doi: 10.17221/124/2016-VETMED

Hyperthermia and not hypoxia may reduce sperm motility and morphology following testicular hyperthermia

J.P. Kastelic[1]*, R.E. Wilde[2], G. Rizzoto[1], J.C. Thundathil[1]

[1]Faculty of Veterinary Medicine, University of Calgary, Calgary, Canada
[2]Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Canada

*Corresponding author: jpkastel@ucalgary.ca

ABSTRACT: The mammalian testis typically operates on the brink of hypoxia; the long-standing dogma is that increased testicular temperature increases metabolism, but blood flow is unaffected and the resulting hypoxia reduces sperm motility and morphology. In rats and mice, oxygen (O_2) content of inspired air affected O_2 content of testes, enabling the latter to range from approximately 50 to more than 200% of physiologic concentrations. A ram model was used to test the hypotheses that hypoxia would disrupt sperm motility and morphology and that hyperoxia would prevent hyperthermia-induced reductions in sperm motility and morphology. Eighteen Canadian Arcott rams (approximately 10 months old) were used in a 2 × 3 factorial, with factors being scrotal insulation (insulated or not insulated) and O_2 concentrations in inspired air (14, 21 or 85%). Six rams, three with and three without scrotal insulation, were placed in each of three enclosed areas for 30 h to expose them to their respective oxygen concentrations, with scrotal insulation removed at the end of the exposure. Semen was collected by electro-ejaculation twice before insulation, bi-weekly for four weeks starting one week after exposures, and then once weekly for two weeks. There were effects of insulation, time and an insulation × time interaction for motile sperm and sperm that had normal morphology or head or midpiece defects ($P < 0.01$ for each). Sperm motility and morphology exhibited alterations between approximately two and five weeks after insulation, with mean progressively motile and morphologically normal sperm decreasing from approximately 55 to 35% and from 80 to 30%, respectively, and with head and midpiece defects increasing from approximately 3 to 50% and from 10 to 20% ($P < 0.05$ for each). The hypotheses that hypoxia would disrupt sperm quality and production, whereas hyperoxia would prevent hyperthermia-induced reductions in sperm quality and production, were not supported. This is apparently the first report that heat-stress induced damage to sperm was due to increased temperature *per se* and not testicular hypoxia, calling into question a long-standing paradigm.

Keywords: scrotal/testicular thermoregulation; scrotal insulation; testicular hypoxia; semen quality; ram

In many mammals, including sheep, it is well established that testis must be 4–5 °C below core body temperature to sustain physiological production of sperm, and to ensure that it is produced in proper numbers, and with appropriate motility and morphology (Skinner and Louw 1966; Kastelic et al. 1995; Saab et al. 2011). Furthermore, the testis normally operates in a condition of near-hypoxia (Waites and Setchell 1964). Increased testicular temperature causes concurrent increases in metabolism and therefore oxygen (O_2) demands. However, in the absence of a concomitant increase in blood flow (Waites and Setchell 1964; Setchell et al. 1995), the testis changes from a near-hypoxic

Supported by the Agriculture and Agri-Food Canada Lethbridge Research Centre.

doi: 10.17221/124/2016-VETMED

state to frank hypoxia. Consequently, the dogma is that decreased sperm motility and an increased proportion of morphologically abnormal sperm following increased testicular temperature are due to hypoxia (Setchell 1998; Paul et al. 2009). Although this view is widely accepted, to our knowledge, this has apparently not been formally tested.

Atmospheric air is approximately 21% O_2, whereas 16.0 and 10.8% O_2 in inspired air constituted mild hypoxia and hypoxia, respectively, in rats (Chen et al. 2007). Mice breathing air with 12.5, 15.0, 21.0, and 100% O_2 had testicular O_2 concentrations of 16, 24, 36, and 102 μmol/l (Baker and Lindop 1970). Similarly, breathing 100% O_2 doubled O_2 saturation in rat testes (Kram et al. 1989). Therefore, we inferred there is an association between the O_2 content of inspired air and O_2 content of testes, enabling testicular oxygen content to be varied from approximately 50 to 200% of physiologic concentrations.

The ability to independently alter testicular O_2 content and testicular temperature provided a novel opportunity to critically test effects of hypoxia and hyperthermia on spermatogenesis. The objective was to determine relative effects of hypoxia versus hyperthermia on sperm quality and production. We used a ram model to test the hypotheses that hypoxia would disrupt sperm motility and morphology and that hyperoxia would prevent hyperthermia-induced reductions in sperm motility and morphology.

MATERIAL AND METHODS

Canadian Arcott rams (n = 18) approximately 10 months of age were used. All rams were designated satisfactory following a standard breeding soundness evaluation (Ott and Memon 1980) and they were randomly allocated into one of six treatment groups. The experiment was a 2 × 3 factorial, with scrotal insulation (insulated or not insulated) and three oxygen concentrations in inspired air (14, 21 or 85%). Scrotal insulation was used to increase testicular temperature and create testicular hyperthermia, as described (Waites and Setchell 1964). Regarding scrotal insulation, the first layer (in apposition with the scrotal skin) was Insul-Bright (The Warm Company, 954 East Union Street, Seattle, USA) which consists of hollow polyester fibres needle-punched through mylar. A small amount of tag cement was applied to the scrotum to secure the insulation in place. Next, a disposable baby diaper was used to provide a second layer of insulation and to help hold the Insul-Bright in place, followed by a "tube" of stretchable fabric material (tubular medical bandage material), held in place with adhesive tape at the top (scrotal neck) and at the bottom, tied with string.

Three enclosed chambers were used to house the rams during 30 h of exposure to variable oxygen concentrations; in each chamber, three of the six rams had insulated scrotums, whereas the remainder did not. Two steel-framed chambers (covered with synthetic "puck board", approximately 7 mm thick) approximately 2.5 × 2.5 × 1.5 m were used for the low- and high-oxygen groups and a wire mesh chamber (covered with a tarpaulin) was used for the ambient oxygen group. The puckboard chambers were virtually air-tight and were supplied with compressed oxygen or nitrogen to maintain an 85 or 14% O_2 environment, respectively. In one corner of the chamber, a barrier was installed (to prevent access by rams); this area housed a dehumidifier with a fan (to decrease relative humidity) and a second circulating fan to promote mixing of supplied gases. Temperature was controlled using a closed-circuit cold water system. Water was chilled with ice in a large reservoir inside a refrigerator located between the two chambers, pumped through a hose into the sheep chamber, through a radiator cooler placed in front of the dehumidifier fan, and returned to the reservoir for re-cooling. Chamber temperature was maintained at approximately 23 °C. The floor of the chamber was covered with rubber matting and bedded with fresh straw, an automatic watering bowl was attached on the wall and fresh hay was also available (to ensure *ad libitum* access to water and feed). Each chamber had a plexiglass (transparent) window on top facilitating viewing of the rams and equipment. Oxygen and temperature monitors were fixed inside the chambers and readings recorded manually every 15 min.

After 30 h, chambers were opened, scrotal insulation was immediately removed and all rams were returned to pens. Semen was collected by electroejaculation on two occasions (14 and seven days before scrotal insulation was applied) and starting at seven days after the onset of scrotal insulation, semen collection was done bi-weekly for four weeks and then weekly for an additional two weeks. All

doi: 10.17221/124/2016-VETMED

procedures involving animals were conducted in accordance with standards of the Canadian Council on Animal Care (1993) and were reviewed and approved by the Animal Care Committee of the Agriculture and Agri-Food Canada Lethbridge Research Centre.

Sperm were assessed subjectively for progressive motility (estimated in increments of 10%) at 400 × and 200 cells were examined (phase-contrast microscopy, 1000 ×) from each sample to determine morphological characteristics. Due to their subjective nature, assessments of motility and morphology were done without knowledge of treatment group. Sperm morphology categories were normal, or defects, which included the head, midpiece or tail, based on morphological abnormalities described for bull sperm (Barth and Oko 1989). All semen data were arc-sine transformed and transformed data were analysed using a Mixed Models procedure for repeated measures, using main effects of insulation, oxygen, time, the three two-way interactions and the three-way interaction for progressively motile sperm, morphologically normal sperm, and for sperm with head, midpiece or tail defects. For all analyses, $P < 0.05$ was considered significant. If there was a significant main effect or interaction, differences were located using a least significant difference test.

RESULTS

For motile sperm and for sperm that had normal morphology, a defective head or defective midpiece, there were effects of insulation, time and an insulation × time interaction ($P < 0.01$ for each; Figure 1). None of the remaining statistical analyses revealed significant effects or interactions ($P > 0.05$).

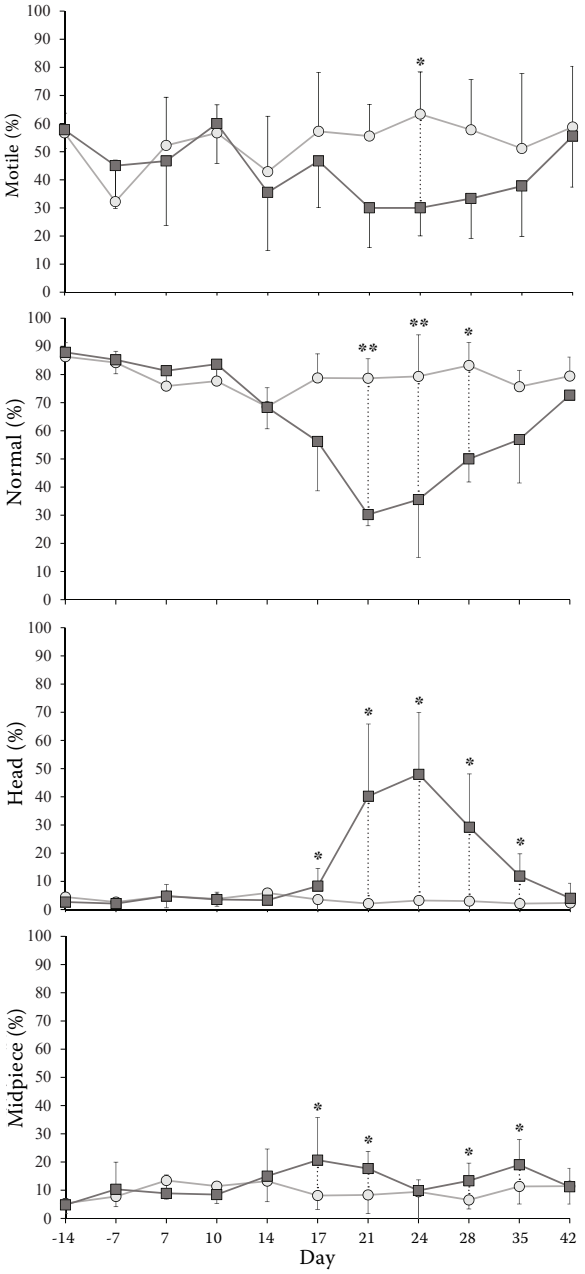

Figure 1. Mean (± SD) sperm characteristics in 18 rams with or without scrotal insulation (square and circle, respectively); insulation was applied on Day 0 and removed after 30 h. For motile sperm and for sperm with normal morphology and for those with a defective head or defective midpiece, there were effects of insulation, time and an insulation × time interaction ($P < 0.01$ for each)
*Difference ($P < 0.05$) between groups on that day of collection

DISCUSSION

In this study, testicular hyperthermia (scrotal insulation) significantly reduced percentages of motile and morphologically normal sperm; these changes were not prevented by hyperoxia nor replicated by hypoxia. Therefore, the hypotheses that hypoxia would disrupt sperm motility and morphology and that hyperoxia would prevent hyperthermia-induced reductions in sperm motility and morphology were not supported. This is apparently the first report that heat-stress induced damage to sperm was due to increased temperature *per se* and not due to testicular hypoxia as reported (Waites and Setchell 1964; Paul et al. 2009; Hamilton et al. 2016).

doi: 10.17221/124/2016-VETMED

Although no attempt was made to determine oxygen delivery to the testis or intratesticular O_2 saturation, this work was based on previous studies in laboratory rodents. Whereas atmospheric air is approximately 21% O_2, rats breathing 16.0 and 10.8% O_2 in inspired air were considered to have mild hypoxia and hypoxia, respectively (Chen et al. 2007). Furthermore, mice breathing air with 12.5, 15.0, 21.0, or 100% O_2 had testicular O_2 concentrations of 16, 24, 36, and 102 μmol/l (Baker and Lindop 1970), and in a rat study, breathing 100% O_2 doubled testicular O_2 saturation (Kram et al. 1989). Therefore, there is apparently an association between the O_2 content of inspired air and O_2 content of testes, enabling testicular oxygen content to be varied from approximately 50 to more than 200% of physiologic concentrations. The present study was conducted using rams, a mid-size animal that facilitated the work, including hyperthermia and hypoxia, and serial collection of semen. Measurement of testicular blood flow and oxygen delivery to the testis was not possible. The study was conducted based on previous studies in laboratory rodents, in which O_2 content of inspired air modulated O_2 delivery to the testes. Therefore, further, more invasive studies are needed to verify that rams have similar physiology.

It is well known that the mammalian testis operates on the brink of hypoxia (Setchell 1998) and that testicular hyperthermia increases metabolism and hence O_2 consumption. However, blood flow is reported to remain relatively constant and therefore the resulting hypoxia is regarded as the basis for decreases in sperm motility and morphology (Waites and Setchell 1964; Paul et al. 2009; Hamilton et al. 2016). Regardless, to the authors' knowledge, this has never been critically tested. In the present study, transient testicular hyperthermia caused substantial reductions in both motility and morphology. It was noteworthy that these changes were not prevented by hyperoxia, nor were they replicated by hypoxia. Therefore, we concluded that effects on motility and morphology were due to hyperthermia *per se* and not due to hyperthermia-induced hypoxia. Although these findings need to be replicated and refined, including direct measurments of O_2 delivery to the testis and ideally more objective means of assessing sperm motility and morphology, they call into question the long-standing paradigm that the effects of testicular hyperthermia on sperm motility and morphology are secondary to hypoxia.

Decreases in sperm motility and increased numbers of sperm with head or midpiece defects were induced by scrotal insulation, with changes first apparent at approximately two weeks after the onset of insulation and persisting for approximately three weeks (i.e., approximately five weeks after the thermal insult). Similar changes occurred at corresponding times in previous studies that involved increased testicular temperatures in bulls and rams (Howarth 1969; Meyerhoeffer et al. 1985; Saab et al. 2011; Rocha et al. 2015; Hamilton et al. 2016) with some apparent differences in the magnitude of changes and the interval from the thermal insult to recovery, attributed to the degree and duration of testicular heating and perhaps species-specific characteristics (Skinner and Louw 1966; Rathore 1970; Brito et al. 2004; Nichi et al. 2006). Furthermore, in this study, sperm damage occurred during the final stages of spermatogenic development in the testis (Howarth 1969; Cardoso and Queiroz 1988; Senger 2003). Although sperm at various stages of development or in the epididymis may be affected by increased testicular temperature (Lue et al. 1999; Perez-Crespo et al. 2008; Hamilton et al. 2016), in our study, due to the delay from testicular heating to appearance of abnormalities, there was no apparent effect on sperm present in the epididymis. However, in a previous study in bulls with scrotal insulation (Vogler et al. 1991), sperm present in the epididymis were apparently unaffected when fresh, unfrozen sperm were evaluated, although changes were evident after sperm cryopreservation, thawing, and incubation for 3 h at 37 °C.

The importance of O_2 availability for sperm differs according to their location (within the testis or within an ejaculate). For testicular sperm, aerobic metabolism is preferred to anaerobic (Voglmayr et al. 1971). Increased O_2 uptake was described for rams during testicular insulation, but there was no apparent difference in lactate concentrations, and therefore, no indication that sperm switched from aerobic to anaerobic metabolism (Waites and Setchell 1964). It is noteworthy that anaerobic metabolism dramatically reduced sperm motility in both bulls and rams (Wilson et al. 1987). In human mountain climbers, chronic hypoxia reduced sperm motility and concentration and increased the number of immature sperm in the ejaculate (Verratti et al. 2008). Similarly, intermittent hypoxia in monkeys disrupted testicular architecture, reduced motility and modified se-

doi: 10.17221/124/2016-VETMED

men pH (Saxena 1995). In contrast, hyperoxia had no apparent effect on testicular histology in rats (Nakada et al. 1986), supporting our interpretation that O_2 availability may not be the primary mechanism responsible for the observed effects. Although some studies have been conducted, there is still a relative lack of information regarding how high and low O_2 availability *in vivo* impact sperm. Furthermore, an increase in reactive oxygen species (ROS) and an impact on sperm mitochondria was observed after long term exposure to increased intra-testicular temperature (approximately 33.5 °C), but no observation on the O_2 availability was taken (Hamilton et al. 2016). Important modifications of the proteomic profile of seminal plasma were related to semen quality parameters after testicular heat stress in rams (Rocha et al. 2015), indicating another potential mechanism for the observed damage that is not necessarily caused by hypoxia.

In the present study, neither low nor high O_2 in the inspired air significantly affected sperm motility or morphology. An *in vivo* model is preferred to understand testicular physiology, as there are autoprotective mechanisms against low O_2 tension in the testis (Elshaari et al. 2012) and natural antioxidant systems (Aitken and Roman 2008; Reyes et al. 2012) that are difficult or impossible to replicate in an *in vitro* model. Furthermore, under heat stress, testicular vasomotion (rhythmic tone variation in blood vessels) is greatly suppressed (Setchell et al. 1995) which can impair testicular function (Lysiak et al. 2000; Aalkjaer et al. 2011).

In conclusion, there were no apparent effects of hypoxia on sperm morphology or motility; furthermore, hyperoxia did not prevent decreases in sperm morphology and motility induced by increased testicular temperature. Therefore, in contrast to long-standing dogma, it appeared that increased temperature and not the hypoxia was the underlying cause of reductions in motile and morphologically normal sperm in rams with testicular hyperthermia.

Acknowledgements

This work was conducted at the Agriculture and Agri-Food Canada Lethbridge Research Centre. The efforts of the animal caretakers at the Lethbridge Research Centre are gratefully acknowledged.

REFERENCES

Aalkjaer C, Boedtkjer D, Matchkov V (2011): Vasomotion – what is currently thought? Acta Physiologica 202, 253–269.

Aitken RJ, Roman SD (2008): Antioxidant systems and oxidative stress in the testes. Oxidative Medicine and Cellular Longevity 1, 15–24.

Baker DJ, Lindop PJ (1970): Oxygen cathode measurements in the mouse testis. Physics in Medicine Biology 15, 263–270.

Barth AD, Oko RJ (eds) (1989): Abnormal Morphology of Bovine Spermatozoa. Iowa State University Press. 285 pp.

Brito LFC, Silva AEDF, Barbosa RT, Kastelic JP (2004): Testicular thermoregulation in Bos indicus, crossbred and Bos taurus bulls: relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production. Theriogenology 61, 511–528.

Canadian Council on Animal Care (1993): Guide to the care and use of experimental animals 1. 2nd edn. Olfert ED, Cross BM, McWilliam AA (eds). Available at www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf.

Cardoso FM, Queiroz GF (1988): Duration of the cycle of the seminiferous epithelium and daily sperm production of Brazilian hairy rams. Animal Reproduction Science 17, 77–84.

Chen XQ, Dong J, Niu CI, Fan JZ (2007): Effects of hypoxia on glucose, insulin, glucagon, and modulation by corticotropin-releasing factor receptor type 1 in the rat. Endocrinology 148, 3271–3278.

Elshaari FA, Elfagihi RI, Sheriff DS, Barassi IF (2012): Testicular torsion-detorsion-histological and biochemical changes in rat testis. Journal of Cytology and Histology 3, 136–142.

Hamilton TRS, Mendes CM, de Castro LS, de Assis PM, Siqueira AFP, Delgado JC, Goissis MD, Muino-Blanco T, Cebrian-Perez JA, Nichi M, Visintin JA, Assumpcao MEOD (2016): Evaluation of lasting effects of heat stress on sperm profile and oxidative status of ram semen and epididymal sperm. Oxidative Medicine and Cellular Longevity 2016, doi: 10.1155/2016/1687657.

Howarth Jr B (1969): Fertility in the ram following exposure to elevated ambient temperature and humidity. Journal of Reproduction and Fertility 19, 179–183.

Kastelic JP, Coulter GH, Cook RB (1995): Scrotal surface, subcutaneous, intratesticular and intraepididymal temperatures in bulls. Theriogenology 44, 147–152.

Kram HB, Miyamoto E, Raijfer J, Appel PL, Shoemaker WC (1989): Testicular oximetry; a new method for the assess-

doi: 10.17221/124/2016-VETMED

ment of tissue perfusion and viability following torsion and detorsion. Journal of Pediatric Surgery 24, 1297–1302.

Lue YH, Hikim APS, Swerdloff RS, Paul IM, Taing KS, Bui T, Leung A, Wang C (1999): Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. Endocrinology 140, 1709–1717.

Lysiak JJ, Nguyen QAT, Turner TT (2000): Fluctuations in rat testicular interstitial oxygen tensions are linked to testicular vasomotion: Persistence after repair of torsion. Biology of Reproduction 63, 1383–1389.

Meyerhoeffer DC, Wettemann RP, Coleman SW, Wells ME (1985): Reproductive criteria of beef bulls during and after exposure to increased ambient temperature. Journal of Animal Science 60, 352–257.

Nakada T, Saito U, Ota K, Saegusa T, Chikenji M, Matsushita T (1986): Serum testosterone, testicular connective tissue protein and testicular histology in rats treated with hyperbaric oxygen. International Urology and Nephrology 18, 439–447.

Nichi M, Bols PEJ, Zuge RM, Barnabe VH, Goovaerts IG, Barnabe RC, Cortada CN (2006): Seasonal variation in semen quality in Bos indicus and Bos taurus bulls raised under tropical conditions. Theriogenology 66, 822–828.

Ott RS, Memon MA (1980): Breeding soundness examinations of rams and bucks, a review. Theriogenology 13, 155–164.

Paul C, Teng S, Saunders PTK (2009): A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. Biology of Reproduction 80, 913–919.

Perez-Crespo M, Pintado B, Gutierrez-Adan A (2008): Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. Molecular Reproduction and Development 75, 40–47.

Rathore AK (1970): Morphological changes in ram spermatozoa due to hot-room exposure for varying periods. British Veterinary Journal 126, 277–281.

Reyes JG, Farias JG, Henriquez-Olavarrieta S, Madrid E, Parraga M, Zepeda AB, Moreno RD (2012): The hypoxic testicle: Physiology and pathophysiology. Oxidative Medicine and Cellular Longevity 15, doi: 10.1155/2012/929285.

Rocha DR, Martins JA, Tilburg MF, Oliveira RV, Moreno FB, Monteiro-Moreira AC, Moreira RA, Araujo AA, Moura AA (2015): Effect of increased testicular temperature on seminal plasma proteome of the ram. Theriogenology 84, 1291–1305.

Saab SA, Sleiman FT, Kallassy N, Darweesh WY, Aad PY (2011): Effect of adaptation and heat stress on reproductive performances of fat-tail Awassi rams in eastern Mediterranean. Lebanese Science Journal 12, 31–44.

Saxena DK (1995): Effect of hypoxia by intermittent altitude exposure on semen characteristics and testicular morphology of male rhesus monkeys. International Journal of Biometeorology 38, 137–140.

Senger PL (2003): Endocrinology of the male and spermatogenesis. In: Senger PL (ed.): Pathways to Pregnancy and Parturition. Cadmus Professional Communications, United States of America. 214–239.

Setchell BP (1998): The parkes lecture heat and the testis. Journal of Reproduction and Fertility 114, 179–194.

Setchell BP, Bergh A, Widmark A, Damber JE (1995): Effect of testicular temperature on vasomotion and blood flow International. Journal of Andrology 18, 120–126.

Skinner JD, Louw GN (1966): Heat stress and spermatogenesis in Bos indicus and Bos taurus cattle. Journal of Applied Physiology 21, 1784–1790.

Verratti V, Berardinelli F, Di Giulio C, Bosco G, Cacchio M, Pellicciotta M, Nicolai M, Martinotti S, Tenaglia R (2008): Evidence that chronic hypoxia causes reversible impairment on male fertility. Asian Journal of Andrology 10, 602–606.

Vogler CJ, Saacke RG, Bame JH, Dejarnette JM, McGilliard ML (1991): Effects of scrotal insulation on viability characteristics of cryopreserved bovine semen. Journal of Dairy Science 74, 3827–3835.

Voglmayr JK, Setchell BP, White IG (1971): The effects of heat on the metabolism and ultrastructure of ram testicular spermatozoa. Journal of Reproduction and Fertility 24, 71–80.

Waites GMH, Setchell BP (1964): Effect of local heating on blood flow and metabolism in the testis of the conscious ram. Journal of Reproduction and Fertility 8, 339–349.

Wilson MC, Harvey JD, Shannon P (1987): Aerobic and anaerobic swimming speeds of spermatozoa investigated by twin beam laser velocimetry. Biophysical Journal 5, 509–512.

Received: August 23, 2016
Accepted after corrections: July 14, 2017