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Defending the Zygote: Search for the Ancestral Animal Block to Polyspermy

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Current Topics in Developmental Biology, Vol. 72

0070-2153/06 $35.00
DOI: 10.1016/S0070-2153(05)72001-9

Fertilization is the union of a single sperm and an egg, an event that results in a diploid embryo. Animals use many mechanisms to achieve this ratio; the most prevalent involves physically blocking the fusion of subsequent sperm. Selective pressures to maintain monospermy have resulted in an elaboration of diverse egg and sperm structures. The processes employed for monospermy are as diverse as the animals that result from this process. Yet, the fundamental molecular requirements for successful monospermic fertilization are similar, implying that animals may have a common ancestral block to polyspermy. Here, we explore this hypothesis, reviewing biochemical, molecular, and genetic discoveries that lend support to a common ancestral mechanism. We also consider the evolution of alternative or radical techniques, including physiological polyspermy, with respect to our ability to describe a parsimonious guide to fertilization.

I. Introduction

Successful embryonic development is enhanced when the zygote is protected from lethal microorganisms or parasites, including additional sperm. Therefore, it is not surprising that all metazoans that reproduce through the fusion of two haploid gametes have adopted a means to establish a postfertilization barrier between the zygote and the environment.

Most physical blocks to polyspermy are derived from material stored by the egg during oogenesis (reviewed in Shapiro *et al.*, 1989).[1] In most animals, construction of a barrier against sperm and other microorganisms uses both the egg's extracellular matrix (ECM) and the egg's secretory vesicles that contain structural proteins and/or enzymes. The terms used for the egg ECM vary widely, including the *zona pellucida* (ZP) (subsequently referred to as *zona* in this chapter) in mammals; the *chorion* in fish; the *vitelline envelope* in amphibians, mollusks, and crustaceans; the *perivitelline layer* in birds; the *vitelline coat* in ascidians; the *vitelline layer* in echinoderms; and the *vitelline membrane* in dipterans. All serve the same basic functions, however. Before

[1]Most animals do not formally ovulate haploid eggs, but oocytes arrested in various stages of meiosis. For clarity, however, the term *egg* is used in this chapter to refer to the cell that is ovulated and the term *oocytes* for the cells that are still developing within the ovary.

fertilization, this matrix provides a supportive substrate for the oocyte or egg. During fertilization, this network of proteins is a primary target for the reception, activation, and binding of sperm. After fertilization, this matrix is often modified to inhibit further sperm binding, thereby avoiding the potentially lethal outcome of polyspermy.

The deleterious effects that multiple cytoplasmic sperm nuclei have on subsequent cleavages maintain a selective pressure in animals to prevent polyspermy. The mechanisms employed to curb supernumerary sperm fusion, however, vary greatly across phyla. For instance, some animal eggs (e.g., those from avians and urodeles) allow multiple sperm to fuse, only later to suppress all but one from merging with the egg pronucleus. Such physiological polyspermy is limited in the animal kingdom; most animals instead employ rapid physical modifications that alter the affinity of sperm for the fertilized egg. A core set of modifications have been described on the molecular level in all animals that use the latter category of physical blocks, suggesting that renovating the egg ECM may follow a common process. If mechanisms to block polyspermy are shared, we would expect that homologous proteins and enzymes are also involved, especially if this type of physical block diverged from a single ancestor. The current state of the field, however, suggests that establishment of a physical block might have convergently evolved through independent routes.

A significant distinction among animal blocks to polyspermy is the composition and modification of the physical barrier. In all vertebrates, members of the ZP protein family comprise most of the egg ECMs (Spargo and Hope, 2003). Thus, any modifications that result in the physical block occur on these ZP proteins. Little is known about the proteins or enzymes responsible for biochemical alterations to the matrix, but the majority of activity is believed to derive from CGs or accessory reproductive organs such as the oviduct. In contrast, very little is known about the constituents of the invertebrate egg ECM. Only a handful of proteins have been isolated and characterized—primarily from ascidians, sea urchins, bivalves, and the dipteran *Drosophila*—and these proteins bear little resemblance to one another or the vertebrate ZP proteins. Variations in the types of morphological modifications that occur are also prominent during animal fertilization, showing dramatic lifting of the egg ECM far from its original location at the egg surface in echinoderms and some anurans, to no observable changes at all in ascidians, mollusks, avians, and mammals. Yet all of these animals exhibit monospermic fertilization. Thus, we immediately see that the evolution of fertilization-related blocks to polyspermy can use a wide variety of mechanisms to achieve the same outcome.

This chapter discusses the role of the egg ECM and CGs during fertilization, focusing on data that implicate molecules and processes involved with the successful transition from an egg ECM to the zygotic block to polyspermy

(see also Bellairs, 1993; Elinson, 1986; Gould and Stephano, 2003; Shapiro *et al.*, 1989; Shur *et al.*, 2004; Yamagami *et al.*, 1992). We draw primarily on observations and molecular data from animals whose process of fertilization is well documented—mammals, amphibians, fish, ascidians, mollusks, and echinoderms—but we include observations from other animals such as birds (see Bellairs, 1993), dipterans (see Bloch Qazi *et al.*, 2003; Fitch *et al.*, 1998), decapods, and the nematode *Caenorhabditis* (reviewed in Singson, 2001), when applicable (see Tables I and II). We also present primary observations and perspectives that highlight the most basic requirements necessary for successful fertilization, consistent with the hypothesis that the present range of species–specific gamete interactions radiated from a common ancestor.

II. Egg Extracellular Matrix

The egg ECM is known by many names in animals. These include the *zona* in mammals, the *chorion* in teleosts, the *perivitelline layer* in birds, the *vitelline envelope* in amphibians, mollusks, and crustaceans, the *vitelline layer* in echinoderms, and the *vitelline membrane* in dipterans. The function of this ECM is multifaceted, providing both a biochemical interface between the oocyte and associated follicle cells and a protective structural barrier to the egg. During oogenesis, the matrix is no more than a thin layer of glycoproteins that defines the boundary between the oocyte and surrounding support cells (Anderson, 1968; Breed and Leigh, 1990; Hedrick and Nishihara, 1991; Mate, 1998; Sinowatz *et al.*, 2001). As oogenesis progresses and oocyte cytoplasm accumulates, the ECM thickens (Figs. 1 and 2), reflecting its increasingly important role in cell–cell signaling between the oocyte, its support cells, and eventually the sperm (Dean, 2004). Outside of the ovary, the primary function of the ECM changes from a supportive to a directive role, acting as a signaling platform for the complex series of events directive up to fertilization, including initiation of the sperm acrosome reaction, sperm orientation, and sperm binding (reviewed in Rankin and Dean, 2000). Following gamete fusion, the egg ECM is modified once more, transforming it into a physical barrier against environmental insults such as additional sperm or microbes. The success of this extracellular block is dependent on the components and their inherent organization within the final structure.

For purposes of this chapter, we focus on the latter events of ECM maturation that result in the protection of the egg from an environment less nurturing than the ovary. Here, we compare the ultrastructure of and highlight the major protein domains associated with each egg ECM used by different animal orders (see Table I). Although this section acts as a primer to familiarize us with the range of descriptives used to define different ECMs, it also introduces potential aspects conserved among the matrices both during the assembly process and

Table I Hierarhy of Select Genera within the Animal Kingdom[a]

Phylum (subphylum)	Class	Subclass	Order	Family	Genus	Animal
CHORDATA (Vertebrata)	Mammalia	Eutheria	Carnivora	Canidae	*Canis*	Dog
					Vulpes	Fox
				Felidae	*Felix*	Cat
				Mustelidae	*Mustela*	Weasel
			Cetartiodactyla	Suidae	*Bos*	Cow
					Sus	Pig
			Lagomorpha	Leporidae	*Oryctolagus*	Rabbit
			Perissodactyla	Equidae	*Equus*	Horse
			Primates	Callitrichidae	*Callithrix*	Marmoset
				Cercopithecidae	*Macaca*	Macaque
					Papio	Baboon
				Hominidae	*Homo*	Human
			Rodentia	Caviidae	*Cavia*	Guinea pig
				Muridae	*Lagarus*	Lemming
					Mesocricetus	Hamster
					Microtus	Vole
					Mus	Mouse, common
					Notomys	Mouse, hopping
					Pseudomys	Mouse, desert
					Rattus	Rat
		Metatheria	Dasyuromorphia	Dasyuridae	*Sminthopsis*	Dunnart
			Didelphimorphia	Didelphidae	*Monodelphis*	Opossum
			Diprotodontia	Macropodidae	*Macropus*	Kangaroo
				Phalangeridae	*Trichosurus*	Possum, brush-tail
				Phascolarctidae	*Phascolarctos*	Koala
				Pseudocheiridae	*Pseudocheirus*	Possum, ring-tail
			Peramelemorphia	Peramelidae	*Isoodon*	Bandicoot
	Aves	Neognathae	Galliformes	Phasianidae	*Coturnix*	Quail
					Gallus	Chicken
	Amphibia	Batrachia	Anura	Bufonidae	*Bufo*	Toad
				Discoglossidae	*Discoglossus*	Frog, painted
				Leptodactylidae	*Eleutherodactylus*	Frog, tree
				Pipidae	*Xenopus*	Frog, clawed
			Caudata	Salamandroidae	*Cynops*	Newt
			Urodela	Hynobiidae	*Hynobius*	Salamander

(*Continued*)

Table I *Continued*

Phylum (subphylum)	Class	Subclass	Order	Family	Genus	Animal
CHORDATA (Vertebrata)	Actinopterygii	Neopterygii (Teleostei)	Anguilliformes	Anguillidae	*Anguilla*	Eel
			Beloniformes	Adrianichthyidae	*Oryzias*	Medaka
			Clupeiformes	Clupeidae	*Clupea*	Herring
			Cypriniformes	Cyprinidae	*Barbus*	Barb, rosy
					Carassius	Goldfish
					Cyprinodon	Pupfish
					Cyprinus	Carp
					Danio	Zebrafish
					Pimephales	Minnow
					Rhodeus	Bitterling
					Tribolodon	Barbel fish
			Perciformes	Gobiidae	*Pomatoschistus*	Goby
				Malacanthidae	*Lopholatilus*	Tilefish
				Sparidae	*Sparus*	Seabream
			Pleuronectiformes	Pleuronectidae	*Pseudopleuronectes*	Flounder
				Scophthalmidae	*Scophthalmus*	Turbot
			Salmoiformes	Salmonidae	*Oncorhynchus*	Trout
					Salmo	Salmon
					Salvelinus	Char
			Scorpaenis forme	Liparidae	*Liparis*	Snailfish
			Jetraodontiformes	Tetraodontidae	*Tetraodon*	Pufferfish
		Chondrostei	Acipenseriformes	Acipenseridae	*Acipenser*	Sturgeon
				Polyodontidae	*Polyodon*	Paddlefish
	Hyperoartia		Petromyzontiformes	Petromyzontidae	*Lampetra*	Lamprey
(Urochordata)	Ascidiacea		Phlebobranchia	Cionidae	*Ciona*	Tunicate
			Stolidobranchia	Pyuridae	*Halocynthia*	Ascidian
ECHINODERMATA	Stelleroidea	Asteroidea	Forcipulatida	Asteriidae	*Asterias*	Starfish
			Paxillosida	Astropectinidae	*Astropecten*	Starfish
			Spinulosida	Asterinidae	*Asterinas*	Bat starfish
	Echinoidea	Echinacea	Arbacoida	Arbadiidae	*Arbacia*	Sea urchin
			Echinoida	Strongylocentrotidae	*Strongylocentrotus*	Sea urchin
			Temnopleuroida	Toxopneustidae	*Lytechinus*	Sea urchin

(Hexapoda)	Insecta	Pterygota	Diptera	Culicidae	*Anopheles*	Mosquito
				Drosophilidae	*Drosophila*	Fly, fruit
				Muscidae	*Musca*	Fly, house
				Tephritidae	*Dacus*	Fly, fruit
ARTHROPODA (Crustacea)	Malacostraca	Eucarida	Decapoda	Palaemonidae	*Palaemon*	Shrimp
				Rhynchocinetidae	*Rhynchocintes*	Prawn
				Sicyoniidae	*Sicyonia*	Shrimp
				Nephropidae	*Homarus*	Lobster
				Majidae	*Libinia* *Maia*	Crab, spider Crab, spider
				Ocypodidae	*Uca*	Crab, fiddler
				Portunidae	*Carcinus*	Crab, common
(Chelicerata)	Merostomata		Xiphosura	Limulidae	*Limulus*	Crab, horseshoe

MOLLUSCA	Gastropoda		Orthogastropoda	Haliotidae	*Haliotis*	Abalone
				Trochidae	*Tegula*	Teguline
	Bivalvia	Hetaroconchie	Veneroida	Mactridae	*Spisula*	Clam
		Heteroconchia	Unionoida	Unionidae	*Unio*	Unio bivalve
		Paleoheterodonta	Mytiloida	Mytilidae	*Mytilus*	Mussel
			Ostreoida	Ostreidae	*Crassostrea*	Oyster
	Cephalopoda		Sepiida	Sepiidae	*Sepia*	Cuttlefish
	Polyplacophora		Neoloricata	Chitonidae	*Tonicia*	Chiton
				Ischnochitonidae	*Challochitin*	Chiton

ANNELIDA	Polychaeta	Palpata	Canalipalpata	Chaetopteridae	*Chaetopterus*	Annelid

NEMATODA	Chromadorea		Rhabditoida	Rhabditoidae	*Caenorhabditis*	Nematode

[a]All genera used in this chapter are listed. Approximate phylogenetic position of each order is listed from most recent (top) to most basal (bottom). Description of animals in the text uses common variants of the phylum, class, order, or family where applicable. Metatherians are referred to as "marsupials" in the text.

Table II Summary of the Molecular Components Predicted to be Involved at Particular Stages of Fertilization in Select Animals

	Decapods	Dipterans	Chiton	Bivalves	Gastropods	*Echinoderms*
Fertilization type	External/internal	Internal	External	External	External	External
% polyspermy	0%	0–11%	0%	0%	0%	0%
Egg ECM(s)	Vitelline envelope (1)	Vitelline membrane Chorion	Vitelline envelope Jelly	Vitelline envelope Jelly	Vitelline envelope Jelly	Vitelline layer Jelly
Source of ECM	Oocyte Follicular cells	Oocyte Follicular cells	Oocyte	Oocyte Follicular cells	Oocyte	Oocyte
ECM components		[dec-1], fc106, 125, 177 [cor-36], sV23/sV17 [fs(2)QJ42], s18, s36		gp180 gp273	VERL	p160 EBR1 Rendezvin^{VL}
Accessory cells ovulated	None	None	Follicle cells	None	None	None
Oviduct contribution	75-kDa trypsin target	Cerotoxin				Jelly Speract, resact ARIS, coARIS, asterosap
Cortical granule contents		n/a			n/a	CGSP1 Ovoperoxidase Transglutaminase Proteoliaisin SFE1 SFE9 Rendezvin^{CG} Hyalin
Epididymal contributions to sperm surface		*N*-acetylglucosaminidase				
Acrosome contents					Lysin	Bindin Metalloprotease
Chemoattractant				Hexapeptides	Amino acids	resact
Egg primary sperm receptor		*N*-acetylglucosamine			VERL	FSP, sialoglycan, speract ARIS, coARIS, asterosap
Sperm primary egg ligand		*N*-acetylglucosaminidase Mannosidase				ERJ1, ERJ2, ERJ3, PC2
Egg secondary sperm receptor					VERL	EBR1
Sperm secondary egg ligand					Lysin	Bindin
Sperm penetration through the ECM	Acrosomal filament	Acrosomal process	Acrosomal filament	Acrosomal filament	16-kDa Lysin	Acrosomal process
Egg contribution to fusion						Metalloprotease
Sperm contribution to fusion					18-kDa Lysin	Bindin
Transient change in membrane potential	Hyperpolarization					Depolarization
Postfertilization egg ECM modifications	Formation of vitelline envelope 2 from cortical granule secretions	Isopeptide bond formation Di-tyrosine cross-linking		Aminopeptidase disruption of sperm receptor		Isopeptide bond formation Di-tyrosine cross-linking Adsorption of cortical granule protein to the ECM

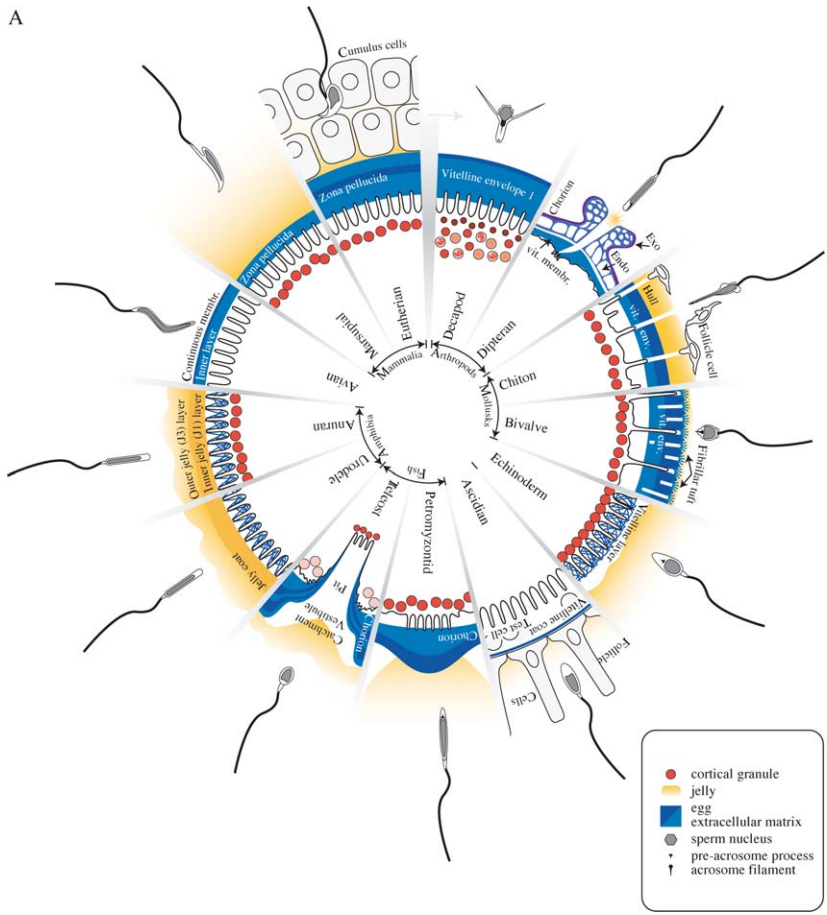
Table II *Continued*

Ascidians	Chondrostreans	Teleosts	Urodeles	Anurans	Avians	Marsupials	Eutherians
External	External	External	External	External	Internal	Internal	Internal
0%	0%	0%	100%	0%	100%	5%	0%
Vitelline coat	Chorion Jelly	Chorion Jelly	Vitelline envelope Jelly	Vitelline envelope Jelly	Perivitelline layer	Zona pellucida	Zona pellucida
		Oocyte Liver	Oocyte Granulosas cells	Oocyte Granulose cells	Oocyte Liver		
VC70	ZP orthologs (>two?)	ZP orthologs (>three)	ZP orthologs (>one?)	ZP orthologs (five)	ZP orthologs (four)	ZP orthologs (>two?)	ZP orthologs (>three?)
Test cells Follicle cells	None	None	None	None	None	Corona radiata	Corona radiata
			Jelly Cleavage of ZPC	Jelly Cleavage of ZPC	Outer egg shell	Glycoproteins	Glycoproteins
n/a	Peroxidase activity	Peroxidase activity Transglutaminase Cystatin FEO-1 FLS CLS Hyosophorin	n/a	Zinc metalloprotease Peroxidase activity XL35/CGL			Protease activity Peroxidase activity HL1 (lectin) p56/AM67 p62
							Spermadhesin
Acrosin Spermosin 20S proteosome 26S proteosome							Acrosin Spermosin TESP5 PH-20 MC41 Proteosome?
SAAF		PMIF		allurin			OGPs progesteone
VC70				ZPC oligosaccharide		PC oligosaccharide	ZPC oligosaccharide
							SVI Mannosidase Hexokinase AWN-1 RSA
	ZP homolog (not ZPA)	ZP homolog (not ZPA)					ZPA-ZPC
							GalTase I TESPs PH-20 sp56/AM67 SED1/p47
Proteolysis	Acrosomal process	n/a			Mechanical oscillation?	Mechanical force	Sperm head oscillation
Metalloprotease							CD9 CD81
				xMDC16			Izumo TRPCs M29 mMN9 Fertilin/PH30 NL1
Depolarization		Freshwater, depolarization Marine None	None	Depolarization	None	None	None
Deglycosylation and proteolysis of VC70		Proteolysis at micropyle Isopeptide bond formation	Hydration-dependent hardening	Calcium-dependent XL35 precipitation ZPA proteolysis [by Zinc metalloprotease] ZP deglycosylation	Application of oviductal coating	Application of oviductal coating	ZPA protolysis ZP deglycosylation

within the organization of the final structure. We focus on the contributions of a selection of protein domains to the egg ECM, using this information to assess whether this structure is conserved on the molecular level. We also ask which types of selective forces associated with fertilization may be influencing the evolution and adaptation of these motifs in different animals.

A. Construction of an Egg Extracellular Matrix

Assembly of a functional egg ECM ultimately depends on the oocyte, but in many animals, this construction requires input from various somatic cells. In some animals, synthesis of some egg ECMs is accomplished entirely by the oocyte (Epifano *et al.*, 1995; Haines *et al.*, 1999; Kanamori, 2000;



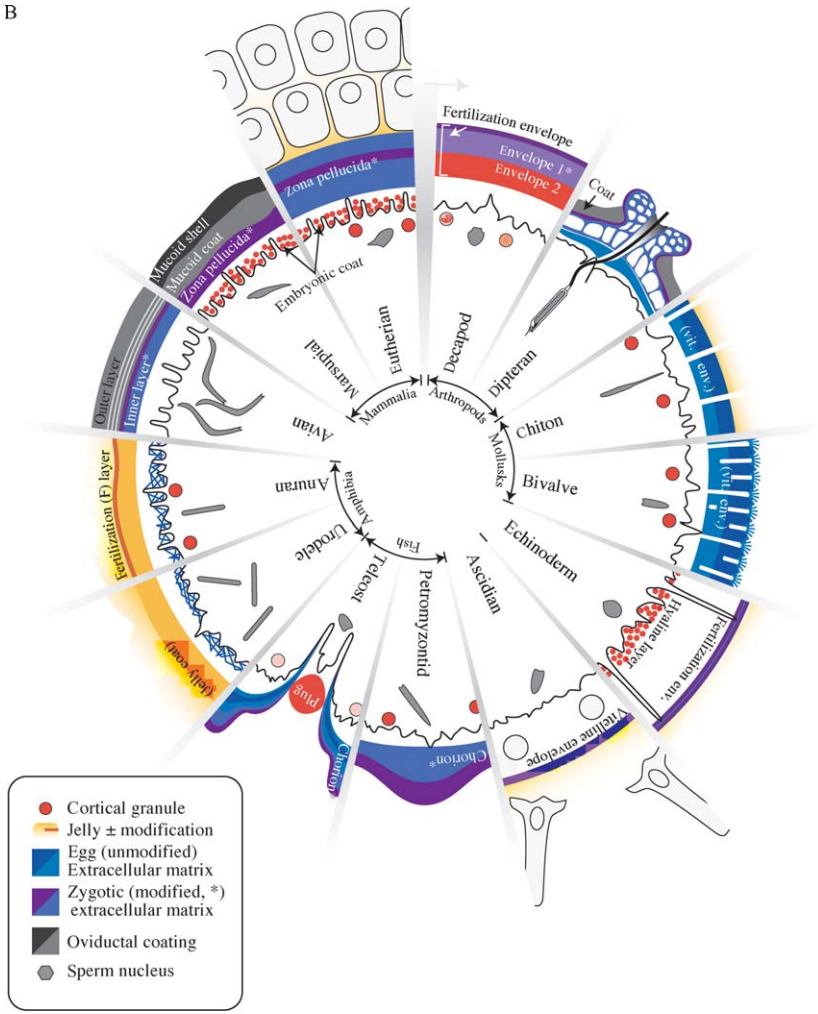


Figure 1 Direct comparison of animal egg cortices before and after fertilization. Representative animal egg cortices before (A) and after fertilization (B). Shown are the most fusion-competent domains of the respective egg (see also Fig. 4). Colors are associated with structures as in the legend: Cortical granule contents are shown in pink/red; the extracellular matrix is indicated in blue (egg) and purple (modified by cortical granule contents); jelly is shown in yellow; and postfertilization coats usually applied by external sources such as oviductal epithelium are in dark gray. Sperm nuclei are shown in light gray. Major structures and ultrastructural divisions, when discernable, are labeled. In (B), structures labeled primarily refer to structures altered at fertilization (including the use of an asterisk if the name itself does not change). endo, endochorion; exo, exochorion; vit. membr., vitelline membrane; vit. env., vitelline envelope.

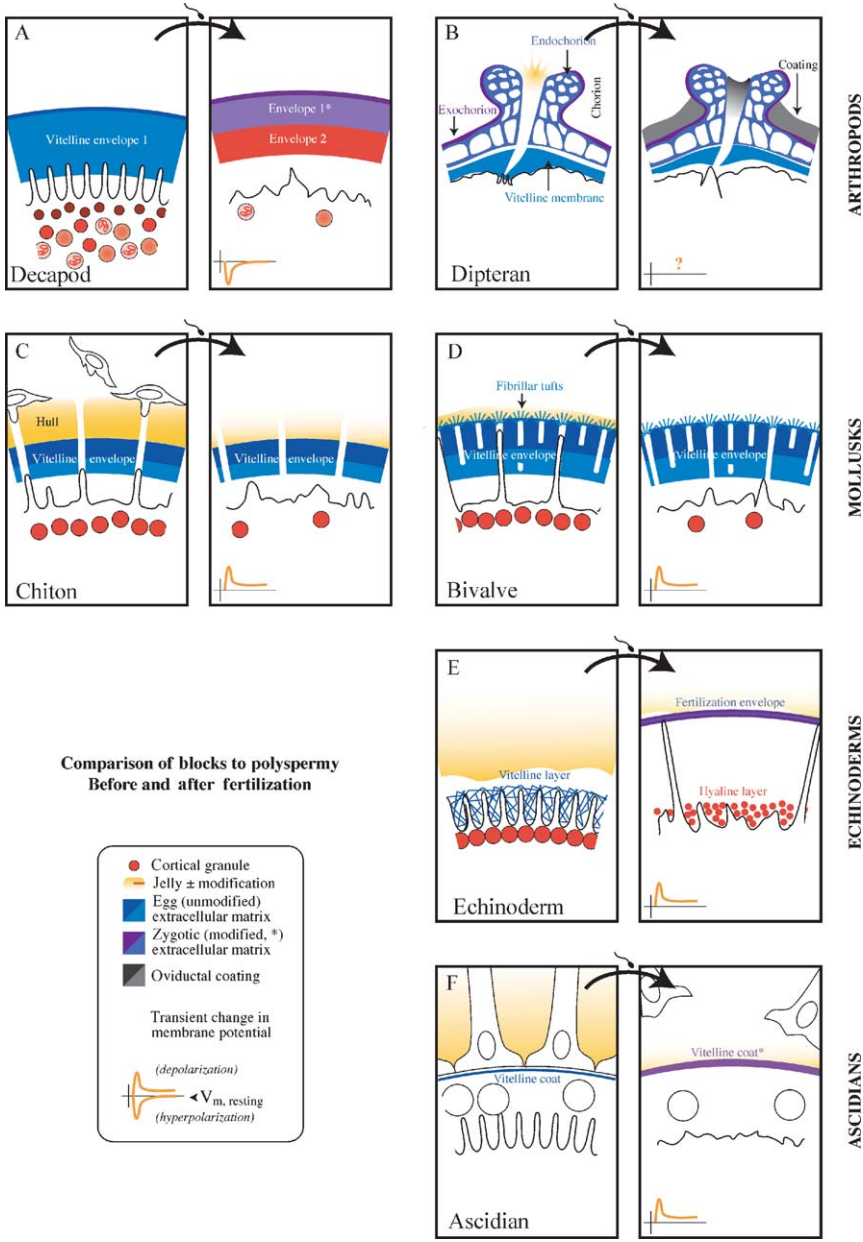


Figure 2 Juxtaposition of egg extracellular matrices before and after fertilization. Detailed side-by-side pairs of animal egg cortices before (left) and after (right) fertilization. Structures and legend generally follow those of Fig. 1. Included are the presence or absence of a fast electrical block to polyspermy (membrane voltage change, in orange). Animal orders separated

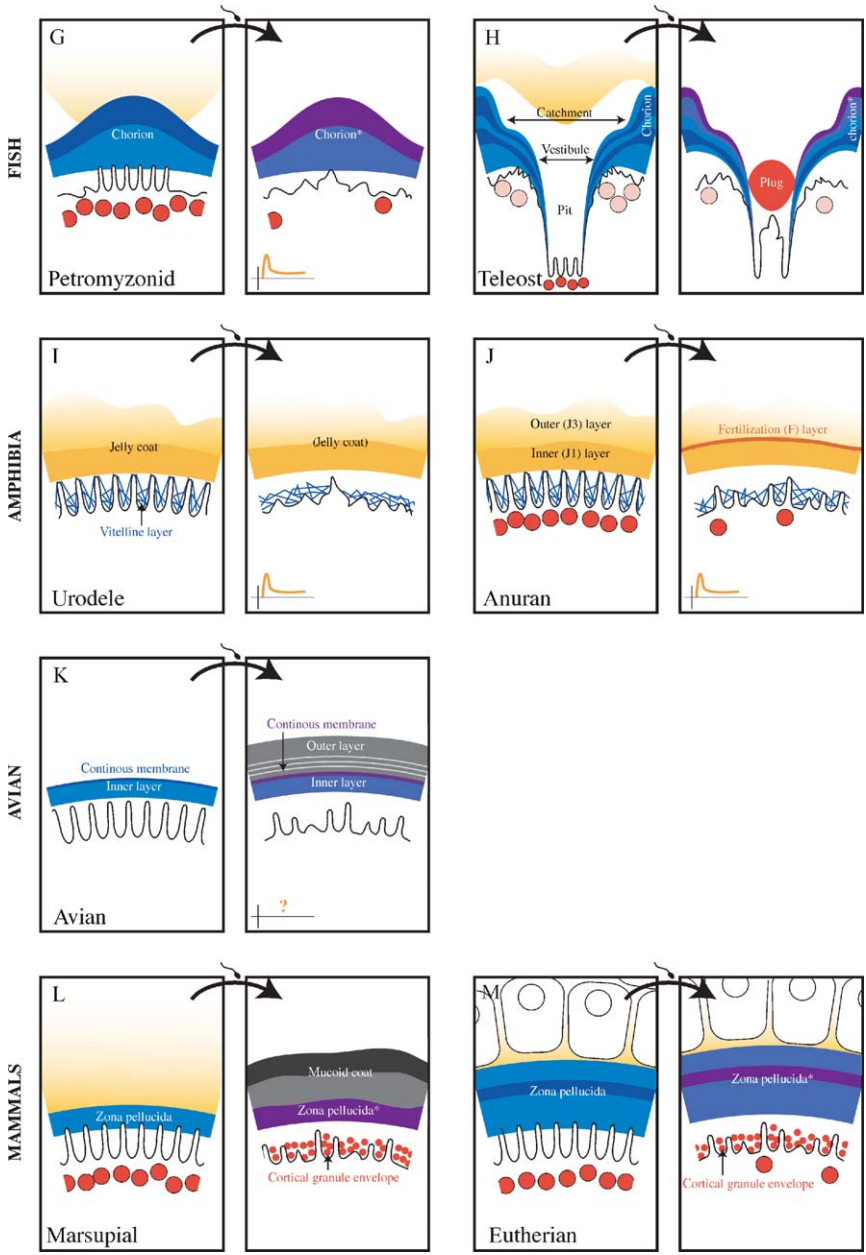


Figure 2 (*Caption continued*) by vertebrate/invertebrate classifications and arranged by their relative phylogenetic position are as follows: decapods (A), dipterans (B), chiton (C), bivalves (D), echinoderms (E), ascidians (F), petromyzontids (G), teleosts (H), urodeles (I), anurans (J), avians (K), marsupials (L), and eutherians (M).

Voyle *et al.*, 1999; Wassarman, 1988; Yamagami *et al.*, 1992), whereas in other animals, synthesis is supplemented by somatic cells (Dunbar *et al.*, 1994; Haines *et al.*, 1999; Kolle *et al.*, 1996; Martinez *et al.*, 1996; Mate, 1998; Okumura *et al.*, 2004; Takeuchi *et al.*, 1999; Vaccaro *et al.*, 2001; Wolgemuth *et al.*, 1984). Such variability of protein sources among animals reflects differences in the robustness and organizational complexity of the specific matrices. In general, simpler ECMs consist of oocyte-derived proteins, whereas the more elaborate matrices may require additional contributions from somatic tissue. The latter situation is often associated with mechanically protective roles, such as resistance to environmental factors like osmotic shock and desiccation. For example, the liver is enlisted in animals whose egg ECMs are extremely robust, such as for avians (Bausek *et al.*, 2000; Okumura *et al.*, 2004) and fish (Chang *et al.*, 1999; Hyllner *et al.*, 2001; Lyons *et al.*, 1993; Murata *et al.*, 1995, 1997; Yamagami *et al.*, 1992). These liver glycoproteins make the long journey to the ovary via the bloodstream, somehow staying in solution until they arrive between the follicle cells and the oolemma, where they self-assemble within the ECM under construction. Whether these distantly–derived proteins are modified by the follicle cells before their incorporation into the egg ECM remains to be determined.

Most ECMs are organized into concentric layers surrounding the egg, which in many cases may reflect its temporal construction. The retention of this lamellar substructure—versus a more homogenized mature ECM—does, however, suggest the possibility that these distinct layers serve a mechanical role in protecting the egg. For example, the eutherian zona can be optically dissected into three layers with orthogonal birefringence: The inner layer possesses radial filaments, the outer tangential, together sandwiching a middle layer of low retardance (Keefe *et al.*, 1997) (Figs. 1–3). This optical deconstruction of the zona agrees with the trilaminar arrangement observed by transmission electron microscopy (TEM) during mouse oogenesis (El-Mestrah *et al.*, 2002) and by immunocytochemical staining for specific glycoproteins in the rabbit zona (Wolgemuth *et al.*, 1984). This alternating, orthogonal organization of parallel fibers arranged in sheets provides greater mechanical strength than a comparably thick mat of parallel fibers (compare layers of plywood vs. fiberboard), supporting the structural role these proteins have in protecting the egg. The teleost chorion layers also appear to be subdivided into a zona radiata interna and externa (Hart and Donova, 1983; Hart *et al.*, 1984). The most inner layer (zona radiata) consists of alternating electron dense and lucent layers, the middle layer is electron lucent, and the thinnest most outer layer (zona externa) is electron dense. Amphibians also possess lamellar vitelline envelopes, a thin structure with at least four subdomains containing two layers of fibers running in parallel with the cell surface, whereas the others are thicker and more scattered (Figs. 1 and 2).

These four layers together participate in initial sperm attraction, binding, and the final block to polyspermy (Arranz and Cabada, 2000; Bonnell *et al.*, 1996; Campanella *et al.*, 1997; Infante *et al.*, 2004; Talevi and Campanella, 1988). Distal to the amphibian egg surface and outside of the vitelline envelope lies a prominent jelly layer composed of a network of fibers and globules (Fig. 3). The lamellar ultrastructure of jelly is a consequence of chronological deposition during the egg's passage through the amphibian oviduct. In anurans, this jelly layer consists of an inner J1 and outer J3 layer sandwiching a concentric stratified middle J2 layer (Bonnell and Chandler, 1996) (Figs. 1 and 3), whereas urodele jelly is composed of four structurally distinct layers with a hyaline or fibrillar ultrastructure (Jego *et al.*, 1986).

In general, the structure of an invertebrate's egg ECM is more variable than the vertebrate analog. One reason for this diversity may lie in the much greater diversity of reproductive methods used by invertebrates, which could result from the wider evolutionary distance separating these animals from the diverged clade of vertebrates (see Table I). Although a vertebrate's egg ECM is a series of glycoprotein shells that surround the egg, those of invertebrates prove more complex (Figs. 1 and 2). An invertebrate egg ECM morphologically most similar to a vertebrate's belongs to echinoderms, the most basal deuterostome order. Its thin vitelline layer consists of a dense fibrillar reticulum proximal to the oolemma that drapes over microvilli, with a second electron-lucent layer found just outside this glycoprotein shell (Bonnell *et al.*, 1994). The entire vitelline layer is synthesized by the oocyte during oogenesis (Runnstrom, 1966). As in amphibians, a more substantial jelly layer is applied by follicle cells over the vitelline layer upon oocyte maturation that serves to attract and activate sperm (Santella *et al.*, 1983; Tegner and Epel, 1976; Tosti, 1994). A similar laminar organization is present in the ascidian vitelline coat, the filamentous glycoprotein layer separated from the egg by a wide perivitelline space (De Santis *et al.*, 1980). Upon ovulation, the follicle cells remain attached to the exterior face of the vitelline coat while the interior perivitelline space is inhabited by test cells, membrane-bound extrusions from the oocyte that appear upon maturation (Rosati, 1985). Follicle cells are thought to regulate the penetrance of sperm during fertilization; the function of test cells, however, is not clear. Retention of follicle cells over the ECM is also seen in chiton. These basal mollusks are spawned with a thin layer of follicle cells surrounding an elaborate jelly hull (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988). Unlike eutherian cumulus cells or ascidian follicle cells, the chiton follicle cells may shrivel upon contact with the seawater, revealing pores in the hull that are nearly continuous with pores of the vitelline envelope. Together, these continuous tunnels facilitate a sperm's access to the egg surface. Consistent with the morphology of the egg ECM in most higher mollusks (e.g., gastropods, bivalves, and cephalopods), the chiton vitelline envelope pores are occupied by elongated microvilli that extend

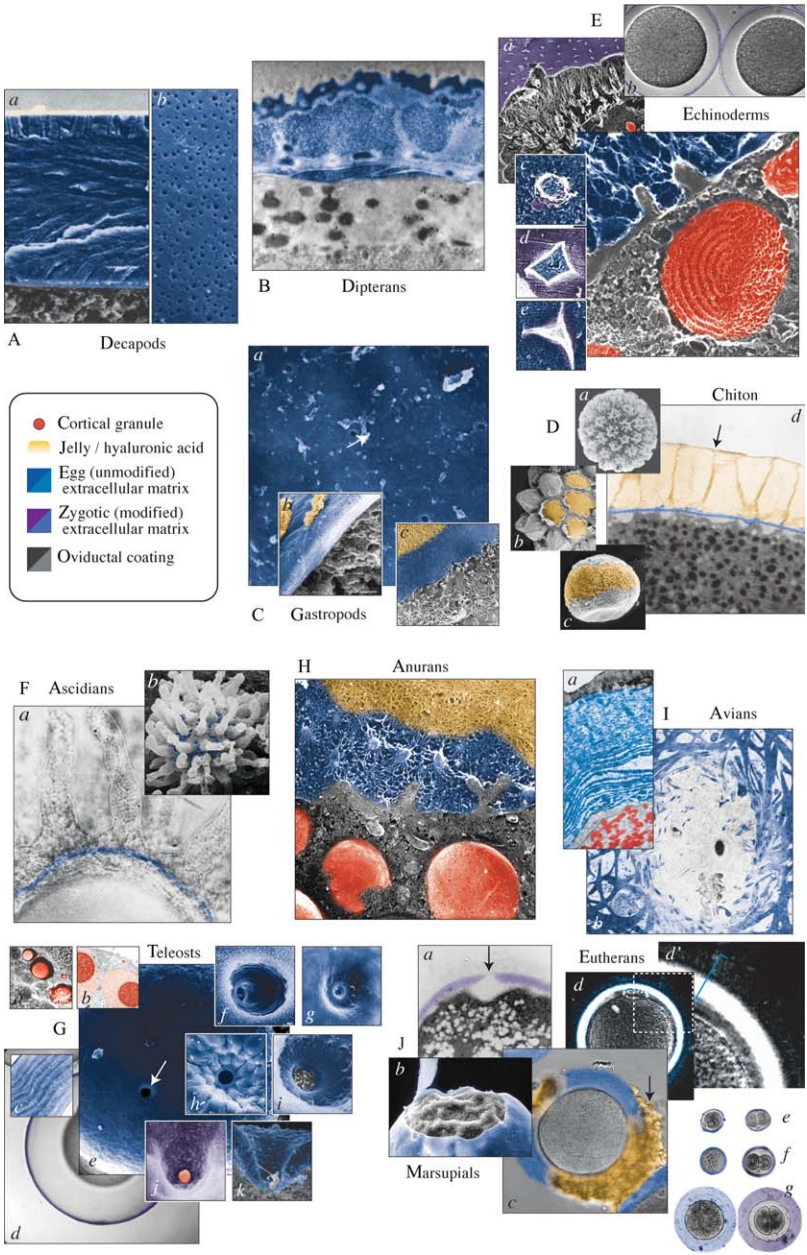


Figure 3 Representative animal egg cortices and extracellular matrices. Photomicrographs of egg cortices and extracellular matrices (ECMs) from light and electron microscopy, clustered by animal order: (A) decapods, (B) dipterans, (C) gastropods, (D) chiton, (E) echinoderms, (F) ascidians, (G) teleosts, (H) anurans, (I) avians, and (J) mammals. Colors indicate specific

to the ECM surface (Alliegro and Wright, 1983; Buckland-Nicks *et al.*, 1988; Mozingo *et al.*, 1995). Thus, passage through the tunnel affords the mollusk sperm direct access to the egg surface. Sperm access is spatially restricted in the bivalve *Unio*, whose vitelline envelope is attached to the egg only at the vegetal pole (Focarelli *et al.*, 1988) (Fig. 4). This attachment point is the sole site of sperm binding and fusion, the functional equivalent to a micropyle. Such a regional specialization of the egg ECM is ubiquitous in dipteran insects, whose eggshell is specifically molded for sperm entry only at the anterior pole (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976). The dipteran chorion is composed of two substructures: the vitelline membrane, an intimate ECM surrounding the insect egg, and the chorion, an outer cavernous structure composed of an egg-proximal endochorion and a distal exochorion (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991;

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organelles, as in Figs. 1 and 2. (A) SEM of a fractured *Limulus* egg cortex (Aa) and a surface view showing the abundance of channels found in the vitelline envelope (Ab; Brown and Humphreys, 1971). (B) Transmission electron microscopy (TEM) cross-section through the anterior region of the *Drosophila* chorion (Pascucci *et al.*, 1996). (C) *Haliotis* egg vitelline envelope, showing surface views of pores (a; arrow) and SEMs of the egg cortex (Cb–c) including the ability to distinguish the ECM from the jelly coat (Mozingo *et al.*, 1995). (D) SEM of chiton hull morphology for *Mopalia* (Da), *Lepidochitona* (Db; Buckland-Nicks, 1993), and *Challochiton* (Dc; Buckland-Nicks and Hodgson, 2000). Also, corresponding *Challochiton* bright-field image (Dd) shows the depth and extensive number of pores within the hull (arrow) (Buckland-Nicks and Hodgson, 2000). (E) Images of the sea urchin egg (f) and zygote (a–e). (Ea) SEM of a fractured *Strongylocentrotus* zygote, including the fertilization envelope (Chandler and Heuser, 1980). (Eb) DIC image of *Lytechinus* zygotes. (Ec–e) SEM series of the ovoperoxidase-dependent transition in the microvillar casts of the *Strongylocentrotus* vitelline layer over time (Larabell and Chandler, 1991). (Ef) Cortical SEM view of a fractured *Strongylocentrotus* egg (courtesy D. E. Chandler). (F) Bright-field (Fa) and SEM (Fb) of *Ciona* eggs, including follicle cells overlying the vitelline coat (De Santis *et al.*, 1980). (G) Collection of images from teleosts, including a DIC (Gd, *Danio*) and a whole chorion SEM (Ge; *Oryzias*, Hart *et al.*, 1984). Arrow indicates location of micropyle. Freeze-fracture SEM of the cortex (Ga; *Danio*, Hart and Collins, 1991). Corresponding cross-sectional TEM image of the cortex (Gb; *Danio*, Hart and Donova, 1983) and chorion (Gc; *Oryzias*, Hart *et al.*, 1984). (Gf–i) SEMs of micropyles from various species (Gf, *Oryzias*; Gg, *Lopholatilus*, both courtesy of N. H. Hart; Gh, *Danio*, Hart and Donova, 1983); Gi, *Rhodeus*, Ohta and Iwamatsu, 1983). Also included is a micropyle populated by sperm, separated from the egg (Gk; *Danio*, Wolenski and Hart, 1987) and an exterior view into the micropyle after fertilization (Gj; *Rhodeus*, Ohta and Iwamatsu, 1983). (H) SEM of fractured *Xenopus* egg cortex (courtesy D. E. Chandler). (I) Images of *Gallus* eggshell, especially a TEM cross-section of the inner layer of the vitelline membrane (Ia; Bellairs *et al.*, 1963) and a surface view of the eggshell overlying the germinal disc (Ib; Okamura and Nishiyama, 1978a). (J) Assorted images of mammalian eggs. (Ja) TEM cross-section of a *Trichosurus* zygotic zona displaying a hole where the sperm penetrated (arrow) (Jungnickel *et al.*, 1999). (Jb) SEM of zona torn from a freshly ovulated *Sminthopsis* egg (Breed *et al.*, 2002). (Jc) DIC image of *Homo* egg, with cumulus cells partially attached (arrow). (Jd) Polarized microscope image of *Homo* egg, and (Jd′) detail showing laminar difference in retardance (courtesy J. Trimarchi). (Je–g) Paired images of egg (left) and two-cell embryo (right) from *Mus* (e), *Mesocricetus* (f), and *Oryctolagus* (g) (Eakin and Behringer, 2004).

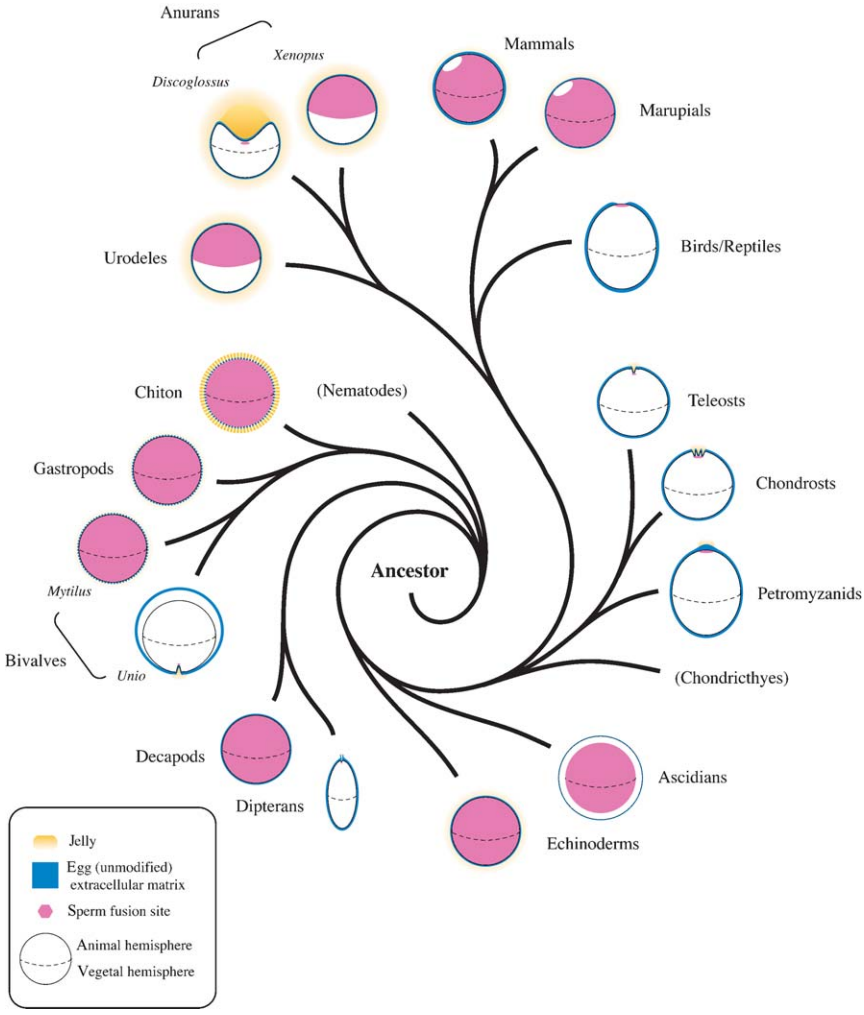


Figure 4 Polarization and fusogenic sites of eggs across animal phylogeny. Representations of eggs of different orders, indicating polarization of sperm fusion sites, superimposed onto a predicted phylogenetic tree of animal evolution. Eggs are not to scale. Where appropriate, genera are shown to represent diversity among animal orders.

Pascucci *et al.*, 1996; Turner and Mahowald, 1976) (Figs. 1 and 2). The chorion is synthesized by surrounding follicle cells and serves to protect the egg from desiccation and mechanical stress after it is laid. Thus, the diversity of egg ECMs includes their ultrastructural appearance, their molecular composition, their functions, the strategy of sperm interaction with them, and their fate

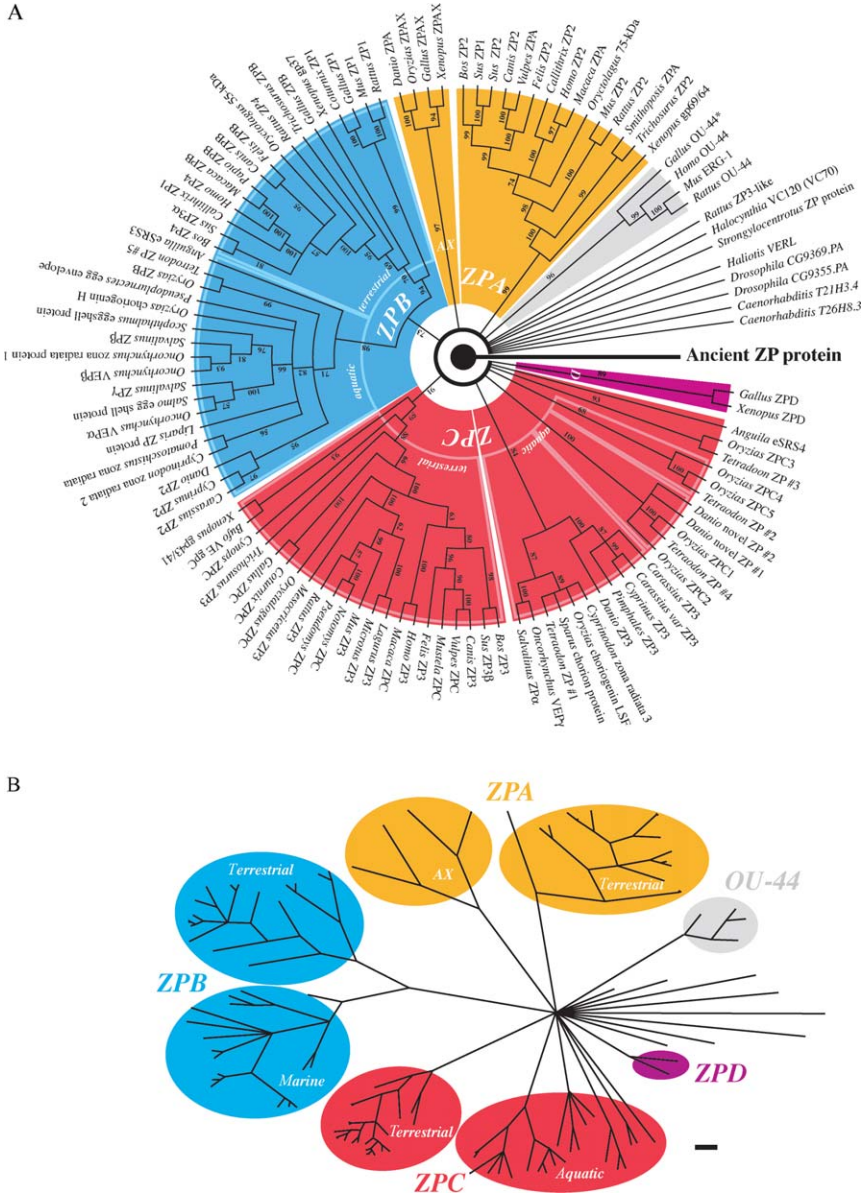
in the zygote. Perhaps, then, it is not surprising that these structures are so varied despite their involvement in a simple event: sperm–egg interaction.

B. Zona Pellucida Homologs

Vertebrate egg ECMs are predominantly composed of proteins with a single ZP domain (Bork and Sander, 1992; Breed *et al.*, 2002; Spargo and Hope, 2003). A ZP domain is about 260 residues in length and contains eight positionally-conserved cysteine residues that disulfide-bond intramolecularly to generate the typical ZP fold (Bork and Sander, 1992). The ZP fold is essential for maintaining the structural integrity of the matrix it is found in, whether the fold is part of the mammalian kidney, pancreas, avian tectorial membrane, or egg ECM (Huynh *et al.*, 2001; Jovine *et al.*, 2002; Leong *et al.*, 2004; Rankin and Dean, 2000). This domain is usually located at the carboxy-terminal end of a highly glycosylated ECM protein. Proper expression and incorporation of ZP proteins in the murine zona requires a conserved hydrophobic patch of residues just upstream of the carboxyl transmembrane domain, presumably to aid in its intracellular trafficking and to promote polymerization (Zhao *et al.*, 2003). Once at the surface of the oocyte, all ZP homologs are cleaved from the cell surface. This enables the polymerization of the ZP proteins in the absence of the steric and electrical hindrances found near the egg surface (Jovine *et al.*, 2002). All ovarian-expressed ZP homologs (Fig. 5; Table III) are cleaved at a site close to the recognition sequence for protein convertase but use a protease distinct from this family of enzymes (Boja *et al.*, 2003). The diffusible amino-terminal ectodomain then rapidly polymerizes with other ZP family members present in the perivitelline space between the oocyte and the follicular cells (Jovine *et al.*, 2002).

Historically, the best-characterized members of the ZP family are those from the protein family's namesake, the *mammalian zona*. This thick ECM (Eakin and Behringer, 2004) (Figs. 1 and 2) gradually accumulates during oogenesis through the synthesis of three major proteins belonging in the ZPA, ZPB, and ZPC subclasses (Bleil and Wassarman, 1980; El-Mestrah *et al.*, 2002; Mate *et al.*, 2003; McCartney and Mate, 1999; Sinowatz *et al.*, 2001; Spargo and Hope, 2003). In most ECMs using ZP family proteins, ZPA heterodimerizes with ZPC and these pairs polymerize into chains, whereas ZPB dimers bridge these protofilaments together via a trefoil motif (Dean, 2004; Moller *et al.*, 1990; Rankin *et al.*, 1999; Wassarman, 1988; Wolgemuth *et al.*, 1984). The major zona constituents, ZPA and ZPC, are freely soluble upon exocytosis (Martic *et al.*, 2004) and are differentially expressed during oogenesis (Epifano *et al.*, 1995), allowing for distinct configurations of the ZPA–ZPC polymers. Release of *Homo* ZPB from the cell surface of a

recombinant expression system, on the other hand, requires coexpression with both ZPA and ZPC (Martic *et al.*, 2004). Surprisingly, ZPB may be dispensable during fertilization: In the absence of murine ZPB, the zona is fully functional at fertilization, albeit morphologically distorted, suggesting that although each ZP member within the egg ECM is structurally and



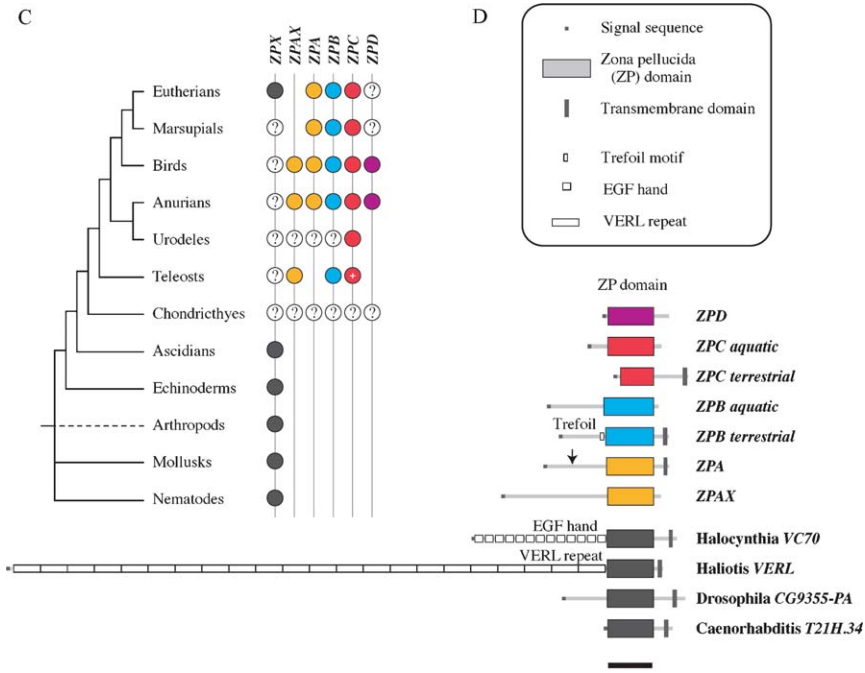


Figure 5 Phylogeny of zona pellucida family members. (A) Unrooted dendrogram of zona pellucida (ZP)–containing proteins in various animal genera. Primarily ZP family members expressed in the ovary were included in the analysis. Most protein sequences used are derived from complementary DNA (cDNA) sequences (see Table III), with the exception of *Drosophila* and *Caenorhabditis* genomic sequences predicted to encode open reading frames. Note that the *Homo* ZP4/B sequence has been annotated as a closer relative to mouse ZP1 (Hughes and Barratt, 1999); no true *Homo* ZP1 sequence has been reported. The four independent subgroups of the aquatic ZPC clade were compiled based on their relationships in other runs using lower bootstrap cutoffs than shown. Numbers represent bootstrap values following 1000 replicates, discarding relationships with a bootstrap value of 60 (60% similar). (B) Unrooted cladogram representing the same phylogenetic data from (A). Bar equals 50 changes. (C) Distribution of different ZP family classes across representative animal orders discussed in the text. (D) Comparison of ZP protein primary structures from the major clades. Colors of the ZP domains correspond to the different clades as in (A). Bar equals 300 residues.

mechanically significant, not all are essential to the fundamental processes of fertilization (Rankin *et al.*, 1999). Thus, the role of ZPB may be to organize the supramolecular structure of the zona in a species-specific fashion (Rankin *et al.*, 2003). Such a function is consistent with the low sequence similarity among the ZPB homologs (Breed *et al.*, 2002; Howarth, 1992; Iwamatsu *et al.*, 1997; Lindsay *et al.*, 2003; Spargo and Hope, 2003).

Molecular evidence from various orders of animals has identified ZP domains within the invertebrate egg ECM as well, although none are

Table III Table of zona Pellucida Homologs Used to Build Phylograms in Fig. 5

Animal	Protein	Accession number
Anguilla japonica	eSRS3	BAA36592.1
	eSRS4	BAA36593.1
	ZP2	BAB21482.1
Bos taurus	ZP3	AAA74385.1
	ZP4	BAB21481.2
Bufo arenarum	VE gpC	AAO25742.1
Caenorhabditis elegans	T21H3.4	AAB65365.2
	T26H8.3	CAB04859.1
Callithrix jacchus	ZP1	CAA71786.1
	ZP2	CAA71740.1
	ZP2	BAA08097.1
Canis familiaris	ZP3	BAA08098.1
	ZPB	AAS78984.1
Carassius auratus	ZP2	CAA96576.1
	ZP3	CAA88838.1
Cyprinus carpio	ZP2	CAA96575.1
	ZP3	CAA88736.1
Coturnix japonica	ZP1	BAB47585.1
	ZPC	BAB86301.1
Cyprinodon variegatus	ZR2	AAT51698.1
	ZR3	AAT51699.1
Cynops pyrrhogaster	ZPC	n/a
	novel ZP #1	CAD60857.1
	novel ZP #2	CAD60856.1
Danio rerio	ZP2	AF095456.1
	ZP3	AF095457.1
	ZPA	AAM34737.1
Drosophila melanogaster	CG9355.PA	NP_511130.2
	CG9369.PA	NP_572747.1
	ZP2	BAA08095.1
Felis catus	ZP3	BAA08096.1
	ZPB	AAA74389.1
	UO-44 (predicted)	XP_421804.1
	ZP1	CAC16087.1
Gallus gallus	ZPAX	CAG27604.1
	ZPB	BAA76739.1
	ZPC	BAA13760.2
	ZPD	BAD13713.2
Haliotis rufescens	VERL	AAL50827.1
Halocynthia roretzi	VC120 (VC70)	BAB72021.1
	UO-44	AAP15461.1
Homo sapiens	ZP2	AAB67599.1
	ZP3	CAA01398.1
	ZP4	AAA74391.1
Lagurus lagurus	ZP3	AAM54024.1
Liparis atlanticus	ZP protein	AAS55643.1
	ZP1	CAA71409.1
Macaca radiata	ZP2	CAA71693.1
	ZP3	CAA57961.1
Mesocricetus auratus	ZP3	AAA37079.1
Microtus brandti	ZP3	AAG18455.1
	ZP1	AAB60507.1
Mus musculus	ZP2	AAA40586.1

Table III *Continued*

	ZP3	AAB18629.1
Mustela erminea	ZPC	AAT67173.1
Notomys alexis	ZPC	AAL79568.1
	zona radiata protein 1	AAD56572.1
Oncorhynchus mykiss	VEPα	AAF71258.1
	VEPβ	AAK97529.1
	VEPγ	AAF71260.1
Oryctolagus cuniculus	55-kDa	AAA31501.1
	75-kDa	AAA31502.1
	ZPC	AAA74392.1
	choriogenin H	AAM91821.1
	choriogenin L	AAM91819.1
	ZPAX	AAN31186.1
	ZPC1	AAN31188.1
Oryzias latipes	ZPC2	AAN31189.1
	ZPC3	AAN31190.1
	ZPC4	AAN31191.1
	ZPC5	AAN31192.1
	ZPC2	AAN31189.1
Papio cynocephalus	ZPB	AAP13260.1
Pimephales promelas	ZP3	AAG28398.1
Pomatoschistus minutus	ZR protein	CAC94865.1
Pseudomys australis	ZPC	AAL79569.1
Pseudopleuronectes americanus	egg envelope	AAC59642.1
	UO-44	AAB71895
	ZP1	BAA24486.1
Rattus norvegicus	ZP2	BAA24487.1
	ZP3	BAA24456.1
	ZP4	AF456325.1
Salmo salar	egg shell protein	CAC04221.1
	ZPα	AAR87393.1
Salvelinus alpinus	ZPβ	AAR87394.1
	ZPγ	AAR87395.1
Sminthopsis crassicaudata	ZPA	AAF73044.1
Sparus aurata	chorion protein	CAA63709.1
Strongylocentrotus purpuratus	ZP protein	n/a
Scophthalmus maximus	eggshell protein	AAP15042.1
	ZP1	AAB33431.2
Sus scrofa	ZP2	BAA08092.1
	ZP3α	AAA50164.1
	ZP3β	BAA08093.1
	ZP #1	CAG11366.1
	ZP #2	CAF93676.1
Tetraodon nigroviridis	ZP #3	CAF93677.1
	ZP #4	CAF97713.1
	ZP #5	CAF92944.1
	ZP2	AAC28737.1
Trichosurus vulpecula	ZP3	AAC28736.1
	ZPA	AAF73043.1
	ZPB	AAF73042.1
Vulpes vulpes	ZPA	AAT37676.1
	ZPC	AAT37677.1
	gp37	BAA90655.1
Xenopus laevis	gp43/41	BAA13117.1
	gp69/64	AAD12172.1
	ZPAX	AF225906.1
	ZPC	AAB39079.1
	ZPD	AAA91467.1

orthologous to the vertebrate ZPs (Fig. 5; Table III). This paradoxical retention of a specific protein fold in the context of completely different proteins implies that specific characteristics of the ZP domain may be critical in all animal egg ECMs. One aspect may be its ability to maintain matrix integrity for proper sperm–egg contact. For example, the ascidian vitelline coat is composed of a dense fibrous network of proteins spatially separated from the egg by a perivitelline space. Of about 20 fucose-rich glycoproteins within the vitelline coat (Rosati, 1985), a single 70-kDa protein purified from the ascidian egg ECM is able to associate with sperm (Lambert, 1989) and to inhibit gamete associations *in vitro* (Matsuura *et al.*, 1995). By mass, this inhibitor is identical to vitelline coat protein VC70, a member of the vertebrate ZP family that contains 12 epidermal growth factor (EGF)-like repeats, a ZP domain, and a carboxy-terminal transmembrane domain (Sawada *et al.*, 2002a). The ZP domain and transmembrane domains are, like vertebrate ZP proteins, separated by a furin-like cleavage site that presumably allows for the separation of ecto-VC70 from the egg surface before its incorporation into the vitelline coat. Analogous to VC70, the abalone vitelline envelope receptor for lysin (VERL) is the major protein present in the vitelline envelope, representing about 30% of the entire mass, and is responsible for sperm associations (Swanson *et al.*, 2001a; Swanson and Vacquier, 1997). This glycoprotein is an oligomer of subunits that each contains 22 nearly homogenized repeats with a carboxy-terminus structurally very similar to the ZP family of proteins, including a predicted ZP domain, a furin-like cleavage sequence, and a transmembrane region positioned in tandem (Galindo *et al.*, 2002; Swanson *et al.*, 2001a). The functional homolog of the abalone lysin receptor is hypothesized to be vertebrate ZPA based on the observation that, upon binding of its sperm-derived ligand lysin, the conformation of VERL and associated proteins is altered to allow the sperm to reach the egg membrane.[2] Finally, a partial cDNA clone of a ZP domain was obtained from a sea urchin ovary expression library (M. L. Leguia and G. M. Wessel, unpublished observations), and genome databases indicate that ZP family homologs are present in echinoderms, dipterans and nematodes (Fig. 5; Table III; data not shown), although the expression profiles are not known. Thus, ZP family members are present in both deuterostomes and protostomes. In most animals, these ZP homologs appear to be used in the construction of the egg ECM, implying that this domain may be critical for gamete recognition.

Assembling the ZP-containing proteins involved in reproduction into a phylogram reveals distinct segregation of all the current, known homologs into five major subclasses (Fig. 5; Table III): ZPA[X], ZPB, ZPC, ZPD, and

[2]The use of the word "receptor" in this chapter refers exclusively to the egg-derived proteins, whereas "ligand" refers to the sperm complement in a receptor-ligand pair.

a more ancient group ZPX. The two largest ZPC subgroups, here listed as independent clades, cluster together 53% of the time (data not shown), whereas the smaller ZPC clades show less similarity to either larger group (Fig. 5B). Inherent in each ZP clade is a separate grouping of orthologs split between terrestrial versus aquatic vertebrates. The primary distinction between these sister clades is the presence or absence of an encoded transmembrane domain: The aquatic ZP genes do not encode transmembrane domains, whereas most terrestrial ZP orthologs do. This characteristic may correspond to the protein's origin in specific animals. For instance, these completely soluble ZPs could be synthesized by somatic cells such as ovarian follicle cells or hepatocytes, and then deposited in the perivitelline space, where they polymerize with nascent oocyte ECM proteins. This is consistent with ZP expression in teleosts, which occurs in the liver and must travel to the ovary via the circulatory system (Chang *et al.*, 1999; Hyllner *et al.*, 2001; Lyons *et al.*, 1993; Murata *et al.*, 1995, 1997; Yamagami *et al.*, 1992). Since the timing of ZP expression likely coincides with vitellogenesis, the soluble ZP proteins can be co-transported to the ovarian follicles with vitellogenic proteins (see Callard *et al.*, 1990a,b; Polzonetti-Magni *et al.*, 2004; Schneider, 1996), thereby minimizing ZP protein precipitation in circulation and maximizing the movement of essential proteins for oogenesis.

What types of selective forces may have resulted in such clear distinctions between the different ZP clades (Fig. 5)? One likely method is functional conservation, specifically the maintenance of orthologs across phlya primarily for their ability to fill a specific role within the ECM. Historically, when mammalian ZP proteins were the best-characterized proteins, this hypothesis held true because *in vitro* studies had clearly defined roles for ZPA, ZPB, and ZPC homologs during fertilization (see Dunbar *et al.*, 1994; Wassarman, 1999). However, these functional distinctions between mammalian ZPA and ZPC subclasses have been questioned. The observation that chimeric *Mus–Homo* zona still exclusively bind sperm homotypically and yield viable embryos suggests that the diversification of these protein sequences is not the sole explanation of their function during fertilization (Dean, 2004; Doren *et al.*, 1999; Rankin *et al.*, 1998, 2003). Similarly, the ZPX subclass contains members from egg ECMs of distantly related animals, including *Drosophila*, abalone (Galindo *et al.*, 2002), sea urchins, ascidians (Sawada, 2002), anurans (Lindsay *et al.*, 2002, 2003), teleosts, and mammals. Members of this group share primary structural organization at the carboxyl-terminus, specifically the ZP domain, but little identity at amino-termini (Fig. 5). Might the ZPX subclass represent the most primitive domains necessary to be an egg ECM protein, relying on other non-ZP proteins to build the ECM? Could the other ZP homologs represent specializations in the vertebrate lineage that correspond to a replacement of non-ZP homologs from the egg ECM?

Compare, for example, the variety of non-ZP proteins and one putative ZPX homolog needed to form the echinoderm vitelline layer (Gache *et al.*, 1983; Haley and Wessel, 2004a; M. L. Leguia, L. M. Varghese, and G. M. Wessel, unpublished observations; Niman *et al.*, 1984), the abalone vitelline envelope (Galindo *et al.*, 2002; Swanson and Vacquier, 1997), or the *Drosophila* eggshell (Nogueron *et al.*, 2000; Pascucci *et al.*, 1996) versus the handful of ZPA, ZPB, and ZPC homologs accounting for nearly the entire vertebrate egg ECM (Breed *et al.*, 2002; Spargo and Hope, 2003). Clearly a single invertebrate ZP homolog is sufficient to achieve monospermic fertilization, so why further limit the diversity of proteins to only ZP homologs? Is the assortment of non-ZP members in invertebrate ECMs extraneous in vertebrates? Might restriction to ZP homologs represent a more efficient process of expression, with diversification of the ZP subfamilies through gene duplication providing the diversity necessary for proper assembly? Or perhaps the ZP domain does not function at all during sperm–egg interactions; rather, it participates in a different essential process during oogenesis or development?

C. Using Homologs to Enhance Structural Diversity

ZP domains interact with each other directly, thereby enhancing the polymerization of ZP-containing proteins (Jovine *et al.*, 2002). Protofilaments formed by such ZP proteins appear to be organized in a conserved fashion, generating a right-handed double helix with frequent branch nodes to create a reticular network. Different ZP sequences can thus interact heterospecifically, allowing for a diverse assembly of proteins within a reticular network of these protofilaments. For example, both a urinary and a cochlear ZP protein are able to incorporate within the mouse zona so long as the entire ZP domain and adjacent carboxy-terminus are unperturbed (Jovine *et al.*, 2002). The auto-aggregation and polymerization of ZP family members provides a distinct advantage for the construction of ECMs because any additional motifs associated with the ZP domain in a particular protein can be incorporated without structurally interfering in matrix assembly. Thus, the quantity of ZP family paralogs expressed by an oocyte may correlate with the variety of different egg ECM ultrastructures and thicknesses represented throughout the animal kingdom. For example, the different ZPB genes might be used to organize the ZPA–ZPC fibers into the discrete layers created in each vertebrate ECM. Compare the thinner nonrefractive internal organization of the mollusk vitelline envelope that is subtly trilaminar in cross-section (Hylander and Summers, 1977; Mozingo *et al.*, 1995) but contains only a single ZPX homolog (Galindo *et al.*, 2002) (Fig. 5) to the clearly birefringent trilaminar organization of the mammalian zona under circularly

polarized light (El-Mestrah *et al.*, 2002; Keefe *et al.*, 1997). The different permutations of homodimers and heterodimers allowed by the mammalian ZPA, ZPB, and ZPC homologs could account for the three essential combinations necessary for the differential zona ultrastructure (Boja *et al.*, 2003; Shabanowitz and O'Rand, 1988). Pulse-chase autoradiography in mice has shown that the zona is constructed radially from the oocyte, laying the inside layers before the outer ones (Wassarman, 1988). Assembly of the zona appears to occur with a stepwise increase in expression of individual ZP homologs, in the chronological order ZPC, ZPB, and ZPA, as observed in the primate *Macaca* (Martinez *et al.*, 1996). Together, these observations predict that ZPC is present throughout the zona, ZPB is necessary for organization of the middle layer, and ZPA is enriched in the most distal layer—all consistent with immunogold labeling of the mouse zona (El-Mestrah *et al.*, 2002). Likewise, the penta-laminar ultrastructure of the teleost chorion (Kudo, 1988) could be built from the increased variety of ZP homologs expressed by these oocytes. More complex structures would require further diversification of the ZPB family or incorporation of other ZP subclasses. This is observed in the complex layering of the *Oryzias* chorion, which incorporates two ZPB paralogs, whereas other teleosts use only one. Alternatively, teleosts may use various ZPCs to distinguish layers of the chorion. Both zebrafish and medaka have at least four ZPC paralogs, allowing each of the chorionic layers to have a different ZPC composition. Combine this with a range of possible ZPX paralogs (Mold *et al.*, 2001), which probably polymerize with the ZPCs in a manner analogous to the ZPA–ZPC fibers formed in mammals, and it is theoretically possible to assemble the various subdomains of a multilaminar chorion without duplicating ZP pairings. As in mammals, this could be achieved by staggering expression of the individual members during the ordered assembly of the chorion from the outside in (Hart *et al.*, 1984; Yamagami *et al.*, 1992).

Is there an evolutionary advantage to the diversification of the teleosts' ZPC subfamily in the absence of the "classic" ZPA (Fig. 5), whereas an equivalent deletion in mammals is lethal (Dean, 2004; Rankin *et al.*, 2001)? Might the selective enrichment and duplication in teleosts for ZPC paralogs, with a subsequent loss in ZPAs, be a consequence of their divergent fertilization technique compared to mammals? The eggs of many fish are released from the ovary immediately into the environment, where they would be fertilized. Unlike internal mammalian fertilization, the fluid that a teleost egg experiences is not isoosmotic to female oviductal fluid. Thus, a sturdier ECM may be necessary to buffer the teleost egg against the immediate osmotic shock. Additional, specialized chorionic layers might provide more protection or at least delay the effects of the new environment until the eggs are fertilized (Gilkey *et al.*, 1978). This delay is critical because the change in osmolarity and cation concentrations is sufficient to spontaneously activate

some fish eggs within 1 min after the change in salinity, thereby preventing fertilization altogether (Lee *et al.*, 1999; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987; Yamamoto, 1954). Of course, the enhanced structural integrity provided by the multiple chorion layers would also provide protection from mechanical forces found in the more turbulent water column into which they are spawned. Thus, the selective advantage of more ZPC proteins in favor of ZPA may be representative of a mechanical advantage: Because the ZP domain alone can dictate how polymerization occurs, heterodimers of ZPC–ZPC paralogs may pack more efficiently than ZPA–ZPC heterodimers. Favoring the incorporation of the smaller ZPC subfamily would thus allow for better compaction of layers than its larger ZPA counterpart, yielding a more resilient ECM.

D. Diversity of Non-ZP Structural Proteins

The exclusive use of ZP homologs in the vertebrate ECM may not be sufficient to restrict monospermic fertilization without the presence of additional physical barriers such as a micropyle or follicle cells. Incorporation of non-ZP family members in the egg ECM for the purpose of restricting the sperm's access to the egg is common in most animals. The process is simplest in vertebrates who, despite an entire egg ECM proper composed of ZP homologs, use oviduct derivatives to enhance monospermy. This process is distinct from the application of jelly, as observed in amphibians and other invertebrates, because the egg ECM itself is modified rather than coated with an additional layer of glycoprotein.

The most common ECM alteration is adsorption of proteins to the matrix, a process that appears to enhance binding efficiency of homospecific sperm to freshly ovulated mammalian zona. For example, oviductal contributions to the ovulated egg are thought to promote fertilization by enlarging the target ECM for sperm, an outcome that is efficacious for both external and internal fertilization. Many externally fertilized eggs are released with additional coats of jelly that serve complementary roles as sperm chemoattractants for the large volume and retardants at close range due to the number of potential sperm that the egg could encounter. Similarly, oviductal contributions are used on internally fertilized eggs, particularly in animals whose egg ECMs are thin such as the marsupial zona (Breed and Leigh, 1990). These additional chemoattractive coats do not directly impact the performance of the ECM, per se, but increase the likelihood of a sperm–egg interaction. Estrogen-inducible oviductal glycoproteins (OGPs) from the mammalian oviduct epithelium, on the other hand, directly intercalate within the zona and can influence how sperm behave toward the ECM. In the absence of OGPs, homotypic sperm binding and capacitation are

reduced, and the rate of polyspermy is enhanced (Buhi, 2002; Buhi *et al.*, 2000; O'Day-Bowman *et al.*, 2002; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997). The participation of the OGP oligosaccharides is critical for maintaining sperm viability, and for regulating sperm–zona binding duration (Buhi, 2002; Dubuc and Sirard, 1995; Rodeheffer and Shur, 2004), suggesting that these sugar moieties may be affecting how sperm respond to zona binding and subsequently determining fertilization success. This is particularly useful in eutherians because estrogen-dependent OGP expression (Buhi, 2002) favors fertilization when the uterus is in the appropriate luteal phase. Thus, the simple presence or absence of a protein adsorbed within an egg ECM can influence fertilization success.

Rather than applying proteins to the ECM *ex post facto*, invertebrates instead intercalate potential sperm-regulating proteins into their ECMs during oogenesis. In contrast to ascidians and abalone, which use ZP family members for homotypic sperm binding, echinoderms employ the egg bindin receptor (EBR1) (Kamei and Glabe, 2003). This 300-kDa glycoprotein contains a metalloendoprotease domain and EBR repeats composed of paired thrombospondin type 1 (TSP-1) repeats and CUB domains (Adams, 1997; Bork and Beckmann, 1993; Kamei and Glabe, 2003). These EBR repeats contain motifs implicated in protein–protein binding and cell aggregation, consistent with the function of EBR1 in gamete interactions: The TSP-1 repeat is a calcium-dependent fold that homo-multimerizes (Adams, 1997); CUB domains may homodimerize or heterodimerize to form carbohydrate-binding pockets or protein-interactive surfaces through anti-parallel β-strands that are stabilized by up to four positionally conserved disulfide bonds (Bork and Beckmann, 1993; Romero *et al.*, 1997; Varela *et al.*, 1997). Together, these binding motifs functionally mimic the polymerizing properties of the ZP domain (Jovine *et al.*, 2002). Like ascidian VC70 (Sawada *et al.*, 2004) and abalone VERL (Galindo *et al.*, 2002; Galindo *et al.*, 2003), sea urchin EBR1 contains a large number of tandem, homogenous repeats that appear to be species specific (Kamei and Glabe, 2003). How such extensive tandem repeats contribute to the function of the invertebrate sperm receptor remains unresolved.

Unlike constituents of the vertebrate ECM, the structural proteins of invertebrate egg ECMs do not appear to be related across animal taxa. The absence of significant molecular data on these proteins, however, limits a rigorous analysis of their phylogeny. Little has been reported on the non–sperm receptor constituents of the ascidian vitelline coat or the abalone vitelline envelope. In the bivalve *Unio*, the single structural glycoprotein gp180 is found throughout the entire vitelline envelope; the sperm receptive gp273 is localized to the crater (Focarelli and Rosati, 1995). Similar roles have been assigned to the products of three genetic loci in the dipteran *Drosophila* that are required for assembly of the vitelline layer and chorion:

the *chorion-36* (*cor-36*), *fs(2)QJ42*, and *defective chorion 1* (*dec-1*) loci (Pascucci *et al.*, 1996; Perotti *et al.*, 2001). The X chromosomal *cor-36* encodes proteins necessary for early chorion formation, whereas *fs(2)-QJ42* encodes the vitelline membrane protein sV23 that is later cleaved into sV17 (Pascucci *et al.*, 1996). The *dec-1* locus encodes an alternatively spliced gene whose products include proteins that share an amino-terminal sequence but vary in their carboxy-terminal motifs (Badciong *et al.*, 2001; Nogueron *et al.*, 2000). The three major *dec-1* proteins—fc106, fc125, and fc177—are expressed differentially during oogenesis and are posttranslationally proteolyzed in a manner that promotes their incorporation into either the vitelline membrane or the endochorion (Nogueron *et al.*, 2000). Finally, two structural protein products of the endochorion, s18 and s36, are synthesized late in oogenesis by the follicle cells to assemble into the chorionic pillars (Pascucci *et al.*, 1996). Most of these *Drosophila* proteins are evenly distributed throughout the eggshell rather than localized at the micropyle, suggesting that these proteins primarily maintain structural integrity rather than participating in gamete interactions, a role supported by the loss-of-function phenotypes associated with the genetic elimination of these loci (Pascucci *et al.*, 1996).

Of the estimated 25 major glycoproteins in the sea urchin vitelline layer (Gache *et al.*, 1983; Longo, 1981; Niman *et al.*, 1984), two have been shown to play a structural role: p160, a 160-kDa, transmembrane vitelline post protein clustered at the tips of microvilli (Haley and Wessel, 2004a) and rendezvin^{VL}, a splice-variant of the oocyte-specific rendezvin gene (Wong and Wessel, 2006). Like the sperm-receptive EBR1, both of these proteins contain CUB domains that may aid assembly and maintenance of the vitelline layer (Bork and Beckmann, 1993; Romero *et al.*, 1997). This enrichment of CUB domains in the sea urchin egg ECM is consistent with the functional sensitivity of the vitelline layer to disulfide bond reducing agents (Aketa and Tsuzuki, 1968). Expression of p160, however, is not oocyte specific. p160 links the vitelline layer to the egg plasma membrane. Cleavage of this linker protein must occur in order for the vitelline layer to separate from the egg surface during the establishment of one sea urchin block to polyspermy. It reappears before the second cell division and persists on the apical ectodermal cell surfaces, suggesting that p160 is not solely involved with the permanent block to polyspermy (Haley and Wessel, 2004a). Instead, this matrix protein may have a more general role in retaining the intimacy between a cell and its ECM. Rendezvin^{VL} (Wong and Wessel, 2006), on the other hand, is retained in the modified egg ECM after it lifts off the egg surface, serving as a core scaffold protein from the vitelline layer that organizes fertilization envelope assembly (Carroll *et al.*, 1986; Kay and Shapiro, 1985; Ruiz-Bravo *et al.*, 1986). Thus, unlike vertebrate eggs, no

clear selective pressure is known to exclude a particular family of structural proteins from the invertebrate egg ECM. Instead, the proteins retained over time appear to be ones that maintain structural integrity of the respective ECM.

E. Divergence or Convergence in Egg ECM Phylogeny?

When compared to the extreme diversity of egg ECMs, the assortment of traits shared among all animal eggs suggests that the selective pressures of speciation that favor divergence of ECM morphology have molded how the ECM evolves. For example, architectural diversity in chiton egg ECM has been proposed as a gauge of evolutionary relatedness among species within the phylogeny of this animal (see Buckland-Nicks and Hodgson, 2000). The organization and articulation of the chiton hull, like anatomical traits, have been scrutinized in order to taxonometrically categorize different species into specific clades. Could such a critical comparison of different egg ECM attributes be used to evaluate the phylogeny of animals? If so, what might this indicate about the relationship between the egg and speciation?

Looking at both the ultrastructural and the molecular components of the egg ECM among taxa, we see a gradual simplification in ECM structure during the progression from protostomes (e.g., decapods, dipterans, and mollusks such as chiton and bivalves) to deuterostomes (e.g., echinoderms, ascidians, and vertebrates) (Fig. 1). The elaboration of microvillar pores in mollusks gives way to a more uniform structure that is devoid of extraneous articulations, as found in fish chorions and the mammalian zona. The palette of proteins utilized is also significantly narrowed, from an array of up to 25 proteins in the echinoderm vitelline layer (Gache *et al.*, 1983; Niman *et al.*, 1984), including a ZP family member (M. L. Leguia, L. M. Varghese, and G. M. Wessel, unpublished observations), to the exclusive use of ZP homologs by most vertebrates. Are these trends merely coincidence, and hence counter to the molecular diversity seen elsewhere in these animals, or do they represent the influence of various selective forces implicit to reproduction? For example, might the diversity observed in egg ECM morphology reflect mechanisms the egg uses to minimize sperm–egg ratios? If so, could these also be related to differences between external and internal fertilization, independent of the position an animal holds on the phylogenetic tree? Broadcast spawners, including most mollusks and echinoderms, often dilute their gamete populations in the ocean. By elongating the microvilli through a sperm-receptive ECM, the eggs of these animals significantly increase their chances of being fertilized. In contrast, the concentrated deposition of sperm

into the uterus or oviduct of internally fertilized animals such as mammals greatly increase the probability that the few eggs ovulated per cycle will be fertilized. To counter the potential onslaught of sperm in these animals, a simple yet substantial ECM is employed to mechanically dilute the number of simultaneous egg encounters.

Numerous exceptions to this simplified comparison of external versus internal fertilization exist, however. Decapod eggs are ovulated with a thick unadorned vitelline envelope surrounding decapod eggs (Talbot and Goudeau, 1988). This ECM most closely resembles the mammalian zona in its simplicity yet requires a significantly greater effort from the sperm to penetrate (Brown and Humphreys, 1971; Goudeau and Becker, 1982; Tsai and Talbot, 1993). The utility of creating such a modification is linked to the method of fertilization employed by decapods, involving the simultaneous release of eggs from the gonophore, whereas sperm are released from storage in the thelycum into the seawater (Lindsay *et al.*, 1992a; Talbot and Goudeau, 1988). This process results in a single decapod egg encountering a large number of sperm at once, hence the selection of a more substantial ECM to "filter" sperm quantities. Another exception to the internal-versus-external hypothesis involves the independent evolution of micropyles in animals such as the mollusk *Unio* (Focarelli *et al.*, 1988), dipterans (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976), and teleosts (Hart, 1990). The micropyle physically restricts the number of sperm capable of binding to an egg, implying that it is particularly useful for limiting sperm–egg ratios. This is likely its role in *Unio*, whose fertilization occurs through the release of gametes into the seawater, followed by concentration of sperm/egg ratio by "sucking" them into the suprabrachial chambers of the gills (Focarelli *et al.*, 1988). Similarly, fish sperm are released externally in concert with a clutch of eggs, so the ratio of sperm to eggs is significantly higher than compared to other broadcast spawners such as echinoderms or ascidians. In contrast, sperm numbers are reduced in internally fertilized dipterans through the spermathecae, a sperm storage organ adjacent to the oviduct that limits the release of sperm to a few at a time (Neubaum and Wolfner, 1999). In these animals, a micropyle is not necessary for limiting sperm–egg encounters, but has still been retained. This likely reflects a separate role for the micropyle, perhaps as a way to ensure that fertilization and embryonic gas exchange occur in the presence of a chorion selected for minimizing desiccation of the embryo following terrestrial oviposition (Li *et al.*, 1996). Thus, while the morphology of an egg ECM generally appears to be governed by the range of sperm concentrations that an egg may encounter, it is not a hard-and-fast rule. Rather, the ecological challenges of reproduction certainly influence the evolutionary selection on the egg ECM.

III. Cortical Granules

Cortical granules (CGs) are secretory vesicles synthesized during oogenesis and released following gamete fusion (reviewed in Cran and Esper, 1990; Wessel *et al.*, 2001). These oocyte- and egg-specific organelles are abundant, ranging from 8000/egg in mice (Ducibella *et al.*, 1994) to 15,000/egg in sea urchins (Laidlaw and Wessel, 1994), and are always enriched within the outermost region of the egg's cortex, subjacent to the plasma membrane. The shear number of granules per egg and their secretion en masse following fertilization implies that CG contents significantly alter the local extracellular environment upon exocytosis, easily transforming a sperm-competent egg ECM into a physical barrier against additional sperm. The mammalian zona, for example, does not undergo any significant histological changes, although biochemical modifications occur following CG exocytosis that reduces its affinity to sperm (Hoodbhoy and Talbot, 1994) (Figs. 1 and 2). In echinoderms, on the other hand, CG exocytosis causes a physical separation of the vitelline layer from the egg plasma membrane, resulting in the formation of a fertilization envelope of approximately fourfold greater surface area than the original vitelline layer (Figs. 1 and 2; reviewed in Kay and Shapiro, 1985). The force necessary for this lifting is thought to be a result of the hydration of mucopolysaccharides derived from the echinoderm CGs (Schuel *et al.*, 1974) but also requires proteolysis of egg–ECM linkages to ensure full release of the vitelline layer (Haley and Wessel, 1999).

The range of ECM modifications that occur as a consequence of CG exocytosis suggests that the contribution of these organelles to the block to polyspermy is both enzymatic and structural in nature. In this section, we review the major constituents of animal CGs (Table II) and briefly describe the functions of some of these proteins after fertilization. A more contextual description of these CG components, however, can be found in Section IX, later in this chapter.

A. Cortical Granule Enzymes

Historically, proteolysis is the one enzymatic activity considered to be of general importance in the block to polyspermy (Boldt *et al.*, 1988; Hatanaka *et al.*, 1992; Hoodbhoy and Talbot, 1994; Moller and Wassarman, 1989). The types of proteases involved may be quite variable among animals, but cleavage or removal of the sperm receptor from the ECM is thought to be a common outcome. Serine protease activity common to the trypsin family has been reported in sea urchins (Haley and Wessel, 1999, 2004b) and mammals (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 1994). The CG serine protease

(CGSP1) is the *only* protease activity detected in the CGs of the sea urchin (Carroll and Epel, 1975b; Haley and Wessel, 1999). This is significant because many functions have been ascribed to this protease, including removal of the sperm receptor, modification of the vitelline layer, and even egg activation (Carroll and Epel, 1975a; Carroll and Jaffe, 1995; Runnstrom, 1966; Vacquier *et al.*, 1973), yet it appears that only CGSP1 is responsible for this extensive range of activities. This CG protease appears to be selective, suggesting that it has specific roles and/or regulators that are not compatible at any other time during development (Haley and Wessel, 1999). For example, it is known that CGSP1 cleaves the vitelline post protein p160, releasing a diffusible 85-kDa ectodomain from its transmembrane domain (Haley and Wessel, 2004a), thus permitting the physical detachment of the vitelline layer from the egg surface during the formation of the fertilization envelope (Kay and Shapiro, 1985). As in sea urchins, one function of mammalian trypsin-like proteases is to facilitate the loss of sperm-binding capabilities on the ECM (Hoodbhoy and Talbot, 1994). The source of one such serine protease activity has been localized to CGs (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 1994), but no further information is known about this mammalian subclass of enzymes.

Other classes of protease activity have also been traced to CGs or to organelles with similar behaviors following fertilization. For example, aminopeptidase activity is detectable around the *Mytilus* egg only after fertilization has occurred, implying that its release is due to CG exocytosis, even though the release of these granules is not documented in detail for this animal (Togo and Morisawa, 1997; Togo *et al.*, 1995). Cathepsin-like substance (CLS) is derived from *Cyprinus* CGs and is presumed to target proteins in the teleost chorion (Chang *et al.*, 1999). Its co-migration with other chorion proteins suggests that any proteolytic activity related to CLS may enhance overall protein aggregation (Chang *et al.*, 1998). On the other hand, *Xenopus* zinc-dependent protease cleaves ZPA, causing a steric shift in the protein fold that results in the hardening of the vitelline envelope (Lindsay and Hedrick, 2004). This conformational change in ZPA is the major proteolytic alteration observed in anurans and eutherians following fertilization, suggesting that zinc-dependent protease orthologs may be functionally conserved in terrestrial vertebrate zygotes (Bauskin *et al.*, 1999; Doren *et al.*, 1999; Moller and Wassarman, 1989; Moos *et al.*, 1994; Shabanowitz and O'Rand, 1988; Tian *et al.*, 1999). This is consistent with the reported insensitivity of mammalian ZPA cleavage to serine protease inhibitors (Hoodbhoy and Talbot, 1994).

Glycosidases also originate from CGs of many taxa. The first CG protein identified from sea urchins was β-1,3-glucanase, an enzyme whose primary substrate is found in algae, not the animal egg itself (Epel *et al.*, 1969). This enzyme has an unusual heritage: Its sequence similarity to bacterium suggests

that sea urchin β-1,3-glucanase was acquired by horizontal gene transfer (Bachman and McClay, 1996). The function of this glycosidase during fertilization is not known, although its accumulation in the perivitelline space following CG exocytosis suggests its target substrates may include constituents of the egg ECM and the perivitelline space (Wessel *et al.*, 1987, 2001). *N*-acetylglucosaminidase activity, on the other hand, has been detected from *Xenopus* (Prody *et al.*, 1985) and mouse CGs (Miller *et al.*, 1993a). Given the participation of oligosaccharides enriched in *N*-acetylglucosamine during vertebrate sperm–egg interactions (Miller *et al.*, 1993b; Vo *et al.*, 2003; see also Section V, later in this chapter), the hypothesized role of this CG derivative is in the abolition of sperm-binding sites within the egg ECM. Whether this occurs remains untested because there has been no definitive report indicating the release of specific sugar residues following fertilization in vertebrates.

One outcome conserved throughout most animal orders is the mechanical transformation of the egg ECM from a flexible network of glycoproteins into a hardened shell. This physical modification is often associated with enzymatic activity detected following CG exocytosis, although the enzyme(s) responsible varies. As previously mentioned, proteolysis of anuran ZPA by a zinc-dependent protease derived from CGs is sufficient to induce hardening of the vitelline envelope (Lindsay and Hedrick, 2004). A similar hardening role is suspected for teleost CLS because detergent-resistant complexes containing *Cyprinus* ZP proteins and other CG derivatives results from this enzyme's activity (Chang *et al.*, 1998, 1999). Thus, the use of protease-dependent hardening of the egg ECM may be common to all vertebrate zygotes. Yet, the exact process that such protease-dependent hardening follows has not been deciphered; only correlations have been made between the changes in physiochemical properties of the ECM in the presence or absence of protease activity (Lindsay and Hedrick, 2004).

A distinct mechanism of matrix hardening involves the formation of covalent bonds between ECM constituents. Such changes typically result from transglutaminase and peroxidase activity, both of which have been found associated with eggs at fertilization. Transglutaminase involvement in the conversion of the egg ECM following fertilization has been reported for decades, as evidenced by the isopeptide amide bonds between glutamine and lysine left in its wake, a process that fuses adjacent proteins to one another (Battaglia and Shapiro, 1988; Cariello *et al.*, 1994; Chang and Huang, 2002; Chang *et al.*, 2002; Lee *et al.*, 1994; Mozingo and Chandler, 1991; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). This extended family of calcium-dependent enzymes generates intermolecular bonds through a cysteine-protease–like catalytic mechanism (reviewed in Lorand and Graham, 2003; Nemes *et al.*, 2005). Only a few candidate egg-derived proteins have been cloned from ovary RNA: one from the teleost *Cyprinus* (Chang *et al.*, 2002) and two from the sea urchin *Strongylocentrotus* (J. L.

Wong and G. M. Wessel, unpublished observations). Yet the subcellular source of this enzyme does not appear to be CGs, even though its activity is intimately associated with CG exocytosis. For example, a zymogenic form of a sea urchin transglutaminase activity has been reported at the egg surface (Battaglia and Shapiro, 1988). This sea urchin transglutaminase is activated within 2 min following CG exocytosis, establishing isopeptide bonds necessary for stabilizing the initial fertilization envelope assembly (Battaglia and Shapiro, 1988; Cariello *et al.*, 1994; Kay and Shapiro, 1985). CG exocytosis is a prerequisite for sea urchin egg transglutaminase activity, suggesting that the zymogenic form is activated by CGSP1 proteolysis, consistent with the observation that its morphological changes are blocked by inhibitors of either transglutaminase or serine proteases (Mozingo and Chandler, 1991). Such a cascade of events is consistent with the requisite proteolytic activation of homologs such as transglutaminase type 2 and plasma coagulation factor XIIIa (reviewed in Lorand and Graham, 2003; Nemes *et al.*, 2005). It is also reminiscent of the activity profile reported for chorionic transglutaminase of *Cyprinus*, which is active only when collected in CG exudate (Chang *et al.*, 2002), and *Tribolodon*, which is activated by a serine-protease–like sialoglycoprotein from CGs (Kudo and Teshima, 1998). In all reported cases, transglutaminase activity is enriched at the periphery of the extracellular chorion (Chang *et al.*, 2002; Kudo and Teshima, 1998; Oppenberntsen *et al.*, 1990). Yet, the *Cyprinus* egg-derived family member (Chang *et al.*, 2002), an ovary-expressed echinoderm transglutaminase originally identified in *Paracentrotus* blastula (Zanetti *et al.*, 2004; J. L. Wong and G. M. Wessel, unpublished observations), and one *Strongylocentrotus* homolog with 35% primary sequence identity to the *Homo* vertebrate type I/keratinocyte isoform (J. L. Wong and G. M. Wessel, unpublished observations) do not possess a signal peptide downstream of their putative initiating methionine—a characteristic of other secreted transglutaminases (Lorand and Graham, 2003). Thus, how these enzymes end up in the extracellular space where their target substrates reside remains a mystery.

Peroxidases catalyze the formation of dityrosine bonds between adjacent proteins through a free radical intermediate (Gross, 1959). Although peroxidase activity is present in mouse CGs and in the perivitelline space following fertilization (Gulyas and Schmell, 1980a,b), the observation that a zinc-dependent protease is sufficient for hardening the amphibian ECM (Lindsay and Hedrick, 2004) suggests that this peroxidase activity may be supplemental and/or specific to murids. Reports of dityrosine residues in the dipteran chorion imply that peroxidases are also responsible for ECM maturation in these invertebrates (Li *et al.*, 1996; Mouzaki *et al.*, 1991). Likewise, peroxidase activity is present in *Tribolon* and *Cyprinus* chorion, in layers that participate in fertilization envelope formation, but whether the activity derives from CGs has yet to be determined (Kudo, 1988). On the other hand,

CG-derived ovoperoxidase is definitely required for the hardening of the sea urchin fertilization envelope, specifically at the intercast regions found between the microvillar caps identifiable within the vitelline layer scaffold (Deits *et al.*, 1984; Foerder and Shapiro, 1977; Hall, 1978; LaFleur *et al.*, 1998; Mozingo and Chandler, 1991; Nomura and Suzuki, 1995; Showman and Foerder, 1979). This myeloperoxidase-like family of enzymes is specifically transcribed in oocytes and packaged into CGs. Upon secretion, it is separated from the egg surface by a tethering protein that keeps it associated with the elevating vitelline layer, thereby restricting its cross-linking activity to the ECM undergoing modification (Mozingo *et al.*, 1994; Somers *et al.*, 1989). In addition to their proposed roles immediately following fertilization, peroxidase activity may act as a temporary antimicrobicide (Klebanoff *et al.*, 1979; Kudo, 1988) whereas transglutaminase activity may alter fertilization envelope adhesivity (Chang *et al.*, 2002; Cheng *et al.*, 1991).

Why is proteolysis sufficient for the hardening of the egg ECM in mammals and anurans, whereas both peroxidase and transglutaminase are required in fish and sea urchins? Consider the differences in environmental complexity that each embryo experiences. Eutherian cumulus cells help buffer the embryo from mechanical forces within the oviduct, at least until implantation. Similarly, the formation of a second calcium-induced precipitate adjacent to the anuran jelly may provide a barrier between the embryo and the environment. But teleosts and sea urchin embryos do not acquire such a supplemental shell. Might the use of both peroxidase and transglutaminases ensure that a hardened barrier will be in place within the first 10 min of gamete fusion? If so, then what other environmental factors could be significantly influencing the selection of enzymatic activities employed during the construction of a physical block to polyspermy?

B. Nonenzymatic Proteins of Cortical Granules

The major protein mass released from an animal's CGs is nonenzymatic, yet it significantly contributes to the ECM remodeling required to establish a permanent block to polyspermy. The most is known about this process in echinoderms and anurans, whose permanent blocks to polyspermy are observable by low-power light microscopy. For example, of the 12 proteins derived from sea urchin CGs (Wessel *et al.*, 2001), the major proteins visible by Coomassie staining are directly incorporated into the fertilization envelope (Wong and Wessel, 2004). The total mass of these proteins account for the fourfold increase in surface area observed as the vitelline layer is dramatically "lifted" from the egg surface during CGs exocytosis (Runnstrom, 1966; Shapiro *et al.*, 1989). Similarly, part of the permanent block in the anuran *Xenopus* is clearly visible as the accumulation of a

refractive CG-derived precipitate between the vitelline envelope and inner jelly (J1) layer (Grey *et al.*, 1974; Shapiro *et al.*, 1989). Using electron microscopy, the contents of decapod CGs can clearly be seen accumulating within the vitelline envelope over the hour-long exocytotic process (Talbot and Goudeau, 1988). Thus, structural proteins that transform the egg ECM are clearly released from CGs. But how conserved are these nonenzymatic content proteins across animal taxa?

The dramatic formation of the sea urchin fertilization envelope is a rich source of raw material for biochemical analysis of CG content proteins. Consequentially, the most is known about the structural proteins responsible for modifying the vitelline layer (Wong and Wessel, 2004). Five genes encode the majority of CG proteins that comprise the fertilization envelope, including *proteoliaisin* (Somers and Shapiro, 1991; Somers *et al.*, 1989), *SFE-1* (Laidlaw and Wessel, 1994; Wessel *et al.*, 2000), *SFE-9* (Laidlaw and Wessel, 1994; Wessel, 1995), and *rendezvin* (Wong and Wessel, 2006). The proteins can rapidly self-assemble within the vitelline layer scaffold to form the fertilization envelope within minutes of their release. This biochemical property is likely due to the tandem arrangement of common protein-binding motifs in all these proteins. Rendezvin contains an abundance of CUB domains that likely participate in protein–protein interactions, perhaps with other CUB domain proteins found in the vitelline layer (Wong and Wessel, 2005b). The high percentage of tyrosine residues in rendezvin^{CG} also suggests that it is a target of ovoperoxidase activity (Wong and Wessel, 2006). Proteoliaisin, SFE-1, and SFE-9, on the other hand, are abundant in low-density-lipoprotein receptor type A (LDLrA) repeats, containing up to 28 tandem LDLrA repeats, in some orthologs (Wessel, 1995; Wessel *et al.*, 2000; Wong and Wessel, 2004). In addition to LDLrA repeats, SFE-1 and SFE-9 contain low-complexity repeats whose sequence and length differ between orthologs (Wong and Wessel, 2004), suggesting that their binding partners may be rapidly changing. Isolation of an SFE-9 ortholog from the starfish *Asterina* (J. L. Wong and G. M. Wessel, unpublished observations), a distant echinoderm ancestor that split from the sea urchin more than 500 million years ago (Hinman *et al.*, 2003), exhibits 45% primary sequence identity to the sea urchin orthologs over the 240 residues initially cloned. Further identification and characterization of other echinoid orthologs such as SFE-9 will provide a great deal of information pertaining to selective pressures influencing the evolution of nonenzymatic CG proteins.

Formation of the anuran fertilization (F) layer also requires the deposition of a significant quantity of CG protein. As in echinoderm fertilization envelope formation (Bryan, 1970b), assembly of this layer requires high concentrations of extracellular calcium (Nishihara *et al.*, 1986). The major protein contributed to the F layer is the *Xenopus* CG-derived lectin XL35/CGL (Chamow and Hedrick, 1986; Chang *et al.*, 2004; Lee *et al.*, 1997; Nishihara

et al., 1986; Quill and Hedrick, 1996). This oligosaccharide-binding protein favors sulfated sugars found along the inner surface of the J1 jelly layer (Bonnell *et al.*, 1996; Tseng *et al.*, 2001), sterically blocking the penetration of sperm into the perivitelline space (Hedrick and Nishihara, 1991; Larabell and Chandler, 1991). A human ortholog of XL35/CGL, HL-1, has been found, but it is not expressed in the ovary (Lee *et al.*, 2001). This observation suggests that, like members of the ZP family of proteins (see Section II.B and II.C, earlier in this chapter), XL35/CGL may be a specialized member of a more ubiquitous lectin family that happens to function at fertilization.

Other than anuran XL35/CGL, the nonenzymatic contents of vertebrate CGs that contribute to the permanent block to polyspermy are not known. The primary reason is a relatively low abundance of protein per CG compared to animals with more significant morphological changes, such as echinoderms and anurans. Even though the teleost chorion is a substantial structure assembled prior to fertilization, it has been difficult to isolate proteins derived from the CGs because most of the content proteins remain in the perivitelline space, leaving only a small fraction that interact with the ZP proteins of the egg ECM (Hart, 1990). Like echinoderms (Runnstrom, 1966; Santella *et al.*, 1983; Wessel *et al.*, 2001), teleost CGs have separate regions of electron density that appear to contain different acidic glycoproteins based on the regional differences in lectin affinity (Hart and Donova, 1983; Hart, 1990). Three *Cyprinus* CG proteins contribute to the postfertilization chorion in a calcium-dependent fashion. These include fertilization envelope outer layer protein-1 (FEO-1) (Chang *et al.*, 1999), fibroin-like substance (FLS) (Chang and Huang, 2002), and cystatin (Chang *et al.*, 1998). FEO-1 is an alternatively expressed protein homologous to a chicken vitelline membrane protein that settles at the outer layer of the fertilization envelope (Chang *et al.*, 1999, 2002). FLS is enriched in glycine, alanine, and serine residues, suggesting that it is extremely elastic and may be responsible for the inherent flexibility of the outer fertilization envelope layer (Chang and Huang, 2002). During its discharge, FLS is believed to associate with cystatin, an inhibitor of cysteine proteases (Chang *et al.*, 1998), and CLS, together forming a complex that facilitates the trapping of FEO-1 within the outer fertilization envelope (Chang *et al.*, 2002). This four-protein complex is directly cross-linked by transglutaminase to the ZP orthologs within the chorion, ensuring the complete transformation of the teleost ECM (Chang *et al.*, 2002).

The precipitation of CG contributions within the egg ECM following fertilization is calcium dependent in three animals (Bryan, 1970b; Chang and Huang, 2002; Chang *et al.*, 2002; Nishihara *et al.*, 1986). Why calcium rather than another cation? Does this divalent cation somehow control the behavior of these proteins? Based on optimal *in vitro* monospermic fertilization of porcine eggs in culture media, an estimated calcium concentration is 1.5–2.0 mM in oviductal fluid (Herrick *et al.*, 2003). Eggs of amphibians and

fish laid in freshwater are exposed to roughly equal concentrations of magnesium and calcium (about 3–5 mM of each) under optimal survival conditions (Godfrey and Sanders, 2004). Thus, the use of one cation versus another in freshwater spawners does not depend on bioavailability. Seawater, on the other hand, contains 53 mM of magnesium versus 10 mM of calcium, making the bioavailability of calcium fivefold less than magnesium. Yet the conservation of calcium-binding LDLrA repeats and EF hands in sea urchin CG proteins implies that the larger ion has been selected for its structural contributions to protein folding (Wong and Wessel, 2004). A similar situation could be predicted for saltwater fish, which experience the same concentrations of magnesium and calcium.

In most animals, CG exocytosis also results in the deposition of proteins essential for embryogenesis. For example, hyalin is released from the echinoderm CGs upon exocytosis (Matsunaga *et al.*, 2002; Vater and Jackson, 1990). This large calcium- and magnesium-sensitive glycoprotein constitutes the bulk of the hyaline layer (Bryan, 1970a; Chandler, 1991; Rimsay and Robinson, 2003; Wessel *et al.*, 1998) and serves as the main substrate for blastomere attachment and cell signaling (Matsunaga *et al.*, 2002; Wessel *et al.*, 1998). Epitope similarities to echinoderm hyalin are also found in vertebrate CGs, including mice and hamsters (Hoodbhoy *et al.*, 2000, 2001). This eutherian homolog was first described by its cross-reactivity with the polyclonal antiserum ABL2, which recognizes CG contents of many species (Hoodbhoy *et al.*, 2001). ABL2 antibodies specifically detect the *Mesocricetus* CG proteins p62 and p56 (Hoodbhoy *et al.*, 2001) and a 75-kDa *Mus* CG protein (Pierce *et al.*, 1990), all found in the CG envelope, an embryonic matrix found in the perivitelline space following fertilization (Figs. 1 and 2). The teleost analog to this family of embryonic signaling molecules is hyosophorin, a heterogeneous protein that possesses high calcium affinity and is deposited along the membrane surface following CG secretion (Hart, 1990; Tsao *et al.*, 1999). Like echinoderm hyalin, hyosophorin is enriched with oligosaccharides and has a tendency to attract water, facilitating hydration of the perivitelline space (Tsao *et al.*, 1999). Its highly repetitive domains are also thought to participate in the gelation process of its embryonic matrix (Tsao *et al.*, 1999). The presence of hyalin-like proteins in the CGs of most deuterostomes reinforces the dual properties of these organelles, participating in both the rapid renovation of the egg ECM and deposition of a new extraembryonic one.

IV. Courtship, Gamete Attraction, and Sperm/Egg Ratios

Selection of a particular type of block to polyspermy depends on a number of factors, including the type of mating used by an animal and the environment within which fertilization occurs. Motile animals have significantly more

mating options than their sessile counterparts, as reflected in the diversity of mating strategies used by mobile organisms. Generally, two types of insemination are employed that can be distinguished by the site of gamete release, "spawning" and "copulation." Subsequent fertilization can thus occur externally or internally, relative to a female's anatomy. *Spawning* is the simplest method of mating, whereby an individual's gametes are released into the environment (usually aquatic) in hopes that a complementary gamete will be nearby. Sessile animals frequently employ this technique, although fish, amphibians, echinoderms, and some mollusks also spawn. Mating choice is often not a consideration in spawning by sessile animals because gamete interaction happens by chance. Their meeting is enhanced by pheromones, which encourage a local female and male spawn at overlapping times, but the union of the individual gametes is dependent on luck. Some vertebrates do, however, actively seek a mate before spawning their gametes into the environment, thereby increasing the probability of fertilization because of the higher sperm/egg ratios (O'Rand, 1988). Fertilization of spawners often occurs externally, although some species such as the bivalve *Unio* draw a mixed population of gametes into a respiratory chamber to undergo fertilization and later release the zygotes into the ocean (Focarelli *et al.*, 1988). *Copulation*, on the other hand, requires deposition of sperm by the male into the female reproductive tract. Individuals of the mating pair involved often seek one another by choice and must come in close contact to complete the transfer of sperm. The sperm is often stored in the female reproductive tract until needed, and the female can use the sperm for either internal or external fertilization (Neubaum and Wolfner, 1999). Internally, sperm-release into the oviduct is controlled by female anatomy and is timed to optimize the chances that the free sperm will encounter a freshly ovulated egg. Externally, sperm are released into the water at the same time as the eggs. Mammals, birds, dipterans, and nematodes usually copulate, with subsequent internal fertilization. Decapods commonly copulate to transfer sperm, but the female simultaneously releases both gametes into the environment when she is ready. Therefore, mating styles and fertilization techniques are interchangeable. In this section, we explore the impact these behaviors have on successful monospermic fertilization.

A. Behavior, Anatomy, and Monospermy

Polyspermy can be achieved in normally monospermic eggs by simply increasing the number of sperm encountering an egg (Alliegro and Wright, 1983; Grey *et al.*, 1982; Lambert *et al.*, 1997; Snook and Markow, 2002; Yu and Wolf, 1981). Several mechanisms are employed by animals to reduce sperm/egg ratios, including the type of insemination and timing of gamete

release. Broadcast spawners naturally minimize sperm/egg ratios by diluting gametes in the environment, a method that does not guarantee that each gamete will encounter its complement. Hence, the more gametes produced and spawned at a given time, the better an individual's success in reproduction. Yet making such large quantities of gametes can prove to be a substantial burden to an animal's energy resources. Strategies that enhance gamete interaction among broadcast spawners with less energy expenditure include locally concentrating the gametes before releasing the zygotes into the open water, as observed in the bivalve *Unio* (Focarelli *et al.*, 1988). Reproductive success of the spawning male also requires a prime location and proper timing to ensure that the largest, fittest eggs are inseminated with his sperm rather than his competitor's (Marshall *et al.*, 2004). An alternative strategy is to actively seek a mate, thereby ensuring that a higher percentage of gametes will be fertilized by one male due to an enhanced sperm/egg insemination ratio (O'Rand, 1988). Of course, one caveat to this behavioral modification is that the ratio is consistently too high, thus favoring polyspermy. Fish and amphibians have minimized this deleterious outcome by incorporating mating choice with external egg insemination. This partially dilutes the sperm to optimize the sperm/egg ratio, but this does not preclude the selection of additional mechanisms on a per-egg basis that further modify the sperm/egg ratio (see Section IV.B and IV.C, later in this chapter).

Deposition of sperm within the female reproductive tract through copulation is the most efficient way a male can enhance reproductive success, but the outcome of such a high sperm/egg ratio could be devastating for the progeny. Consequently, female anatomy has evolved methods to cope with this plethora of sperm, namely by controlling the release of sperm per ovulation. Like other aquatic vertebrates, decapods use the additional dilution factor provided by spawning into the environment to reduce the number of sperm available per egg (Talbot and Goudeau, 1988). Terrestrial animals, on the other hand, control the quantity of sperm released from storage through reproductive organs (Neubaum and Wolfner, 1999). For example, mammalian oviductal crypts store sperm along the oviduct epithelium until ovulation occurs, when only fertilization-competent sperm are released into the lumen (Eisenbach, 1999; Rodger and Bedford, 1982a). Spermathecae, a sperm storage organ that can release only from one to a few sperm per ovulation, are used by nematodes (Singson, 2001), dipterans (Fitch *et al.*, 1998; Snook and Markow, 2002; Turner and Mahowald, 1976), and urodeles (Elinson, 1986) to limit the sperm/egg ratio within the oviduct. Again, further specializations in the morphology or biochemistry of the egg ECM can significantly pare down the sperm/egg ratio almost to unity to maximize successful fertilization (see Singson, 2001).

B. Egg Attraction

Successful fertilization requires the union of two compatible gametes, a conspecific pairing of sperm and egg. Behavior and mating are crude methods to ensure that individuals of the same species will copulate; broadcast spawning has little guarantee of success unless the timing and distance of spawning is optimal. To overcome some of these negative influences, eggs use soluble chemical factors to guide conspecific sperm toward them. Thus, sperm have been selected for their phenotypic response toward a chemoattractant gradient (Brown, 1976; Eisenbach, 1999; Elinson, 1986; Garbers *et al.*, 1986; Hansbrough and Garbers, 1981; Hirohashi and Vacquier, 2002a,b; Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Oda *et al.*, 1995, 1998; Olson *et al.*, 2001; Ramarao *et al.*, 1990; Riffell *et al.*, 2002, 2004; Suzuki *et al.*, 1988; Zatylny *et al.*, 2002).

Most gametes are released into aqueous environments, making microfluid dynamics a significant factor in the behavior of chemoattractants released by an egg. The efficacy of a particular sperm chemoattractant is dictated by the chemical's diffusion constant (mass vs. solvation properties) and local fluid turbulence (Xiang *et al.*, 2005). These two variables determine how steep and how long a gradient can be maintained in a form that is conducive to attracting homotypic sperm toward an egg. Additional factors that may influence the gradient itself include the source and abundance of the chemoattractant. For example, egg jelly is used as a chemoattractant in many animals. This glycoprotein coat is often applied over the egg ECM by ovarian follicle cells (Buckland-Nicks and Hodgson, 2000; Santella *et al.*, 1983) or oviduct epithelium as the egg travels toward the uterus (Elinson, 1986; Hedrick and Nishihara, 1991; Jego *et al.*, 1986; Lindsay *et al.*, 2003; Olson *et al.*, 2001; Schmidt *et al.*, 1997). Upon ovulation, jelly immediately, albeit passively, dissolves into the environment because of local convections and its relatively low chemoattractant retention constant (Arranz and Cabada, 2000; Olson *et al.*, 2001; Ward *et al.*, 1985; Xiang *et al.*, 2005). The single layer of jelly means that only a finite amount of chemoattractant is available, limiting the duration that the egg will be attractive to sperm. This is in contrast to actively released chemoattractants, such as the amino acids used by gastropods (Riffell *et al.*, 2002). In these mollusks, attraction gradients can be maintained for extended periods and may be modified in response to the environment to enhance the likelihood of attracting the correct sperm.

Eggs and oocytes of both protostomes and deuterostomes use a range of substances to attract conspecific sperm. Molecularly, the simplest chemoattractant is L-tryptophan, used by the gastropod *Haliotis rufescens* (Riffell *et al.*, 2002, 2004). The uniform presence of L-tryptophan actively released by the egg

activates *H. rufescens* sperm motility and orients it toward the source (Riffell *et al.*, 2002). Yet sperm from the closely related abalone *H. fulgens* are indifferent to L-tryptophan, the first indication that L-tryptophan is a bona fide chemoattractant that facilitates conspecific gamete interaction in animals whose spawning geographies may overlap (Riffell *et al.*, 2004). To further the complexity, and hence the specificity, some animals use peptides and small proteins as chemoattractants. For example, the cuttlefish *Sepia officinalis* egg uses the modified pentapeptide PIPGVamide to attract sperm toward the egg (Zatylny *et al.*, 2002). Surprisingly, sperm behavior toward this peptide can work both ways: The concentration gradient of *S. officinalis* PIPGVamide initially attracts sperm released from the female copulatory pouch toward the freshly spawned egg, but following fertilization or egg activation, this same peptide is trapped in the surrounding capsule, causing the accumulation of the peptide to concentrations that are repulsive to sperm (Zatylny *et al.*, 2002). This biphasic sperm-response elegantly toggles between promoting sperm interactions and inhibiting them to avert polyspermy.

Sperm chemoattraction also plays a critical role in deuterostome fertilization. Starfish asterosap, a glutamine-rich tetracontapeptide found in the jelly coat, can activate and reorient sperm through receptors along the sperm tail (Hoshi *et al.*, 2000; Neill and Vacquier, 2004). Its functional homologs in sea urchin eggs include members of the speract (Hansbrough and Garbers, 1981; Ramarao *et al.*, 1990; Suzuki *et al.*, 1988) and resact families of peptides (Garbers *et al.*, 1986; Ward *et al.*, 1985). Both of these sea urchin peptides originate from the jelly coat and diffuse away from the egg. When bound by sperm receptors found on the tail and midpiece of sperm, both peptides conspecifically increase sperm respiration, but only resact can reorient the sperm toward the source of the chemoattractant (Kaupp *et al.*, 2003; Neill and Vacquier, 2004; Ward *et al.*, 1985). In contrast to echinoderms, sperm chemoattractant has only recently been identified in higher deuterostomes. For example, chemotaxis of *Xenopus* sperm is achieved using the 21-kDa allurin, a glycoprotein member of the cysteine-rich secretory protein (CRISP) family of sperm-binding proteins (Olson *et al.*, 2001). It is responsible for 88% of sperm the chemoattractive behavior elicited by unfractionated egg jelly (Arranz and Cabada, 2000; Bonnell *et al.*, 1996; Olson *et al.*, 2001). This anuran chemoattractant diffuses from the outermost layer of *Xenopus* jelly (J3) (Figs. 1 and 2) into the surrounding fluid at a rate similar to smaller chemoattractants (Xiang *et al.*, 2005). Together, these observations imply that *Xenopus* allurin is a bona fide sperm attractant. A similar activation profile is observed for the *Ciona* sperm activating and attracting factor (SAAF), a sulfated steroid derivative that serves as a chemoattractant, albeit as a heterospecific one because the identical molecules are used by *C. intestinalis* and *C. savignyi* (Yoshida *et al.*, 2002). Such absence of conspecificity, however, may be balanced by the follicle cells

attached to the vitelline coat surface since these cellular gatekeepers regulate which sperm may interact with the ascidian vitelline coat (Lambert, 2000) (Figs. 1 and 2). Acid hydrolysis of the sulfur groups on SAAF abolishes activity, suggesting that the additional charges provide hydrophilicity to the steroid, thereby allowing it to participate in signaling on the sperm surface (Yoshida *et al.*, 2002). Use of the steroid progesterone is also implicated in chemoattraction toward mammalian eggs, but the data do not indicate that this ubiquitous steroid triggers chemotaxis per se; it may only activate sperm (Eisenbach and Tur-Kaspa, 1999). Could the addition of sulfate groups to mammalian progesterone convert this steroid into a functional chemoattractant? Or might progesterone represent a speract homolog, with a more potent chemotactic factor also required to reorient the sperm?

The involvement of various classes of molecules in chemoattraction points to the enormous diversity acquired during the initial phases of gamete interaction. Yet, a pattern exists throughout phylogeny with regards to the classes of chemoattractants used: Lower protostomes, who are often broadcast spawners, use single molecules (amino acids or modified peptides) to achieve a conspecific chemotactic response from sperm, whereas more recently evolved deuterostomes use more complex molecules (groups of peptides, proteins, steroids). Might chemoattractant complexity be linked to organism diversity, under the presumption that species specificity will increase upon addition of more variable in the mix of chemoattractants? How does the type of mating used by an animal factor into the selection of chemoattractant properties? Broadcast spawning might favor simpler molecules, such as L-tryptophan or peptides, because of the reduced cost of synthesizing them in bulk. Meanwhile, the pressure to co-evolve species-specific chemoattractive molecules may have relaxed as a consequence of reproductive isolation, either through geographical separation of populations (e.g., ascidians) or by acquisition of conspecific mating behavior (e.g., vertebrates). Additional data from other taxa that implicate specific molecules in conspecific versus generic sperm chemotaxis will be critical to assess how gamete chemoattraction ranks within the hierarchy of gamete interactions and prefertilization events.

C. Cell-Mediated Reduction in Sperm Quantity

Chemoattractants guide sperm toward a receptive egg, but an appropriate outcome falls within a defined range of sperm/egg ratios to achieve monospermy. Thus, a balance exists between effective chemoattraction and limited sperm number. In many animal eggs, the same structure that distributes the chemoattractant is responsible for retarding the progression of sperm toward the egg. The thick jelly coat of amphibian, echinoderm, xiphosurid,

and mollusk eggs helps to orient sperm perpendicular to the egg membrane. In the process, the proteoglycan constituents invariably retard sperm progress, reducing the number of sperm that successfully reach the egg surface at one time, delaying fertilization (Brown and Humphreys, 1971; Elinson, 1986) and/or selecting against prematurely activated sperm (see Section V, later in this chapter) (Hylander and Summers, 1977; Mah *et al.*, 2005). Chiton use their elaborate jelly coat hull, a structure synthesized and shaped by follicle cells in the ovary, as a chemoattractant and an obstacle that masks available sites of sperm binding (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988) (Figs. 1 and 2).

In a select few animals, follicle cells ovulated with the egg also participate in reducing the sperm/egg ratio. For example, the same follicle cells that construct the chiton's hull are ovulated with the egg. In some species, these follicle cells retract upon contact with the hyperosmotic seawater, revealing evenly ordered channels that guide sperm toward the receptive egg surfaces; in other species they remain steadfast, directing sperm to those productive tunnel openings found between cells (Buckland-Nicks, 1993; Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988). In both situations, chiton follicle cells passively obstruct sperm access to the egg ECM (Figs. 1–3). Ascidian follicle cells, on the other hand, actively participate in promoting sperm–egg interactions. The ascidian egg is spawned with a vitelline coat separated from the egg by a significant perivitelline space and a tight epithelial layer of follicle cells coating the outer surface of the vitelline coat (Figs. 1 and 2). Upon insemination, sperm must pass through the layer of follicle cells to access the ECM. To do so, two mechanisms have been proposed where sperm are either phagocytosed by the cells and transported across to contact the vitelline coat (De Santis *et al.*, 1980) or they penetrate through lateral junctions shared by adjacent cells (Lambert, 1989); neither has been observed *in vivo*. Regardless of which method is used to transit this cellular barrier, an active decision is made by the follicle cell on a per–sperm basis, and part of this decision is dependent on the species of the sperm (Lambert, 2000). Eutherian sperm are also required to transit a stratified layer of cumulus cells before contacting the zona (Figs. 1–3). Unlike ascidian or chiton follicle cells, the stratified organization of these cumulus cells helps them surround themselves with a viscous ECM enriched in hyaluronic acid and chondroitin sulfate A (Tatemoto *et al.*, 2005). The presence of these two matrix molecules impedes sperm progression through the cumulus layer and requires functional hyaluronidase, PH-20, on the sperm head (Dean, 2004; Hunnicutt *et al.*, 1996b; Myles and Primakoff, 1997; Primakoff and Myles, 2002; Tatemoto *et al.*, 2005). It is quite probable that other molecules on the sperm surface are co-opted by the egg to both retard and survey incoming sperm, thereby optimizing the sperm/egg ratio for successful fertilization.

Many animals utilize other methods to minimize sperm quantities that do not involve cellular gatekeepers, but utilize spatial restrictions instead. In birds, an egg ovulates from the ovarian capsule with the germinal disc, the preferred site of sperm binding and fusion, facing the infundibulum (Bramwell and Howarth, 1992; Okamura and Nishiyama, 1978b). Because fertilization occurs at the time of rupturing, the remainder of the large egg is anatomically blocked from sperm access, thereby avoiding polyspermy from occurring at a site other than the germinal disc (Bramwell and Howarth, 1992; Harper, 1904). Limiting the site of sperm fusion along the egg surface is common in many animals (see Section VII.A, later in this chapter) (Fig. 4). Often this spatial restriction is associated with complementary morphological modifications to the egg ECM. Some eggs have evolved radical polarizations in their ECM architecture compared to their sister taxa. For example, the anuran *Discoglossus* designates a patch of membrane at the animal pole as the major site of gamete fusion (Campanella *et al.*, 1992; Caputo *et al.*, 2001). Sperm are directed to this patch of microvillar-rich membrane by an extensive, chemoattractive jelly plug impinging on the animal hemisphere (Campanella *et al.*, 1997; Talevi and Campanella, 1988) (Fig. 4). The chemoattractive role of this jelly is most obvious when comparing fertilization to dejellied *Discoglossus* eggs, in which sperm are found to bind anywhere along the vitelline envelope but can fuse only at the dimple (Caputo *et al.*, 2001). A similarly radical polarization can be found in the bivalve mollusk *Unio*, whose egg is attached to the vitelline envelope only at the vegetal-most tip (Focarelli *et al.*, 1988) (Fig. 4). Here lies an elaboration of the ECM, where a crater composed exclusively of the sperm receptive gp273 marks the only fusogenic region of the egg; the remainder of the vitelline envelope consists of the inert structural glycoprotein gp180 (Focarelli and Rosati, 1995; Focarelli *et al.*, 1988).

The most elaborate reduction in sperm-accessible surface is found in eggs with polarized, impenetrable ECMs with a narrow channel, or micropyle, that guides sperm to the only receptive site on the egg. Such a specialization has convergently evolved in at least two animal orders with surprisingly different methods of insemination, namely dipterans and teleosts. Dipteran eggs are fertilized internally as the egg traverses down the oviduct. Sperm/egg ratios are limited anatomically by the controlled release of sperm from the spermatheca (Bloch Qazi *et al.*, 2003; Neubaum and Wolfner, 1999), so it is surprising to find that dipterans such as *Drosophila*, *Dacus*, and *Musca* utilize a micropyle (Degrugillier and Leopold, 1976; Mouzaki *et al.*, 1991; Turner and Mahowald, 1976) (Figs. 1 and 2). This paradox likely reflects the role of the chorion in minimizing desiccation following egg deposition (Li *et al.*, 1996), leaving the micropyle not as an elaboration whose primary role is not to block polyspermy, but as a feature that enhances gamete interactions while favoring gas exchange during embryogenesis. This

micropyle is marked by a thickened chorion, formed with the help of follicle cells, and a tuft of glycoproteins that distally seal the micropylar pore from the oviduct (Mouzaki *et al.*, 1991; Perotti *et al.*, 1990; Turner and Mahowald, 1976). To achieve fertilization, sperm must pierce this tuft before traveling through the canal separating the endochorion and the vitelline membrane (Degrugillier and Leopold, 1976). The molecular composition of this tuft suggests that it is the initial site of species-specific gamete interaction (Cattaneo *et al.*, 1997, 2002).

Unlike their dipteran counterparts, female teleosts do not have the ability to anatomically regulate the number of sperm per insemination; rather, the localized receptivity and the narrowness of the chorionic micropyle must be sufficient to deter excessive sperm entry. How might this have evolved, particularly because the more ancient chondroichthyes use sperm storage and anatomically regulated release of sperm (Neubaum and Wolfner, 1999)? The lineage of present-day fish provides some clues to this evolutionary process (see Fig. 4). In the most primitive bony fish, such as the petromyzontid *Lampetra*, the animal pole is covered with a small region of thickened chorion decorated with a tuft of jelly (Figs. 1 and 2). This jelly guides and orients the sperm toward the most fusogenic patch of egg membrane (Kobayashi and Yamamoto, 1994). Chondrostean fish such as *Acipenser* or *Polyodon* use a more advanced specialization, specifically a cluster of multiple channels that transect the chorion (Ciereszko *et al.*, 2000; Hart, 1990) (Fig. 4). The micropylar catchment of each channel participates in sperm attraction and binding (Cherr and Clark, 1986). Finally, the most recently diverged teleosts, whose eggs meiotically activate when they transit from the oviduct into the environment regardless of the state of fertilization, possess a single micropylar interruption in their chorion (Hart, 1990; Lee *et al.*, 1999; Yamagami *et al.*, 1992). Only the micropylar region is attractive to sperm and remains so for only a short window of time outside of the body before environmental activation, a time scale on par with the tens of seconds–long period of sperm motility (Hart, 1990; Tosti, 1994; Wolenski and Hart, 1987). Osmotic egg activation results in complete hardening of the chorion, as well as a depression or loss in micropylar chemoattractiveness to sperm (Amanze and Iyengar, 1990; Iwamatsu *et al.*, 1997; Wolenski and Hart, 1987).

The morphology of the fish micropyle is dependent on the activity of a micropylar follicular cell found in the ovary. This cell dictates how the chorion will be locally molded to form the micropyle (reviewed in Hart, 1990). Micropyle architecture ranges across the species from a simple tunnel traversing the chorion, marked by a local elevation of the zona radiata externa (Hart *et al.*, 1984), to a more elaborate structure consisting of an outer sperm catchment area or vestibule that funnels the sperm into a canal or pit (Amanze and Iyengar, 1990; Cherr and Clark, 1986; Hart, 1990; Yamagami *et al.*, 1992). The nuances of micropylar adornments within each

region vary among species (Fig. 3). For example, the catchment of *Barbus* has 7–10 grooves and ridges that radiate from the micropylar pit, accounting for 0.01% of the total surface area of the rosy barb chorion (Amanze and Iyengar, 1990). On the other hand, the *Danio* vestibule is covered with folds arranged in a right-hand spiral toward the pit (Hart and Donova, 1983). The pit ends above a circle of egg membrane enriched in fusogenic microvilli (see Section VII.A, later in this chapter) (Hart, 1990; Hart and Donova, 1983; Ohta and Iwamatsu, 1983). The pit diameter is only wide enough to accommodate a single sperm head, thus making monospermic fusion more likely (Amanze and Iyengar, 1990; Cherr and Clark, 1986; Hart, 1990; Hart and Donova, 1983; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987).

Teleost sperm use different intracellular signaling cascades to distinguish where they are within the micropyle, shifting the sperm behavior from a "seek" to a "follow" mode as necessary (Iwamatsu *et al.*, 1997; Murata *et al.*, 1995). *Clupea* sperm use the chemokinetic molecule herring sperm activating protein (HSAP) to initiate the "seek" mode of sperm (Oda *et al.*, 1995, 1998). HSAP is an 8-kDa water-soluble protein that readily diffuses from the outer chorion layer and can alter sperm motility at short ranges (Oda *et al.*, 1995). HSAP is found throughout the chorion, except at the micropyle. Its ability to initiate chemokinetic activity in sperm is consistent with a role in directing sperm *away* from the less productive chorion proper and *toward* the micropyle (Oda *et al.*, 1998). Following their activation, the sperm are attracted to the micropylar pit by a gradient of insoluble sperm motility initiation factor (SMIF) immobilized within the micropylar catchment (Griffin *et al.*, 1996). The ability of sperm to bind a solubilized form of herring SMIF *in vitro* (Griffin *et al.*, 1996) suggests that this normally immobile glycoprotein can facilitate the sperm's switch to "follow" mode, tracking along the grooves or folds lining the catchment to enter the vestibule and micropyle, as documented in other teleosts (Amanze and Iyengar, 1990; Hart and Donova, 1983). Might these structural micropylar grooves expose other deeper layers of the chorion, providing additional molecules for the sperm to maintain contact during the "following" stage? If so, then the participation of additional ZPC homologs (see Fig. 5; Section II.B and II.C, earlier in this chapter) could be a factor in the sperm's behavioral changes.

Is one of the mechanical obstacles, including viscous jelly, micropyles, and cellular gatekeepers, more effective than the others at keeping conspecific sperm/egg ratios low? Considering the wide range of methods used by species within the same taxon (Figs. 1, 2, and 4), the best mechanism is clearly the most adapted for a particular mating style. The dual chemoattractant–retardant properties of jelly serve spawning animals well because dilution of sperm in the media sufficiently lowers the ratio of interacting gametes per volume, particularly at the rate gametes are spawned in the wild. The use of a single micropyle ensures a sperm/egg ratio of unity but does not guarantee

that every egg will be fertilized (Hart, 1990; Snook and Markow, 2002). Likewise, chemoattraction and insemination do not guarantee that conspecific sperm will fertilize the egg; these processes only favor this outcome. The involvement of follicle cells in distinguishing between nonspecific and conspecific sperm (Lambert, 2000) clearly trumps the limitations imposed by the passive micropyle. Even though cellular gatekeeping requires more energy expenditure per egg than micropyles, the selectivity afforded by the initial sperm–follicle cell recognition phase may outweigh such costs, particularly in sessile animals like ascidians, for whom heterospecific gamete interactions are more likely (Lambert *et al.*, 2002). It is important to point out that selecting for a slightly higher sperm/egg ratio may be favorable to yield high fertilization percentages because of the successive series of gamete recognition steps that must occur before fusion.

V. Initial Gamete Contact

The ECM is a critical mediator of cell–cell communication in many tissues, including gamete interactions. The initial contact with the egg ECM triggers a cascade of changes in the sperm, including increased metabolism, greater motility, and the acrosome reaction, when the contents of the sperm's only secretory vesicle are released into the local environment (reviewed in Neill and Vacquier, 2004; Okamura and Nishiyama, 1978a; Tulsiani *et al.*, 1998; Wassarman, 1999). In most animals, these events are the first indications of successful homotypic recognition between gametes. Among animals, the types of molecules required to achieve this state of sperm activity vary significantly and often involve a combination of overlapping receptor–ligand interactions. This complexity not only reinforces species specificity between gametes prior to fertilization, but also proves to be more difficult for the egg to deal with when a block to polyspermy must be established.

In this section, we survey the major receptors and ligands responsible for initial gamete recognition (see also Table II). Particular emphasis is made on the candidates in the egg ECM or at the sperm surface that are likely modified during the establishment of a block to polyspermy. We also discuss the impact of these essential proteins on speciation and radiation within the animal kingdom.

A. Variability in Locations of Initial Sperm Contact

The site of initial conspecific sperm–egg interaction is highly variable among animals (Figs. 1 and 2). For example, a sperm's chemokinetic and chemotactic response to attraction factors (see Section IV.B, earlier in this chapter) are often indistinguishable from metabolic changes resulting from initial

sperm–egg contact. The critical difference between attraction and physical contact lies in the status of the sperm acrosome: Only primary sperm–egg contact will initiate the appropriate cascade of events that result in the acrosome reaction. Thus, the acrosome reaction has become a benchmark for the full activation of sperm and the achievement of initial gamete contact. The only taxon exhibiting an exception to this generality is teleosts, whose sperm do not possess acrosomes (Hart, 1990) (Figs. 1 and 6).

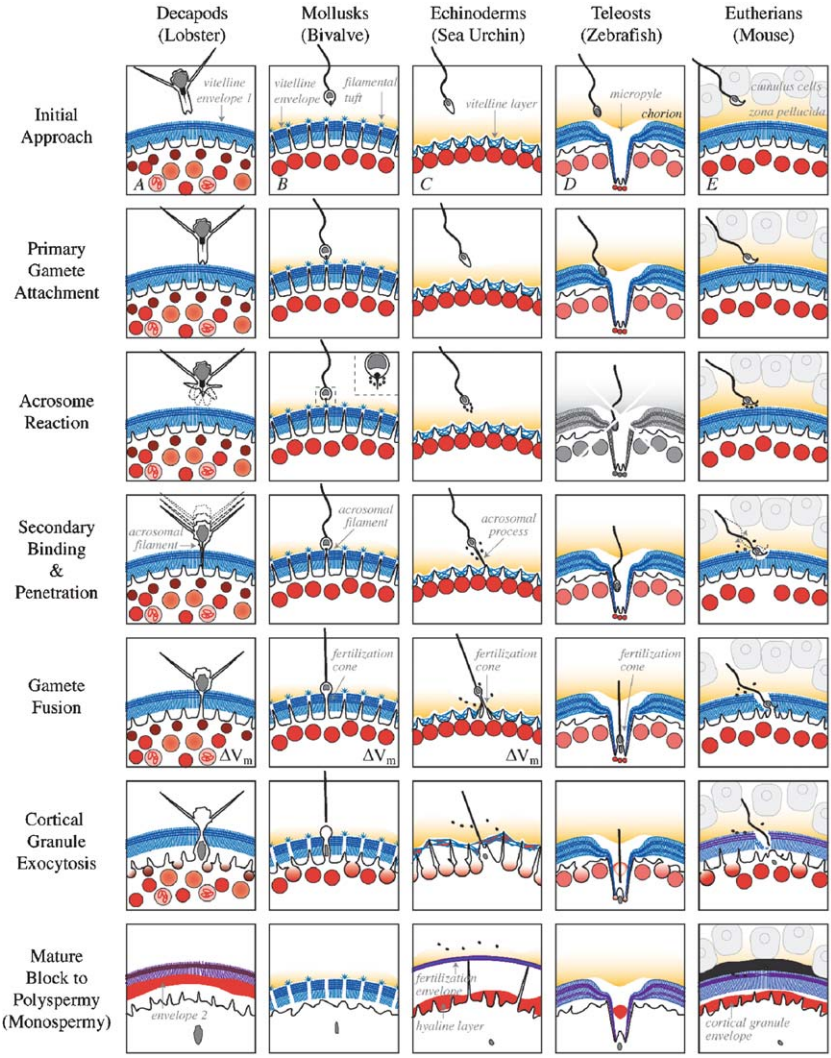


Figure 6 *Continued*

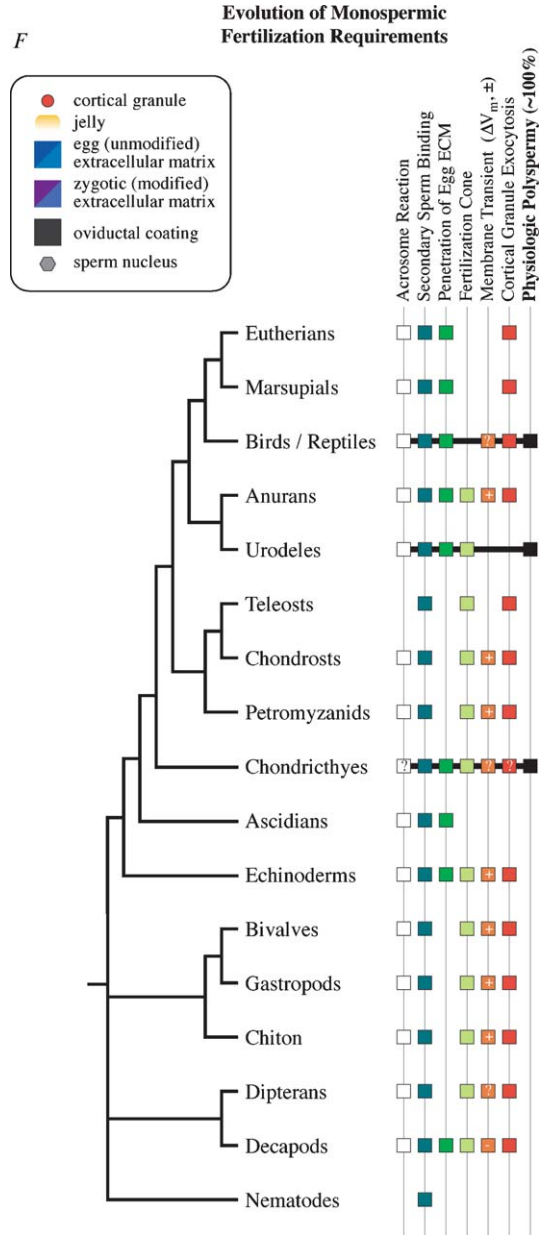


Figure 6 Diversity of mechanisms used during animal fertilization. Representations of sequential steps required for fertilization used by a selection of animals, including decapods (A), bivalves (B), echinoderms (C), teleosts (D), and eutherians (E). Steps are listed vertically in chronological order. Colors are as represented in the figure legend, following the scheme of

Egg jelly is often bifunctional, acting as both chemoattractant and sperm activator. The different effects of echinoderm jelly on sperm activity have been traced to separate molecules found in the heterogeneous mix of jelly glycoproteins (reviewed in Neill and Vacquier, 2004). The chemoattractive peptides encoded by starfish asterosap increase sperm motility in a heterospecific fashion and potentiate the acrosome reaction (see Section IV.B, earlier in this chapter) (Hoshi *et al.*, 2000; Matsumoto *et al.*, 2000). Starfish simultaneously present a greater than 2000-kDa acrosome reaction–inducing substance (ARIS) also found within the jelly coat that activates sperm conspecifically (Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Miller and Crawford, 1994). The essential fragment of pronase-digested *Asterias* ARIS contains at least 10 repeats of a sulfated pentasaccharide oligomer that can compete with full-length ARIS for binding to a 0.2-μm patch of the anterior sperm head (Hoshi *et al.*, 2000; Koyota *et al.*, 1997; Longo *et al.*, 1995). Under physiological conditions, however, ARIS alone cannot induce the acrosome reaction; normal activation is achieved only when sperm are presented with a complex containing asterosap, ARIS, and coARIS, a class of saponins covalently linked to sulfated steroids (Hoshi *et al.*, 2000; Matsumoto *et al.*, 2000). A triad of sea urchin jelly components is also essential for maximum rates of sperm activation. Like starfish asterosap, the speract family of peptides initiates sea urchin sperm chemokinesis (see Section IV.B, earlier in this chapter; Garbers *et al.*, 1986; Hansbrough and Garbers, 1981; Ramarao *et al.*, 1990; Suzuki *et al.*, 1988; Ward *et al.*, 1985). The acrosome reaction, however, is conspecifically potentiated by a glycoprotein complex anchored within the jelly by the 380-kDa fucose sulfate polymer (FSP) (Bonnell *et al.*, 1994; Hirohashi and Vacquier, 2002b; Keller and Vacquier, 1994; Vacquier and Moy, 1997; Vilela-Silva *et al.*, 1999). FSP contains more than 50% carbohydrate by mass, and 97% of this is fucose (Vacquier and Moy, 1997). Like its starfish analog ARIS, FSP alone can trigger the acrosome reaction species specifically at a pH of 8.0 (Hirohashi and Vacquier, 2002a; SeGall and Lennarz, 1979). In the presence of additional globular sialoglycans that reside on the branches of FSP, the acrosome reaction can be initiated at a pH of 7.8 (Bonnell *et al.*, 1994; Hirohashi and Vacquier, 2002a,b; Keller and Vacquier, 1994). When presented with speract, the FSP complex is able to trigger the acrosome reaction and to maintain sperm activity in more than 90% of all sperm, all at a significantly lower pH of 7.0 (Hirohashi and Vacquier, 2002a). This pH sensitivity is

Figs. 1 and 2. Grayed image indicates an absence of that particular step during fertilization in the respective animal. (F) Phylogenetic comparison of steps or structures used by different animals during fertilization. Where applicable, colors correspond to panels A–E; green tones indicate additional processes. ?, no data reported; −/+, direction of the fast electrical block (?V_m), either hyperpolarizing (−) or depolarizing (+).

curious because the pH of sea urchin egg jelly is identical to the surrounding media (Holland and Cross, 1983). The neutrality of the paleontological ocean is hypothesized to be a major force that selected for the maintenance of this triad of egg jelly proteins for sustaining sperm activation (Hirohashi and Vacquier, 2002a).

Based on the functional parallels between starfish and sea urchin jelly components (asterosap-speract; ARIS-FSP; coARIS-sialoglycans), it is tempting to speculate that jelly may be partly responsible for the divergence of these echinoderms. Both asterosap and speract represent families of peptides that increase sperm activity, yet sequence and size differences among these functional homologs are indicative of divergence. Similarly, the high degree of glycosylation in ARIS and FSP is probably held over from a common ancestor, whereas the size of each subunit could be a consequence of speciation. The synergists coARIS and sialoglycan, however, share little resemblance: The active backbone of coARIS is a sulfated steroid-liked saponin (Hoshi *et al.*, 2000), whereas sialoglycans predominantly consist of oligosaccharides (Hirohashi and Vacquier, 2002b). Yet the effects of each synergist on sperm activity are similar: Co-presentation of ARIS and coARIS increases intracellular calcium levels (Hoshi *et al.*, 2000), whereas co-presentation of FSP and sialoglycans raises intracellular pH and potentiates calcium-dependent acrosome exocytosis (Hirohashi and Vacquier, 2002b). Could speciation alone be responsible for the divergence in the synergists used? Might this switch be a memento of the earliest changes that resulted in the separation of these sister taxa more than 500 million years ago (Hinman *et al.*, 2003), with the divergence of ARIS and FSP following soon after? Perhaps the expression profile of sperm has also influenced the use of saponins versus sialoglycans. The absence of definitive sperm ligands for each molecule of the triad, however, makes this hypothesis difficult to evaluate.

Diffusion, the one factor that makes jelly a good chemoattractant, limits its utility as a trigger of sperm activation (Xiang *et al.*, 2005). In echinoderms, jelly is generously applied over each egg even though only a thin layer at the egg surface is necessary for successful fertilization; the excess is probably used to establish a large chemoattractive territory to lure other spawned sperm. A similar bifunctional role in both sperm attraction and activation has been proposed for anuran jelly. Such duality is supported by the absence of fertilization in anuran eggs that have been exposed to spawning media too long, a phenotype attributed to the visibly swollen and thinned jelly (Hedrick and Nishihara, 1991). The observation that mechanically dejellied anuran eggs are fertilization incompetent also strongly suggests that jelly is a significant contributor to initial sperm binding (Caputo *et al.*, 2001; Elinson, 1986). In *Discoglossus*, the most distal jelly layer (J3) is responsible for triggering the acrosome reaction, whereas the glycoproteins

that comprise the jelly plug primarily direct the reacted sperm toward the dimple (Campanella *et al.*, 1997; Talevi and Campanella, 1988). Similarly, cross-species fertilization can be achieved by simply passing a *Xenopus* egg through the oviduct of the species akin to the desired sperm donor (Elinson, 1986). Yet all anurans do not appear to use jelly to initiate a conspecific acrosome reaction. Only the vitelline envelope proteins are capable of species-specific exocytosis of the acrosome in *Bufo* (Barisone *et al.*, 2002) and the presence of jelly only enhances fertilization by 10% in *Eleutherodactylus* (Toro and Michael, 2004). One reason for this difference in jelly-mediated activation may lie in the aquatic versus terrestrial environment that anurans inhabit; semiaquatic frogs may use the outer jelly layer to induce the acrosome reaction because the spawned sperm are less numerous upon contact with the jelly compared to their terrestrial counterparts. By using the outer jelly layer, the egg establishes its attractiveness to the more dilute sperm earlier in the process, thereby favoring its own fertilization. In contrast, the role of jelly in terrestrial anuran eggs may be to prepare the sperm for interaction with the egg ECM, perhaps enhancing the sperm's affinity for the components found within the vitelline envelope, while reducing the number of sperm that finally contact the egg by selecting against prematurely acrosome-reacted individuals.

Consistent with terrestrial anurans, sperm activation and the acrosome reaction in most animals occur at the closely associated ECM made by the oocyte. More often than not, mechanical removal of jelly or other extraneous glycoprotein coats does not drastically impair sperm activation or fertilization *in vitro*, but removal of the ECM does (Talbot and Chacon, 1982). Thus, components in the egg ECM of most animal taxa mediate conspecific sperm–egg interactions. In many vertebrates, the major glycoproteins responsible for primary sperm–egg interactions are members of the ZPC subfamily (Infante *et al.*, 2004; Iwamatsu *et al.*, 1997; Kerr *et al.*, 2002; Mengerink and Vacquier, 2001; Moller *et al.*, 1990; Murata *et al.*, 1995; Tulsiani, 2000a; Vo and Hedrick, 2000; Wassarman, 1987, 1999) (Fig. 5). Two regions of ZPC, found at the most amino- and carboxy-terminal domains of the ectodomain, are under positive selection, whereas the primary sequence of the whole protein is under negative selective pressure (Swanson *et al.*, 2001b). Such negative selection on the ZP domain suggests that its conformation is essential for ZPC to intercalate within the ECM, a model supported by experimental deletions (Jovine *et al.*, 2002) and exon swapping (Kinloch *et al.*, 1995). Thus, the orientation of ZPC within the ECM may be essential for successful sperm binding, perhaps using the ZP domain to anchor itself within the matrix while the divergent regions are left exposed. Conformational dependence of ZPC for sperm-activating behavior is supported by the requirement for oviductal modification prior to achieving sperm binding to the egg ECM in anurans (Gerton and Hedrick, 1986; Infante

et al., 2004; Kubo *et al.*, 1999), urodeles (Makabe-Kobayashi *et al.*, 2003), marsupials (Breed *et al.*, 2002), and mammals (Boja *et al.*, 2003; Kiefer and Saling, 2002).

Additional factors found within the ECM may also participate in optimizing access of incoming sperm to the appropriate sperm receptor. For example, adsorption of oviductal glycoproteins to freshly ovulated eggs can enhance conspecific sperm–egg binding efficiency (Brown and Cheng, 1986; Buhi, 2002; Buhi *et al.*, 2000; O'Day-Bowman *et al.*, 2002; O'Rand, 1988; Schmidt *et al.*, 1997), possibly by modulating the binding affinities of ZPC (Rodeheffer and Shur, 2004). Endogenous ZP proteins may also be critical for their proper exposure: Oviductal cleavage of ZPA is required for functional sperm binding to the anuran vitelline envelope (Caputo *et al.*, 2001; Infante *et al.*, 2004; Tian *et al.*, 1997, 1999; Vaccaro *et al.*, 2001), and the simultaneous presentation of ZPB–ZPC dimers is required for sperm binding to *Sus* zona (Yurewicz *et al.*, 1998). The identification of ZPD family members (Fig. 5) also supports a model of increased complexity in sperm-binding conformations through heterodimerization of ZP proteins (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). The chicken perivitelline layer, for example, is primarily composed of three major ZP homologs: ZP1 (Bausek *et al.*, 2000), ZPC (Takeuchi *et al.*, 1999), and ZPD (Okumura *et al.*, 2004) (a minor fourth protein can be detected [Okumura *et al.*, 2004] and may represent the ZPAX ortholog identified in the *Gallus* genome, but this link has not been made experimentally). Of these three, ZPD is readily removed from the ECM by sonication; ZP1 and ZPC require acid and chaotropic denaturation for separation (Okumura *et al.*, 2004). Yet only dimeric ZP1 and monomeric ZPD can induce the acrosome reaction in sperm at 50% and 95% of the frequency of the intact perivitelline layer, respectively (Okumura *et al.*, 2004). As with *Xenopus* ZPD, the presence of the small molecular weight *Gallus* ZPD was initially masked by an overwhelming quantity of ZPC under non-reducing gel electrophoretic conditions (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). Because initial sperm-binding and sperm-activating assays in vertebrates were accomplished with gel-purified ZPC, the ZPD contaminant was never considered functionally relevant. Although chicken ZPC follows the pattern of the archetypical mammalian ZPC ortholog, namely homology and abundance in the perivitelline layer, its inability to induce the acrosome reaction *in vitro* leaves room for other contributing ECM components such as ZPD (Lindsay *et al.*, 2002; Okumura *et al.*, 2004). The exclusive presence of ZPD orthologs in avians and anurans (Fig. 5), however, suggests that vertebrates procured ZPD late in evolution as animals became terrestrial but was subsequently lost in the mammals. One hypothesis for this loss may be the transition to a broader sperm-receptive surface on the egg (see Section VII.A, later in this chapter) (Fig. 4): Avian eggs fertilize primarily at the germ disc (Bramwell and Howarth, 1992; Okamura and Nishiyama,

1978b), anurans fertilize in a restricted region of the animal pole (Elinson, 1986), but mammalian eggs can be fertilized anywhere along the egg surface except over the metaphase spindle (Gulyas, 1980; Myles, 1993; Wassarman, 1999) (Fig. 4). In animals that retain ZPD expression (Fig. 5), this soluble protein might work in conjunction with ZPC to enhance sperm receptivity by locally lowering the threshold for sperm activation (Okumura *et al.*, 2004).

Where does the acrosome reaction occur in invertebrate eggs that also contain bona fide ZP homologs in their egg ECM? In hermaphroditic ascidians, the acrosome reaction is triggered upon contact between the sperm and vitelline coat (De Santis *et al.*, 1980; Rosati, 1985). Immobilized VC70, the major protein of the vitelline coat, is able to bind conspecific sperm *in vitro* (Sawada *et al.*, 2004), whereas soluble VC70 can block sperm–vitelline coat binding (Matsuura *et al.*, 1995). In addition to the carboxy-terminal ZP domains, VC70 has six paired EGF–like repeats separated by regions that exhibit a high frequency of allelic polymorphisms among individuals (Sawada *et al.*, 2004). The potential of each polymorphic linker region to modulate how one allele of VC70 folds is fundamental to discriminate between self and nonself gametes, a property significantly more stringent than the species specificity required for sperm–ECM interactions in other animals. Similarly, the acrosome of abalone sperm remains intact until the gamete interacts with conspecific VERL filaments (Mozingo *et al.*, 1995). VERL, a member of the ZPX subfamily (Fig. 5), contains 28 tandem repeats of 150 residues in length (Galindo *et al.*, 2002). Between species, only the first two VERL repeats exhibit positive selection compared to the neutral purifying selection occurring over the remaining 26 repeats (Galindo *et al.*, 2003; Swanson *et al.*, 2001a; Swanson and Vacquier, 1998). Thus, like vertebrate ZPCs, both ascidian and abalone sperm receptors are diversifying while maintaining their overall structure by limiting sequence modifications to only a few regions selected for sperm interaction.

What does regional diversification in primary sperm receptor sequence imply about the assembly and evolution of egg ECMs, particularly in light of sexual conflict? The sexual conflict hypothesis postulates that an endless coevolutionary chase between the sexes is driving speciation through behavioral, anatomical, and molecular interactions (see Haygood, 2004; Swanson *et al.*, 2001b). Viewing the final criterion in light of gamete interactions, the egg should be dictating the diversification of a species because eggs are so outnumbered by sperm. Yet, critically analyzing the phylogeny of primary sperm receptors questions the penetrance of this process in gamete evolution. The ZP family contains at least three members that serve as primary sperm receptors throughout phylogeny, namely mammalian/anuran ZPC, ascidian VC70, and abalone VERL. As is required for species specificity in gamete interactions, these proteins exhibit regional positive selection in their sequences. Yet the diversifying domains are extremely restricted and are

found between otherwise conserved structural folds such as ZP domains, EF hands, and VERL repeats. In keeping with the overall conservation of the ECM assembly process within animal orders, the selection to retain the sperm receptor's overall structure clearly outweighs the need to diversify. But are the scattered regions under positive selection large enough to sweep into a population such that their sequences will lead to speciation? In the abalone, the 300 amino terminal residues of VERL repeats no. 1 and 2 are clearly sufficient to promote diversification without detriment to the remaining 3400 residues (Galindo *et al.*, 2002), and evidence from its complementary ligand shows that sperm are able to keep up with the changes (see Kresge *et al.*, 2000b; Lee *et al.*, 1995; Vacquier *et al.*, 1997). But what about the two patches of ZPC that together represent nearly 20% of the whole primary sequence? Is the conservation of the ZP domain fold sufficient to preserve protofilament assembly, even if most of these sequences are altered? Experimental evidence suggests that this is possible so long as none of the 260 residues encoding the ZP domain are affected (Jovine *et al.*, 2002), and natural selection would favor the loss of eggs that were not able to sustain such conservation (Dean, 2004; Rankin and Dean, 2000). Thus, the egg successfully maintains its advantage in the arms race against sperm by elegantly incorporating potentially sweeping changes into an otherwise conserved structure.

B. Oligosaccharide Sperm Receptors

The minimal sequence diversity in conserved sperm receptors cannot fully account for the plethora of animal species. Do other molecular components participate, specifically ones that significantly alter the surface profile of the sperm receptor without compromising the protein's structural conformation? Of all the protein modifications possible, animal eggs appear to rely on oligosaccharides to readily increase diversity. Given the appropriate transferases, a single chain of 1,6-linked sugar units can be synthesized using any available monosaccharide. Further complexity can be gained along the same polysaccharide backbone by alternative branching positions, covalent linkages made at 1,3 and 1,4 positions of a terminal or internal sugar unit that expands the oligosaccharide into the second dimension. Within the Golgi apparatus, branched or linear oligosaccharides are often covalently linked to proteins at asparagine (*N*-linked), serine, or threonine (both *O*-linked) residues found at select surfaces along a folded protein. The permutations available for oligosaccharide structure alone are more than sufficient to account for the number of species in most animal orders. But if monosaccharide composition and branch structure do not provide enough diversity, a simple nonsynonymous mutation to a putative *N*- or *O*-linked residue or a change in glycosylation

position could dramatically alter the surface profile and glycosylation pattern of a protein. Furthermore, specializations including acetylation, methylation, and sulfation of sugar residues are also possible, modifying the electrical charge on the oligosaccharide in a manner that could modulate ligand affinities. Thus, it should not be surprising that animals utilize specific oligosaccharides as the cofactors that mediate initial sperm–egg interaction (reviewed in Mengerink and Vacquier, 2001; Shalgi and Raz, 1997; Tanghe *et al.*, 2004; Tulsiani, 2000a).

A single protein can be linked to any number of oligosaccharides. Identifying which ones are responsible for sperm reception requires careful dissection of the composition of the protein. Similar methods have been used in all animals to assess which fragments and oligosaccharides may be critical for binding. These include the following binding assays: (1) competition, where sperm are challenged with oligosaccharides or glycopeptide fragments while assessing binding affinities to endogenous ECM proteins; (2) sperm activation, where sperm are preincubated with oligosaccharide or glycopeptides followed by scoring their degree of specific binding or the frequency of sperm activation as indicated by acrosome exocytosis; (3) loss of binding, where the ECM glycoprotein is enzymatically stripped of oligosaccharides and asked how effective they are at competing for sperm binding to control eggs; and (4) chimeras, where transgenic animals are created to test the role of specific protein backbones in the context of another species' glycosylation machinery. Once a fragment or an oligosaccharide has been identified functionally, the structure of the sugar may be determined using (5) lectin affinity analysis, which tests various plant lectins to determine the branching pattern and approximate composition of the candidate oligosaccharide, (6) monosaccharide release following glycosidase treatment, or (7) mass spectroscopy or NMR to establish a more detailed map of the molecule, including information on composition and branch points. The extensive data from this field of gamete glycobiology is beyond the scope of this chapter, but we include a partial listing of key findings from different animals (Table IV). In the following paragraphs, we highlight some of the observations made that further extend our understanding of the conservation of initial sperm–egg interactions and the sexual conflict of gamete receptor–ligand pairs.

The oligosaccharides presented to sperm are frequently composed of a variety of specific sugar residues. This enrichment reflects a process unique to oogenesis because the recombinant ZPC expressed in conspecific nonovarian cell lines is not sperm receptive (Martic *et al.*, 2004), whereas *in situ* expression in eggs of heterospecific ZPC does yield functional protein (Doren *et al.*, 1999; Kinloch *et al.*, 1992; Moller *et al.*, 1990; Rankin *et al.*, 1998, 2003). Although it is theoretically possible to generate these moieties *de novo*, evidence suggests that animals directly incorporate dietary monosaccharides into their glycoproteins (Berger *et al.*, 1998; Martin *et al.*, 1998).

Table IV Table of Monosaccharides in Animal Egg Extracellular Matrices: Monosaccharide Reported to be Present in the Structure of Specific Molecules of Animal Egg[a]

Genus	Source	Linkage	L-Fucose	D-Galactose	Galactosamine	*N*-acetylgalactosamine	Glucosamine	*N*-acetylglucosamine	D-Mannose	D-Xylose	*N*-acetylneuraminic acid	*N*-glycolyneuraminic acid	Sialic acid	Other	Assay	Citation
Sus	ZPB	N-	■					■	■						5	Kudo *et al.*, 1998
	ZPC	N-		■				■					*		5	Yurewicz *et al.*, 1991
		N- (a)		■				■	■		■	■	■		5	Noguchi and Nakano, 1992
		N- (n)		■				■	■						5	Noguchi *et al.*, 1992
		O-		■		■	■								5	Yurewicz *et al.*, 1991
Oryctolagus	zona		■											D-Glucose*	1, 5	O'Rand *et al.*, 1988
Homo	zona		■												5	Lucas *et al.*, 1994
									■					D-Fructose	1	Mori *et al.*, 1993
Mus	n/a					■		■	■						2	Loeser and Tulsiani, 1999
	zona							■	■						1	Cornwall *et al.*, 1991
	ZPA	N-	■	■				■	■				■		5	Tulsiani, 2000
	ZPA / C	O-		■					■		■	■	■		5, 7	Easton *et al.*, 2000
	ZPC	N-	■	■			■	■					■		5	Tulsiani, 2000
		O-				■									1, 3	Florman and Wassarman, 1985
		O-	■	■											1, 6	Bleil and Wassarman, 1988
		O-													4	Kinloch *et al.*, 1995
		O-													1, 4	Chen *et al.*, 1998
Rattus	zona		■						m						1	Shalgi *et al.*, 1986
Sminthopsis	zona			■		■	■	■	■		■				5	Chapman *et al.*, 2000
Monodelphis	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Macropus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Trichosurus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Phascolarctus	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Pseudocherius	zona			■		■		■	■		■				5	Chapman et al., 2000
Isoodon	zona			■		■		■	■		■				5	Chapman *et al.*, 2000
Discoglossus	ZPA		■	■		■		■	■		■				5	Caputo *et al.*, 2001
		N-							■						5	Vaccaro *et al.*, 2001
		O-				■									5	Vaccaro *et al.*, 2001
	ZPC			■		■		■	■		■				5	Caputo *et al.*, 2001
	gp106														5	Maturi *et al.*, 1998
			■	■		■		■	■		■				5	Caputo *et al.*, 2001
	gp118		■												5	Maturi *et al.*, 1998
				■		■			■		■				5	Caputo *et al.*, 2001
	gp200, 260		■												5	Maturi *et al.*, 1998

Xenopus	jelly (J2, J3)							■			■				5	Bonnell and Chandler, 1996
	ZPC	N-	■	■				■	■		■				6	Vo *et al.*, 2003
Oncorhyncus	micropyle				■	■								Ceramide	1, 3	Yu *et al.*, 2002
Ciona	vit. coat		■				■	■	■		■				5	Rosati *et al.*, 1978
							■		■						5	De Santis *et al.*, 1980
			■												1	Rosati, 1985
Halocynthia	vit. coat	N-				■		■	■						1, 5	Baginski *et al.*, 1999
		O-		*		■		■		*				D-Arabinose	1, 5	Baginski *et al.*, 1999
Asterias	ARIS		*	■	■					■				D-Glucopyranose	2, 7	Koyota *et al.*, 1997
	coARIS		■	■	■					■				D-Glucopyranose	7	Koyota *et al.*, 1997
Arbacia	FSP		*												2, 7	Alves *et al.*, 1997
	FSP	O-	■												2, 3, 7	SeGall and Lennarz, 1979
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
Echinometra	FSP													L-Galactose	2, 7	Alves *et al.*, 1997
Lytechinus	FSP		■												2, 7	Alves *et al.*, 1997
		O-	*												2, 3,	SeGall and Lennarz, 1979
			■												2	Biermann *et al.*, 2004
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
Strongylocentrotus	FSP		■												2, 7	Keller and Vacquier, 1994
			*												2, 7	Vilela-Silva *et al.*, 1999
			■												2	Biermann *et al.*, 2004
		O-	*												2, 3, 7	SeGall and Lennarz, 1979
	sialoglycan		■										■		2, 3, 7	SeGall and Lennarz, 1979
				■		■		■	■						2, 7	Keller and Vacquier, 1994
Drosophila	micropyle		■	■		■		■	■		■				5	Perotti *et al.*, 1990
	vit. memb.							■	■						5	Perotti *et al.*, 1990
Limulus	vit. env.		■					■							1, 5	Barnum and Brown, 1983
Haliotis	VERL		■	■			■		■					D-Glucose	7	Swanson and Vacquier, 1997
Unio	gp273	N- (n)					■	■	■		■				7	Di Patrizi *et al.*, 2001
		O-													3	Focarelli and Rosati, 1995
		O-	■	■		■									7	Di Patrizi *et al.*, 2001

[a]Grayed boxes indicate the presence of the sugar residue.

(a) = acidic oligosaccharide chains; (n) = neutral oligosaccharide chains; * = sulfated residue; m = methylated residue; Fuc = L-fucose; Gal = D-galactose; GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; Gln = Glucan; Glt = Galactan; (L-galactose); Glc = Glucosamine; Glu = Glucose; Man = D-mannose; NeuNAc = *N*-acetylneuraminic acid; NeuNGc = *N*-glycolyneuraminic acid; Sial = Sialic acid; * = Sulfated; Cer = Ceramide; Xyl = D-xylose; Gan = Galactosamine.

(1) Competition; (2) Sperm activation w/acrosome reaction; (3) Loss of binding to stripped ECM; (4) Chimera; (5) Lectin affinity analysis; (6) Monosaccharide release; (7) Gas chromatography, mass spectroscopy, or NMR.

In light of the abundance of rare monosaccharides in the animal egg ECM, such as L-fucose and *N*-acetylglucosamine (GlcNAc) (Table IV), might direct utilization of dietary saccharides be favored by oocytes? Such a hypothesis is consistent with the correlations reported between animal fitness and reproduction—specifically, the more physically fit an animal is, the more likely it will produce viable offspring. Because one significant influence of animal fitness is the quality of the diet, the observed enrichment of dietary sugars in the egg ECM suggests one method that gametes may use to exhibit the fitness of the individual they came from. For example, the predominant monosaccharide used by sea urchins is L-fucose (Alves *et al.*, 1997; Biermann *et al.*, 2004; Keller and Vacquier, 1994; SeGall and Lennarz, 1979; Vilela-Silva *et al.*, 1999). Because the main source of food for sea urchins is algae, a prominent source of L-fucose (Vanhooren and Vandamme, 1999), the selective enrichment of this hexose in the primary sperm receptor FSP (see Section V.A, earlier in this chapter) is compatible with the dietary and fitness contributions to fecundity.

The ability of sea urchins to conspecifically regulate the interaction of gametes based on the branching chain geometry of fucan (Alves *et al.*, 1997; Biermann *et al.*, 2004) represents an elegant form of oligosaccharide-based interactivity that is likely extended to other animals by the use of more complex glycan chains. It has been reported that most animals use L-fucose in their egg ECMs (Table IV). This hexose alone may serve as a backbone for further elaboration, consistent with the ability to generate more than 750 different isomers using a single tetra-fucan root (Alves *et al.*, 1997). Variability in the expression pattern of fucosyltransferase orthologs among different animals (Staudacher *et al.*, 1999) is consistent with distinct fucosylation profiles on the egg surface. The presence of D-galactose, D-xylose, and D-glucopyranose in addition to L-fucose on the primary starfish sperm receptive proteins ARIS and coARIS (see Section V.A, earlier in this chapter) (Baginski *et al.*, 1999; Koyota *et al.*, 1997) further enhances gamete selectivity between these two echinoderm families. Incorporation of monosaccharides such as GlcNAc and D-mannose, as observed in many other animal ECMs, may reflect further diversification because use of these common dietary sugars can generate significantly more elaborate oligosaccharide moieties. Incorporation of more unusual sugars, such as L-rhabanose and L-arabinose (Baginski *et al.*, 1999) or L-galactose (Alves *et al.*, 1997), would only serve to expand the possible permutations. In the case of the hermaphroditic ascidian, the incorporation of such ecologically rare sugars facilitates the process of allorecognition already used in their receptor VC70 to distinguish self from nonself (Sawada *et al.*, 2004). Similarly, the coexpression of the nonfucosylated sea urchin sialoglycan (Keller and Vacquier, 1994) might reflect requirements for additional factors to enhance both gamete specificity and the potency of sea urchin jelly (Hirohashi and Vacquier, 2002a). Finally,

modification of the oligosaccharide by sulfation or methylation could ensure that species-specific interactions occur at the egg surface.

The additional bias in monosaccharide use across different orders (Table IV) could also reflect changes in diet that certain animals were required to make during their transition to different habitats, thus allowing speciation to follow reproductive isolation. For example, with the move to land comes a different source and abundance of essential sugars, two factors that have a direct impact on the complexity of an egg's ECM. If true, then the use of a specific collection of monosaccharides in the egg ECMs of a single family of animals could suggest evolutionary relatedness. Such a transition can be seen in anurans, who prominently display L-fucose, D-mannose, GlcNAc, and *N*-acetylneuraminic acid on the egg surface (Caputo *et al.*, 2001; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo *et al.*, 2003; Vo and Hedrick, 2000). This set appears to have been maintained through the separation of terrestrial marsupials (Chapman *et al.*, 2000) but later expanded upon in eutherians with the inclusion of *N*-glucosylneuraminic acid and sialic acid moieties (Easton *et al.*, 2000a; Noguchi and Nakano, 1992; Tulsiani, 2000b; Yurewicz *et al.*, 1991). Another event that favors additional complexity in sperm receptors may be occurring in teleosts, as suggested by the incorporation of ceramide in the moiety galactose-*N*-acetyl-β4-galactose-β4-glucan-β1-ceramide that is enriched at the micropylar entrance of *Oncorhyncus* eggs (Yu *et al.*, 2002). Thus, the collection of monosaccharides present on a functional sperm receptor may provide a rough estimation of the relationship between taxa.

C. Sperm Ligand for the Egg

The vast array of oligosaccharides used by eggs for sperm binding presents a challenge to the complementary gamete. How have sperm managed to keep up? One method is by maintaining diversity in their expressed carbohydrate-binding ligands (reviewed in O'Rand, 1988; Shalgi and Raz, 1997; Sinowatz *et al.*, 1995; Topfer-Petersen *et al.*, 1998, 2000). Unlike the egg, sperm have fewer constraints requiring them to maintain one class of ligands over another because sperm contribute few cytoplasmic or surface proteins to the zygote. Nevertheless, sperm appear to prefer catalytically inactive glycolytic enzymes and lectin-like molecules to function as egg ligands.

One primary advantage to using glycosidases as the ligand of the egg's receptor(s) is the complementary nature of the interaction. Although the specific sequence of events that lead to the final glycosylation pattern of the egg receptor for sperm is specific to oocytes, it is likely that the enzymes responsible for this process are conserved in other organs or even identical in the testis. Thus, little effort would be required for sperm to adopt posttranslational,

synthesis-related glycosidases for the purposes of egg binding. The major caveat to the exploitation of these enzymes, however, is the possibility for their activation. Most candidate glycosidases have counterparts in the Golgi, an acidic organelle whose major function is the conjugation of oligosaccharides to proteins destined for the extracellular environment. Hence, the optimal pH of most of these glycosidases tends to be acidic. Considering that the normal extracellular pH is higher than 6.5, the activity of such enzymes would likely be depressed, making them respectable ligands for mediating sperm–egg interactions.

Several major glycosidases with putative monosaccharide targets on the egg ECM can be found on the sperm surface. Using enzyme activity assays, the presence of surface glycosidases has been documented in animals such as dipterans (Cattaneo *et al.*, 1997), ascidians (Lambert, 1989), and eutherians (Loeser and Tulsiani, 1999; Rebeiz and Miller, 1999; Tulsiani *et al.*, 1990). The best-studied candidate is mammalian β-(1,4)-galactosyltransferase (GalTase). Under the appropriate conditions, this surface-expressed sperm isoform is capable of binding terminal GlcNAc residues on the respective egg sperm receptor (Table IV) and covalently attaching its endogenous substrate UDP-galactose (Loeser and Tulsiani, 1999; Rebeiz and Miller, 1999). Clustering of the GalTase also effectively activates sperm, inducing acrosome exocytosis (Macek *et al.*, 1991), a phenotype that is exacerbated when GalTase is overexpressed on the sperm surface (Youakim *et al.*, 1994). Yet in the absence of this enzyme, sperm are still fertile, albeit at a lower percentage than their comparable wild-type counterparts (Lu and Shur, 1997). Thus, the role of GalTase during murine fertilization is likely complemented by other surface proteins, a model consistent with the reported low- or no GalTase-associated activity in other eutherian sperm (Larson and Miller, 1997; Tulsiani *et al.*, 1990). Alternative glycosidases found on sperm include α-D-mannosidase in rats and humans (Cornwall *et al.*, 1991; Mori *et al.*, 1993; Tulsiani *et al.*, 1989, 1990), a mouse 95/116-kDa hexokinase (Kalab *et al.*, 1994; Leyton and Saling, 1989), and *N*-acetylglucosaminidase in ascidian (Lambert, 1989) and dipterans (Cattaneo *et al.*, 1997, 2002; Perotti *et al.*, 2001). Each of these candidates participates in species-specific binding to the egg ECM, especially to the sperm receptor derivatives, but like GalTase, they do not appear to be essential for sperm binding. In fact, *Drosophila* β-*N*-acetylglucosaminidase may be active *in vivo*, suggesting that its catalytic behavior participates in sperm competition at the micropyle by removing sperm-receptive proteins before subsequent sperm may enter and activate (Cattaneo *et al.*, 1997, 2002; Pasini *et al.*, 1999; Perotti *et al.*, 2001). Thus, the participation of sperm surface glycosidases appears to be supplementary during gamete interactions, facilitating the process of recognition rather than initiating the process of sperm activation.

Another mechanism for binding oligosaccharides uses more versatile sugar-binding ligands. Like their plant-derived lectin homologs, these low molecular weight molecules exhibit a high affinity for specific sugar moieties or oligomers. One such protein first described in *Mus* is sperm EGF-discoidin protein 1 (SED1) (Ensslin and Shur, 2003). This protein is synthesized by and localized on the surface of sperm heads, specifically to regions overlying the intact acrosome. SED1 is thought to interact with both ZPA and ZPC via one of its carboxy-terminal discoidin domains (Ensslin and Shur, 2003). The absence of a taxon-specific zona binding by SED1 reminds us that the purpose of some ligands may simply be to retain sperm attachment to the egg ECM rather than for recognition or conspecificity. Another generic oligosaccharide-binding family prefers the sulfated fucose-rich proteins found within the zona of mammalian eggs (O'Rand, 1988; O'Rand *et al.*, 1985, 1988; O'Rand and Fisher, 1987) (Table IV). These ligands were first identified in a screen of autoimmune serum raised against a cluster of 13–15 kDa rabbit sperm autoantigens (RSAs) (O'Rand *et al.*, 1988). Insemination in the presence of anti-RSA Fabs causes 60% inhibition of *in vitro* sperm–zona attachment and *in vivo* fertility (O'Rand, 1981). This is consistent with the ability of purified RSA to compete for sperm–egg associations at the zona surface (O'Rand *et al.*, 1988). Upon sperm–zona binding, RSA is believed to cluster along the sperm surface, thereby initiating a signaling cascade that facilitates acrosome exocytosis (O'Rand and Fisher, 1987). Thus, as predicted for GalTase and other glycosidases, RSA may act as a liaison between the zona surface and signaling ligands.

Spermadhesins represent a second major family of sperm-specific lectin-like proteins that participate in initial gamete attachment. Zona binding by the 12–16 kDa spermadhesins occurs via their single CUB domain (Topfer-Petersen *et al.*, 1998). The conformation of these β-strand CUB domains is maintained by positionally conserved disulfide bonds, creating a structure that can either homodimerize or heterodimerize to form a carbohydrate-binding pocket or multimerize in a side-by-side configuration to create a binding plane (Romero *et al.*, 1997; Varela *et al.*, 1997). They prefer branching oligosaccharides containing D-galactose, D-glucosamine, and D-mannose (Calvete *et al.*, 1996; Dostalova *et al.*, 1995a,b; Solis *et al.*, 1998). Most spermadhesins adsorb to the surface of the sperm during ejaculation but are lost during their transit through the oviduct. Thus, most spermadhesins are thought to prevent premature activation of sperm (Dostalova *et al.*, 1994). A minor fraction of glycosylated AWN-1, AQN-3, and HSP-7 is retained at the sperm plasma membrane over the apical third of the acrosomal cap, however, suggesting that they may participate in sperm–zona interactions (Calvete *et al.*, 1994, 1996; Dostalova *et al.*, 1995a,b; Reinert *et al.*, 1996; Robinson *et al.*, 1987). Unlike other family members secreted by accessory glands, this minor population of spermadhesins are also synthesized during

spermatogenesis and are presumed to remain with the sperm through direct interactions with specific phospholipids found in the sperm plasma membrane (Calvete *et al.*, 1994; Dostalova *et al.*, 1994; Sinowatz *et al.*, 1995). This subset of sperm-derived proteins also has a high affinity for heparin-like glycoproteins found in the zona (Calvete *et al.*, 1994, 1996; Reinert *et al.*, 1996), suggesting that AWN-1 and AQN-3 may participate in carbohydrate-dependent gamete interactions involving glucosamine moieties such as GlcNAc (Topfer-Petersen *et al.*, 1998). This is consistent with the ability of anti-AWN serum to inhibit the *in vitro* association of gametes (Sanz *et al.*, 1992) but has not been observed *in vivo* (Topfer-Petersen *et al.*, 1998). Interestingly, expression of spermadhesins is limited to ungulates such as *Sus* and *Equus*, suggesting that this family of proteins evolved after the split of ungulates from other eutherians (Dostalova *et al.*, 1994; Reinert *et al.*, 1996; Topfer-Petersen *et al.*, 1998). Such restricted expression in animals is likely a consequence of changes in both female anatomy and egg ECM glycobiology.

The range of sperm ligand classes among animal taxa reinforces the dynamic relationship between evolving gametes. Thus far, diversification or specialization in sperm expressed ligands appears to be limited to a few clusters of animal classes, in whom the types and sequences of these proteins are often highly conserved (e.g., conservation of RSA epitopes in many eutherians [O'Rand *et al.*, 1985] or greater than 98% sequence similarity between spermadhesins among ungulates [Reinert *et al.*, 1996]). Yet distinctions must also occur at the species level, possibly using ligands that are just as diverse as the target sperm receptor. Teleost sperm appear to have responded to the inherent complexity ingrained in the glycobiology of the sperm receptor (see Section VI.C, later in this chapter) by using oligosaccharides as the complementary ligand. Conspecific gamete binding at the *Oncorhyncus* micropyle is achieved with sperm ligand 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid $\alpha 2 \rightarrow$3-galactose-β4-galactose-β4-glucan-β1-ceramide (Yu *et al.*, 2002). Thus, involvement of sperm carbohydrates at fertilization may also reflect how male fitness is evaluated during fertilization (see Section V.B, earlier in this chapter). This may be a phenomenon specific to teleosts, however, because their sperm lack an acrosome, the one organelle that could influence the effectiveness of a second round of species specificity analysis (Hart, 1990).

Perhaps sperm do not combat the diversity in the ECM with a single ligand but instead assemble a functional complex in response to egg receptor binding. Echinoderm sperm appear to use such a clustering mechanism to recognize the egg jelly triad responsible for initiating sperm chemotaxis (see Section V.A, earlier in this chapter). Sea urchins, for example, use a family of carbohydrate-binding proteins that preferentially associate with egg jelly. This family of receptors for egg jelly/polycystins (REJ/PCs) is represented by many members, including three original REJ proteins found

on the sperm as well as more distantly related polycystin proteins (Galindo *et al.*, 2004; Hughes *et al.*, 1999; Mengerink *et al.*, 2002; Moy *et al.*, 1996; Neill *et al.*, 2004; Neill and Vacquier, 2004). The core motifs of this family include a lectin domain, a calcium-dependent carbohydrate-recognition domain (CRD), a 1000-residue REJ module containing a carboxy-terminal G-protein–coupled receptor proteolytic cleavage site (GPS) and at least one carboxy-terminal transmembrane domain. The various members encode additional motifs that likely have distinct functions during sperm activation, including one additional CRD in REJ1 (Moy *et al.*, 1996), a single region of polycystic kidney disease (PKD) repeats and two transmembrane domains in REJ2 (Galindo *et al.*, 2004), or a series of tandem PKD repeats and 11 transmembrane domains that form a cluster homologous to ion channels in REJ3 (Mengerink *et al.*, 2002). Both REJ1 and REJ3 are cleaved at the GPS site, but the extracellular portion remains associated with the plasma membrane overlying the sperm acrosome (Mengerink *et al.*, 2002; Moy *et al.*, 1996; Trimmer *et al.*, 1985). It is hypothesized that these ectodomains remain associated with the plasma membrane via heterogenic protein–protein interactions, likely anchored to the membrane via unmodified transmembrane REJ/PC members (Mengerink *et al.*, 2002; Neill *et al.*, 2004).

The role of REJ/PC proteins during sperm activation is twofold. Original observations show that REJ1 directly binds the sulfated sea urchin jelly fucan (see Section V.B, earlier in this chapter) (Alves *et al.*, 1997; Biermann *et al.*, 2004; Bonnell *et al.*, 1994; SeGall and Lennarz, 1979; Vacquier and Moy, 1997). The specificity required by this interaction (Alves *et al.*, 1997; Biermann *et al.*, 2005; SeGall and Lennarz, 1979) is probably dictated by the CRDs because clusters of positively selected residues are found on the extended loops of CRD thought to participate in receptor recognition (Mah *et al.*, 2004). A high degree of nonsynonymous polymorphisms cluster at very specific sites along the protein is observed among individuals (Mah *et al.*, 2005)—clear evidence that an individual's sperm sustain a high level of adaptation to remain compatible with eggs (see Section V.A, earlier in this chapter). Further, species selectivity may be achieved by including REJ3 into the equation because of its preference for L-galactose of sialoglycan (Hirohashi and Vacquier, 2002b; Mengerink *et al.*, 2002). Upon binding of its specific FSP-sialoglycan receptive complex, REJ1–REJ3 is thought to participate in a single transduction event that leads to sperm activation. The stable association of this REJ1–REJ3 pair might induce extracellular calcium influx via the ectodomain's association with sea urchin PC2 (Neill *et al.*, 2004) or a putative transmembrane calcium channel (Mengerink *et al.*, 2002). The similarity of the transmembrane domains encoded by REJ3 and PC2 to transient receptor potential channels (TRPCs), a family of proteins intimately involved with the maintenance of cytoplasmic calcium levels via

extracellular sources (Zhu *et al.*, 1996), suggests an elegant mechanism that accounts for the immediate activation of sperm upon exposure to egg jelly (Vacquier, 1979). This is also consistent with the reported participation of *Mus* TRPC2 in calcium-dependent sperm activation at the zona (Jungnickel *et al.*, 2001).

D. Common Thread to Initial Sperm–Egg Interactions?

The initial interaction between sperm and egg involves a complex set of highly divergent molecules. The ability to modify the glycosylation status of the egg receptor on a whim provides a challenging situation for sperm, whose respective males have clearly adapted to such conditions by introducing a high frequency of individual polymorphisms within critical ligand domains (Mah *et al.*, 2005). Indeed, this may be one reason for the advantage in large populations of cheaply produced sperm; although individual sperm are extremely diverse, the cohort as a whole exhibits enhanced recognition and subsequent activation efficiency to favor fertilization of any conspecific egg encountered. The cofactors employed are not restricted to carbohydrate-binding molecules, either. For example, a 15-kDa sperm seminal vesicle proteinase inhibitor (SVI) receptor found embedded in the murine sperm plasma membrane can, upon association with ZPC, activate a pertussis toxin–sensitive G-protein–coupled signaling cascade that results in acrosome exocytosis (Aarons *et al.*, 1991; Boettger-Tong *et al.*, 1992; Robinson *et al.*, 1987). Thus, sperm have confronted the high level of uncertainty associated with each generation of egg receptor oligosaccharide moieties by (1) increasing the polymorphism of ligands at those specific interaction surfaces that recognize divergent regions of the receptors and (2) assembling ligand complexes that, together, have a greater probability of receptor recognition over each ligand alone. An egg's response to the quantity of sperm that now have the potential to fertilize it may be the incorporation of oviduct-derived glycoproteins into the ECM, partly to foster sperm attraction, but also to act as a distraction that serves to prolong the duration of sperm–egg interaction at the egg surface (Buhi, 2002; Mate *et al.*, 2000; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997).

VI. After the Acrosome Reaction

Sperm activation causes two major changes in sperm behavior. The first is a switch to chemotactic motility toward the ECM (see Eisenbach, 1999; Kaupp *et al.*, 2003; Neill and Vacquier, 2004). The second involves release

of the sperm acrosome, whereby a collection of new membrane-associated proteins from the luminal face of the acrosome is exposed, promoting additional sperm–egg ECM binding partners, whereas soluble content proteins are released into the local environment. Some of the soluble components are known to facilitate the progression of sperm through the ECM. Here, we review the events involved with acrosome exocytosis and the consequences of its release on sperm–egg interactions (see Table II). In particular, we focus on the content of proteins released and the role of each in advancing the sperm towards the egg plasma membrane.

A. Secondary Sperm Contact

Secondary sperm binding is an important opportunity available after the acrosome reaction because it raises the stringency of species specificity. Acrosome exocytosis requires that the delimiting acrosome membrane fuse with the plasma membrane, resulting in the transformation of surface ligands on the sperm. Many of these ligands are contributed by the inner acrosome matrix, the content proteins most closely juxtaposed to the luminal face of the acrosome membrane, but some may also migrate from other unaffected sites on the sperm head. This alteration in the surface profile of the sperm provides both gametes with a second checkpoint for conspecificity.

Abalone sperm prominently display their acrosome contents on the surface, allowing these proteins to participate in subsequent interactions. Although chemoattraction and sperm activation are conspecifically regulated in these animals (Riffell *et al.*, 2002), it is still possible for heterospecific gamete interactions to occur because of the distribution of various species within the same habitat (Riffell *et al.*, 2004). Abalone sperm have selected for the acrosome-derived lysin, a 16-kDa protein with a high affinity for the filamentous VERL of the vitelline envelope. The lysin-VERL dissolution process is often conspecific in a mixed population of animals (Hellberg and Vacquier, 1999; Vacquier and Lee, 1993; Vacquier *et al.*, 1990). Specificity of this interaction is enhanced through positive selection at most of the exposed surfaces of the lysin fold (Lee *et al.*, 1995; Vacquier and Lee, 1993; Yang *et al.*, 2000) and in the first and second VERL repeats on the receptor (Galindo *et al.*, 2003). Under situations of heterospecific insemination, significantly more lysin is required to bind and dissociate VERL (Vacquier *et al.*, 1990), a quantity too high to be released by the paltry mollusk acrosome (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977; Togo and Morisawa, 1999; Usui, 1987); thus, heterospecific gamete fusion is averted.

The major acrosome-derived echinoderm protein responsible for secondary gamete contact is bindin, a 30-kDa protein homologous in at least 70%

of the echinoid orders (Vacquier and Moy, 1977; Vacquier *et al.*, 1995; Zigler and Lessios, 2003). Bindin contains a highly conserved core sequence enriched in nonpolar residues, flanked by two hypervariable domains, including a carboxy-terminal domain of repeats whose quantity differs in sister species (Biermann, 1998; Metz and Palumbi, 1996; Minor *et al.*, 1991; Palumbi, 1999; Zigler and Lessios, 2003; Zigler *et al.*, 2003). The carboxy-terminal tandem repeats of bindin homodimerize to form a lectin-like binding pocket with high affinity for sulfated fucan polymers (DeAngelis and Glabe, 1987; Glabe *et al.*, 1982; Ruiz-Bravo *et al.*, 1986; Vacquier and Moy, 1977; Vacquier *et al.*, 1995). The predicted characteristics of this protein match its egg receptor, EBR1, the sea urchin egg bindin receptor. EBR1 is a 350-kDa sulfated glycoconjugate found within the vitelline layer whose oligosaccharides are enriched with L-fucose, D-mannose, D-galactose, and galactosamine (Aketa *et al.*, 1968; DeAngelis and Glabe, 1987; Dhume and Lennarz, 1995; Foltz and Lennarz, 1990; Foltz *et al.*, 1993; Kamei and Glabe, 2003; Ohlendieck *et al.*, 1993; Rossignol *et al.*, 1984; Ruiz-Bravo *et al.*, 1986). The functional motifs of EBR1 include thrombospondin type 1 (TSP-1) repeats (Adams, 1997), CUB domains (Bork and Beckmann, 1993), and a highly conserved cytoplasmic tail that may participate in intracellular signaling (Foltz and Lennarz, 1993; Foltz *et al.*, 1993). Receptor aggregation is thought to occur via the TSP-1 and CUB domains (Romero *et al.*, 1997; Varela *et al.*, 1997), whereas its selectivity for bindin is linked to the type of protein interaction domains encoded by the carboxy-terminal segment of the receptor's ectodomain (Foltz and Lennarz, 1990; Foltz *et al.*, 1993; Kamei and Glabe, 2000, 2003; Ruiz-Bravo *et al.*, 1986). For example, *Strongylocentrotus franciscanus* EBR1 contains homogenous EBR1-specific repeats consisting of TSP-1/CUB tandems, whereas the orthologous region in *S. purpuratus* encodes hyalin-like repeats (Wessel *et al.*, 1998). Such complete divergence between these repeat motifs may also be responsible for the variable *O*-linked oligosaccharide composition and branching patterns observed among species (Foltz and Lennarz, 1990; Hirohashi and Lennarz, 2001) and is a likely contributor to the species specificity of the EBR1-bindin interaction (Glabe and Vacquier, 1977b; Lopez *et al.*, 1993; Minor *et al.*, 1991; Ohlendieck *et al.*, 1993; Ruiz-Bravo *et al.*, 1986). In some species, however, carbohydrate moieties do not significantly affect the affinity of the bindin–EBR1 pairing (Kamei and Glabe, 2000); in such cases, specificity may be more effectively determined at a more distal site to the egg, such as in the jelly (see Section V.A, earlier in this chapter) (Biermann *et al.*, 2004).

Vertebrate ZPA is an egg-derived glycoprotein thought to be responsible for binding acrosome-reacted sperm. This preference, however, varies across taxa. Teleosts, whose sperm lack acrosomes, may have selected variants (e.g., ZPAX) or duplications of ZPX and ZPC to replace the activity of ZPA proposed for other vertebrates (Fig. 5). In most other animals,

acrosome-containing sperm are reported to preferentially bind ZPA glycoproteins at specific sites along the sperm head, including the acrosome-derived membrane of anuran sperm (Barisone *et al.*, 2002; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo and Hedrick, 2000; Vo *et al.*, 2003) and the postacrosomal domain and midpiece of acrosome-reacted murine sperm (Kerr *et al.*, 2002; Tsubamoto *et al.*, 1999). Positive selection has been observed in a region totaling about 4% of the ZPA gene (Swanson *et al.*, 2001b), implying that like ZPC and sea urchin EBR1, this member participates in species-specific discrimination. The comparative affinity of acrosome-reacted murine sperm for ZPA, however, is significantly lower than for ZPC (K_d of about 200 nM, compared to 63 nM for ZPC) (Kerr *et al.*, 2002), suggesting that the reported sperm–ZP protein preferences are not absolute. Is it possible for a relatively large sperm head to distinguish between individual ZP members within a long oligomer of ZPA–ZPC pairs (see Section II.B, earlier in this chapter)? Probably not since the acrosome-membrane–associated sperm protein sp56/AM67, named for its orthologs from mouse (sp56) and guinea pig (AM67), preferentially binds ZPC (Bleil and Wassarman, 1990; Bookbinder *et al.*, 1995; Easton *et al.*, 2000b; Foster *et al.*, 1997). Yet, this protein is exposed to the zona only after the acrosome reaction (Foster *et al.*, 1997), where it is believed to multimerize via its β-strand Sushi domains to achieve a lectin-like binding pocket that specifically recognizes ZPC oligosaccharides (Cheng *et al.*, 1994). Consistent with the participation of both ZPA and ZPC in sperm binding is the higher sperm–zona interactions of ZPB-null compared to wild-type eggs, indicative of more accessible ZPA–ZPC filaments for sperm attachment (Rankin *et al.*, 1999). Similarly, the acrosome-intact sperm surface protein SED1 does not discriminate among ZPA or ZPC proteins in the zona (Ensslin and Shur, 2003), whereas acrosome-intact *Sus* sperm show a synergistic enhancement in zona binding in the presence of the ZPB–ZPC dimer (Yurewicz *et al.*, 1998). Also consider the sperm hyaluronidase PH-20. Originally found on the posterior surface of acrosome-intact guinea pig sperm and used to penetrate the cumulus cell complex, PH-20 is thought to also be invloved with secondary sperm-binding reactions (Hunnicutt *et al.*, 1996b; Myles and Primakoff, 1997). After the acrosome reaction, a fraction of the remaining membrane-bound PH-20 migrates to the inner acrosome matrix and is cleaved (perhaps by one of the acrosome-derived proteases) (see Section VI.B, later in this chapter), exposing a nonenzymatic carboxy-terminal domain. Antibody studies suggest that the fragment of PH-20 still associated with the plasma membrane participates in secondary sperm binding (Hunnicutt *et al.*, 1996a). It is also possible that the nonenzymatic domain of PH-20 is involved with sperm attachment to the same zona receptor pre-acrosome and postacrosome reaction, thereby obviating a need for the sperm to reattach to the ECM surface after acrosome exocytosis.

It is clear that sperm do not exhibit single-receptor preferences at any stage of sperm–egg ECM interaction. This is advantageous for both gametes because the wider range of receptor-ligand pairs used will bias for conspecific fertilization. The observation that most egg receptors evolve with regional positive selection (Swanson *et al.*, 2001b) further enhances the argument that the most effective method to achieve species-specific gamete interactions enlists a collection of selective molecular mechanisms.

B. Acrosomal Proteases

The egg ECM is a fibrillar matrix that does not, at first, appear to be readily penetrable (Fig. 3), yet sperm do regularly penetrate this matrix. One hypothesis to explain this phenomenon is that sperm gain access to the egg membrane through hydrolytic digestion of the ECM fibrils, gradually creating a tunnel that eventually allows for complete penetration. Thus, it was assumed that acrosomal contents included proteases that would participate in the lysis of the egg ECM. One caveat associated with the digestion of a tunnel through the mammalian zona to accommodate the sperm head is the potential loss of secondary sperm-binding epitopes. Nevertheless, the model for proteolytic digestion of the ECM is supported by the observed depression in fertilization rates associated with the including of protease inhibitors during insemination (Ciereszko *et al.*, 1994, 1996, 2000; Dabrowski *et al.*, 2004; Jones *et al.*, 1996; Rios and Barros, 1997; Sawada *et al.*, 1984b; Takano *et al.*, 1993; Takizawa *et al.*, 1993; Yamagata *et al.*, 1998a). Hence, the search for acrosome-derived proteases was initiated.

The first candidate protease described was acrosin, a 55-kDa serine protease containing a single disulfide linkage between the amino- and carboxy-terminus of its catalytic domain (see Sawada, 2002; Topfer-Petersen *et al.*, 1990). Acrosin is stored in the acrosome in its inactive zymogenic form. Upon release into a more alkaline environment, the enzyme is activated by autocatalysis just downstream of the amino-terminal cysteine, freeing the catalytic domain from its constricted conformation without the loss of the light chain from the proteolytic heavy chain. Among ascidians and eutherians, the animals in whom acrosin is most conserved, the only major variation in primary sequence is the addition of two CUB domains at the carboxy-terminus of the ascidian ortholog (Kodama *et al.*, 2001; Sawada, 2002). Ascidian acrosin is thought to bind the vitelline coat via its CUB domains, yet the predicted separation of these CUB domains from the active chains of acrosin leaves the true function of these binding motifs in question (Kodama *et al.*, 2001). Instead, both ascidian and eutherian acrosin attach to the ECM via positionally conserved basic residues in its amino-terminus, possibly binding through sulfated fucans within their respective egg ECMs

(Baginski *et al.*, 1999; Howes and Jones, 2002; Howes *et al.*, 2001; Jansen *et al.*, 1998; Jones, 1991; Jones *et al.*, 1996; Moreno and Barros, 2000; Richardson and O'Rand, 1996; Topfer-Petersen *et al.*, 1990; Urch and Patel, 1991).

The participation of acrosin proteolysis during sperm penetration, however, remains unclear. First, no evidence has been reported that clearly shows acrosin-dependent digestion of the ascidian vitelline coat (Sawada *et al.*, 1984a). Similarly, *in vitro* insemination with a wide range of serine protease inhibitors never completely inhibits eutherian fertilization (Takano *et al.*, 1993), and acrosin-null sperm are still able to fertilize eggs, albeit with a 30-min delay in sperm penetration compared to heterozygous or wild type genotypes (Adham *et al.*, 1997; Baba *et al.*, 1994; Honda *et al.*, 2002; Nayernia *et al.*, 2002). The retention of acrosin on the surface of acrosome-reacted sperm, particularly at domains most available for ECM binding, suggests an alternative role for acrosin as a tether that maintains sperm–egg interactions (Castellani-Ceresa *et al.*, 1983; Howes *et al.*, 2001; Huang and Yanagimachi, 1984; Jones, 1991; Noguchi and Nakano, 1992; Sawada, 2002; Urch and Patel, 1991). Acrosin could also be involved with the dispersal and/or activation of acrosome proteins (Honda *et al.*, 2002; Takano *et al.*, 1993; Yamagata *et al.*, 1998b) because the presence of endogenous levels of activity during fertilization is consistently associated with a selective advantage over sperm with low levels of activity, as observed in mice (Adham *et al.*, 1997; Yamagata *et al.*, 1998a) and humans (Cui *et al.*, 2000; Shimizu *et al.*, 1997).

In light of the fertilization rates observed in acrosin-compromised sperm, a search for other acrosome-derived proteases has also identified candidates that could be involved with the protease inhibitor–dependent phenotype. One protease present in both ascidians and eutherians is spermosin, another serine protease released from the acrosome (Kodama *et al.*, 2002; Sawada, 2002). Spermosin contains a proline-rich domain at its amino-terminus and a carboxyl ECD (Glu-Cys-Asp) motif thought to enhance the enzyme's association with the ECM. This is best documented by the *in vitro* association between ascidian spermosin and both a 28-kDa vitelline coat protein and VC70 (Kodama *et al.*, 2002; Sawada, 2002; Sawada *et al.*, 1996). Like acrosin, however, the absence of detectable vitelline coat debris following spermosin exposure leaves its role in sperm penetration in question (Sawada *et al.*, 1984a). Another family of proteins postulated to participate in sperm penetration is the testicular serine proteases (TESPs), specifically TESP1, TESP2, TESP4, and TESP5 (Honda *et al.*, 2002). Of particular interest is murine TESP5, a protease that co-migrates with a suspected 42-kDa trypsin-like activity responsible for digesting the zona of both wild-type and acrosin-null mice that is present in sperm lipid rafts, structures thought to be involved with cell–cell signaling (Honda *et al.*, 2002). TESP5 is limited to

Mus, however, suggesting that this animal has co-opted the use of two different trypsin-like proteases, perhaps selecting for allotypes with lower activity compared to other eutherians (Honda *et al.*, 2002).

The first evidence that sperm-dependent ECM degradation occurs was documented during ascidian fertilization (Lambert, 1989). One mechanism for this process depends on a 35-kDa chymotrypsin-like protease that specifically depletes the outer electron-dense layer of the *Ciona* vitelline coat (Marino *et al.*, 1992; Sawada *et al.*, 1998). Extracellular proteasomes have also been implicated in the digestion of the vitelline coat (Saitoh *et al.*, 1993; Sakai *et al.*, 2003; Sawada *et al.*, 1998, 2002a,b). This family of multienzyme complexes is normally found in the cytoplasm of most cells, where it participates in general housekeeping and cell homeostasis via its ubiquitin-mediated method of degradation. Yet even the extracellular members, such as the 20S (620-kDa) and cognate 26S (930-kDa) ascidian proteasomes, are able to efficiently degrade polyubiquitinated targets like the ascidian sperm receptor VC70 (Saitoh *et al.*, 1993; Sakai *et al.*, 2003; Sawada *et al.*, 2002a,b). The conservation of extracellular proteasome in deuterostomes is implied by additional reports from sea urchin (Matsumura and Aketa, 1991) and human sperm (Morales *et al.*, 2004; Rossato *et al.*, 1999; Wojcik *et al.*, 2000). In deuterostomes, proteasome activity is associated with specific regions of the sperm head (Morales *et al.*, 2004; Sawada *et al.*, 1996; Wojcik *et al.*, 2000). Also, micrograms per milliliter of free ubiquitin, an essential cofactor of proteasomes, have been found in human seminal fluid (Lippert *et al.*, 1993; Wojcik *et al.*, 2000); application of free ATP, as suggested by the *in vivo* rise in oviductal ATP upon ovulation, to sperm during *in vitro* fertilization increases success with infertile males (Rossato *et al.*, 1999); and anti-proteasome antibodies are found in the seminal fluid of clinically infertile males (Bohring *et al.*, 2001). Yet in contrast to a direct role for proteasome-dependent degradation in ascidians (see later discussion), no ultrastructural evidence for requisite ECM proteolysis exists in mammals.

The exact mechanism of protease-dependent dissolution of the ascidian vitelline coat may proceed as follows (Lambert, 1989): Upon exocytosis of the small apical acrosome (Fig. 6), the sperm plasma membrane becomes intimately attached to the vitelline coat (De Santis *et al.*, 1980; Lambert, 1989). Following digestion of a physical hole in the vitelline coat by the proteasome, the sperm cytoplasm and nucleus are extruded, leaving the mitochondrion and the tail extending along the extracellular surface of the vitelline coat. Acrosin and spermosin may act to sterically mask potential sperm-binding sites along the vitelline coat that would otherwise impede the progression of the acrosome-reacted sperm plasma membrane as it slides through the hole, perhaps via the detached CUB domains of acrosin (Kodama *et al.*, 2001) and/or by associations along the plasma membrane that also mask or modify potential ECM-associating ligands.

A similar mechanism of sperm penetration requiring the creation of a hole in the ECM is documented in modern decapods, suggesting that another animal order depends on direct proteolysis of the egg ECM (Fig. 6). Decapod sperm lack flagellum, or any other motile organelle; their mobility is restricted to the reaction initiated by acrosome exocytosis (Hinsch, 1971; Medina and Rodriguez, 1992; Tsai and Talbot, 1993). Following primary contact with the egg ECM, the decapod sperm acrosome dehisces, causing violent hydration of its contents outside the sperm (Tsai and Talbot, 1993). The next phase of the acrosome reaction involves a circular contraction along the tip of the former anterior sperm cap, forcing the remaining plasma membrane to evert (Medina and Rodriguez, 1992; Tsai and Talbot, 1993). This process causes a preformed filament stored in the posterior end of the sperm to extend forward, carrying the nucleus with it. Thus, the sperm tip travels some 10 μm closer to the egg surface within seconds of initial contact with the ECM (Tsai and Talbot, 1993). Penetration of these immotile sperm through a dense fibrous ECM requires lysis of the matrix to create a tunnel that facilitates the sperm's progress (Hinsch, 1971; Rios and Barros, 1997). The source of this lysis is thought to be a protease released from the acrosome. In fact, a trypsin-like activity that specifically degrades a 72-kDa protein from the shrimp *Rhynochocinetes* vitelline envelope has been reported, but the absence of a definitive acrosome in its sperm leaves the source of this enzyme in question (Rios and Barros, 1997), a situation historically reminiscent of the status of the ascidian sperm until the discovery of the minute ascidian acrosome (De Santis *et al.*, 1980). Still, even at low concentrations of enzyme, the proteolytic of sperm-penetration model remains plausible in shrimp, whose acrosomal filament is not ejected until 10 min after initial sperm–ECM contact (Lindsay *et al.*, 1992a). In contrast, the rate of sperm penetration in *Homarus* and *Uca* gametes *in vitro* (on the order of seconds from primary sperm binding to complete eversion [Tsai and Talbot, 1993]) is too fast to accommodate the activity of enzymatic degradation, suggesting that a complement of processes may be required for rapid vitelline envelope penetration in these decapods. For example, these sperm may utilize the combination of a chaotropic ECM softener or hyperactive protease and the mechanical force of eversion and/or acrosomal filament extension to successfully penetrate the vitelline envelope.

C. Penetration of Egg ECM in the Absence of Proteolysis

With the exception of ascidians and *Rhynochocinetes*, the absence of identified sperm-derived protease substrates from the egg ECM brings into question the actual mechanism used by sperm to penetrate the egg ECM (see Section VI.B, earlier in this chapter). The simplest method of penetration in

the absence of protein degradation is observed in animals whose eggs possess micropyles. In teleosts such as *Danio* (Mengerink and Vacquier, 2001; Wolenski and Hart, 1987) and *Rhodeus* (Ohta and Iwamatsu, 1983), no sperm acrosome exists, so the involvement of acrosome-derived proteases is moot (Figs. 1 and 6). Instead, the process of penetration is likely a consequence of modifications in intracellular signaling cascades in response to the relative position of the sperm within the micropyle (Yu *et al.*, 2002). The same may even hold true for more primitive fish such as chondrosteans, whose sperm exocytose their acrosome at the micropylar entrance, followed by extension of an acrosomal process through the pit to rapidly contact the fusogenic egg membrane at the other end (Cherr and Clark, 1986; Kobayashi *et al.*, 1994; Kobayashi and Yamamoto, 1994). A similar reaction occurs in dipterans, whose acrosome is also released upon contact with the glycoprotein tuft at the micropylar entrance (Figs. 1 and 2), exposing an acrosomal rod that participates in sperm penetration of the tuft (Degrugillier and Leopold, 1976). The observed sperm-dependent loss in surface glycosidases following the acrosome reaction likely facilitates passage of the sperm by removing high-affinity receptors along the micropylar entrance (Cattaneo *et al.*, 1997, 2002; Perotti *et al.*, 2001).

Animals that use preestablished tunnels similar to a micropyle also do not require mechanisms to dramatically alter the integrity of the ECM. For example, the journey of flagellate mollusk sperm is made easier by channels in the vitelline envelope that terminate in fusogenic microvilli (Figs. 1–4). These sperm also use a preformed microtubule filament that extends away from the anterior face of the nucleus and transects the anterior acrosome (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977; Togo and Morisawa, 1999; Usui, 1987). Following the acrosome reaction, the membrane-delimited filament is exposed and coated with the acrosome content proteins (Lewis *et al.*, 1982; Usui, 1987). In general, the acrosome filament is slightly shorter than the distance from the ECM surface to the microvillar occupant, so the acrosome-reacted sperm need only push the filament a short distance into the vitelline envelope to contact the egg membrane (Buckland-Nicks and Hodgson, 2000; Hylander and Summers, 1977). Indenting the vitelline envelope surface requires more than just mechanical force, however. In the case of gastropods such as teguline or abalone, the evolutionarily divergent lysin chaotropically dissociates the vitelline envelope to facilitate the sperm head's progress through the ECM (Hellberg and Vacquier, 1999; Lewis *et al.*, 1982). The 16-kDa acrosome-derived lysin irreversibly binds its oligomeric glycoprotein receptor via hydrophilic residues found on its α-helices, thereby converting a high-affinity hydrogen-bonded interactive surface along the VERL repeat into a hydrophilic interface that is easily separated (Galindo *et al.*, 2002; Kresge *et al.*, 2000a,b, 2001). This process essentially unzips VERL macromolecules,

separating them into individual subunits, a process that can domino through the vitelline layer to provide sperm full access to the microvilli at the terminus if enough lysin is present (Kresge *et al.*, 2001; Lewis *et al.*, 1982; Vacquier *et al.*, 1990). Given the concerted evolution of the VERL repeats (Galindo *et al.*, 2002, 2003), however, this domino effect could prove detrimental because fortuitous separation of VERL along its first two repeats by a heterospecific sperm lysin could unzip the remainder of the molecule (see Section VI.A, earlier in this chapter) (Swanson and Vacquier, 1997; Vacquier and Lee, 1993; Vacquier *et al.*, 1990). Thus, the heterospecific sperm would quickly gain access to the egg and possibly fertilize it, leaving at least one embryo with incompatible haploid genomes (Kresge *et al.*, 2001; Swanson and Vacquier, 1997).

Most animals do not synthesize eggs with preestablished sites for sperm access in their ECM or use single molecules that offer lenient safeguards against heterospecific secondary sperm binding (see Section VI.A, earlier in this chapter). Instead, sperm must mechanically penetrate the matrix to access the egg plasma membrane. A dramatic example of this process occurs during fertilization of the primitive crustacean *Limulus*, whose flagellate sperm uses an acrosome filament with stored mechanical energy (Tilney, 1975). Upon binding fucan residues exposed on the vitelline envelope (Barnum and Brown, 1983; Brown, 1976), *Limulus* sperm acrosome react, forming an anterior collar that remains attached to the surface of the ECM while the actin-based acrosome filament elongates from the subacrosomal domain with a right-handed helical turn (Tilney, 1975; Tilney *et al.*, 1979). The curvature of the filament allows the sperm to screw itself into the matrix ahead, carrying the sperm nucleus closer to the egg surface while the flagellum and collar remain perpendicular and peripheral to the ECM (Brown, 1976; Tilney, 1975). Like a corkscrew inserting into cork, the actin filament would mechanically displace and fray the ECM fibers. No ultrastructural evidence for such displacement has been reported, however, suggesting either that limited fragmentation occurs or that acrosome contents participate in the dispersal of the ECM glycoproteins.

Echinoderm sperm use both the force of their acrosomal processes and the flagellar movement to penetrate the jelly and to contact the delicate vitelline layer draped over the egg's microvilli (Anderson, 1968; Chandler and Heuser, 1980; Chandler and Kazilek, 1986; Glabe and Vacquier, 1977a; Larabell and Chandler, 1991; Runnstrom, 1966) (Fig. 2). Within seconds after the acrosome reaction, cytoplasmic calcium concentrations and pH rise in echinoid sperm (Neill and Vacquier, 2004). This causes, respectively, acrosome exocytosis and polymerization of actin monomers originating from the actomere, an actin-organizing center found anterior to the nucleus and posterior to the acrosome proper (Dan *et al.*, 1964; Schatten and Mazia, 1976; Tilney, 1978). Polymerization results in the extension of a membrane-delimited acrosomal

process toward the egg surface (Dan *et al.*, 1964; Schroeder and Christen, 1982; Tilney *et al.*, 1978) (Fig. 6). The acrosomal contents coating the acrosomal process thus gain immediate access to its cognate receptors found in the vitelline layer (Moy and Vacquier, 1979). The primary protein responsible for coating this acrosomal process is bindin (Vacquier and Moy, 1977; Vacquier *et al.*, 1995). Bindin preferentially associates with EBR1 within the vitelline layer (see Section VI.A, earlier in this chapter) (DeAngelis and Glabe, 1987; Kamei and Glabe, 2003; Ohlendieck *et al.*, 1993; Rossignol *et al.*, 1984). Conspecific association of bindin and EBR1 is thought to facilitate agglutination of the gametes, whereby the sperm and egg are drawn together through the adhesive and mutually attractive forces of this receptor–ligand pair (Glabe, 1985a; Glabe and Vacquier, 1977b; Glabe *et al.*, 1982; Lopez *et al.*, 1993; Vacquier and Moy, 1977). The egg jelly protein FSP responsible for triggering the acrosome reaction is also rich in fucose (Keller and Vacquier, 1994) (Table IV). Thus, premature acrosome reaction in the jelly could retard the progression of sperm toward the egg. Given the brief 20-sec half-life a sperm has from the time of acrosome reaction to successfully fuse (Vacquier, 1979), any retardation of progress in the jelly could prove detrimental to the sperm's success yet is advantageous for monospermic fertilization since fewer competent sperm will interact with the egg.

Mechanical penetration of vertebrate egg ECMs is also possible, despite their relatively thick fibrillar matrices. The acrosome reaction of many anuran sperm occurs within the jelly (see Section V.A, earlier in this chapter) (Campanella *et al.*, 1997; Elinson, 1986; Maturi *et al.*, 1998; Vaccaro *et al.*, 2001; Vo and Hedrick, 2000; Vo *et al.*, 2003). In these animals, penetration through the remaining jelly coat likely relies on the morphology of the acrosome-reacted sperm head, a long tapered cone or pointed rod that can be used to stab through the fibrillar ECM (Arranz and Cabada, 2000; Campanella *et al.*, 1997). Such a model is also parsimonious for all anurans, even those taxa whose sperm remain acrosome intact until reaching the vitelline envelope (Arranz and Cabada, 2000; Barisone *et al.*, 2002; Toro and Michael, 2004). Further evidence for the application of mechanical force during sperm penetration of the vertebrate egg ECM has been reported in avians and mammals. Holes can be found in the avian perivitelline layer, yet the morphology of these holes is indicative of the dispersion of avian perivitelline layer filaments rather than proteolysis (Howarth, 1990; Okamura and Nishiyama, 1978a) (Fig. 3). Similarly, frayed tunnels were reported in some marsupial zonae following insemination (Breed and Leigh, 1990; Jungnickel *et al.*, 1999; Rodger and Bedford, 1982b) (Fig. 3). Combined with the observations from anurans, the absence of detectable proteolytic fragments from these vertebrates has resulted in an alternative model for vertebrate sperm penetration of the egg ECM (Breed and Leigh, 1990):

Following the acrosome reaction, content proteins such as acrosin (see Section VI.B, earlier in this chapter) or β-*N*-acetylglucosaminidase (Miller *et al.*, 1993b) adsorb to their appropriate receptors, sterically masking these sites within the ECM from the sperm to limit otherwise retardant interactions between the gametes. The morphology of the acrosome-reacted sperm head would dictate how the sperm mechanically penetrates the ECM, namely by piercing through with a long narrow head or by pushing filaments aside using an oscillating motion, as predicted for the scythe-shaped head of murine sperm (Bedford, 1998) (Figs. 1 and 6). In this way, the sperm gains access to the egg membrane without severely altering the integrity or the mass of the ECM, a result that may be essential for the assembly of a permanent block to polyspermy using components of this very same egg ECM.

VII. Climax of Fertilization

Once the sperm head has successfully penetrated the egg ECM, its remaining obstacle before completing fertilization is the egg plasma membrane. The theoretical energy necessary for fusion of two inflexible planar membranes is upwards of 93 kcal/mol for mammals at physiological temperatures (Siegel, 1993). The composition of the membrane significantly impacts both the absolute energy required to achieve such intermediates (Basanez, 2002; Kozlovsky and Kozlov, 2002; Kuzmin *et al.*, 2001; Markin and Albanesi, 2002). This is best observed in zona-free hamster eggs, who normally can fuse with all species of mammalian sperm (Talbot and Chacon, 1982). Pretreatment of these eggs with phospholipase C (PLC), however, abolishes this promiscuity, presumably as a consequence of the resultant alteration to membrane composition (Boldt *et al.*, 1988).

Catalysts have a significant impact on the rate of membrane fusion. The most effective protein catalysts include the SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) protein complexes (Bentz and Mittal, 2000; Lang *et al.*, 2001; Lentz *et al.*, 2000), viral membrane glycoproteins (Basanez, 2002; Lentz *et al.*, 2000; Shemer and Podbilewicz, 2003), and homologs of the nematode fusogenic protein *eff-1* (Shemer and Podbilewicz, 2003). The participation of such protein catalysts is consistent with the loss-of-fusion phenotypes following pronase pretreatment of acrosome-reacted human sperm (Arts *et al.*, 1997).

Two additional components that appear to be necessary for fusion include active proteases (Boldt *et al.*, 1988; De Santis *et al.*, 1992; Kato *et al.*, 1998; Roe *et al.*, 1988; Takano *et al.*, 1993) and extracellular calcium, as suggested by the reduced efficiency of fusion in the absence of this divalent cation (Glabe, 1985b; Tilney *et al.*, 1978; Yanagimachi, 1978). This cation-dependence is consistent with the participation of calcium in other

membrane-fusion events (Abbott and Ducibella, 2001; Ales *et al.*, 1999; Crabb and Jackson, 1985; Horsley and Pavlath, 2004; Lang *et al.*, 2001; Lentz *et al.*, 2000). Here, we survey the mechanisms of membrane fusion in the context of fertilization, focusing on catalysts likely responsible for the union of a sperm and an egg (see Table II).

A. Sites of Fusion and Membrane Properties

Even though a single sperm has gained access to the egg membrane, it must be near a fusion-competent site to complete fertilization. Most animal eggs use microvilli, specifically microvillar tips, as the preferred site of fusion (Buckland-Nicks *et al.*, 1988; Evans, 1999; Hart, 1990; Hylander and Summers, 1977; Longo *et al.*, 1986; Myles, 1993; Talbot and Chacon, 1982; Vigil, 1989; Wolenski and Hart, 1987). These cell-surface extensions provide the egg with two features that dramatically improve fusion efficiency: (1) an increase in overall surface area that is proportional to the square of the radial gain provided by average microvillus length and (2) discrete sites to localize and concentrate fusion machinery. One added benefit is the intimate association of microvilli with the actin cytoskeleton, which allows for rapid remodeling of the membrane in ways that can significantly increase the rate of sperm incorporation and the surface area of plasma membrane participating in a single fusion event. For example, many animals exhibit the formation of a fertilization cone at the site of sperm fusion, an extension of many local microvilli that together engulf the sperm head to rapidly complete fusion (Buckland-Nicks and Hodgson, 2000; Goudeau and Becker, 1982; Hart, 1990; Schatten and Mazia, 1976; Wolenski and Hart, 1987) (Fig. 6). In some teleosts, the extensiveness of this cone is sufficient to plug the micropylar canal, thereby ensuring that the egg is monospermic (Ohta and Iwamatsu, 1983) (Figs. 3 and 6).

Animal eggs use a wide range of fusion-competent domains along their membranes (Figs. 3 and 6). The method selected for may be related to what the sperm/egg ratios of insemination are *in vivo* (see Section IV, earlier in this chapter). For example, eggs of broadcast spawners such as mollusks and echinoderms show no distinct preferences for where the sperm may fuse, possibly because their effective dilution of gametes in the open ocean significantly reduces the number of sperm that may encounter an egg. One exception, is the bivalve *Unio* egg, which has a very small fusogenic domain that likely evolved in response to the high sperm/egg ratios encountered following gamete concentration in the suprabrachial chambers of the gills after spawning (Focarelli *et al.*, 1988). Like echinoderms, mammalian eggs are receptive over the majority of the egg surface, with the exception of the region overlying the meiotic spindle (Evans, 1999; Myles, 1993). Such a large

promiscuous fusogenic surface implies that the mammalian oviduct and egg are extremely efficient at regulating the sperm/egg ratio. Amphibians, whose method of insemination significantly increases the sperm/egg ratio, produce eggs that preferentially fuse at the animal hemisphere (Elinson, 1986). Again, the large surface area primed for fusion may be a consequence of the efficacy of the surrounding jelly in limiting the number of sperm that reach the egg surface. One exception to this generalization lies with *Discoglossus*, whose egg restricts sperm fusion to a single depression at the animal pole (Talevi and Campanella, 1988). Thus, the most restrictive sites are associated with animals that either spawn in close quarters (avian, petromyzontids) or utilize micropyles (dipterans, chondrosteans, teleosts) (Cherr and Clark, 1986; Harper, 1904; Hart, 1990; Mouzaki *et al.*, 1991; Neubaum and Wolfner, 1999; Okamura and Nishiyama, 1978b).

Sperm also appear to possess discrete fusion-competent domains. Teleost sperm, for example, generally fuse along the equatorial band (Hart, 1990; Wolenski and Hart, 1987). Similarly, echinoderms and mollusks preferentially initiate fusion at the apical tips of their acrosomal projections, although fusion can proceed anywhere along the side (Buckland-Nicks *et al.*, 1988; Hylander and Summers, 1977; Schatten and Mazia, 1976). The echinoderm sperm head, for example, is drawn towards and rotated parallel to the egg surface following initial membrane contact, presumably to increase the total surface area available to rapidly complete fusion (Schatten and Mazia, 1976). Acrosome-reacted eutherian sperm, however, prefer the equatorial segment and anterior third of the postacrosomal region (Arts *et al.*, 1993; Clark and Koehler, 1990; Myles, 1993; Talbot and Chacon, 1982; Vigil, 1989). This β-hydroxysterol–rich equatorial segment (Clark and Koehler, 1990) significantly lowers the energy threshold for fusion by increasing membrane flexibility (Basanez, 2002; Markin and Albanesi, 2002).

B. Egg Contributions to Fusion

Protein-dependent catalysis of plasma membrane fusion requires contributions from both membranes. Because the major effect these proteins have on fusion is a reduction in the energy requirements for membrane deformation, it is hypothesized that these catalysts are associated with the plasma membrane or complex with other membrane-affiliated proteins (Basanez, 2002; Bentz, 2000; Bentz and Mittal, 2000; Lentz *et al.*, 2000). Only a few candidate molecules fit these criteria in eggs. The first is the sea urchin sperm receptor EBR1 (egg bindin receptor). This freely soluble receptor is a member of the "a disintegrin and metalloprotease" (ADAM; also known as "metalloprotease/disintegrin/cysteine-rich," or MDC) family of proteins (Evans, 1999; Kamei and Glabe, 2003). This family of transmembrane

proteins classically encode a metalloendoprotease domain followed by a disintegrin loop, a cysteine-rich motif, and an EGF–like repeat within the ectodomain (Evans, 1999). EBR1 encodes a metalloprotease domain but lacks the disintegrin repeat. Association of EBR1 with the vitelline layer likely occurs through its CUB and/or thrombospondin type 1 (TSP-1) repeats (Kamei and Glabe, 2003). Its candidacy as a member of the gamete fusion complex comes first from its sequence, specifically the metalloprotease domain whose putative enzymatic activity is critical for sperm–egg fusion in sea urchins (Kato *et al.*, 1998) and ascidians (De Santis *et al.*, 1992). Second, EBR1 associates with sperm bindin, making it a possible coordinator of the bindin-dependent agglutination and fusion observed *in vitro* (see Section VII.C, later in this chapter) (Glabe, 1985a,b; Glabe and Vacquier, 1977b).

Another female-contributed protein important for membrane fusion is egg CD9, a mammalian member of the tetraspanin protein family (reviewed in Hemler, 2003; Kaji and Kudo, 2004). Tetraspanins are integral membrane proteins containing four transmembrane domains and a large central extracellular loop thought to participate in direct protein–protein interactions with other membrane-associated receptors and signaling enzymes. A subclass of tetraspanins is implicated in the process of cell–cell fusion, including *late bloomer*, involved in establishment of neuromuscular junctions in *Drosophila* (Kopczynski *et al.*, 1996); the interacting pair CD82/CD81 (C33/M38) that facilitates human T-cell leukemia virus type 1–dependent T-lymphocyte fusion (Imai and Yoshie, 1993); and CD9/CD81, which promotes myoblast fusion (Tachibana and Hemler, 1999). Within the egg, CD9 localizes specifically at the microvillar tips on the egg (Chen *et al.*, 1999). The role of CD9 in fertilization was first observed in knockout mice, whose most significant phenotype is sterility because of a failure of sperm–egg fusion (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000; Zhu *et al.*, 2002). Overexpression of CD81, a putative binding partner also implicated in plasma membrane fusion events, rescues the CD9-null phenotype (Kaji *et al.*, 2002). As with other tetraspanins, the extracellular loop—especially residues Ser^{173}-Phe^{174}-Gln^{175}—is essential for retaining CD9-dependent fusion (Zhu *et al.*, 2002). It is thought that CD9 is required for organizing egg membrane proteins like integrins and other tetraspanins, thereby enhancing cell aggregation and potentiating fusion (Maecker *et al.*, 1997; Zhu *et al.*, 2002). Such coordinated activity of CD9 would ultimately facilitate the initial stage of sperm–egg fusion by localizing all the necessary cell–cell contact and fusion machinery to a cluster of microvilli near the site of sperm–egg membrane attachment (Chen *et al.*, 1999; Kaji *et al.*, 2000, 2002).

Based on their potential interactions with the actin cytoskeleton and membrane-associated tetraspanins, the superfamily of integrins is also suspected to participate in gamete fusion (reviewed in Berditchevski, 2001;

Evans, 2001; Hemler, 2003; Maecker *et al.*, 1997). Each member of the integrin family is a heterologous receptor composed of an α-subunit and a β-subunit. Several members have been detected on the egg surface, including eutherian α_2, α_3, α_5, α_6, α_9, α_M, α_V, β_1, β_2, β_3, β_4, β_5, and β_6 (He *et al.*, 2003; Sengoku *et al.*, 2004) and sea urchin α_B- and β_C-subunits (Murray *et al.*, 2000). Based on observations made in other fusogenic mammalian cells, only the paired $\alpha_6\beta_1$ and $\alpha_9\beta_1$ heterodimeric pairs are believed to participate in CD9-dependent gamete fusion. Yet functional and genetic knockdown experiments of every integrin pairing possible in eutherian eggs, via antibody or genetic manipulations, have shown that none of the known egg surface integrins is essential for fertilization (Eto *et al.*, 2002; He *et al.*, 2003; Kaji and Kudo, 2004; Miller *et al.*, 2000; Sengoku *et al.*, 2004; Zhu and Evans, 2002). No additional reports have been presented for the sea urchin integrin, but based on the results from eutherian experiments, their participation in fusion is unlikely.

C. Sperm Contributions to Fusion

As observed in all other gamete receptor–ligand pairs, the list of candidate sperm catalysts that may participate in gamete fusion is extremely diverse compared to the shorter list of shared egg contributors. One candidate is part of the SNAREs, a family of proteins that often assemble into specific multimeric complex at the future site of vesicle-plasma membrane fusion (Bentz, 2000; Chamberlain *et al.*, 2001; Conner *et al.*, 1997; Ikebuchi *et al.*, 1998; Lang *et al.*, 2001; Pelham, 2001; Tahara *et al.*, 1998). The sperm-specific Vam6p (vesicle-associated membrane protein type-6) is present in both acrosome-intact and acrosome-reacted sperm, although its localization changes dramatically upon sperm activation (Brahmaraju *et al.*, 2004). The abundance of Vam6p over the acrosome implies that, with the assistance of SNAP, this protein participates in acrosome fusion during sperm activation. Following the acrosome reaction, the only detectable Vam6p is found at the fusogenic equatorial band (Brahmaraju *et al.*, 2004). Based on the current models of SNARE-mediated membrane fusion, Vam6p would have to lie between the two membrane faces (Bentz, 2000; Lentz *et al.*, 2000). Antibody inhibition of Vam6p blocks sperm–egg binding, consistent with an ectopic orientation (Brahmaraju *et al.*, 2004). But this phenotype does not directly implicate it in the process of fusion because it is not clear how the normally cytoplasmic vesicle-associated protein flips across the membrane to become an extracellular protein capable of binding the egg.

A broader list of sperm-derived candidates has been identified through fusion-inhibiting screens using monoclonal antibody raised against antigens from the highly-fusogenic regions of *Mus* and *Cavia* sperm (see Myles, 1993).

Two of these antigens, (IgM) M29 (Saling *et al.*, 1983) and mMN9 (Toshimori *et al.*, 1998), localize exclusively to the equatorial region of acrosome-reacted sperm. Their respective immunoglobulins inhibit gamete fusion, but not membrane attachment between the gametes (Saling *et al.*, 1983; Toshimori *et al.*, 1998). The target antigen of mMN9 is the acrosome-derived equitorin, and is functional as a contraceptive that blocks sperm fusion mechanisms (Toshimori *et al.*, 1998; Yoshinaga *et al.*, 2001). A third candidate from such a screen is PH-30, the α-subunit of eutherian fertilin (Primakoff *et al.*, 1987). Fertilin is a mammalian heterodimeric member of the ADAMs family of proteins; thus, both sperm and eggs could contribute the necessary metalloendoprotease activity required for fusion (see Section VII.B, earlier in this chapter) (De Santis *et al.*, 1992; Evans, 1999; Roe *et al.*, 1988). Fertilin consists of an α-(ADAM1) and a β-(ADAM2) subunit that both retain their ability to bind the egg surface via their disintegrin domains, together forming one potential candidate egg receptor part of the CD9-dependent signaling network (Blobel *et al.*, 1990; Eto *et al.*, 2002; Lum and Blobel, 1997; Takahashi *et al.*, 2001; Zhu and Evans, 2002). Within these domains are two functionally conserved motifs thought to participate in gamete fusion: the β-subunit's functional ECD (Glu-Cys-Asp) protein-binding motif and the α-subunit's "fusion peptide," a stretch of hydrophobic residues homologous to a viral fusion protein (Blobel *et al.*, 1990; Evans *et al.*, 1997a,b; Konkar *et al.*, 2004; Lum and Blobel, 1997; Myles and Primakoff, 1997; Nishimura *et al.*, 2002; Primakoff *et al.*, 1987; Zhu *et al.*, 2000). The acidic residues of the conserved ECD motif are necessary and sufficient for sperm–egg association (Konkar *et al.*, 2004; Zhu *et al.*, 2000), whereas the fusion peptide of fertilin α is thought to provide the impetus for membrane deformation and pore formation during fusion (Basanez, 2002; Myles and Primakoff, 1997). In the absence of functional *Mus* sperm fertilin β, both sperm binding to zona-free eggs and membrane fusion are impaired compared to wild type, although the 50% reduction in fusion is probably a compounded effect of the 13% reduction in gamete binding (Cho *et al.*, 1998; Myles and Primakoff, 1997; Nishimura *et al.*, 2001). Though not as penetrant as a CD9 knockout (see Section VII.B, earlier in this chapter), this reduced-fertility phenotype further supports the hypothesis that fertilin is involved with the final stage of fertilization (Bronson *et al.*, 1999; Nishimura *et al.*, 2001; Zhu and Evans, 2002; Zhu *et al.*, 2000).

The persistence of low levels of fusion competency following loss of sperm fertilin raises the question of how redundant the ADAMs protein function is along the surface of an acrosome-reacted sperm. Initially, the low sequence similarity among fertilin orthologs within the fusion peptide domain and the nonfunctional human fertilin α pseudogene suggests that fertilin proper is likely not involved in fusion of all eutherian gametes (see Jury *et al.*, 1997, 1998; Myles and Primakoff, 1997). The discovery of complete

fertilin α paralogs in mice (Nishimura *et al.*, 2002) and primates (Hooft van Huijsduijnen, 1998; Jury *et al.*, 1998), however, indicates that alternate forms of the fertilin heterodimer can exist. These neighboring intron-less genes encode fertilin α paralogs of different mass with one paralog displaying a carboxy-terminal truncation while another in humans lacks an appropriate active catalytic histidine (Hooft van Huijsduijnen, 1998; Jury *et al.*, 1998; Nishimura *et al.*, 2002). These isoforms are predicted to be distinctly and alternatively expressed during spermatogenesis (Nishimura *et al.*, 2002). Yet, even though deletion of any of the fertilin α paralogs is detrimental to the surface expression of the fertilin heterodimer, the observed retention of fusion competency of these sperm suggests that non-fertilin alternatives are compensatory (Nishimura *et al.*, 2004; Primakoff and Myles, 2002).

One of the proteins thought to supplement and/or compensate for fertilin during gamete fusion is the ADAM cyritestin (ADAM3) (Evans, 1999, 2001). As with fertilin, the extracellular binding loop of cyritestin is hypothesized to be critical for fusion, as shown by peptide competition assays using its extracellular loop sequence (Kaji and Kudo, 2004; Takahashi *et al.*, 2001). Although elimination of cyritestin from the sperm surface completely abolishes *in situ* fertilization following mating, these knockout sperm are still able to fuse with zona-free eggs *in vitro* (Nishimura *et al.*, 2001; Shamsadin *et al.*, 1999). These knockout results in mice are consistent with data in humans that show no impaired fertility in the absence of functional *cyritestin* transcript (Frayne and Hall, 1998; Grzmil *et al.*, 2001). Furthermore, the absence of both *Mus* fertilin and cyritestin does not further impair the fertilin β–null fusion phenotype during insemination of zona-free eggs (Cho *et al.*, 1998; Nishimura *et al.*, 2001), suggesting that cyritestin functions in the oviduct prior to fusion (Nishimura *et al.*, 2001, 2004; Shamsadin *et al.*, 1999).

Production of functional sperm ADAMs is also linked, as documented by the impaired surface expression of fertilin or cyritestin when the reciprocal protein is knocked out (Nishimura *et al.*, 2001, 2004). This sensitivity to surface protein levels suggests the importance of ADAMs in fusion (Bronson *et al.*, 1999; Evans, 2001; Takahashi *et al.*, 2001). This is further supported by competition assays that report impaired *in vitro* fertilization when the highly conserved ADAM disintegrin loop sequence RX_8DLPEF is present (Eto *et al.*, 2002). Although no known egg integrin is directly responsible for mediating cell fusion, the possibility remains that an egg-specific integrin or a mimetic found at the microvillar tips is involved (Myles and Primakoff, 1997). Localization of fertilin and cyritestin to lipid rafts (Nishimura *et al.*, 2001) implies that eutherian fusion is dependent on intracellular signaling to coordinate the complex sequence of steps. Thus, an initial sketch of the molecular mechanisms involved in gamete fusion could be described as thus:

Binding of the disintegrin loop to its egg receptor initiates a series of cytoskeletal modifications that remodel the location of tetraspanins such as CD9 or CD81. This would reorient neighboring microvilli toward the site of the bound sperm. In total, these rearrangements move the necessary proteins to sites of successive fusion events. Given the correct ADAM subunit, a functional fusion peptide could be used to initiate the membrane deformation required for fusion. Such a model is consistent with the close proximity of tetraspanins and ADAMs binding domains, as observed by the steric inhibition fertilin- or cyritestin-dependent binding using antibodies against CD9 or CD81 (Chen *et al.*, 1999; Maecker *et al.*, 1997; Takahashi *et al.*, 2001). This signaling-dependent mechanism of action is consistent with the reported ability to activate *Xenopus* eggs upon exposure to the disintegrin loop encoded by the *Xenopus* ADAM ortholog xMDC16 (Shilling *et al.*, 1998).

Although the reported involvement of metalloendoproteases during fusion of deuterostome gametes is also consistent with a role for ADAM members at fertilization (De Santis *et al.*, 1992; Kato *et al.*, 1998; Roe *et al.*, 1988), the current ADAM candidates do not appear to be likely candidates for this proteolysis. Processing during spermatogenesis is thought to cleave the metalloendoprotease domains from both fertilin subunits before encountering the egg (Blobel *et al.*, 1990; Lum and Blobel, 1997). It is possible that the fertilin β metalloendoprotease domain remains tethered to the surface by association with its transmembrane domain or other surface binding partners, as shown to occur with other sperm proteins (Neill *et al.*, 2004). But the absence of tethering evidence requires that the proteolytically competent fertilin β be ignored for now. NL1, a soluble alternative to the ADAMs, may have been selected for in eutherians instead. NL1 is a secreted sperm-specific zinc metalloendoprotease that preferentially degrades neuropeptides enriched with aliphatic and aromatic residue (Ghaddar *et al.*, 2000). Its participation in fertilization is evident from the reduced fertility of male mice lacking functional NL1 (Carpentier *et al.*, 2004). One hypothesized role for NL1 is in sperm–egg signaling, possibly via its activity on proenkephalin derivatives found in the sperm acrosome (Carpentier *et al.*, 2004; Ghaddar *et al.*, 2000; Kew *et al.*, 1990). The involvement of NL1 in cell signaling is consistent with the model that sperm proteases may be active during the rapid reorganization of the egg cortex in preparation for fusion. Alternatively, the essential enzymatic activity could be associated with the egg, as observed in sea urchins. The final step prior to fusion in these animals requires the participation of the ADAM protein EBR1 (see Section VII.B, earlier in this chapter) (Kamei and Glabe, 2003). EBR1, however, only contains a canonical metalloendoprotease domain; no disintegrin domain is present. Thus, in echinoderms, reunification of the egg metalloendoprotease with a sperm-contributed disintegrin domain might trigger the

release of a fusion peptide into the appropriate plasma membranes, thereby initiating membrane deformation and fusion.

The retention of fertilization in the absence of metalloendoproteases still implies that other proteins participate in the actual fusion event. One such candidate is Izumo, the antigen of the potent fusion-blocking monoclonal antibody OBF13 (Inoue *et al.*, 2005). Izumo is a sperm transmembrane protein containing a single extracellular immunoglobulin-like domain. It is only egg-accessible after the acrosome reaction, when it is found over the most fusogenic region of the sperm head (Inoue *et al.*, 2005). Unlike sperm ADAMs, deletion of the functional *Izumo* gene does not impair expression of other candidate sperm fusion proteins (Inoue *et al.*, 2005). Yet sperm from these knock-out males are fusion-incompetent, suggesting that Izumo is necessary and sufficient to mediate mammalian sperm–egg fusion (Inoue *et al.*, 2005). The corresponding receptor for Izumo is hypothesized to be egg CD9 (see Section VII.B, earlier in this chapter), based on this tetraspanin's promiscuous associations with other immunoglobulin family members (Hemler, 2003). Association of Izumo with other, perhaps compensatory, egg sperm receptors is likely because deletion of CD9 still retains a low percentage of fertility (Kaji *et al.*, 2000; Miyado *et al.*, 2000), whereas sperm lacking Izumo are incapable of fusing with eggs *in vitro* or *in vivo* (Inoue *et al.*, 2005).

Invertebrate gamete fusion also appears to be dependent on the activity of nonenzymatic proteins from the acrosome (see Section VI, earlier in this chapter). For example, the participation of sea urchin EBR1 in fusion depends entirely on the properties of its sperm ligand bindin; in abalone, it depends on VERL and its sperm ligand sp18, a divergent 18-kDa paralog to sperm lysin (Swanson and Vacquier, 1995a,b). Both proteins localize to the extended tips of their respective acrosomal process or filament, the structures most likely to make first contact with the egg microvilli (Buckland-Nicks *et al.*, 1988; Moy and Vacquier, 1979; Mozingo *et al.*, 1995; Swanson and Vacquier, 1995b). Bindin alone promotes cell–cell aggregation (Glabe and Vacquier, 1977b; Lopez *et al.*, 1993); both proteins can induce mixed-phase liposome aggregation followed by direct fusion (Glabe, 1985b; Hong and Vacquier, 1986; Swanson and Vacquier, 1995b). In sea urchin bindin, this latter activity is directed by the hydrophobic 18-residue core "fusion peptide" conserved in 70% of all echinoderms (Vacquier *et al.*, 1995; Zigler and Lessios, 2003), possibly by a mechanism common to viral- or SNARE protein–dependent membrane fusions (Bentz, 2000; Bentz and Mittal, 2000; Knecht and Grubmuller, 2003; Lentz *et al.*, 2000). The fusogenic properties of abalone sp18, on the other hand, are attributed to its amphipathic fold rather than a specific fusion domain (Swanson and Vacquier, 1995b). The hydrophobicity of each respective domain suggests that these fusion catalysts act to overcome the electrostatic repulsion of membranes, holding them in an intermediate

state of deformation that thermodynamically favors mixed-phase lipid bilayer fusion (Glabe, 1985b; Hong and Vacquier, 1986). Both sperm-derived proteins prefer the negatively charged fluid-phase lipid phosphatidylserine but are reciprocally affected by the presence of divalent cations; bindin-mediated fusion rates are enhanced by Zn^{2+} whereas sp18 rates are retarded by most divalent cations (Glabe, 1985b; Hong and Vacquier, 1986; Swanson and Vacquier, 1995b), properties that may be consequences of their different modes of operation. Whether the transmembrane egg receptors for bindin or lysin, EBR1 or VERL, respectively, participate in the aggregation events has not been addressed experimentally. The proposed role of sea urchin EBR1 as a metalloendoprotease (see Section VII.B, earlier in this chapter), however, suggests that proteolytic activity could initiate a conformational change that releases bindin's fusion peptide towards the egg and plasma membranes. Thus, the hydrophobic properties of bindin alone could be sufficient to achieve fusion.

D. Extracellular Calcium

The role of extracellular calcium during fusion remains questionable. Although many fusion catalyzing complexes have been shown to be activated by calcium (Conner *et al.*, 1997; Crabb and Jackson, 1985; Lentz *et al.*, 2000; Tahara *et al.*, 1998), the ubiquitous presence of calcium in most extracellular media (see Sections III.B, earlier in this chapter, and Section IX.A, later in this chapter) suggests that the functional target for calcium is intracellular. One family of proteins that could participate in the influx of such calcium may be the TRPCs. These canonical ion channels are responsible for the restoration and maintenance of intracellular calcium stores in a voltage-independent fashion, a mechanism referred to as "capacitative calcium entry" (Putney and Ribeiro, 2000; Zhu *et al.*, 1996). TRPC members have been found along the surface of the sperm head, specifically overlying the midpiece and sperm acrosome (Castellano *et al.*, 2003; Mengerink *et al.*, 2002; Neill *et al.*, 2004). Thus, a primary role of TRPC homologs may be in the activation of sperm motility (Castellano *et al.*, 2003) and during the acrosome reaction when they are required to maintain high intracellular calcium levels long enough to complete exocytosis (see Section V.C, earlier in this chapter) (Jungnickel *et al.*, 2001; Mengerink *et al.*, 2002; Neill *et al.*, 2004). A secondary role is postulated for a TRPC member during fusion based on the failure of *Caenorhabditis* sperm to fuse with the egg in the absence of TRPC-3 (Xu and Sternberg, 2003). Nematode sperm lack acrosomes (Singson, 2001), thereby obviating the need for a bona fide calcium channel that facilitates vesicle exocytosis. Of all the known TRPC members, only the TRPC-3 homolog has been implicated in gamete fusion

(Castellano *et al.*, 2003; Jungnickel *et al.*, 2001; Mengerink *et al.*, 2002; Neill *et al.*, 2004; Trevino *et al.*, 2001). Thus, one hypothesis is that the influx of ions through TRPC-3 may be responsible for local influx of calcium into the sperm head just before fusion. Because of the presence of TRPCs in lipid rafts of the sperm head (Trevino *et al.*, 2001), TRPC-3 could be a target of specific signaling pathways that respond to egg receptors or to other extracellular interactions, as proposed for sea urchin TRPC homologs (Mengerink *et al.*, 2002; Neill *et al.*, 2004). Opening of the TRPCs would promote the local elevation of calcium, leading to conformational changes in membrane-associated proteins, such as SNARE proteins, which might be involved with membrane deformation. Such deformations in the sperm plasma membrane would be translated directly to extracellular machinery, thereby lowering the threshold of energy required to complete fusion.

VIII. A Denouement

Gamete fusion is correlated with the activation of many signaling cascades that contribute to the block to polyspermy and to egg activation. Sperm alone are capable of inducing such processes in the egg, suggesting that contributions from their membrane and/or cytoplasm are sufficient to release the egg from quiescence. For example, initiation of gamete fusion in sea urchins has been correlated with a local loss of CGs as the fertilization cone expands to accommodate the sperm pronucleus (Buckland-Nicks and Hodgson, 2000; Goudeau and Becker, 1982; Longo *et al.*, 1986; Schatten and Mazia, 1976). In some animals, the rapid flux of intracellular calcium necessary for these cortical changes may also be responsible for electrically altering the voltage potential across the membrane, resulting in the temporary activation of a series of voltage-dependent ion channels that are responsible for establishing an initial block to supernumerary sperm fusion. Elevation of intracellular calcium concentrations in the zygote is also required to completely activate the beginning of development. Here, we briefly review the conservation of these processes immediately following animal fertilization, focusing on those events responsible for enhancing monospermy. More detailed coverage of the signaling events at fertilization may be found elsewhere (see Bement, 1992; Jaffe *et al.*, 2001; Santella *et al.*, 2004).

A. Fast Electrical Block to Polyspermy

The fast electrical block to polyspermy present in many animals is dependent on changes to the voltage potential across the zygotic plasma membrane. The flux of specific monovalent ions through transmembrane channels is

the major contributor to the fast electrical block, and dictates whether the membrane potential (V_m) rises (depolarization) or falls (hyperpolarization) from the egg's resting state. Upon fertilization, most animals exhibit a depolarization of the membrane (see Fig. 6) including most marine animals (Dufresne-Dube *et al.*, 1983; Goudeau *et al.*, 1994; Gould and Stephano, 2003; Hagiwara and Jaffe, 1979; Jaffe, 1976; Moccia *et al.*, 2004; Togo and Morisawa, 1999; Togo *et al.*, 1995), primitive fish (Kobayashi *et al.*, 1994), primitive urodeles (Iwao, 1989), and anurans (Charbonneau *et al.*, 1983; Cross and Elinson, 1980; Glahn and Nuccitelli, 2003; Jaffe and Schlichter, 1985; Jaffe *et al.*, 1983a; Nuccitelli *et al.*, 1988). Decapod embryos, however, exhibit a transient hyperpolarization (Goudeau and Goudeau, 1986, 1989, 1996; Gould and Stephano, 2003). The absolute change in V_m is often greater than 30 mV and may persist for 60 s to 10 min, depending on the animal and the combinations of ion channels employed (reviewed in Gould and Stephano, 2003; Hagiwara and Jaffe, 1979), but must subside before egg activation and development can progress (Iwao and Jaffe, 1989).

The ion flux that changes V_m involves specific chloride, sodium, and/or potassium channels found in the egg plasma membrane (Cross and Elinson, 1980; Dufresne-Dube *et al.*, 1983; Goudeau and Goudeau, 1986, 1989; Grey *et al.*, 1982; Iwao, 1989; Iwao and Jaffe, 1989; Jaffe and Schlichter, 1985; Kobayashi *et al.*, 1994; Nuccitelli *et al.*, 1988; Obata and Kuroda, 1987; Togo and Morisawa, 1999; Togo *et al.*, 1995). The participation of sperm-derived factors likely initiates the fast electrical block because changes in V_m are usually triggered at the site of gamete fusion and propagate rapidly and uniformly along the entire egg membrane (Fall *et al.*, 2004; Iwao and Jaffe, 1989; McCulloh and Chambers, 1992). In *Discoglossus* eggs, however, the arrangement of ion channels within the fusogenic dimple mechanically restricts the change in V_m to a region slightly wider than the dimple (Nuccitelli *et al.*, 1988; Talevi and Campanella, 1988). Thus, the potency of a fast electrical block is geographically optimized at the membrane surfaces responsible for fertilization while minimally affecting global ion homeostasis. Voltage-clamp studies of other anuran eggs, which do not exhibit such specializations for fusion, have shown that the efficacy of depolarization is instead dependent on its maximum amplitude. For example, the peak V_m achieved by one species is optimized to block supernumerary fusion of only conspecific sperm, and is not sufficient to repel less sensitive heterospecific sperm who require a higher voltage potential to be deterred or who are simply insensitive to membrane voltage potentials (Iwao and Jaffe, 1989; Jaffe *et al.*, 1983a).

To establish a timely fast electrical block requires a rapid signaling cascade that likely originates from the sperm itself (Iwao and Jaffe, 1989). In determining which factors may be responsible for initiating the fast electrical block, it is important to distinguish between electrophysiological continuity

of the membranes versus cytoplasmic continuity. Although both result in capacitance changes, the former only requires outer leaflet continuity, whereas the latter requires fusion of both leaflets (Basanez, 2002; Markin and Albanesi, 2002). Thus, the time between cytoplasmic continuity and the onset of the fast electrical block may be much shorter than reported. Nevertheless, the time delay between fusion and the onset of a change in V_m is sufficient for its initiation by a soluble acrosome-derived sperm factor that can regulate an egg's ion channels (Gould and Stephano, 1987) or by a sperm ion channel contributed to the site of membrane fusion (Gould and Stephano, 2003; McCulloh and Chambers, 1992), such as the TRPC family of calcium channels (see Section VII.D, earlier in this chapter) (Xu and Sternberg, 2003). One simple trigger of the fast electrical block could be the flux of ions from the sperm into the egg following the establishment of cytoplasmic continuity. In this case, the influx of sperm-derived calcium or protons—initially accumulated from the extracellular fluid during events leading up to the acrosome reaction (see Section V.C, earlier in this chapter) (Hirohashi and Vacquier, 2002a; Neill and Vacquier, 2004; Runft *et al.*, 2002; Shapiro *et al.*, 1990; Tosti, 1994)—could be responsible for the initial change in V_m that releases a voltage-dependent fast electrical block over the egg.

A fast electrical block has not been observed in mammals (Gianaroli *et al.*, 1994; Jaffe *et al.*, 1983b; Kline and Stewart-Savage, 1994), teleosts (Nuccitelli, 1980), or common urodeles (Charbonneau *et al.*, 1983). Gradual changes in membrane potential have been reported in mammals, however, including hyperpolarizations in hamster (Igusa *et al.*, 1983; Kline and Stewart-Savage, 1994; Miyazaki and Igusa, 1981), mouse (Igusa *et al.*, 1983), and human eggs (Gianaroli *et al.*, 1994) or a prolonged minor depolarization in rabbits (McCulloh *et al.*, 1983). But the periodicity and duration of these oscillations are too late to reasonably block the fusion of supernumerary sperm. Instead, such gradual changes may be a consequence of intracellular calcium waves that result from egg activation (see Section VIII.B, later in this chapter) (Gianaroli *et al.*, 1994; Goudeau and Goudeau, 1996; Igusa *et al.*, 1983; Kline and Stewart-Savage, 1994; McCulloh *et al.*, 1983). Both hamster and mouse eggs also lose membrane resistance rapidly following fertilization (Jaffe *et al.*, 1983b; Miyazaki and Igusa, 1982). Might this membrane change be sufficient to inhibit additional sperm from binding the egg membrane, specifically by altering global membrane flexibility (Horvath *et al.*, 1993; Lee *et al.*, 1988; Tatone *et al.*, 1994; Wolf and Hamada, 1979)?

One likely source of the different penetrance of a fast electrical block across animal phyla may lie with the environment of fertilization per taxon. Because the concentration gradient of ions across the plasma membrane influences the direction of ion flow, a major factor in the survivorship of the

zygote utilizing a fast electrical block is significantly influenced by the ion concentration of the insemination media, particularly extracellular concentrations of ions used to establish current across biological membranes, such as sodium, potassium, and chloride (Gianaroli *et al.*, 1994; Grey *et al.*, 1982). In general, individual ion concentrations in bodies of water are significantly higher than oviductal fluid (see Section III.B, earlier in this chapter, and Section IX.A, later in this chapter), so those eggs that are fertilized in higher ionic environments are more likely to use the ion potentials that exist across their plasma membranes than eggs fertilized internally. Such a model agrees with the observed changes in V_m described for most eggs, specifically the presence of a fast electrical block in animals that spawn (decapods, echinoderms, ascidians, primitive fish, and anurans; see previous discussion) versus an absence in those that undergo internal fertilization (urodeles, mammals). The major exception is with teleosts, but the physical limitations established by the micropyle are likely sufficient to achieve monospermy, so a fast electrical block was not retained (see Section IV.C, earlier in this chapter).

B. Zygotic Intracellular Calcium-Dependent Signaling

Upon gamete fusion, the egg undergoes a series of changes that release it from quiescence to incorporate the sperm nucleus and to initiate embryogenesis (Bement, 1992; Ben-Yosef and Shalgi, 1998; Dumollard *et al.*, 2004; Mellor and Parker, 1998; Miyazaki *et al.*, 1993; Patel, 2004; Talmor-Cohen *et al.*, 2002). The universal trigger of this activation process is a cytoplasmic increase in calcium levels (see Carroll, 2001; Hart, 1990; Hogben *et al.*, 1998; Kaji *et al.*, 2000; Machaty *et al.*, 2000; Miyazaki *et al.*, 1993; Pecorella *et al.*, 1993; Runft *et al.*, 2002; Santella *et al.*, 2004; Stricker, 1999; Witton *et al.*, 1999). This calcium originates from intracellular stores enriched at the egg cortex or from mitochondria (Dumollard *et al.*, 2004; Halet, 2004; Leckie *et al.*, 2003; Liu *et al.*, 2001; Putney and Ribeiro, 2000; Shen, 1995; Stricker, 1999; Thaler and Epel, 2003). Distinct patterns of calcium release have been observed in different animals, ranging from a single prolonged wave that travels across the egg along its cortex from the point of sperm entry to oscillations of high and low calcium release that persist at least until first cleavage (Runft *et al.*, 2002; Stricker, 1999). The ability to propagate and maintain such patterns of intracellular calcium requires a network of calcium storage that acts synchronously in response to the activation status of a neighboring site (Machaca, 2004; Nuccitelli *et al.*, 1988; Sardet *et al.*, 2002).

Initiation of calcium release involves a universal signaling cascade that begins with PLC (reviewed in Dumollard *et al.*, 2004; Runft *et al.*, 2002, 2004). This membrane-associated enzyme converts phosphoinositol found in the plasma membrane into the secondary messengers inositol-3-phosphate

(IP3) and diacylglycerol (DAG). IP3 directly affects the level of intracellular calcium by triggering the release of stored calcium in the cortical endoplasmic reticulum upon binding its receptor, which also acts as a calcium channel. This initial cytoplasmic flux is sufficient to propagate local calcium release along the cortex that results in calcium waves of short duration (Dupont and Dumollard, 2004; Fall *et al.*, 2004). IP3, however, is quickly inactivated, a process that promotes the re-sequestration of calcium into stores and results in a rapid lag phase at the end of each oscillation. Prolonged waves of calcium require the additional participation of calcium-induced calcium responsive (CICR) channels (reviewed in Abbott and Ducibella, 2001; Ben-Yosef and Shalgi, 1998; Dumollard *et al.*, 2004; Galione *et al.*, 1991; Hart, 1990; Lawrence *et al.*, 1997; Miller *et al.*, 1994; Putney and Ribeiro, 2000; Stricker, 1999). IP3-dependent or neighboring CICR-mediated calcium release is often sufficient to trigger an extended flux of calcium from CICR stores. A separate mechanism that controls calcium release is dependent on the outcome of DAG activity. This second messenger facilitates the phosphatidylserine-dependent activation of conventional and novel protein kinase C (PKC) signaling. In addition to its role in cytoplasmic calcium dynamics (Putney and Ribeiro, 2000; Stricker, 1999), the phosphorylation activity of specific PKC isozymes also selectively initiates downstream signaling cascades, enzymes, and processes involved with cytoskeletal reorganization and nuclear function during early development (reviewed in Bement, 1992; Halet, 2004; Mellor and Parker, 1998; Page Baluch *et al.*, 2004; Talmor-Cohen *et al.*, 2002). Unlike the transient nature of the fusion-dependent IP3 signaling, the DAG–PKC relationship has more enduring effects on early development.

Based on the required timing of PLC-dependent calcium release following gamete fusion, one general hypothesis is that the sperm activates PLC. In echinoderms, the process of activation requires the gamma isoform of PLC (PLC_γ). The translocation of normally cytoplasmic PLC_γ to the plasma membrane is thought to be triggered by a Src-like kinase via tyrosine phosphorylation (Dumollard *et al.*, 2004; Runft *et al.*, 2002, 2004). How this egg kinase is activated by the sperm, however, is still debated (Runft *et al.*, 2002; Santella *et al.*, 2004). One source of such activation could be the sperm itself, as described in mammals. Upon fusion, the soluble sperm-specific zeta isoform of PLC (PLC_ζ) is released into the egg cytoplasm, where it rapidly triggers IP3-dependent calcium release (reviewed in Kurokawa *et al.*, 2004; Swann *et al.*, 2004). Under physiological concentrations, exogenous PLC_ζ is able to initiate calcium signaling and progress through early stages of development in a wide range of deuterostome eggs (Cox *et al.*, 2002; Kouchi *et al.*, 2004; Saunders *et al.*, 2002; Yoda *et al.*, 2004). Furthermore, depletion of PLC_ζ by RNAi from murine sperm significantly affects the pattern of calcium oscillations following fusion (Knott *et al.*, 2005). The repression of its

activity by calcium concentrations higher resting levels in eggs (Kouchi *et al.*, 2004) suggests that PLC_ζ is optimally active upon dilution into the egg at fusion. The absence of a membrane targeting domain on PLC_ζ (Saunders *et al.*, 2002) implies that it is also soluble in the sperm, at least until the calcium-dependent process of sperm activation when its calcium-binding domain might target it to a sperm membrane. The persistence of high cytoplasmic sperm calcium levels following activation would keep PLC_ζ in a primed, but not active, state at the membrane. Only upon dilution of the local calcium levels following fusion could PLC_ζ then catalyze the degradation of phosphoinositol to initiate the IP3-dependent calcium release in the egg, a process that likely shuts off the sperm-derived PLC again.

A similar sperm-derived contribution effectively activates echinoderm eggs and, like mammalian PLC_ζ, can mark the site of sperm entry. Nicotinic acid adenine dinucleotide phosphate (NAADP), a molecule that permanently binds its target receptor, initiates a rapid rise in cortical calcium (the "cortical flash") via membrane-associated voltage-gated calcium channels located on the surface of the egg (Churchill *et al.*, 2003; Moccia *et al.*, 2004; Patel, 2004; Santella *et al.*, 2004). This sharp peak in calcium concentration likely activates the egg's PLC_γ signaling cascade, although CICR channels may be triggered as a consequence of the cross-talk among calcium-sensitive calcium channels found in the endoplasmic reticulum and/or the plasma membrane (Patel, 2004; Santella *et al.*, 2004). The involvement of plasma membrane–associated calcium channels could also occur via TRPCs, whose presence in the sperm plasma places them in a prominent position to play an active role during early zygotic calcium signaling (see Section VII.C, earlier in this chapter).

The first sequence of calcium transients following fusion is usually responsible for the translocation, when necessary, and secretion of CGs, as well as initial events essential for the transition to embryogenesis (Ben-Yosef and Shalgi, 1998; Cran and Esper, 1990; Ducibella *et al.*, 2002; Goudeau and Goudeau, 1996; Goudeau *et al.*, 1991). The timing and duration of CG release is species-dependent, but universally requires calcium (Abbott and Ducibella, 2001; Cran and Esper, 1990; Gilkey *et al.*, 1978; Goudeau *et al.*, 1991; Wessel *et al.*, 2001). CG exocytosis is most likely governed by calcium-responsive proteins embedded within the membrane of the organelles and plasma membrane (Crabb and Jackson, 1985), particularly cysteine string proteins (Gundersen *et al.*, 2001) and the SNARE complex, including the calcium-sensitive proteins synaptotagmin, rab3, and rabphilin-3A (Abbott and Ducibella, 2001; Conner *et al.*, 1997; Ikebuchi *et al.*, 1998; Tahara *et al.*, 1998; Wessel *et al.*, 2001). Thus, conformational changes in the protein fusion machinery induced upon calcium binding could be directly translated into forces needed to initiate membrane deformation, membrane fusion, and content exocytosis (reviewed in Bentz and Mittal, 2000).

IX. Producing the Physical Block to Polyspermy

The most effective mechanism to block supernumerary sperm from fusing with an egg is to establish a physical barrier separating the monospermic zygote from the sperm. Constructing such an obstacle at the zygote's surface is impossible without molecules, specifically enzymes or structural proteins, which alter or mask the egg's sperm-receptive ECM (see Table II). In most animals, the source of these converting factors resides at the egg cortex. The secretory granules found at the periphery of most eggs are released immediately after fertilization, usually in response to the initial prolonged elevation of intracellular calcium (see Section VIII.B, earlier in this chapter). Prior to secretion, the contents of these CGs are often organized into paracrystalline arrays whose architecture is often species specific (see Bannon and Brown, 1980; Campanella *et al.*, 1992; Cran and Esper, 1990; Hart, 1990; Talbot and Goudeau, 1988; Wong and Wessel, 2004). During exocytosis, subcomplexes of this paracrystalline architecture are expelled nearly intact, but rapidly hydrate and evenly incorporate into the egg ECM (Bryan, 1970b; Carroll *et al.*, 1986; Gulyas, 1980; Talbot and Goudeau, 1988).

The duration of CG exocytosis varies significantly across animal phyla, requiring anywhere from seconds to hours to complete (Brown and Clapper, 1980; Campanella *et al.*, 1992; Elinson, 1986; Gilkey *et al.*, 1978; Goudeau and Becker, 1982; Gould and Stephano, 2003; Hart, 1990; Kline and Stewart-Savage, 1994; Matese *et al.*, 1997; Talbot and Goudeau, 1988; Whalley *et al.*, 1995). Although the duration of CG exocytosis tends to give a good estimate of how long the permanent block to polyspermy takes to establish, the reported duration of hours required for decapods clearly suggests that construction of the physical block is not the only role of CG contents after fertilization (see Section III.B, earlier in this chapter) (Brown and Clapper, 1980; Santella and Ianora, 1992; Talbot and Goudeau, 1988).

In this section, we summarize the various biochemical modifications that occur at the egg ECM after CG exocytosis. We review and compare the different methods used by various taxa, including a discussion of those exceptional eggs whose physical blocks are constructed without the contribution of CGs.

A. Cortical Granule Contents + Egg ECM = ?

Establishment of a physical block to polyspermy requires the combination of proteins from two distinct structures originally synthesized in the egg, specifically the ECM and CGs (Table II). Although the effect of CG contents on the egg ECM is usually significant, the ultrastructural changes that occur

across phylogeny range from minor to radical. Radical modifications are inherently associated with a complete loss of sperm recognition because of the severity of the changes to the ECM surface. In those ECMs exhibiting insignificant ultrastructural changes, however, biochemical modifications prove to be critical because they are solely responsible for the reduction in sperm affinity associated with a permanent block to polyspermy. Thus, a predictable relationship exists between the probability of polyspermy and the degree of morphological change to which the CG contents contribute.

Although mammalian CG exocytosis significantly alters the receptivity of sperm at the ECM, little morphological modification is observed (Shapiro *et al.*, 1989) (Figs. 1–3). In most mammals, CG exocytosis results in a slightly greater distance between the zona and the zygotic plasma membrane, as well as the accumulation of a thin intimate CG envelope overlying the zygotic plasma membrane (Breed and Leigh, 1992; Dandekar and Talbot, 1992; Dandekar *et al.*, 1995; Hoodbhoy and Talbot, 2001; Hoodbhoy *et al.*, 2001; Jungnickel *et al.*, 1999; Talbot and Dandekar, 2003). Many lectin-reactive proteins derived from CGs are deposited at the surface of the egg, accumulate within the perivitelline space in mice (Lee *et al.*, 1988), and sometimes intercalate into the ECM, as observed in hamsters (Cherr *et al.*, 1988; Hoodbhoy and Talbot, 2001). One such glycoprotein is the antigen of the ABL2 antibody, a CG envelope protein that remains adherent to the embryo until hatching and may participate in establishing a membrane-level block to polyspermy (Hoodbhoy *et al.*, 2001; Talbot and Dandekar, 2003). Unlike eutherians eggs that remain surrounded by a cumulus cell layer, marsupial zygotes acquire two dense mucoid layers external to the zona, structures thought to be applied by oviduct epithelium as the zygote travels toward the uterus (Selwood, 1992).

The most noticeable change to the anuran egg ECM at fertilization occurs to the jelly layer (Figs. 1 and 2). As in mammals, the vitelline envelope is released from the plasma membrane but remains a smooth shell compacted against the jelly layer (Larabell and Chandler, 1991). Within minutes of CG exocytosis, a precipitate forms along the boundary between the elevated vitelline envelope and the inner J1 jelly layer (Hedrick and Nishihara, 1991; Larabell and Chandler, 1991). This fertilization (F) layer is the result of the CG-derived lectin XL35/CGL (or its orthologs) preferentially binding a sulfated galactose-rich oligosaccharide present in the J1 layer (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001). Pretreating anuran eggs with total CG exudate inhibits fertilization only when the jelly layer is intact, providing direct evidence that, in addition to its role in sperm attraction, jelly also contributes to the block to polyspermy (Barisone *et al.*, 2002; Prody *et al.*, 1985). In *Discoglossus*, on the other hand, CG exocytosis is responsible for the dissolution of the jelly plug (Campanella

et al., 1992). This process is functionally analogous to the physical obstruction of the sperm's path via the F layer produced in other anurans (Hedrick and Nishihara, 1991) because the chemoattractive jelly plug is required to guide sperm to the fusogenic dimple of the animal pole (see Section IV.C, earlier in this chapter).

Most CG-dependent morphological changes occur at the teleost micropyle, consistent with the need to alter the most fusogenic regions of an egg after fertilization (Figs. 1 and 2). For example, *Danio* eggs contain three types of CGs associated with the egg membrane, each type distinguishable by size and location in the animal or vegetal hemispheres (Hart and Donova, 1983). The patch of membrane directly below the micropylar canal is enriched in microvilli and devoid of CGs; surrounding this void, however, is a specialized cluster of smaller CGs that lie in the shadow of the micropylar tunnel (Gilkey *et al.*, 1978; Hart, 1990). These small CGs are thought to establish the modifications necessary for plugging the micropyle. Although most teleosts exhibit a propagated pattern of CG release starting at the site of fusion (Gilkey *et al.*, 1978; Hart, 1990), the micropylar population in *Danio* is the last to exocytose after environmental activation or fertilization, postponed sufficiently to allow the short-lived sperm to penetrate before the fertilization plug is formed (Hart and Donova, 1983; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987). In the end, the global outcome of teleost CG exocytosis results in both the physical separation of the chorion from the egg surface, via hydration of the CG contents secreted into the perivitelline space (Hart, 1990; Ohta and Iwamatsu, 1983; Wolenski and Hart, 1987), and the physical obstruction of the micropyle using CG lectins that form the fertilization plug (Hart, 1990) or using protease-dependent collapse of the overlying chorion (Iwamatsu *et al.*, 1997). Additional CG-dependent modifications occur over the nonmicropylar chorion, events that have been partly worked out in *Cyprinus*. Following CG exocytosis, FLS, CLS, and cystatin polymerize in a cation-dependent fashion within the chorion and/or perivitelline space (Chang and Huang, 2002; Chang *et al.*, 1998). Different FLS isoforms are spatially separated during oogenesis but polymerize during fertilization envelope formation; only this homo-oligomeric complex is able to bind CLS and cystatin. As the FLS–CLS–cystatin triad diffuses away from the egg surface, it is trapped by ZPA–ZPC protofilaments at the outer edge of the chorion, where the triad then tethers fertilization envelope outer layer protein-1 (FEO-1) to the chorion (Chang and Huang, 2002; Chang *et al.*, 1998, 1999). Such a peripheral location of cystatin is also optimal for its role as an antifungal protein that serves to protect the zygote chemically and structurally (Chang *et al.*, 1998).

As in some teleosts, the exocytosis of decapod CGs is a hierarchal process that modifies the ECM in stages. Decapod eggs synthesize at least four populations of cortical vesicles that are packaged independent of the Golgi

apparatus (Brown and Clapper, 1980; Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Santella and Ianora, 1992; Talbot and Goudeau, 1988) (Figs. 1, 2, and 6). The smallest, most peripheral electron-dense granules are released within 20 min after fertilization (Bannon and Brown, 1980; Talbot and Goudeau, 1988). The contents of these first granules are thought to separate the chorion from the plasma membrane as they adsorb or intercalate within the inner layer of the chorion. Upon completing small CG exocytosis, gradual exocytosis of the remaining high-density, medium-density and ring granules—the ''secondary'' granules—follows. These secondary CGs contribute to the more intimate electron-dense embryonic envelope deposited at the surface of the zygotic membrane and hardened by mechanical processes (Bannon and Brown, 1980; Brown and Humphreys, 1971; Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Hinsch, 1971; Santella and Ianora, 1992; Talbot and Goudeau, 1988). CG release can last for hours in decapods (Goudeau and Becker, 1982; Goudeau *et al.*, 1991; Santella and Ianora, 1992), suggesting that the contribution of these vesicles, particularly the secondary CGs, to an immediate physical block to polyspermy is negligible. Compared to the original vitelline envelope, however, the differences in physical characteristics of the zygotic ECM following CG exocytosis support the involvement of early CG secretion in establishing a protective barrier for development (Goudeau and Becker, 1982; Talbot and Goudeau, 1988).

The most dramatic change to egg ECMs following CG exocytosis is observed in echinoderms (reviewed in Kay and Shapiro, 1985). A nearly fourfold increase in surface area develops during the transformation of the vitelline layer into the fertilization (Figs. 1–3), a structure that is lifted from the plasma membrane by the hydration of CG-derived glucosaminoglycans (Harvey, 1909; Larabell and Chandler, 1991; Runnstrom, 1966; Tegner and Epel, 1976). Based on the sheer abundance of CG protein mass relative to the vitelline layer, it is almost guaranteed that these proteins interact with one another, sticking wherever possible along the vitelline layer scaffold (Inoue and Hardy, 1971). These protein–protein interactions probably use the abundant LDLrA repeats and CUB domains encoded by both the CG and the vitelline layer components (see Sections II.D and III.B, earlier in this chapter) (Wong and Wessel, 2004, 2006). The network of binding is not random: Initial studies reported proteoliaisin binding to the vitelline layer and ovoperoxidase (Weidman and Shapiro, 1987). Further analysis showed that the vitelline layer component of rendezvin promiscuously binds all the CG content proteins through high-affinity ionic interactions (Wong and Wessel, 2006). This implies that CUB domains of the vitelline layer are seeds that coordinate the interaction of all the CG proteins during their rapid, autonomous assembly. The role of the sea urchin LDLrA repeats, however, is not clear. Evidence from other proteins that contain similar tandem arrays of LDLrA repeats clearly indicates that these repeat motifs are essential for

the function of the ECMs they compose (Kallunki and Tryggvason, 1992; Yochem *et al.*, 1999).

A common theme in the assembly of structural components necessary for a physical block to polyspermy is the participation of calcium (see Section III.B, earlier in this chapter). Ascidians modify their plasma membrane using electron-dense, calcium-rich granules extruded from subcortical cytoplasmic vesicles (Rosati *et al.*, 1977). XL35/CGL binds a terminally sulfated, galactose-rich 250-kDa mucin of the anuran J1 jelly layer in a calcium-dependent fashion, making calcium essential to the stable precipitation of the fertilization (F) layer at the interface of the vitelline membrane and jelly coat following fertilization (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001; Wolf *et al.*, 1976). Calcium chelation motifs are also abundant in all the sea urchin fertilization envelope structural proteins (Wong and Wessel, 2004). In both teleosts (Chang and Huang, 2002) and echinoderms (Bryan, 1970b), free calcium is essential to establish a crystalline matrix within the renovated ECM that can withstand chemical and physical abuse (Harvey, 1909; Zotin, 1958). The difference lies in how this cation is primarily used: Teleosts need calcium for enzymatic activity (Yamagami *et al.*, 1992), whereas echinoderms need it to maintain the structural integrity of the matrix (Bryan, 1970b; Wong and Wessel, 2004). As observed in anurans, protein precipitation at the ECM–jelly interface may occur in sea urchins during fertilization envelope elevation, with the jelly providing a mold for CG structural proteins to efficiently assemble against (J. L. Wong and G. M. Wessel, unpublished observations). Retention of a thin layer of jelly may also prove to be important for sea urchin development, especially to reduce the agglutination of embryos and attachment of microbes (Mah *et al.*, 2005). The need to create a durable barrier within a calcium-rich environment could account for the selection of calcium-chelating motifs for structural elements by these spawning animals. For internally fertilized animals such as eutherians, on the other hand, pressure to maintain these attributes diminishes because oviductal fluid is not as abundant in calcium (~1.7 mM calcium vs. 3–5 mM in freshwater, 10 mM in seawater) (see Section III.B, earlier in this chapter). Instead, other associated modifications to the ECM or the eutherian cumulus cells likely provide equivalent protection to the developing embryo until implantation.

B. Modifying the Egg ECM by Destruction

In most animals, CGs contribute the enzymes essential for assembling a permanent block to polyspermy. The most common activity associated with the modification involves destruction of the ECM to minimize its attractiveness to tardy sperm.

Proteinases are essential for the construction of barriers that are histologically distinct from the original egg ECM. Successful development of petromyzontids to two-cell embryos requires a chymotrypsin-like activity (Dabrowski *et al.*, 2004), suggesting that this family of egg-derived enzymes participates in the permanent block to polyspermy (Kobayashi and Yamamoto, 1994). A more definitive reduction in molecular weight of major teleosts chorion components following CG exocytosis suggests that proteolysis may occur along the inner layer, facilitating the hardening process by exposing hydrophobic amino acid residues for targeted cross-linking (see Section IX.D, later in this chapter) (Yamagami *et al.*, 1992). Alternatively, proteases may facilitate the collapse of the micropylar vestibule to physically block sperm from reaching the egg (Iwamatsu *et al.*, 1997). A similarly dramatic histological change is the assembly of an echinoderm fertilization envelope, whose formation requires the separation of the egg ECM from the egg plasma membrane. Detachment of the vitelline layer is facilitated by the sea urchin serine-like protease CGSP1, which cleaves target proteins on the surface of the egg membrane (Carroll and Epel, 1975a,b; Haley and Wessel, 1999) such as the vitelline post protein p160 (Haley and Wessel, 2004a) and the 350-kDa EBR1 sperm receptor, whose fragments can be found buried in the core of the mature fertilization envelope (Carroll *et al.*, 1986; Ruiz-Bravo *et al.*, 1986).

A strong case for the involvement of proteolysis during the transformation of the egg ECM can be found in the cleavage of ZPA from anurans (Barisone *et al.*, 2002; Infante *et al.*, 2004; Lindsay and Hedrick, 2004; Wolf *et al.*, 1976) and eutherians (Bauskin *et al.*, 1999; Moller and Wassarman, 1989). One candidate originally thought to be responsible for the degradation of ZPA was anuran ovochymase, an extracellular chymotrypsin-like protease active in the perivitelline space of the *Xenopus* zygote (Lindsay *et al.*, 1992b, 1999; Lindsay and Hedrick, 1995). The ovochymase zymogen contains a single, amino-terminal CUB domain that may anchor it to the vitelline envelope until its activation (Lindsay *et al.*, 1999). A trypsin-like enzyme released upon CG exocytosis is thought to activate 0.01% of the bound ovochymase zymogen functional at fertilization (Lindsay and Hedrick, 1989; Lindsay *et al.*, 1992b, 1999). Such a weak activation potential of this CG-derived trypsin-like enzyme on ovochymase suggests that ovochymase and its sibling ovotryptases do not participate in vitelline envelope remodeling (Lindsay *et al.*, 1999). Rather, the protease responsible for cleavage of anuran ZPA is a zinc-dependent CG protease similar to bone morphogenic protein-1 (BMP-1) (Lindsay and Hedrick, 2004). This family of enzymes cleaves approximately 28 residues from the ZPA amino-terminus at a consensus sequence (X|DD/E) found in most vertebrate ZPA orthologs (Lindsay and Hedrick, 2004). Proteolysis likely disrupts the conformation of ZPA by relaxing the fold normally retained by an intramolecular disulfide

bond between the amino- and carboxyl-termini. This site-specific hydrolysis of ZPA accounts for the observed retention of the entire ZPA protein following ECM modification, albeit its electrophoretic mobility could change (Bauskin *et al.*, 1999; Lindsay and Hedrick, 2004; Moller and Wassarman, 1989; Moller *et al.*, 1990). Consequences of this structural relaxation in ZPA likely initiate an avalanche of systemic conformational changes along a ZP protofilament, ultimately terminating in the acquisition of chemical and protease resistance throughout the postfertilization ECM (Lindsay and Hedrick, 2004; Sun *et al.*, 2003).

Does the model of non-proteolytic sperm penetration fit with the hypothesized ZPA-dependent mechanism of ECM conversion in anurans? Under the non-proteolytic model of penetration, the integrity of the ECM remains the same (see Section VI.C, earlier in this chapter). Because every ZP protofilament (Jovine *et al.*, 2002) along the sperm's path through the ECM also retains its native configuration, reassembly and modification of the fibers occurs efficiently because no holes would have to be patched during remodeling. By simply rearranging the displaced protofilaments through changes in ZPA conformation, sperm tunnels are eradicated and sperm receptors are masked without the integration of new material (Barisone *et al.*, 2002; Infante *et al.*, 2004; Rankin *et al.*, 2003). This radical change in conformation would also allow for the acquisition of mechanical resilience association with the zygotic ECM (Sun *et al.*, 2003). Thus, the intramolecular conformation of ZPA affects both primary and secondary sperm receptivity by regulating ZPC and ZPA accessibility to sperm both before and after fertilization (Rankin *et al.*, 2003). Such simple protease-dependent conversion is also parsimonious with the ECM conversion in other animals. For example, cleavage of the homologous glycoproteins at the *Oryzias* micropylar catchment leaves a thin compacted outer chorion layer that is unattractive to teleost sperm (Iwamatsu *et al.*, 1997). In ascidians, too, proteasomes specifically target the putative sperm binding protein, VC70, for degradation (Sawada *et al.*, 2002a)—although the contribution of this cleavage is male derived, making its proteasome activity more like a sperm competition mechanism than a block to polyspermy (Lambert, 2000). Finally, the mollusk *Mytilus* uses an aminopeptidase to disrupt the sperm-binding affinity of the vitelline envelope proteins (Togo and Morisawa, 1997; Togo *et al.*, 1995). Unlike the proteases used in other animals, no significant morphological or biochemical modification other than loss of sperm binding has been reported in this bivalve.

Given the high degree of complexity and overlap often observed at fertilization, it is not surprising to find that other CG-derived enzymes alter the animal ECM. The universal involvement of oligosaccharides in sperm–egg interaction (see Section V.B, earlier in this chapter) provides one likely target of enzymatic modification. The source of this alteration is not known, but

the process is hypothesized to be essential for a permanent block to polyspermy (Mahowald *et al.*, 1983). This model is consistent with the reported change in lectin affinities during the transition from egg to zygotic ECM in *Drosophila*, whose vitelline membrane loses α-mannose and sperm-binding β-*N*-acetylglucosamine following fertilization (Perotti *et al.*, 1990, 2001). In some animals, the loss of primary sperm binding has been attributed to deglycosidases presumably stored in the CGs (Florman *et al.*, 1984; Prody *et al.*, 1985; Talbot and Dandekar, 2003; Vo *et al.*, 2003) but alternatively may be stored in the perivitelline space, such as in ascidian test cells or in follicle cells external to the vitelline coat (Lambert, 2000; Lambert *et al.*, 1997; Rosati, 1985; Rosati *et al.*, 1977). For example, soluble *N*-acetylglucosaminidase activity is associated with the ascidian egg surface and is thought to remove sperm-binding sugar residues to eliminate vitelline coat receptivity (Lambert and Goode, 1992; Lambert *et al.*, 1997; Matsuura *et al.*, 1993). *N*-acetylglucosaminidase activity has also been purified from CGs in *Xenopus* (Prody *et al.*, 1985) and *Mus* (Miller *et al.*, 1993a). The enzyme is active in *Xenopus*, causing a significant loss in ZPC oligosaccharide mass (Vo *et al.*, 2003). The functional eutherian ortholog, however, does not contribute the same degree of modification because the electrophoretic mobility of ZPC is unaltered following CG exocytosis (Bauskin *et al.*, 1999; Miller *et al.*, 1993a). The absence of substrate modification or byproducts associated with its activity suggests that, like the sperm ligand (see Section V.C, earlier in this chapter), either reversible ionic interactions between the *N*-acetylglucosaminidase and its preferred oligosaccharides or another steric modification blocks gamete interactions in eutherians.

C. Modifying the Egg ECM by Addition

The identification of nonenzymatic lectin-like proteins within CGs (see Section III.B, earlier in this chapter) suggests that steric masking of essential sugar moieties may supplement deglycosidase activity or may alone be sufficient to inhibit sperm binding in some animals. Two prominent examples include the oligomerizing XL35/CGL from *Xenopus* (Chamow and Hedrick, 1986; Chang *et al.*, 2004; Nishihara *et al.*, 1986) and potential carbohydrate-binding pocket motifs such as dimerized CUB domains in sea urchins (Bork and Beckmann, 1993; Romero *et al.*, 1997; Varela *et al.*, 1997; Wong and Wessel, 2006). The precipitation of XL35/CGL is dependent on binding of a galactose-rich oligosaccharide found in the *Xenopus* J1 jelly layer, resulting in its local precipitation and the formation of an impenetrable calcium-rich barrier (see Section IX.A, earlier in this chapter) (Arranz-Plaza *et al.*, 2002; Chang *et al.*, 2004; Hedrick and Nishihara, 1991; Nishihara *et al.*, 1986; Quill and Hedrick, 1996; Tseng *et al.*, 2001).

In the sea urchin, the egg bindin receptor EBR1 is retained in the vitelline layer core of the fertilization envelope but is probably masked by the adsorption of non-enzymatic CG proteins (Carroll *et al.*, 1986; Ruiz-Bravo *et al.*, 1986). This mechanism is similar to the loss of lectin-accessible sugars of the vitelline layer immediately following fertilization, including a 50% decrease in wheat germ agglutinin-binding saccharides such as *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuNAc) (Kitamura *et al.*, 2003). In mammals, a lectin-like protein could interfere with sperm–zona binding by blocking sperm-binding sites. Alternatively, proteolysis of ZPA and subsequent conformational changes to the zona (see Section IX.B, earlier in this chapter) may be prerequisite for proper lectin-like epitope masking, as demonstrated by the retention of sperm-binding capacity when ZPA is not cleaved (Rankin *et al.*, 2003).

Although the oligosaccharide-binding molecules are likely derived from CGs, such an adaptation may not be necessary. In marsupials, for example, CG exocytosis does not provide a full block to polyspermy; instead, the CG-derived permanent block is supplemented by the application of a mucoid shell over the modified zona during its travels across secretory epithelium of the oviduct isthmus (Figs. 1 and 2). This coating is believed to mask all unoccupied sperm receptors and to trap any supernumerary, zona-bound sperm prior to membrane binding (Breed and Leigh, 1990, 1992; Jungnickel *et al.*, 1999; Rodger and Bedford, 1982a,b; Selwood, 1992). Such a mechanism may be successful because of the apparent low stoichiometry of sperm-to-ovum within the oviduct (Rodger and Bedford, 1982a) and the low binding frequency of capacitated sperm to zonae *in vitro* (Mate *et al.*, 2000). Not surprisingly, application of such a distal physical block does not prevent the persistence of multiple sperm in the perivitelline space (Jungnickel *et al.*, 1999), a phenomenon consistent with the 5% rate of polyspermy in some species (Breed and Leigh, 1990). Distinct from the oviductal glycoproteins that matriculate into the zona and enhance homologous sperm binding (see Section II.D, earlier in this chapter) (Buhi, 2002; O'Day-Bowman *et al.*, 2002; Rodeheffer and Shur, 2004; Schmidt *et al.*, 1997), these zygotic mucoid coatings contain epitopes antigenically conserved with oviductal glycoproteins that supplement the CG-derived zona modification in all mammals. Their biochemical functions include physically blocking sperm penetration, stabilizing the modified zona, and/or acting as an antimicrobicide or spermicide as the embryo travels toward the uterus (Brown and Cheng, 1986; Hoodbhoy and Talbot, 1994; Roberts *et al.*, 1997; Selwood, 1992; Wang *et al.*, 2003). An analogous protective function is attributed to the coatings applied to *Drosophila* and avian embryos by female reproductive organs. The carbohydrate profile of the zygotic *Drosophila* micropyle is enriched in low sperm-affinity sugars such as α-galactose (Gal) and *N*-acetylneuraminic acid (NeuNAc) (Perotti *et al.*, 1990) while the

remaining chorion is coated with anti-bactericidal ceratoxins (Marchini *et al.*, 1997). Similarly, an outer coat is deposited peripheral to the avian perivitelline layer during the zygote's journey through the infundibulum and magnum of the oviduct (Bellairs, 1993; Bellairs *et al.*, 1963; Harper, 1904) (Figs. 1 and 2). Because fertilization at the germinal disc occurs soon after ovulation, this avian coat does not prevent polyspermy; rather it protects the embryo from microbes by coating the outer layer with avidin, lysozyme, and a 62-kDa anti-microbicidal lectin (Cook *et al.*, 1985; Harper, 1904; Marchini *et al.*, 1997; Okamura and Nishiyama, 1978a).

D. Modifying the Egg ECM by Transmogrification

An alternative to physically masking the egg ECM by protein addition is to toughen the old matrix. This "hardening" process is representative of a change in the physical properties of the matrix, including the acquisition of mechanical resilience, protease insensitivity, and chemical resistance (Harvey, 1909; Lindsay and Hedrick, 2004; Sun *et al.*, 2003; Wong *et al.*, 2004; Zotin, 1958). This could be achieved by simple ECM hydration, as observed in the primitive urodele *Hynobius* (Iwao and Jaffe, 1989), by oviduct contributions, as in *Drosophila* (Bloch Qazi *et al.*, 2003), or by complete reorganization and compaction of the ECM contents, as observed in anurans through ZPA proteolysis (see Section IX.B, earlier in this chapter) (Lindsay and Hedrick, 2004). Yet some animals rely on physically cross-linking adjacent proteins via the creation of covalent bonds between adjacent proteins. The most common enzymes that specifically generate these covalent bonds are peroxidases and transglutaminases. Peroxidases generate dityrosine bonds between neighboring proteins (Deits *et al.*, 1984; Gulyas and Schmell, 1980a; LaFleur *et al.*, 1998; Nomura and Suzuki, 1995), whereas transglutaminases create isopeptide amide bonds between glutamine and lysine (Battaglia and Shapiro, 1988; Chang *et al.*, 2002; Lee *et al.*, 1994; Lorand and Graham, 2003; Nemes *et al.*, 2005; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). Both affect the structural integrity of the matrix by adding covalent, intermolecular braces and by fusing polymers into a unified surface. This is most noticeable in scanning electron micrographs of the sea urchin fertilization envelope, where the glycoprotein fibers remain loose when either 3-aminotriazole or glycine ethyl ester are used to inhibit ovoperoxidase or transglutaminase activities, respectively, but stiffen and align under normal conditions (Battaglia and Shapiro, 1988; Deits *et al.*, 1984; Foerder and Shapiro, 1977; Mozingo and Chandler, 1991; Veron *et al.*, 1977) (Fig. 3).

Bridging of proteins via covalent cross-links has the potential to block sperm-binding sites through conformational changes in the surrounding matrix. The specific enzymatic activity utilized, however, depends on the

organism. Unlike sea urchins, most animals have either peroxidase or transglutaminase. For example, peroxidase activity has been localized to mouse CGs and to the outer surface of the zona (Gulyas and Schmell, 1980a). Although further characterization of this activity has not been reported, such a modification suggests that peroxidase-like enzymes may supplement the zinc-dependent proteolysis of ZPA during ECM hardening (Lindsay and Hedrick, 2004). Similarly, peroxidase activity is found in specific chorionic layers of the teleost *Tribolodon* (Kudo, 1988). More concrete evidence for the participation of peroxidase-dependent cross-linking, comes from studies that identified the *o,o*-dityrosine products of this enzyme within the modified ECM. The typical mechanism of peroxidases involves production of free radicals at the *ortho* position of adjacent tyrosine phenyl rings, followed by collapse of these extra electrons to form a single carbon–carbon bond (Chance, 1949; Ljunggrn, 1966; Marquez and Dunford, 1995; Morrison and Schonbaum, 1976). The covalent linkage changes the physical properties of the tyrosine residues, allowing them to yield more blue light (420 nm) when excited with ultraviolet (325 nm) light compared to their monomeric counterparts (Gross, 1959; Heinecke *et al.*, 1993a,b). This characteristic has led to the hypothesis that peroxidases are also responsible for cross-linking eggshells of the dipterans *Anopheles* (Li *et al.*, 1996) and *Dacus* (Mouzaki *et al.*, 1991).

Transglutaminase cross-linking, on the other hand, occurs in various species of dipterans, echinoderms, and teleosts. For example, products of the *defective chorion* (*dec-1*) gene (see Section II.D, earlier in this chapter) are bound within the *Drosophila* chorion through isopeptide amide bonds (Badciong *et al.*, 2001). These γ-glutamyl-ε-lysine bonds are also reported to be in the chorion of teleosts (Chang *et al.*, 2002; Kudo and Teshima, 1998; Lee *et al.*, 1994; Oppen-Berntsen *et al.*, 1990; Yamagami *et al.*, 1992). In *Oryzias*, this enzyme is thought to partially dehydrate and, hence, compact the chorion (Lee *et al.*, 1994). Such a process may occur through the calcium-dependent, cadaverin-sensitive cross-linking of *Gadus* chorion proteins (Oppen-Berntsen *et al.*, 1990), of *Cyprinus* ZPB and ZPC (Chang *et al.*, 2002), or of *Pseudopleuronectes* ZPB, specifically at its $(PQQ)_4PKY$ repeats (Lyons *et al.*, 1993). In echinoderms such as sea urchins, transglutaminase activity organizes and stabilizes self-assembly of CG structural proteins within the vitelline layer scaffold (Battaglia and Shapiro, 1988; Kay and Shapiro, 1985). The isopeptide bonds made by this enzyme are essential for retaining CG content proteins in the fertilization envelope and contribute to the permeability barrier of the mature matrix (Cheng *et al.*, 1991; Kay and Shapiro, 1985; J. L. Wong and G. M. Wessel, unpublished observations).

The selective advantage for one type of cross-linking activity over another may depend on additional factors introduced upon zygotic activation. Such conditions include the availability of catalytic cations such as calcium (see

Section IX.A, earlier in this chapter), signal transduction within the zygote that regulates substrate availability (see Section IX.E, later in this chapter), and modifications to the ECM that would affect the accessibility of target substrates. Yet the final products—covalent cross-links that irreversibly alter the physical attributes of the egg ECM—are the same. Why would an animal select for peroxidases, with the additional requirements of a hydrogen peroxide–generating source (Heinecke and Shapiro, 1989; Takahashi *et al.*, 1989; Wong *et al.*, 2004), over a single enzyme like transglutaminase? Perhaps retention of ovoperoxidase activity is more a result of the presence and calcium-dependent activation of a hydrogen peroxide generating system initially maintained for early cleavage and cell proliferation processes (see Burdon, 1995; Chen *et al.*, 2004; Kamata and Hirata, 1999; Maulik and Das, 2002; Stone and Collins, 2002; Wong and Wessel, 2005), a holdover that facilitates the reduction of otherwise toxic hydrogen peroxide released during the postfertilization calcium wave. The consequential selection for tyrosine-rich structural proteins (Wong and Wessel, 2004, 2006), however, proves more difficult to reconcile, particularly considering the glutamate and lysine residues used by transglutaminase are generally more abundant. Perhaps further work in sea urchins, who require both transglutaminase and ovoperoxidase activity to complete fertilization envelope maturation (Battaglia and Shapiro, 1988; Deits *et al.*, 1984; Foerder and Shapiro, 1977; Veron *et al.*, 1977), will provide an answer.

E. Regulation of ECM-Modifying Enzymes

Regardless of enzyme–substrate specificity, the absence of regulated proteinase, peroxidase, and transglutaminase activity during construction of the physical block could be just as catastrophic to the zygote as not using any modifiers at all. Thus, it should be expected that the individual enzymes possess various methods of regulation, particularly because they are released into the extracellular environment and are expected to perform for a brief period only within the egg ECM.

Most of what is known about the enzymatic regulation of CG-derived proteins relates to sea urchins. CG lumens are acidic, approximately pH 5.5 (Haley and Wessel, 2004b). Because the pH of seawater lies between 7.5 and 8.0, CG contents experience a rapid and dramatic change in their environment upon exocytosis. This pH shift is thought to regulate the activity of the CG serine protease (CGSP1) (Haley and Wessel, 1999, 2004b) and ovoperoxidase (Deits and Shapiro, 1985; Deits and Shapiro, 1986). For CGSP1, the acidic CG environment maintains the enzyme in its pro–form; upon alkalization of its environment, the enzyme autoactivates (Haley and Wessel,

2004b). This conversion occurs in seconds, allowing for the rapid cleavage of proteins that attach the vitelline layer to the egg membrane and that help construct the fertilization envelope (Haley and Wessel, 1999, 2004a,b). Ovoperoxidase undergoes a similar change from an inactive to an active form upon alkalization of its environment, but this enzyme requires about 10 min to complete its conformational hysteresis (Deits and Shapiro, 1985, 1986) and can be suppressed by proteolysis (Haley and Wessel, 2004b). Thus, the egg has two independent methods that remove the potentially toxic activity of ovoperoxidase from its surface before nonspecific cross-linking activity begins: tethered separation from the surface by proteoliaisin (Somers *et al.*, 1989; Weidman *et al.*, 1985; Weidman and Shapiro, 1987) and CGSP1–specific repression (Haley and Wessel, 2004b).

A second means of regulating ovoperoxidase in the sea urchin is to control the source of its primary substrate, hydrogen peroxide (Boldt *et al.*, 1981). This NADPH-dependent oxidase activity, observed first in sea urchins (Warburg, 1908), is the main source of hydrogen peroxide in the zygote (Foerder *et al.*, 1978; Heinecke and Shapiro, 1989, 1992; Wong *et al.*, 2004). It is a member of the dual oxidase family of enzymes, containing an amino-terminal peroxidase domain, a carboxy-terminal NADPH reductase domain, and a cytoplasmic linker with two calcium-binding EF hands (Wong *et al.*, 2004). This sea urchin egg dual oxidase (Udx1) resides at the egg cortex and is sensitive to intracellular calcium concentrations, PKC phosphorylation, and intracellular pH (Foerder *et al.*, 1978; Heinecke and Shapiro, 1989, 1992; Wong *et al.*, 2004). Udx1 regulation complements the hysteretic delay in ovoperoxidase activation (Deits and Shapiro, 1985), where a similar lag time is expected for PKC activation of the reductase component *in vivo* (see Section VIII.B, earlier in this chapter). As cytoplasmic pH rises (Johnson and Epel, 1976, 1981; Shen and Steinhardt, 1978), Udx1 activity is depressed, decreasing hydrogen peroxide production, and consequentially down-regulates ovoperoxidase activity by depleting its major substrate (Wong *et al.*, 2004). The amino-terminal peroxidase of Udx1 is hypothesized to play a protective role against rampant hydrogen peroxide diffusion toward the zygote, similar to the scavenging activity of catalase (Wong *et al.*, 2004) and ovothiol (Turner *et al.*, 1986, 1987, 1988). The identification of ovothiols in many marine invertebrate eggs (Turner *et al.*, 1987) suggests that hydrogen peroxide production, possibly by Udx1 orthologs, is a conserved event in the block to polyspermy.

Is it also critical to shut off ovoperoxidase activity after complete hardening? Prolonged (120-min) exposure to hydrogen peroxide causes the purified enzyme to auto-inactivate (Deits *et al.*, 1984). The corresponding shutdown of Udx1 activity (Wong *et al.*, 2004) prevents this long-term exposure, possibly to retain the anti-microbicidal activity of ovoperoxidase within the fertilization envelope (Klebanoff *et al.*, 1979), whose source of hydrogen

peroxide could be zygotic Udx1 (Wong and Wessel, 2005). Thus, at least in sea urchins, the downstream effects of phospholipase C activity become important in the initiation and completion of the permanent block to polyspermy, as well as in the survival of the early embryo. The IP3-dependent calcium wave is essential for CG exocytosis to release the structural proteins and enzymes necessary for modifying the vitelline layer and for regulation of Udx1, whereas DAG is essential for PKC activity that modulates the NADPH-oxidase activity. Without these three components, the sea urchin fertilization envelope would remain unfinished, leaving the embryo exposed to the whims of its environment.

F. Unusual Suspects

A minor cluster of animal eggs exhibits significant biochemical and morphological changes to their egg ECM that qualify as a true physical block to polyspermy, yet the source of these alterations is not known. Although most of these eggs release the contents of their CGs upon fertilization, the contribution of these vesicles toward the ECM is negligible. For example, marsupials primarily rely on oviductal glycoproteins to coat and mask the zona from sperm rather than promoting the CG-dependent modifications observed in eutherians (see Section IX.C, earlier in this chapter) (Breed *et al.*, 2002). Other animals completely lack CGs, such as mollusks (Hylander and Summers, 1977; Togo *et al.*, 1995), ascidians (Lambert *et al.*, 1997; Rosati *et al.*, 1977), and nematodes (Singson, 2001). Zygotes of mollusks and ascidians depend on subtle changes to avoid supernumerary fusions, including the alteration of plasma membrane conductance and/or topology. One such source of these enzymes may originate from ascidian test cells, who are thought to contribute to postfertilization events that establish the permanent block to polyspermy (Rosati *et al.*, 1977), thus displaying a life cycle similar to that of serum platelets, anucleate blood cells filled with secretory vesicles whose content participate in inflammation (reviewed in King and Reed, 2002). Nematodes, on the other hand, somehow ensure monospermy at the level of the spermatheca. Soon after fertilization, zygotes subsequently acquire a chitinous eggshell as they pass through the uterus, thereby hindering further sperm–egg interactions (Singson, 2001).

Anurans use a variety of methods to establish a mechanically sound block to polyspermy. For example, some anurans simply rely on ECM reorganization and hydroscopic swelling of the outer jelly layers to create a resilient barrier against sperm (Elinson, 1986; Hedrick and Nishihara, 1991; Wolf *et al.*, 1976). In *Discoglossus*, CGs are exocytosed within 5 min of egg activation, because of the initial calcium wave (Nuccitelli *et al.*, 1988), but no changes to the egg ECM are observable until 20 min after egg activation,

when the overlying jelly plug begins to liquefy because of peroxidase-like activity (Campanella *et al.*, 1992; Pitari *et al.*, 1993). Dissolution of the plug by oxidative loss of disulfide bonds ensures that the once-ordered plug structure is destroyed, thereby eliminating access of additional sperm to the dimple (Campanella *et al.*, 1992; Pitari *et al.*, 1993). The enzyme responsible for this liquefaction derives from vacuoles released after the cortical reaction has passed (Campanella *et al.*, 1992). Thus, while all anuran zygotes appear to modify their egg jelly layers to establish the permanent block to polyspermy, they utilize extremely diverse mechanisms.

Physical blocks are also observed in animals whose eggs do not undergo a standard CG release or completely lack CGs, or even secretory granules that remotely resemble such organelles. Examples of these animals include the mollusks chiton (Buckland-Nicks *et al.*, 1988), abalone (Vacquier and Lee, 1993), and bivalves (Togo and Morisawa, 1999), the dipteran *Drosophila* (Mahowald *et al.*, 1983), urodeles (Charbonneau *et al.*, 1983; Iwao, 1989; Jego *et al.*, 1986; Makabe-Kobayashi *et al.*, 2003), ascidians (Rosati *et al.*, 1977), and nematodes (Singson, 2001). Are plasma membrane-associated electrical changes sufficient to prevent supernumerary sperm fusion, as suspected in the primitive urodele *Hynobius* (Iwao, 1989) and mollusks *Crassostrea* (Alliegro and Wright, 1983; Togo and Morisawa, 1999), *Tonicella* (Buckland-Nicks *et al.*, 1988), and *Callochitin* (Buckland-Nicks and Hodgson, 2000)? If not, then how do these externally fertilized eggs cope with the high risk of polyspermy? Do they have nonelectrical mechanisms—such as secretion of modifying enzymes from secondary vacuoles (Campanella *et al.*, 1992; Pitari *et al.*, 1993), supernumerary sperm extrusion (Yu and Wolf, 1981), or female pronuclear choice (Gould and Stephano, 2003)—to prevent or reject additional sperm at the surface or in the cytoplasm? One dramatic change in ascidians is the release of follicle cells upon fertilization (De Santis *et al.*, 1980). During the courting process, sperm must pass through tightly apposed follicle cells attached to the vitelline coat, using a mechanism that may require active participation of these cells (De Santis *et al.*, 1980). Might the follicle cells direct the sperm to a favorable region on the vitelline coat or maintain sperm-receptive tufts of fibers on its outer surface? If so, then their loss after fertilization could be the primary physical alteration that inhibits supernumerary sperm binding (Rosati, 1985; Rosati *et al.*, 1977). The use of glycosidases to abolish sperm receptivity along the vitelline coat surface has been postulated to supplement the loss of follicle cells, thereby enhancing the physical block to polyspermy (Lambert, 2000; Lambert *et al.*, 1997).

Alternatively, a nonelectrical plasma membrane block in many animal zygotes has been documented to be sufficient to block polyspermy. For example, modification of integral components along the zygotic plasma membrane has been proposed to account for membrane blocks in mammals

(Hoodbhoy and Talbot, 1994). One model is that the CG-derived glycoproteins that form the mammalian CG envelope (Figs. 1 and 2) establish a significantly charged surface that repels sperm still trapped in the perivitelline space. Such a mechanism is consistent with the bias against sperm fusion overlying the mammalian metaphase II spindle and polar body, which colocalizes with prematurely released CGs (Cran and Esper, 1990). Unfortunately, the timing of CG envelope formation may not be early enough to establish an effective block if sperm are abundant in the perivitelline space prior to CG release. An alternative mechanism lies at the plasma membrane itself: Retraction or morphological alteration of all fusogenic microvilli or physical detachment of the old receptive membrane from the zygote have been observed in the annelid *Chaetopterus* (Eckberg and Anderson, 1985), chiton (Buckland-Nicks and Hodgson, 2000; Buckland-Nicks *et al.*, 1988), crustaceans (Brown and Clapper, 1980; Goudeau and Becker, 1982), dipterans (Mahowald *et al.*, 1983), sea urchins (Longo *et al.*, 1974, 1986), anurans (Talevi and Campanella, 1988), marsupials (Breed and Leigh, 1992), and at the membrane subjacent to the micropyle of teleosts *Danio* (Wolenski and Hart, 1987) and *Rhodeus* (Ohta and Iwamatsu, 1983). Could cortical remodeling, perhaps via an integrin-dependent cytoskeletal reorganization mediated at gamete fusion (see Sections VII.B and VII.C, earlier in this chapter), be sufficient to block polyspermy? Mouse and hamster zygotes utilize both plasma membrane and zona blocking methods (Cherr *et al.*, 1988; Tatone *et al.*, 1994; Wolf and Hamada, 1979); rabbit zygotes do not display any significant modifications to their zona or plasma membrane (Overstreet and Bedford, 1974), yet all their eggs remain monospermic. Could there be even more types of blocks to polyspermy used throughout the animal kingdom?

X. Descendents of Requisite Polyspermy?

Physiological polyspermy is the condition when multiple sperm are allowed to fuse with the egg, but subsequently only one male pronucleus is merged with the haploid egg nucleus. This mechanism of fertilization is prevalent in some orders of animals. Occasional physiological polyspermy is observed in domesticated *Drosophila* species, some naturally ovulating between 1 and 10% polyspermic zygotes (Fitch *et al.*, 1998; Snook and Markow, 2002), whereas the marsupial *Sminthopsis* exhibits at least 5% polyspermy in the oviduct (Breed and Leigh, 1990). Urodeles fail to release monospermic zygotes, despite the use of a spermatheca (Elinson, 1986), and birds and reptiles, both with large eggs, almost require physiological polyspermy to ensure the "certainty of fertilization" (Harper, 1904).

Common urodeles such as newts and salamanders (excluding *Hynobius*) produce eggs lacking CGs, and are incapable of generating a fast electrical block to polyspermy (Jego *et al.*, 1986). Although a physical block eventually forms between jelly layers—because of the hydration and consequential precipitation of lectins as the laid eggs age—this is not on a time-scale that favors monospermy because nearly 90–100% of all eggs are polyspermic (Elinson, 1986; Iwao, 1989; Jego *et al.*, 1986). This ultraslow physical block may have evolved to protect the new embryo from a second wave of sperm insemination and/or microbes rather than the second sperm to encounter the egg ECM (Jego *et al.*, 1986). Rather than committing the resources to generate a timely physical block, urodele zygotes use mechanisms that suppress all cytoplasmic sperm nuclei that do not fuse with the egg pronucleus, pushing them away from the animal hemisphere into the vegetal to avoid further developmental complications (Elinson, 1986; Fankhauser, 1932; Iwao, 1989; Iwao and Elinson, 1990). This activity is predicted to involve a local concentration of factors around the egg pronucleus/zygotic nucleus that controls the timing of cell cycle reentry; other amphibians lack cytoplasmic localization of this factor and are thereby thought to be incapable of recovering from polyspermy (Iwao and Elinson, 1990).

As expected for a zygote that undergoes requisite physiological polyspermy, modifications to the intimate avian vitelline membrane and egg plasma membrane do not change immediately after fertilization (Bellairs *et al.*, 1963). Upon ovulation, the perivitelline layer is weakened over the germinal disc where the sperm bore visible tunnels through the fibrillar meshwork (Bramwell and Howarth, 1992; Okamura and Nishiyama, 1978a), a process that promotes supernumerary sperm penetration. These holes are subsequently patched by the oviductal application of an outer layer eggshell coat, but the process of glycoprotein adsorption is not rapid enough to restrict the quantity of sperm fusing (Okamura and Nishiyama, 1978a). Avian zygotes instead limit the number of pronuclear fusion events to unity by expelling the accessory sperm pronuclei and their associated centrioles from the germ disc into extraembryonic regions punctuated by yolk granules (Bellairs, 1993; Harper, 1904; Okamura and Nishiyama, 1978b). There, the pronuclei continue to undergo asynchronous division through the early stages of cleavage, but eventually degenerate (Harper, 1904).

Among vertebrates, the use of physiological polyspermy is generally restricted to more ancient lineages, for example, reptiles, birds, urodeles (Elinson, 1986), and chondroichthyes such as sharks and chimera (Hart, 1990). At face value, this suggests that methods to cope with polyspermy may be older than the complexity associated with establishment of a block to polyspermy. Yet the essential components of the egg ECM between purely monospermic and physiologically polyspermic animals are often homologous, suggesting that only a fine distinction exists between the selection of

ECM components for sperm binding versus their coevolution as postfertilization scaffold proteins necessary to establish a block to polyspermy. Is it really more efficient for an egg to package a self-assembling barrier into granules that must be coordinately released soon after fusion than to partition a select group of cytoplasmic molecules that can "choose" a dominant sperm pronucleus? Broadcast spawners would clearly benefit by retaining self-assembling barriers because such structures provide both a physical block to polyspermy *and* a protective shell for embryogenesis. Internally fertilized animals, particularly ones that use oviductal contributions to create a protective shell for development, benefit as much from such an egg-derived barrier. In fact, the energy expenditure required to synthesize CGs would likely selectively decrease the quantity of eggs produced to favor the survival of the potential zygote. Thus, one major factor influencing the selection of a cytoplasmic (physiologic) or extracellular (monospermic) block to polyspermy could be the type of insemination used (internal or external).

Within a deuterostome order, it is possible to trace phylogenetic paths that map the more ancient mechanisms to cytoplasmic blocks, whereas the most recently diverged taxa use extracellular blocks (see Figs. 4 and 6). For example, amphibians are represented by urodeles and anurans. Most urodeles exhibit internal fertilization, whereas anurans display both internal and external (see Section V.A, earlier in this chapter) (Elinson, 1986; Toro and Michael, 2004). Urodeles also exhibit a cytoplasmic block to polyspermy, despite the rare presence of a fast electrical block in the more primitive members (e.g. *Hynobius*) (Charbonneau *et al.*, 1983; Iwao, 1989; Iwao and Jaffe, 1989). Most urodele species also lack CGs (Elinson, 1986). On the other hand, most anurans possess both a fast electrical block and CGs, regardless of the type of insemination used (Elinson, 1986; Iwao, 1989; Iwao and Jaffe, 1989). Thus, monospermy is favored through extracellular modifications. In *Discoglossus*, monospermy is further enhanced by severe polarization of egg, but relies on both an efficient fast electrical block and a delayed CG-dependent modification of the ECM to ensure monospermy (Campanella *et al.*, 1992; Nuccitelli *et al.*, 1988; Pitari *et al.*, 1993; Talevi and Campanella, 1988). Under the rare circumstances when polyspermy does occur in *Discoglossus*, the zygote can rapidly eliminate the supernumerary sperm before its pronucleus has completely penetrated the cytoplasm (Talevi and Campanella, 1988). Based on the hypothesis that the cytoplasmic block is more ancient than an extracellular block, urodeles would be placed basal to anurans, with *Discoglossus* more basal to the other anurans such as *Xenopus*, *Bufo*, and *Eleutherodactylus*. Unfortunately, this organization cannot be tested against the pedigree because amphibians are thought to be diphylogenetic, where urodeles and anurans may have arisen from different lineages (Elinson, 1986). Within anurans, however, *Discoglossus* is thought to occupy a more basal position than the others listed. Thus, the

transition from a cytoplasmic block to an extracellular block may have required the sequential acquisition of a fast electrical block, followed by a more robust CG contribution to the zygotic ECM in anurans.

A more robust comparison can be made using the phylogeny of fish. Sharks and chimeras utilize spermathecae during internal fertilization, but still require a cytoplasmic block at fertilization (Hart, 1990; Neubaum and Wolfner, 1999). Other orders of spawning fish release highly polarized eggs that exhibit increasingly more sophisticated specializations (see Fig. 4): Petromyzontids use the earliest signs of a localized site for enhanced fusogenicity, namely a tuft of sperm-attractive jelly, extension of an acrosomal process following acrosome exocytosis, as well as both fast electrical and permanent blocks to polyspermy initiated by specialized CGs below the site of sperm fusion (Dabrowski *et al.*, 2004; Kobayashi and Yamamoto, 1994; Kobayashi *et al.*, 1994). Next are the chondrosteans, whose use of a cluster of multiple micropyles and an acrosomal process reduces sperm/egg ratios but does not ensure monospermy as elegantly as the single micropyle found in teleosts (Hart, 1990; Cherr and Clark, 1986). Thus, fish eggs exhibit a gradient of complexity along its pedigree (Fig. 4). Sharks and chimeras are phylogenetically basal to other bony fish, which rank in the order petromyzontids, chondrosteans, to teleosts from most ancient to most recently diverged. Thus, as in anurans, cytoplasmic blocks presumably evolved before extracellular blocks in fish. The rapid specialization in ECM morphology from a polarized site of sperm entry to a single micropyle is also an intriguing transition. Ironically, with the progression toward a single micropyle comes a reduction in the number of CGs contributing to zygotic ECM modifications (Hart, 1990; Hart and Donova, 1983) and the loss of the sperm acrosome and acrosomal process—two features hypothesized to be advantageous for spawning animals (see Section IV, earlier in this chapter). Thus, in fish, extracellular blocks to polyspermy appear to be undergoing minimization in the overall energy expenditure during gametogenesis. Yet selection of this specific method to favor monospermy requires a significant degree of morphological and molecular complexity in the egg ECM. Does this progress, then, truly represent advancement? For spawners, yes; for animals using internal fertilization, not really.

Could the association between cytoplasmic blocks and internal fertilization indicate an overall trend in the progressive evolution of an animal lineage? Nematodes fertilize internally and display requisite monospermy in the absence of any ECM modifications (Singson, 2001). Most dipterans use micropyles to achieve monospermy during internal fertilization (Snook and Markow, 2002). Mammalian eggs are internally fertilized and achieve monospermy with the help of CGs and associated cumulus and/or oviductal cells (Selwood, 1992). Mollusks spawn, but only require a change in membrane and cytoskeletal organization to maintain monospermic conditions

(Dufresne-Dube *et al.*, 1983; Hylander and Summers, 1977). Decapods also spawn, using a fast hyperpolarization and an elaborate hierarchy of CGs to establish monospermic conditions (Gould and Stephano, 2003; Talbot and Goudeau, 1988). Echinoderms spawn, but use the full spectrum of ECM modifications to sustain monospermy (Shapiro *et al.*, 1989). Separating these monospermic animals based on their method of insemination, a similar trend of increasing complexity in the type of extracellular monospermic blocks can be distinguished: Within either internal or external fertilizers lies a gradient of increasing ECM complexity that parallels the predicted phylogenetic position of the taxon. Hence, more ancient animals possess the fewest plasma membrane or ECM modifications, whereas the more recently diverged animals have acquired the most (Fig. 6).

Yet consider all animals originated from a hypothetical "common" ancestral egg that possessed all of the aforementioned mechanisms involved in the permanent block to polyspermy, namely a fast electrical block, abundant CG contributions to the ECM, and contributions from the oviduct if the animal is internally fertilized. Next, consider the amount of time each taxon has had to individually refine its eggs from this common ancestor. In such a hypothetical situation, selection would favor the most efficient mechanism of monospermy, one that requires minimal expenditure of resources while still ensuring a high rate of fertilization success. In such a scenario, the taxa with the least amount of time to refine the process of fertilization would look most like the common ancestor. Conversely, those around longer would have had time to rework the process, eventually losing most of the extraneous parameters in favor of a streamlined process. One result of this time-dependent evolution would be the complete loss of extracellular blocking mechanisms in favor of physiological polyspermy because it represents the most efficient mechanism and requires the least expenditure of energy: namely, the intracellular localization of specific factors that choose the sperm pronucleus to fuse with. With such simplification may come the adoption of an embryonic coat for protection during development. Mapping these criteria based on time of separation from the common ancestor would yield a cladogram of animal phylogeny quite similar to our current working model. Granted, the hypothetical scenario would miscalculate the attributes of a few animals, such as echinoderms and dipterans, but the overall pattern would be strikingly familiar. Therefore, it is quite possible that the process of animal fertilization may have a common ancestor.

XI. Perspective

Dynamics between the egg ECM and sperm are essential to achieve monospermic fertilization. Common structural motifs are retained in egg matrices of many different animal phyla, as well as overlapping enzymatic

contributions from the CGs postfertilization. A common method of ECM modifications was most likely used during fertilization in the most common ancestor to animals, so what were the original requirements? Based on our current understanding, common ultrastructure, molecular motifs, and mechanisms are used during specific stages of fertilization and the block to polyspermy, including (1) the indiscriminate auto-polymerizing ZP domain as a common building block of the egg ECM (Jovine *et al.*, 2002); (2) species-specific sperm receptors composed of signature branched oligosaccharides bound to homologous proteins found in both the egg ECM and the oviductal enhancements; (3) the interaction of at least one transmembrane protein with its complement to initiate sperm–egg fusion; (4) the use of CG–derived proteases, protein–protein cross-linking enzymes, and lectins to render the egg ECM incapable of further sperm binding after fertilization; and (5) exchange of the egg ECM with a more intimate zygotic ECM of different composition than the original that can serve as a substratum for embryonic development and signaling (Bellairs *et al.*, 1963; Matsunaga *et al.*, 2002; Selwood, 1992).

A clear distinction exists, however, between internally and externally fertilizing animals: Internally fertilizing animals have also retained the participation of oviduct epithelial contributions toward sperm retention and deposition of a mechanical mucoid coat onto the surface of the zygote. These anatomical contributions to fertilization may have relaxed the selective pressure for more robust ECM-associated mechanisms that are prominent in externally fertilizing animals. Thus, although further characterization of the proteins composing the egg ECM and CGs must be made, we speculate that an essential set of orthologs has been retained throughout evolution for the purpose of blocking polyspermy. Modifications and adaptations of these original processes were made by each animal pedigree, thus achieving the present diversity in mechanisms of monospermic fertilization.

Acknowledgments

We would like to thank Drs. Mark Johnson and David Rand for their critical analysis and discussions. Also thanks to Drs. Doug Chandler, Nathan Hart, and Jim Trimarchi for generously providing photomicrographs for our use. The authors were supported by grants from the National Institutes of Health and the National Science Foundation.

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