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

4 C. J. Byrne^{1, 2}, S. Fair³, A.M. English^{1, 3}, S.A. Holden³, J.R. Dick⁴, P. Lonergan², and
5 D.A. Kenny^{1, 2*}

6 ¹*Animal and Bioscience Research Department, Teagasc, Dunsany, Co. Meath,*
7 *Ireland.* ²*School of Agriculture and Food Science, University College Dublin, Belfield,*
8 *Dublin 4, Ireland.* ³*Laboratory of Animal Reproduction, Department of Life Sciences,*
9 *University of Limerick, Limerick, Ireland.* ⁴*Institute of Aquaculture, School of Natural*
10 *Sciences, University of Stirling, Stirling, Scotland, UK.*

11 *Corresponding author: David A. Kenny, Animal and Bioscience Research
12 Department, Teagasc, Dunsany, Co. Meath, Ireland, C15 PW93.
13 (david.kenny@teagasc.ie)

14 **Abstract**

15 The aim of this study was to examine the effects of dietary supplementation with
16 rumen protected n-6 or n-3 polyunsaturated fatty acids (PUFA) on the quantity and
17 quality of semen from young post-pubertal dairy bulls. Pubertal Holstein-Friesian
18 (n=43) and Jersey (n=7) bulls with a mean \pm s.e.m. age and bodyweight of 420.1 \pm
19 5.86 days and 382 \pm 8.94 kg, respectively, were blocked on breed, weight, age and
20 semen quality (based on the outcomes of two pre-trial ejaculates) and randomly
21 assigned to one of three treatments: (i) a non-supplemented control (CTL, n=15), (ii)
22 rumen-protected safflower (SO, n=15), (iii) rumen-protected n-3 PUFA-enriched fish
23 oil (FO, n=20). Bulls were fed their respective diets, *ad libitum* for 12 weeks;
24 individual intakes were recorded using an electronic feeding system for the initial 6

25 weeks of the feeding period. Semen was collected via electro-ejaculation at weeks -
26 2, -1, 0, 7, 10, 11 and 12 relative to the beginning of the trial period (week 0). On
27 collection, semen volume, sperm concentration and progressive linear motility (PLM)
28 were assessed. On weeks -2, -1, 0, 10, 11, 12, semen was packaged into 0.25 mL
29 straws and frozen using a programmable freezer. On weeks -1, 7 and 11; a sub-
30 sample of semen was separated into sperm and seminal plasma, by centrifugation
31 and stored at – 20°C until analysis of lipid composition. Semen from 10 bulls per
32 treatment were used for post-thaw analysis at weeks 10, 11 and 12 (3 straws per
33 ejaculate). Sperm motility was analysed by computer assisted semen analysis
34 (CASA). In addition, membrane fluidity, acrosome reaction and oxidative stress were
35 assessed using flow cytometry. Sperm from bulls fed SO had a 1.2 fold higher total
36 n-6 PUFA content at week 11 compared to week -1 ($P<0.01$) while bulls fed FO had
37 a 1.3 fold higher total n-3 PUFA content, in sperm by week 11 ($P<0.01$). There was
38 no effect of diet on semen volume, concentration or PLM of sperm when assessed
39 either immediately following collection or post-thawing. Membrane fluidity and
40 oxidative stress of sperm were also not affected by diet. The percentage of sperm
41 with intact-acrosomes was lower in CTL bulls compared to those fed SO ($P<0.01$). In
42 conclusion, while the lipid composition of semen was altered following dietary
43 supplementation with either n-6 or n-3 based PUFA, this did not lead to measurable
44 improvements in the quantity or quality of semen produced by young post-pubertal
45 dairy bulls.

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

47 1. Introduction

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

60 Genomic selection has led to more accurate identification of elite sires, resulting
61 in increased demand for their semen at a much younger age. This demand
62 necessitates that bulls reach puberty as early as possible and produce an adequate
63 volume of high quality semen, to meet this demand. Dietary supplementation with n-
64 3 PUFA, derived from fish oil (FO) has been reported to improve certain semen
65 parameters including sperm concentration in rams [7], as well as progressive motility
66 and percentage of normal sperm in boars [10]. Other studies [6] however, found no
67 improvement in semen quantity or quality in boars. Similarly, there are conflicting
68 data from bulls in the literature regarding the effects of dietary n-3 PUFA
69 supplementation.

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

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

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

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

93 **2. Material and Methods**

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

97 1876, as amended by European Communities regulations 2002 and 2005) and the
98 European Community Directive 86/609/EC.

99 **2.1. Animal Management**

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

115 **2.2. Semen collection**

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

121 microscope incorporating a heated stage at 37 °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 80×10^6 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, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL,
150 $\mu\text{m/s}$), 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 3×10^5 /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**

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

236 **2.6.3. Oxidative stress**

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

240 presence of MSXR (4 mM) for 15 min. MSXR is an intracellular stain that fluoresces
241 in the presence of the superoxide anion. Following this, the nucleic dead stain YP
242 was added to give a final concentration of 25 nM and again, incubated at 37°C in the
243 presence of MSXR for 15 min. The fluorescence of MSXR was analysed via the red
244 (690/50 BP) detector and YP via the green detector (525/30 BP). Minor computed
245 compensation was carried out. The presence of superoxide was calculated as the
246 percentage of MSXR positive sperm of the YP negative (MSXR +ve/ YP -ve)
247 population, as initially gated, as P01.Population, based on controls, FSC and SSC.

248 **2.7. Statistical analysis**

249 Data were analysed using appropriate procedures of Statistical Analysis Software
250 (SAS version 9.3, Cary, NC, USA). Data were tested for normality (UNIVARIATE
251 procedure) and, where appropriate, transformed to the power of lambda
252 (TRANSREG procedure). Data were analysed using ANOVA (MIXED procedure).
253 Diet, block, sampling time and their interactions, were included in the model. The
254 interaction term, if not statistically significant ($P > 0.05$), was subsequently excluded
255 from the final model. The covariance matrix was determined for each variable by
256 examining the Bayesian Information Criteria (**BIC**) (smaller is better) value. Animal
257 was the experimental unit. Sampling time (week of collection) was included in the
258 statistical models as a repeated term. Multiple regression analysis was used (REG
259 and STEPWISE procedure) to identify statistically significant predictor variables for
260 concentration, motility of both fresh and frozen-thawed semen and all parameters
261 measured by flow cytometer. The fixed effects of diet and week were corrected for in
262 the model. Dietary total n-6 and n-3 intake, percentage lipid content of total n-3 and
263 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 \pm 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 \pm 0.37 kg, 9.54 \pm 0.31 kg, 9.34 \pm 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 \pm 0.19, 1.4 \pm 0.17 and 1.6 \pm 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 \pm 0.008, 0.13 \pm 0.009 and 0.13 \pm 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

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

292 In SP, there was a diet by week interaction detected for myristic acid (Table 5.
293 $P<0.01$); bulls fed the FO diet had higher myristic acid on week 11 in comparison to
294 those fed the CTL diet ($P<0.001$). On week 7, bulls fed the FO diet also tended to
295 have higher myristic acid ($P=0.06$) than bulls fed the SO diet. There was also a diet
296 by week interaction for arachidic acid ($P<0.001$) in SP. A higher level of arachidic
297 acid ($P<0.01$) was observed in SP of bulls fed both the CTL and SO diets compared
298 to those fed the FO diet on week -1; this difference was no longer evident on weeks
299 7 or 11. There was a strong tendency ($P=0.06$) for SP concentrations of palmitic acid
300 to be higher at week -1 than on week 11. There was no effect of diet or week of
301 sampling on total SFA concentrations of SP.

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

309 Monounsaturated fatty acids in SP were unaffected by diet and week with the
310 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

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

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

359 There was a diet by week interaction for SP concentrations of DPA (n-3;
360 $P < 0.001$). 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 ($P<0.001$). 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 ($P<0.05$).

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

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

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

429 **4. Discussion**

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

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

434 Before assessing the effect of dietary supplementation with specific nutrients on
435 semen quality, it is important to quantify the level of incorporation of the biochemical
436 of interest into the spermatozoa. It is also important to consider the duration of the
437 spermatogenesis cycle (61 days) of the bull [28] in order to allow adequate time for
438 PUFA supplementation to have an effect on all stages of developing spermatozoa. In
439 the current study, the change in FA composition of both the spermatozoa and SP
440 has been described in detail. Total SFA concentration of either spermatozoa or SP
441 was not altered by supplementation with either SO or FO, when compared to the
442 CTL. However, across diets, total SFA concentration in sperm decreased 1.2-fold
443 between weeks -1 and 11, with the decrease first evident on week 7. It has been
444 shown that changes in sperm FA composition following dietary supplementation of
445 bulls can take up to 35 days [29]. However, the change in SFA concentrations in the
446 current study is in contrast to the findings of the previous study, where FO, flaxseed
447 and vegetable oil (high in C16:0) were fed to mature (6 yrs.) and semi-mature (2 yrs.)
448 bulls and none of these supplements resulted in a change in total SFA concentration
449 [29].

450 The overall 1.8-fold decrease in total MUFA in sperm in the current study is
451 higher than that observed in a similar study in pigs (1.2-fold decrease) when the n-6:
452 n-3 ratio was also altered [30]. Few changes were detected in MUFA composition of
453 SP; only oleic acid exhibited a significant (2.2-fold) decrease; following an alteration
454 of the dietary n-6 to n-3 ratio. In the small number of other studies in bulls and pigs
455 where SP MUFA composition has been quantified, none report changes over time
456 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.

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

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

501 Despite dietary-induced changes to lipid composition of both spermatozoa
502 and SP, no differences in either the quantity or quality of semen produced were
503 observed between treatment groups. Similar findings have previously been reported
504 for bulls [12] where feeding a DHA-enriched supplement for nine weeks resulted in
505 no difference in semen volume or spermatozoa concentration. Although a subjective
506 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.

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

543 **4.1. Conclusion**

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

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553 **References**

- 554 [1] Esmaeili V, Shahverdi AH, Moghadasian MH, Alizadeh AR. Dietary fatty acids
555 affect semen quality: a review. *Andrology*. 2015;3:450-61.
- 556 [2] Martinez-Soto JC, Landeras J, Gadea J. Spermatozoa and seminal plasma fatty
557 acids as predictors of cryopreservation success. *Andrology*. 2013;1:365-75.
- 558 [3] Wathes DC, Abayasekara DR, Aitken RJ. Polyunsaturated fatty acids in male and
559 female reproduction. *Biol Reprod*. 2007;77:190-201.
- 560 [4] Mattos R, Staples CR, Thatcher WW. Effects of dietary fatty acids on
561 reproduction in ruminants. *Rev Reprod*. 2000;5:38-45.
- 562 [5] Attaman JA, Toth TL, Furtado J, Campos H, Hauser R, Chavarro JE. Dietary fat
563 and semen quality among men attending a fertility clinic. *Hum Reprod*.
564 2012;27:1466-74.
- 565 [6] Castellano CA, Audet I, Bailey JL, Chouinard PY, Laforest JP, Matte JJ. Effect of
566 dietary n-3 fatty acids (fish oils) on boar reproduction and semen quality. *J*
567 *Anim Sci*. 2010;88:2346-55.
- 568 [7] Fair S, Doyle DN, Diskin MG, Hennessy AA, Kenny DA. The effect of dietary n-3
569 polyunsaturated fatty acids supplementation of rams on semen quality and
570 subsequent quality of liquid stored semen. *Theriogenology*. 2014;81:210-9.
- 571 [8] Childs S, Hennessy AA, Sreenan JM, Wathes DC, Cheng Z, Stanton C, et al.
572 Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on
573 systemic and tissue fatty acid concentrations and on selected reproductive
574 variables in cattle. *Theriogenology*. 2008;70:595-611.
- 575 [9] Ashes JR, Siebert BD, Gulati SK, Cuthbertson AZ, Scott TW. Incorporation of n-3
576 fatty acids of fish oil into tissue and serum lipids of ruminants. *Lipids*.
577 1992;27:629-31.
- 578 [10] Rooke JA, Shao CC, Speake BK. Effects of feeding tuna oil on the lipid
579 composition of pig spermatozoa and in vitro characteristics of semen.
580 *Reproduction*. 2001;121:315-22.
- 581 [11] Kelso K, Redpath A, Noble R, Speake B. Lipid and antioxidant changes in
582 spermatozoa and seminal plasma throughout the reproductive period of bulls.
583 *J Reprod Fertil*. 1997;109:1-6.
- 584 [12] Gholami H, Chamani M, Towhidi A, Fazeli MH. Effect of feeding a
585 docosahexaenoic acid-enriched nutraceutical on the quality of fresh and
586 frozen-thawed semen in Holstein bulls. *Theriogenology*. 2010;74:1548-58.
- 587 [13] Khoshvaght A, Towhidi A, Zare-Shahneh A, Noroozi M, Zhandi M, Dadashpour
588 Davachi N, et al. Dietary n-3 PUFAs improve fresh and post-thaw semen
589 quality in Holstein bulls via alteration of sperm fatty acid composition.
590 *Theriogenology*. 2016;85:807-12.
- 591 [14] Gürler H, Calisici O, Calisici D, Bollwein H. Effects of feeding omega-3-fatty
592 acids on fatty acid composition and quality of bovine sperm and on
593 antioxidative capacity of bovine seminal plasma. *Anim Reprod Sci*.
594 2015;160:97-104.
- 595 [15] Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in
596 human spermatozoa, spermicidal properties of fatty acid peroxides, and
597 protective action of seminal plasma. *Fertil Steril*. 1979;31:531-7.
- 598 [16] Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the
599 measurement of malondialdehyde and 4-hydroxyalkenals in human
600 spermatozoa: relationships with semen quality and sperm function. *Int J*
601 *Androl*. 1998;21:81-94.
- 602 [17] Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization
603 and pregnancy. *Cell Tissue Res*. 2005;322:33-41.

- 604 [18] Aitken RJ. Founders' Lecture. Human spermatozoa: fruits of creation, seeds of
605 doubt. *Reprod Fertil Dev.* 2004;16:655-64.
- 606 [19] Wolf F, Almquist J, Hale E. Prepuberal behavior and puberal characteristics of
607 beef bulls on high nutrient allowance. *J Anim Sci.* 1965;24:761-5.
- 608 [20] Sweeney RA. Generic combustion method for determination of crude protein in
609 feeds: collaborative study. *Journal of AOAC International* 1988;72:770-4.
- 610 [21] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and
611 purification of total lipides from animal tissues. *J Biol Chem.* 1957;226:497-
612 509.
- 613 [22] Christie WW. *Lipid Analysis*,. 3rd Edition ed. Bridgewater, UK: The Oily Press;
614 2003.
- 615 [23] Ghioni C, Bell J, Sargent J. Polyunsaturated fatty acids in neutral lipids and
616 phospholipids of some freshwater insects. *Comparative Biochemistry and*
617 *Physiology Part B: Biochemistry and Molecular Biology.* 1996;114:161-70.
- 618 [24] Murphy C, English AM, Holden SA, Fair S. Cholesterol-loaded-cyclodextrins
619 improve the post-thaw quality of stallion sperm. *Anim Reprod Sci.*
620 2014;145:123-9.
- 621 [25] Murphy C, Holden SA, Murphy EM, Cromie AR, Lonergan P, Fair S. The impact
622 of storage temperature and sperm number on the fertility of liquid-stored bull
623 semen. *Reprod Fertil Dev.* 2015;28:1349-59.
- 624 [26] Petrunkina AM, Harrison RAP. Fluorescence Technologies for Evaluating Male
625 Gamete (Dys)Function. *Reproduction in Domestic Animals.* 2013;48:11-24.
- 626 [27] Kiernan M, Fahey AG, Fair S. The effect of the in vitro supplementation of
627 exogenous long-chain fatty acids on bovine sperm cell function. *Reprod Fertil*
628 *Dev.* 2013;25:947-54.
- 629 [28] Johnson L, Varner D, Roberts M, Smith T, Keillor G, Scrutchfield W. Efficiency
630 of spermatogenesis: a comparative approach. *Anim Reprod Sci.* 2000;60:471-
631 80.
- 632 [29] Moallem U, Neta N, Zeron Y, Zachut M, Roth Z. Dietary alpha-linolenic acid from
633 flaxseed oil or eicosapentaenoic and docosahexaenoic acids from fish oil
634 differentially alter fatty acid composition and characteristics of fresh and
635 frozen-thawed bull semen. *Theriogenology.* 2015;83:1110-20.
- 636 [30] Liu Q, Zhou YF, Duan RJ, Wei HK, Jiang SW, Peng J. Effects of dietary n-6:n-3
637 fatty acid ratio and vitamin E on semen quality, fatty acid composition and
638 antioxidant status in boars. *Anim Reprod Sci.* 2015;162:11-9.
- 639 [31] Esmaeili V, Shahverdi AH, Alizadeh AR, Alipour H, Chehrazi M. Saturated,
640 omega-6 and omega-3 dietary fatty acid effects on the characteristics of fresh,
641 frozen-thawed semen and blood parameters in rams. *Andrologia.* 2014;46:42-
642 9.
- 643 [32] Cook HW, McMaster CR. *Fatty acid desaturation and chain elongation in*
644 *eukaryotes: Elsevier: New York, NY, USA; 2002.*
- 645 [33] Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty
646 acids. *Biomed Pharmacother.* 2002;56:365-79.
- 647 [34] de Graaf SP, Peake K, Maxwell WMC, O'Brien JK, Evans G. Influence of
648 supplementing diet with Oleic and Linoleic acid on the freezing ability and sex-
649 sorting parameters of ram semen. *Livest Sci.* 2007;110:166-73.
- 650 [35] Sellem E, Broekhuijse ML, Chevrier L, Camugli S, Schmitt E, Schibler L, et al.
651 Use of combinations of in vitro quality assessments to predict fertility of bovine
652 semen. *Theriogenology.* 2015;84:1447-54.e5.

653 [36] James PS, Wolfe CA, Mackie A, Ladha S, Prentice A, Jones R. Lipid dynamics
654 in the plasma membrane of fresh and cryopreserved human spermatozoa.
655 Hum Reprod. 1999;14:1827-32.

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658 Tables

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

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

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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 \pm s.e.m.).

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

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

^atotal n-6/total n-3

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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 \pm s.e.m.).

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

^atotal 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

¹Intake 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 \pm s.e.m.).

	Diet			Week			Significance ¹		
	CTL	SO	FO	10	11	12	Diet	Week	Diet by week
CASA Total Motile (%)	29.2 \pm 2.37	35.7 \pm 2.92	30.9 \pm 2.66	22.4 \pm 2.38 ^a	24.5 \pm 3.04 ^a	28.7 \pm 2.06 ^b	NS	***	NS
CASA PLM (%)	21.9 \pm 2.25	28.8 \pm 2.75	25.1 \pm 2.54	28.5 \pm 2.61 ^a	31.3 \pm 3.05 ^a	36.0 \pm 2.21 ^b	NS	**	NS
Curvilinear velocity (μ m/s)	77.3 \pm 4.15	89.3 \pm 3.08	82.0 \pm 4.52	83.1 \pm 3.89	79.4 \pm 4.51	86.1 \pm 3.61	NS	NS	NS
Straight-line velocity (μ m/s)	56.3 \pm 3.78	64.5 \pm 2.80	60.6 \pm 3.80	60.1 \pm 3.65	58.2 \pm 3.97	63.1 \pm 2.87	NS	NS	NS
Average path velocity (μ m/s)	66.1 \pm 3.97	75.7 \pm 2.95	70.5 \pm 4.23	70.4 \pm 3.82	68.1 \pm 4.28	73.7 \pm 3.20	NS	NS	NS
Linearity	58.8 \pm 2.43	61.6 \pm 1.70	62.3 \pm 2.73	59.6 \pm 2.40	58.5 \pm 2.69	64.6 \pm 1.61	NS	NS	NS
Straightness	70.9 \pm 2.04	74.6 \pm 1.30	75.3 \pm 1.65	72.4 \pm 1.98	71.6 \pm 1.62	76.7 \pm 1.39	NS	NS	NS
Amplitude of lateral head displacement (μ m)	2.1 \pm 0.08 ^a	3.3 \pm 0.92 ^b	2.1 \pm 0.10 ^{ab}	2.2 \pm 0.08	3.0 \pm 0.93	2.3 \pm 0.09	*	NS	NS
Beat cross frequency (Hz)	5.9 \pm 0.28	6.9 \pm 0.23	6.5 \pm 0.35	6.4 \pm 0.30	6.1 \pm 0.34	6.9 \pm 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 ($\Sigma R^2 = 0.09$; y-intercept = 24.4)			
Total n-6 PUFA intake ¹	0.06	0.09	<0.01
CASA PLM ($\Sigma R^2 = 0.09$; y-intercept = 18.2)			
Total n-6 PUFA intake	0.06	0.09	<0.01
Viability ($\Sigma R^2 = 0.38$; 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 ($\Sigma R^2 = 0.27$; y-intercept = 75.6)			
Total n-6 PUFA intake	0.04	0.27	<0.001
Membrane fluidity ($\Sigma R^2 = 0.21$; 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

ΣR^2 : overall model R²

¹Intake recorded for initial six weeks of feeding period.

DHA: docosahexaenoic acid

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783 **Figure legends**

784

785 Figure 1. Effect of dietary supplementation with either safflower (SO; n=15) or fish oil
786 (FO; n=20) vs a control (CTL; n=15) diet on semen volume, progressive linear motility
787 (PLM) and sperm concentration collected from young post-pubertal dairy bulls after
788 10 weeks of feeding. ^{xy}Different superscripts indicate a significant difference between
789 weeks. Vertical bars represent s.e.m.

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

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

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