Lentivirus-mediated *in vitro* gene transfer into Nile tilapia spermatogonial stem cells

S.M.S.N. Lacerda[1], F.M.P. Tonelli[2], M.A. Silva[1], G.M.J. Costa[1], T.C.A.S. Bicalho[1], R.R. Resende[2], L.R. França[1]

[1]Department of Morphology, [2]Departament Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

Spermatogonial stem cells (SSCs) are the foundation of the spermatogenic process and are unique stem cells in the adult organism due to their ability to transmit the genetic information to the subsequent generation. Importantly, the genetic modification of these cells provides a great opportunity to study the biology of their complex self-renewal and differentiation processes, as well as to enable the generation of transgenic animals in a wide range of species, including fish. In Nile tilapia (*Oreochromis niloticus*), we have previously developed a competent SSC culture system and also an efficient methodology for SSCs transplantation into the testes of adult fish. Here, we investigated the ability of lentiviral vectors to transduce Nile tilapia SSCs *in vitro* by inserting the DsRed2 transgene that encodes the red fluorescent protein. The DsRed2 sequence was cloned into pLenti6.3/V5-TOPO®; this recombinant vector was transformed into One Shot® Stbl3™ Competent *E. coli* and these bacterial cells were submitted to maxiprep. The recombinant vector was sequenced and used to transform 293 FT cells by Lipofectamine 2000. The viral particles were obtained after 72 hours into the supernatant of the complete medium (high glucose D-MEM). Nile tilapia SSCs were exposed to lentivirus for 12 hours under pre-established optimal culture conditions, selected with Blasticidin, and formerly analysed for the expression of RFP under fluorescent microscopy. Transduction of the reporter gene encoding the RFP was monitored in *in vitro* cultivated SSCs for 9 days with the first observation at 24h post-infection. Although in low intensity, at that time a substantial fraction of cells expressed the transgene. The percentage of RFP-positive cells increased during the *in vitro* cultivation, with gradual overgrowth of RFP-positive colonies until one week. In addition, using long-term fluorescence analysis and DNA sequencing we confirmed the randomic and stable integration of the transgene into SSCs genome. In order to obtain transgenic offspring, such investigations are essential before transplanting transduced SSCs into the tilapia recipient testis. The data obtained here indicate that, similar to mammals, viral transduction represents an efficient method to introduce genes into the fish male germline. Therefore, these findings provide the first step in establishing a system that will allow genetic manipulation of fish SSCs, representing an important progress towards the production of transgenic fish lines and new biotechnologies in aquaculture.

Financial support: FAPEMIG and CNPq

E-mail: lacerdasmsn@icb.ufmg.br

Influence of cyclic AMP and PKA on modulation of gene expression, ERK1/2 and AKT phosphorylation by FSH, in rat Sertoli cells

A.R. Nascimento, T.F.G. Lucas, C.S. Porto, M.F.M. Lazari

Section of Experimental Endocrinology, Department of Pharmacology, Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo, SP, Brazil.

Immature Sertoli cells proliferate and several factors affect this process, including testosterone, FSH, estradiol, and a number of paracrine factors. Timing of transition from a proliferative to a differentiation stage is important to determine the appropriate number of Sertoli cells and the support of germ cells in adult life. Sertoli cells from 15-day-old rats proliferate, but they are close to differentiate. In this transition phase we found that FSH strongly increases cyclic AMP production, stimulates the transcription factor CREB, inhibits phosphorylation of ERK1/2 and AKT, and changes gene expression in a complex way, suggesting that FSH starts to direct cells for differentiation. In contrast, the paracrine factor relaxin inhibits cyclic AMP production and stimulates MEK/ERK1/2 and PI3K/AKT pathways to promote proliferation of Sertoli cells at this age. We tested whether the inhibitory effect of FSH on ERK1/2 and AKT phosphorylation depends on PKA and cyclic AMP, and whether relaxin blocks this effect of FSH. Sertoli cells were removed from 15 day old Wistar rats and were cultured for 5 days. Cells were incubated with the PKA inhibitor H89 (2 µM, 2 h), the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mM, 30 min), or vehicle, and then treated with FSH (100 ng/mL) or relaxin (50 ng/mL), or a combination of both, for 5 min. Phosphorylation of ERK1/2 and AKT was measured by Western blot. As expected, FSH inhibited phosphorylation of ERK1/2 and AKT. Relaxin but not H89 blunted FSH effect on ERK1/2. The combination of H89 and relaxin did not further blunt the reduction of ERK1/2 phosphorylation. Relaxin and H89 tended to revert FSH inhibition of AKT phosphorylation. The combination of H89 and relaxin was more efficient to inhibit the FSH effect on AKT phosphorylation. The inhibition of phosphorylation of ERK1/2 and AKT induced by FSH was potentiated when cyclic AMP degradation was inhibited by IBMX. Relaxin did not affect this response to FSH with IBMX. We also tested if inhibition of PKA and of the CREB transcription factor reduces the effect of FSH on expression of the cell cycle genes inhibin A (*Inba*) and *Bcl2*. Sertoli cells were incubated with the PKA inhibitor H89 (2 µM, 2 h), the inhibitor of CREB-CBP interaction, KG-501 (25 µM, 30 min), or vehicle, followed by incubation with FSH (100 ng/mL) for 4 h. Cell cycle genes were measured by quantitative real time PCR. FSH increased *Inba* and reduced *Bcl2* expression. These effects were resistant to H89, but were reduced by KG-501. In conclusion, cyclic AMP but not activation of PKA is important for the reduction of phosphorylation of ERK1/2 induced by FSH, whereas both cyclic AMP and PKA seem to contribute to the reduction of phosphorylation of AKT. However, the effects of FSH on gene expression do not seem to involve PKA, but depend on CREB activation. The signaling pathways involved on FSH regulation of gene expression at this transition time need to be explored. This study was approved by the Ethics Committee from the Universidade Federal de São Paulo (CEP: 1311/11).

Financial support: FAPESP and CNPq

E-mail: alinenascmento@gmail.com

Survival of domestic cat oocytes within ovarian cortex after prolonged storage and vitrification

J.L.A. Martins[1], M.D. Lopes[1], K. Jewgenow[2]

[1]Laboratory of Reproduction of small and wild animal FMVZ/UNESP Botucatu, Brazil; [2]Department Reproduction Biology, Leibniz-Institute for Zoo and Wildlife Research, Berlin, Germany.

Assisted Reproduction Techniques (ART) have a great potential for preservation of endangered wildlife species. They allow population management by using genetic material regardless of time and space. The domestic cat has been shown to be an excellent model for the development of ART for endangered feline species. In the case of females, ovaries contain a huge genetic reserve – the primordial and primary follicles (PAF), which correspond to almost 90% of the oocyte pool. To preserve this gamete pool, we investigated the possibility to cryopreserve ovarian tissues by vitrification and the impact of prolonged ovary transportation on PAF viability. Using a vitrification protocol developed by Keros et al. (2009, *Hum Reprod, 24, 1670-83*) for human ovarian tissue preservation, we examined the effect of storage time at 5°C on survival of PAF obtained from domestic cat ovarian cortex, before and after vitrification. Ovaries from twelve domestic cats were obtained after ovariohysterectomia and transported in DPBS to the lab within 2-6 h. Upon arrival, parts of the ovaries were stored at +5°C for another 24 h or 72 h. After storage (2-6 h control group, 24 h and 72 h experimental groups) the ovarian cortex was dissected as described by Wiedemann et al. (2013, *BMC Vet Res*, 9**,** 37) and subjected to either viability assessment or vitrification. For vitrification, pieces of ~0.5 square cm were obtained and vitrified according to Keros et al (2009). For viability assessment, the ovarian cortex was further dissected with 2mm biopsy punches into equal size pieces. From each group, a piece was subjected to Neutral Red (NR) stain for live-dead discrimination of intraovarian follicles. Tissue samples were incubated with NR in DPBS (1:40) at 37.5°C, 5% CO2 for 16 h. NR viability assessment was performed under inverted microscope. The mean number of NR stained follicles (± standard deviation) per ovarian piece before vitrification was 83. ± 85.3 (n = 12, 2-6 h); 55.8 ± 51.1 (n = 12, 24 h) and 15.9 ± 19.4 (n = 12, 72 h), respectively. After thawing the number of viable follicles per piece was 33.7 ± 26.3 (n = 15; 2-6 h), 23.0 ± 18.1 (n = 14, 24 h) and 10.7± 12.7 (n = 12, 72 h) with maximum numbers of 303 (2-6 h), 127(24 h) and 111 (72 h). After NR assessment, each sample was fixed in Bouin solution and embedded in paraffin. Serial sections (3 µm thickness, every 30 µm) were stained with Haematoxilyn/Eosin. The calculated number of intact follicles (CFN) was determined as described before by Wiedemann et al. (2013). No difference was found between control and experimental groups. Another piece was fixed with Karnovski's solution and subjected to Transmission Electronic Microscopy (TEM). In TEM, the integrity of mitochondria, of plasma and basal membranes, as well as the presence of pregranulosa cells were seen in PAF of all groups. Our results showed that feline PAF can survive a prolonged storage (72 h) at 5°C followed by vitrification. No impact of vitrification on PAF survival was observed, regardless of storage time. As expected, with prolonged storage the viability drops down with significant differences found after 72 h. To conclude, the vitrification protocol applied ensured the survival of PAF for domestic cats. The next step of research should focus on the preservation of developmental competence of the frozen-thawed follicles and the adaptation of the technique to endangered wildlife cat species in Brazil.

This project was supported by Science without Borders Program, IZW and CAPES

E-mail: jlamartins@yahoo.com.br

The effect of astaxanthin on oxidative status of bovine oocytes submitted to heat shock

J. Ispada[1], P.H.B. Risolia[2], D.R. Gonçalves[1], T.A. Rodrigues[1], M. Nichi[3], D. Rettori[1], F.F. Paula-Lopes[1,2]

[1]Departament of Biological Sciences, Institute of Environmental Sciences, Chemistry and Pharmacology, Federal University of São Paulo, Diadema-SP, Brazil; [2]Institute of Biosciences, São Paulo State University, Botucatu-SP, Brazil; [3]Department of Animal Reproduction, University of São Paulo, São Paulo-SP, Brazil.

The series of events associated with oocyte growth and maturation are susceptible to disruption by elevated temperature compromising the ability of the oocyte to undergo adequate fertilization and embryonic development. However, the cellular mechanisms triggered by elevated temperatures and the role of oxidative stress in this context is poorly understood. Recent studies indicated that exposure of bovine embryos to heat stress/shock increased intracellular concentration of reactive oxygen species (ROS). Moreover, antioxidants such as astaxanthin (AST) can reverse the deleterious effects of heat shock on preimplantation embryonic development. Therefore, the aim of the current study was to evaluate the effects of AST on the redox state of bovine oocytes and cumulus cells (CCs) exposed to heat shock during in vitro maturation (IVM). Slaughterhouse derived cumulus-oocyte complex (COCs) were subjected to IVM in Tissue Culture Medium 199 [TCM 199 – bicarbonate containing 50 μg/mL gentamicin, 0.2 mM pyruvate, 2 μg/mL estradiol, 20 μg/mL follicle stimulating hormone (FSH), 10 μg/mL luteinizing hormone (LH), 6 mg/mL essentially fatty-acid free bovine serum albumin] supplemented with 0, 12.5 or 25 nM AST at 38.5 or 41°C for 14 hours followed by 38.5°C for 8 hours. The CCs were removed by denudation, then the CCs, oocytes and IVM medium were stored at -80°C. Briefly, all analyzes were made with spectrophotometer, and the quantification of the activity of glutathione peroxidase (GPX) was done indirectly through the conversion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP) on 340 nm at 37°C; superoxide dismutase (SOD) by cytochrome c reduction by O^{2-}, since both compete for the anion on 550 nm at 25°C; catalase (CAT) was based on the consumption and degradation of H_2O_2 evaluated on 230 nm at 30°C. The product of lipid peroxidation was determined by reaction with thiobarbituric acid (TBARS) measured in a spectrophotometer on 532 nm at room temperature. Heat shock did not affect oocyte and cumulus cells GPX, SOD and CAT activity or the rate of lipid peroxidation in the IVM medium. However, 12.5 ($P= 0.09$) and 25 nM ($P< 0.05$) AST increased GPX activity in heat-shocked oocytes as compared to heat shocked oocytes matured in 0 nM AST. In contrast, 25 nM AST decreased ($P< 0.05$) GPX activity on cumulus cells regardless of temperature. Addition of 12.5 nM AST to IVM media increased ($P< 0.05$) SOD activity on heat shocked oocytes when compared to 12.5 nM AST control oocytes. SOD activity was also increased ($P< 0.05$) in control temperature oocytes matured with 25 nM AST when compared to 0 nM AST. Cumulus cells SOD activity profile varied with dose. While 12.5 nM AST inhibited SOD on heat shocked CCs, a higher dose of 25 nM increased the SOD activity on heat shocked CCs. There was a significant increase ($P= 0.09$) on CAT activity of in heat-shocked oocytes matured in 12.5 nM AST. In conclusion, AST increased antioxidant capacity in heat-shocked oocytes. It is possible that the beneficial effect of AST on oocyte developmental capacity described previously (Ispada et al. Biol of Reprod. 2013:87:199) was mediated by this increase of antioxidative capacity in oocytes subjected to heat shock. The pathways by which the AST affects this antioxidative capacity only during heat shock was not fully understood.

Financial support: FAPESP 2011/05454-4 and CNPq 482371/2011-0

Email: paula.lopes29@unifesp.br

Differential expression of several key testis marker genes in iNOS deficient mice during perinatal development and in adulthood

N.L.M. Lara[1], S.M.S.N. Lacerda[1], C.F.A. Oliveira[1], R.R. Resende[2], R.M. Sharpe[3], L.R. França[1]

[1]Department of Morphology and [2]Department of Biochemistry, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil; [3]Centre for Reproductive Health, The Queen's Medical Research Institute, University of Edinburgh, UK.

Nitric oxide (NO) is an important mediator of intra and extra-cellular processes, and plays a crucial role in several physiological and pathological conditions; several studies have shown that NO can inhibit testicular steroidogenesis *in vitro* and *in vivo* in rodents. In the testis, constitutive expression of iNOS (inducible nitric oxide synthase) is found in Leydig (LC), Sertoli (SC) and germ cells (GC), suggesting a regulatory function of this enzyme in both spermatogenesis and steroidogenesis. Recent studies from our laboratory have shown that adult iNOS deficient mice ($iNOS^{-/-}$) exhibit lower germ cell apoptosis, higher number of Sertoli and Leydig cells, and also higher sperm production; these changes are probably due to a higher proliferation index of SC and LC during the perinatal period. Our aims in the present study were to characterize and evaluate, by immunohistochemistry and qPCR, the expression of important markers of testis function in wild type and $iNOS^{-/-}$ mice, during perinatal testis development and in adulthood. Wild type (WT) and $iNOS^{-/-}$ mice were evaluated on e18.5 (embryonic day), and on Pnd1, Pnd5, Pnd10, Pnd15, Pnd20 and Pnd70 (postnatal days). For this purpose, the testes of these mice were either fixed in Bouin's solution and routinely prepared for immunohistochemistry analysis, or frozen for later quantitative PCR. The qPCR data were analyzed by taking an average of four duplicates, normalized to β-Actin expression and corrected for the volume density of the corresponding cell type in which the marker gene is expressed. Immunohistochemistry data showed that the expression of aromatase and 3β-HSD protein was observed in Leydig cells of WT and $iNOS^{-/-}$ mice at all ages analyzed. However, aromatase gene expression was significantly reduced in testes of $iNOS^{-/-}$males in the perinatal period (i.e. at e18.5, Pnd1 and Pnd5), suggesting that estrogen production could be modulated by NO perinatally. In $iNOS^{-/-}$ male Leydig cell expression of certainsteroidogenic enzymes was altered. 3β-HSD expression was reduced ($p<0.05$) at Pnd1, Pnd5, Pnd15, Pnd20 and Pnd70, whilst StAR gene expression was reduced ($p<0.05$) in the $iNOS^{-/-}$ mice testis at e18.5, Pnd1, Pnd5, Pnd20 and Pnd70. As StAR expression at Pnd10 and Pnd15 was similar in both groups, this could be related to the period in which adult Leydig cells appear for the first time in mice. In contrast to these findings, Sox9 mRNA levels were significantly higher in the $iNOS^{-/-}$ testis at Pnd5 and Pnd70. According to the literature, this gene is essential for sex determination and for differentiation and function of SC. Although the expression of these and other important markers is currently being quantified using qPCR and immunohistochemistry pixel intensity, the preliminary data herein obtained demonstrate high variation in the expression of these genes during development, as well as their differential expression in wild type and $iNOS^{-/-}$ mice. We are currently investigating if these findings are related to the modulation of somatic cell proliferation during perinatal testis development and/orthe regulation of GC apoptosis in the adult testis.

Financial support: Capes, CNPq and FAPEMIG

E-mail: lrfranca@icb.ufmg.br

Effect of BSP1 (Binder of Sperm Protein-1) addition into fertilization media on in vitro production of bovine embryos

P. Rodríguez-Villamil[1], V. Hoyos Marulanda[1], L.H. Aguiar[2], A.N. Oliveira[2], M.F. Van Tilburg[2], M. Bertolini[2], A.A. Moura[1]

[1]Animal Physiology Laboratory, Federal University of Ceara, Fortaleza, Brazil; [2]Molecular and Developmental Biology Laboratory, University of Fortaleza, Fortaleza, Brazil.

The BSP1 (Binder of Sperm Protein-1) is the major protein in bull seminal plasma and mediates sperm capacitation and binding to the oviduct epithelium, among other events. BSP1 is a phospholipid-binding protein, with fibronectin type-II domains, that has the ability to remove both cholesterol and phospholipids from the membrane. Therefore, the aim of this study was to evaluate the effect of BSP1 supplementation into the fertilization media on the in vitro production of bovine embryos. Cumulus-oocyte complexes (COCs; n = 902) were obtained from slaughterhouse ovaries, matured in TCM- 199 and supplemented with 0.4 % BSA for 24 h in a controlled atmosphere. Then, COCs were selected and randomized into four different groups to be fertilized with frozen semen in the presence of different concentrations of BSP1. Control group: COCs fertilized in Fert-TALP medium with heparin; Group 1: COCs fertilized in Fert-TALP medium without heparin + 10 µg BPS1/mL; Group 2: COCs fertilized in Fert-TALP medium without heparin + 20 µg of BPS1/mL; Group 3: oocytes fertilized in Fert-TALP medium without heparin + 40 µg of BPS1/mL. Semen from threee *Bos taurus* var. *indicus* bulls was used for IVF. BSP1 was purified from bovine seminal vesicle fluid by affinity chromatography. Zygotes were cultured in SOF medium for 7 days at 38.8°C in a humidified incubator with 5% CO_2 and 5% O_2. Cleavage and embryo development were evaluated on Days 2 and 7 after fertilization. Data for cleavage and blastocyst rates were analyzed by ANOVA. Cleavage and blastocyst rates associated with the lower concentration of BSP (10 µg/mL; 76.9 ± 4 and 34.3 ± 5.5%, respectively) and (20µg/mL; 73.3 ± 2.5 and 21.4 ± 2.7%, respectively) were similar to those obtained in the control group (72.2 ± 3.2 and 39 ± 7%, respectively). However, higher concentrations of BSP-1 (40µg/mL) significantly decreased both the cleavage and blastocyst rates (63.2 ± 2.6 and 13.6 ± 3.5, respectively), in comparison with the control group (72.2 ± 3.2 and 39 ± 7, respectively). In conclusion, BSP-1 at the concentration of 10µg/mL had a capacitating effect similar to heparin, associated with proper cleavage and embryo development. The deleterious effect caused by higher concentrations of BSP-1 may be the results of BSP-1-induced damage on sperm, oocytes and/or the embryo.

Financial support: CNPq

E-mail: arlindo.moura@gmail.com

Effect of the vitamin E-analog Trolox on ovarian tissue autograft of the squirrel monkey (*Saimiri collinsi*)

S.R.R.A. Scalercio[1,5], D.C.C. Brito[1,2], S.F.S. Domingues[1,5], C.A.Amorim[4], R.R.Santos[1,3]

[1]Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Castanhal, Pará, Brazil; [2]Laboratory of Animal Cytogenetics, Federal University of Pará, Brazil; [3]Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [4]Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Brussels, Belgium; [5]National Primate Centre, Secretary of Health Policy, Ministry of Health, Ananindeua, Pará, Brazil.

Despite the successful results obtained with cryopreservation and autotransplantation of ovarian tissue, it is important to highlight the large follicle loss resulting from the latter procedure probably due to the avascular transplantation of the ovarian tissue, and consecutive oxidative stress. The aim of the present study was to evaluate tissue pretreatment with Trolox followed by heterotopic transplantation of *Saimiri collinsi* ovarian tissue. To evaluate tissue health and functionality, immunohistochemical analysis on the stroma and follicular cells was performed. For this, we used ovaries from non-human primates. Ovarian fragments from five adult squirrel monkeys were incubated in Trolox solution before being autografted. After one week, relative areas of fibrosis were evaluated using Masson's trichrome-stained, apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to detect DNA fragmentation, and following markers were selected to assess cell proliferation, follicle growth and function, stromal cell apoptosis and graft vascularization: Ki67, anti-Müllerian hormone (AMH), growth differentiation factor-9 (GDF-9), c-kit, and cluster of differentiation 31 (CD31). The quality of stromal tissue and influence of the Trolox incubation and autografted were also evaluated. Tissue vascularization was confirmed macroscopically and by CD31 staining. Fibrotic areas increased significantly in grafted + Trolox ovarian tissue, when compared with the other groups. Our results showed that after Trolox incubation and grafting, follicles were able to grow and maintain their function as illustrated by Ki67, growth differentiation factor-9, anti-Müllerian hormone and c-kit immunostainings. In conclusion, this study shows that Trolox pre-incubation improves follicular survival after grafting, decreases apoptosis rates in stromal cells, but also results in increased fibrotic areas. Our results suggest that fresh ovarian tissue can be incubated and grafted with no major impact on early follicular growth and morphology after short-term subcutaneous auto-transplantation.

Email: daniellecalado@ymail.com

The parental age affects the sex ratio of the offspring in horses

M.M. Santos[1], D.M. Nobre[2], J.F. Oliveira Neto[3], T.R. Garcia[3], M.C.G.R. Lage[1], M.I.V. Melo[1], G.R. Valle[1]

[1]Department of Veterinary Medicine, Pontifical Catholic University of Minas Gerais, Brazil; [2]Medicine School, Federal University of Ouro Preto, Brazil; [3]Brazilian Association of Mangalarga Marchador Horse Breeders, Brazil.

The gender of the offspring in mammals is primarily determined at the time of fertilization and secondarily by the conceptus survival until labor. The Trivers-Willard hypothesis states that the female, according to the evolutionary strategies of each species, is the final arbiter, influencing the X or Y-bearing spermatozoa chances to fertilize or influencing the conceptus survival inside their genital tract. This hypothesis has been observed in some species, but not yet in horses, despite our preliminary observation in a limited (n=262) equine group (Maia et al., XXº Congresso Brasileiro de Reprodução Animal, 2013). The aim of this work was to demonstrate, in a representative equine population, that the Trivers-Willard hypothesis is true for horses. The data of 78,182 Mangalarga Marchador horses born in Brazil (registered at the Brazilian Association of Mangalarga Marchador Horse Breeders – ABCCMM) from 1990 to 2011 were analyzed, using only parental DNA certified foals. The sex ratios of the offspring were compared among groups according to mare and stallion ages (from 3 to 25 years). On the first stage, the mare and stallion age groups were stated as: below 5; 5 to 10; 10 to 15; 15 to 20; over 20 years old. Crossing all parental groups, 5 *vs* 5 matrix of mare age groups *vs* stallion age groups were done. On the second stage the curves of offspring sex ratios were observed throughout the aging of the mares and stallions, year-by-year, regardless of the partner age. All of these analyses, on first and second stages, were done for natural (NAT) and embryo transfer (ET) derived offspring. On the third stage, the recipient mares of ET derived offspring were distributed along the same age groups attributed to mares, ignoring the stallion's age, and the sex ratio of these groups were compared. All the sex ratio comparisons were performed using Qui-square ($P<0.01$). The sex ratio of the registered population did not represent the sex ratio of the entire Mangalarga Marchador population born in Brazil, since not all of the males born in farms were registered at ABCCMM, because of the breeder's interest or not for registration of each one. Because of this, if the entirety of registered animals were used the results would not be reliable. To avoid data misinterpretation, 1:1 male:female sex ratio populations were obtained for NAT (29,976 foals of each gender) and for ET (9,115 foals of each gender). These numbers were determined by the total number of males registered, and the same numbers of females were randomly obtained. For the first step, the overall results showed that more foals were born from younger mares and more fillies were born from older mares for NAT offspring in all stallion age groups. However, for ET offspring group, this sex:ratio deviation tendency was slightly attenuated. For comparisons among the stallion age groups, more foals were born from younger stallions and more fillies were born from older stallions for NAT offspring, but with low magnitude compared to the same situation for mare age study. For ET offspring group, this sex:ratio deviation tendency for stallion age was almost completely abolished. On the second stage, larger sex:ratio were observed with 6 year old mares (1.34:1-MN; 1.53:1-ET) and smaller with 23 and 24 year old mares (0.56:1-MN and 0.71:1-ET, respectively). For both MN and ET, the decreasing of sex:ratio were continuous, and similar to each other. For stallions, a variation around the 1:1 sex ratio was observed both for MN and ET, with above 1:1 from 4 to 9-10 year olds; and varying around 1:1, but predominantly below 1:1, from 9-10 to 25 years old. On the third stage, the sex:ratio of the offspring were similar among recipient mare age groups, revealing that their age did not influence the sex:ratio of the offspring. To our knowledge, the literature attributes the bias of the offspring sex ratio in mammals to the nutritional status and age of the mother, influencing the primarily and/or secondarily sex determination of the offspring by affecting the X or Y-bearing spermatozoa ability to fertilize the oocyte, and because of differences in XX or XY embryo survival in the female genital tract. Our data showed that the aging of mares contributes to the birth of more fillies than foals, and that the gender at birth is mostly determined before 8-9 days (day of embryo transference to the recipient mares), as similar sex ratios were observed when ET was performed. Also, the stallion aging seems to have some influence in offspring gender, but smaller than mare's influence.

Financial support: FIP PUC Minas (# 2013/7563-1S)

E-mail: guilhermerv@pucminas.br