ABSTRACTS FOR POSTER PRESENTATION

198 BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN CELL LINES USED FOR SOMATIC CELL CLONING

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Most isolates of BVDV cause unapparent infections in cultured cells. Fetuses, postnatal animals or fetal bovine serum are possible sources of the virus for cultivated cells used as karyoplasts in cloning. Routine screening by veterinary diagnostic laboratories of 39 fetal fibroblast cell lines used in cloning research had revealed that 15 (39%) were positive for BVDV by various assays including RT-nPCR. As some were valuable transgenic cell lines, a rigorous protocol for evaluation of each line was undertaken to confirm infection with BVDV. A cryopreserved vial of each line was thawed, medium discarded and cells incubated (38.5°C in 5% CO_2 and air) through 2 passages (6–10 days) in α-MEM supplemented with 10% equine serum. At the end of the second passage, cells were separated from medium, washed and assayed for presence of BVDV using virus isolation in 2 sequential passages in Madin Darby Bovine Kidney Cells and RT-nPCR. Available lots of fetal bovine serum and medium that had been used to culture the cells also were tested for BVDV. When the virus was detected, the RT-nPCR products were sequenced and compared. Also, an attempt was made to evaluate the earliest available cryopreserved passage of any positive cell lines. Results indicated that just 5 of 39 of the original cells tested (13%) were positive. Since cryopreserved earlier passages of 4 of the cell lines were available, they were assayed with the result that 2 of the 4 were not infected at the earliest passage. Further, BVDV was isolated from one lot of fetal bovine serum that was used to culture one of the cell lines. Sequence analysis verified that only 2 of these 4 cell lines were infected with the same isolate of BVDV, and one isolate was identical to the virus found in the fetal bovine serum used in medium to culture it. The discrepancy between our viral detection and that of the diagnostic laboratories is explained in part by the presumed test protocols. All BVDV-positive cells, as reported by the diagnostic laboratories, were positive by RT-nPCR. We presume that they did not separate medium from cells before assays. Thus, any noninfectious viral RNA that was in the medium (e.g. as would be expected in many lots of irradiated serum) would have been reported positive. The only possible sources for BVDV in these cell lines were the fetuses from which they originated or fetal bovine serum used in medium. Sequence analysis confirmed that serum was the source of viral infection in one line. The likely source of virus for 2 other lines was serum, since they were not infected at earlier passages. The 2 remaining cell lines were positive at the earliest available passages, so the fetuses from which cells were harvested could not be discounted as the source of BVDV. This report highlights the risks of introducing BVDV in embryo technologies and the difficulties that can be encountered in attempting accurate diagnosis of the presence of infectious virus.

Exotic Species

199 CHARACTERISTICS OF CHAMOIS (*RUPICAPRA PYRENAICA PARVA*) EPIDIDYMAL SPERMATOZOA DEPENDING ON TIME POSTMORTEM

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The Spanish Cantabrian chamois (*Rupicapra pyrenaica parva*) is a wild ruminant of the Cantabric Mountains (North of Spain). It is not an endangered species, but it is vulnerable to sarcoptic mange outbreaks and it is appreciated as a hunting trophy. The aim of the present study, as part of a project for establishing genetic resource banks for wild species in the North of Spain, was to determine the effect of postmortem time (PT) on epididymal sperm quality. We obtained 37 sets of testes from males hunted during the breeding season (autumn). The samples, with testes inside of the scrotum to prevent drying, were cooled to 5°C, and processed at 4 different PTs (0–30 h, 30–60 h, 60–90 h, 90–120 h). Sperm was obtained from incisions made in the caudal epididymis. Osmolality (OSM) and pH of the undiluted fluid were measured with a cryoscopic osmometer (Osmomat-030,

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Gonotec™; Berlin) and an electronic pH-meter (CG 837, Schott™; Mainz, Germany), respectively. Sperm motility (M) and progressive motility (PM) were assessed subjectively at 37°C. An aliquot of sperm was fixed in glutaraldehyde and used to evaluate acrosome integrity (ACR) and abnormal forms (heads, AH; midpieces, AM; tails, AT). Membrane functionality (MF) was assessed by means of the HOS test (100 mOsm kg^{-1}, 18 min). All analyses were carried out using a phase-contrast microscope (×100 for motility and ×400 for other analyses). We obtained the Spearman correlation coefficients between the analyzed parameters and PT. Samples were graded on sperm quality parameters (PM, ACR and MF: >60%, high; 60–30%, medium; <30%, low), and their distribution among PT groups was compared (χ^2, $P < 0.05$). The following parameters correlated significantly with PT (r and *P* are shown): pH (0.37, 0.008), OSM (0.52, < 0.001), M (−0.51, < 0.001), PM (−0.62, < 0.001), MF (−0.44, 0.001), ACR (−0.33, 0.02), AT (0.41, 0.005). The correlations of pH and osmolality indicate changes in epididymal fluid composition which could impair sperm viability. In fact, sperm motility, acrosome integrity and membrane functionality showed negative correlations. Also, AT increased with PT, which could be related to membrane damage (along with MF decrease). The distribution of the samples in quality groups is shown in the Table 1. There were no low quality samples in the 0–30 h group, but the proportion of such samples increased significantly with PT. In conclusion, epididymal sperm characteristics changed with PT, showing a remarkable loss of quality after the first 30 h. Even so, it was still possible to find samples of acceptable quality after several days. This is an important observation when collecting samples for genome resource banking. However, it will be necessary to assess the fertilizing ability of sperm stored for extended periods postmortem to confirm the utility of these findings.

Table 1. Variation of sperm quality (PM, ACR and MF: >60, high; 60%–30%, medium; < 30%, low) with PT

Time	0–30 h	30–60 h	60–90 h	90–120 h
High	75.0[a]	0.0[b]	0.0[b]	0.0[b]
Medium	25.0	38.5	22.2	12.5
Low	0.0[a]	61.5[ab]	77.8[b]	87.5[b]

In the same row, different superscripts indicate significant differences ($P < 0.05$).

200 OVARIAN STIMULATION, LAPAROSCOPIC OOCYTE RETRIEVAL, IVF AND BLASTOCYST PRODUCTION USING SEQUENTIAL MEDIA IN THE AFRICAN LION (*PANTHERA LEO*)

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The objective of this study was to test the efficacy of Gardner's Sequential Medium on in vitro embryo production in lions. Three young (3–4 yr) nulliparous lionesses were treated with a total of 50 IU purified porcine FSH (Sioux Biochemical, Sioux Center, IA) in a decreasing regimen over 4 days. At 80 h, they were chemically immobilized with a combination of tiletamine and zolazepam (Zoletil; Virbac) and a single deslorelin implant (Ovuplant, Peptech Animal Health Pty, Ltd., North Ryda, New South Wales, Australia) was placed (s.c.) in the lateral thorax over the scapula to stimulate final oocyte maturation. Approximately 24 h later, the lionesses were again chemically immobilized (Zoletil); ovaries were viewed, structures recorded and oocytes retrieved laparoscopically (Table 1).

Just prior to oocyte retrieval, four lions were chemically immobilized. Semen was collected by rectal probe electrostimulation and diluted 1:10 in five media (TL HEPES, modified Tyrodes + 6 mg mL^{-1} BSA + 10% FCS, HEPES-Hams F10 + 10% FCS, Biladyl and Tyrodes + 5% lion serum) and incubated at room temperature and ambient atmosphere for 6 h before evaluation and use in IVF. Since there were no differences in sperm motility in any of these media (>90% after 6 h), spermatozoa from each male were pooled from all treatments for IVF. A total of 6, 10 and 3 grade A oocytes (73%) from Lionesses #1, 2 and 3, respectively, were inseminated with approximately 1×10^6 lion sperm/mL in 0.5-mL wells containing G1 Sequential Medium and cultured for 16 h in humidified 6% CO_2 at 38°C. Then, presumptive zygotes were washed and cultured in 50-μL microdrops of G1 under oil. After 72 h, embryos were washed and cultured in 50-μL microdrops of G2 under oil. At 110 h post-insemination, 10 had cleaved (53%) and, of these, 5 (50%) developed to the morula stage and 3 (30%) to the blastocyst stage. In conclusion, the present study has shown that lion oocytes can be successfully fertilized in vitro. Furthermore, the in vitro-produced embryos can be cultured to the blastocyst stage in G1/G2 Sequential Media.

Table 1. Ovarian structures observed and oocytes collected from three lionesses

Lioness	Ovarian follicles/ corpora hemorrhagica (CH)			Other	Oocytes collected
	2–4 mm	6–8 mm	10–12 mm		
1	13	8	9	2 luteinized, 1 CH	12
2	8	3	15	3 14–15 mm, 3 CH	11
3	22	3	0	3 luteinized	3

201 RED DEER (*CERVUS ELAPHUS*) CALVES BORN FROM IN VITRO-PRODUCED BLASTOCYSTS FERTILIZED AND CULTURED IN DEER SYNTHETIC OVIDUCT FLUID

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Deer Synthetic Oviduct Fluid medium (DSOF; Berg and Asher 2003 Theriogenology 59, 189–205), based upon the composition of red deer oviduct fluid, has been shown to support routine in vitro fertilization and blastocyst development (15%) of in vitro-matured red deer oocytes without the use of somatic cell co-culture or serum supplementation. However, pregnancy establishment and fetal survival remained to be determined. The objectives of this study were to transfer in vitro red deer blastocysts produced by the DSOF culture system, follow fetal survival through calving and investigate trans-cervical embryo transfer for red deer. Red deer hinds of mixed age were synchronized using a 12-day CIDR (Pharmacia & Upjohn, Auckland, NZ) synchrony program (Berg DK *et al*. 2002 Ani. Reprod. Sci. 70, 85–98). Onset of estrus was synchronized to the day of IVF and embryos were transferred 7 days later. in vitro red deer blastocysts were produced after aspirating oocytes from abattoir-sourced ovaries. Selected COCs were matured and fertilized and presumptive zygotes cultured in vitro (Berg and Asher 2003 Theriogenology 59, 189–205) with modified Ca^{2+} concentrations: 3.0 mM and 1.5 mM for early and late DSOF, respectively. in vitro blastocyst development was 14.7% (21/143) on Day 7 and 22.4% (32/143) on Day 8. Ten blastocysts (grade 1 and 2) were selected for transfer on Day 7 (post-IVF) and placed into Emcare embryo holding medium (ICPbio, Auckland, NZ). Blastocysts were loaded into 0.25-cc straws ($n = 5$) or tom cat catheters ($n = 5$) and transported to the Ruakura Deer Unit at 25°C. Hinds were restrained and sedated as described for OPU (Berg and Asher 2003 Theriogenology 59, 189–205) and an attempt was made to pass a cattle transfer pistolette through the cervix. If unsuccessful, the hind underwent laparoscopic uterine transfer. Serial serum progesterone values diagnosed pregnancies at Day 21 and fetal survival was determined using rectal ultrasonography on Day 35, 45, 60 and 90. Seven single-embryo transfers were completed; 2 of 5 trans-cervical attempts and 5 using the laparoscopic method. Serum progesterone levels confirmed 57% (4/7) of the hinds were pregnant on Day 21; 2/2 (100%) from trans-cervical and 2/5 (40%) from laparoscopic transfers. No pregnancy losses occurred after Day 21. Four calves, 1 male and 3 female, were born unassisted after 230 to 233 days of gestation. Birth weights ranged from 7.3 to 10 kg. Our results indicate that in vitro red deer blastocysts produced using the DSOF culture system can establish pregnancies after transfer and result in normal healthy calves.

202 FERTILIZING ABILITY OF EPIDIDYMAL CAT SPERMATOZOA AFTER CRYOPRESERVATION OR STORAGE AT 4°C.

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The possibility of harvesting cat epididymal spermatozoa from excised testis represents a potentially important tool for preserving valuable genetic material from males that die unexpectedly. The purpose of this study was to evaluate plasma membrane integrity, acrosomal status and in vitro fertilizing capability of epididymal cat sperm after short- and long-term storage at 4°C and after cryopreservation. Spermatozoa were collected by flushing the cauda epididymis and the vasa deferentia removed from adult cats undergoing orchiectomy. Spermatozoa were split into three aliquots: A-fresh, B-frozen, and C-cooled. The A portion was immediately tested on the day of collection (control), the B portion was frozen in pellets in Test Yolk Buffer (TYB) with 8% glycerol, while the C portion was extended in TYB and stored at 4°C for 1 day, 2 days or 7 days. Cat oocytes recovered from minced ovaries were matured for 24 h in TCM 199 + 1 UI/mL hCG + 0.5 UI/mL FSH + 0.3% BSA at 38.5°C in 5% CO_2. In vitro fertilization (IVF) of in vitro-matured (IVM) oocytes was performed using spermatozoa from portions A, B or C in modified Tyrode's solution supplemented with 0.6% BSA at 38.5°C in 5% in air. Before IVF, aliquots of sperm samples from the three portions were stained simultaneously with fluorescein isothiocyanate-labelled Pisum Sativum agglutinin (FITC-PSA) and propidium iodide to evaluate the percentage of plasma membrane-intact spermatozoa with acrosomes present. At least 200 spermatozoa were counted in duplicate for each sample. After 24 h of incubation, to evaluate fertilization rate, the oocytes were stained with aceto-lacmoid. Oocytes with two pronuclei, presumably male and female, were classified as fertilized. The percentages of spermatozoa maintaining plasma membrane integrity and intact acrosomes after 1 day or 2 days of storage at 4°C were 75% and 65%, respectively, and were similar to that of fresh epididymal spermatozoa (78%). However, the percentages of spermatozoa with intact plasma membranes that had intact acrosomes after 7 days of storage at 4°C (52%) or after cryopreservation (48%) were significantly lower ($P < 0.01$, ANOVA) than those of samples stored for 1 or 2 days at 4°C. As shown in the Table, fertilization rates of oocytes inseminated with fresh spermatozoa and spermatozoa stored for 1 or 2 days were similar. In contrast, the fertilization rates of oocytes inseminated with spermatozoa that had been cryopreserved or stored for 7 days were significantly lower than those obtained with spermatozoa used immediately after collection or storage for 1 day. From our results, we suggest that storage of epididymal spermatozoa at 4°C may be a potentially useful method for temporary storage of spermatozoa from endangered cats, and may allow more efficient use and long-distance transport of genetically important germplasm. This work was supported by MIUR (ex 40%).

Table 1. In vitro fertilization of in vitro-matured cat oocytes with fresh, stored at 4°C and cryopreserved epididymal cat spermatozoa

Sperm treatment	Oocytes, *n*	Mature oocytes, *n*	Fertilized oocytes, *n*
A-fresh	65	51	48 (94.11)[a]
B-frozen	69	53	34 (64.1%)[b]
C-cooled			
1 day	66	49	40 (81.6%)[ad]
2 days	63	48	37 (77.1%)[bcd]
7 days	53	41	24 (58.5%)[bce]

Values with different superscripts are significantly different, ANOVA ($P < 0.05$; a v. bce $P < 0.01$). Three replicates were performed.

203 TIMING OF IN VITRO OOCYTE MATURATION IN SPRINGBOK (*ANTIDORCAS MARSUPIALIS*), BLACK WILDEBEEST (*CONNECHAETES GNU*), BLESBOK (*DAMALISCUS DORCUS PHILLIPSI*), AND REEDBUCK (*REDUNCA ARUNDINUM*)

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With an increased need for genetic diversity within populations, assisted reproductive technology is becoming an important tool for banking semen and embryos, with the possibility of future AI or embryo transfer between distant populations. Previous research has demonstrated that the broad approach of applying bovine IVM/F/C protocols to African antelope is inefficient for embryo production. The purpose of this study was to determine the timing of oocyte maturation (to telophase or metaphase II) in vitro in springbok (*Antidorcas marsupialis*; $n = 84$), black wildebeest (*Connechaetes gnu*; $n = 18$), blesbok (*Damaliscus dorcus phillipsi*; $n = 9$), and reedbuck (*Redunca arundinum*; $n = 1$). Ovaries were collected within 4 h of death from culled animals on game reserves in South Africa, placed into warm SOF-HEPES, sliced and cumulus-oocyte complexes (COCs) collected. Complexes were placed into GMat medium supplemented with 0.01 U mL^{-1} each FSH and LH, 50 ng mL^{-1} EGF, and 1.0% (v/v) PSA (100 U penicillin mL^{-1}, 100 μg streptomycin mL^{-1}, 0.25 ng amphotercin mL^{-1}). Oocytes were removed from medium after 16, 20, 24, 28 or 32 h of maturation in 5% CO_2 in air at 39°C. After removal, complexes were denuded with hyaluronidase, mounted on a slide and placed into 3 : 1 (ethanol : glacial acetic acid) fixative until time of analysis. Oocytes were stained with aceto-orcein, and nuclear maturation was evaluated with a phase contrast microscope. As shown in the Table 1, in springbok ($n = 311$), the percentage of mature oocytes peaked at 28 h of maturation and was not different ($P < 0.05$) from oocytes matured for 32 h. Fewer springbok oocytes were mature at 16, 20 and 24 h. Black Wildebeest ($n = 88$) oocytes were mature by 24 h, with no additional increase in maturation at 28 or 32 h. Blesbok oocytes ($n = 42$) were not mature at 16 or 20 h, and there was no difference ($P > 0.05$) in the percentage of oocytes mature at 24 or 28 h. Observational data in reedbuck ($n = 10$) indicated that no oocytes were mature at 20 or 24 h, but 25.0% were mature at 28 h. The low occurrence of mature oocytes at 28 h in blesbok and reedbuck suggests that additional time points should be examined later in maturation. This study demonstrates that oocytes of these species can be successfully matured in vitro, but the rate of maturation and thus the optimal time of insemination for IVF is species specific.

Table 1. Timing of in vitro oocyte nuclear maturation to telophase or metaphase II in springbok, black wildebeest and blesbok

	% Mature				
Species	16 h	20 h	24 h	28 h	32 h
Springbok	3.3 ± 3.3^{a}	6.5 ± 2.5^{a}	35.9 ± 7.1^{b}	65.1 ± 9.8^{c}	72.4 ± 10.9^{c}
Black Wildebeest	12.5 ± 0.0^{a}	$22.5 \pm 2.5^{a,b}$	52.4 ± 12.6^{b}	48.2 ± 8.3^{b}	$25.0 \pm 25.0^{a,b}$
Blesbok	0.0 ± 0.0	0.0 ± 0.0	38.4 ± 9.6^{a}	20.0 ± 20.0^{a}	

[a,b,c]Different superscripts represent statistical differences ($P < 0.05$) between time points within species.

204 THE PRODUCTION OF INTRACYTOPLASMIC SPERM INJECTION LION (*PANTHERA LEO*) EMBYROS USING SPERMATOZOA COLLECTED BY PERCUTANEOUS EPIDIDYMAL SPERM ASPIRATION FROM VASECTOMIZED MALES

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Contraception and/or sterilization methods have become an essential component of many captive animal management programs. Sterilization techniques are considered to be the last resort if a viable contraceptive cannot be attained and are generally not considered reversible. The African lion (*Panthera leo*) is one species in which sterilization techniques have been routinely applied. The objective of this study was to develop and evaluate a method for the collection of spermatozoa from male lions that have been previously rendered sterile by vasectomy. Percutaneous epididymal sperm aspiration (PESA) is a technique in which spermatozoa are aspirated from the epididymis and no surgical incision is required. In the present study, two lions (12 and 19 yrs old) were anesthetized and PESA was attempted. A 21-gauge needle attached to a 10-mL syringe (Norm-Ject) filled with 2–3 mL of Tyrodes HEPES medium was gently inserted into the head of the epididymis and aspirated gently until spermatozoa were noted. Spermatozoa were visually assessed for motility (grade 1–5; 1 = few motile sperm to 5 = all motile), sperm concentrations were determined and then the sperm were cryopreserved. The total sperm concentration collected from the older (19 yr) male was lower than that obtained from the younger (12 yr) lion (0.08×106 sperm/mL v. 65.5×106 sperm/mL, respectively). Furthermore, more motile spermatozoa (grade 3) were collected from the younger individual compared to the older male (grade 1). Sperm samples from the 12-yr-old lion were frozen by multi-step addition of TEST

yolk buffer + glycerol. Lionesses ($n = 3$) were subjected to laparoscopic oocyte retrieval after gonadotropin treatment. A total of 38 oocytes were retrieved and 74% (28/38) were mature as determined by extrusion of the first polar body. Mature oocytes were subjected to ICSI using frozen-thawed spermatozoa obtained by PESA. More than 60% (17/28) of the injected oocytes cleaved and 100% (17/17) reached the morula stage by Day 5 or 6 of IVC. Embryos were cryopreserved and were subsequently transferred ($n = 15$) into one lioness. We have demonstrated that it is possible to collect viable spermatozoa from sterile male lions using the PESA technique. Spermatozoa collected were motile and could be cryopreserved and functional for assisted reproductive techniques. This technique could be applied to other infertile or sterile males whose genetic background would benefit a current conservation program.

205 A GAME OF CAT AND MOUSE: XENOGRAFTING OF TESTIS TISSUE FROM DOMESTIC KITTENS RESULTS IN COMPLETE CAT SPERMATOGENESIS IN A MOUSE HOST

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Loss of genetic diversity due to infertility or the premature death of valuable individuals is a significant problem in the conservation of rare and endangered felid species, as well as in the maintenance of lines of cats used to study inherited feline and human disease. Attempts to overcome loss of genetic diversity have focused on freezing sperm; however, sperm cannot be collected from immature males. Previously, we reported completion of spermatogenesis in testis tissue from newborn pigs and goats grafted ectopically into host mice (Honaramooz A *et al.*, 2002 Nature 418, 778–781). Xenografting of testis tissue not only serves as a powerful system for the study of spermatogenesis and testicular maturation, but it also provides a previously unavailable system to obtain sperm from immature animals. The objective of the present study was to extend the technique of testis tissue xenografting to the domestic cat as a model animal for felid species. Testes from 1- to 5-wk-old domestic short-haired kittens ($n = 8$) were cut into small fragments (about 1×3 mm each), and up to eight fragments were grafted under the back skin of each castrated immunodeficient host mouse ($n = 12$). Histological examination of the testis xenografts was performed between 14 and 36 weeks post-transplantation. From 8 of the 12 recipient mice, 93% of testis tissue grafts were recovered. No grafts were recovered from the remaining 4 mice. At the time of grafting, the seminiferous cords of the donor testis tissue contained only immature Sertoli cells and gonocytes. At 14 weeks after grafting, tubular expansion was evident, caused by the proliferation of Sertoli cells and tubular lumen formation. By 18 weeks after transplantation, the seminiferous epithelium contained spermatocytes, and by 20 weeks, round spermatids were the most advanced types of germ cells. By 36 weeks after transplantation, xenografts of cat testis tissue had completed spermatogenesis and mature sperm were present. In all recipient animals where xenografts were recovered, the weight of the seminal vesicles in the castrated host mice was restored to pre-castration values, indicating that xenografts were capable of releasing biologically active testosterone. These results demonstrate the potential of xenografting to achieve full spermatogenesis in testis tissue from kittens without the necessity of exogenous hormonal stimulation. It was shown previously that sperm recovered from testis xenografts can support fertilization and development (Shinohara T *et al.*, 2002 Hum. Reprod. 17, 3039–3045; Schlatt S *et al.*, 2003 Biol. Reprod. 68, 2331–2335). Therefore, sperm production in a mouse host can provide an alternative for germ line preservation from immature felids where sperm cryopreservation is not an option. Sperm recovered from xenografts can be used for assisted reproduction, thereby making it possible to produce offspring from immature males.

206 POSTNATAL MANAGEMENT OF CHRYPTORCHID BANTENG CALVES CLONED BY NUCLEAR TRANSFER UTILIZING FROZEN FIBROBLAST CULTURES AND ENUCLEATED COW OVA

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A multi-institutional collaboration was developed to evaluate the potential for conservation management of genetic variation in captive banteng calves via nuclear transfer cloning of a deceased genetically valuable individual. Fibroblast cultures frozen in 1978 were fused with enucleated cow ova and resulting embryos were transferred to domestic beef cattle for gestation. Two banteng (*Bos javanicus*) calves produced by heterologous nuclear transfer were delivered by standing Cesarean section on April 1 and 3, 2003. Birth weights for the two calves were 23.13 and 38.64 kg, respectively. The larger calf failed to gain strength and died on April 8. The surviving calf was transferred from Sioux Center, IA, to the San Diego Wild Animal Park in Escondido, CA on April 18, 2003. The calf was offered a goat's milk-based formula containing 21.9 kJ metabolizable energy (ME) and 22% total solids. The total solids fraction consisted of 26.3% crude protein, 29.8% crude fat, 33.5% total carbohydrate. Intake and growth rate for this calf was compared to other hand-reared bovid calves produced by normal mating. Average daily gain from birth to 16 weeks of age was 767 g day^{-1}. A routine neonatal physical examination revealed that the surviving calf was bilaterally cryptorchid. The large calf that did not survive was noted to have one abdominally retained testicle, whereas the other testicle was scrotal. Ultrasound examination of the healthy banteng calf confirmed abdominal localization of the retained testicles. Both testes were located in the abdominal cavity about 4–6 cm from the caudal pole of the kidneys. Therapeutic intervention was considered because of the genetic value of the calf and because the cell donor exhibited normal scrotal testicles. In July, 2003, orchipexy was conducted on this calf. The left testis was exteriorized through a small abdominal flank incision. After freeing it from tissue holding it in place, it was relocated through a subcutaneous tunnel to a pouch created just in front of the scrotum. The attachments to the testis were not long enough to allow the testis to be placed fully in the scrotum. At a follow-up examination conducted four weeks after the surgery, the left testicle was palpable in the subcutaneous pouch just cranial to the scrotum. Under ultrasound examination, the left testicle was easily identified less than 1 cm deep with echogenic texture of a testis. Color flow Doppler ultrasound analysis demonstrated pulsatile flow in the center of the testis synchronous with the heart beat. These findings indicate testicular viability and blood supply.

207 OVARIAN STIMULATION, TRANSVAGINAL, ULTRASOUND-GUIDED OOCYTE RETRIEVAL, ICSI AND BLASTOCYST PRODUCTION IN SEQUENTIAL MEDIA IN THE WESTERN LOWLAND GORILLA (*GORILLA GORILLA GORILLA*)

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Two young (ages: 15 and 16 yr; studbook # 947 and # 939, respectively) parous female gorillas were initially primed with daily oral monophasic estrogen/progesterone treatment (Ovcon 35: Bristol-Myers Squibb Co., Princeton, NJ, USA) for 3 mo to synchronize their menstrual cycles. After withdrawing treatment, urine was tested daily for occult blood (Hemastix; Baxter Healthcare Corp., Deerfield, IL, USA). On days 3–10 following the onset of menses (Day 1), ovarian activity was stimulated with 225 IU human FSH im (Repronex; Ferring Pharmaceuticals, New South Wales, Australia). Then on Days 6–8 this treatment was combined with 25 mg GnRH antagonist im (Antagon; Organon, West Orange, NJ, USA) to prevent premature endogenous LH release. Final oocyte maturation was stimulated 36 h after the last FSH/GnRH treatment with 10,000 IU hCG im (Profasi; Serono Lab., Hingham/Rockland, MA, USA) and oocyte retrieval was performed 36 h post-hCG administration in sternal recumbency (knee-chest) using a 3–6 mHz probe, 17-ga needles and 87 mm Hg (VMAR-5000 Regulated Vacuum Pump; Cook Veterinary Products). Both gorillas displayed a thickened endometrium, and approximately 6 (# 947) to 10 (# 939) maturing follicles (10–15 mm) were detected in each animal. In female # 947, one oocyte was collected but peritoneal fluid and pathology (hydrosalpinx) were also diagnosed and the right ovary showed no follicular development. A total of 3 oocytes were recovered in highly viscous follicular fluid containing massive amounts of cumulus cells. They were transported in a HEPES-buffered transport medium in a portable incubator (CryoLogic, Mulgrave, Victoria, Australia) at 37°C by airline immediately from Brownsville to Dallas, TX, USA, and within 6-h post-retrieval were fertilized by ICSI using cryopreserved sperm collected by rectal probe electrostimulation (age: est. 40 yr ; studbook # 268). The fertilized oocytes were cultured in Gardner's Sequential Medium at 37°C in 6% CO_2 all three cleaved and developed to blastocysts by 115 h post-ICSI. One high-quality expanding blastocyst was transported back to Brownsville, TX, and transferred transcervically into the oocyte donor (studbook # 939), and two fair-quality blastocysts were transported overnight to Omaha, NE, and transferred into a synchronized gorilla recipient (age: 29 yr; studbook # 543/91). Weekly urine samples from the two embryo transfer recipients are being tested for pregnancy diagnosis using OvuQuick test strips (Quidel, San Diego, CA, USA). Acknowledgments: This research was supported in part by the Morris Animal Foundation (Ruth Morris Keesling Animal Health Study).

208 EFFECT OF REFRIGERATION AND CRYOPRESERVATION ON THE QUALITY OF LION EPIDIDYMAL SPERMATOZOA

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Epididymal spermatozoa from harvested wild animals is potentially useful for conservation purposes, as it can be used for subsequent artificial insemination or stored in Biological Resource Banks for future use. The potential of sperm banking is of particular interest for use in lion (*Panthera leo*) populations maintained in small National Parks, as translocation of males to effect gene-flow is often problematic, resulting in the translocated lion being killed by resident pride males. We measured the change in sperm quality over time during cool storage (at 4°C) and after thawing of samples cryopreserved at −196°C. Also, we present a correlation between sperm plasma membrane integrity and mitochondrial activity as measured by fluorescent analysis. The testes from a pride lion were removed and transported to the laboratory (at 4°C) within 6 h. The epididymides were removed and both cauda epididymides were flushed with 1 mL of Tris-citrate egg yolk extender (Fraction A, Biladyl, Minitub, Germany). The sample containing 2930×10^6 cells mL^{-1} was washed (20 mM HEPES, 355 mM sucrose, 10 mM glucose, 2.5 mM KOH; 400 mOsm/kg, pH 7; Sigma, South Africa) and after centrifugation (5 min. at 600*g*), the pellet was resuspended in 0.5 mL of washing solution (with 197 mM NaCl instead of sucrose). One aliquot of spermatozoa was kept at 4°C and evaluated at 24 h intervals for 7 days. A second aliquot of the sperm sample was extended in Tris-citrate egg yolk extender with glycerol (Fraction B, Biladyl), frozen in liquid nitrogen (LN) vapor and stored in LN. The frozen sample was later thawed and evaluated as for the cooled samples. Percentages of motile (MS) and progressive (PS) spermatozoa were assessed using a phase contrast microscope (×200; stage at 37°C). Sperm plasma membrane damage was assessed by determining the percentage of cells exhibiting red fluoresence after staining with propidium iodide (PI, 50 ng/mL; 10 min RT). Spermatozoa that did not stain red in PI were classified as plasma membrane intact (PMI). Resilience to hypo-osmotic shock and plasma membrane integrity were evaluated by incubating a portion of the sample in a 100 mOsm/kg solution (10 nM glucose, 20 nM HEPES, 30 nM NaCl) containing PI for 15 min at room temperature. The percentage of sperm cells with active mitochondria (MIT) was determined by counting spermatozoa showing orange fluoresence over the mid-piece after staining with JC-1(7.5 uM Sigma) for 30 min at 37°C. At collection, MS was 15% and did not show a significant decrease during the 7-day storage period. Initially, PS was 10% and dropped to 5% after 7 days, with values fluctuating during the storage period. Both PMI and HOSPMI were 80% on Day 1, gradually decreasing to 75% on Day 7 of storage. PMI and MIT showed a highly significant correlation ($r = 0.88$; $P = 0.003$; $n = 8$). In frozen-thawed sperm samples, MS fell from a pre-freeze value of 15% to 5% after thawing. Similarly, PS fell from 10% in pre-freeze to 3% in frozen-thawed samples. Likewise, PMI, HOSPMI and MIT values were 80% and 45%, 87% and 45% and 89% and 49%, respectively. Our study showed that lion sperm PMI and MIT remained high after 7 days at 4°C. MS and PS, although low, did not vary during this same period. PI and JC-1 showed a significant correlation,

suggesting that both might be affected by the same deleterious factors. Although PMI, HOSPMI and MIT values decreased approximately 40% after freezing, we feel that such sperm samples could be used for in vitro embryo production, if not by IVF, by ICSI. Of course, additional studies are needed to validate our suggestion.

209 FUNCTIONAL ASSESSMENT OF WHITE RHINOCEROS *CERATHOTERIUM SIMUM* EPIDIDYMAL SPERMATOZOA BEFORE AND AFTER CRYOPRESERVATION

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Biological Resource Banks represent a potentially valuable tool for species conservation. It is, however, necessary to understand the species-specific cryopreservation process and its consequences for spermatozoa to aid in the development of assisted reproduction as a future conservation tool. The aim of this study was to assess the in vitro functionality of white rhinoceros *Cerathoterium simum* epididymal spermatozoa both before and after cryopreservation. Testes from a harvested white rhino bull were removed and transported at 5°C to the laboratory within 4 h. The cauda epididymis was dissected out and flushed with 2 mL of Tris-citrate egg yolk extender (fraction A, Biladyl, Minitüb, Germany). A 0.1 mL aliquot was removed for analysis and the balance (9 mL; 2 mL fraction A + 7 mL sperm sample) mixed with an additional 27.2 mL of Tris-citrate egg yolk with glycerol (fraction B, Bidadyl). The extended sample was allowed to cool to 4°C over a 6-h period before an additional 29.2 mL of cooled fraction B were added (final sperm concentration = 150×10^6 mL^{-1}). Sperm samples were loaded into 0.25-mL straws and frozen over LN_2 vapor (4 cm for 20 min) for later assessment. Sperm straws were thawed by placing the straws in water at 37°C for 30 s. Pre-freeze and post-thaw evaluations were carried out in the same manner. Media used included: HEPES for washing (20 mM HEPES, 355 mM sucrose, 10 mM glucose, 2.5 mM KOH) and HEPES saline (197 mM NaCl, instead of sucrose). An aliquot was diluted with HEPES (washing) and centrifuged for 5 min at 600 × g; the pellet was resuspended in HEPES saline. Sperm motility (total motility %, TM; and progressive motility %, PM) was assessed using phase contrast microscopy (×200; 37°C). Sperm plasma membrane status was assessed using the fluorescent dye, propidium iodide (50 ng mL^{-1} in HEPES saline; 10 min, RT). Percentage of cells with plasma membranes intact (unstained; PMI) was recorded. Mitochondrial status was assessed with the fluorescent dye, JC-1 (7.5 μM in HEPES saline; 30 min, 37°C). The % of cells with an orange-stained midpiece was recorded (active mitochondria; MIT). Resilience to hypoosmotic shock (HOS test) was assessed by diluting a sample in 100 mOsm/kg HEPES saline (1 : 20; 15 min, RT). An aliquot was stained with PI to assess plasma membrane status (HOSPMI), and the rest was fixed with formaldehyde, and % coiled tails (positive endosmosis; HOST) was estimated using phase contrast microscopy (×400). Evaluations of PMI, MIT and HOSPMI were performed using fluorescence microscopy (×400, 450–490 nm excitation filter). The results indicated that quality was good pre-freezing (TM: 60%; PMI: 86%; MIT: 100%), except for a PM value of 15%. After thawing, although there was a drop in TM (30%), there was no decrease in PM (20%). Our in vitro functional assessment indicated a loss of quality between the pre-freeze and post-thaw evaluations, but PMI and MIT maintained their pre-thaw levels (60% and 72%, respectively). The HOS test, which indicates plasma membrane integrity, decreased from the pre-freeze level (91%) to a post-thaw value of 70%. HOSTPMI was 72% pre-freeze, and decreased to 54% post-thaw. In conclusion, epididymal spermatozoa from the white rhino may retain its functionality after cryopreservation in a commerically available cryo-extender (Bidadyl). The use of assisted reproduction techniques could someday play a role in the management and conservation of the white rhinoceros and related species.

210 INCREASE IN OVULATION RATE AFTER IMMUNIZATION OF MALPURA EWES AGAINST A SYNTHETIC PEPTIDE SEQUENCE OF THE α-SUBUNIT OF BOVINE INHIBIN

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Unlike many other breeds of sheep (e.g. Boroola, Romney or Merino) which have high fecundity, the Malpura ewe, an Indian breed of sheep, is marked by an ovulation rate of one and a low incidence of twinning. Active immunization against a number of inhibin-based synthetic peptides has been reported to increase ovulation rates in these high fecundity breeds of sheep. The objective of the present study was to explore the possibility of increasing ovulation rates in Malpura ewes by active immunization against a synthetic peptide replica of the N-terminal sequence of the bovine inhibin. Adult Malpura ewes ($n = 5$) were actively immunized against a synthetic peptide that corresponded to the N-terminus of the α-subunit of bovine inhibin [$bI_\alpha(1–29)Tyr^{30}$]. The peptide was conjugated to ovalbumin, with a peptide-to-ovalbumin ratio of around 20 moles mole^{-1}, to increase its antigenicity. Control ewes ($n = 5$) were immunized against ovalbumin. On the day of primary immunization, 400 μg of peptide-ovalbumin conjugate or ovalbumin were dissolved in 1 mL of isotonic saline, emulsified with an equal volume of Freund's complete adjuvant and injected at four sites in each ewe. Following this, boosters 1, 2 and 3 were given on Days 28, 56 and 84, respectively, of the experiment (Day 0 = day of primary immunization); boosters were 200 μg of peptide-ovalbumin conjugate or ovalbumin dissolved in 1 mL of isotonic saline and emulsified with an equal volume of Freund's incomplete adjuvant. Estrus was synchronized by a double injection schedule of $PGF_{2\alpha}$ (7.5 mg Lutalyse, once each on Days 35 and 45). The animals were subsequently allowed to undergo normal cyclicity until the end of the experiment. Ovulation rate was determined by counting the number of corpora lutea observed during laparoscopic examinations approximately 5 days after estrus during three estrous cyles following treatment. The ovulation rate between control and immunized groups was compared by repeated measures ANOVA. Immunization of the Malpura ewes against the synthetic peptide sequence of the α-subunit of bovine inhibin [$bI_\alpha(1–29)Tyr^{30}$] increased ovulation rate over 5-fold

compared to that of controls (Table 1). In conclusion, we have shown that inhibin-based fecundity vaccines have the potential of increasing ovulation rates in the Malpura breed of sheep.

Table 1.

Immunogen	Ovulation rate			
	Estrous cycle 1	Estrous cycle 2	Estrous cycle 3	Overall
Ovalbumin (control)	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	0.60 ± 0.24^{a}	0.87 ± 0.09^{a}
$bI_{\alpha}(1–29)Tyr^{30}$	4.80 ± 1.02^{b}	5.00 ± 1.05^{b}	5.00 ± 0.45^{b}	4.93 ± 0.47^{b}

Values with different superscripts in the same column differ significantly ($P < 0.01$).

211 QUALITY CHANGES IN BLUE WILDEBEEST (*CONNOCHAETES TAURINUS*) EPIDIDYMAL SPERMATOZOA MAINTAINED AT 4°C

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Wildlife management in southern Africa often involves the harvesting of animals on ranches and reserves, providing unique opportunities to collect and assess the quality of epididymal spermatozoa for possible future conservation actions. The black wildebeest (*Connochaetes gnu*) is facing renewed threats to its survival, including the production of fertile hybrids from crossing with the more common blue wildebeest (*Connochaetes taurinus*). The close relationship between the two wildebeest species allows for the blue wildebeest to be used as a model to assess epididymal sperm quality over time while maintained at 4°C. Field conditions often preclude the immediate availability of liquid nitrogen, necessitating the development of alternative short-term storage methods. All chemicals were provided by Sigma (South Africa) unless otherwise stated. Testes were harvested from 6 blue wildebeest bulls at a local game farm, Savannah, and kept at 5°C during transportation to the lab. Epididymides were dissected out and spermatozoa were flushed out of the cauda epididymis using 1 mL of Tris-citrate egg yolk extender (Fraction A, Biladyl, Minitub, Germany), followed by storage at 4°C and assessment at 12 h intervals. At each interval, an aliquot was removed, washed with a modified buffered HEPES solution (20 mM HEPES, 355 mM sucrose, 10 mM glucose, 2.5 mM KOH; 400 mOsm/kg, pH 7; Sigma) and visually assessed with a phase contrast microscope (×200, at 37°C) to determine the percentage of motile (MS) and progressive motile (PS) spermatozoa. In addition, plasma membrane integrity (PMI) was assessed with eosin-nigrosin staining and active mitochondrial status (MIT) assessed with an epifluorescent microscope (×400) using the fluorescent probe JC-1 (Molecular Probes, The Netherlands; 7.5 μM; 30 min at 37°C). Resilience to hypo-osmotic shock was also evaluated by incubating the sample in a modified hypo-osmotic medium (100 mOsm kg^{-1}; 15 min RT), and staining with PI to assess plasma membrane integrity (HOSPMI). A summary of results is presented in the table 1. The MS, MIT and HOSPMI did not decrease significantly during the 48 h storage period. The only parameters that showed a significant decrease were PS and PMI ($P < 0.01$, Kruskall-Wallis test). However, PMI showed a slow but steady decrease (13%), whereas PS underwent a significant drop (52%). In conclusion, epididymal spermatozoa from the blue wildebeest, kept at 4°C for 48 h, may still be useful for some assisted-reproduction techniques. The use of spermatozoa from a common but closely related wildebeest species allows for the development of assisted-reproduction techniques that may one day aid the conservation of threatened wildebeest species. Additional research is needed to confirm these findings and to test the effect of longer storage times on spermatozoa of this species as well as closely related endangered species.

Table 1. Parameters measured during the 12-h time periods (mean ± SD)

Parameters	T = 0	T = 12	T = 24	T = 36	T = 48
MS	85.0 ± 8.9	77.5 ± 6.9	83.3 ± 5.2	77.5 ± 6.1	85.8 ± 3.8
PS	60.8 ± 19.6	37.5 ± 9.4	32.5 ± 21.6	28.3 ± 26.4	9.2 ± 4.9
MIT	90.3 ± 5.9	86.2 ± 10.1	80.2 ± 14.6	86.7 ± 8.7	84.2 ± 7.9
HOSVIT	57.8 ± 11.0	61.2 ± 13.9	58.7 ± 16.4	58.5 ± 19.8	55.7 ± 12.7
VIT	81.2 ± 3.7	78.2 ± 4.6	76.3 ± 4.9	72.2 ± 4.3	68.3 ± 5.9

212 EFFECT OF CRYOPRESERVATION METHODS AND PRE-CRYOPRESERVATION STORAGE ON BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATES*) SPERMATOZOA

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In conjunction with artificial insemination (AI) and sperm preservation, sperm sexing technology has great potential as a population management strategy for captive bottlenose dolphins. Successful AI using fresh spermatozoa (Robeck TR *et al.* 2001 CRC Marine Mammal Medicine 193–226) and flow cytometric analysis of bottlenose dolphin spermatozoa (Garner DL and Seidel GE Jr 2002 CSAS Symposium 2–13) support this approach. For sperm sexing, methods for short-term storage of semen in a liquid state are required to enable transport of spermatozoa to the sorting laboratory. In addition, cryopreservation techniques must be optimized for long-term storage of sexed spermatozoa. Our objectives were to

assess: (i) 3 cryopreservation methods × 2 straw sizes × 3 thawing rates (Exp. 1) and (ii) effects of liquid storage for 24 h (pre-cryopreservation) and sperm concentration at freezing (Exp. 2) on post-thaw characteristics (PT) of bottlenose dolphin spermatozoa. For Exp. 1 and 2, 4 ejaculates (collected by manual stimulation) × 3 males (aged 14–34 yr) × 4 replicates were used. For Exp. 1, semen was frozen in 0.25-mL (SM) and 0.5-mL straws (LG) by 3 methods (Mt) (Mt1: lactose, egg yolk, $-32°C\ min^{-1}$; Mt2: lactose, egg yolk, 1.5% Equex STM (Nova Chemical, Calgary, Canada), $-19.7°C\ min^{-1}$; Mt3: Test yolk buffer (TYB), $-116°C\ min^{-1}$). All Mt had 3% glycerol. Samples were thawed using a slow (S: $2.8°C\ s^{-1}$), medium (M: $8.8°C\ s^{-1}$) or fast (F: $21°C\ s^{-1}$) rate. In Exp. 2, ejaculates were divided into 4 aliquots for dilution (1:1) and stored at 4°C with EquiPro® (EP4°C, Minitube, Verona, WI, USA) and TYB (TYB4°C) or at 21°C with Androhep Enduraguard™ (AH21°C, Minitube) or no dilution (NEAT21°C). After 24 h, samples were frozen and thawed using Mt3 × SM × F at 10×10^6 sperm mL^{-1} (LOW) or 100×10^6 sperm mL^{-1} (STD). PT evaluations of motility (total motility [TM], % progressive motility [PPM], kinetic rating [KR, 0 to 5]) and acrosomal status (Spermac® , Minitube) were performed at 30 min and 6 h after dilution (1:1) with AH at 21°C. For statistical analysis (ANOVA), a sperm motility index (SMI = TM × PPM × KR) was calculated and expressed as % of initial SMI. For all ejaculates, initial TM and PPM were greater than 85% and KR was 5. In Exp. 1, at 6 h PT, %SMI was highest for Mt3 × LG × M (45.5 ± 8.7) and Mt3 × SM × F (44.8 ± 11.9). For Exp. 2, %SMI at 0 h PT was higher for samples stored at 4°C than at 21°C (TYB4°C 41.0 ± 8.4, EP4°C: 36.7 ± 7.7, NEAT21°C: 23.8 ± 8.6, AH21°C: 14.8 ± 8.6, $P < 0.001$) and, with the exception of AH21°C, was similar between the LOW and STD concentration. At 6 h PT, %SMI for all treatments was higher for STD than LOW concentration ($P < 0.05$). Acrosome integrity was similar across treatments. In summary, a semen cryopreservation protocol maintained high levels of the initial characteristics of ejaculated spermatozoa. Transport of semen for sex pre-selection and cryopreservation within 24 h may be feasible, but impact of storage time on functional capacity of dolphin spermatozoa is unknown.

213 EFFECTS OF TIME OF MATURATION AND SHEEP SERUM ON IN VITRO FERTILIZATION IN THE ENDANGERED ELD'S DEER (*CERVUS ELDI THAMIN*) OOCYTES

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The Eld's deer, native to Southeast Asia, is threatened with extinction. Although artificial insemination is effective for offspring production, in vitro fertilization (IVF) would be more useful for rapidly disseminating genetic material from valuable founders. The objectives of this study were to: 1) determine if oocytes recovered from exogenous gonadotropin-treated hinds require additional in vitro maturation; and 2) assess if fertilization is enhanced by supplementing Deer Synthetic Oviduct Fluid (DSOF; Berg DK *et al.*, 2003 Theriogenology 59, 189–205) with 1-day postestrus sheep serum (SS). Estrous cycles in Eld's deer hinds ($n = 10$) were synchronized with PGF2α analog (Lutalyse™, 500 mg), followed by a 14-day intravaginal CIDR-G insertion; ovine FSH (Ovagen™; 0.05 unit × 8 injections) was administered at 12-h intervals beginning 84 h before CIDR-removal. COCs ($n = 160$) were retrieved laparoscopically 40–46 h post-CIDR-removal and either fixed or matured in vitro (for 12 h v. 24 h) in TCM-199 (Earle's salt) supplemented with 0.33 mM pyruvate, 2 mM glutamine, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 10% fetal calf serum, 5 μg mL^{-1} FSH and LH and 1 μg mL^{-1} E_2 (5% CO_2, 38.5°C). After 12- or 24-h IVM, cumulus cells were partially removed and oocytes ($n = 110$) fertilized in DSOF with pooled frozen-thawed sperm (3 males; 2×10^6 motile sperm mL^{-1}), in the absence or presence of SS (20%, v/v). Additional oocytes ($n = 18$) were used for parthenogenetic control. At 20-h postinsemination, presumptive zygotes were fixed and stained (Hoechst 33342) to assess fertilization success (presence of two pronuclei). Data were analyzed by ANOVA. Overall, 16.0 ± 2.6 (mean ± SEM) COCs were recovered/female. The majority of COCs were of excellent quality (grade I; $67.7 \pm 3.8\%$). At time of aspiration, 85% of the oocytes ($n = 11/13$) were in metaphase I stage, 7.5% in telophase and 7.5% degenerate. No parthenogenic activation was observed. Likewise, no polyspermy was observed in any treatment. Fertilization was higher ($P < 0.05$) in oocytes matured for 24 h and fertilized in the absence ($64.4 \pm 3.1\%$) compared to presence ($26.9 \pm 11.2\%$) of SS. In the absence of SS, a higher ($P < 0.05$) proportion of oocytes were fertilized after 24 h ($64.4 \pm 3.1\%$) compared to 12 h ($27.1 \pm 9.0\%$) IVM. There was no effect ($P > 0.05$) of SS on fertilization among oocytes subjected to 12-h IVM ($27.1 \pm 9.0\%$ v. $12.5 \pm 9.5\%$). When SS was present during fertilization, no difference ($P > 0.05$) was observed among oocytes matured for 12 or 24 h. Results demonstrate that: 1) Eld's deer oocytes require an additional 24-h IVM to complete maturation; 2) DSOF supports sperm-oocyte interaction; and 3) SS is not essential for successful fertilization. (Supported by Morris Animal Foundation.)

214 USE OF FLOURESCENT PROBES TO ACCESS EPIDIDYMAL SPERMATOZOA OF THE BLUE WILDEBEEST *CONNOCHAETES TAURINUS* AND IMPALA ANTELOPE *AEPYCEROS MELAMPUS MELAMPUS*

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Sperm quality assessment may be a useful tool not only for evaluating the reproductive health of free-ranging populations, but also for selecting individuals for future assisted-reproduction technology programs. The aim of this study was to assess the functionality of epididymal spermatozoa collected from blue wildebeest (*Connochaetes taurinus*) and impala (*Aepyceros melampus melampus*) during the non-breeding season, using the fluorescent probes, propidium ioide (PI; Sigma, South Africa) and JC-1 (Molecular Probes, The Netherlands). Six blue wildebeest and eight impala were harvested as part of a wildlife management program on a game ranch in South Africa. Testes were removed and transported to the laboratory within 6 hours while being maintained at 4°C. The cauda epididymides were removed and flushed with 1 mL of Tris-citrate egg yolk extender (fraction A, Biladyl; Minitüb, Germany). The sperm sample was diluted 1:4 in HEPES washing medium (Sigma; 20 mM HEPES, 355 mM sucrose, 10 mM glucose, 2.5 mM KOH; 400 mOsm/kg, pH 7), and centrifuged for 5 min at 600*g*, followed by re-suspending the pellet in 0.1 mL of

HEPES saline medium (Sigma; as for washing medium, except 197 mM NaCl instead of sucrose). The percentage of motile (MS) and progressively motile (PS) spermatozoa were determined using phase contrast microscopy (×200, 37°C). Sperm plasma membrane integrity and mitochondrial status were assessed using fluorescence microscopy (×400, 450–490 nm excitation filter, 510 nm dichroic-beam splitter, 520 nm barrier filter) after staining with PI (50 ng mL^{-1}; 10 min, RT) and JC-1 (7.5 μM; 30 min, 37°C), respectively. Spermatozoa with damaged plasma membranes showed a red fluorescence and spermatozoa with active and inactive mitochondria (MIT) fluoresced orange and green, respectively. Spearman correlation coefficients were calculated between spermatozoa with intact plasma membranes (IPM) and MIT, and with motility (Statistica™ package). A summary of the results is shown in the table 1. Although samples were not collected during the breeding season, sperm quality appeared to be good for the blue wildebeest, but less so for the impala. In general, impala results were more varied. Significant correlations were found for impala ($n = 8$, $P < 0.05$) MS-IPM: 0.75; IPM-MIT: 0.83, and for blue wildebeest ($n = 6$, $P < 0.05$), MS-IPM: 0.84; IPM-MIT: 0.81, and for pooled data ($n = 14$, $P < 0.01$), MS-IPM: 0.93; MS-MIT: 0.87; PS-IPM: 0.67; PS-MIT: 0.66; IPM-MIT: 0.95. These correlations suggest a relationship of functional parameters to sperm motility. Both membrane integrity and mitochondrial status are important for sperm flagellar activity. The correlation between IPM and MIT indicates a relationship or the effect of common factors. In conclusion, sperm collected from blue wildebeest and impala during the non-breeding season appear functional, a fact that may be useful for future conservation programs based on assisted reproduction technology or for assessing the reproductive health status of free-ranging wildlife populations. The fluorescent probes PI and JC-1 appear useful for assessing sperm quality in these two species and should be considered for further sperm quality assessment studies in other antelope species.

Table 1. Results of the analyses, showing mean ± SD (max.–min.)

	MS	PS	IPM	MIT
Blue wildebeest	85 ± 8.9 (90–70)	60.8 ± 19.6 (90–40)	84.7 ± 7.9 (96–74)	90.3 ± 5.9 (97–81)
Impala	54.4 ± 12.7 (75–40)	23.1 ± 8.8 (30–10)	44.3 ± 19.4 (71–15)	54.5 ± 16.9 (84–30)

215 SUCCESSFUL OUT-OF-BREEDING SEASON ESTRUS SYNCHRONIZATION FOLLOWED BY FIXED TIME INSEMINATION IN WATERBUFFALO (*BUBALUS BUBALIS*)

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The most important barrier to the increase of buffalo productivity is an overall poor reproductive efficiency, characterized by late sexual maturity, seasonal anestrus and long periods of postpartum ovarian inactivity resulting in extended calving intervals and poor expression of estrus behavior (Singh J *et al.*, 2000, Anim. Reprod. Sci. 60–61, 593–604). Buffaloes are seasonal breeders with the highest reproductive activities during winter (short day lengths) and a high frequency of anestrus during the summer months (Singh G *et al.* 1985, Ind. J. Anim. Res. 19, 57–60). Recent research demonstrated that a combination of progesterone, estradiol benzoate and equine chorionic gonadotropin (eCG) was effective for estrus induction and synchronization in buffalo heifers under Mediterranean conditions (Barile *et al.* 2001, Livestock Prod. Sci. 68, 283–287). The aim of the present study was to investigate the impact of an estrus synchronization protocol on reproductive efficiency of water buffalo during out of the normal breeding season. A total of six heifers (21 to 23 months of age) and three cows (5, 6 and 18 years of age) were enrolled in an estrus synchronization protocol lasting for 12 days. All animals were kept under tropical conditions in the coastal part of Paraná (Antonina), about 450 km south of São Paulo. The experiment was performed in December, 2002, during the Brazilian summer season, when reproductive efficiency of buffaloes is greatly reduced. On the first day of the protocol (Day 0), animals were implanted with an intravaginal device containing 1 g of progesterone (DIB, Syntex SA, Buenos Aires, Argentina) and injected with 10 mg estradiol benzoate (Estrogin, Famavet, São Paulo, Brazil). On Day 9, the DIB implant was removed and the animals received 150 μg (i.m.) of cloprostenol (Prolise, Syntex SA, Buenos Aires, Argentina) and 2500 IU of eCG (Novormon, Syntex SA, Buenos Aires, Argentina). On Day 11, all animals received 1500 IU of hCG (Vetecor, Lab. Calier, Spain). Artificial insemination (AI) was performed on Day 12 using frozen-thawed semen from a bull of proven fertility. Only one AI was performed per heifer/cow. Pregnancies were determined by ultrasound examination at 53 days following AI and confirmed by rectal palpation at 90 days post AI. The use of this estrus synchronization protocol, followed by fixed-time insemination, resulted in four pregnant heifers (66%) and three pregnant cows (100%). Our results demonstrate that buffalo reproduction can be successful during out-of-breeding season when adequate hormonal treatment is used. Additional experiments should be done to validate the protocol.