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Dietary polyunsaturated fatty acid supplementation of young post-pubertal dairy bulls alters the fatty acid composition of seminal plasma and spermatozoa but has no effect on semen volume or sperm quality

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Abstract

The aim of this study was to examine the effects of dietary supplementation with rumen protected n-6 or n-3 polyunsaturated fatty acids (PUFA) on the quantity and quality of semen from young post-pubertal dairy bulls. Pubertal Holstein-Friesian (n=43) and Jersey (n=7) bulls with a mean ± s.e.m. age and bodyweight of 420.1 ± 5.86 days and 382 ± 8.94 kg, respectively, were blocked on breed, weight, age and semen quality (based on the outcomes of two pre-trial ejaculates) and randomly assigned to one of three treatments: (i) a non-supplemented control (CTL, n=15), (ii) rumen-protected safflower (SO, n=15), (iii) rumen-protected n-3 PUFA-enriched fish oil (FO, n=20). Bulls were fed their respective diets, *ad libitum* for 12 weeks; individual intakes were recorded using an electronic feeding system for the initial 6

weeks of the feeding period. Semen was collected via electro-ejaculation at weeks -2, -1, 0, 7, 10, 11 and 12 relative to the beginning of the trial period (week 0). On collection, semen volume, sperm concentration and progressive linear motility (PLM) were assessed. On weeks -2, -1, 0, 10, 11, 12, semen was packaged into 0.25 mL straws and frozen using a programmable freezer. On weeks -1, 7 and 11; a sub-sample of semen was separated into sperm and seminal plasma, by centrifugation and stored at – 20˚C until analysis of lipid composition. Semen from 10 bulls per treatment were used for post-thaw analysis at weeks 10, 11 and 12 (3 straws per ejaculate). Sperm motility was analysed by computer assisted semen analysis (CASA). In addition, membrane fluidity, acrosome reaction and oxidative stress were assessed using flow cytometry. Sperm from bulls fed SO had a 1.2 fold higher total n-6 PUFA content at week 11 compared to week -1 ($P<0.01$) while bulls fed FO had a 1.3 fold higher total n-3 PUFA content, in sperm by week 11 ($P<0.01$). There was no effect of diet on semen volume, concentration or PLM of sperm when assessed either immediately following collection or post-thawing. Membrane fluidity and oxidative stress of sperm were also not affected by diet. The percentage of sperm with intact-acrosomes was lower in CTL bulls compared to those fed SO ($P<0.01$). In conclusion, while the lipid composition of semen was altered following dietary supplementation with either n-6 or n-3 based PUFA, this did not lead to measurable improvements in the quantity or quality of semen produced by young post-pubertal dairy bulls.

Keywords: Fertility, Semen, PUFA, Lipid composition, Fish oil, Safflower.

1. Introduction

Polyunsaturated fatty acids (PUFA) are important components of cell membranes, and play an integral role in oocyte fertilization [1]. Fertile mammalian spermatozoa are characterized by a higher proportion of PUFA compared to saturated fatty acids (SFA) [2]. Sperm utilise PUFA, in particular n-3 PUFA, to maintain membrane fluidity required for normal cell function [3]. Ruminants cannot synthesize n-6 or n-3 PUFA *de novo* as they lack the necessary fatty acid (FA) de-saturase enzymes. Thus, these animals must obtain PUFA, or their pre-cursors, from dietary sources [4]. Diet-derived PUFA are known to have positive effects on FA composition of spermatozoa in humans [5] as well as a variety of farm animals including pigs [6], sheep [7] and cattle [8]. In order to ensure that sufficient PUFA bypass the ruminal microbial mediated bio-hydrogenation process, they must be chemically protected [9].

Genomic selection has led to more accurate identification of elite sires, resulting in increased demand for their semen at a much younger age. This demand necessitates that bulls reach puberty as early as possible and produce an adequate volume of high quality semen, to meet this demand. Dietary supplementation with n-3 PUFA, derived from fish oil (FO) has been reported to improve certain semen parameters including sperm concentration in rams [7], as well as progressive motility and percentage of normal sperm in boars [10]. Other studies [6] however, found no improvement in semen quantity or quality in boars. Similarly, there are conflicting data from bulls in the literature regarding the effects of dietary n-3 PUFA supplementation.

The motility of fresh semen was improved in bulls supplemented with dietary DHA but there was no improvement detected in frozen-thawed semen in the same study [12]. Positive effects on progressive motility, morphology and viability in frozen-

thawed sperm following FO supplementation of bulls [13], have also been reported. In contrast, supplementation of bulls with linolenic acid, a n-3 PUFA, using linseed oil, resulted in no improvement in fresh semen quality but did improve plasma membrane integrity post-thawing [14].

Although some positive effects of PUFA supplementation on semen quality have been detected, increasing dietary PUFA intake can also cause vulnerability of spermatozoa to reactive oxygen species (ROS) damage, leading to an increase in lipid peroxidation [15]. In humans, increased levels of lipid peroxidation have been associated with loss of sperm motility [16] and thus is likely to have a negative impact on fertility. Increases in oxidative stress are also associated with DNA damage [17] and damage to DNA of spermatozoa can reduce fertilizing ability as well as leading to an increase pre-implantation early embryo loss [18]. In addition, a significant reduction in sperm PUFA concentration, particularly in docosahexaenoic acid (DHA; C22:6n-3), has been reported with increasing age In bulls [11]. This has stimulated commercial interest in the use of dietary supplementation to alter the PUFA content of sperm, and increase reproductive potential.

Given the conflicting nature of in the published literature on the consequences of dietary PUFA supplementation on semen characteristics of cattle, the aim of this study was to examine the effects of dietary rumen-protected n-6 and n-3 PUFA on semen quantity and quality in young post-pubertal dairy bulls.

2. Material and Methods

All animal procedures performed in this study were conducted under experimental licence from the Irish Department of Health and Children (licence number B100/2869). Protocols were in accordance with the Cruelty to Animals Act (Ireland

1876, as amended by European Communities regulations 2002 and 2005) and the European Community Directive 86/609/EC.

2.1. Animal Management

Holstein-Friesian (n=43) and Jersey (n=7) bulls with a mean ± s.e.m. age and bodyweight of 420.1 ± 5.86 days and 382.0 ± 8.94 kg, respectively, were blocked on breed, weight, age and semen quality (based on the outcomes of two pre-trial ejaculates) and randomly assigned to one of three concentrate-based dietary treatments (Table 1), namely: (i) a non-supplemented control (CTL, n=15), (ii) rumen-protected safflower (Safflower; SO, n=15), or (iii) rumen-protected n-3 PUFA-enriched FO (Incromega; FO, n=20). Both fat supplements were supplied by Trouw Nutrition; Belfast, Ireland. All diets were isonitrogenous and isocaloric (Table 2). Animals were housed in a concrete slatted floor shed and individually fed using an electronic feeding system (Calan Inc., Northwood, NH, USA) for the initial six weeks of the feeding period, followed by group feeding (5 bulls per treatment/pen), for the remaining six weeks. Animals were allowed two weeks to acclimatise to the individual feeding facility followed by ten days acclimatisation to their respective diets and were then offered diets *ad libitum* for 12 weeks. All animals received 5 kg (fresh weight) of grass silage daily.

2.2. Semen collection

Semen collections were carried out in the summer, between June and August. Semen was collected using the trans-rectal electro-ejaculation (Pulsator, Lanes, CO, USA) technique [19] at weeks -2, -1, 0, 7, 10, 11 and 12 relative to the beginning of the trial period (week 0.). Following collection, semen volume was recorded and progressive linear motility (PLM) was assessed subjectively using a phase contrast

121 microscope incorporating a heated stage at 37 ^{o}C (100 sperm per assessment).
122 Spermatozoa concentration was assessed using a photometer (Minitub, Tiefenbach,
123 Germany). On weeks -2, -1, 0, 10, 11, and 12, semen was diluted to 80 x 10^6 sperm
124 per mL in Bioxcell (IMV, L'Aigle, France) and loaded into 0.25 mL straws (IMV).
125 Straws were cooled gradually from room temperature to 4˚C over a period of 90 min
126 and allowed to equilibrate at 4˚C for 3 h. They were then frozen to -140˚C over a 9
127 min period (-15.5˚C/min) in a programmable freezer (Planar, Birmingham, UK)
128 followed by immersion and storage in liquid nitrogen, pending further laboratory
129 analysis. At weeks -1, 7 and 11 a sub-sample of fresh semen was centrifuged at
130 2000 g for 10 minutes at 4˚C. The seminal plasma (SP) was removed and the sperm
131 pellet was resuspended in 3 mL of phosphate buffered saline (PBS) and centrifuged
132 at 2000 g for 10 minutes at 4˚C. The supernatant was again removed and the sperm
133 pellet was resuspended in 500 mL of cold PBS. Both SP and sperm were snap-
134 frozen in liquid nitrogen, and stored at -80˚C. For post-thaw semen assessments and
135 lipid analysis, straws from 10 bulls were selected from each dietary treatment.
136 Selection was based on bulls with the most consistent feed intake pattern during the
137 six weeks of individual feed intake recording.

138 **2.3. Post-thaw semen analysis**

139 Straws were thawed at 37˚C for 30 seconds. Following thawing, post-thaw motility
140 and kinematic parameters (n=3 straws assessed per ejaculate per bull) were
141 measured out using computer-assisted semen analysis (CASA, Sperm Class
142 Analyser, Microptic S.L., Barcelona, Spain). After a 1:1 dilution, in PBS, 5 µl of
143 semen was placed on a pre-warmed glass slide, covered with a pre-warmed cover
144 slip and viewed using a phase-contrast microscope at 100X fitted with a pre-warmed

145 stage at 37˚C. A minimum of five microscopic fields were analysed in each sample
146 and objects incorrectly identified as sperm were edited out using the playback
147 function. The CASA derived motility characteristics assessed were total motility (%)
148 and progressive motility (%), while the kinematic parameters were average path
149 velocity (VAP, µm/s,), straight-line velocity (VSL, µm/s,), curvilinear velocity (VCL,
150 µm/s,), amplitude of lateral head displacement (ALH, µm,), beat/cross frequency
151 (BCF, Hz,), straightness (STR) and linearity (LIN).

152 **2.4. Feed sample collection and analysis**

153 Samples of the treatment rations as well as silage were collected weekly and stored
154 at -20˚C. Weekly samples were then composited into monthly samples and sub-
155 sampled. Sub-samples were used to determine dry matter content by drying the
156 ration at 98˚C for 16 h and silage at 85˚C for 16 h. A second sub-sample was dried
157 at 40˚C for 48 h for chemical analysis. Both silage and ration samples were milled
158 through a 1 mm sieve and subsequently analysed for crude protein, acid detergent
159 fibre, neutral detergent fibre, ash, ether and gross energy. Crude protein was
160 determined using the method of [20] with a Leco FP 528 nitrogen analyser (Leco
161 instruments UK Ltd, Cheshire, UK). Acid detergent fibre and neutral detergent fire
162 were determined using the Ankom method (Ankom Technologies, NY, USA). Ash
163 was determined after ignition of a known weight of ground sample in a furnace
164 (Carbolite Gero, Derbyshire, United Kingdom) at 550˚C for 4 h. The gross energy of
165 diets and silage samples was determined using an adiabatic bomb calorimeter (Parr
166 Instruments, IL, USA). The remaining undried composite was used for FA analysis
167 (Table 3).

168 **2.5. Fatty acid analysis of feed, sperm and seminal plasma**

169 Fatty acid analysis was conducted following extraction of total lipid, using gas liquid
170 chromatography (GLC; Thermo Fisher Trace, Hemel Hempstead, Hertfordshire, UK)
171 procedures. Briefly, total lipids were extracted from the full re-suspended sperm
172 pellet, 1 mL of SP and 10 g of both feed samples, according to the method of Folch
173 et al., [21] which removes non-lipid impurities. Fatty acid methyl esters (FAME) were
174 prepared by acid-catalysed trans esterification of total lipids according to the method
175 of Christie et al. [22]. Extraction and purification of FAME was performed as
176 described by Ghioni et al. [23]. FAME were separated by GLC fitted with a flame
177 ionisation detector using 60 m × 0.32 mm i.d. × 0.25 µm film thickness capillary
178 column (ZB Wax; Phenomenex, Macclesfield, Cheshire, UK) and hydrogen as a
179 carrier gas (4.0mL/min). The column oven temperature gradient was from 50 to
180 150˚C at 40˚C/min and then to 195˚C at 1.5˚C/min and finally to 220˚C at 2˚C/min.
181 Individual methyl esters were identified by reference to published data (Ackman,
182 1980). Data were collected and processed using the Chromcard for Windows
183 (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy). The
184 percentage of individual FA was calculated according to the area of an individual
185 peak relative to the total area. All FA data are presented as means ± s.e.m in
186 percentage (%) of total FA.

187 **2.6. Flow cytometry analysis**

188 Flow cytometry was used for assessment of sperm for specific intracellular markers
189 of viability, membrane fluidity, acrosome integrity and oxidative stress, in frozen-
190 thawed samples from weeks 10, 11 and 12 of feeding. Samples were diluted to a
191 concentration of 3 x 10^5 /spermatozoa per mL in PBS and were analysed on a flow
192 cytometer (Guava easyCyte 6HT-2 L; Merck Millipore Billerica, MA, USA) equipped

193 with both a krypton laser (642 nm) and an argon laser (488 nm). Appropriate single-
194 colour controls were prepared to establish the respective fluorescent peaks of the
195 individual stains. These were used in conjunction with the forward scatter (FSC) and
196 side scatter (SSC) signals to discriminate sperm from debris in a population known
197 as P01.Population. Fluorescent events were recorded using GuavaSoft (Version 2.7;
198 Merck Millipore) and all variables were assessed using logarithmic amplification. In
199 each sample 10,000 gated events were captured.

200 **2.6.1. Membrane fluidity**

201 Membrane fluidity was assessed using a dual staining protocol using Yo-Pro-1 (YP;
202 Ex/Em 491⁄509; Life Technologies, Carlsbad, CA, USA) and Merocyanine 540
203 (M540; Ex/Em 540/578 nm; Sigma-Aldrich), adapted from Murphy *et al.* [24]. Yo-Pro
204 identifies apoptotic cells via green fluorescence visible on the green detector (525/30
205 nm band pass (BP). Merocyanine 540 preferentially binds to highly disordered lipids,
206 thus indicating increased membrane fluidity. Emission spectra for M540 are visible in
207 the yellow detector (583/23 nm BP). Samples were first incubated with YP in the
208 dark at 32˚C for 10 min and a final working concentration of 25 nM. M540 was then
209 added at a final working concentration of 10 µM. Samples were then incubated in the
210 dark at 32˚C for 15 min. Sperm with high membrane fluidity were defined as cells
211 negative for YP and positive for M540 (M540 +ve/ YP -ve) and calculated as a
212 percentage of the total viable population (YP –ve population). Membrane fluidity was
213 calculated as the percentage of M540-positive sperm of the Yo-Pro-1-negative
214 population, as initially gated as P01.Population, based on controls, FSC and SSC.

215 **2.6.2. Acrosome integrity and viability**

Acrosome status was assessed using the fluorescent stain Alexa Fluor 647 PNA (AF647; Ex/Em 650/668 nm; Life Technologies) and a method adapted from Murphy *et al.* [25]. AF647 consists of an AF647 fluorochrome conjugated with lectin PNA from *Arachis hypogaea* (peanut). Briefly, AF647 was added to 500 µL of sperm diluted to 3 x 10^5 sperm per mL in PBS to give a final concentration of 6 mg/mL and was then incubated at 37˚C for 15 min. Peanut agglutinin binds to the inner surface of the outer acrosomal membrane, which is only accessable post acrosome reaction as reviewed by Petrunkina and Harrison [26] . Following this, the nuclear stain SYTO16 (S16; Ex/Em 488/518; Life Technologies) was added at a final working concentration of 100nM and incubated for 10 min. Finally, the fluorescent stain propidium iodide (PI; Ex/Em; 493/636; Life Technologies) was added to the sample at a final concentration of 12 mM and incubated for a further 5 min at 37˚C. PI is selectively taken up by membrane-compromised cells, thus indicating a loss of viability. The fluorescence of AF647 was analysed via the red2 (664/20 BP) detector; S16 fluorescence was measured via the green detector (525/30 nm BP) and PI via the yellow detector (583/26 BP). No compensation was needed. The percentage of viable sperm with intact acrosomes was calculated as the percentage of AF647-negative cells of the PI-negative S16-positive (AF647 –ve/ S16 +ve/ PI –ve) population, as initially gated, as P01.Population, based on controls, FSC and SSC (Figure 3).

2.6.3. Oxidative stress

The generation of the superoxide anion was assessed using the fluorescent stain MitoSOX Red (MSXR; Ex/Em 510/580; Life Technologies) using a method adapted from Kiernan *et al.* [27]. Briefly, diluted samples were incubated at 37˚C in the

presence of MSXR (4 mM) for 15 min. MSXR is an intracellular stain that fluoresces in the presence of the superoxide anion. Following this, the nucleic dead stain YP was added to give a final concentration of 25 nM and again, incubated at 37˚C in the presence of MSXR for 15 min. The fluorescence of MSXR was analysed via the red (690/50 BP) detector and YP via the green detector (525/30 BP). Minor computed compensation was carried out. The presence of superoxide was calculated as the percentage of MSXR positive sperm of the YP negative (MSXR +ve/ YP –ve) population, as initially gated, as P01.Population, based on controls, FSC and SSC.

2.7. Statistical analysis

Data were analysed using appropriate procedures of Statistical Analysis Software (SAS version 9.3, Cary, NC, USA). Data were tested for normality (UNIVARIATE procedure) and, where appropriate, transformed to the power of lambda (TRANSREG procedure). Data were analysed using ANOVA (MIXED procedure). Diet, block, sampling time and their interactions, were included in the model. The interaction term, if not statistically significant ($P > 0.05$), was subsequently excluded from the final model. The covariance matrix was determined for each variable by examining the Bayesian Information Criteria (**BIC**) (smaller is better) value. Animal was the experimental unit. Sampling time (week of collection) was included in the statistical models as a repeated term. Multiple regression analysis was used (REG and STEPWISE procedure) to identify statistically significant predictor variables for concentration, motility of both fresh and frozen-thawed semen and all parameters measured by flow cytometer. The fixed effects of diet and week were corrected for in the model. Dietary total n-6 and n-3 intake, percentage lipid content of total n-3 and n-6, n-6 to n-3 ratio and DHA content of both sperm and SP were used as

264 independent variables. Multiple regression analysis was also used to identify suitable
265 predictor variables for total n-3 and n-6 PUFA content of sperm using total saturated,
266 monounsaturated, n-3 and n-6 intakes as independent variables, The analysis was
267 conducted separately for each of the three timepoints (weeks -1, 7, 11). All results
268 are presented as mean ± s.e.m., unless otherwise stated.

269 ## 3. Results

270 ### 3.1 Animal performance and intake

271 There was no difference in concentrate intake during the first six weeks of the trial
272 period across the three diets with bulls on the CTL, SO and FO diets consuming 9.54
273 ± 0.37 kg, 9.54 ± 0.31 kg, 9.34 ± 0.35 kg DM per day, respectively. Similarly, there
274 was no difference in average daily gain between diets, with CTL, SO and FO bulls
275 gaining 1.4 ± 0.19, 1.4 ± 0.17 and 1.6 ± 0.29 kg per day, respectively. There was no
276 effect of diet on FCE during the initial six weeks of supplementation (CTL, SO and
277 FO: 0.13 ± 0.008, 0.13 ± 0.009 and 0.13 ± 0.009 kg liveweight per kg of concentrate
278 consumed, respectively).

279 ### 3.2. Seminal plasma and sperm fatty acid concentration

280 The effects of diet on the FA concentration of spermatozoa and SP are presented in
281 Tables 4 and 5, respectively. In the interest of brevity, only FA which have a positive
282 role in fertility or contribute substantially to the overall FA composition are reported in
283 the text.

284 There was no diet by week interaction for concentration of the various
285 saturated fatty acids (SFA; Table 4) measured in spermatozoa, nor was there any
286 effect of diet. Week affected concentration of SFA ($P<0.01$) in spermatozoa, with the

concentration of most SFA declining, across treatment, from weeks -1 to 7 and remaining at this level to week 11, with the exception of arachidic acid which did not decline until week 11. Concentrations of myristic acid in spermatozoa increased by week 7 ($P<0.001$) and remained elevated to week 11. Total SFA in spermatozoa declined ($P<0.001$) from weeks -1 to 7 and then plateaued.

In SP, there was a diet by week interaction detected for myristic acid (Table 5. $P<0.01$); bulls fed the FO diet had higher myristic acid on week 11 in comparison to those fed the CTL diet ($P<0.001$). On week 7, bulls fed the FO diet also tended to have higher myristic acid ($P=0.06$) than bulls fed the SO diet. There was also a diet by week interaction for arachidic acid ($P<0.001$) in SP. A higher level of arachidic acid ($P<0.01$) was observed in SP of bulls fed both the CTL and SO diets compared to those fed the FO diet on week -1; this difference was no longer evident on weeks 7 or 11. There was a strong tendency ($P=0.06$) for SP concentrations of palmitic acid to be higher at week -1 than on week 11. There was no effect of diet or week of sampling on total SFA concentrations of SP.

There were no diet by week interactions for the various *monounsaturated fatty acids (*MUFA; Table 4;), assessed in spermatozoa. Palmitoleic (n-7), palmitoleic (n-9) and oleic acid decreased from weeks -1 to 11 ($P<0.001$). There was a quadratic effect of week on vaccenic and nervonic acid ($P<0.01$); concentration of sperm vaccenic acid increased from weeks -1 to 7 and then decreased from weeks 7 to 11. The opposite trend was observed for sperm nervonic acid concentration. There was a linear decrease in sperm total MUFA ($P<0.01$) from weeks -1 to 11.

Monounsaturated fatty acids in SP were unaffected by diet and week with the exception of oleic acid which decreased from weeks -1 to 11 ($P<0.05$). This

311 difference contributed to a tendency for total MUFA in SP to decrease from weeks -1
312 to 11 (P=0.09).

313 In spermatozoa, there was a diet by week interaction for eicosadienoic acid
314 (P<0.01). This PUFA was higher in SO bulls than in CTL bulls at weeks 7 and 11
315 (P<0.05), but concentrations for both diets were similar for FO. There was a diet by
316 week interaction for adrenic acid concentration in spermatozoa (P<0.001). Bulls on
317 SO had a higher concentration of adrenic acid than either CTL or FO at week 11
318 (P<0.001). There was also a diet by week interaction for docosapentaenoic acid
319 (DPA, n-6) concentration (P<0.001). Bulls on CTL had higher concentrations of DPA
320 (n-6) in sperm on weeks -1 and 7 compared to FO (P<0.001), while SO had higher
321 DPA (n-6) in sperm, at week 11 than either the CTL (P<0.05) or FO (P<0.001) bulls.
322 There was a diet by week interaction for total n-6 PUFA concentration in sperm
323 (P<0.001). Total n-6 PUFA concentrations were higher in CTL bulls compared to FO
324 bulls at week 11 (P<0.001). The SO bulls tended to have higher total n-6 PUFA
325 concentrations in spermatozoa on week 7 compared to FO bulls (P=0.09); this
326 difference reached statistical significance on week 11 (P<0.001). Stepwise
327 regression models using total saturated, monounsaturated, n-3 and n-6 PUFA
328 intakes as independent variables showed that there is an increase in the amount of
329 variation of total n-6 PUFA content in sperm, that can be explained overtime (Table
330 6). On week -1; none of the variability was accounted for. However, by week 7 total
331 n-3 PUFA intake accounted for 37% of the variability in total n-6 PUFA content of
332 sperm. By week 11, n-3 PUFA and MUFA intake account for 68 and 6% of the
333 variation in total n-6 PUFA content of sperm, respectively.

334 In SP, there was a diet by week interaction for adrenic acid (P<0.001) as a
335 result of higher concentrations on week 11, in both CTL and SO bulls compared to

FO bulls (P<0.001). The CTL and SO bulls were not different. There was also a diet by week interaction for DPA (n-6) in SP (P<0.001). The DPA (n-6) concentration was lower in FO bulls on weeks 7 and 11 compared to either CTL or SO bulls. There was a diet by week interaction for total n-6 PUFA (P<0.001), in SP. Overall n-6 concentrations were lower in FO on weeks 7 (P<0.05) and 11 (P<0.001) compared to either CTL or SO. There was an effect of diet on SP dihommo-gamma-linolenic acid (DGLA; P<0.05). Bulls on CTL had higher DGLA concentrations than those on FO (P<0.05); SO were different to either of these diets. The concentrations of eicosadienoic, ɣ-linolenic and arachidonic acids in SP were affected by week (P<0.01) as the latter two both declined from week -1 to week 11, across diets, while concentrations of eicosadienoic increased in the same period (Table 4).

There was a diet by week interaction detected for sperm DPA (n-3) (P<0.001). Sperm from bulls fed the FO diet had higher concentrations on weeks 7 and 11 in comparison to the CTL (P<0.01) and SO (P<0.001) bulls. At week 7 and 11, eicosapentoenoic (EPA) was undetectable in sperm from either CTL or SO bulls, while low concentrations were detected in FO bulls. There was also a diet by week interaction for sperm DHA, with a higher concentration detected for FO than CTL (P<0.01) or SO (P<0.001) bulls on week 11 but no difference, between diets, detected at either weeks -1 or 7. There was an effect of week on concentrations of linolenic acid (P<0.001), which decreased from weeks -1 to 11. There was a diet by week interaction for total n-3 PUFA (P<0.05) with bulls on FO having a higher overall n-3 PUFA concentrations in comparison to CTL (P<0.01) or SO (P<0.001) bulls on week 11, but again no difference between diets at either weeks -1 or 7.

There was a diet by week interaction for SP concentrations of DPA (n-3; P<0.001). Concentrations of DPA (n-3) were higher in CTL bulls at week -1 than

361 those on FO (P<0.05); however, by week 11 this had reversed and FO had higher
362 DPA (n-3) than CTL bulls (P<0.001). The FO bulls had higher DPA (n-3) than the SO
363 bulls at weeks 7 and 11 (P<0.01). There was a diet by week interaction for SP
364 concentration of DHA (P<0001). Bulls fed FO had higher DHA on week 7 (P<0.05)
365 and on week 11 (P<0.001) compared to those on either CTL or SO diets. There was
366 a tendency for an interaction of diet by week for EPA concentration in SP (P=0.09).
367 Bulls fed FO had higher concentrations of EPA compared to SO bulls on week 7
368 (P<0.01) and tended (P=0.06) to have higher EPA compared to CTL bulls at the
369 same time-point. There was an effect of diet (P<0.05) on linolenic acid concentration
370 of SP with FO tending to have higher linolenic acid than CTL (P=0.09) or SO
371 (P=0.06) bulls. There was a diet by week interaction for total n-3 PUFA (P<0.001)
372 manifested as bulls on FO having higher concentrations of n-3 PUFA on weeks 7
373 (P<0.05) and 11 (P<0.001) compared with those on the CTL and SO diets.

374 There was a diet by week interaction for n-6 to n-3 ratio (P<0.001). The ratio
375 of n-6 to n-3 FA was lower in FO on week 11 compared to either CTL or SO bulls
376 (P<0.001), consistent with the design of the study. Also, at week 11 the n-6 to n-3
377 ratio in sperm tended (P=0.06) to be lower in FO compared to CTL bulls. In SP, there
378 was an interaction of diet by week for n-6 to n-3 ratio (P<0.05); FO supplementation
379 led to a significant drop in n-6 PUFA concentration, evidenced by lower n-6 to n-3
380 ratio on weeks 7 (P<0.05) and 11 (P<0.001) in FO bulls compared to either CTL or
381 SO bulls. There was no diet by week interaction or effect of diet on total PUFA
382 concentration in spermatozoa. However, there was an effect of week (P<0.001); total
383 spermatozoa PUFA concentration increased from weeks -1 to 7 (P<0.01) and
384 remained at this level until week 11.

385 There was no diet by week interaction or effect of week on total PUFA in SP
386 There was an effect of diet (P<0.05); total PUFA were lower in CTL (P<0.05) and
387 tended to be lower in SO (P=0.09) in comparison to FO bulls (Table 5).

388 Stepwise regression models using total saturated, monounsaturated, n-3 and
389 n-6 intakes as independent variables shows that there is an increase in the amount
390 of variability in total n-3 PUFA content in sperm, that can be explained overtime
391 (Table 6). At week 1 none of the variability can be accounted for however by week 7
392 total n-3 PUFA intake accounts for 27%. At week 11 both n-3 PUFA and MUFA
393 intake account for 60 and 7% of the variation in total n-3 PUFA content in sperm,
394 respectively.

395 **3.4. Fresh semen assessment**

396 There was no diet by week interaction or effect of diet on semen volume, sperm
397 concentration or PLM. After decreasing from weeks -2 to -1 and 0 (P<0.05) both
398 semen volume (Figure 1) and concentration (Figure 1) increased again by week 10,
399 remaining at this level to weeks 11 and 12. Week of collection also had an effect on
400 PLM (P<0.01; Figure 1); PLM increased from weeks -2 to -1 and bulls maintained
401 this level of PLM for the remainder of the experiment.

402 **3.5. Post-thaw semen assessment**

403 There was no effect of diet on post-thaw spermatozoa total motility using
404 CASA; Table 7). There was an effect of week (P<0.05) on PLM and motility which
405 were higher on week 12 compared to weeks 10 or 11. There was no effect of week
406 or week by diet interaction on VCL, VSL, VAP, LIN, STR, ALH or BCF. Higher ALH
407 was recorded when bulls were offered the SO diet compared to the CTL (P<0.05).

Stepwise regression models, show that total n-6 PUFA intake explained 9% of the variability in both total and PLM, post-thaw motility (Table 8).

There was an effect of diet on the percentage of viable spermatozoa post-thawing ($P<0.05$). Bulls on SO tended to have a higher percentage of viable cells compared to those on CTL at week 10 ($P=0.06$; Figure 2(a)), but similar to FO. By week 11 all diets were the same.. There was no diet by week interaction, nor was membrane fluidity of spermatozoa affected by diet or week (Figure 2(b)). There was a diet by week interaction on the percentage of live spermatozoa with intact acrosomes ($P<0.01$; Figure 2(c)). At week 10 both SO and FO bulls had a higher percentage of acrosome-intact spermatozoa compared to the CTL bulls. This difference between CTL and SO bulls remained until week 11; however there were no differences in acrosome status, between diets on week 12 of the study. There was no effect of diet, week or their interaction on oxidative stress (Figure 2(d)).

Stepwise regression, using FA intake and sperm FA composition as independent variables showed that 38% of the variability in viability, 27% in acrosome integrity and 21% membrane fluidity could be explained (Table 8). The n-6 PUFA intake of bulls accounted for 18 and 27% of the variation in viability and acrosome integrity, respectively. The DHA composition of SP tended to accounted for 20% ($P=0.09$) of variability in viability, while a small but statistically significant portion of the variability in membrane fluidity was explained by the n-6/n-3 ratio in sperm (8%) and by dietary n-3 intake (13%).

4. Discussion

This study shows that dietary supplementation with SO and FO alters the n-6 and n-3 PUFA composition, respectively, of spermatozoa and SP of young post-pubertal,

dairy bulls. However, these changes were not associated with improvements in the quantity of semen produced or quality of either fresh or frozen-thawed spermatozoa.

Before assessing the effect of dietary supplementation with specific nutrients on semen quality, it is important to quantify the level of incorporation of the biochemical of interest into the spermatozoa. It is also important to consider the duration of the spermatogenesis cycle (61 days) of the bull [28] in order to allow adequate time for PUFA supplementation to have an effect on all stages of developing spermatozoa. In the current study, the change in FA composition of both the spermatozoa and SP has been described in detail. Total SFA concentration of either spermatozoa or SP was not altered by supplementation with either SO or FO, when compared to the CTL. However, across diets, total SFA concentration in sperm decreased 1.2-fold between weeks -1 and 11, with the decrease first evident on week 7. It has been shown that changes in sperm FA composition following dietary supplementation of bulls can take up to 35 days [29]. However, the change in SFA concentrations in the current study is in contrast to the findings of the previous study, where FO, flaxseed and vegetable oil (high in C16:0) were fed to mature (6 yrs.) and semi-mature (2 yrs.) bulls and none of these supplements resulted in a change in total SFA concentration [29].

The overall 1.8-fold decrease in total MUFA in sperm in the current study is higher than that observed in a similar study in pigs (1.2-fold decrease) when the n-6: n-3 ratio was also altered [30]. Few changes were detected in MUFA composition of SP; only oleic acid exhibited a significant (2.2-fold) decrease; following an alteration of the dietary n-6 to n-3 ratio. In the small number of other studies in bulls and pigs where SP MUFA composition has been quantified, none report changes over time and, in many, oleic acid was the only MUFA detectable [6, 14].

457 There are very few studies in which n-6 PUFA have been supplemented to
458 ruminants and effects on semen quantity and quality assessed. In one such study
459 [31], in which rams were fed sunflower oil as a source of n-6 PUFA, the level of
460 incorporation into animal tissues was not reported. In our study, the concentration of
461 n-6 PUFA in spermatozoa was higher in CTL bulls on week 7 and SO bulls at week 7
462 and 11 compared to FO bulls in which a 1.5-fold decrease was observed by week 11
463 compared to week -1. By week 11; 74% of the variation in total n-6 PUFA in sperm
464 could be explained by the dietary intake of n-3 PUFA and MUFA. The rise in n-6
465 PUFA in sperm from bulls on SO was more modest than expected given that the
466 linoleic acid was included in the SO diet at almost twice the level of either CTL or FO
467 diets. The level of DPA (n-6) in the SO diet was similar to that of linoleic acid and
468 incorporation of DPA (n-6) into both spermatozoa and SP was much greater than for
469 linoleic acid. It is likely that the dietary linoleic acid consumed underwent elongation
470 to synthesize DPA [32].

471 Total n-3 PUFA concentrations increased throughout the dietary supplementation
472 period, in both spermatozoa and SP, with the increase being highest on week 11 in
473 FO bulls, compared with either CTL or SO bulls. Regression models also show this
474 increase over time as the explanation of variation increases from 0 to 67% between
475 weeks -1 and 11 for total n-3 PUFA in sperm; explained by total n-3 PUFA and
476 MUFA intake. Alpha-linolenic acid (ALA), also a precursor of long-chain n-3 PUFA,
477 decreased suggesting that this FA was used to synthesize both DPA (n-3) and DHA.
478 Changes over time in ALA following FA supplementation have not been well
479 documented. In rams, no change in sperm ALA concentrations was found following
480 FO supplementation [7]. This is in contrast to the findings of the current study where
481 there was a 3.6-fold reduction in ALA across all diets.

We observed a 10% increase in spermatozoa DHA concentration from weeks 1 to 11 when FO was fed to bulls which resulted in 6% higher DHA concentrations on week 11 compared to either CTL or SO bulls. Following 11 weeks of FO supplementation at 1.2% total DM; 10% differences in DHA concentrations between FO and non-supplemented bulls have been reported [13]. However, based on percentage of total lipids, the 11 weeks of FO supplementation implemented in our study resulted in higher DHA incorporation into the spermatozoa than reported by others who have supplemented bulls with FO [29]. The DHA increase in SP was higher and more evident earlier than in spermatozoa; bulls on FO had 14% higher DHA in SP at 7 weeks compared to either CTL or SO bulls. In spermatozoa, FO bulls had 7% higher DHA than CTL and SO bulls; though this difference was not observed until week 11. The earlier incorporation of FA into SP compared to spermatozoa (35 vs 42 days) is consistent with a previous report in bulls [29]. That study [29] reported a similar difference (5%) between SFA and FO bulls, as we observed between CTL and FO bulls.

The importance of dietary n-6:n-3 ratio has been reviewed [33] and all evidence points towards benefits for both fertility and health when this ratio is reduced. Indeed, in the current study, the n-6:n-3 ratio of both sperm and SP was reduced by almost 50% when bulls were supplemented with FO.

Despite dietary-induced changes to lipid composition of both spermatozoa and SP, no differences in either the quantity or quality of semen produced were observed between treatment groups. Similar findings have previously been reported for bulls [12] where feeding a DHA-enriched supplement for nine weeks resulted in no difference in semen volume or spermatozoa concentration. Although a subjective examination of spermatozoa motility found a greater percentage of motile

507 spermatozoa in DHA supplemented bulls [12], a subsequent, objective assessment
508 using CASA found no difference in motility between treatments. The main
509 improvements in semen quality in that study were seen when the DHA-enriched diet
510 was fed to bulls, resulting in a higher percentage of hypo-osmotic swell test (HOST)-
511 positive bulls, suggesting an improvement in cell membrane integrity.

512 No changes to membrane fluidity were detected following the dietary PUFA
513 supplementation strategies employed in this study. The presence of n-3 long chain
514 PUFA in spermatozoa is important for maintaining spermatozoa plasma membrane
515 fluidity which facilitates membrane fusion with the oocyte [3]. However, our findings
516 suggest that increasing the long chain n-3 PUFA concentration of bovine
517 spermatozoa does not result in appreciable improvements to plasma membrane
518 fluidity when compared to a basal control diet. There were a higher percentage of
519 acrosome-intact spermatozoa in both the SO and FO bulls at week 10 but by week
520 12 all three diets had a similar percentage of acrosome-intact spermatozoa. In
521 agreement with our week 12 finding, dietary supplementation of rams with linoleic
522 acid (n-6 PUFA) and subsequent sex-sorting of the spermatozoa did not result in any
523 alteration of the percentage of acrosome-intact spermatozoa in comparison to non-
524 supplemented contemporaries [34]. *In vitro* measurements of spermatozoa, such as
525 CASA and flow cytometry, have been correlated with non-return rate in bull field
526 fertility (adjusted r^2= 0.40) [35]. Based on the CASA and flow cytometry data in our
527 study, we conclude that supplementation of bulls with dietary PUFA is unlikely to
528 affect fertility. Total n-6 intake of bulls appeared to account for significant, albeit a
529 low degree of explained variation in an array of functional semen analyses (Table 8).
530 Given that there are a very few studies that have examined dietary supplementation
531 of with n-6 PUFA, in bulls; their effects on fertility require further study.

In humans, it has been shown that cryopreservation causes a significant reduction in the lipid composition of spermatozoa [36]. Based on this evidence, one could reasonably hypothesize that bulls with a higher PUFA content would produce an ejaculate that could maintain a higher level of spermatozoa quality post-thawing. Our results show that this is not necessarily the case. For example, despite a 10% increase in DHA (most abundant FA in mammalian spermatozoa), FO supplemented bulls in this study did not have higher post-thaw semen quality compared to un-supplemented bulls. It should also be noted that the bulls used in this experiment had normal fertility potential based on semen characteristics measured. Perhaps dietary PUFA supplementation to bulls of poor semen quality would result in positive effects of on semen characteristics.

4.1. Conclusion

Consistent with the initial design of our study, we successfully generated divergence in the n-6 and n-3 PUFA concentrations of both spermatozoa and SP of bulls within the context of a full cycle of spermatogenesis. However, despite significantly altering the lipid composition of bull spermatozoa we failed to observe any appreciable difference in an array of *in vitro* fertility-related parameters for either fresh or frozen-thawed spermatozoa.

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658 Tables

659 **Table 1.** Composition of ration offered to young post-pubertal dairy bulls for 12 weeks.

Ingredient	%	660
Rolled barley	25	
Maize	20	661
Soya bean	15	
Beet pulp	17	662
Soya hulls	12	
Oil	4	663
Minerals/Vitamins	2	
Molasses	5	664

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Table 2. Chemical composition of diets offered to young post-pubertal dairy bulls for 12 weeks (mean as g/kg, unless otherwise stated).

	Ration				Silage	
	CTL	SO	FO	s.e.m.		s.e.m.
DM	829	837	838	177.8	230	0.8
Crude protein	17.9	15.7	19.2	0.65	10.9	0.63
ADF	107.6	101.0	78.0	5.35	368.3	4.09
NDF	211.2	199.5	161.2	11.6	580.6	8.33
Ash	78.9	110.1	112.0	9.73	88.9	3.84
Ether extract	1.30	2.65	2.56	0.261	3.04	0.26
Gross energy (MJ/kg DM)	16.46	15.89	15.73	0.178	16.8	0.05

DM: dry matter, ADF: acid detergent fibre, NDF: neutral detergent fibre, CTL: control diet, SO: safflower oil diet, FO: fish oil diet.

Table 3. Fatty acid composition of experimental rations and silage offered to young post-pubertal dairy bulls for 12 weeks (% of total fatty acids; mean ± s.e.m.).

Fatty acid (%)	CTL	SO	FO	Silage
Myristic (C14:0)	0.30 ± 0.006	0.23 ± 0.003	0.39 ± 0.028	0.69 ± 0.035
Pentadecylic (C15:0)	0.21 ± 0.015	0.12 ± 0.012	0.14 ± 0.003	0.28 ± 0.027
Palmitic (16:0)	22.01 ± 0.457	12.77 ± 0.709	12.31 ± 0.119	17.81 ± 0.255
Stearic (18:0)	2.28 ± 0.050	2.71 ± 0.067	3.36 ± 0.109	2.48 ± 0.142
Arachidic (C20:0)	0.25 ± 0.009	0.32 ± 0.007	0.69 ± 0.020	0.64 ± 0.037
Behenic (C22:0)	0.38 ± 0.064	0.24 ± 0.006	0.35 ± 0.023	1.19 ± 0.017
Lignoceric (C24:0)	0.27 ± 0.023	0.11 ± 0.024	0.15 ± 0.020	0.79 ± 0.075
Total saturated	25.70 ± 0.548	16.50 ± 0.666	17.40 ± 0.103	23.87 ± 0.408
Palmitoleic (C16:1 n-9)	0.13 ± 0.007	0.08 ± 0.012	0.10 ± 0.015	6.42 ± 0.096
Palmitoleic (C16:1 n-7)	0.39 ± 0.038	0.26 ± 0.023	0.80 ± 0.081	1.00 ± 0.187
Oleic (C18:1 n-9)	13.11 ± 0.469	13.13 ± 0.105	11.53 ± 0.105	3.13 ± 0.289
Vaccenic (C18:1 n-7)	1.12 ± 0.009	0.90 ± 0.026	2.04 ± 0.092	0.95 ± 0.129
Gadoleic acid (20:1 n-11)	ND	ND	0.23 ± 0.006	ND
Gondoic (C20:1 n-9)	0.57 ± 0.006	0.42 ± 0.015	1.98 ± 0.102	0.16 ± 0.026
Paullinic acid (20:1 n-7)	ND	ND	0.21 ± 0.009	ND
Cetoleic acid (22:1 n-11)	0.15 ± 0.045	0.23 ± 0.096	1.54 ± 0.150	ND
Erucic (C22:1 n-9cis)	0.24 ± 0.081	ND	0.39 ± 0.019	ND
Nervonic (C24:1 n-9)	ND	0.08 ± 0.020	0.28 ± 0.052	0.12 ± 0.005
Total monounsaturated	15.74 ± 0.391	15.08 ± 0.137	19.10 ± 0.324	11.72 ± 0.598
Linoleic (C18:2 n-6)	53.07 ± 0.463	64.95 ± 0.700	31.73 ± 1.022	15.15 ± 0.250
Gamma-Linolenic (C18:3 n-6)	ND	ND	0.07 ± 0.006	ND
Eicosadienoic (20:2 n-6)	0.10 ± 0.010	0.06 ± 0.003	0.25 ± 0.013	ND
Dihomo-gamma-linolenic (C20:3 n6)	ND	ND	0.15 ± 0.003	ND
Arachidonic (C20:4 n-6)	ND	ND	0.94 ± 0.048	ND
Adrenic (C22:4 n-6)	ND	ND	0.06 ± 0.003	ND
Docosapentaenoic (C22:5 n-6)	ND	ND	0.25 ± 0.009	ND
Total n-6	53.13 ± 0.433	65.02 ± 0.697	33.46 ± 0.970	15.23 ± 0.189
Alpha-linolenic (C18:3 n-3)	5.34 ± 0.236	2.53 ± 0.097	3.53 ± 0.121	46.79 ± 1.056
Stearidonic acid (18:4 n-3)	ND	ND	0.91 ± 0.081	ND
Eicosatrienoic acid (20:3 n-3)	ND	ND	0.12 ± 0.003	0.13 ± 0.020
Eicosatetraenoic acid (20:4 n-3)	ND	ND	0.74 ± 0.027	ND
Eicosapenteanoic (20:5 n-3)	ND	0.46 ± 0.035	13.06 ± 0.451	ND
Heneicosapenteanoic (21:5 n-3)	ND	ND	0.57 ± 0.015	ND
Docosapentaenoic (C22:5 n-3)	ND	ND	1.61 ± 0.046	ND
Docosahexaenoic (C22:6 n-3)	ND	0.37 ± 0.025	9.28 ± 0.228	ND
Total n-3	5.37 ± 0.216	3.40 ± 0.028	29.83 ± 0.667	46.88 ± 1.082
Total PUFA	58.56 ± 0.609	68.42 ± 0.699	63.50 ± 0.403	62.12 ± 0.955

Limit of quantification = 0.06%; ND = not detectable. CTL: control diet, SO: safflower oil diet: FO:

fish oil diet.

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Table 4. Effect of dietary polyunsaturated fatty acid supplementation on fatty acid concentration of sperm from bulls offered a control, safflower or fish oil diet for 12 weeks on weeks -1, 7 and 11 of the experimental period (% total fatty acids; mean ± s.e.m.).

Diet	CTL (n = 10)			SO (n = 10)			FO (n = 10)			Significance, P value		
Week	-1	7	11	-1	7	11	-1	7	11	Diet	Week	Diet by Week
Myristic (C14:0)	5.19 ± 0.602	5.92 ± 0.592	6.05 ± 0.545	5.16 ± 0.382	5.68 ± 0.445	6.49 ± 0.367	5.09 ± 0.457	6.82 ± 0.290	6.49 ± 0.507	NS	<0.001	NS
Pentadecylic (C15:0)	0.23 ± 0.047	0.11 ± 0.017	0.12 ± 0.013	0.21 ± 0.066	0.13 ± 0.016	0.12 ± 0.010	0.24 ± 0.051	0.11 ± 0.014	0.09 ± 0.006	NS	<0.01	NS
Palmitic (16:0)	17.04 ± 1.776	13.18 ± 0.702	13.00 ± 0.520	14.65 ± 0.972	13.40 ± 0.548	12.53 ± 0.246	16.23 ± 1.552	12.69 ± 0.548	12.28 ± 0.316	NS	<0.001	NS
Stearic (16:0)	8.11 ± 0.832	6.43 ± 0.363	5.50 ± 0.602	7.81 ± 0.892	6.82 ± 0.330	6.35 ± 0.142	7.80 ± 0.607	6.26 ± 0.258	5.95 ± 0.182	NS	<0.001	NS
Arachidic (C20:0)	0.52 ± 0.153	0.20 ± 0.032	0.20 ± 0.021	0.44 ± 0.141	0.27 ± 0.038	0.18 ± 0.011	0.56 ± 0.118	0.25 ± 0.043	0.19 ± 0.020	NS	<0.001	0.10
Behenic (C22:0)	2.02 ± 0.659	0.57 ± 0.112	0.55 ± 0.067	1.41 ± 0.422	0.82 ± 0.147	0.47 ± 0.039	2.16 ± 0.523	0.74 ± 0.174	0.54 ± 0.065	NS	<0.001	NS
Lignoceric (C24:0)	1.07 ± 0.359	ND	ND	0.84 ± 0.384	0.57 ± 0.129	ND	0.78 ± 0.215	0.48 ± 0.195	ND	NS	NS	NS
Total saturated	33.75 ± 3.431	26.43 ± 1.171	25.42 ± 0.883	30.17 ± 2.567	27.27 ± 1.083	26.17 ± 0.373	32.77 ± 2.773	26.96 ± 1.098	25.54 ± 0.460	NS	<0.001	NS
Palmitoleic (C16:1 n-9)	0.13 ± 0.004	0.12 ± 0.007	0.11 ± 0.004	0.16 ± 0.025	0.13 ± 0.003	0.11 ± 0.004	0.15 ± 0.008	0.13 ± 0.005	0.12 ± 0.003	NS	<0.001	NS
Palmitoleic (C16:1 n-7)	0.34 ± 0.081	0.23 ± 0.043	0.19 ± 0.017	0.46 ± 0.222	0.21 ± 0.023	0.17 ± 0.017	0.32 ± 0.069	0.22 ± 0.024	0.16 ± 0.010	NS	<0.001	NS
Oleic (C18:1 n-9)	3.94 ± 1.034	2.10 ± 0.367	1.88 ± 0.171	4.35 ± 1.795	2.38 ± 0.298	1.58 ± 0.086	4.44 ± 0.841	2.11 ± 0.268	1.52 ± 0.136	NS	<0.001	0.09
Vaccenic (C18:1 n-7)	2.00 ± 0.072	2.14 ± 0.137	1.96 ± 0.078	2.41 ± 0.427	2.20 ± 0.074	1.94 ± 0.037	2.02 ± 0.100	2.10 ± 0.042	1.89 ± 0.064	NS	<0.01	NS
Gondomic (C20:1 n-9)	0.14 ± 0.024	0.10 ± 0.018	0.11 ± 0.017	0.21 ± 0.087	0.12 ± 0.013	0.09 ± 0.006	0.17 ± 0.027	0.10 ± 0.008	0.08 ± 0.006	NS	<0.001	NS
Erucic (C22:1 n-9cis)	0.13 ± 0.035	ND	0.07 ± 0.013	0.21 ± 0.117	0.09 ± 0.008	0.09 ± 0.028	0.13 ± 0.027	0.08 ± 0.006	0.07 ± 0.004	NS	<0.05	NS
Nervonic (C24:1 n-9)	0.26 ± 0.077	0.09 ± 0.015	0.14 ± 0.016	0.29 ± 0.079	0.12 ± 0.014	0.16 ± 0.018	0.38 ± 0.091	0.10 ± 0.009	0.27 ± 0.129	NS	<0.001	NS
Total monounsaturated	6.86 ± 1.224	4.79 ± 0.581	4.38 ± 0.269	7.96 ± 2.672	5.19 ± 0.418	4.07 ± 0.138	7.57 ± 1.000	4.76 ± 0.316	4.03 ± 0.271	NS	<0.01	NS
Linoleic (C18:2 n-6)	4.82 ± 0.522	3.78 ± 0.264	3.77 ± 0.155	4.43 ± 0.349	4.45 ± 0.274	4.00 ± 0.192	5.43 ± 0.522	4.03 ± 0.149	3.65 ± 0.150	NS	<0.001	0.08
Gamma-Linolenic (C18:3 n-6)	0.11 ± 0.017	ND	ND	0.12 ± 0.043	ND	0.07 ± 0.010	0.10 ± 0.023	ND	ND	NS	<0.05	NS
Eicosadienoic (20:2 n-6)	0.25 ± 0.021	0.22 ± 0.020	0.27 ± 0.022	0.23 ± 0.029	0.30 ± 0.025	0.37 ± 0.035	0.24 ± 0.015	0.22 ± 0.011	0.30 ± 0.014	0.09	<0.001	<0.01
Dihomo-gamma-Linolenic (C20:3 n6)	0.46 ± 0.046	0.55 ± 0.033	0.51 ± 0.032	0.51 ± 0.044	0.60 ±0.032	0.53 ± 0.029	0.43 ± 0.021	0.54 ± 0.031	0.53 ± 0.040	NS	<0.05	NS
Arachidonic (C20:4 n-6)	2.95 ± 0.207	3.26 ± 0.185	3.02 ± 0.117	3.11 ± 0.285	3.51 ± 0.145	3.37 ± 0.130	3.03 ± 0.249	3.40 ± 0.072	2.95 ± 0.096	<0.05	<0.001	NS
Adrenic (C22:4 n-6)	0.27 ± 0.055	0.26 ± 0.037	0.29 ± 0.034	0.27 ± 0.063	0.27 ± 0.031	0.40 ± 0.036	0.23 ± 0.038	0.15 ± 0.010	0.14 ± 0.033	NS	NS	<0.001
Docosapentaenoic (C22:5 n-6)	5.41 ± 1.468	4.76 ± 1.026	5.88 ± 0.971	5.20 ± 1.651	5.38 ± 0.928	8.21 ± 0.916	3.33 ± 0.905	0.94 ± 0.156	1.18 ± 0.846	<0.001	<0.01	<0.001
Total n-6	14.18 ± 1.555	12.83 ± 1.110	13.74 ± 1.004	13.78 ± 1.853	14.50 ± 1.097	16.86 ± 0.940	12.74 ± 1.205	9.29 ± 0.192	8.77 ± 0.876	0.06	<0.01	<0.001
Alpha-linolenic (C18:3 n-3)	0.26 ± 0.087	0.10 ± 0.014	0.08 ± 0.014	0.24 ± 0.096	0.11 ± 0.014	0.08 ± 0.009	0.32 ± 0.072	0.12 ± 0.016	0.07 ± 0.004	NS	<0.001	NS
Eicosapenteanoic (20:5 n-3)	0.10 ± 0.010	ND	ND	0.09 ± 0.030	ND	ND	ND	0.08 ± 0.007	0.10 ± 0.008	-	-	-
Docosapentaenoic (C22:5 n-3)	0.57 ± 0.052	0.55 ± 0.018	0.64 ± 0.058	0.57 ± 0.014	0.51 ± 0.014	0.52 ± 0.016	0.57 ± 0.045	0.81 ± 0.032	0.92 ± 0.057	<0.01	<0.01	<0.01
Docosahexaenoic (C22:6 n-3)	27.80 ± 3.051	34.35 ± 1.158	35.03 ± 0.895	30.06 ± 3.313	33.53 ± 1.667	32.11 ± 0.906	29.71 ± 2.518	38.44 ± 0.863	39.83 ± 1.057	NS	<0.001	<0.01
Total n-3	28.68 ± 3.011	34.99 ± 1.167	35.83 ± 0.945	30.90 ± 3.232	34.16 ± 1.669	32.67 ± 0.912	30.60 ± 2.488	39.45 ± 0.844	40.88 ± 1.106	NS	<0.01	<0.05
n-6 to n-3 ratio[a]	0.57 ± 0.093	0.38 ± 0.041	0.39 ± 0.039	0.54 ± 0.107	0.45 ± 0.062	0.53 ± 0.046	0.46 ± 0.078	0.24 ± 0.007	0.22 ± 0.033	NS	<0.001	<0.001

Total PUFA	42.86 ± 2.855	47.82 ± 0.989	49.57 ± 0.726	44.68 ± 3.304	48.66 ± 0.855	49.53 ± 0.272	43.33 ± 2.442	48.74 ± 0.899	49.65 ± 0.430	NS	<0.001	NS

CTL = control; SO = safflower oil; FO = fish oil; NS = not significant; week 0 indicates start of dietary supplementation.
Limit of quantification = 0.06%; ND = not detectable.
[a] total n-6/total n-3

Table 5. Effect of dietary polyunsaturated fatty acid supplementation on fatty acid concentration of seminal plasma from bulls offered a control, safflower or fish oil diet for 12 weeks on week -1, 7 and 11 of the feeding period (% total fatty acids; mean ± s.e.m.).

Diet	CTL (n=10)			SO (n=10)			FO (n=10)			Significance		
Week	-1	7	11	-1	7	11	-1	7	11	Diet	Week	Diet by week
Myristic (C14:0)	4.60 ± 0.635	4.28 ± 0.486	3.73 ± 0.296	4.38 ± 0.255	4.47 ± 0.276	4.34 ± 0.301	3.99 ± 0.646	5.53 ± 0.409	5.79 ± 0.382	0.09	NS	<0.01
Pentadecylic (C15:0)	2.64 ± 2.462	0.13 ± 0.014	0.51 ± 0.367	0.26 ± 0.094	0.15 ± 0.016	0.14 ± 0.016	0.49 ± 0.265	0.17 ± 0.052	0.16 ± 0.020	NS	NS	NS
Palmitic (16:0)	16.52 ± 1.969	20.05 ± 0.883	19.34 ± 0.499	18.96 ± 0.684	19.06 ± 0.520	20.85 ± 0.425	18.42 ± 1.095	18.32 ± 0.479	19.51 ± 0.894	NS	0.06	0.07
Stearic (16:0)	10.25 ± 1.436	9.29 ± 0.972	9.31 ± 0.733	8.62 ± 1.141	8.64 ± 0.743	9.17 ± 0.625	10.04 ± 1.123	7.48 ± 0.396	7.61 ± 0.499	0.10	NS	NS
Arachidic (C20:0)	0.14 ± 0.018	0.13 ± 0.018	0.15 ± 0.025	0.14 ± 0.010	0.15 ± 0.016	0.15 ± 0.006	0.30 ± 0.096	0.17 ± 0.019	0.14 ± 0.017	<0.01	0.10	<0.001
Behenic (C22:0)	0.46 ± 0.060	0.48 ± 0.062	0.58 ± 0.143	0.44 ± 0.059	0.47 ± 0.047	0.48 ± 0.065	0.93 ± 0.303	0.47 ± 0.036	0.42 ± 0.049	NS	NS	NS
Lignoceric (C24:0)	0.39 ± 0.064	0.54 ± 0.300	0.36 ± 0.069	0.53 ± 0.107	0.36 ± 0.046	ND	0.62 ± 0.126	0.56 ± 0.105	0.38 ± 0.059	NS	NS	NS
Total saturated	33.05 ± 0.635	34.47 ± 0.936	33.76 ± 0.685	32.93 ± 1.087	33.02 ± 1.011	35.09 ± 0.901	34.36 ± 1.312	32.08 ± 0.557	33.69 ± 0.931	NS	0.08	NS
Palmitoleic (C16:1 n-9)	0.27 ± 0.035	0.21 ± 0.022	0.24 ± 0.041	0.22 ± 0.020	0.23 ± 0.022	0.21 ± 0.031	0.26 ± 0.035	0.26 ± 0.035	0.25 ± 0.039	NS	NS	NS
Palmitoleic (C16:1 n-7)	0.70 ± 0.240	0.22 ± 0.030	0.22 ± 0.047	0.27 ± 0.088	0.46 ± 0.235	0.37 ± 0.176	0.71 ± 0.375	0.50 ± 0.209	0.27 ± 0.041	NS	NS	NS
Oleic (C18:1 n-9)	3.43 ± 0.419	3.63 ± 0.446	3.57 ± 0.591	3.27 ± 0.617	2.85 ± 0.259	2.59 ± 0.224	5.78 ± 1.199	3.20 ± 0.485	2.64 ± 0.268	NS	<0.05	NS
Vaccenic (C18:1 n-7)	0.98 ± 0.051	1.16 ± 0.135	1.27 ± 0.214	0.98 ± 0.057	1.08 ± 0.061	1.12 ± 0.112	1.17 ± 0.119	1.01 ± 0.032	1.05 ± 0.042	NS	NS	NS
Gondomic (C20:1 n-9)	0.15 ± 0.011	0.17 ± 0.024	0.16 ± 0.020	0.15 ± 0.047	0.12 ± 0.012	0.17 ± 0.054	0.21 ± 0.048	0.13 ± 0.039	0.13 ± 0.023	NS	NS	NS
Erucic (C22:1 n-9cis)	ND	0.13 ± 0.031	0.10 ± 0.023	0.20 ± 0.010	0.12 ± 0.037	0.11 ± 0.028	0.15 ± 0.032	0.08 ± 0.005	0.25 ± 0.130	NS	NS	NS
Nervonic (C24:1 n-9)	1.15 ± 0.208	1.51 ± 0.631	1.06 ± 0.193	0.98 ± 0.346	0.71 ± 0.119	0.67 ± 0.161	1.96 ± 0.703	0.72 ± 0.134	0.84 ± 0.328	NS	NS	NS
Total monounsaturated	6.65 ± 0.698	6.91 ± 0.857	6.53 ± 1.002	5.87 ± 1.076	5.39 ± 0.462	5.08 ± 0.543	10.04 ± 1.476	5.70 ± 0.717	5.11 ± 0.636	NS	0.09	NS
Linoleic (C18:2 n-6)	4.85 ± 0.812	4.71 ± 0.912	5.67 ± 1.001	3.92 ± 0.973	4.75 ± 0.725	5.49 ± 0.500	7.36 ± 1.880	3.59 ± 0.377	4.24 ± 0.627	NS	0.09	<0.05
Gamma-Linolenic sperm (C18:3 n-6)	0.84 ± 0.252	0.47 ± 0.205	0.11 ± 0.005	0.43 ± 0.348	0.91 ± 0.419	0.13 ± 0.045	1.17 ± 0.631	0.81 ± 0.493	0.31 ± 0.045	NS	<0.01	0.09
Eicosadienoic (20:2 n-6)	0.45 ± 0.082	0.46 ± 0.085	0.58 ± 0.091	0.38 ± 0.072	0.55 ± 0.077	0.66 ± 0.047	0.42 ± 0.088	0.54 ± 0.189	0.47 ± 0.074	NS	<0.001	NS
Dihomo-gamma-linolenic (C20:3 n6)	1.66 ± 0.328	1.64 ± 0.281	1.76 ± 0.278	1.68 ± 0.443	1.64 ± 0.282	1.70 ± 0.230	1.88 ± 0.388	1.23 ± 0.172	1.07 ± 0.131	<0.05	NS	NS
Arachidonic (C20:4 n-6)	1.74 ± 0.080	1.70 ± 0.178	1.50 ± 0.051	1.58 ± 0.064	1.63 ± 0.197	1.44 ± 0.086	1.60 ± 0.224	1.41 ± 0.066	1.36 ± 0.074	NS	<0.001	NS
Adrenic (C22:4 n-6)	0.45 ± 0.073	0.39 ± 0.051	0.43 ± 0.044	0.41 ± 0.071	0.39 ± 0.048	0.47 ± 0.024	0.42 ± 0.062	0.25 ± 0.059	0.14 ± 0.018	<0.01	<0.01	<0.001
Docosapentaenoic (C22:5 n-6)	5.09 ± 1.404	3.71 ± 0.846	4.99 ± 0.718	5.03 ± 1.648	4.54 ± 0.950	6.30 ± 0.736	2.58 ± 0.960	0.80 ± 0.137	0.56 ± 0.284	<0.001	<0.01	<0.001
Total n-6	14.61 ± 1.418	12.72 ± 1.330	14.94 ± 1.066	13.13 ± 1.936	13.77 ± 1.322	16.07 ± 0.733	19.77 ± 4.531	8.20 ± 0.882	7.82 ± 0.854	<0.01	<0.001	<0.01
Alpha-linolenic (C18:3 n-3)	0.24 ± 0.149	ND	0.12 ± 0.025	0.18 ± 0.051	ND	0.08 ± 0.012	0.96 ± 0.616	0.19 ± 0.057	0.17 ± 0.065	<0.05	NS	NS
Eicosapenteanoic (20:5 n-3)	0.21 ± 0.078	0.12 ± 0.009	0.26 ± 0.076	0.11 ± 0.010	0.09 ± 0.013	1.22 ± 1.094	0.21 ± 0.072	0.59 ± 0.197	0.69 ± 0.155	<0.001	<0.05	NS
Docosapentaenoic (C22:5 n-3)	1.46 ± 0.113	1.32 ± 0.104	1.28 ± 0.086	1.23 ± 0.084	1.11 ± 0.082	1.13 ± 0.053	1.24 ± 0.180	1.75 ± 0.070	1.94 ± 0.116	<0.01	<0.01	<0.001
Docosahexaenoic (C22:6 n-3)	28.17 ± 1.753	28.39 ± 1.980	27.39 ± 1.620	29.75 ± 2.597	28.77 ± 1.561	26.33 ± 1.468	21.62 ± 3.508	35.65 ± 0.862	35.59 ± 0.925	NS	<0.05	<0.001
Total n-3	29.84 ± 1.757	29.78 ± 1.958	28.83 ± 1.574	31.17 ± 2.539	29.94 ± 1.557	27.86 ± 1.411	23.59 ± 3.435	37.91 ± 0.754	38.10 ± 0.911	NS	<0.05	<0.001

n-6 to n-3 ratio	0.53 ± 0.079	0.46 ± 0.067	0.55 ± 0.060	0.51 ± 0.118	0.49 ± 0.071	0.60 ± 0.050	0.68 ± 0.182	0.22 ± 0.026	0.21 ± 0.026	0.08	<0.05	<0.05
Total PUFA	44.45 ± 0.591	42.50 ± 0.062	43.77 ± 0.619	44.30 ± 1.089	43.71 ± 1.189	43.92 ± 0.914	43.37 ± 1.780	46.11 ± 0.593	45.93 ± 0.692	<0.05	NS	NS

CON = control; SO = safflower oil; FO = fish oil; NS = not significant; week 0 indicates start of dietary supplementation
Limit of quantification = 0.06%; ND = not detectable.
[a] total n-6/total n-3

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Table 6. Stepwise regression models for total n-3 and n6 PUFA in sperm using total saturated, monounsaturated, n-3 and n-6 intakes as independent variables.

	Slope			R^2			P-value		
	-1	7	11	-1	7	11	-1	7	11
Total n-3 PUFA in sperm									
Total n-3 PUFA intake[1]		0.76	0.15		0.27	0.60	ns	<0.01	<0.001
Total MUFA intake			-0.15			0.07	ns	ns	<0.05
Total n-6 in sperm									
Total n-3 PUFA intake		-0.07	-0.15		0.37	0.68	ns	<0.001	<0.001
Total MUFA intake			0.14			0.06	ns	ns	<0.05

[1]Intake recorded for initial six weeks of feeding period.

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Table 7. Effect of dietary polyunsaturated fatty acid supplementation post-thaw sperm motility parameters from bulls offered a control, safflower or fish oil for 12 weeks (mean ± s.e.m.).

	Diet			Week			Significance[1]		
	CTL	SO	FO	10	11	12	Diet	Week	Diet by week
CASA Total Motile (%)	29.2 ± 2.37	35.7 ± 2.92	30.9 ± 2.66	22.4 ± 2.38[a]	24.5 ± 3.04[a]	28.7 ± 2.06[b]	NS	***	NS
CASA PLM (%)	21.9 ± 2.25	28.8 ± 2.75	25.1 ± 2.54	28.5 ± 2.61[a]	31.3 ± 3.05[a]	36.0 ± 2.21[b]	NS	**	NS
Curvilinear velocity (µm/s)	77.3 ± 4.15	89.3 ± 3.08	82.0 ± 4.52	83.1 ± 3.89	79.4 ± 4.51	86.1 ± 3.61	NS	NS	NS
Straight-line velocity (µm/s)	56.3 ± 3.78	64.5 ± 2.80	60.6 ± 3.80	60.1 ± 3.65	58.2 ± 3.97	63.1 ± 2.87	NS	NS	NS
Average path velocity (µm/s)	66.1 ± 3.97	75.7 ± 2.95	70.5 ± 4.23	70.4 ± 3.82	68.1 ± 4.28	73.7 ± 3.20	NS	NS	NS
Linearity	58.8 ± 2.43	61.6 ± 1.70	62.3 ± 2.73	59.6 ± 2.40	58.5 ± 2.69	64.6 ± 1.61	NS	NS	NS
Straightness	70.9 ± 2.04	74.6 ± 1.30	75.3 ± 1.65	72.4 ± 1.98	71.6 ± 1.62	76.7 ± 1.39	NS	NS	NS
Amplitude of lateral head displacement (µm)	2.1 ± 0.08[a]	3.3 ± 0.92[b]	2.1 ± 0.10[ab]	2.2 ± 0.08	3.0 ± 0.93	2.3 ± 0.09	*	NS	NS
Beat cross frequency (Hz)	5.9 ± 0.28	6.9 ± 0.23	6.5 ± 0.35	6.4 ± 0.30	6.1 ± 0.34	6.9 ± 0.23	ns	ns	ns

[abc]Different superscripts differ significantly within row [1]*=P<0.05; **=P<0.01; ***=P<0.001; ns= not significant (P>0.05). CON = control; SO = safflower oil; FO = fish oil; NS = not significant. CASA = computer assisted semen analysis. PLM =progressive linear motility.

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Table 8. Stepwise regression models for computer assisted semen analysis (CASA) of total motility, progressive linear motility (PLM), sperm viability, acrosome integrity, and membrane fluidity of frozen-thawed semen using dietary total n-6 and n-3 intakes, percentage lipid content of total n-3 and n-6, n-6 to n-3 ratio and DHA content of both sperm and seminal plasma (SP) as independent variables.

	Slope	Individual R^2	P-value
CASA total motility ($\sum R^2$ = 0.09; *y*-intercept = 24.4)			
Total n-6 PUFA intake[1]	0.06	0.09	<0.01
CASA PLM ($\sum R^2$ = 0.09; *y*-intercept = 18.2)			
Total n-6 PUFA intake	0.06	0.09	<0.01
Viability ($\sum R^2$ = 0.38; *y*-intercept = 3.6)			
Total n-6 PUFA intake	0.04	0.18	<0.001
DHA in SP	0.09	0.20	0.09
Acrosome integrity ($\sum R^2$ = 0.27; *y*-intercept = 75.6)			
Total n-6 PUFA intake	0.04	0.27	<0.001
Membrane fluidity ($\sum R^2$ = 0.21; *y*-intercept = 2.5)			
n-6 to n-3 ratio in sperm	11.4	0.08	<0.01
Total n-3 PUFA intake	0.05	0.13	<0.05

$\sum R^2$: overall model R^2
[1]Intake recorded for initial six weeks of feeding period.
DHA: docosahexaenoic acid

Figure legends

Figure 1. Effect of dietary supplementation with either safflower (SO; n=15) or fish oil (FO; n=20) vs a control (CTL; n=15) diet onsemen volume, progressive linear motility (PLM) and sperm concentration collected from young post-pubertal dairy bulls after 10 weeks of feeding. [xy]Different superscripts indicate a significant difference between weeks. Vertical bars represent s.e.m.

Figure 2. Effect of dietary supplementation with either safflower (SO; n=10) or fish oil (FO; n=10) vs a control (CTL; n=10) diet on viability (a), membrane fluidity (b), acrosome integrity (c) and presence of superoxide anion (d) of frozen-thawed semen collected from young post-pubertal dairy bulls after 10 weeks of feeding. Vertical bars represent s.e.m. [ab]Different superscripts differ significantly within week. [xy] Different superscripts indicate a significant difference between weeks. *SO diet tends to be greater than CTL (P=0.06). ǂWeek 11 tends to be lower than week 12 (P=0.06).

Figure3. Fluorescent dot plot and univariate histograms showing the distribution of Alexa Fluor 647 (AF647), Syto16 (S16) and Propidium Iodide (PI) fluorescence in frozen-thawed bull sperm as determined by flow cytometry. The population of sperm was identified based on the forward scatter and side scatter variables and discriminated from debris, known as P01.Population. The fluorescence dot plot (a) reports the sperm population positive and negative for AF647 and S16. The univariate histogram (b) represents the S16 single colour control and displays the proportion of negative (unstained) and positive events for S16 in the Green detector. The univariate histogram (c) represents a PI single colour control and displays the proportion of negative (unstained) and positive events for PI in the Yellow detector. The univariate histogram (d) represents the AF647 single colour control and displays the proportion of negative (unstained) and positive events for AF647 Red2 fluorescence in the Red2 Detector.