cumulus–oocyte complexes (COCs; $n = 750$) from slaughterhouse ovaries were cultured in 199 $HNaCO_3$ with polyvinyl alcohol (PVA) 0.1 mg mL^{-1} as a basic medium. Culture was made in two steps, a 24 h meiotic arrest (roscovitine 25 μM), and a subsequent *in vitro* maturation period with FSH-LH for 24 h. Testosterone (T-86500, Sigma-Aldrich, St. Louis, MO, USA) was added throughout the entire oocyte culture at 0, 30, 300, and 1500 nM. After *in vitro* fertilization (Day 0), zygotes were freed of cumulus cells by pipetting, and subsequently cultured in SOF + 6 g L^{-1} BSA up to Day 3. At this time, embryo development was recorded, and all embryos having 3 or more cells were treated with pronase to remove the zona pellucida. Zona-free embryos were washed in PBS containing PVA 0.1 mg mL^{-1} and individually frozen at −80°C until sex analysis by PCR (Bermejo-Alvarez P *et al.* 2008 Biol. Reprod. doi:10.1095/biolreprod.108.070169). A total of 252 embryos from 5 replicates were sexed. Data for development and sex-ratio are presented as % LSM ± SD. There were no interactions between testosterone treatment, embryonic sex, and embryonic stage analyzed. Testosterone did not affect development rates ($P > 0.05$) at any stage: cleavage (47.8 ± 6.8, 56.5 ± 6.8; 50.9 ± 6.8; 62.2 ± 6.8), 3 to 4 cells (40.6 ± 5.2, 45.8 ± 5.2; 37.8 ± 5.2; 47.7 ± 5.2) and >5 cells rates (24.5 ± 4; 27.3 ± 4; 21.3 ± 4; 25.3 ± 4) for 0, 30, 300, and 1500 nM testosterone, respectively. Cumulative percentages of male embryos were as follows: 53 ± 8 ($n = 56$), 42.6 ± 8 ($n = 52$), 53.6 ± 6 ($n = 81$) and 57.6 ± 8 ($n = 63$) for 0, 30, 300, and 1500 nM groups respectively ($P > 0.05$). These results show that the testosterone effects on oocyte ability to select Y-chromosome bearing spermatozoa are not reproducible *in vitro* under the present experimental conditions.

Grant support: MEC, project AGL2008-01530; RTA2008-0082; M. Muoz is supported by FICYT.

265 SEXING OF BOVINE EMBRYOS BASED ON PCR TECHNIQUE

E. Koban, A. Tas, Ö. Aslan, T. Akkoc, S. Arat, and H. Bagis

TUBITAK Marmara Research Center, Gebze, Kocaeli, Turkey

Turkish cattle breeds are well adapted to harsh environmental and poor feeding conditions. However, their productivity is low. Increasing fertility rate and obtaining a high number of progeny from high-quality animals are important parameters in animal husbandry. The objective of the present project is to produce sexed embryos and cryopreserve them for subsequent transfer. The birth of the calves produced from the transferred embryos with sex determined prior to transfer by PCR are additional objectives of the study. To develop and optimize the PCR method, DNA was first isolated by using standard phenol-chloroform extraction from blood samples of cows and bulls to use as positive control. Then two multiplex PCR methods were developed using one autosomal (bovine 1.715 satellite locus which produces 216 bp long PCR product), and two Y-chromosome specific loci BRY4.a (300 bp) and BRY1 (300 bp). Both multiplexes include bovine 1.715 satellite locus, and they either include BRY4.a or BRY.1 as the second locus. Female individuals produce one PCR band, whereas male individuals produce two PCR bands. Bovine parthenogenetic blastocysts were used to test these two multiplex PCR methods. Immature bovine oocytes were aspirated from slaughterhouse material and *in vitro* matured in tissue culture medium-199 (TCM-199) supplemented with 10% FCS, sodium pyruvate, EGF, bLH, bFSH and penicillin/streptomycin for 18 h at 39°C and 5% CO_2 in humidified air. After removing the cumulus cells of matured oocytes (MII), meiotic spindles and first polar bodies were removed. Oocyte-cell complexes were fused by one 30 μs pulse of 133V/500 μM. All fusion units were subjected to chemical activation. Afterwards, parthenogenetic oocytes were cultured in Sage cleavage® medium supplemented with 8 mg mL^{-1} BSA for 72 h and then developing embryos were cultured in Sage blastocyst® media supplemented with 4 mg mL^{-1} BSA + 5% FCS for 4 additional days. Then they were stored at −20°C until DNA extraction. For DNA extraction two methods (Park *et al.* 2001 Theriogenology **55**, 1843–1853; Tshimangadzo *et al.* 2004 Biol. Reprod. **71**, 1671–1676) were employed to test their efficiency in our laboratory conditions, and we got better results with the former method. Repeated PCR tests of these parthenogenetic blastocysts were carried out and tests revealed only one PCR product of 216 bp corresponding to the 1.715 satellite locus as expected. The multiplex PCR methods will also be employed for Nuclear Transfer and IVF embryos.

This project is supported by Turkish Scientific and Technical Research Council-TOVAG (project no: KAMAG 107G027).

266 WHOLE GENOME AMPLIFICATION ON BLASTOMERS OF POST-BIOPSY BOVINE EMBRYO

J. Polisseni[A,B], *M. A. Machado*[A], *A. L. Souza*[A], *R. Domingues*[A], *M. O. Guerra*[B], *R. V. Serapiao*[A], *M. M. Pereira*[A,B], *W. F. Sa*[A], *B. C. Carvalho*[C], *L. S. A. Camargo*[A], *J. H. M. Viana*[A], *and V. M. Peters*[B]

[A]Embrapa Gado de Leite, Juiz de Fora, MG, Brazil;
[B]Universidade Federal de Juiz de Fora, Juiz de Fora, MG, Brazil;
[C]Empresa de Pesquisa Agropecuaria de Minas Gerais, Juiz de Fora, MG, Brazil

Biopsy of embryos is very useful for choosing the desired sex and for production of cloned and transgenic livestock. However, only a small amount of genomic DNA is available to perform genetic studies. Alternatively, methodologies using whole genome amplification (WGA) have been developed. The aims of this study were to evaluate the effect of WGA on blastomeres removed from 8- to 16-cell bovine embryos and to determine the sex of blastomeres. Oocytes obtained from slaughterhouse ovaries were *in vitro* matured and fertilized. On the fourth day after fertilization, 8- to 16-cell bovine embryos were biopsied, and one-fourth of an embryo was removed. The blastomeres ($n = 56$) were submitted to WGA followed by PCR. Prior to the whole genome amplification, male and female bovine DNA samples were serially diluted (30 ng μL^{-1}, 3.0 ng μL^{-1}, 0.3 ng μL^{-1}, 0.03 ng μL^{-1}, 0.003 ng μL^{-1}, 0.0003 ng μL^{-1}) and embryos of various development stages (2, $n = 6$; 4–7, $n = 5$; ≥8-cell, $n = 5$; blastocyst $n = 27$) were used to standardize PCR protocols and set the amplification limits. To digest the cellular cytoplasm and release the genomic DNA, embryos and blastomeres were submitted a 3 mg mL^{-1} proteinase K before PCR. Next, blastomeres were submitted to the GenomiPhi DNA Amplification Kit

SP 4470
P.145

(GE Healthcare) according to manufacturer's instructions. The product (1 μL) was electrophoresed on a 1% agarose gel stained with 3.0 μg mL^{-1} ethidium bromide. The reaction mixture was added to the material to be amplified (2 mM MgCl2, 5X PCR buffer, 0.2 mM each dNTPs, 0.05 U μL^{-1} GoTaq DNA polymerase, 0.25 μM of primer). The products were submitted to electrophoresis on 8% polyacrilamide gel and stained with silver nitrate procedure. The chi-square test was used for statistic evaluation of the results to test the WGA efficiency and to determine the sex rates of bovine embryos and biopsied samples submitted to PCR. It was possible to achieve 98% efficiency in amplifying blastomeres using the WGA kit. Amplified samples showed approximately 400 ng of DNA generated from an estimated initial amount of 12 pg of DNA resulting from two cells per embryo. In whole embryos from different stages, no diffference was detected in the proportion of sexes ($P > 0.05$). However, a greater number of female samples was noted in biopsied material (76%, 25/33) ($P < 0.05$). PCR efficiency in blastocysts (93%, 2/27) was statistically greater ($P < 0.05$) than embryos in early stages of development (83%, 5/6), and biopsied material to 2, 4–7 and ≥8-cell (40%, 2/5; 60%, 3/5; and 59%, 33/56; respectively). These differences could be related to a sex-chromosomal mosaicism or absence of a nucleus in biopsied samples. The WGA creates a DNA stock sample that could be used for various gene profiling and sex determination analyses.

Financial support: Fapemig, CNPq.

267 DIFFERENCES IN BULL SPERM NUCLEAR SHAPE BETWEEN FLOW-SORTED AND NON-SORTED SPERM USING FOURIER HARMONIC ANALYSIS

J. R. Schindler, R. L. Monson, K. L. Willenburg, and J. J. Parrish

Department of Animal Sciences, University of Wisconsin, Madison, WI

The objective of the study was to evaluate sperm nuclear shape in sex-sorted and non-sorted semen using Fourier Harmonic Amplitudes (FHA). Frozen semen was obtained from a single commercially available source. Mature bulls ($n = 15$) from the same breed with an average age of 4.42 ± 2.08 years were collected and the semen was either frozen or X-chromosome sorted using a flow cytometer and then frozen. Frozen straws were transported to the lab and analyzed for FHA. Briefly, straws were thawed and cells were incubated with 1.6 μM Hoechst 33342. Cells were then washed, fixed, dried to a slide and analyzed for nuclear head shape. Harmonic amplitudes 0 to 5 (HA0–HA5), derived from FHA, were previously shown to be an accurate, objective, and repeatable measure of sperm nuclear shape. HA0 describes the overall nuclear size of the sperm, whereas HA1 describes the anterior head, HA2 the length of the sperm along the longitudinal axis, and HA3 to 5 the distal, post-nuclear curvature of the sperm head. Each unit of semen was evaluated for motility and FHA. There was a significant decrease in motility in the sorted group (77 ± 1% *v.* 54 ± 3%; $P < 0.0001$). Multivariate ANOVA showed that there were differences between the sorted and non-sorted groups in HA1 to 4 ($P < 0.02$). Harmonic amplitude means ± SD (microns) for sorted and non-sorted treatments are as follows: HA1 (0.117 ± 0.003 *v.* 0.109 ± 0.003), HA2 (1.087 ± 0.005 *v.* 1.063 ± 0.005), HA3 (0.139 ± 0.003 *v.* 0.130 ± 0.003), and HA4 (0.201 ± 0.004 *v.* 0.191 ± 0.004), respectively. The nuclear shape of X-sorted sperm is longer and more pinched in both the anterior and posterior head. Interestingly there was no difference in HA0 ($P = 0.119$) indicating that the overall size of the sperm head is not affected by the sorting process. The differences in harmonic amplitudes may be due to the size and a restricted location of the X *v.* Y chromosome in the sperm nucleus.

268 IDENTIFICATION OF X- AND Y-BEARING SPERMATOZOA IN SORTED BUFFALO (*BUBALUS BUBALIS*) SEMEN BY FLUORESCENCE *IN SITU* HYBRIDIZATION

M. Zhang[A,B], *X. J. Zhuang*[A,B], *Y. Q. Lu*[A,B], *C. H. Hu*[A,B], *S. S. Lu*[A,B], *and K. H. Lu*[A,B]

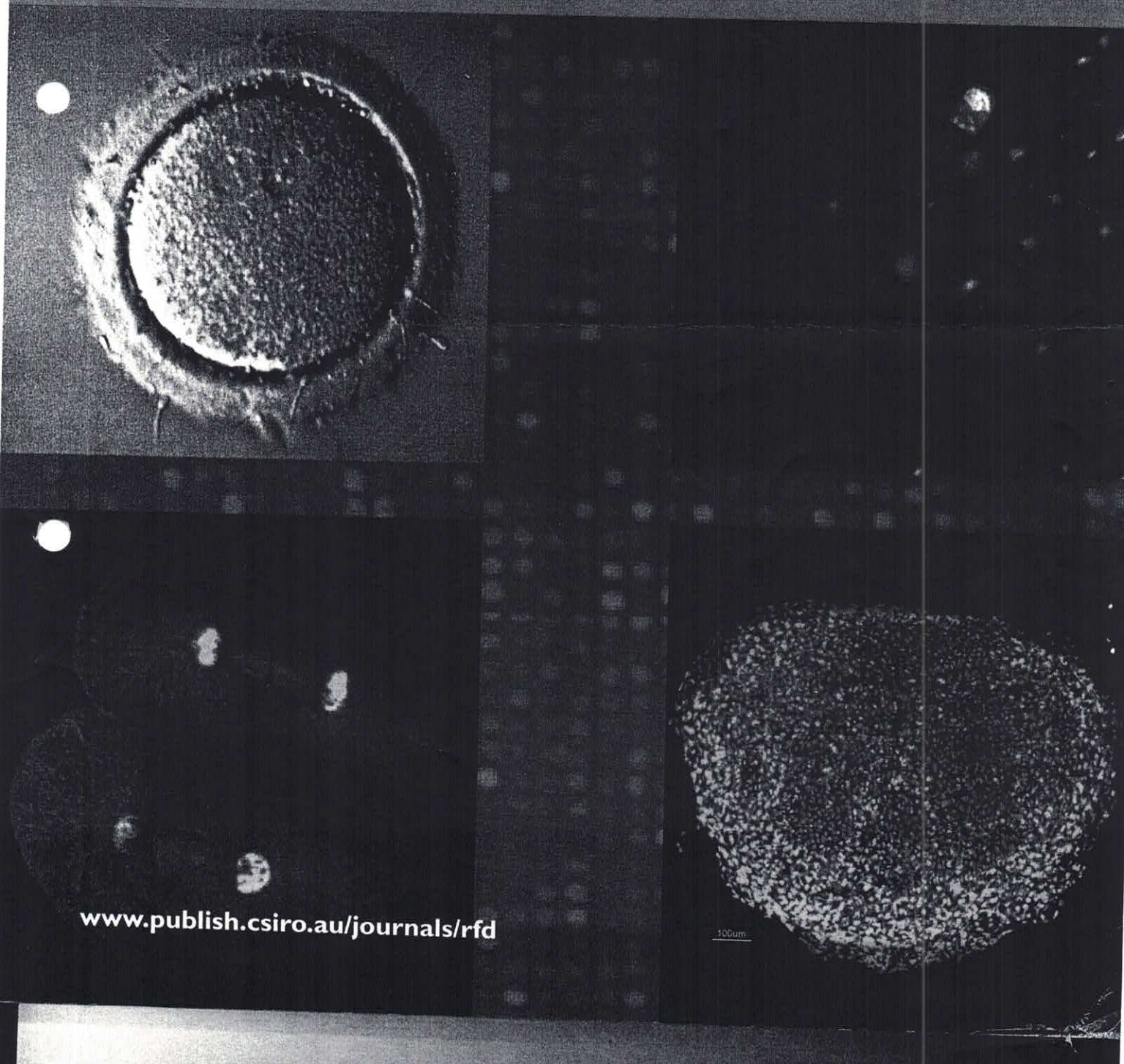
[A]Guangxi Key Laboratory of Subtropical Bio-Resource Conservation and Utilization;
[B]Animal Reproduction Institute, Guangxi University, Nanning, China

Flow cytometry sorting technology has been successfully used to sort the X- and Y-chromosome bearing sperm. Previous studies showed that fluorescence *in situ* hybridization (FISH) method was a simple and reliable procedure for assessing the effectiveness of separation of X- and Y-sperm in the swine (Kawarasaki T *et al.* 1998 Theriogenology **50**, 625–635) and the bovine (Rens W *et al.* 2001 Reproduction **121**, 541–546). Reports of sex-preselection by flow-cytometry sorting of the X- and Y-sperm were also seen in the buffalo (Presicce GA *et al.* 2005 Reprod. Dom. Anim. **40**, 73–75; Lu YQ *et al.* 2006 Anim. Reprod. Sci. **100**, 192–196). There was, however, no report to date for using the FISH method to assess the purity of the sorted buffalo sperm. The objective of the present study was to verify the purity of flow cytometrically-sorted buffalo X- and Y-sperm by FISH using bovine X- and Y- chromosome painting probes prepared by microdissection. The X- and Y- chromosomes of bovidea were microdissected respectively from the metaphase spreads of Holstein blood cells with a glass needle controlled by a micromanipulator and amplified by degenerate oligo-nucleotide primer-PCR (DOP-PCR) (Mariela N *et al.* 2005 Genet. Mol. Res. **4**, 675–683). The DOP-PCR products of X- and Y- chromosome were labeled with CY3-dUTP and Biotin-11-dUTP, respectively. The buffalo X- or Y-sperm DNA from unsorted semen and sorted semen were hybridized to the labeled probes, respectively. The results showed that the hybridized signals were clearly visible in the metaphase karyotype of bovine and buffalo semen samples. About 47.7% (594/1246) and 48.9% (683/1396) of the unsorted buffalo sperm emitted strong fluorescent signals when assessed by Y- and X-chromosome painting probes, respectively, which was conformed to the sex ratio in normal buffalo sperm (50%:50%). About 86.1% (1529/1776) hybridization signals of the sperm in the sorted X-semen assessed by X-chromosome painting probes were detected, while 82.2% (2232/2716) of the Y-sorted buffalo sperm emitted strong fluorescent signals when assessed by Y-chromosome painting probe. The results of the flow cytometer re-analysis revealed that the proportions of X- and Y-bearing sperm in the sorted semen were 89.6% and 86.7%, respectively. There were no apparent differences between the two assessment methods of sperm separation by flow cytometry re-analysis and by FISH with the

Reproduction, Fertility and Development

Proceedings of the Annual Conference of the International Embryo Transfer Society, San Diego, California, USA, 3–7 January 2009

Volume 21(1) 2009



www.publish.csiro.au/journals/rfd