The effect of dietary *n*-3 polyunsaturated fatty acids supplementation of rams on semen quality and subsequent quality of liquid stored semen

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Abstract:

The objective of this study was to examine the effect of dietary *n*-3 polyunsaturated fatty acid (*n*-3 PUFA) supplementation of rams on semen quality and subsequent sperm function of liquid stored semen. Mature rams of proven fertility were individually housed and were blocked by breed, bodyweight and body condition score and randomly allocated within block to one of two dietary treatments (n=7/treatment). Rams were offered a base diet of hay and concentrate, with the concentrate enriched with either (i) saturated palmitic acid (control; CON) or (ii) high *n*-3 PUFA fish oil (FO) supplements. Both lipid supplements were added at

2% (w/w) of the total diet as fed and both were partially rumen protected. The animals were fed their respective diets for a total of 9 weeks and blood samples were collected on weeks 0 (pre-experimental), 4 and 9, relative to initial allocation of diet (week 0), for measurement of plasma concentration of fatty acids, metabolites, IGF-1 and insulin. Semen was collected from each ram (on one day in each week) in weeks 4, 5, 7, 8 and 9 and each ejaculate was assessed for volume, wave motion and concentration of sperm, following which it was diluted in a skim milk based extender and stored at 4 ^{o}C. A second ejaculate was collected on weeks 4, 7 and 9 centrifuged and the sperm frozen for subsequent lipid analysis. A sample of semen from each ram was assessed at 24, 48 and 72 h post collection for sperm progressive linear motion (PLM), ability to penetrate artificial mucus as well as the ability to resist lipid peroxidation (at 24 and 48 h only) using the thiobarbituric acid reactive substances assay. There was no effect of diet on plasma insulin concentrations or on any of the metabolites measured, however, there was a diet by week interaction for plasma IGF-1 concentration ($P < 0.05$). This was manifested as the FO supplemented rams having higher IGF-1 concentrations on week 9 compared to the CON treatment ($P < 0.05$), but not at the earlier sampling dates. Compared to the pre-experimental values supplementation with FO increased plasma concentrations of total *n*-3 PUFA's by 3.1 fold and decreased *n*-6 PUFA's concentrations by 1.84 fold. Consequently, the ratio of *n*-6 to *n*-3 PUFA was decreased in the FO supplemented rams ($P < 0.001$). Dietary supplementation with FO increased the concentration of eicosapentaenoic in sperm from week 4 to 9 by 2.7 fold ($P < 0.05$) leading to a 1.5 fold increase in total *n*-3 PUFA in the same period. Ejaculates collected from rams supplemented with FO yielded a higher semen concentration ($P < 0.05$), however, there was no difference between diets on any of the other semen quality parameters including semen volume, wave motion, PLM, ability to penetrate artificial mucus or ability to resist lipid peroxidation. In conclusion, dietary supplementation of rams with *n*-3 PUFA successfully increased the n-3

PUFA content of both plasma and sperm but has limited effects on the quality of liquid stored semen.

Keywords:

Fertility, Sheep, Sperm, PUFA, Lipid Peroxidation, Mucus Penetration

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1.0. Introduction:

It is well established that dietary supplementation of lipid can increase the dietary energy density (as reviewed by Staples et al. [1]) but recently the inclusion of specific polyunsaturated fatty acids (PUFA) has been shown to have a number of positive benefits for the reproductive performance of female ruminants including enhanced steroidogenesis [2-5], greater ovarian follicle and luteal size [3, 6], increased embryo survival [7] and gene expression [2, 8]. PUFA of the *n*-3 and *n*-6 series are essential fatty acids, since they cannot be synthesized *de novo* in mammals and therefore, must be provided by the diet. Childs et al. [9] demonstrated that supplementation of heifers with a semi-rumen protected high *n*-3 PUFA supplement significantly enhanced the concentration of *n*-3 PUFA's in a number of reproductive tissues compared with unsupplemented controls, while others using the same

model [2, 8, 10] observed differential expression of a number of genes critical to prostaglandin $F_{2\alpha}$ in the bovine endometrium.

Like all mammalian cells, sperm have a plasma membrane which is made up of a phospholipid bilayer and this contains large amounts of PUFA's. PUFA's are involved in physicochemical modifications of the sperm head during capacitation [11] and gives the sperm membrane the fluidity it needs to participate in the membrane fusion events associated with fertilization [12]. However, these PUFA are extremely vulnerable to oxidative damage generated by reactive oxygen species (ROS) [13-15]. While ROS are involved in cell signaling at low levels, excessive production of ROS can lead to oxidative stress, lipid peroxidation, DNA damage and associated impairment of sperm function [16]. In addition, sperm have a relatively small amount of cytoplasm which contains low concentrations of antioxidants [17]. Therefore, there is a critical balance between lipids, ROS and the antioxidant system in the environment surrounding the sperm, which is required to ensure their efficient functionality. It is perhaps this complex interaction that has led to conflicting reports in the scientific literature on the relationship between the fatty acid profile of sperm and subsequent fertility. There are two main approaches to date which have been used to investigate the role of fatty acids in sperm cell function, namely; the comparison of the fatty acid profile of high and low fertility males, and dietary supplementation with PUFA to alter the milieu in which spermatogenesis occurs and therefore, the composition of the sperm membrane [18] .

Sperm from infertile men have been reported to have higher concentrations of saturated fatty acids [19] and lower levels of *n*-3 fatty acids [20]. Similarly, Am-in et al. [21] compared the sperm fatty acid profiles from boars having normal or low sperm motility, and reported that

both the level of saturated fatty acids and the ratio of *n*-6: *n*-3 PUFA was negatively correlated with sperm motility, viability, morphology and plasma membrane integrity.

While dietary supplementation with a wide range of PUFA supplements have been shown to alter the sperm fatty acid profile there are conflicting results on the effect of this on both fresh and frozen-thawed sperm quality. Studies with rams [22], bulls [23], fowl [24, 25] and boars [26-29], have suggested benefits following dietary supplementation of *n*-3 fatty acids on male reproductive parameters, whereas, other studies in rams [30], humans [31], turkeys [32], chickens [33], rabbits [18] and boars [34, 35] failed to show any significant effect on semen quality. Despite this, and to the best of our knowledge, there is no study investigating the effect of dietary *n*-3 PUFA supplementation of rams on the subsequent quality of liquid stored semen.

Thus, the objective of this study was to examine the effect of dietary *n*-3 PUFA supplementation of rams on the incorporation of PUFA into ejaculated sperm and subsequently evaluate its effect in stored liquid semen through assessments of progressive linear motion, ability to penetrate artificial mucus and ability to resist lipid peroxidation.

2.0. Materials and Methods:

2.1. Experimental design

The study was carried at the Animal Production Research Centre, Teagasc, Athenry, Co Galway, Ireland. Mature (11-36 month old) Belclare (n = 6), Suffolk (n = 5) and Texel (n = 3) rams of proven fertility and with a mean ± S.E.M. live-weight of 67.0 ± 4.43 kg and body

condition score (BCS) of 3.6 ± 0.09 units were used for the experiment. Rams were individually housed and were blocked by breed, bodyweight and body condition score and randomly allocated within block to one of two treatments (n = 7 per treatment); Palmitic acid (CON; 2% (w/w) saturated fat control) and Fish oil (FO; 2% (w/w) fish oil). Both lipid supplements were added at 2% (w/w) of the total diet as fed and rams were fed their respective diets for a total of 64 days (9 weeks). Blood samples were collected on weeks 0 (pre-experiment), 4 and 9 (Fig 1) for measurement of plasma concentration of fatty acids as well as a number of blood metabolites. Semen was collected from each ram (on one day in each week) in weeks 4, 5, 7, 8 and 9 and assessed for volume, concentration and wave motion after which it was diluted and stored for up to 72 h at 4^{o}C. Progressive linear motion (PLM) and the ability of stored sperm from each ram to penetrate artificial mucus was assessed at 24, 48 and 72 h post semen collection, while lipid peroxidation was assessed at 24 and 48 h post semen collection. Fatty acid analysis of sperm samples was performed on samples collected on weeks 4, 7 and 9.

2.2. Composition of the diets and feeding regime

Prior to the commencement of the trial, to allow for acclimatisation, all rams were fed a standard diet for a period of 5 days. All rams had *ad libitium* access to good quality hay and clean drinking water throughout the experimental period and were supplemented with their respective concentrate diets. The control diet consisted of a standard sheep concentrate containing palmitic acid (CON; Palmit 80®; Trouw Nutrition, 36 Ship Street, Belfast BT15 1JL: Table 1) while the fish oil (FO) diet consisted of a standard sheep concentrate containing partially rumen protected fish oil (Trouw Nutrition, 36 Ship Street, Belfast BT15 1JL; EPA:DHA, 1.5:1; Table 1 [9]). Due to the range in age and thus live-weight, rams less than

one year of age were offered 0.48 kg DM, while rams greater than one year of age were offered 0.72 kg DM, of their respective concentrate diets. Rams received their daily allocation of concentrate each day at approximately 08.30 h and throughout the trial period rams were monitored closely on a daily basis to ensure complete consumption of allocated feed occurred.

Rumen protection was achieved via encapsulation in a pH sensitive matrix which remains intact at rumen pH but degrades at the lower pH in the abomasum releasing the constituents for absorption. The FO was derived from anchovy, sardine and salmon oil, however, the oil was distilled in order to concentrate the EPA and DHA content. The ingredient composition and chemical analysis of the lipid supplemented experimental rations are presented in Table 1, while the fatty acid composition of the two experimental concentrates are presented in Table 2. All diets were formulated to be isonitrogenous (14% (w/w) crude protein in total diet) and isolipid.

2.3. Liveweight and body condition scoring

Animals were weighed and body condition scored on week 0 and on week 9 (Week 1 = start of experimental diets and week 9 = last week animals were fed experimental diets). Body condition was estimated on a linear scale of 1-5 (1 = emaciated, 5 = extremely obese) and was carried out by the same trained technician on each occasion.

2.4. Feed sampling and nutritional analysis

A concentrate and hay sample were collected on week 0 and on week 9 and were stored at -20^{o}C until analysed for DM, crude protein (CP), crude fibre (CF), ether extract, ash and gross energy (GE). Samples were milled through a 1 mm screen using a Christy and Norris hammer mill (Christy and Norris Process Engineers Ltd., Chelmsford, UK). Dry matter was determined by oven drying at 104 °C to a constant weight for a minimum of 16 h. Ash was determined on all materials after ignition of a known weight of ground material in a muffle furnace (Nabertherm, Bremen, Germany) at 550 °C for 4 h. Crude fibre was determined on all samples using a Fibertec extraction unit (Tecator, Hoganas, Sweden) according to the method of [36]. Crude protein (total nitrogen × 6.25) was determined using the method of [37] on a Leco FP 528 nitrogen analyser (Leco Instruments UK Ltd., Newby Road, Hazel Grove, Stockport SK7 5DA, Cheshire). Ether extract was determined using a Sortex instrument (Tecator, Hoganas, Sweden) while the gross energy of the samples was determined using a Parr 1201 oxygen bomb calorimeter (Parr, Moline, IL, USA).

2.5. Blood Sampling

Blood samples were collected in the morning prior to feeding on week 0 (pre-experiment), week 4 and week 9 for measurement of plasma concentration of insulin like growth factor 1 (IGF-1), fatty acids, cholesterol, triglycerides, glucose, urea, non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA). Blood was collected by jugular veinipuncture under license in conjunction with the European Community Directive, 86-609-EC. Samples were collected into lithium heparinised vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK). Following collection, samples were immediately stored on ice-water and centrifuged at 1500 x *g* at 4^{o}C for 15 min. Plasma was removed and stored at -20^{o}C until analysed.

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200 2.6. Semen collection

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202 Two ejaculates were collected (>3 h apart) from each ram on one day in each of weeks 4, 5, 7
203 and 9 of the experiment by an experienced handler using an artificial vagina fitted with a 15
204 mL graduated test tube. After collection, each ejaculate was assessed for volume, wave
205 motion on a 6 point scale of 0-5 (0 = no currents; 5 = numerous rapid and vigorous currents)
206 and sperm concentration using a photometer calibrated for ovine semen. The first ejaculate
207 was diluted to a final sperm concentration of 800×10^6 sperm per mL in a standard skim-milk
208 egg-yolk diluent [38] without antioxidants and was stored at 4^{o}C for up to 72 h. The diluted
209 semen from each individual ram was kept separate throughout. The second ejaculate from
210 each ram was used to determine the fatty acid profile of the sperm. The ejaculate was
211 centrifuged at 1500 *g* for 10 min at 32^{o}C. The seminal plasma was removed and the sperm
212 pellet was resuspended in 3 mL of phosphate buffered saline (PBS) and centrifuged at 1000 *g*
213 for 10 min at 4^{o}C. The supernatant was removed and the sperm pellet was resuspended in 500
214 µL of cold PBS, snap frozen in liquid nitrogen and stored at -80^{o}C.

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216 2.7. Thiobarbituric acid reactive substances

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218 A sample (1 mL) of stored diluted semen from each ram was assessed at 24 and at 48 h post
219 semen collection for lipid peroxidation using the thiobarbituric acid reactive substances
220 (TBARS) assay [39]. Washed ram sperm were diluted to 200 x 10^6 sperm/mL and incubated
221 for 1 h at 37 ^{o}C in 600 µL Tyrosine Albumin Lactate Pyruvate (TALP) media containing 473
222 µM sodium ascorbate and 12 µM ferrous sulphate. Following incubation, 600 µL of 40%
223 (v/v) ice-cold trichloroacetic acid was added, left on ice for 10 min and centrifuged at 1600 *g*

for 12 min @ 4 ^{o}C. The supernatant (1 mL) was added to 500 µL of 2% (v/v) thiobarbituric acid in 0.2 N NaOH and boiled in a water bath at 95°C for 10 min. The samples were cooled on ice for 10 min, centrifuged at 3,500 *g* for 10 min and the absorbance of the supernatant was read at 534 nm using a spectrophotometer.

2.8. Sperm motility and mucus penetration test

A sample of chilled semen from each ram was assessed at 24, 48 and 72 h post semen collection for progressive linear motion (a minimum of 50 sperm were assessed) and the ability to penetrate artificial mucus (10 mg/mL sodium hyaluronate; MAP-5, Bioniche, Animal Health, Clonee, Ireland) [40]. A sample of semen from each ram was diluted to a final sperm concentration of 20 x 10^6 sperm /mL in Biggers, Whitten and Whittingham (BWW) media and was stained with Hoechst 33342 for 5 min at 37 °C to aid visibility. Flat capillary tubes (0.3 mm x 3.0 mm x 100 mm; Composite Metal Services, UK) were marked at 10, 30 and 70 mm intervals along the tube, loaded with artificial mucus and placed vertically (two capillary tubes per ram per day) in a 1.5 mL ependorf tube containing 250 µL of the stained sperm solution and incubated at 37 °C for 10 min. After incubation the tubes were placed on a hotplate at 50 ºC for 30 sec to kill and therefore immobilize the sperm. The numbers of spermatozoa in two fields of view at each mark were counted using a fluorescent microscope (400 X; Olympus BX 60). The two fields of view were at the centre of the capillary tube to avoid sperm which may swim against the capillary tube wall.

2.9. Fatty acid analysis of feed, plasma and sperm

All blood samples taken (Fig 1) were analysed, while only sperm samples collected on weeks 4, 7 and 9 were analysed for fatty acid content. Total lipids were extracted from 1 mL of plasma, from 6 g of the feed sample and from the full resuspended sperm cell pellet using chloroform methanol (2:1, v/v) as described previously [41]. Methylation was carried out for all samples by *in situ* transesterification with 0.5 N methanolic NaOH followed by 14% (v/v) boron trifluoride in methanol as described by Park and Goins [42]. The fatty acid methyl esters (FAME) were separated using a CP Sil 88 column (100 m x 0.25 mm i.d., 0.20 mm film thickness; Chrompack, Middleburg, The Netherlands) and quantified using a gas liquid chromatograph (GLC; 3400; Varian, Harbor City, CA, USA). The GLC was calibrated using a range of commercial fatty acid standards (Sigma Aldrich). The GLC was fitted with a flame ionization detector and helium (37 psi) was used as the carrier gas. The injector temperature was held isothermally at 225 ^{o}C for 10 min and the detector temperature was 250 ^{o}C. The column oven was held at an initial temperature of 140 ^{o}C for 8 min and then programmed to increase at a rate of 8.5 ^{o}C/min to a final temperature of 200^{o}C, which was held for 41 min. Data were recorded and analysed on a Minichrom PC system (VG Data System, Manchester, UK) and expressed as g/100g fatty acid methyl ester (FAME).

2.10. Plasma metabolite analysis

Plasma glucose, triglycerides, NEFA, BHBA, urea, and cholesterol concentrations were analysed using commercial biochemical assay kits (Boehringer Mannheim, Mannheim, Germany, and Randox Private Ltd., Crumlin UK) on an automated biochemical analyzer (ABX Mira, Cedex 4, France).

Plasma IGF-1 concentrations were determined by radioimmunoassay following an acid–ethanol extraction procedure as described previously by [43]. The mean inter- and intra-assay

coefficients of variation for samples containing low (45.24 ± 4.43 ng/mL^{-1}), medium (174.9 ± 13.80 ng/mL^{-1}) and high (466.4 ± 2.14 ng/mL^{-1}) IGF-1 concentrations were 9.8 and 20.2% (low), 7.9 and 15.5% (medium) and 0.5 and 12.6% (high).

Insulin concentration in plasma was determined using a human immunometric assay kit (AutoDELFIA Insulin, Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland; catalogue no. B080-101) on an automated time-resolved fluoroimmunoassay (TR-FIA) system (Wallac 1235 AutoDELFIA, Wallac Oy, Turku, Finland) as previously described by Ting et al. [44]. The mean inter and intra assay coefficients of variation for samples containing low (5.1 ± 0.62 pmol/L^{-1}), medium (10.2 ± 0.88 pmol/L^{-1}) and high (150.9 ± 7.04 pmol/L^{-1}) insulin concentrations were 12.2 and 16.1% (low), 8.6 and 11.57% (medium), and 4.7 % and 5.7% (high), respectively.

3.0. Statistical analysis

All data were analyzed using Statistical Analysis Software (SAS, version 9.1). Data were examined for normality and homogeneity of variance by histograms, quantile-quantile plots, and formal statistical tests as part of the UNIVARIATE procedure of SAS. Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate P-values. However, the corresponding least squares means and SE of the non-transformed data are presented in the results for clarity. Variables having more than 1 observation per subject, such as plasma and semen analytes, were analyzed using repeated measures linear mixed effects model ANOVA (MIXED procedure), with terms for diet, block, day (or time) of

sampling, and their interaction included in the model. The interaction term if not statistically significant ($P > 0.05$) was subsequently excluded from the final model. The type of variance-covariance structure used was chosen depending on the magnitude of the Akaike information criterion (AIC) for models run under compound symmetry, unstructured, autoregressive, or Toeplitz variance-covariance structures. The model with the least AIC value was selected. In all analyses the individual animal was denoted as the experimental unit, and animal within dietary treatment was set as the error term.

4.0. Results

4.1. Animal Performance, Metabolites, and Metabolic Hormones

There was no difference in ADG for rams fed the control and the FO diets nor was there any difference in the change in BCS between treatments during the experimental period ($P > 0.05$).

There was no diet by week interaction for any of the plasma metabolites measured (Table 3). In addition, there was no effect of diet or week on the metabolites measured with the exception of urea which increased from week 0 to week 9 ($P < 0.001$; Table 3). Plasma insulin concentrations declined linearly from the pre-experimental samples to the final samples ($P = 0.05$; Fig 2). Insulin was not affected by diet ($P = 0.11$) and there was no diet by sampling week interaction ($P = 0.78$). There was a diet by week interaction for IGF-1 ($P < 0.05$; Fig 3) and this was manifested in no difference in plasma concentrations of IGF-1 between treatment diets at the start of the experiment nor at the 4 week sampling. However,

concentrations were higher in FO supplemented animals on week 9 compared to the control treatment ($P < 0.05$; Fig 3).

4.2. Plasma and Sperm Fatty Acid Concentration

The effect of diet on the fatty acid concentration of plasma and sperm assessed are presented in Tables 4 and 5, respectively. However, in the interest of brevity we will only discuss changes in plasma and sperm concentrations of selected fatty acids which are of biological significance.

There was a diet by week interaction for plasma concentration of palmitic acid ($P < 0.05$) with levels higher in both the control and FO treatments on week 9 compared to the pre-experimental levels ($P < 0.01$). Plasma stearic acid concentrations declined in both treatment diets with week ($P < 0.001$) and tended to be lower in rams on the FO diet ($P = 0.06$). The difference between groups prior to the start of the experimental period in plasma oleic acid concentrations approached significance ($P = 0.09$), and was different on weeks 4 and 9 ($P < 0.001$), with the levels in the FO supplemented rams being lower than the control on both occasions (Table 4). Dietary supplementation with FO led to a 2.1 fold reduction in the plasma concentration of linoleic acid compared to the pre-experimental values ($P < 0.001$), while plasma concentrations remained elevated in the CON over the experimental period. There was a significant diet by week interaction in plasma arachidonic acid concentrations, with FO supplemented rams experiencing a 1.6 fold increase from pre-experimental levels to week 4, however, unexpectedly concentrations declined on week 9. Plasma concentrations of EPA and DHA in the CON increased by 4.47 and 3.6 fold, respectively. In contrast, dietary supplementation with FO led to a 9.3 fold increase in the plasma concentration of EPA and a

5.7 fold increase in the plasma concentration of DHA ($P < 0.001$) over the experimental period. As a result, supplementation with FO increased plasma concentrations of total *n*-3 PUFA's by 3.1 fold and decreased *n*-6 PUFA concentrations by 1.84 fold. Consequently, the ratio of *n*-6 to *n*-3 PUFA's decreased significantly in the FO supplemented rams ($P < 0.001$).

Sperm stearic acid and palmitic acid content declined with increasing weeks on the trial ($P < 0.01$) but were not affected by diet. There was a diet by week interaction ($P < 0.001$) for sperm oleic acid concentrations which was in line with plasma concentration trends described above, this manifested itself in a 2.87 fold decline in the FO treatment on week 9 compared to the week 4 values. There was a diet by week interaction for sperm concentration of EPA ($P < 0.05$), with dietary supplementation of FO leading to a 2.7 fold increase between weeks 4 and 9, while there was a 50 fold reduction in the CON fed group over the same period. There was no diet by week interaction or treatment effect on sperm DHA concentrations, however, there was an effect of week ($P < 0.001$). This was represented by an increase in sperm DHA concentration up to week 7 and a decline thereafter. There was a diet by treatment interaction on the effect of sperm arachidonic acid concentrations ($P < 0.01$). This was represented by a reduction in sperm arachidonic acid levels in the FO supplemented rams on week 7, however, they increased to near pre-experimental concentrations again on week 9. Sperm arachidonic acid concentrations in the CON were the same across all weeks. Consequently, from week 4 to 9, supplementation with FO increased sperm concentrations of total *n*-3 and *n*-6 PUFA's by 1.5 and 1.31 fold, respectively.

4.3. Semen Quality at Collection

There was no diet by week interaction on sperm concentration, however, there was an effect of diet on concentration with the FO supplemented rams yielding higher values than the control (4.15 ± 1.627 vs 3.61 ± 1.709 x 10^6 sperm/mL, respectively; $P < 0.05$). There was a quadratic effect of week on sperm concentration ($P < 0.01$), in which concentration increased from week 4 to 5 and declined on weeks 7, 8 and 9. There was no diet by week interaction, nor was there an effect of week of collection for semen volume. However, the animals on the CON diet had a strong tendency towards a higher volume than animals on the FO diet (0.95 ± 0.070 vs 0.77 ± 0.066 Ml, respectively; $P = 0.06$). Diet did not have any effect on total sperm number ($P = 0.95$), however, there was a tendency for an effect of week ($P = 0.09$). Wave motion was not affected by diet nor was there a diet by week interaction, however, in line with the change in semen concentration, there was a quadratic effect of week ($P < 0.05$) which was represented by an increase in wave motion from week 4 to 5 and then a steady decrease to week 9.

4.4. PLM, Mucus Penetration and Lipid Peroxidation

There was no diet by week interaction or effect of diet on PLM, however, there was a linear decline in PLM from week 5 to 9 of the experiment, in line with the ending of the normal sheep breeding season for the breeds used in this study (54.6 ± 4.32 and 45.8 ± 4.12 %, respectively; $P < 0.05$). There was a number of 2 and 3 way interactions between the main factors employed in the swim up study; namely treatment, week of study, hours post semen collection and position in the capillary tube. Consequently, there was no clear pattern of biological significance on the effect of these variables on the ability of sperm to penetrate artificial mucus. There was no effect of treatment on lipid peroxidation as assessed by the TBARS assay, but there was a week by hour interaction ($P < 0.001$), however, similar to the

mucus penetration assay there was no clear pattern of biological significance towards an effect of diet.

5.0. Discussion:

The current study has demonstrated that enrichment of the diet of rams with *n*-3 PUFA, can alter the fatty acid profile of plasma and to a more limited extent sperm. This had a knock on effect in improving sperm concentration but had no effect on the quality of liquid stored ram sperm as assessed by PLM, the ability to penetrate artificial mucus or on the ability to resist lipid peroxidation.

All rams consistently consumed their feed over the duration of the experiment and dietary supplementation did not influence ADG, BCS (as planned) or any of the plasma metabolites measured including BHBA, cholesterol, glucose, NEFA, triglycerides or urea, which is in agreement with studies in cattle fed a similar dietary supplement [5, 9, 23, 45]. Similarly, insulin concentrations were not affected by *n*-3 PUFA supplementation, which is in agreement with studies in lactating dairy cows [46] and boars [47]. However, in the current study, the plasma concentrations of the metabolic hormone IGF-1 increased significantly on week 9 in the FO treatment compared to the control. IGF-I concentrations in rams has been previously shown to be affected by diet [48] and to be positively correlated with sperm function across a range of species [49-51].

The most prominent fatty acids in ram sperm in the current study in descending order were, palmitic, DHA, oleic, stearic, myristic and myristoleic acids, which broadly agree with other studies [52]. However, the level of DHA (mean 17.8% in the week 4 samples) was most

notably lower than in other studies which assessed ram sperm [53-56]. It is unlikely that differences in the genetic profile or the nutrition of the rams would explain such substantial differences in sperm DHA content and is more likely to be due to the way in which concentrations are expressed. Given that the fatty acid profile is expressed in most studies as a proportion of the total fatty acids measured in sperm, the disparity in DHA content is most likely due to the more extensive number of fatty acids profiled in sperm in the current study (21 fatty acids in total compared to approximately half this in other studies). Interestingly, sperm DHA concentrations were in the order of 30 fold higher than plasma DHA concentrations (on week 4 of the experiment), indicating an extensive localising effect in the testes [57, 58]. Supplementation with FO increased DHA concentrations further in both plasma and sperm and in line with the objectives of the current study, led to an increase in the plasma and sperm concentrations of EPA, respectively; as well as a parallel decrease in plasma linoleic concentrations. This consequently led to a reduction in the *n*-6:*n*-3 PUFA ratio in plasma, and to a lesser extent in sperm, a trend which is in agreement with previous fertility studies which used the same FO dietary supplement and focused on reproductive tissues in heifers [9, 45].

Supplementation with FO increased semen concentration, but not semen volume, total sperm number or wave motion. The differences in semen concentration indicate that the rate of spermatogenesis may be hastened in rams supplemented with FO, which would agree with other studies which suggest that *n*-3 PUFA, especially DHA, has an important role in the formation of functional sperm [59, 60]. Despite this, there was no beneficial effect of FO supplementation on subsequent sperm quality during liquid storage at 4 ^{o}C as assessed by PLM, ability to penetrate artificial mucus or resist lipid peroxidation. There are a number of reports on the effect of a range of dietary PUFA supplements on the subsequent success of

cryopreservation of ram, bull and boar semen. De Graaf et al. [30] reported that dietary supplementation of rams with oleic acid, or linoleic acid, did not improve the cryosurvival of ram spermatozoa, whether it had been processed for sex-sorting by flow cytometry or not. Gholami et al. [23] fed a DHA enriched diet to Holstein bulls and reported an improvement in the *in vitro* (as assessed by computer assisted sperm analysis) quality of fresh but not frozen-thawed semen. Selvaraju et al. [22] examined the effect of different sources of dietary energy and fed rams a maize diet or a sunflower oil diet (linoleic acid) and found that PUFA enrichment influenced sperm quality by stabilising membrane integrity but had no effect of the ability of sperm to fertilise oocytes *in vitro*. Castellano et al. carried out a number of detailed studies on the effect of dietary FO supplementation of boars and reported that such supplementation had no effect on sperm output [35], on frozen-thawed semen quality [61] but had a minor beneficial effect on liquid stored semen [61].

In conclusion, dietary supplementation with FO successfully modified the plasma *n*-6: *n*-3 ratio, but had limited effects on the corresponding sperm fatty acid profile. While supplementation increased semen concentration it had no effect on the quality of liquid stored semen. It should, however, be noted that this study used rams of normal fertility and it may be that PUFA supplementation, may be more beneficial to rams with poor semen quality. Nevertheless these results provide an insight into the relationship between nutrition and sperm function and further research to elucidate the mechanisms by which diet manipulation affects the integrity of the sperm membrane and subsequent sperm quality is warranted.

6.0. References:

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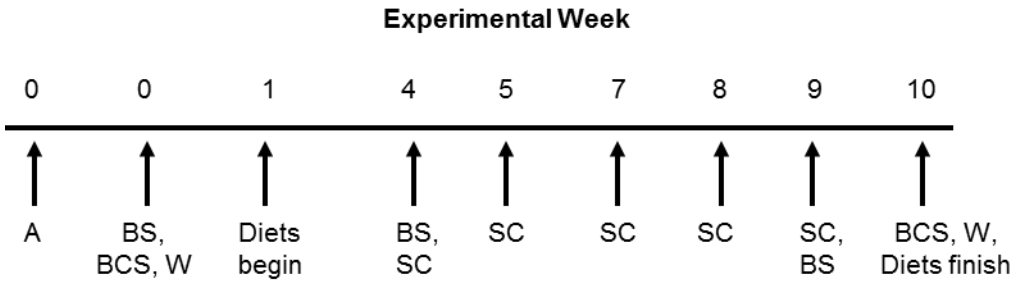

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665 **Fig 1:** Schedule of tasks undertaken for experiment; BS = Blood sample; A = acclimatisation;

666 W = weigh; BCS = body condition score; SC = semen collection

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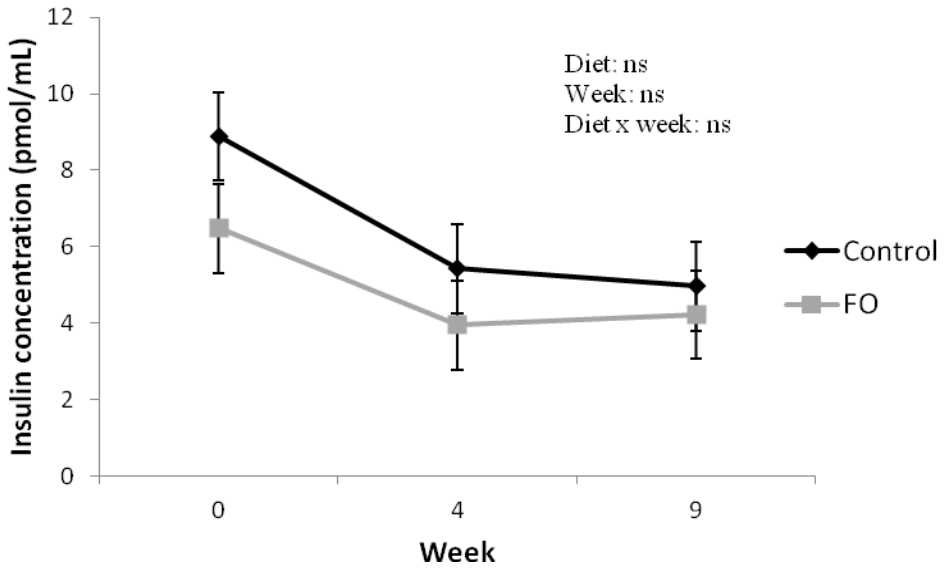
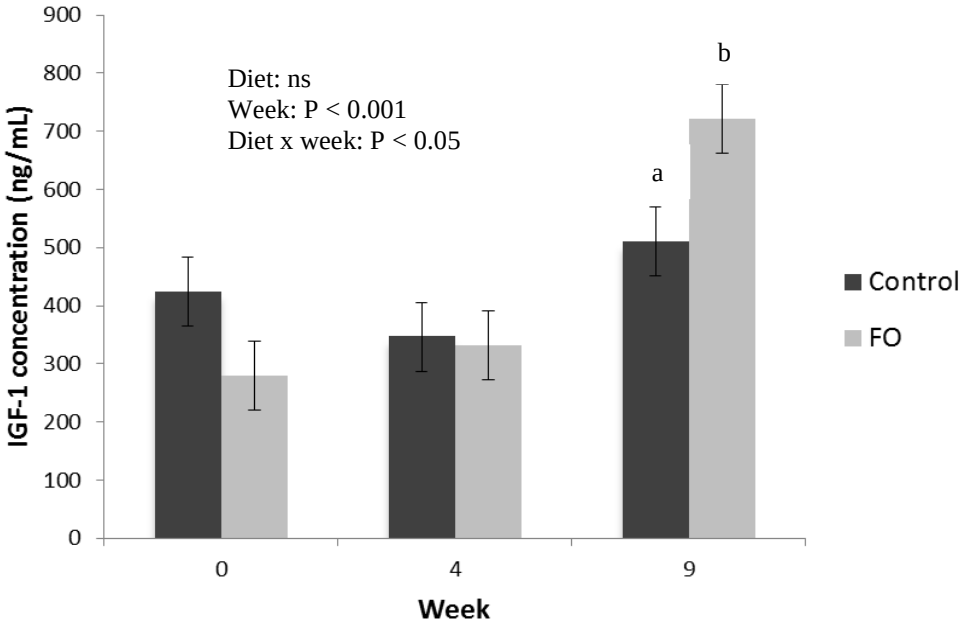


Fig 2: The effect of diet on plasma insulin concentration (pmol/mL) on weeks 0, 4 and 9 of the experimental period. Vertical bars represent standard error of the mean



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704 **Fig 3:** The effect of diet on plasma IGF-1 concentrations (ng/mL) on weeks 0, 4 and 9 of the
705 experimental period. Vertical bars represent standard error of the mean. $^{ab}P < 0.05$.

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721 **Table 1:** Ingredient composition (g/kg as fed) and chemical analysis (expressed as g/kg of
722 dry matter unless otherwise stated) of the control ration, fish oil ration and hay

Ingredient	Control	Fish Oil	Hay
Rolled Barley	350	310	
Soya bean Meal (48%)	210	220	
Molassed sugar beet pulp	340	300	
[a]Palmit 80 prills	50		
EPA/DHA supplement		120	
[b]Vitamin mineral Premix	50	50	
DM (g/kg)	959	962	948
Crude Protein	180.7	186.4	95.5
Crude Fibre	50.2	31.1	311.0
Ash	98.7	158.7	53.5
Ether Extract	70.51	72.0	20.9
Gross energy (MJ/kg DM)	17.48	16.33	17.12

723 [a]Minimum content of palmitic acid (C16:0) = 80%; myristic acid (C14:0) < 2%; stearic acid
724 (C18:0) = 4–7%; oleic acid (C18:1) = 8–12%; linoleic acid < 3%.

725 [b]Premix supplied per kilogram of supplement: 500000 IU of vitamin A; 100000 IU of vitamin
726 D3; 750 mg of vitamin E as alpha tocopherol; 25 mg Selenium; 261 g calcium; 300 g
727 phosphorus; 120 g sodium.

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734 **Table 2:** Fatty acid profile of concentrates fed (g/100 g FAME)

	CON	**FO**
Myristic (C14:0)	3.68	0.27
Palmitic (C16:0)	71.92	8.16
Stearic (C18:0)	1.49	3.98
Oleic (C18:1)	8.57	9.96
n-6 PUFA		
Linoleic (C18:2)	11.12	13.20
Arachidonic (C20:4)	0.01	0.99
Eicosatrienoic (C20:3)	0.02	0.25
n-3 PUFA		
Linolenic (C18:3)	1.34	2.15
EPA (C20:5)	0.21	26.11
DPA (C22:5)	0.50	4.92
DHA (C22:6)	0.12	16.89
n-3 family[a]	1.67	45.15
n-6 family[b]	11.13	14.19
n-6 to *n*-3 ratio	6.66	0.31

735 [a] C18:3 + C20:5 + C22:6.
736 [b] C18:2 + C20:4.

Table 3: The effect of diet on plasma metabolite (mmol/l) concentrations on weeks 0 and 9 of the experimental period

	Treatment			**Week**			**P values****	
	CON	**FO**	**SED***	**0**	**9**	**SED***	**Trt**	**Week**
BHBA	0.21	0.22	0.004	0.20	0.23	0.023	0.853	0.247
Cholesterol	2.04	1.83	0.134	2.03	1.84	0.134	0.121	0.167
Glucose	3.60	3.62	0.159	3.76	3.46	0.159	0.898	0.073
NEFA	0.78	0.74	0.096	0.79	0.73	0.796	0.645	0.544
Triglycerides	0.18	0.15	0.020	0.17	0.16	0.020	0.272	0.412
Urea	6.89	7.01	0.346	5.09	8.81	0.346	0.744	<0.001

*Standard error of the difference. **No significant treatment x week interaction for any variable

Table 4: Effect of diet on fatty acid concentration of plasma sampled pre-trial and on weeks 4 and 9 of the experimental period (mean ± s.e.m)

Fatty Acid	**Diet**						**Statistical significance P value**		
	CON, week			**FO, week**			**Diet**	**Week**	**Diet x week**
	Pre	4	9	Pre	4	9			
Butyric (C4:0)	0.53±0.019	0.38±0.019	0.35±0.019	0.38±0.0177	0.43±0.0177	0.45±0.018	0.9608	0.0049	<0.0001
Caprylic acid(C8:0)	0.28±0.037	0.31±0.037	0.36±0.037	0.32±0.037	0.31±0.037	0.41±0.037	0.3448	0.0462	NS
C9:t11CLA	0.55±0.085	0.28±0.085	0.12±0.085	0.49±0.085	0.05±0.085	0.01±0.01	0.5842	0.0625	NS
Myristic (C14:0)	6.49±0.661	4.63±0.661	5.62±0.661	3.60±0.661	1.65±0.661	4.49±0.661	0.0004	0.0278	NS
Pentadecylic acid (C15:0)	0.62±0.039	0.50±0.039	0.51±0.039	0.65±0.035	0.73±0.035	0.64±0.035	0.0011	0.3091	0.0590
Palmitic (C16:0)	23.92±0.576	28.12±0.576	26.87±0.576	23.83±0.573	26.56±0.573	29.53±0.573	0.3077	<.0001	0.0110
Stearic (C18:0)	21.62±0.488	18.39±0.488	19.09±0.488	21.83±0.488	17.66±0.488	17.20±0.488	0.0626	<.0001	NS
Heptadecenoic acid (C17:1)	0.41±0.065	0.61±0.065	0.42±0.065	0.61±0.058	0.05±0.058	0.23±0.058	0.0060	0.0005	<.0001
Oleic (C18:1c9)	16.00±0.879	16.41±0.879	12.62±0.879	18.17±0.879	7.65±0.879	6.35±0.879	<.0001	<.0001	<.0001
Vaccenic (t11C18: 1)	1.96±0.260	0.97±0.260	2.28±0.260	2.15±0.260	5.22±0.260	4.74±0.260	<.0001	<.0001	<.0001
n-6 PUFA									
Linoleic (C18:2)	10.56±0.62	12.91±0.62	10.88±0.62	11.36±0.621	5.00±0.621	5.47±0.621	<.0001	0.0001	<.0001
Arachidonic (C20:4)	1.83±0.182	1.71±0.182	1.82±0.182	1.91±0.182	3.00±0.182	1.69±0.182	0.0654	0.0312	0.0073

Fatty Acid	Diet						Statistical significance P value		
	CON, week			FO, week			Diet	Week	Diet x week
DGLA (C20:3)	0.22±0.052	0.17±0.052	0.17±0.052	0.14±0.052	0.21±0.052	0.11±0.052	0.4604	0.6037	0.5454
n-3 PUFA									
Linolenic (C18:3)	1.75±0.133	1.93±0.133	2.00±0.133	2.19±0.133	1.05±0.133	1.33±0.133	0.0105	0.0026	<.0001
EPA (C20:5)	0.87±0.521	1.07±0.521	3.89±0.521	0.98±0.521	13.68±0.521	9.08±0.521	<.0001	<.0001	<.0001
DPA (C22:5)	1.06±0.089	1.06±0.089	1.37±0.089	1.20±0.089	1.61±0.089	1.37±0.089	0.0143	0.0188	0.0095
DHA (C22:6)	0.59±0.191	0.71±0.191	2.14±0.191	0.60±0.191	4.21±0.191	3.41±0.191	<.0001	<.0001	<.0001
Plasma Total FA	97.12±0.226	96.76±0.226	96.99±0.226	96.95±0.226	97.52±0.226	96.86±0.226	0.4248	0.6485	0.0867
n-3 family[a]	4.27±0.710	4.77±0.710	9.39±0.710	4.97±0.710	20.55±0.710	15.19±0.710	<.0001	<.0001	<.0001
n-6 family[b]	12.61±0.695	14.79±0.695	12.86±0.695	13.41±0.695	8.21±0.695	7.27±0.695	<.0001	0.0004	<.0001
n-6 to *n*-3 ratio	3.07±0.224	3.11±0.224	1.53±0.224	2.84±0.224	0.48±0.224	0.48±0.224	<.0001	0.0004	<.0001

Table 5: Effect of diet on fatty acid concentration of sperm sampled on weeks 4, 7 and 9 of the experimental period (mean ± s.e.m)

Fatty Acid	Diet						Statistical significance P value		
	CON, week			FO, week			Diet	Week	Diet x week
	4	7	9	4	7	9			
Enanthic acid (C7:0)	0.87±0.092	0.32±0.076	0.22±0.066	0.32±0.059	-0.006±0.059	0.13±0.065	0.0004	<.0001	0.0153
Lauric acid (C12:0)	0.59+0.084	0.37±0.071	0.61±0.071	0.57±0.066	0.35±0.066	0.45±0.070	0.3919	0.0019	NS
Tridecylic acid (C13:0)	0.26±1.295	2.8±1.079	3.33±1.079	4.27±1.004	1.69±1.004	3.00±1.071	0.3066	0.7354	NS
Margaric acid (C17_0)	0.30±0.029	0.26±0.024	0.25±0.024	0.30±0.022	0.22±0.022	0.24±0.0238	0.2497	0.0419	NS
Myristoleic Acid (C14:1t)	4.70±0.688	7.78±0.573	7.51±0.573	6.10±0.533	8.83±0.533	8.12±0.570	0.1089	<.0001	NS
Myristoleic Acid (C14:1c)	1.07±0.149	0.78±0.122	0.71±0.122	0.80±0.113	0.63±0.113	0.60±0.122	0.102	0.081	NS
C16:1t	0.65±0.048	0.79±0.039	0.73±0.039	0.43±0.036	0.57±0.036	0.62±0.039	<.0001	0.0027	NS
C16_1c	0.93±0.171	0.50±0.141	0.57±0.141	0.77±0.130	0.29±0.130	0.15±0.140	0.0172	0.0054	NS
C18_1c9	2.87±0.185	2.18±0.151	2.05±0.151	1.62±0.140	1.36±0.140	1.44±0.151	<.0001	0.0156	NS
C18_1t	0.91±0.125	1.35±0.105	1.04±0.106	0.91±0.099	1.41±0.099	1.34±0.105	0.3001	<.0001	NS
Caprylic acid (C8:0)	0.66±0.097	0.30±0.081	0.47±0.081	0.53±0.075	0.14±0.075	0.31±0.080	0.0127	0.0012	NS
Myristic (C14:0)	11.23±1.388	5.63±1.154	5.03±1.154	8.49±1.072	2.77±1.072	1.36±1.147	0.0012	<.0001	NS
Palmitic (C16:0)	33.58±2.202	26.19±1.798	31.21±1.798	33.53±1.272	28.42±1.665	30.43±1.798	0.7131	0.0061	NS
Stearic (C18:0)	18.71±1.406	14.21±1.202	17.14±1.1202	17.31±1.131	13.87±1.131	15.43±1.194	0.4348	0.0009	NS
Oleic (C18:1c-9)*	16.00±0.879	16.41±0.879	12.62±0.879	18.17±0.879	7.6543±0.879	6.35±0.879	<.0001	<.0001	<.0001

Fatty Acid	Diet						Statistical significance P value		
	CON, week			FO, week			Diet	Week	Diet x week
n-6 PUFA									
Linoleic (C18:2)	1.45±0.153	1.59±0.128	1.29±0.128	1.06±0.120	1.20±0.120	1.08±0.127	0.0347	0.1369	NS
Arachidonic (C20:4)	0.87±0.021	0.88±0.021	0.87±0.021	0.86±0.021	0.76±0.021	0.88±0.021	0.0654	0.0312	0.0073
n-3 PUFA									
Linolenic (C18:3)	0.29±0.0168	0.31±0.014	0.32±0.014	0.30±0.013	0.27±0.013	0.30±0.014	0.2203	0.3239	NS
EPA (C20:5)	0.10±0.048	0.06±0.040	0.002±0.040	0.09±0.037	0.23±0.037	0.24±0.040	0.0058	0.3891	0.0104
DPA (C22:5)	0.44±0.066	0.40±0.056	0.38±0.056	0.40±0.053	0.71±0.053	0.58±0.056	0.025	0.027	0.005
DHA (C22:6)	16.61±2.937	27.37±2.524	22.96±2.524	18.96±2.379	32.51±2.379	28.53±2.507	0.1395	<.0001	NS
Sperm Total FA	98.37±0.588	96.09±0.502	97.46±0.502	98.12±0.469	97.71±0.469	97.16±0.494	0.1559	0.1305	NS
n-3 family[a]	17.44±2.994	28.14±2.573	23.66±2.573	19.75±2.426	33.72±2.426	29.65±2.426	0.1396	<.0001	0.6080
n-6 family[b]	2.60±0.347	3.40±0.296	2.77±0.296	2.23±0.279	3.47±0.279	2.92±0.294	0.8826	0.0011	0.5139
n-6 to *n*-3 ratio	0.15±0.010	0.12±0.009	0.12±0.009	0.11±0.008	0.10±0.008	0.10±0.009	0.0103	0.0285	0.6039