Sperm-Oocyte Membrane Interactions during Fertilization in the Nematode Caenorhabditis elegans

Alissa Gale Richmond

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SPERM-OCYTE MEMBRANE INTERACTIONS DURING FERTILIZATION IN THE NEMATODE *CAENORHABDITIS ELEGANS*

A Thesis

Presented to
The Faculty of the Department of Biology
The College of William and Mary

In Partial Fulfillment
Of the Requirements for the Degree of

Master of Arts

by
Alissa Richmond
2004
APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts

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Approved by the Committee, July 2004

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ABSTRACT

The objective of this research is to elucidate the mechanism of gamete membrane interaction during fertilization in the nematode Caenorhabditis elegans. Currently, fusion and endocytosis are the only documented models of sperm-oocyte interaction in nematodes. To analyze this stage of fertilization in C. elegans, I have determined the localization of three sperm components in newly fertilized oocytes: 1) a centriolar component, SPD-2, 2) a transmembrane protein, SPE-9, and 3) membranous organelles (MOs). According to my predictions, the localization pattern of these sperm components should correlate with either endocytosis or fusion. Primarily, the results of this research suggest that fusion is the mechanism of sperm-oocyte membrane interaction in C. elegans. Secondarily, this study has provided information about how various sperm components behave in the early embryo and relative to each other.
SPERM-OOCYTE MEMBRANE INTERACTIONS DURING FERTILIZATION IN
THE NEMATODE *CAENORHABDTIS ELEGANS*
Fertilization Review

Fertilization is a process that encompasses a range of events beginning with the formation of gametes and ending with the initiation of zygotic development. The major aim of this research, however, is to elucidate the mechanism of gamete membrane interaction at the point of sperm-oocyte contact. The following mini-review discusses the stages immediately prior to this point, gamete recognition and binding/fusion, as well as the stage immediately following this point, the transition from egg to zygote. The gamete recognition section addresses how sperm and eggs/oocytes find each other. The gamete binding and fusion section addresses what happens when sperm and egg/oocyte do find each other. The last section addresses the events following sperm-egg fusion which mark the initiation of zygotic development. Throughout this review, representative organisms, from plants to mice, have been chosen to provide varying views of these crucial sub-stages of fertilization.

Chemotaxis as a method of gamete recognition

Chemotaxis is the positive (chemoattractive) or negative (chemorepulsive) response of cells to a gradient of a specific diffusible substance. Examples of chemotaxis include the guided migration of axonal growth cones to their target cells (reviewed in Kolodkin, 1996) and the aggregation of unicellular Dictyostelium cells into a multicellular organism (reviewed in Firtel and Chung, 2000). In the case of gamete recognition, chemotaxis refers to the attraction of male gametes (sperm) to female gametes (eggs). Several organisms, including plants, sea urchins and frogs, use species-
specific chemoattraction to facilitate recognition of compatible gametes that are often separated by great physical distances. For instance, sea urchins shed their gametes directly into large bodies of water making it difficult for sperm to encounter an egg without active attraction mechanisms. Gamete chemotaxis also occurs in internally fertilizing animals such as mice and humans, where follicular fluids of the female reproductive tract provide a positive attractant for sperm (Ralt et al., 1994; Eisenbach and Tur-Kaspa, 1999; Oliveira et al., 1999). In all cases, these chemoattractants serve to instruct sperm on its location relative to the egg. Although this phenomenon takes place in a wide range of metazoans (for a comprehensive review see Miller, 1985), plants, sea urchins and frogs represent three well studied but distinct examples.

Pollen tube guidance

For fertilization to occur in plants, the newly germinated pollen tube (PT) must first locate the entrance to the style and then migrate down its length to reach the ovary (Fig. 1). Over 50 years ago, researchers found that secretions from lily stigmas stimulated directional growth of PTs in vitro (Miki, 1954; Welk et al., 1965), suggesting that these secretions guide PTs across the broad stigma to the entrance of the style. More recently, PTs have been shown to reorient themselves towards a specific component of stigma secretions (Kim et al., 2003). Through a series of protein fractionation/purification steps and chemotropism assays, the protein responsible for the chemotactic properties of lily stigma secretions was identified as chemocyanin.

The next step in guiding the PT through the style and towards the ovary can be divided into two phases: sporophytic and gametophytic (for review see Lord and Russell, 2002). During the sporophytic phase, secretions of the transmitting tract epithelium...
(TTE) internally guide the PT along the length of the style (Lord and Russell, 2002). During the gametophytic phase, the PT first exits from the TTE onto the funiculus (ovary stalk) and subsequently grows to the micropyle (ovule opening) (Lord and Russell, 2002). Although no specific peptide or protein has been discovered, both stages of the gametophytic phase seem to be controlled by a chemoattractant secreted from ovules (Hülskamp et al., 1995; Higashiyama et al., 1998) and, more specifically, the female gametophyte or embryo sac proper (Ray et al., 1997; Shimizu and Okada, 2000; Higashiyama et al., 2001).

As in animals (Ward et al., 1985; Ralt et al., 1994; Al-Anzi and Chandler, 1998), the large, sessile female gamete, in this case the ovules, secretes a substance that attracts the smaller, motile gamete, sperm or PTs containing sperm. To test for the involvement of ovules in attracting PTs, Hülskamp and colleagues (1995) examined Arabidopsis thaliana mutants defective in ovule development. Ovules with defective sporophytic (integument) and gametophytic (embryo sac) tissues never get fertilized since the PT neither exits onto the funiculus nor grows towards the micropyle (Hülskamp et al., 1995). On the other hand, mutants defective only in embryo sac development have PTs that successfully grow along funiculi but fail to enter the micropyle (Shimizu and Okada, 2000). The above-mentioned studies and others like it (Ray et al., 1997; Higashiyama et al., 1998; Higashiyama et al., 2001) strongly suggest that at least two tissues guide PTs upon their exit from the TTE: sporophytic tissues direct PTs along the funiculus, and gametophytic tissues direct PTs to the micropyle.
Figure 1: **Gamete recognition in the pistil.** The pollen grain (PG) lands and germinates on the stigma of the pistil. Cues from the transmitting tract epithelium (TTE) signal the pollen tube (PT) to grow down its length towards the ovary. The pollen tube then exits onto the funiculus (ovary stalk) and grows towards an individual ovule. After entering the ovule through the micropyle, the pollen tube delivers its sperm cells. Specific tissues direct each stage of growth of the pollen tube (see text). Adapted from Cheung and Wu (2001).
Chemotaxis of sea urchin and Xenopus sperm

In organisms that employ external fertilization, chemoattraction is essential for efficient gamete recognition. In two species of sea urchin, Arbacia punctulata and Strongylocentrotus purpuratus, the egg jelly peptides resact and speract, respectively, function as chemoattractants (Suzuki and Garbers, 1984; Ward et al., 1985). In chemotaxis assays, sperm will swim up a peptide gradient of their respective chemoattractant (Suzuki and Garbers, 1984; Ward et al., 1985) unless they have been preincubated in high concentrations of chemoattractant (resact or speract), suggesting that a gradient is necessary and chemotaxis is saturable (Ward et al., 1985).

In an analogous manner, the egg jelly of Xenopus laevis contains a protein with functions similar to resact (Al-Anzi and Chandler, 1998; Olson et al., 2001). Just as in sea urchins, Xenopus sperm chemotax in gradients of concentrated egg water (water conditioned with frog egg jelly), implying frog egg jelly contains a chemoattractant (Al-Anzi and Chandler, 1998). Biochemical studies by Olson and colleagues (2001) revealed allurin as the key chemoattractive protein in Xenopus egg jelly.

Although chemocyanin, resact and allurin play analogous roles in attracting the male gamete, they are three molecularly distinct polypeptides. Chemocyanin, a 9 kDa, acidic protein is a plantacyanin, a family of cell wall proteins with unknown function (Kim et al., 2003). In contrast, speract and resact are both small, glycine rich peptides (Suzuki and Garbers, 1984; Ward et al., 1985). Finally, allurin, a 21 kDa, basic protein, is thought to belong to the CRISP (cysteine-rich secretory protein) family of proteins involved in aiding the transition of sperm through various stages of development and fertilization (Olson et al., 2001). While molecularly diverse polypeptides are used, a
broad range of organisms (including, but not limited to, plants, echinoderms and vertebrates) employ sperm chemoattraction as a major method of gamete recognition.

But to what extent is chemoattraction used as a mechanism for species-specific gamete recognition? In the context of this discussion, two species of sea urchin, within the class Echinoidea, utilize the previously mentioned chemoattractants, resact and speract, in a species-specific manner. This suggests that animals that broadcast their gametes may frequently use chemoattraction for species-specific gamete recognition. On the other hand, follicular fluid, the chemoattractant in mammals, functions across species within the class Mammalia (Sun et al., 2003). This suggests that species barriers in mammals, and perhaps in other internally fertilizing vertebrates, occur at levels other than gamete chemoattraction.

**Gamete binding and fusion**

Binding and fusion are two closely linked processes involved in cellular events such as membrane trafficking, morphogenesis, cell signaling and fertilization. During binding, two cellular membranes become physically attached to one another. During fusion, the two bound lipid bilayers physically merge into one. Examples of intracellular binding include vesicular docking in which SNARE proteins interact to bring vesicle membranes in close contact (Lodish et al., 2000; Blumenthal et al., 2003). Examples of intercellular binding include tight junctions and flagellar adhesion. In epithelial cells, tight junctions attach not only neighboring cells but also form physical barriers that serve to polarize cells (Anderson and Van Itallie, 1999; Lodish et al., 2000). Likewise, there are intracellular and intercellular examples of fusion. After endocytosis, the glycoprotein hemagglutinin mediates fusion of the influenza viral membrane with the endosomal
membrane enabling the virus to unload its genetic material (Lodish et al., 2000; Eckert and Kim, 2001). On the intercellular level, uninucleate myoblasts (muscle cell precursors) fuse to form mature, multinucleate muscle cells (Dworak and Sink, 2002; Blumenthal et al., 2003). In Caenorhabditis elegans cell-cell fusions are essential in forming the hypodermis as well as more specialized organs such as the vulva (reviewed in Shemer and Podbilewicz, 2003).

The process of creating a diploid zygote through fertilization requires both binding and fusion of the sperm and egg plasma membranes; both are essential for allowing the male gamete to insert its paternal genome into the egg. Although the exact molecular mechanisms of these two processes remain to be determined, candidate molecules include integrins and disintegrin-like proteins (Takahashi et al., 2000). Furthermore, researchers agree on a primary mechanism of action: membrane bound sperm receptors interact with egg ligands to facilitate gamete binding and then the same or different proteins facilitate gamete fusion. Although these events take place in most organisms, the general principles can be illustrated in Chlamydomonas, sea urchin and mice.

**Non-mammalian organisms: Chlamydomonas and Sea urchin**

Gamete binding in the green alga Chlamydomonas reinhardi occurs on two levels, adhesion of the flagella and adhesion of the mating structures. Similar to but on a larger scale than the coiling of SNARE proteins, flagella of Chlamydomonas gametes adhere and intertwine to bring together opposing, mt+ and mt-, gametes (reviewed in Goodenough et al., 1995). Flagellar adhesion facilitates the second level of binding by orienting gametes such that mt+ and mt- mating structures face each other. Tightly
coupled with mating structure binding is gamete fusion during which the mating structure membranes become continuous and eventually lead to a quadriflagellated zygote (Goodenough et al., 1995). Originally, researchers thought that fringe, a glycoprotein-rich material surrounding mating structures, mediated *Chlamydomonas* gamete fusion (Goodenough et al., 1982; Ferris et al., 1996). Ultrastructural studies show that mutants with drastically lowered levels of fringe (imp-1 and imp-11) are defective in gamete fusion (Goodenough et al., 1982). Later studies demonstrate that these mutants regain their fusion ability when transformed with a normal copy of the fringe gene, *fus1* (Ferris et al., 1996). However recent studies suggest that the Fus1 protein functions not in fusion but rather mating structure adhesion (Misamore et al., 2003). Docking and fusion assays of gametes mutant for *fus1* show that both processes are severely inhibited (Misamore et al., 1996). If gametes are unable to bind, it follows that they would be unable to fuse. Although it may be difficult to separate *Chlamydomonas* gamete binding and/or fusion, evidence suggests that Fus1 is essential for both processes.

In sea urchins, gamete binding is mediated by the interactions of a sperm protein, bindin and the corresponding receptor on the egg. The acrosome reaction of sperm exposes and deposits bindin on the surface of the sperm head allowing it to now interact with the egg receptor (reviewed in Foltz and Lennarz, 1993). Egg agglutination assays show that bindin functions in sea urchin species-specific sperm-egg adhesion (Glabe and Vacquier, 1977; Glabe and Lennarz, 1979). In these agglutination assays, adding isolated bindin protein causes eggs to agglutinate, or bind to each other thus forming aggregates. Furthermore, immunolocalization experiments show that bindin localizes to the area of contact between agglutinated eggs (Glabe and Lennarz, 1979). The receptor for bindin is
a 350-kDa glycoprotein located within the egg jelly (Hirohashi and Lennarz, 2001; Kamei and Glabe, 2003). Sperm binding assays show that this sperm binding protein functions in species-specific sperm-egg adhesion (Hirohashi and Lennarz, 2001; Kamei and Glabe, 2003). Beads coated with the egg glycoprotein from one species bound acrosome reacted sperm from that same species but not that from other sea urchin species (Hirohashi and Lennarz, 2001). Kamei and Glabe (2003) sequenced the gene for this egg glycoprotein and named it egg bindin receptor 1, EBR1, for its ability to bind to the sperm adhesive component bindin. Anti-EBR1 antibodies reduce fertilization in a concentration dependent manner suggesting that EBR1 is required for sperm-egg interactions (Kamei and Glabe, 2003).

**Mammalian organisms: Mice**

Similar to the sperm-egg binding and/or fusion molecules involved in invertebrates those in mammals can be divided into two groups: sperm disintegrins and egg integrins. In mammalian sperm, disintegrins, primarily fertilin β and cyritestin, belong to the larger ADAM (a disintegrin and a metalloprotease) family of proteins which are thought to promote cell-cell adhesion by binding to integrins (reviewed in Huovila et al., 1996). The proposal that fertilin β and cyritestin play essential roles in gamete binding/fusion is based on studies in which either peptide fragments or function blocking antibodies were found to strongly inhibit fertilization (extensively reviewed in Evans 1999).

In an even more convincing set of experiments, “knockout” mice lacking either fertilin β (fertilin β -/-) or cyritestin (cyrn -/-) are infertile despite producing sperm that are morphologically and behaviorally normal (Cho et al., 1998; Shamsadin et al., 1999;
For fertilin $\beta^{-/-}$ sperm, the severe reduction in fertility can be attributed to a parallel reduction in sperm binding to both zona-free and zona-intact oocytes (Cho et al., 1998). In addition to binding defects, fertilin $\beta^{-/-}$ sperm have a fusion rate 45-50% that of wild-type (Cho et al., 1998). Cym $^{-/-}$ sperm show the same reduction in binding to zona-intact oocytes (Shamsadin et al., 1999; Nishimura et al., 2001). However their ability to bind zona-free oocytes remains unclear due to conflicting results. Unlike the reduction in fusion rate of fertilin $^{-/-}$ sperm, cym $^{-/-}$ sperm have fusion rates comparable to cym $^{+/+}$ sperm (Shamsadin et al., 1999; Nishimura et al., 2001). Collectively, the evidence mentioned above and reviewed in Evans (1999), leads to three conclusions: 1) fertilin and cyritestin are required for sperm binding to the zona-pellucida, 2) fertilin is required for sperm binding to the oocyte plasma membrane, and 3) fertilin plays a minor role in membrane fusion while cyritestin does not.

If disintegrin proteins such as fertilin and cyritestin indeed act as the sperm component(s) in binding and/or fusion, then it is likely that they interact with integrins on the egg’s surface. The $\alpha6\beta1$ integrin has received the most attention in studies both supporting and refuting its role in sperm-egg binding (reviewed in Talbot et al., 2003). Incubating zona-free oocytes with GoH3, an $\alpha6$ integrin function blocking antibody, severely reduces sperm binding while a non-function blocking antibody has no effect on sperm-egg binding (Almeida et al., 1995). Furthermore, cells that do not normally express $\alpha6$ integrins develop the ability to bind sperm when transfected with $\alpha6$ or $\beta1$; this binding capacity can be specifically inhibited with GoH3 (Almeida et al., 1995). In a complementary experiment, egg lysates were incubated with sperm in a binding assay to identify egg proteins that physically interact with sperm (Takahashi et al., 2000). In these
experiments, a 135-kDa protein tightly associated with sperm; this association was lost when egg lysates were preincubated with GoH3 (Takahashi et al., 2000). In this report, Takahashi and colleagues (2000) concluded that the 135-kDa protein is indeed α6β1.

Though the evidence seems to support an egg integrin’s role in sperm-egg binding, there also exists evidence that supports the idea that integrins do not function in either binding or fusion (reviewed in Evans, 1999 and Talbot et al., 2003). One of the most compelling arguments against α6β1’s function in binding and/or fusion is that zona-free α6 -/- and α6 +/+ oocytes do not differ in their fertilization rates, fertilization indices or sperm binding capacity (Miller et al., 2000). Contrary to previous results (Almeida et al., 1995), preincubating wild type oocytes (α6 +/) with GoH3 does not reduce the fertilization rate (Miller et al., 2000). One explanation for this discrepancy is that other egg integrins have the ability to compensate for α6 (Zhu and Evans, 2002). However, a recent study utilizing a series of complex knockouts and antibody inhibition experiments suggests that compensation between various egg-expressed integrins does not occur (He at al., 2003).

Although the precise role of integrins in binding and/or fusion remains uncertain, current data strongly suggest that CD9, a member of the tetraspanins, is the egg component that functions in fusion, but not binding, of the sperm and egg plasma membranes (Kaji et al., 2000; LeNaour et al., 2000; Miller et al., 2000; Miyado et al., 2000; Kaji et al., 2002). Immunoblotting and immunofluorescence experiments show that CD9 localizes to the oocyte plasma membrane (Kaji et al., 2000; Miyado et al., 2000) and becomes concentrated opposite the acrosomal region of sperm during fertilization (Kaji et al., 2000). Thus, CD9 is at the right place and time to function in sperm-egg fusion.
Furthermore CD9 -/- mice generated by targeted gene disruption produce fertile males and infertile females (Kaji et al., 2000; LeNaour et al., 2000; Miyado et al., 2000). However, these mice are normal in development, mating behavior, and oocyte maturation and ovulation. Although CD9 -/- oocytes are fully competent to bind sperm (Kaji et al., 2000; Miyado et al., 2000), only 0.6% formed pronuclei, showing a lack of fusion (Miyado et al., 2000). Likewise, treatment of wild-type oocytes with anti-CD9 monoclonal antibodies had no effect on sperm binding but severely disrupted sperm-egg fusion (Miller et al., 2000; Miyado et al., 2000). The requirement of CD9 in fusion is further confirmed by the lack of calcium oscillations in CD9 -/- oocytes incubated with sperm (Kaji et al., 2000). Importantly, the fertilization defects of CD9 -/- eggs can be fully rescued by injections of poly-adenylated forms of CD9 (Kaji et al., 2000).

**From egg to zygote**

Though crucial steps in fertilization, gamete recognition, binding, and fusion alone are not enough to form a single celled zygote with the potential to create a new individual. Sperm-egg fusion triggers a progression of events that includes the resumption and completion of meiosis, inhibition of polyspermy, formation/fusion of pronuclei, and the formation of the first mitotic spindle (Gilbert 2000). These events, collectively termed egg activation, characterize the developmental transition of an egg into an embryo. However, the first sign of egg activation is the well-characterized calcium increase observed in all animals (Whitaker and Swann, 1993; Stricker 1999; Gilbert 2000) and some plants (Antoine et al., 2001). This calcium increase, sparked by sperm-egg fusion, can be manifested as a single calcium spike in jellyfish and frogs, as multiple calcium spikes in some bivalves, annelids and mammals, or as a combination of
the two in echinoderms (reviewed in Stricker 1999). Regardless of the type of spike, the resulting increase in intracellular calcium is thought to regulate events of egg activation such as establishing the block to polyspermy, reinitiating the cell cycle, and forming pronuclei (Whitaker and Steinhardt, 1983; Runft et al., 2002). For example, injecting a calcium chelator into *Xenopus* eggs before fertilization inhibits cortical granule exocytosis (discussed below), meiotic resumption and the formation of sperm and egg pronuclei (Kline, 1988). How does the calcium increase affect events of egg activation? The inositol-lipid signaling pathway and its players, PLC, IP₃ and DAG, have been implicated in activating downstream signaling pathways that ultimately start egg activation (Carroll et al., 1999, 2000; Jaffe et al., 2001; Runft et al., 2002).

*Block to polyspermy*

In order for a diploid zygote to have the correct genomic number one haploid sperm must fuse with one haploid egg. Out of necessity, organisms in which hundreds of sperm contact a single egg have developed ways to prevent polyspermy, or fertilization by more than one sperm. Extensively studied in sea urchin, frogs and mice, the block to polyspermy in various animals employs at least one of three distinct changes (Fig. 2), physiological (fast), morphological (slow) and molecular, in the newly fertilized egg (reviewed in Jaffe and Gould, 1985). At the level of the plasma membrane, the fast or physiological block to polyspermy typically involves a change in the membrane potential from negative to positive (Jaffe and Gould, 1985; Longo et al., 1986; Gilbert 2000). In both sea urchin and frog, if the membrane potential is artificially clamped at a negative value, the zygote is fertilized by multiple sperm (Jaffe and Gould, 1985; Gilbert 2000). Conversely, if the membrane potential is clamped at a positive value, fertilization is
Figure 2: Polyspermy prevention mechanisms. A) Fast block: fertilization triggers a change in membrane potential from positive in the unfertilized egg to negative in the fertilized egg. B) Slow block: fertilization triggers cortical granule exocytosis. The released contents of these cortical granules form a physical barrier. C) Permanent block: fertilization triggers enzymatic modification of egg plasma membrane glycoproteins. Glycolytic processing of specific sugar residues (blue) in turn changes the conformation of the glycoprotein, such that subsequent sperm are no longer able to recognize the egg. Adapted from Gilbert 2001.
blocked altogether. Normally the egg plasma membrane restores its resting potential soon after the fast block, thus morphological changes of the egg plasma membrane must reinforce the initial electrical response. This slower morphological block to polyspermy involves the cortical granule reaction, which creates a physical barrier around the fertilized egg (Schuel 1985; Gilbert 2000). Cortical granules, located directly under the egg plasma membrane, fuse with and unload their contents causing the space between the vitelline envelope and plasma membrane to swell (Gilbert 2000). Mucopolysaccharides, hyalin enzyme, and peroxidase enzyme, substances inside the cortical granules, form and harden the fertilization envelope, thus blocking other sperm from binding to and fusing with the egg plasma membrane (Jaffe and Gould, 1985; Gilbert 2000). Polyspermy occurs in eggs defective in cortical granule exocytosis (Kline 1988). The last mechanism of polyspermy prevention involves molecular changes of gamete recognition molecules. In sea urchin, proteases released by cortical granules clip the sperm protein bindin such that excess sperm neither bind nor fuse (Gilbert 2000; Jaffe and Gould, 1985). Similarly, mammalian cortical granule enzymes modify zona pellucida glycoproteins such that sperm can no longer bind (Aviles et al., 1996; Gilbert 2000). In both cases, molecular modification to gamete recognition proteins results in a block to polyspermy.

Resumption of meiosis

Following an initial prophase arrest (Masui and Clarke, 1979), oocytes/eggs from almost all species undergo a second meiotic arrest before fertilization (Masui, 1985). The exact stage of second meiotic arrest varies depending on the organism (Fig. 3). For example, some bivalves arrest at prophase I while ascidians and some polychaete worms arrest at metaphase I. Fish, frogs and mammals arrest at metaphase II (Whitaker
Figure 3: Stage of meiotic arrest prior to fertilization varies by organism. A) Prefertilization starfish oocytes arrest between G2 (the gap phase following replication) and meiosis when the chromosomes are still fully decondensed. B) In some bivalves, prefertilization oocytes arrest in meiotic prophase with the homologs condensed and paired and the nuclear envelope partially broken down. C) In ascidian oocytes, condensed chromosomes are already lined up on the first metaphase plate at the time of fertilization. D) Fish, frogs and mammals have completed meiosis I and are arrested in metaphase II at the time of fertilization. E) Sea urchin oocytes complete both meiotic divisions prior to fertilization. Adapted from Stricker 1999.
1996; Stricker 1999). At both extremes, starfish oocytes do not start meiotic divisions until fertilization takes place while sea urchin oocytes complete both meiotic divisions prior to fertilization (reviewed in Stricker 1999). These second meiotic arrest points are maintained by repressing cell cycle activators and stabilizing cell cycle inhibitors such as maturation/mitosis promoting factor (MPF) and cytostatic factor (CSF), respectively (Whitaker 1996; Yamashita 1998; Maller et al., 2002). Regardless of the stage of meiotic arrest at fertilization, sperm-egg fusion triggers the completion of the oocyte's meiotic divisions and the formation of the oocyte pronucleus.

Formation and fusion of pronuclei

While the oocyte completes its meiotic divisions, the sperm nucleus changes dramatically in preparation for DNA replication and its union with the oocyte chromatin into a single, merged genome. Like the rest of the spermatozoa, the sperm nucleus is a specialized structure characterized by a mass of tightly condensed, transcriptionally inactive chromatin. Following fertilization, the sperm nucleus morphologically and molecularly metamorphoses into a pronucleus, an organelle analogous to a typical nucleus but with half the chromosome number. Transmission electron microscopy studies revealed morphological changes common to many organisms: 1) dissolution of the sperm nuclear envelope, 2) a gradual decondensation of sperm chromatin, and 3) formation of the pronuclear envelope (reviewed in Longo 1985; Imschenetzky et al., 2003; McLay and Clarke, 2003). Although the molecular changes associated with these morphological alterations employ similar mechanisms in sea urchins and mammals, the actual molecules involved differ. Chromatin of sea urchin sperm is wrapped around sperm specific histones while chromatin of mammals is wrapped around protamines.
To mediate chromatin decondensation, maternally derived histone variants replace sperm specific histones in sea urchin (reviewed in McLay and Clarke, 2003), while maternal histones replace protamines in mammals (reviewed in Immenschenetzky et al., 2003). Additional events, such as polyadenylation and phosphorylation of sperm histones, proteolysis of sperm basic nuclear proteins, and import of kinetochores and transcription factors into the pronucleus, aid in remodeling sperm chromatin (Immenschenetzky et al., 2003; McLay and Clarke, 2003).

The formation of the female pronucleus requires much less remodeling of the oocyte chromatin. Following meiotic completion, chromosome decondensation and DNA synthesis, the diffuse oocyte chromatin becomes surrounded by vesiculated plasma membranes, which ultimately fuse together to form the mature female pronuclear envelope (Longo 1985). In order to create a diploid zygote ready for the ensuing mitotic divisions, male and female pronuclei must associate such that oocyte and sperm chromatin are able to directly interact. Pronuclei associate either by fusion of their nuclear envelopes or intermixing of maternal and paternal chromosomes following pronuclear envelope breakdown (Longo 1985). In either case, the genomes merge prior to metaphase of mitosis when the combined complement of maternally and paternally contributed chromosomes line up in a metaphase plate.

**Paternal contributions and formation of the first mitotic spindle**

Most oocyte/egg meiotic divisions occur in a spindle lacking centrosomes (Albertson and Thomson, 1993), while the first mitotic division of the zygote occurs in a spindle formed from two centrosomes. After pronuclear association, the fertilized egg
forms a mitotic spindle using centrosomes contributed by the sperm (reviewed in Sutovsky and Schatten, 2000). Paternal inheritance of the centrosomes occurs in many organisms including algae (Nagasato et al., 1999), *Drosophila* (Callaini and Riparbelli, 1996; Riparbelli et al., 1997), starfish (Zhang et al., 2004) and mammals (Sutovsky and Schatten, 2000). In rodent species, however, centrosomal inheritance is maternal as evidenced by the presence of centrosomal components in unfertilized eggs (Schatten et al., 1991; Sutovsky and Schatten, 2000) and the casting off of centrosomes during murine spermiogenesis (Manandhar et al., 1998). Polyspermic eggs of the brown alga *Fucus distichus* form a multipolar spindle, two centrosomes for each fertilizing sperm, suggesting that sperm are indeed the main contributors to the mitotic spindle (Nagasato et al., 1999).
**Introduction**

Fertilization is a process by which the formation of a new diploid individual results from the union of two haploid gametes, sperm and egg. As discussed in the fertilization review, the many individual steps in the fertilization process have been well-studied in plants, algae, echinoderms, amphibians and mammals. More specifically, sperm-egg plasma membrane interactions during fertilization have been extensively researched in sea urchin and mouse (Foltz and Lennarz, 1993; Evans, 1999) but less well-studied in invertebrates such as arthropods and nematodes. In this classic view, fertilization involves the fusion of the sperm and egg membranes (Gilbert, 2000). But is this true of nematodes? To date, *Ascaris lumbricoides*, the pig intestinal parasite, and *Dirofilaria immitis*, the dog heartworm, are the only two nematodes in which the actual process of fertilization has been examined. Although both are parasitic and closely related evolutionarily (Blaxter et al., 1998), *A. lumbricoides* and *D. immitis* provide examples of two differing mechanisms of gamete membrane interactions. Early ultrastructural studies of *A. lumbricoides* indicate fusion as a mechanism of sperm-oocyte membrane interaction (Foor, 1968). In this study, electron micrographs show varying stages of fusion in which the sperm and oocyte plasma membranes become continuous with one another. In contrast, a recent study of *D. immitis*, suggests endocytosis as an alternative mechanism of sperm-oocyte membrane interaction (Sacchi et al., 2002). In this study, electron micrographs show sperm inside oocyte cytoplasm as well as being engulfed by pseudopodial-like projections of the oocyte plasma membrane.
Which of these mechanisms is employed by the model nematode *Caenorhabditis elegans*? *C. elegans* is a one millimeter long, non-parasitic nematode that lives in the soil. It is a well-established system for genetic studies and its genome is completely sequenced. In addition, it 1) is easily maintainable in a laboratory setting, 2) is anatomically simple yet complex; it has a complete digestive system, reproductive system and nervous system, 3) produces hermaphrodites and males, and 4) has the ability to create hundreds of progeny from a single adult. Studies to date have taught us a great deal about events prior to and following fertilization (for extensive reviews see Kimble and Ward, 1988 and Wood, 1988). Fertilization occurs within the adult hermaphrodite gonad (Fig. 4) where oocytes are fertilized one at a time in an assembly line fashion. The sperm contributes not only DNA but also centrioles to the acentriolar oocyte (Albertson, 1984). The sperm may also function in activating the developmental program of the newly formed embryo, and one strictly paternal effect lethal mutation, spe-11, has been identified (L'Hernault et al., 1988; Hill et al., 1989; Browing and Strome, 1996).

In contrast, nothing is known about the physical mechanics of *C. elegans* fertilization; that is, how a single sperm actually fertilizes an oocyte in the spermatheca. The goal of this thesis is to provide information about how fertilization is achieved in *C. elegans*; more specifically, how the sperm and oocyte plasma membranes interact during fertilization. Is *C. elegans* fertilization more similar to that of *A. lumbricoides* (fusion) or of *D. immitis* (endocytosis)? Previously, two technical difficulties have precluded this type of study. First, a study of gamete membrane interaction requires observations of
Figure 4: The *Caenorhabditis elegans* adult hermaphrodite gonad. A) A Normaski (DIC) view of a whole worm (top) and a corresponding line diagram (bottom). The line diagram shows the outline of the bilobed gonad. B) A closer view of one-half the adult hermaphrodite gonad. Outlined in orange is the distal portion of the gonad which consists of three regions: 1) a small mitotically dividing region of germ cells, 2) a transition zone in which these germ cells transition from mitosis to meiosis, and 3) a larger region of pachytene nuclei. Outlined in green is a portion of the proximal gonad arm in which meiotic oocytes are growing and maturing in a linear sequence. Outlined in blue is the spermatheca which doubles as a sperm storage compartment and the site of fertilization. Outlined in pink is a portion of the common uterus, which contains fertilized embryos of various stages. Fertilization occurs in an assembly line like fashion; one oocyte per gonad arm is fertilized at a time. Contractions of overlying sheath cells pushes the most mature oocyte into the spermatheca where a single sperm fertilizes the oocyte. The newly fertilized oocyte exits the spermatheca and enters the uterus where it will undergo several cleavage divisions before being laid. Figure generated by Dr. Penny Sadler.
embryos that have just been fertilized. Since fertilization occurs rapidly in *C. elegans* and is in assembly line like fashion, large numbers of early post-fertilization embryos are challenging to obtain. Second, membrane markers and/or markers of fusion or endocytosis have been unavailable.

While an early live-imaging study of *C. elegans* fertilization concluded that oocytes engulf sperm, this study failed to provide experimental data regarding either the details or mechanisms (Ward and Carrel, 1979). The present study employs the unique features of nematode sperm (Fig. 5) in combination with the immunofluorescence analysis of various sperm markers to distinguish between endocytosis and fusion. This fertilization study can be divided into the following elements: 1) optimizing the isolation of early post-fertilization embryos, 2) examining the localization of a sperm contributed sperm centriolar protein, SPD-2, and 3) examining the localization of two distinct sperm membrane markers, SPE-9 and the 1CB4 antigen. While determining the localization of sperm contributed centrioles, SPD-2, in early embryos does not directly address the question of endocytosis or fusion, it does allow us to analyze one of the important paternal contributions of sperm. On the other hand, determining the post-fertilization localization patterns of SPE-9 (a sperm transmembrane protein) and 1CB4 (a membranous organelle, MO, marker) distinguishes between the endocytosis and fusion models of fertilization. If the oocyte engulfs sperm, these two markers should exhibit a staining pattern within the oocyte cytoplasm that resembles their staining pattern in an intact sperm. However, if the sperm and oocyte plasma membranes fuse, these two markers should localize to a patch on the membrane of newly fertilized oocytes (Fig. 6).
Our data strongly suggest that fusion is indeed the mechanisms by which sperm and oocyte membranes interact during fertilization in *C. elegans*. 
Figure 5: Electron micrographs of *C. elegans* sperm. A) Undifferentiated spermatids are symmetrical and spherical in shape. The immature membranous organelles (MOs) (arrows) have not yet fused with the plasma membrane. B) A bilobed, immature MO at high magnification showing the empty head region (arrow), the membranous body (asterisk) and the electron dense collar (arrowheads). C) Mature, motile spermatozoa are asymmetrical with a distinct cell body (CB) which contains organelles such as the nucleus (N), mitochondria (M), and fused MOs (arrows). The pseudopod (P) is free of organelles and is used for motility. D) A fused MO at higher magnification. The electron dense collar (arrowheads) and membranous body (asterisk) are still present in spermatozoa. E) A cartoon representation of spermatozoa, showing both the pseudopod and cell body. The sperm specific transmembrane protein SPE-9 (red ovals) is restricted to the pseudopod while MOs (green) are restricted to the cell body. Centrioles (red oval) lie adjacent to and are closely associated with the highly condensed sperm chromatin (blue). A,C) 12,800 X and B,D) 66,800 X
Figure 6: *C. elegans* gamete membrane interactions hypotheses. If the oocyte engulfs the sperm (endocytosis), we would predict the staining patterns of anti-SPE-9 (red) and 1CB4 (green) to be the same or similar to those in an intact sperm. If the oocyte and sperm plasma membranes fuse (fusion), we would predict the SPE-9 protein to be in the newly fertilized oocyte plasma membrane. We would also predict that 1CB4 would stain both regions that are continuous with the embryonic membrane as well as small vesicles within the oocyte that lie in the vicinity of the sperm chromatin mass.
Materials and Methods

Nematode culturing

All Caenorhabditis elegans strains were cultured on MYOB plates (Church et al., 1995) spotted with the OP50 strain of E. coli. Wild-type (N2 strain) adult hermaphrodites were raised at 16°C or 20°C essentially as described by Brenner (1974). Hermaphrodites with a mutation in a germline specific gene (fog-2 (q71)) only produce oocytes and are essentially female while males are unaffected (Schedl and Kimble, 1988). These fog-2 (q71) females were maintained at 20°C with fog-2 males. Hermaphrodites and males with mutations in the non-conditional spe-9 (eb19) allele were raised at 16°C (Singson et al., 1998).

Dissection, freeze cracking and fixation

On a poly-lysine coated slide, adult hermaphrodites were placed in a 10 μl drop of Edgar’s buffer (60 mM NaCl, 32 mM KCl, 3 mM Na2HPO4, 2 mM MgCl2, 2 mM CaCl2, 5 mM HEPES, 0.2% glucose, pH 7.2; Boyd et al., 1996). These hermaphrodites were then cut at the vulva with a 27.5 gauge needle to release embryos contained within the uterus. Following dissection, a 24x40 mm coverslip was gently lowered crosswise over the sample. These slides were then carefully placed in liquid nitrogen and then freeze cracked by popping off the coverslip. Freeze cracked slides were allowed to fix overnight in -20°C methanol.
**Immunocytochemistry**

A basic immunofluorescence protocol can be found in Miller and Shakes (1995). In coplin jars, fixed samples were washed three times, ten minutes each, in phosphate buffered saline (PBS). For anti-SPE-9, samples were treated for five minutes with 0.2% Triton-X-100 (Fischer Scientific) and then washed in PBS. All samples were blocked in PBS containing 0.04% sodium azide, 0.1% Tween 20 and 0.5% BSA. All antibodies, primary and secondary, were diluted in this blocking solution. Following blocking, slides were incubated with primary antibody for 1-1.5 hours at room temperature in a humid chamber by placing a 30μl drop of antibody directly onto the sample. The polyclonal anti-SPD-2 antibody (generously provided by the O'Connell Lab) was used at a 1:15,000 dilution. The polyclonal anti-SPE-9 antibody (generously provided by the Singson Lab) was used at a 1:600 dilution. The monoclonal 1CB4 antibody (generously provided by the L'Hemault Lab) was used at a 1:50 dilution. After primary antibody incubation, slides were washed three times, for five minutes each, in coplin jars containing PBS. As follows, all samples were incubated at room temperature in a 30μl drop of the appropriate secondary antibody: 1) SPD-2: a 1:800-1:1000 dilution of Cy3 conjugated affinity purified goat anti-rabbit (Jackson Immunoresearch), 2) SPE-9: a 1:100 dilution of TRITC or FITC conjugated affinity purified goat anti-rabbit (Jackson Immunoresearch), 3) 1CB4: a 1:100 dilution of Alexa 488 conjugated goat anti-mouse (Molecular Probes). Slides were mounted in Gel/Mount (Biomedia Corp.) containing DAPI (1:1000) after being briefly washed (dipped) in PBS. Slides were then scored using Differential Interference Contrast (Nomarski) optics or under epifluorescence on an Olympus BX60 microscope attached to a black and white Cooke CCD camera.
Electron Microscopy

The following protocol was adapted from Drs. Joe Scott and Penny Sadler. In a watchglass, adult hermaphrodites were dissected with a 27.5 gauge needle in 200μl Edgar’s Buffer. An equal volume of a 5% paraformaldehyde, 4% glutaraldehyde fixative was added to the watchglass for a final concentration of 2.5% paraformaldehyde, 2% glutaraldehyde. Samples were allowed to fix at room temperature for approximately 30 minutes, then transferred to 4°C for 1-2 hours. Following fixation, the samples in watchglasses were washed by carefully pipetting off fixative and replacing with 1ml of 0.1M cacodylate buffer. This step was repeated twice. Under a dissecting microscope, samples were trimmed with a dissecting needle to either separate carcasses from gonads or to free embryos from gonads. Samples were then transferred onto a 2% agarose pad by using a micropipette. On these agar pads, embryos or gonads were gathered by using an eyelash tool. Samples were then gently covered with 1% low melt agarose and trimmed with a razor blade into 1mm² blocks. The corners of these blocks were marked with black India ink to aid in visualization and orientation in the following steps. The agar blocks were then transferred to EM vials containing 1ml 0.1M cacodylate buffer and allowed to rinse for at least 15 minutes at room temperature or overnight at 4°C. After post-fixation in 1% OsO₄ for one hour at 4°C, the samples were rinsed with dH₂O three times, 15 minutes each, by carefully pipetting off post-fix or water and replacing it with 1ml dH₂O. Following a graded series of room temperature ethanol dehydration steps (30%, 50%, 70%, 70% + uranyl acetate 1-4 hours at 4°C, 90%), 15-30 minutes each, samples were further dehydrated in 100% acetone. Continuing in small glass vials, sample blocks were infiltrated in a graded series of acetone-resin (Embed 812, Electron...
Microscopy Sciences) mixtures (2:1, 1:1, and 1:2) for 15-120 minutes each. Resin infiltrated sample blocks were then embedded in foil pan molds containing 100% resin. Blocks were arranged appropriately with a wooden applicator stick. Embedded samples were incubated overnight at 60-70°C. Following sectioning by the EM technician, thin sections were stained 1 minute with lead citrate and mounted on formvar coated one-hole grids. Samples were analyzed using a Zeiss 109 Transmission Electron Microscope and images were obtained of T-Max 100 Kodak film.
**Results**

*Cooled temperatures provide a reliable source of meiotic stage embryos*

One of the many advantages of using *C. elegans* as a model organism is the ability to regulate developmental timing by varying culture temperature. Lower temperatures correlate with slower growth while higher temperatures correlate with faster growth. For example, at 16°C, it takes 74.5 hours for L1 larvae to develop into young adults, or complete all larval molts, whereas it takes approximately half this time at 25°C (Wood et al., 1980). Fertilization in *C. elegans* hermaphrodites occurs in an assembly line like fashion (see introduction), thus the uterus of each individual hermaphrodite contains embryos at various developmental stages rather than an array of same-age embryos. In the absence of developing successful *C. elegans in vitro* fertilization techniques, it is impossible to alter the “one-at-a-time” nature of *C. elegans* fertilization. However, given that these fertilization studies require the analysis of a large number of early, post-fertilization, meiotic stage embryos, we sought to determine whether or not cooler growth temperatures could be used to increase the average number of meiotic-stage embryos per uterus in populations of wild type *C. elegans* hermaphrodites.

To test if cooler temperatures generate wild-type hermaphrodites with more meiotic embryos, we shifted 10 L4 hermaphrodites that had been raised at 16°C to 12°C, 10°C, or 8°C respectively and then scored their uteri for the presence of meiotic-stage 1-cell embryos using Nomarski (DIC) optics. Control worms were maintained continuously at 16°C but analyzed at the same age. At 16°C, hermaphrodites typically had 0-1 meiotic
embryos per uterus, and similar numbers were observed in the 10°C hermaphrodites (Table 1). In contrast, the 12 and 8°C hermaphrodites typically had 1-2 meiotic embryos per uterus (Table 1). However, upon further examination of the morphology of 8°C embryos, we observed various abnormalities including fully mature oocytes that retained prominent nucleoli and numerous 3-cell embryos. Because the 12°C hermaphrodites had elevated numbers of meiotic embryos but not obvious abnormalities, we chose to perform our fertilization studies on adult hermaphrodites cultured at 12°C.

SPD-2 closely associates with sperm chromatin in early post-fertilization embryos

The first mitotic division of a C. elegans zygote occurs on a centriolar spindle; each spindle pole contains a pair of centrioles in a centrosome that nucleates the spindle microtubules (Albertson 1984). In newly fertilized oocytes, however, meiotic divisions of the oocyte chromosomes occur on an acentriolar spindle (Albertson and Thomson, 1993). The pair of centrioles that ultimately set up the first mitotic spindle are contributed by the sperm upon fertilization (Albertson, 1984; Albertson and Thomson, 1993). While defective centrioles do not prevent C. elegans sperm from fertilizing oocytes, the resulting zygote has abnormal anterior-posterior polarity as well as impaired mitotic spindle formation (O’Connell et al., 2000). On the other hand, zygotes fertilized by sperm that lack DNA yet retain centrioles accurately partition anterior-posterior markers and form properly oriented spindles (Sadler and Shakes, 2000); further demonstrating the importance of sperm contributed centrioles.

To further analyze the contribution of this key sperm contributed factor, we have used the anti-SPD-2 antibody (O’Connell et al., 2000) to determine the localization of
<table>
<thead>
<tr>
<th>Temperature</th>
<th># meiotic embryos/uterus (Av +/- St. dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16°C</td>
<td>0.7 +/- 0.7</td>
</tr>
<tr>
<td>12°C</td>
<td>1.4 +/- 0.8</td>
</tr>
<tr>
<td>10°C</td>
<td>0.8 +/- 0.7</td>
</tr>
<tr>
<td>8°C</td>
<td>1.8 +/- 0.5</td>
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Table 1. Abundance of meiotic stage 1-cell embryos relative to growth temperature.
sperm centriolar components in early post-fertilization embryos. In *C. elegans* spermatids (Fig. 7A) and spermatozoa (data not shown), anti-SPD-2 antibody recognizes a single spot immediately adjacent to the sperm chromatin mass. At this stage, the quiescent sperm centrosome is not actively nucleating microtubules (Ward et al., 1981; Albertson 1984; O’Connell et al., 2000). Similar to staining in sperm, meiotic stage embryos show anti-SPD-2 associated but not colocalized with the condensed chromatin mass (Fig. 8, meta I, ana I, meta II). Note that throughout meiosis, anti-SPD-2 remains closely associated with the highly condensed sperm chromatin. In contrast, neither oocyte cytoplasm nor dividing oocyte chromatin is associated with anti-SPD-2 staining, further demonstrating the absence of centriolar components in the oocyte meiotic spindle. Following meiosis, the sperm centrioles duplicate and two spots of anti-SPD-2 can be observed adjacent to the sperm pronucleus (Fig. 8, pro). Other studies have shown that this post-meiotic transition is associated with microtubule nucleation from previously quiescent centrosomes (reviewed in O’Connell, 2000). In interphase 2-cell embryos (Fig. 8, 2-cell), two spots of SPD-2 associate with each nucleus. Similar to meiotic embryos, post-meiotic embryos show centriolar components closely associated with the pronucleus or nucleus of each cell (Fig. 8, pro and 2-cell). SPD-2 is not found in other regions of the embryo such as the cell cortex or throughout the cytoplasm; the diffuse red staining throughout the embryo is background staining. Ultimately, SPD-2, and other centriolar proteins, will make up the centrosome of each spindle pole (for each nucleus) in mitotically dividing embryos.
Figure 7: The antibodies SPD-2, anti-SPE-9, and 1CB4 recognize sperm components. A) DAPI stains DNA and SPD-2 stains centriolar components in spermatids (Tids) and spermatozoa (data not shown). The merge panel shows both DNA (blue) and SPD-2 (red). SPD-2 associates closely, but does not colocalize, with the condensed sperm chromatin mass. B) In spermatids (Tids), 1CB4 stains the vesicular organelles termed MOs, which abut the plasma membrane. In spermatozoa (Zoa), the MOs form permanent fusion pores with the cell membrane and are restricted to the cell body. 1CB4 staining patterns reflect the localization and morphology of MOs. In spermatids, SPE-9 is dispersed over the entire cell surface. In spermatozoa, SPE-9 is restricted to the pseudopod. The merge picture shows the differential localization of 1CB4 (green) and SPE-9 (red) in both spermatids and spermatozoa (see text). Enlargements, 4X
Figure 8: SPD-2 associates with sperm chromatin in early 1-cell embryos. DNA = DAPI, metaphase I = meta I, anaphase I = ana I, metaphase II = meta II, pronuclear stage = pro. Oocyte chromosome morphology, to the left of each panel, is used to assess sub-stages of the meiotic divisions. As in sperm, SPD-2 (arrowhead) closely associates, but does not colocalize, with sperm chromatin (arrow) in metaphase I, anaphase I, metaphase II, and pronuclear staged embryos. The merge picture, with enlargements, shows this association of SPD-2 (red) and sperm chromatin (blue). At the pronuclear stage, two spots of SPD-2 can be observed. At the 2-cell stage, each nucleus is associated with two SPD-2 spots.
A pseudopod localized transmembrane protein serves as a marker for sperm-oocyte membrane interactions

Two temperature sensitive alleles of the spe-9 gene were first discovered in a screen for spermatogenesis defective mutants (L’Hernault et al., 1988). Despite having ovulation rates comparable to wild-type, spe-9 mutant hermaphrodites exhibit self-sterility; that is, they are unable to create self progeny but generate viable offspring upon mating with wild-type males (L’Hernault et al., 1988; Singson et al., 1998). Because oocytes are phenotypically and functionally wild-type, this suggests that the spe-9 gene is only required for proper sperm function. Unlike many other spermatogenesis defective mutants such as fer-1, fer-2, and spe-4 (reviewed in L’Hernault, 1997), sperm from spe-9 males are not only morphologically normal but they activate (form pseudopods), crawl to the spermatheca, and make contact with ovulating oocytes (Singson et al., 1998). The spe-9 gene encodes a single pass transmembrane protein with several EGF (epidermal growth factor) repeats and structural homology to Notch/Lin-12/GLP-1 ligands in Drosophila, C. elegans, and mammals (Singson et al., 1998). Because of its fertilization defect and similarity to known adhesion and signaling proteins, the SPE-9 protein is hypothesized to function in C. elegans sperm-oocyte binding and/or fusion (Singson et al., 1998). As a transmembrane protein known to be specific for the spermatozoa pseudopod, SPE-9 provides an excellent marker to distinguish between fusion and endocytotic models of C. elegans fertilization.

To test whether the oocyte engulfs a single sperm or whether the two gamete membranes fuse, we have determined the localization of SPE-9 in sperm and early post-fertilization embryos with the polyclonal anti-SPE-9 antibody. In agreement with previous studies (Zannoni et al., 2003), we show that SPE-9 localizes differentially in
immature spermatids and mature spermatozoa. In spermatids, SPE-9 disperses evenly over the cell surface while spermatozoa show SPE-9 concentrated in the pseudopod (Fig. 7B). We were interested in what happened to this protein once fertilization had taken place. Does SPE-9 remain as a concentrated mass in the embryonic cytoplasm or is it found in the embryonic plasma membrane (see Fig. 6)?

All 1-cell embryos, from meiosis I to the first mitotic division, show discrete bright foci of anti-SPE-9 staining over the non-specific background staining (Fig. 9, approximately 700 meiotic embryos examined). Surprisingly, there was no graded distribution or higher concentration of SPE-9 in the embryonic posterior. To show that sperm are indeed introducing SPE-9 at the time of fertilization instead of oocytes containing endogenous SPE-9 protein or synthesizing SPE-9 de novo, we performed the same SPE-9 antibody experiments on both wild-type and spe-9 mutant hermaphrodite gonads. In both cases, the unfertilized oocytes lacked detectable anti-SPE-9 staining (Fig. 10). In contrast, the same wild-type gonads contain sperm that stain brightly with anti-SPE-9, showing that this lack of staining was not a consequence of faulty primary antibody or errors in procedure. Unfertilized oocyte controls, like those mentioned above, rule out the possibility that the anti-SPE-9 we see in early embryos is a result of pre-existing protein.
Figure 9: Early embryos show a punctate pattern of SPE-9. In the DAPI column, oocyte chromatin is to the left of each panel and sperm chromatin is to the right. At all stages, meiosis I through the first cell cycle, SPE-9 distributes over the entire embryo surface. The merge picture shows DNA (blue) and SPE-9 (red). Prometaphase = prometa, anaphase I = ana I, metaphase II = meta II, anaphase II = ana II, and first mitotic division = Mito I.
**Figure 10: Oocytes do not endogenously contain SPE-9 protein.** Unfertilized oocytes from wild-type adult hermaphrodite worms do not stain with the anti-SPE-9 antibody. Sperm and fertilized embryos from the same hermaphrodites do stain with the anti-SPE-9 antibody.
wt oocytes

DAPI

SPE-9
Perhaps SPE-9 only remains in the embryonic posterior shortly after fertilization and then immediately diffuses throughout the plasma membrane. Following entry into the spermatheca, it takes an oocyte/embryo approximately 17 minutes to complete meiosis I and 26 minutes to complete meiosis II (McCarter et al., 1999). Using this timeline in combination with the rough calculation that 0.5-1% of all meiotic embryos are actually in prometaphase (see Table 2), we estimate that embryos reach metaphase I two minutes post-fertilization. Even at these earliest stages (prometaphase), less than two minutes after fertilization, SPE-9 foci are distributed over the entire embryo surface (Fig. 9). According to our predictions (Fig. 6), these results suggest that *C. elegans* sperm and oocyte plasma membranes fuse upon fertilization. The highly concentrated SPE-9 protein in the sperm pseudopodial membrane appears to be dispersing rapidly in the oocyte membrane rather than remaining in the same pattern as an intact sperm.

*MOs localize as a membrane patch at the site of sperm entry in newly fertilized oocytes*

Membranous organelles (MOs) are specialized organelles in the sperm (see introduction) believed to function in the partitioning of cellular components during spermatogenesis and spermiogenesis. Several mutations that affect the differentiation of MOs have been well characterized and all result in *spe* (spermatogenesis defective) phenotypes (reviewed in L'Hernault, 1997). Some mutations, such as *spe-4* and *spe-5*, cause early spermatogenesis defects in which defective MO formation is accompanied by a terminal spermatocyte arrest; spermatids never form (L'Hernault and Arduengo, 1992; Machaca and L'Hernault, 1997; Arduengo et al., 1998). Others, such as *fer-1*, produce infertile spermatozoa with short pseudopods and defective MO fusion (Ward et al., 1981; Achanzar and Ward, 1997). While the study of MOs has been directed towards
<table>
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<th>Stage of Embryo</th>
<th>Number of Embryos/Experiment (SPE-9 or 1CB4)</th>
<th>Percent of All Meiotic Embryos</th>
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<tr>
<td>Prometaphase</td>
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<td>.9</td>
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<td>Metaphase I</td>
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<td>33</td>
</tr>
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<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>113</td>
<td>99.9</td>
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Table 2. **Number of embryos in each meiotic stage.** A total of approximately 700 meiotic embryos were examined per antibody (SPE-9 or 1CB4). Approximately 100-200 embryos were examined per experiment for a total of 5 experiments. This table is one representative experiment.
understanding the relationship between spermiogenesis and fertilization (L'Hernault 1997), we used these caveolae-like structures (Alberts et al., 1994) as markers to determine the mechanism of sperm-oocyte membrane interaction during fertilization in *C. elegans*.

To determine the localization pattern of MOs and their components in sperm and early post-fertilization embryos, we used the monoclonal antibody 1CB4 (Okamoto and Thomson 1985). Within spherical spermatids, 1CB4 is found in unfused MOs which lie adjacent to all regions of the cell cortex (Figs. 7B and 5A). In polarized spermatozoa, however, MOs in their fused conformation are restricted specifically to the cell body rather than the pseudopod (Figs. 7B and 5C). Comparison of the merged images reveals that 1CB4 and anti-SPE-9 localize to distinct surfaces during the transformation of sessile, spherical spermatids into bipolar, crawling spermatozoa.

In the parasitic nematode *Ascaris lumbricoides*, the membranous region of the MOs remain intact in newly fertilized embryos following fusion of the two gamete membranes (Foor 1968). Similarly, MO components persist in all *C. elegans* embryos during the first meiotic division up to approximately 17 minutes after fertilization. 1CB4, the MO marker, localizes adjacent to the sperm chromatin mass in both small internal vesicles and as a cortical membrane patch (Fig. 11A). Enlargements of the anterior cortex (AC) and posterior cortex (PC) show that cortical membrane staining is restricted to the embryonic posterior, or the site of sperm entry. As predicted, the pattern of MOs in early embryos is consistent with the fusion mechanism of gamete membrane interaction.
**Figure 11:** The MO marker, 1CB4, localizes as a membrane patch at the site of sperm entry. A) Oocyte chromatin (arrow) marks the future anterior of embryos while sperm chromatin (arrowhead) marks the future posterior. 1CB4 stains both a membrane patch as well as small cytoplasmic vesicles in the region of the sperm chromatin. The merge picture shows both 1CB4 (green) and DNA (blue). Enlargements of the anterior cortex (AC) and posterior cortex (PC) show that 1CB4 membrane staining is restricted to the embryonic posterior. B) The pre-fertilization oocytes of fog-2 “females” and wild-type (wt) hermaphrodites do not stain with 1CB4. Since fog-2 females lack sperm there is no staining with DAPI or 1CB4. However, in wt gonads, spermatheca stain with both DAPI and 1CB4 (arrows). Embryos not exposed to primary antibody result in no 1CB4 staining (meta I wt embryo).
To rule out the possibility that MO components are endogenously present in unfertilized oocytes, we examined the proximal gonads of wild-type hermaphrodites and fog-2 (q71) females, which lack sperm and therefore should not stain with 1CB4. In both cases, 1CB4 staining is undetectable within the unfertilized oocytes (Fig. 1B) showing that sperm do indeed introduce MO components at the time of fertilization rather than the oocyte having its own supply of MOs and/or MO components. In contrast, the same wild-type gonads contain sperm that stain with 1CB4, showing that this lack of staining was not a consequence of faulty primary antibody or errors in procedure. To show that 1CB4 staining is not a product of nonspecific secondary antibody staining (see Materials and Methods), we did not expose a subset of embryo samples to primary antibody. Without the primary antibody, secondary antibodies should not stain. In these embryos, there is no 1CB4 staining (Fig. 1B) which indicates the specificity of the secondary antibody to the MO marker, 1CB4.

Many proteins specifically stabilize and/or localize to various regions of the 1-cell embryo and/or to specific blastomeres thereafter: P-granules (Strome and Wood, 1982, 1983), PARs (Kemphues, 2000), and other asymmetrically localized proteins. To analyze differential distribution and stability of MOs, we examined their staining pattern in the stages following meiosis I. In post-meiosis I embryos, the cortical 1CB4 staining is no longer detectable (Fig. 12). Unlike the rapid dispersal of SPE-9, 1CB4-staining vesicles remain concentrated within the posterior cytoplasm of embryos in their second meiotic division (Fig. 12, meta II and ana II). Following completion of both oocyte meiotic divisions, 1CB4 staining vesicles distribute uniformly throughout the 1-cell embryo, in pronuclear and mitotic stage embryos (Fig 12, pro and Mito I). As embryonic
development proceeds, vesicular 1CB4 staining remains detectable in all blastomeres of 2- and 4-cell embryos but ultimately dissipates as development proceeds (Fig. 13).
Figure 12: **1CB4 staining is dynamic in early embryos.** Cortical 1CB4 staining is no longer detectable in stages following meiosis I (meta II, ana II, pro, and Mito I). 1CB4 staining vesicles remain concentrated in the posterior during meiosis II (meta II and ana II). Following meiosis (pro and Mito I), 1CB4 staining vesicles distribute uniformly. The merge column shows 1CB4 (green) and DNA (blue) together. Metaphase II = meta II, anaphase II = ana II, pronuclear stage = pro, and first mitotic division = Mito I.
Figure 13: 1CB4 staining in early and late cleavage stage embryos. Vesicular 1CB4 staining remains detectable in all blastomeres of 2- and 4-cell embryos. In older embryos (multi-cell), 1CB4 staining vesicles are no longer detectable. Merge, 1CB4 = green and DAPI/DNA = blue
Discussion

Summary of the results

Analyzing the localization of sperm components in early, post-fertilization embryos has allowed us to determine the mechanism of sperm-oocyte membrane interaction during fertilization in *C. elegans*. Previously, such studies were limited by the inability to obtain an ample sample size of pre-meiotic embryos and the unavailability of gamete membrane markers. In this study, we have solved the first limitation by culturing wild-type hermaphrodites at 12°C; well below the standard culturing temperature range 16°C-20°C. At this lower temperature, adult hermaphrodites consistently contain more meiotic embryos than their 16°C raised counterparts. These meiotic embryos were then used in immunofluorescence studies to examine the localization of anti-SPD-2 (a centriolar marker), anti-SPE-9 (a sperm transmembrane protein), and 1CB4 (a MO marker). While the analysis of centriolar components does not address gamete membrane interactions, it does provide information on how this essential sperm contributed factor behaves in newly fertilized embryos. Our analysis of anti-SPD-2 in post-fertilization embryos shows that centriolar components 1) closely associate with DNA in meiotic embryos or with nuclei in post-meiotic embryos and 2) only duplicate following completion of the oocyte meiotic divisions. The staining pattern of anti-SPE-9 and 1CB4 (MOs) addresses the question of gamete membrane interaction more directly by examining exactly where sperm membrane components end up in the newly fertilized embryo. The punctate pattern of SPE-9 over the entire embryo surface and the cortical
membrane patch of MO components supports the fusion hypothesis while refuting the endocytosis hypothesis.

_Nematodes employ distinct mechanisms of sperm-oocyte membrane interactions during fertilization_

Ultrastructural studies of fertilization in _Ascaris lumbricoides_ (Poor, 1968) and _Dirofilaria immitis_ (Sacchi et al., 2002) in combination with our immunofluorescence study of _C. elegans_ fertilization demonstrate the variability of gamete membrane interactions within the Phylum Nematoda. Surprisingly, _A. lumbricoides_ and _D. immitis_, both parasites, are closely related molecularly (Blaxter et al., 1998) but employ differing mechanisms of gamete membrane interactions. In contrast, _C. elegans_ is molecularly distant from _A. lumbricoides_ (Blaxter et al., 1998), but both employ fusion as a mechanism of sperm-oocyte membrane interaction. Why do these differences exist? Perhaps the patterning of the hermaphrodite or female gonad predicts the mechanism by which sperm-oocyte membranes interact in nematodes. Unlike _C. elegans_ hermaphrodites, _D. immitis_ females lack a true, specialized sperm storage compartment (Delves et al., 1986). Instead, the distal portion of the uterus, termed the seminal receptacle, houses mature sperm (Delves et al., 1986). A comparison of gonad anatomy and point of fertilization shows that _C. elegans_ oocytes must squeeze one-by-one through the physically constrained space of the spermatheca to be fertilized. In contrast, multiple oocytes are surrounded by sperm in the much larger seminal receptacle of _D. immitis_ (Delves et al., 1986; Sacchi et al., 2002). In that environment, perhaps _D. immitis_ oocytes must play a more active role in insuring their own fertilization. One way that an oocyte could play a more active role would be to physically engulf the sperm, in other words, use an endocytotic mechanism of gamete membrane interaction. Conversely, within the
C. elegans spermatheca, the sperm may actively compete to fertilize each oocyte as it passes through. Whichever sperm can bind and fuse first, successfully fertilizes the incoming oocyte. While differences in gonad anatomy may not completely explain the differences in gamete membrane interaction, they do provide some explanation. The possibility that fertilization mechanism (endocytosis vs. fusion) is dependent on gonad anatomy could be clarified or strengthened by ultrastructural evidence from a broader range of nematodes. Furthermore, these TEM studies may provide evidence for alternative mechanisms of gamete membrane interaction.

Membrane fluidity functions in the localization of sperm components in newly fertilized embryos

Sperm and oocytes, like other typical cells, are enclosed by a dynamically fluid phospholipid bilayer, also known as the plasma membrane. The fluidity of this membrane, dependent upon lipid composition and temperature, allows cells to move and change shape (Lodish et al., 2000), two incredibly important actions for sperm and oocytes. The plasma membrane of C. elegans oocytes appears to be highly fluid as evidenced by the rapid disappearance of EGG-1, an oocyte plasma membrane protein, between fertilization and the end of meiosis II (A. Singson unpublished observations). As a Notch-like protein (Singson et al., 1998), SPE-9 is likely to function in cell signaling and/or adhesion (Singson et al., 1998). But does it just function in gamete interactions? If SPE-9 has a post-fertilization function, its rapid dispersion throughout the entire embryo would predict that it plays a global role. Because it has similarities to signaling proteins, perhaps it signals events of egg activation such as resumption of the cell cycle, secretion of the eggshell, or polyspermy prevention.
An interesting future study would analyze the distribution of both EGG-1 and SPE-9 in early embryos to 1) compare their dispersion rates and 2) to determine if the two colocalize. Perhaps the fluidity of the oocyte plasma membrane, as indicated by EGG-1, accounts for the rapid dispersal of SPE-9. Furthermore, to test if SPE-9 does trigger egg activation, the wild-type copy of the *spe-9* gene could be ectopically expressed in unfertilized oocytes. If this is indeed the function of SPE-9, these oocytes should either prematurely 1) re-enter the cell cycle, 2) secrete an eggshell or 3) activate the block to polyspermy; all of which would inhibit fertilization. Notably, nothing is currently known about how polyspermy is blocked in *C. elegans*.

In contrast to SPE-9’s rapid dispersion, 1CB4 staining revealed that MO components are stable in post-fertilization embryos. Although they eventually diffuse throughout the embryo, glycoproteins of the MOs (containing the 1CB4 antigen) persist in the embryonic posterior as a cortical membrane patch and as vesicular cytoplasmic staining. If the MO components have any post-fertilization role, their persistence could allow them to function along with sperm centrioles as a polarity cue in the 1-cell embryo. However, any such role would be limited to the initial establishment of anterior-posterior polarity as MO components eventually diffuse throughout the embryo without localizing to specific blastomeres. The localization of other MO markers in early embryos, such as *spe-4* and *fer-1*, could determine whether all or only some MO proteins or components, such as the electron dense collar, exhibit this slow dispersion pattern. Since MOs are believed to be in highly stable lipid rafts, do proteins within the MOs behave in a similar fashion?
Future studies

In future studies, we would like to corroborate our finding that fusion is the mechanism by which sperm-oocyte plasma membranes interact in *C. elegans*. The most definitive evidence for the fusion hypothesis will be ultrastructural proof as seen with the TEM study of *Ascaris lumbricoides* fertilization (Foer, 1968). In addition to the studies discussed in this paper, we would like to analyze the embryonic localization of SPE-38 and SPE-42, two sperm specific proteins not only known to have similar phenotypes to *spe-9* but also predicted to have similar gamete binding/fusion functions. Do they colocalize in sperm? If so, is this colocalization maintained in newly fertilized embryos? However, if SPE-9, SPE-38, and SPE-42 do not associate in the embryo, what are their relative dispersion rates? Does one protein diffuse faster than the others? Do SPE-38 and SPE-42 polarize in the embryo and if so, what function might they have? Another way to elucidate the mechanism of gamete membrane interaction would be to mark the sperm membrane with fluorescent dyes and examine where sperm membrane components end up in the fertilized embryo. To extend our analysis beyond the current study, we would like to examine the localization of non-membrane sperm components in the early embryo both ultrastructurally and immunofluorescently. What happens to pseudopod components such as MSP? Perhaps sperm components, organelles or proteins, function in post-fertilization events as well as in fertilization itself. These types of studies may be the foundation for discovering paternal contributions during development in *C. elegans* embryogenesis.
References


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**Vita**

Alissa Gale Richmond grew up in Newport News, VA where she attended Warwick High School. Following completion of the International Baccalaureate Program there in 1998, she attended the University of Virginia where she earned a B.A in Biology. In the Fall of 2002, she entered the master’s degree program in Biology at the College of William and Mary. In the lab of Dr. Diane Shakes, she learned about the biology of *Caenorhabditis elegans* and honed her skills as a research scientist. Alissa currently resides in Newport News, VA and she is currently employed in the Shakes’ Lab as a Research Assistant.