Title

The Effect of Dietary Supplementation of Algae Rich in Docosahexaenoic Acid on Boar Fertility

Authors

E.M Murphy[a], C. Stanton[b], C. O'Brien[b], C. Murphy[a], S. Holden[a], R.P. Murphy[c], P. Varley[c], M.P. Boland[d], *S. Fair[a].

Affiliations

[a]Laboratory of Animal Reproduction, Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland. [b]Teagasc Biotechnology Centre, Moorepark Food Research Centre, Fermoy, Co. Cork, Ireland. [c]Hermitage Pig Genetics Ltd, Callan, Co. Kilkenny, Ireland. [d]Alltech, Bioscience Centre, Dunboyne, Co Meath, Ireland

*Corresponding Author: Dr Sean Fair, Department of Life Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland. Tel: + 353 61 202548, Fax: + 353 61 331490, E-mail sean.fair@ul.ie

Keywords:

DHA, Sperm, Cryopreservation, PUFA, Farrowing rate

Abstract

The objective of this study was to assess the effects of dietary supplementation of a commercial algal product rich in docosahexaenoic acid (DHA) on boar fertility as assessed *in vitro* and *in vivo*. Boars were fed one of three experimental diets for 19 weeks: (i) Control (Ctl) diet (n=31), (ii) Ctl diet plus 75g All-G-Rich per day (n=31) or (iii) Ctl diet plus 150g All-G-Rich per day (n=30). Parameters assessed were (i) raw semen quality; volume, sperm concentration, total motility and morphology (ii) liquid semen quality; progressive motility, viability, hypotonic resistance and acrosomal integrity (iii) frozen-thawed semen quality; motility, thermal stress, viability, membrane fluidity and mitochondrial activity (iv) sperm and seminal plasma (SP) fatty acid composition (FAC) (v) total antioxidant capacity (TAC) of SP and (vi) farrowing rates and litter sizes of sows (n=1,158) inseminated with liquid semen. Boars consuming 75g All-G-Rich had a larger semen volume ($P<0.05$) and a higher total sperm number ($P<0.01$) than the Ctl treatment, however, there was no effect of treatment on any other semen quality parameter ($P>0.05$). There was no effect of dietary treatment on the FAC and TAC of SP or on farrowing rate and litter size ($P>0.05$). There was an effect of dietary treatment on the FAC of sperm, represented by an 1.72 and 1.60 fold increase in the DHA content for 75g and 150g treatments, respectively, compared to the Ctl treatment. In conclusion, dietary supplementation of algae altered the FAC of sperm, increased semen volume and total sperm numbers but had no effect on any other semen quality parameter as assessed *in vitro* or *in vivo*.

1. Introduction

Polyunsaturated fatty acids (PUFAs), especially omega-3 fatty acids, have been widely reported to have positive effects on human health, such as reducing the risk of both

cardiovascular disease [1] and breast cancer [2] through their anti-inflammatory and chemo-preventative activities. They have been shown to have beneficial effects on female fertility through supporting foetal development [3] and, more recently, have been shown to positively influence male fertility in both human [4] and animal models, including; the boar [5, 6], ram [7], bull [8], stallion [9] and chicken [10]. Dietary supplementation of PUFA's have been shown to alter the (FAC) of the sperm and seminal plasma, increase libido, sperm concentration and, thus, total sperm numbers [6], decrease morphological abnormalities [11] and also increase sperm motility [12]. These benefits may be due to the influence of PUFA's on steroid production pathways, such as increasing testosterone concentration [13], the number of gonadotrophin receptors involved in the regulation of steroidogenesis and also as a result of modifying the phospholipid profile of the sperm membrane facilitating an increase in the fluidity and flexibility of the sperm membrane [14].

Docosahexaenoic acid (DHA) is a long chain omega-3 PUFA and is the most prevalent unsaturated fatty acid found in mammalian sperm cells, comprising approximately, 38% of phospholipids and 20-30% of total omega-3 fatty acids in boar sperm [15]. The highest concentration of DHA in sperm is located within the tail region (99%) rather than the head (1%; [16]), giving the plasma membrane of the sperm tail a significant degree of flexibility and elasticity, facilitating the flagella movement required for motility [15]. The inclusion of DHA in the diet has been shown to contribute to sperm membrane fluidity and flexibility [17], enabling sperm to undergo membrane associated events such as capacitation and the acrosome reaction [15]. Two main approaches have been used to investigate the role of fatty acids in sperm cell function, namely; to define the lipid composition of both sperm and seminal plasma in normozoospermic and asthenozoospermic males [14, 18]. The second approach is the addition of specific fatty acids to the male diet in an attempt to increase the

rate of spermatogenesis and alter the fatty acid profile of sperm cell membranes and therefore, improve sperm quality [5, 19].

Although most studies feeding PUFAs, from fish or vegetable oil sources, have reported a positive modification in the sperm lipid composition, in particular increasing the proportion of DHA and decreasing the level of saturated fatty acids [12, 20], not all studies observed similar results in relation to sperm production, quality and function [11, 20]. By far the most promising results are from studies which have supplemented DHA in the diet or through the addition of various oil types [5, 8, 20]. Rooke et al. [5] supplemented boars with 30g tuna oil diet for a period of 6 weeks and reported a decrease in the percentage of boar sperm with morphological abnormalities and an increase in progressive motility while Gholami et al. [8] reported an improvement in the viability and motility of fresh bovine semen. However, contradiction in the literature may be related to differences in breeds, boar age, sources of omega-3 PUFAs and the duration of supplementation. Most of these studies are focused on either the *in vitro* analysis of liquid or frozen-thawed semen and are not supported with field data. However, studies which have reported farrowing rate and litter size from boars, supplemented PUFAs in the diet, are confounded by small sample size [21].

Fish oils are the major commercial source of omega-3 fatty acids, specifically DHA [22]. However, due to increases in global demand and the price of fish oils, the development of fish oil alternatives is imperative [24]. Microalgae mass culture is a renewable production technology at an industrial scale which enables the production of omega-3 fatty acids, particularly DHA, from algal sources [25] and is now providing an important source of DHA within the food industry [26]. However, there is no published study on the effect of the

dietary supplementation of algal DHA on male fertility in any species. The objective of this study was to assess the effects of dietary supplementation of a commercial algal product rich in docosahexaenoic acid (DHA) on boar fertility as assessed *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Experimental Design

Purebred maternal (Landrace; LR; n=36, Largewhite; LW; n=35) and terminal line boars (Maxgro; MG; n=21; Hermitage Genetics 2014) of proven fertility, ranging between 10 and 18 months of age were used in this study. All boars were balanced across treatments according to age, breed and pre-experimental semen quality records from the boar stud (total motility, sperm concentration and morphology). Boars were fed one of three experimental diets for 19 weeks. These diets included (i) Control diet (Ctl; standard commercial grain based diet; n=31 boars), (ii) Ctl diet plus 75g of All-G-Rich per day (75g; n=31 boars) and (iii) Ctl diet plus 150g of All-G-Rich per day (150g; n=30 boars). All-G-Rich is a DHA-rich algal commercial supplement (Hower 2014: Filer 2014) which contains the antioxidant ethoxyquin (Table 2: Alltech, Dunboyne, Co Meath, Ireland). Ethoxyquin, is a synthetic antioxidant, commonly used in animal feed to protect against lipid peroxidation but is also known for having a high antioxidant capacity [28]. This study was approved by the University of Limerick eithics committee (2013_12_1_ULAEC).

Semen was collected from boars once per week throughout the supplementation period and assessed on farm for volume, sperm concentration as well as motility and morphology (microscopy-based; Figure 1) from week 1 to 14. Laboratory technicians were blind to treatments. On 3 occasions (replicates; Figure 1), between week 8 and 14, liquid semen from

a subset of boars (n=12 per treatment) was assessed on Days 1, 3 and 6 post collection for progressive motility using a phase-contrast microscope and viability, hypotonic resistance and acrosomal integrity via flow cytometry. Motility and viability were performed on liquid semen as these are standard parameters to assess semen quality while acrosomal integrity and hypotonic resistance were conducted to assess the versatility of sperm to increased duration of storage. Throughout the experimental period sows (n=1,158) were inseminated with liquid semen on commercial farms (n=27) and farrowing rates and litter sizes were captured. On 3 occasions (replicates; Figure 1) between week 14 and 19, semen was collected and frozen from 6 boars per treatment. Frozen-thawed semen was assessed post-thaw for motility pre- and post-thermal stress using Computer Assisted Sperm Analysis (CASA; SCA Evolution, Microptics, Barcelona, Spain) as well as viability, membrane fluidity, mitochondrial activity and oxidative stress via flow cytometry. These tests were performed on frozen-thawed semen as again motility and viability are basic parameters to assess semen quality while thermal stress test, membrane fluidity and oxidative stress test were conducted to best assess if dietary supplementation reduced the damage sustained to sperm membrane due to the stress associated with the freeze-thaw process. On weeks 1, 8 and 18 (Figure 1), ejaculates were collected from 12 boars per treatment, centrifuged and sperm and seminal plasma (SP) were harvested and frozen. The SP was later analysed for total antioxidant capacity (TAC) while the sperm and SP samples were analysed for their fatty acid profile (week 8 only).

2.2 Composition of Diets and Feeding Regime

All boars were located at one commercial boar stud in County Kilkenny, Ireland, individually housed and fed and maintained under similar management and feeding conditions. Boars were individually fed approximately 3 kg of Ctl diet daily as per routine procedure (depending on size and age), between 08.30 and 09.00 hrs, and had *ad libitum* access to

145 water. The All-G-Rich product was top-dressed onto the Ctl diet, and throughout the trial
146 period, boars were monitored on a daily basis to ensure that complete consumption of
147 allocated feed occurred. The chemical analysis of the Ctl diet and the All-G-Rich supplement
148 are presented in Table 1. The FAC of the Ctl diet and All-G-Rich supplement are presented in
149 Table 2.

150

151 *2.3 Semen Collection and Processing*

152 Semen was collected from all boars using the glove-hand technique [5] and following
153 collection, the gel portion and sperm-poor fraction of each ejaculate was discarded so that
154 only the sperm-rich fraction was used for processing. Ejaculates were kept separate
155 throughout and were initially partially extended in pre-warmed (37 ^{o}C) TRIXcell + swine
156 extender (IMV Technologies, Normandy, France) immediately post collection to allow for
157 initial on farm analysis. On farm analysis was conducted to assess semen volume (based on
158 weight), sperm concentration via colorimeter (Sherwood 254; SciChem, Co. Cork, Ireland)
159 and total motility (5 point scale; 1 = < 20% motile sperm; 5 = 81 – 100% motile sperm) and
160 morphology via microscopy, to ensure all semen samples were of a commercial standard ($\geq$ 3
161 and < 20% abnormalities). Abnormalities were divided into primary (clumps and detached
162 heads) and secondary (distal droplets, proximal droplets and long, bent or broken tails)
163 abnormalities [57]. Total sperm numbers were calculated by multiplying sperm concentration
164 (10^6/mL) by semen volume (mL). Each ejaculate was then fully extended to achieve 2.7 x 10^9
165 sperm per 75 mL dose, via colorimeter. Semen was packaged into individual doses using a
166 GTB 1000 instrument (IMV Technologies, Normandy, France) following which these were
167 sealed and placed in a temperature controlled cooler box for transport at 17 ^{o}C.

168

Upon collection of semen samples for cryopreservation, a portion of the raw ejaculate (15 mL) was partially extended with TRIXcell+ while the remainder of the ejaculate was processed for liquid semen. Semen samples (15 mL) were assessed for sperm concentration, total motility and morphology as described above. Only ejaculates achieving a morphology score of > 85% and a motility score of > 4.5 were used for cryopreservation. Following *in vitro* assessments, the semen was fully extended with pre-warmed TRIXcell+ to achieve a concentration of 3 x 10^9 sperm per mL and was subsequently cooled to room temperature (15 – 17^{o}C) over approximately 60 min. Samples were then centrifuged at 1000 *g* for 25 min at 15 ^{o}C, the supernatant was removed, leaving 3 mL of a pellet (1 x 10^9 sperm/mL). A cooling diluent (12 mL; BOARCIPHOS A; IMV Technologies, Normandy, France), at 15 ^{o}C, was added slowly to re-suspend the sperm pellet, to give a working concentration of 200 x 10^6 sperm per mL. Samples were refrigerated at 4 ^{o}C for 60 min, following which, a further 15 mL of freezing diluent (BOARCIPHOS B; IMV Technologies, Normandy, France), at 4^{o}C, was added to give a final concentration of 100 x 10^6 sperm per mL. Samples were then packaged into 0.5 mL straws (IMV Technologies, Normandy, France), at 4^{o}C (10 straws per ejaculate) and sealed using polyvinyl alcohol powder. Straws were frozen to -140 ^{o}C as follows: 3^{o}C per min from +5^{o}C to -5^{o}C and thereafter 40^{o}C per min from -5^{o}C to -140^{o}C [30] in a programmable freezer (IMV Technologies, Normandy, France), followed by submersion and storage in liquid nitrogen at -196 ^{o}C until use.

2.4 Sperm Functional Assessments

2.4.1 In Vitro Microscopic Analysis of Liquid and Frozen-Thawed Semen

2.4.1.1 Standard Microscopic Techniques:

This method was used for the assessment of progressive motility of diluted liquid semen on Days 1, 3 and 6 post semen collection. Motility of liquid semen was assessed using a phase contrast microscope (CX31; Olympus, Centre Valley, PA, USA) at a magnification of 400 X. A drop (5 µL) of diluted semen was placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (37 ^{o}C) and assessed by counting a minimum of 100 sperm, over at least five different fields of view, for each dietary treatment on each assessment day. Motility was expressed as the percentage of motile sperm in the total sperm population (motile and non-motile).

2.4.1.2 CASA:

Motility of frozen-thawed sperm samples was assessed using a CASA system. Straws (n=4 per ejaculate) were thawed at 70 ^{o}C for 8 sec and placed into 3 mL of pre-warmed Beltsville Thawing Solution (BTS; 37 ^{o}C). Samples were centrifuged at 400 *g* for 6 min at 32 ^{o}C. The supernatant was removed, sperm pellet re-suspended in 1 mL BTS and incubated in a heated block at 37 ^{o}C. A drop (5 µL) of diluted semen was placed on a pre-warmed chamber (37 ^{o}C; Leja counting chambers, depth 20 µm; Microptics, Barcelona, Spain) and analysed for sperm motion and kinematic characteristics immediately post-thaw. A minimum of five microscopic fields with at least 300 sperm were analysed in each sample using a phase-contrast microscope at 100X fitted with a pre-warmed stage at 37˚C. Objects incorrectly identified as sperm were edited out using the playback function. The CASA derived motility and kinematic characteristics assessed were total motility, progressive motility, average path

velocity (VAP above 10 μm/s), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF; [31]).

2.4.1.3 Thermal Stress Test

A thermal stress test [56] was conducted on only frozen-thawed sperm using CASA. Sperm were diluted 2:1 in BTS to achieve 50 x 10^6 sperm/mL. Briefly, the motility of frozen-thawed sperm were assessed immediately post-dilution (T0) and again following a 30 min incubation (T30) of the diluted sperm at 37 ^{o}C. The difference in motility immediately post-dilution and after 30 min at 37 ^{o}C was recorded.

2.4.2 In Vitro Flow Cytometric Analysis of Liquid and Frozen-Thawed Semen

Before flow cytometric analysis, sperm samples were diluted to a final working concentration of 300 x 10^5 sperm/mL in either TRIXcell+ (liquid semen samples) or BTS (frozen-thawed semen samples). Samples were analysed on a flow cytometer (Guava easyCyte 6HT-2L, Merck Millipore, Billerica, MA, USA) equipped with both a Krypton Laser (642 nm) and an Argon Laser (488 nm). Appropriate single colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in conjunction with the forward scatter (FSC) and side scatter (SSC) signals to discriminate sperm from debris (P0.1 Population). Fluorescent events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample 10,000 gated events were captured. All fluorochromes used were prepared as standard procedure using dimethyl sulfoxide (DMSO).

241 2.4.2.1 Assessment of Viability

242 Viability was assessed for both liquid and frozen-thawed semen using two fluorescent probes;
243 a nucleic acid probe, SYTO 16 (Ex/Em: 488/518; Life Technologies, Carlsbad, CA USA) is a
244 cell permeant probe which fluoresces upon binding to nucleic acids. Propidium iodide (PI;
245 Ex/Em: 535/617; Life Technologies, Carlsbad, CA, USA) is selectively taken up by
246 membrane compromised cells thus indicating a loss of viability [32]. SYTO 16 (5 µL) was
247 added to diluted sperm (300 x 10^5 sperm/mL) to give a final concentration of 100 nM and
248 incubated at 37 ^oC in the dark for 10 min. Subsequently, PI was added at a final concentration
249 of 15 µM and incubated for a further 5 min. SYTO 16 emission was detected via the Green
250 photomultiplier (PMT; 525/30 nm BP filter) and PI was read with the Red1 PMT (690/50 nm
251 BP filter) no compensation was carried out. Viability was defined as the percentage of sperm
252 positive for SYTO 16 but negative for PI. All percentages were calculated as part of the total
253 gated sample, P0.1 Population.

254

255 2.4.2.2 Assessment of Hypotonic Resistance

256 Hypotonic resistance was evaluated as the percentage of viable cells with intact membranes
257 after incubation in a hypo-osmotic medium. Hypo-osmotic TRIXcell + media were prepared
258 by diluting iso-osmotic TRIXcell+ (300 mOsm) with dH_2O to produce a range of hypo-
259 osmotic media, namely 300 (Control), 240, 210, 180, 150, 120, 90 and 30 mOsm.
260 Osmolarities were confirmed using an osmometer (VAPRO, Wescor, Utah, USA) and were
261 accurate to ± 3 mOsm. Sperm (300 x 10^5 sperm/mL) were incubated for 15 min in 500 µL of
262 hypo-osmotic media (TRIXcell+) and were subsequently assessed for viability, as described
263 above. The percentage of membrane intact sperm in each sample after hypo-osmotic
264 incubation was normalised against the percentage of membrane intact sperm in the iso-
265 osmotic control (300 mOsm).

2.4.2.3 Assessment of Acrosomal Integrity

Acrosomal integrity was assessed by incubating sperm with SYTO 16 and PI, as described above, followed by incubation with Alexa Flour 647 (AF647; Ex/Em: 650/668; Life Technologies, Carlsbad, CA, USA) to identify sperm which had undergone the acrosome reaction [32]. Alexa Fluor 647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. Alexa Flour 647 (3 µL) was added to diluted sperm (300 x 10^5 sperm/mL) to a final concentration of 4.6 µM and incubated in the dark for 15 min at 37 ^{o}C. AF647 positive events were read on the Red2 PMT (661/19 nm BP filter) with no compensation carried out. The percentage of acrosome-intact sperm in the live population was expressed as a percentage of the sperm negative for Alexa Flour 647 and positive for SYTO 16 as part of the total gated sample, P0.1 Population.

2.4.2.4 Assessment of Mitochondrial Activity

Mitochondrial activity was assessed on frozen-thawed sperm samples only. Diluted sperm from each treatment were washed once in BTS (3 mL at 400 *g* for 6 min at 32 ^{o}C) and were subsequently re-suspended in BTS (1 mL). To assess mitochondrial activity, the fluorescent probe JC-1 (5 µL; Ex/Em: 514/529; Life Technologies, Carlsbad, CA, USA) was added to 300 x 10^5 sperm/mL to give a final concentration of 1.5 µM. JC-1 fluoresces red upon accumulation in the mitochondria, thus indicating high mitochondrial activity. The samples were incubated in the dark for 15 min at 37 ^{o}C. At low levels of mitochondrial activity (intracellular JC-1 exists as a monomer) the fluorescence of the JC-1 probe was read with the Green PMT (525/30 nm BP filter) and at high levels of mitochondrial activity (formation of JC-1 aggregates) the fluorescence was read using the Yellow PMT (583/26 nm BP filter).

High mitochondrial activity was defined as the percentage of cells positive for the formation of JC-1 aggregates from the total gated population.

2.4.2.5 Assessment of Membrane Fluidity

Frozen-thawed sperm were washed as above and membrane fluidity was assessed using the fluorescent probe Merocyanine 540 (M540; Ex/Em:540/578; Sigma, Arklow, Wicklow, Ireland). This probe becomes more fluorescent as membrane fluidity increases due to intercalation. The viability probe SYTO 62 (5 µL; Ex/Em: 652/676; Life Technologies, Carlsbad, CA, USA) works in a similar manner to SYTO 16, however, fluoresces at a high wavelength. SYTO 62 was firstly added to the samples to give a final concentration of 200 nM and incubated in the dark for 15 min at 37 ^{o}C. Immediately post incubation, M540 (5 µL) was added to the give a final concentration of 10 µM and incubated for a further 15 min. Fluorescence of SYTO 62 was read with the Red2 PMT (661/19 nm BP filter) and M540 with the Yellow PMT (583/26 nm BP filter). High membrane fluidity was defined as the percentage of viable sperm positive for M540, from the total gated population, P0.1 Population.

2.5 Fatty Acid Analysis of Sperm, Seminal Plasma and Feed

Semen was collected and 10 mL of raw ejaculate was centrifuged at 2000 *g* for 10 min at 4 ^{o}C. The top 4 mL of SP was snap frozen in liquid nitrogen in 1 mL aliquots and the remainder of the SP was discarded. Phosphate Buffered Saline (PBS; 5 mL) was slowly added and the sperm pellet disturbed, following which, each sperm sample was centrifuged as above, to remove any excess seminal plasma. The sperm pellet was then snap frozen in liquid nitrogen and stored at -80 ^{o}C until use. A representative sample of feed (50g) of both the Ctl diet and

All-G-Rich supplement were taken on weeks 1, 8 and 19 and frozen. The 3 feed samples within diet/supplement were pooled prior to analysis.

Fatty acid analysis was conducted using gas liquid chromatography (GLC; Varian 3400; JVA Analytical) procedures. Briefly, total lipids were extracted from the full re-suspended sperm pellet, 1 mL of SP and 10g of both feed samples and they were purified with chloroform-methanol (2:1, v/v; Thermo Scientific) according to the method of Folch, Lees and Sloane-Stanley [33]. Methylation of fatty acid methyl esters (FAME) was performed using in situ trans-esterification; firstly, with 10 mL of 0.5 NaOH (Sigma Aldrich, Arklow, Wicklow, Ireland) in methanol for 10 min at 90 ^{o}C, followed by 10 mL of 14 % boron trifluoride (BF_3) in methanol (Sigma Aldrich, Arklow, Wicklow, Ireland) for 10 min at 90 ^{o}C as described by [34]. FAME were recovered with hexane (Fisher Scientific, Dublin, Ireland). Before GLC analysis, samples were dried over 0.5 mg anhydrous sodium sulphate (Sigma Aldrich, Arklow, Wicklow, Ireland) for 1 h and stored at -20 ^{o}C. FAME were separated by GLC fitted with a flame ionisation detector using a Chrompack CP Sil 88 column (100m x 0.25 mm internal diameter and 0.20 um film thickness, Chrompack; JVA Analytical) and Helium as a carrier gas. The column oven was programmed initially at 80 ^{o}C for 8 min and then increased by 8.5 ^{o}C per min to a final column temperature of 200 ^{o}C. The injection volume used was 1 µL with an automatic sample injection of SPI 1093 splitless on-column temperature-programmable injector. Peaks were integrated by using the Varian Star Chromatography Workstation (version 6.0; Agilent Technologies, CA, USA) and identified by comparison of retention times with pure FAME standards (Nu-Chek Prep). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100 %). All fatty acid data are presented as means ± s.e.m in g/100g FAME.

338 2.6 Total Antioxidant Capacity

339 SP was analysed for TAC using an adapted version of the 2,2'-azino-bis-3-
340 ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) radical. A commercial antioxidant assay kit
341 (Sigma-Aldrich, Arkliw, Co. Wicklow, Ireland), which included ABTS reagent, equine heart
342 myoglobin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and 3%
343 hydrogen peroxide (H_2O_2) was used to determine TAC. Trolox was used for standard
344 antioxidant solutions, ranging from 0 to 0.42 mM, for assay, calibration and preparation of a
345 standard curve. SP samples were thawed at ambient temperature. In a 96-well plate,
346 myoglobin was added to standards or thawed samples (2:1), whereas H_2O_2 was added to the
347 ABTS substrate to activate it before its addition to each well. The plate was incubated for 5
348 min at ambient temperature before absorbance was assessed at 405 nm by using an ultra
349 microplate reader (BioTek, Winooski, VT, USA). The absorbance of each sample was
350 assessed in triplicate with the average value inserted in the equation of the line for the Trolox
351 calibration curve to determine the TAC of the samples, expressed as millimolar Trolox
352 equivalents per litre of SP.

353

354 *2.7 In Vivo* Fertility

355 Liquid semen from 60 boars was used (n = 20, 21 and 19 for the Ctl, 75g and 150g All-G-
356 Rich treatments, respectively) and sows were inseminated twice, on consecutive days, with
357 semen from the same boar under commercial conditions. Field fertility data was recorded
358 from 1,158 sows inseminated with liquid semen (n = 427, 400 and 331 for the Ctl, 75g and
359 150g All-G-Rich treatments, respectively) across 27 commercial farms. Inseminations
360 conducted pre week 8 were excluded from the statistical analysis to allow for a complete
361 spermatogenetic cycle to be complete [54]. Litter size was recorded as total number of piglets

born per litter for both primiparous (n=144) and multiparous (n=1,014) sows. Farrowing rate (number of farrowings/number of inseminations * 100) and litter size data were based on inseminations carried out between weeks 8 and 19 of the trial.

2.8 Statistical analysis

Statistical analysis was conducted on all data post week 8 (indicated by a broken vertical red line in Figures 2 and 3). Data were examined for normality of distribution (Shapiro-Wilk test), tested for homogeneity of variance, transformed where appropriate and analysed in the Statistical Package for the Social Sciences (SPSS software; version 20.0, IBM, Chicago, IL). Sperm viability data for liquid semen were transformed using a Box-Cox transformation (λ = 3.07). The transformed data were used to calculate the P values, however, the corresponding estimated marginal means and the standard error of the non-transformed data are presented in the results. Data were considered to be statistically significant with a P value of < 0.05. Analysis of variance (ANOVA) was used to analyse both on farm and post-thaw *in vitro* sperm assessments, fatty acid profile, TAC and litter size data. Repeated measures ANOVA was used for liquid semen *in vitro* sperm assessments and chi-square for farrowing rate data. Correlations between sperm DHA content were conducted for all *in vitro* and *in vivo* parameters (Pearson correlation – Parametric data; Spearman correlation – Non-parametric data). Post hoc tests were carried out using the Bonferroni test, and results reported as the mean ± the standard error of the mean (s.e.m). Fixed effects included in the model were treatment, day of storage, boar, breed, week and each fixed effect was assessed for an interaction with treatment. If not statistically significant ($P > 0.05$), effects were subsequently excluded from the model. The final model for all six analysis included; (i) on farm collection records; treatment, week, and treatment by week interaction, (ii) i*n vitro* analysis of liquid semen; treatment, day, day by treatment interactions, (iii) *in vitro* analysis of frozen-thawed

semen; treatment, (iv) fatty acid analysis of sperm and seminal plasma; treatment, (v) total antioxidant capacity; treatment, day and treatment by day interaction and (vi) *in vivo* fertility; treatment, boar, breed and farm.

3. Results

3.1 Semen Quality Immediately Post Collection

There was no effect of week by treatment interaction on semen volume, sperm concentration and or total sperm number ($P < 0.05$). Although, there was a numerical increase in semen volume over the duration of the trial for both 75g and 150g All-G-Rich treatments, there was no effect of week ($P > 0.05$). There was an effect of treatment on semen volume with boars consuming 75g All-G-Rich producing a larger semen volume than the Ctl treatment ($P < 0.05$; Figure 2). There was no effect of treatment on sperm concentration ($P > 0.05$). In line with the differences in semen volume, there was an effect of treatment on total sperm number ($P < 0.01$; Figure 1) as boars consuming 75g All-G-Rich had a higher total sperm number than the Ctl treatment ($P < 0.01$). There was no effect of treatment on gross motility or on the percentage of abnormalities ($P > 0.05$). There was no effect of treatment on the percentage of primary and or secondary abnormalities ($P > 0.05$).

3.2 In Vitro Analysis of Liquid Semen

There was no day by treatment interaction on motility, viability and acrosomal integrity ($P > 0.05$). There was no effect of treatment on liquid semen quality as assessed *in vitro* ($P > 0.05$). However, viability, acrosomal integrity (Figure 2) and motility of sperm declined with increasing duration of storage in all treatments ($P < 0.05$). While there was no effect of treatment on hypotonic resistance ($P > 0.05$), the 180 mOsm treatment had the greatest

411 variation between treatments and hence, was selected for further analysis. When assessed at
412 180 mOsm, there was no effect of day of storage, treatment, or treatment x day interaction ($P > 0.05$)
413 on the hypotonic resistance of sperm in all three treatments. Unexpectedly, the
414 percentage of hypotonic resistant sperm in all treatments increased from Day 1 of storage to
415 Day 3 ($P < 0.01$) but declined thereafter ($P < 0.01$).

416

417 *3.3 In Vitro* Analysis of Frozen-Thawed Semen

418 There was no week by treatment interaction on post-thaw motility, motility post thermal
419 stress, viability, mitochondrial activity or membrane fluidity of frozen-thawed semen ($P >$
420 0.05). There was no effect of treatment on any frozen-thawed semen parameters assessed any
421 frozen-thawed semen parameters assessed ($P > 0.05$). As expected, there was an effect of
422 time on thermal stress ($P < 0.01$) with an average total motility at T0 of 29.9 ± 2.49, 26.6 ±
423 2.35 and 27.2 ± 1.89% versus 22.6 ± 1.77, 18.9 ± 1.44 and 18.9 ± 1.23% at T30, for the Ctl,
424 75g and 150g All-G-Rich treatments, respectively.

425

426 3.4 Fatty Acid Composition of Seminal Plasma and Sperm

427 There was an effect of treatment on the fatty acid profile of SP ($P < 0.05$) with a decrease in
428 adrenic and palmitoleic acid ($P < 0.05$) in the 75g and 150g All-G-Rich treatments in
429 comparison to the Ctl treatment. Palmitic acid was the main SFA in SP followed by steric
430 acid, while, DHA and linoleic acid were the most abundant omega-3 and omega-6 fatty acids,
431 respectively (Table 3). DHA content in SP was low (< 2%) and although it was numerically
432 higher in the 150g All-G-Rich treatment compared to the Ctl this was not significant. There
433 was an effect of treatment on the FAC of sperm, which was represented by an increase in
434 DHA ($P < 0.01$) and a decrease in docosapentaenoic acid (DPA; $P < 0.01$) in the 75g and

150g All-G-Rich treatments compared to the Ctl (Table 4). The DPA:DHA ratio of the 75g and 150g All-G-Rich treatments was more than quarter of that of the Ctl treatment ($P < 0.01$). In line with the objectives of the study, the largest alteration in sperm FAC was a 1.72 and 1.60 fold increase in sperm DHA content for 75g and 150g All-G-Rich treatments, respectively, in comparison to the Ctl treatment. However, there was no correlation between the sperm DHA content and motility, viability, acrosomal integrity, mitochondrial activity, membrane fluidity or farrowing rate ($P > 0.05$). The supplementation of All-G-Rich decreased sperm concentrations of total n-3 PUFAs by 1.09 fold and decreased n-6 PUFA concentrations by 1.10 fold. Consequently, the ratio of n-6 to n-3 PUFAs decreased significantly in the 75g and 150g treatments in comparison to the Ctl treatment ($P < 0.01$).

3.5 Total Antioxidant Capacity of Seminal Plasma

There was no week by treatment interaction on TAC of SP ($P > 0.05$). There was no effect of treatment on TAC of SP ($P > 0.05$). Surprisingly, there was an increase in TAC over the duration of the supplementation period in all treatments ($P < 0.01$) with an increase of 0.49 ± 0.029 to 0.65 ± 0.060, 0.47 ± 0.023 to 0.67 ± 0.043 and 0.44 ± 0.019 to 0.67 ± 0.038 mM Trolox equivalents for the Ctl, 75g and 150g All-G-Rich treatments from week 0 to 18, respectively.

3.6 Field Fertility

There was no effect of treatment on farrowing rate or litter size ($P > 0.05$). The mean farrowing rate for the 75g and 150g All-G-Rich treatments were 77 and 75%, respectively compared to 73% for the Ctl treatment with an average farrowing rate of 75 ± 1.3%. The total

piglets boar (dead and or alive) were 13.8 ± 0.22, 13.4 ± 0.23 and 13.4 ± 0.25 piglets for the Ctl, 75g and 150g All-G-Rich treatments, respectively.

4. Discussion

This study takes advantage of an animal model which facilitates the reliable assessment of the *in vivo* fertility of males combined with detailed cellular assessments of sperm function. This novel study has demonstrated that dietary supplementation of All-G-Rich, is effective at incorporating DHA into sperm, and while there was no effect of dietary supplementation on semen quality as assessed *in vitro* or *in vivo*, boars supplemented with 75g All-G-Rich daily, had a significantly higher semen volume and thus, total sperm numbers compared to the control treatment.

In this current study, the addition of both 75 and 150g All-G-Rich in the diet of boars significantly increased the FAC of sperm cells by 1.72 and 1.60 fold, respectively, compared to the Ctl treatment, which is in agreement with Castellano et al. [36], Am-In et al. [14] and Rooke et al. [5]. Although an effect on the level of DHA was recorded in sperm there was no effect observed in SP, thus, indicating a concentrating effect, most likely in the sperm membrane. This increase in sperm DHA content resulted in a significant reduction in docosapentaenoic acid (DPA) which may indicate that both fatty acids have similar metabolic pathways [37]. This is similar to the findings of Castellano et al. [20] who reported that the inclusion of 3 different fish oil diets, containing a variety of omega-3 sources, resulted in an increase in the proportion of omega-3 FAs in sperm, specifically DHA.

In agreement with the current work a number of studies which have supplemented the diet of boars with various fish oils such as tuna oil [5], menhaden oil [20, 37] and shark liver oil [12] have successfully increased the DHA content of sperm. However, the aforementioned studies have inconsistent results in relation to the effect of dietary supplementation of fish oil on sperm number as well as *in vitro* quality and critically, none of these studies assessed *in vivo* fertility. A number of studies have also shown that a high proportion of DHA in boar sperm has been positively correlated with increased sperm concentration and total sperm number [15, 38] as well as increased motility, viability and normal morphology [14]. Although, sperm concentration, motility and the percentage of abnormalities did not differ in this current study, immediately post-collection, a significant increase in semen volume was evident in the 75g All-G-Rich treatment, leading to an approximately 10% increase in total sperm number. Considering that a typical AI dose contains between 2 and 3 x 10^9 sperm this would provide 3-4 more semen doses per ejaculation. These results are supported by the findings of Maldjian et al. [38] and Estienne et al. [6] which demonstrated that dietary supplementation of boars with 3% fish oil and an omega-3 supplement, respectively, resulted in a significant increase in the total number of sperm per ejaculate, thus, resulting in an increase in the number of potential AI doses. A possible explanation for an increase in semen volume and thus, total sperm number, may be due to an increase in ejaculation time, as reported by Estienne et al. [6]. However, as sperm are not isolated entities it is important to note that dietary supplementation of omega-3 fatty acids may also play a role on testicular development and function such as the regulation of steroidogenesis and so a change in sexual behaviour such as increased libido as a result of enhancing the production of testosterone may be another explanation for increased semen volume and total sperm number [37]. However, neither ejaculation time nor testosterone concentrations were recorded in this study.

Despite the significant alteration in lipid composition in the current study there was no effect of dietary supplementation of All-G-Rich on semen quality as assessed *in vitro.* Motility, viability, acrosomal integrity and hypotonic resistance declined as expected with the duration of *in vitro* storage of liquid semen, but was not affected by dietary treatment. This decrease in sperm quality with increasing duration of storage is well described in the literature [39, 40] and is in part due to the production of reactive oxygen species (ROS) associated with sperm ageing [41]. In the current study dietary supplementation of All-G-Rich had no effect on sperm motility or on post-thaw semen quality as assessed *in vitro*. No beneficial action of PUFAs on the freezability of boar semen was exhibited as the cryogenic process, regardless of treatment, was found to be detrimental to the sperm cells. This resulted in a significantly lower viable and motile sperm population in comparison to liquid semen, thus, supporting the observations of Paulenz et al. [42]. However, centrifugation, although is a necessary step in the boar sperm cryopreservation protocol as it facilitates the removal of SP and concentrates sperm for redilution with cryopreservation extender, is also damaging to sperm [43]. Thus, centrifugation may have contributed to the lower viability and motility socres recorded for frozen-thawed semen in this study. It should also be noted, that this study used boars of proven fertility and, therefore, it may be that the supplementation of algae may be more beneficial to boars with poor semen quality.

In comparison to liquid semen samples, post-thaw motility and viability of frozen-thawed semen samples was lower for all treatments. Although viability and mitochondrial activity of frozen-thawed semen was low, membrane fluidity was high for all treatments. This membrane stabilisation was also evident in the liquid stored semen as the number of sperm with intact acrosomes remained high throughout the duration of storage, irrespective of treatment. Previous studies, involving the addition of omega-3 fatty acids in the diet of boars,

such as those conducted by Paulenz et al. [42], Maldjian et al. [38], Waterhouse et al. [44] and Castellano et al. [36] have indicated that the cryopreservation process has a detrimental impact on sperm characteristics such as motility, viability and acrosomal integrity in comparison to liquid semen, thus, supporting the findings in this study. However, Kaeoket et al. [45] reported that the *in vitro* addition of fish oil into boar semen freezing extender had a beneficial effect on subsequent post-thaw motility, viability and acrosomal integrity suggesting it may be beneficial to add an omega-3 supplement directly into the semen extender rather than through dietary supplementation alone.

A high proportion of PUFAs, especially DHA, within sperm membranes has been demonstrated to render the cell susceptible to oxidative damage by ROS [46], due to the presence of double bonds, ultimately leading to the impairment of sperm function ([47], [48]). It is well known that PUFA's play a role in improving the TAC of sperm as a balanced omega-6 to omega-3 fatty acid ratio in the diet of breeding boars enhances, not only the development of the testis, but also accessory sex gland function [58]. The DHA packed dietary supplement used in this study, All-G-Rich, also contains Ethoxyquin, which is a synthetic antioxidant and is also known for having a high antioxidant capacity [28]. However, the level of TAC in this study increased irrespective of treatment. It is difficult to explain why TAC increased for the Ctl treatment but it may be due to seasonal changes in antioxidant defence systems within seminal plasma as described by Koziorowska-Gilun et al. [49] as the trial commenced in early spring and continued until early summer.

In this current study, dietary supplementation with All-G-Rich did not affect either farrowing rate or litter size following insemination with liquid semen. This result was not surprising as

555 the *in vitro* analysis, of raw, liquid and frozen-thawed semen demonstrated no beneficial
556 sperm quality effects. There are a limited number of studies which have assessed the *in vivo*
557 fertility in boars following dietary supplementation of omega-3 PUFAs and all studies to-date
558 have focused on fish oil as the source of omega-3 fatty acids. In this study, overall farrowing
559 rate (~75%) was marginally lower, in comparison to the average farrowing rate recorded
560 within the pig industry for liquid semen (80-85%; [50]). This lower farrowing rate is likely to
561 be due to the use of homospermic semen in the current study compared to the routine
562 practise, of the use of heterospermic semen [51], which has been shown to enhance
563 reproductive performance [52]. Results in the current study correspond to Alm et al. [53] who
564 reported a farrowing rate of 75.8% using homospermic inseminations. It should also be noted
565 that inseminations were conducted mainly on purebred sows of both maternal and terminal
566 lines and therefore, lower farrowing rates would be expect due to the lack of heterosis in sow
567 fertility traits. The average litter size of ~14 piglets is comparable to the industry norm [55]
568 and is greater than the homospermic litter size of 12.4 ± 0.4 piglets reported by Ferreira et al.
569 [51].

570

571 **5. Conclusion**

572 In conclusion, this study provides important insights into the relationship between nutrition,
573 semen composition, sperm quality and fertility. Dietary supplementation of algae altered the
574 FAC of the sperm, increased semen volume and total sperm numbers but had no effect on any
575 other semen quality parameter as assessed *in vitro* or *in vivo*. The ability of sperm to
576 incorporate DHA into the membrane indicates a concentrating effect of DHA within the
577 sperm as this increase was not observed in SP. A significant increase in semen volume and
578 total sperm number seen with boars supplemented 75g All-G-Rich daily resulted in an

increase in production of 3-4 more doses per ejaculate, therefore, indicating that the feeding regime described within this study has the potential for increasing the output of boar studs.

Funding

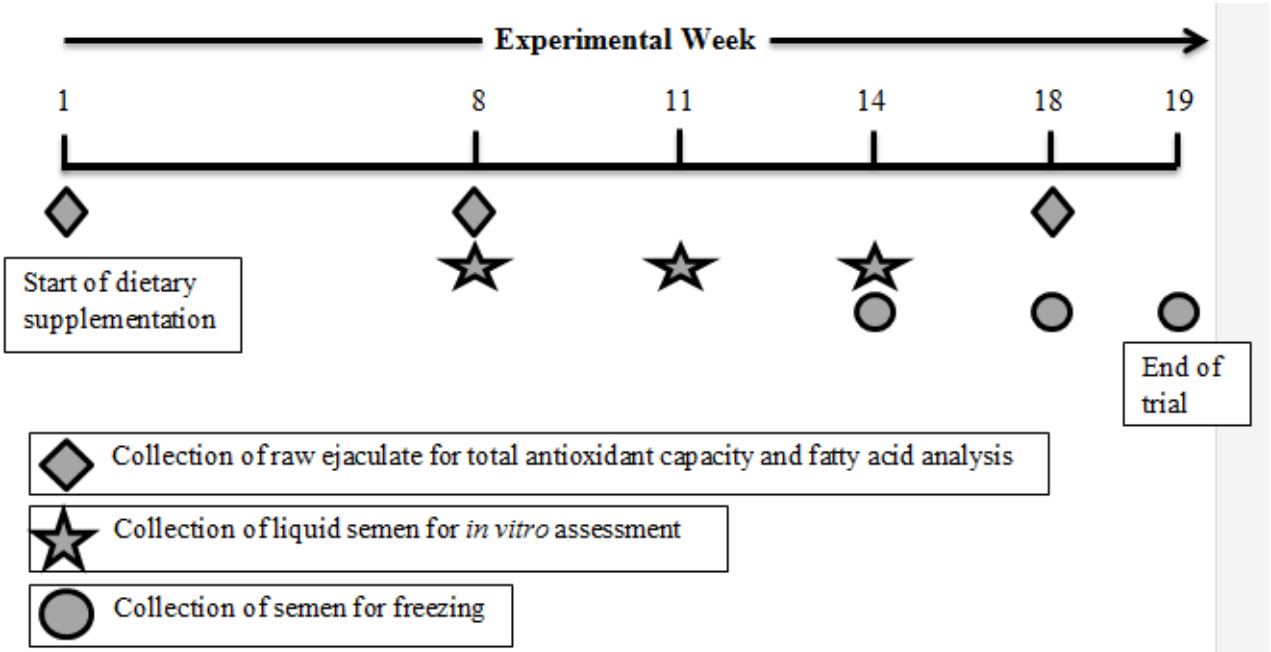
This work was supported by Alltech, Bioscience Centre, Dunboyne, Co Meath, Ireland

Acknowledgements:

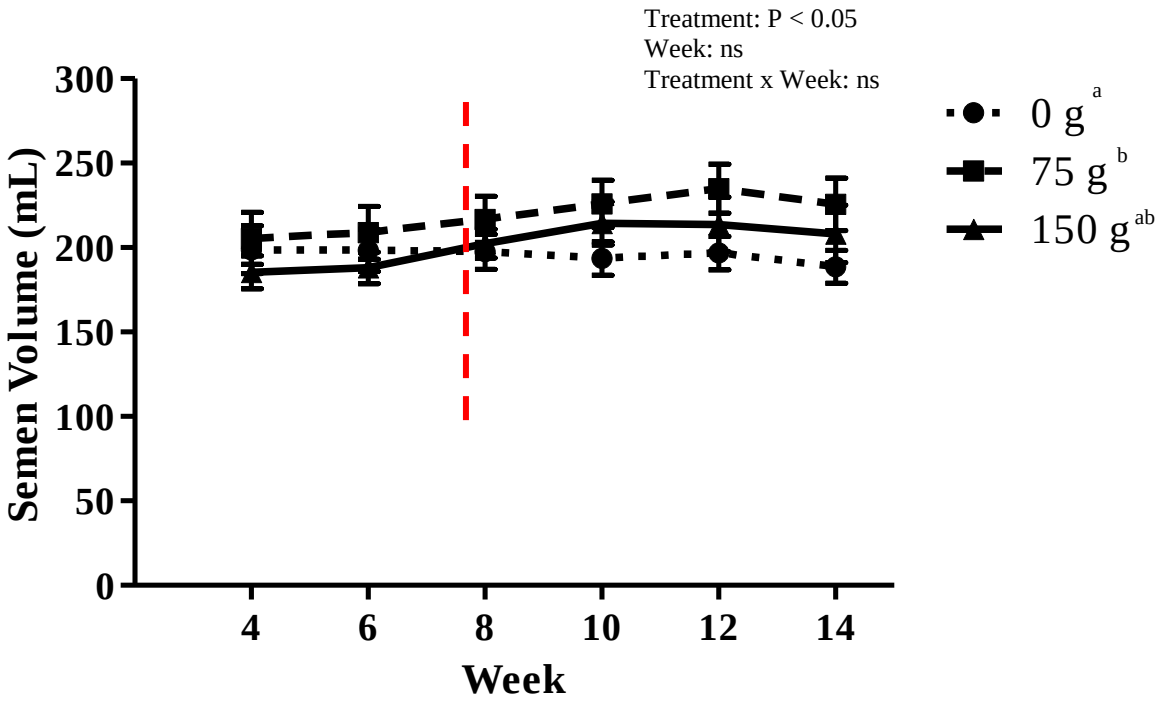
The authors gratefully acknowledge the contribution of Mr Colin Byrne, Teagasc BioScience Centre, Grange, Co. Meath, Ireland for the proximate analysis of the feed samples and Mr. James Dick, University of Stirling, Stirling, England for advice and input on the fatty acid analysis

Figures

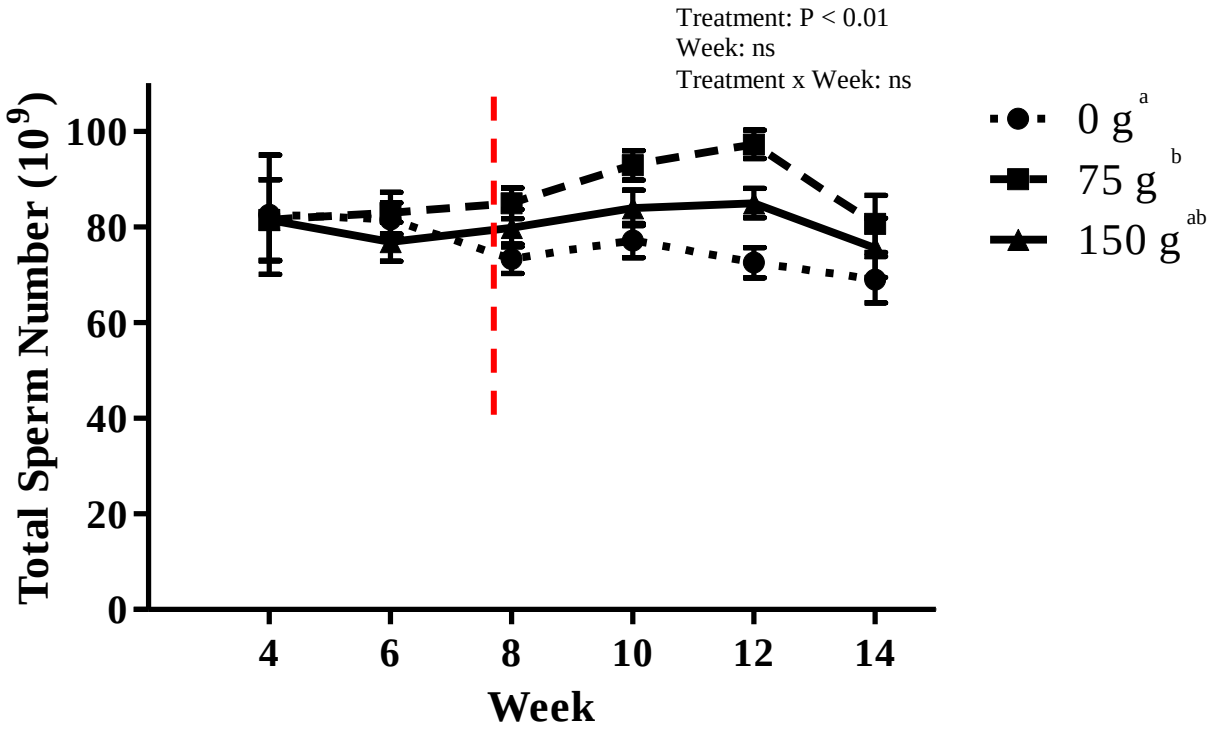
Murphy et al, Figure 1.



620 Murphy et al, Figure 2.



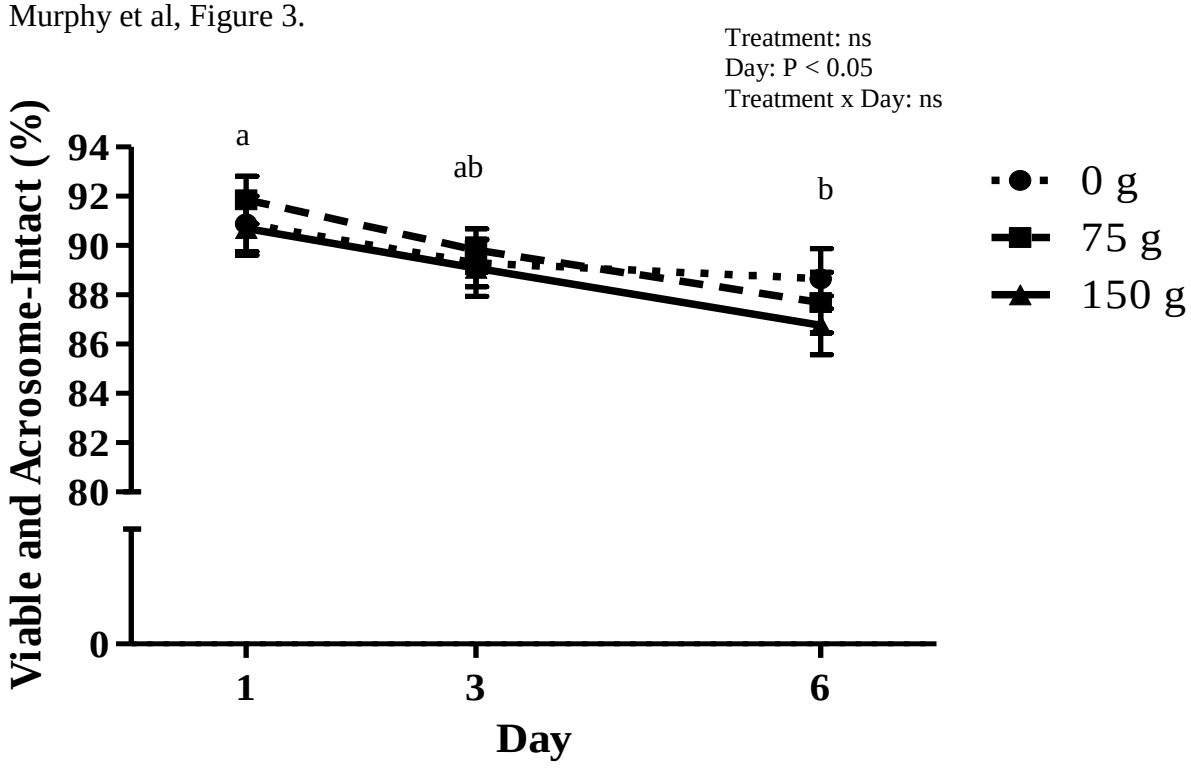
621



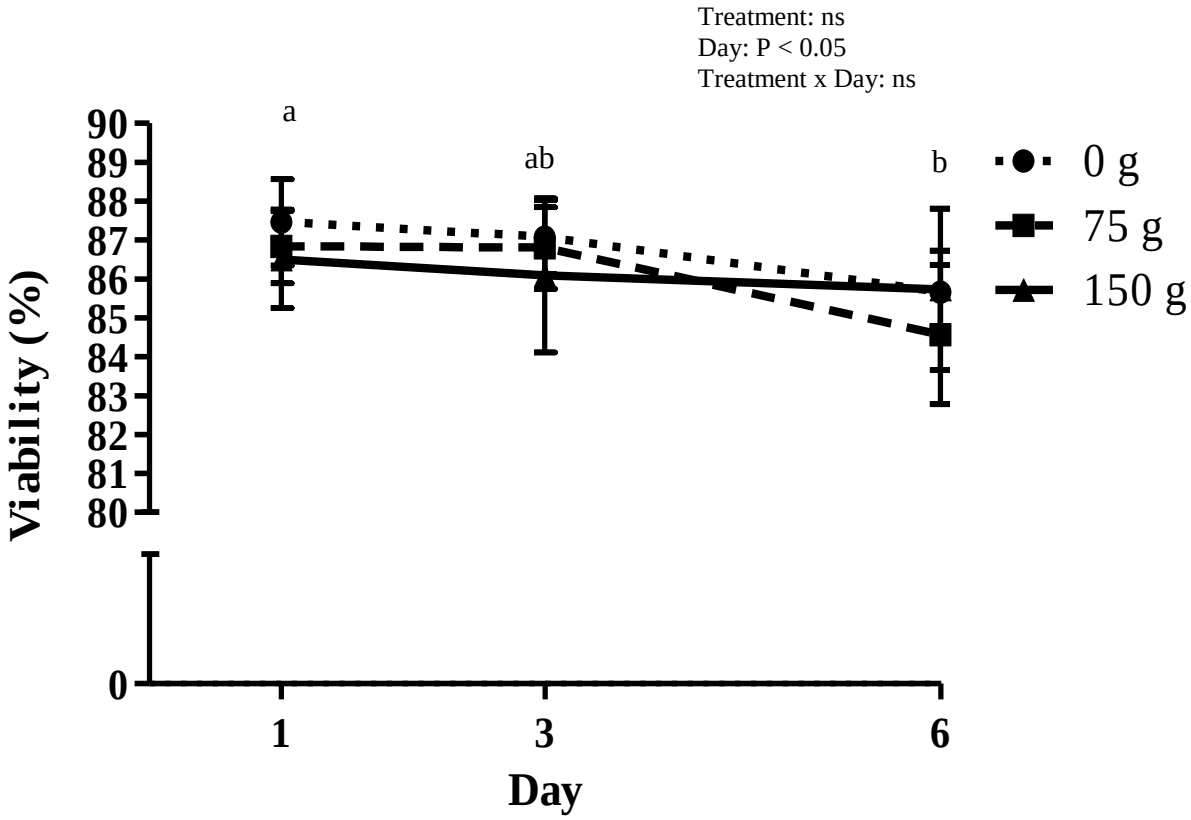
622
623

624

625 Murphy et al, Figure 3.



626
627



628
629

630

631

632 **Tables**

633 Murphy et al. Table 1.

Ingredient	Control Diet	All-G-Rich
DM (%)	87.4	97.3
Crude Protein (g/kg)	16.5	10.7
Crude Fibre (g/kg)	38.2	16.8
Ash (g/kg)	59.58	44.1
Acid Detergent Fibre (g/kg)	66.26	64.21
Neutral Detergent Fibre (g/kg)	104.47	81.04
Ether Extract (g/kg)	2.3	33.2
Gross Energy (MJ/kg DM)	17.0	31.8

634

635

636

637

638

639

640

641

642

643

644

645

646

647 Murphy et al. Table 2.

Fatty Acid	Control Diet (%)	All-G-Rich (%)
Lauric acid (C12:0)	0.29	< 0.10
Myristic acid (C14:0)	0.02	4.27
Myristoleic acid (C14:1c)	nd	1.60
Palmitoleic acid (C16:1t)	0.19	< 0.10
Palmitoleic acid (16:1c)	0.20	nd
Palmitic acid (C16:0)	16.66	54.69
Margaric acid (C17:0)	nd	0.63
Steric acid (C18:0)	4.00	1.80
Elaidic acid (C18:1t)	nd	< 0.10
Oleic acid (C18:1 n-9)	29.48	0.36
Linoleic (C18:2)	43.98	< 0.10
α-linolenic acid (C18:3)	3.21	< 0.10
Arachidic acid (C20:0)	nd	0.24
Paullinic acid (C20:1 n-7)	0.52	nd
Arachidonic acid (C20:4)	nd	1.11
Eicosapentaenoic acid (C20:5)	0.88	0.33
Erucic acid (C22:1)	nd	0.53
Docosapentaenoic acid (C22:5)	nd	0.07
Docosahexaenoic acid (C22:6)	0.48	33.55

648 Abbreviations: nd = not detectable; FAME, fatty acid methyl ester.

649

650

651 Murphy et al. Table 3.

Fatty Acid	Treatment			P value
	Seminal Plasma			Effect of Treatment
	0g (%)	75g (%)	150g (%)	
Enanthic acid (C7:0)	1.1 ± 0.81	2.3 ± 0.73	2.8 ± 1.26	ns
Lauric acid (C12:0)	3.8 ± 0.56	4.9 ± 0.38	3.5 ± 0.38	ns
Tridecuclic acid (C13:0)	7.3 ± 10.37	1.8 ± 2.98	3.7 ± 10.88	ns
Dimethylacetals (DMA)	18.3 ± 3.39	18.9 ± 2.59	23.3 ± 3.71	ns
Myristoleic acid (C14:1t)	1.3 ± 0.57	1.6 ± 0.30	2.0 ± 0.36	ns
Myristoleic acid (C14:1c)	0.1 ± 0.16	0.1 ± 0.10	0.2 ± 0.05	ns
Palmitolic acid (C16:1t)	15.6 ± 3.71	16.2 ± 1.76	12.4 ± 1.94	ns
Palmitoleic acid (C16:1c)	3.9 ± 1.14[a]	1.9 ± 0.35[b]	1.8 ± 0.41[b]	P < 0.05
Elaidic acid (C18:1t)	0.1	0.3 ± 0.10	0.4 ± 0.07	ns
Myristic acid (C14:0)	2.7 ± 0.44	2.3 ± 0.23	2.8 ± 0.43	ns
Palmitic acid (C16:0)	22.8 ± 2.43	24.3 ± 2.02	21.6 ± 1.78	ns
Stearic acid (18:0)	9.2 ± 1.03	10.7 ± 0.71	9.6 ± 0.74	ns
Oleic acid (C18:1 n-9)	7.2 ± 1.21	7.6 ± 0.77	8.4 ± 0.74	ns
n-6 PUFA				
Adrenic acid (C22:4)	0.6 ± 0.33[a]	0.3 ± 0.05[b]	0.6 ± 0.11[ab]	P < 0.05
Linoleic acid (C18:2)	1.5 ± 0.23	1.5 ± 0.12	1.9 ± 0.32	ns
Arachidonic acid (C20:4)	0.6 ± 0.23	0.5 ± 0.13	0.9 ± 0.12	ns
n-3 PUFA				
a-Linolenic acid (C18:3)	0.3 ± 0.12	0.2 ± 0.09	0.2 ± .013	ns
DPA (C22:5)	1.2 ± 0.79	nd	0.1	ns
DHA (C22:6)	1.0 ± 0.39	0.8 ± 0.11	1.4 ± 0.28	ns
n-6 family[x]	2.7 ± 0.03[a]	2.4 ± 0.03[b]	3.4 ± 0.07[c]	P < 0.01
n-3 family[y]	2.5 ± 0.19[a]	1.1 ± 0.03[b]	1.7 ± 0.06[c]	P < 0.01
n-6 to n-3 ratio	1.1 ± 0.21[a]	2.2 ± 0.06[b]	2.0 ± 0.02[c]	P < 0.01

652 Abbreviations: NS, not significant; PUFA, polyunsaturated fatty acid; nd, not detected; FAME, fatty acid

653 methyl ester.

654 [x]C18:2 + C20:4 + C22:4

655 [y]C18:3 + C22:5 + C22:6

656

657

Murphy et al. Table 4.

Fatty Acid	Treatment			P value
	Sperm			Effect of Treatment
	0g (%)	75g (%)	150g (%)	
Enanthic acid (C7:0)	0.2 ± 0.06	0.3 ± 0.05	0.4 ± 0.09	ns
Lauric acid (C12:0)	0.9 ± 0.15	0.8 ± 0.07	0.8 ± 0.14	ns
Tridecuclic acid (C13:0)	2.2 ± 2.20	2.6 ± 3.48	3.2 ± 2.74	ns
Dimethylacetals (DMA)	11.4 ± 2.05	15.0 ± 2.01	15.4 ± 2.6	ns
Myristoleic acid (C14:1t)	1.2 ± 0.31	1.8 ± 0.33	1.7 ± .29	ns
Myristoleic acid (C14:1c)	0.1 ± 0.07	0.2 ± 0.04	0.2 ± .06	ns
Palmitolic acid (C16:1t)	1.0 ± 0.33	0.4 ± 0.08	0.3 ± 0.33	ns
Palmitoleic acid (C16:1c)	1.6 ± 0.48	1.1 ± 0.16	1.1 ± 0.31	ns
Elaidic acid (C18:1t)	1.9 ± 0.52	2.3 ± 0.15	2.7 ± 0.31	ns
Myristic acid (C14:0)	13.7 ± 0.87	12.2 ± 0.98	12.8 ± 1.31	ns
Palmitic acid (C16:0)	16.4 ± 0.76	15.4 ± 1.05	16.5 ± 0.87	ns
Stearic acid (18:0)	6.3 ± 0.36	6.2 ± 0.38	5.6 ± 0.37	ns
Oleic acid (C18:1 n-9)	2.0 ± 0.40	1.6 ± 0.28	1.1 ± 0.14	ns
Mead acid (C20:3 n -9)	1.0 ± 0.07	1.0 ± 0.06	0.9 ± 0.09	ns
n-6 PUFA				
Adrenic acid (C22:4)	1.1 ± 0.07	0.7 ± 0.08	0.9 ± 0.07	ns
Linoleic acid (C18:2)	1.5 ± 0.13	1.4 ± 0.09	1.4 ± 0.10	ns
Arachidonic acid (C20:4)	1.7 ± 0.18	1.7 ± 0.12	1.6 ± 0.16	ns
n-3 PUFA				
a-Linolenic acid (C18:3)	0.2 ± 0.04	0.3 ± 0.03	0.3 ± 0.02	ns
DPA (C22:5)	19.9 ± 1.61[a]	8.7 ± 0.73[b]	8.1 ± 0.79[b]	P < 0.01
DHA (C22:6)	14.9 ± 1.49[a]	25.6 ± 2.03[b]	23.8 ± 1.73[b]	P < 0.01
n-6 family[x]	4.3 ± 0.03[a]	3.9 ± 0.01[b]	4.0 ± 0.03[c]	P < 0.01
n-3 family[y]	35.1 ± 0.50[a]	34.7 ± 0.59[b]	32.2 ± 0.49[c]	P < 0.01
n-6 to n-3 ratio	0.1 ± 0.01[a]	0.1 ± 0.02[b]	0.1 ± 0.03[c]	P < 0.01
DPA:DHA	1.3 ± 0.06[a]	0.3 ± 0.65[b]	0.3 ± 0.47[c]	P < 0.01

Abbreviations: NS, not significant; PUFA, polyunsaturated fatty acid; nd, not detected; FAME, fatty acid methyl ester.

[x]C18:2 + C20:4 + C22:4

[y]C18:3 + C22:5 + C22:6

Figure Captions

Figure 1: Schedule of tasks undertaken throughout the experimental period (Weeks).

Figure 2: Semen volume (upper panel) and total sperm number (lower panel) from boars supplemented with 0 (Ctl), 75g and 150g All-G-Rich (a DHA-rich algal commercial supplement) per day over a 14 week period. Data to the right of the broken line (indicates the completion of one spermatogenic cycle) were used for statistical analysis. Vertical bars represent ± s.e.m. [ab] Differing superscripts differ between treatments ($P < 0.01$).

Figure 3: Viability (upper panel) and acrosomal integrity (lower panel) of sperm stored as liquid semen collected form boars supplemented with 0 (Ctl), 75g and 150g All-G-Rich (a DHA-rich algal commercial supplement) per day and assessed on Days 1, 3 and 6 post collection. Vertical bars represent ± s.e.m. [ab] Differing superscripts differ between days ($P < 0.01$).

Table Captions

Table 1: Chemical analysis (expressed as g/kg of dry matter (DM) unless otherwise stated) of the control diet (a standard commercial grain based diet) and the All-G-Rich supplement (a DHA-rich algal commercial supplement).

Table 2: Fatty acid profile of the control diet (a standard commercial grain based diet) and the All-G-Rich diet (a DHA-rich algal commercial supplement). Values are g/100 g Fatty Acid Methyl Esters.

691 **Table 3:** Fatty acid composition (mean ± s.e.m) of seminal plasma collected on Day 126 of
692 the trial from boars supplemented with 0g, 75g and 150g All-G-Rich (a DHA-rich algal
693 commercial supplement) per day. Values are g/100 g FAME. [abc] Differing superscripts differ
694 between treatments ($P < 0.01$).

695

696 **Table 4:** Fatty acid composition (mean ± s.e.m) of sperm collected on Day 126 of the trial
697 from boars supplemented 0g, 75g and 150g All-G-Rich (a DHA-rich algal commercial
698 supplement) per day. Values are g/100 g FAME. [abc] Differing superscripts differ between
699 treatments ($P < 0.01$).

700

701

702

703

704

705

706

707

708

709

710

711

712

713

714

715

References

1. Cottin, S., T. Sanders, and W. Hall, The differential effects of EPA and DHA on cardiovascular risk factors. Proceedings of the Nutrition Society, 2011;70:215-231.
2. Rose, D.P. and J.M. Connolly, Omega-3 fatty acids as cancer chemopreventive agents. Pharmacology & therapeutics, 1999;83:217-44.
3. Connor, W.E., Importance of n− 3 fatty acids in health and disease. The American journal of clinical nutrition, 2000;71:171-75.
4. Wathes, D.C., D.R.E. Abayasekara, and R.J. Aitken, Polyunsaturated fatty acids in male and female reproduction. Biology of reproduction, 2007;77:190-201.
5. Rooke, J., C. Shao, and B. Speake, Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. Reproduction, 2001;121:315-22.
6. Estienne, M., A. Harper, and R. Crawford, Dietary supplementation with a source of omega-3 fatty acids increases sperm number and the duration of ejaculation in boars. Theriogenology, 2008;70:70-76.
7. Fair, S., D. Doyle, M. Diskin, A. Hennessy, and D. Kenny, The effect of dietary n-3 polyunsaturated fatty acids supplementation of rams on semen quality and subsequent quality of liquid stored semen. Theriogenology, 2014;81:210-19.
8. Gholami, H., M. Chamani, A. Towhidi, and M. Fazeli, Effect of feeding a docosahexaenoic acid-enriched nutriceutical on the quality of fresh and frozen-thawed semen in Holstein bulls. Theriogenology, 2010;74:1548-58.
9. Brinsko, S.P., D.D. Varner, C.C. Love, T.L. Blanchard, B.C. Day, and M.E. Wilson, Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology, 2005;63:1519-27.
10. Surai, P. and N. Sparks, Tissue-specific fatty acid and α-tocopherol profiles in male chickens depending on dietary tuna oil and vitamin E provision. Poultry Science, 2000;79:1132-42.
11. Yeste, M., X. Barrera, D. Coll, and S. Bonet, The effects on boar sperm quality of dietary supplementation with omega-3 polyunsaturated fatty acids differ among porcine breeds. Theriogenology, 2011;76:184-96.
12. Mitre, R., C. Cheminade, P. Allaume, P. Legrand, and A.B. Legrand, Oral intake of shark liver oil modifies lipid composition and improves motility and velocity of boar sperm. Theriogenology, 2004;62:1557-66.

13. Walker, W.H., Molecular mechanisms of testosterone action in spermatogenesis. Steroids, 2009;74:602-7.
14. Am-In, N., R. Kirkwood, M. Techakumphu, and W. Tantasuparuk, Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology, 2011;75:897-903.
15. Speake, B.K., P.F. Surai, J.A. Rooke, S. De Vriese, and A. Christophe, Regulation of avian and mammalian sperm production by dietary fatty acids. Male Fertility and Lipid Metabolism. AOCS Press, Champaign, IL, 2003;96-117.
16. Connor, W.E., D.S. Lin, D. Wolf, and M. Alexander, Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. Journal of lipid research, 1998;39:1404-11.
17. Lenzi, A., M. Picardo, L. Gandini, and F. Dondero, Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Human Reproduction Update, 1996;2:246-56.
18. Aksoy, Y., H. Aksoy, K. Altınkaynak, H.R. Aydın, and A. Özkan, Sperm fatty acid composition in subfertile men. Prostaglandins, Leukotrienes and Essential fatty acids, 2006;75:75-79.
19. Comhaire, F.H., A.B. Christophe, A.A. Zalata, W.S. Dhooge, A.M.A. Mahmoud, and C.E. Depuydt, The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 2000;63:159-65.
20. Castellano, C.-A., I. Audet, J. Bailey, P. Chouinard, J.-P. Laforest, and J. Matte, Effect of dietary n-3 fatty acids (fish oils) on boar reproduction and semen quality. Journal of animal science, 2010;88:2346-55.
21. Paulenz, H., O. Taugbøl, P. Hofmo, and K. Saarem, A preliminary study on the effect of dietary supplementation with cod liver oil on the polyunsaturated fatty acid composition of boar semen. Veterinary research communications, 1995;19:273-84.
22. Pyle, D.J., R.A. Garcia, and Z. Wen, Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. Journal of Agricultural and Food Chemistry, 2008;56:3933-39.
23. Chi, Z., D. Pyle, Z. Wen, C. Frear, and S. Chen, A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process Biochemistry, 2007;42:1537-45.

24. Lenihan-Geels, G., K.S. Bishop, and L.R. Ferguson, Alternative sources of omega-3 fats: can we find a sustainable substitute for fish? Nutrients, 2013;5:1301-15.
25. Chi, Z., Y. Liu, C. Frear, and S. Chen, Study of a two-stage growth of DHA-producing marine algae Schizochytrium limacinum SR21 with shifting dissolved oxygen level. Applied microbiology and biotechnology, 2009;81:1141-48.
26. Ward, O.P. and A. Singh, Omega-3/6 fatty acids: alternative sources of production. Process Biochemistry, 2005;40:3627-52.
27. De Swaaf, M., J. Pronk, and L. Sijtsma, Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga Crypthecodinium cohnii on ethanol. Applied microbiology and biotechnology, 2003;61:40-43.
28. Brannegan, D.R., Analysis of ethoxyquin and its oxidation products using supercritical fluid extraction and high performance liquid chromatography with chemiluminescent nitrogen detection. 2000.
29. Huang, Y.-T. and R.K. Johnson, Effect of selection for size of testes in boars on semen and testis traits. Journal of animal science, 1996;74:750-60.
30. Pena, F., A. Johannisson, M. Wallgren, and H.R. Martinez, Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Animal reproduction science, 2003;78:85-98.
31. Mortimer, S.T. and W.C. Maxwell, Effect of medium on the kinematics of frozen–thawed ram spermatozoa. Reproduction, 2004;127:285-91.
32. Murphy, C., S.A. Holden, E.M. Murphy, A.R. Cromie, P. Lonergan, and S. Fair, The impact of storage temperature and sperm number on the fertility of liquid-stored bull semen. Reproduction, Fertility and Development, 2015.
33. Folch, J., M. Lees, and G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues. J biol chem, 1957;226:497-509.
34. Parks, P. and R. Goins, In situ preparation of fatty acid methyl esters for analysis of fatty acid composition in food. Jounal of Food Science, 1994;59:1262-66.
35. Murphy, C., A. Fahey, A. Shafat, and S. Fair, Reducing sperm concentration is critical to limiting the oxidative stress challenge in liquid bull semen. Journal of dairy science, 2013;96:4447-54.
36. Castellano, C.A., I. Audet, J.L. Bailey, J.P. Laforest, and J.J. Matte, Dietary omega-3 fatty acids (fish oils) have limited effects on boar semen stored at 17 °C or cryopreserved. Theriogenology, 2010;74:1482-90.

37. Castellano, C.-A., I. Audet, J.-P. Laforest, J.J. Matte, and M. Suh, Fish oil diets alter the phospholipid balance, fatty acid composition, and steroid hormone concentrations in testes of adult pigs. Theriogenology, 2011;76:1134-45.
38. Maldjian, A., F. Pizzi, T. Gliozzi, S. Cerolini, P. Penny, and R. Noble, Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology, 2005;63:411-21.
39. Johnson, L., K. Weitze, P. Fiser, and W. Maxwell, Storage of boar semen. Animal reproduction science, 2000;62:143-72.
40. Waberski, D., K. Weitze, T. Gleumes, M. Schwarz, T. Willmen, and R. Petzoldt, Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. Theriogenology, 1994;42:831-40.
41. Kumaresan, A., G. Kadirvel, K. Bujarbaruah, R. Bardoloi, A. Das, S. Kumar, and S. Naskar, Preservation of boar semen at 18 C induces lipid peroxidation and apoptosis like changes in spermatozoa. Animal reproduction science, 2009;110:162-71.
42. Paulenz, H., O. Taugbøl, E. Kommisrud, and I. Grevle, Effect of dietary supplementation with cod liver oil on cold shock and freezability of boar semen. Reproduction in Domestic Animals, 1999;34:431-35.
43. Carvajal, G., C. Cuello, M. Ruiz, J.M. Vázquez, E.A. Martínez, and J. Roca, Effects of centrifugation before freezing on boar sperm cryosurvival. Journal of andrology, 2004;25:389-96.
44. Waterhouse, K., P. Hofmo, A. Tverdal, and R. Miller, Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction, 2006;131:887-94.
45. Kaeoket, K., P. Sang-urai, A. Thamniyom, P. Chanapiwat, and M. Techakumphu, Effect of docosahexaenoic acid on quality of cryopreserved boar semen in different breeds. Reproduction in domestic animals, 2010;45:458-63.
46. Tavilani, H., M.T. Goodarzi, A. Vaisi-Raygani, S. Salimi, and T. Hassanzadeh, Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa. International braz j urol, 2008;34:485-91.
47. Agarwal, A., R.K. Sharma, K.P. Nallella, A.J. Thomas, J.G. Alvarez, and S.C. Sikka, Reactive oxygen species as an independent marker of male factor infertility. Fertility and sterility, 2006;86:878-85.
48. Vernet, P., R. Aitken, and J. Drevet, Antioxidant strategies in the epididymis. Molecular and cellular endocrinology, 2004;216:31-39.

851 49. Koziorowska-Gilun, M., M. Koziorowski, J. Strzeżek, and L. Fraser, Seasonal
852 changes in antioxidant defence systems in seminal plasma and fluids of the boar
853 reproductive tract. Reproductive biology, 2011;11:37-47.
854 50. Roca, J., M. Hernández, G. Carvajal, J. Vázquez, and E. Martinez, Factors influencing
855 boar sperm cryosurvival. Journal of animal science, 2006;84:2692-99.
856 51. Ferreira, C., D. Sávio, A. Guarise, M. Flach, G. Gastal, A. Gonçalves, O. Dellagostin,
857 R. Alonso, I. Bianchi, and C. Corcini, Contribution of boars to reproductive
858 performance and paternity after homospermic and heterospermic artificial
859 insemination. Reproduction, Fertility and Development, 2015;27:1012-19.
860 52. Haugan, T., O. Reksen, Y. Gröhn, A. Gaustad, and P. Hofmo, A retrospective study
861 on effects of storage time of liquid boar semen on reproductive performance in
862 Norwegian swine. Theriogenology, 2005;64:891-901.
863 53. Alm, K., O. Peltoniemi, E. Koskinen, and M. Andersson, Porcine field fertility with
864 two different insemination doses and the effect of sperm morphology. Reproduction
865 in domestic animals, 2006;41:210-13.

866 54. França, L., G, Avelar and F, Almedia, Spermatogenesis and sperm transit through the
867 epididymis in mammals with emphasis on pigs, 2005;63:300-18

868 55. Teagasc. National Pig Herd Performance Report.
869 http://www.teagasc.ie/publications/2015/3651/Pig Development Report 2014
870 web.pdf/;2014 [assessed 20.08.15]

871 56. Bacinoglu, S., M. Taş, Ü. Cirit, Ö. Özdas, and K. Ak, The potential fertility
872 estimation capacity of the hypoosmotic swelling test, the thermal stress test and a
873 modified cervical mucus penetration test in the bovine. Animal reproduction science,
874 2008;104:38-46

875 57. Maes, D., A. Rodriguez, T. Rihsselaere, P. Vyt, and A. Soom, Artificial insemination
876 in pigs. Artificial insemination in farm animals, 2011;79-94

877 58. Yan, L., X. Cheng, J. Mao, D. Wu, B. Ren, S. Xu, Z. Fang, L. Che, C. Wu, and J. Li,
878 Effects of different dietary n-6/n-3 polyunsaturated fatty acid ratios on boar
879 reproduction. Lipids in health and disease, 2016;15:31