Oviductin sets the species-specificity of the mammalian zona pellucida

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Short title: **OVGP1 drives species-specific fertilization**

Abstract

The zona pellucida (ZP) is vital for species-specific fertilization as this barrier mediates sperm-oocyte binding. Here, we determined whether sperm from distant mammalian orders (Carnivora, Primates, and Rodentia) could penetrate bovine oocytes by examining the role of bovine oviductal fluid and species-specific oviductal glycoprotein (OVGP1 or oviductin) from bovine, murine, or human sources in modulating the species specificity of bovine and murine oocytes. Sperm from all the species were found to penetrate intact bovine ovarian oocytes to form hybrid embryos. However, contact with oviductal fluid or bovine, murine, or human OVGP1, conferred the ZP species specificity, allowing only the penetration of the corresponding sperm regardless of the ZP's origin. Glycolytic and microstructural analyses revealed that OVGP1 covers the pores present in the ZP and that OVGP1 glycosylation determines sperm specificity. This suggests specific fertilization capacity is acquired in the oviduct through the ZP's incorporation of specific oviductin.

Teaser

The oocyte zona pellucida needs to interact with an oviduct protein called OVGP1 to ensure that only sperm from the same species can fertilize the egg.

Introduction

Interaction between the sperm and egg is generally assumed to be a species-specific event mediated by the recognition and binding of complementary molecules on the sperm plasma membrane to both the zona pellucida (ZP) and the oocyte oolemma (*1, 2*). However, it remains unclear whether both the oocyte's ZP and plasma membrane are necessary to determine species specificity. In evolutionary terms, the ZP is the oldest among the coatings that envelop vertebrate oocytes and conceptuses. This barrier is responsible for preventing polyspermy, facilitating preimplantation development, and mediating species-restricted recognition between the gametes (*3*). ZP proteins and sperm-oocyte fusion protein are usually found to evolve under selection pressure. It is proposed that this rapid evolution plays an important role in the reproductive isolation of diverging taxa (*4, 5*). The mechanism of fusion between the sperm and oolemma membranes is not fully understood, although various candidate molecules have been identified (*6-8*). The species specificity of interactions between gamete membranes in mammals is not always apparent. The significance of the ZP in maintaining species boundaries is revealed by the fact that its removal allows for heterospecific sperm binding and penetration of the oocyte plasma membrane. For example, sperm from humans, mice, rats, guinea pigs, goats, bulls, horses, pigs, rabbits, and dolphins can all successfully penetrate hamster oocytes lacking their ZP coat (*2, 9*). This phenomenon was demonstrated in the hamster egg penetration test (HEPT) developed by Yanagimachi et al. (*10*). In this test, researchers observed that upon removal of the ZP from hamster ova, the eggs allowed for the penetration of sperm from other species (*10*). However, it remains unknown why zona-free hamster eggs are readily penetrated by heterologous spermatozoa, as this characteristic is not shared by the ova of other species. For instance, human spermatozoa will not penetrate zona-free rat, rabbit, or mouse ova (*11*). The ability of sperm from different species to penetrate the hamster oocyte suggests that the ZP could be a key factor in preventing heterologous fertilization between different species.

The successful *in vitro* fertilization (IVF) of zona-free bovine oocytes with deer spermatozoa has been reported (*12*). The IVF of zona-intact bovine oocytes with endangered African antelope spermatozoa was also described in 1998 (*13*). Further heterologous gamete interactions have been documented between porcine or equine sperm and ZP-intact bovine oocytes in large domestic animals (*14*), as have interspecific interactions between equine and porcine gametes (*15*). However, other research groups have reported complete species-specificity for IVF between heterologous gametes in pigs and cattle (*16*). In prior work, we observed in zona-intact bovine oocytes obtained directly from ovaries, the sperm fertility of other species of the same order, such as ram (*17*), common bottlenose dolphins (*18*), and wild goats (*19*). This suggests that species within the order Artiodactyla share similar fertilization mechanisms. However, it appears that this capacity is not restricted solely to this order. Both, our group and other researchers have

found that horse (order Perissodactyla) and guinea pig spermatozoa (order Rodentia) are also capable of fertilizing zona-intact bovine oocytes (*20-22*).

In the present study, we used spermatozoa from the orders Carnivora (cats), Primates (humans), and Rodentia (mice, Muridae family) to examine whether species from other mammalian orders can also fertilize zona-intact bovine oocytes. We also explored the role of bovine oviductal fluid and murine, human, or bovine oviductal glycoprotein 1 (OVGP1, also known as oviductin) in modulating the sperm-binding species specificity of the bovine and murine ZP.

Results

Human, mouse, and cat spermatozoa can fertilize bovine ovarian oocytes

We first examined whether human spermatozoa could penetrate bovine oocytes obtained directly from the ovary, without prior exposure to oviductal fluids. For this purpose, human spermatozoa were capacitated in two different media: G-IVF™ PLUS (HeA) and Fert (HeB), commonly used for the capacitation of human spermatozoa and bovine spermatozoa, respectively. As presented in **table S1** and depicted in **Figures 1A** and **S1**, human spermatozoa were able to penetrate the bovine oocytes, which went on to develop a pronucleus and eventually underwent cleavage into the two-cell stage. Heterologous fertilization outcomes were improved when the G-IVF™ PLUS medium (HeA) was used instead of Fert (HeB). However, these outcomes were still worse than those obtained for homologous fertilization. No differences were observed in the number of spermatozoa remaining attached to the ZP, or in pronuclear formation up to 12 hours post-insemination (hpi). These results indicate that human spermatozoa can bind to and penetrate both the bovine oocyte's ZP and plasma membrane. Human spermatozoa were also found capable of inducing the first embryo cleavage.

As shown in **table S2** and illustrated in **Figures 1A** and **S2**, mouse spermatozoa showed a similar capacity to human spermatozoa to fertilize bovine ovarian oocytes. Following sperm penetration of the oocyte, the sperm nucleus decondenses, forming a pronucleus, which ultimately induces cleavage into two-cell stage bovine zygote. Pronucleus formation and cleavage rates were, however, lower than those obtained for homologous fertilization. Nonetheless, these findings confirm that mouse spermatozoa can also bind to and penetrate both the bovine oocyte ZP and plasma membrane, and are also capable of inducing the first embryo cleavage.

Similar results were also obtained with cat sperm. As presented in **table S3** and depicted in **Figures 1A** and **S3**, feline spermatozoa were found to bind to and penetrate bovine oocytes, thereby initiating the first cellular division of the hybrid embryo. Pronucleus formation and cleavage rates were lower than those observed for homologous bovine fertilization.

To explore if the same effect was produced using oocytes from another species, we collected oocytes from the ovaries of mice following PMSG-induced superstimulation. The ovarian murine oocytes were matured *in vitro* and then fertilized with bovine sperm. The results obtained were similar to those obtained with bovine oocytes in that both the ZP and plasma membrane of the mouse oocytes could be penetrated by the bovine sperm, which then underwent decondensation giving rise to two-cell cleavage stage embryos (**Fig 1B**).

Oviductal fluid inhibits the heterologous fertilization of bovine ovarian oocytes

We then went on to investigate whether the interaction of oocytes with oviductal fluid would hinder the heterologous fertilization of bovine oocytes with human spermatozoa. In this experiment, oocytes were subjected to 30 minutes of incubation with oviductal fluid derived from the oviducts of slaughtered heifers. Subsequently, these oocytes were inseminated with either human or bovine spermatozoa. Results showed that oocytes co-incubated with oviductal fluid could only be fertilized by bovine spermatozoa, with 86% of these oocytes cleaving into two cells. In contrast, bovine oocytes that had been exposed to oviductal fluid were impervious to the penetration by human spermatozoa when using either of the two IVF media (only 3% of these oocytes cleaved into two cells, a similar rate to that recorded in the non-fertilized parthenogenesis group) (see **table S4** and **Fig. 1C).** These findings suggest that the ZP and/or oolema undergo certain alterations upon contact with oviductal fluid, rendering them penetrable exclusively by spermatozoa of the same species. No sperm were found in the perivitelline space, suggesting that the changes occur in the ZP.

Oviductal fluid sets the species specificity of the zona pellucida

To validate our hypothesis that the ZP undergoes modifications within the oviduct that confer species specificity, we used murine oocytes sourced both from the ovary and oviduct. We extracted the cytoplasmic contents from the oocytes, keeping the ZP (**Fig. S4**), and then subjected them to either homologous IVF with mouse sperm or heterologous IVF with bovine or human sperm over 4 hours. Because this experiment was done on empty ZPs, we called this test "empty zona penetration test" (EZPT). Our results showed that the ZPs of oocytes obtained directly from the ovary were susceptible to penetration by the sperm of all three species, but once exposed to oviductal fluids, they could only be penetrated by sperm of their homologous species (see **table S5** and **Fig. 1D-G**). While mouse spermatozoa were found to adhere to the ZP of oocytes obtained both from the ovary and oviduct, more sperm cells appeared on the ZP of oocytes from the ovary. Further, very few bovine and human spermatozoa adhered to the mice ZP of oocytes obtained from the ovary or oviduct (**table S5** and **Fig. 1D, E**).

To test whether oviductal fluid indeed determines the species specificity of bovine oocytes and ascertain whether bovine oviductal fluid affects the ZP, the oocyte, or both, we extracted the cytoplasmic contents from the bovine ZP oocytes (**Fig. S4**) and exposed the empty ZP to oviductal fluid for 30 minutes. Subsequently, we co-incubated ZP with either bovine, human or murine spermatozoa for a period of 6 hours in an EZPT. As observed in **Figure 2**, when ZPs were obtained from ovarian oocytes and not exposed to the oviductal fluid, although the numbers of spermatozoa penetrating the ZP were higher in the homologous IVF group, penetration percentages were similar for human, murine or bovine spermatozoa (see **table S6** and **Fig. 2A, C, E, G**). However, when ZP devoid of oocyte cytoplasmic contents were exposed to oviductal fluid for 30 minutes, these were only susceptible to penetration by bovine spermatozoa (see **table S6** and **Fig. 2B, D, F, H**),

thereby confirming that the effect of oviductal fluid on the ZP is sufficient to determine the specificity of penetrating sperm.

With our EZPT, we were able to determine that all spermatozoa penetrating the ZP lacked an acrosome, while the majority of those that failed to penetrate the egg coat retained an intact acrosome (see **Fig. 2I-Q**). To assess acrosomal status, the fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) method was used (*23*). This observation suggests that during the penetration of the ZP, bovine, murine, and human spermatozoa undergo the acrosomal reaction. Additionally, it should be noted that, regardless of co-incubation with oviductal fluid, a higher proportion of bovine spermatozoa remained bound to the ZP (see **table S6** and **Fig. 2C-H**). It may be seen in **table S6** and **Figure 2A-B**, that more bovine spermatozoa bound to the ZP than human ones, though numbers were similar to murine sperm and there was no difference between numbers of bovine sperm bound to ZP treated or not with oviductal fluid. Also, for human spermatozoa, no difference was found between those bound to ZPs treated or not with oviductal fluid, but for mice sperm, a greater number attached to the bovine ZP when it had not been in contact with oviductal fluid.

Homology of bovine, human and murine OVGP1 proteins and fusion of recombinant OVGP1 proteins with murine or bovine ZPs

To investigate the role of OVGP1 on the species specificity of the ZP, we prepared histidine-tagged cow and mouse recombinant OVGP1 glycoproteins and purchased DDK-tagged human recombinant OVGP1 glycoprotein. As depicted in **Fig. 3A**, the sequences of these three OVGP1 have five distinct regions (A, B, C, D and E) (*24*). Regions A and D are conserved in the different mammals. Region A, corresponding to the amino terminal end, shows high identity among monotremes, marsupials and placentals. Region B features low identity among the different mammals and contains multiple insertions/deletions. Region C is an insertion present only in the mouse (Mus) and region E is typical of human oviductin. Details of OVGP1 sequence alignments for the three species can be seen in **Figure S5**. Clear differences may be seen in predicted N-glycosylation sites and chitin-binding sites (**Fig. S5**) as well as in predicted O-glycosylation sites (**Table S7**).

Proteins were expressed in mammalian cells, separated by SDS-PAGE and analyzed by immunoblotting using rabbit polyclonal antibody to human OVGP1. Additionally, rabbit monoclonal antibody against His-tag, and mouse monoclonal antibody against Flag (DDK)-tag immunoblots were performed to test the recombinant OVGP1 proteins (data not shown). Western blots of the three OVGP1 recombinants indicated expected sizes based on those of the proteins: 75 kDa for human and murine OVGP1 and around 60 kDa for bovine OVGP1 (**Fig. 3B**). Immunoblots were prepared of bovine and murine oviductal fluid protein extracts from animals in estrus (mice) or ovulated (cow) versus anestrous animals. In **Figure 3B**, an arrow indicates the presence of the expected band of OVGP1 found in the oviductal fluid of female mice in estrus or oviduct of ovulated cows, compared to the oviductal fluid of animals in anestrous. Murine oviductal fluids displayed the expected 75kDa band and a more intense 50kDa band, but only the 75kDa band corresponded to

OVGP1 glycoprotein, as confirmed by proteomics of the bands along with PEAKS Studio v11.5 search engine peptide identification software.

OVGP1 recombinant proteins were also assessed for their ability to bind murine or bovine ZPs obtained from *in vitro* matured (IVM) ovarian oocytes compared to ZPs derived from murine oocytes from superovulated females. Oocytes were incubated for 30 minutes at room temperature with bovine (bOVGP1), murine (mOVGP1), or human (hOVGP1) OVGP1, and were then fixed and imaged by confocal fluorescence and DIC microscopy using rabbit polyclonal antibody to human OVGP1 for bOVGP1 and endogenous OVGP1, and mouse monoclonal antibody against Flag (DDK)-tag for hOVGP1 and mOVGP1. All three recombinant proteins were able to bind to the murine and bovine ZPs (**Fig. 3C-D;** controls **in Fig. S6**). No differences were observed in the ability of the three OVGP1s to attach and penetrate the mouse ZPs (**Fig. 3C** and **Fig S6A**). However, while binding of bOVGP1 was observed throughout the thickness of the bovine ZP, mOVGP1 and hOVGP1 preferentially bound to the outer bovine ZP surface without penetrating the coat (**Fig. 3D** and **Fig S6B**).

OVGP1 plays a species-specific role in setting the specificity of the zona pellucida

To investigate whether oviductal protein OVGP1 had a similar effect to oviductal fluid in determining the species specificity of bovine ZP during fertilization, we conducted the EZPT using ZPs from bovine oocytes that were either treated with bOVGP1 or left untreated (for 30 minutes). In this experiment, we used homologous (bovine) and heterologous sperm (human and murine). As illustrated in **Figure 4**, when the ZPs from ovarian oocytes had not been exposed to bovine OVGP1, both homologous and heterologous sperm could penetrate the ZPs (**table S9** and **Fig. 4A**). However, after ZPs were treated with bOVGP1 for 30 minutes, only bovine sperm were capable of penetrating the ZP, while human or mouse sperm were unable to do this (**table S9** and **Fig. 4B**). Similar effects were found in terms of numbers of penetrated sperm per ZP (**Fig 4A, B**).

ZPs that had not been exposed to bovine OVGP1 all showed attached sperm of the three species; bovine sperm sticking most, followed by mouse sperm and then human sperm (**Fig. 4A**). However, when ZPs had been exposed to bovine OVGP1, only bovine sperm were found to bind to bovine ZPs (**Fig. 4B**).

Scanning electron microscopy (SEM) observation of the effect of oviductal fluid and homologous or heterologous OVGP1 on bovine zona pellucida structure

During *in vitro* maturation (IVM), oocytes displayed a porous ZP structure characterized by fine-meshed reticular pores; the pores were circular or elliptical, arbitrarily distributed, and accompanied by deep pore holes (**Fig. 5A-B**). Upon exposure to bovine OVGP1 for 30 minutes, bOVGP1 deposition on the outer ZP surface was observed (**Fig. 5C-D**). Pores were larger and more numerous for IVM oocytes that had not been in contact with bOVGP1. A similar effect was observed with human or murine OVGP1 (**Fig. S7**). In this

SEM study, we were thus able to visualize the morphological changes occurring on the outer ZP surface of IVM bovine oocytes following their exposure to OVGP1.

Only species-specific OVGP1 confers the zona pellucida complete species specificity

We then conducted an experiment to assess whether oviductin from a species different from the origin of the ZP would confer the same specificity as sperm from the same species as that of the ZP or that of the oviductin. Through the EZPT several combinations were tested whereby ZPs from bovine or murine ovarian oocytes were co-incubated with bovine, murine, or human OVGP1 and then exposed to sperm of the three species. As shown in **Figure 6A** and **table S10**, for bovine ZPs and bOVGP1, penetration of the empty ZP was only possible in the case of bovine sperm, such that the entry of sperm of the other two species was completely blocked. For the combination bovine ZP/mOVGP1, human sperm were fully blocked, while some penetration (approximately 60%) by bovine sperm (homologous to the ZP) or murine sperm (homologous to OVGP1) was possible. Similar results were observed for bovine ZP/hOVGP1, sperm homologous to the ZP (bovine) and their pretreatment with human OVGP1, giving rise to partial penetration capacity (around 60%), indicating that OVGP1 non-homologous to the ZP allow the partial passage of sperm of the ZP's species of origin and of the OVGP1-providing species. Similar penetration results were obtained using murine ZPs in combination with the three oviductin and sperm species (**Fig. 6B, table S11**), indicating that results were not specific to bovine ZP.

Results corresponding to mean numbers of penetrated sperm (**Fig. 6A and B, center graphs**) were consistent with these previous findings reflecting a clear reduction (from approximately 17 to 5 sperm cells) when the OVGP1 source was not homologous to that of the ZP, but the sperm used belonged to one of these two species. When there was no species homology among ZP, OVGP1, or sperm, the mean number of sperm recorded was less than one.

When we examined mean numbers of sperm bound to the ZP (**Fig. 6A and B, right graphs**), results paralleled those of penetration. Hence, when there was homology among the sperm, ZP, and OVGP1, only sperm of the homolog species were able to bind to the ZP; and when there was only homology between sperm and either OVGP1 or ZP, there was a reduction in the number of sperm found attached to the ZP. Interestingly, species homology between OVGP1 and sperm resulted in more bound sperm than when there was only homology between ZP and sperm, indicating the involvement of OVGP1 in sperm-ZP binding. These differences in sperm binding were, nevertheless, not reflected in the penetration rate.

Impact of neuraminidase on the EZPT in bovine and murine oocytes pre and post OVGP1 treatment

The enzyme neuraminidase (NMase) cleaves sialic acid residues commonly found in glycoproteins and glycolipids, and is thus able to modify the surface properties of cells and consequently affect their interactions with other molecules or cells. While the involvement of sialic acid in sperm–ZP binding (*25, 26*) is generally accepted, studies reaching this

conclusion have not compared the ZPs of oocytes that have been in contact or not with OVGP1. The aim of this experiment was to examine the influence of NMase treatment on the sperm's ability to bind and to penetrate the ZPs of both bovine and murine oocytes before and after exposure to OVGP1 (**Fig. 7**).

The results indicate that, regardless of whether ZPs had been or not in contact with OVGP1, after NMase treatment, sperm penetration was completely blocked, and the number of sperm adhering to the ZP was reduced by 70%, suggesting that both nonspecific sperm-ZP binding and species-specific binding occurring after contact with OVGP1 are prevented by NMase treatment, thus impeding sperm penetration (**Fig. 7**). It is important to note that despite completely blocking penetration, the binding of some sperm to the ZP likely indicates their ability to nonspecifically adhere, which could occur, for example, when the acrosome is damaged.

Discussion

The zona pellucida (ZP) exhibits different characteristics before and after ovulation and fertilization (*3*). The present study provides evidence that both bovine and murine oocytes acquire species-specific fertilization capacity within the oviduct. Oocytes from the ovaries of cows and mice can be fertilized by sperm from distant mammalian species, yet they acquire species specificity upon contact with oviductal fluid or specific OVGP1 protein. Our results unveil two distinct mechanisms for sperm to recognize and penetrate the ZP. One is nonspecific and acts when oocytes come directly from the ovary, while the other is specific and operates when oocytes reach the oviduct and encounter oviductal fluid or its main secreted constituent, OVGP1. In the nonspecific mechanism, carbohydrate residues present in the ZP, particularly those containing sialic acid, are necessary, allowing sperm to penetrate through the ZP's matrix. For the species-restricted mechanism, oligosaccharides chains containing sialic acids from OVGP1 must be recognized by sperm for them to bind and penetrate the ZP. These findings indicate that species-specific OVGP1 determines the species specificity of the ZP in mammals. Further, our results suggest that heterologous IVF using ovarian oocytes or their ZPs could be an excellent way to assess sperm capacitation and fertility in mammalian species. This study contributes to our understanding of reproductive biology and sheds light on the molecular mechanisms driving cross-species fertilization.

The ZP is an extracellular matrix that surrounds the oocyte and preimplantation embryo in mammals (*27, 28*). This matrix acts as a barrier, separating cumulus cells from the oocyte, facilitating species-restricted recognition during fertilization, and preventing polyspermy, contributing in this way to preimplantation embryo development (*3*). ZP matrix proteins are glycosylated and specific oligosaccharide residues have been linked to their role as sperm receptors (*29*). The diverse glycoconjugate composition of the ZP is thought to be unique to each species (*3, 30*). In cattle, the main components (77%) of the carbohydrate chains of ZP glycoproteins are acidic chains containing sialic acids (*31*): mainly Neu5Ac (84.5%) and Neu5Gc (15.5%) linked to Galβ1-4GlcNAc and GalNAc by α2,3- and α2,6-linkages on N- and O-glycans respectively (*25*). Oviductal fluid composition plays a crucial role in fertilization (*24*) and subsequent embryo development (*32-34*). Oviductal proteins have been proposed to induce changes in the ZP, influencing sperm-oocyte binding specificity (*35, 36*). These changes may impact ZP maturation, sperm-ZP binding, and the prevention of polyspermy (*30*). Modifications induced by oviductal proteins could alter the ZP's composition by affecting its carbohydrates or proteins. One set of proteins found in oviductal fluid is a family of glycosylated proteins collectively known as oviduct-specific glycoprotein (OVGP1) or oviductin. This protein has been identified in various mammalian species, including humans. Oviductin has been associated with the ZP of ovarian oocytes post-ovulation (*37*). OVGP1, along with heparin-like glycosaminoglycans (GAGs), plays a role in functionally modifying the ZP in pigs and cows, making it more resilient to enzyme digestion and sperm penetration, thereby helping prevent polyspermy (*38, 39*). However, it remains uncertain whether the ZP's sperm

receptor is consistent across all mammalian species. This raises questions about how sperm from different mammalian orders are able to fertilize cow oocytes, and whether the lack of contact between cow oocytes from slaughterhouse ovaries and oviductal proteins prevents the changes in the ZP necessary for bull sperm recognition.

The empty zona penetration test (EZPT) enables heterologous sperm to overcome the oocyte's second barrier, the plasma membrane or oolema. While the exact mechanism of fusion between sperm and oolemma membranes is only partially understood, several molecules such as IZUMO, TMEM95, SPACA6, and SOF1 have been incriminated (*6-8*). However, species-specific interactions between gamete membranes in mammals are not always clear-cut. The test known as HEPT, which employs ZP-free oocytes, has shown that the hamster oocyte membrane can fuse with the sperm membrane of many mammalian species but the presence of ZP prevents sperm penetration. Our study reveals that bovine and murine ovarian oocytes permit the penetration of both the ZP and oolema of various mammalian orders. This suggests that, prior to reaching the oviduct, a similar mechanism is used across mammalian species for heterologous sperm to recognize and penetrate the ZP and oocyte plasma membrane. However, when the oviduct is reached, some components play a crucial role in inducing species-specific alterations in the ZP, preventing the recognition and/or penetration of the sperm of other species.

During folliculogenesis and transit through the oviduct, the ZP of ovarian oocytes undergoes maturation in preparation for interactions with spermatozoa in the female reproductive tract (*38, 40, 41*). These changes occur when ovulated oocytes are exposed to oviductal fluid, although the exact molecular mechanisms are incompletely understood, it was previously reported changes in carbohydrate composition and incorporation of proteins (*40, 42*). It has been observed that immature oocytes possess a smooth, compact ZP surface with few pores, while IVM oocytes exhibit a net-like porous surface with a rough pattern on the zona matrix (*43, 44*). Here, we observed that IVM ZPs exposed to OVGP1, undergo a complete transformation, oviductin covering most of the ZP and its pores. This suggests that OVGP1 contributes to the maturation of the oviductal ZP by influencing matrix morphology. At the point of fertilization, OVGP1 is found as the main non-serum glycoprotein in oviductal fluid and its relationship with the ZP was noted early on (*45*). OVGP1 plays a role in strengthening the ZP, rendering it resistant to proteolytic digestion and sperm penetration (*38*), and these processes are regulated by OVGP1's C-terminal region (*46*). The C-terminal region of OVGP1 is the region which mainly differ among species (*24*) and this fact may explain the species-specificity of sperm penetrability.

In mammals, it is accepted that fertilization typically occurs within the ampulla of the oviduct, where sperm bind to the oocyte through interactions with ZP via N- and O-glycoproteins (*47*). Over time, the proposed molecular model explaining sperm-oocyte interactions has evolved. Initially, studies suggested ZP2 and ZP3 acted as primary sperm receptors (*48-50*). Later, it was proposed that sperm-ZP interactions might depend more on the supramolecular structure of the oocyte coat rather than specific ZP subunits (*51*). More recent research points to a nuanced perspective, highlighting a central role for a specific domain of ZP2 in gamete recognition, which physically interacts with complementary

molecules on sperm (*52*). Despite these advances, the exact roles of ZP and OVGP1 glycosylation and sialic acid residues (Sias) in fertilization are poorly understood. After ovulation, the changes reported in the carbohydrate composition of the ZP (*3*, *25*) are likely induced by the addition of glycoproteins of oviductal origin, as we have seen here with OVGP1. By employing NMase, to remove sialic acid residues from ZP glycoproteins, we here ascertain that OVGP1 plays a pivotal role in the modifications to the carbohydrate composition of the ZP necessary for species-specific sperm fertilization. In conclusion, our results indicate that the oocyte ZP must interact with the contents of the oviductal fluid, specifically with OVGP1 protein, to ensure that only sperm from the same species can fertilize the egg. This determines that OVGP1 modifications are critical to define the barrier among the different species of mammals.

Materials and Methods

Reagents

All media components were purchased from Sigma–Aldrich, except where stated otherwise.

Sperm collection and capacitation

Human semen samples were obtained from a normozoospermic donor with their written informed consent. Approval for the study protocol was obtained from the Hospital Clinico San Carlos Research Ethics Review Committee (Madrid, Spain) and the FivCenter fertility clinic (Aravaca, Madrid, Spain) in accordance with the principles of the Declaration of Helsinki. Informed consent for patients was overseen by the Spanish Fertility Society. Donor semen samples were obtained by masturbation after 2-3 days of abstinence and analyzed in the fertility clinic's laboratory. Samples were left to liquefy and then subjected to basic seminogram analysis following WHO guidelines (*53*). Sperm concentration and motility assessments were conducted using a Makler® counting chamber (BioCare Europe, Rome, Italy).

Semen samples were resuspended at a 1:1 ratio with the freezing medium (TEST Yolk Buffer Fujifilm, Irvine Scientific) and stored for 40 min at 4ºC. After this time, the samples were frozen into pellets and stored in liquid nitrogen at -196ºC or lower.

For thawing, the desired pellets were transferred to a 15 mL Falcon tube and kept at 37°C for 20 minutes. Next, the samples were washed by diluting 1:1 in human G-IVF™ PLUS medium (Vitrolife, Igenomix) and centrifuging at 1800 rpm for 10 minutes. After discarding the supernatant, 200-220 µL of G-IVF™ PLUS medium was added to the sediment and the sample stored at 38.5ºC in a 5% CO2 atmosphere for 45-60 minutes. After this time, 90 µL of the upper culture medium layer was removed using a sterile pipette. This aliquot was ready for use following standard swim-up procedures.

Sperm from B6CBAF1 (C57BL/6xCBA) male mice (aged 8-20 weeks) were used for the heterologous and homologous IVF experiments. The animals were maintained under conditions of 14 hours of light and 10 hours of darkness, with no food or water restrictions. Male mice were individually isolated for a period of 2 weeks before sperm collection. Euthanasia was performed by cervical dislocation. Each cauda epididymis was placed in M2 to wash them out and remove the artery of the vas deferens, followed by washing out with HTF medium (human tubal fluid) (2.04 mM CaCl2, 101.6 mM NaCl, 4.69 mM KCl, 0.37 mM KH2PO4, 0.2 mM MgSO4, 21.4 mM sodium lactate, 0.33 mM sodium pyruvate, 2.78 mM glucose, 25 mM NaHCO3, 100 U/mL penicillin, 50 µg/mL streptomycin and 0.001% (w/v) phenol red); supplemented with 1% (w/v) BSA). Spermatozoa were collected by gently squeezing the dissected vas deferens and cauda epididymis, and were then incubated to swim-out in a 500 µL droplet of HTF medium, which supports capacitation, for 30 minutes at 37°C in the 5%CO2 incubator. The concentration of the sperm sample was determined using a Thoma cell counting chamber. Sperm motility

quality was assessed following placement of 6 µL of sperm suspension in a Mackler® chamber on the stage of a microscope heated to 37ºC (Nikon Eclipse E400). Fertilization was conducted in a final concentration of 2x10^5 capacitated spermatozoa per mL (*54*). All studies involving mice were approved by the Ethics Committee on Animal Experimentation of the INIA (Madrid, Spain), and were registered with the Dirección General de Agricultura y Ganadería of the Comunidad de Madrid (Spain) under PROEX 137.2/21.

Cat spermatozoa were collected from the cauda epididymis by modified retrograde flushing (*55*). Collected spermatozoa were incubated in 200 µL of medium, and their concentration determined using a Neubauer cell counting chamber. Next, 50 µL of spermatozoa suspension (4 x 10^6 spermatozoa/mL) were stored in liquid nitrogen vapor for 2 hours to reach a temperature of 4ºC. The frozen semen straws were thawed at 37ºC in a water bath for 1 minute and then centrifuged for 8 minutes at 300 g using a gradient of 1 mL of 40% and 1 mL of 80% BoviPure (Nidacon Laboratories AB, Göthenborg, Sweden) according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of BoviWash (Nidacon Laboratories AB, Göthenborg, Sweden) by centrifugation at 300 g for 5 minutes. The pellet was re-suspended in the remaining 80 µL of BoviWash. Fertilization was carried out in a final concentration of 2x10^6 capacitated spermatozoa/mL.

Homologous and heterologous IVF of bovine oocytes

Ovaries from mature heifers were collected at a local slaughterhouse, and immature cumulus–oocyte complexes (COCs) obtained by aspirating follicles of diameter 2 to 8 mm (*56*). Groups of 50 COCs were cultured in four-well dishes (Nunc) for 24 hours at 38.5°C under 5% CO_2 in air, using 500 µL of maturation medium. This medium was composed of TCM 199 supplemented with 10% fetal calf serum (FCS) and 10 ng/mL of epidermal growth factor (EGF).

For homologous fertilization, frozen/thawed semen (0.25 mL) from a single Asturian Valley bull was treated by density gradient centrifugation (BoviPure®, Nidacon International, Sweden), and matured COCs where then co-incubated with selected sperm at a final concentration of 1 × 10^6 spermatozoa per mL for 18–22 hours in 500 µL of fertilization medium (*57*). This medium consisted of Tyrode's medium containing 22 mM sodium lactate, 25 mM bicarbonate, 1 mM sodium pyruvate, and 6 mg/mL fatty acid-free BSA supplemented with 10 µg/mL heparin sodium salt (Calbiochem). Co-incubations took place in four-well dishes as groups of 50 COCs per well at 38.5°C under an atmosphere of 5% CO2 in air and maximum humidity.

For heterologous IVF, the bovine mature COCs were co-incubated with spermatozoa from other species (cat, human, mouse, and rabbit). Sperm-egg interactions were assessed through a sperm-ZP binding assay at 2.5 hours post-insemination (hpi). For this purpose, COCs were vortexed for 3 minutes, fixed in 4% paraformaldehyde for 30 minutes, washed with cold PBS, and stained with Hoechst 33342 to count the number of sperm that remained bound to the ZP. This was done using a widefield fluorescence microscope with

structured illumination (ApoTome, Zeiss) and UV-2E/C excitation: 340–380 nm and emission: 435–485 nm. In addition, pronuclear formation was assessed at 6, 12, 18, and 22 hpi. For this, presumptive zygotes were treated and examined following the procedures explained above for sperm-ZP binding. The times used in this study for hybrid embryo analysis were similar to those used in co-incubation studies performed in other species (*18*, *22*).

In vitro culture of presumptive zygotes to first cleavage embryos on Day 2

After 22 hours co-incubation, presumptive zygotes or COCs were denuded of cumulus cells by vortexing for 3 minutes and then cultured in groups of approximately 20 in 25 µL droplets of G-IVF™ PLUS medium, or 25 µL droplets of synthetic oviductal fluid (SOF). The SOF contained 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 mL/mL BME amino acids, 10 mL/mL minimum essential medium (MEM) amino acids, and 1 mg/mL phenol red. These cultures were supplemented with 5% FCS and placed under mineral oil in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, and maximum humidity at 38.5°C (cow) or 37°C (human, mouse, and rabbit). Forty eight hours after the start of co-incubation, cleavage was assessed under a stereomicroscope by checking for the presence of two cells per embryo.

Collection of oviductal fluid

Five stage I (Days 1 to 4) oviducts and another five stage II (Days 5 to 9) were selected from slaughtered heifers. The stage of the estrous cycle was estimated based on the morphology of ovarian structures, especially the corpus luteum (CL). Briefly, in stage I, the CL is small and red, and the epithelium has not yet covered the point of follicular rupture. In stage II, the CL is somewhat bigger, vascularization is observed around it, the apex is reddish in color, and the epithelium has already covered the rupture point. Each tract was independently deposited in a plastic bag and transported to the laboratory on ice in a polystyrene box within 2 hours of collection. The oviducts were washed with cold water to remove blood traces and then in cold Ca2+ and Mg2+-free phosphate-buffered saline (PBS-). Each oviduct was strengthen by removing the adjacent tissues and flushed with 1 mL of cold PBS− from the ampulla to the isthmus. All manipulations were performed at 4°C. The fluid from the five oviducts was pooled and then centrifuged once at 300 × g for 7 minutes. Subsequently, the supernatant was centrifuged again at 10,000 × g for 30 minutes at 4°C to remove debris (*32*). The resulting supernatant was aliquoted and stored at -80°C.

Collection of oviductal fluid from mice

Oviducts from four estrous female mice were collected and washed by flushing from the ampulla to the isthmus with 0.2 mL of Ca2+ and Mg2+-free phosphate-buffered saline. Female mice were sacrificed sequentially and oviductal fluid was washed out with the same PBS- and oviducts kept on ice after each flushing. This was followed by storage at -80ºC.

Preparation of empty zona pellucidae from bovine ovarian oocytes

COCs obtained from ovaries from slaughtered heifers and matured in vitro underwent a thorough washing process before their immersion in 30 mL of Tyrode's medium. The oocytes were then mechanically denuded by vortexing and gentle pipetting. The denuded oocytes were subjected to three washing cycles using either G-IVF™ PLUS (Vitrolife, Igenomix) or FERT medium, depending on the experimental requirements. Batches of approximately 30 oocytes were then carefully placed in 400-µL droplets of the selected medium and covered with mineral oil. For the removal of cytoplasmic contents, a micromanipulator was used following the procedure outlined in **Figure S5**. The oocyte was secured using a glass capillary holder, while an ICSI needle, attached to a piezoelectric manipulator, was used to create a small opening in the ZP. This opening facilitated the penetration of the needle, allowing for the complete aspiration of the oocyte's cytoplasm. The aspiration needle was then retrieved from the ZP to expel the extracted cytoplasm. Following this, the empty ZP underwent two washing cycles using G-IVF™ PLUS or FERT medium. These prepared zona pellucidae were either frozen at -20°C for a duration of several months (3-9 months) or directly utilized in the sperm penetration experiments.

Collection and *in vitro* maturation of ovarian cumulus-oocyte complexes in mice

Female mice aged 8 to 10 weeks were superovulated by intraperitoneal injections of 0.1 mL of PMSG (pregnant mare serum gonadotropin, Folligon, 5 IU, INIA, Madrid, Spain). Fifty hours after PMSG supplementation, the females were euthanized by cervical dislocation, and their ovaries removed. Collected ovaries were cleaned of any connective tissue and placed in M2 handling medium supplemented with 4 mg/mL of bovine serum albumin fraction V. Antral follicles were gently punctured with 30-gauge needles, and COCs with at least 3 compact cumulus cells layers were collected in handling medium and matured for 16-17 hours in TCM199 supplemented with 10% FCS and 10 ng/mL EGF (epidermal growth factor) (*58*) and kept at 37°C under an atmosphere of 5% CO2 in air with maximum humidity. Matured oocytes were isolated and placed in an appropriate medium; HTF for IVF and M2 for ZP isolation and later protein co-incubation.

Isolation of superovulated mice oocytes

Metaphase II oocytes were collected from the oviducts of 6- to 8-week-old female mice superovulated with 7.5 IU of eCG (equine chorionic gonadotropin), and an equivalent dose of hCG (human chorionic gonadotropin) 48 hours later. Briefly, 14 hours post-chorionic gonadotropin administration, oviducts were removed from the superovulated female mice and placed in a petri dish containing M2 at room temperature. After washing, collected oviducts were placed in fresh M2 medium, and COCs previously in contact with oviductal fluid were released from the ampulla with the aid of Dumont #55 forceps and washed in fresh M2 medium until cytoplasm removal.

Preparation of zona pellucidae from mice oocytes

After obtaining the COCs using the two methods described, cumulus cells were dispersed by 3 to 5 minutes of incubation in M2 medium containing 350 IU/mL of hyaluronidase

(Sigma-Aldrich H4272). After washing, oocytes were kept in KSOM medium at 37°C in an atmosphere of 5% CO_2 until use.

To obtain empty zona pellucidae, cytoplasmic contents of the oocytes were removed using a micromanipulator, following the procedure outlined in **Figure S5.** The protocol used was similar to that employed for the bovine oocytes with the help of a holding pipette and a blunt microinjection pipette. The decumulated oocytes were placed in a drop of M2 medium, and with the assistance of a piezoelectric manipulator, each zona pellucida was penetrated, followed by the removal of the cytoplasm using the blunt microinjection pipette.

In vitro fertilization, sperm penetration, and binding assessment in mice

Four experimental groups were established. Each of the two groups of oocytes described was used for heterologous fertilization with bovine semen that had been recovered and capacitated via the procedure detailed earlier. For each group, a positive control was set up through homologous fertilization with murine semen.

In vitro fertilization was carried out under the same conditions for all four groups. The zona pellucidae were co-incubated with sperm, and the sperm concentration was determined by counting these cells in a Thoma chamber and then adjusting to a final concentration of 1x10^6 sperm/mL. Male and female gametes were then co-incubated for 6 hours in 50 µL of HTF fertilization medium covered with mineral oil in a 4-well plate at 38.5°C in a 5% CO_2 maximum humidity atmosphere.

Six hours after insemination, the zona pellucidae were transferred from the 50 µL drop of fertilization medium to a new drop to correctly assess both sperm penetration of the zona pellucida and sperm binding to the zona. Once the zonas were in the new drop, the spermatozoa inside every zona pellucida were individually counted and differentiated from binding spermatozoa, which were also quantified.

Origins of bovine, murine and human OVGP1 recombinants

The coding sequences of bovine OVGP1 (NM_001080216.1) and mouse OVGP1 (NM_007696.2), an additional leader peptide sequence for mammalian cell expression (MGWSCIILFLVATATGVHS) and the sequence to express six-His residues were cloned into a pcDNA3.1(+) expression vector from GenScript Giotech (Rijswijk, NL) containing a promoter for bacteriophage T7 RNA polymerase. Tagged OVGP1 protein was produced by transient expression using a Vaccinia/T7 system (*59*) in BHK-21 cells (ATCC CCL-10) grown in G-MEM BHK-21 medium (Gibco) supplemented with 5% FBS (Gibco), 10% tryptose phosphate medium (Gibco), 20 mM Hepes (Fisher Scientific), and supplemented with 2 mM L-glutamine (Lonza), 0.1 µg/ml penicillin, and 0.1 µg/ml streptomycin (Lonza). BHK-21 cells grown in 6-well plates were infected with a crude stock of vaccinia virus expressing T7 polymerase (vTF7-3) (*60*) at a multiplicity of infection (m.o.i.) of 5 pfu (plaque-forming unit)/cell in 2% FBS EMEM (Eagle's minimum essential medium)

medium. After a 90-minute adsorption period, the virus inoculum was removed, cell monolayers were washed with EMEM medium and subsequently transfected with pH224 plasmid. Transfection for each well was performed with a mixture of 2 µg of DNA and 6 µL of Fugene HD reagent (Promega). The DNA/fugene mixture was incubated for 15 minutes at room temperature prior to its drop-wise addition to the infected culture. At 3 days post-infection, the cell culture was harvested and cells were sedimented by low speed centrifugation. Supernatant was used in subsequent experiments.

Bovine OVGP1 (bOVGP1) and murine OVGP1 (mOVGP1) recombinant proteins were purified using the Amicon Pro Purification System (Merck Millipore Ltd) by combining metal chelation chromatography with an Amicon concentrator membrane. Mammalian cell supernatants were incubated under constant agitation for 2 hours with the Ni-NTA His-Bind resin, previously equilibrated with binding buffer. Then, following the manufacturer's protocol specifications and after washing and elution centrifugation steps, His-tagged proteins were concentrated in an Amicon Ultra-0.5 device, and eluted in 25 µL of the desired buffer (50 mM Tris-HCl, 500 mM NaCl, 5% glycerol, pH=8) and stored at -80ºC.

Human recombinant oviductin protein was obtained from Origene Technologies Inc. (human Cat. No. TP321684, murine Cat. No. Tp521693; Rockville, US). A sequence expressing FLAG-tagged epitope proteins (DYKDDDDK) was cloned into an expression vector. The recombinant proteins were produced by transient transfection in HEK293T, obtained from the cell culture supernatant, and captured on an affinity column followed by conventional chromatography steps.

Western blotting of OVGP1

Proteins were run on an SDS-PAGE gel of 10% acrylamide, loading per well 300 ng of total purified commercial protein from Origene, an equivalent amount of the in-house produced proteins from oviductal fluid, and 10 µL of bovine and 50 µL of murine proteins. The resultant gel was transferred to a nitrocellulose membrane for immunoblotting following standard procedures. Membranes were blocked with 3% BSA in PBS-T (0.05% Tween in 1X PBS) and incubated with shaking overnight at 4ºC with anti-OVGP1 rabbit monoclonal antibody (1:750) (NBP1-76939). Incubation was continued for 2 hours at room temperature using as secondary antibodies for OVGP1, goat anti-rabbit IgG-HRP (Cat. No. GTX213110-01, GeneTex), or goat anti-mouse IgG-HRP, diluted 1:5000. As loading control, membranes were incubated for 2 hours at room temperature with monoclonal anti-β-actin HRP-conjugated mouse antibody (A3854, Sigma-Aldrich). Membranes were developed with Immobilon® Forte Western HRP Substrate (Cat. No. WBLUF0100, Millipore). The chemiluminescence signal was digitalized using an ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare Life Sciences, USA, 29005063).

OVGP1 immunofluorescence

Zona pellucidae, previously incubated with OVGP1 proteins from several species and murine oviductal fluid for 30 minutes at room temperature, were fixed in 4% PFA (paraformaldehyde) in PBS 1X 1% BSA for 20 minutes at room temperature. The zonas

were washed out twice with PBS 1X 1% BSA and incubated with blocking buffer (5% FBS in PBS 1X) for 45 minutes. After blocking, primary antibody was prepared at 1:50 in 20% blocking buffer in PBS 1X 1% BSA followed by incubation in a humid atmosphere at 4ºC overnight. ZPs were washed out twice in PBS 1X 1% BSA and incubated for 2 hours at room temperature with the secondary antibody 1:200 (in 20% blocking buffer in PBS 1X 1% BSA). After 3 wash-outs in PBS 1X 1% BSA, the ZPs were embedded in Fluoromount Aqueous Mounting Medium (F4680, Sigma-Aldrich) and observed under structured illumination in a Zeiss Axio Observer microscope equipped with ApoTome.2 software. The primary antibodies used were anti-OVGP1, rabbit monoclonal (NBP1-76939) for endogenous OVGP1 and bOVGP1 and Anti- Flag M2 antibody, mouse monoclonal (F1804, Sigma-Aldrich) for exogenous recombinant hOVGP1 and mOVGP1. The secondary antibodies used were Alexa Fluor 647 goat anti-rabbit (H+L) (A21245, Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (H+L) (A11029, Invitrogen) (*61*).

Proteomics identification of murine OVGP1

Oviductal fluid from 5 female mice was obtained in PBS as previously described, and 50 µL aliquots loaded on an 8% polyacrylamide gel for SDS-PAGE. Some lanes of the gel were blotted onto a nitrocellulose membrane for immunoblotting against OVGP1 antibody, and other lanes were fixed for 30 minutes in methanol:acetic acid (50:10) and rinsed with distilled water. Fixed bands were stained with Coomassie Brilliant Blue, and 75kDa and 50kDa bands (compared to the lanes immunoblotted for OVGP1) were cut, destained in acetonitrile:water (ACN:H2O, 1:1), digested with trypsin, and subjected to reverse phase-liquid chromatography RP-LC-MS/MS mass scan analysis (*62*). Murine oviductin identification was performed with the PEAKS Studio v11.5 search engine (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) against the Uniprot Mus musculus database using a filter of peptides and proteins with a FDR<=1% (*63*).

Scanning Electron Microscopy and Image Processing

Oocytes were denuded as detailed above and prepared for SEM as previously described (*64*) with minor modifications. Briefly, oocytes were placed in fixation medium (2.5% glutaraldehyde [v/v] and 0.1 mol/L sodium cacodylate buffer) for 1 hour at 4°C. They were then washed with 0.1 mol/L sodium cacodylate buffer and kept in the buffer for 1 hour at 4°C, followed by washing in distilled water for 5 minutes. Thereafter, the oocytes were dehydrated with increasing concentrations of acetone at room temperature. After their dehydration, they were CO_2-critical point dried in a stainless chamber (Denton Vacuum DCP-1, Denton Vacuum, LLC, Moorestown, NJ, USA), mounted onto aluminum stubs, and coated with platinum sputter-coated with 5.0 nm thin layer (Leica EM ACE 600). Photomicrographs of the ZP of oocytes were taken a FESEM electron microscope. FESEM analyses were performed with a FE-SEM (ApreoS Lovac IML Thermofisher).

Incubation of ZP with neuraminidase (NMase)

Two hundred isolated ZPs were treated with *Clostridium perfringens* neuraminidase (type V, Sigma) in 0.1M acetate buffer at pH 4.5, as three separate replicates. The enzyme was

tested at concentrations of 1 and 10 IU/mL for an incubation time of 17 hours at 38.5°C. As control, ZPs were incubated under the same conditions in the absence of NMase. The NMase used shows broad specific activity, cleaving terminal sialic acid residues which are α-2,3- α-2,6- or α-2,8-linked to Gal, GlcNac or GalNAc oligosaccharides, glycolipids or glycoproteins (*65*).

Statistical analysis

Statistical analysis was carried out using the software package SigmaStat (Jandel Scientific, San Rafael, CA). Results are provided as means ± standard error of the mean (SEM). Means were compared and analyzed by repeated measures one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Significance was set at $p < 0.05$.

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Author contributions: D.F., M.M., Y.N.C., K.C.-B. performed fertilization experiments, R.F.-G. performed micromanipulations to purify the ZPs. A.M.-M. performed heterologous fertilization with cat sperm. J. M. S.-P. and R. B. undertook recombinant OVP1 production. P.C. and M.A. performed the electron microscopy study. A.G.-A. and D.R. designed the project and supervised the experiments and data analysis. All authors discussed the results of the study. A.G.-A. and D.F. wrote the paper with contributions from the other authors.

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Data and materials availability: All data needed to support the conclusions of this study are present in the paper and/or the Supplementary Materials.

Fig. 1. Heterologous IVF of bovine oocytes, mouse oocytes, or empty mouse ZPs, using human, mouse, or cat sperm, before and after contact with oviductal fluid. (A) Embryo cleavage rates resulting from the IVF of bovine oocytes with human, murine, or feline sperm. (**B**) Sperm penetration rate after the IVF of IVM mouse oocytes with murine or bovine sperm. **(C)** Embryo cleavage rates resulting from the IVF of bovine oocytes, treated 30 minutes with bovine oviductal fluid, with human sperm (IVF medium a = G-IVF™ PLUS medium (HeA); IVF medium b = Fert (HeB). **(D)** Penetration rates and average numbers of sperm bound to ZP after the empty zona penetration test (EZPT) using mouse ovarian oocytes and murine or bovine sperm. **(E)** Penetration rates and average numbers of sperm bound to ZP after the EZPT using mouse oviductal oocytes and murine or bovine sperm. **(F)** Picture of an empty zona pellucida obtained from a mouse ovarian oocyte after the EZPT using bovine sperm. Note the sperm has penetrated the zona. (**G**) Picture of an empty zona pellucida from a mouse oviductal oocyte after the EZPT using bovine sperm. Note sperm only appear outside the zona. Scale bar for ZP pictures = 50 µm

Fig. 2. Co-incubation of empty ZPs obtained from bovine ovarian oocytes with oviductal fluid determines the specificity of spermatozoa capable of penetrating the zona. The empty zona penetration test (EZPT) was performed after homologous (bovine sperm) or heterologous (human sperm) fertilization. Similar outcomes were observed when ZPs were not treated with oviductal fluid (**A**), but after co-incubation with oviductal fluid for 30 minutes, human sperm were unable to penetrate the bovine ZPs (**B**) (6 replicates per medium per semen sample). **(C-I**) Representative pictures of empty bovine ZPs penetrated by bovine sperm illustrating that the penetrating sperm (**H**) lack an acrosome, while those unable to penetrate the zona maintain the acrosome (**I**). (**E-L**) Empty bovine ZP penetrated by human sperm revealing that the penetrating sperm have lost the acrosome (**K**), whereas those not penetrating the zona maintain the acrosome (**L**). In the absence of oviductal fluid, the empty bovine ZP can be penetrated by bovine (**C, G**) or human (**E, J**) sperm; however, when the ZP has been in contact with oviductal fluid, it can only be penetrated by bovine sperm (**D**), and not by human sperm (**F**). Scale bar for ZP pictures = 50 µm. Scale bar for sperm pictures = 5 µm.

Fig. 3. Structure of oviductin, Western blots of OVGP1 recombinants and localization of these recombinants at the bovine or murine ZPs. (**A**) Diagram showing the five regions (A, B, C, D and E) of the oviductin protein in human (hOVGP1), murine (mOVGP1), and bovine (bOVGP1) mammalian species (Figure taken from (*24*)). Regions A and D are conserved in the different mammals. Region A corresponding to the amino terminal end shows high identity in monotremes, marsupials and placentals. Region B shows a low identity among the different mammals and contains multiple

insertions/deletions. Region C is an insertion present only in the mouse. Region E is typical of human oviductin. (**B**) Western blots of the three OVGP1 recombinants used in this study (human, murine, and bovine). Proteins were expressed in mammalian cells, separated by SDS-PAGE and analyzed by immunoblotting using rabbit polyclonal antibody to the human OVGP1. Oviductal fluid of mice in estrus and anoestrus indicating the presence in estrous of the OVGP1 band. Oviductal fluid from ovulated cows or anoestrus cows. ZPs from IVM bovine (**C**) and murine (**D**) oocytes were incubated for 30 minutes at RT with bOVGP1, mOVGP1, and hOVGP1. ZPs were fixed and imaged by confocal fluorescence and DIC microscopy using rabbit polyclonal antibody to the human OVGP1 for bOVGP1, and a monoclonal antibody against Flag-tag for hOVGP1 and mOVGP1. Scale bars = 20 μm.

Fig. 4. Co-incubation of empty ZPs obtained from bovine ovarian oocytes with OVGP1 determines the specificity of spermatozoa capable of penetrating the zona. The empty zona penetration test (EZPT) was performed after homologous (bovine sperm) or heterologous (human or murine sperm) fertilization. Similar penetration rates were observed when ZPs were not treated with bOVGP1 (**A**), but after co-incubation with bOVGP1 for 30 minutes, human or murine sperm were unable to penetrate the bovine ZPs (**B**). A drastic reduction was also observed for fertilization by heterologous sperm, both in the average number of sperm penetrating the ZPs, and in the average number of sperm binding to the ZPs, when fertilization without pretreatment of the ZP with bOVGP1 (A) was compared to fertilization after the zona had been in contact with bOVGP1 (B). (6 replicates per medium per semen sample).

Fig. 5. Scanning electron micrographs (SEM) of the outer surface of the bovine ZP treated or not with OVGP1. The ZP of an in vitro matured (IVM) bovine oocyte: Magnification x2000 (**A**) and x4000 (**B**); and bovine oocytes IVM in the presence of bovine OVGP1 (bOVGP1): Magnification x2000 (**C**) and x4000 (**D**). High magnification reveals the ultrastructural characteristics of the ZP's pores on the IVM oocytes without bOVGP1 (**B**) and with bOVGP1 (**D**). Scale bars = 10 μm.

Fig. 6. Species-specific OVGP1 confers complete species-specificity to the zona pellucida. Experiment using the EZPT to analyze several combinations whereby ZPs from bovine and murine oocytes were co-incubated with bovine, murine, or human oviductin, and exposed to sperm of all three species. The variables ZP penetration rate, average number of sperm penetrating the ZPs, and average number of sperm bound to ZPs were examined using bovine ZPs (**A**) or murine ZPs (**B**) and combinations of the three oviductins with sperm of the three species. Only by combining ZP, OVGP1 and sperm of the same species, was species-specific fertilization possible. However, when the same species was matched only with the oviductin and sperm, or only with the ZP and sperm,

penetration and binding to the ZP of the spermatozoa also occurred, although in much smaller measure. No penetration was observed when only the ZP and OVGP1 species matched.

Fig. 7. Effect of neuraminidase (NMase) treatment of bovine or murine ZPs before and after contact with OVGP1 on sperm penetration. Penetration and sperm binding rates were measured after homologous EZPT with bovine sperm using ZPs not subjected (**A**) or subjected (**B**) to 30 minutes of co-incubation with bOVGP1. Penetration and sperm binding rates were measured after homologous EZPT with murine sperm using ZPs not subjected (**C**) or subjected (**D**) to 30 minutes of co-incubation with mOVGP1. ZPs of both species were co-incubated with acetate buffer (PH 4.5) at 38ºC for 18 hours in the presence or absence of neuraminidase diluted at 10 µ/mL.

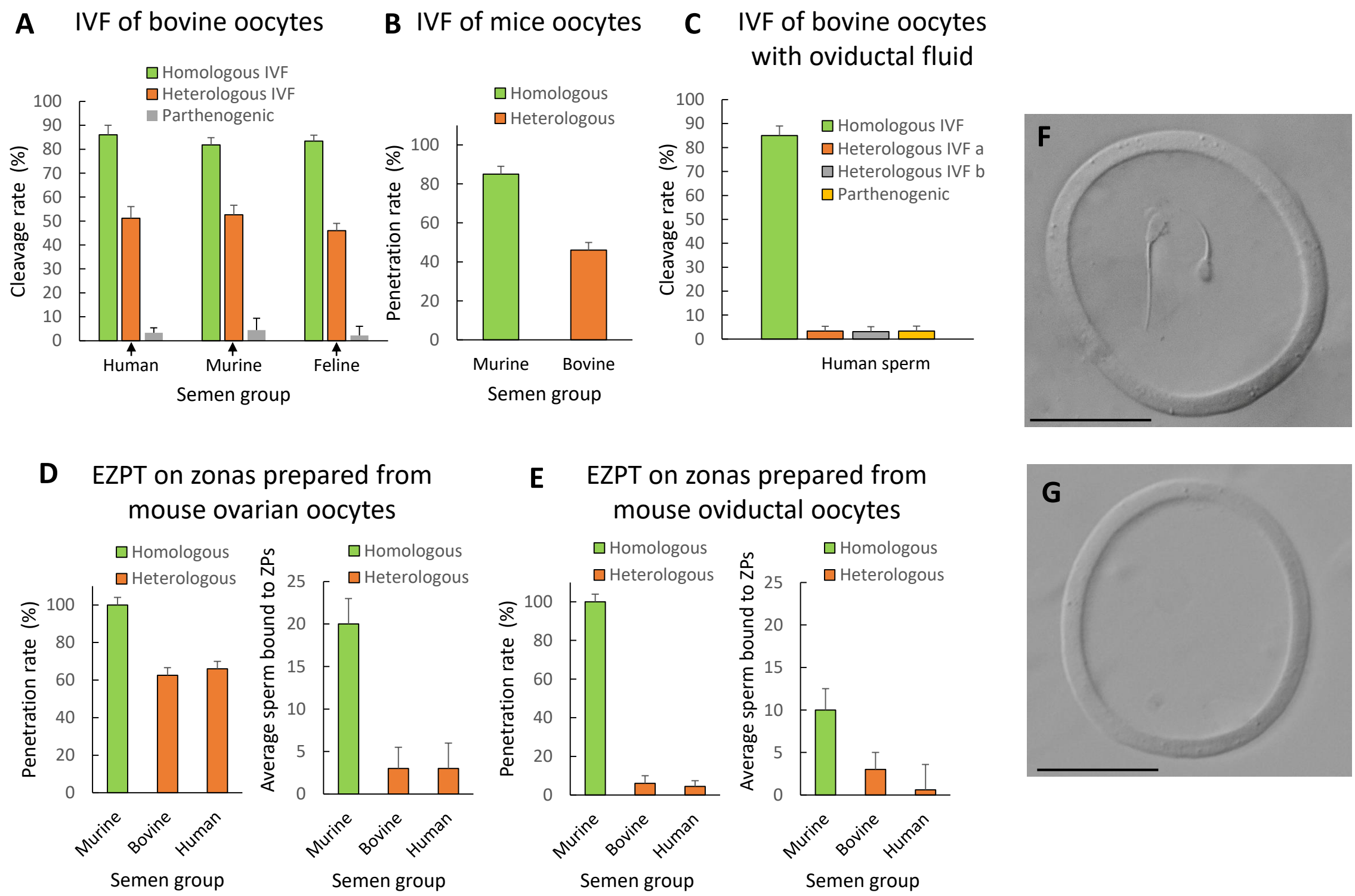

A
IVF of bovine oocytes
Homologous IVF
Heterologous IVF
Parthenogenic
Cleavage rate (%)
Human
Murine
Feline
Semen group
B
IVF of mice oocytes
Homologous
Heterologous
Penetration rate (%)
Murine
Bovine
Semen group
C
IVF of bovine oocytes with oviductal fluid
Homologous IVF
Heterologous IVF a
Heterologous IVF b
Parthenogenic
Cleavage rate (%)
Human sperm
F
G
D
EZPT on zonas prepared from mouse ovarian oocytes
Homologous
Heterologous
Penetration rate (%)
Average sperm bound to ZPs
Murine
Bovine
Human
Semen group
E
EZPT on zonas prepared from mouse oviductal oocytes
Homologous
Heterologous
Penetration rate (%)
Average sperm bound to ZPs
Murine
Bovine
Human
Semen group

Fig. 1

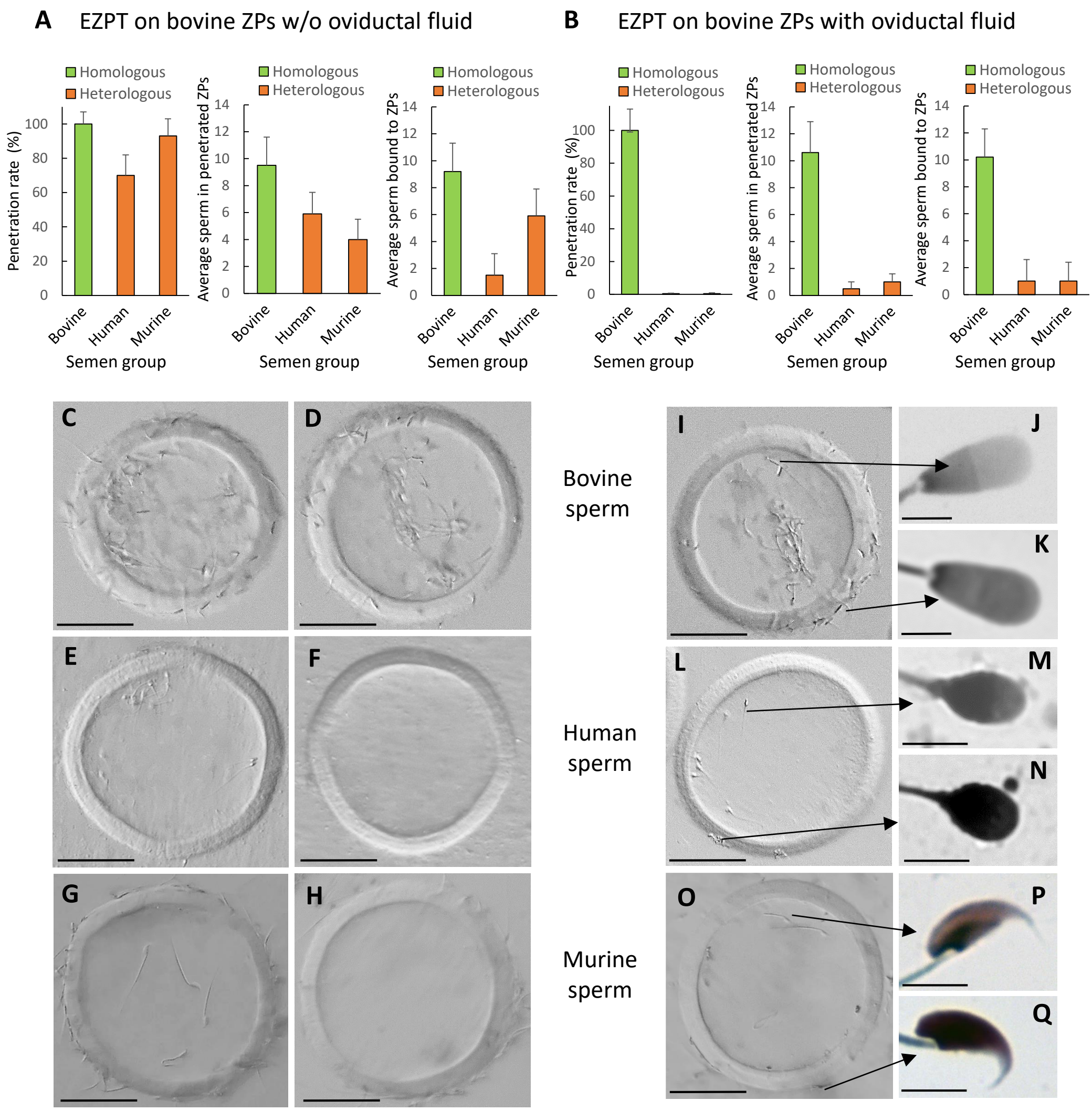

A
EZPT on bovine ZPs w/o oviductal fluid
B
EZPT on bovine ZPs with oviductal fluid
Homologous
Heterologous
Penetration rate (%)
Average sperm in penetrated ZPs
Average sperm bound to ZPs
Bovine
Human
Murine
Semen group
C
D
E
F
G
H
Bovine sperm
Human sperm
Murine sperm
I
J
K
L
M
N
O
P
Q

Fig. 2

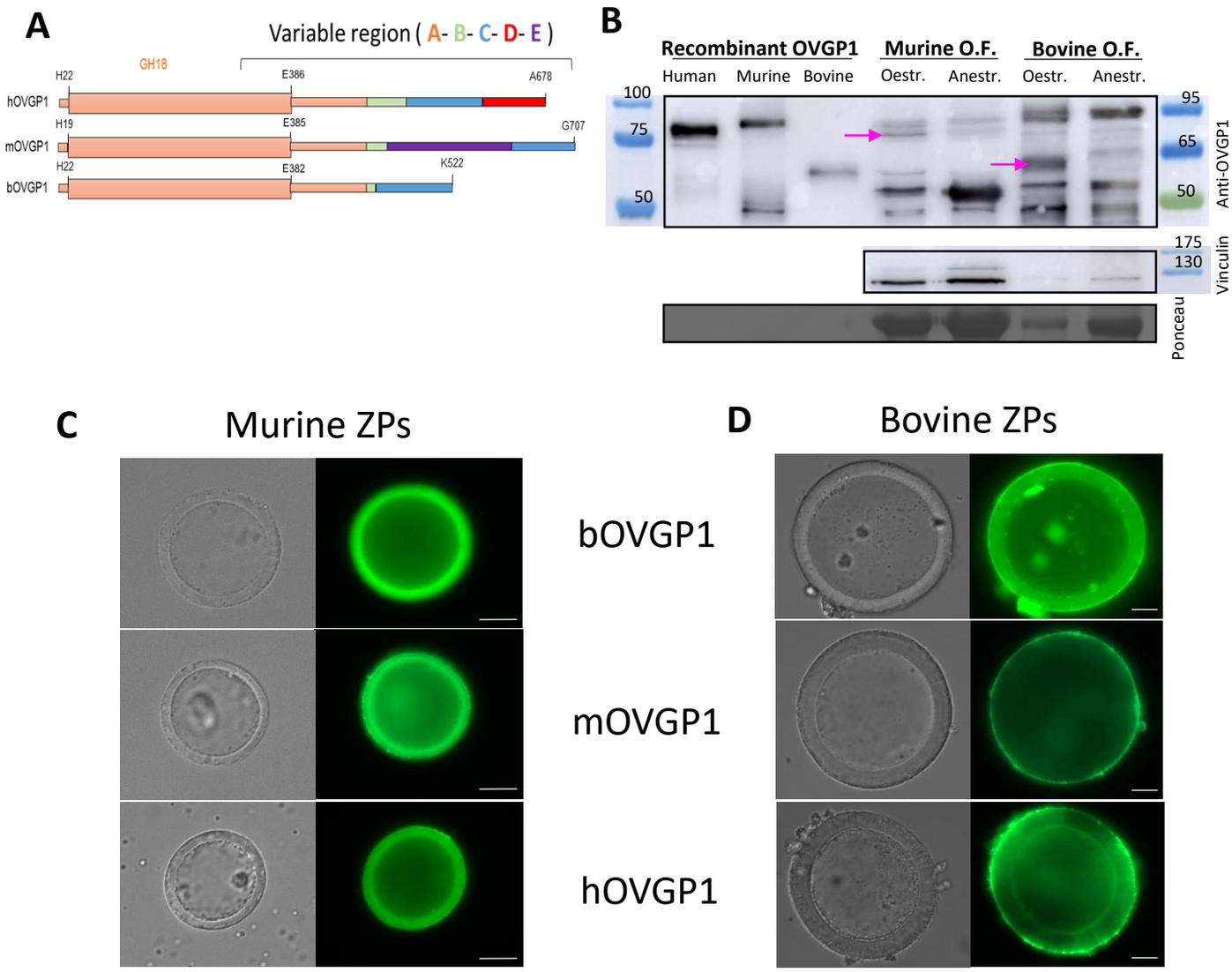

A
Variable region (A- B- C- D- E)
GH18
H22
E386
A678
hOVGP1
H19
E385
G707
mOVGP1
H22
E382
K522
bOVGP1
B
Recombinant OVGP1
Murine O.F.
Bovine O.F.
Human
Murine
Bovine
Oestr.
Anestr.
Oestr.
Anestr.
100
75
50
95
65
50
Anti-OVGP1
175
130
Vinculin
Ponceau
C
Murine ZPs
D
Bovine ZPs
bOVGP1
mOVGP1
hOVGP1

Fig. 3

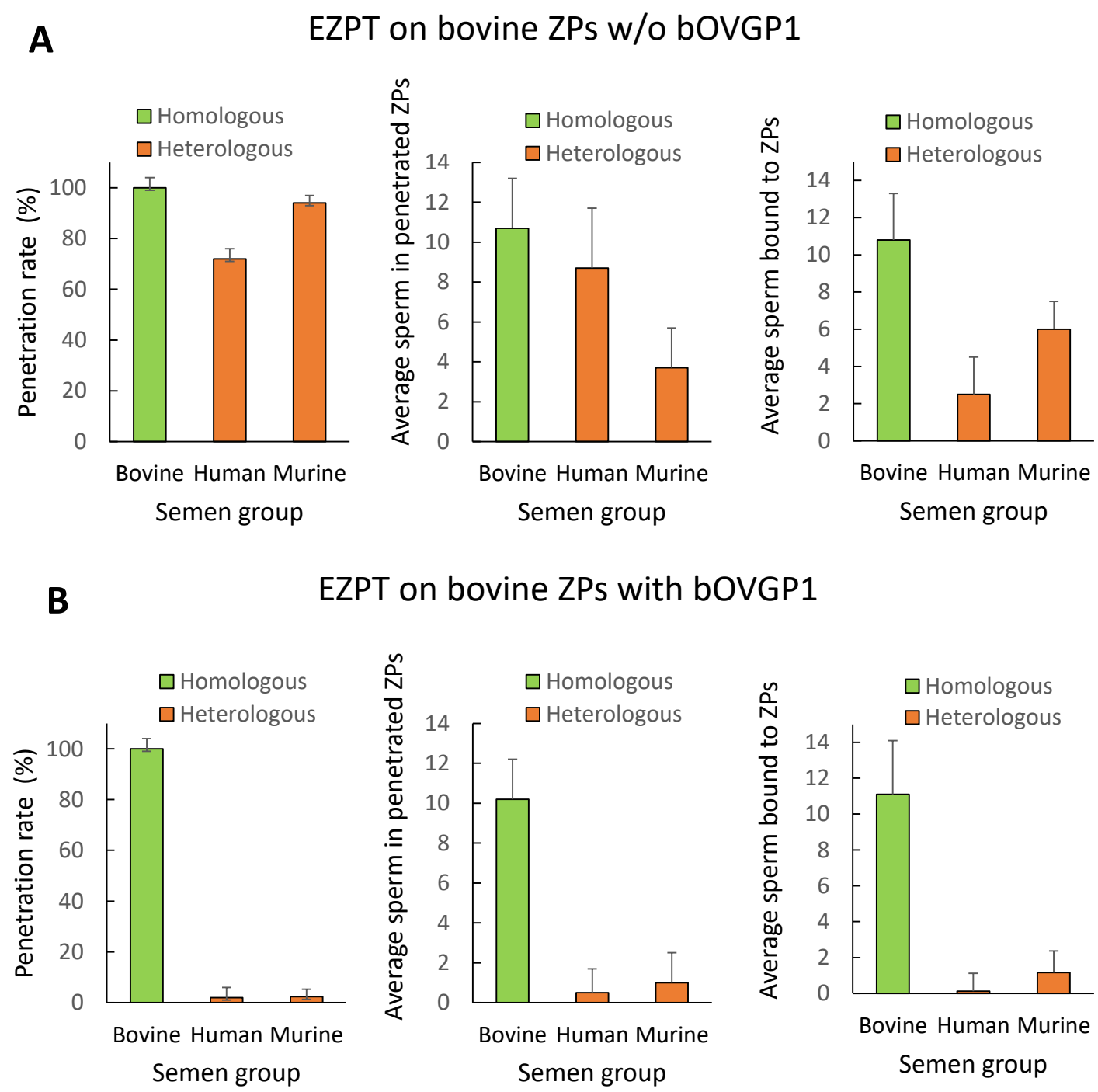

A
EZPT on bovine ZPs w/o bOVGP1
Homologous
Heterologous
Penetration rate (%)
Average sperm in penetrated ZPs
Average sperm bound to ZPs
Bovine Human Murine
Semen group
B
EZPT on bovine ZPs with bOVGP1
Homologous
Heterologous
Penetration rate (%)
Average sperm in penetrated ZPs
Average sperm bound to ZPs
Bovine Human Murine
Semen group

Fig. 4

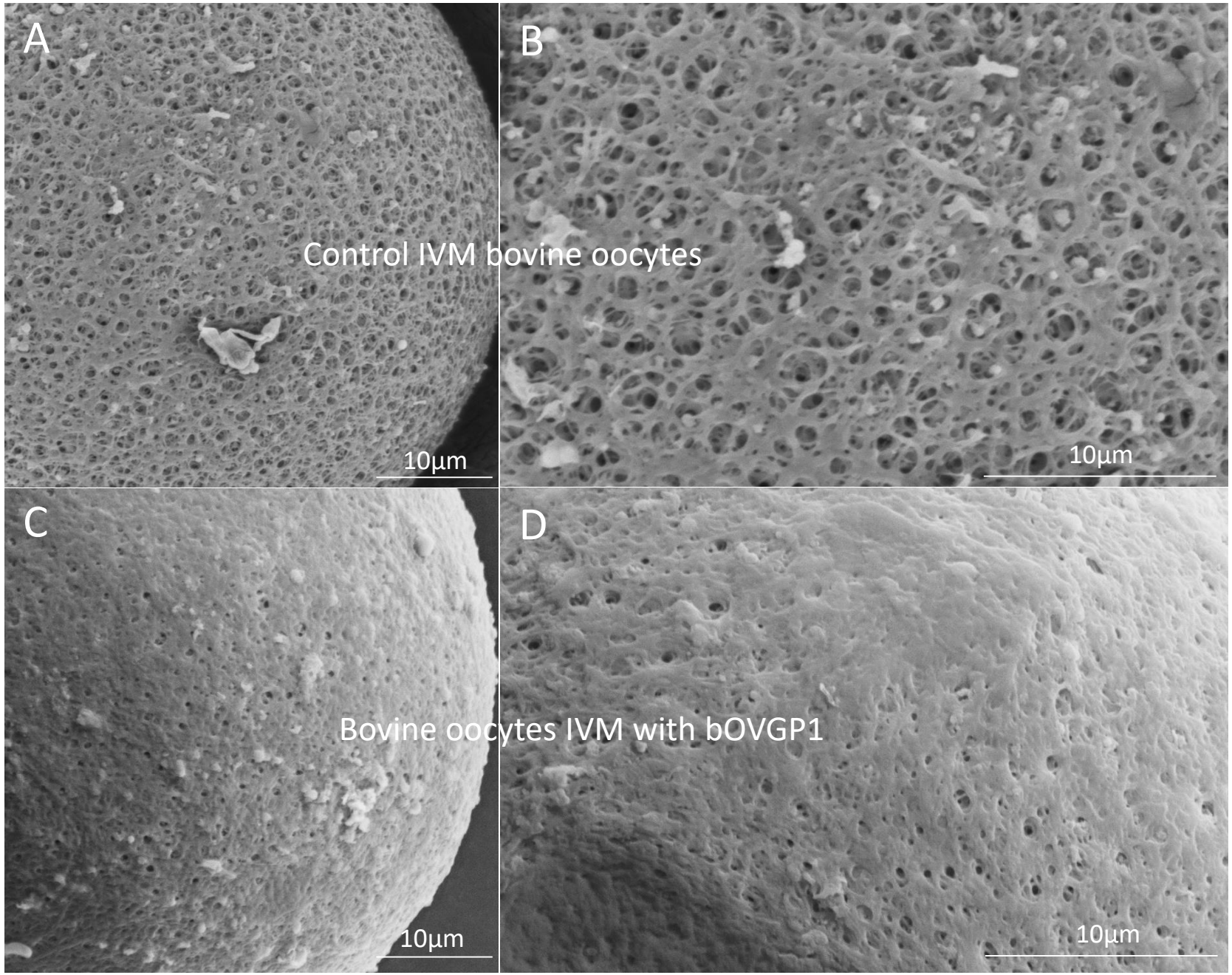

A
B
Control IVM bovine oocytes
10µm
10µm
C
D
Bovine oocytes IVM with bOVGP1
10µm
10µm

Fig. 5

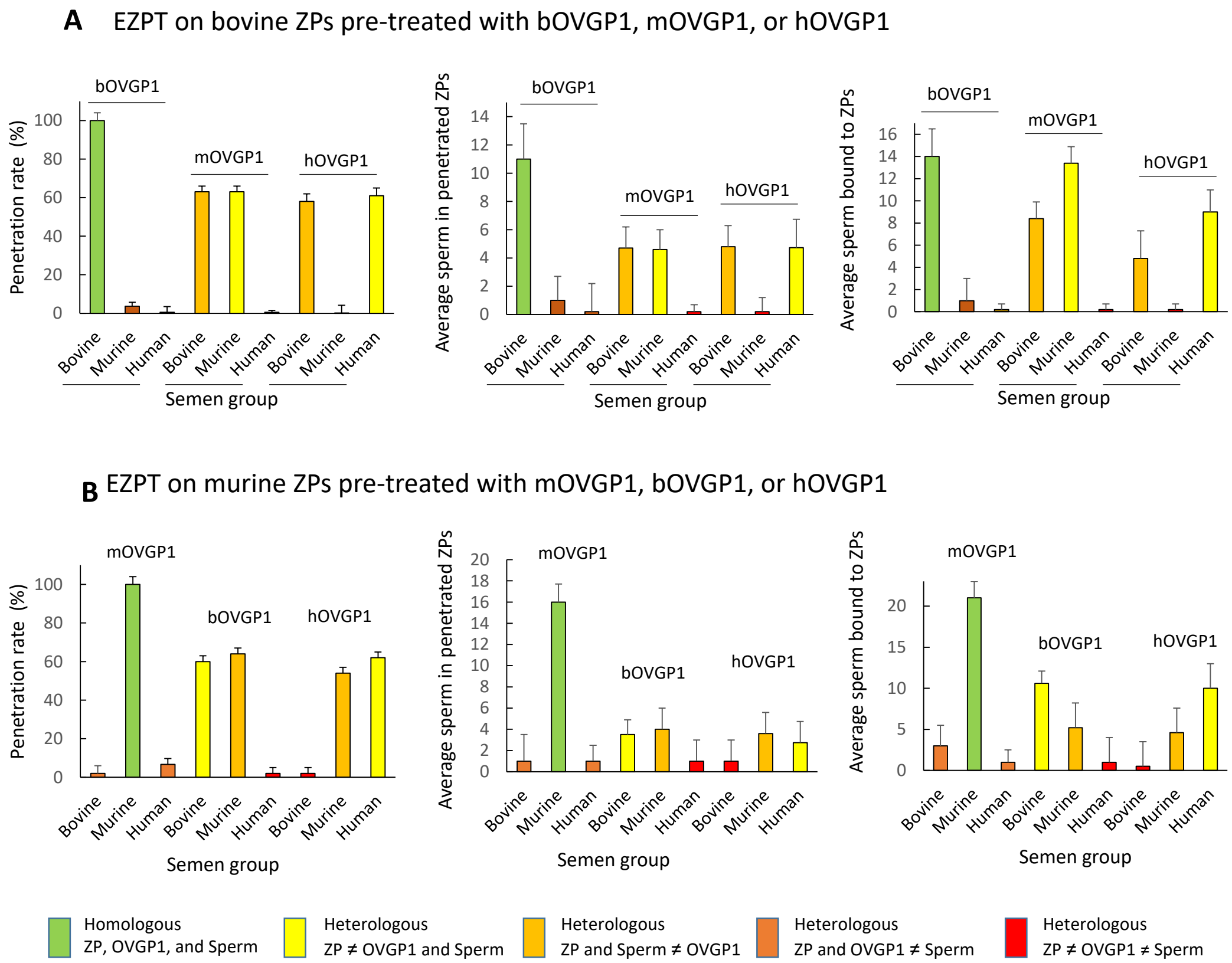

A EZPT on bovine ZPs pre-treated with bOVGP1, mOVGP1, or hOVGP1
Penetration rate (%)
Average sperm in penetrated ZPs
Average sperm bound to ZPs
bOVGP1
mOVGP1
hOVGP1
Bovine
Murine
Human
Semen group
B EZPT on murine ZPs pre-treated with mOVGP1, bOVGP1, or hOVGP1
Homologous
ZP, OVGP1, and Sperm
Heterologous
ZP ≠ OVGP1 and Sperm
Heterologous
ZP and Sperm ≠ OVGP1
Heterologous
ZP and OVGP1 ≠ Sperm
Heterologous
ZP ≠ OVGP1 ≠ Sperm

Fig. 6

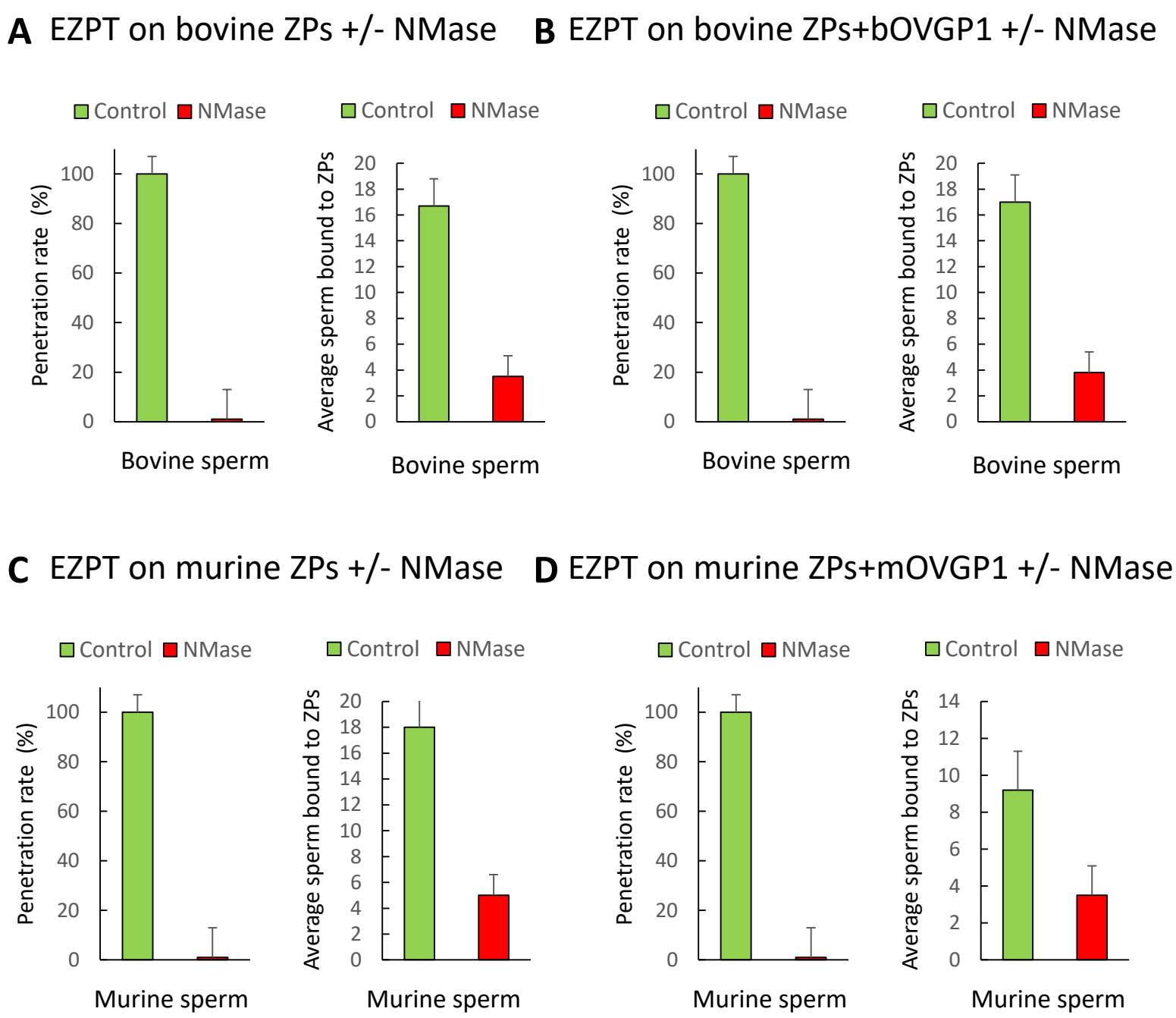
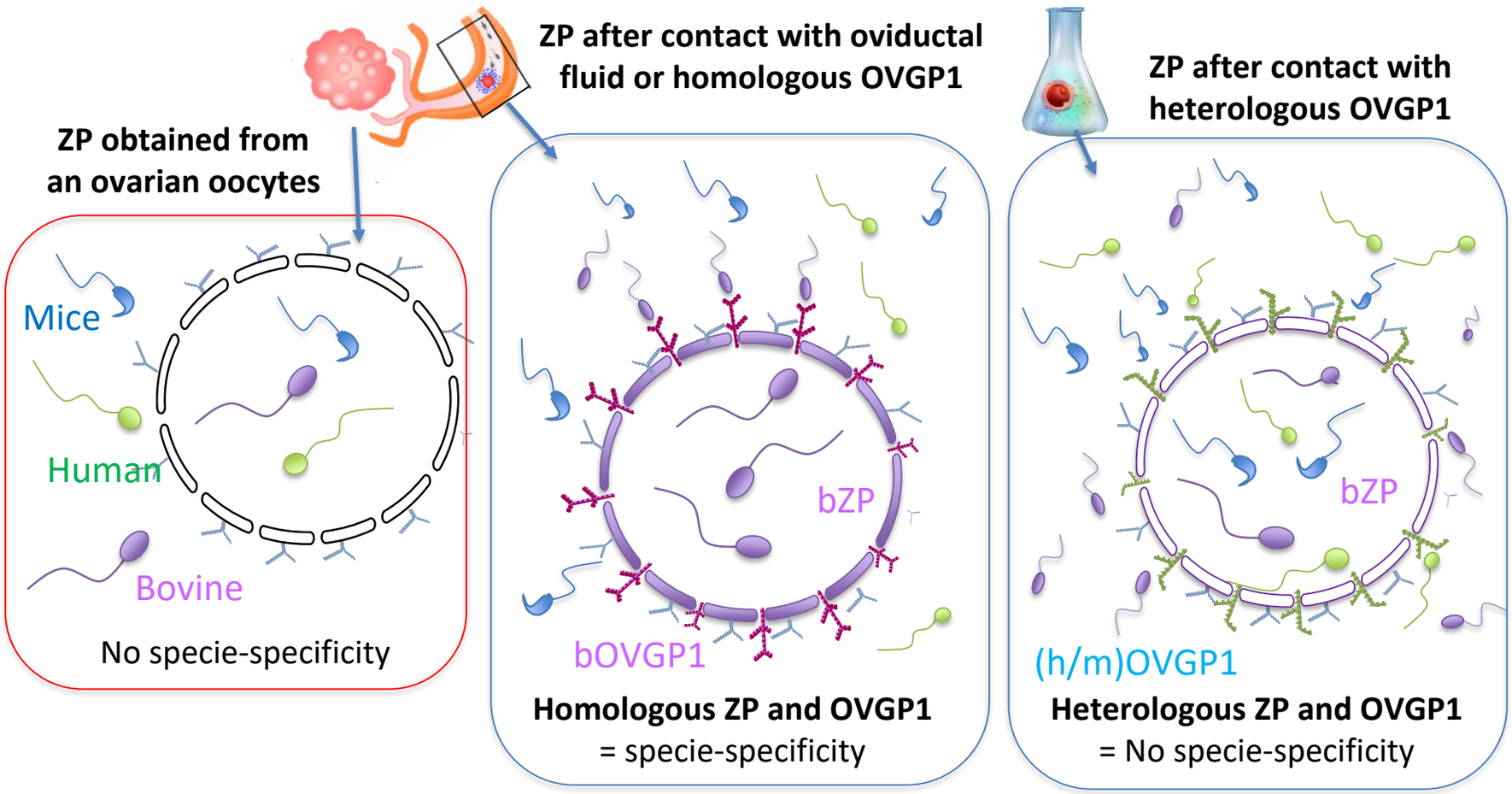

A EZPT on bovine ZPs +/- NMase
B EZPT on bovine ZPs+bOVGP1 +/- NMase
C EZPT on murine ZPs +/- NMase
D EZPT on murine ZPs+mOVGP1 +/- NMase
Control
NMase
Penetration rate (%)
Average sperm bound to ZPs
Bovine sperm
Murine sperm

Fig. 7


ZP obtained from
an ovarian oocytes
Mice
Human
Bovine
No specie-specificity
ZP after contact with oviductal
fluid or homologous OVGP1
bZP
bOVGP1
Homologous ZP and OVGP1
= specie-specificity
ZP after contact with
heterologous OVGP1
bZP
(h/m)OVGP1
Heterologous ZP and OVGP1
= No specie-specificity

Model