REPRODUCTION
RESEARCH

Season-induced variation in lipid composition is associated with semen quality in Holstein bulls

N Argov-Argaman, K Mahgrefthe, Y Zeron[1] and Z Roth

The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University, Rehovot 76100, Israel and [1]Sion Artificial Insemination Center, Hafetz-Haim, Israel

Correspondence should be addressed to Z Roth; Email: roth@agri.huji.ac.il

Abstract

Season-induced variation in fatty acid and cholesterol composition in bovine semen has been associated with semen quality. Given the specific roles of the various semen compartments (seminal fluids, sperm head, and sperm tail) in fertilization, we hypothesized that environmental-stress-induced alterations in the lipid composition of a specific compartment might impair semen quality and sperm function. Semen samples were collected from five mature Holstein–Friesian bulls during the summer (August to September) and winter (December to January). Semen was evaluated by computerized sperm-quality analyzer, calibrated for bulls' semen, and centrifuged to separate the spermatozoa from the seminal fluids. The spermatozoal fraction was sonicated to separate the sperm head and tail compartments. Cold lipid extraction was performed with chloroform:methanol (2:1, vol/vol). Lipids were identified and quantified by gas chromatography. Seasonal variation was found in both physiological and structural parameters. The proportion of spermatozoa defined as morphologically normal was higher in the winter, with higher motility, progressive motility, and velocity relative to summer samples. Lipid composition within fractions varied between seasons with prominent impairment in the tail compartment, characterized by high saturated fatty acid, low polyunsaturated fatty acid, and low cholesterol concentrations during the summer. Given the association between alterations in lipid composition and reduced sperm motility and velocity during the summer, it is suggested that lipid composition might serve to predict sperm quality.

Reproduction (2013) **145** 479–489

Introduction

The season in which bovine semen is collected is associated with semen quality (Menendez-Buxadera *et al*. 1984). Exposure to environmental thermal stress increases the proportion of morphologically abnormal spermatozoa and attenuates sperm motility in sheep (Ibrahim 1997, Gundogan & Elitok 2004), decreases total sperm count in ejaculates and reduces fertility in humans (Levine 1999, Sinclair 2000), and decreases sperm viability in cattle (Meyerhoeffer *et al*. 1985, Ax *et al*. 1987, Argov *et al*. 2007). The molecular and structural mechanisms underlying these alterations remain unclear.

Traditionally, physiological characteristics of sperm, such as sperm concentration, morphology, and motility, have been used to evaluate semen quality. Sperm concentration is considered a weak predictor, whereas sperm motility is highly associated with fertility competence (Comhaire *et al*. 1987). Computerized instruments such as the computer-assisted semen analyzer (Verstegen *et al*. 2002) and the sperm-quality analyzer (SQA; Rodriguez *et al*. 2011) are used to evaluate semen quality. The computer-assisted semen analyzer provides sperm velocity and motility parameters analyzed from multiple digital images of sperm tracks. The SQA converts signal variations in optical density into electronic signals that are translated into sperm physiological parameters. Although widely used to evaluate semen quality, physiological parameters are limited in predicting semen fertilization capacity (Comhaire *et al*. 1987, Roudebush & Diehl 2001, Argov *et al*. 2007, Lewis 2007). For example, a subjective evaluation of semen collected from fertile men revealed that 54.9% of the individuals had a lower value in at least one parameter than the minimal values established by the WHO (de los Rios *et al*. 2004). Additional evaluation methods and markers are therefore suggested, such as platelet-activating factor (PAF). The concentration of this phospholipid mediator was positively associated with fertility in boar (Roudebush & Diehl 2001), and its activity was positively correlated with sperm motility in men (Zhu *et al*. 2006). An additional indicator for sperm quality is its mitochondrial DNA content, as determined by its gene ratio to β-globin using real-time PCR, which was shown to be associated with abnormal sperm characteristics in infertile human patients (May-Panloup *et al*. 2003).

In a previous study, we showed seasonal variation in fatty acid and cholesterol composition in both sperm

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-12-0498
Online version via www.reproduction-online.org

membrane and seminal fluids (Argov *et al*. 2007). For example, semen collected during the summer had reduced levels of arachidonic acid (20:4), and poly-unsaturated fatty acids (PUFA) in the cell compartments, and reduced cholesterol and fatty acid concentrations in the seminal fluids. Such alterations in lipid composition might explain, in part, the high sensitivity of semen collected during the summer to cryopreservation and its reduced motility and progressive motility (PM) following the freeze–thaw process (Orgal *et al*. 2012). Nevertheless, information about a seasonal effect on a wide range of fatty acids in the sperm is still lacking. Moreover, given the specific role of each semen compartment (seminal fluids, sperm head, and sperm tail) in fertilization (Ahluwalia & Holman 1969, Zalata *et al*. 1998), we hypothesized that environmental-stress induced alterations in the lipid composition of a specific compartment might impair semen quality and sperm function. A relatively low proportion of cholesterol and high proportion of PUFA in the sperm head are suggested to improve the capacitation process and the acrosome reaction (Lenzi *et al*. 2000, Furland *et al*. 2007). The lipid profile in the sperm tail membrane is associated with physical properties that facilitate sperm motility and velocity (Connor *et al*. 1998). Antioxidant (Lenzi *et al*. 2000, Strzeżek *et al*. 2004) and cholesterol contents in the seminal fluids (Huacuja *et al*. 1981, Morales *et al*. 2008, Saez *et al*. 2011), as well as cholesterol acceptors that facilitate cholesterol efflux from the sperm during capacitation (Saez *et al*. 2011), positively affect sperm quality.

This study examines lipid composition in the sperm functional compartments (seminal fluids, sperm head, and sperm tail). In particular, the study aims to determine whether lipid composition is differentially modulated by season, whether lipid composition among the semen compartments is associated with sperm physiological parameters, and if so, whether lipid composition can be used to evaluate semen *in vitro* physiological parameters.

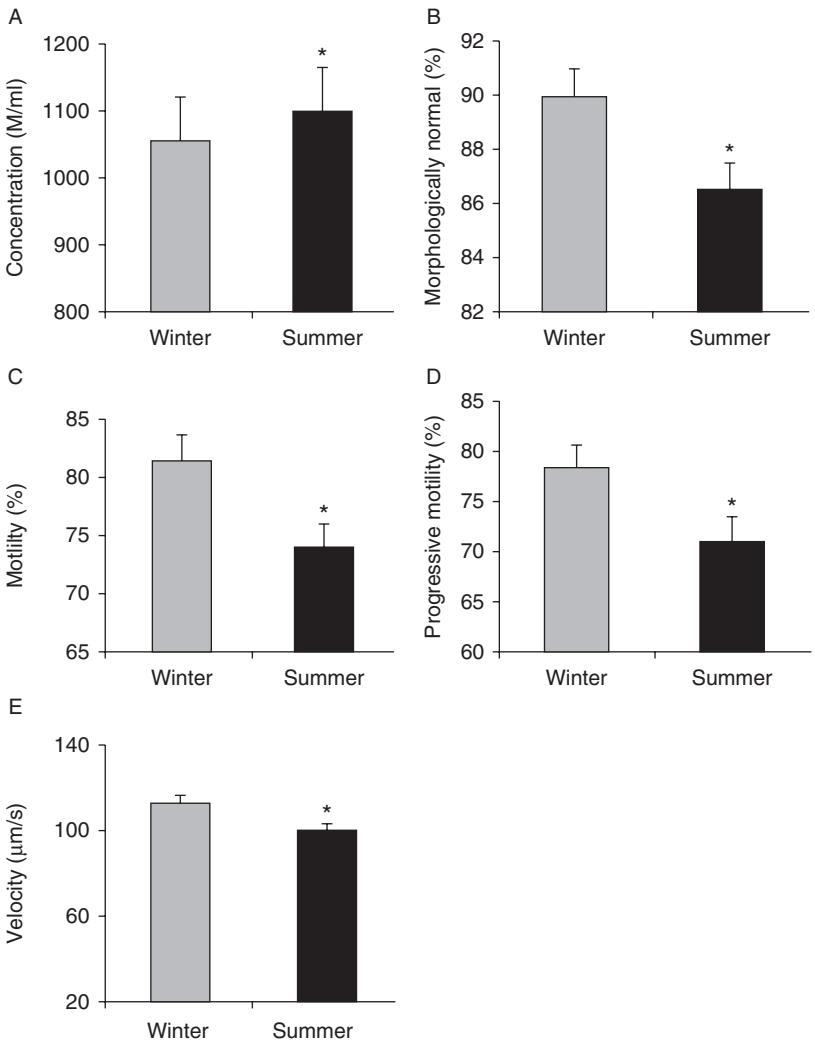

Figure 1 Seasonal variations in sperm traits. Semen was collected during the winter (gray bars) and summer (black bars). Sperm concentration (A), morphology (B), motility (C), progressive motility (D), and velocity (E) were evaluated by SQA-Vb. Data are presented as mean ± S.E.M. *$P<0.05$.

Results

Environmental factors

Average daily means of air temperatures varied between 31.3±0.2 and 22.6±0.6 °C during the summer and between 21.5±1.3 and 10.6±0.8 °C in winter. Maximum and minimum air humidity varied between 80.1±0.4 and 49±1.5% during the summer and between 93±0.4 and 47.6±1.1% in the winter.

Semen physiological parameters

The physiological parameters of semen collected during the summer and winter are shown in Fig. 1. Sperm concentration was lower in winter than in summer ($P<0.02$), but the proportion of spermatozoa defined as morphologically normal was higher ($P<0.02$). The motility, PM, and velocity of spermatozoa were higher in the winter than in the summer samples ($P<0.02$). The average volume of ejaculates did not differ ($P=0.79$) between seasons: 8.67±0.49 and 8.24±0.48 ml for winter and summer samples respectively. The total sperm number per ejaculate did not differ ($P=0.53$) between seasons and was $8950\pm905\times10^6$ and $10\,435\pm2169\times10^6$ for summer and winter samples respectively.

Fatty acid composition in the sperm compartments

Fatty acid compositions of semen samples and their functional compartments (seminal fluids, intact spermatozoa, heads, and tails) in both summer and winter are presented in Table 1. The concentrations of six major fatty acids varied between seasons. In general, the proportion of docosahexaenoic acid (DHA, c22:6n3) was about 30% of the total fatty acids in semen samples in both seasons. However, DHA content in intact spermatozoa was higher ($P<0.001$) in summer than in winter, whereas in the tail compartment, DHA content was lower ($P<0.01$) in summer than in winter. Content of the PUFA eicosapentaenoic acid (EPA, c20:5n3) in the intact spermatozoa was approximately fivefold higher in winter than in summer ($P<0.001$). EPA content in the seminal fluids was fourfold higher in winter than in summer ($P<0.001$), whereas in the tail compartment, it was twofold lower in winter than in summer ($P<0.001$). Arachidonic acid (c20:4n6) content in both intact spermatozoa and seminal fluids was higher in summer than in winter ($P<0.001$ and 0.01 respectively). On the other hand, the relative concentration of arachidonic acid in the tail and head compartments did not differ ($P=0.53$) between seasons. The proportion of palmitate (c16:0) in the semen compartments ranged between 16 and 37% of total fatty acids. Relative palmitate concentration in the tail compartment was 10% higher in summer vs winter samples ($P<0.001$). Myristic acid (c14:0) content in intact spermatozoa was 5% higher in winter than in summer ($P<0.01$) but did not differ between seasons in the other semen compartments ($P=0.09$, 0.58, and 0.8 for tail, seminal fluid, and head compartments respectively).

Fatty acids were grouped according to their biochemical characteristics to gain an understanding of the cellular functionalities of specific fatty acid compositions (Fig. 2). Concentration of saturated fatty acids (SFA) in both intact spermatozoa and seminal fluids was lower ($P<0.0001$ and $P=0.008$ respectively) in summer than in winter, whereas in the tail compartment, it was higher ($P<0.0001$) in summer than in winter. The composition of monounsaturated fatty acids (MUFA) was lower ($P=0.0049$) in summer than in winter in the tail, whereas in the seminal fluids and intact spermatozoa, MUFA concentration was higher in summer. The proportion of PUFA in both intact spermatozoa and seminal fluids was higher ($P<0.0001$ and $P=0.003$ respectively) in summer

Table 1 Seasonal effect on fatty acid profiles in sperm compartments (spermatozoa, seminal fluids, tail, and head).

	Spermatozoa		Seminal fluids		Tail		Head	
FA	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
c12:0	0.25±0.01‡	0.16±0.01	0.14±0.01	0.15±0.03	1.48±0.44	0.71±0.10	1.05±0.28	0.70±0.29
c14:0	21.85±1.02†	16.92±1.02	9.50±0.31	9.13±0.47	5.32±0.38	6.57±0.54	14.34±0.98	14.01±0.97
c16:0	24.78±0.44	24.20±1.06	37.57±0.69	36.00±0.95	16.81±0.74‡	25.53±1.21	22.94±0.73	23.34±0.75
c16:1n7	0.29±0.01	0.26±0.01	0.14±0.01‡	0.21±0.01	0.72±0.04†	0.97±0.08	0.40±0.03	0.48±0.06
c18:0	9.05±0.16	9.19±0.20	12.99±0.42	13.36±0.64	11.75±0.44	12.74±0.31	12.50±0.54*	11.11±0.32
c18:1n9	2.44±0.06*	3.17±0.34	5.77±0.48	5.89±0.43	6.40±0.43	7.12±0.50	2.57±0.30*	3.89±0.46
c18:1n7	3.64±0.12	3.35±0.14	1.69±0.19*	1.97±0.06	5.95±0.24‡	4.49±0.21	3.21±0.34	2.76±0.09
c18:2n6	6.55±0.49	6.14±0.23	7.50±0.54	7.62±0.69	12.87±0.32‡	10.36±0.45	3.82±0.42	4.46±0.29
c18:3n3	0.06±0.01	0.06±0.00	0.09±0.01	0.08±0.01	0.83±0.18*	0.36±0.04	2.26±0.70†	0.80±0.27
c20:0	0.18±0.01‡	0.14±0.01	0.28±0.01†	0.18±0.03	1.05±0.12*	0.75±0.12	0.43±0.07	0.57±0.06
c20:1n9	0.11±0.01‡	0.17±0.02	0.26±0.02	0.31±0.03	0.60±0.14	0.35±0.05	0.74±0.19*	0.31±0.10
c20:4n6	4.62±0.09‡	5.33±0.15	1.90±0.04†	2.76±0.28	8.58±0.42	7.85±0.44	3.56±0.57	3.37±0.15
c20:5n3	0.15±0.09‡	0.03±0.00	0.37±0.02‡	0.08±0.01	0.19±0.04‡	0.53±0.05	0.23±0.06	0.34±0.08
c22:1n9	0.11±0.01‡	0.17±0.02	0.21±0.01‡	0.09±0.02	2.73±0.35‡	0.81±0.11	0.48±0.10	0.57±0.15
c22:4n6	0.97±0.05‡	1.23±0.03	1.04±0.04‡	1.27±0.03	1.14±0.11	0.96±0.06	1.75±0.57	1.44±0.06
c22:6n3	24.94±0.74‡	30.05±0.74	18.78±0.63*	20.37±0.96	23.79±0.86†	19.06±1.04	26.26±2.28	30.41±1.30
c24:0	0.17±0.01†	0.09±0.01	0.94±0.06‡	0.21±0.02	0.80±0.09†	0.42±0.07	1.67±0.59	0.74±0.13

Values are presented as mean±S.E.M. *$P<0.05$, †$P<0.01$, ‡$P<0.001$.

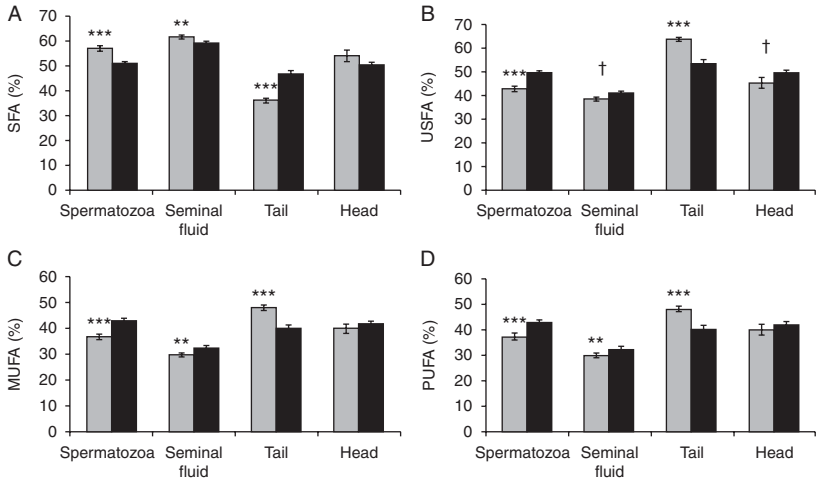

Figure 2 Seasonal variations in fatty acid composition. Semen samples were collected during the winter (gray bars) and summer (black bars). Seminal fluids and spermatozoa were separated and spermatozoa were further fractionated into tails and heads. (A) Sum of mol% values of the saturated fatty acids (SFA) C8:0, C10:0, C12:0, C14:0, C16:0, C1:0, C20:0, C22:0, and C24:0. (B) Sum of mol% values of the unsaturated fatty acids (USFA) C16:1n7, C18:1n9, C18:1n7, C18:2n6, C18:3n6, C18:3n3, C20:1n9, C20:4n4, C20:5n3, C22:1n9, C22:4n6, and C22:6n3. (C) Sum of mol% values of monounsaturated fatty acids (MUFA) C16:1n7, C18:1n9, C18:1n7, C20:1n9, and C22:1n9. (D) Sum of mol% values of the polyunsaturated fatty acids (PUFA) C18:2n6, C18:3n6, C18:3n3, C20:4n6, C20:5n3, C22:4n6, and C22:6n3. Data are mean±S.E.M. $^{\dagger}P<0.10$, $^{**}P<0.01$, $^{***}P<0.001$.

than in winter, whereas in the tail compartment, it was lower ($P=0.0006$) in summer than in winter.

The concentrations of omega-6 and omega-3 PUFA subfamilies in the tail compartment differed between seasons (Fig. 3), with lower concentrations of omega-6 ($P=0.0054$) and omega-3 ($P=0.0007$) in the summer compared with winter samples. While non-significant, numerical differences in omega-6 and omega-3 concentrations were found between seasons in the intact spermatozoa, with a higher proportion in summer than in winter. Nevertheless, the ratio between omega-6 and omega-3 fatty acid concentrations did not differ ($P=0.13$) between seasons.

Indicators of elongation and desaturation activity

The indicator value for palmitate desaturation activity was higher ($P<0.001$) in the seminal fluids whereas that for stearate desaturation was higher ($P<0.003$) in the head compartment in summer compared with winter (Fig. 4). On the other hand, elongation activity values were consistently higher in the tail compartment in the winter compared with the summer samples. The activity indicator values of palmitoleate (c16:1n7), stearate (c18:0), and oleate (c18:1n9) in the tail compartment were higher in winter than in summer ($P=0.04$). Elongase activity indicator values in the seminal fluids were higher for stearate ($P=0.0006$) in winter compared with summer. The oleate elongation activity indicator value for seminal fluids did not ($P=0.34$) differ between seasons. The elongation activity in intact spermatozoa differed between seasons, with higher indicator values for stearate and lower ones for oleate ($P=0.0006$ and 0.03 respectively) in winter. Activity indicator values in the head compartment were higher ($P=0.03$) for oleate in winter.

Seasonal variations in cholesterol concentrations in semen functional compartments

Cholesterol concentrations in summer and winter samples are presented in Fig. 5. In both intact spermatozoa and seminal fluids, cholesterol concentration was higher ($P=0.008$ and 0.0001 respectively) in the summer vs winter samples, by fourfold in the seminal fluids. The tail compartment in the winter samples was richer in cholesterol than the corresponding summer samples ($P=0.01$).

Correlations between physiological parameters and lipid profile

The association between physiological parameters and lipid profiles of the functional semen compartments was determined by correlation analysis. In general, more correlations were found between the physical properties and lipid composition in the intact spermatozoa. Due to the large amount of data, only the main findings are presented in Table 2, showing positive or negative correlations for each of the examined compartments (intact spermatozoa, seminal fluids, sperm tail, and sperm head).

Desaturase activity markers negatively correlated with motility, velocity, and motile sperm concentration (MSC) in the intact spermatozoa ($P=0.006$, 0.005, and 0.03 respectively) in the summer samples. In general, MUFA concentration was associated with motility features in summer samples. In particular, MUFA concentration negatively correlated with motility and PM in the tail compartment and with motility, MSC, progressive MSC (PMSC), and velocity in the intact spermatozoa. Omega-6 in the seminal fluid negatively correlated with velocity in both summer and winter ($P=0.05$ and 0.04 respectively). PUFA concentration

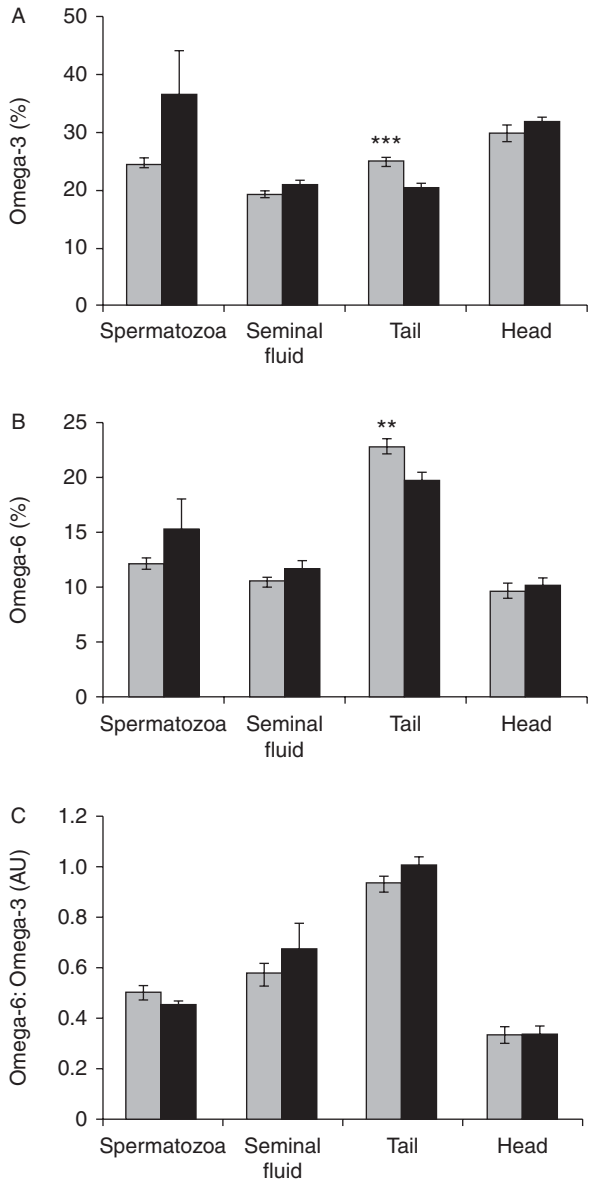

Figure 3 Seasonal variations in polyunsaturated fatty acid composition. Semen samples were collected during the winter (gray bars) and summer (black bars). Seminal fluids and spermatozoa were isolated and spermatozoa were further fractionated into tails and heads. Fatty acids were grouped according to the position of the first double bond from the methyl end of the fatty acid molecule. For each compartment, omega-3 group (A) was calculated as the sum of the relative concentrations of c18:3n3, c20:5n3, and c22:6n3. omega-6 group (B) was calculated as the sum of the relative concentrations of c18:2n6, c18:3n6, c20:4n6, and c22:4n6; (C) omega-6:omega-3 ratio. Data are mean ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$.

tended to positively correlate with motility and velocity ($P=0.06$) in the intact spermatozoa in summer samples. The total sperm number per ejaculate negatively correlated with MUFA ($P=0.003$) in the intact spermatozoa in the winter samples and tended to positively correlate with omega-6 ($P=0.08$) in the head compartments of winter samples.

Discussion

Seasonal variations in sperm quality have been well studied. However, while most of the studies focus on physiological and morphological alterations, seasonal effects on semen lipid profile and their association with sperm physiological properties are rarely discussed. Herein, we compared the composition of a wide range of fatty of acids and cholesterol concentration in Holstein–Friesian bull semen collected during the summer and winter. Findings indicated that semen functional traits vary between seasons in association with alterations in lipid composition. An illustration of composition based on the fatty acid profile, rather than on total extracted fat, revealed differential alterations in lipid composition among the sperm compartments. A prominent effect was found on the lipid composition of sperm tails in association with sperm motility. It should be noted that seasonal effects on lipid composition in the sperm might include different nutrition and feed contents; however, this is not the case in the current study as bulls were fed the same standard feed in both summer and winter. Nonetheless, seasonal alterations in food intake, digestion, and absorption cannot be ruled out. These parameters were not examined, as they were beyond the scope of this study.

Analysis by SQA-Vb revealed that the semen collected during the winter is of higher quality than that collected during the summer, at least according to the *in vitro* parameters: sperm motility, PM, and velocity, as well as the proportion of cells with normal morphology. A similar reduction in sperm motility during the summer (July and August) was reported by Argov *et al.* (2007) and was associated with increased proportion of rejected ejaculates (sperm motility index <70). In the current study, sperm concentration was higher in summer vs winter samples, in contrast to results reported by Mathevon *et al.* (1998). The association between sperm quality and concentration has yet to be determined.

Given the limitations of morphological characterizations in determining sperm quality and predicting fertilization success, additional factors are required for better evaluation. Lipid composition of spermatozoa and seminal fluids plays a significant role in male fertility (Tavilani *et al.* 2006, Wathes *et al.* 2007). In bovines, seasonal variations in total lipid and cholesterol concentrations in spermatozoa are associated with reduced sperm motility (Argov *et al.* 2007). In rams, seasonally reduced sperm motility was associated with total lipid and cholesterol concentration in the seminal fluids (Gundogan & Elitok 2004). As the sperm tail is the functional compartment that determines motility and velocity parameters, it is reasonable to assume that seasonal variation in the lipid composition of this compartment will correlate with its function. The findings of the current study support this assumption: the most significant seasonal variations in lipid

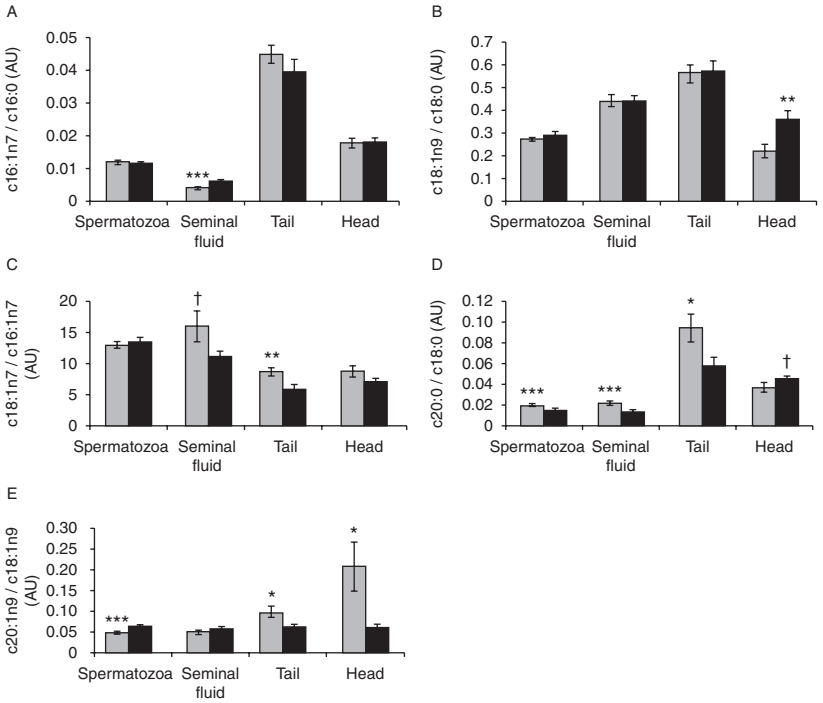

Figure 4 Seasonal variations in indicators of desaturase and elongase activity. Semen samples were collected during the winter (gray bars) and summer (black bars). Seminal fluids and spermatozoa were isolated and spermatozoa were further fractionated into tails and heads. For each semen compartment, the desaturation activity was calculated as the ratio between the mol% of the substrate and product: (A) c16:1n7/c16:0 and (B) c18:1n9/c18:0. Elongation activity was calculated as the ratio between the mol% of the substrate and product: (C) c18:1n7/c16:1n7; (D) c20:0/c18:0; and (E) c20:1n9/c18:1n9. Data are mean±S.E.M. $^{\dagger}P<0.10$, $*P<0.05$, $**P<0.01$, $***P<0.001$.

composition were observed in the tail compartment rather than in the intact sperm or sperm head. Nonetheless, the seminal fluid compositions of the fatty acid biochemical groups also differ extensively between seasons and principally showed opposite trends than those found in the tail, in particular, that of saturated, monounsaturated, and polyunsaturated. These opposite trends could be attributed to the fact that seminal fluids provide the spermatozoa with various energy and structural molecules (Lenzi *et al*. 1996, 2000). Moreover, while the tail compartment expressed an opposite pattern than that of the intact spermatozoa, the intact spermatozoa composition seemed to correspond to that of the seminal fluid. Although not clear enough, these findings might reflect a mechanism for directing lipid constituents to the various cell compartments that is specifically compromised by season. Alternatively, the differences between intact spermatozoa and tail compositions, and their interaction with seminal fluid composition, could be attributed to the different proportion of lipids between compartments.

The proportion of fatty acids in the tail compartment differed between seasons, with a lower proportion of palmitate in the winter vs summer and the opposite pattern for DHA (22:4n6). Palmitate and DHA were found to be the most abundant fatty acids in the sperm samples, accounting for more than 40% of the total tail fatty acids, suggesting that both physical and mechanical properties of these fatty acids play a pivotal role in determining the spermatozoa's tail membrane composition. A similar positive association between DHA tail concentration, motility and flagellum-banding capacity was reported by Tavilani *et al*. (2007). Taken together, season-induced alterations in the proportion of DHA in spermatozoa tails, as reported here, might explain, in part, the reduced motility and velocity recorded for summer samples.

DHA is the most abundant PUFA in spermatozoa (Poulos *et al*. 1973). Approximately 95% of monkey spermatozoa DHA is located in the spermatozoa tail

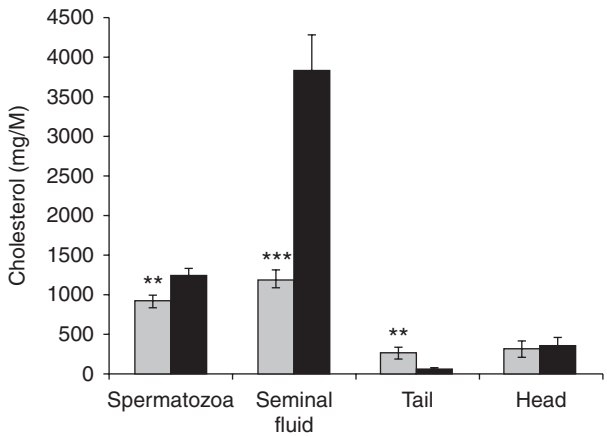

Figure 5 Seasonal variations in cholesterol concentration. Semen samples were collected during the winter (gray bars) and summer (black bars). Seminal fluids and spermatozoa were isolated and spermatozoa were further fractionated into tails and heads. Cholesterol concentration was determined for each of the semen compartments and normalized for 1 million spermatozoa. Data are mean±S.E.M. $**P<0.01$, $***P<0.001$.

Table 2 Seasonal correlations between semen traits and lipid composition in seminal fluid and various sperm compartments (intact spermatozoa, head, and tail).

Sperm compartment	Sperm trait	Lipid composition	Summer		Winter	
			Correlation	*P*	Correlation	*P*
Head	Motility	SFA	0.80	0.05	0.20	0.70
		Omega-6	−0.90	0.03	−0.98	0.002
	PM	SFA	−0.90	0.02	0.20	0.70
		Omega-6	−0.80	0.06	−0.9	0.002
	Morphology	SFA	0.90	0.02	0.20	0.70
		Omega-6	−0.80	0.06	−0.98	0.002
	MSC	C18:1n9/C18:0	−0.80	0.08	0.70	0.15
	Velocity	SFA	0.90	0.02	−0.04	0.90
		Omega-6	−0.70	0.13	−0.88	0.04
	Cells/ejaculation	Omega-6	−0.04	0.90	0.82	0.08
Tail	Motility	MUFA	−0.89	0.01	−0.08	0.90
	PM	MUFA	−0.87	0.002	−0.80	0.80
	MSC	MUFA	−0.70	0.10	0.50	0.30
	PMSC	PUFA	0.40	0.30	−0.80	0.09
Intact spermatozoa	Motility	C18:1n9/C18:0	−0.90	0.006	0.20	0.70
		MUFA	−0.87	0.02	0.50	0.30
		PUFA	0.80	0.06	−0.70	0.15
	Morphology	C18:1/C18:0	−0.90	0.02	0.18	0.70
		MUFA	−0.80	0.01	−0.70	0.14
	MSC	C18:1n9/C18:0	−0.80	0.03	−0.60	0.20
		SAT	−0.20	0.60	0.80	0.05
		MUFA	−0.80	0.02	−0.20	0.67
		PUFA	0.60	0.20	−0.80	0.07
	PMSC	C18:1n9/C18:0	−0.80	0.03	−0.60	0.30
		MUFA	−0.80	0.02	−0.40	0.40
	Velocity	C18:1n9/C18:0	−0.90	0.005	0.20	0.70
		MUFA	−0.80	0.02	0.50	0.30
		PUFA	0.80	0.06	−0.70	0.15
	Cells/ejaculation	MUFA	−0.35	0.40	−0.98	0.003
Seminal fluid	Motility	Omega-6	−0.40	0.40	−0.90	0.05
	PM	Omega-6	−0.20	0.60	−0.87	0.05
	Morphology	Omega-6	−0.60	0.27	−0.80	0.05
	Velocity	MUFA	−0.93	0.02	−0.20	0.76
		Omega-6	−0.88	0.04	−0.87	0.05

Sperm traits are morphology, progressive motility (PM), motile sperm concentration (MSC), progressive motile sperm concentration (PMSC), and total cell number per ejaculation and velocity. Lipid composition includes omega-6, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and C18:1n9/C18:0.

(Connor *et al*. 1998). A comparison of normozoospermic and asthenozoospermic men revealed that sperm morphology and motility are positively associated with PUFA concentration in intact spermatozoa (Tavilani *et al*. 2007). Therefore, the seasonal PUFA variation in the tail compartment reported in the current study seems to be a reliable indicator of sperm motility. In particular, PUFA concentration in the tail in winter samples (i.e. high-quality semen) was 10% higher than that in summer samples (i.e. inferior semen), whereas SFA concentration in winter was 15% lower than that in summer. In support of this, the concentrations of both omega-3 and omega-6 PUFA subgroups were higher in the tail compartment of winter vs summer samples. However, the omega-6: omega-3 ratio did not differ between the seasons in the tail or other semen compartments. In humans, the omega-6:omega-3 ratio is considered a major indicator of sperm quality (Safarinejad *et al*. 2010). While not entirely clear, it is possible that this ratio is subject to regulatory mechanisms that are not affected by environmental changes but might change under extreme stress. Taken together, it seems that alterations in the lipid composition of the sperm tail (i.e. high level of SFA and low level of PUFA) underlie the compromised motility of sperm during the summer.

The ratio between the relative concentration of substrates and products might serve as a reliable indicator of the fatty acid flux through elongation and desaturation pathways (Zivkovic *et al*. 2009). The findings of the current study suggest seasonal variations of elongation and desaturation product distribution among the sperm compartments. The indicator values for elongase activity on palmitate (c16:0) as a substrate in the winter vs summer were higher in the sperm tail but not in the intact spermatozoa, seminal fluids, or sperm head. The indicator values for desaturase activity did not differ between the seasons in intact spermatozoa or sperm tail but were higher in the sperm heads for stearate (c18:0) in the summer. The indicator value for desaturase activity was also higher in the seminal fluids for palmitate in the summer, suggesting that the season induces changes in desaturase activity not only in the spermatozoa's cellular

compartments but also in the accessory sex glands (i.e. prostate, vesicular glands, and Cowper's glands) whose products play a pivotal role in seminal fluid composition. It should be noted, however, that fatty acid metabolism through desaturation and elongation pathways is dominant in tissues with active lipid metabolism (Wang *et al*. 2005). Thus, the activity indicators for desaturation and elongation pathways might reflect seasonally induced alterations in fatty acid metabolism in the liver or adipose tissues or both (do Amaral *et al*. 2009) and substrate availability in the male reproductive tract, and not necessarily local processes in the reproductive system (i.e. indirect effect). In the current study, sperm motility, MSC, and PMSC negatively correlated with desaturase activity marker in the intact spermatozoa in the summer samples. While the precise site of the altered metabolism has not been established, these findings suggest a strong association between desaturation pathway and sperm motility features.

Another prominent seasonal effect found in this study was the alteration in cholesterol concentration: higher levels in the intact spermatozoa in the summer relative to winter samples and the opposite pattern in the sperm tails. Cholesterol concentration has been associated with semen quality (Flesch *et al*. 2001, Travis & Kopf 2002, Haila & Tulsiani 2009). A high cholesterol concentration in the sperm membrane has been found to reduce its fluidity (Flesch *et al*. 2001). The possibility that ambient temperature may dictate the amount of cholesterol in the membrane is supported by the known biphasic response of the membrane fluidity to its cholesterol concentration at different temperatures. On the other hand, instability of membrane caused by high content of PUFA can be modulated by cholesterol (Van Hoeven *et al*. 1975, Darin-Bennett & White 1997). Moreover, while a threshold level of cholesterol is required for sperm membrane stability (Nolan & Hammerstedt 1997), high cholesterol concentration can potentially inhibit capacitation and impair acrosome reaction (Travis & Kopf 2002, Scharenberg *et al*. 2009). In this study, both cholesterol and PUFA concentrations were higher in the tail compartment in winter samples. It is therefore possible that an alteration in the cholesterol-to-PUFA ratio in the sperm tail is also involved in spermatozoa's reduced motility in summer. Moreover, the increased cholesterol concentration in the summer sample's seminal fluids might negatively affect the capacitation process, presumably by compromising cholesterol efflux in the spermatozoa (Flesch *et al*. 2001, Travis & Kopf 2002, Scharenberg *et al*. 2009). A similar inhibitory effect of cholesterol on human sperm acrosome reaction has been reported by Khorasani *et al*. (2000).

In summary, semen functional traits vary between seasons in association with alterations in lipid composition. Analysis of specific sperm compartments revealed that the sperm tail is highly sensitive to seasonal changes: while some alterations were found in the intact spermatozoa and the seminal fluid, prominent impairments in lipid composition (high SFA, low PUFA, and low cholesterol concentrations) were found in the tail compartment during the summer. Such alterations might explain, in part, the reduced motility and velocity of spermatozoa in this season. Further *in vitro* and *in vivo* studies might clarify whether the association between lipid composition and sperm physiological features reported here can also predict sperm fertilization competence.

Materials and Methods

Environmental data collection and animals

Experiments were performed at the Israeli artificial insemination center ('Sion', Hafetz-Haim, Israel), in accordance with the 1994 Israeli guidelines for animal welfare and experimentation. Environmental data were obtained from the central meteorological station in Bet-Dagan, Israel. Semen samples were collected from mature Holstein–Friesian bulls (7.3 ± 0.6 years of age; $n=5$ per season) during the summer (August to September) and the following winter (December to January). Bulls were fed the same total mixed ration throughout the experiment, in both summer and winter, containing 7.2% (wt/wt) protein, 36.2% (wt/wt) neutral detergent fiber, 20.0% (wt/wt) acidic detergent fiber, 1.45 Mcal/kg net energy (NE_L), and 3.5 g minerals/kg (NaCl, Ca, and P) on a DM basis.

Semen collection and initial evaluation of physiological parameters

The animals were ejaculated four times a week, on Sunday, Monday, Wednesday, and Thursday. However, the examined ejaculate was collected only once a week, on Sunday ($n=5$ samples per season per bull). Moreover, to eliminate any potential differences in sperm quality within serial ejaculates, samples were taken only from the first ejaculate of the collection 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 laboratory and the semen was evaluated by the computerized SQA, calibrated for bulls' semen (SQA-Vb, Medical Electronic Systems, Caesarea, Israel) for the following physiological characteristics: volume (ml), concentration (million spermatozoa/ml), total motility (motility, %), PM (%), morphologically normal spermatozoa (%), MSC (million/ml), PMSC (million/ml), and velocity (μm/s). As per routine procedure at Sion, samples with a concentration $>650\times10^6$ cell/ml and motility $>70\%$ were defined as being of good quality.

Sperm handling

For each collection, 2 ml of the total collected semen volume were divided into two tubes and centrifuged (800 ***g***) for 10 min at room temperature to separate the spermatozoa from the seminal fluids. The supernatant (i.e. seminal fluids) was collected and kept at −20 °C until further analysis. The pellet

was resuspended and washed twice in 1 ml physiological solution (saline; 0.9% NaCl in double-distilled water, wt/wt) and centrifuged again to remove any remaining seminal fluids. The pellet from one tube was suspended in 200 µl saline and used for lipid profile analysis, and the pellet from the second tube was suspended in 2 ml saline and subjected to separation of the sperm head and tail fractions.

Separation of spermatozoa into head and tail compartments

Spermatozoa were separated into head and tail compartments as described by Zalata *et al.* (1998) with minor modifications. Briefly, samples were sonicated for 2 min at maximal power (Misonix Microson XL2000 Ultrasonic Processor, Cole Parmer, Vernon Hills, IL, USA). One drop of the sonicated liquid was evaluated by light microscopy (Eclipse TE2000, Nikon, Tokyo, Japan) to determine the fractionation rate (i.e. proportion of spermatozoa separated into heads and tails). A 0.5 ml aliquot of the sonicated sample was gently layered on top of a 2 ml 90% Percoll column (GE Healthcare Bio-Science AB, Piscataway, NJ, USA) and slowly centrifuged (500 ***g***, 20 min) to separate the two compartments: the sperm tails remained as a solid layer at the top of the Percoll column and the sperm heads sedimented at the bottom. Note that while the head fraction was easily recovered, it was harder to get a tight pellet of the tail fraction, compromising recovery rates for this fraction. Eventually, both fractions were diluted in 100 µl physiological saline and held at −20 °C for further analysis.

Analysis of lipid profiles in spermatozoa, seminal fluids, sperm heads, and sperm tails

Chemicals and reagents

All solvents used for lipid extraction and analysis were of analytical grade. Methanol and chloroform were purchased from Bio-Lab Ltd. Laboratories (Jerusalem, Israel). Petroleum ether was purchased from Gadot Laboratory Supplies (Netanya, Israel) and sulfuric acid was from Bet-Dekel (Ra'anana, Israel). External fatty acid methyl ester (FAME) standards (FAME Mix C4-C24 and FAME mix C16-C22) were purchased from Sigma–Aldrich Israel.

Lipid extraction, fatty acid, and cholesterol analysis

Cold lipid extraction was performed with chloroform:methanol (2:1, vol/vol) as described previously by Argov *et al.* (2007). The lipid-rich chloroform fraction was collected and evaporated under vacuum at 65 °C. The dry lipid fraction was transmethylated at 65 °C for 1 h (5% sulfuric acid in methanol, vol/vol) to generate FAME. After methylation, FAME were extracted with petroleum ether, which was also used as the injection solvent for the GC analysis. FAME separation, identification, and quantification were performed with a 6890N gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with flame-ionizing detector and a fused-silica capillary column (DB-23, 60 m×0.25 mm ID, 0.25 µm film, Agilent Technologies) under the following conditions: the oven temperature was programmed from 170 to 215 °C at a rate of 2.75 °C/min, from 215 to 250 °C at 40 °C/min, and held at 250 °C for 5 min. Peak identification was based on relative retention times of the two external FAME standards. Relative concentrations of fatty acids were determined as molar percentage of total fatty acids within each sample (mol %). Fatty acids with the same chemical composition were grouped (sum of mol% values) into SFA (C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C22:0, and C24:0), unsaturated fatty acids (USFA; C16:1n7, C18:1n9, C18:1n7, C18:2n6, C18:3n6, C18:3n3, C20:1n9, C20:4n4, C20:5n3, C22:1n9, C22:4n6, and C22:6n3), MUFA (C16:1n7, C18:1n9, C18:1n7, C20:1n9, and C22:1n9), and PUFA (C18:2n6, C18:3n6, C18:3n3, C20:4n6, C20:5n3, C22:4n6, and C22:6n3). In addition, fatty acids were grouped into omega-3 (C18:3n3, C20:5n3, and C22:6n3) and omega-6 (C18:2n6, C18:3n6, C20:4n6, and C22:4n6) fatty acids. Cholesterol concentration was determined according to an internal standard, used as a normalization factor.

Elongation and desaturation indicators

Elongation and desaturation activities were determined as the ratio between the relative concentration of substrates and the product outcomes of the desaturation and elongation pathways (Zivkovic *et al.* 2009). Indicators of desaturation were the ratios between c16:1n7 and c16:0 and between c18:1n9 and c18:0. Indicators of elongation were the ratios between c18:1n7 and c16:1n7, c20:0 and c18:0, c20:1n9 and c18:1n9, and c22:0 and c20:0.

Statistical analysis

JMP software version 7 (SAS Institute, Cary, NC, USA) was used for statistical analyses. Data are presented as means±S.E.M. (semen volume, concentration, velocity, and lipid profile) or as percentage means±S.E.M. (sperm morphology, motility, and PM). Outliers were defined as distant from the mean by more than two S.D. and were deleted. Outliers (one sample for each season) were excluded from the analysis only if the sample deviated from the mean in all physiological measurements (i.e. concentration, velocity, and morphology). Comparison of seasons was performed with repeated-measures ANOVA. The effects in the model were season, sampling week nested within season, and bull (random). Normality was established based on the distribution of all the residual data for each variable. Association was expressed by Pearson's correlation coefficient test; data were the means of repeated measures for each bull. Significance was set at $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Cattle Division of the Ministry of Agriculture, Israel (project #820-0272-08).

Acknowledgements

The authors are grateful to the staff of the Israeli artificial insemination center ('Sion', Hafetz-Haim, Israel) for helping with semen collection and evaluation.

References

Ahluwalia B & Holman RT 1969 Fatty acid composition of lipids of bull, boar, rabbit and human semen. *Journal of Reproduction and Fertility* **18** 431–437. (doi:10.1530/jrf.0.0180431)

do Amaral BC, Connor EE, Tao S, Hayen J, Bubolz J & Dahl GE 2009 Heat-stress abatement during the dry period: does cooling improve transition into lactation? *Journal of Dairy Science* **92** 5988–5999. (doi:10.3168/jds.2009-2343)

Argov N, Sklan D, Zeron Y & Roth Z 2007 Association between seasonal changes in fatty-acid composition, expression of VLDL receptor and bovine sperm quality. *Theriogenology* **67** 878–885. (doi:10.1016/j.theriogenology.2006.10.018)

Ax RL, Gilbert GR & Shook GE 1987 Sperm in poor quality semen from bulls during heat stress have a lower affinity for binding hydrogen-3 heparin. *Journal of Dairy Science* **70** 195–200. (doi:10.3168/jds.S0022-0302(87)79994-9)

Comhaire FH, Vermeulen L & Schoonjans F 1987 Reassessment of the accuracy of traditional sperm characteristics and adenosine triphosphate (ATP) in estimating the fertilizing potential of human semen *in vivo*. *International Journal of Andrology* **10** 653–662. (doi:10.1111/j.1365-2605.1987.tb00366.x)

Connor WE, Lin DS, Wolf DP & Alexander M 1998 Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. *Journal of Lipid Research* **39** 1404–1411.

Darin-Bennett A & White IG 1997 Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology* **14** 466–470. (doi:10.1016/0011-2240(77)90008-6)

Flesch FM, Brouwers JFHM, Nievelstein PFEM, Verkleij AJ, van Golde LMG, Colenbrander B & Gadella BM 2001 Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of Cell Science* **114** 3543–3555.

Furland NE, Oresti GM, Antollini SS, Venturino A, Maldonado EN & Aveldaño MI 2007 Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa. *Journal of Biological Chemistry* **282** 18151–18161. (doi:10.1074/jbc.M700709200)

Gundogan M & Elitok B 2004 Seasonal changes in reproductive parameters and seminal plasma constituents of rams in Afyon province of Turkey. *Deutsche Tierärztliche Wochenschrift* **111** 158–161.

Haila AA & Tulsiani DRP 2009 Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Archives in Biochemistry and Biophysics* **485** 72–81. (doi:10.1016/j.abb.2009.02.003)

Huacuja L, Delgado NM, Calzada L, Wens A, Reyes R, Pedron N & Rosado A 1981 Exchange of lipids between spermatozoa and seminal plasma in normal and pathological human semen. *Archives of Andrology* **7** 343–349. (doi:10.3109/01485018108999329)

Ibrahim SA 1997 Seasonal variations in semen quality of local and crossbred rams raised in the United Arab Emirates. *Animal Reproduction Science* **49** 161–167. (doi:10.1016/S0378-4320(97)00063-8)

Khorasani AM, Cheung AP & Lee CYG 2000 Cholesterol inhibition effects on human sperm induced acrosome reaction. *Journal of Andrology* **21** 586–594. (doi:10.1002/j.1939-4640.2000.tb02124.x)

Lenzi A, Picardo M, Gandini L & Dondero F 1996 Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Human Reproduction Update* **2** 246–256. (doi:10.1093/humupd/2.3.246)

Lenzi A, Gandini L, Picardo M, Tramer F, Sandri G & Panfili E 2000 Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): scavenger mechanisms and possible scavenger therapies. *Frontiers in Bioscience* **5** 1–15. (doi:10.2741/Lenzi)

Levine RJ 1999 Seasonal variation of semen quality and fertility. *Scandinavian Journal of Work Environmental and Health* **25** 34–37.

Lewis SEM 2007 Is sperm evaluation useful in predicting human fertility? *Reproduction* **134** 31–40. (doi:10.1530/REP-07-0152)

Mathevon M, Buhr MM & Dekkers JCM 1998 Environmental, management and genetic factors affecting semen production in Holstein bulls. *Journal of Dairy Science* **81** 3321–3330. (doi:10.3168/jds.S0022-0302(98)75898-9)

May-Panloup P, Chreâtien MF, Savagner F, Vasseur C, Jean M, Malthieáry Y & Reynier P 2003 Increased sperm mitochondrial DNA content in male infertility. *Human Reproduction* **18** 550–556. (doi:10.1093/humrep/deg096)

Menendez-Buxadera A, Morales JR, Perez AP & Guerra YD 1984 Seasonal variation in semen production of Holstein, Zebu and Criollo bulls under artificial insemination conditions in Cuba. Reproduction des ruminants en zone tropicale, Reunion international, Pointe a Pitre, Guadeloupe, France. *Les colloques de l'INRA* **20** 239–249.

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** 352–357.

Morales CR, Marat AL, Ni Y, Yu X, Oko R, Smith BT & Argraves WS 2008 ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa. *Biochemical and Biophysical Research Communications* **376** 472–477. (doi:10.1016/j.bbrc.2008.09.009)

Nolan JP & Hammerstedt RH 1997 Regulation of membrane stability and the acrosome reaction in mammalian sperm. *FASEB Journal* **11** 670–682.

Orgal S, Zeron Y, Elior N, Biran D, Fridman E, Druker S & Roth Z 2012 Season-induced changes in bovine sperm motility following freeze–thaw procedure. *Journal of Reproduction and Development* **58** 212–218. (doi:10.1262/jrd.10-149N)

Poulos A, Voglmayr JK & Whute JG 1973 Phospholipid changes in spermatozoa during passage through the genital tract of the bull. *Biochimica et Biophysica Acta* **306** 194–202. (doi:10.1016/0005-2760(73)90225-7)

de los Rios J, Cardona WD, Berdugo JA, Correa C, Arenas A, Olivera-Angel M, Peña RB & Cadavid AP 2004 Sperm parameters in 113 subjects after recent fatherhood did not correlate with WHO standards. *Archivos Españoles de Urologia* **57** 147–152.

Rodriguez AL, Rijsselaere T, Bijttebier J, Vyt P, Soom AV & Maes D 2011 Effectiveness of the sperm quality analyzer (SQA-Vp) for porcine semen analysis. *Theriogenology* **75** 972–977. (doi:10.1016/j.theriogenology.2010.10.024)

Roudebush WE & Diehl JR 2001 Platelet-activating factor content in boar spermatozoa correlates with fertility. *Theriogenology* **55** 1633–1638. (doi:10.1016/S0093-691X(01)00508-8)

Saez F, Ouvrier A & Drevet JR 2011 Epididymis cholesterol homeostasis and sperm fertilizing ability. *Asian Journal of Andrology* **13** 11–17. (doi:10.1038/aja.2010.64)

Safarinejad MR, Hosseini SY, Dadkhah F & Asgari MA 2010 Relationship of omega-3 and omega-6 fatty acids with semen characteristics, and anti-oxidant status of seminal plasma: a comparison between fertile and infertile men. *Clinical Nutrition* **29** 100–105. (doi:10.1016/j.clnu.2009.07.008)

Scharenberg C, Mannowetz N, Robey RW, Brendel C, Repges P, Sahrhage T, Jähn T & Wennemuth G 2009 ABCG2 is expressed in late spermatogenesis and is associated with the acrosome. *Biochemical and Biophysical Research Communications* **378** 302–307. (doi:10.1016/j.bbrc.2008.11.058)

Sinclair S 2000 Male infertility: nutritional and environmental considerations. *Alternative Medicine Review* **5** 28–38.

Strzeżek J, Fraser L, Kuklińska M, Dziekońska A & Lecewicz M 2004 Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. *Reproductive Biology* **4** 271–287.

Tavilani H, Doosti M, Abdi K, Vaisiraygani A & Joshaghani HR 2006 Decreased polyunsaturated and increased saturated fatty acid concentration in spermatozoa from asthenozoospermic males as compared with normozoospermic males. *Andrologia* **38** 173–178. (doi:10.1111/j.1439-0272.2006.00735.x)

Tavilani H, Doosti M, Nourmohammadi I, Mahjub H, Vaisiraygani A, Salimi S & Hosseinipanah SM 2007 Lipid composition of spermatozoa in normozoospermic and asthenozoospermic males. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **77** 45–50. (doi:10.1016/j.plefa.2007.07.001)

Travis AJ & Kopf GS 2002 The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *Journal of Clinical Investigation* **110** 731–736. (doi:10.1172/JCI16392)

Van Hoeven RP, Emmelot P, Krol JH & Oomen-Meulemans EPM 1975 Studies on plasma membranes. II. Fatty acid profiles of lipid classes in plasma membranes of rat and mouse livers and hepatomas. *Biochimica et Biophysica Acta* **380** 1–11. (doi:10.1016/0005-2760(75)90039-9)

Verstegen J, Igur-Ouada M & Onclin K 2002 Computer assisted analyzer in andrology research and veterinary practice. *Theriogenology* **57** 149–179. (doi:10.1016/S0093-691X(01)00664-1)

Wang Y, Botolin D, Christian B, Busik J, Xu J & Jump DB 2005 Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. *Journal of Lipid Research* **46** 706–715. (doi:10.1194/jlr.M400335-JLR200)

Wathes DC, Abayasekara DRE & Aitken RJ 2007 Polyunsaturated fatty acids in male and female reproduction. *Biology of Reproduction* **77** 190–201. (doi:10.1095/biolreprod.107.060558)

Zalata AA, Christophe AB, Depuydt CE, Schoonjans F & Comhaire FH 1998 The fatty acid composition of phospholipids of spermatozoa from infertile patients. *Molecular Human Reproduction* **4** 111–118. (doi:10.1093/molehr/4.2.111)

Zhu J, Massey JB, Mitchell-Leef D, Elsner CW, Kort HI & Roudebush WE 2006 Platelet-activating factor acetylhydrolase activity affects sperm motility and serves as a decapacitation factor. *Journal of Andrology* **27** 429–433. (doi:10.2164/jandrol.05159)

Zivkovic AM, German JB, Esfandiari F & Halsted CH 2009 Quantitative lipid metabolomic changes in alcoholic micropigs with fatty liver disease. *Alcoholism, Clinical and Experimental Research* **33** 751–758. (doi:10.1111/j.1530-0277.2008.00892.x)

Received 10 December 2012
First decision 11 January 2013
Accepted 11 March 2013