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ABSTRACT

Young Scientist Competition

YSC 1 | Anti-Müllerian hormone under short and long photoperiods in female cats

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Anti-Müllerian hormone (AMH) is a glycoprotein belonging to the transforming growth factors (TGF-β). In females, AMH is secreted by granulosa cells of small follicles in the ovary. In most seasonal species AMH varies throughout the different reproductive seasons. Although the domestic cat has been classified as a reproductively seasonal species, nothing is known about the effect of photoperiod on AMH serum concentrations in queens. The objective of this study was to describe and compare AMH serum concentrations under a short (SP) vs a long (LP) artificial photoperiod. Five, 2.5 years female cats were housed free in a windowless room with LED lamps under a short (SP, 6:18 L/D) and a long photoperiod (LP, 16:8 L/D) for 3 months (IACUC). A blood sample was collected from the jugular vein at the end of the 2nd and 3rd month of both SP and LP at 6 h am. Serum was stored frozen at −70°C until AMH analysis (Elecsys, Cobas, Roche Diagnostics International Ltd., Switzerland). The sensibility and the intraassay CV of the kit were 0.01 n/mL and <5%, respectively. Normality of AMH serum values was confirmed by Shapiro–Wilk test. AMH concentrations were analysed by paired and unpaired Student t tests. The level of significance was set at $p < .05$. AMH serum concentrations had a tendency to decrease in the 2nd sample of the SP ($p < .1$), but it did not vary during the LP ($p > .1$). Although serum AMH concentrations were 32% lower in LP than in SP (2.76 ± 0.54 vs. 3.66 ± 0.49 ng/mL; $p = 0.2$) this difference was not significant. Lack of significance might be because of a low number of animals. This lower AMH levels could be explained by the presence of large antral ovarian follicles during this stimulating photoperiod.

YSC 2 | The influence of intra-venous glucose administration during in-vivo embryo production process in dairy cattle

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The aim of the study was to determine the effect of intravenous Glucose (IVG) treatment, starting with Follicle Stimulating Hormone (FSH) administration during the superovulation process, on follicle diameters, the number of follicles, corpus luteum (CL), and embryos, as well as FSH and Leptine plasma concentrations. A total of 14 (7 control (CG)/7 experimental (EG)) Holstein cows were utilized. 160–200 mL IVG was administered to EG, prior to FSH application, which is included in the superovulation protocol. Blood samples were taken for 4 days, 6 times a day (3 times morning/evening, each) at 2-h intervals. Follicle diameters, number of follicles and CLs were measured by ultrasonograpyh (USG). Kruskal Wallis method was used for the analysis of variance between the groups. Plasma FSH and Leptine levels were measured by a commercial Elisa-Kit. Repeated measures-multivariante analysis and One-way ANOVA were used for comparison of hormone plasma concentrations. The mean number of follicles and diameters, CLs, and embryos were as follows 13.71 ± 1.169, 1.17 ± 0.039, 10.00 ± 0.976 and 5.00 ± 0.218 respectively, in EG. However, in CG, the mean number of follicles, their diameters, CLs, and embryos were 9.86 ± 1.010, 1.15 ± 0.039, 6.86 ± 0.857, 3.14 ± 0.595, respectively. There was a statistical difference between the groups in terms of numbers of follicle, CL, and embryo ($p < .05$). Furthermore, FSH levels on the 2nd and 3rd day ($p < .05$) were significantly higher in EG. Also, Leptin levels were significantly higher on the 2nd, 3rd, and 4th days in EG. In conclusion, IVG treatment, prior to FSH administration during the superovulation process, had a positive effect on the number of follicles, CLs, embryos and plasma concentrations of FSH and Leptin.

YSC 3 | Cow uterine fluid extracellular vesicles show oestrous cycle phase dependant proteomic changes affecting embryo development in vitro

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Extracellular vesicles (EVs) in the uterine fluid (UF) are known to regulate early embryo development. Moreover, the changes in UF-EV proteome during the bovine oestrous cycle and the effects of these proteins on embryo development are yet to be discovered. We used mass-spectrometry based shotgun quantitative proteomics to compare UF-EV proteomes at day 0, 7 and 16 of the oestrous cycle ($n=4$ per group). Also, we supplemented follicular and luteal phase UF-EVs to group embryo cultures to evaluate their impact on embryo development. Proteomics data was analysed using LFQ-analyst platform, while the differences of blastocyst rates were evaluated with logistic regression analysis. Proteomic analysis revealed pathways which are important for early embryo development and its nutritional needs, such as antioxidant activity, cell morphology and cycle, cellular homeostasis, cell adhesion and carbohydrate metabolic process. Furthermore, 159 UF-EV proteins differentially enriched at different timepoints. These proteins were involved in pathways related to antioxidant activity, actin cytoskeleton organization, immune processes, gene expression regulation and metabolic functions. The luteal phase UF-EVs supplementation to the embryo culture media increased blastocyst rates from 25.0±5.9% to 41.0±4.0% ($p=.03$). Overall, our findings suggest that there are significant differences in bovine UF-EV proteome throughout the oestrous cycle and UF-EVs improve in vitro embryo production, however further studies are required to identify the exact UF-EV cargo for optimal embryo development.

YSC 4 | Effect of equilibration temperature and time on feline ovarian tissue vitrification

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The entire members of Felidae are currently classified as endangered except for the domestic cat (*Felis catus*) making it an excellent model for conservation studies. Vitrification of feline ovarian tissue is an emerging conservation technique suitable in field conditions however, not yet standardized. Thus, the aim was to establish a suitable vitrification protocol for feline ovarian tissue in field conditions. Feline ovarian tissue fragments were punched with a biopsy punch (1.5 mm diameter) and divided into 4 groups. Group 1 was directly placed in culture (Fresh control – FC), while the other three were placed on 30G needles (4 fragments/needle) and vitrified using 3 protocols (A, B, C). Protocol A involved two step equilibrations for 10 min each at 4°C and then vitrification [1]. Protocol B involved three step equilibrations for 14 min in total at room temperature [2], while protocol C was the same with protocol B except the equilibration timings which were reduced by half. Fragments were warmed and placed in culture [1] for 6 days. Follicular morphology, cellular proliferation (expression of Ki-67, MCM-7) and apoptosis (expression of caspase 3) were evaluated. Data were analysed using Chi square test. Proportions of morphological intact follicles were higher in FC ($p=.0001$) and protocol C ($p=.0383$) in comparison to the other protocols at the sixth day of culture. Generally, most follicles remained at primordial state which was confirmed by the low expression of ki-67, MCM-7 markers. In conclusion, protocol C, which has lower equilibration time at room temperature, can be used for vitrification of feline ovarian tissue. [1] Mouttham, Cryobiology 2016;73:187; [2] Amorim, Human Reproduction, 2013;28: 2146.

YSC 5 | Stress during the transition period shortens telomere length in dairy cows

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Telomere length (TL) has long been recognized as a biomarker of ageing. In humans, physiological stress is known to impact health and longevity, manifested by an accelerated shortening of TL. In cattle, the transition period from pregnancy to lactation is considered a crucial stage, during which cows undergo significant metabolic and physiological changes. We hypothesize that telomeres shorten during this critical period, due to oxidative and metabolic stress. Seventy-one Holstein Friesian cows, on one farm, were followed up during the transition period and blood and milk samples were collected at regular time points. Average relative leukocyte TL was measured by a modified quantitative real-time PCR (qPCR) protocol at 7 days before and 21 days after parturition. Oxidative, inflammatory, and metabolic parameters were also determined. From 7 days prior to 21 days after parturition, a paired t-test showed a significant decrease in TL of 0.08±0.243 ($p=.022$). Multiple linear models, built in R, were used to assess factors influencing TL shortening. Both oxidized glutathione in blood (GSSG, oxidative

parameter) and beta-hydroxybutyrate in milk (BHB, metabolic parameter) were associated with an increased TL shortening. For each percentage increase in GSSG and for each 0.01 mmol/L increase of BHB, TL shortening tended to be 0.016 greater ($p = .051$ and .097 respectively). The preliminary findings of this study revealed a significant shortening of TL during the transition period in dairy cattle. In addition, higher levels of oxidative and metabolic stress parameters were associated with a greater TL shortening.

YSC 6 | Adipokines expression profiles in pituitary gland and plasma levels during the estrous cycle of large white and Meishan sows

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The relationship between reproduction and energy metabolism of domestic animals is very close. Previously data showed differences in adipokines levels in white adipose tissue in normal weight Large White (LW) and obese Meishan (MS) pigs. However, comparison of central-pituitary levels of adipokines during the estrous between LW and MS is still unknown. The aim of the study was to compare plasma concentrations (ELISA) and gene expression (RT-qPCR) of adipokines: adipolin, adiponectin, chemerin, visfatin, apelin, omentin, vaspin and their receptors in the anterior pituitary (AP) in LW and MS ($n = 5$) during the estrous. Statistical analysis was performed by two-way ANOVA, Tukey's post-hoc test, and Pearson correlation coefficient, two-tailed $p \leq .05$. Our results showed increased levels of apelin and adiponectin while decreased chemerin with receptors, adipolin, visfatin, omentin in AP of LW vs. MS. There are no differences in adipolin and visfatin plasma concentration, but increased levels of chemerin, vaspin in MS and omentin in LW depending on the phase of the estrous. We observed correlations between apelin expression and plasma level on days 2–3 in MS and 10–12 in LW. The LH concentration in plasma was positively correlated with visfatin level in LW and negatively correlated with adiponectin in MS during late luteal phase. There was no correlation between plasma FSH and any adipokine. These results demonstrated the pattern of expression of selected adipokines and their receptors in AP and plasma concentrations depending on the animal metabolic status and day of the estrous suggesting adipokines' action on pituitary level. Funding: Supported by NCN 2020/37/B/NZ9/01154; we thank the team of porcine experimental unit and the slaughterhouse of INRAE Centre Val de Loire.

YSC 7 | The role of phoenixin-14 on endocrine function of porcine corpus luteum, an in vitro study

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Phoenixin (PNX-14) is a neuropeptide which modulates ovarian follicular cells steroidogenesis and oocytes maturation. Our previous study showed expression of PNX-14 and its receptor GPR173 in the porcine corpus luteum (CL). However, the role of PNX-14 in luteal cells physiology has never been studied, so the aim of the study was to determine the in vitro effect of PNX-14 on endocrine function of CL during the estrus cycle. Porcine luteal cells were isolated from CL on days 10–12 of the estrus cycle and cells were stimulated with PNX-14 (1–1000 nM) with or without LH (100 ng/mL). The concentration of steroid hormone: P4 and E2 and prostaglandins (PGE2 and PGF2α) was determined by ELISA assays. The transcript and protein levels of StAR, CYP11A1, 3βHSD, CYP19A1, PTGER2 and PTGFR as well as expression of GPR173 and kinases ERK1/2 and PKA were analysed by RT-qPCR and Western-blot. Moreover, the involvement of GPR173 (by siRNA) and ERK1/2 and PKA (by pharmacological inhibitors) was studied in PNX-14 effect on luteal endocrinology. The results showed a dose-dependent stimulatory effect of PNX-14 on the secretion of P4, E2, and PGE2 and inhibitory action on PGF2α. PNX-14 induced modulatory effect on LH-induced steroid synthesis. Moreover, PNX-14 increased the expression of steroidogenic enzymes and exerted a modulatory effect on prostaglandin receptors. In addition, PNX-14 stimulated expression of GPR173 and activation of ERK1/2, while downregulating PKA. In conclusion, we demonstrated that PNX-14 improved steroidogenesis and inhibited PGF2α via GPR173 and ERK1/2 pathways, suggesting a direct role of this neuropeptide on the porcine luteal endocrinology. Supported by NSC: 2020/37/N/NZ9/00981.

YSC 8 | Fertility in a randomized controlled study of customized voluntary waiting period in primiparous dairy cows

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Previous studies have shown that primiparous cows in general, but not all individuals, are suited for extended voluntary waiting period (VWP), however, cows with less persistent lactation risk a long dry period and high BCS at the end of lactation. Customizing the VWP may reduce the risk of undesirable effects of extended VWP. This

study was performed in 18 commercial dairy herds in Sweden. Three criteria were used to choose cows expected to be suited for extended VWP. (1) the 10% cows with the highest genomic (lactation curve) persistency index, (2) cows with difficult calving or a disease event the first month after calving, and (3) cows with an average daily yield (between DIM 4–33) above the herd average for primiparous cows. Cows meeting at least one of the criteria were randomized to and received either conventional VWP of <90 days (Conv group, $n = 139$), or extended VWP of >184 days (Ext group, $n = 120$). Preliminary results showed that first service conception rate, analysed with a binomial model with breed as fixed factor and farm as random factor, was higher in the Ext than in the Conv group (60 vs. 45%, $p = .04$). Number of inseminations per pregnancy, calculated per VWP group on each farm and analysed with a negative binomial model with farm as a random factor, was lower in the Ext compared to the Conv group (1.67 vs. 2.19, $p = .02$). Moreover, the 305 days yield, analysed with a linear mixed model with breed as fix and farm as random factor, was higher in the Ext than in the Conv group (10,371 ± 10 vs. 9812 ± 204, $p < .001$). These results suggest that customizing the VWP by using genomic persistency index, diseases, and calving problems, or average milk yield in early lactation, may improve fertility and 305 days yield for primiparous dairy cows.

YSC 9 | The ex vivo response of canine pregnant myometrium to exogenous PGF2α

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In the pregnant bitch the sharp decline of progesterone, signalling the initiation of parturition, is accompanied by a concomitant increase of PGF2α. PGF2α plays an important role in luteolysis, but is also postulated to induce myometrial contractions necessary for fetal expulsion. In this study we aimed to investigate the effect of exogenous PGF2α on canine myometrial contractility ex vivo. Four strips each of the circular (CM) and longitudinal (LM) myometrial layer of canine uterine interplacental tissue samples ($n = 7$ dogs) obtained during Caesarean section without prior medial intervention were placed in an organ bath set up. After equilibration, with some strips already showing spontaneous contractions, strips were challenged twice with dinoprost (50 pM, 0.5 μM, 50 μM PGF2α) and washed out in between. Per layer, each strip received only one concentration of PGF2α and one strip served as untreated control. Contractions were recorded and evaluated for average amplitude, mean force, area under curve and frequency of contractions. Results were statistically analysed by ANOVA for each stimulation separately, using GraphPad Prism. Responses to PGF2α were separately investigated in each layer and compared to the respective controls. In the CM, 50 μM PGF2α significantly increased amplitude, mean force and area under curve compared to untreated strips and other concentrations. This effect was reproducible upon restimulation. However, the frequency was not changed significantly. The LM did not respond to PGF2α at all. Since the in vivo dosage, corresponding to 50 μM in our experiment, is presumably above the LD50 of the dog and the effect was limited to the CM, PGF2α does not seem to be appropriate in dystocia management.

YSC 10 | In vitro effects of heavy metals on boar sperm motility

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Industrial toxicants like heavy metals interfere with male reproductive functions and fertility. The aim of this study was to evaluate the in vitro effects of different concentrations of cadmium, lead, and arsenic on boar sperm motility parameters. Extended boar (Finnish Landrace × Large white) semen ($n = 15$) acquired from the breeding station were incubated in different concentrations of heavy metals; cadmium (0, 3.75, 18.75, 37.50, 75.0 μg/mL), lead, (0, 3.75, 37.50, 75.0, 150 μg/mL) and arsenic (0, 37.50, 75.0, 112.50, 150 μg/mL). Before the exposure, the semen samples were stored in semen extender (MR-A®, Kubus, Spain) at 16–18°C for 24 h after ejaculation. Samples stored in extender without the addition of heavy metals served as controls (con). Sperm parameters were analysed with computerized sperm analysis (Androvision™ Software Version 3.5.6.2, CASA, Minitube, Germany) after incubation at 16–18°C with a heavy metal (Cd, Pb, As) for 0 h, 1 h, 2 h, 4 h. Differences in sperm motility between treatment and control samples at different concentrations, and differences in motility between incubation time and among metals were calculated by ANOVA on R Studio version 1.4.0. Results are expressed as mean ± SE. There was no significant difference in motility between incubation times. Progressive and total motility (%) were significantly reduced in spermatozoa exposed to higher concentrations (37.50 μg/mL–150 μg/mL) compared to con in all tested metals. They were recorded respectively as cadmium 75.0 μg/mL and 37.50 μg/mL (12.1 ± 1.2 and 16.7 ± 1.8), arsenic 150 μg/mL and 112.50 μg/mL (11.6 ± 1.9 and 16.3 ± 1.9), lead 150 μg/mL and 75.0 μg/mL (10.4 ± 1.5 and 17.1 ± 1.3) at the last incubation time point (4 h). Our results show direct harmful effects of heavy metals on sperm motility.

YSC 11 | In vitro evaluation of antimicrobial effect of ozone against bacteria responsible for equine endometritis

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Infectious endometritis is observed in up to 60% of barren mares and requires intrauterine or systemic antibiotic therapy. The goal of the present study was to characterize the in vitro antimicrobial properties of two different ozone preparations (O_3) in respect of the most common bacteria linked to endometritis. Accordingly, we tested strains of *Streptococcus equi subsp. zooepidemicus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolated from the uterus of mares diagnosed with infectious endometritis. Briefly, bacterial suspensions were prepared and adjusted to 0.5 McFarland turbidity in sterile saline solution and inoculated onto Tryptic Soy Agar or Blood Agar plates. The cultures were divided in different groups: untreated positive control, distilled water, continuous gas flow of O_3 at a concentration of 15 μg/mL and 40 μg/mL for three time points (1, 3 and 5 min) and distilled water ozonated at 40 μg/mL for 10 min. Gaseous ozone drastically reduced the growth of bacteria in all concentrations and times tested, resulting in a minimal residual growth (complete inhibition or maximum 10 CFU/plate) after 5 min of continuous gas flow at 40 μg/mL. On the contrary, ozonated water did not reduce bacterial growth since bacterial density was similar to the one observed in the control groups. In conclusion, gaseous O_3 represents a promising therapy for infectious endometritis and could prevent or scale down the use of antibiotics, which are routinely used in broodmares, while water ozonated at the concentration and time used in this study did not seem to be effective.

YSC 12 | Fluorescent dyes to assess sperm competition in female north African houbara bustard (*Chlamydotis undulata*): Impact on sperm motility and viability

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Female birds can store sperm for weeks in their oviduct. In polyandrous species, last male precedence (LMP) allows the last male copulating to sire the offspring. Little is known about mechanisms leading to LMP. Finding suitable staining molecules to visualize different males' sperm in storage sites is challenging, it must permit good microscopic observation without impairing sperm function, and efficacies can be species dependent. We compared the effects of two fluorescent dyes on sperm motility and viability over time using beta regressions, to further investigate sperm competition and sperm storage in the oviduct of a wild-polyandrous bird, Chlamydotis undulata. Twenty semen samples were stained with 2 fluorescent dyes, Hoechst 33,342, NUCLEAR-ID® Red DNA (NR), plus a blank control. Motility (4°C) and viability (4°C & 40°C) were evaluated after 20 min, 24 h, 48 h, and 72 h after staining. Fluorescence signal was checked by microscopy for both dyes at each interval. Both dyes gave good results for microscopic observations of fluorescent signal up to 72 h. Hoechst dye did not impact sperm motility ($z = 0.90$, $p = .37$) or viability over time (4°C: $z = 0.98$, $p = .32$; 40°C: $z = -2.17$, $p = .08$), but the NR stain impaired sperm motility after 48 h ($z = -2.89$, $p < .01$) and viability after 24 h (4°C: $z = -2.29$, $p = .02$, 40°C: $z = -6.21$, $p < .01$). Hoechst preserved key sperm characteristics for artificial insemination (AI) and so, can be tested in vivo. NR stain, however, may only be used for shorter time incubation experiments. In vivo studies are necessary to verify fluorescent signals from sperm cells in the female tissue after AI.

YSC 13 | Alterations of Sertoli cells in dogs with chronic asymptomatic orchitis

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Chronic asymptomatic orchitis (CAO) is the most common finding in male dogs with acquired infertility and is associated with severe histopathological changes including a spermatogenic arrest and immune cell infiltration. Its aetiology is unknown due to the lack of clinical signs, of abnormal bacterial or endocrine findings. As Sertoli cells play an important role for spermatogenesis and the testicular micromilieu, alterations in Sertoli cell number and function might be involved in disruption of spermatogenesis in CAO. Results were obtained from bilateral testicular samples of 15 CAO dogs (biopsies for diagnostic purpose) and compared to those of 15 normospermic healthy controls (CG) (castrated on owners request). Number of Vimentin-positive Sertoli cells were counted in 20 seminiferous tubules per sample at 40× magnification. Results were statistically compared using GraphPadPrism. Significantly less Sertoli cells were found in CAO compared to CG ($p < .0001$). Besides, immunohistochemistry was performed using antibodies against GDNF (Glial cell line-derived neurotrophic factor) and bFGF (basic fibroblast growth factor) to study Sertoli cell function. Sertoli cells stained GDNF and bFGF positive in both groups, as did the blood vessels. Besides, some peritubular cells stained GDNF immunopositive. Despite the significant reduction in number, Sertoli cells in CAO still express GDNF and bFGF indicating (at least partial) functionality of Sertoli cells for stem cell replication and maintenance. Further studies should focus on additional markers to better characterize Sertoli cell maturation status and function, also for a possible spermatogonial stem cell

therapeutic approach to potentially (re-) initialize spermatogenesis. Pauline Rehder receives a GkF scholarship.

YSC 14 | AMH expression in cat ovaries and correlation with animal age and weight

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The aim of this study was to determine plasma AMH concentration and ovarian AMH expression in cats and analyse correlations with follicular growth, antral follicle count (AFC) the presence of corpora lutea (CL) and ovarian cysts as well as age and weight. Ovaries were collected from 99 healthy cats presented for ovariectomy. Age of the cats was 4 months to 9 years and weight between 1.5 and 4.7 kg. Before surgery, blood was collected for AMH and progesterone (P4) analysis. Removed ovaries were examined for AMH expression by immunohistochemistry, follicular development (AFC and follicle size), CL and pathologies. Correlation analysis and comparisons between age and weight groups by ANOVA were made with the SPSS statistics program. Corpora lutea were present in 35% of the cats and the frequency of CL increased with age and weight of the cats. Ovarian cysts (fluid-filled structures >3.5 mm in diameter) were detected in 22% of the cats, irrespective of age. The AMH protein was expressed in all follicles and in cysts independent of size, 79% of CL and 28% of atretic follicles. Plasma AMH concentration was positively correlated with follicle count ($r = 0.39$, $p < .001$) but there was no significant correlation with cat age and weight and with P4 concentration. Plasma AMH concentration was not altered in cats with cysts and not influenced by season of the year. In conclusion, findings confirm spontaneous ovulations in female cats and these occur irrespective of season. The expression of AMH is not restricted to small antral follicles in cat ovaries. The concentration of AMH in plasma was constant and neither affected by the presence of CL nor ovarian cysts.

YSC 15 | Prolonged cold-preservation outcome of feline ovarian tissue is improved by intra- and extracellular solutions but impaired by the fragmentation of ovary

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Assisted reproduction techniques are most effective when feline ovaries are stored up to 24 h at 4°C. Yet, transporting ovaries to specialized labs exceeds this timeframe, hindering cryo-banking of endangered species' ovarian tissue. Therefore, our objective was to determine if commercial transportation solutions could enhance the yield of feline ovarian storage for longer than 24 h. To address this, we investigated preservation capabilities of three transportation solutions: Dulbecco's Phosphate Buffered Saline supplemented with glutathione (DPBS+GSH), intracellular BELZER UW® (UW), and extracellular StoreProtectPlus® (SP+). Ovaries from 10 domestic cats were stored intact (control group in DPBS) or fragmented (in three experimental solutions) at 4°C for 48 h and 72 h. We assessed follicular morphology, apoptosis rates (caspase-3 expression, DNA fragmentation by TUNEL), and performed statistical analysis using ANOVA. The results revealed that ovary fragmentation impaired follicular morphology compared to intact ovaries ($p < .001$). Preservation rates for follicular morphology averaged 60 ± 6% vs. 51 ± 4% for 48 h and 51 ± 7% vs. 37 ± 2% for 72 h storage in intact and fragmented ovaries, respectively. UW solution demonstrated significantly superior follicular morphology and lower DNA fragmentation at 48 h compared to SP+ and DPBS+GSH ($p < .05$). Furthermore, both UW solution and SP+ exhibited improved follicular morphology preservation after 72 h compared to DPBS+GSH ($p < .05$). In conclusion, complex transportation solutions enhanced preservation during prolonged storage of feline ovarian fragments. However, further studies are required to validate the benefits for whole ovaries, as our findings suggest that feline ovaries should not be fragmented prior to transportation.