Document downloaded from:

http://hdl.handle.net/10251/68533

This paper must be cited as:

Baeza Ariño, R.; Peñaranda, D.; Vilchez Olivencia, MC.; Tveiten, H.; Pérez Igualada, LM.; Asturiano Nemesio, JF. (2015). Exploring correlations between sex steroids and fatty acids and their potential roles in the induced maturation of the male European eel. Aquaculture. 435:328-335. doi:10.1016/j.aquaculture.2014.10.016.

The final publication is available at

https://dx.doi.org/10.1016/j.aquaculture.2014.10.016

Copyright Elsevier

Additional Information

*Manuscript
Click here to view linked References

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Exploring correlations between sex steroids and fatty acids and their potential roles in the induced maturation of the male European eel

R. Baeza[1], D.S. Peñaranda[1,2], M.C. Vílchez[1], H. Tveiten[2], L. Pérez[1], J.F. Asturiano[1,]*

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n 46022 Valencia (Spain).
[2]Norwegian Institute of Fisheries and Aquaculture, 9037 Tromsø, Norway.

*Corresponding author:

Juan F. Asturiano, PhD
Universitat Politècnica de València
Instituto de Ciencia y Tecnología Animal (Edificio 7G)
46022 Valencia (Spain)
e-mail: jfastu@dca.upv.es
Phone:+34 96 387 93 85
Fax: +34 963877439

Abstract

The present study was undertaken to evaluate the correlations between the fatty acids in the liver and testis and the plasma levels of the hormonal steroids used during eel spermatogenesis, in order to clarify the physiological roles fatty acids play in the spermatogenetic process. The stages of testis development (S1-S5) were assessed by histological observations in order to classify the different phases of hormonally-induced spermatogenesis and evaluate the possible relationships between the hormones and fatty acids in each stage.

The highest plasma levels of 17β-Estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT) were found in S1, when spermatogonial proliferation occurs. A correlation was found between 17α-20β-dihydroxy-4-pregnen-3-one (DHP) levels and some fatty acids during the proliferation and growing phases (S1-2), suggesting that DHP might modulate lipid metabolism in the liver during early spermatogenesis. The DHP levels increased significantly during the growing phase (S2) and remained at high levels throughout the subsequent development stages (S3-S5).

Similar to results found in mammals, our results show that in the eel there are regulatory mechanisms, including eicosapentaenoic acid (20:5-n3, EPA) and docosahexaenoic acid (22:6-n3, DHA), which act as modulators in the synthesis of androgens, particularly during the final phase of sperm maturation. Our results suggest that the fact that EPA, ARA and DHA concentrations in the eel testis remain constant/stable during spermiation could be related to the subsequent union of the spermatozoa and the egg. The findings from this research provide new insights for further studies about the possible effect of steroids on desaturase activity and highlight the importance of the effect of lipid metabolism during male eel spermatogenesis.

Keywords:

Steroid hormone, desaturase, fatty acid, prostaglandins, spermatogenesis

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Introduction

European eel (*Anguilla anguilla*) is seriously under threat and has declined notably in recent years (Nielsen and Prouzet, 2008). The decline in the health of the spawners occurs as a consequence of pollution, diseases, migration barriers, restriction of habitat, etc. Together these stressors decrease the chances of successful migration and reproduction, thus affecting, egg and larval development in wild (ICES 2013). Eel is an valued product with a high commercial demand in both Europe and Asia, and recently standardized artificial fertilization techniques have been developed for this species (Butts et al., 2014).

To date, eels do not mature spontaneously in captivity, and many studies have focused on achieving this goal. However, maturity can be induced by long-term hormonal treatments in female (Dufour et al., 2003; Mazzeo et al., 2012; Peñaranda et al., 2013; Pérez et al., 2011) and male broodstock (Asturiano et al., 2006; Gallego et al., 2012; Müller et al., 2005; Peñaranda, et al., 2010).

Eels do not feed during their transoceanic migration, as such the food available during the growing phase of their life cycle provides them with the fat stores which they later use for their reproductive migration and gonadal development (Van Ginneken and Van den Thillart, 2000). Fatty acids affect gametogenesis, and we know that in males they have two specific functions: to regulate steroid production and to ensure the appropriate composition of the sperm cell membranes (Dupont et al., 2014). Unsaturated fatty acids provide the sperm plasma membrane with the fluidity required for membrane fusion, an event associated with fertilization (Whates et al., 2007). Numerous studies have focused on the modulatory effects dietary fatty acids have on steroid production in terrestrial animals (Castellano et al., 2011; Kelton et al., 2013; Zhang et al., 1992) and aquatic animals (Asturiano et al., 2000; Cerdá et al., 1995, 1997; Navas et al., 1998; Martin et al., 2009). In terms of steroidogenesis, most research has focused on arachidonic acid (20:4-n6, ARA) because there is clear evidence that it can influence steroid output at a cellular level and is considered to be one of the most important factors in successful fish reproduction (Alorend, 2004; Furuita et al., 2003; Norambuena et al., 2013). ARA is the precursor for some prostaglandins which are active biological substances involved in reproduction (Sargent et al., 2002). For instance, in vitro, ARA promotes testicular synthesis of testosterone in goldfish (*Carassius auratus*) stimulating prostaglandin synthesis (Wade et al., 1994). Asturiano et al. (2000) demonstrated that in European sea bass (*Dicentrarchus labrax*) ARA stimulated a significant increase in prostaglandin E_2

production, in a dose- and time-dependent manner, and suggested that it may have important effects on steroidogenesis and spermiation. On the other hand, series 3 fatty acids can influence both the prostaglandin and steroid pathways involved in the regulation of the reproductive function, as well as the fatty acid composition and fertilizing capacity of sperm (Whates et al., 2007).

Another important factor is the influence of steroids on the metabolism of fatty acids. In the case of tilapia (*Oreochromis mossambicus*), an increase in desaturase activity was seen in fish treated with E2 injections. This was reflected in the decrease in the saturated fatty acids and the increase in the monounsaturated fatty acids (Hsieh et al., 2004). In rats, testosterone can cause an increase or decrease in various desaturase activities, thus modifying the fatty acid profile in the testis (Hurtado and Gómez, 2005). In humans, Burdge (2006) suggested that estrogen has a regulatory effect on the conversion of linolenic acid (18:3n3, ALA) to eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA).

The objective of this research was to investigate the correlations between liver and testis fatty acids and sex steroids at different stages of gonadal development (S1-S5) during hormonally-induced sexual maturation in male European eels.

2 Material and methods

2.1 Fish acclimatization and hormonal treatment

Three hundred and seventeen male eels (mean body weight 100 ± 2 g) from the fish farm Valenciana de Acuicultura, S.A., (Puçol, Valencia; East coast Spain) were transported to the Aquaculture Laboratory at the Polytechnic University of Valencia. They were housed in six 200 L aquaria, each equipped with separate recirculation systems and covered to maintain constant darkness. The fish were gradually acclimatised over the course of two weeks from freshwater to seawater (37 ± 0.3 g L^{-1}). Once a week they were anesthetized with benzocaine (60 ppm) and weighed before being administered human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Argent Chemical Laboratories. USA) by intraperitoneal injection (Pérez et al., 2000).

2.2 Thermal regimes

The fish underwent three thermal regimes: T10 (10 ºC for the first 6 weeks, 15 ºC for the next 3 weeks and 20 ºC for the last 6 weeks); T15 (15 ºC for the first 6 weeks and 20 ºC for the last 9 weeks) and T20 (20 ºC throughout the whole experimental period;

Gallego et al., 2012). Two aquaria were used for each treatment and 50 eels per aquaria, thus, 100 eels per thermal regime.

When the means of the fatty acids and of the hormones in each of the thermal regimes were compared by stage of development, no differences between the thermal treatments were found (P-value <0.05, see Supplementary Tables 1-3). Thus, in order to increase the number of samples and having checked beforehand that there were no differences, all the analyses were carried out independently of the thermal regime.

2.3 Sampling

When the animals arrived at our facilities, and before starting any treatment, eight animals were sacrificed as freshwater controls. Fish sacrifice was carried out by decapitation, after having previously been anesthetized with benzocaine (60 ppm). Blood samples were collected in heparinized vials and centrifuged at 3000 r.p.m. for 5 min, and the blood plasma was stored at -80 ºC until analysis.

A small sample of testis (0.5 g) from each male was preserved in 10% buffered formalin for histology processing. For each thermal regime, liver and testis samples from eight animals were obtained during the first eight weeks of treatment, and from five animals in the last five weeks. The first testis samples were collected as soon as they had reached an appropriate size for analysis. All the testis and liver samples were stored at -80 ºC until lipid extraction and fatty acid quantification.

2.4 Fat extraction and fatty acid quantification

The fat extraction of the liver and testis was carried out using a modified version of the method described by Baeza et al. (2014). The fatty acid quantification was carried out by gas chromatography as described by Baeza et al. (2014).

2.5 Steroids

Plasma concentrations of 17α-20β-dihydroxy-4-pregen-3-one (DHP), 17β–estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) were measured by means of radioimmunoassay, as described previously (Schulz, 1985; Frantzen et al., 2004). Assay characteristics and cross-reactivities of the E2 and T antisera have previously been examined by Frantzen et al. (2004) and further validated for eel plasma by Mazzeo et al. (2014). The characteristics and cross-reactivities of the DHP assay have previously been described by Tveiten et al. (2010) and validated for eel plasma by Peñaranda et al.

(2010). The cross-reactivities of a new 11-KT antiserum used in this work have previously been described by Johnsen et al. (2013). To validate the recovery of 11-KT from plasma in the eel assay, a plasma pool was spiked with 45ng 11-KT pr. ml of plasma and then underwent ether extraction as described below. The resulting product was then assayed by the 11-KT RIA at three different dilutions. The dilutions were found to be parallel to the standard assay curve. Steroid recovery after ether extraction was 71.9±2.8.0%. The 11-KT values were corrected for recovery losses. The inter and intra assay coefficients of variation (CV) for the 11-KT assay were 15.4% (n=7) and 5.3% (n=10), respectively.

2.6 Gonad histology

After fixation in 10% buffer formalin (pH 7.4), a small section of testis was dehydrated in ethanol and embedded in paraffin. The samples were sectioned to thicknesses of 5 and 10 μm. The sections were stained using the current haematoxylin and eosin method. The slides were observed using a Nikon Eclipse E-400 microscope and the images were taken with a Nikon DS-5M camera.

The maturation stages were determined using the following criteria: Stage 1 (S1) was characterized by the dominance of spermatogonia; some spermatocytes can be present but not dominant; Stage 2 (S2), with spermatocytes as the dominant cell. Some spermatids can be present in low numbers. The dominant process in this stage is meiosis; Stage 3 (S3) was characterized by the dominance of spermatids. The dominant process in this stage is spermiogenesis (spermatid maturation). Males in non-spermiating stage; if some milt was produced, it is of a low volume (< 0.5 ml) and low motility (<10 %); Stage 4 (S4), abundant sperm cells present inside the tubule lumen. Tubule lumen delimited by a multiple germ cell layer. Males in early spermiation stage; Stage 5 (S5), was characterized by a dominance of spermatozoa and a low proportion of other germ cells and luminal fusion. Males showing high sperm motility and high sperm volume. Stage of maximal spermiation. (Figure 1).

Once the fatty acid and steroid analyses had been carried out, the results were classified into the different development stages of the testis. These were assigned once the animals had been sacrificed.

2.7 Statistical analysis

The differences between the sex steroids at the different stages of testis development were analysed by one way ANOVA . Pearson´s correlations ($P<0.05$) were then used to find the correlations between each fatty acid in the liver and testis and the steroid hormone. When the fatty acids and the hormone levels were correlated for each testis development stage, it was observed that the number of samples in each stage was very low when the three thermal treatments (T10, T15 and T20) were considered separately. The absence of statistical differences was checked by one way ANOVA, comparing the means of the fatty acids (with the thermal regimes considered seperately) at each stage of testis development. So, considering the absence of statistical differences and with the knowledge that all the animals were at the same development stage, correlations were carried out independently of the initial thermal regime. This decision was made in order to give us a higher number of samples for the correlations. Finally, Pearson´s correlations ($P<0.05$) were also used to find the correlations between the fatty acids at each stage of testis development. All the ANOVA were followed by a post-hoc multiple Newman-Keuls comparison test at a significance level of $P<0.05$. All he statistical procedures were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Stage 1: proliferation phase

Throughout the experimental period a total of 103 eels were found in S1, 75% of which were registered during the first three weeks of the hormonal treatment.

During S1, T, 11-KT and E2 reached their highest plasma levels, while DHP had the lowest plasma level of this stage (Table 1). In the liver, a high correlation ($r= 0.83$ with $P<0.01$, Table 4) was found between Linoleic acid (18:2-n6, LA) and α-Linolenic acid (18:3-n3, ALA) at S1.

Table 2 shows that in S1, E2 was negatively correlated and DHP was positively correlated with several liver fatty acids.

Due to the small size of the testis samples during S1, correlations between the testis fatty acids and steroid levels were not carried out.

3.2. Stage 2: growing phase

Over the course of the treatment a total of 46 eels were observed in S2. Table 1 shows that T and 11-KT plasma levels do not vary significantly from those of stage 1, while E2 decreased significantly in this stage. On the other hand, DHP increased significantly during this testis development stage, in comparison to S1. The correlation between ALA and LA levels in the liver remained high during stage 2 ($r=0.87$ with $P<0.01$, Table 4). Regarding the correlations found between liver fatty acids and hormones, Table 2 shows how only DHP displayed a significant correlation with palmitic acid (16:0).

In this stage of testis development, when cellular growth occurs, a very high correlation was found between Oleic acid (18:1-n9) and LA levels in the testis ($r=0.94$ with $P<0.01$, Table 5). This was in fact the highest correlation found between testis fatty acids during previous S2.

3.3. Stage 3: maturation phase

Over the course of the experiment a total of 31 male eels were found to be in S3. During this stage a significant decrease was registered in 11-KT plasma levels (Table 1) however there were no differences observed in the rest of the steroids analyzed in comparison to the previous stage. Correlations between some saturated and monounsaturated fatty acids in the liver were higher than those found during the previous stages of testis development. The correlation between LA and ALA was the highest correlation registered ($r=0.91$ with $P<0.01$, Table 4).

On the other hand, following the dynamics of the previous stage, among all the correlations found between the testis fatty acids, the highest correlation registered in S3 was between 18:1-n9 and LA ($r=0.94$ with $P<0.01$, Table 5). With regards to the correlations observed between the decreasing plasma levels of androgens (Table 1) and the testis fatty acids, a significant correlation was found between 11-KT and 16:0 and 16:1, and also between T and 16:1 (Table 3).

3.4. Stage 4: initial spermiation

Over the course of the experiment a total of 27 males were classed as being in S4.

T levels decreased significantly in S4 whereas the rest of steroids analyzed remained at the same concentration as the previous stage.

Compared with the previous stages, in stage 4 the correlations between saturated and monounsaturated fatty acids from liver were lower (Table 4). Once again, as we saw in

the other development stages, the highest registered correlation found between all the liver fatty acids was between LA and ALA (r=0.87 P<0.01, Table 4).

High correlations were found between the testis fatty acids during S4, with the highest correlation being between 18:1-n9 and LA (r=0.98 P<0.01, Table 5). In this stage, eicosapentaenoic acid (20:5-n3, EPA), docosahexaenoic acid (22:6-n3, DHA) and arachidonic acid (20:4-n6, ARA) only correlated with each other, but not with any of the other fatty acids (Table 5).

3.5. Stage 5: advanced spermiation

The histology results showed 49 animals in S5 over the course of the experiment. With regards to the steroidogenic hormones, T and 11-KT plasma levels were significantly lower during this advanced stage of testis development (Table 1). Significant positive correlations in the testis were found between T and several n-3 series polyunsaturated fatty acids such as EPA and DHA, and also between 11-KT and DHA (Table 3).

The correlations found between the fatty acids in the liver (Table 4) and those in the testis (Table 5) were very similar to the correlations found during initial spermiation (S4). In particular a very high correlation was observed between 18:1-n9 and LA in the testis (r=0.97 with P<0.01, Table 5) and also in testis 16:1 and 18:1-n9 (r=0.97 with P<0.01, Table 5).

4. Discussion

As in most teleost, European eel spermatogenesis goes through an initial phase of mitotic proliferation, developing from spermatogonial cells to differentiated spermatogonia showing a defined nucleus (Schulz and Miura, 2002). Miura et al. (1991a) described how in Japanese eel a marked activation of Sertoli and Leydig cells was followed by a proliferation of spermatogonia, beginning three days after the administering of a single dose of hCG. During S1, gonadotropin stimulates the Leydig cells to produce steroids such as T and 11-KT, the major androgen in teleost fish (Miura et al., 1991b). In addition, 11-KT is one of the factors involved in the initiation of spermatogonial proliferation and the start of meiosis (Miura et al., 1991b; Kobayashi et al., 1991; Amer et al., 2001; Peñaranda et al., 2013). Our experiment corroborates these findings, with the highest T and 11KT plasma levels being registered in S1 (Table 1), when spermatogonial proliferation occurred. We had expected to find a correlation between ARA and T plasma levels in the testis during S1 because literature has

described the regulatory role of ARA in prostaglandin production (Asturiano et al., 2000; Norambuena et al., 2012, 2013) and, subsequently, through the prostaglandins, ARA stimulates production of T (Wade et al., 1994). However, this correlation between ARA and T was not observed in our experiments. A possible explanation is that this correlation probably occurred in a very early phase, before there was enough testis tissue to allow its collection for the fatty acid analysis.

Continuing with the evolution of T and 11-KT plasma levels, a correlation was registered between these androgens and 16:0 and 16:1 in the testis during the sperm maturation phase (S3). During this stage genetic diversity is generated by meiosis II, the result of which is four haploid cells, and the formation of spermatids. T and 11-KT are also involved in this process of maturation (Schulz et al., 2010). These correlations found in S3 can be explained by the fact that 16:0 and consequently 16:1, are the main products of the *de novo* synthesis of the fatty acids (Cook and Mc Master, 2002) and as we know, fatty acids, especially polyunsaturated fatty acids, are essential in the formation of sperm cell membranes (Whates et al., 2007). Constant membrane synthesis is required for the production of spermatozoa and also to maintain spermatozoa quality (Dupont et al., 2014). It seems that the correlations registered between the androgens and 16:0 and 16:1 is probably related to the fact that although the males were in the same maturation stage (S3), a great variation was found between them. Some males displayed formed spermatids, while others were already producing spermatozoa (although still showing low sperm volume and quality). Therefore, less developed S3 males, had higher 16:0 and 16:1 levels in the testis (due to an intensive *de novo* biosynthesis of fatty acids for the process of membrane formation) together with higher androgen plasma levels. On the other hand, the more developed S3 males showed lower 16:0 and 16:1 plasma levels (probably, because they had been converted to other fatty acids in the process of membrane formation) and lower androgen plasma levels (due to the fact that their importance is mainly at beginning of maturation).

At advanced spermiation (S5) some correlations were found in the testis between the androgens and several n3-series fatty acids, including EPA and DHA. Although T is thought to play its most important role in the previous stages of testis development in fish, in particular when spermatogonial proliferation occurs (Nagahama, 1994), our results suggest that in S5, when mature and differentiated sperm are present, EPA and DHA could modulate the production of T, just as Wade et al. (1994) reported for goldfish. EPA is a substrate for the biosynthesis of 3-series prostaglandins, which have

modest effects on T production (Tocher, 2003). In our case, the male eels with the higher T and 11-KT plasma levels also had higher EPA and DHA concentrations in the testis. This supports our hypothesis of fatty acids having a modulatory effect on the synthesis of androgens in eels with mature sperm. Castellano et al. (2011) discovered that a DHA or EPA rich diet fed to pigs modifies the fatty acid composition of testicular tissue, and diets rich in DHA reduce T concentrations. Moreover, and also corroborating our present results, a decrease in total serum T concentrations has been observed in men fed a diet rich in n-3 series fatty acids (Nagata et al., 2000). Recently, in a study of Senegalese sole (*Solea senegalensis*), it was suggested that high dietary EPA content could explain the high levels of of 3-series prostaglandins, and could consequently affect T production (Norambuena et al., 2013).

Regarding E2, its role during the synthesis of yolk protein is well known (Arukwe and Goksøyr, 2003), but the fact that it is also important in male spermatogenesis is also recognised (Miura and Miura, 2003). In our experiment, the highest E2 plasma levels were also found in S1 and they were negatively correlated with many of the fatty acids in the liver. The oxidation of fatty acids releases two carbon units in an active form (acetil-CoA), which end up entering into the citric acid cycle. This process involves the synthesis of ATP, and this provides energy to the cell (Melo and Cuamatzi, 2007). Rossato et al. (2001) demonstrated that extracellular ATP promotes the secretion of E2 in rats. Our results suggest that the negative correlations found between some of the fatty acids and E2 could be due to the fact that the oxidation of fatty acids generates ATP, which could be being used in the synthesis of E2, specifically in S1, when E2 reaches its highest plasma levels.

Moreover, and regarding DHP plasma levels, Miura et al. (2006) demonstrated a new function of DHP using eel testis *in vitro*. They showed that not only is it a factorin the regulation of final maturation, but also in the early stages of spermatogenesis, especially the initiation of meiosis. Schulz et al. (2010) also suggested that DHP is an essential hormone in the initiation of meiosis during spermatogenesis in teleost fish. In our experiment, the lowest plasma levels of the progestin DHP were registered in S1 and increased significantly after the growing phase (S2), reaching their highest concentrations in the maturation (S3) and spermiation (S4-S5) phases. This thus coincides with the profile previously described in other species (Asturiano et al., 2002; Nagahama, 1994). During the initial stages of testis development (S1-S2), DHP plasma levels correlated with several liver fatty acids (LA, ALA, ARA and DHA in S1; 16:0 in

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

both stages). Our results show in particular a positive correlation between 16:0 from the liver, and DHP during the proliferation (S1) and growing (S2) phases. This suggests a possible link between DHP and the process of *de novo* biosynthesis of fatty acids in the liver. The main fatty acid synthetized by the cells is 16:0, and other fatty acids are synthetized by modifications of 16:0. This synthesis takes place through enzymatic reactions. Fatty acid synthase (FAS) is a multifunctional enzyme that acts as a catalyst in all the stages of fatty acid synthesis and is expressed mainly in the liver and adipose tissue (Favarger, 1965). Lacasa et al. (2001) found that progesterone is able to stimulate FAS in the adipose tissue of rats. Recently, it has been demonstrated that progesterone stimulates the gene expression of lipogenic enzymes, also in the adipose tissue of rats (Stelmanska and Swierklzynski, 2013).

The discovery of this correlation between 16:0 from the liver and DHP during the proliferation (S1) and growing (S2) phases, coinciding with an increase in the activity of cellular division and multiplication, suggests that DHP could modulate the lipid metabolism of the liver. The levels of this progestin could control the range of production of fatty acids, which later are used to form sperm cell membranes during early spermatogenesis. However, further research would be needed to define the real physiological mechanism.

In terms of the correlations found between the different fatty acids present in the liver during testis maturation (Table 4), a high correlation was found between LA and ALA in several development stages. This could be linked to desaturase activity. Hurtado and Gómez (2005), after observing a decrease in the enzyme activity in which desaturase is involved (i.e. the conversion of LA into ALA) after T administration, suggested that T has a modulatory effect on desaturase activity in rats.. Animal studies carried out on rats by Marra and Alaniz (1989) demonstrated a significant inhibition of Δ6 desaturase enzymes in the liver after T administration. In a review about animal experiments, cell culture studies and cultured human trials, Decsi and Kennedy (2011) concluded that estrogen stimulates, whereas T inhibits, the conversion of essential fatty acids into their long-chained metabolites. Our results appear to agree, as they suggest that the high correlations found between liver fatty acids may be related to the demonstrated modulatory effect of hormones on desaturase activity.

The highest correlations between saturated and monounsaturated fatty acids (i.e.: 16:0 was correlated with 16:1, r=0.87** $P<0.01$; Supplementary Table 3) in the liver were registered during the maturation phase (S3). This suggests a new phase of hepatic *de*

novo synthesis of fatty acids since 16:0, and consequently 16:1, are their main products (Cook and Mc Master, 2002). Similar to these findings, Baeza et al. (2014) suggested *de novo* biosynthesis of hepatic fatty acids when eels began to produce sperm.

Regarding the correlations found between the testis fatty acids, a very high correlation (r>0.9) between 18:1-n9 and LA was registered in all the stages of testis development (Table 4). There is little information available about the pathways to elongate and desaturate fatty acids in eel, and this is a common area of study in fish nowadays and has been reviewed in marine fish recently (Monroig et al., 2013). The high correlation found between 18:1-n9 and LA could be the *de novo* biosynthesis of fatty acids in the testis. Several studies seem to agree, showing that fatty acids can be synthetized *de novo* in the testis (Conglio, 1994; Lenzi et al., 2000).

Several testis fatty acids were highly correlated between themselves during initial spermiation (S4). EPA, ARA and DHA behave differently compared to the rest of the fatty acids in this stage, correlating only with one another. This can be explained by the stability of EPA, ARA and DHA concentrations and the decrease in the rest of the testis fatty acids when initial spermiation occurs, as reported by Baeza et al. (in press). Lamirande et al. (1997) suggested that lipid peroxidation on the sperm cell membrane promotes binding to the zona pellucida, making the connection between the egg and spermatozoa possible. The compound lipids, which are mainly present in biological membranes which contain abundant unsaturated fatty acids, are very vulnerable to oxidation (Melo and Cuamatzi, 2007). Therefore, our results suggest that the maintenance of EPA, ARA and DHA levels (highly unsaturated fatty acids) in eel testis may be related to the ease at which they subsequently oxidate and therefore to sperm capacitation.

From our results we conclude that several fatty acids are involved in the process of spermatogenesis, although their regulatory role in steroid production and on fatty acids may be different depending on the phase of testis development. DHP, as in other teleost species, seems to be an essential element in the initiation of the early stages of spermatogenesis and our results provide substantial evidence that DHP might modulate the metabolism of lipids in the liver during early spermatogenesis. Moreover, one possible theory is the oxidation of fatty acids in order to produce available energy necessary for E2 production during the proliferation phase of testis development. Finally, it appears there are mechanisms in the eel which are similar to those reported in mammals, with EPA and DHA acting as modulators of androgen synthesis, particularly

during the final phase of sperm maturation. Overall, the findings from this research give new insights which can be used to continue with further studies about the roles and interactions between fatty acids and steroids in fish spermatogenesis.

Acknowledgements

Funded by the European Community's 7th Framework Programme under the Theme 2 "Food, Agriculture and Fisheries, and Biotechnology", grant agreement n°245257 (PRO-EEL). D.S.P. had a contract co-financed by MICINN and UPV (PTA2011-4948-I) and received a Short-term Scientific Mission grant from COST Office (Food and Agriculture COST Action FA1205: AQUAGAMETE) to carry out the steroids analyses in Norway.

References

Alorend, E., 2004. The effect of dietary arachidonic acid concentration on Atlantic halibut (*Hippoglossus hippoglossus*) broodstock performance, assessment of egg, milt and larval quality. PhD dissertation, University of Stirling,

Amer, M.A., Miura, T., Miura, C., Yamauchi, K., 2001. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). Biol. Reprod. 65, 1057–1066.

Arukwe, A., Goksøyr, A., 2003. Egg shell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comparative Hepatology 2, 4.

Asturiano, J.F., Sorbera, L.A., Zanuy, S., Carrillo, M., 2000. Effects of polyunsaturated fatty acids on prostaglandins series E production in a primary testis cell culture system for the European sea bass. Journal of Fish Biology 57, 1563-1574.

Asturiano, J.F., Sorbera, L.A., Ramos, J., Kime, D.E., Carrillo, M., Zanuy, S. 2002. Group-synchronous ovarian development ovulation and spermiation in the European sea bass (*Dicentrachus labrax*) could be regulated by shifts in gonadal steroideogenesis. Scientia Marina 66(3), 273-282.

Baeza, R., Mazzeo, I., Vílchez, M.C., Gallego, V., Peñaranda, D.S., Pérez, L., Asturiano, J.F., 2014. Effect of thermal regime on fatty acid dynamics in male European

eel (*Anguilla anguilla*, L.) during hormonally-induced spermatogenesis. Aquaculture 430, 86-97.

Burdge, G.C., 2006. Metabolism of a-linolenic acid in humans. Prostaglandin Leukotrienes and Essential Fatty Acids 75, 161–8.

Butts, I.A.E., Sørensen ,S.R., Politis, S.N., Pitcher, T.E., Tomkiewicz, J., 2014. Standardization of fertilization protocols for the European eel, *Anguilla anguilla*. Aquaculture. 426-427, 9-13.

Castellano, C.A., Audet, I., Laforest, J.P., Matte, J.J., Suh, M., 2011. Fish oil diets alter phospholipid balance, fatty acid composition, and steroid hormone concentrations in testes of adult pigs. Theriogenology 76, 1134-1145.

Cerdá, J., Zanuy, S., Carrillo, M., 1997. Evidence for dietary effects on plasma levels of sexual steroids during spermatogenesis in the sea bass. Aquaculture International 5, 473-477.

Cerdá, J., Zanuy, S., Carrillo, M., Ramos, J., Serrano, R., 1995. Short and long term dietary effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profile of lipids and sex steroids in relation to reproduction. Comparative Biochemistry and Physiology 111, 83-91.

Conglio, J.G., 1994. Testicular lipids. Progress in lipid research 33, 387-401.

Cook, H.W., Mc Master, C.R., 2002. Fatty acid desaturation and chain elongation in eukaryotes. In: Vance, D.E, Vance, J.F. (Eds.). Biochemistry of lipids, lipoproteins and membranes: Elsevier, pp. 181-204.

Decsi, T., Kennedy, K., 2011. Sex-specific differences in essential fatty acid metabolism. American Journal of Clinical Nutrition 94, 1914-1919.

Dufour, S., Burzawa-Gerard, E., Le Belle, N., Sbaihi, M., Vidal, B., 2003. Reproductive endocrinology of the European eel, *Anguilla anguilla*, in: Aida, K. Tsukamoto, K. Yamauchi, K. (Eds.), Eel Biology: Springer, pp. 373–383.

Dupont, J., Reverchon, M., Bertolo, M.J., Froment, P., 2014. Nutritional signals and reproduction. Molecular and Cellular Endocrinology 382, 527-537.

Favarger, P., 1965. Relative importance of different tissues in the synthesis of fatty acids, In: Renold, A.E., and Cahill, G.F., Adipose tissue eds, Washington D.C., p 19.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissue. Journal of Biological Chemistry. 226, 497-509.

Frankel, E.N., 2005. Lipid oxidation. Oily press lipid library series, USA: ISBN 0 9531949 8 1, 488p.

Frantzen, M., Arnesen, A.M., Damsgård, B., Tveiten, H., Johnsen, H.K., 2004. Effects of photoperiod on sex steroids and gonad maturation in Artic charr. Aquaculture 240, 561-576.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N., Takeuchi, T., 2003. Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder *Paralichthys olivaceus*. Aquaculture 220, 725–735.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354, 7-16.

Gnoni, G.V., Muci, M.R., 1990. *De novo* fatty acid synthesis in eel-liver cytosol. Comparative Biochemistry and Physiology B 95, 153–158.

Hsieh, S.L, Chang, H.T., Wu, C.H., Kuo, C.M., 2004. Cloning, tissue distribution and hormonal regulation of stearoyl-CoA desaturase in tilapia, *Oreochromis mossambicus*. Aquaculture 230, 527-546.

Hurtado, G.E., Gómez, I.N.T., 2005. Influence of testosterone on polyunsaturated fatty acid biosynthesis in Sertoli cells in culture. Cell Biochemistry and Function 23, 175-180.

ICES. 2013. ICES Working Group on Eels. Report of the ICES Advisory Committee on Fisheries Management 18, 253 pp.

Johnsen, H., Tveiten, H., Torgersen, J.S., Andersen, O., 2013. Divergent and sex-dimorphic expression of the paralogs of the Sox9-Amh-Cyp19a1 regulatory cascade in

developing and adult Atlantic cod (*Gadus morhua* L.). Molecular Reproduction and Development 80, 358-370.

Kelton, D., Lysecki, C., Aukema, H., Anderson, B., Kang, J. X., Ma, D.W.L., 2013. Endogenous synthesis of n-3 PUFA modifies fatty acid composition of kidney phospholipids and eicosanoids levels in the fat-1 mouse. Prostaglandins, Leukotrienes and Essential Fatty acids 89, 169-177.

Kobayashi, M., Aida, K., Stacey, N.E., 1991. Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zoological Science 8, 389-393.

Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxigen species and sperm physiology. Reviews of Reproduction 2, 48-54.

Lenzi, A., Gandini, L., Maresca, V., Rago, R., Sgrò, P., Dondero, F., Picardo, M., 2000. Fatty acid composition of spermatozoa and inmature germ cells. Molecular Human Reproduction 6, 226-231.

Marra, C.A., Alaniz, M.J.T., 1989. Influence of testosterone administration on the biosynthesis of unsaturated fatty acids in male and female rats. Lipids 24, 1014–1019.

Martín, M.V., Rodríguez, C., Cejas, J.R., Pérez, M.J., Jerez, S., Lorenzo, A., 2009. Body lipid and fatty acid composition in male gilthead sea bream broodstock at different stages of the reproductive cycle: effects of a diet lacking n-3 and n-6 HUFA. Aquaculture Nutrition 15, 60-72.

Mazzeo, I., Peñaranda, D.S., Gallego, V., Hidahl, J., Noarizadeh-Lillabadi, R., Asturiano, J.F., Pérez, L., Weltzien, F.A., 2012. Variations in the gene expression of zona pellucida proteins, zpb and zpc, in female European eel (*Anguilla anguilla*) during induced sexual maturation. General and Comparative Endocrinology 178, 338-346.

Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R., Tveiten, H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature modulates the progression of vitellogenesis in European eel. Aquaculture, *in press*.

Melo, V., Cuamatzi, O., 2007. Bioquímica de los procesos metabólicos, Second ed. Reverte, S.A., Barcelona. ISBN: 968-6708-61-8. (In Spanish).

Miura, T., Miura, C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiology and Biochemistry 28, 181-186.

Miura, T., Higuchi, M., Ozaki, Y., Ohta, T., Miura, C., 2006. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. Proceedings of the National Academy of Sciences 103, 7333-7338.

Miura, T., Yamauchi, K., Nagahama, Y., Takahashi, H., 1991a. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zoology Science 8, 63–73.

Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991b. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci. USA 88, 5774–5778.

Monroig, O., Tocher, D.R., Navarro, J.C., 2013 Biosynthesis of polyunsaturated fatty acids in marine invertebrates: Recent advances in molecular mechanisms. Marine Drugs 11, 3998-4018.

Müller, T., Baska, F., Niklesz, C., Horn, P., Váradi, B., Bercsényi, M., 2005. The testis histology of artificially matured European eel (*Anguilla anguilla* L.) at the end of sexual maturation, and spermatozoa ultrastructure in freshwater rearing. Acta Biologica Hungarica 56, 169–172.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. International Journal of Developmental Biology 38, 217-229.

Nagata, C., Takatsuka, N., Kawakami, N., Shimizu, H., 2000. Relationships between types of fat consumed and serum estrogen and androgen concentrations in Japanese men. Nutrition and Cancer 38, 163–7.

Navas, J.M., Mañanós, E., Thrush, M., Ramos, J., Zanuy, S., Carrillo, M., Zohar, Y., Bromage, N., 1998. Effect of dietary composition on vitellogenin, 17β-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (*Dicentrarchus labrax* L.). Aquaculture 165, 65-79.

Nielsen, T.; Prouzet, P. 2008. Capture-based aquaculture of the wild European eel (*Anguilla anguilla*). In A. Lovatelli and P.F. Holthus (eds). Capture-based aquaculture. Global overview. FAO Fisheries Technical Paper. No. 508. Rome, FAO. pp. 141-168.

Norambuena, F., Estevéz, A., Mañanós, E., Bell, J.G., Carazo, I., Duncan, N., 2013. Effects of graded levels of arachidonic acid on reproductive physiology of Solea sole

(*Solea senegalensis*): Fatty acid composition, prostaglandins and steroid levels in the blood of broodstock bred in captivity. General and Comparative Endocrinology 191, 92–101.

Norambuena, F., Mackenzie, S., Bell, J.G., Callol, A., Estevéz, A., Duncan, N., 2012. Prostaglandin (F and E, 2-and 3-series) production and cyclooxygenase (COX-2) gene expression of wild and cultured broodstock of Senegalese sole (*Solea senegalensis*). General and Comparative Endocrinology 177, 256–262.

Pérez, L., Asturiano, J.F., Tomás, A., Zegrari, S., Barrera, R., Espinós, F.J., Navarro, J.C., Jover, M., 2000. Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. Journal of Fish Biology 57, 1488-1504.

Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., Van den Thillart, G.E.E.J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*). General and Comparative Endocrinology 174, 51-59.

Peñaranda, D.S., Mazzeo, I., Hidahl, J., Gallego, V., Nourizadeh-Lilabadi, R., Pérez, L., Asturiano, J.F., Weltzien, F.A., 2013. Molecular characterization of three GnRH receptor paralogs in the European eel, *Anguilla anguilla*: tissue distribution and changes in transcript abundance during artificially induced sexual development. Molecular and Cellular Endocrinology 369, 1-14.

Peñaranda, D.S., Morini, M., Vílchez, M.C., Gallego, V., Mazzeo, I., Dirks, R.P., Van den Thillart, G.E.E.J.M., Tveiten, H., Pérez, L., Asturiano, J.F., 2013. Temperature affects the European eel spermiation modulating the testis steroidogenesis. 17th International Congress of Comparative Endocrinology. ICCE 2013. Barcelona, 2013. Book of abstracts (digital).

Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F., 2010. Molecular and physiological study of the artificial maturation process in European eel males: from brain to testis. General and Comparative Endocrinology 166, 160-171.

Rossato, M., Merico, M., Bettella, A., Bordon, P., Foresta, C., 2001. Extracellular ATP stimulates stradiol secretion in rat Sertoli cells in vitro: modulation by external sodium. Molecular and Cellular Endocrinology 178, 181-187.

Schulz, R.W., França, L.R., Lareyre, J.J., Le Gac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. General and Comparative Endocrinology 165, 390-411.

Schulz, R.W., 1985. Measurement of five androgens in the blood of immature and maturing male rainbow trout, *Salmo gairdneri* (Richardson). Steroids 46, 717-726.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiology and Biochemistry 26, 43–56.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tveiten, H., Frantzen, M., Scott, A.M., Scott, A.P., 2010. Synthesis of 17, 20beta, 21-trihydroxypregn-4-en-3-one by ovaries of reproductively mature Atlantic cod *Gadus morhua*. Journal of Fish Biology 77, 33-53.

Van Ginneken, V., Van den Thillart, G., 2000. Eel fat stores are enough to reach the Sargasso. Nature 403, 156-157.

Wade, M.G., Van der Kraak, G., Gerrits, M.F., Ballantyne, J.S., 1994. Release and steroidogenic actions of polyunsaturated fatty acids in the goldfish testis. Biology of Reproduction 51, 131-139.

Whates, D.C., Abayasekara, D.R.E., Aiken, R.J., 2007. Polyunsaturated fatty acids in male and female reproduction. Biology of Reproduction 77, 190-201.

Zhang, Z., Benson, B., Logan, J.L., 1992. Dietary fish oil delays puberty in female rats. Biology of Reproduction 47, 998-1003.

Table legend

Table 1. Plasma levels (in ng/mL) of different hormones throughout the different eel testis development stages**:** Testosterone (n_{S1}= 94; n_{S2}= 43; n_{S3}= 26; n_{S4} = 24; n_{S5}= 39); 11-Ketotestosterone (n_{S1}= 81; n_{S2}= 43; n_{S3}= 25; n_{S4} = 23; n_{S5}= 36); 17β-Estradiol (n_{S1}= 82; n_{S2}= 45; n_{S3}= 25; n_{S4} = 23; n_{S5}= 40); DHP (n_{S1}= 95; n_{S2}= 44; n_{S3}= 28; n_{S4} = 25; n_{S5}= 45). Small letters show significant differences in each hormone plasma levels between different stages of testis development. Results show as mean ± SEM (P<0.05).

Table 2. Correlations between liver fatty acids and steroidogenic hormones during different testis development stages. Asterisks indicate significant correlations (*, P<0.05; **, P<0.01). Number of samples correlated in each stage, n_{S1}= 80-95; n_{S2}= 44.

Table 3. Correlations between testis fatty and steroidogenic hormones during different testis development stages. Asterisks indicate significant correlations (*, P<0.05; **, P<0.01). Number of samples correlated in each stage: n_{S3}= 24; n_{S5}= 39.

Table 4. Correlations between liver fatty acids in different stages of testis development. *Indicate significant correlations between fatty acids in each development stage (*P<0.05; **P<0.01). Number of samples correlated: n_{S1}= 95; n_{S2}= 33 n_{S3}= 26; n_{S4}= 24; n_{S5}= 38.

Table 5. Correlations between testis fatty acids in different stages of testis development. *Indicate significant correlations between fatty acids in each development stage (*P<0.05; **P<0.01). Number of samples correlated: n_{S2}= 17; n_{S3}= 24 n_{S4}= 24; n_{S4}= 38.

Table 1

Hormone (ng/mL)	Stage of testis development				
	S1	S2	S3	S4	S5
Testosterone	4.28^{c}	3.90^{c}	3.23^{c}	2.26^{b}	1.64^{a}
11-Ketotestosterone	77.94^{c}	71.51^{c}	35.05^{b}	22.90^{b}	12.32^{a}
17β-Estradiol	1.74^{b}	0.97^{a}	0.76^{a}	0.76^{a}	0.76^{a}
DHP	0.59^{a}	0.97^{b}	1.16^{b}	1.06^{b}	1.02^{b}

Table 2

Liver fatty acids	Stage of testis development		
	S1		S2
	17β-Estradiol	DHP	DHP
16:0	-.276*	.321**	.350*
16:1	-.329**		
18:1n9	-.228*		
18:2n6	-.250*	.275**	
18:3n3		.216*	
ARA		.226*	
DHA		.222*	

Table 3

Testis fatty acids	Stage of testis development			
	S3		S5	
	Testosterone	11-KT	Testosterone	11-KT
16:0		.502*		
16:1	.488*	.548**		
EPA			.359*	
DHA			.404**	.407**

Table 4

Liver fatty acids	Stage 1	Satge 2	Stage 3	Stage 4	Stage 5
16:0 – 16:1	.498**	.457**	.877**	.529**	.665**
16:0 – 18:0	.234*		.587**	.407*	
16:0 – 18:1-n9	.594**	.447**	.813**	.684**	.556**
16:0 – 18:2-n6	.577**			.469*	.344*
16:0 – 20:1	.225*	.382*	.771**		.511**
16:0 – 18:3-n3	.435**			.529**	.328*
16:0 – ARA	-.145				
16:0 – EPA	.387**				.578**
16:0 – 22:5-n3	.407**			.455*	
16:0 - DHA	.205*				.408**
16:1 – 18:0	-.416**	-.353*			
16:1 – 18:1-n9	.772**	.673**	.851**	.602**	.638**
16:1 – 18:2-n6	.476**	.448**	.416*	.502*	.462**
16:1 – 20:1	.475**	.427**	.823**	.645**	.729**
16:1 – 18:3-n3	.392**	.477**		.597**	.474**
16:1 – ARA	-.401**	-.460**	-.472*		-.508**
16:1 – EPA	.244*			.583**	.547**
16:1 – 22:5-n3	.522**	.335*		.418*	.418**
16:1 - DHA	.254*				.353*
18:0 – 18:1-n9			.477*		
18:0 – 18:2-n6		-.375*			
18:0 – 20:1		.322*	.602**		
18:0 – 18:3-n3		-.429**			
18:0 – ARA	.421**				.572**
18:0 – EPA		-.393**			
18:0 – 22:5-n3	-.301**	-.425**	-.404*		-.323*
18:0 - DHA	-.229*	-.449**	-.467*		
18:1-n9 – 18:2-n6	.640**	.338*	.433*	.794**	.577**
18:1-n9 – 20:1	.444**	.494**	.743**		
18:1-n9 – 18:3-n3	.560**	.345*		.623**	.531**
18:1-n9 – ARA	-.465**	-.506**	-.384*		-.332*
18:1-n9 – EPA					.410**
18:1-n9 – 22:5-n3	.569**			.648**	.356*
18:1-n9 - DHA	.				.420**
18:2-n6 – 20:1					
18:2-n6 – 18:3-n3	.834**	.879**	.911**	.877**	.761**
18:2-n6 – ARA					
18:2-n6 – EPA	.427**			.446*	
18:2-n6 – 22:5-n3	.550**	.417**	.433*	.693**	.532**
18:2-n6 - DHA	.400**			.709**	.550**
20:1 – 18:3-n3		-.330*			
20:1 – ARA	-.600**	-.462**		-.584**	-.526**
20:1 – EPA		-0.1			.492**
20:1 – 22:5-n3		-.311*			
20:1 - DHA	-.272**	-.467**			

Liver fatty acids	Stage 1	Satge 2	Stage 3	Stage 4	Stage 5
18:3-n3 – ARA	-0.17				
18:3-n3 – EPA	.468**	.374*	.494**	.646**	.320*
18:3-n3 – 22:5-n3	.576**	.568**	.507**	.715**	.593**
18:3-n3 - DHA	.439**	.420**	.423*	.765**	.438**
ARA – EPA					
ARA – 22:5-n3	-.217*		-.407*		
ARA - DHA	.275**	.318*			
EPA – 22:5-n3	.408**	.432**		.495*	.470**
EPA – DHA	.596**	.651**	.636**	.742**	.635**
22:5-n3 - DHA	.651**	.730**	.561**	.760**	.789**

Table 5

Testis fatty acids	**Satge 2**	**Stage 3**	**Stage 4**	**Stage 5**
16:0 – 16:1	$.775^{**}$	$.856^{**}$	$.960^{**}$	$.926^{**}$
16:0 – 18:0	$.794^{**}$	$.556^{**}$	$.690^{**}$	$.518^{**}$
16:0 – 18:1-n9	$.827^{**}$	$.687^{**}$	$.949^{**}$	$.933^{**}$
16:0 – 18:2-n6	$.677^{**}$	$.548^{**}$	$.908^{**}$	$.905^{**}$
16:0 – 20:1	$.640^{**}$		$.902^{**}$	$.829^{**}$
16:0 – 18:3-n3		$.666^{**}$	$.914^{**}$	$.896^{**}$
16:0 – ARA				$-.388^{**}$
16:0 – EPA				
16:0 – 22:5-n3	$.696^{**}$	$.843^{**}$	$.940^{**}$	$.923^{**}$
16:0 - DHA		$.578^{**}$		
16:1 – 18:0	$.602^{*}$		$.620^{**}$	$.382^{*}$
16:1 – 18:1-n9	$.768^{**}$	$.842^{**}$	$.953^{**}$	$.977^{**}$
16:1 – 18:2-n6	$.689^{**}$	$.739^{**}$	$.909^{**}$	$.937^{**}$
16:1 – 20:1	$.674^{**}$	$.668^{**}$	$.956^{**}$	$.935^{**}$
16:1 – 18:3-n3	$.609^{**}$	$.761^{**}$	$.920^{**}$	$.876^{**}$
16:1 – ARA				$-.639^{**}$
16:1 – EPA				$-.422^{**}$
16:1 – 22:5-n3		$.741^{**}$	$.912^{**}$	$.886^{**}$
16:1 - DHA				$-.360^{*}$
18:0 – 18:1-n9	$.918^{**}$	$.485^{*}$	$.720^{**}$	$.427^{**}$
18:0 – 18:2-n6	$.854^{**}$	$.433^{*}$	$.731^{**}$	$.441^{**}$
18:0 – 20:1			$.557^{**}$	$.358^{*}$
18:0 – 18:3-n3	$.589^{*}$		$.656^{**}$	$.428^{**}$
18:0 – ARA				
18:0 – EPA				$.315^{*}$
18:0 – 22:5-n3	$.783^{**}$	$.477^{*}$	$.725^{**}$	$.519^{**}$
18:0 - DHA				$.321^{*}$
18:1-n9 – 18:2-n6	$.942^{**}$	$.943^{**}$	$.983^{**}$	$.977^{**}$
18:1-n9 – 20:1		$.769^{**}$	$.896^{**}$	$.898^{**}$
18:1-n9 – 18:3-n3	$.663^{**}$	$.793^{**}$	$.952^{**}$	$.916^{**}$
18:1-n9 – ARA		$-.411^{*}$		$-.578^{**}$
18:1-n9 – EPA				$-.429^{**}$
18:1-n9 – 22:5-n3	$.831^{**}$	$.708^{**}$	$.952^{**}$	$.917^{**}$
18:1-n9 - DHA				$-.330^{*}$
18:2-n6 – 20:1		$.655^{**}$	$.832^{**}$	$.843^{**}$
18:2-n6 – 18:3-n3	$.813^{**}$	$.832^{**}$	$.964^{**}$	$.946^{**}$
18:2-n6 – ARA				$-.514^{**}$
18:2-n6 – EPA				$-.446^{**}$
18:2-n6 – 22:5-n3	$.854^{**}$	$.667^{**}$	$.951^{**}$	$.922^{**}$
18:2-n6 - DHA				$-.327^{*}$
20:1 – 18:3-n3			$.806^{**}$	$.719^{**}$
20:1 – ARA	$-.520^{*}$	$-.675^{**}$		$-.709^{**}$
20:1 – EPA				$-.484^{**}$
20:1 – 22:5-n3			$.829^{**}$	$.814^{**}$
20:1 - DHA				$-.396^{**}$
Testis fatty acids	**Satge 2**	**Stage 3**	**Stage 4**	**Stage 5**
18:3-n3 – ARA				$-.410^{**}$

18:3-n3 – EPA				
18:3-n3 – 22:5-n3	.663**	.741**	.940**	.900**
18:3-n3 - DHA				
ARA – EPA	.595*	.633**		.738**
ARA – 22:5-n3				-.367*
ARA - DHA	.687**	.752**	.438*	.699**
EPA – 22:5-n3				
EPA – DHA	.712**	.465*	.686**	.847**
22:5-n3 - DHA	.512*	.661**		

Figure(s)

Figure legend

Figure 1. Histological sections of testis in different development stages. A, B: testis at stage 1; C: testis at stage 2; D: testis at stage 3, E: testis at stage 4; F: testis at stage 5. Scale bar: A, E= 100µm; B, C, D= 50µm; F= 200µm. SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: spermatozoa.

Figure 1