—Original Article—

Season-Induced Changes in Bovine Sperm Motility Following a Freeze-Thaw Procedure

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Abstract. Decreased conception rate of dairy cows in the summer is mainly associated with the deleterious effects of environmental thermal stress on the female reproductive tract. Here, we suggest that decreased reproductive performance might be partially due to inferior-quality semen. Semen from five representative bulls was collected in summer (August to September) and winter (December to January) and evaluated with a computerized sperm-quality analyzer for bulls (SQA-Vb). No seasonal effect was found in fresh ejaculate, but sperm examined post-thawing showed lower velocity, motility and progressive motility ($P < 0.04$) in summer vs. winter samples. Element concentrations in the seminal plasma, determined by inductively coupled plasma-atomic emission spectrometry, differed between seasons, with higher ($P < 0.01$) concentration values of K, Mg, Na and S elements in winter vs. summer samples. Therefore, season-induced alterations in seminal plasma element concentration should be taken into account when using an extender for cryopreservation. Acrosome integrity was assessed by a triple-fluorescence test using Hoechst 33342, fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) and propidium iodide. Acrosome reaction was examined by a one-step staining method using FITC-PSA. The proportion of sperm cells with a damaged acrosome post-thawing tended to be higher ($P < 0.07$) in semen collected during the summer vs. winter. Such alterations suggest that seasonal reductions in sperm function might also be involved in the decreased conception rate of dairy cows in summer.

Key words: Element concentration, Seasonality, Sperm motility

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Decreased fertility of dairy cows during the summer is mainly associated with the deleterious effects of environmental thermal stress on the female reproductive tract. However, the effects of heat stress on semen quality may also contribute to the phenomenon [1, 2].

Mature mammalian spermatozoa are quiescent in the male reproductive tract. Upon ejaculation, they undergo capacitation, develop hyperactive motility and acquire acrosomal ability to react [3]. These events involve sperm membrane hyperpolarization, activation of plasma-membrane ion channels and changes in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$ [4, 5]. Thus, season-induced alterations in one of these events might compromise spermatozoon fertilization competence.

In bovines, the sperm flagellum contains cyclic nucleotide-gated (CNG) channels—nonselective cation channels that regulate Na^+ , Mg^{2+} and Ca^{2+} influx under cGMP and cAMP control [4–6]. CNGA3 (formerly CNG α 3) ion channels are expressed along the entire sperm flagellum, whereas CNGB1 (formerly CNG β 1) channels are localized to its principal part [4, 7, 8]. Activation of CNG channels leads to depolarization of the membrane voltage and a concomitant increase in cytosolic cation concentrations [9]. In

addition, calcium flux through sperm inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) located in the sperm's head and connecting piece plays a pivotal role in acrosomal exocytosis, as binding of IP_3 to IP_3Rs triggers release of acrosomal calcium during the acrosome reaction [5].

Here, we hypothesize that bovine spermatozoon motility is negatively affected by the hot season. We also postulate that season-induced alterations in element concentrations might affect the sperm's motility and its ability to undergo the acrosome reaction. Characterizing such alterations might enable evaluation of the effect of environmental thermal stress on sperm quality.

Materials and Methods

All chemicals, unless otherwise specified, were from Sigma-Aldrich (Rehovot, Israel). Sperm-Tyrode's lactate (SP-TL) was prepared in our laboratory. SP-TL was supplemented with 0.6% (wt/vol) BSA, 1 mM sodium pyruvate and 0.2 mg/ml gentamicin (SP-TALP). Fluoromount was from Diagnostic BioSystems (Pleasanton, CA, USA).

Animals

The experiment was performed at the Israeli Artificial Insemination Center ('Sion', Hafetz-Haim, Israel) in accordance with the 1994 Israeli guidelines for animal welfare. The study included five representative 5-year-old Holstein Friesian bulls (# 3904, 7053, 7060, 7122, and 7085) from the Israeli bull board that

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were already on a collection schedule when the experiment was begun. According to the 2008 Israeli Herdbook (unpublished), the bulls' average conception rates did not differ from those recorded for the Israeli herd, $39.05 \pm 0.70\%$ and $18.81 \pm 0.70\%$ for winter and summer, respectively, indicating a clear seasonal variation in conception rate. It should be noted, however, that the routine management of semen collection at 'Sion' does not include any seasonal records, and therefore, seasonal variations within and between bulls, seasonal effects on semen quality and their contribution to the decline in conception rate are unknown.

Bulls were fed the same total mixed ration (TMR) throughout the experiment, in both summer and winter, containing 68.4% (wt/wt) DM, 7.2% (wt/wt) protein, 36.2% (wt/wt) NDF, 20.0% (wt/wt) ADF, 1.45 NE Mcal/kg and 3.5 g minerals/kg (NaCl, Ca and P) on a DM basis. Samples were collected throughout the summer (August to September) and winter (December to January). Environmental data were obtained from the central meteorological station in Bet-Dagan, Israel. The maximum average air temperature and maximum relative humidity were 31.8 ± 1.4 C and $84.3 \pm 3.8\%$ in the summer and 16.8 ± 2.3 C and $46.0 \pm 15.0\%$ in the winter, respectively.

Semen collection

Semen was collected routinely twice a week. The first weekly collection was taken for analysis. To eliminate any potential differences in sperm quality or in seminal plasma element concentrations in serial ejaculates, samples were obtained only once a day. Bulls were mounted on a live teaser, and semen was collected into a disposable tube using a heated (38 C), sterile artificial vagina. The ejaculate was immediately transferred to a nearby lab. Semen was evaluated by the computerized sperm-quality analyzer for bulls (SQA-Vb, Medical Electronic Systems, Caesarea, Israel), including measurements of volume, concentration, and velocity, motility, and progressive motility rates. According to the routine procedure at 'Sion', samples with a concentration greater than 650×10^6 cell/ml and motility greater than 70% were defined as being of good quality. For each collection ($n = 5$ per season) from each bull ($n = 5$), 2 ml out of the total volume of the collected semen was centrifuged (1100 g) for 5 min at room temperature to separate the sperm from the seminal plasma. The seminal plasma was examined for element concentrations (see the spectrometry procedure below). The remaining volume (4–7 ml/bull per collection) of fresh semen was cryopreserved for further post-thaw examination, according to the routine procedure at 'Sion'. Briefly, samples were diluted (1:10 vol/vol at room temperature—22–25 C) with an extender containing (per ml) 10% (vol/vol) glycerol, 20% (wt/vol) egg yolk, 20 mg lactose, 1000 IU penicillin and 500 mg streptomycin. Diluted semen was chilled for 3 h to 4 C and inserted into 0.25-ml chilled straws. Straws were then separated on racks and cooled for 10 min to -95 C in a programmed box with a vapor nitrogen-saturated atmosphere followed by plunging into liquid nitrogen. After 6 months of cryopreservation, representative samples (three straws per bull from each collection in each season) were thawed by plunging into warm water (37 C) for 30 sec and evaluated by the SQA-Vb system as described above.

Element concentrations in the seminal plasma

Element concentrations in the seminal plasma were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES; Spectro Genesis, Spectro Analytical Instruments GmbH, Kleve, Germany) using scandium as the internal standard and a blank control in parallel. Briefly, 0.8 ml seminal plasma samples were digested in Teflon vessels containing 1 ml of 65% HNO_3 , using a Milestone ETHOS One sample digestion system under high temperature and pressure. Samples were exposed to microwave radiation for 25 min. The temperature inside the vessels was maintained at 180 C. Thereafter, liquid residues were taken up in deionized water and adjusted to 15 ml. Analysis was performed for 28 different elements by using an End-On-Plasma ICP-AES (Spectro ARCOS, Spectro Analytical Instruments GmbH, Kleve, Germany). Calibration was performed with standards for ICP (Merck) every 10 samples to check stability. Samples exceeding the linear dynamic range were diluted using calibrated pipettes and reanalyzed.

Assessment of cell viability and acrosome-membrane integrity

Acrosome integrity was assessed by a triple-fluorescence test modified from that described by Sa-Ardrit *et al.* [10] using Hoechst 33342, fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) and propidium iodide (PI). Thawed samples were centrifuged (1100 g for 5 min) to separate the extender and seminal plasma from the cells. The pellet was then incubated at 35 C in the dark with Hoechst 33342 at a final concentration of 200 $\mu\text{g/ml}$ and FITC-PSA at a final concentration of 10 $\mu\text{g/ml}$ for 20 min. At this point, 2 μl PI was added, and samples were then incubated for an additional 10 min at 35 C in the dark. Cell viability and acrosome integrity were determined under a fluorescent microscope equipped with a digital camera (DXM1200F; Nikon, Tokyo, Japan) and connected to a computer microscope at $400 \times$ magnification with excitation at 450 to 490 nm and emission at 520 nm. The procedure was repeated three times with a minimum of 200 cells from five ejaculates per bull per season. Cells with damaged acrosomes fluoresced green, dead cells fluoresced red and cell nuclei fluoresced blue.

Assessment of acrosome reaction

An acrosome reaction assay was performed by a one-step staining method using FITC-PSA as described by Mendoza *et al.* [11]. Briefly, semen samples were purified by Percoll gradient (45/90) and incubated with 0.5 ml SP-TALP for 4 h at 38 C. At this point, 2 μl of the ionophore A23187 at a final concentration of 20 μM was added, and samples were then incubated at 38 C for 20 min. Thereafter, 10 μl of ionophore-treated samples were smeared on a slide, air-dried and dipped in absolute methanol for 10 min. Samples were then treated with 35 μl FITC-PSA at a final concentration of 50 $\mu\text{g/ml}$ and incubated for 35 min at room temperature in a moisture chamber. Following incubation, slides were washed in distilled water, dried in the dark and covered with Fluoromount. Acrosome reaction was examined under a fluorescent microscope as described above. A minimum of 200 cells taken from three to five ejaculates per bull, per season were examined.

Table 1. Seasonal effect on characteristics of fresh and post-freeze-thawed semen

	Volume (ml)	Concentration (millions/ml)	Motility (%)	Progressive motility (%)	Velocity ($\mu\text{m}/\text{sec}$)
F					
Winter	$7.89 \pm 0.3^*$	$895 \pm 36^{**}$	85.2 ± 1.0	82.2 ± 1.0	116.7 ± 1.4
Summer	8.72 ± 0.3	1017 ± 36	85.9 ± 1.1	83.0 ± 1.0	117.8 ± 1.5
PF					
Winter	Straw volume	52.3 ± 1.4	$62.8 \pm 3.2^{**}$	$30.7 \pm 2.0^{**}$	$42.9 \pm 3.4^{**}$
Summer	Straw volume	48.0 ± 1.4	52.5 ± 3.2	24.6 ± 2.0	31.6 ± 3.4

Data are presented as means $\pm$ SEM for five samples per bull ($n = 5$) per season (winter, summer), per status (F = fresh, PF = post-freeze-thaw). * and **: $P < 0.06$ and $P < 0.04$ within a column within a status, respectively.

Statistical analysis

Differences between seasons in volume, concentration, velocity, motility, progressive motility and seminal plasma ion concentrations were analyzed by one-way ANOVA with repeated measures using JMP 7.0 (SAS Institute, Cary, NC, USA). Element concentrations were analyzed by one-way ANOVA. The Student's *t*-test was used to compare acrosome integrity and viability of post-thaw semen among the experimental groups. Data are presented as means $\pm$ SEM. For all analyses, $P < 0.05$ was considered significant, and *P*-values between 0.05 and 0.1 were also reported as trends that may be real and worthy of note.

Results

Seasonal effect on semen physiological characteristics

Motility, progressive motility and velocity of fresh semen did not differ between seasons, averaging $85.5 \pm 1.0\%$, $82.6 \pm 1.0\%$ and $117 \pm 1.5 \mu\text{m}/\text{sec}$, respectively (Table 1). The ejaculate volume

tended to be higher ($P < 0.06$), and more concentrated ($P < 0.04$), in semen collected during the summer *vs.* that collected during the winter (Table 1). It should be noted, however, that there was wide seasonal variation between bulls (Table 2). Two out of the five bulls examined (#3904, 7060) had higher sperm concentrations ($P < 0.03$) in the summer than in the winter. One bull (#7053) had higher ($P < 0.03$) semen volume in the summer than in the winter, but this was not associated with any differences in the other examined parameters (concentration, motility, or progressive motility). Another bull (#7085) showed higher ($P < 0.03$) sperm motility and higher ($P < 0.03$) progressive motility in the summer *vs.* winter sample. On the other hand, bull #3904 tended ($P < 0.1$) to show higher motility and higher progressive motility in the winter *vs.* summer sample.

Although there was no seasonal effect on motility, progressive motility, or velocity of fresh semen, examination of semen post freeze and thaw revealed differences between seasons in all examined parameters (Table 1). There was a significant effect on samples collected during the summer, showing lower motility,

Table 2. Seasonal variation in fresh semen characteristics among bulls; volume and concentration (2A); and motility, progressive motility, and velocity (2B)

2A		Volume (ml)	Concentration (millions/ml)		
Bull (#)	Winter	Summer	Winter	Summer	
3904	8.2 ± 0.6	8.8 ± 0.4	860 ± 45**	1151 ± 30	
7053	7.6 ± 0.7**	9.9 ± 0.7	658 ± 31	685 ± 68	
7060	7.7 ± 0.9	8.5 ± 0.5	1155 ± 38**	1277 ± 18	
7085	5.9 ± 0.4	7.3 ± 0.8	1080 ± 49	1160 ± 48	
7122	10.1 ± 0.4	9.2 ± 0.4	724 ± 41	780 ± 52	

2B		Motility (%)	Progressive motility (%)		Velocity (µm/sec)	
Bull (#)	Winter	Summer	Winter	Summer	Winter	Summer
3904	82.7 ± 2.4*	77.7 ± 1.5	79.8 ± 2.3*	75.1 ± 1.4	113.2 ± 3.3*	106.5 ± 2.0
7053	88.6 ± 1.7	89.4 ± 1.4	85.5 ± 1.4	86.4 ± 1.5	121.4 ± 2.4	122.0 ± 1.5
7060	80.1 ± 2.8	81.8 ± 3.0	77.3 ± 2.7	79.0 ± 2.9	109.6 ± 3.9	112.0 ± 4.2
7085	89.6 ± 0.9**	92.2 ± 0.2	86.5 ± 0.9**	89.1 ± 0.2	122.9 ± 1.4**	126.5 ± 0.3
7122	85.2 ± 1.9	89.0 ± 1.4	82.1 ± 1.9	85.8 ± 1.3	116.5 ± 2.6	121.9 ± 1.9

Data are presented as means $\pm$ SEM for five samples per bull ($n = 5$) per season (winter, summer). * and **: $P < 0.1$ and $P < 0.03$ within a column within a status, respectively.

Table 3. Seasonal variation in freeze-thawed semen characteristics among bulls

Bull (#)	Motility (%)		Progressive motility (%)		Velocity ($\mu\text{m}/\text{sec}$)	
	Winter	Summer	Winter	Summer	Winter	Summer
3904	64.6 $\pm$ 2.8	57.0 $\pm$ 4.6	30.4 $\pm$ 3.0	25.4 $\pm$ 3.0	52.5 $\pm$ 4.2	42.5 $\pm$ 4.2
7053	58.6 $\pm$ 4.2**	34.2 $\pm$ 4.4	28.3 $\pm$ 4.0	15.3 $\pm$ 6.8	34.4 $\pm$ 3.8*	16.5 $\pm$ 4.0
7060	55.6 $\pm$ 3.8	57.9 $\pm$ 3.8	25.1 $\pm$ 2.6	28.3 $\pm$ 2.6	36.0 $\pm$ 3.9	32.0 $\pm$ 3.9
7085	84.1 $\pm$ 3.9**	62.2 $\pm$ 3.9	45.5 $\pm$ 2.3**	31.8 $\pm$ 2.3	63.5 $\pm$ 3.3**	37.3 $\pm$ 3.3
7122	52.8 $\pm$ 3.7	51.3 $\pm$ 3.7	24.1 $\pm$ 2.3	22.0 $\pm$ 2.3	28.0 $\pm$ 3.7	29.5 $\pm$ 3.7

Data are presented as means $\pm$ SEM for five samples per bull (n = 5) per season (winter, summer). * and **: P<0.06 and P<0.04 within a column within a status, respectively.

Table 4. Element concentrations (mg/l) in the seminal plasma of semen collected in the winter and summer. Data are presented as means $\pm$ SEM.

P-value	Summer	Winter	Elements
<0.27	364 $\pm$ 12	383 $\pm$ 12	Ca
<0.0002	1439 $\pm$ 89	1944 $\pm$ 89	K
<0.0001	80.4 $\pm$ 1	90.0 $\pm$ 1	Mg
<0.0001	1330 $\pm$ 53	1918 $\pm$ 53	Na
<0.059	959 $\pm$ 36	862 $\pm$ 36	P
<0.0014	1147 $\pm$ 26	1270 $\pm$ 26	S

lower progressive motility, and lower velocity than those collected during the winter (P<0.04, Table 1). It should be noted, however, that there was seasonal variation between the bulls' semen post freeze and thaw (Table 3). In three out of the five examined bulls, there were no differences in sperm cryosurvival. On the other hand, one out of the five examined bulls (#7085) exhibited higher motility, higher progressive motility, and higher velocity (P<0.04) in cryopreserved samples collected during the winter vs. summer. Note that this latter bull expressed a reciprocal pattern when its fresh semen was examined (Table 2). Another bull (#7053) showed higher motility (P<0.04) and a tendency toward higher velocity (P<0.06) in samples collected during the winter vs. those collected in summer (Table 3).

Seasonal effect on elements concentrations in the seminal plasma

The results of analysis of the major elements in the seminal plasma are presented in Table 4. In general, elements concentrations (average of five ejaculates/bull per season) differed between seasons, with higher (P<0.01) K, Mg, Na, and S concentrations in the winter vs. summer samples. On the other hand, Ca and P concentrations in the seminal plasma did not differ between seasons. Additional analysis of elements concentrations per bull revealed variations between bulls, with differences in Ca, K, and Na concentrations between seasons (Table 5). For instance, all of the bulls had a higher Na concentration in the seminal plasma of semen collected in the winter vs. summer; three of the five examined bulls (#3904, 7060, 7085) had a higher Ca concentration in the seminal plasma of semen collected in the winter vs. summer. In contrast, the Ca concentration in the seminal plasma of bull #7053 was higher during the summer. In addition, three (#3904, 7060, 7122) out of

Table 5. Element concentrations (mg/l) in the seminal plasma of semen collected in the winter and summer

Bull	Element concentration (mg/l)			
	Winter	Summer	Winter	Summer
Ca				
7085	374 $\pm$ 2*	311 $\pm$ 45	2426 $\pm$ 148**	1584 $\pm$ 148
7060	380 $\pm$ 3**	268 $\pm$ 27	1393 $\pm$ 70**	942 $\pm$ 70
3904	383 $\pm$ 3	292 $\pm$ 80	1186 $\pm$ 60**	894 $\pm$ 188
7053	397 $\pm$ 15**	539 $\pm$ 15	2734 $\pm$ 129**	1935 $\pm$ 129
7122	383 $\pm$ 2	411 $\pm$ 55	1852 $\pm$ 123**	1297 $\pm$ 123
K				
7085	745 $\pm$ 73	934 $\pm$ 805	1208 $\pm$ 68	1177 $\pm$ 68
7060	2447 $\pm$ 55**	1791 $\pm$ 55	955 $\pm$ 178	1034 $\pm$ 57
3904	2635 $\pm$ 71**	2123 $\pm$ 71	794 $\pm$ 78*	1055 $\pm$ 78
7053	986 $\pm$ 532	745 $\pm$ 86	608 $\pm$ 90	589 $\pm$ 90
7122	2905 $\pm$ 240**	1602 $\pm$ 240	742 $\pm$ 84	938 $\pm$ 267
Mg				
7085	8 $\pm$ 3	7 $\pm$ 3	1326 $\pm$ 64	1180 $\pm$ 64
7060	86 $\pm$ 3	8 $\pm$ 3	1081 $\pm$ 46	1046 $\pm$ 46
3904	7 $\pm$ 3	6 $\pm$ 10	959 $\pm$ 55	921 $\pm$ 55
7053	115 $\pm$ 4*	9 $\pm$ 4	1515 $\pm$ 58	1340 $\pm$ 58
7122	9 $\pm$ 11*	8 $\pm$ 2	1472 $\pm$ 62*	1247 $\pm$ 62

Data are presented as means $\pm$ SEM for five samples per bull (n = 5) per season (winter, summer). * and **: P<0.05 and P<0.01 within a row within a ion, respectively.

the five examined bulls showed a higher K concentration in the winter than in the summer (Table 5).

Seasonal effect on sperm viability, acrosome integrity, and acrosome reaction in post-thaw semen

For each bull in each season, semen samples were taken from three to five different ejaculates and evaluated for cell viability and acrosome integrity post freeze-thaw procedure (Fig. 1). While not significant, the proportion of dead cells (fluorescing red) in samples collected during the summer was numerically higher than that detected in the winter samples (56.1 $\pm$ 3.6 vs. 53.6 $\pm$ 2.3%, respectively; P<0.11). Correspondingly, there was a tendency (P<0.07) for a higher proportion of sperm cells with damaged acrosomes (fluorescing green; Fig. 1D) in the summer than in the winter (54.2 $\pm$ 3.5 vs. 51.4 $\pm$ 1.9%, respectively). Note

Fig. 1. Representative picture of post-freeze-thaw sperm cells. A: Nuclei of all cells fluoresce blue. B: Dead cells fluoresce red. C: Merge of 1A and 1B. D, D': Cells with damaged acrosome membrane fluoresce green. E, E': Dead cells with damaged acrosome membrane (fluoresce red and green). F: Representative picture of another set of post-freeze-thaw sperm cells that underwent acrosome reaction (yellow arrowhead).

that all sperm with damaged acrosomes (fluorescing green) were dead (fluorescing red), whereas some of the dead sperm had an intact acrosome membrane (Fig. 1E), suggesting that additional cell damage occurred upon cryopreservation. While not significant, examination of the acrosome reaction in Percoll-purified semen revealed that the proportion of sperm expressing the acrosome reaction post cryopreservation was numerically higher (by 5%) in semen collected during the winter vs. summer (76.8 ± 2.15 and 72.7 ± 1.82 , respectively; $P < 0.13$; Fig. 1F).

Discussion

Reduced male fertility has become a major concern in both humans and farm animals. With respect to high-lactating cows, the inseminating bull is considered a risk factor affecting fertility [12]. However, although the deleterious effects of thermal stress on the female reproductive tract have long been known, the effect of environmental thermal stress on semen quality and its association with low fertility during the hot season are unclear. In a previous study, we showed that semen collected during the summer is in-

ferior in terms of its metabolic characteristics [13], as expressed by seasonal alterations in fatty acid composition and cholesterol concentration. In addition, we documented seasonal variations in the level of mRNA for very-low-density lipoprotein receptor (VLDL-R), indicating modifications associated with extracellular lipid utilization. In the present study, post-thaw examinations revealed that semen collected during the summer has reduced motility, impaired progressive motility, and a tendency toward a higher proportion of sperm with damaged acrosomes relative to semen collected in the winter. Taken together, these findings suggest that semen collected during the summer is of inferior quality. It is therefore possible that the use of inferior-quality semen may also contribute to the decreased reproductive performance of dairy cows during the hot season. Due to variation between bulls, and the fact that some of the examined bulls were more sensitive to environmental changes, further *in vivo* and *in vitro* fertility studies are required to confirm the association between these season-induced alterations and low fertility. In particular, a 2×2 factorial study in which semen collected during the summer and winter is used in both seasons is suggested.

Mature mammalian spermatozoa are quiescent in the male reproductive tract and become motile upon ejaculation. Fertilization competence is gained upon their movement through the reproductive tract [3]. Therefore, exposing males to an elevated temperature can subsequently compromise spermatozoon competence [2]. In the current study, features of mobility were not affected by season when examined at time of collection (i.e., fresh semen). However, further examination following the freeze-thaw process revealed that semen collected during the summer is highly sensitive to cryopreservation, as expressed by reduced motility, progressive motility, and velocity. In support of this, the period of semen collection has been found to significantly influence post-thaw survival of Leccese ram spermatozoa [1]: the post-thaw proportion of live spermatozoa was higher when semen had been collected in the winter and spring and was positively correlated with acrosome integrity [1]. In light of these findings, it is suggested that the characterization of semen quality would be more accurate if examinations were performed post freeze-thaw rather than at collection in fresh semen. Given the variations between bulls, attention to individual seasonal sensitivity is also recommended.

The sperm's ability to survive cryopreservation can be determined by examining its motility and membrane integrity throughout the freeze-thaw process. During cryopreservation, sperm are exposed to hypertonic solutions, and these may affect both membrane integrity and sperm motility. In the current study, such chilling injuries were more prominent in semen collected during the summer vs. winter. Nevertheless, as the proportion of sperm undergoing acrosome activity did not differ between summer and winter samples, it is more likely that season-induced alteration involves changes in the mechanism responsible for sperm motility rather than membrane integrity. In particular, in the current study, semen collected during the summer had reduced motility and progressive motility post thawing. However, Ca in the seminal plasma, which plays a pivotal role in sperm motility, did not differ between seasons. Distinct regional expression of channel subtypes along the flagellum is believed to produce a spatiotemporal profile of Ca^{2+} that regulates flagellar beating patterns to create hyperactivated motility, i.e., asymmetrical, high-amplitude, and lower-frequency flagellar beating [14, 15]. This pattern of motility and elevation of Ca^{2+} in the sperm flagellum is also required for penetration through the zona pellucida [16]. In mature bovine sperm, CNGA3 is observed along the entire flagellum, whereas CNGB1 is restricted to its principal part [5]. Although not examined in the current study, it is reasonable to assume that season-induced alteration in progressive motility is a result of impairment in the CNG channels, which in turn might lead to alterations in post-thaw membrane depolarization and in cytosolic Ca^{2+} increase. Supporting this assumption, cryopreservation has been shown to affect the expression of two glucose transporters (GLUT-3 and GLUT-5) in boar spermatozoa in association with lower percentages of progressively motile spermatozoa [17], suggesting that cryopreservation impairs some membrane receptors and RNA populations associated with sperm motility. In this respect, it is also possible that such alterations are accelerated upon previous exposure to environmental thermal stress.

Knowledge of the seminal plasma ion composition is essential to improving storage conditions and predicting the storage tolerance

of spermatozoa. Seminal plasma is an extremely complex fluid containing a large variety of both organic and inorganic chemical constituents. The inorganic ion composition varies from species to species, but in all cases investigated to date, these components have important effects on sperm function. The acrosome is a membrane-bound organelle located in the head of sperm cells that contains a store of hydrolytic enzymes. Accumulation of Ca^{2+} during the capacitation and release of acrosomal calcium plays a pivotal role in hyperactivated motility [15] and acrosome function [18, 19]. Thus, season-induced acrosome damage might indicate alterations in events associated with the dialogue between the sperm and oocyte, i.e., the acrosome reaction. Nevertheless, in the current study, the proportion of sperm cells with a damaged acrosome was only numerically higher in winter semen but did not significantly differ from that of the summer season. Moreover, examination of the seminal plasma element concentrations did not reveal any alterations in Ca concentration in samples collected during the summer. Taken together, it appears that accumulation of Ca and acrosome membrane integrity are less likely to be involved in season-induced alterations in sperm function. On the other hand, a significant decrease was found in K, Mg, Na, and S concentrations in the seminal plasma of summer ejaculates. As animals were fed the same ration in both summer and winter, alterations are most likely due to seasonal differences (i.e., environmental thermal stress) rather than changes in feed administration. Moreover, semen was collected once a day, ruling out differences in elements composition between the first and subsequent ejaculations. Such alterations in element concentrations might have direct or indirect effects on post-ejaculation sperm functions. For instance, capacitation-associated hyperpolarization is ascribed in part to an increase in K^+ permeability [20] and blockage of the epithelial Na^+ channel [21]. K^+ channels are also involved in sperm-volume regulation [22], which is known to occur upon dramatic changes in environmental osmolality. In rams, $[\text{Mg}^{2+}]$ in the seminal plasma is positively correlated with the sperm concentration [23]. In stallions, a higher $[\text{Mg}^{2+}]$ was found in the sperm-poor fraction, i.e., with low sperm concentration [24]. However, in the current study, the opposite pattern was found in bovine semen collected during the hot season, exhibiting a lower Mg and higher sperm concentration (i.e., negative correlation). In humans, abnormal $[\text{Mg}^{2+}]$ in the seminal plasma correlates with infertility [25]. Given the dissimilarity between species, a direct association between the seasonal variations observed here in the K, Mg, Na and S concentrations and reduced bovine sperm quality is not sufficiently clear. Nevertheless, alterations in seminal plasma element composition might affect the sperm's external pH and osmolality, which in turn might affect sperm metabolism as well as sperm function [26]. Moreover, osmotic pressure, rather than the level of a particular ion, has a greater effect on sperm survival. Therefore, season-induced alterations in seminal plasma elements concentrations should be taken into account when using an extender for cryopreservation to achieve the proper balance in ionic composition. Basing semen resuspension on the sperm concentration rather than on the seminal plasma ion concentration might reduce the ionic concentration enough for it to have an influence on sperm function. This might be highly relevant for semen collected during the summer, which is characterized by a high sperm concentration

but low ion concentration in the seminal plasma.

In summary, the present study suggests a seasonal effect on semen quality: ejaculate collected during the summer was more sensitive to cryopreservation than that collected in the winter, as reflected by reduced motility, and impaired progressive motility in post-thawing semen. While not entirely clear, it appears that the molecular mechanism underlying these alterations involves reduced elements concentrations in the seminal plasma. Given the importance of the ion concentration in the seminal plasma and due to variation between bulls, further *in vivo* and *in vitro* fertility studies are required to confirm the association between these season-induced alterations and low fertility.

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References

1. D'Alessandro AG, Martemucci G. Post-thaw survival and acrosome integrity of spermatozoa of Lecce rams frozen in different seasons with a milk-egg yolk extender. *Italian J Anim Sci* 2005; **4**: 139–148.
2. Yaeram J, Setchell BP, Maddocks S. Effect of heat stress on the fertility of male mice *in vivo* and *in vitro*. *Reprod Fertil Dev* 2006; **18**: 647–653. [Medline] [CrossRef]
3. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), *Physiology of Reproduction*. New York: Raven Press; 1994: Vol. 1 189–317.
4. Darszon A, Nishigaki T, Wood C, Treviño CL, Felix R, Beltrán C. Calcium channels and Ca^{2+} fluctuations in sperm physiology. *Int Rev Cytol* 2005; **243**: 79–172. [Medline] [CrossRef]
5. Darszon A, Acevedo JJ, Galindo BE, Hernández-González EO, Nishigaki T, Treviño CL, Wood C, Beltrán C. Sperm channel diversity and functional multiplicity. *Reproduction* 2006; **131**: 977–988. [Medline] [CrossRef]
6. Darszon A, Treviño CL, Wood C, Galindo B, Rodríguez-Miranda E, Acevedo JJ, Hernández-González EO, Beltrán C, Martínez-López P, Nishigaki T. Ion channels in sperm motility and capacitation. *Soc Reprod Fertil Suppl* 2007; **65**(Suppl): 229–244. [Medline]
7. Weyand I, Godde M, Frings S, Weiner J, Muller F, Altenhofen W, Hatt H, Kaupp UB. Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. *Nature* 1994; **368**: 859–863. [Medline] [CrossRef]
8. Wiesner B, Weiner J, Middendorff R, Hagen V, Kaupp UB, Weyand I. Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. *J Cell Biol* 1998; **142**: 473–484. [Medline] [CrossRef]
9. Dzeja C, Hagen V, Kaupp UB, Frings S. Ca^{2+} permeation in cyclic nucleotide-gated channels. *EMBO J* 1999; **18**: 131–144. [Medline] [CrossRef]
10. Sa-Ardrit M, Saikhun J, Thongtip N, Danyang M, Mahasawangkul S, Angkawanish T, Jansittiwate S, Faisaikarm T, Kitiyanant Y, Pavasuthipaisit K, Pinyopummin A. Ultrastructural alterations of frozen-thawed Asian elephant (*Elephas maximus*) spermatozoa. *Int J Androl* 2006; **29**: 346–352. [Medline] [CrossRef]
11. Mendoza C, Carreras A, Moos J, Tesarik J. Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. *J Reprod Fertil* 1992; **95**: 755–763. [Medline] [CrossRef]
12. García-Ispuerto I, López-Gatius F, Santolaria P, Yaniz JL, Nogareda C, Lopez-Bejar M. Factors affecting the fertility of high producing dairy herd in northeastern Spain. *Theriogenology* 2007; **67**: 632–638. [Medline] [CrossRef]
13. Argov N, Sklan D, Zeron Y, Roth Z. Association between seasonal changes in fatty-acid composition, expression of VLDL receptor and bovine sperm quality. *Theriogenology* 2007; **67**: 878–885. [Medline] [CrossRef]
14. Suarez SS, Varosi SM, Dai X. Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *Proc Natl Acad Sci USA* 1993; **90**: 4660–4664. [Medline] [CrossRef]
15. Ho HC, Suarez SS. An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca^{2+} store is involved in regulating sperm hyperactivated motility. *Biol Reprod* 2001; **65**: 1606–1615. [Medline] [CrossRef]
16. Lindemann CB, Goltz JS. Calcium regulation of flagellar curvature and swimming pattern in Triton X-100-extracted rat sperm. *Cell Motil Cytoskeleton* 1988; **10**: 420–431. [Medline] [CrossRef]
17. Sancho S, Casas I, Ekwall H, Saravia F, Rodríguez-Martínez H, Rodríguez-Gil JE, Flores E, Pinart E, Briz M, García-Gil N, Bassols J, Pruneda A, Bussalleu E, Yeste M, Bonet S. Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. *Reproduction* 2007; **134**: 111–121. [Medline] [CrossRef]
18. Walensky LD, Snyder SH. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J Cell Biol* 1995; **130**: 857–869. [Medline] [CrossRef]
19. De Blas G, Michaut M, Treviño CL, Tomes CN, Yunes R, Darszon A, Mayorga LS. The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. *J Biol Chem* 2002; **277**: 49326–49331. [Medline] [CrossRef]
20. Zeng Y, Clark EN, Florman HM. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Dev Biol* 1995; **171**: 554–563. [Medline] [CrossRef]
21. Hernández-González EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, López-González I, Demarco I, Wertheimer E, Darszon A, Visconti PE. Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. *J Biol Chem* 2005; **281**: 5623–5633. [Medline] [CrossRef]
22. Di Zhang D, Gopalakrishnan M. Sperm ion channels: molecular targets for the next generation of contraceptive medicines? *J Androl* 2005; **26**: 643–653. [Medline] [CrossRef]
23. Abdel-Rahman HA, El-Beley MS, Al-Qarawi AA, El-Mougy SA. The relationship between semen quality and mineral composition of semen in various ram breeds. *Small Rumin Res* 2000; **38**: 45–49. [Medline] [CrossRef]
24. Kareskoski AM, Reilas T, Sankari S, Andersson I M, Guven K, Katila T. Alkaline and acid phosphatase, beta-glucuronidase and electrolyte levels in fractionated stallion ejaculates. *Reprod Dom Anim* 2010; **45**: e369–374. [CrossRef]
25. Pandey VK, Parmeshwaran M, Soman SD, Dacosta JC. Concentrations of morphologically normal, motile spermatozoa: Mg, Ca and Zn in the semen of infertile men. *Sci Total Environ* 1983; **27**: 49–52. [Medline] [CrossRef]
26. Hamamah S, Jean-Luc G. Role of the ionic environment and internal pH on sperm activity. *Hum Reprod* 1998; **13**(Suppl 4): 20–30. [Medline]