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The 3rd CZU Prague hybrid seminar "Biotechnology in small ruminant reproduction: an international experience"

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The Book Of Abstracts

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Dear colleagues,

Dear students,

We were very pleased to welcome you again, either online or in person, to the Czech University of Life Sciences Prague on the occasion of the 3rd Seminar dedicated to the topic of "Biotechnology in small ruminant reproduction: an international experience". We are delighted that many distinguished speakers responded favorably to our call for contributions! We thank all our invited speakers; we thank all the attendees of the seminar! We thank the Czech University of Life Sciences Prague, the Faculty of Agrobiology, Food, and Natural Resources, and the Department of Animal Science for providing the rooms and equipment for organizing the seminar. Furthermore, we would like to express thanks to the company Avantor, which again sponsored this event.

In conclusion, we do believe that our main aims (to give the younger generation of students and scientists an overview of the modern methods currently used in top-level laboratories around the world and to build even tight bonds between several laboratories working in a similar field) were successfully fulfilled, same as in previous years.

We thank you once again! We hope to see you next year to attend the planned 4th CZU hybrid seminar!

Sincerely,

Filipp Georgijevič Savvulidi & **Martin Ptáček,**

Seminar Organizing Committee.

Direct link to the web-page of the 3rd Seminar "Biotechnology in small ruminant reproduction: an international experience":

Abstracts

Title: **Endocrine and molecular basis of small ruminant sperm cell cryoresistance**

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AIM

Fertility results after artificial insemination using frozen-thawed spermatozoa are not always acceptable in small ruminants. The underlying molecular causes of sperm cryoresistance variations, depending on seasonality, endocrine status, sperm source, etc., are not well known. Acquiring a sound knowledge of the environmental and physiological causes that affect sperm cryoresistance will improve the effectiveness of cryopreservation techniques. The role of endocrine status (seasonal variations of testosterone, prolactin and thyroid hormones), and the sperm proteome has been studied in some wild and domestic species to better understand the underlying mechanism of the annual variation in ruminant sperm cryoresistance. Because some aquaporins (AQPs, membrane proteins involved with osmoregulation) may change their locations in different membrane domains of the sperm cell in response to different osmotic conditions, we have also examined whether differences in freezability could even involve changes in location and expression of AQP3, AQP7, AQP9 and AQP10 in small ruminants spermatozoa.

RESULTS

Changes in the sperm proteome concerning cryopreservation have been described in the wild ancestor of goat (ibex). Furthermore, seasonal changes in testosterone and prolactin plasma concentrations seem to contribute to different sperm cryotolerance in wild and domestic ruminants. Experimental modification of testosterone concentrations confirmed the negative effect of high testosterone levels on the sperm response to the freeze-thawing process, which explains the seasonal variations in sperm freezability, independent of the quality of the fresh sperm. In vitro supplementation with prolactin reduces the post-thaw acrosome integrity of ram and buck sperm. Induced high plasma prolactin concentrations by sulpiride administration seem to negatively influence sperm freezability. Together, these results suggest that high levels of prolactin negatively affect the cryoresistance. The implication of thyroid hormones in sperm cryodamage is not clear. Whereas in Gabon bucks there was no relationship between thyroxine concentrations and sperm cryoresistance, in Florida breed bucks, a negative correlation was found between plasma thyroxine concentrations and the cryoresistance ratios for many sperm variables. The negative influence of testosterone on sperm cryoresistance might be mediated, at least in part, by AQP3, AQP7, and AQP10 expression in the acrosome and midpiece during the rutting season. Immunolabeling of AQP3 in the midpiece of freshly ejaculated from rams was lower in samples displaying good freezability than in poor freezability samples.

CONCLUSION

The donor endocrine status at the moment of sperm collection and changes in the sperm proteome according to the sperm source appear to explain changes in the sperm cryoresistance of small wild ruminants. Our results also confirmed changes in AQP3 relocalisation could be linked to an increase the osmo-adaptative capacity of ejaculates with better capacity to withstand freeze-thawing processes, suggesting AQP3 expression could be used as a biomarker for potential sperm freezability in small ruminants.

Keywords: ram, goat, wild ruminants, aquaporins, testosterone, prolactin

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ACKNOWLEDGMENT: This work was supported by PID2020-113288RB-I00/AEI/10

Presentation available:

Title: **Coping with the cold: how does the ovine cervix respond to frozen sperm?**

Names: **J. P Rickard[1], S. Warr[1], S. P. de Graaf[1] and T. Pini[2]**

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AIM

The fertility of frozen ram sperm is limited following cervical artificial insemination due to its inability to successfully penetrate the ewes' cervix. However, if deposited in the uterus, its fertility is not compromised. The failed ability of frozen sperm to cross the ewe's cervix is thought to be related to freezing induced changes to sperm surface characteristics previously modified by seminal plasma at ejaculation. To date, extensive work has focused on characterising proteomic sperm traits prior to and following seminal plasma exposure and in vitro processing. However, it remains unclear how cervical tissue responds to different sperm phenotypes, and whether there is a transcriptomic response impeding cervical transit. As such, an ex vivo cell culture model was used to assess the transcriptomic response of cervical endometrial explants harvested from the reproductive tract of oestrus-synchronised ewes (*n*=6) to; epididymal sperm, epididymal sperm exposed to seminal plasma, frozen-thawed sperm and seminal plasma alone from rams (*n*=3).

RESULTS

Explants were co-cultured under CO_2 for 6h. Analysis of differentially expressed genes by RNA sequencing revealed that sperm with exposure to seminal plasma significantly activated pathways related to integrin cell surface and extracellular matrix signalling, compared to epididymal sperm alone. Exposure of cervical explants to cryopreserved sperm activated NFKB signalling, multiple interleukin signalling pathways (IL-1, 2, 6, 8 and 15), miRNA biogenesis and estrogen receptor signalling.

CONCLUSION

These results suggest that cervical gene expression is altered in response to spermatozoa and seminal plasma, and that cryopreservation may further modify this interaction. Identifying pathways of interest could highlight potential supplements for frozen sperm which could improve their ability to transit the cervix and achieve fertilisation following cervical artificial insemination.

Keywords: Cryopreservation, ram, cervical artificial insemination, reproductive technologies

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The authors would like to acknowledge Sydney Informatics Hub, The University of Sydney for their assistance with transcriptomics and QCIF for its support in this research.

Title: **The melatonin receptor mt1 in rams as a tool to improve reproductive parameters of the sheep flock**

Names: **Pérez-Pe, R*.; Peña-Delgado, V.; Noya, A.; Canto, F.; Carvajal-Serna, M.; Casao, A. and Abecia, J.A.**

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AIM

Melatonin in mammals is a molecule with hormonal properties and has multiple functions. In some species, such as ovine, it is also involved in the control of reproductive seasonality. This seasonality is a limiting factor that affects the productive potential of most sheep breeds, although it is less marked in the ram than in the ewe, and sperm production is continuous throughout the year. However, during the non-reproductive season, there is a reduction in sexual behavior, a decrease in testicular weight and volume, in sperm quality and changes in the seminal plasma composition. Although melatonin can exert its actions by directly crossing the cell membrane, some are mediated by its binding to specific receptors, MT1 and MT2. The MT1 receptor appears to be involved in the seasonality control in several species, including the sheep. The gene coding for MT1 is the *MTNR1A* and has two exons divided by a long intron. Several polymorphic sites related to certain reproductive traits have been found in Exon II, such as *Rsa*I and *Mnl*I. In *Rsa*I, a C is substituted by a T, and in *Mnl*I, a G is substituted by an A. So, animals with C/C, C/T or T/T genotypes based on the first polymorphism, and G/G, G/A or A/A based on the second polymorphism can be found. In ewes of several breeds, some of these genotypes have been related to a shorter anestric period, different responses to melatonin treatments and higher fertility rates. So, the relationship between the *MTNR1A* polymorphisms and the reproductive performance in ewes has been thoroughly tested. However, the available information on the ram is scarce. Therefore, the objective of our last research project focused on studying the influence of these polymorphisms on ram reproduction.

RESULTS

In the Rasa Aragonesa breed, T/T (*Rsa*I) and G/G (*Mnl*I) seem to be related to a less marked reproductive seasonality in rams since lambs born in autumn carrying one of those genotypes have a greater ability to reproduce in their first spring and adult T/T or G/G rams exhibit a more intense reproductive behavior also in spring. On the other hand, for the *Rsa*I polymorphism, TT rams led to a lower fertility rate than CC and CT, with no differences between these two genotypes. For *Mnl*I, significant differences were observed between genotypes, with AA being the one that was associated with the lowest fertility and GA with the highest. Significant differences among genotypes were observed only during the reproductive season. When seminal quality was evaluated, TT rams for *Rsa*I and AA for *Mnl*I polymorphisms presented lower quality, particularly during the reproductive season.

CONCLUSION

The genotyping of rams based on melatonin receptor 1 (MT1) gene polymorphisms could be a valuable tool for more correct and rational use of animals in farming.

Keywords: ovine, polymorphisms, *MTNR1A* gene, seasonality, seminal quality, fertility.

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Presentation available:

Title: **Improvement strategies in ovine artificial insemination: the moroccan case**

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AIM
The aim of this abstrct is to explore in detail the most factors and challenges facing the success of ovine artificial insemination and to suggest strategies and best practices for its improvement. The Moroccan case will be presented as an example.

RESULTS
Artificial insemination (AI) is crucial in sheep breeding programs. However, due to irregular and low fertility results, as well as difficulties in using enhancements like frozen-thawed sperm, this technology is not as widespread compared to that in other domestic species. In many cases, the AI is judged based on the fertility rate, while it has to be considered as a complex equation. It includes endogenous factors such as breed, age, fertility traits, genetic disorders, and cervical anatomy in ewes that togather should be considered while evaluating the AI success. Besides, exogenous factors, including techniques like estrus induction, synchronization, semen handling methods (fresh, chilled, or frozen), insemination time, and methods (cervical or laparoscopic), could be also implicated in the outcome of AI. Moreover, the present work highlights the environmental factors and climatic conditions directly impacting the success of AI. The Moroccan case has been presented as a successful example, as the performed trials led to good fertility rates (60 to 70%). As good strategies, overcoming the anatomical obstacles of the ovine cervix and improving fertility rates with frozen-thawed semen are crucial for the widespread adoption of AI in commercial breeding programs.

CONCLUSION
Continued research and optimization of techniques, such as combined protocols using modified catheters and dilator substances and producing new extenders to maintain semen quality for a long time, may pave the way for more efficient and practical artificial insemination in sheep and improve fertility rates.

Keywords: Artificial insemination, sheep, factors, challenges, improvements

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ACKNOWLEDGMENT: The author thank all PhD students, farmers and organizations that largely contributed to generate the knowledge presented in this work.

Presentation available:

Title: **Photoperiodic treatments to control seasonal sperm production in sheep and goats**

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AIM

Seasonality is a major drawback for sheep and goat artificial insemination (AI) centers which face seasonal sexual rest of males. Under the inhibitory influence of natural photoperiod, from end winter to end summer the hypothalamus-pituitary-gonadal (HPG) activity decreases. The low yield of spermatogenetic processes and the low stimulation of intra-testicular Leydig cells provoke this dramatic decrease in male sexual activity. Thus, males are difficult to collect with the artificial vagina, they produce less semen and its quality and fertilizing ability is severely impaired. However, spring and summer are generally the main seasons when farmers want to fertilize their ewes and goats by AI.

RESULTS

Different artificial photoperiodic treatments are available to control seasonal sperm production in both species. All are based on the same principles of specific alternations between long days (LD) and short days (SD). They can be applied in light-proof or in open barns and can be divided in two main groups relatively to their objective: (a) semen during a relatively brief period in spring and/or summer, or (b) semen all the year round.

(a) For a short period of high sexual activity in open barns, it is necessary to provide 2 months of LD, (16h) by a simple additional lighting during winter (i.e. Nov.-Dec.) then leave males under natural photoperiod where they perceive SD. This triggers a “short sexual season” (high and good semen production, testosterone, libido and odor) which starts about 30 (rams) or 45 (bucks) days after the end of LD and continues for about two months. The number of AI doses is increased and the fertility rates of ewes is increased of about 10 points. When season advances, it may be necessary to use either melatonin implants or permanent light (prohibited in Europe) after the end of LD. This light-schedule LD->SD can also be used in light-proof buildings.

(b) For obtaining semen all the year round, permanent and rapid alternations between LD and SD (1 month LD/ 1 month SD) completely prevent the seasonal inhibition of HPG activity and allow rams and bucks to maintain a high sexual activity (semen production, testosterone and libido) even during the usual season of sexual rest. Rams and bucks submitted to such treatments in light-proof buildings produced semen all the year round allowing a tremendous increase in the total AI doses produced compared to males left under natural photoperiod. They were applied for at least three consecutive years without any negative effect on the animals. They are used since > 20 years in the only AI center in France that produces goat semen. Very recently it has been demonstrated that bucks receiving in open barns the alternation 1 month LD/ 1 month permanent light experienced the same results as above.

CONCLUSION

Alleviating seasonality of reproductive activity in rams and bucks in AI centers is now possible by either inducing a short artificial breeding season during spring and/or summer, or completely suppressing the usual rest season.

Keywords: seasonality, photoperiod, libido, sires, bucks, rams

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Presentation available:

Title: **Alternative techniques for preservation of small ruminant spermatozoa without liquid nitrogen**

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AIM

To date, the most widely utilized method worldwide for sperm preservation is cryopreservation using liquid nitrogen (LN). A dose of frozen semen maintains viability for several decades and, once thawed, remains capable of producing offspring. However, LN production carries a significant environmental footprint, prompting exploration into alternative methods such as spermatozoa freeze-drying.

RESULTS

Although freeze-drying of spermatozoa presents itself as a potential alternative to cryopreservation, current findings reveal that rehydrated spermatozoa remain immotile. However, in small mammal species like mice and rats, these spermatozoa have demonstrated the ability to produce offspring via ICSI fertilization.

In contrast, freeze-dried ram semen lacks the capacity to independently activate oocytes. Consequently, chemical activation of the oocyte is necessary to achieve embryo development up to the blastocyst stage. Ongoing research aimed at refining freeze-drying techniques has culminated in the elimination of liquid nitrogen from the process. This refinement has led to notable advancements in embryonic development, with blastocyst attainment reaching 13%.

Moreover, alternative freeze-drying methodologies, like spin-drying, have demonstrated enhanced preservation of sheep spermatozoa in an anhydrous state. These spermatozoa have shown the capability to generate embryos at the pre-implantation stage even after two years of storage at room temperature.

CONCLUSION

As of now, freeze-drying of seeds stands as a viable alternative to storage in liquid nitrogen. This topic warrants increased attention, given the limited scope of scientific efforts thus far, largely confined to small mammals.

Keywords: freeze-dry, ICSI, Ram, Spermatozoa, DNA fragmentation
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Presentation available:

Title: **Antifreeze proteins: potential cryoprotectant of gametes and embryos from small ruminants**

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AIM
One of the main challenges in cryopreservation is the reduction of cryodamages that may occur during the preservation of gametes and embryos. In this sense, several substances, such as the antifreeze proteins (AFPs), have been tested to improve cryopreservation outcomes. The AFPs are a subgroup of ice-binding proteins identified first in some antarctic fishes. Although a vast diversity of AFPs exist, with variable structural characteristics, these proteins act by reducing the freezing point below the melting point, in a thermal hysteresis gap, and ice recrystallization inhibition. Based on the results achieved over the last decades, supplementing these proteins in different protocols could be considered a potential strategy to mitigate cellular damage that may occur in cryopreservation. Thus, here we compile the main advances in the scientific literature regarding the supplementation of different antifreeze proteins in the cryopreservation of gametes and embryos in small ruminants.

RESULTS
It has been demonstrated that 0.1 µg/mL of AFP type I has beneficial effects in sheep semen, enhancing sperm kinetics, plasma membrane integrity, and sperm morphology, but does not appear to increase ejaculate cryoresistance. In goat semen, the addition of 1 µg/mL of AFP type III AFP was capable of enhancing the post-thaw motility, membrane and acrosome integrity, and reducing DNA damage in sperm. For oocytes, results are scarce, however, it has already been demonstrated that immature ovine oocytes vitrified in the presence of 0.25 µg/mL of AFP type I can maintain a molecular pattern similar to that of non-vitrified oocytes. For ovine embryos, the supplementation of 10 µg/mL ApAFP914 can increase the hatching rate of vitrified embryos, while, 0.1 µg/mL of AFP type I in slow freezing solution has been demonstrated to enhance mitochondrial activity *in vitro*.

CONCLUSION
The addition of different AFP types at low concentrations can be a potential strategy for cryopreservation of small ruminant gametes and embryos. However, some points still need to be clarified regarding the outcome of the cryosurvival from small ruminant gametes and embryos.

Keywords: cryodamage, goat, ice crystal, preservation, sheep

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ACKNOWLEDGMENT: FAPERJ and CNPq.

Presentation available:

Title: **Use of H_2S to improve ram sperm cryopreservation: preliminary results**

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AIM

Since the use of the gasotransmitter hydrogen sulphide (H_2S) has been reported as an innovative tool to protect sperm against oxidative stress due to its effect mediated by persulfidation of the enzymes involved in the antioxidan response, it has become essential to understand whether H_2S plays a protective role in ram sperm function against oxidative stress caused by the cryopreservation process. Thus, to address this general aim, three specific objectives were proposed, consisting of: (1) to characterize the presence and location of H_2S-releasing enzymes (cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH), and 3-mercaptopyruvate sulfurtransferase (MST)) in ram testes and spermatozoa, (2) to assess H_2S supplementation on fresh ram sperm quality and (3) to assess H_2S supplementation after cryopreservation on ram sperm motility.

RESULTS

The histological and molecular assays carried out (immunofluorescence, immunocitochemistry and western blot) characterize for the first time the presence and location of H2S-releasing enzymes in testes and epididymal spermatozoa of ram.

Moreover, exogenous supplementation of fresh sperm with H_2S-donor significantly ($p<0.05$) reduced ROS levels when used at concentrations of 15 μM and 50 μM at different incubation times (5, 30, 60 and 120 minutes) while it increased significantly ($p<0.05$) the sperm population with intact acrosomes as well as the population with active mitochondria.

On the other hand, when H_2S was added to frozen-thawed ram sperm, 3 sperm subpopulations were identified based on objective motility parameters: fast and linear sperm (SPI), slow and non-linear sperm (SPII) and slow and linear sperm (SPIII). Furthermore, compared to the control, this exogenous supplementation on cryopreserved samples significantly improved ($p<0.05$) the percentage of sperm belonged to SPI with concentrations of 2 μM and 15 μM of H_2S-donor after 30 min of incubation, whereas decreased percentage of sperm from SPII.

CONCLUSION

These findings suggest that the gasotransmitter H_2S plays a key role in the male reproduction of small ruminants. Its supplementation on ram sperm, applied before or after cryopreservation,

is expected to improve sperm quality and, subsequently, *in vitro* and *in vivo* fertilization capacity, protecting cells against oxidative stress and increasing the fast and linear spermatozoa.

Keywords: hydrogen sulphide, oxidative stress, ROS, small ruminants

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Presentation available:

Title: **Molecular and biochemical factors contributing to ewe breed differences in cervical sperm transport**

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AIM
In sheep, both vaginal and cervical artificial insemination (AI) with frozen-thawed semen has consistently yielded unacceptably low pregnancy rates worldwide. The exception is in Norway where vaginal AI to a natural oestrus yields non-return rates in excess of 60%, which has been attributed to the ewe breed used there. In this work, we used a novel sheep model consisting of four ewe breeds with known differences in cervical sperm transport following cervical AI with frozen-thawed semen to interrogate molecular and biochemical markers in the cervix and its secretions that are responsible for cervical sperm transport between ewe breeds.

MATERIAL AND METHODS
Cervical mucus (n = 28 to 30 ewes/breed) and cervical post mortem tissue samples were collected from two Irish ewe breeds (Belclare and Suffolk; medium and low fertility, respectively) and from two Norwegian ewe breeds (Norwegian White Sheep (NWS) and Fur; both high fertility compared to both Irish breeds) at the follicular phase of both a natural and synchronised oestrous cycle (3 replicates/ewe). The biochemical characterization of the cervical mucus was performed by ultra-performance liquid chromatography (UPLC) and mass spectrometry (MS)/MS methods. High quality mRNA extracted from cervical biopsies was analysed by RNA-sequencing following which differential gene expression and gene ontology (GO) were assessed. Finally, to study the possible effect of the cervical microbiome on sperm transport, cervical swabs were analysed by 16S rRNA gene sequencing on an Illumina MiSeq platform.

RESULTS
We identified for the first time the main *O*-glycans in sheep cervical mucus and more specifically differences in sialylated glycans between breeds. The low fertility Suffolk breed had higher levels of sialic acid compared to high fertility NWS and Fur ewes. Using RNA-sequencing, we also detected higher expression of sialic acid related genes in the cervical epithelium of the Suffolk. In addition, there was a more active immune response in Suffolk which differed significantly to the other ewe breeds. This molecular signature was evident in the metabolomic composition of cervical mucus, presenting reduced levels of lipids associated with the resolution of inflammation, and an elevated number of amino acids derived from mixed anaerobic bacteria in the Suffolk breed. Regarding the cervical microbiota, we also identified higher microbial abundance and diversity in the low fertility Suffolk breed compared to high fertility breeds.

CONCLUSION

The results of this study indicate that compromised cervical sperm transport in the low fertility Suffolk breed following cervical AI using frozen-thawed semen is due to a combination of higher content of sialic acid in mucus, a pro-inflammatory cervical enviroment and a higher bacterial load.

Keywords: cervix, ovine, sperm interaction.

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Presentation available:

Title: **Molecular aspects of fertilization**

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AIM

In my presentation I will give an overview of the processes that take place at the molecular level in the membranes of the sperm cell and the oocyte in the ferilization cascade. Mostly this journey has been studied in the pig as model species. We also work on rodents, small and large ruminants, cats, dogs and primates as other mammalian species.

THE FERTILIZATION CASCADE FROM A CELLULAR MEMBRANE PERSPECTIVE IN GAMETES

Once a sperm cell is released from the Sertoli cell into the lumen of the seminiferous tubule it has terminated a number of cellular processes such as transcription and translation, vesicular mediated transport, also the endoplasmic reticulum, Golgi complexes, peroxisomes, ribosomes and the majority of the cytoplamsma has been removed. The testicular sperm cell will start a journey through the male and genital tract where the diverse epithelia and secretion as well as adsorption of factor to and from sperm cells will control their sequence of chainges that are characterisc for mammals.

1. In the epididymis sperm cells will as a consequence change their metabolism which reults in the generation of sperm motility. During this maturation process sperm also will shed of the cytoplasmic droplet (a remainder of the cytoplasmic bridges that synchronized the elongation of spermatids). In the cauda epididymis sperm will also attract zona binding proteins to their surface and thus acquire fertilization properties.
2. During ejaculation epididymidal sperm and fluids will be mixed with screte fluids from the diverse accessory sex glands and pumped out of the male into the female. The added fluids contain nutrients, innate defense molecules and mucus like factors that stabilize sperm cells and making them stealth for white blood cells in the uterus.
3. When sperm cells reach the oviduct they first shed of these stabilizing factors and swim into the oviduct fluid, where sperm will first bind to the oviduct epithelium and after some hours will become capacitated. Typically such cells have a hyperactivated motility and certain surface alterations enable the sperm to interact appropriately with the cumulus oocyte complex.
4. At the apical ridge area of the sperm head a set of zona pellucida binding proteins concentrate and they are needed for the recognition of the zona pellucida (primary zona binding). The phenomenon coincides with the docking of the sperm plasma membrane and the outer acrosomal membrane at the apical ridge area of the sperm head.
5. Once the sperm cell binds to the zona pellucida the acrosome reaction (acrosomal membrane fusions) will be induced (and thus enable sondary zona binding and local lyusis of the zona matrix), while the hyperactive sperm can immediately start to drill through the zona pellucida.
6. After drilling through the zona pellucida the sperm cell will orient in parallel to the oolemma where the equatorial area of the sperm head is able to bind and fuse with the oocyte (fertilization fusion). A specific structure of 50 nm diameter and 1 μm fingershaped structures are formed here between plasma membrane and outer acrosomal membrane and are likely involved in this ferilization fusion.

7. The sperm internal (cytosolic) structures are now exposed to the interior milieu of the oocyte. Especially the high reducing capacity of the oocyte (in sharp contrast to that of the sperm cell) allows solubilization of inert condensed protein structures of the sperm cell: the perinuclear theca, outerdense fibers and the fibrous sheath. Our current research focusses on the post-fertiolization release effects of perinuclear proteins that in the oocyte activation, the removal of intra-oocyte sperm structures and the involvement in paternal genom epigenetic imprinting.

CONCLUSION

Likely, the exact timing and organization of these processes will be different in small ruminents but in gross similar processes will be relevant and crucial for the fertilization and activation of small ruminant eggs.

Keywords: spermatozoa, oocyte, gamete membranes, adhesion, secretion, fusion

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Presentation available:

Title: **The female immune response to the presence of spermatozoa in the swine model: scientific inspiration for research in small ruminants**

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AIM

The study presented here summarizes pre/peri-ovulation expression changes of immune-related genes along the internal female genital tract (cervix to infundibulum).

MATERIALS AND METHODS

Tissues were collected 24 h after either (i) mating, (ii) cervical insemination of P1-semen extended in Beltsville Thawing Solution (BTS) or (iii) cervical deposition of sperm-free seminal plasma (SP) from the P1-fraction of the ejaculate, or of the (iv) whole SP (from the entire ejaculate) and assayed against untreated controls. Equal amounts of total RNA (Trizol, 250 ng) from each sample were used to make cDNA using GeneChip®Whole Transcript Plus reagent kit (Affymetrix) following the manufacturer protocol, until loaded on the array chip (GeneChip® Porcine Gene 1.0 ST Array, Affymetrix), subjected to washing and staining using a GeneChip® Fluidics Station 450 (Affymetrix), to be finally scanned using the Affymetrix GeneChip® scanner GCS3000. The intensity data of each array chip was processed using the robust multi-array average (RMA) normalization, computing average intensity values by background adjustment, quantile normalization among arrays and finally log2 transformation for extracting the expression values of each transcript in the probe set, as implemented in the official Transcriptome Analysis Console (TAC v4.0, Affymetrix).

RESULTS

Mating was the treatment which, along the tract, had the highest effect on immune-related genes, even when compared to the P1-AI treatment. Of the 154 annotated immune-related genes (excluding repeating genes in all tissues), 117 of them (65%) were up- or down-regulated ($p < 0.05$) 24 h after treatment, as compared to controls. Comparing the presence of semen (entire ejaculate or only the P1-fraction) with the sperm-free SP infusions, it was evident that the latter induced the expression of fewer genes (35 genes differentially expressed in SP-Ejac (17 up-regulated and 28 down-regulated) and 75 genes differentially expressed in SP-P1 (26 up-regulated and 49 down-regulated)). Of note, more genes were down-regulated than up-regulated up to the UTJ (130 vs. 103) when mating was involved, but not when only the sperm-peak fraction (P1-AI) was used. Of interest, infusion of SP from the entire ejaculate (SP-Ejac) was neither able to modify the expression of any immune-related genes in the UTJ, nor to downregulate genes in the adjacent isthmic or ampullar segments. Infusion of only the SP-P1 fraction was, on the other hand, able to modify expression (UTJ: 5 up-regulated and 8 downregulated; Isthmus: 4 up-regulated and 5 down-regulated; Ampulla: 4 up-regulated and 5 down-regulated).

CONCLUSION
The findings indicate that there is a concerted action of spermatozoa and SP affecting gene expression in the pig female genital tract towards down-regulation during the pre-/peri-ovulatory stage. Spermatozoa, at least those in the vanguard sperm-peak fraction, seem to have an up-regulatory effect, visible in the upper (oviductal) segments. Sperm-free SP, on the other hand, did not seem to play major effects on gene expression, despite the clinical notion that SP mitigates inflammatory reactivity by the female after mating/AI.

Keywords: transcriptomics; bioinformatics; spermatozoa; seminal plasma; immuneregulation; pig.

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Presentation available:

Title: **Quality of European red deer spermatozoa stored in the epididymides and in a liquid state at 5 degrees Celsius**

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AIM

The aim of this study was to compare the biological properties of European red deer spermatozoa stored in the epididymides and in a liquid state at a temperature of 5 °C. The following biological properties of spermatozoa stored for up to 144 h were assessed: motility, viability and integrity of acrosomal membranes, apoptotic-like changes, mitochondrial activity and DNA integrity.

MATERIALS AND METHODS

The experimental material (testicles with epididymides stored in the scrotum sack) was collected from 36 hunter-harvested European red deer stags. Sperm samples were collected from the cauda of the epididymis. In the first variant, the samples were diluted in two extenders (Bovidyl® and Salomon's), and were stored at 5 °C for up to 144 h. In the second variant, spermatozoa were stored in the epididymides at 5 °C for up to 144 h, and then diluted in the same extenders. The biological properties of spermatozoa were examined after 0, 48, 96, and 144 h of storage. Motility was determined with the CASA system (Hamilton Thorne IVOS v. 12.3 motion analyzer). Sperm viability, acrosomal membrane integrity, apoptotic-like changes, mitochondrial activity, and DNA integrity were assessed using fluorochromes and a fluorescence microscope.

RESULTS

The study demonstrated that spermatozoa stored in a liquid state were characterized by higher motility than sperm stored in the epididymides, regardless of the applied extender. Significant differences ($P \leq 0.05$) in the majority of sperm parameters were observed between storage variants at 144 h of storage, regardless of the applied extender. Total motility (TMOT), viability, and mitochondrial activity decreased below 50% of baseline values in the spermatozoa stored in the epididymides but remained above 70% of baseline values in the spermatozoa stored in a liquid state at 144 h. The compared storage variants did not differ in TMOT, mitochondrial activity, or the percentage of viable spermatozoa without apoptotic-like changes up to 96 h of storage, regardless of the applied extender. Differences ($P \leq 0.05$) in sperm viability and integrity of acrosomal membranes between the variants were found after 48 h of storage in Bovidyl extender. In spermatozoa stored in a liquid state, significant differences in progressive motility was noted between extenders. Bovidyl contributed to maintaining progressive movement.

CONCLUSION

In conclusion: European red deer spermatozoa can be stored in the epididymides in the scrotum sack at 5 °C for up to 96 h. However, storing sperm in the epididymides, compared with liquid storage, causes earlier structural and functional damage to cells, thus deteriorating their motility. Liquid storage is recommended for short-term preservation of epididymal spermatozoa. In turn, extenders improve the biological properties of stored epididymal sperm.

Keywords: red deer, epididymal sperm, short-term storage methods, sperm parameters

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Presentation available:

Title: **Gene banking of Hungarian local sheep breeds**

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AIM
As the Sustainable Development Indicator SDG 2.5.1b about the number of local or transboundary domestic animal breeds with sufficient genetic material stored for breed reconstruction (https://www.fao.org/dad-is/sdg-251/en/) indicates, in most cases either there is no material or no information available, either at global or at national level. The aim of the project presented was to establish an *in vitro* gene bank based on cryopreserved sperm of native Hungarian sheep breeds.

MATERIAL AND METHODS
A total of 199 sperm batches were cryopreserved from 11 Cigaja, 7 Cikta and 6 Racka rams (92, 60 and 47 doses, resp.). Besides the routine post-thaw sperm quality control with CASA and Kovacs-Foote viability staining on 73 representative thawed batches, we tested the applicability of Feulgen staining to reveal disturbances in sperm chromatin condensation as well as to do sperm head morphometry analysis with ImageJ.

RESULTS
Feulgen staining patterns did not reveal sperm chromatin condensation problem in any of the semen batches. Sperm head area and permimeter mean values did not differ significantly between breeds, however, we detected a significant ($p<0.05$) difference in the SD of sperm head area (0.97, 0.93 and 1.03 μm^2 median SD in Cigaja, Cikta and Racka, resp.) indicating a breed dependent difference in intramale sperm head size variation.

CONCLUSION
The current local *in vitro* sheep gene bank is planned to be expanded to contain more representative samples from each breed. Feulgen staining provides useful additional information about breed-specific differences in intramale sperm size variation and further studies are planned to apply more precise and quicker, automatized flow cytometric approaches to measure sperm head morphometry.

Keywords: Ram, sperm morphology, morphometry, chromatin condensation

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Presentation available:

Title: **Non-surgical artificial insemination, embryo recovery and transfer in small ruminants**

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AIM

Both artificial insemination (AI) and *in vivo* embryo production, also known as multiple ovulation and embryo transfer programs, play crucial roles in advancing the propagation of genetically and economically superior goats and sheep. This review aims to provide an overview of current transcervical AI and embryo recovery (NSER) procedures in small ruminants.

RESULTS

The limited body size of small ruminants, which prevents rectal palpation, along with the highly restricted penetrability of the uterine cervix (mainly in sheep), are primary factors contributing to the rare use of nonsurgical assisted reproduction techniques in these species. Consequently, AI and embryo collection procedures in sheep predominantly involve more invasive approaches, such as laparoscopy or laparotomy. In goats, the Embrapa AI method enables high rates of intrauterine semen deposition, yielding pregnancy rates from 50 to 60% under field conditions using frozen-thawed semen. Furthermore, after administering a low dose of d-cloprostenol 12-16 hours before the procedure, transcervical embryo recovery is realistic in goats, with a cervical penetration rate nearing 100%. Although there is limited data on the efficacy of nonsurgical AI utilizing frozen-thawed semen in sheep, adequate results have been reported with fresh or cooled semen. In ewes, the application of the NSER technique has seen significant advancements in the past decade. Cervical penetration rates of up to 95% can now be achieved by implementing a hormonal cervical dilation protocol involving d-cloprostenol, oxytocin, and/or estradiol ester (such as estradiol benzoate). Depending on the breed, the estradiol is not required. A few studies have demonstrated that embryo recovery efficiency by NSER is similar to that obtained by traditional laparotomy. Moreover, NSER emerges as the preferred approach when considering animal welfare.

CONCLUSION

Continued enhancements are essential to ensure the safe application of these technologies and to maximize productivity in the small ruminant market. As a result of several improvements in the NSER technique, it stands nowadays as a viable alternative to surgical procedures. With ongoing advancements, it holds the potential to become the primary, if not sole, embryo recovery technique for small ruminants globally.

Keywords: AI, MOET, transcervical embryo collection

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