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Effect of thermal regime on fatty acid mobilization in male European eels (*Anguilla anguilla*) during hormonally-induced spermatogenesis

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Abstract

Little is known about the role of fat and fatty acids in European eel spermatogenesis. The aim of this work was to study the changes in fat content and to carry out a quantitative analysis of the fatty acid composition of the muscle, liver and gonad of European male eels through hormonally induced sexual maturation under three different thermal regimes (two of them variables: T10 and T15; and one constant: T20) considering the changes of temperature suffered by these fish during their transoceanic reproductive migration. The European eel reached spermatogenesis earlier in treatment T20, suggesting that spermatogenesis in this species is closely regulated by water temperature. Although eels lose body mass due to the fasting period that accompanies the gonadal growth, no significant changes were found in the fat and fatty acid content in the muscle during the experimental period.

With regards to the liver, the levels of palmitic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids increased significantly with the start of the spermiation process in all the thermal treatments.

In the testis, EPA, araquidonic acid (ARA) and DHA remained constant during the maturation process while the levels of the rest of the fatty acids decreased significantly. The stability of ARA and EPA levels in the testis may have a physiological significance, whereas the stability of DHA levels may have a structural one. The results suggest that the progress of spermiation is influenced by water temperature and demonstrate the importance of the roles of EPA, ARA and DHA in European eel reproduction. This study makes it clear that complementary studies focused on lipid composition of commercial diets could improve sperm quality in this species.

Keywords

Muscle; Liver; Gonad; Spermatogenesis; Diets; PUFA

1. Introduction

European eel (*Anguilla anguilla* L.) populations have declined steadily. After high levels in the late 1950s, there was a rapid decrease that still continues to the present day (ICES 2011). The causes for the decline of the eel population are habitat reduction, overfishing, pollution and infections, among others (Feunteun, 2002).

To date, eels do not mature spontaneously in captivity, so the sexual maturation of males must be induced using long-term hormonal treatments (Asturiano et al., 2005; Gallego et al., 2012; Huang et al., 2009; Ohta et al., 1997; Pérez et al., 2000).

The European eel is a catadromous species which moves from freshwater to the sea when sexual maturation starts, and then begins a transoceanic migration of 4-6000 km from Europe to their spawning grounds in the Sargasso Sea. Although many details about their migration still remain unknown, several environmental factors (including light, pressure, temperature) may be important to their sexual maturation, as, during their trip, they can swim in depths of between 200-1000m (Aarestrup et al., 2009, Tesch, 2003), meaning a high variation in environmental conditions. Moreover, the temperature range in which the migration occurs is extremely variable (Bruijs and Durif, 2009). Furthermore, in the case of European eel, it is known that the temperature of the probable spawning area in the Sargasso Sea is about 20 ºC but, due to the fact their migration takes several months, it seems probable that gonadal development happens during the journey, at low temperatures, whereas the spawning takes place at high temperatures. In one intend to imitate approximately what happens in the nature we decided to test three different thermal regimes, two with low and variable temperatures (one from 10 to 20 ºC and another from 15 to 20 ºC) versus a constant temperature (20 ºC), which is the usual method used with eel males. In many teleost fish, including the European eel, gonadal maturation and reproduction is accompanied by a starvation period in which they carry out long and exhausting migrations. For their reproductive migration to the Sargasso Sea, the eel fat stores, which can be as much as 30% of their body weight at the silver stage, can be very useful to complete their long journey. Laboratory studies have shown that 40% of the fat accumulated by silver eels will be used for the reproductive migration, while the remaining 60% will be used for gonadal growth (Van Ginneken and Van den Thillart, 2000).

Essential fatty acids act in metabolism as energy sources for growth and movement, including migration, and have also been shown to play an important role in the regulation of reproduction in many aquatic animals (Bell and Sargent 2003; Tocher,

2003). Fatty acids are important for the reproduction of male and female fish. In females, the lipids are stored mainly in the muscle and liver and are transported as lipoproteins to the ovary during gonadal maturation (Almansa et al., 2001; Cejas et al., 2004; Mourente et al., 2002; Ozaki et al., 2008). In males, fatty acid composition provides the sperm cell membrane with the necessary fluidity, conditioning the functionality of the membrane which is associated with the fertilization process (Wathes et al., 2007). Polyunsaturated fatty acids are precursors of eicosanoids, whose metabolites (e.g., prostaglandins) have an important role in fish reproduction. ARA has been shown to be a key substrate for the production of the 2-series prostanoid, whereas EPA plays a modulatory role in their synthesis (Asturiano et al., 2000; Sargent et al., 2002).

Spermatozoa and egg lipids can come from dietary lipids, body reserves or *de novo* synthesized lipids. Many studies have proven the importance of broodstock nutrition in the reproductive performance of fish (Asturiano, 1999; Bobe and Labeé, 2010; Glencross, 2009; Izquierdo et al., 2001). The development of optimum diets for European eel broodstocks seems to be a key challenge in order to improve the chances of its reproduction under captivity, by ensuring a high gamete quality. The importance of dietary lipid in reproduction has been extensively reported (Bruce et al., 1999; Fernandez-Palacios et al., 1995; Navas et al., 1997; Norambuena et al., 2013; Rodriguez et al., 1998; Zhou et al., 2011). However, nutritional experiments often focus on female broodstock and egg quality, forgetting the effects of diet on sperm and male reproductive performance. Studies with goldfish (*Carassius auratus*; Wade et al., 1994), rainbow trout (*Oncorhynchus mykiss*; Labbé et al., 1993, 1995; Pustowka et al., 2000; Vassallo-Agius et al., 2001) and European sea bass (*Dicentrarchus labrax*; Asturiano et al., 1999; 2001; Bell et al., 1996) have demonstrated the relationship between the fatty acid content of the broodstock diet and the sperm fatty acid composition and suggested that consequently, fertilization could be affected. Asturiano et al. (2001) showed that the diet of the European sea bass male affected the survival of embryos and larvae, thus indicating a long-term effect of diet-mediated sperm quality.

Little is known about the role of fatty acids in the reproductive performance of European eel and, from the different environmental factors which we could mimic in order to know more things about the eel reproduction, we decided to focus this study on temperature, as it plays an important role in gonad development of many fish species (Garcia-Lopez et al., 2006; Lim et al., 2003; Pankhurst and Munday, 2011; Van Der

Kraak and Pankhurst, 1997). So, the aim of this work was to quantify the fatty acid levels in male muscle, liver and testes during induced sexual maturation under different thermal regimes, to determine the dynamics of fat and fatty acid mobilization under different temperatures and which fatty acids might be the most important in testis development and spermatogenesis. All this information could be useful for developing suitable diets to improve sperm quality and subsequently, larval development in this species.

2. Material and methods

2.1 Animal origin and acclimatization

The study was carried out during the months of September to December after moving 317 European eel males (mean body weight 100±2 g) from the fish farm Valenciana de Acuicultura S.A. (Puzol, Valencia, Spain) to our facilities at the Universitat Politècnica de València (Spain).

The animals were distributed in six 200 L aquaria (approximately 50 animals per aquaria and two aquaria per treatment), covered to maintain constant shade. The aquaria were equipped with separate recirculation systems, coolers and thermostats to control the temperature. The fish were not fed throughout the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

The transition from fresh water to sea water (37 ± 0.3 g L^{-1}) was conducted during the first two weeks. It was done adding 3 g $L^{-1}$of commercial aquarium salt per day during the first week and, 4 g L^{-1} per day during the second acclimatization week.

2.2 Thermal regimes and hormonal treatment

Each treatment consisted of a different thermal regime: T10, (10 ºC first 6 weeks, 15 ºC next 3 weeks and 20 ºC last 6 weeks); T15, (15 ºC first 6 weeks and 20 ºC last 9 weeks); and T20, (20 ºC throughout the whole experimental period; Fig. 1).

For 13 weeks, males were injected weekly with human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Argent Chemical Laboratories. USA) as it was previously described by Pérez et al. (2000).

2.3 Measurements and sampling

When the animals arrived to our facilities, before starting any treatment, eight specimens were sacrificed as freshwater controls. Eight fish per treatment were sacrificed weekly during the first eight weeks of the experiment, and later five animals per treatment were sacrificed weekly during the last five weeks of the experiment.

Fish sacrifice was done by decapitation, after having previously been anesthetized with benzocaine (60 ppm). During dissection, the total body, liver and gonad were weighed to calculate the hepatosomatic index (HSI = (Liver mass ⁄ Total body mass)*100) and gonadosomatic index (GSI = (Gonad mass ⁄ Total body mass)*100).

From the first week of the experiment (0), muscle and liver samples were collected. Because of their small size, testis samples were not collected for further analysis until: T10 (the 7^{th} week, GSI=0.28); T15 (the 4^{th} week, GSI=0.34); and T20 (the 3^{rd} week, GSI=0.92).

The muscle was crushed in a meat grinder and homogenized before storage. All the testis, liver and muscle samples were stored at -80 ºC until lipid extraction and fatty acid quantification.

2.4 Gonad histology

A small sample of testis from each male was preserved in 10% buffered formalin for histology processing, while the rest of the tissue was used for the subsequent analysis of fat and fatty acids. All formalin fixed tissues were routinely dehydrated in ethanol and embedded in paraffin as per standard histological techniques. Transverse sections 5-10 µm thick were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin for examination. The slides were observed using a Nikon Eclipse E-400 microscope and the images were taken with a Nikon DS-5M camera.

The stages of spermatogenesis were determined following the description made by Peñaranda et al. (2010): Stage 1 (S1) was characterized by the presence of spermatogonia; stage 2 (S2), by the presence of spermatogonia and spermatocytes; stage 3 (S3), by the appearance of spermatids in the testis: stage 4 (S4), by the appearance of spermatozoa in small lumen; stage 5 (S5), by the increase in the number of spermatozoa, as well as lumen size; and stage 6 (S6), by a dominance of spermatozoa, a low proportion of other germ cells, and luminal fusion.

Once the fatty acid analyses were done, the results were classified considering the different development stages of the testis previously determined by histology.

2.5 Lipid extraction

In order to get the best homogenization, the muscle samples were first lyophilized. Crude fat was extracted using 0.3 g of lyophilized muscle. Fat extraction was done with an organic solvent (diethyl ether). Muscle tissue was placed in a cellulose cartridge that was slowly filled with warm solvent and fat was dissolved and extracted from the sample in a metal glass. When the extraction finished the solvent was condensed in a Soxtec extraction unit (1043, Tecator). The remaining solvent was then evaporated and the recovered fat was drying for 2 h at 110 ºC. The water content of the muscle samples was determined in triplicate. To determine it, 0.5 g (w/w) of the sample were weighed and maintained at 110 °C for 24 h. After this period, samples were weighed again calculating the moisture by the weight difference.

Because of the small size of the testis and liver during the test, a different method for lipid extraction was adapted. The total lipids were extracted from the testis and the liver using a modified Folch method (Folch et al., 1956). The total pure lipids were extracted with dichloromethane/methanol (2:1, v/v) containing 0.05% butylated hydroxitoluene (BHT) as an antioxidant. The fresh testis samples were weighed and added 2:1 (v/v) to the dichloromethane/methanol mixture. Homogenization of the different tissues was carried out in a glass tube with an Ultra-turrax type of homogenizer. The homogenate was filtered through fat-free paper into another glass tube. 3 ml of saline solution was added (7.45 g KCl/ L ultrapure water) to separate it into two parts: one with lipid and another with the non-lipid substances, and the glass tube was preserved in the refrigerator. Between 8 to 48 h later, the two parts were formed and the upper part/half with non-lipid substances was removed with a vacuum pump. Finally with the help of a centrifuge vacuum concentrator (Scan Speed MaxiVac Alpha), the oil was transferred into Pyrex tubes which were kept at -80 ºC until synthesis of the fatty acids methyl esters (FAME).

2.6 Fatty acid quantification

From the 27 fatty acids detected, 20 were used for quantification (Table 1). The fatty acids considered were divided into three classes: SFA (Saturated Fatty Acids), MUFA (Monounsaturated Fatty Acids) and PUFA (Polyunsaturated Fatty Acids).

A direct method of FAME synthesis was performed as per O´Fallon et al. (2007). The analysis of the muscle was carried out with 20-30 mg of freeze-dried sample and for the testis and liver weighing 10-30 mg of extracted oil. First, 1 ml of tridecanoic acid

(C13:0) was used as internal standard (0.5 mg of C13:0 / mL of methanol). We added also, 0.7 ml of KOH 10 N and 5.3 ml of HPLC quality methanol (High Performance Liquid Chromatography). Tubes were incubated at 55 ºC in a thermoblock for 1.5 h and underwent vigorous shaking for 5 s every 20 min. After cooling in a room temperature water bath, 0.58 ml of H_2SO_4 24 N was added. The tubes were mixed by inversion and were incubated again at 55 ºC in a thermoblock for 1.5 h and shaken for 5 s every 20 min. After cooling in a room temperature water bath, 1.5 ml of HPLC quality hexane was added to the reaction tubes, which were vortex-mixed and centrifuged at 1006 g for 5 min and the hexane layer, containing the FAME, was placed into vials for gas chromatography. The vials were kept at -80 ºC until gas chromatography was performed.

The FAME were analyzed in a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. Separation of the methyl esters was performed in a fused silica capillary column SPTM 2560 (Supelco, PA, USA) (100 m x 0.25 mm x 0.2 μm film thickness). Helium was used as the carrier gas at flow rate of 20 cm s^{-1}. Samples were injected with a split ratio of 1/100.

The initial oven temperature was set at 140 ºC held for 5 min and increased up to 240 by 4 ºC min^{-1} and finally maintained at that temperature for 30 min. Fatty acids were identified by comparing their retention times with standards supplied by Supelco. Fatty acid amounts are reported as percentages in 100 g of fat and in each tissue only those fatty acids present at minimum levels of 0.1% were considered. To quantify them, we used data from the sample weight used in the analysis to calculate g of fatty acids per 100 g of sample and with the fat content of the sample we transformed to g of fatty acids per 100 g of fat.

2.7 Statistical analysis

After establishing data normality using the asymmetry standard coefficient and Curtosis coefficient, analysis of variance (General Linear Model, GLM) was carried out to compare the results of body mass, GSI, HSI and fatty acid percentages. Comparison of means was done using a Newman –Kewls multiple comparison tests. Differences were considered significant when p values <0.05. Two statistical analyses were carried out with the results: the first to evaluate the differences between each thermic treatment over the development stages, and the second to evaluate the differences between each treatment over time. In addition we also analyzed the differences between each

265 treatment over the stages.. These statistical analyses were carried out using Statgraphics
266 Plus® 5.1.
267 A principal component analysis (PCA) was carried out. First, to estimate the fatty acid
268 distribution in the three tissues and later, to determine the contribution of the individual
269 fatty acids to the different development stages in each tissue. Score plots illustrate the
270 relationship between individual cases (stage groups) and the variables (fatty acids), and
271 help in the analysis of data by showing graphical associations. In the present study,
272 factor scores were analyzed by one-way ANOVA. PCA was performed using the
273 statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL,
274 USA).
275
276 **3. Results**
277 **3.1. Analysis of morphometric parameters and testis development stages**
278 With regard to the morphometric parameters, only under the highest temperature
279 regime, T20 did the body weight of the males decrease significantly during the
280 treatment period, from 108.9±5.6 to 77.1±7.1 g (data not shown). In parallel, the weight
281 of the testis increased (Fig. 2) in all the treatments. The mean GSI of fish from
282 treatment T20 increased gradually throughout the weeks, reaching the highest value
283 (11.3±0.9%) in the 11th week. In treatment T15, the maximum GSI (8.1±0.7%)
284 appeared in the 10th week, while T10 males showed the slowest increase, without
285 reaching a significantly higher GSI compared to basal levels until the 8th week.
286 There were no significant differences in HSI over the course of the weeks in treatments
287 T10 and T15, and only under the highest temperature did HSI increased.
288 Regarding the evolution of the testis maturation stages (Fig. 3), T10 males reached stage
289 S1 during the first three weeks, with this treatment being the slowest in terms of gonad
290 development. Treatment T15 eels reached S2 from the third week, and spermatozoa
291 (S4) were not found until the 6th week. The treatment with the highest temperature
292 (T20) resulted in the fastest gonad development. In the last week of the test (13^{th} week),
293 all T20 males in the testis were at stage S6.
294
295 **3.2. Changes in total fat content of tissues**
296 Treatment T20 induced a sudden and significant (from S2) increase in fat content in the
297 muscle (Fig. 4A1), whereas the differences were not significant in the other treatments.
298 The evolution of the fat content of the liver throughout all the testis development stages

was very different depending on the thermic treatment. In treatments T15 and T20 the fat content increased significantly from S1 to stages S3 and S4, respectively (Fig. 4B1). The fat content in T10-treated fish did not change through the testis development stages (Fig. 4B2), but when the fat content results were analyzed in relation to the week of treatment (data not shown), a significant decrease of fat content (from 14.0±1.4 to 5.0±1.8%) was found from the 7^{th} to 8^{th} week. Between these weeks (7-8^{th}) a second water temperature change was applied as part of treatment T10 and this is probably closely related to this decrease in fat.

The fat content in the testis decreased as development progressed in treatments T10 and T15, with a sharp decrease from S2 to S3 (Fig. 4C1 and 4C2). The fat content in the testis at stage 2 was higher in T15-treated fish than in treatments T10 and T20, while, at S5, T20-treated fish showed higher fat values than T15 treated fish. When we analyzed the differences between the fat content in testis in the same stage, Figure 1C shows a significant higher fat content of T15 respect to T20 in S2.

3.3 Muscle

Significant increases or decreases in the total amounts of SFA, MUFA and PUFA between the different testis development stages were not noticed under the different treatments. However, the proportions of these three fatty acids groups were different, with MUFA (at 36%) being the most abundant, followed by SFA (23%) and PUFA (16%). In particular, oleic (18:1n-9) and palmitoleic (16:1) acids were the most abundant type of MUFA with concentrations of around 20 and 7%, respectively.

3.4. Liver

MUFA represented close to 30% of 100 g of fat in the liver, whereas SFA and PUFA were found in quantities of around 20-25% each. The most abundant fatty acids in the liver were palmitic (16:0), oleic (18:1n-9) and docosahexaenoic acids (22:6n-3, DHA) with approximately 16, 15 and 12%, respectively.

There was a clear increase in liver SFA with the progression of the testis development stages (Fig. 5A1 and 5A2). In both T15 and T20 the highest values were observed from S4 onwards, while in T10 they increased later, in S6.

Figure 5B1 and 5B2 shows the levels of MUFA in the liver. No significant differences in MUFA percentages between the different developmental stages were found in T10 treated fish. MUFA levels varied in treatment T20, with significant differences from S1

to S2 (a reduction from 25.8±1.5 to 21.8±0.9%) and increases from S4. MUFA levels in T15-treated males increased significantly in S3 when compared to S1.

Regarding PUFA levels, significant differences in the development stages were only found in T20-treated fish with an increase from 21.4±1.4% in S1 to 26.8±1.1% in S4 (Fig. 5C1). In T10 treatment higher values of PUFA were found in S2 (Fig 5C2).

3.5. Testis

Figure 6 shows the evolution of the different fatty acid groups through the testis development. The dominant classes of fatty acids in the testis were: MUFA (25%) followed by SFA (20%) and PUFA (15%). The most abundant fatty acids were palmitic (16:0) and oleic (18:1n-9) acids representing, jointly, 25% of the total fatty acids detected. DHA was the PUFA with the highest percentage detected in the gonad (5%; data not shown). Figure 6 shows that the percentages of quantified fatty acids changed depending on the testis development stages. In the final development stages, the sum of SFA MUFA and PUFA in testis represents less than 30% of total fat.

Regarding the fatty acid classes and their relationship with the development stages, no differences in PUFA content were found in T15 treated fish (Fig. 6C1) but this treatment did result in a significant decrease in MUFA (Fig. 6B1) when the males reached S5 and S6. In contrast, both PUFA and MUFA content decreased significantly in treatment T20 (although without significant differences in the last case). T10 treatments showed a significant decrease in all fatty acid classes in testis during development stages (Fig 6A2, 6B2 and 6C2). With regards to SFA (Fig 6A1), T20 treatment resulted in a significant decrease coinciding with stages S5 and S6. When analyzing the differences between treatments in the same stage, there were significant differences in PUFA in S3, where treatment T15 levels were higher than those of T20 ($p<0.001$).

3.6. Principal component analyses

The PCA demonstrated different patterns in fatty acid distribution in the testis, liver and muscle. In the first component from the score plot (Fig. 7A), all the muscle samples are located to the right in the diagram, while the gonad samples are located to the left. The corresponding component plot (Fig. 7B) suggests that SFA and MUFA (on the right of the diagram) in particular, could be related with muscle samples. Furthermore, in score plot (Fig. 7A) all the liver samples are located on the positive axis of the second

component explaining a different pattern. The second component of the corresponding component plot (Fig. 7B) shows DHA located on the positive axis of the second component and could be related with liver samples. The variables found between the positive axis of the first and the second component keeps a similar pattern in liver and muscle. The values corresponding to stearic acid (18:0) are near the origin in component plot graph, indicating that it follow a similar pattern in the three tissues.

The PCA of the liver fatty acids in the three treatments resulted in similar plots, and only results from treatment T20 are shown here (Fig. 8). A substantial change in the fatty acid composition of the liver during the maturation stages was found in this study. In the scores plot from the PCA of the liver fatty acids (Fig. 8A), the samples from S4-S6 stages, located to the right in the diagram, were significantly separated from the S1-S2 samples, located to the left in the diagram. The corresponding component plot (Fig. 8B) suggests that the fatty acids located on the right of the diagram were related with more advanced development stages (S4-S6) in the liver. In accordance with the results obtained from the PCA of the liver, Table 3 shows fatty acids in the liver over the development stages. Table 3 shows that, especially palmitic acid (16:0) and EPA (which fall on the right of the first component, Fig. 9B) increased significantly in the liver when the males began to produce spermatozoa (S4-S6).

We carried out a PCA and variance analysis of the testis fatty acids, and considered all the collected samples, independently of the thermal regime (Figure 9 and Table 3). This was due to the lower amount of analyzed testis samples (T10, n=27; T15, n=48, T20, n=63) in comparison with the rest of tissues considered in the experiment. Evaluated separately (one analysis for each thermal treatment), the number of animals that reached the different developmental stages was very low, making it difficult to find clear tendencies. However, when considering all the collated data together, the first two axes on the analysis efficiently summarize the variation in the data set accounting for 63.9 and 14% of the total variation respectively (Fig. 9). In the scores plot of the testis (Fig.9A), the first component from the PCA of the testis fatty acids shows that the samples from S5-S6 stages were located to the left in the diagram, while the rest of the samples (S2-S3) were located to the right in the diagram. The corresponding component plot on the testis fatty acids (Fig. 9B) suggests that all the fatty acids located to the right of the diagram are related with the stages S2-S3. Furthermore, EPA, ARA and DHA, located on the positive axis of the second component from the component plot suggest that these fatty acids explain the different causes of the variation in the analysis.

Regarding these results, Table 4 shows the fatty acids in the testis over the development stages, suggesting they are used in differing amounts through the spermatogenesis process. Table 4 shows a general decrease in the fatty acids, represented in the first component of figure 9B, when the animals produce more sperm (S5-S6). EPA, ARA and DHA, seen on the second component, remained constant throughout the testis development stages.

4. Discussion

Temperature is one of the main environmental factors affecting the reproductive performance of fish (Pankhurst and Munday, 2011). Gallego et al. (2012) conducted a parallel study with these same animals, focusing on the sperm quality obtained under the three thermal regimes. It was observed that T20 males began spermiating earlier and showed higher percentages of spermiating males in all the weeks, compared to the alternative thermal treatments (T10 and T15). In the present study, the male eels which were maintained at the lower temperatures (T10, T15) did not begin to produce sperm until they had spent 1-2 weeks at 20 ºC, proving the importance of temperature in the final stages of the eel male maturation process.

Moreover, about the use of lipid reserves, its known that in European eels the fat in muscle increase from 8 to 28% between the yellow and silver stages (Larsson et al., 1990). Our experimental males were fed on a fish farm and this is probably the reason why their total fat percentage is higher (approximately 35%). Muscle is the main storage organ in fatty fish (Sheridan, 1988; Shulman, 1974) and during the fasting of fatty fish the muscle reserves are the first to mobilize but, Larsson and Lewander (1973) demonstrated that in eels an initial reduction of hepatic lipids occurs and only subsequently, are the muscle lipids mobilized.

Besides, under fasting conditions eel usually show a body (muscle) mass decrease but although this was also observed in this study, the fat content in the muscle increased through maturation. There are two possible explanations for this: Firstly, as Lovern (1940) demonstrated, eels lose weight mainly due to use of protein for energy during starvation, while the fat content is not exhausted. In our case, a decrease in the muscle protein of the eels cannot be confirmed because protein analyses were not carried out. However, the dry matter analysis carried out on the muscle samples showed that the water content did not change significantly during the treatment. This last fact suggests

that the observed increase in fat content in the muscle might more likely be due to a decrease in the proportion of another component, probably protein. Also, it is known that protein plays an important role in satisfying the energy demands of starving fish (Godavarthy and Kumari, 2012). The second reason for the increase in muscle fat could be the increase in the proportion of red muscle to improve the aerobic capacity during eel silvering and migration. Pankhurst (1982) showed that this increase in red muscle volume is mainly due to an increase in fat and mitochondria.

Given the fat percentages in liver and HSI, our results were similar to those found by Mazzeo et al. (2010) for European eel males. In terms of HSI, this increased in the highest thermal treatment (T20) and was apparently due more to a relative body mass loss, than to a real liver mass increase. In terms of the testis, the results corroborated those of Mazzeo et al. (2010), with an increase in GSI due to an increase in testis mass through the course of the treatments.

Regarding the distribution of fatty acids in these three different tissues, throughout he PCA (Fig. 7) we could interpret that, samples of muscle (on the right of the first component, Fig. 7A) were characterized for higher quantities of SFA and MUFA (on the right of the first component, Fig. 7B) being oleic acid (18:1n-9) the fatty acid found in the highest percentage. In particular, the high quantities of MUFA in muscle, is linked to the composition of the diet provided in the fish farm. Long chain MUFA in particular, like 20:1n-9 and 22:1n-9, are abundant in the fish oils and fish meals found in the formulated diets for eels. In a comparative study of the composition of wild and farmed European eel females, Støttrup et al. (2012) also observed that MUFA were the most abundant fatty acids in cultured eel and suggested that it is due to the more abundant levels of n-9 monomers often used to produce the formulated diets. The percentage of fat in the testis was very low (1-6%) and the samples fall in left part of the first component (Fig. 7A) being possible to explain it because the testis have a similar profile of fatty acids as muscle but in lower quantities. About liver fatty acid composition, PCA (Fig. 7) shows that the liver samples fall on the positive axis of second component (Fig 7A) explain a different composition of liver fatty acids comparing with muscle and testis. These variations could be explain due to the liver have higher amounts of DHA (fall on the positive axis of the second component, Fig.7B), than muscle and testis.

Regarding the variations found in fatty acids of three different tissues during spermatogenesis in the present study, as Mazzeo et al. (2010) previously reported,

European eel males induced to maturation at 20 ºC did not show any fatty acid content variations in the muscle, and the proportions of fatty acids remained the same, therefore it was impossible to detect a preferential use of specific fatty acids. In the liver, our results differ partially from those reported by Mazzeo et al. (2010). In the latter study a decrease in liver MUFA during sexual maturation was observed, but the methodology used in both studies was different. Mazzeo et al. (2010) quantified MUFA per 100 g of fatty acids. In that study they presented the results as, if the summation of SFA, MUFA and PUFA was the 100% of detected fatty acids. In our study quantification is done per 100 g of fat. So, the apparent decrease in MUFA observed by Mazzeo et al. (2010) must be due to an increase in the proportion of other components such as SFA and PUFA, as is evidenced here. The results showed an increase in several fatty acids in the liver when the eels began to produce sperm in stages S4-S6 (Table 3). Palmitic acid (16:0) and EPA in particular, increased significantly. Our results did not reveal a mobilization of 16:0 and EPA from the muscle to the liver, so these most likely would have been synthesized *de novo* in the liver. There is scarce information available on the enzymatic control of fatty acid biosynthesis in the eel, but it is known that the European eel maintains its ability to synthesize lipids in the liver from endogenous sources, even during a prolonged period of fasting (Abraham et al., 1984; Giudetti et al., 2001; Gnoni and Muci, 1990). The increase in the amount of palmitic acid found in the liver when the eels produced sperm (S4-S6) can be explained by the fact that it is the main product of fatty acid biosynthesis *de novo* (Cook and Mc Master, 2002). An increase in EPA may be due to the fact that freshwater fish have the ability to produce PUFA from linoleic acid (18:3n-3) to satisfy EPA and DHA requirements (Bell and Tocher, 2009). Several studies have proven that EPA and DHA are produced from 18:3n-3 in hepatocytes of several other species of freshwater fish such as Atlantic salmon (*Salmo salar*; Tocher et al., 1997), Arctic charr (*Salvelinus alpinus*; Tocher et al., 2001a), brown trout (*Salmo trutta*; Tocher et al., 2001a), tilapia (*Oreochromis niloticus*; Tocher et al., 2001b) and zebrafish (*Danio rerio*; Tocher et al., 2001b). Vertebrates, including fish, lack the Δ12 and Δ15 desaturases and so cannot form 18:2n-6 and 18:3n-3, respectively, from 18:1n-9. However, 18:2n-6 and 18:3n-3 can, with varying efficiencies depending on the fish species, be further desaturated and elongated to form ARA, EPA and DHA. Seawater and freshwater fish species have different levels of efficiency when performing this conversion. The inability of seawater fish to produce long chain PUFA (LC-PUFA) such as ARA, EPA and DHA from 18:2n-6 and 18:3n-3

is thought to be related to an evolutionary adaptation to LC-PUFA rich marine ecosystems where such a conversion was less advantageous. However, for freshwater fish it has been necessary to maintain this ability of conversion (from 18:2n-6 and 18:3n-3 to LC-PUFA) to have a good ARA, EPA and DHA levels (Bell and Tocher, 2009), as the freshwater environment is not so rich in LC-PUFA. Recently, this widely accepted paradigm was revised after the discovery of another pathway of synthesis of LC-PUFA in two marine vertebrates, *Siganus canalicalatus* (Li et al., 2010) and *Solea senegalensis* (Morais et al., 2012) so, further investigations would be needed to find different biosynthesis pathways. After viewing the great ability of the eel to synthesize PUFA in the liver during spermiation, studies on the isolation, cloning, and characterization of European eel fatty desaturases and elongases could be an important goal of future research. Finally, eel testis showed a decrease of fatty acids coinciding with the most advanced developmental stages (Fig. 6). It could be explain because, with FAME method only saponificable lipids, which contain fatty acids in their molecular structure, can be extracted. But unsaponificable lipids are present as well in the testis samples. These lipids cannot be extracted with FAME transformation and do not have fatty acids in their molecular structure. So, unsaponificable lipids, as eicosanoids and steroids, could be part of the total fat not quantified as fatty acid, being in a higher proportion in the testis because of its importance during the final maturation.

Among quantified fatty acids, a decrease could be observed also during the most advanced development stages. The classes of fatty acids that especially decreased in all the thermal treatments were SFA and MUFA. Only EPA, DHA and ARA remained constant, while the rest of fatty acids detected in the gonad decreased when males began to produce sperm (Table 4). These differences can be explained by the selective use of fatty acids of the sperm; while some PUFA are used to maintain the basic structures of the cell, all the rest of the fatty acids are used to produce energy through oxidative processes. The maintenance of quantities of ARA, EPA and DHA can be explained by the fact that the membrane of the sperm contains a high concentration of PUFA and plays an important role in regulating the fluidity and permeability of the sperm membrane, as well as in maintaining their capacity for fertilization of the oocyte (Wathes et al., 2007). The conservation of ARA and EPA levels may have a physiological significance, whereas the constant levels of DHA may have a structural one. In fish, high proportions of DHA have been found in sperm **(**Bell et al., 1996; Labbé et al., 1993, 1995; Pérez et al., 2000, 2007; Pustowka et al., 2000) indicating it

536 has a large structural function. On the other hand, ARA and EPA are the major
537 eicosanoid precursors in fish cells, including prostaglandins, thromboxans and
538 leukotrienes. EPA is known to be precursor of 3-series prostaglandins (PGE_3) and ARA
539 forms 2-series prostaglandins (PGE_2) (Sargent et al., 2002; Tocher, 2003). Therefore,
540 the EPA/ARA ratio modulates steroidogenesis in the testis increasing testosterone
541 production and any change in the ratio or in the levels of ARA and EPA in the gonad
542 may influence the prostaglandins and steroid production. In vitro ARA stimulates the
543 production of testicular testosterone in goldfish through its conversion to PGE_2,
544 whereas EPA may function as an inhibitory regulator (Wade et al., 1994; Wade & Van
545 Der Kraak, 1993). Asturiano et al. (2000) found similar results in European sea bass
546 males, indicating that PUFA are capable of regulating prostaglandin and androgen
547 production. Mercure and Van Der Kraak (1995), in their studies on in vitro of ovarian
548 follicles in goldfish and rainbow trout showed that EPA inhibed gonadotrophin-
549 stimulated testosterone production, whereas ARA was only weakly inhibitory. Sorbera
550 et al. (2001) showed that ARA and its metabolites, PGE_2, stimulate European sea bass
551 oocyte maturation.
552 PUFA conservation in European eel testis during spermiation suggests that the
553 spermatozoa membrane contains a high concentration of PUFA. Our results are
554 corroborated by Mazzeo et al. (2010) who reported PUFA as the main components of
555 European eel milt and PUFA also found in other fish species such as rainbow trout
556 (Labbé et al., 1993), European sea bass (Asturiano et al., 2001; Bell et al., 1996) or
557 herring (Huynh et al., 2007).
558 The fatty acids in fish sperm is affected by diet. Studies of goldfish (Wade et al., 1994),
559 rainbow trout (Labbé et al., 1993, 1995; Pustowka et al., 2000; Vassallo-Agius et al.,
560 2001) and European sea bass (Asturiano et al., 1999; 2001; Bell et al., 1996) have
561 showed the relationship between the fatty acid content of the broodstock diet with the
562 fatty acid composition of sperm and suggest that consequently, fertilization could be
563 affected. The preliminary results of the fatty acid composition of European eel testis
564 during sexual maturation have shown the possibility of using diets with appropriate
565 fatty acid profiles to improve sperm quantity and quality (I.A.E. Butts, R. Baeza, L.
566 Pérez, J.G. Støttrup, M. Krüger-Johnsen, C. Jacobsen, J. Tomkiewicz, J.F. Asturiano;
567 unpublished results).
568 In summary, the results have shown that temperature affects the sexual maturation of
569 the male eel, with sperm production being reached earlier with the highest temperature

(20 ºC). The achieved results cohere with the importance of PUFA in teleost reproduction due to their role in male maturation and sperm composition. EPA and DHA biosynthesis have been observed in the liver, probably due to their subsequent mobilization to the testis because of their important function during steroidogenesis. In the testis, the maintenance of ARA and EPA levels may have a physiological significance, whereas the maintenance of DHA levels may have a structural one. Further research on the relationship of fatty acids and sperm quality should be considered. Additionally, because eel starve during sexual maturation, the state in which eels reach maturity is very important, thus the present results suggest that complementary studies focusing on the lipid composition of the commercial diets could well improve sperm quality.

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800

Table legend

Table 1. Identified fatty acids

Table 2 . Fatty acid composition in the liver of T20 treated fish by development stage. Small letters show significant differences in each fatty acid over the development stages. Results represent means ± SEM ($P < 0.05$). Results are shown as percentage of fatty acids in 100 g of fat.

Table 3. Fatty acid composition in the testis by development stage, independently of the thermal regime. Small letters show significant differences in each fatty acid over the development stages. Results represent means ± SEM ($P < 0.05$). Results are shown as percentage of fatty acids in 100 g of fat.

816 **Figure Legends**

817

818 **Figure 1.** Water temperature treatments (T10, T15 and T20) during induced sexual
819 maturation in male eels. Arrows indicate the first injection with human chorionic
820 gonadotropin (hCG).

821

822 **Figure 2.** Gonadosomatic index (GSI) throughout hormonal treatment under three
823 thermal regimes (T10, T15, and T20). Number of samples: T10, n_{0-7} = 8; n_{8-12} = 5; n_{13} =
824 4; T15, n_{0-7} = 8; n_{8-13} = 5; T20, n_{0-7} = 8; n_{8-13} = 5. Different letters mean significant
825 statistical differences between weeks in each treatment.

826

827 **Figure 3.** Percentage of fish reaching the different stages of testis development
828 throughout the hormonal treatment in each thermal regime: A) T10; B) T15; C) T20.
829 Number of samples in each stage under different thermal regimes: T10, n_{S1}= 28; n_{S2}=
830 29; n_{S3}= 22; n_{S4} = 6; n_{S5}= 3; n_{S6} = 1; T15, n_{S1}= 19; n_{S2}= 14; n_{S3}= 18; n_{S4} = 6; n_{S5}= 17;
831 n_{S6} = 9; T20, n_{S1}= 8; n_{S2}= 19; n_{S3}= 15; n_{S4} = 13; n_{S5}= 9; n_{S6} = 28. Stages S1-S6 were
832 described by Peñaranda et al. (2010). Temperature changes are described in Figure 1.

833

834 **Figure 4.** Fat content in percentage of wet weight (% w w) in A1) muscle, B1) liver and
835 C1) testis of T15 and T20 treatment (A2, B2 and C2 shows the same but in T10
836 treatment with S4-S6 stages grouped as S4-6) shown in relation to the different testis
837 development stages. Small letters show significant differences in the same treatment
838 between the different development stages; capital letters show significant differences in
839 the same stage between different thermal treatments. Results show as mean ± SEM ($P <$
840 0.05). Number of samples in each tissue under different thermal regimes: Muscle T10,
841 n_{S1}= 28; n_{S2}= 29; n_{S3}= 22; n_{S4} = 10; Muscle T15, n_{S1}= 19; n_{S2}= 14; n_{S3}= 18; n_{S4} = 6;
842 n_{S5}= 17; n_{S6} = 9; Muscle T20, n_{S1}= 8; n_{S2}= 19; n_{S3}= 15; n_{S4} = 13; n_{S5}= 9; n_{S6} = 28;
843 Liver T10, n_{S1}= 28; n_{S2}= 28; n_{S3}= 22; n_{S4} = 8; Liver T15, n_{S1}= 18; n_{S2}= 14; n_{S3}= 18;
844 n_{S4} = 6; n_{S5}= 17; n_{S6} = 8; Liver T20, n_{S1}= 8; n_{S2}= 19; n_{S3}= 15; n_{S4} = 13; n_{S5}= 9; n_{S6} =
845 28; Gonad T10, n_{S2}= 4; n_{S3}= 13; n_{S4} = 10; Gonad T15, n_{S2}= 3; n_{S3}= 14; n_{S4} = 6; n_{S5}=
846 17; n_{S6} = 9; Gonad T20, n_{S2}= 5; n_{S3}= 15; n_{S4} = 10; n_{S5}= 7; n_{S6} = 26.

847

848 **Figure 5.** Classes of fatty acids in the liver during the development stages: A1)
849 Saturated fatty acids (SFA); B1) Monounsaturated fatty acids (MUFA) and C1)

Polyunsaturated fatty acids (PUFA) in T15 and T20 treatment. A2, B2 and C2 Shows the same but in T10 treatment with S4-S6 grouped as S4-6. Small letters show significant differences in the same treatment between different development stages. Capital letters show significant differences in the same stage between different thermal treatments. Results are shown as mean ± SEM ($P < 0.05$). Number of samples: T10, $n_{S1}= 28$; $n_{S2}= 28$; $n_{S3}= 22$; $n_{S4} = 8$; T15, $n_{S1}= 18$; $n_{S2}= 14$; $n_{S3}= 18$; $n_{S4} = 6$; $n_{S5}= 17$; $n_{S6} = 8$; T20, $n_{S1}= 8$; $n_{S2}= 19$; $n_{S3}= 15$; $n_{S4} = 13$; $n_{S5}= 9$; $n_{S6} = 28$.

Figure 6. Classes of fatty acids in the gonad during the development stages: A1) Saturated fatty acids (SFA); B1) Monounsaturated fatty acids (MUFA) and C1) Polyunsaturated fatty acids (PUFA) in T15 and T20 treatment. A2, B2 and C2 Shows the same but in T10 treatment with S4-S6 grouped as S4-6. Small letters show significant differences in the same treatment between different development stages. Capital letters show significant differences in the same stage between different thermal treatments. Results are shown as mean ± SEM ($P < 0.05$). Number of samples: T10, $n_{S2}= 4$; $n_{S3}= 13$; $n_{S4} = 10$; T15, $n_{S2}= 3$; $n_{S3}= 14$; $n_{S4} = 6$; $n_{S5}= 17$; $n_{S6} = 9$; T20, $n_{S2}= 5$; $n_{S3}= 15$; $n_{S4} = 10$; $n_{S5}= 7$; $n_{S6} = 26$.

Figure 7. Component plot (A) and factor score plot (B) from principal component analysis on the most abundant fatty acid composition from the muscle, liver and gonad of European eel. n= 699.

Figure 8. Component plot (A) and score plot (B) from principal component analysis on fatty acid composition of the liver of European eel in T20 treatment, at each development stage. n= 93.

Figure 9. Component plot (a) and score plot (b) from principal component analysis on fatty acid composition of the gonad of European eel (independently of the thermal regime), at each development stage. n=139.

Table 1

SFA (saturated fatty acids)	**MUFA** (monounsaturated fatty acids)	**PUFA** (polyunsaturated fatty acids)
14:0 Myristic	16:1 Palmitoleic	18:2n-6 Linoleic
15:0 Pentadecanoic	18:1n-7 Cis-vaccenic	18:3n-6 γ -Linolenic
16:0 Palmitic	18:1n-9 Oleic	18:3n-3 Linolenic
17:0 Heptadecanoic	20:1 Eicosenoic	20:2 Eicosadienoic
18:0 Stearic	22:1n-9 Erucic	20:3n-3 Eicosatrienoic
		20:4n-6 Arachidonic (AA)
		20:5n-3 Eicosapentaenoic (EPA)
		22:4n-6 Docosateraenoic
		22:5n-3 Docosapentaenoic
		22:6n-3 Docosahexaenoic (DHA)

885 **Table 2**

886

Fatty acids	Stages					
	1	2	3	4	5	6
14:0	2.4±0.2a	2.2±0.1a	2.8±0.1b	3.3±0.1b	3.0±0.2b	3.3±0.1b
15:0	0.2±0.0a	0.2±0.0ab	0.3±0.0ab	0.3±0.0b	0.3±0.0b	0.3±0.0b
16:0	14.3±1.0a	15.4±0.6ab	17.4±0.7bc	18.4±0.8c	18.6±0.9c	18.9±0.5c
17:0	-	-	-	-	-	-
18:0	3.1±0.2a	3.1±0.1a	2.5±0.1b	2.5±0.1b	2.4±0.1b	2.3±0.1b
16:1	4.5±0.3a	4.3±0.2a	4.9±0.2ab	5.6±0.2b	5.4±0.2b	5.4±0.1b
18:1n-7	3.6±0.2ab	3.3±0.1a	3.6±0.1ab	4.0±0.1b	3.8±0.2ab	4.0±0.1b
18:1n-9	14.2±0.9ab	12.1±0.6a	13.3±0.6ab	13.8±0.7ab	13.8±0.8ab	15.6±0.5b
20:1	3.4±0.3	2.5±0.2	2.8±0.2	3.1±0.2	3.0±0.3	2.8±0.2
22:1n-9	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
18:2n-6	2.4±0.2ab	2.1±0.1a	2.6±0.1abc	3.1±0.1bcd	2.9±0.2cd	3.3±0.1d
18:3n-3	0.5±0.0ab	0.4±0.0a	0.5±0.0ab	0.6±0.0b	0.5±0.0ab	0.6±0.0b
18:3n-6	0.8±0.1	0.6±0.1	0.6±0.1	0.6±0.1	0.6±0.1	0.6±0.0
20:2	0.5±0.1	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.1	0.4±0.0
20:3n-6	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0
20:4n-6 (ARA)	0.7±0.1	0.7±0.0	0.6±0.0	0.7±0.0	0.7±0.0	0.6±0.0
20:5n-3 (EPA)	2.7±0.3a	2.9±0.2a	3.9±0.2b	4.9±0.2c	4.3±0.3bc	4.6±0.2bc
22:4n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.2	0.2±0.2
22:5n-3	2.4±0.1a	2.0±0.1ab	1.9±0.1ab	2.0±0.1ab	1.7±0.1b	2.0±0.1ab
22:6n-3 (DHA)	11.0±0.9a	10.8±0.6a	11.6±0.7ab	13.7±0.7bc	12.1±0.8abc	14.4±0.5c
∑ SFA	19.6±1.1a	20.9±0.8ab	23.0±0.7bc	24.5±0.9c	24.4±1.0c	25.0±0.6c
∑ MUFA	25.8±1.5a	21.8±0.9b	24.8±1.0ab	26.7±1.1a	26.2±1.4a	28.1±0.8a
∑ PUFA	21.4±1.4a	20.1±0.9a	22.5±1.0a	26.4±1.1b	23.4±1.3ab	26.8±0.7b

887

888

889 **Table 3**
890

Fatty acids	Stages				
	2	3	4	5	6
14:0	3.4±0.3a	2.8±0.2ab	2.5±0.2bc	1.8±0.2c	1.8±0.2c
15:0	0.2±0.0a	0.2±0.0ab	0.1±0.0b	0.1±0.0b	0.1±0.0b
16:0	11.2±0.7a	10.1±0.3ab	9.3±0.5bc	8.1±0.4cd	7.5±0.4d
17:0	-	-	-	-	-
18:0	2.8±0.1a	2.5±0.0ab	2.5±0.1b	2.3±0.1b	2.4±0.1b
16:1	4.0±0.4a	3.4±0.2ab	2.7±0.3bc	2.1±0.2c	1.9±0.2c
18:1n-7	3.0±0.2a	2.6±0.1b	2.4±0.1bc	2.0±0.1c	1.9±0.1c
18:1n-9	13.4±1.1a	10.7±0.6ab	9.3±0.8bc	7.5±0.7c	7.5±0.6c
20:1	3.8±0.4a	3.6±0.2ab	3.4±0.3ab	2.6±0.3ab	2.5±0.2b
22:1n-9	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0
18:2n-6	2.6±0.2a	2.0±0.1b	1.7±0.2bc	1.3±0.1c	1.4±0.1c
18:3n-3	0.4±0.0a	0.3±0.0b	0.2±0.0c	0.2±0.0c	0.2±0.0c
18:3n-6	0.5±0.1a	0.5±0.0a	0.3±0.0ab	0.3±0.0ab	0.2±0.0b
20:2	0.5±0.0a	0.3±0.0b	0.3±0.0b	0.3±0.0b	0.3±0.0b
20:3n-6	-	-	-	-	-
20:4n-6 (ARA)	0.6±0.1	0.6±0.0	0.6±0.0	0.7±0.0	0.8±0.0
20:5n-3 (EPA)	2.5±0.1	2.6±0.1	2.6±0.1	2.7±0.1	2.6±0.1
22:4n-6	-	-	-	-	-
22:5n-3	1.4±0.1a	1.2±0.0ab	1.1±0.1bc	0.9±0.1c	0.9±0.1c
22:6n-3 (DHA)	5.3±0.3	5.5±0.2	5.5±0.2	5.6±0.2	5.3±0.2
∑ SFA	17.6±1.0a	15.6±0.5ab	14.4±0.7bc	12.4±0.7c	12.0±0.6c
∑ MUFA	24.4±2.1a	20.5±1.1ab	17.9±1.5bc	14.3±1.3c	14.0±1.2c
∑ PUFA	14.0±0.6a	13.2±0.3ab	12.5±0.4b	12.1±0.4b	11.7±0.3b

891

892

893 **Figure 1**

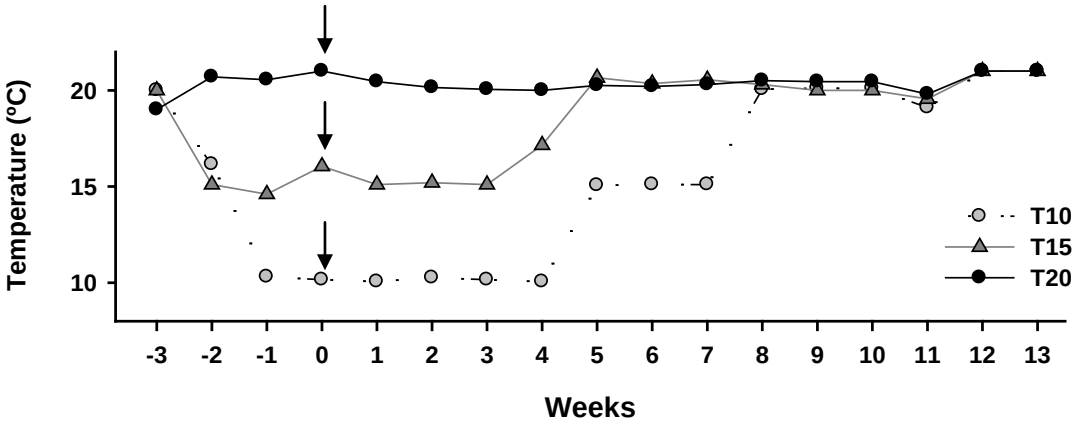

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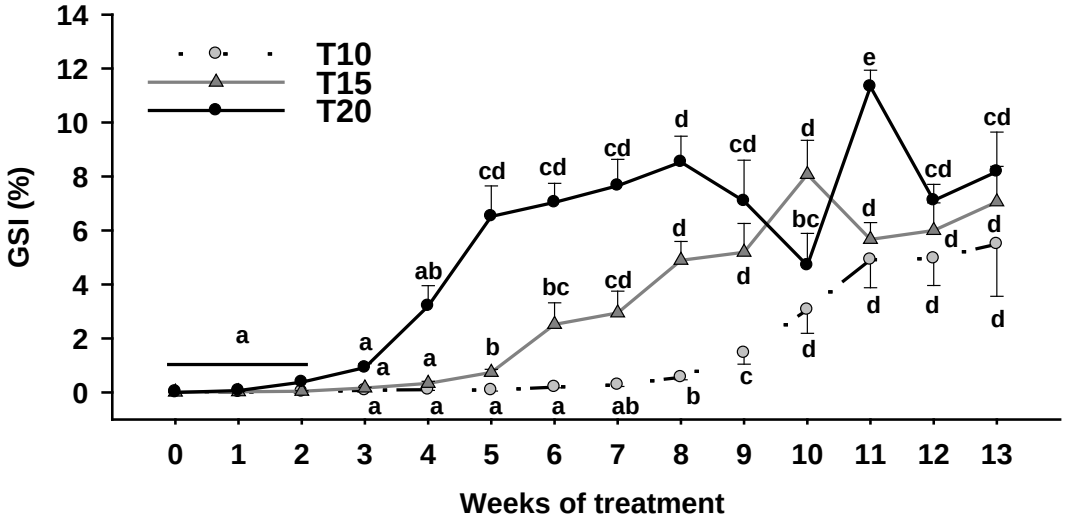
Figure 2



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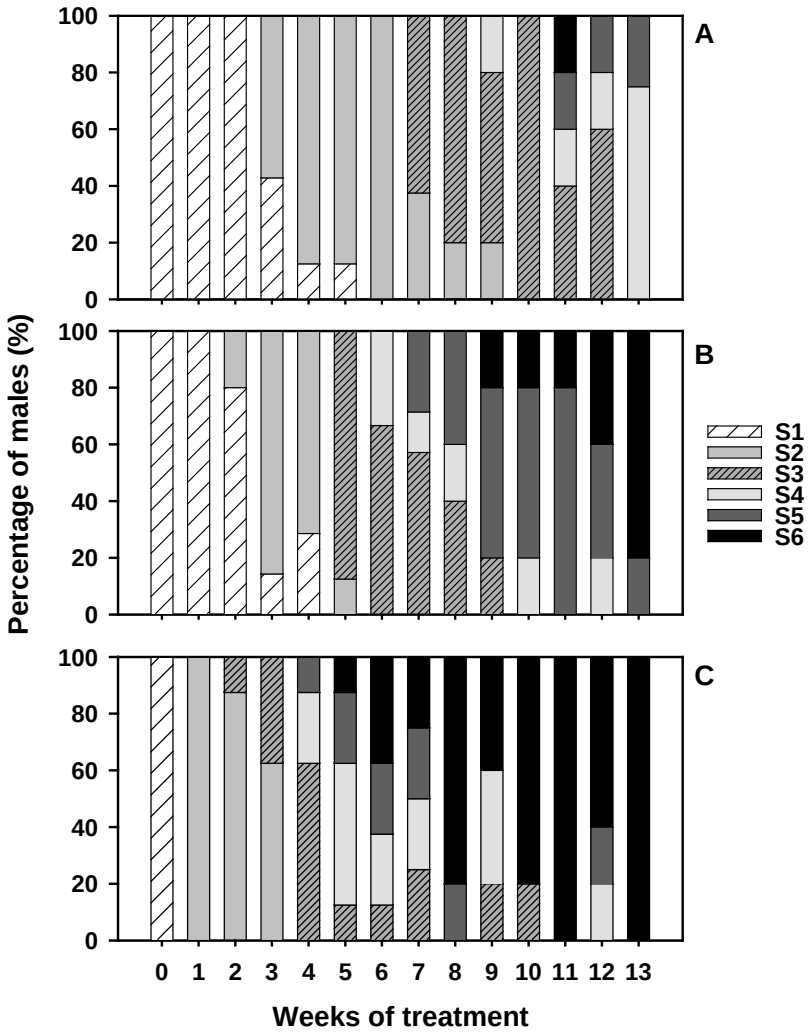

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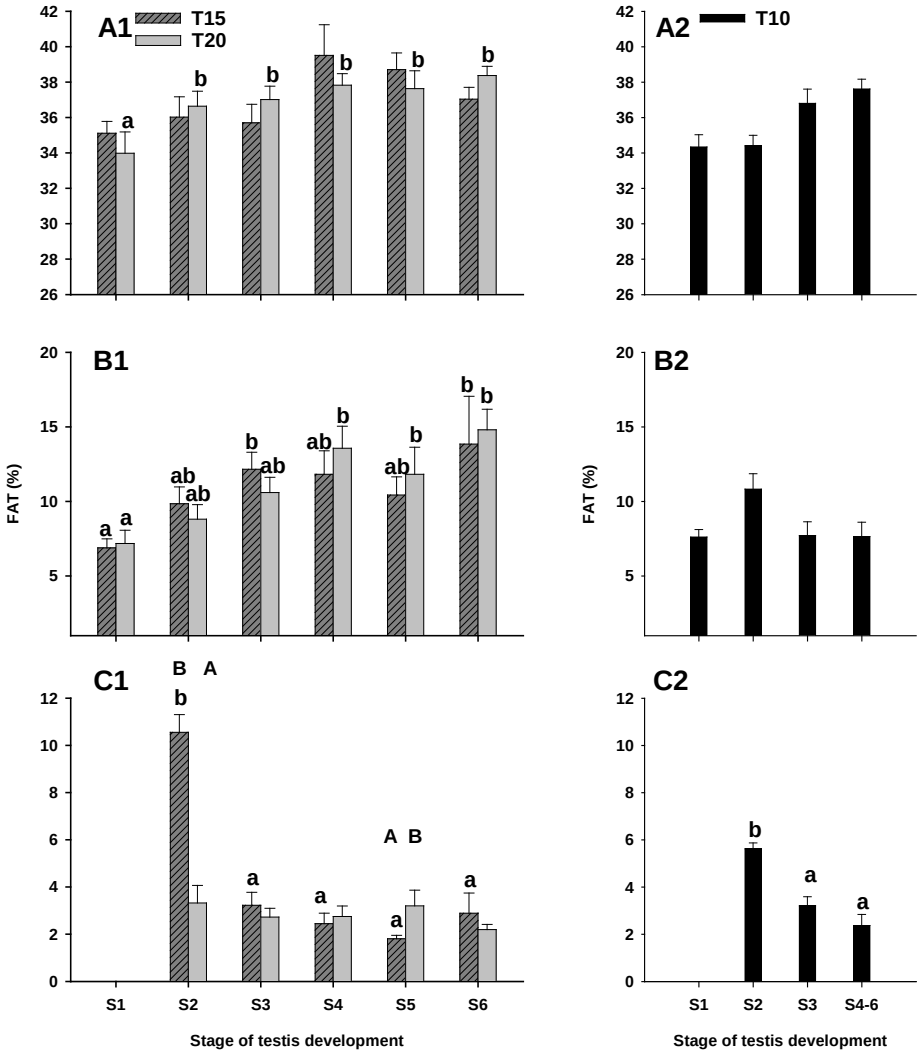
Figure 4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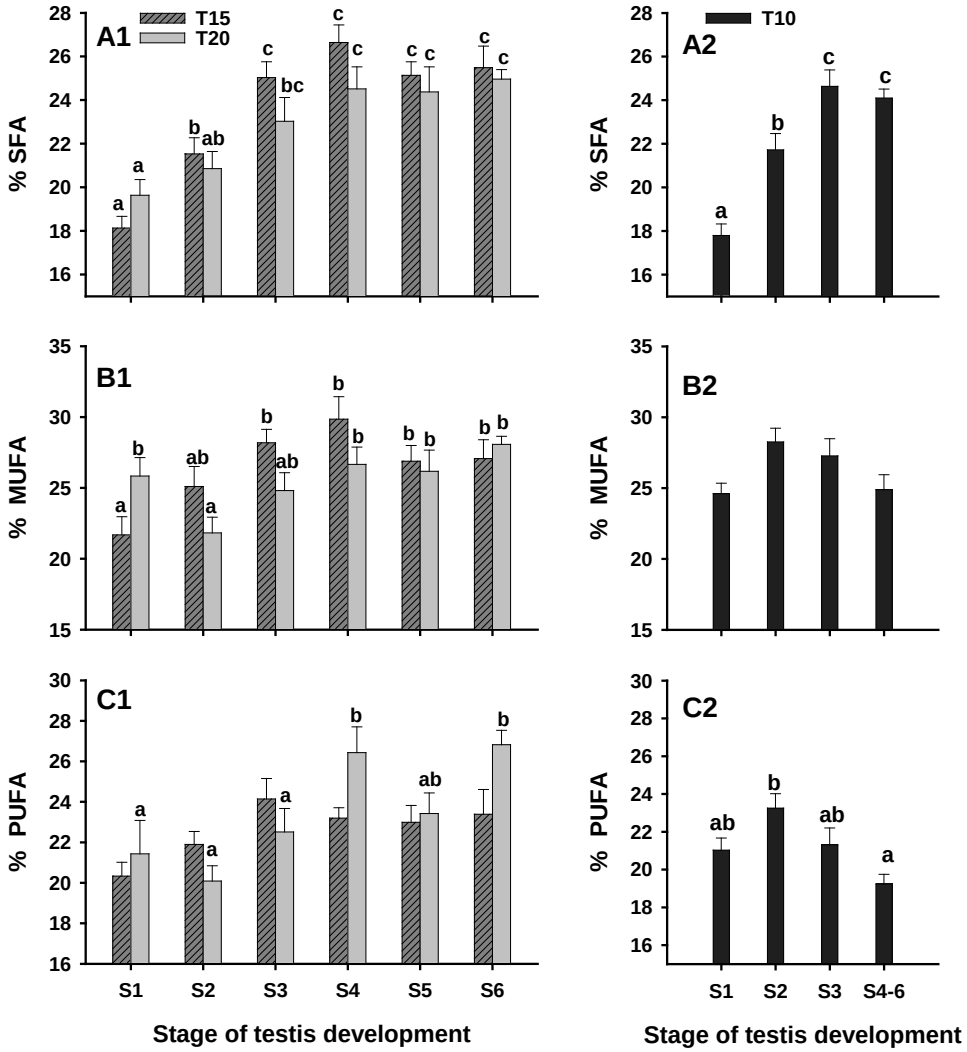
T15
T20
A2
T10
B1
B2
C1
C2
FAT (%)
Stage of testis development
S1
S2
S3
S4
S5
S6
S4-6

Figure 5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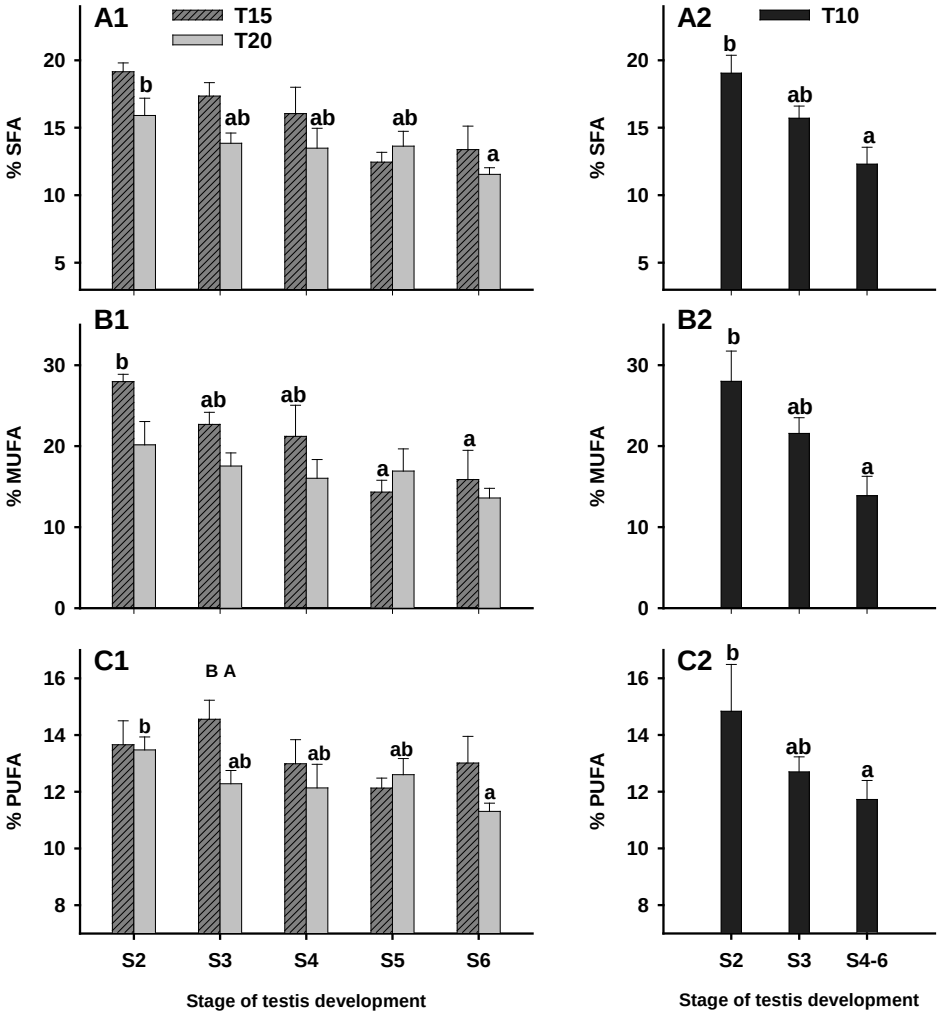
T15
T20
A2
T10
B1
B2
C1
C2
% SFA
% MUFA
% PUFA
S1
S2
S3
S4
S5
S6
S4-6
Stage of testis development
Stage of testis development

Figure 6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T15
T20
% SFA
b
ab
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A2
T10
b
ab
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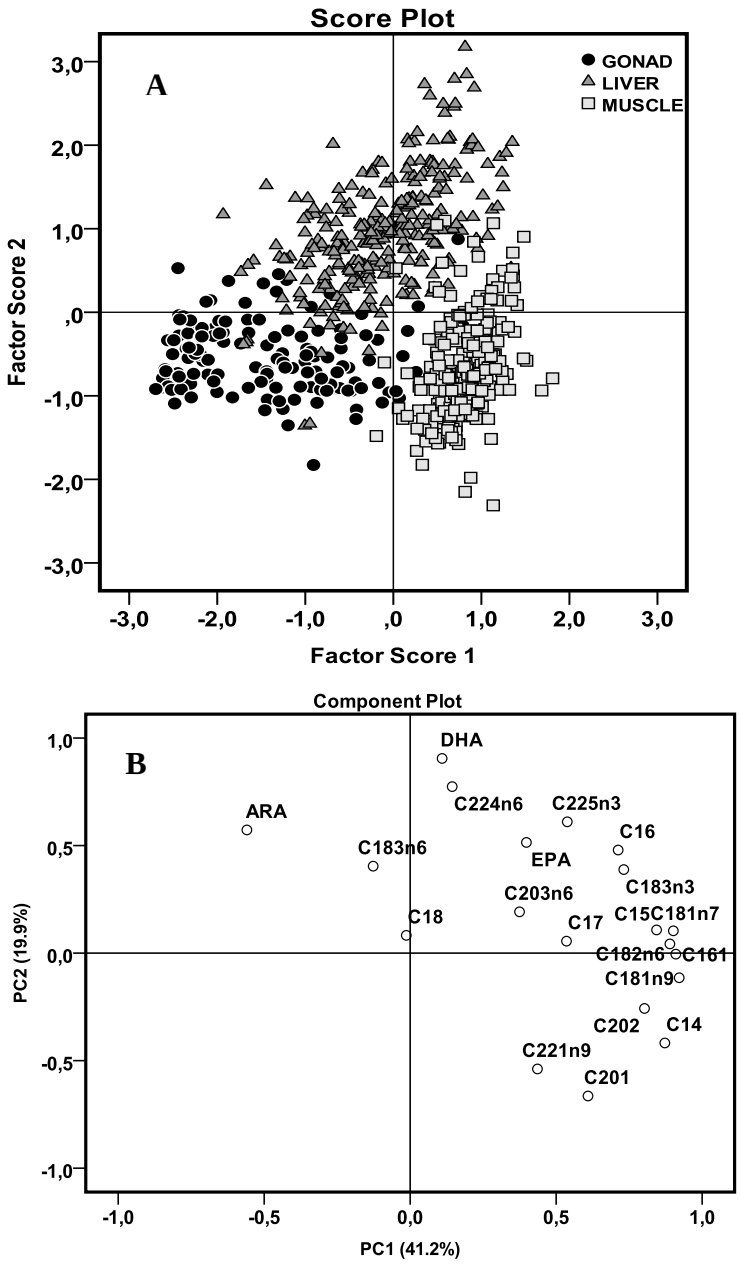
b
ab
ab
a
a
B2
C1
% PUFA
B A
C2
S2
S3
S4
S5
S6
S4-6
Stage of testis development

921 **Figure 7**
922



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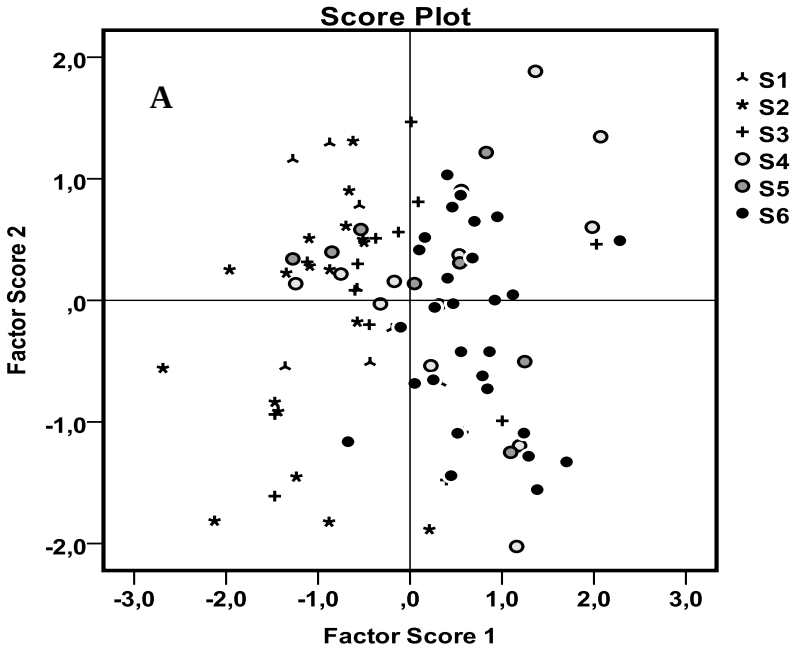
926 **Figure 8**



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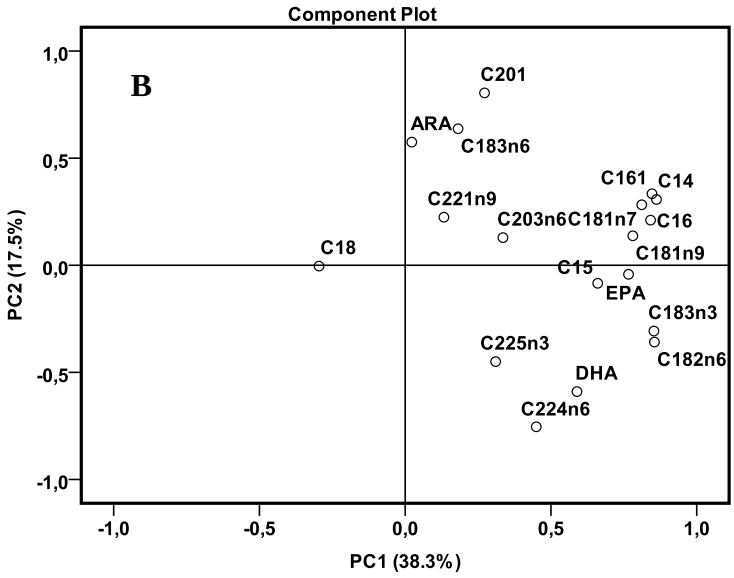

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930 **Figure 9**
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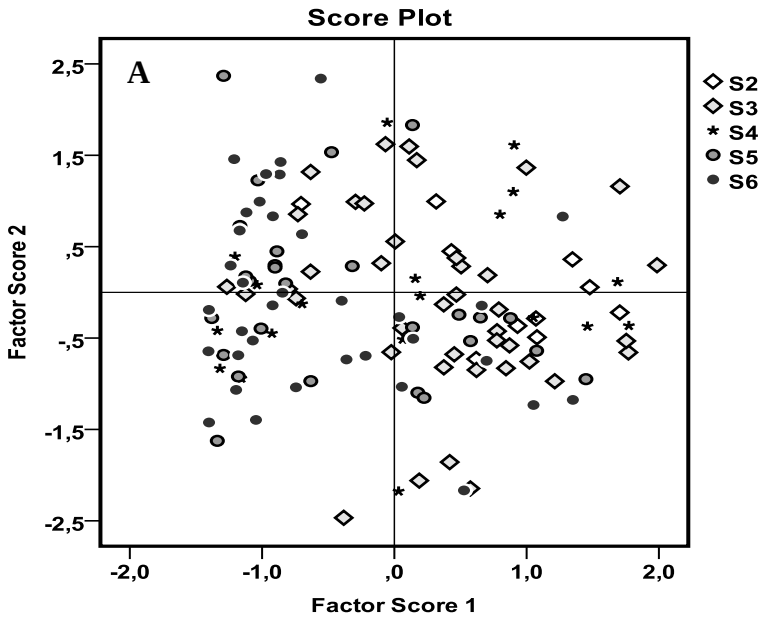

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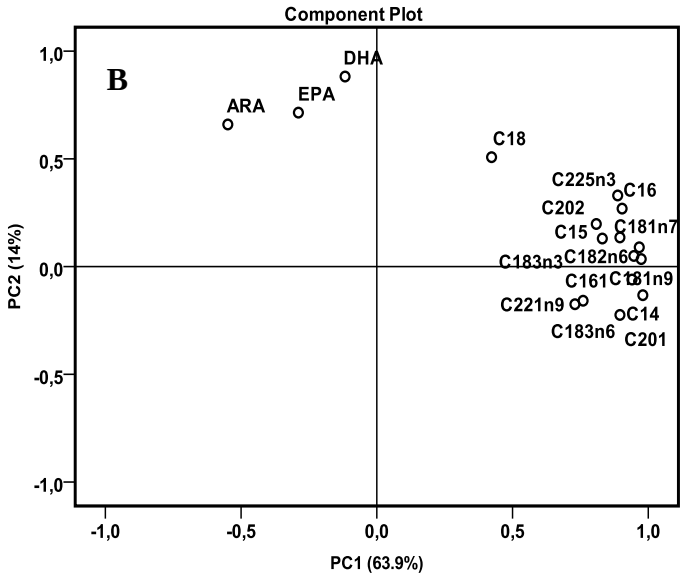

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