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Coevolution of Animal Gametes

Koevoluce gamet živočichů

BACHELOR THESIS

Student: Palina Prakapovich

Supervisor: Doc. RNDr. Kateřina Komrsková, Ph.D.

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Abstract: Genes of gamete surface proteins play a critical role in reproductive isolation and are therefore a significant factor in speciation. Sperm competition, sex conflict and reinforcement are major drivers of gamete surface protein selection producing recurrent evolutionary patterns recapitulated at different taxonomic levels. The protein surface participating in sperm-egg interaction, necessary for ferilisation, represents the major hotspot of positive selection, as male and female sexes compete continuously, each being selected for a different optimal level of fertilisation efficiency. Signals of positive selection have been repeatedly observed in gamete surface proteins, especially in their protein-interacting domains, in line with proposed evolutionary models of sex conflict. The choice of sperm exerted by eggs extends beyond fertilisation-specific proteins presented on the sperm surface and includes other fitness factors, such as the MHC complex. Many of the genes encoding gamete surface proteins have evolved via the duplication-neofunctionalisation mechanism, indicating that many of the yet unidentified genes may be orthologs or paralogs of the known reproductive and non-reproductive genes in other species. Specific evolutionary signatures, such as signals of positive selection, can help to identify novel reproductive genes which are translated into proteins with undiscovered functions. Combined with genetic and biochemistry analysis, the evolutionary studies of these candidates may inform reproductive biology of new targets for infertility research.

Keywords: coevolution, gametes, fertilization, polyspermy, sex conflict, IZUMO1-JUNO, fertilin, MHC complex.

Abstract: Geny kódující povrchové proteiny hrají rozhodující roli v reprodukční izolaci a jsou důležitým faktorem speciace. Kompetice spermií, sexuální konflikt a posílení speciace jsou hlavním hnacím motorem selekce povrchových proteinů gamet, vyvolávají opakující se evoluční trendy na taxonomických úrovních. Povrchové proteiny účastnící se interakce spermie a vajíčka, a jsou nezbytné pro oplození, reprezentují hlavní hot-spot pozitivní selekce, díky kompetici pohlaví pro které jsou rozdílné hodnoty účinné selekce. Signály pozitivní selekce byly opakovaně pozorovány u povrchových proteinů gamet, hlavně v jejich protein interagujících doménách, a to podle navrhovaných evolučních modelů sexuálního konfliktu. Výběr spermie vajíčkem neprobíhá jenom na základě pro oplození specifických proteinů, které se nacházejí na povrchů spermie, ale zahrnuje i jiné faktory ovlivňující reprodukční fitnes, jako např. MHC komplex. Hodně genů kódujících povrchové proteiny se vyvinulo prostřednictvím neofunktionalizačním mechanismům duplikace, což naznačuje, že mnohé z neidentifikovaných genů mohou být ortology či paralogy genů jiných druhů. Specificky charakteristický evoluční rys jako pozitivní selekce může být užitečný pro identifikaci nových reprodukčních genů, které kódují proteiny s doposud nepopsanou funkcí v reprodukci. Z toho důvodu, kombinací genetických a biochemických analýz, studium jejich evoluce, může poskytnut nové znalosti, které se lze uplatnit v reproduktivní biologie.

Klíčová slova: koevoluce, gamety, fertilizace, polyspermie, sexualní konflikt, IZUMO1-JUNO, fertilin, MHC komplex.

List of Abbreviations

APC/c	Anaphase Promoting Complex
CaMKII	Ca^{2+}/Calmodulin-Dependent Protein Kinase II
DAG	Diacyl Glycerol
ER	Endoplasmic Reticulum
EGF	Epidermal Growth Factor
IP_3	1,4,5-triphosphate
MHC	Major Histocompatibility Complex
MPF	Maturation-Promoting Factor
PLC	Phospholipase C
ZP	*Zona Pellucida*

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Introduction

Sexual reproduction is common among multicellular organisms and is frequently postulated to have evolved as a mechanism to enhance genetic diversity and hence the adaptive potential of both individuals (through their offspring) and populations (Gray & Goddard, 2012). Processes involving horizontal gene transfer, similar to sexual reproduction, are also observed in unicellular species, including bacteria (Heuer & Smalla, 2007), highlighting the ubiquitous adaptive benefits of genetic diversity for life on Earth.

Depending on the presence of differential parental investment (both behavioural and physiological) among sexually reproducing organisms in populations, the corresponding species can be broadly classified into hermaphroditic (with no sex differences) and gonochoric (with two distinct sexes). Noteworthy, the majority of animals (and all mammalian species) belong to the latter group (Jarne & Auld, 2006). A mechanism of sexual reproduction that involves two distinct sexes, drastically differing in the nature of gametes that they produce, confers two principal evolutionary advantages (Kodric-Brown & Brown, 1987; Scudo, 1967). The first advantage is that the female sex can specialize in storing substantial amount of nutrients in a small number of large gametes, thus providing future zygotes with ample food resources during initial development. The second advantage stems from the fact that taken together males produce vastly more relatively uncostly gametes than needed to fertilise all the eggs in a population, allowing for the possibility of intrasexual competition between males and sexual selection by females. This sexual selection operates in addition to environmental selection forces and causes corresponding evolutionary adaptations in male physiology, anatomy and behaviour. Evolutionary pressures are however exerted on the female sex as well, in particular because females invest a lot of their resources in producing large nutrient-rich gametes and often engage in other activities facilitating offspring survival and development. Females cannot afford squandering their limited resources on creating and rearing offspring with suboptimal genotypes and thus the female sex is normally highly selective with regards to male genes to be potentially passed on to their offspring. This female sex selection can operate both pre- and post-copulation. As the mechanisms of female sex selection evolve, so do adaptive responses of males, striving to overcome selective barriers set by the female sex.

In addition to intrasexual competition, sexual reproduction creates a need for inter-species barriers for fertilization, as hybrid offspring is often non-viable or otherwise unfit (Barreto et al., 2015). Consequently, sexually reproducing species have evolved a variety of mechanisms which preclude hybrid fertilization at different levels, including both behavioural barriers and cross-species gamete incompatibility (Anholt et al., 2020; Lenz et al., 2018). Furthermore, the nature of the fertilisation process, involving the creation of a diploid zygote from two haploid gametes entails the necessity of mechanisms preventing zygote polyploidy (and in particular polyspermy), which is either lethal or highly suboptimal (Jacobs et al., 1978; Zaragoza et al., 2000). Thus, both sexes are locked in an evolutionary arms race, wherein they are trying to reconcile their competing evolutionary interests with a common goal of maximising the fitness of their offspring and preventing gene contamination from other species. One of the results caused by these selective forces is coevolution of gametes, which is particularly relevant for fertilisation, during which gametes interact and fuse. Insights gained from the evolutionary analysis of gamete proteins may

be instrumental for a deeper understanding of the underlying molecular mechanisms and developing new infertility treatments and birth control solutions (Turner & Hoekstra, 2004).

1. General trends in gamete surface proteins evolution

The evolution of gamete surface proteins is shaped by an interplay of multiple selective pressures acting simultaneously or cyclically. Compared to intricately organised protein networks governing mating behaviour and physiology, gamete surface proteins represent a relatively simple system for discerning mating-related evolutionary patterns. Studies of these patterns in gamete surface proteins of free-spawning animals have provided valuable insights into the selection processes relevant to mating, which could potentially be generalised to other species. Moreover, due to the fundamental and indispensable role of sexual reproduction in horizontal gene transfer within and between populations, understanding the evolution of reproductive proteins may shed light on the mechanisms of reproductive isolation and speciation (Coyne & Orr, 2004).

At the core of selective pressure operating on gamete surface proteins lies the fundamental conflict between sexes (Swanson & Vacquier, 2002). Males benefit from their sperm successfully fertilising the maximum number of eggs and therefore are under pressure to maximise the fertilisation efficiency of their sperm. A major part of fertilisation efficiency depends on the recognition and interaction between sperm and egg surface proteins, which may be reflected in their molecular traits. For instance, if a relatively recent mutation in a gene encoding a sperm surface protein confers a significant advantage in fertilisation efficiency, this mutation will be under a strong positive selection. Such strong positive selection will cause an evolutionary sweep, characterised by a high ratio of amino acid substitutions to silent mutations between and within species as well as low population polymorphism (Hughes & Nei, 1988, 1989). A pattern consistent with recent evolutionary sweep was found for lysin - one of the sperm surface proteins of a marine snail abalone (Metz et al., 1998). Overall, reproductive proteins have been found to be among the fastest-evolving classes of proteins, in line with the possibility of past evolutionary sweeps across many taxa (Swanson & Vacquier, 2002).

Fast-paced evolution of gamete surface proteins has been proposed as an explanation for another frequently observed phylogenetic phenomenon - reciprocal monophyly of the gamete receptor alleles in closely related species. For instance, sea urchins *Echinometra oblonga* and *E. mathaei* are mutually monophyletic with regard to alleles of one of the genes encoding an egg surface protein, whereas alleles at other loci are polyphyletic (Palumbi, 1999). This trend could be explained by rapid coevolution of separate sets of compatible egg and sperm surface proteins, driven by the tendency of sperm surface proteins to evolve towards higher fertilisation efficiency. Once divergence of gamete surface proteins has reached a certain level it will cause reproductive isolation. In sympatric species partial reproductive isolation can become reinforced by reproductive character displacement, wherein hybrid offspring will be selected against due to decreased fitness, causing more rapid gamete protein divergence than in allopatric species (Palumbi, 2009). Two mechanisms have been proposed to drive rapid sympatric divergence of gamete surface proteins. One mechanism entails initial ecological niche separation between diverging species promoting subsequent reproductive separation due to decreased hybrid fitness (Gavrilets & Waxman, 2002). Another model proposes that divergence in egg surface proteins may cause separation of eggs into distinct reproductive classes, causing the sperm to evolve into different compatible reproductive classes as well, eventually leading to complete reproductive separation even without apparent ecological divergence (Doorn et al., 2001).

At a certain point, evolution of sperm fertilisation efficiency may result in frequent polyspermy, thus incurring costs on female fitness due to the waste of the limited egg reserve (Levitan & Ferrell, 2006). This issue becomes particularly relevant in the conditions of high sperm density. Consequently, females are under the pressure to evolve mechanisms slowing down fertilisation. This pressure may favour rare alleles encoding sperm receptor proteins, for which sperm surface proteins are not optimised. Due to high relative fitness rare alleles of egg surface protein-encoding genes will become more widespread, promoting selection for matching sperm alleles (Levitan & Ferrell, 2006). Once corresponding alleles of sperm surface protein-encoding genes become sufficiently frequent in the population, the matching alleles of genes encoding egg surface proteins will lose their evolutionary advantage and start being selected against. Such rare allele advantage would cause cyclical changes in allele frequencies and is consistent with the maintenance of low-frequency alleles in the population (Fig. 1, Levitan & Ferrell, 2006). As a result, some alleles of genes encoding gamete surface proteins may persist across divergent species to a higher extent than alleles of non-reproductive genes (Takahata, 1990) and a high level of polymorphism will be observed in the population for a given reproductive gene. The gene encoding the sperm surface protein bindin in sea urchin genus *Echinometra* provides a prominent example of such persistent cross-species polymorphism (Palumbi, 1999)**.**

These tendencies are expected in case of high sperm densities, while under the circumstances of low sperm densities both males and females benefit from efficient fertilisation and common gamete phenotypes are hence more advantageous (Fig. 1, Levitan & Ferrell, 2006).

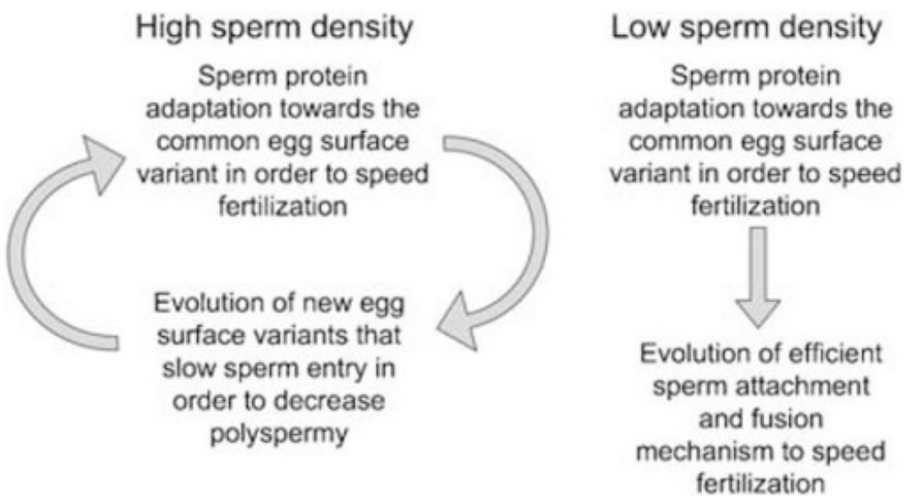

Figure 1. Different modes of gamete coevolution depending on sperm density

From Palumbi, 2009

An additional explanation accounting for polymorphism observed at some loci coding for gamete surface proteins is heterozygote superiority. If gamete surface proteins form polymers on gamete surface, it is conceivable that heteropolymers derived from two distinct alleles could confer higher fertilisation efficiency than homopolymers produced by identical alleles. This is only possible if both alleles are expressed on the surface of haploid gametes, i.e. their expression occurs prior to meiosis. Experiments performed on *Echinometra oblonga* and *E. mathaei* have demonstrated that both bindin alleles are expressed on sperm surface (Palumbi, 2009), suggesting the possibility of another mechanism preserving rare alleles of gamete surface protein genes.

An interesting property of some invertebrate egg surface proteins is their repetitive organisation. Examples include abalone egg envelope protein VERL, which contains 22 tandem repeats (Galindo et al., 2002) and sea urchin egg receptor EBR1 with 19 tandem repeats (Jovine et al., 2005). This architecture has been suggested to underlie concerted evolution of repeats, wherein a mutation in a single repeat might drive rapid changes in other repeats via gene conversion, forcing the sperm binding partner to adapt quickly (Swanson & Vacquier, 1998).

2. Evolutionary trends in the recognition step of gamete interaction

2.1 The role of ZP proteins in species-specific fertilisation and their evolution

Fertilisation is a multi-step process (Fig. 2), which has been extensively studied both *in vitro* and *in vivo*. In mammals fertilisation consists of gamete recognition, adhesion and fusion (Bhakta et al., 2019; Okabe, 2013). One of the structures particularly relevant to the recognition step is *zona pellucida* (ZP). *Zona pellucida* surrounds the oocyte with the polar body and consists of proteins ZP1-ZP3 in mice and ZP1-ZP4 in humans (Boja et al., 2003; Harris et al., 2009; Lefièvre et al., 2004). These proteins and associated glycans have been long considered to be central to the gamete recognition process. Null mutations in *ZP1-ZP3* lead to subfertility or infertility in mice (T. Rankin et al., 1996, 1999; T. L. Rankin et al., 2001). Similarly, mutations in human *ZP* genes were identified in some case studies of human female infertility, namely a missense c.400 G to A (p.Ala134Thr) mutation in *ZP3* and (T. Chen et al., 2017) and a frameshift deletion of 8 bp spanning nucleotides 1169 through 1176 in *ZP1* (Huang et al., 2014). According to the classical model, during recognition sperm bind to *zona pellucida* which elicits a signal cascade in the sperm eventually leading to the acrosome reaction (Okabe, 2013). The acrosome reaction is an exocytosis event, wherein the anterior sperm plasma membrane fuses with the acrosome outer membrane resulting in the release from the acrosome of digestive enzymes and other proteins needed to penetrate *zona pellucida*. All the sperm cells that crossed *zona pellucida* in guinea-pig, mouse and rabbit were acrosome-reacted, underscoring the importance of the acrosome reaction for fertilisation (Fleming & Yanagimachi, 1982; Inoue et al., 2011; Kuzan et al., 1984). Correspondingly, there has been an extensive search for the factors responsible for sperm binding.

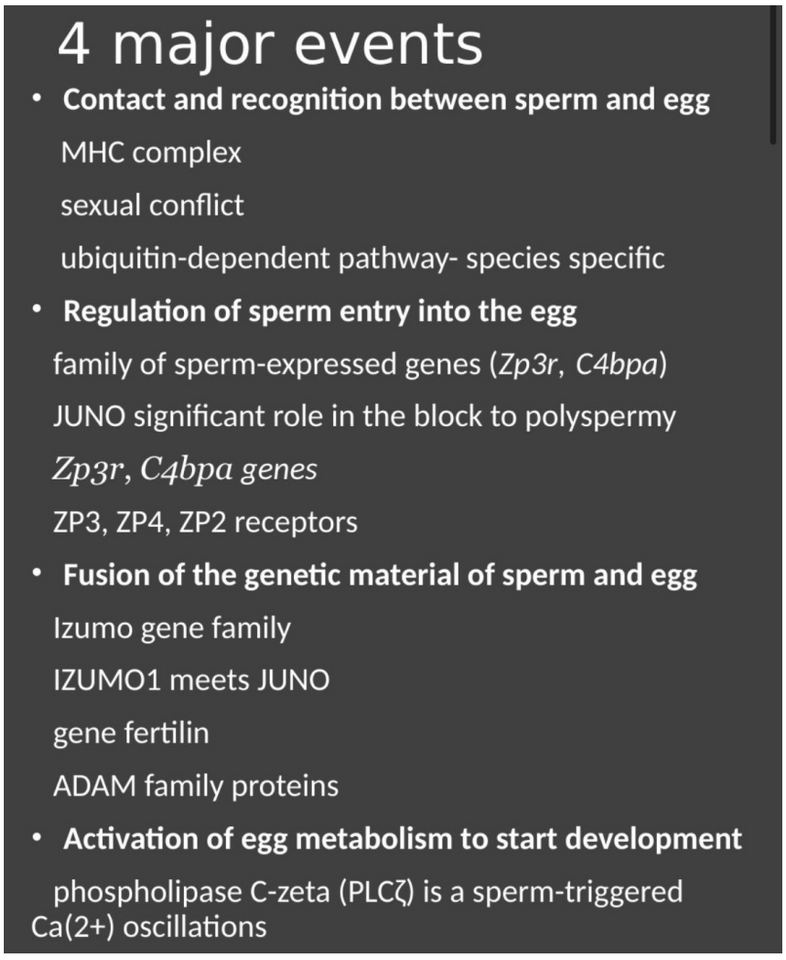

Figure 2. Major steps in mammalian fertilisation

In vitro competitive sperm binding assays using soluble mouse ZP proteins have demonstrated that only ZP3 was capable of interfering with mouse sperm-to-egg binding (Bleil & Wassarman, 1980). Furthermore, soluble ZP3 has been demonstrated to set off acrosome reaction in sperm (Bleil & Wassarman, 1990). Consequently, ZP3 and later, the O-glycans attached to ZP3 Ser^{332} and Ser^{334} were widely accepted as the crucial sperm binding factors (J. Chen et al., 1998; Florman & Wassarman', 1985). Namely, the N-acetylglucosamine and the α1,3-galactose were postulated to play the key role in ZP recognition by sperm (Bleil & Wassarman, 1988; Miller et al., 1992). However, this model was later challenged when it was discovered that α1,3-galactose-lacking transgenic mice were still fertile (Thall et al., 1995) and that in native mouse zona ZP3 is devoid of any glycans at Ser^{332} and Ser^{334} (Boja et al., 2003). Furthermore, it has been found that sperm are not able to bind to *zona pellucida* of two-cell embryos, meanwhile, the only change in ZP upon fertilisation is ZP2 cleavage (Bleil et al., 1981), suggesting the role of intact ZP2, rather than ZP3, in sperm binding.

In the meantime, an alternative model has been proposed, claiming that sperm binding was dispensable for *zona pellucida* penetration. The conventional model has been contested based on the studies of the mouse sperm surface protein ADAM3. Although male *Adam3*$^{-/-}$ mice are sterile and their sperm are incapable of ZP-binding, these sperm are still fertilisation-competent *in vitro* (Yamaguchi et al., 2009). A number of other genes, such as *Clgn, Calr3, Pdilt, Adam1a,* and *Adam2* behave similarly and share the common property: their deletion leads to the disappearance of ADAM3 protein from the sperm surface (Cho et al., 1998; Ikawa et al., 1997, 2011; Nishimura et al., 2004; Shamsadin et al., 1999; Tokuhiro et al., 2012). In addition, the sperm of *Pdilt*-mutated mice were not able to cross the uterotubal junction, however when these sperm were directly injected into the oviduct ampulla they managed to fertilise eggs (Hasuwa et al., 2010). Thus, although acrosome reaction is vital for successful fertilisation, its timing and dependency on ZP binding is still a matter of heated scientific debate.

ZP proteins were implicated in another crucial aspect of fertilisation - cross-species fertilisation barrier. Thus, human sperm cells are incapable of binding mouse ZP and *vice versa* (Bedford, 1981). However, human sperm was able to bind ZP of transgenic mice, in which mouse *ZP2* was replaced by human *ZP2* (Baibakov et al., 2012). Noteworthy, when either N- or C-terminus of the mouse ZP2 was replaced by a human counterpart, only mouse egg cells expressing ZP2 with the human N-terminus could bind human sperm. Thus, the N-terminus of ZP2 appears to be necessary and sufficient for species-specific sperm-to-egg binding. Interestingly, one of the mechanisms preventing polyspermy in human and mouse fertilised eggs involves the release of the enzyme ovastacin which cleaves off the N-terminus of ZP2 (Burkart et al., 2012). In conclusion, ZP2 has evolved at the intersection of two evolutionary demands - species-specificity of fertilisation and polyspermy prevention.

Similarly to the previously discussed male gamete surface proteins, ZP2 and ZP3 have been found to be a subject of positive selection in the past (Swanson et al., 2001). This conclusion was based on the prevalence of amino acid substitutions over neutral mutations in several mammalian species. For *ZP2* gene sequences from *Homo sapiens*, *Macaca radiata* (bonnet macaque), *Callithrix jacchus* (common marmoset), *Felis catus* (cat), *Sus scrofa* (wild boar)*, Canis familiaris* (dog)*, Mus musculus* (mouse) and *Rattus norvegicus* (rat) were analysed, while for *ZP3* the

sequences were taken from *M. musculus*, *Rattus rattus*, *H. sapiens*, marmosets, *M. radiata*, *C. familiaris*, *F. catus* and *S. scrofa*. In ZP3 a portion of the sites identified to have been under positive selection are known to be involved in sperm-egg interaction. Another cluster of positively-selected sites was located close to the signal peptide (Fig. 3).

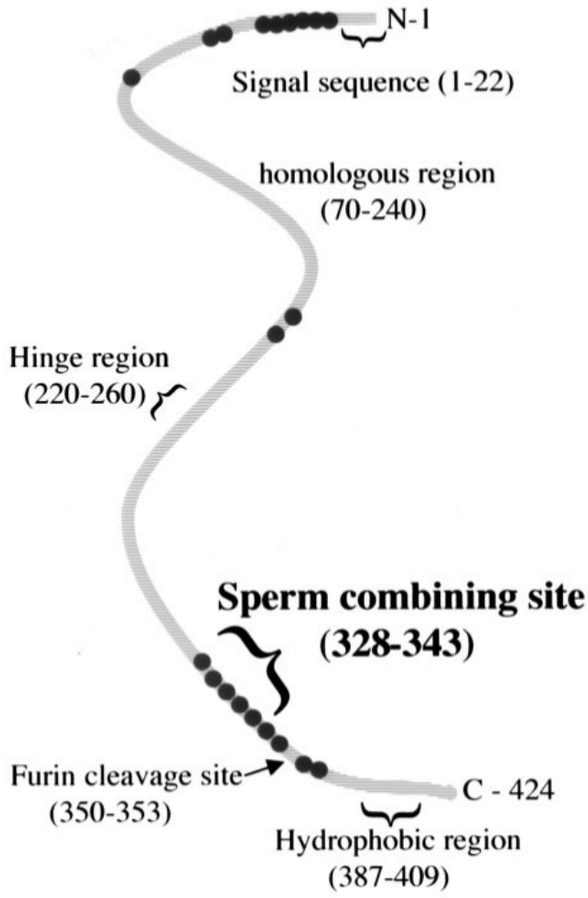

Figure 3. A schematic of ZP3 functional regions with positively-selected sites marked by black circles

From Swanson et al., 2001

A different study looked at the evolution of ZP2 and ZP3 proteins in the 15 species of the *Peromyscus* (deer mice) genus (Turner & Hoekstra, 2006). Similarly to the earlier Swanson et al. study, the study performed on deer mice has identified signs of positive selection in *ZP2* and *ZP3*, consistent with a relatively recent adaptive evolution within the genus. Noteworthy, in deer mice the phylogenetic pattern of *ZP2* and *ZP3* divergence was drastically different from that of non-reproductive genes *Mc1r* and *Lcat* which were taken for comparison. Whereas *Mc1r* and *Lcat* showed monophyletic topology, the evolutionary trees for *ZP2* and *ZP3* demonstrated a high level of intraspecific variation and a significant degree of allele sharing between species. The observed pattern could be indicative of selection forces supporting polymorphism of reproductive alleles in the population, similarly to the previously discussed bindin in sea urchins. As in the previous analysis, a major part of positively-selected sites were identified in the ZP3 sperm-combining region. Interestingly, convergent evolution was detected for some ZP3 sites in a number of *Peromyscus* lineages suggesting that available evolutionary pathways might be limited for these sites. Meanwhile, an analysis of the evolution of ZP3 carboxy-terminal region in Australian and New Guinean murine taxa revealed signs of positive selection in some of the lineages (Swann et

al., 2007). Given that this region participates in sperm-egg interaction (Williams et al., 2006), this finding corroborates the general pattern of sex conflict-driven positive selection being predominantly localised to the protein-interacting domains of gamete surface proteins. Overall, the results of these studies demonstrate that the evolutionary patterns observed for the ZP proteins at the level of mammals is recapitulated in lower-ranking taxa.

Contrary to the aforementioned general pattern of mammalian ZP protein evolution, the evolution of ZP2 and ZP3 in the *Bovini* tribe is characterised by a low degree of intraspecific polymorphism and interspecific divergence (S. Chen et al., 2011). As opposed to the signals of positive selection in ZP2 and ZP3 observed in the previously mentioned studies, signs of purifying selection in the *Bovini* ZP2 and ZP3 were detected instead. Thus, the evolution of *Bovini* ZP2 and ZP3 has hardly contributed to reproductive isolation, demonstrating a remarkable exception to the generally observed pattern. Further evolutionary studies of reproductive proteins complemented with functional *in vitro* studies would facilitate gaining a more coherent and detailed understanding of the connections between sexual conflict and sperm competition on the one hand and the evolution of functionally relevant parts of reproductive proteins on the other.

2.2 Egg-mediated sexual selection of optimal sperm MHC genes

Selective fertilisation serves not only to ensure species-specific gamete fusion but also as a post-copulatory barrier for suboptimal sperm haplotypes. Such a barrier facilitates desirable mating outcomes not only in species which indulge in indiscriminate promiscuous mating, but also in species with highly selective mating preferences. Even when a female succeeds in finding a genetically optimal mating partner, each meiosis-derived egg and spermatozoon harbours a specific haplotype, not all of which are optimally compatible. Consequently, cryptic haplotype-specific gamete selection has evolved to prevent sperm with contextually suboptimal haplotypes from successful fertilisation of an egg (Firman et al., 2017). One of such examples is MHC-dependent selection of sperm haplotypes in various species of vertebrates. MHC is a cluster of genes which encode proteins important for antigen presentation to T lymphocytes (Rock et al., 2016). To fight efficiently against versatile pathogens the immune system should possess a sufficient repertoire of diverse MHC genes. Therefore, a number of mechanisms have evolved to promote the choice of MHC-compatible mates. Such mechanisms were postulated both at the behavioural and post-copulatory levels (Milinski, 2006). For instance, it was found that in some species, such as Atlantic salmon (Yeates et al., 2009) and Chinook salmon (Gessner et al., 2017) the eggs were biased towards fertilisation with MHC-identical sperm, while in red junglefowl the preference towards MHC-dissimilar sperm was observed (Løvlie et al., 2013). In three- spined stickleback fertilisation outcomes were skewed in the direction of intermediate individual MHC diversity, which also turned out to be close to the mean population MHC divergence (Lenz et al., 2018). An intermediate MHC diversity may represent an optimal trade-off between a sufficiently versatile MHC repertoire and a high enough number of mature T cells. It has been postulated that if individual MHC diversity becomes too high, then it increases the chances of a T-cell receptor finding an MHC variant that hyperactivates it (Woelfing et al., 2009). A too large proportion of immature T-lymphocytes become hyperactivated during maturation, leading to their elimination and depletion of the T cell repertoire.

3. Evolutionary patterns in gamete-adhesion proteins

3.1 Coevolution of the IZUMO1-JUNO binding pair

Once a sperm traverses *zona pellucida*, the gamete adhesion and gamete fusion steps ensue. Gamete adhesion involves a close contact between the membranes of an egg and an acrosome-reacted sperm. The loss-of-function assays performed in transgenic mice identified two sperm proteins participating in gamete adhesion - IZUMO1 and SPACA6 (Inoue et al., 2005; Lorenzetti et al., 2014). IZUMO1 belongs to the immunoglobulin protein superfamily and the *Izumo1* gene has several paralogs in mammalian genome that are expressed in testes - *Izumo2*, *Izumo3*, and *Izumo4*, all of which possess a sequence encoding the immunoglobulin-like domain (Ellerman et al., 2009; Inoue et al., 2005). *Izumo4* is the only member of the family that is expressed in other tissues as well as testes. Upon sequence analysis of human, rat, mouse, dog and bull *Izumo* genes (human, mouse, rat and guinea pig for *Izumo3*) all of the four paralogs were found to contain eight conserved cysteine residues with four α-helix regions postulated to be present between these residues. Null mutation in *Izumo1* renders male mice sterile, resulting in their sperm accumulating in the perivitelline space (between *zona pellucida* and egg plasma membrane called oolemma) (Inoue et al., 2005). However, intra-cytoplasmic sperm injection of *Izumo1*$^{-/-}$ spermatozoa into a mouse ooplasm results in viable offspring, suggesting that IZUMO1 is required solely for gamete adhesion and fusion.

Despite the apparent lack of a consistent pattern of *Izumo* gene selection across all mammals, P. Grayson and A. Civetta managed to provide evidence for group-specific selection signals (Grayson & Civetta, 2012). Their analysis suggests a history of bouts of positive selection of *Izumo1* in the Laurasiatheria clade. On average species in this group diverged approximately 80 million years ago (Kumar & Hedges, 2011), suggesting that the bouts of positive selection occurred early in their evolution and do not pertain to species-specific adaptations (Grayson & Civetta, 2012). An extensive bioinformatics analysis conducted by P. Grayson revealed the existence of *Izumo1* orthologs in vertebrate clades other than mammals (Grayson, 2015). Another study identified possible signs of coevolution between IZUMO1 and an egg surface protein CD9 in rodent species (Vicens & Roldan, 2014), although no direct interaction between the two proteins has been reported so far.

Following acrosome reaction, IZUMO1 spreads to the equatorial region of the sperm (Satouh et al., 2012). In 2014 an IZUMO1-interacting partner was identified on the egg membrane (oolemma) - JUNO (previously called FOLR4) (Bianchi et al., 2014). JUNO is indispensable for gamete adhesion and fertilisation. Three members of the folate receptor family - *Fol1*, *Fol2* and *Fol4* are present across many mammalian species and *Fol3* is found in exclusively primates (Elwood, 1989; Petronella & Drouin, 2014; Shen et al., 1994; Spiegelstein et al., 2000). Despite its similarity to folate receptors, JUNO is incapable of binding folate (Bianchi et al., 2014), suggesting that *Juno* evolved by gene duplication followed by repurposing of one of the duplicates. The notion that a duplication and repurposing event took place in *Juno* evolution is consistent with the fact that *Juno* orthologs are only found in mammals (Grayson, 2015). In contrast to *Izumo1*, signs of positive selection of *Juno* were only found in Primates (Grayson, 2015). *Izumo1* and *Juno* show

particularly robust coevolution signals in Primates and some evidence of coevolution in members of Laurasiatheria and Glires clades.

According to structural X-ray crystallography studies, human IZUMO1$^{22-254}$ and JUNO$^{20-228}$ interact directly and in a stable fashion (Aydin et al., 2016; Ohto et al., 2016). The interaction occurs between 19 amino acid residues of JUNO and 20 amino acid residues of IZUMO1. Some evidence suggests that the IZUMO1-JUNO interaction promotes IZUMO1 dimerization which facilitates IZUMO1 binding to another yet undiscovered receptor on the oolemma (Inoue et al., 2015).

Using avidity-based extracellular interaction screen it has been demonstrated that IZUMO1-JUNO interaction is conserved within mammals, such as humans, pigs, mice and opossums (Bianchi et al., 2014). The same assay has also shown that among all pairwise combinations of mouse paralogs, only IZUMO1-JUNO interact, further confirming that the interaction is specific and evolutionary conserved (Bianchi et al., 2014). In an intriguing exception to this rule, human, mouse and pig IZUMO1 were capable of interacting with Syrian golden hamster (*Mesocricetus auratus*) JUNO, which likely explains the ability of acrosome-reacted sperm of the respective species to fuse with ZP-free hamster eggs, highlighting the role of IZUMO1-JUNO interaction in gamete fusion (Bianchi & Wright, 2015). Thus, IZUMO1 and JUNO have coevolved to maintain species-specific and paralog-specific interaction, mediating gamete adhesion.

It is worth noting that IZUMO1-JUNO interaction is necessary but not sufficient for cell fusion, since COS-7 cells, ectopically expressing *Izumo1* and *Juno* do not fuse (Inoue et al., 2015). Thus, other proteins might be participating in gamete fusion, putative candidates including egg CD9 and sperm SPACA6. Further, upon fertilisation, oolemma sheds JUNO resulting in membrane block of fertilisation, since sperm are no longer able to adhere to the fertilised egg (Bianchi et al., 2014). This fertilisation block functions in addition to the zona blocks mediated by zinc and ovastacin released from egg cortical granules following fertilisation (Duncan et al., 2016).

3.2 Evolution of fertilin

Fertilin alpha/beta is a dimeric protein on the sperm surface that consists of two subunits ADAM1 and ADAM2 (Cho et al., 2000; Evans et al., 1995). Initially discovered in guinea pigs (Primakoff et al., 1987), both subunits belong to the disintegrin metalloproteinase (ADAM) family, which includes a multitude of members expressed in mammalian tissues (Cho, 2012). The members of the ADAM family are characterised by the presence of metalloprotease domain as well as disintegrin, cysteine-rich, EGF-like, transmembrane, and cytoplasmic domains (Primakoff & Myles, 2000; Schlöndorff & Blobel, 1999). ADAM proteins are expressed in both reproductive and nonreproductive tissues and based on their tissue expression profile can be broadly subdivided into somatic ADAMs (sADAMs) mostly expressed in somatic tissues and testicular ADAMs (tADAMs), predominantly expressed in testes (Bahudhanapati et al., 2015). Phylogenetically ADAMs can be classified into three groups, with group I and II falling into the tADAM category and group III comprising the sADAM category. With few exceptions, *ADAM* genes in the first group contain no introns in the coding region, suggesting that they might have arisen via retrotransposition from a mature mRNA. The members of group II and group III contain introns in the coding region, with group II genes being expressed in testes and group III genes - in

epididymis (Cho, 2012). To date 34 *ADAM* genes were identified in the mouse genome and 27 *ADAM* genes in the human genome (Cho, 2012).

A study conducted by Shuo Wei group has found that putative group I and group II *ADAMs* are present in amniotes (Bahudhanapati et al., 2015). Evidently, these *tADAMs* have arisen from *ADAM9* or its close paralog *ADAM9-like*. Both gene duplication and retrotransposition appear to have been the driving force behind the evolution of the *ADAM* gene family in vertebrates.

Interestingly, mice possess two genes for ADAM1 - *Adam1a* and *Adam1b* (Nishimura et al., 2002). Both precursors of ADAM1a and ADAM1b form heterodimers with precursor of ADAM2 in the endoplasmic reticulum of germ cells, however only the mature ADAM1b isoform was found on sperm cell surface (E. Kim et al., 2003). Based on the structural studies of fertilin combined with the fact that *Adam2*$^{-/-}$ male mice are sterile it was initially proposed that fertilin is required for egg-sperm fusion through the adhesion between ADAM2 disintegrin domain ECD sequence and egg integrin (Almeida et al., 1995; Cho et al., 1998; Snell & White, 1996; Zhu et al., 2000). However, subsequent research challenged this notion, as it was discovered that *Adam1b*$^{-/-}$ mice are fertile (Nishimura et al., 2004). It was also found that fertilin participates in the localization of ADAM3 protein on the sperm surface. Considering the essential role of ADAM3 in fertilisation (Nishimura et al., 2001; Shamsadin et al., 1999), the connection between fertilin and ADAM3 sperm surface localization was speculated to underlie the role of fertilin in fertilisation (Nishimura et al., 2004). However, both fertilin- and ADAM3-deficient sperm were later found to be capable of fertilisation, when deposited directly into the oviduct, suggesting that the sterility in fertilin-lacking sperm was in fact due to impaired ability to cross uterotubal junction (E. Kim et al., 2006; Tokuhiro et al., 2012).

Although *ADAM2* is a functional gene in humans (Vidaeus et al., 1997), human *ADAM1* is a pseudogene (Jury et al., 1997) as opposed to some other primates, such as *Macaca fascicularis* (Perry et al., 1995). In contrast to mice who possess a single copy of *Adam3* (Lemaire et al., 1994), two human *ADAM3* genes have been identified, both of which turned out to be non-functional, with their functions supposedly assumed by *ADAM21* and *ADAM30* (Grzmil et al., 2001). It would be interesting to find out, whether the unique properties of human reproductive ADAMs have contributed to or have been shaped by the evolution of idiosyncratic human mating biology.

A high ratio of nonsynonymous to synonymous amino acid substitutions detected in both fertilin subunits of several mammalian species implies that the evolution of ADAM1 and ADAM2 was under the influence of positive selection, similarly to other ADAM family members involved in fertilisation (Civetta, 2003; Glassey & Civetta, 2004). A broader-scale follow-up study revealed several notable patterns in the evolution of ADAM proteins (Finn & Civetta, 2010). Positively-selected sites were restricted to the adhesion domain of the studied ADAM proteins, suggesting that ADAMs coevolved accordingly with their interacting partners on the egg. Also, clade-specific evolution rates for ADAM adhesion sites were heterogeneous. Interestingly, in closely related primate species ADAM evolution rates were found to positively correlate with the degree of postmating sexual selection competition, consistent with the idea of mating strategy affecting gamete protein evolution. An in-depth phylogenetic analysis of rodent *ADAM* family genes,

including *Adam2*, revealed an important role of duplication and neofunctionalisation in the evolution of these genes in rodents, highlighting that not all of the observed positive evolution signals in male gamete surface proteins are necessarily linked to sex competition and mating behaviour (Grayson & Civetta, 2013)

4. Gamete coevolution at the level of post-fertilisation

4.1 Sex conflict and evolution of mechanisms preventing polyspermy

As noted previously, polyspermy is a highly undesirable scenario, associated with zygotic lethality, which may severely undermine the prospects of successful procreation, particularly for females. Therefore, there exists a considerable selective pressure for females to evolve mechanisms preventing polyspermy. On the contrary, for males polyspermy is a minor side-effect of the evolutionary drive to accelerate their fertilisation speed and ability.

In mammals at least three mechanisms participate in the block of polyspermy (Fig. 4). The so-called membrane block of polyspermy becomes effective within minutes (Wolf & Hamada, 1977). Interestingly, this block is not set off by sperm entry into the cytoplasm, since intracytoplasmic sperm injection did not prevent fertilisation by other sperm (Wolf & Hamada, 1977). Some data suggests that the membrane block involves JUNO shedding from the oolema, which prevents IZUMO1-JUNO interaction and thus impairs gamete adhesion and fusion (Bianchi et al., 2014). It should be noted, this understanding of the mammalian membrane block is not exhaustive, as JUNO has been found to remain on the oolemma for 40 minutes post-fertilisation. In addition, a decrease in membrane CD9 as well as reorganisation of microvilli on the oolema have been implicated in the mammalian membrane block (Żyłkiewicz et al., 2010). In contrast to some invertebrates, such as starfish and sea urchin, the mammalian membrane block does not rely on rapid membrane depolarization (Jaffe et al., 1983). The efficiency of the mammalian membrane block varies depending on the species. For instance, dozens of sperm can be seen in the perivitelline space of zygotes harvested from rabbit, pocket gopher and mole, while in dogs, sheep, mice, pigs, rats and humans perivitelline sperm are hardly ever detected (Gardner et al., 2005). This observation suggests a presence of a stronger membrane block in the first group compared to the second, considering the evolutionary pressures involved.

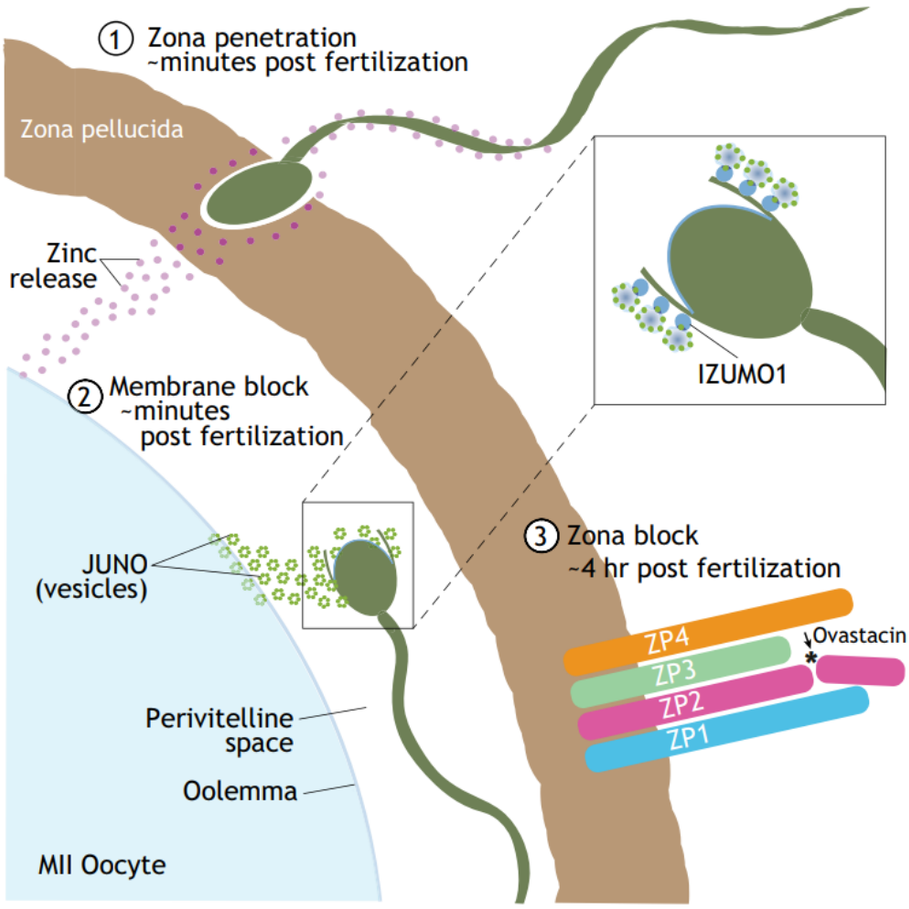

Figure 4. Mammalian polyspermy blocks

Within minutes zinc released from cortical vesicles starts to inhibit sperm motility (1). Simultaneously, the membrane block goes into effect by shedding JUNO from the oolemma as well as via other mechanisms (2). Ovastacin from cortical granules cleaves off ZP2 protein thus rendering ZP unable to bind to sperm, effectively imposing a prolonged zona block (3). From Bhakta et al., 2019

During the so-called zona block ZP loses its ability to interact with sperm, usually upon hours since fertilisation (Bleil et al., 1981). The zona block is initiated by a calcium spike, triggered by gamete fusion (Ducibella et al., 2002). The increased calcium concentration leads to the fusion of egg cortical granules with oolemma, releasing their contents into the surroundings. These granules enclose multiple enzymes, such as trypsin-like proteases, ovoperoxidase, N-acetylglycosaminidase ovastacin as well as zinc. Ovastacin has been found to play a particularly prominent role in the zona block, as upon being released from the granules it cleaves the N-termimus of ZP2 protein, rendering it unable to bind sperm anymore (Burkart et al., 2012). This cleavage is species-specific and mutation-sensitive, as mutation of the diacidic cleavage site on ZP2 results in abrogated ZP2 cleavage and impaired zona block (Baibakov et al., 2007; Gahlay et al., 2010).

Zinc release is believed to play an important, yet transient, role in the zona block (Que et al., 2017). According to one hypothesis, zinc alters the ZP density, resulting in diminished ability of ZP to interact with sperm. Another proposition holds that zinc decreases sperm motility (Tokuhiro & Dean, 2018).

4.2 Sperm-dependent activation of egg metabolism

In the chain of events giving rise to a new life it is hard to overstate the importance of egg metabolism activation upon gamete fusion. The contrast between the resting physiological state of an unfertilized egg in anticipation of an upcoming sperm and a full-tilt fast-paced development of a zygote is remarkable. Not in the least because it provides an example of how a single cell, such as an egg, has evolved to switch between two starkly different metabolic programs. Furthermore, egg metabolism activation is associated with a transition from a highly differentiated state into a totipotent cell. In order to ensure a successful transition of such scale and complexity, the processes underlying egg metabolism activation have to proceed in a tightly controlled and intricately coordinated fashion.

Activation of egg metabolism is believed to be triggered via distinct mechanisms in different species (Horner & Wolfner, 2008). For instance, in vertebrates egg activation is caused by fertilisation, whereas in insects - by mechanical stimulation, and in star fishes - by altered Na^+ concentration in the surroundings. However, in all species studied thus far egg activation is associated with an increase of intracellular Ca^{2+} (Fig. 5, Krauchunas & Wolfner, 2013). In vertebrates an increase of Ca^{2+} concentration in the egg mediates all of the processes of egg activation (Horner & Wolfner, 2008; Wakai et al., 2011). In mammalian eggs the increase of Ca^{2+} concentration depends on the sperm enzyme phospholipase C (PLC□) (Nomikos et al., 2012; Saunders et al., 2002; Wakai et al., 2011). Upon gamete fusion PLC□ converts phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 interacts with IP_3-receptor (IP_3-R1) on the ER, causing the efflux of free Ca^{2+} from the ER into the cytosol (Wakai et al., 2011). It has been found that for mouse eggs Ca^{2+} inflow from the exterior is also required for proper egg activation (Miao et al., 2012). So far, a sperm PLC has been only found in mammals (Krauchunas & Wolfner, 2013).

In some species Ca^{2+} concentration oscillates continuously after fertilisation. In mammals these oscillatory patterns can span as long as several hours (Stricker, 1999). The significance of Ca^{2+} level oscillations in egg activation is not fully understood (Wakai et al., 2011). For instance, in mouse eggs the number of Ca^{2+} concentration spikes needed to initiate the processes of egg activation is lower than the number required to drive egg activation to a completion (Ducibella et al., 2002). Meanwhile, a single high-level Ca^{2+} spike elicited by ethanol was sufficient to produce fully-developed embryos from an egg (Rogers et al., 2006; Suzuki, Suzuki, et al., 2010). Therefore, some authors have argued that the overall increase of Ca^{2+} concentration rather than Ca^{2+} oscillations is crucial for egg activation (Ducibella et al., 2006; Ozil et al., 2006). Upon an increase of Ca^{2+} concentration the events of egg activation (see Fig. 5) are believed to be carried out by Ca^{2+}-dependent proteins.

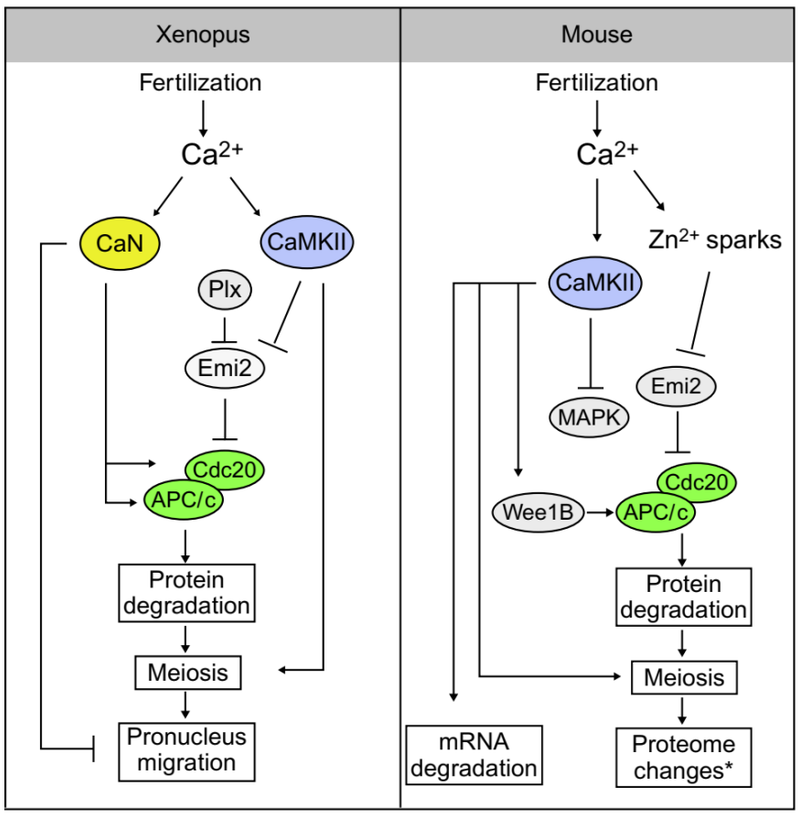

Figure 5. Ca^{2+}-dependent egg activation pathways in various species

CaN - calcineurin. See main text for further details. Modified from Krauchunas & Wolfner, 2013

Another ion participating in mammalian egg activation is Zn^{2+}. In contrast to Ca^{2+}, the level of Zn^{2+} decreases upon egg activation. Following fertilisation Zn^{2+} is rapidly removed from the egg via 1-5 rounds of exocytosis, which bear the name of "zinc sparks" (A. M. Kim et al., 2011). Such sparks have been observed in mice as well as *Macaca mulatta* (rhesus macaque) and *Macaca fascicularis* (crab-eating macaque). When Ca^{2+} increase is prevented by a chelating agent, Zn^{2+} sparks do not occur, suggesting that Zn^{2+} sparks are dependent on the calcium signal. Zn^{2+} sparks appear to be important for degradation of cyclin B and the release from meiotic arrest (Suzuki, Yoshida, et al., 2010). In both, *Xenopus* and mice the mechanism of the meiotic release involves APC/c activator Cdc20 and inhibitor Emi2 (see Fig. 5; Schmidt et al., 2005; Shoji et al., 2006), the latter being dependent on Zn^{2+} for its activity (Bernhardt et al., 2012). Overall, in mammals the decrease of Zn^{2+} level appears to act downstream of Ca^{2+} signal (see Fig. 5) and to be both necessary and sufficient for all of the egg activation events.

Egg activation is accompanied by drastic proteome changes. Due to the absence of a comparable level of concurrent transcriptome activation these changes are largely attributed to posttranscriptional and posttranslational modifications (Krauchunas & Wolfner, 2013). A significant proportion of the observed proteome changes in mouse activated eggs is due to protein degradation and phosphorylation (Pfeiffer et al., 2011; Wang et al., 2010; Yurttas et al., 2010). Protein degradation has been also detected in sea urchin eggs, where proteasome activity appears to be indispensable for entry into mitosis upon fertilisation (Kawahara et al., 2000). Some of the important protein targets, which are marked for proteasomal degradation by APC/c, include securin and cyclins, which prevent the unfertilised oocyte from the metaphase exit (McLean et al., 2011).

As mentioned previously, proteome alterations during egg activation are not limited to the protein degradation alone. For example, in Drosophila, maternal sumoylation was shown to be crucial for eggshell patterning and mitosis at the early stages of development (Nie et al., 2009). A major part of research effort on proteome changes during egg activation has been devoted to protein phosphorylation. In a remarkable example, the number of phosphorylated proteins in sea urchin eggs doubles two minutes following fertilisation (Roux et al., 2006). In Drosophila the phosphorylation status of at least 311 proteins was altered upon transition from a mature oocyte to an unfertilised activated egg (Krauchunas et al., 2012). The significance of phosphorylation in egg activation can be inferred from the fact that phosphorylation modulators Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calcineurin are vital for egg activation.

CaMKII is serine/threonine protein kinase that requires calcium and calcium-binding protein calmodulin for its function (Hudmon & Schulman, 2002). Due to its reliance on Ca^{2+} CaMKII has been considered as one of the key integrators of the Ca^{2+}-dependent signal cascade in eggs (see Fig. 5). In line with this notion, CaMKII activity is elevated upon an increase of Ca^{2+} concentration in both Xenopus and mouse eggs (Liu & Maller, 2005; Tatone et al., 2002). A constitutively active mutant form of CaMKII has been shown to induce egg activation and meiosis resumption even in the absence of a Ca^{2+} rise (Knott et al., 2006). CaMKII together with another kinase Plx1 has been implicated in metaphase arrest relief via the degradation of Emi2 in Xenopus eggs (see Fig. 5; Liu & Maller, 2005). In addition, in *Xenopus laevis* CaMKII provokes meiotic spindle depolymerisation independently of APC/c (Reber et al., 2008). Meanwhile, in mouse eggs CaMKII promotes progression from metaphase to anaphase by phosphorylating Wee1b which then phosphorylates and deactivates Cdc2 thus inhibiting MPF (Oh et al., 2011).

Another prominent transducer of Ca^{2+}-signal in activated eggs is calcineurin. Calcineurin is a two-subunit protein phosphatase, dependent on Ca^{2+} and calmodulin for its activity (Rusnak & Mertz, 2000). In Xenopus eggs the increase of Ca^{2+} level is accompanied by a rise of calcineurin activity (Mochida & Hunt, 2007; Nishiyama et al., 2007). During egg activation the phosphatase activity of calcineurin is important for inhibition of Cdc2, degradation of cyclin B and securin as well as successful exit from the metaphase arrest (Nishiyama et al., 2007). Evidence suggests that in Xenopus calcineurin is directly involved in APC/c regulation (Mochida & Hunt, 2007). The transient activity of both CaMKII and calcineurin are indispensable for cell cycle progression in activated Xenopus eggs, unless a constitutively active form of CaMKII is provided (Nishiyama et al., 2007). Calcineurin has also been shown to be instrumental for Drosophila egg activation (Takeo et al., 2010). Surprisingly, mammalian calcineurin does not appear to play any significant part in mammalian egg activation (Suzuki, et al., 2010).

The current understanding of animal reproductive biology sees Ca^{2+} at the centre of the egg activation process. The intricate regulatory network that has emerged around calcium signalling to control egg activation together with the Ca^{2+} spike-triggering sperm-borne protein PLC□ serve as a testimony to the fact that immensely complex systems can arise from cooperative evolution between sexes.

Conclusions

A vast body of empirical research referenced in this work indicates that gamete surface protein evolution is to a large extent governed by a set of overarching principles. Some of these principles are a direct consequence of the conflicting reproductive strategies of the two sexes. It could be stipulated that females prioritise the quality of their offspring, while males prioritise the quantity, often at the expense of the quality. Although, such oversimplification may appear crude, it can provide a general conceptual framework for understanding of the basic properties of the sex conflict.

A substantial amount of data has been gathered pointing out the signals of positive selection in gamete surface proteins of both free-spawning and internally-fertilising species. In the context of reproductive protein evolution positive selection, most reliably inferred from a high amino acid substitution to neutral mutation ratio, is considered to be an indication of sex conflict. Although, one might expect that females of internally-fertilising species would rely mostly on pre-fertilisation selection strategies to filter out undesirable mating partners, positive selection of gamete surface proteins in these species suggests otherwise. Consistent with evolutionary expectations, positive selection seems to correlate with the degree of postmating competition, as exemplified by the evolutionary patterns observed in the ADAM family in primates (Finn & Civetta, 2010).

Apart from spanning across both vertebrates and invertebrates in the animal kingdom, positive selection of gamete surface proteins is also recapitulated at different taxonomic levels, as seen in a lot of examples of mammalian reproductive proteins. For this reason, reproductive protein evolution is often considered to play one of the key roles at the initial stages of reproductive isolation in many species. Subsequently, such isolation might be reinforced by reproductive character displacement.

Another prominent pattern in gamete surface protein evolution is the connection between the hotspots of positive selection and the functional role of the corresponding amino acid sites in the protein. Recurrently, such hotspots have been found to participate in sperm-egg interaction, examples including mammalian ZP proteins (Swanson et al., 2001) as well as ADAM family proteins (Finn & Civetta, 2010). These findings highlight the particular importance of combining the evolutionary studies with functional analysis, as both approaches can help to identify promising targets for each other and also provide an integrated understanding of reproductive biology. Such considerations may be particularly relevant for the clinical reproductive research, where novel alleles conferring infertility may be identified by searching for reproductive proteins based on their evolutionary signatures followed by the functional dissection of the candidate proteins.

One of the recurrent themes observed in the evolution of genes encoding gamete surface proteins is their repeated duplication followed by neofunctionalisation. The *ADAM* gene family provide a prominent example of this pattern. An entire family of mammalian male reproductive genes appears to have emerged from a series of duplications starting from an ancestral *ADAM9* gene (Bahudhanapati et al., 2015). Another example is the *JUNO* gene which participates in gamete interactions and has apparently originated in an ancestral mammalian species via a duplication

from some folate receptor gene (Grayson, 2015). Therefore, in search for novel reproductive gene candidates it might be helpful to consider looking for paralogs of the known reproductive genes.

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