona domain; SC, sperm-combining region; FC, furin cleavage site; TM, transmembrane domain. Star indicates an additional cluster of positively selected sites in exon 1 identified in studies of diverse mammals (Swanson* et al., *2001b) and rodents (Jansa* et al., *2003).*

stages of fertilization (reviewed in Clark *et al.*, 2006) and at different levels of taxonomic divergence. Genomic approaches have documented high evolutionary rates of sperm proteins (Torgerson *et al.*, 2002), seminal fluid proteins (Clark and Swanson, 2005), proteins expressed during spermatogenesis (Good and Nachman, 2005), and proteins having testis-specific expression (Turner *et al.*, in press). All of these genomic studies provide evidence for positive selection (Table 1). Studies of candidate genes, chosen because they play a role in fertilization, also have identified several targets of selection (Table 1; Singh and Kulathinal, 2000; Swanson and Vacquier, 2002b; Clark *et al.*, 2006). Taken together, these data show that selection acts on a functionally diverse set of mammalian reproductive proteins.

Until recently, research on reproductive protein evolution in mammals involved comparing sequences among only distantly related species. However, processes occurring within species and among closely related species are more relevant to understanding how amino acid changes affect fertilization and ultimately reproductive isolation. New and interesting patterns are emerging as more data become available about the evolution of reproductive proteins on shorter timescales. Here, we first describe in detail the evolution of the egg protein ZP3, in which several of these patterns are evident, then relate these findings to data from other mammalian reproductive proteins.

Evolution of ZP3 in mammals

Zona pellucida glycoprotein 3 (ZP3) is one of the best-characterized mammalian fertilization proteins, from both a functional and evolutionary perspective. ZP3 is one of three or four proteins that make up the zona pellucida, or egg coat, in mammals. ZP3 has been implicated in sperm binding in the mouse, although there are contradictory data from *in vitro* binding assays and transgenic experiments on the specific role of ZP3 (Rankin *et al.*, 1998; Wassarman, 1999; Rankin *et al.*, 2003). As a result, two models of sperm-ZP binding have been proposed. The first posits that ZP3 is the primary sperm receptor, binding sperm in a species-specific manner (reviewed in Wassarman, 1999). The alternative model proposes that sperm do not bind to only ZP3, but instead recognize the supramolecular structure of the zona pellucida (Dean, 2004). Further, ZP3 is a glycoprotein and its attached carbohydrates may also affect sperm binding (Clark and Dell, 2006). Additional evidence is required to resolve these conflicts, but a critical role for ZP3 in sperm binding in the mouse is unequivocal. ZP-sperm binding in other mammals is not as well characterized, but there are clearly taxonomic differences in the way that ZP proteins interact with sperm (Sinowatz *et al.*, 2001; Nixon *et al.*, 2007).

ZP3 was one of the first fertilization proteins that showed evidence for positive selection in mammals (Swanson *et al.*, 2001b). This early study aligned DNA sequences from eight phylogenetically diverse mammals and identified several amino acid sites as targets of selection. These target sites are clustered in two regions of ZP3. The first cluster lies in an N-terminal region with unknown function and the second lies in and around a region described as the "sperm-combining" region (Fig. 3; Kinloch *et al.*, 1995; Swanson *et al.*, 2001b; but see Berlin and Smith, 2005).

More recently, several studies have documented adaptive evolution of ZP3, in particular the sperm-combining region, in rodents (*Mus*, Jansa *et al.*, 2003; *Peromyscus*, Turner and Hoekstra, 2006; Australasian rodents, Swann *et al.*, 2007). Taken together, results from these studies make a compelling case for adaptive evolution of the sperm-combining region (encoded by exon 7) of ZP3. First, amino acid sites identified as targets of selection in these studies are clustered together in and around the sperm-combining region (Fig. 3). In many cases, these sites are adjacent or even identical in comparisons among species. Second, within groups and even between more distant lineages, in which the starting amino acid sometimes differs, substitution to the same amino acid has occurred independently (e.g., X324I, X341A, W344R; Fig. 3). These sites with convergent change are of particular interest because they may affect ZP3-sperm interaction. Characterizing the functional effects of substitutions at these sites (and others) will help us understand the molecular mechanisms governing ZP-sperm binding in rodents.

At least in one group, amino acid polymorphism in the sperm-combining region *within* several species has been found (Turner and Hoekstra, 2006). A more detailed study of ZP3 in *Peromyscus truei* showed that divergent alleles of ZP3 are present at similar frequencies in geographically distant populations, a result consistent with balancing selection maintaining ZP3 variation in this species (Turner and Hoekstra, 2008), although additional studies are needed to rule out other selective and demographic scenarios.

Thus far, several studies of ZP3 evolution have found evidence for positive selection, and amino acid changes in the region putatively important for egg-sperm binding. But has ZP3 evolution played any role in reproductive isolation? Within the genus *Mus*, none of the variable sites near the sperm-combining region differ between sister species (Jansa *et al.*, 2003). Similarly, divergence among closely related species of Australasian rodents is low (Swann *et al.*, 2007). In contrast, there is extensive amino acid sequence variation in ZP3 within and among closely related species in *Peromyscus* (Turner and Hoekstra, 2006). It is possible, however, that one or few changes in the sperm-combining region could drastically affect ZP3-sperm binding. Functional assays to test the affects of ZP3 allelic variation on egg-sperm binding and fertilization success are needed.

Other fertilization proteins in mammals

Characterizing evolutionary patterns in a small but functionally diverse set of reproductive proteins has identified new targets of selection in the mammalian fertilization process. In females, several proteins show evidence for positive selection among recently diverged species. For example, ZP2 is another major protein component of the egg coat and has sperm-binding activity, although its function is not well characterized (Wassarman *et al.*, 1999). ZP2 evolves adaptively and has several parallel amino acid substitutions among species of *Peromyscus* (Turner and Hoekstra, 2006).

On the male side, elevated rates of adaptive amino acid substitution in groups of closely related species have been reported for proteins involved in different stages of sperm production and function. For example, RHOX5 (also called PEM), a homeobox transcription factor expressed only in reproductive tissue, is required for normal sperm production, morphology, and motility in mice (MacLean *et al.*, 2005). In rodents, RHOX5
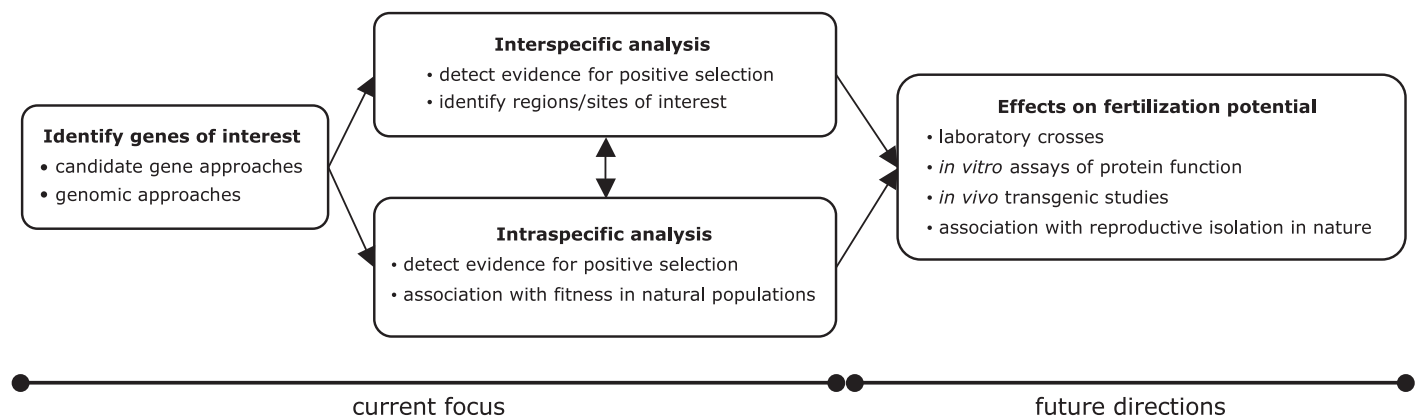
Fig. 4. Common approaches and new directions in the study of reproductive protein evolution.

evolves rapidly and is subject to positive selection (Sutton and Wilkinson, 1997), a particularly surprising result since homeodomains are generally highly conserved. Protamines, the sperm-specific DNA packaging proteins that are members of the generally conserved DNA-binding protein class, evolve rapidly in primates (Retief *et al.*, 1993; but see Clark and Civetta, 2000). Intriguingly, the rate of protamine evolution is positively correlated with the degree of promiscuity (and thus sperm competition) in primates (Wyckoff *et al.*, 2000).

Protein length as well as composition can vary adaptively among closely related species. Length variation in the sperm motility protein CATSPER1 in rodents (Podlaha *et al.*, 2005) and the seminal plug proteins SEMG1 and SEMG2 in primates (Jensen-Seaman and Li, 2003; Kingan *et al.*, 2003) is subject to positive selection. As in protamines, SEMG1/SEMG2 evolution is positively correlated with promiscuity (Jensen-Seaman and Li, 2003; Kingan *et al.*, 2003; Dorus *et al.*, 2004).

There have been surprisingly few intraspecific studies of reproductive proteins in mammals. To our knowledge, data are limited to five male reproductive proteins in primates and one study of ZP3 in *Peromyscus truei* (Turner and Hoekstra, 2008). ESX1, another homeobox transcription factor active during spermatogenesis, shows significant evidence of recent selective sweeps in humans and rapid adaptive amino acid substitution among a diverse sample of primates (Wang and Zhang, 2007). SEMG2, one of the seminal plug proteins described above, shows signs of selective sweeps in both chimps and humans (Kingan *et al.*, 2003). Like ZP3 in *P. truei*, the other three male proteins have high levels of intraspecific amino acid variation. NYD-SP12 is a protein involved in acrosome formation during spermatogenesis that shows significant signs of positive selection among primates, particularly along the chimp and human lineages (Zhang *et al.*, 2007).

Evidence for adaptive evolution of two candidate egg-binding sperm proteins, PKDREJ and zonadhesin, has been reported in primates and humans (Gasper and Swanson, 2006; Hamm *et al.*, 2007; Herlyn and Zischler, 2008). In both cases, there is evidence for balancing selection maintaining amino acid polymorphisms. Interestingly, sites that are polymorphic within humans are found in the same regions as sites that are positively selected across primates. These adaptively evolving regions include both domains implicated in ZP-binding and domains without known

functions. In contrast to patterns in protamines and seminal plug proteins, there is no evidence that rates of evolution of these egg-binding proteins correlate with the degree of sperm competition. Evidence for adaptive sequence variation in these proteins among humans raises the possibility that these proteins may contribute to variation in fertility among individuals, although associations between allelic variation and fertilization success have not been tested. Data on patterns of intraspecific variation of egg coat proteins in humans may show whether egg-sperm binding pairs are coevolving, a finding that could help identify the causes of infertility in couples.

These studies of reproductive protein evolution in mammals suggest some general conclusions. First, many reproductive proteins diverge rapidly among closely related mammals, raising the possibility that change in these proteins contribute to reproductive isolation, although functional tests of natural variation are awaited. Second, studies in diverse species suggest that the same proteins, and even the same amino acid sites, have been repeatedly targeted by selection. Finally, amino acid polymorphism within species has been reported for several fertilization proteins, raising the possibility that gametes interact differently and contribute to variation in fertility among individuals of a species. However, such intraspecific data are scarce — our conclusions are based on just a handful of proteins in only two mammalian groups, rodents and primates.

The nexus of evolution and development

Tremendous advances in understanding both the mechanics and evolution of fertilization will be possible with the integration of developmental and evolutionary biology. To achieve this, evolutionary biologists must go beyond the statistical analysis of protein evolution, and developmental biologists must go beyond studying single strains within species.

Many evolutionary biologists have a standard procedure for studying selection from DNA or protein sequences (Fig. 4). First, candidate genes are identified and sequenced in several taxa. Next, statistical tests are used to determine if the genes are evolving adaptively and, if so, to identify the specific sites subject to selection. These tests have been criticized because they can detect selection only when adaptation has occurred via multiple amino acid substitutions in a single protein (Hughes, 2007).

However, rapid divergence of reproductive proteins is not rare, as we have seen, particularly among proteins mediating interactions between male and female gametes. Certainly tests for positive selection based on nucleotide sequence data will miss important cases of adaptation. Nevertheless, these tests have been successful at identifying functionally important regions of proteins in the absence of any *a priori* information that these regions were important (e.g., Lyon and Vacquier, 1999; Yang, 2005). Despite these limitations, these are currently the best tests available for identifying candidate sites affecting protein function.

While such comparative studies can identify interesting genes and gene regions, they reveal little about the functional effects of evolutionary change on fertilization potential, which cannot be reached through statistical inference alone. An integrative approach, combining evolutionary analyses of natural populations, traditional genetics, and, most important, tests of molecular function, is required to determine the consequences of change in reproductive proteins for fertility and species divergence.

Fertilization is a difficult process to study mechanistically – it requires the interaction of cells and proteins from two individuals and gametes are not amenable to cell culture. Nevertheless, many new molecular tools are available to help the evolutionary biologist study the actual effects of protein variation on fertilization potential. For example, the effects of amino acid changes at positively selected sites could be tested using site-directed mutagenesis and *in vitro* binding assays (e.g., Kinloch *et al.*, 1995; Chen *et al.*, 1998; Hickox *et al.*, 2001; Williams *et al.*, 2003, 2006). Alternatively, manipulation of genes could be performed *in vivo* using transgenics or targeted gene transfer (Flotte, 2007; Gao *et al.*, 2007; Waehler *et al.*, 2007).

Evolutionary biologists readily adopted genomic tools, such as microarrays and high through-put genotyping, however gene manipulation techniques, experiments in cell culture, *in vitro* protein assays and *in vivo* transgenic approaches have not been adopted for the wholesale study of evolutionary questions. The effects on protein function of evolutionary changes in protein sequence, then, have been neglected (Dean and Thornton, 2007). This is particularly important in studying speciation, for which divergence of gamete interactions could be an important source of reproductive isolation. We want to know whether divergence in protein sequence has led to gametic isolation, and there is no way to understand this without functional analysis. Clearly, this is one of the most fruitful areas of inquiry in which evolution and development intersect.

Developmental biologists often study fertilization in inbred lines, and have either neglected genetic variation as an annoyance, or as a source of experimental error. Yet this very variation can inform the study of the mechanics of fertilization. Evolutionary studies, for example, have shown that natural variation among individuals in fertilization proteins is common, is sometimes maintained by natural selection, and can have dramatic effects on fertilization success. Further, while laboratory experiments are often designed to minimize environmental variation, studies of natural populations have shown that fertilization success varies depending on the environment where gametes interact (e.g., high versus low sperm density).

Variation among species, especially those that are closely related, also can inform many developmental studies. Interspecific comparison of DNA sequences, for example, can identify not only novel reproductive proteins, but also proteins and protein regions likely to play important roles in fertilization. Such comparative sequence analyses will only get easier and faster as more genomes become available. Furthermore, a comparative approach at a functional level can also inform the study of fertilization. For example, comparing functional data across several species will determine the generalities of the fertilization process as well as elucidate the mechanisms that, in some cases, cause the binding of egg and sperm to be species-specific.

In summary, the time is ripe for the cross-fertilization of developmental and evolutionary biology. Evolutionary biologists can use the developmental methodologies of *in vitro* and *in vivo* gene manipulation techniques to test the molecular effects of protein substitution, and developmental biologists can take advantage of naturally occurring genetic variation to identify novel proteins and candidate protein regions that play important roles in fertilization. This integrated approach will help answer long-standing questions about fertilization and the origin and preservation of species.

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