Polyspermy prevention: facts and artifacts?

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Abstract The purpose of this review is to open a debate as to whether or not oocytes actively repel supernumerary sperm or in nature final sperm : oocyte ratios are so low that polyspermy preventing mechanisms are not necessary. Before encountering the oocyte, spermatozoa need to be primed, either by environmental factors as in animals exhibiting external fertilization, or by factors from the female reproductive tract, as in mammals. The spermatozoon must then recognize and interact with the outer layers of the oocyte and progression of the fertilizing spermatozoon through these layers is further controlled and modulated by a precise sequence of signals in situ. Removal of these outer coats may not inhibit fertilization, however does interfere with the dynamics of sperm-oocyte interaction. We propose that monospermy in mammals and sea urchins, under natural conditions, is ensured by the controlled and gradual encounter of a minimum number of spermatozoa with the oocyte and that fine tuning is ensured by the structural and molecular organization of the oocyte and its surrounding coats. We suggest that laboratory experiments using oocytes deprived of their investments and exposed to unnaturally high concentrations of spermatozoa are artifactual and argue that the conclusions leading to the hypothesis of a fast electrical block to polyspermy are unfounded. Under laboratory conditions the majority of spermatozoa, although motile and capable of attaching to the oocyte surface, are either physiologically incompetent or attach to areas of the oocyte surface that do not support entry.

Capsule The review opens a debate as to whether oocytes actively repel supernumerary spermatozoa or in nature sperm : oocyte ratios are so low that polyspermy prevention is not necessary.

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Introduction

Most of our information on the process of fertilization has come from laboratory experiments on two deuterostomes, the echinoderms and the mammals. Despite the difference in the site of fertilization, the gametes from these animals are remarkably similar, easy to collect from the adults and amenable to laboratory conditions and manipulations [28]. For example, it is common practice to bypass the initial gamete interactions by removing the extracellular coats of the oocyte or microinjecting the spermatozoon directly into the oocyte cytoplasm. In eukaryotes, if more than one sperm nucleus interacts with the female nucleus abnormal cleavage will occur and death of the embryo ensues. Laboratory images of oocytes with many spermatozoa attached to their surface have led to the idea that following interaction with the successful spermatozoon mechanisms located in the oocyte membrane are activated that repel supernumerary spermatozoa (see reviews [14, 39, 45, 75, 81]). The purpose of this paper is to review what is known about the kinetics of sperm oocyte interaction in these two animal groups under natural conditions and to summarize those activation events reported to be polyspermy preventing mechanisms. Since, the dynamics of fertilization in these two deuterostome groups are significantly different and more is known about sea urchins than mammals, it has not always been possible to make a direct comparison at all stages of gamete interaction.

Internal fertilization in mammals

Passage of spermatozoa through the female tracts

In mammals, the sperm : oocyte ratio at origin can be as high as $10^9:1$ [113]. Despite this, behavioural adaptations are required to ensure fertilization, such as the deposition of

sperm in the female tract and the synchrony of mating. Regardless of whether spermatozoa are deposited in the vagina (e.g., humans) or directly into the uterus (e.g., mice), the vast majority are rapidly eliminated from the tract [94, 113]. Only a minute fraction successfully migrate to the site of fertilization, the ampulla or ampulla-isthmic junction and there is evidence that the female tract tends to prevent morphologically abnormal sperm from reaching the ampullae [77, 94].

In mice, the utero-tubal junction is the major barrier for sperm ascent. Spermatozoa are then sequestered in the lower part of the oviductal isthmus until ovulation begins when they are progressively released. Sperm ascent and oocyte descent to the ampulla occur synchronously, avoiding premature aging in the oocyte, which would lead to abnormal embryonic development. In humans, the first barrier is the highly folded mucus filled cervix, which retains sperm for later migration to the upper tract. The release of spermatozoa from the cervix may continue for days [47]. Sperm ascend through the uterus primarily by the contractile activity of the uterine wall, and also here sperm appear to be sequestered in the lower isthmus until ovulation (see [52, 53] for domestic animals). In the pig, spermatozoa stored in the isthmus are in close contact with the epithelium [37]. The mechanism by which isthmus bound sperm are selectively released is not clear; however it seems to be associated with changes in the sperm head plasma membrane and capacitation (see also [44]). Migration from the isthmus to the ampullae appears to be due to sperm motility and contractile activity of the oviduct [53]. In mammals, as the sperm passes through the female tract a series of progressive changes to its physiology occur, termed capacitation, which are a pre-requisite for fertilization. This process is essentially the acquisition of fertilizing potential of the spermatozoon and involves cell membrane alterations, changes in protein phosphorylation and modulation to intracellular Ca^{2+} .

In studies *in situ*, 700 spermatozoa were found in sheep ampullae [8] and five in man [34], while in rodents, sperm : oocyte ratios at the site of fertilization are usually unity or below [54, 92, 113].

In conclusion, the female reproductive tract in mammals modulates and controls the gradual encounter of spermatozoa with oocytes often leading to unity.

Sperm-oocyte interaction in mammals

After reaching the oocyte in the ampullae, the spermatozoa must traverse and interact with the outer oocyte investment, the cumulus oophorus and then bind to and penetrate the zona pellucida before finally fusing with the plasma membrane. Although the extracellular coats may be

removed in some animals without inhibiting fertilization and these initial gamete interactions may be bypassed by micro-injecting the spermatozoon directly into the oocyte, these events cannot and are not bypassed in nature. The zona pellucida composed of several glycoproteins (ZP) that differ in number between species [4, 38, 42, 57, 79, 106] serves to modulate sperm binding and to protect the embryo during early development. Whether or not mammalian spermatozoa respond to chemotactic stimuli is very much open to debate [110]; however there is data to suggest that an odorant receptor gene expressed in the testis may be involved in sperm chemotaxis in humans [90, 105]. Progression through the outer layers of the oocyte depends on successive molecular interactions that change sperm physiology step-by step promoting fertilization competence. Although there is much information in the literature on the genetics and biochemistry of the outer layers, in particular the zona pellucida [4, 38, 42, 57, 79, 106], we know little about their topographical constitution and if indeed sperm entry is piloted to a specific site. Recently chemical modulation of the zona by oviductal-specific glycoproteins before the oocyte encounters the spermatozoon has been described and this may also be involved in the fine tuning of sperm-oocyte interactions in mammals [14, 15]. Finally, although the plasma membrane of the human metaphase II oocyte appears at the scanning electron microscope to be unpolarized, a study with surface lectins may be instrumental in showing whether or not spermatozoa may fuse at any site around the plasma membrane [87]. In contrast to the situation in the human, the plasma membrane in other mammalian oocytes is polarized with a flat microvillus-free area overlying the metaphase spindle. In mouse rat and hamster, spermatozoa do not fuse with this area of the plasma membrane [60, 78, 89].

The first indication of activation in mammalian oocytes is an electrical change at the plasma membrane followed by the release of Ca^{2+} from intracellular stores [93, 98–100]. As in other animals, this increase in intracellular calcium triggers the activation of the cell cycle, through the degradation of MPF and the exocytosis of cortical granules. The cortical reaction in mammals is less dramatic and slower than in its invertebrate counterparts, but appears to follow the same principles. For example in the mouse oocyte plasma membrane, the lateral diffusion of proteins and lipids is strongly restricted after fertilization [59]. A protease released from the cortical granules partially hydrolyses the zona pellucida glycoproteins, ZP2 and ZP3, removing sperm binding capacity but also leading to a general hardening of the zona pellucida, even though transient [35, 38, 73]. The cortical reaction is a slow structural change that changes the receptive outer investment of the oocyte into a hardened protective layer for the developing embryo.

Timing of fertilization in mammals is critical to avoid oocyte aging and abnormal activation and development [111]. Timing is also of the essence *in situ* in mammals, since insemination of post ovulatory pigs may also lead to an increase in polyspermy, either by oocyte aging or the dynamics of sperm transport in the oviduct [51]. Polyspermy is a fact in aged oocytes *in vivo* and *in vitro*, whereas the concept of “polyspermy-blocking mechanisms” in mammalian oocytes is mainly based on experiments *in vitro* using oocytes deprived of their external coats. Within the mammals there are of course species differences with regards to the constitution of the zona pellucida [42] and this reflects in the kinetics and final outcome of the zona reaction. It is the general consensus that polyspermy blocking mechanisms vary between species being either located at the plasma membrane, as in the rabbit [8, 102], at the zona pellucida as in the sheep and pig [3], or a combination of both as in the mouse [3]. Since such conclusions were originally based on experiments using oocytes deprived of their outer investments we would like to offer an alternative explanation. That is, that the species difference is not due to the different location of different sperm repelling mechanisms, but to the different behaviour of gametes under experimental conditions. In particular, how sperm react physiologically to an incorrect sequence of oocyte signals due to investment removal, how oocytes age differently, and how sperm are able to capacitate *in vitro*.

Since the kinetics of the activation events do not depend on whether one spermatozoon or several spermatozoa approach the oocyte, we suggest they serve solely to change the quiescent oocyte into a dynamic zygote.

External fertilization in sea urchins

Dilution and chemotaxis

A commonly held misconception is that fertilization in marine organisms is a haphazard “free for all with small eggs bombarded by hoards of fertilization ready sperm”. Fertilization in all animals is not a first order chemical reaction but a fine tuned, gradual, and controlled encounter of gametes, where each gamete, in order to progress, must receive a correct sequence of signals from its partner [16].

In marine animals, whether or not fertilization occurs depends on environmental cues, behavioural adaptations and chemotaxis. Sperm concentration in the sea urchin testis is similar to that in mammals, however many more oocytes are produced than in mammals, consequently the problem faced by these animals is making sure there are enough spermatozoa to satisfy all the oocytes spawned. In sea urchins, the sperm : oocyte ratio at source is $10^4:1$ [48]

and although there is little information on the final sperm : oocyte ratio in sea urchins after spawning, there appears to be much wastage of unfertilized gametes [55]. To ensure gamete encounter, spermatozoa are attracted towards the oocyte or gonangial extract by specific chemo-attractants [16, 61, 71, 75]. Sea urchin sperm motility is first activated upon spawning by exposure to the higher pH of the sea water which leads to the activation of a Na^+/H^+ exchanger and a Dynein ATPase [29]. Sperm behavior is then finely modulated by factors released from the extracellular coats as first described by Lillie [64]. Since then, biochemical studies have led to the identification of many sperm-activating peptides (SAP's) in the jelly layer, for example, Speract and Resact [96]. SAPs have been shown to stimulate sperm motility and respiration by a cascade of intracellular signaling events that involve cyclic nucleotides, pH and Ca [13, 95–97, 108].

The jelly layer

Before interacting with the vitelline membrane the spermatozoa must traverse and interact with the outer oocyte investment, which in sea urchins is the jelly coat. The sperm must then bind to and penetrate the vitelline coat before finally fusing with the plasma membrane. Progression through these outer layers depends on successive molecular interactions that change sperm physiology promoting fertilization competence.

The sea urchin oocyte jelly coat is often considered an unnecessary accessory in the laboratory and may be removed with acid sea water. This manipulation has led to confusion since conditions for removal are variable and it is difficult to know if all jelly coat molecules have in fact been removed. For example, in *Lytechinus pictus* jelly solutions enhance fertilization of de-jellied oocytes, whereas in *Strongylocentrotus purpuratus* they decrease fertilizability [76, 102, 104]. *Arbacia punctulata* jelly coat solutions at the normal pH of sea water and the isolated jelly hulls of *Pseudocentrotus depressus* oocytes fail to induce the acrosome reaction in spermatozoa [1, 30], whereas in other species the solubilized jelly coat is used routinely to artificially induce the acrosome reaction. Solubilizing the coat at low pH may expose molecules not usually exposed in the gel configuration. Hagstrom [46] showed that even at relatively high sperm: oocyte ratios, 90% of spermatozoa are unable to penetrate the jelly layer and remain immobilized at various depths within the jelly. Those that pass through must arrive in a physiological condition that both promotes binding and subsequently penetration of the vitelline membrane.

The jelly layer around oocytes, although an essential modulator of sperm progression, is, owing to its nature, difficult to analyze *in situ*. Jelly organization has been best

studied in another deuterostome group, the amphibians, where a fibrillar matrix of high molecular weight glycoproteins is interspersed with globular proteins of lower molecular weight [5]. At the time of deposition the amphibian jelly layer is also extremely rich in ions, containing about 70 mM Na⁺, 30 mM K⁺, 6 mM Ca²⁺ and 7 mM Mg²⁺ [70]. Leaching these ions, in particular calcium and the low molecular glycoproteins, in hypotonic medium, leads to a marked yet reversible decrease in fertilizability. Considering that in aurans spermatozoa enter the oocyte in the animal hemisphere [9, 43] it is probable that the jelly layer differs from antipode to antipode.

The jelly layer in echinoderms is composed mainly of high molecular weight fucose-sulphate-rich glycoconjugates, however little is known about its structure in situ. The pH in the jelly layer of some species is lower (6.3 to 7.6) than that of the surrounding sea water (7.8 to 8.2; [12]), which may play a role both in maintaining the metabolic repression of the oocyte and regulating sperm-oocyte interaction. It needs to be clarified whether the jelly layer is sub-divided into micro-environments that differ with regard to sperm recognition and progression. It has also been suggested that the sea urchin jelly coat, in situ, is an organized structure both radially and topographically. In fact the schools of Boveri at the beginning of the 20th century and Runnstrom in the 1960's considered the jelly coat to be a barrier to spermatozoa with a preferential entry site, the jelly canal at the animal pole, analogous to a micropyle [7, 84, 85].

Timing and cytoplasmic maturity

In the life history of the oocyte, maturation sets in motion a chain of physiological and structural changes giving rise to a cell unit geared to interact with the fertilizing spermatozoon at a particular moment in time. However, this condition is a transient one; should the oocyte not be fertilized, the maturation processes continue and the cell ages. Cytoplasmic aging is often independent of nuclear events, as first shown by Delage [33], and may lead to polyspermy, parthenogenesis, apoptosis, early extrusion of cortical granules, a decrease in MPF and MAPK, and changes in histone acetylation.

The phenomenon of aging of marine invertebrate oocytes is well documented [48]. Oocytes may age in the ovary following spontaneous ovulation, or after removal from the ovary; however in the latter situation the process is considerably accelerated. Borei [6] showed that the rate of oxygen consumption of sea urchin oocytes removed from the ovary rapidly and steadily declines, whereas over-mature oocytes respond differently to mature oocytes when exposed to hypertonic insults [86] indicating alterations to membrane permeability and the cytoskeleton. The jelly

layer in sea urchin oocytes spontaneously dissolves after several hours in sea water and many synthetic processes may be initiated precociously leading to the partial dissolution of cortical granules. In aged echinoderm oocytes, the elevation of the fertilization membrane is delayed by up to 60 s [72]. Thus aged sea urchin oocytes have altered receptivity to spermatozoa owing to dissolution of the jelly coat and different reactivity due to precocious activation, leading, in part, to a delay in cortical granule exocytosis, both processes leading to polyspermy.

The fast polyspermy block idea

Rothschild and Swann [82, 83] on the assumption that sperm: oocyte ratios in sea urchins was high, designed a series of laboratory experiments that led them to suggest that the fertilizing spermatozoon induced a fast, yet partial, change in the oocyte surface that reduced sperm receptivity by 1/20th. In this historic work, the authors, by treating the fertilization reaction as a first order chemical reaction, generated an impressive mathematical model based on the experimental observation that the rate of re-fertilization of oocytes was much lower than the rate of initial fertilization. Although the rationale and mathematical bases for this model have been criticized in depth [31], the pleasingly aesthetical idea of a rapid change in receptivity in the oocyte surface induced by the fertilizing spermatozoon has prevailed in the literature.

Fertilization is not a first order chemical reaction, but, as we have argued, a fine tuned, gradual, and controlled encounter of gametes. Despite this fact, the fast block appealed to many and some suggested that, at least in sea urchins, it was electrically mediated. The fertilizing spermatozoon caused a rapid overshooting depolarization across the oocyte plasma membrane that rendered the oocyte unreceptive to supernumerary spermatozoa [58]. Our considerations are purely observational. First, there is no evidence that a sea urchin or mammalian oocyte, inundated with spermatozoa in the laboratory, has been synchronously exposed to a multitude of fertilization competent spermatozoa, or indeed that spermatozoa attached to the oocyte have located a site compatible with sperm entry. Second, if we observe echinoderm oocytes during fertilization it is not the first spermatozoon that arrives that activates and enters the oocyte [17, 20, 80]. The electrical response to fertilization in sea urchin oocytes is biphasic [24]. Several methods have been used to show that the first small initial step-like event in the oocyte [24] is temporally correlated with fusion of the spermatozoon [17, 20, 31, 65, 68]. and that this event precedes the larger depolarizing fertilization potential by several seconds [23]. In this "latent period" many spermatozoa may bind to the oocyte, some fusing and generating further steps, others

only binding [17]. Thus the overshooting depolarization is a late event and in any case too late to prevent supernumerary spermatozoa from fusing.

If voltage does control sperm entry then holding cells at positive potentials should block sperm entry, while holding cells at negative values should lead to multiple sperm entry. Unfortunately, most experiments were carried out using an inappropriate configuration of current clamp [58], erroneously reported as voltage clamp, where the oocytes were held positive by uncontrolled and unmeasured amounts of positive current. Voltage clamping, either by a whole cell clamp electrode or two intracellular electrodes, is the correct configuration both to “hold” the membrane potential at a particular voltage and to measure the resulting transmembrane currents. Since sea urchin oocytes voltage clamped at negative membrane potentials were shown to be monospermic [50, 69], we believe it unlikely that depolarization of the oocyte membrane at activation has evolved to prevent polyspermy.

The role of electrical events at activation

If, as we sustain, the electrical depolarization at fertilization has not evolved as a polyspermy blocking mechanism what is the purpose of this activation event? The difference in ionic balance between the cytoplasm and the extracellular environment in all cells is maintained by ion channels and transporters located in the plasma membrane, which account for 15–30% of all membrane proteins [2]. Gametes and somatic cells have many voltage-gated channels and transporters in common, while novel ligand-gated channels are specific to the cell type [23]. The type, number, and topographical distribution of channels and transporters change continually during gametogenesis through fertilization to early embryonic cleavage stages, indicating both the importance of ionic homeostasis and the role of second messengers in early development.

The importance of ion fluxes across the plasma membrane in the process of oocyte activation has been recognized for over 50 years. Measurements of the fertilization current in marine deuterostome oocytes, using the whole cell voltage clamp technique, showed the current to be inward and bell-shaped reaching a peak of 1 nA after about 30 s [22]. This means that a total charge of 10^{-9} Coloumbes per second may flow into the oocyte at activation, that if localized and composed mainly of Na^+ and Ca^{2+} , may lead to the entry of 10^{10} ions (Ca^{2+} carries twice the charge of Na^+ , but this is ignored in order of magnitude calculations). If this number of ions were to localize to 1% of the oocyte volume in the sub-cortical cytoplasm, the concentration would be 10 mM. Thus the fertilization current alone may drastically change the sub-cortical concentration of free cations,

priming or modulating the subsequent intracellular Ca^{2+} release mechanisms.

The current is generated by the gating of large, non-specific ion channels of up to 400 pS single channel conductance, possibly activated by a soluble sperm factor via the ADPr/NO pathway [21, 27, 56, 109]. In contrast, in higher deuterostomes the fertilization channel is a calcium-gated potassium channel [26, 41]. Knowing the total conductance change at fertilization, the single channel conductance and the probability of a channel being open, we estimated that a spermatozoon opens 200–2000 fertilization channels around the point of sperm-oocyte fusion [22, 32]. Finally, it has been shown that when fertilization channels are inhibited in the ascidian oocyte by naturally occurring bioactive aldehydes development is abnormal [101].

The cortical reaction

Oocytes are programmed to interact with the fertilizing spermatozoon at a particular moment in time. Once activated the emphasis passes from that of a quiescent cell concerned with enticing its male counterpart to a metabolically dynamic zygote that must progress through early development with the minimum hindrance or interference from the environment. Activation involves many surface events; perhaps the most dramatic being the cortical reaction.

Sea urchin oocytes contain about 20,000 cortical granules that are derived from the golgi apparatus and migrate to the cell periphery during maturation. Each granule measures about one micron in diameter and contains, amongst many other proteins, hyaline, a serine protease, a peroxidase and several sulphated mucopolysaccharides. Following interaction with the fertilizing spermatozoon, the granules fuse to the plasma membrane releasing their contents into the perivitelline space. Fusion starts at the site of sperm entry and then traverses the oocyte in a wave to the antipode taking about 30 s to complete. The hyaline layer forms a tightly adhering protective layer around the oocyte [40, 46, 66], while the peroxidase hardens the elevated fertilization membrane by cross linking the tyrosine residues of the vitelline coat aided by hydrogen peroxide. Fusion of the cortical granules with the plasma membrane causes an increase in the total surface area of the latter which is accommodated as a transient increase in length of microvilli and presumably changes the permeability and exchange with the environment [10, 11, 62, 103]. The cortical reaction is a slow structural change that changes the receptive outer investment of the oocyte into a hardened protective layer for the developing embryo. We forward the idea that this surface reaction in oocytes has evolved solely for this purpose and not as is commonly believed to repel “late coming spermatozoa” [75].

An alternative idea: Pre-determined sperm entry sites

All animals are to some extent models for others. Although we have restricted this essay to deuterostomes, we would like to mention the organization of insect and teleost oocytes. In these animals the oocytes lack cortical granules and in fact are surrounded by a pre-formed impenetrable protective coat, the chorion. Here spermatozoa enter the oocyte through a pre-formed entry site, the micropyle [16].

We have argued that few spermatozoa actually reach the oocyte in nature and those that do are modulated by the extracellular coats, gaining competence as they progress closer to their goal. It is not easy to design experiments to study why only one spermatozoon enters the oocyte. Perhaps the best is observation. In monospermic fertilization in the jelly-free sea urchin oocyte, many spermatozoa may attach to the oocyte surface, before one, the fertilizing spermatozoon, distinguishes itself by gyrating around its point of attachment. Three seconds later the step depolarization event occurs, with no change in the morphology of the oocyte surface or sperm behaviour, until a further 9 s later when the larger fertilization potential starts [24]. At this time the sperm stops gyrating, the tail stiffens and cortical granule exocytosis is seen around the point of sperm fusion. The fertilization cone then starts to engulf the sperm head and the cortical reaction is completed during the falling phase of the fertilization potential [18]. The period between the two electrical events is in fact equivalent to the latent period [66] during which a microfilament dependent process appears to occur [19].

Germinal vesicle stage oocytes do not have cortical granules and are naturally polyspermic. Here the step depolarization also occurs within 5 s of insemination, however the fertilizing spermatozoa gyrate for 30 to 60 s before stiffening and being engulfed by the fertilization cones. Unsuccessful spermatozoa do not generate the step event and may continue gyrating for several minutes until their energy resources are depleted and they fall limp to the oocyte surface. A second category of unsuccessful spermatozoa generate a step depolarization that subsequently turn off without generating a response, while TEM shows that 95% of attached spermatozoa at 20 s insemination are not acrosome reacted [20]. When electrophysiological recordings were made with jelly intact oocytes the step depolarization occurred after 5 s in the germinal vesicle stage oocytes but was delayed to 13 s in the mature oocyte [17]. Similar failures are observed in mature oocytes capable of fertilization in which the step closes prematurely [24]. These failed steps are analogous to the well known “kiss and run” phenomenon observed for vesicle fusion [49].

The idea that microfilaments are involved in sperm entry is not new [36, 88]. Changes in the actin filaments just beneath the plasma membrane that are involved in the re-

organization of the oocyte surface and are also related to activation has been known for decades [91, 107]. The former authors speculated that the G-actin, sequestered on the inner surface of the unfertilized oocyte plasma membrane, may be induced to polymerize upon fertilization during the first few seconds of sperm—egg interaction. Another echinoderm, the starfish, is a particularly useful model to study sperm-oocyte interaction, since the jelly layer is compact, difficult to remove and the acrosome reaction can be seen at the light microscope to occur at the outer surface of the jelly layer [25]. A long acrosomal tubule is then generated from the tip of the spermatozoon that perforates the jelly and the vitelline coat and then fuses with the plasma membrane. By using confocal and digitally enhanced video microscopy it has been shown that the successful spermatozoon triggers a localized sub-cortical polymerization of actin, beneath the fertilization cone, that “draws” the spermatozoon into the oocyte [80]. Under laboratory conditions of high sperm densities several sperm may undergo the acrosome reaction fuse with the plasma membrane and cause transient but abortive localized activation events, but only the successful spermatozoon that “locates” an underlying actin anchor is actually able to enter the oocyte [80]. Finally, although a point for conjecture, we would like to forward the concept that spermatozoa only successfully interact and enter oocytes through pre-determined actin-rich sites.

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