The in vitro addition of docosahexaenoic acid (DHA) improves the quality of cooled but not frozen-thawed stallion semen

In vitro addition of DHA to stallion semen

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ABSTRACT

The aim of this study was to assess the effect of the addition of docosahexaenoic acid (DHA) on the in vitro quality of cooled and frozen-thawed stallion semen. In Experiment 1, semen from 10 stallions was collected (3 ejaculates per stallion). Semen was diluted to 100 x 10\textsuperscript{6} spermatozoa/mL with 0.02 mM of vitamin E (VE) and 0, 1, 10 or 20 ng of DHA/mL and frozen. Semen was thawed and total motility (TM), acrosome integrity and morphology were assessed. In Experiment 2, semen from 3 stallions was collected (3 ejaculates per stallion) and frozen as in Experiment 1, but VE and DHA were added after thawing. Total motility and progressive linear motility (PLM) were assessed at 30, 60 and 120 min and viability, acrosome integrity and membrane fluidity at 30 min. In Experiment 3, semen from 5 stallions was collected (1-3 ejaculates per stallion), diluted to 20 x 10\textsuperscript{6} spermatozoa/mL and stored at 4\textdegree C. After 1, 24, 48 and 72 h, TM, PLM, viability, membrane fluidity and lipid peroxidation were assessed. The addition of DHA had no effect on frozen semen (Experiments 1 and 2) but improved TM, PLM and membrane fluidity in cooled stallion semen.

Keywords: Equine, Sperm, PUFA, Fertility
1. Introduction

Artificial insemination with cooled and frozen-thawed semen is widely used in the equine sporthorse breeding industry (Aurich and Aurich, 2006). To achieve acceptable pregnancy rates with cooled stallion semen it must be inseminated within 24-36 h of semen collection (Lindahl et al., 2012) compared to 3 days with liquid bull semen (Murphy et al., 2015) and 5 days with boar semen (Johnson et al., 2000). The pregnancy rates achieved following the use of frozen-thawed stallion semen are lower (approximately 45%; Miller, 2008) than those in cattle (about 54%; Odhiambo et al., 2014) but are also highly dependent on the individual stallion (Haadem et al., 2015).

During temperature decreases stallion spermatozoa undergo a membrane lipid phase change, in which it transitions from a liquid to a gel phase with peak phospholipid transition thought to occur in stallion spermatozoa at approximately 20°C (Parks and Lynch, 1992). The cholesterol/phospholipid ratio is thought to influence spermatozoa membrane fluidity (Klein et al., 1995) and affect the stability of the membrane during spermatozoa cooling, freezing and subsequent re-thawing (Darin-Bennett and White, 1977). Thus intact lipid molecules are necessary for a good functional spermatozoa membrane (Bustamante-Filho et al., 2014) which can survive the freeze-thaw process. In addition, there are differences in the lipid composition of the plasma membrane between different stallions, which may help explain the variations in spermatozoa resistance to the cooling and as well as the freeze-thaw process (Battelier et al., 2001).

The phospholipid profile of the stallion spermatozoa plasma membrane is similar to that of the boar. It contains high levels of docosapentaenoic acid (DPA), an omega-6 polyunsaturated fatty acid (PUFA), and docosahexaenoic acid (DHA), an omega-3-PUFA (Parks and Lynch, 1992). While it may be desirable to increase the omega-3-PUFA content of spermatozoa using dietary or in vitro supplementation, so as to increase membrane fluidity, this strategy may also promote susceptibility of the spermatozoon to lipid peroxidation resulting in membrane damage (Schmid-Lausigk and Aurich, 2014). Thus, beneficial and detrimental effects of PUFA enrichment are closely balanced in stallions (and other species) due to sensitivity to reactive oxygen species (ROS; Pena et al., 2011). The relationship between lipid profile to the plasma membrane and spermatozoa quality in stallions remains poorly understood. In contrast, this relationship has been studied in humans (Lewis, 2007; Aitken et al., 2012), bulls
A number of studies supplementing PUFA’s in the diet have demonstrated a beneficial effect on semen quantity and quality in bulls (Gürler et al., 2015; Moallem et al., 2015), boars (Liu et al., 2015) and rams (Fair et al., 2014). Brinsko et al., (2005) reported beneficial effects of dietary supplementation of stallions with a DHA nutraceutical on both frozen-thawed and cooled semen quality, with the most notable improvements in stallions whose semen did not tolerate cooling well. In an attempt to compensate for the damages caused to stallion spermatozoa during storage, researchers have studied the effect of the addition of substances to semen during processing. A number of studies have shown that the *in vitro* addition of fatty acids to semen improved the quality of cryopreserved (Büyükleblebici et al., 2014; Kaka et al., 2015a; Sampaio et al.) and liquid stored bull semen (Kiernan et al., 2013) as well as cryopreserved boar semen (Chanapiwat et al., 2009) but there is no published study on the effect of the exogenous DHA addition to stallion semen.

Thus, the objective of this study was to assess the effect of the *in vitro* addition of DHA to stallion semen before freezing, after thawing and before cooling on a range of *in vitro* spermatozoa quality parameters.

2. **Materials and method**

2.1 Experimental design

*Animal ethics*

All experiments were performed according to appropriate ethical and legal standard under the approval number: 2014_11_11_ULAEC (University of Limerick, Ireland).

*Experiment 1: Effect of the addition of docosahexaenoic acid to stallion semen prior to freezing*

The aim of this experiment was to assess the effect of the addition of DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid, Sigma, Arklow, Ireland, 25 mg) and vitamin E (VE; α-Tocopherol, Arklow, Ireland, Sigma) to semen before freezing. Semen from 10 Irish Sport Horse stallions of proven fertility, ranging between 13 and 28 years of age,
was collected at a commercial stud in Ireland using an artificial vagina (3 ejaculates from each stallion with a rest interval of at least 3 days between ejaculates) and all ejaculates were processed individually. Following collection, the gel fraction was removed following which total motility (TM) was assessed subjectively using a phase contrast microscope (minimum TM of 70% was used; results not presented). The ejaculate was diluted in a 1:1 ratio of INRA 96 extender (IMV Technologies, L’Aigle, France) and centrifuged at 600 g for 10 min at 32°C following which the concentration of the spermatozoa in the pellet was assessed using a photometer (SDM6, Minitube, Tiefenbach, Germany). The pellet was diluted to 100 x 10^6 spermatozoa/mL in Gent freezing extender (Minitube), in the presence of (i) 0 ng of DHA/mL + 0.02 mM of VE (control; T0VE), (ii) 1 ng of DHA/mL + 0.02 mM of VE (T1VE), (iii) 10 ng of DHA/mL + 0.02 mM of VE (T10VE) or (iv) 20 ng of DHA/mL + 0.02 mM of VE (T20VE). The concentrations of DHA and VE were adapted from Nasiri et al. (2012) as this study demonstrated a positive effect on spermatozoa characteristics following the addition of 0.02 mM of VE and 10 ng of DHA/mL to frozen-thawed bull semen. The diluted semen was cooled slowly to 4°C over 60 min and then packaged into 0.5 mL straws (Minitube) and sealed using polyvinyl alcohol (PVA) powder (Minitube). Straws were frozen to -110°C (13.9°C/min) in a programmable freezer (IceCube 14S, Minitube, Germany) following which they were plunged into liquid nitrogen at -196°C. The sperm concentration within straws was confirmed using a haemocytometer and was ±10% of the target concentration.

One straw of each treatment was thawed at 37°C for 30 sec (10 stallions with 3 ejaculates per stallion = 30 ejaculates) and maintained at 32°C in a heated-block until TM and kinematic parameters were analysed using computer assisted sperm analysis (CASA). A further two straws of each treatment were thawed (5 stallions with 3 ejaculates per stallion = 15 ejaculates) and assessed for acrosome integrity and membrane fluidity using flow cytometry. Another straw of each treatment was thawed (5 stallions with 3 ejaculates per stallion = 15 ejaculates) and assessed for morphology.

**Experiment 2: Effect of the addition of docosahexaenoic acid to stallion semen after thawing**

The aim of this experiment was to assess the effect of the addition of DHA to semen after thawing. Semen from 3 Irish Sport Horse stallions of proven fertility, ranging between 7 and 17 years of age, was collected at a commercial stud in Ireland
using an artificial vagina (3 ejaculates from each stallion), processed and frozen as per
Experiment 1 but without the addition of DHA or VE. Two straws were thawed per
ejaculate at 37°C for 30 sec and semen was diluted to a final concentration of 25 x 10^6
spermatozoa/mL in INRA 96 containing the following: (i) 0 ng of DHA/mL (control; T0),
(ii) control + 0.02 mM of VE (T0VE), (iii) 1 ng of DHA/mL + 0.02 mM of VE
(T1VE), (iv) 10 ng of DHA/mL + 0.02 mM of VE (T10VE) and (v) 20 ng of DHA/mL +
0.02 mM of VE (T20VE). All treatments were maintained at 32°C until analysis were
completed (3 stallions with 3 ejaculates per stallion = 9 ejaculates). Total motility,
progressive linear motility (PLM) and kinematic parameters were assessed at 30, 60 and
120 min following the addition of DHA using CASA (as per Experiment 1) while
viability, acrosome integrity and membrane fluidity were assessed at 30 min following
the addition of DHA using flow cytometry.

Experiment 3: Effect of the addition of docosahexaenoic acid to stallion semen
prior to cooling

The aim of this experiment was to assess the effect of the addition of DHA to semen before cooling. Semen from 5 Irish Sport Horse stallions of proven fertility, ranging between 7 and 20 years of age, were collected, diluted and centrifuged as per
Experiment 2. The pellet was resuspended to 20 x 10^6 spermatozoa/mL in INRA 96 using the same treatments as per Experiment 2. Semen was maintained at 15°C for 2 h, packaged in 0.5 mL straws, sealed using PVA powder and following a gradual
temperature reduction were stored at 4°C. After 1 h (Day 0), one straw from each
treatment was warmed to 32°C, following which motility (TM and PLM) and kinematic
parameters were assessed (5 stallions with 1 to 3 ejaculates per stallion = 12 ejaculates)
using CASA (as per Experiment 1). Another straw from each treatment was warmed to
32°C following which viability, membrane fluidity and lipid peroxidation were assessed
(3 stallions with 3 ejaculates per stallion = 9 ejaculates) after 24 (Day 1), 48 (Day 2) and
72 h (Day 3) using flow cytometry.

2.2 Sperm Functional Assessments

Computer assisted sperm analysis

Total motility and kinematic parameters were analysed using negative phase
contrast (100X) brightfield microscopy on an Olympus BX60 fitted with a CASA
system (Spermatozoa Class Analyser, SCA, Microptic, Viladomat, Barcelona, Spain). A drop (5 μL) of diluted semen was placed on a pre-warmed chamber (37°C; Leja counting chambers; Microptic) and analysed for spermatozoa motion and kinematic characteristics immediately post-thaw using SCA Evolution software pre-set to record stallion parameters (SCA Evolution, Microptic, Viladomat, Barcelona, Spain). A minimum of five microscopic fields with at least 100 spermatozoa 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 spermatozoa were edited out using the playback function. The CASA derived motility and kinematic characteristics assessed were TM, PLM, 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). None of the treatments in all three experiments significantly affected any of the kinematic parameters and therefore these results are not presented.

**Morphology**

Spermatozoa were fixed with 0.2% glutaraldehyde following which 10 μL of the solution was placed on a slide and covered with a coverslip. After placing a drop of immersion oil on the coverslip, percentage of morphologically normal and abnormal spermatozoa was assessed using a phase-contrast microscope at 1000X. At least 100 spermatozoa were assessed in each sample.

**Assessment of acrosome integrity, membrane fluidity, viability and lipid peroxidation**

Samples were diluted using phosphate buffered saline (PBS) medium to a concentration of 6 x 10⁶ spermatozoa/mL and were analysed using a flow cytometer (Guava EasyCyte 6HT-2L, Merck Millipore, Billerica, USA) equipped with both a krypton (640 nm) and an argon (488 nm) laser. 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 spermatozoa from debris. 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.
Acrosome integrity was assessed using a method adapted from Murphy et al. (2015). Briefly the fluorescent stains Alexa Fluor 647 PNA (AF647; lectin peanut agglutinin from *Arachis hypogaea*; Ex/Em: 650/688; Life Technologies) was added to a final concentration of 6 µg/mL and was incubated at 32°C in the dark for 15 min. AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. SYTO 16 (Ex/Em: 488/518 nm; Life Technologies, Carlsbad, USA) was added to the sample at a final concentration of 100 nM and incubated in the dark at 32°C for 15 min. SYTO 16 works by binding to nucleic acids. Following this incubation period the fluorescent stain propidium iodide (PI; Ex/Em: 535/617 nm; Life Technologies) was added to the sample at a final concentration of 15 µM and incubated for further 15 min. Since PI can not permeate live cells it is used to detect dead cells, PI binds to DNA by intercalating between the bases with little or no sequence preference. Post incubation, samples (200 µL) were transferred to a 96-well microplate and analysed. The fluorescence of AF647, SYTO 16 and PI was analysed via the photodetector 661/19, 525/30 and 583/23 nm BP filter, respectively, no compensation was needed. The percentage of viable spermatozoa with intact acrosomes was calculated as the percentage of AF647 negative cells of the PI negative population as initially gated based on controls, FSC and SSC.

Membrane fluidity was assessed using a method adapted from Murphy et al. (2014). The apoptotic stain Yo-Pro-1 (Ex/Em: 491/509; Life Technologies) was added to a final concentration of 50 nM and incubated at 32°C in the dark for 10 min. Yo-Pro-1 works by identifying apoptotic cells. Following the incubation period, to assess membrane fluidity, the fluorescent probe merocyanine 540 (M540; Ex/Em: 555/576; Sigma, Wicklow, Ireland) was added to a final concentration of 10 µM and incubated in the dark for a further 15 min. M540 binds to the surface of polarized membranes and fluoresces upon membrane depolarisation, thus indicating increased membrane fluidity. Post incubation, samples (200 µL) were transferred to a 96-well microplate and analysed. Fluorescence of Yo-Pro-1 and M540 were read with the photodetector (525/30 nm BP filter). High membrane fluidity was defined as the percentage of viable cells (Yo-Pro-1 negative) positive for M540.
Viability was assessed using two fluorescent stains: SYTO 16 and PI using a method adapted from Murphy et al. (2015). SYTO 16 was added to a final concentration of 100 nM and incubated at 32°C in the dark for 15 min. Subsequently, PI was added at a final concentration of 15 μM and incubated for a further 15 min. Post incubation, samples (200 μL) were transferred to a 96-well microplate (Corning Inc., Corning, NY, USA) and analysed. SYTO 16 was read with the photodetector (525/30 nm BP filter) and PI was read with the photodetector (583/23 nm BP filter), no compensation was needed. The percentage of viable cells was expressed as the percentage of cells positive for SYTO 16, but negative for PI.

Lipid peroxidation was assessed using two fluorescent stains: BODIPY C<sub>11</sub> (Ex/Em: 581/591; Life Technologies) and PI. BODIPY C<sub>11</sub> was added to a final concentration of 2 nM and incubated at 32°C in the dark for 15 min. BODIPY C<sub>11</sub> works by detecting ROS in cells and membranes. Following the incubation period, PI was added to a final concentration of 15 μM and incubated for further 15 min. Post incubation, samples (200 μL) were transferred to a 96-well microplate and analysed. Fluorescence of BODIPY C<sub>11</sub> and PI were read with the photodetector (583/23 nm BP filter). Lipid peroxidation was defined as the percentage of viable cells (PI negative) positive for BODIPY C<sub>11</sub>.

2.3 Statistical analysis

Data were examined for normality of distribution, tested for homogeneity of variance and analysed using an Analysis of Variance (ANOVA; Experiments 1 and 2) or repeated measures ANOVA (Experiments 2 and 3) in the Statistical Package for the Social Sciences (SPSS; version 22.0, IBM, Armonk, USA). The final statistical model employed, included the main effects of treatment, incubation period, stallion and their interactions. Post hoc tests were conducted using the Tukey test and P<0.05 was deemed to be statistically significant. All results are reported as the mean ± the standard error of the mean (s.e.m.).

3. Results
3.1 Experiment 1: Effect of the addition of docosahexaenoic acid to stallion semen prior to freezing

There was no effect of treatment on any of the *in vitro* parameters assessed (P>0.05), with an overall post-thaw TM of 50.3 ± 4.02%, percentage of spermatozoa with intact acrosomes in the live population of 95.7 ± 0.73%, percentage of spermatozoa with high membrane fluidity in the live population of 31.0 ± 3.49% and percentage of spermatozoa with normal morphology of 74.6 ± 2.26%. There was effect of stallion (P<0.001) on all motility and flow cytometric parameters assessed, but there was no treatment by stallion interaction.

3.2 Experiment 2: Effect of the addition of docosahexaenoic acid to stallion semen after thawing

There was no effect of treatment, stallion or their interactions on TM or PLM (P>0.05). Over all the time points assessed (30, 60 and 120 min following the addition of DHA) TM was 29.6 ± 5.07% and PLM was 14.4 ± 4.49%. There was an effect of incubation period with TM and PLM (P<0.001) decreasing over time. There was an interaction between incubation period and stallion in both TM and PLM (P<0.001).

There was no effect of treatment, stallion or their interaction on viability (P>0.05), while there was no effect of treatment or treatment by stallion interaction on acrosome integrity and membrane fluidity (P>0.05). Overall viability was 30.4 ± 4.24%, percentage of spermatozoa with intact acrosomes in the live population was 97.6 ± 0.29% and percentage of spermatozoa with high membrane fluidity in the live population was 52.9 ± 4.85%. There was effect of stallion (P<0.001) on acrosome integrity and membrane fluidity.

3.3 Experiment 3: Effect of the addition of docosahexaenoic acid to stallion semen prior to cooling

There was an effect of treatment on TM, with the T20VE having greater TM than the T0 (P<0.05; Figure 1). TM declined with day of storage (P<0.001) but this was not affected by treatment (P>0.05). There was an effect of stallion on TM (P<0.001), but no stallion by treatment interaction (P>0.05). There was an effect of treatment on PLM, with
all the DHA treatments having greater PLM than both T0 and T0VE (P<0.001; Figure 2).

PLM declined with day of storage (P<0.01) but was not affected by treatment (P>0.05). There was an effect of stallion on PLM (P<0.01) but no stallion by treatment interaction (P>0.05).

There was no effect of treatment, day of storage or their interaction on viability (P>0.05), with overall viability (on Days 0 to 3) of 55.2 ± 7.10%. There was an effect of stallion (P<0.05) but no stallion by treatment interaction (P>0.05).

There was an effect of treatment on membrane fluidity (P<0.001), with T10VE (28.7 ± 4.35%) and T20VE (29.4 ± 5.18%) having a greater percentage of spermatozoa with high membrane fluidity in the live population than the other treatments (19.0 ± 3.85% over Days 0 to 3). There was an effect of day of storage, day by treatment interaction (P<0.001), stallion and stallion by day interaction (P<0.001) but no treatment by stallion interaction (P>0.05).

There was an effect of treatment on lipid peroxidation (P<0.05), with T0VE (13.0 ± 4.40%) having lower lipid peroxidation than T10VE (21.3 ± 5.20%). While there was an effect of day of storage (P<0.001), there was no treatment by day interaction (P>0.05). There was an effect of stallion (P<0.001) but no stallion by treatment interaction (P>0.05).

4. Discussion

This is the first published study to assess the effect of the \textit{in vitro} addition of exogenous DHA to stallion spermatozoa and has demonstrated beneficial effects on cooled semen but not when added prior to freezing or after thawing.

In contrast to the findings in other species, the addition of DHA to stallion semen prior to freezing did not affect the spermatozoa quality in any of the parameters assessed. Increased post-thaw motility (Kaka et al., 2015b; Nasiri et al., 2012; Towhidi and Parks, 2012), improved morphology, acrosome integrity and membrane integrity (Kaka et al., 2015b) have been reported after the \textit{in vitro} addition of DHA to bull semen prior to freezing and it may be as a result of DHA accumulation in the membrane of the spermazoon and consequently increased resistance to degradation caused by ice crystal formation (Nasiri et al., 2012). Others have used a semen extender supplemented with DHA from fish oil (Kaeoket et al., 2010) or a combination of L-cysteine and DHA-enriched hen egg yolk (Chanapiwat et al., 2009) prior to freezing boar spermatozoa and
reported improved post-thaw motility and acrosomal integrity. It may be that stallion spermatozoa membranes are more sensitive to the freeze-thaw process than other farm animal species and the in vitro addition of DHA to semen was not enough to promote improvements in the semen quality. It is plausible that higher concentrations of DHA, or a more prolonged incubation period, may be required for stallion spermatozoa so as to avoid the disruption caused by ice crystal formation during the cryopreservation process.

Addition of DHA to stallion semen after thawing did not affect spermatozoa quality in any parameters analysed. There is a dearth of published studies which added DHA to semen after thawing. However, after dietary supplementation with a nutraceutical rich in DHA, improvements in freezability consequently resulting in increased motion characteristics have been reported in frozen-thawed stallion semen (Brinsko et al., 2005). It is not known why dietary supplementation of DHA seems to be more effective in improving frozen-thawed stallion semen quality than in vitro addition of DHA to frozen-thawed stallion semen but it may be due the duration of exposure of the spermatozoa to DHA or even the low concentrations of exogenous DHA. Future studies should consider keeping DHA in contact with thawed semen for longer period than the period used in the present study and/or using higher concentrations of DHA.

The improvements observed in the current study in TM, PLM and membrane fluidity when DHA was added to cooled semen are likely related with the DHA incorporation into the spermatozoa membrane thus protecting the membrane against the damage caused due the temperature changes (Nasiri et al., 2012). Therefore, DHA seems to be important to preserve the membrane functionality when the temperature change is not severe, from 37°C to 4°C, but the concentrations of DHA used in this study was not enough to protect the membrane when sub zero temperature were applied (as in Experiments 1 and 2). In contrast, there was no effect on TM in cooled stallion semen after dietary supplementation with a DHA-enriched nutriceutical (Brinsko et al., 2005), this suggests that the in vitro addition of DHA to cooled stallion semen is more efficient in improving TM than supplementation of DHA in the diet.

In the current study, the addition of DHA to cooled stallion semen yielded higher PLM than the controls up to Day 3 of storage, which may be associated with the capacity of DHA in preventing the disruption of lipid membranes (Meryman, 1966). Similarly, an improvement in PLM was observed on both Day 1 and 2 of cooled semen storage when the stallions with <40% initial PLM had their diets supplemented with
DHA (Brinsko et al., 2005). On the other hand the *in vitro* addition of exogenous DHA to cooled bull semen had detrimental effects on PLM and viability (Kiernan et al., 2013) and may be due to DHA accelerating the production of ROS. Therefore, the use of antioxidants such as VE along with PUFA supplementation to protect spermatozoa seems essential.

The addition of the exogenous DHA to cooled stallion semen yielded a greater percentage of spermatozoa with high membrane fluidity and it may be explained by the several roles of PUFAs, for example their ability to confer upon the spermatozoa plasma membrane the fluidity it needs to fertilise the oocyte (Whates et al., 2007). The percentage of spermatozoa with high membrane fluidity decreased from Day 0 to Day 1 which may be due to stallion spermatozoa being susceptible to the drop in temperature, due to the phase transition of lipids which can damage the plasma membrane (Moran et al., 1992). After Day 1, spermatozoa with high membrane fluidity survived while spermatozoa with low membrane fluidity did not, therefore it would appear that the percentage of spermatozoa with high membrane fluidity in the live population increased after Day 1.

Similarly to this study, Kaka et al. (2015b) found that lipid peroxidation increased as the DHA concentration added increased. The presence of high levels of PUFA requires efficient antioxidant levels to protect spermatozoa against lipid peroxidation (Aitken and Baker, 2004) and it seems that the 0.02 mMol of VE used in the current study was not sufficient to prevent lipid peroxidation.

In conclusion, the present *in vitro* study has demonstrated that the addition of DHA had a positive effect on the quality of cooled stallion semen, in terms of increased TM, PLM and percentage of spermatozoa with high membrane fluidity, however, there was no effect on frozen-thawed semen. A field fertility trial is required to establish if the *in vitro* addition of DHA to cooled stallion semen can increase *in vivo* fertility.

**Conflict of interest**

The authors have no conflicts of interest to declare.

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Figure 1. Effect of the addition of docosahexaenoic acid (DHA) in the presence of Vitamin E (VE) prior to cooling to 4°C on total motility of stallion semen (n=5 stallions). Vertical bars represent s.e.m.

Figure 2. Effect of the addition of docosahexaenoic acid (DHA) in the presence of Vitamin E (VE) prior to cooling to 4°C on progressive linear motility (PLM) of stallion semen (n=5 stallions). Vertical bars represent s.e.m.
References


