Eur J Wildl Res (2009) 55:1–6
DOI 10.1007/s10344-008-0207-6

ORIGINAL PAPER

Capacitation status of frozen–thawed spermatozoa from wild ruminant species

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Received: 8 February 2008 / Revised: 4 June 2008 / Accepted: 10 June 2008 / Published online: 17 July 2008

Abstract Semen from Barbary sheep (*Ammotragus lervia*), Bighorn sheep (*Ovis canadensis*), Mouflon sheep (*Ovis musimon*), Fallow deer (*Dama dama*), collected by electroejaculation, and semen from Wildebeest antelope (*Connochaetes taurinus*), collected post mortem, were frozen using a standardized technique and a commercial freezing medium (Triladyl). Sperm quality was assessed by measuring their capacitation status (chlortetracycline assay) in addition to classic assessment: motility, viability (plasma membrane integrity), and acrosome integrity. Sperm cryosurvival measured in terms of recovery (from the initial, pre-freeze values) of motility, viability, and acrosome integrity was relatively high in most species. However, proportion of premature-capacitated spermatozoa (B+AR patterns) increased many times in relation to that of fresh semen: from 8% to 78% in Barbary sheep; from 27% to 61% in Bighorn sheep; and from 12% to 68% in Mouflon sheep. The use of a standardized technique for freezing of spermatozoa from wild ruminant species produced, in most of the cases, acceptable results. This approach may be extensively used to carry out sperm cryopreservation in field conditions. Chlortetracycline assay allowed identifying the same fluorescent patterns observed in spermatozoa from domestic animals and produced additional information on sperm cryosurvival. That is, it revealed those cells that survive freeze–thawing and are potentially fertile: non-capacitated (F pattern) and capacitated acrosome-intact spermatozoa (B pattern).

Keywords Semen · Cryopreservation · Wild sheep · Antelope · Deer

Communicated by W. Lutz

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Introduction

Biodiversity is currently being threatened due to gradual loss of ecosystems and loss of genetic diversity, mainly by human impact. As natural habitats disappear, populations of organisms remaining in fragmented natural areas are reduced to small, inbred populations that lose genetic diversity over time, leading to lower viability and fecundity. Without genetic variation between individuals, a population is more vulnerable to new hazards and challenges. A consequence of the decrease of genetic variability is the limitation of their adaptation capacity, which is vital due to the frequent changes in ecosystems and global environment (Lacy 1987). Genetic banks which include spermatozoa, oocytes, embryo, and tissue cryopreservation represent a very useful strategy in order to preserve genetic biodiversity and, therefore, to be used in the future to re-establish threatened species (Gilmore et al. 1998). Cryopreservation of sperm in liquid nitrogen has many advantages such as the movement of genetic material between populations, long-term storage, and use in assisted reproduction and maintenance of small populations of wild animals without the risk

that genetic diversity is altered (Howard 1999). Recently, Fickel et al. (2007) reviewed these and other aspects around semen preservation of threatened animal species.

Cryopreservation reduces sperm survival and motility about 50%; besides, spermatozoa suffer premature capacitation that shortens their lifespan (Watson 1995). Mammal spermatozoa acquire their fertile capacity when completing several biochemical and morphological changes, a process called capacitation which allows the plasma membrane to fuse with the external acrosomal membrane, as well as a change in flagellar movement known as hyperactivation (Bedford 1983). All these changes together will enable spermatozoa to fuse with the oocyte (Flesh and Gadella 2000; Gadella et al. 2001).

To study capacitation and acrosome reaction, Ward and Storey (1984) described the use of chlortetracycline (CTC); this fluorescent stain allows the classification of spermatozoa in non-capacitated (F pattern), capacitated with intact acrosome (B pattern), and capacitated acrosome-reacted (AR pattern). Also, this assay has been used to assess premature capacitation during cryopreservation (Green and Watson 2001). Thus, CTC assay together to routine semen assessment tests may produce extra information to take vital decisions about the final use of spermatozoa and to improve freezing protocols.

When dealing with semen from wildlife species one of the main problems is the limited number of individuals and ejaculates available to investigate basic aspects such as cryoprotectans and critical temperatures that restricts the information about individual requirements (Asher et al. 2000); thus, it would be desirable to establish a simple but effective methodology easily adaptable to in field conditions to increase cryopreservation as a tool for species conservation. Triladyl is a commercial extender widely used to freeze semen from several animal species (Johnston et al. 2002; Garde et al. 2003; Soler et al. 2003; Herold et al. 2004); its use is very convenient due to its availability and easy preparation.

In this work, semen samples from five wild ruminant species were obtained for freeze–thawing. One of these species was Bighorn sheep (*Ovis canadensis*), included in a Mexican Special Protection Program by the Mexican Official Law NOM-059-ECOL-2001 and as a part of the Project "Assisted Reproduction of Bighorn Sheep" and included in the appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora; the European Mouflon (*Ovis musimon*) was included since Irapuato Zoo (Guanajuato, Mexico) interest to maintain genetic variability into its animal collection and as an animal model to study interspecies gestation (Palma 2005).

The objective of this work was to test a simple cryopreservation protocol, adapted from domestic to wild ruminant species that may be carried out in field conditions. In addition to common sperm quality assessment (motility, viability, acrosome integrity, and morphology), sperm premature capacitation was assessed.

Materials and methods

Semen was obtained from two different zoological institutions: Chapultepec Zoo (Mexico City) and Irapuato Zoo (Guanajuato, Mexico); sperm thawing and assessment were carried out at the Laboratory of Reproduction of the Faculty of Superior Studies—Cuautitlan, National Autonomous University of Mexico.

Animal species, management, and semen collection

Anesthesia was based on either a mix of (1) 2 mg/kg of xylazine and 5 mg/kg of ketamine for Bighorn sheep, European Mouflon, and Barbary sheep or (2) 1.5 mg/kg xylazine and 2.5 mg/kg of tiletamine–zolacepam (Zoletil, Virbac) for Fallow deer, within a dart thrown by air pistol. After cleaning the penis region and removal of feces, a conical rectal probe (2.5 cm diameter, 20 cm length; Bailey Ejaculator, USA) was inserted into the anus about 10 cm; after two stimuli of 7 s each and 7 s rest period, semen was obtained.

Bighorn sheep (Ovis canadensis) Semen from two individuals, 3 and 5 years old, 80 kg live weight approximately, and housed at Chapultepec Zoo, was obtained by electroejaculation; rams were previously anesthetized as described. A third sample was obtained by flushing the cauda epididymidis from a hunted individual in Sonora (Mexico).

European Mouflon (O. musimon) Semen from two males, 3 and 6 years old, 50 kg live weight approximately, from Irapuato Zoo, was obtained by electroejaculation. Anesthesia was carried out as described.

Barbary sheep (Ammotragus lervia) Semen from two males, 2 and 4 years old, 70 kg live weight approximately, from Irapuato Zoo, was obtained by electroejaculation; anesthesia was carried out as described.

Fallow deer (Dama dama) Semen from two adult males, 2 years old, 45 kg live weight approximately, and housed at Irapuato Zoo, was obtained by electroejaculation; anesthesia was carried out as described.

Wildebeest antelope (Connochaetes taurinus) Spermatozoa from one male, from Irapuato Zoo, were obtained 2 h post mortem: testicles were removed, cauda epididymides were

then washed, and several deep cuts were made to obtain spermatozoa.

Semen assessment, freezing, and thawing

Once ejaculates were obtained, motility, volume, concentration, and color were assessed. Semen was diluted in 1:1 (*v*/*v* semen:extender) at 37°C in a commercial extender (Triladyl, Minitube, Germany), prepared according to the manufacturer. Briefly, Triladyl, egg yolk, and tri-distilled water were mixed (1:1:3 *v*/*v*), filtered, and kept at 37°C. Diluted semen were packaged in plastic straws (0.25 and 0.5 ml) sealed with polyvinyl acetate, cooled to 5°C for 2 h, frozen for 8 min at −90°C approximately over nitrogen vapor 4 cm above the nitrogen level, and plunged finally in liquid nitrogen.

Thawing was carried out 2 months later; straws were warmed at 37°C in a water bath for 30 s. One drop of thawed spermatozoa was viewed in an optical microscope at ×100 magnification to estimate progressive motility; Blom's eosin–nigrosine stain (Barth and Oko 1989) was used to assess plasma membrane intact cells and sperm abnormalities; proportion of acrosome-intact spermatozoa was estimated by phase-contrast microscopy at ×1,000 magnification and cells were fixed in glutaraldehyde (Barth and Oko 1989).

Semen filtration

After thawing, semen was filtered through a sephadex column (G-50, Sigma., St. Louis, MO, USA) to remove dead and immotile spermatozoa (Perez et al. 1996; Ahmad et al. 2003); thus, CTC patterns were assessed in live cells only. Previously, columns were washed four times with phosphate-buffered saline at 37°C to warm them and avoid a possible thermal shock to the spermatozoa. Filtered semen was collected in plastic vials and sperm motility was immediately assessed.

Chlortetracycline assay

Chlortetracycline stain was carried out as described by Green and Watson (2001); briefly, 100 μl of filtered semen was added to 100 μl of a chlortetracycline solution and mixed for 30 s; 20 μl of glutaraldehyde solution (0.2% *v*/*v* in distilled water) was then added and mixed for 10 s. Ten microliters of this solution was put on a slide with a drop of DABCO (220 mM in glycerol, Sigma); a cover slide was gently pushed over this preparation and was protected from light. Two smears per ejaculate were prepared and 100 cells were counted from each slide classifying them according to CTC fluorescence patterns: (1) F, with uniform fluorescence over the whole head, considered characteristic of uncapacitated acrosome-intact spermatozoa; (2) B, with fluorescence-free band in the post-acrosomal region, considered characteristic of capacitated, acrosome-intact spermatozoa; or (3) AR, with almost no fluorescence over the whole head except for a band of fluorescence in the equatorial segment, considered characteristic of capacitated acrosome-reacted spermatozoa (Fraser 1995).

Statistical analysis

Data from each species were analyzed only by descriptive statistics since comparisons between males and ejaculates were inappropriate (only one ejaculate per male). The statistic package Stats Soft (UK) version 5 was used.

Results

Table 1 shows the initial pre-freeze and post-thaw sperm characteristics from the five wild ruminant species. In all these species, CTC assay allowed identifying all reported patterns: F, B, and AR. Regarding Barbary sheep (*A. lervia*), most of spermatozoa (92%) in fresh semen displayed F pattern; the rest showed either B or AR pattern. After thawing, F pattern was present only in 24% and 20% of cells while proportion of B+AR patterns was in 76% and 80% of cells from males 1 and 2, respectively. Motile, live, and acrosome-intact spermatozoa showed high values of recovery with respect to the initial values (Table 1). In the same way, in Bighorn sheep (*O. canadensis*), about 73% of spermatozoa in fresh semen displayed the F while the rest showed the other patterns. After thawing, F pattern was present only in 38%, 41%, and 37% of cells while proportion of B+AR patterns was in 62%, 59%, and 63% of cells from males 1, 2, and 3, respectively. Motile, live, and acrosome-intact spermatozoa displayed high values of recovery (Table 1). In European Mouflon sheep (*O. musimon*), majority of spermatozoa (88%) in fresh semen displayed F pattern; this proportion decreased to about 32% in frozen–thawed cells while proportion of B+AR patterns was about 68%; motile, live, and acrosome-intact spermatozoa displayed high values of recovery (Table 1). In Fallow deer (*D. dama*) spermatozoa, it was not possible to carry out CTC assay before freezing. Nevertheless, after thawing, F pattern was present only in about 30% of cells while proportion of B+AR patterns was in 70% of spermatozoa (Table 1). Motile spermatozoa displayed high values of recovery; the other sperm variables showed high values regardless pre-freeze initial values are lacking. Sperm freezing in Wildebeest antelope (*C. taurinus*) was carried out from spermatozoa collected from epididymis

Table 1 Fresh and thawed sperm variables from five wild ruminant species

Animal species	Semen status	Sperm concentration $\times 10^6$ ml/volume of ejaculate (ml)	Motility (%)	Live sperm (%)	Acrosome-intact sperm (%)	Normal sperm (%)	Non-capacitated sperm F pattern (%)	Capacitated sperm B+AR pattern (%)
Barbary sheep (*Ammotragus lervia*)	Fresh	4,010/2.0	90	92	90	96	92	8
	Thawed	Male $1^{(5)}$	74±5.5	79±4.6	81±2.4	92±4.0	24±6.8	76±6.8
		Male $2^{(4)}$	71±6.3	75±8.4	82±3.4	94±1.7	20±5.5	80±5.5
Bighorn sheep (*Ovis canadensis*)	Fresh	3,031/1.4	84	85	92	92	73	27
	Thawed	Male $1^{(3)}$	78±2.5	82±2.9	84±5.1	89±2.5	38±5.7	62±5.7
		Male $2^{(3)}$	79±10.1	85±8.4	89±4.9	92±2.0	41±8.4	59±8.4
		Male $3^{(3)}$	73±9.3	84±7.6	92±2.3	93±2.1	37±8.5	63±8.5
European Mouflon sheep (*Ovis musimon*)	Fresh	3,009/1.2	85	90	96	95	88	12
	Thawed	Male $1^{(4)}$	72±5.2	79±7.6	87±5.2	86±2.5	34±6.2	66±6.2
		Male $2^{(5)}$	73±4.2	76±5.9	91±4.2	93±1.5	30±5.5	70±5.5
Fallow deer (*Dama dama*)	Fresh	2,703/1.3	83	No	No	No	No	No
	Thawed	Male $1^{(6)}$	74±13.7	85±3.0	91±3.9	92±1.8	27±8.8	73±8.8
		Male $2^{(6)}$	61±7.7	79±6.6	90±2.5	88±2.7	30±4.8	70±4.8
Wildebeest antelope (*Connochaetes taurinus*)	Fresh	3,199/0.6	70	No	No	No	No	No
	Thawed	Male $1^{(7)}$	46±9.3	69±9.3	92±3.3	74±6.1	27±4.9	73±4.9

Values are means±SD. Male $^{(n)}$ number of frozen–thawed straws assessed

cauda few hours post mortem; thus, fresh sperm assessment was not possible, except for motility (70%). After thawing, F pattern was present only in 27% of cells while proportion of B+AR patterns was in 73% of cells (Table 1); after thawing, motility declined to 46%.

Discussion

The objective of this work was to validate a simple semen cryopreservation protocol applicable to a great variety of wild animal species for implementing genetic resource banks from endangered species. One important concern is the very limited number of animals and ejaculates for experimentation; besides, semen collection is sometimes carried out in very difficult conditions, for instance in hunting farms. In addition, most of research on sperm cryopreservation from wild animal species lacks results from in vivo fertility trials. Fickel et al. (2007) have listed a number of non-domestic mammals in which successful AI or IVF using frozen/thawed spermatozoa have been performed (13 out of 62 species). Chlortetracycline assay may be considered an additional tool for the assessment of sperm cryosurvival, since traditional evaluation of frozen–thawed spermatozoa usually considers a few attributes: motility, viability, and acrosome integrity. Since CTC assay, to assess sperm capacitation status, has been reported in domestic animals and some non-human primates, we were interested in the first place in observing whether those patterns were present in spermatozoa from the species used in this study; therefore, we were able to identify all described patterns in all studied species. CTC patterns changed according to the dynamic described for other species: B and AR patterns increased at thawing while F pattern decreased. Gillan et al. (1997) observed a 2.4 times increase in the proportion of capacitated patterns (B+AR) in sheep spermatozoa: from 39% to 93% in fresh and frozen–thawed spermatozoa, respectively. Rios et al. (2004) observed an increase of capacitated patterns (B+AR) in domestic sheep of 2.6 times; in the present study, we observed an increase of 2.3 times in Bighorn sheep. This suggests domestic and wild sheep spermatozoa survive similarly to freeze–thawing protocols. Sperm cryosurvival obtained with our technique was successful in all the studied species of wild ruminants, particularly in both Bighorn sheep and Fallow deer; similar results have been reported using different freezing protocols in semen from Fallow deer (Asher et al. 2000). In Barbary sheep, the proportion of capacitated spermatozoa after thawing was about 80%; this would reduce fertility to very low levels if this semen were used for artificial insemination in cervix or vagina. In contrast, in Bighorn sheep, the population of capacitated spermatozoa increased 2.3 times (from 27% to 61%). In this species, one of the semen samples was obtained by post mortem epididymis flushing after hunting; it is worth noting that sperm cryosurvival was similar to that of samples obtained by electroejaculation. Considering capacitated sperm population was about 61%, the use of intrauterine artificial insemination would be justified, given the great value of this species. Artificial insemination by laparoscopy, using semen frozen with our protocol, produced 54% and 38% of fertility in domestic and Mouflon

sheep, respectively, applying 70 million of spermatozoa in each uterine horn (Palma 2005). Regarding European Mouflon, population of capacitated spermatozoa increased only 5.7 times (from 12% to 68%). Ptak et al. (2002) carried out in vitro fertilization using frozen–thawed Mouflon spermatozoa and obtained 34% of embryos (blastocyst); this suggests sperm cryosurvival was relatively high and similar to that obtained in our work. In addition, Berlinguer et al. (2005) reported Mouflon spermatozoa were efficiently cryopreserved, without reduction in viability, confirmed by both in vivo and in vitro fertility trials. In Fallow deer, a complete assessment of quality of fresh semen was not possible; high rates of fertility in several deer species using frozen semen have been reported (Asher et al. 1988; Jacobson et al. 1989; Asher et al. 2000), even using low dosages of semen. Sperm collection from Blue wildebeest was performed in difficult conditions, as testicles were removed post mortem and they had to pass through some temperature changes; however, proportion of motile cells was acceptable (70%). At thawing, motility decreased to 46%; this value is similar to that obtained in frozen–thawed spermatozoa from African buffalo (53%), collected from epididymis and diluted in Triladyl (Herold et al. 2004). Herrick et al. (2004) observed about 65% of initial, pre-freeze motility could be recovered after cryopreservation of epididymal spermatozoa from black wildebeest (*Connochaetes gnou*); this value is similar to that obtained in this work (66%). Taking into account sperm motility was not dramatically reduced in none of the wild ruminants after cryopreservation and considering sperm assessment was only based on traditional variables, we would have had incomplete information. In this way, the assessment of premature capacitation may provide additional and valuable information to design appropriate freeze–thawing protocols for wild animal species.

Acknowledgements This project was partially supported by the National Autonomous University of Mexico (UNAM), Irapuato Zoo, and Chapultepec Zoo. M. Mejia was supported by a scholarship from the Mexican government (CONACYT). All experiments comply with the Institutional Subcommittee for Care of Animals in Experimentation from the National Autonomous University of Mexico (Subcomité Institucional para el Cuidado de Animales en Experimentación, Universidad Nacional Autónoma de México).

References

Ahmad Z, Anzar M, Shahab M, Ahmad N, Andrabi SMH (2003) Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low-grade buffalo semen ejaculates. Theriogenology 59:1189–1202 doi:10.1016/S0093-691X(02)01159-7

Asher GW, Adam JL, Otway W, Bowmar P, Mackintosh CG, Dratch P et al (1988) Hybridization of Pere David's deer (*Elaphurus davidianus*) and red deer (*Cervus elaphus*) by artificial insemination. J Zool (Lond) 215:197–203

Asher GW, Berg DK, Evans G (2000) Storage of semen and artificial insemination in deer. Anim Reprod Sci 62:195–211 doi:10.1016/S0378-4320(00)00159-7

Barth AD, Oko RJ (1989) Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames

Bedford JM (1983) Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biol Reprod 28:108–120 doi:10.1095/biolreprod28.1.108

Berlinguer F, Leoni GG, Bogliolo L, Bebbere D, Succu S, Ledda S et al (2005) In vivo and in vitro fertilizing capacity of cryopreserved European mouflon (*Ovis gmelini musimon*) spermatozoa used to restore genetically rare and isolated populations. Theriogenology 63:902–911 doi:10.1016/j.theriogenology.2004.05.006

Fickel J, Wagener A, Ludwig A (2007) Semen cryopreservation and the conservation of endangered species. Eur J Wildl Res 53:81–89 doi:10.1007/s10344-007-0089-z

Flesh FM, Gadella BM (2000) Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469:197–235

Fraser LR (1995) Ionic control of sperm function. Reprod Fertil Dev 7:905–925 doi:10.1071/RD9950905

Gadella BM, Rathi R, Brouwers JH, Stout TAE, Colebrander B (2001) Capacitation and the acrosome reaction in equine sperm. Anim Reprod Sci 68:249–265 doi:10.1016/S0378-4320(01)00161-0

Garde JJ, Soler AJ, Crespo C, Malo F, Espeso G, Gomendio M et al (2003) Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri*, *G. dama mhorr* and *G. dorcas neglecta*). Biol Reprod 69:602–611 doi:10.1095/biolreprod.102.012914

Gillan L, Evans G, Maxwell MC (1997) Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. Reprod Fertil Dev 9:481–487 doi:10.1071/R96046

Gilmore JA, McGann LE, Ashworth E, Acker JP, Raath JP, Bush M et al (1998) Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. Anim Reprod Sci 53:277–297 doi:10.1016/S0378-4320(98)00118-3

Green CE, Watson PF (2001) Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reproduction 122:889–898 doi:10.1530/rep.0.1220889

Herold FC, Aurich JE, Gerber D (2004) Epididymal sperm from the African buffalo (*Syncerus caffer*) can be frozen successfully with AndroMed and with Triladyl but the addition of bovine seminal plasma is detrimental. Theriogenology 61:715–724 doi:10.1016/S0093-691X(03)00256-5

Herrick JR, Bartels P, Krisher RL (2004) Postthaw evaluation of in vitro function of epididymal spermatozoa from four species of free-ranging African bovids. Biol Reprod 71:948–958 doi:10.1095/biolreprod.103.026831

Howard J (1999) Assisted reproductive techniques in nondomestic carnivores. In: Fowler ME, Miller RE (eds) Zoo and wild animal medicine current therapy 4. WB Saunders, Philadelphia, pp 1047–1053

Jacobson HA, Bearden HJ, Whitehouse DB (1989) Artificial insemination trials with white-tailed deer. J Wildl Manage 53:224–227 doi:10.2307/3801338

Johnston SD, McGowan MR, Blyde D (2002) Birth of a Banteng (*Bos javanicus*) calf at Western Plains Zoo after fixed time artificial insemination. Aust Vet J 80:94–95

Lacy RC (1987) Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection and population subdivision. Conserv Biol 1:143–157 doi:10.1111/j.1523-1739.1987.tb00023.x

Palma M (2005) Monitoreo de gestacion de borregas muflon (*Ovis musimon*) y domesticas (*Ovis aries*), inseminadas con borrego cimarron (*Ovis canadensis*). MSc. Thesis, Universidad Autonoma Metropolitana—Xochimilco, Mexico

Perez JL, Valcarcel A, De las Heras MA, Moses D, Baldassarre H (1996) Evidence that frozen/thawed ram spermatozoa show accelerated capacitation in vitro as assessed by chlortetracycline assay. Theriogenology 46:131–140 doi:10.1016/0093-691X(96)00148-3

Ptak G, Clinton M, Barboni B, Muzzeddu M, Cappai P, Tischner M et al (2002) Preservation of the wild European Mouflon: the first example of genetic management using a complete program of reproduction biotechnologies. Biol Reprod 66:796–801 doi:10.1095/biolreprod66.3.796

Rios E, Lopez S, Palacios P, Medrano A (2004) Pre-freeze cooling below 0°C improves ram sperm cryosurvival. 15th International Congress on Animal Reproduction, Brazil, Abs 2:480

Soler AJ, Pérez-Guzmán M, Garde J (2003) Storage of red deer epididymides for four days at 5°: Effects on sperm motility, viability and morphological integrity. J Exp Zool 295A:188–199 doi:10.1002/jez.a.10194

Ward CR, Storey BT (1984) Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 104:287–296 doi:10.1016/0012-1606(84)90084-8

Watson PF (1995) Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 7:871–891 doi:10.1071/RD9950871