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Impact of dietary fatty acids on muscle composition, liver lipids, milt composition and sperm performance in European eel

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ABSTRACT

In order for European eel aquaculture to be sustainable, the life cycle should be completed in captivity. Development of broodstock diets may improve the species' reproductive success in captivity, through the production of high-quality gametes. Here, our aim was to evaluate the influence of dietary regime on muscle composition, and liver lipids prior to induced maturation, and the resulting sperm composition and performance. To accomplish this fish were reared on three "enhanced" diets and one commercial diet, each with different levels of fatty acids, arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Neutral lipids from the muscle and liver incorporated the majority of the fatty acid profile, while phospholipids incorporated only certain fatty acids. Diet had an effect on the majority of sperm fatty acids, on the total volume of extractable milt, and on the percentage of motile sperm. Here, our results suggest that the total volume of extractable milt is a DHA-dependent process, as we found the diet with the highest DHA level induced the most milt while the diet with the lowest DHA level induced the least amount of milt. The diet with the highest level of ARA induced medium milt volumes but had the highest sperm motility. EPA also seems important for sperm quality parameters since diets with higher EPA percentages had a higher volume of milt and higher sperm motility. In conclusion, dietary fatty acids had an influence on fatty acids in the tissues of male eel and this impacted sperm performance.

Keywords: Eicosapentaenoic acid, Docosahexaenoic acid, Arachidonic acid, Broodstock diet, Fatty acid composition, Sperm

1. Introduction

Proper nutrition is essential for rearing healthy broodstock and for the production of high-quality gametes and viable offspring (reviewed in Izquierdo et al., 2001). Most studies conducted in the literature on dietary impacts have focused on female rather than male broodstock. However, when dietary regimes are administered to fish and tested in properly designed experiments, they have been shown to account for a significant portion of variation in phenotypic expression and reproductive traits in males (Asturiano et al., 2001; Alavi et al., 2009; Henrotte et al., 2010; Nyina-Wamwizaet et al., 2012; Norambuena et al., 2013). For instance, dietary fatty acids affected sperm morphology and sperm velocity in common barbel, *Barbus barbus* (Alavi et al., 2009); biochemical composition of sperm in Eurasian perch, *Perca fluviatilis* (Henrotte et al., 2010) was altered by dietary components; Nyina-Wamwiza et al. (2012) found higher milt volumes and improved sperm velocity in African catfish, *Clarias gariepinus*, fed a diet where fishmeal was completely substituted by agricultural products and consequently had higher levels of n-6 series fatty acids; and there was an effect of dietary arachidonic acid [ARA; C20:4(n-6)] levels on steroid production in Senegalense sole, *Solea senegalensis* (Norambuena et al., 2013). Furthermore, European sea bass, *Dicentrarchus labrax*, exhibited enhanced reproductive performance (i.e. increased sperm quality and fertilization success) when males were fed polyunsaturated fatty acids (PUFA)-enriched diets (Asturiano et al., 2001). Together, these studies (among others) demonstrate that broodstock diet is an important factor for male reproductive performance, thus dietary influences on male as well as female broodstock gamete production and quality need attention.

European eel, *Anguilla anguilla*, has long been a highly valued species targeted for aquaculture production (Ottolenghi et al., 2004; Nielsen and Prouzet, 2008). In order for eel aquaculture to be sustainable, the life cycle should be completed in captivity. Unfortunately, this is not an easy task as the reproductive cycle of the eel is quite complex including a transoceanic migration, where conditions are still relatively unknown and the natural spawning process has never been observed. However, we know that when eels reach sexual maturation they undergo an approx. 5000 km spawning migration from Europe to arrive 6 to 7 months later at their spawning site, which is presumed to be in the Sargasso Sea (Schmidt, 1922; Tesch, 2003). In this regard, the development of 'optimal' broodstock diets may improve the species' reproductive success in captivity, through the production of high-quality gametes and viable offspring from farmed fish (Heinsbroek et al., 2013; Støttrup et al., 2013). After studying sperm quality parameters and fatty acid composition during hormone-induced sexual maturation, Baeza et al. (2013) found significant correlations between several fatty acids and sperm quality parameters in male European eel. They mainly found a relationship between eicosapentaenoic acid [EPA; C20:5(n-3)] and sperm volume; sperm motility and PUFA precursors; and ARA and sperm velocity parameters.

In this study, male European eel were reared on three "enhanced" diets and one commercial diet, each with different levels of dietary fatty acids, ARA, EPA and docosahexaenoic acid [DHA; C22:6(n-3)]. Here, our aim was to evaluate the influence of these diets on muscle composition, and liver lipids prior to induced maturation, and the resulting sperm composition and performance (i.e. total volume and motility). Together, this information will prove useful for the development of

sustainable aquaculture for European eel, through the development of diets that enhance sperm quality, fertilization success, and larval production.

2. Materials and methods

2.1. Fish husbandry, treatments, and experimental procedures

Male broodstock were reared in four separate freshwater recirculation aquaculture systems (RAS) at ~25°C at Stensgård Eel Farm A/S, Denmark (55.655461N : 9.20051E) on three "enhanced" diets (PRO-EEL1, PRO-EEL2, PRO-EEL3) and one commercial diet (DAN-EX 2848; BioMar A/S, Brande, Denmark) (Table 1). To briefly summarize, PRO-EEL1 had 3.81%, 3.98%, 2.31% for EPA, DHA, ARA respectively; PRO-EEL2 had 18.02%, 9.94%, 3.21% for EPA, DHA, ARA, respectively; PRO-EEL3 had 7.90%, 7.47%, 0.52% for EPA, DHA, ARA, respectively and the commercial DAN-EX diet had 8.24, 8.38, 0.45% for EPA, DHA, ARA, respectively (Table 1). Eels were fed these experimental diets from December 2010 until mid-September 2011, equalling 38 weeks. All fish were fed the DAN-EX diet from the fingerling stage until the start of the feeding experiment.

Male fish (n = 71) were selected in the range of 31 to 45 g and 83 to 178 cm and transported to an experimental facility of the Technical University of Denmark (DTU), Denmark (55.407444N : 9.403414E) where they were housed in 300 L tanks equipped with a closed re-circulation system. No feed was provided during experimentation as eels in the silvering stage cease feeding (Dollerup and Graver, 1985). Acclimatization to saltwater took place over a 14-day period. Salinity was

adjusted artificially using Tropic Marin Sea Salt (Dr. Biener GmbH, Wartenberg, Germany).

After acclimatization, 32 males (8 randomly selected per diet) were sacrificed for morphometric records, histology prior to hormonal treatment, and muscle and liver lipid analyses (see below). The remaining male eels (n = 39) received weekly injections of human chorionic gonadotropin at 1.5 IU/g fish (Sigma Aldrich Denmark A/S); body morphology was recorded weekly (see below). Prior to hormonal treatment males were anaesthetized (benzocaine, 60 mg/L) and tagged with a passive integrated transponder in the dorsal muscle. During maturation, male eels were kept at a density of ≤ 30 kg per m^3. Salinity and temperature ranged from 36.7 to 37.3% and 19.5 to 20.5 °C, respectively. After the 11^{th} injection, milt was sampled for analyses of lipid composition as well as sperm performance and males were subsequently sacrificed for analyses of testes mass and histological testes development (see below).

2.2. Data collection

2.2.1. Body morphology

Prior to the onset of hormonal treatment, body mass and length were recorded in the sacrificed fish (8 fish per diet, as previously indicated). Furthermore, samples of liver and muscle tissue (right filet) were obtained for analyses of lipid composition. Samples were frozen at -20 ^{o}C at the experimental facility and then transferred to -40 ^{o}C at the National Food Institute, DTU. Testes were weighed and preserved in formaldehyde buffered with NaH_2PO_4-H_2O and Na_2HPO_4-$2H_2O$ for histological analysis of reproductive development.

143 For the hormonally treated males, total body mass and length of each fish was
144 recorded at the time of first injection (22 September 2011) and then at weekly
145 intervals for 10 weeks (last injection was 28 November 2011). On 29 November
146 2011, i.e. ~24 h after the last treatment, the males were stripped to obtain all available
147 milt. Sperm analyses were performed (see below) and subsamples frozen as above for
148 lipid analysis. The fish were subsequently sacrificed and dissected. Total body mass
149 and testes weight was recorded and testes preserved for histological analysis of
150 reproductive development (see above). Gonadosomatic index (GSI = (testes
151 mass/total body mass) × 100) was later calculated and used as the proxy for male
152 reproductive investment.
153
154 2.2.2. Sperm quality analyses
155
156 Milt was collected from 27 males 24 h after administration of the 11th hormone
157 injection, as previous studies (i.e. Pérez et al., 2000; Asturiano et al., 2005) showed
158 that this is the time when the highest sperm quality is found. Prior to harvest, males
159 were anaesthetized with benzocaine (60 mg/L) and the genital pore was wiped dry
160 after cleaning with deionized water. Milt was collected by applying light pressure on
161 the abdomen, and stored at 4 ^{o}C. Milt was filled into graduated tubes to calculate the
162 total volume stripped per male (expressed as mL 100 g/fish).
163 Sperm motility was assessed according to Gallego et al. (2013). In brief, individual
164 sperm samples were evaluated by checking the percentage of motile spermatozoa. The
165 sperm cells were activated by mixing 2 μL of milt with 200 μL of artificial seawater
166 [Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH adjusted to 8.2; Peñaranda et
167 al., 2010]. The sperm suspension (2 μL) was then pipetted into a Makler reusable

chamber (10 μm deep; Sefi Medical Instruments, Haifa, Israel) and motility was observed between 15 and 30 s after activation using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon DS-Fi1 camera head and and 40× objective lens. All equipment was maintained at room temperature (~20 °C). Samples were performed in triplicate and analyzed by the same trained observer to avoid subjective differences in the motility evaluation.

2.2.3. Lipid analyses

Lipid extraction and determination of lipid content

Lipids in feed samples, homogenized muscle and liver (~2 g samples) as well as sperm (~1 g samples) were extracted with a homogeneous mixture of chloroform, methanol, and water (2:2:1.8), following the method of Bligh and Dyer (1959). The method was modified to use a smaller volume of solvents, but the original ratio between chloroform, methanol, and water was maintained. The lipid extracts were used for the subsequent lipid class fractionation and determination of fatty acid composition and lipid content. The lipid content was determined by gravimetry after evaporation of chloroform. Duplicate analyses of each sample were performed.

Lipid class separation

Lipids were separated into neutral lipids (NL) and phospholipids (PL) by chromatography on a solid phase consisting of aminopropyl modified silica. Solvents with increasing polarity were used to separate the lipid classes. A lipid extract

corresponding to 10 to 100 mg lipid was used for the lipid class separation. Solvents from the lipid extraction were evaporated and the extract was resolubilized in 0.5 mL chloroform and transferred to a Sep-Pak column (Waters Corporation, Milford, Massachusetts). NL were eluted using 4 mL chloroform/2-propanol (2:1), and PL were eluted with 6 mL methanol. The elutes were evaporated to almost dryness (NL) or to 1-2 mL (PL) under nitrogen.

Preparation of fatty acid methyl esters and analysis of fatty acid composition

Elutes from lipid class separation of tissue and sperm extracts as well as lipid extracts from feed were used for the preparation of fatty acid methyl esters (Anon, 1998). C23:0 methylester was used as the internal standard. Fatty acid methyl esters were analysed on a HP 5890A gas chromatograph (Hewlett-Packard, Palo Aalto, CA) equipped with an Omegawax 320 (30 m × 3.2 mm × 0.25 μm) column from Supelco (Bellefonte, PA) using AOCS method Ce 1b-89 (Anon, 1998). The oven temperature programme was 15 ^{o}C/min to 160 ^{o}C, hold 2 min, 3 ^{o}C/min to 200 ^{o}C, hold 1 min, 3 ^{o}C/min to 220 ^{o}C, hold 17 min. A split ratio of 1:25 was used. Fatty acids were identified by comparison of retention times with a mixture of standards, containing all the fatty acids identified in this study. Each fatty acid was quantified by calculating its peak area relative to the total peak area. These values are referred to as fatty acid content (% weight of total fatty acids) throughout the paper.

2.2.4. Histological analyses

For the assessment of testes development, lobes sampled from the middle part of testes were dehydrated and embedded in paraffin and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H & E, VWR - Bie & Berntsen A/S, Denmark). The histological sections were photographed (Olympus DP 71 digital camera) at 200× magnification for identification of gamete development stages and tissue types. Testes tissues were categorized according to cell types and their relative area fraction (F) estimated (Tomkiewicz et al., 2011). Cell types included testicular somatic cells (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz) (Fig. 1). Excluded areas included areas with no tissue and lumen (Lu). The area fractions (F) of the different tissue categories were estimated by placing a point grid (48 points) on the images (photomicrographs) using the software ImageJ plugin Analyze. The categories were marked and counted using Cell Counter.

The progression of spermatogenesis was assessed using a spermatogenic maturity index (SMI) (Tomkiewicz et al., 2011):

$$\mathrm{SMI} = 0F_{\mathrm{Ts}} + 0.25F_{\mathrm{Sg}} + 0.5F_{\mathrm{Sc}} + 0.75F_{\mathrm{St}} + 1F_{\mathrm{Sz}}$$

The index ranges from 0 for only testicular somatic cells present to 1 for all germinal cells transformed into spermatozoa. SMI was estimated for each of the testes images in order to compare the morphological development of the testes tissue in males receiving different diets prior to onset hormonal treatment and after 11 weeks.

2.3. Statistical analyses

Data were analysed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA). Residuals were tested for normality

(Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Data were transformed to meet assumptions of normality, and homoscedasticity when necessary. Alpha was set at 0.05 for main effects and interactions. Treatment means were contrasted using the Tukey's test.

2.3.1. Body morphology

Prior to the onset of hormonal treatment, body mass was compared between the dietary groups using a one-way ANOVA model. For the hormonally treated males, temporal changes in total body mass were analysed using repeated measures mixed-model ANOVAs:

$$Y_{ipn} = \mu + M_i + A_p + MA_{ip} + \varepsilon_{n(ip)}$$

where μ is the true mean; M_i is the diet effect (where i = PRO-EEL1, PRO-EEL2, PRO-EEL3, and the DAN-EX diet); A_p is the effect of sampling week (where p = number of weeks); MA_{ip} is the diet × sampling week interaction; and $\varepsilon_{n(ip)}$ is the residual error. When a non-significant first-order diet × sampling week interaction was detected, main effects were interpreted. Diet and sampling week were fixed factors, while female was random and included as the subject in the REPEATED statement in SAS PROC MIXED (Littell et al., 1996; SAS, 2003). The Kenward-Roger procedure was used to approximate the denominator degrees of freedom for all F-tests (Spilke et al., 2005). The repeated statement was used to model the covariance structure within subjects (Littell et al., 1996). Three covariance structures were modeled: compound symmetry (type = cs), autoregressive order one (type = ar(1)), and “unstructured” (type = un). Akaike’s (AIC) and Bayesian (BIC) information

model-fit criterion were used to assist in final model inference determination (Littell et al., 1996). One-way ANOVA was used to compare GSI between the dietary groups.

2.3.2. Lipid analyses

One-way ANOVAs were used to compare fatty acids, total n-3, total n-6, n-3/n-6 ratio, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), and total PUFA in the muscle, liver tissue and sperm between the dietary groups; for statistical comparisons we examined the fatty acid composition as a percentage. For each fatty acid, and for each tissue type, separate one-way ANOVA models were run for PL and NL.

2.3.3. Sperm quality and histological analyses

One-way ANOVA models were used to compare total milt volume and sperm motility between the dietary groups. Furthermore, one-way ANOVA models were used to compare SMI between the diets prior onset to hormonal treatment and after 11 weeks.

3. Results

3.1. Body morphology

Prior to the onset of hormonal treatment, total body mass for the 8 fish sacrificed per diet (these fish were used for lipid analyses) ranged from 86 to 174 g and there

292 was no significant difference in body mass between the fish fed the four diets ($P > 0.05$). For the hormonally treated males, body mass ranged from 83 to 187 g, 87 to 159 g, 90 to 156 g, and 92 to 143 g for the dietary groups (Fig. 2). The broodstock diet × sampling week interaction ($P > 0.05$) and broodstock diet main effect ($P > 0.05$) both had no significant impact on total body mass of the fish throughout the experimental sampling period (Fig. 2A). On the contrary, the sampling week main effect was significant ($P < 0.0001$; Fig. 2B), such that total mass of the fish declined over the sampling period.

300 Diet composition had no effect on GSI of the males after hormonal treatment ($P > 0.05$; PRO-EEL1 = 8.3 ± 1.3%; PRO-EEL2 = 10.1 ± 1.3%; PRO-EEL3 = 8.9 ± 1.0%; DAN-EX diet = 11.8 ± 1.0%). Furthermore, the testes development of males sacrificed prior to treatment (week 0) was uniform in all groups with only spermatogonia present (Fig. 3A), but no spermatocytes. After hormonal treatment, the testes of all analysed males had spermatozoa in the tubuli, while at the same time showing continued generation of spermatocytes and spermatids (Fig. 3B). The SMI of hormonally treated males neither differed between diet groups prior to treatment (ranged from 0.07 to 0.24; $P > 0.05$) and/or after hormonal treatment (ranged from 0.50 to 0.87; $P > 0.05$).

310

311 3.2. Lipid analyses

312

313 3.2.1. Muscle composition

314

315 After 38 weeks of feeding on the various diets and prior to maturation, MUFA represented 47.0 to 51.8% of the NL fraction of the muscle tissue, whereas SFA and

317 PUFA were found in quantities of 19.8 to 25.8% (Table 2). The most abundant fatty
318 acids in the NL fraction of muscle tissue where palmitic (C16:0) and oleic acid
319 [C18:1(n-9)], with 14.6 to 17.0% and 21.4 to 28.0%, respectively; reflecting their high
320 dietary content (Table 1). Diet had a significant effect on the majority of fatty acids in
321 the NL fraction of the muscle ($P < 0.05$; Table 2). Here, PRO-EEL2 had the highest
322 percentage of ARA (1.0%) and EPA (5.7%), while DHA was significantly lower in
323 PRO-EEL1 (5.2%) compared to PRO-EEL2 (6.4%; Table 2).

324 The dominant classes of fatty acids in the PL fraction of the muscle were PUFA
325 (46.5 to 51.0%), followed by SFA (24.2 to 26.3%) and MUFA (17.1 to 22.0%; Table
326 3). The most abundant fatty acids were DHA, representing 22.7 to 26.8%, and
327 palmitic acid, representing 16.1 to 17.2% (Table 3). Diet had a significant effect on
328 the majority of fatty acids in the PL fraction of muscle ($P < 0.05$; Table 3). Here, the
329 essential fatty acids, ARA, EPA, and DHA were impacted by diet (ARA: $P < 0.0001$;
330 EPA: $P < 0.0001$; DHA: $P < 0.0001$). PRO-EEL1 had the highest levels of ARA
331 (8.0%), the DAN-EX diet had the highest levels of EPA (15.9%), and PRO-EEL3 as
332 well as the DAN-EX diet had the highest level of DHA (26.1 and 26.8%,
333 respectively) in the PL fraction of muscle tissue (Table 3).

334

335 3.2.2. Liver lipid

336

337 After 38 weeks of feeding on the various diets and prior to maturation, diet had no
338 impact on levels of total SFA in liver NL. On the contrary, PRO-EEL1 had higher
339 levels of MUFA than PRO-EEL2 or the DAN-EX diet, while PRO-EEL2 had the
340 highest level of PUFA ($P < 0.05$; Table 4) in liver NL. Palmitic acid and oleic acid
341 were the most abundant fatty acids in the NL fraction of liver. In particular, for oleic

342 acid the percentage of total fatty acids was highest in PRO-EEL1 and PRO-EEL3 ($P <$
343 0.01), while no significant difference was detected for palmitic acid in NL fraction of
344 the liver (Table 4). Diet had a significant effect on ARA, EPA and DHA levels in
345 liver NL (ARA: $P < 0.0001$; EPA: $P < 0.0001$; DHA: $P < 0.0001$), where the PRO-
346 EEL2 diet typically had the highest level of these fatty acids (Table 4).
347 DHA and palmitic acid were the most abundant fatty acids in the PL fraction of the
348 liver (Table 5). The dominant classes of fatty acids in the PL fraction of the liver were
349 PUFA (48.3 to 53.8%), followed by SFA (27.6 to 31.2%) and MUFA (8.8 to 11.5%;
350 Table 5). Diet had a significant effect on ARA, EPA and DHA levels in liver PL
351 (ARA: $P < 0.0001$; EPA: $P < 0.0001$; DHA: $P < 0.0001$; Table 5). Here, PRO-EEL1
352 had the highest ARA, DAN-EX had the highest EPA, and PRO-EEL3 had the highest
353 level of DHA (Table 5).
354
355 3.2.3. Sperm composition
356
357 DHA and palmitic acid were the most abundant fatty acids in eel sperm, followed
358 by oleic acid and EPA (Table 6). PRO-EEL2 had higher levels of SFA than the DAN-
359 EX diet, while MUFA levels were higher in PRO-EEL1 than PRO-EEL2; no other
360 significant differences were observed between the other diets for total SFA and total
361 MUFA (Table 6). Dietary effects had an influence on the relative content of the
362 majority of the sperm fatty acids ($P < 0.05$). Here, PRO-EEL1 and PRO-EEL2 had the
363 highest levels of ARA, while PRO-EEL1 had the lowest level of EPA (Table 6).
364
365 3.3. Sperm quality analyses
366

Mean ± SEM total extractable milt volume from the males stripped in week 11 was 3.69 ± 0.4 mL/100 g fish. Diet had a significant influence on sperm volume ($P < 0.05$), such that fish reared on the DAN-EX diet produced significantly more milt volume than fish reared on PRO-EEL1, while PRO-EEL 2 and PRO-EEL 3 showed intermediate values (Fig. 4A).

Mean sperm motility for the diets ranged from 42.8 ± 8.1 to 72.5 ± 7.2%. Diet had a significant effect on sperm motility ($P < 0.05$; Fig. 4B), such that PRO-EEL2 had significantly higher motility than PRO-EEL1; no significant differences in motility were observed between the other diets.

4. Discussion

The results of the present study on broodstock nutrition for the European eel suggests that the lipid composition in the diet should be optimized with regard to the content of ARA, EPA and DHA as it affects both the total volume of extractable milt produced and sperm motility. To date, most broodstock nutrition studies focus on female reproductive output but our results emphasize the need to address both male and female dietary needs to improve the reproductive performance for European eel.

Males reared on the PRO-EEL1 diet had “sub-optimal” sperm quality parameters; i.e. lowest mean milt volume and sperm motility. This diet also had the lowest n-3/n-6 ratio (1.04), due to a low percentage of n-3 series fatty acids and a high percentage of n-6 series fatty acids [consequently, high linoleic acid, (LA, 18:2(n-6)), and ARA levels]. Nowadays, the global shortages in fish oil are forcing the aquaculture feed industry to use alternative oil sources. Such dietary supplementations with vegetable oils contain large amounts of n-6 series fatty acids, such as LA. Thus, our findings

392 further exemplify the need to control and optimize the ratio of n-3/n-6 fatty acids in
393 formulated diets to improve reproductive performance in eel. Moreover, it has been
394 demonstrated that EPA and DHA are major components of membrane phospholipids
395 and their presence helps to facilitate the quantity and mobility of ejaculated sperm
396 (Sargent et al., 2002; Henrotte et al., 2010). Thus, the low sperm motility reported for
397 males reared on PRO-EEL1 could be linked to the lower levels of n-3 series fatty
398 acids in this diet (mainly EPA), when compared to the other treatments. Similarly,
399 Vassallo-Agius et al. (2001) found that rainbow trout fed with deficient n-3 series
400 fatty acids, showed lower sperm motility.

401 The PRO-EEL2 diet, with the highest level of ARA, induced medium milt volumes
402 but had the highest mean sperm motility. This coincides with the previously proposed
403 role of ARA metabolites during the sperm maturation process; this includes
404 prostaglandins, which essentially modulate the sperm maturation (Asturiano et al.,
405 2000; Baeza et al., 2014a; Norambuena et al., 2013). Furthermore, PRO-EEL2 had the
406 highest percentage of n-3 fatty acids levels, especially with regard to EPA content. In
407 this regard, PRO-EEL2 diet had a 2-fold higher EPA content in comparison to PRO-
408 EEL3 and the DAN-EX diet, however the milt samples of these three experimental
409 groups after induced maturation did not show any differences in EPA percentage.
410 Recently, studies have suggested a preferential utilization of dietary EPA as an energy
411 source for sperm (Senadheera et al., 2011; Al-Souti et al., 2012; Wing-Keong et al.,
412 2013); thus, it could be related to higher sperm motility for the fish fed diets with a
413 high percentage of this fatty acid and producing sperm with higher EPA levels. Baeza
414 et al. (2014a) suggested the existence of a synthesis of PUFA in the liver (especially
415 EPA) from their precursors to be sent to the testis to increase sperm energy for
416 motility and highlighted the importance of EPA during spermatogenesis. Baeza et al.

(2014b) found a negative correlation between EPA from the liver and sperm volume, suggesting its synthesis in the liver and its subsequent mobilization to the testis to be used to produce sperm cell membranes. Finally, correlations between EPA and androgens during the final phase of sperm maturation, exemplifies the modulatory effect of EPA on the synthesis of androgens (Baeza et al., 2015). Together, these studies highlight the importance of EPA in male eel reproduction and compliment our findings; our study shows that PRO-EEL2, PRO-EEL3 and DAN-EX diet, having the highest percentage of EPA in sperm, showed the highest sperm motility. Thus, EPA seems to be important for European eel with respect to male reproductive performance.

In our study, eels fed PRO-EEL2 had milt with a significantly higher proportion of saturated fatty acids and sperm showed the highest motility. Contradictorily, Beirão et al. (2012a), analyzing sperm of gilthead seabream, *Sparus aurata*, found a negative correlation between the proportion of saturated fatty acids and sperm quality parameters, i.e. viability and motility. Thus, these findings suggest that the amount of saturated fatty acids in sperm is not the only determinant for male performance, but it is the ratio between unsaturated/saturated fatty acids in the cell that may provides more information and will be affected by diet. For further confirmation, studies should continue to adjust this ratio between the diets.

Results from our study suggest that the total volume of extractable milt available is a DHA-dependent process. Here, we showed that diets with the highest DHA levels provided the most milt, while the diet with the lowest DHA level (PRO-EEL1 diet) induced the least amount of milt. Together, this supports the hypothesis that DHA has a structural role in sperm membrane formation (Baeza et al., 2014a), which is important for sperm morphology and function.

PRO-EEL2 diet had the highest level of palmitic acid, but no differences were detected with respect to the percentage of palmitic in liver NL between the experimental groups. Among others, palmitic acid is the predominant source of metabolic energy (Tocher et al., 2003) so this could be due to utilization of this fatty acid to obtain energy; similar to the results found by Caballero et al. (2002) for rainbow trout. PRO-EEL1 and PRO-EEL3 had the highest levels of oleic acid. Eels fed these diets seem to accumulate oleic acid in the muscle and liver NL, and it is possibly due to an excess of this fatty acid in the diet or may be due to the use of other fatty acids to obtain energy (Caballero et al., 2002).

PRO-EEL3 and the DAN-EX diet had similar percentages of ARA, EPA and DHA, but very different quantities of LA and alpha-linolenic acid (ALA, 18:3n-3) and consequently, PRO-EEL3 diet had a higher percentage of total PUFA. The different composition in PRO-EEL3 (higher LA and ALA and similar ARA, EPA and DHA), compared to the DAN-EX diet resulted in significant differences in tissue composition, especially in regard to liver NL. The results obtained in liver NL from eels fed PRO-EEL3 showed that the high LA and ALA provided in this diet could have an influence on fatty acid metabolism. Fatty acid chain elongation is actively present in the mitochondria of the eel hepatocytes (Giudetti et al., 2001), and enzymatic activity involved in lipid metabolism seems to be dependent on the fatty acids provided in the diets. Several authors hypothesized that competition between n-3 and n-6 series fatty acids, for the same enzyme (Δ-6 desaturase), may manifest due to an inappropriate dietary LA and ALA ratio (Caballero et al., 2002; Al-Souti et al., 2012). Recently, Wang et al. (2014) conducted the first study on the PUFA biosynthesis pathway in Japanese eel. Here, they demonstrated a freshwater pattern for this species, thus this focus area should also be explored for the European eel.

467 The PRO-EEL3 diet (higher LA and ALA) induced higher levels of n-3 series fatty
468 acids in liver NL (especially DHA content) than the DAN-EX diet. Thus, the results
469 from the present study, suggest an effect of high percentages of LA and ALA due to
470 different fatty acid elongation activity in the PRO-EEL3 diet, as explained above.
471 The allocation of resources from the muscle to the testes was evident from an
472 increase in GSI from week 0 to week 11, which was accompanied by spermatogenesis
473 induced by weekly hormonal treatment. The diets did not influence the progression of
474 spermatogenesis among groups, and all males were still actively producing sperm and
475 milt at the end of the experiment. Muscle and liver fatty acids were analyzed prior to
476 the fasting period, but eel have the ability to reallocate and synthesize lipids from
477 their endogenous sources, even after a prolonged period of fasting (Gnoni et al.,
478 1990). Therefore, although in our experiment the eels were fasted for >70 days, the
479 analyses of sperm fatty acid after the hormonal treatment still reflected the effect of
480 diet previously supplied. But, despite the differences between the compositions of
481 diets, the sperm fatty acids did not show differences in total PUFA. Long chain
482 PUFAs give the sperm plasma membranes the fluidity it needs to participate in
483 membrane fusion events associated with fertilization (Whates et al., 2007). Recently,
484 in gilthead seabream it was reported that membrane unsaturated fatty acids from the
485 sperm head and flagella were positively correlated with motility parameters (Beirao et
486 al., 2012b). Here, our results did not show a direct correlation between unsaturated
487 fatty acids and sperm quality parameters, but independent of the diet supplied, all
488 experimental groups registered a high content of unsaturated fatty acids in the sperm;
489 highlighting their importance in normal sperm function. Independent of diet, eels
490 reached the same PUFA levels probably due to their mobilization from other tissues.
491 Baeza et al. (2014a) found in European eel that stored fatty acids in the liver can be

transferred to the gonad, contributing to sperm membrane formation. Thus, defining the adequate PUFA percentage in diets is an important issue to improve sperm quality, since several studies have highlighted the negative impacts that occur if inadequate PUFA percentages are supplied in diets for both males and female reproductive performance. Accordingly, an excessive n-3 PUFA supplementation in rats decreased both sperm density and motility (Yan et al., 2013). Furuita et al. (2002) also found higher n-3 series PUFA in broodstock diets impaired reproduction in female Japanese flounder, *Paralichthys olivaceus*.

Eels fed PRO-EEL1 had the lowest levels of total n-3 fatty acids in the analyzed sperm samples, and also had the lowest sperm performance (motility and volume). As well as other species like European sea bass (Asturiano et al., 2001), rainbow trout, *Oncorhynchus mykiss* (Vassallo-Agius et al., 2001), Eurasian perch (Henrotte et al., 2010), Atlantic cod (*Gadus morhua*; Butts et al., 2011) and Arctic char (*Salvelinus alpinus*; Mansour et al., 2011), DHA was the most abundant PUFA registered in sperm fatty acids, which supports the hypothesis that DHA has a structural role in spermatozoa membrane formation (Baeza et al., 2014). Moreover, a high accumulation of ARA in sperm fatty acids was found as previously described in rainbow trout by Vassallo-Agius et al. (2001).

5. Conclusions

Overall, our results suggest that an optimal feed for eel should contain high levels of DHA to induce the production of high sperm volumes, high EPA levels (even >7%) to induce high sperm motility, medium levels of ARA, and should have n-3/n-6 ratio close to or >2. We also conclude that LA and ALA levels have importance in

517 diets and their inclusion can fundamentally influence liver lipid metabolism. Taken
518 together, this information will be useful to develop broodstock diets to improve the
519 sperm quality and subsequently, larval production for this species.
520

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522

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533

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671

672 **Figure captions**

673

674 Fig. 1 Photomicrographs of histological testes tissue sections of European eel,
675 *Anguilla anguilla*, illustrating testes development and cell types after 38 weeks of
676 feeding, prior to hormonal treatment (A) and after 11 weeks of hormonal treatment
677 (B). Ts = testicular somatic cells, Sg = spermatogonia, Sc = spermatocytes, St =
678 spermatids, Sz = spermatozoa, Lu = lumen.

679

680 Fig. 2 Effect of diet (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet) on
681 total body mass of hormonally treated European eel, *Anguilla anguilla,* broodstock
682 throughout the sampling period (A). Data were analyzed using a repeated measures
683 ANOVA model. The broodstock diet × sampling week interaction and broodstock
684 diet main effect both had no significant impact on body mass, while the sampling
685 week main effect was significant (B). Error bars represent standard errors. Bars
686 without a common superscript differed significantly ($P < 0.05$).

687

688 Fig. 3 Testes development of European eel, *Anguilla anguilla* males after 38 weeks of
689 feeding on different diets (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet)
690 illustrated as the average area fractions, *F*, of different tissue types in males sacrificed
691 prior to hormonal treatment (A) or after 11 weeks of hormonal treatment in (B).

692

693 Fig. 4 Effect of diet (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet) on
694 total milt volume (A) and percent motile sperm (B) of European eel, *Anguilla*
695 *anguilla*. Error bars represent standard errors. Bars without a common superscript
696 differed significantly ($P < 0.05$).

697 Table 1. Composition of the diets (% of total fatty acids) used to examine the impact
698 of dietary fatty acids on muscle, liver and milt lipid composition, and sperm
699 performance in male European eel, *Anguilla anguilla.* ARA = arachidonic acid, EPA
700 = eicosapentaenoic acid, DHA = docosahexaenoic acid

701
702

Fatty acids	**Experimental diets**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	4.13	7.14	3.27	6.13
C16:00	10.63	18.61	11.17	14.03
C16:1(n-7)	5.46	7.72	3.44	7.01
C18:00	1.98	4.01	2.60	1.77
C18:1(n-9)	29.19	8.75	35.05	12.44
C18:1(n-7)	2.80	3.01	2.82	2.93
C18:2(n-6)	9.41	3.26	12.65	3.45
C18:3(n-3)	3.10	0.77	4.59	1.16
C20:1(n-7)	9.71	0.80	1.04	10.24
C20:4(n-6) (ARA)	2.31	3.21	0.52	0.45
C20:5(n-3) (EPA)	3.81	18.02	7.90	8.24
C22:1(n-11)	7.61	0.28	0.39	9.58
C22:5(n-3)	0.33	2.19	1.01	0.89
C22:6(n-3) (DHA)	3.98	9.94	7.47	8.38
total n-3	12.88	36.91	23.61	22.03
total n-6	12.36	7.39	13.45	4.36
n-3/n-6	1.04	4.99	1.76	5.06
total SFA	17.44	30.71	17.90	22.93
total MUFA	56.56	21.49	43.64	44.45
total PUFA	26.00	47.79	38.46	27.37

703 Table 2. Effect of diet on fatty acid composition (%) in neutral lipids of muscle tissue
704 of male European eel, *Anguilla anguilla,* after 38 weeks of feeding. Small letters show
705 significant differences in each fatty acid over the dietary regimes. Results represent
706 means ± SEM ($P < 0.05$).

707

Fatty acids	**Dietary treatment**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	5.71±0.10	5.53±0.80	5.59±0.11	6.10±0.10
C16:0	15.88±0.12 b	17.03±0.10 c	14.58±0.19 a	16.23±0.11 b
C16:1(n-7)	7.76±0.11 b	8.41±0.13 c	7.13±0.14 a	8.31±0.08 c
C18:00	2.24±0.74 ab	2.58±0.15 b	2.04±0.07 a	2.16±0.07 a
C18:1(n-9)	26.64±0.44 b	21.44±0.52 a	28.01±0.51 b	22.73±0.50 a
C18:1(n-7)	3.56±0.07	3.55±0.06	3.58±0.05	3.60±0.06
C18:2(n-6)	4.31±0.13 ab	3.04±0.05 a	4.99±0.71 b	3.58±0.07 a
C18:3(n-3)	0.92±0.05 ab	0.59±0.01 a	1.13±0.20 b	0.74±0.02 ab
C20:1(n-7)	0.35±0.01 b	0.33±0.02 b	0.20±0.04 a	0.34±0.01 b
C20:4(n-6)	0.81±0.05 b	1.01±0.06 c	0.51±0.01 a	0.40±0.01 a
C20:5(n-3)	3.47±0.14 a	5.73±0.21 c	4.24±0.09 b	4.21±0.16 b
C22:1(n-11)	2.61±0.30 a	2.31±0.21 a	2.24±0.15 a	3.56 ±0.14 b
C22:5(n-3)	1.46±0.04 a	2.39±0.32 b	1.65±0.20 ab	1.71±0.05 ab
C22:6(n-3)	5.15±0.10 a	6.39±0.58 b	6.25±0.09 ab	6.23±0.12 ab
total n-3	12.68±0.23 a	17.23±1.02 c	15.12±0.34 bc	14.97±0.32 b
total n-6	5.95±0.19 ab	4.75±0.05 a	6.26±0.72 b	4.60±0.08 a
n-3/n-6	2.15±0.08	3.62±0.20	3.11±0.92	3.26±0.10
total SFA	24.43±0.11 ab	25.77±0.83 b	22.81±0.29 a	25.14±0.10 b
total MUFA	51.83±0.44 b	46.97±1.33 a	49.69±0.29 b	50.69±0.32 b
total PUFA	19.77±0.28 a	23.71±1.06 c	23.02±0.75b c	20.84±0.33 ab

708
709

710 Table 3. Effect of diet on fatty acid composition (%) of phospholipids in muscle tissue
711 of male European eel, *Anguilla anguilla,* after 38 weeks of feeding. Small letters show
712 significant differences in each fatty acid over the dietary regimes. Results represent
713 means ± SEM ($P < 0.05$).

714

Fatty acids	**Dietary treatment**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	4.07±0.10	4.02±0.38	3.43±0.20	3.80±0.16
C16:00	16.73±0.42	17.16±0.25	16.28±0.26	16.09±0.32
C16:1(n-7)	1.68±0.14 a	1.96±0.11 a	1.57±0.08 a	2.74±0.35 b
C18:00	4.20±0.29	4.57±0.24	3.82±0.16	3.85±0.63
C18:1(n-9)	12.06±0.25 b	9.40±0.26 a	12.34±0.24 b	9.13±0.21 a
C18:1(n-7)	2.40±0.08 ab	2.17±0.06 a	2.53±0.10 b	2.39±0.08 ab
C18:2(n-6)	2.20±0.11 b	1.01±0.06 a	2.52±0.21 b	1.39±0.05 a
C18:3(n-3)	0.34±0.03 b	0.16±0.01 a	0.40±0.07 b	0.26±0.03 ab
C20:1(n-7)	0.06±0.01 ab	0.05±0.01 ab	0.01±0.01 a	0.07±0.02 b
C20:4(n-6)	7.97±0.58 d	6.37±0.31 c	3.98±0.16 b	2.38±0.10 a
C20:5(n-3)	9.56±0.43 a	14.11±0.38 c	11.45±0.30 b	15.85±0.29 d
C22:1(n-11)	0.58±0.06 b	0.24±0.02 a	0.20±0.02 a	0.70 ±0.15 b
C22:5(n-3)	1.68±0.03 a	2.69±0.12 c	1.92±0.09 ab	2.04±0.10 b
C22:6(n-3)	22.73±0.62 a	24.42±0.45 ab	26.13±0.44 bc	26.77±0.74 c
total n-3	34.97±0.84 a	42.07±0.63 b	40.50±0.43 b	45.94±0.67 c
total n-6	10.88±0.55 c	7.74±0.25 b	7.16±0.34 b	4.3±0.12 a
n-3/n-6	3.28±0.21 a	5.47±0.15 ab	5.76±0.32 ab	10.74±0.25 b
total SFA	25.52±0.45 ab	26.27±0.36 b	24.20±0.42 a	24.21±0.70 a
total MUFA	22.02±0.63 b	17.08±1.48 a	20.32±0.40 b	19.67±1.01 ab
total PUFA	46.50±0.98 a	50.77±0.82 b	48.42±0.52 ab	51.00±0.72 b

715 Table 4. Effect of diet on fatty acid composition (%) of liver neutral lipids in male
716 European eel, *Anguilla anguilla,* after 38 weeks of feeding. Small letters show
717 significant differences in each fatty acid over the dietary regimes. Results represent
718 means ± SEM ($P < 0.05$).

Fatty acids	**Dietary treatment**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	3.95±0.18a	3.68±0.11a	3.94±0.17b	4.83±0.33b
C16:00	19.07±0.54	18.83±0.28	18.48±0.64	18.67±0.81
C16:1(n-7)	7.59±0.32b	7.67±0.24b	6.54±0.19a	7.47 ±0.19b
C18:00	2.38±0.34	2.79±0.37	2.15±0.12	4.86±2.26
C18:1(n-9)	29.29±1.08c	20.09±1.05a	27.21±0.70bc	21.58±2.57ab
C18:1(n-7)	3.42±0.14	3.15±0.19	3.82±0.08	3.39±0.48
C18:2(n-6)	4.14±0.13b	2.48±0.10a	5.49±0.23c	2.72±0.09a
C18:3(n-3)	0.81±0.04b	0.57±0.03ab	1.22±0.07c	0.44±0.10a
C20:1(n-7)	0.26±0.03bc	0.17±0.02ab	0.13±0.02a	0.33±0.06c
C20:4(n-6)	1.12±0.09b	1.38±0.09b	0.76±0.02a	0.53 ±0.07a
C20:5(n-3)	3.20±0.39a	8.05±0.35b	4.03±0.29a	2.87±0.19a
C22:1(n-11)	0.95±0.07b	0.47±0.06a	0.54±0.04ab	2.20 ±0.21c
C22:5(n-3)	1.80±0.17a	4.36±0.17c	2.81±0.11b	1.88±0.15a
C22:6(n-3)	5.85±0.69a	12.76±0.60c	10.18±0.57b	6.65±0.64a
total n-3	11.66±1.25a	25.74±0.83c	18.23±0.93b	11.82±0.50a
total n-6	5.26±0.20b	3.86±0.11a	6.25±0.24c	3.25±0.13a
n-3/n-6	2.19±0.18a	6.69±0.24c	2.95±0.18ab	3.68±0.23b
total SFA	25.40±0.69	25.29±0.49	24.57±0.74	28.36±3.06
total MUFA	41.50±0.89c	31.54±0.78a	38.24±0.67bc	34.96±3.10ab
total PUFA	16.92±1.42a	29.60±0.88c	24.48±1.01b	15.07±0.50a

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721 Table 5. Effect of diets on fatty acid composition (%) of liver phospholipids in male
722 European eel, *Anguilla anguilla,* after 38 weeks of feeding. Small letters show
723 significant differences in each fatty acid over the dietary regimes. Results represent
724 means ± SEM ($P < 0.05$).

Fatty acids	**Dietary treatments**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	1.34±0.08b	1.43±0.04b	1.05±0.05a	1.51±0.03b
C16:00	20.54±0.38ab	21.81±0.45b	19.66±0.63a	20.90±0.47ab
C16:1(n-7)	1.53±0.11ab	1.67±0.07bc	1.25±0.03a	1.91±0.08c
C18:00	7.64±0.31	7.97±0.42	6.93±0.99	6.23±0.27
C18:1(n-9)	7.47±0.27b	5.25±0.12a	7.38±0.16b	6.75±0.17b
C18:1(n-7)	1.90±0.04	1.80±0.04	2.40±0.73	1.88±0.05
C18:2(n-6)	1.07±0.06b	0.48±0.02a	1.28±0.08c	0.95±0.03b
C18:3(n-3)	0.16±0.02a	0.08±0.00a	0.27±0.05b	0.13±0.01a
C20:1(n-7)	0.12±0.01b	0.07±0.01a	0.07±0.01a	0.11±0.01b
C20:4(n-6)	6.56±0.54c	4.65±0.19b	3.68±0.18ab	2.89 ±0.12a
C20:5(n-3)	6.97±0.26a	10.27±0.50b	9.44±0.23b	12.40±0.53c
C22:1(n-11)	0.49±0.30	0.06±0.02	0.05±0.01	0.31±0.02
C22:5(n-3)	2.48±0.47	2.23±0.15	2.14±0.11	2.55±0.11
C22:6(n-3)	31.05±1.04a	34.32±0.49b	36.96±0.80c	31.91±0.64ab
total n-3	40.65±0.63a	46.90±0.58b	48.81±0.90b	46.99±0.64b
total n-6	7.63±0.58c	5.13±0.17b	4.96±0.24ab	3.84±0.14a
n-3/n-6	5.62±0.55a	9.22±0.36b	10.04±0.59b	12.34±0.45c
total SFA	29.51±0.56ab	31.20±0.45b	27.64±1.46a	28.64±0.62ab
total MUFA	11.51±0.26b	8.84±0.16a	11.14±0.81b	10.97±0.23b
total PUFA	48.28±0.83a	52.02±0.59bc	53.76±0.89c	50.83±0.67ab

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726 Table 6. Effect of diet on fatty acid composition (% of total fatty acids) in milt of
727 male European eel, *Anguilla anguilla*. Small letters show significant differences in
728 each fatty acid over the dietary regimes. Results represent means ± SEM ($P < 0.05$).

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Fatty acid	**Dietary treatment**			
	PRO-EEL1	**PRO-EEL2**	**PRO-EEL3**	**DAN-EX**
C14:0	0.83±0.21 b	0.73±0.12 ab	0.63±0.12 a	0.64±0.07 ab
C16:00	18.89±1.23	19.39±0.85	18.69±1.09	18.38±0.72
C16:1(n-7)	1.55±0.15	1.71±0.21	1.53±0.16	1.64±0.12
C18:00	5.65±0.30 a	6.35±0.27 b	6.08±0.34 ab	5.79±0.43 a
C18:1(n-9)	12.89±2.12 ab	10.92±0.86 ab	12.90±2.26 b	10.65±0.84 a
C18:1(n-7)	3.25±0.08	3.52±0.22	3.33±0.19	3.28±0.13
C18:2(n-6)	2.70±0.60 bc	1.58±0.32 a	2.73±0.44 bc	2.05±0.27 ab
C18:3(n-3)	0.12±0.04	0.10±0.03	0.08±0.02	0.08±0.02
C20:1(n-7)	5.94±1.11 b	3.98±0.61 a	4.26±0.65 a	5.74±0.43 b
C20:4(n-6)	7.01±0.55 c	7.13±0.72 c	5.29±0.53 b	4.03±0.43 a
C20:5(n-3)	13.18±1.50 a	18.21±1.79 b	16.66±2.02 b	18.25±1.43 b
C22:1(n-11)	0.35±0.19 b	0.21±0.03 ab	0.18±0.04 a	0.24±0.03 ab
C22:5(n-3)	1.60±0.23 a	2.02±0.28 b	1.73±0.17 ab	1.64±0.21 a
C22:6(n-3)	18.92±2.62 ab	17.04±2.67 a	19.01±0.91 ab	20.59±1.55 b
total n-3	34.64±1.76 a	38.00±1.76 b	38.13±2.23 b	41.21±0.75 c
total n-6	10.52±0.47 c	9.32±0.94 bc	8.90±0.65 b	6.92±0.73 a
n-3/n-6	3.30±0.21	4.12±0.49	4.3±0.40	6.01±0.61
total SFA	25.56±1.20 ab	26.71±0.97 b	25.61±1.09 ab	24.99±0.88 a
total MUFA	24.79±1.64 b	20.75±1.68 a	22.49±2.26 ab	22.04±0.73 ab
total PUFA	45.78±1.80	47.70±1.63	47.54±2.29	48.57±0.95

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732 Fig. 1
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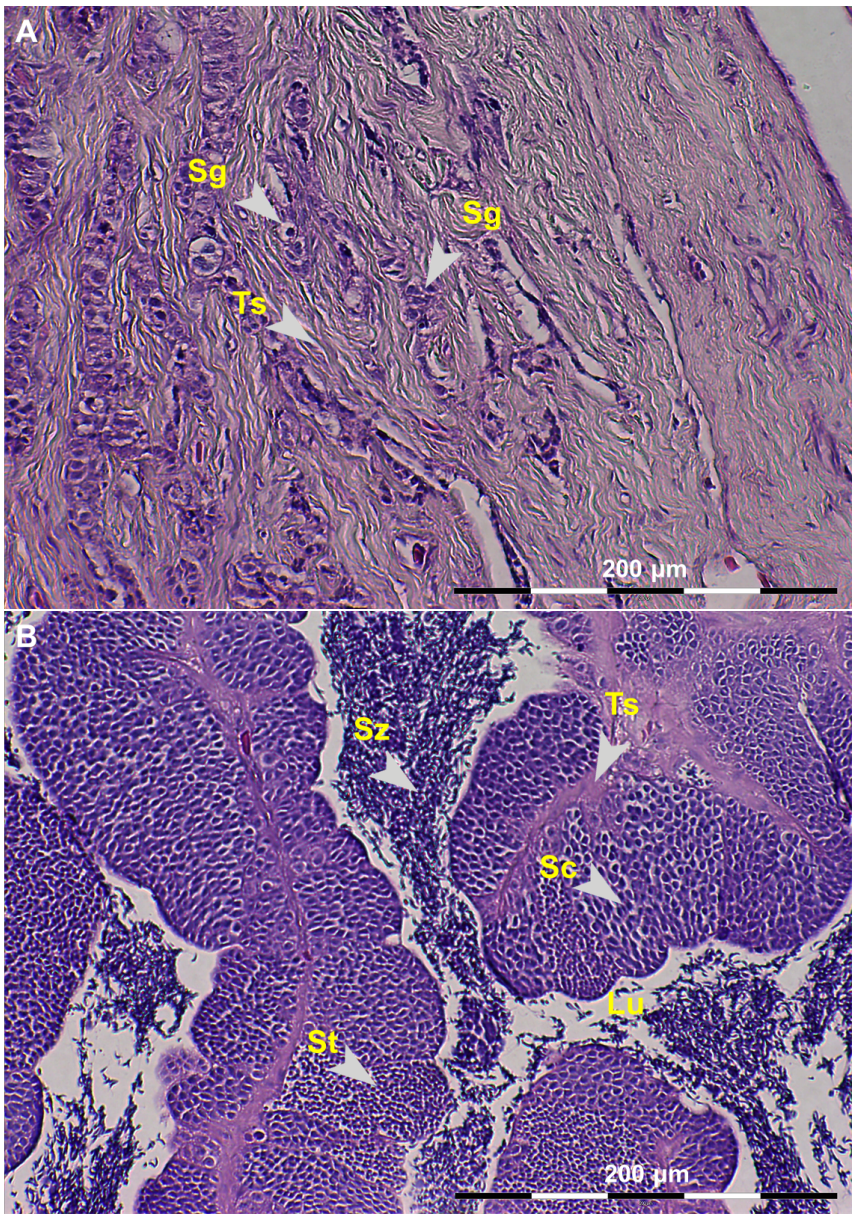

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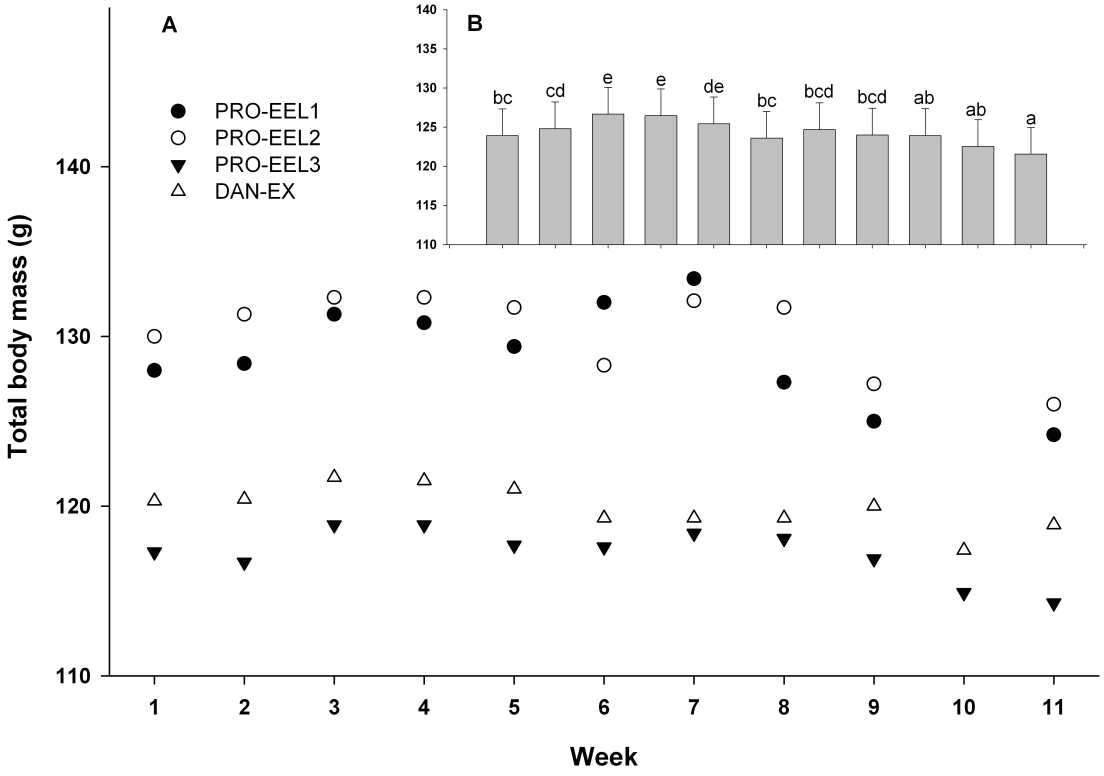
737 Fig. 2



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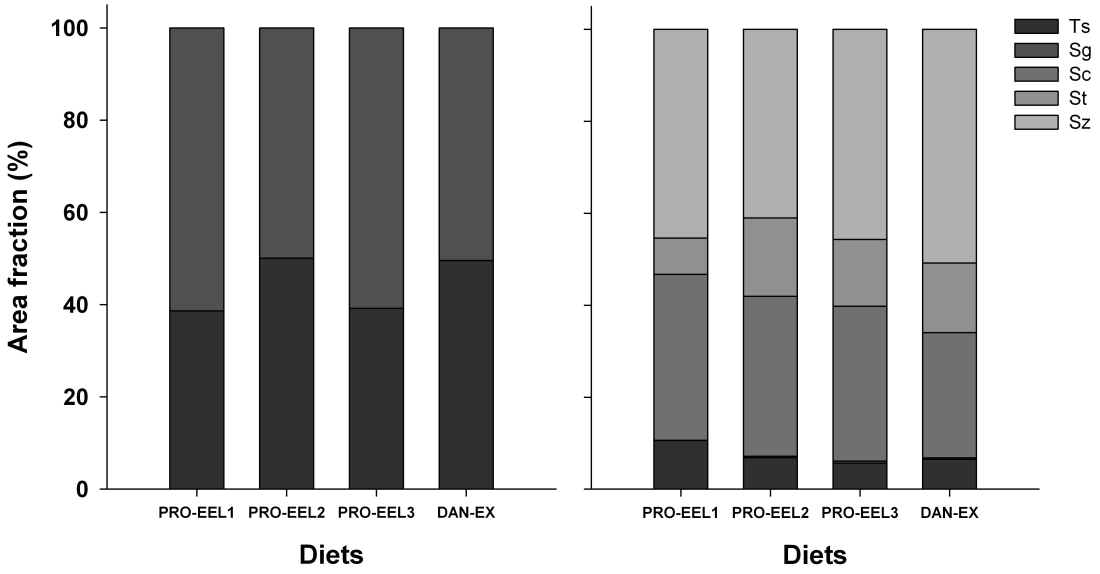
753 Fig. 3



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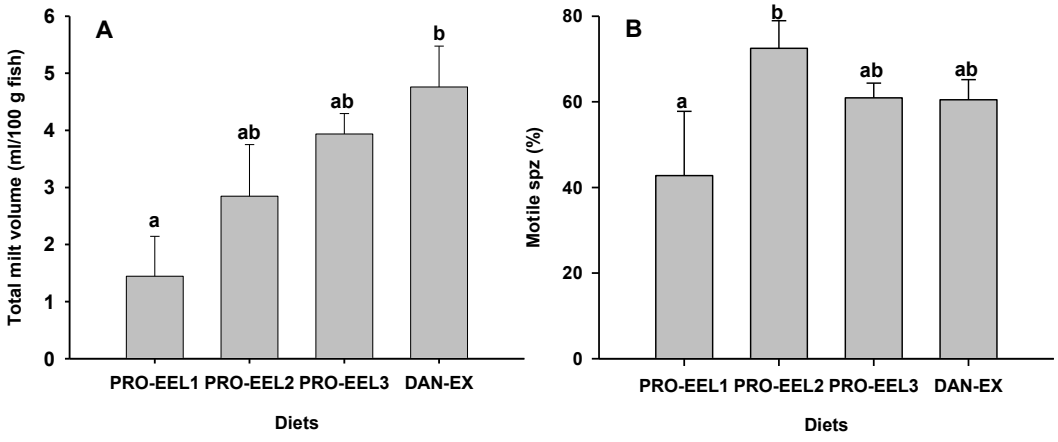
778 Fig. 4



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