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Variation in lipid profiles within semen compartments—the bovine model of aging

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ABSTRACT

Semen lipid composition was examined in young and mature bulls. Given the specific roles of various semen compartments (i.e., seminal fluid, sperm head, and sperm tail) during fertilization, we hypothesized that altered fatty acid and cholesterol composition of a specific compartment might impair semen quality and sperm function. Semen samples were collected from five mature and five young Holstein Friesian bulls during the winter (December–January). Semen was evaluated by computerized sperm-quality analyzer for bulls and was centrifuged to separate the sperm from the seminal fluid. The sperm fraction was sonicated to separate its head and tail compartments. Cold extraction of lipids was performed, and fatty acids and cholesterol were identified and quantified by gas chromatography. Semen physiological features (concentration, motility, and progressive motility) did not differ between mature and young bulls. However, lipid composition within fractions varied between groups, with prominent impairments in the head compartment. In particular, the proportions of polyunsaturated fatty acids, omega-3 fatty acids, and docosahexaenoic acid in the intact sperm; seminal fluid; and sperm head were lower in semen collected from mature bulls than in that from young bulls. The finding suggests an age-differential absorption and/or metabolism through spermatogenesis. Reduced proportions of major fatty acids in mature bulls might reduce membrane fluidity, which in turn might affect the ability to undergo cryopreservation and/or oocyte-sperm fusion through fertilization.

1. Introduction

In modern dairy herds, where reproductive management is on the basis of artificial insemination, the bull is considered a risk factor for fertility [1]. In that respect, the association between bull age and ejaculate characteristics has become highly relevant for the study of reproduction. Ejaculate volume and total sperm number increase with age and have been found to vary among bovine breeds [2–4]. On the other hand, advancing bull age is associated with a decrease in sperm motility and an increase in sperm defects [5] and sperm concentration [6]. In contrast, Hallap et al. [7] reported an increase in sperm motility and membrane integrity and a higher proportion of sperm with normal tail and acrosome morphology as bull age increases. Nevertheless, the mechanism underlying these alterations through aging is not clear.

Lipid and fatty acid composition is associated with semen physiological characteristics, which is considered as important reproductive predictor. For instance, Cerolini et al. [8] found that increased concentrations of free cholesterol, free fatty acids, triacylglycerol, and cholesterol ester are associated with decreased sperm motility and fertility. Similarly, in chickens, reduced male fertility was associated with low lipid content in the seminal fluid and reduced content of polyunsaturated fatty acids (PUFAs) in the sperm membrane, most notably arachidonic acid and docosahexaenoic acid (DHA) [6]. The high concentration of PUFA in sperm makes it extremely vulnerable to

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nonenzymatic oxidation processes. The extent of PUFA (i.e., antioxidation capacity), determined by the presence of antioxidant agents, is reduced through aging in both seminal fluid [6] and epididymal sperm [9] in association with reduced activity of the antioxidant enzyme glutathione peroxidase 1 (GPX1). Cholesterol, the most abundant lipid molecule in the sperm membrane, is also sensitive to peroxidation. Reduced cholesterol concentration in the membrane impairs membrane stability, resulting in premature deterioration of the sperm cells [10]. Taken together, it is suggested that an adequate lipid and fatty acid composition with appropriate antioxidant activity plays a role in protecting the sperm and maximizing its fertilization potential.

Given the specific role and physical properties of each of the semen compartments required for successful fertilization, and due to the uneven distribution of fatty acids between sperm heads and tails [11,12], we hypothesized that altered fatty acid and cholesterol composition of a specific compartment might impair semen features and sperm function. The aim of the present study was to provide a wide lipid profile to determine whether age-related alterations in bull semen are associated with sperm characteristics. For this purpose, lipid composition was examined in different semen fractions: whole sperm, sperm head, sperm tail, and seminal fluid.

2. Materials and methods

2.1. Animals

Experiments were performed at the Israeli Artificial Insemination Center ("Sion," Hafetz-Haim, Israel), in accordance with the Israeli guidelines for animal welfare and experimentation (1994). Semen samples were collected from five mature (7.3 ± 0.6 years) and five young (1.8 ± 0.1 years) Holstein Friesian bulls during the winter to avoid any deleterious effects of summer heat stress. All animals were fertile bulls from the "Sion inseminating bulls" list. Bulls were routinely ejaculated at the same interval, and samples were taken only from the first ejaculate of the collection day. Bulls were fed the same total mixed ration throughout the experiment, containing 68.4% (wt/wt) dry matter (DM), 7.2% (wt/wt) protein, 36.2% (wt/wt) neutral detergent fiber, 20.0% (wt/wt) acidic detergent fiber, 1.45 net energy Mcal/kg, and 3.5 g minerals/kg (NaCl, Ca, and P) on a DM basis.

2.2. Semen collection and initial evaluation of physiological characteristics

Semen was collected routinely once a week ($n = 5$ samples per bull). To eliminate any potential differences in sperm quality due to serial ejaculates, the 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 laboratory, and the semen was evaluated by computerized sperm-quality analyzer for bulls (SQA-Vb, Medical Electronic Systems, Caesarea, Israel). The physiological characteristics included semen volume (mL), concentration (million sperm/mL), motility (%), progressive motility (%), morphologically normal sperm (%), motile sperm concentration (MSC, million/mL), progressive motile sperm concentration (PMSC, million/mL), and velocity, that is, the average velocity of the progressively motile sperm (μm/s). According to routine procedures at "Sion," samples with a concentration higher than 650×10^6 cell/mL, motility higher than 70%, and progressive motility higher than 60% were defined as being of good quality.

2.3. Sperm handling

At each collection, 2 mL of the total volume of collected semen was centrifuged (800 × *g*) for 10 minutes at room temperature to separate the sperm from the seminal fluid. The supernatant (i.e., seminal fluid) 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 fluid. The pellet from one tube was suspended in 200 μL saline and used for lipid profile analysis, whereas the pellet from the second tube was suspended in 2 mL saline and subjected to separation into sperm head and tail fractions.

2.4. Separation of spermatozoa into head and tail compartments

Spermatozoa were separated into head and tail compartments as previously described by Zalata et al. [13] with minor modifications. Briefly, samples were sonicated for 2 minutes at maximal power (Misonix Microson XL2000

Table 1
Fatty acid composition of bulls' rations.

Fatty acid	Mol%
c8:0	0.06
c12:0	0.21
c14:0	0.66
c15:0	0.23
c16:0	20.7
c16:1	0.85
c17:0	0.22
c18:0	4.04
c18:1	17.7
c18:2n6	32.5
c18:3n3	6.3
c20:0	0.63
c20:1	0.35
c20:2	0.3
c20:4n6	0.29
c20:5n3	0.4
c21:0	0.2
c22:0	0.8
c22:1	1.3
c22:2	0.2
c22:3	0.3
c22:5n3	0.1
c22:6n3	0.3
c23:0	0.3
c24:0	0.8
c24:1	0.3

Values are presented as mol% of each fatty acid from the total identified fatty acids in the sample.

Table 2
Physiological and morphological semen characteristics.

Semen characteristics	Young	Mature
Volume (mL)	4.5 ± 0.3	8.5 ± 0.6**
Motility (%)	86.0 ± 2.1	80.3 ± 2.7
Progressive motility (%)	83.0 ± 2.0	77.5 ± 2.6
Morphologically normal (%)	91.7 ± 0.8	89.6 ± 1.0
Velocity (μm/s)	120.9 ± 3.3	111.8 ± 4.3*

Semen was collected during the winter from mature and young Holstein bulls. Semen volume, and sperm concentration, motility, progressive motility, morphology, and velocity were evaluated by SQA-Vb. Data are presented as mean ± SE.
* $P < 0.10$.
** $P < 0.001$.

Ultrasonic Processor, Newtown, CT, USA). One drop of the sonicated liquid was evaluated by light microscopy (Eclipse TE2000, Nikon, Tokyo, Japan) to determine the fractionation rates (i.e., proportion of sperm separated into heads and tails). The sonicated sample (0.5 mL) was gently layered on top of a 2-mL 90% Percoll column (Sigma Aldrich Rehovot, Israel) and slowly centrifuged (500 × *g*, 20 minutes) to separate the two compartments: the sperm tails remained as a solid layer at the top of the Percoll column, whereas the sperm heads sedimented to the bottom. Note that although the head fraction was easily recovered, the tail fraction was relatively tightly bound and was therefore washed twice with 2 mL saline. Eventually, both fractions were diluted in 100-μL physiological saline and kept at −20 °C for further analysis.

2.5. Analysis of lipid profile in sperm, seminal fluid, sperm head, and sperm tail

2.5.1. 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 Lab Supplies (Haifa, Israel) and sulfuric acid was from Bet-Dekel (Netanya, Israel). External standard of fatty acid methyl esters (FAME Mix C4-C24, FAME mix C16-C22) was purchased from Sigma Aldrich Israel.

2.5.2. Lipid extraction and fatty acid and cholesterol analysis

Lipid-cold extraction was performed with chloroform:methanol (2:1; vol/vol) as previously described by Argov et al. [14]. The lipid-rich chloroform fraction was collected and evaporated under vacuum conditions at 65 °C. The dry lipid fraction was trans-methylated at 65 °C for 1 hour (5% sulfuric acid in methanol, vol/vol) to generate FAME. FAME separation, identification, and quantification were performed with a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization 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 °C to 215 °C at a rate of 2.75 °C/minute, from 215 °C to 250 °C at a rate of 40 °C/minute, and held at 250 °C for 5 minutes. Peak identification was based on relative retention times of two external standards. Fatty acid relative concentrations were determined as molar percentage (mol%) of total fatty acids within each sample. Fatty acids with the same chemical composition were grouped (sum of mol% values) into saturated fatty acids (SFAs) (C8:0, C10:0, C12:0, C14:0, C16:0, C1:0, C20:0, C22:0, and C24:0) and unsaturated fatty acids (USFAs) (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); the latter were further divided into monounsaturated fatty acids (MUFAs) (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, relative concentrations of fatty acids were grouped as omega-3 (C18:3n3, C20:5n3, and C22:6n3) and omega-6 (C18:2n6, C18:3n6, C20:4n6, and C22:4n6). Cholesterol concentration was determined according to an internal standard, used as a normalization factor.

Table 3
Age effects on fatty acid profile in the sperm compartments (intact sperm, seminal fluid, sperm tail, and sperm head).

Fatty acid	Head		Tail		Seminal fluid		Intact sperm	
	Young	Mature	Young	Mature	Young	Mature	Young	Mature
c12:0	1.00 ± 0.16	2.13 ± 1.11	0.73 ± 0.11	1.40 ± 0.46	0.24 ± 0.08	0.14 ± 0.01	0.21 ± 0.01	0.25 ± 0.01*
c14:0	12.77 ± 1.15	15.10 ± 1.19	3.57 ± 0.27	5.02 ± 0.25†	7.25 ± 0.57	9.50 ± 0.31†	15.62 ± 0.73	21.85 ± 1.02†
c16:0	22.87 ± 1.16	22.74 ± 0.70	17.39 ± 0.54	16.66 ± 0.2	36.95 ± 0.87	37.5 ± 0.68	23.9 ± 0.64	24.76 ± 2.18
c18:1n9	2.40 ± 0.30	2.70 ± 0.31	7.04 ± 0.52	6.40 ± 0.43	4.94 ± 0.28	5.77 ± 0.48	3.08 ± 0.20	2.44 ± 0.06†
c18:1n7	4.31 ± 0.74	2.99 ± 0.38	5.06 ± 0.22	5.95 ± 0.25**	1.50 ± 0.05	1.86 ± 0.19*	3.08 ± 0.20	2.44 ± 0.06
c18:2n6	2.77 ± 0.26	3.68 ± 0.41***	10.94 ± 0.38	12.87 ± 0.32†	6.25 ± 0.50	7.50 ± 0.54*	5.32 ± 0.23	6.55 ± 0.49*
c20:0	0.32 ± 0.07	0.49 ± 0.06*	1.57 ± 0.32	1.05 ± 0.12	0.30 ± 0.03	0.28 ± 0.01	0.19 ± 0.02	0.19 ± 0.01
c20:1n9	0.51 ± 0.20	0.62 ± 0.13	0.52 ± 0.06	0.46 ± 0.04	0.17 ± 0.01	0.26 ± 0.02†	0.12 ± 0.01	0.11 ± 0.01
c20:4n6	3.34 ± 0.28	2.63 ± 0.25*	8.00 ± 0.30	8.80 ± 0.37	2.29 ± 0.18	1.90 ± 0.04**	4.72 ± 0.14	4.65 ± 0.09
c20:5n3	ND	ND	0.30 ± 0.03	0.20 ± 0.04*	0.38 ± 0.04	0.37 ± 0.08	0.15 ± 0.01	0.15 ± 0.01
c22:1n9	0.71 ± 0.18	0.50 ± 0.11	3.34 ± 0.30	2.69 ± 0.37**	0.21 ± 0.03	0.21 ± 0.01	0.13 ± 0.02	0.11 ± 0.01
c22:4n6	0.89 ± 0.14	1.90 ± 0.65	0.75 ± 0.07	1.10 ± 0.08***	0.69 ± 0.04	1.04 ± 0.04†	0.63 ± 0.01	0.98 ± 0.05†
c24:0	1.29 ± 0.27	1.17 ± 0.36	0.69 ± 0.10	0.73 ± 0.06	0.72 ± 0.07	0.94 ± 0.06**	0.22 ± 0.03	0.20 ± 0.03
c22:6n3	34.25 ± 2.19	27.63 ± 2.04**	26.60 ± 1.30	24.37 ± 0.68	23.97 ± 0.67	18.78 ± 0.63†	31.91 ± 1.09	24.35 ± 0.71†

The values are presented as mean ± SE.
* $P < 0.10$.
** $P < 0.05$.
*** $P < 0.01$.
† $P < 0.001$.

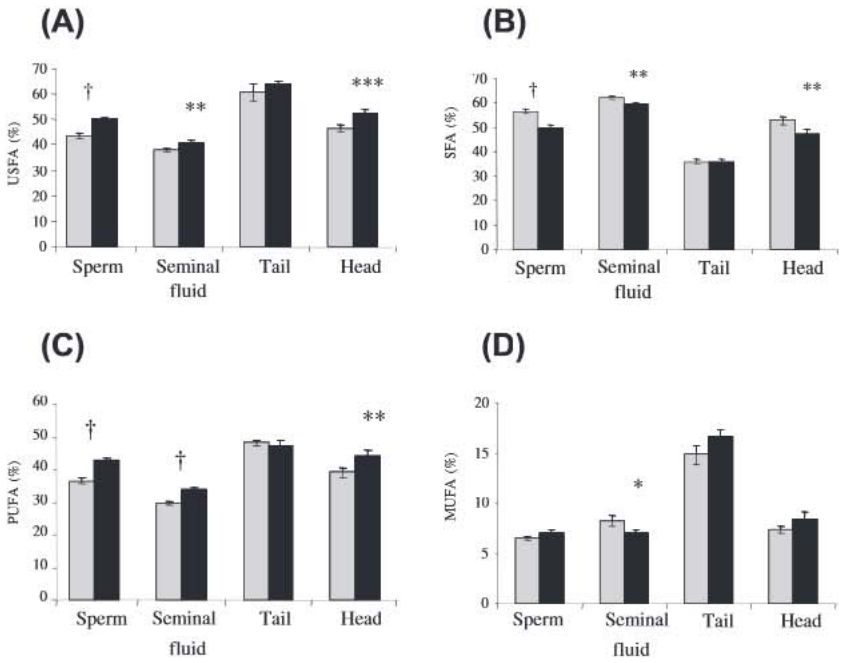

Fig. 1. Age-related variations in fatty acid composition. Semen samples were collected from mature (gray bars) and young bulls (black bars). Seminal fluids and sperm were separated and sperm were further fractionated into tails and heads. (A) Sum of mol% values of the USFAs C16:1n7, C18:1n9, C18:1n7, C18:2n6, C18:3n6, C18:3n3, C20:1n9, C20:4n4, C20:5n3, C22:1n9, C22:4n6, and C22:6n3. (B) Sum of mol% values of the SFA C8:0, C10:0, C12:0, C14:0, C16:0, C1:0, C20:0, C22:0, and C24:0. (C) Sum of mol% values of the PUFA C18:2n6, C18:3n6, C18:3n3, C20:4n6, C20:5n3, C22:4n6, and C22:6n3. Data are mean ± SE. * $P < 0.10$, ** $P < 0.05$, *** $P < 0.01$, † $P < 0.001$. (D) Sum of mol% values of the MUFA C16:1n7, C18:1n9, C18:1n7, C20:1n9, and C22:1n9.

2.5.3. Elongation and desaturation markers

Elongation and desaturation activities were determined as the ratio between the relative concentrations of substrates and products of the desaturation and elongation pathways [15]. Desaturation indicators were the ratios between c16:1n7 and c16:0 and between c18:1n9 and c18:0. Elongation indicators 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.

2.6. Statistical analysis

Statistical procedures were performed using one-way ANOVA in JMP software version 7 (SAS Institute Inc., Cary, NC, USA). The model included age, sample, and animal. Data are presented as means ± SE (semen volume, concentration, velocity, and lipid profiles) or as percentage means ± SE (sperm morphology, motility, and progressive motility). The differences between the variables in mature and young animals were tested by Student's *t*-test. Significance was set at $P < 0.05$. Outliers from the mean were defined as data with >2 SD. Dependent variables were checked for normality, and correlation analysis was performed between variables using the "multivariant and correlation" function of JMP.

3. Results

3.1. Environmental and nutritional features

The average minimum and maximum air temperatures during the winter were 10.6 ± 0.8 °C and 21.5 ± 1.3 °C, respectively, and the average minimum and maximum air humidity was 47.6 ± 1.1% and 93 ± 0.4%, respectively. The feeding-ration composition was 75.0% DM and 1.6% total fat. The fatty acid composition in the feeding ration is presented in Table 1.

3.2. Semen physiological characteristics

Semen collected from mature bulls did not differ in motility, progressive motility, or morphological features from that of young bulls. However, the velocity tended to be higher ($P = 0.1$) in semen collected from young bulls compared with that of mature bulls (Table 2). On the other hand, the average volume of ejaculates collected from young bulls was lower than that collected from the mature animals ($P = 0.001$).

3.3. Fatty acid composition in the sperm compartments

Fatty acid compositions of semen samples and their functional compartments (intact sperm, seminal fluid, sperm heads, and sperm tails) for both mature and young bulls are presented in Table 3. The proportion of a major SFA, myristic acid (c14:0) differed between the experimental groups with approximately 30% higher concentration ($P < 0.0001$) in intact sperm, seminal fluid, and tail compartments of mature bulls compared with the respective compartments in young bulls. Palmitic acid (c16:0) did not differ between the groups in any of the analyzed compartments. The proportion of linoleic acid (c18:2n6) was higher in sperm heads and tails ($P < 0.05$ and

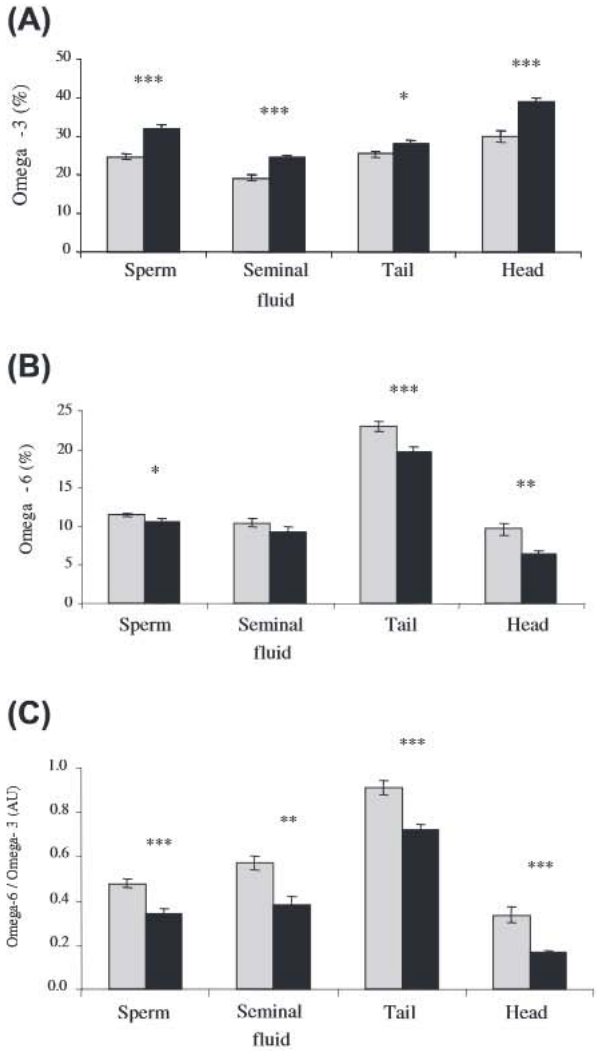

Fig. 2. Comparison of (A) omega-3, (B) omega-6, and (C) omega-6:3 ratio in semen obtained from mature bulls (gray column) versus that obtained from young bulls (black column) in four semen fractions (sperm, seminal fluid, sperm tail, and head). Values in (A) and (B) are presented as percentage mean ± SE and in (C) as mean ± SE. * $P < 0.10$, ** $P < 0.01$, *** $P < 0.001$.

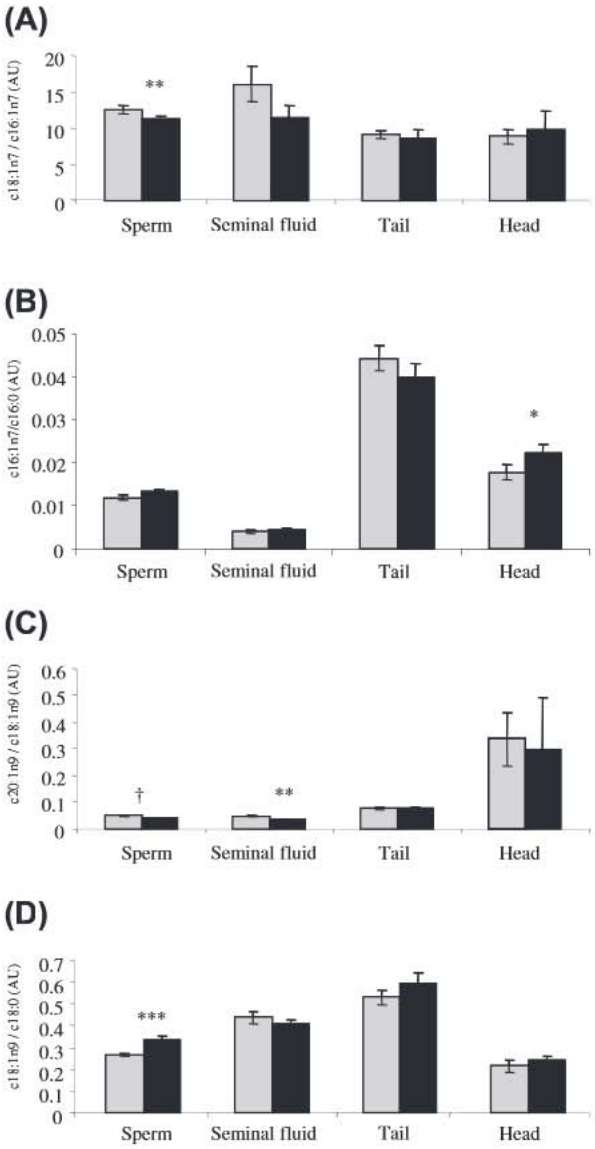

Fig. 3. Comparison of (A) c18:1n7/c16:1n7 ratio, (B) c16:1n7/c16:0 ratio, (C) c20:1n9/c18:1n9 ratio, and (D) c18:1n9/c18:0 ratio between semen obtained from mature bulls (gray column) and semen obtained from young bulls (black column) in four semen fractions (sperm, seminal fluid, sperm tail, and head). Values are presented as mean ± SE. * $P < 0.10$, ** $P < 0.05$, *** $P < 0.01$, † $P < 0.001$.

$P < 0.001$, respectively) and tended ($P = 0.09$) to be higher in intact sperm and seminal fluid of mature bulls relative to young bulls. In addition, the concentration of oleic acid (c18:1n9) was higher ($P < 0.005$) in intact sperm of young versus mature bulls but did not differ between age groups in the other examined compartments. The proportion of DHA (c22:6n3) was lower in intact sperm, seminal fluid ($P < 0.0001$), and sperm heads ($P < 0.005$) isolated from semen of mature bulls relative to that of young bulls. The proportion of adrenic acid (c22:4n6) was higher in intact sperm, seminal fluid ($P < 0.0001$), and sperm tails ($P < 0.01$) of mature bulls relative to young bulls.

Fatty acid composition was analyzed according to the acids' biochemical characteristics to gain a biochemical understanding of the cellular functionalities with a specific fatty acid composition (Fig. 1). SFA concentrations in intact sperm, seminal fluid, and sperm heads were higher in mature versus young bulls ($P = 0.0001$, $P = 0.03$, and $P = 0.02$, respectively; Fig. 1B). UNFAs and PUFAs were lower ($P = 0.03$ and $P = 0.002$, respectively; Fig. 1A, C), and MUFA tended to be higher ($P < 0.1$; Fig. 1D) in the seminal fluid of mature bulls relative to that of young bulls. Lipid profile in the sperm tail did not differ between groups (Fig. 1A–D).

The concentrations of the PUFA subfamilies omega-6 and omega-3 differed between groups (Fig. 2A, B) with a lower ($P = 0.001$) concentration of omega-3 in the intact sperm, seminal fluid, and sperm head and a tendency ($P < 0.1$) toward lower concentration in sperm tails of mature bulls relative to young bulls. The concentration of omega-6

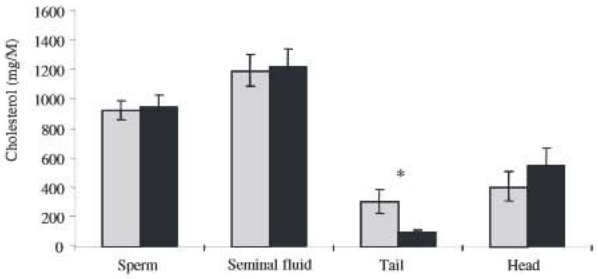

Fig. 4. Comparison of cholesterol amounts in semen obtained from mature bulls (gray column) and young bulls (black column) in four semen fractions (sperm, seminal fluid, sperm tail, and head). Values are presented as mean ± SE. * $P < 0.01$.

was higher ($P = 0.01$) in the sperm tail and sperm head and tended to be higher ($P < 0.1$) in the intact sperm isolated from the semen of mature versus young bulls. Respectively, the ratio between omega-6 and omega-3 was higher ($P < 0.01$) in all examined compartments isolated from the semen of mature versus young bulls (Fig. 2C).

3.4. Elongation and desaturation activity indicators

Indicators for desaturation and elongation activity in all semen compartments were determined by calculating the ratio between the concentration of substrates and products (i.e., activity indicator value). The value of the elongase activity indicator c18:1n7/c16:1n7 in intact sperm was higher ($P < 0.05$) in mature bulls than in young bulls (Fig. 3A). The Δ9 desaturase activity indicator value, calculated for c16:1n7/c16:0, tended to be higher ($P < 0.1$) in the sperm heads of young versus mature bulls (Fig. 3B). Elongase activity indicator c20:1n9/c18:1n9 was higher in the intact sperm and in the seminal fluid ($P < 0.05$) of mature versus young bulls (Fig. 3C). The Δ9 desaturase activity indicator c18:1n9/c18:0 was higher ($P < 0.05$) in the intact sperm of young versus mature bulls (Fig. 3D).

3.5. Age-associated variations in cholesterol concentrations in semen compartments

Cholesterol concentrations in mature and young samples are presented in Figure 4. In intact sperm, seminal fluid, and sperm head compartments, the cholesterol concentration did not differ between mature and young bulls ($P = 0.095$). However, the cholesterol concentration in the tail compartment was higher ($P = 0.02$) in mature versus young bulls.

3.6. Correlations between physiological characteristics and lipid profile

Associations between physiological features and lipid profiles of the functional semen compartments were determined by correlation analysis. Due to the large amount of data, only the main findings are presented; in particular, positive or negative correlations for each of the examined compartments (intact spermatozoa, seminal fluid, and sperm tails and heads) are shown in Table 4.

More correlations between physical properties and lipid composition were found in intact sperm, with opposite directions for mature bulls and young bulls (Table 4A). For example, semen concentration was negatively correlated ($P = 0.026$) with SFA in mature bulls but not in young bulls. Semen concentration was positively correlated with MUFA in mature bulls but negatively correlated with MUFA in young bulls ($P = 0.05$ and $P = 0.001$, respectively). The omega-6:3 ratio in the intact sperm of mature bulls was positively correlated with sperm concentration ($P = 0.014$), but this correlation tended to be negative ($P = 0.07$) in young bulls. The value of the elongase activity indicator c18:1n7/c16:1n7 was positively correlated with semen concentration in mature bulls ($P = 0.005$), whereas that of c18:1n9/c18:0 was negatively correlated in young bulls ($P = 0.0001$). In addition, motility and progressive motility were negatively correlated with MUFA ($P = 0.007$) and omega-6:3 in the mature bulls ($P = 0.004$), but these correlations were positive in the young bulls ($P = 0.004$ and $P = 0.008$, respectively).

Opposite correlations between physical properties and lipid composition were also evident in the seminal fluids of mature and young bulls (Table 4B). Negative correlations between motility or progressive motility and c18:1n9/c18:0 ($P = 0.04$ and $P = 0.037$, respectively) were found in the sperm tails of mature bulls (Table 4C). The most prominent correlations were found in the sperm head compartment of young bulls (Table 4D). In particular, semen concentration was negatively correlated with omega-6:3 ($P = 0.049$), and motility and progressive motility were negatively correlated with USFAs ($P = 0.02$), PUFA ($P = 0.019$), and omega-3 concentrations ($P = 0.013$).

Cholesterol level was positively correlated with MSC and PMSC in intact sperm of both mature bulls ($P = 0.015$ and $P = 0.012$, respectively) and young bulls ($P = 0.0003$ and $P = 0.0004$, respectively; Table 5A). Cholesterol was only positively correlated with sperm concentration in young bulls ($P = 0.0003$; Table 5A). Cholesterol level in the seminal fluid was positively correlated with sperm concentration in young bulls ($P = 0.013$) but not with that in mature bulls ($P = 0.57$; Table 5B). Cholesterol level in the sperm head (Table 5D) was positively correlated with both MSC and PMSC ($P = 0.017$ and $P = 0.016$, respectively) only in the young bulls.

4. Discussion

Semen quality is considered a risk factor for low fertility in dairy herds. Previous studies have reported that advancing bull age is associated with a decrease in sperm motility and an increase in sperm defects [5] and sperm concentration [6]. Other studies have shown that lipid profile and membrane fatty acid composition directly affect sperm properties (i.e., integrity, fluidity, stability, and permeability), which in turn affect fertilization capacity [16]. Moreover, lipid composition can modulate gene expression and metabolism, thereby influencing cellular function [17]. In light of these, the current study examined the association between ejaculates' lipid composition and bulls' age.

Table 4
Correlations between fatty acid profiles and semen parameters in all four semen fractions: intact sperm (A), seminal fluid (B), sperm tail (C), and sperm head (D).

Variable by variable		Young	Mature
		Correlation	Correlation
(A) Intact sperm			
Concentration	SFA	0.10	−0.44**
Concentration	USFA	−0.10	0.44***
Concentration	MUFA	−0.66***	0.39**
Concentration	Omega-6	−0.44**	0.63***
Concentration	Omega-6:3	−0.41*	0.48***
Concentration	c18:1n9/c18:0	−0.88***	0.08
Concentration	c18:1n7/c16:1n7	−0.14	0.53***
Motility	MUFA	0.45***	−0.62***
Motility	Omega-6	0.06	−0.88***
Motility	Omega-6:3	0.38**	−0.65***
Motility	c18:1n7/c16:1n7	0.29	−0.68***
P. motility	MUFA	0.45***	−0.63***
P. motility	Omega-6	0.06	−0.88***
P. motility	Omega-6:3	0.38**	−0.65***
P. motility	c18:1n7/c16:1n7	0.30	−0.69***
Morphology	MUFA	0.46***	−0.71***
Morphology	Omega-6	0.06	−0.93***
Morphology	Omega-6:3	0.38**	−0.73***
Morphology	c18:1n7/c16:1n7	0.29	−0.69***
Morphology	20:0/18:0	0.1	−0.34**
(B) Seminal fluid			
Concentration	c16:1n7/c16:0	−0.42**	0.02
Motility	c18:1n9/c18:0	−0.24	0.34**
P. motility	c18:1n9/c18:0	−0.25	0.35**
Morphology	c18:1n9/c18:0	−0.25	0.42***
MSC	MUFA	−0.52***	0.06
MSC	Omega-3	0.38**	−0.37**
MSC	Omega-6	−0.41**	0.57***
MSC	Omega-6:3	−0.50***	0.47***
MSC	c18:1n9/c18:0	−0.41**	0.27
MSC	c18:1n9/c18:1n7	−0.53***	0.34
PMSC	MUFA	−0.53***	0.05
PMSC	Omega-3	0.38**	−0.38**
PMSC	Omega-6	−0.40**	0.57***
PMSC	Omega-6:3	−0.49***	0.47***
PMSC	c16:1n7/c16:0	−0.44***	0.02
PMSC	c18:1n9/c18:0	−0.42**	0.27
PMSC	c18:1n9/c18:1n7	−0.54***	0.35**
(C) Sperm tail			
Concentration	PUFA	0.59***	−0.03
Concentration	Omega-3	0.65***	−0.08
Concentration	Omega-6:3	−0.49***	0.13
Motility	c18:1n9/c18:0	0.16	−0.44**
Motility	c20:1n9/c18:1n9	−0.42**	0.26
Motility	c18:1n9/c18:1n7	0.28	−0.67***
P. motility	c18:1n9/c18:0	0.15	−0.45**
P. motility	c18:1n9/c18:1n7	0.27	−0.68***
Morphology	c18:1n9/c18:0	0.15	−0.55***
Morphology	c20:1n9/c18:1n9	−0.42**	0.21
Morphology	c18:1n9/c18:1n7	0.27	−0.78***
MSC	Omega-6:3	−0.52***	0.15
MSC	c16:1n7/c16:0	−0.58***	0.01
PMSC	Omega-6:3	−0.50***	0.14
PMSC	c16:1n7/c16:0	−0.57***	0.02
Velocity	c18:1n9/c18:0	0.14	−0.41**
Velocity	c20:1n9/c18:1n9	−0.41**	0.26
Velocity	c18:1n9/c18:1n7	0.26	−0.64***
(D) Sperm head			
Concentration	Omega-6:3	−0.51**	−0.06
Concentration	c18:1n9/c18:0	−0.66***	0.23
Concentration	c18:1n7/c16:1n7	0.50**	0.23
Concentration	c20:0/c18:0	−0.53***	−0.25
Concentration	c18:1n9/c18:1n7	−0.51***	−0.22
Motility	USFA	−0.49**	0.08
Motility	PUFA	−0.50**	0.08
Motility	Omega-3	−0.53**	0.07

(*continued on next page*)

Table 4 (*continued*)

Variable by variable		Young	Mature
		Correlation	Correlation
P. motility	USFA	−0.49**	0.07
P. motility	PUFA	−0.50**	0.06
P. motility	Omega-3	−0.53***	0.04
Morphology	USFA	−0.49**	0.06
Morphology	PUFA	−0.50**	0.05
Morphology	Omega-3	−0.53***	0.03
MSC	Omega-6:3	−0.50**	0.12
MSC	c18:1n9/c18:0	−0.73***	0.12
MSC	c18:1n7/c16:1n7	0.66***	0.36
MSC	c20:0/c18:0	−0.56***	−0.05
MSC	c18:1n9/c18:1n7	−0.53***	−0.12
PMSC	c18:1n9/c18:0	−0.73***	0.12
PMSC	c18:1n7/c16:1n7	0.65***	0.36
PMSC	c20:0/c18:0	−0.55***	−0.04
PMSC	c18:1n9/c18:1n7	−0.52***	−0.11
Velocity	USFA	−0.50**	0.06
Velocity	PUFA	−0.51***	0.06
Velocity	Omega-3	−0.54***	0.04

Examined parameters are motility, progressive motility (P. motility), concentration, morphology, and velocity.
* $P < 0.10$.
** $P < 0.05$.
** $P < 0.01$.

The sperm cell is a unique cell with two main compartments, head and tail, which play different roles in the cascade of events through the fertilization process. Whereas the tail is mostly associated with sperm movement, the head function is associated with acrosome reaction and membrane fusion [18]. Given their specific role and the uneven distribution of lipids between compartments [11,12], we examined lipid composition in the intact sperm, sperm head, sperm tail, and seminal fluid. The findings suggest age-related variations in lipid profile in bulls' semen. Correlation analysis revealed more significant correlations between semen physiological features and lipid composition in young versus mature bulls. These alterations were evident for each of the examined compartments, therefore, suggested to be involved in the mechanism governing the age-related reduction in semen quality.

The findings also suggest that the differences in lipid composition, which constitute the biochemical basis for physiological activities in the functional parts of the semen. The most prominent difference between young and mature bulls was the reduced concentration of myristic acid (c14:0) and the increased concentration of DHA (c22:6n3), which were found in both the seminal fluid and intact sperm. Similarly, we have recently shown season-induced changes in sperm quality characterized by inverse fatty acid patterns between seasons, in both the seminal fluid and sperm [19]. Nevertheless, given the differences in the mode of alteration induced by seasonality and aging, it is possible that such impairments involve different mechanisms.

Beyond their role in the membrane's physical properties [20,21], PUFAs have a variety of bioactivities such as gene expression [22], regulation of protein expression [23], and protein activation [24]. High sperm quality has been associated with a high concentration of long-chain n-3 fatty acids. In particular, DHA is an essential membrane component for the bending and flexing of sperm flagellum, that is, sperm motility [25]. In the present study, a lower concentration of DHA was found in intact sperms of mature bulls. It should be noted, however, that this reduction was mostly attributed to the changes found in the head rather than tail compartments. Moreover, neither the low DHA concentration nor the low proportion of PUFA in the intact sperm was associated with reduced sperm motility or progressive motility in mature bulls. The balance of PUFA, SFA, and cholesterol concentrations directly affects membrane physical properties, such fluidity and fusion. Thus, it is possible that the age-associated alterations in SFA concentration reported for mature bulls impaired sperm membrane fluidity. This in turn might affect Ca^{2+} influx, capacitation, and acrosome reaction through fertilization [17,26,27]. Taken together, it is reasonable to assume that the low proportion of PUFA in the intact sperm of mature bulls reflects, to some extent, semen of inferior quality. Supporting this assumption is the inverse pattern found in young bulls, with increased DHA concentration in the head compartment and higher PUFA concentration in both intact sperm and sperm heads.

PUFAs are the main targets of membrane oxidation. Therefore, the proportion of PUFAs has been suggested as an indicator of semen oxidative state [28]. Although a certain degree of USFA in the membrane is required to maintain cell equilibrium [6,28], a high proportion of PUFA might result in membrane injury upon exposure to oxidative stress. It is therefore suggested that the above-discussed decrease in the total amount of PUFA and DHA indicates a higher oxidative state with aging. An imbalance between pro- and antioxidant factors has been shown to alter sperm quality and is considered one of the features of aging [28,29]. Interestingly, whereas n-3 PUFA concentrations were higher in the tail and head compartments of young bulls, those of omega-6 were higher in mature bulls. Most notably, c22:4n6 was ~ 30% higher in intact sperm, seminal fluid, and the sperm head compartment of mature bulls.

Table 5
Correlations between cholesterol amount and semen parameters in all four semen fractions: intact sperm (A), seminal fluid (B), sperm tail (C), and sperm head (D).

Variable by variable		Young	Mature
		Correlation	Correlation
(A) Intact sperm			
Cholesterol	Vol.	−0.14	0.09
Cholesterol	Conc.	0.72**	−0.14
Cholesterol	M	−0.36	0.62**
Cholesterol	PM	−0.37	0.62**
Cholesterol	Morpho.	−0.37	0.64**
Cholesterol	MSC	0.71*	0.015**
Cholesterol	PMSC	0.73*	0.012**
Cholesterol	Velocity	−0.33	0.61**
(B) Seminal fluid			
Cholesterol	Vol.	−0.03	−0.19
Cholesterol	Conc.	0.54*	−0.44
Cholesterol	M	0.20	0.30
Cholesterol	PM	0.19	0.30
Cholesterol	Morpho.	0.19	0.27
Cholesterol	MSC	0.65*	−0.18
Cholesterol	PMSC	0.66*	−0.19
Cholesterol	Velocity	0.18	0.30
(C) Sperm tail			
Cholesterol	Vol.	−0.02	−0.56**
Cholesterol	Conc.	0.03	−0.24
Cholesterol	M	−0.16	0.22
Cholesterol	PM	−0.16	0.22
Cholesterol	Morpho.	−0.16	0.21
Cholesterol	MSC	−0.02	0.001
Cholesterol	PMSC	−0.02	0.02
Cholesterol	Velocity	−0.14	0.24
(D) Sperm head			
Cholesterol	Vol.	−0.37	0.004
Cholesterol	Conc.	0.01	−0.15
Cholesterol	M	0.11	−0.03
Cholesterol	PM	0.10	−0.007
Cholesterol	Morpho.	0.11	0.005
Cholesterol	MSC	0.10*	−0.20
Cholesterol	PMSC	0.08*	−0.16
Cholesterol	Velocity	0.13	−0.01

Abbreviations: Vol., volume; Conc. (M/mL), concentration; M, motility; PM, progressive motility; Morpho, morphology.
* $P < 0.05$.
** $P < 0.01$.

Both n-3 and n-6 PUFA subfamilies are subjected to the same metabolic pathways in the cell. However, it should be pointed out that the structural differences are those that determine their metabolic status, metabolites' potency, and bioactivity (e.g., n-6 and n-3 prostaglandins) [22]. The omega-6:3 is considered to be a major indicator of sperm quality in humans [30], therefore, suggested to be involved in the reduction in sperm quality with age. In the present study, a higher n-3 and lower n-6 PUFA concentration was found in young compared with mature bulls, also reflected in a lower ratio between omega-6 and omega-3 in all the examined fractions.

Elongation and desaturation processes can modulate cellular fatty acid composition by increasing the carbon chain lengths and saturation levels of fatty acids. The ratios between the product and substrate concentrations in these processes are commonly used as activity indicators for elongase and desaturase enzymes [15]. In intact sperm of mature bulls, the values of the elongase activity indicators values were higher than in young bulls, whereas in young bulls, those of desaturase were higher. Increased desaturase activity might underlie the higher concentration of oleic and erucic acids (c18:1n9 and c22:1n9, respectively) in the intact sperm and sperm tails of young bulls. In support, a high concentration of oleic acid might improve acrosome reaction in young bulls, as previously reported for boar semen [27]. On the other hand, incubation of boar semen with oleic fatty acid improves sperm motility [27]. Nevertheless, in the current study, such an alteration was not associated with better motility or progressive motility in young bulls.

Cholesterol is a key factor in semen quality and function. For example, a high amount of cholesterol can disrupt sperm maturation and impair its membrane's fluidity, which in turn negatively affects sperm flexibility and motility, in particular, its hyperactivity following capacitation [26,31]. In the present study, a higher cholesterol concentration was found in the tail compartment of mature bulls. This difference may be reflected in the tendency toward lower motility found in mature compared with young bulls.

In summary, the findings indicate age-associated alterations in semen lipid profile. Analysis of sperm compartments revealed a decrease in the total amount of PUFA and DHA in the intact sperm, sperm heads, and seminal fluid of mature bulls, suggesting a high oxidation process. Such alterations might affect the semen's capacity to successfully undergo the cryopreservation procedures, which are widely used in intensive reproduction management; as such, they might also affect fertilization competence. This information is highly relevant in supporting the use of semen from young versus mature bulls for each subpopulation of cows, that is, heifers, primiparous, and multiparous cows. Further *in-vitro* and *in-vivo* fertilization studies are required to confirm the association between age, sperm membrane lipid composition, and fertilization competence.

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