Roles of lipids and fatty acids through the spermatogenesis of European eel (*Anguilla anguilla*)

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UNIVERSITAT POLITÈCNICA DE VALÈNCIA

D. **Juan F. Asturiano**, Profesor Titular de la Universitat Politècnica de València.

Informa:

De que la Tesis Doctoral titulada **Roles of lipids and fatty acids through the spermatogenesis of European eel (*Anguilla anguilla*)** ha sido realizada por Dña. **Rosa Baeza Ariño** en el Departamento de Ciencia Animal bajo su dirección y que, una vez revisado y comprobado el trabajo, considera que reúne los requisitos necesarios para la obtención del grado de Doctor, por lo que autoriza su presentación.

Y para que así conste firman el presente informe en Valencia a veintisiete de Marzo de dos mil quince.

Fdo. Dr. Juan F. Asturiano

...Avanzar por caminos construidos

es negarse la oportunidad

de construir nuevos caminos...

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Desde bien pequeña he tenido miedo al mar. Con el tiempo descubrí que no era miedo lo que sentía sino curiosidad. Era todo un misterio para mí lo que ocultaba el océano y esa intriga a lo desconocido, es lo que de pequeña confundía con miedo. No todo el mundo es capaz de enfrentarse a sus temores y yo me siento muy afortunada de haber tenido la oportunidad de que aquello que tanta expectación me ha causado siempre, se haya convertido en el área de estudio de mi tesis doctoral. Algunas personas han sido partícipes de que lo consiga y otras, han formado parte de estos años que para mí han sido toda una aventura. Todas ellas han colaborado para que señale esta etapa de mi vida como "inolvidable" y es por eso que siento la necesidad de agradecérselo.

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Summary

During the last 25 years, eel populations have declined considerably, this species is considered outside safe biological limits. The life cycle of the eel is quite complex including transoceanic migration whose conditions are still so unknown that even the natural spawning process has never been observed.

Eels cease feeding during migration and that is why their energy reserves such as lipids play a crucial role and thus their in deep study is essential to obtain gametes (eggs and sperm) quality as the first step for their reproduction in captivity. In the last years, many efforts have been directed at the study of the influence of fatty acids on the reproductive performance in females, but not in male eels.

The present manuscript describes the results obtained in two experiments. The first experiment (which led to the three first articles included in the memory) focused on the effect of fatty acids along maturation, their relationship with steroid hormones and their effect on sperm quality parameters. The second experiment involved the comparison of different diets designed, considering the previously acquired insights into the most influential fatty acids on male eel reproduction, and the assessment of their influence on the reproductive performance of males.

The first experiment was used to assess the changes in the fat content, as well as fatty acids, in different tissues of male eels hormonally induced to sexual maturation under different thermal regimes (two variables and one constant). Males finished spermatogenesis earlier with a constant temperature of 20 °C, suggesting that eel spermatogenesis is tightly regulated by temperature. The fat content did not change significantly in the muscle, but was increased in the liver and decreased in testes during testicular development. With regard to fatty acids, during sperm maturation, the liver was highlighted as the main site of synthesis. Finally, EPA, ARA and DHA remained constant in testes, while the level of the rest of fatty acids decreased significantly.

Using samples from the same experiment, the correlation between the main steroid hormones and fatty acids at different stages of testicular development were investigated. Similarly as occurs in mammals, EPA and DHA were highlighted as possible modulators of androgen synthesis. The set of the results obtained suggests new perspectives concerning the functions and interactions between fatty acids and steroids in fish spermatogenesis.

Also, with samples from the first experiment, a study was carried out seeking to establish the possible correlations between different fatty acids and several sperm quality parameters. Correlations between several highly unsaturated fatty acids with parameters such as volume, the percentage of motile sperm and sperm velocity were found.

Finally, with the acquired knowledge so far, a second experiment was carried out designing feeds with different fatty acid percentages in order to evaluate the influence of the diets on sperm quality. The results showed that high levels of DHA and EPA in the diets induce higher volumes and high sperm motility. This study allowed obtaining results applicable to the design of optimum broodstock diets for this species.

Resumen

Durante los últimos 25 años las poblaciones de anguila han disminuido considerablemente, de modo que la especie se considera fuera de los límites biológicos de seguridad. El ciclo vital de la anguila es bastante complejo incluyendo una migración transoceánica cuyas condiciones son todavía tan desconocidas que incluso el proceso natural de desove no se ha observado nunca.

Las anguilas dejan de alimentarse durante la migración y es por ello que sus reservas energéticas en forma de lípidos tienen un papel crucial y su estudio en profundidad es importante para obtener gametos (huevos y esperma) de calidad como primer paso para su reproducción en cautividad. En los últimos años se han dirigido muchos esfuerzos al estudio de la influencia de los ácidos grasos en el rendimiento reproductivo de las hembras, pero no en el de los machos de anguila.

El presente documento describe los resultados obtenidos en dos experimentos. El primer experimento (que dio lugar a los tres primeros artículos incluidos en la memoria) se centró en el efecto de los ácidos grasos durante la maduración, su relación con las hormonas esteroideas y su efecto sobre los parámetros de calidad espermática. El segundo experimento consistió en la comparación de diferentes dietas, diseñadas considerando los conocimientos adquiridos previamente sobre los ácidos grasos más influyentes en la reproducción de machos de anguila, y en la evaluación de su influencia en el rendimiento reproductivo de los machos.

El primer experimento sirvió para evaluar los cambios en el contenido graso, así como en los ácidos grasos, en diferentes tejidos de machos de anguila hormonalmente inducidos a la maduración sexual bajo diferentes regímenes térmicos (dos variables y uno constante). Los machos concluyeron la espermatogénesis más temprano a una temperatura contante de 20 ºC, sugiriendo que la espermatogénesis de anguila está estrechamente regulada por la temperatura. El contenido en grasa no varió significativamente en el músculo, se incrementó en el hígado y bajó en el testículo a lo largo del desarrollo

testicular. Con respecto a los ácidos grasos, durante la maduración del esperma, el hígado destacó como el principal sitio de síntesis. Finalmente, EPA, ARA y DHA permanecieron constantes en el testículo, mientras que el nivel del resto de ácidos grasos bajó significativamente.

Usando muestras del mismo experimento, se investigaron las correlaciones entre las principales hormonas esteroideas y los ácidos grasos en los diferentes estadios del desarrollo testicular. De forma similar a lo que ocurre en mamíferos, EPA y DHA destacaron como posibles moduladores de la síntesis de andrógenos. El conjunto de los resultados obtenidos sugiere nuevas perspectivas sobre las funciones y las interacciones entre los ácidos grasos y los esteroides en la espermatogénesis de peces.

También con muestras del primer experimento, se llevó a cabo un estudio que buscó las posibles correlaciones de los diferentes ácidos grasos con varios parámetros de calidad espermática. Se encontraron correlaciones entre varios ácidos grasos altamente insaturados con parámetros como el volumen, el porcentaje de espermatozoides móviles y la velocidad del esperma.

Finalmente, con los conocimientos adquiridos hasta el momento, se realizó un segundo experimento para el que se diseñaron piensos con diferentes porcentajes de ácidos grasos con el fin de evaluar la influencia de las dietas sobre la calidad del esperma. Los resultados mostraron que niveles altos de DHA y EPA en las dietas inducen mayores volúmenes y una alta motilidad del esperma. Este estudio permitió obtener resultados aplicables al diseño de dietas óptimas para reproductores de esta especie.

Resum

Durant els últims 25 anys les poblacions d´anguila han disminuït considerablement, de manera que l´espècie es considera fora dels límits biològics de seguretat. El cicle vital de l´anguila és bastant complex incloint una migració transoceànica les condicions de la qual són encara tan desconegudes que fins i tot el procés natural de fresa mai ha estat observat.

Les anguiles deixen d'alimentar-se durant la migració i és per això que les seves reserves energètiques en forma de lípids tenen un paper crucial i el seu estudi en profunditat és important per a obtenir gamets (ous i esperma) de qualitat com a primer pas per a la reproducció en captivitat. En els últims anys s´han dirigit molts esforços a l´estudi de la influència dels àcids grassos en el rendiment reproductiu de les femelles, però no en el dels mascles d´anguila.

El present document descriu els resultats obtinguts en dos experiments. El primer (que dona lloc als tres primers articles inclosos en la memòria) es va centrar en l´efecte dels àcids grassos durant la maduració, la seva relació amb les hormones esteroides i el seu efecte sobre els paràmetres de qualitat espermàtica. El segon experiment va consistir en la comparació de diferents dietes, dissenyades considerant els coneixements adquirits prèviament sobre els àcids grasos més influents en la reproducció dels mascles d´anguila, i en l´avaluació de la seva influència en el rendiment reproductiu dels mascles.

El primer experiment va servir per avaluar els canvis en el contingut de gras, així com en els àcids grassos, en els diferents teixits de mascles d´anguila hormonalment induïts a la maduració sexual baix diferents règims tèrmics (dos variables i un constant). Els mascles van concloure la espermatogènesis més prompte a una temperatura de 20 ºC, suggerint que l´espermatogènesis d´anguila està estretament regulada per la temperatura. El contingut en greix, no va variar significativament en el múscul, es va incrementar en el fetge i va baixar en els testicles al llarg del desenvolupament testicular. Pel que fa als àcids grassos, durant la maduració de l´esperma, el fetge va

destacar com al principal lloc de síntesi. Finalment, EPA, ARA y DHA van romandre constants en el testicle, mentre que el nivell de la resta d´àcids grassos va baixar significativament.

Emprant mostres del mateix experiment, es van investigar les correlacions entre les principals hormones esteroides i els àcids grassos en els diferents estadis de desenvolupament testicular. De manera semblant al que ocorre en mamífers, EPA i DHA van destacar com a possibles moduladors de la síntesi d´andròGens. El conjunt dels resultats obtinguts suggerixen noves prespectives sobre les funcions i les interaccions entre els àcids grassos i els esteroides en l´espermatogènesi dels peixos.

També amb mostres del primer experiment, es va dur a terme un estudi que va buscar les possibles correlacions dels diferents àcids grassos amb diversos paràmetres de qualitat espermàtica. Es van trobar correlacions entre diversos àcids grassos altament insaturats amb paràmetres com el volum, el percentatge d´espermatozoides mòbils i la velocitat de l´esperma.

Finalment, amb els coneixements adquirits fins al moment, es va realitzar un experiment per al qual es van dissenyar pinsos mb diferents percentatges d´àcids grassos per tal d´avaluar la influència de les dietes sobre la qualitat de l´esperma. Els resultats van mostrar que nivells alts de DHA i EPA en les dietes indueixen a majors volums i una alta motilitat de l´esperma. Aquest estudi va permetre obtenir resultats aplicables al disseny de dietes òptimes per a reproductors d´aquesta espècie.

GENERAL INTRODUCTION

1. Lipids and fatty acids

Lipids are found in all living creatures and play an essential role in the maintenance of life. Lipids are a diverse group of organic molecules which are characterized mainly by being insoluble in water due to their chemical structure. They tend to be molecules with a high number of carbon atoms, with abundant hydrogen and few oxygen atoms (Melo and Cuamatzi, 2007).

Animal lipids can be broadly classified into two groups: polar and neutral lipids. Polar lipids mainly include phospholipids (PLs) while trylglycerols (TAGs) are the principal components of neutral lipids (Figure 1).

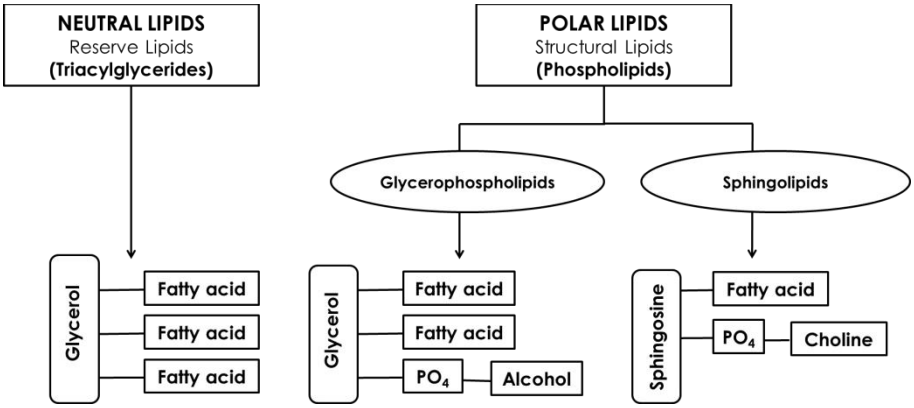

Figure 1. Main lipid classes

PLs is a general term that includes all lipids containing phosphorous. PLs are key components in cellular membranes. PLs can be interconverted when homeoviscous adaptation of biological membranes occurs due to a temperature change. PLs are important precursors for a range of highly biologically active mediators of metabolism and physiology including eicosanoids (Tocher et al., 2008). Eicosanoids are implicated in many physiological processes such as immune and inflammatory responses, neural functions and reproduction (Tocher et al., 2003).

TAGs act as energy storage in animals and are the most abundant lipid class even if they are not components of biological membranes. TAGs contain fatty acids as constituent elements and adipose tissue constitutes that is called "fat depots". Fat depots are ways of storing carbon and energy. TAGs in form of lipoproteins serve as transport, allowing ingested fatty acids to be distributed within the body. This type of lipids, also provides physical protection and thermal insulation to diverse body organs. TAG oxidation generates ATP that causes a lot of metabolic processes (Melo and Cuatzi, 2007).

Both PLs and TAGs are composed of fatty acids. Fatty acids are constituted by a polar tail group (hydrophobic), generally ranging from 14 to 22 carbons and a polar head group (hydrophilic; Figure 2).

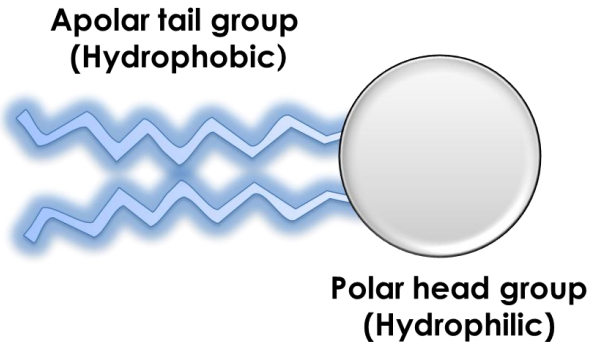

Figure 2. General fatty acid structure

The length of the carbon chain and the site and number of double bonds determine the properties of the fatty acid. In saturated fatty acids (SFAs) all the carbon atoms of the chain are linked by a simple bond, while in unsaturated fatty acids (UFAs) they have one or more double bonds between the carbons forming the chain, and can be classified as monounsaturated fatty acids or MUFAs (one double bond) and polyunsaturated fatty acids (PUFAs) if they have more than one double bond (Figure 3).

PUFAs include highly unsaturated fatty acids or HUFAs (≥3 double bonds, ≥20 carbon atoms). Fatty acids with one or more double bonds are also classified according to the position of the first double

bond from the carboxyl termination. Hence, there are mainly fatty acid series of: n-9, n-6 and n-3.

The conformational structure of the fatty acid molecule determines the properties of the fatty acid. The length of the carbon chain and the site and the number of double bonds determines the properties of the fatty acid. As explained above, SFAs have no double bonds and have a straight structure, whereas UFAs have up to six double bonds and various conformational structures.

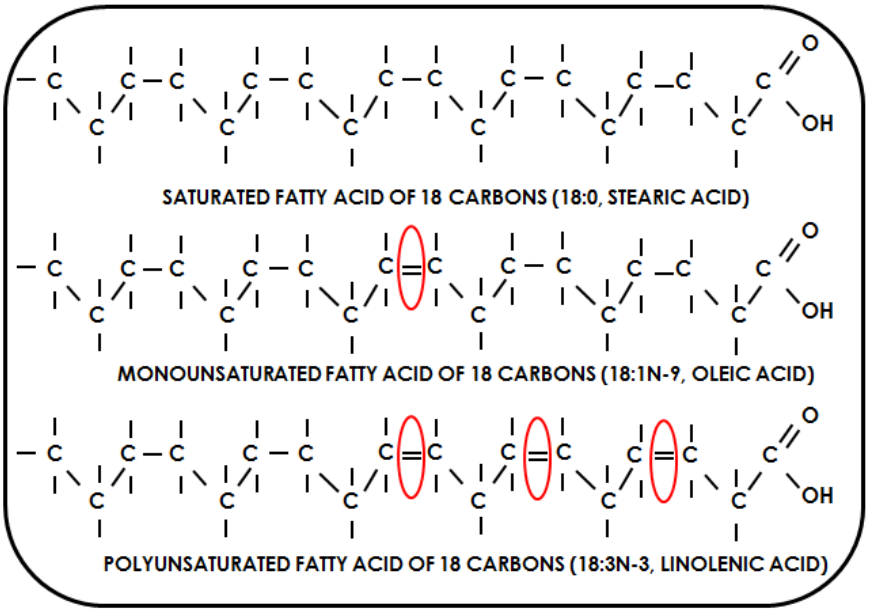

Figure 3. Structure of saturated, mono- and polyunsaturated fatty acids.

The straight molecular structures of SFAs result in close intramolecular interactions and, consequently, in molecules with a high melting point. Fatty acids with double bonds confer molecules with lower melting points as intramolecular interactions are much weaker (Rustan and Drevon, 2005).

Depending on their structure and properties, fatty acids are used for a number of different functions in the body. Some fatty acids are termed essential fatty acids and they need to be included in the diet

because they cannot be synthetized *de novo* in the body. In vertebrates, α-linolenic acid (18:3n-3, LA) and linoleic acid (18:2n-6, ALA) are considered essential fatty acids (Melo and Cuamatzi, 2007).

2. Lipids and fatty acids in fish

Fatty acids and other lipids in fish are essential in many ways, including among others: they affect on growth, reproduction, vision, osmoregulation, membrane fluidity (thermal adaptation), and the immune response (Arts and Kohler, 2009).

The contribution of lipids in the diet of fish, just as in mammals, is fundamental to satisfy the requirements in essential fatty acids. In fish, essential fatty acids are necessary for cellular metabolism (for prostaglandin synthesis and similar compounds) as well as to maintain the integrity of membrane structures (membrane fluidity). Lipids also serve as vectors of liposoluble vitamins and carotenoids upon intestinal absorption. Finally, lipids also play a key role in the energy supply, an important function in fish, because many digest complex carbohydrates poorly (Corraze, 2002).

Of all the effects of fatty acid in fish cited above, most importantly, they are the main source of metabolic energy for growth and reproduction. Several fatty acids (especially SFAs and MUFAs) are heavily catabolized for energy in fish because they are all consumed in large amounts during growth. Tocher (2003) highlighted that, for successful reproduction, the stored oil must support not only the immediate energy requirements of the parent fish but also, the future requirements of the progeny. The high content of HUFAs in embryos and larvae are required for rapid cell proliferation and biomembranes, mainly by brain and nervous tissue (Tocher et al., 1997).

In general, the diverse functions of fatty acids provided by the diet depend on the lipid class to which they belong. In this way, PLs represent a relatively constant part of tissues and consequently of the fish biomass, since they are essentially located in cellular membranes and in the membranes of cellular organelles. TAGs are used mainly for

energy storage and both, PLs and TAGs, through oxidation, represent a source of energy production.

The location of fat depots in fish varies greatly according to species and the following nutritional approach can be differentiated:

- "White" fish (or nonfat) formed by less active species (benthonic species of shallow water), usually tend to be sedentary and detritivores or omnivores.

- "Blue" fish (or fatty) formed by active species (pelagic and good swimmers), with a high development of thruster muscles, generally migratory species, and most being carnivorous.

Many studies have reported that TAGs are much more influenced by dietary fatty acid composition in comparison with PLs. As a result, tissues with a high TAGs content, such as lipid stores, may be more affected by dietary fatty acid composition than tissues which are low in TAGs. PLs are less influenced by dietary fatty acids, confirming their role as a membrane lipid (Budge et al., 2011; Jobling, 2003; Tocher, 2003; Trushenski et al., 2008).

The lipid composition, more than the storage sites and the importance of the reserves, differ considerably between fish and higher terrestrial vertebrates. While the lipids from mammals and birds contain mainly SFAs and MUFAs, fish contain always an elevated percentage of PUFAs. The aquatic medium is characterized by a wealth of PUFAs and particularly of HUFAs. The most important requirements in fish correspond to HUFAs of the -n3 series, contrarily to terrestrial vertebrates. This particular enrichment in fatty acids of the -n3 series in fish have been confirmed to have beneficial effects on human health (Ruxton et al., 2004; Siriwardhana et al., 2012; Wang et al., 2006).

2.1. Essential fatty acids of marine and freshwater fish

Fatty acids requirements are very different when freshwater and marine fish are compared. This difference is related to the distinct enzymatic activity of elongases and desaturases. These enzymes are responsible for both, increasing the number of carbon atoms as well

as, adding double bonds to the precursor molecules of 18 carbon atoms. This ability for elongation and desaturation is considered to be more effective in freshwater fish than in marine fish. Freshwater fish have comparable abilities to mammals and the only really necessary fatty acids for these animals are those of 18 carbon atoms (ALA and LA). For marine fish, HUFAs are essential fatty acids such as eicosapentaenoic acid (20:5-n3, EPA), docosahexaenoic acid (22:6-n3, DHA) and arachidonic acid (20:4-n6, ARA) (Corraze, 2002).

In marine fish, the mechanism of PUFA formation is poorly understood and it has been suggested that marine fish have lost this ability or that it is severally repressed due to the high content of HUFAs already present in their natural diet. Sargent et al. (1995) showed the influence of the dietary availability of long chain PUFAs (LCPUFAs) on marine and freshwater fish, demonstrating that, the conversion of α-linoleic acid (18:3-n3, ALA) to EPA and DHA occurs poorly in marine fish when compared to freshwater fish (Table 1).

Table 1. Bioconversion ability of 18:3-n3 (Kanazawa et al., 1979)

SPECIES	RELATIVE ABILITY OF BIOCONVERSION
Rainbow Trout (*Salmo gairdnerii*)	100
Ayu (*Plecoglossus altivelis*)	36
Eel (*Japanese eel*)	20
Red Seabream (*Chrysophrys major*)	15
Globefish (*Fugu rubripes*)	13
Rockfish (*Sebastiscus marmoratus*)	7

In recent years, numerous studies on the abilities of fatty acid bioconversion in freshwater and marine fish have been carried out (Monroig et al., 2012, 2013; Morais et al., 2012; Fonseca-Madrigal et al., 2014). It has been demonstrated that a freshwater fish may have a marine pattern of fatty acid elongation and desaturation or vice versa.

An interesting case is that of diadromous species such as the species under study in the present thesis, the eel. Diadromous are migratory fish that move between sea and fresh water. The eel is a catadromous species (born in the sea, living in fresh water and reproducing in the sea) which has recently been demonstrated to present a freshwater fish pattern (Wang et al., 2014). Other studies in anadromous species (born in freshwater, living in the sea and reproducing in freshwater), have caused controversy in the conventional classification about the ability of fatty acid bioconversion in marine and freshwater fish (Morais et al., 2009; Monroig et al., 2013).

2.2. Biosynthesis elongation and desaturation

Fish are able to endogenously synthesize SFA 16:0 and 18:0 and desaturate them to 16:1-n7 and 18:1 –n9, by removal of 2 carbon acetyl units (Sargent, 1976). Like other vertebrates, fish have the absolute requirement for –n3 and –n6 PUFAs in their diets such us LA and ALA. They have the ability of desaturation and chain elongation but are incapable of *de novo* synthesis of C_{20} and C_{22} PUFAs.

Sargent et al. (1989) demonstrated that the cytosolic enzyme system "fatty acid synthase (FAS) multienzyme complex" in fish is responsible for the *de novo* biosynthesis of fatty acids of up to C_{16} and, its synthesis occurs mainly in the liver.

Fatty acid desaturation is an aerobic reaction catalyzed by terminal oxygenase ("desaturase"), introducing a double bond(or unsaturation) into fatty acyl chains, whereas fatty acid elongation is a reaction that occurs in four steps, each catalyzed by a specific enzyme ("elongase") and as their name indicates, elongate a preexisting fatty acid chain by two carbon atoms (Tocher, 2003).

DHA rather than EPA is the main end product of desaturation and elongation of ALA, whereas ARA is the end product of desaturation and elongation of LA. The degree of HUFA synthesis from C_{18} PUFA is dependent on enzymatic activities from desaturases and elongases (Bell and Tocher, 2009).

Desaturation and elongation of C_{20} and C_{22} metabolites to their end products ARA, EPA and DHA depend on two crucial enzymes (Δ5 and Δ6). These fatty acids have essential physiological roles. ARA and EPA are precursors of eicosanoids, biologically active compounds that regulate and modulate several physiological processes. DHA is an essential component of cell membrane lipids. The synthesis of LCPUFA occurs in the microsomal fraction of the liver except for the chain shortening from 24:6-n3 to 22:6-n3 and from 24:5-n6 to 22:5-n6, which occurs in the peroxisomes by β-oxidation (Figure 4) (Monroig et al., 2011).

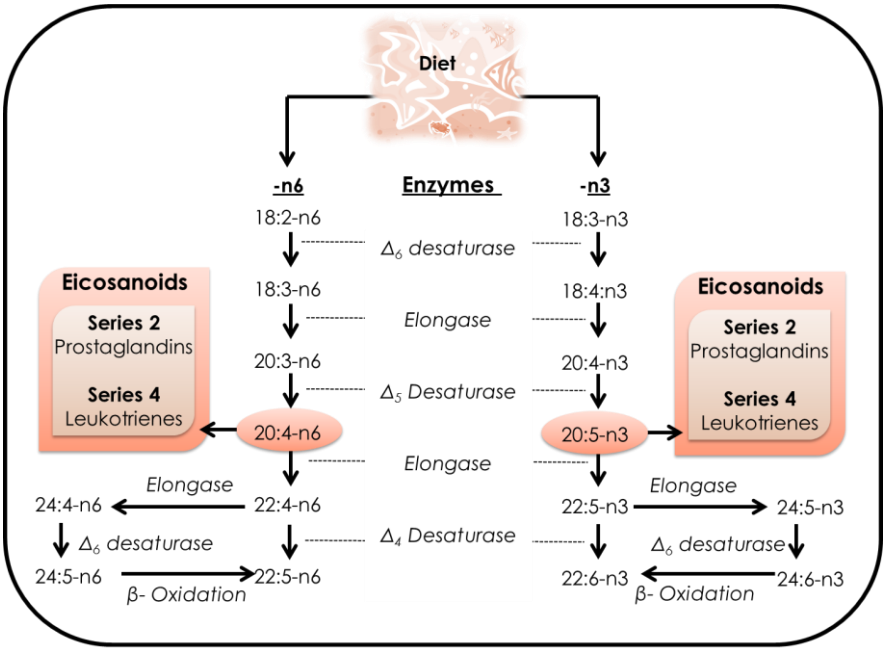

Figure 4. Elongation and desaturation pathways of –n6 and –n3 fatty acids and the precursors of eicosanoids. (Adapted from Monroig et al., 2011)

Recently, an alternative more direct way for DHA biosynthesis has been shown by the presence of fatty acyl desaturase with Δ4 activity, demonstrating that an alternative pathway via direct Δ4-desaturation of 22:5-n3 was possible for the production of DHA from EPA in two marine fish (*Siganus canaliculatus*; Li et al., 2010 and *Solea senegalensis*; Morais et al., 2012).

Diet, as explained above, provides fish with the essential fatty acids for their metabolism. However, in some fish species such as eel, reproduction is associated with a period of starvation when there is no supply of essential fatty acids (explained in section 3.4). In these cases, the species has developed a series of metabolic and behavioral changes in order to adapt to the prevailing conditions. Turchini et al. (2006) represented schematically the whole-body fatty acid balance model when fatty acid intake does not occur, as in the case during eel reproduction. They described that when there is no fatty acid intake, a change in the fatty acid profile occurs in the body through appearance or disappearance by means of: deposition, elongation, desaturation and oxidation (Figure 5, fatty acid metabolic model during fasting highlighted in blue color). This shows that during fasting, fatty acid elongation, endogenous synthesis and desaturation are vital for the fish to meet their needs.

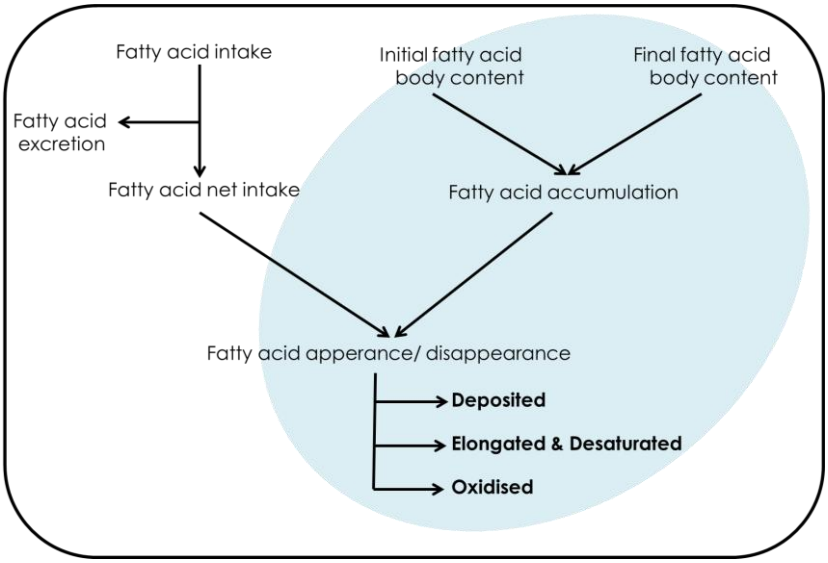

Figure 5. A schematic fatty acid metabolic model (Turchini et al., 2006). Fatty acid metabolic model during fasting (in blue).

2.3 Fatty acids in fish reproduction

Lipids transferred from parents (male and female) to the larvae are the reserves that they use during their development as energy source for their metabolism as well as structural components of their cell membranes. The type and the content of the lipids of these reserves determine larvae quality. Therefore, it is necessary to understand the transfer of energy and structural components to the gonads. The quantity and the quality that will finally be transferred to the eggs determine largely the size of the larvae at the moment of the first feeding and the amount of time available to find food. Both will directly affect their development and survival (Tocher et al., 2010).

During their life cycle, as, for example, the eel, gonad development occurs in a period of no feeding which corresponds to migration. In this case, TAGs are stored in the reserves, which are mobilized to supply energy and used in gonad formation. These reserves play an essential role in reproduction depending on the sex. In the female fish, the vitellogenin is a lipoprotein synthesized in the liver and sent to the ovaries in the early stages of oogenesis. This high density protein can be found along the female eel's development and in the culmination of maturation. It is made of 80% protein and 20% lipids, essentially PLs. The vitellogenin is transported to the ovaries and constitutes the reserve material of the eggs, the yolk. It will be the reserve used during embryonic and larval development (Sargent, 1995). Mainly for this reason, most efforts are focused on elucidating the influence of fatty acids in reproducing females, ignoring the equally important influence of these molecules in male reproduction.

Fatty acids with C_{20}, ARA and EPA, produce eicosanoids whose metabolites, prostaglandins (see Figure 5 in Section 2.2) play an important role in male reproduction. ARA generates 2-series prostaglandins, and EPA competes with ARA in eicosanoid production and is itself converted into 3-series prostaglandins which are less biologically active than those produced by ARA (Sargent et al., 2002). Eicosanoids modulate steroid synthesis and spermiation during sexual maturation (Wade et al., 1994; Asturiano et al., 2000; Norambuena et al., 2013).

Furthermore, constant membrane synthesis is required for spermatozoa production. The spermatozoa membrane contains a high concentration of PUFAs (Whates et al., 2007). Fatty acids are important in male reproduction and it has been demonstrated that they can influence several aspects such as spermiation periods, sperm quality parameters, sperm peroxidation and also the sperm viability in different fish species such as the European eel (*Anguilla Anguilla*; Asturiano et al., 2001; Pérez et al., 2000), gilthead seabream (*Sparus aurata*; Beirão et al., 2012a), Atlantic cod (*Gadus morhua*; Butts et al., 2011), European sea bass (*Dicentrarchus labrax*; Martínez-Páramo et al., 2012).

3. The European eel

3.1 Status of target species

The European eel has a long and complex biological cycle, many of its aspects are still poorly understood or undocumented. In the past 30 years there has been a dramatic decline of the population, which has led to it being declared "outside its safe biological limits". The species is listed as "Critically Endangered" on the IUCN Red List of Threatened Species and has received attention from both the European Union and the Convention on International Trade in Endangered Species of Wild Fauna and Flora. European eel stock has decreased by 95-99%, compared to its levels in 1960-80. Entry of juvenile eels to European waters has also fallen dramatically (Figure 6) (Gollock, 2011; ICES, 2013).

There are a number of suggested causal factors that have been implicated in the decline and impede the management and conservation of the species and can affect every life stage. Overfishing, pollution, parasites, viruses, hydropower station, pumping stations and barriers such as culverts, weirs, bridges, etc. are some of the causes of the decrease (ICES 2013).

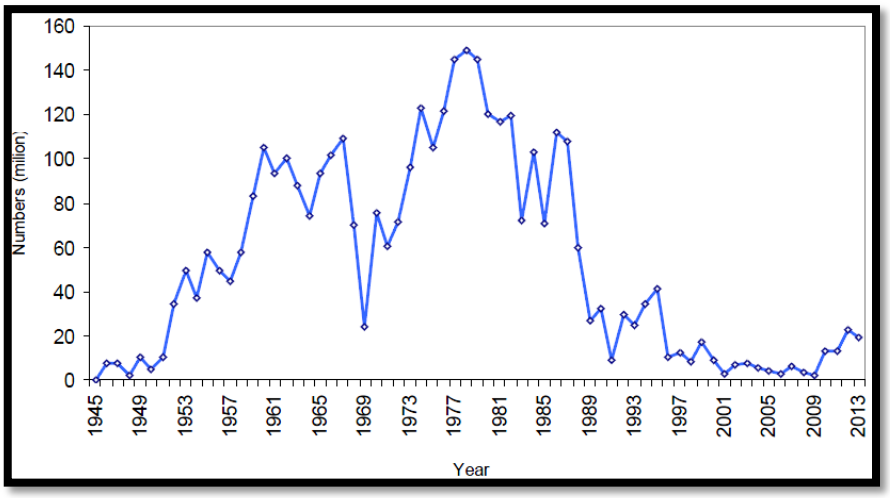

Figure 6. Reported stocking of glass eel in Europe (ICES, 2013).

3.2 The life cycle of the European eel

The life cycle of the European eel is very complex and still poorly understood. In the Sargasso Sea area the smallest larvae have been found and, it is presumably the site where spawning take place.

Larvae (leptocephalus) of progressively larger size are found between the Sargasso Sea and European continental shelf waters. These are shaped like a willow leaf and the head is very small. The body is laterally compressed and is completely transparent. Larvae spend some months drifting on oceanic currents.

While approaching the continent, the leptocephalus turns into a glass eel. At that time its shape is already anguilliform but unpigmented and it grows to 80 mm. Glass eels migrate into coastal waters and estuaries. Upon arrival in estuaries, they go through nine pigmentation stages, ranging from transparent to another fully pigmented stadium. In its first year following recruitment from the ocean, the young eels are called elver (Figure 7).

The elver is metamorphosed into the yellow eel when entering rivers. This stage lasts for up to 20 or more years. In the yellow-eel stage, the eel is characterized by its blackish back, while its belly is yellowish. The

eyes are small. During this stage, eels may occupy freshwater or marine and estuarine areas. This is often defined as a sedentary phase, but migration within and between rivers occurs. Males grow to 200 g and females reach 2000 g).

Sexual differentiation occurs when the eels are partly grown, depending probably on local stock density. The reproductive maturation and migration, metamorphosis takes place by acquiring silver tones, called the silver eel. Their eyes grow with maturation. Female silver eels are twice as large and also may be twice as old as males (Gómez-Juaristi and Salvador, 2011; Tesch, 2003; van de Thillart and Dufour, 2009).

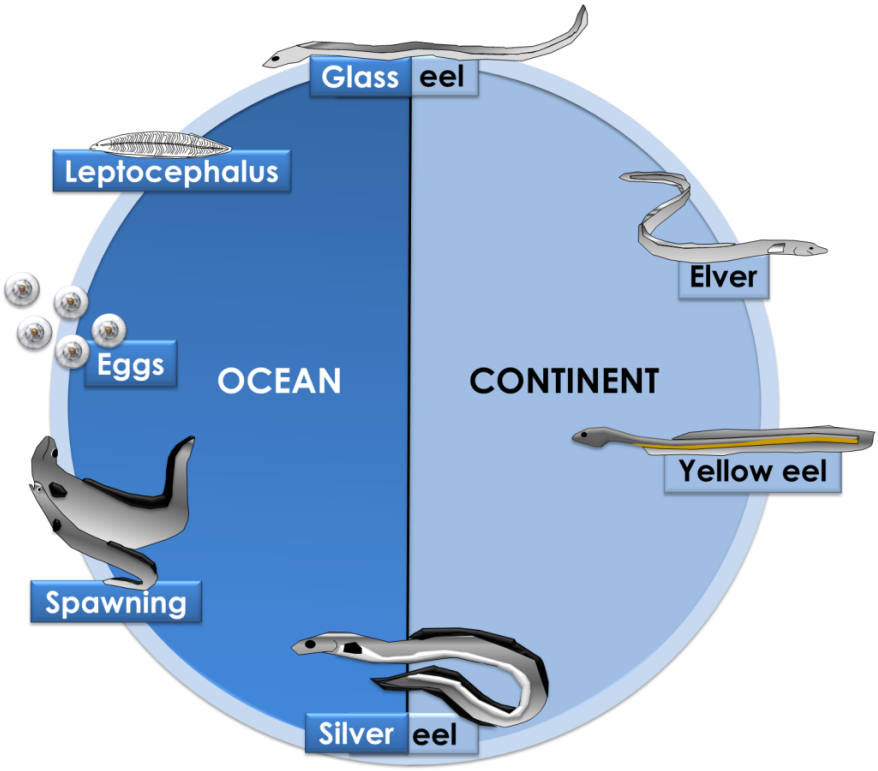

Figure 7. Life cycle of the European eel (*Anguilla anguilla*)

3.3 Eel reproduction

Natural eel reproduction has been characterized since Aristoteles times by something magical, as there were fantastic beliefs about the origin of eels. It was not until the late nineteenth century, when the Italian ichthyologists Grassi and Calandruccio tested in an aquarium that some specimens of a fish known as leptocephalus were transformed in elvers. Subsequently, Dr. Schmidt, after 11 years of tracking down the mysterious leptocephalus, in 1992 showed that the eel reproduction site was the Sargasso Sea. When European eels reach sexual maturation, they undertake a ~5000 km spawning migration from Europe to that area (Figure 8).

Breeding eels leave the continental waters of Europe and the north and taking advantage of the currents from the Canary Islands and the northern equator to arrive, after 6-7 months of traveling at the spawning sites. The return of the leptocephalus larvae occurs following the Gulf Stream and lasts 8-12 months (Ginneken and Maes, 2006).

It is known that the temperature of the probable spawning area in the Sargasso Sea is about 20 °C but, due to the fact that their migration takes several months, it seems probable that gonadal development happens during the journey, at low temperatures, whereas spawning takes place at high temperatures (Mazzeo et al., 2014). Aarestrup et al. (2009) using a miniaturized satellite transmitter followed eels up to 1300 km and provided some interesting information. The speed rate varied from 5 to 25 km/day, and they concluded that the velocity required to reach the spawning areas should be 35 km/day. Consequently, they hypothesized that eels gain travel speed and increase travel efficiency by entering the south and west-flowing currents. In that study it was also elucidated that the eel encountered a diverse range of environments. During the night, eels try to find warm water (300 m deep, and 12 °C approx.), and during the day, eels support cooler temperatures (600 m deep, and 9 °C approx.).

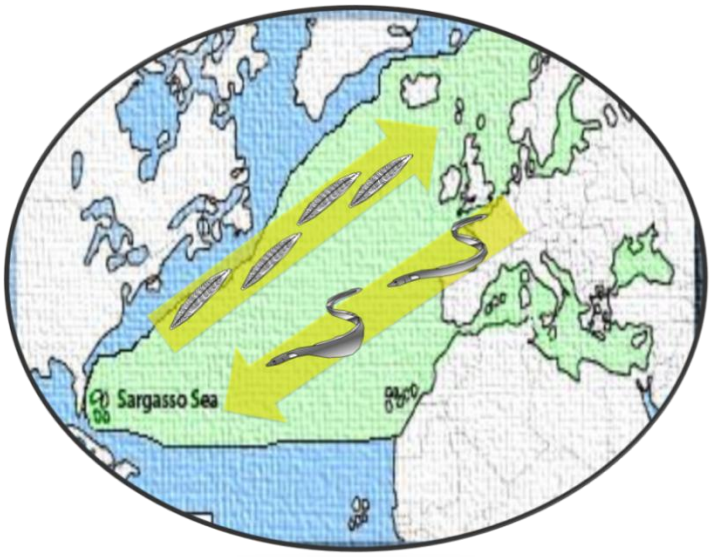

Figure 8. Distribution (in green) and migration route of European eel.

No eggs or spawning adults have been collected in the supposed spawning area and many efforts have been made to try to reproduce this species in captivity. Boëtius and Boëtius (1980) were the first to fertilize the eggs of the European eel and a few years later, Bezdenezhnykh et al. (1984) obtained the first larvae, but they died within a few days after hatching.

In Spain, Asturiano et al. (2002) induced the first ovulation and spawn of the European eel. Later, the EU "PRO-EEL" project, implemented from 2010 to 2014, tried to expand the current knowledge on eel reproduction and develop standardized protocols for production of high quality gametes, viable embryos and feeding larvae of the European eel. Pérez et al., one of the research groups involved in PRO-EEL, achieved the first fertilization, hatching and larvae in Spain, which remained alive for 3-4 days (Figure 9) (Pérez et al., 2012; Vílchez et al., 2014). Finally, Towkiewicz et al. (also as part of the PRO-EEL project) were able to produce larvae, which were keep alive for up to 22 days and carried out pilot feeding experiments (Butts et al., 2014).

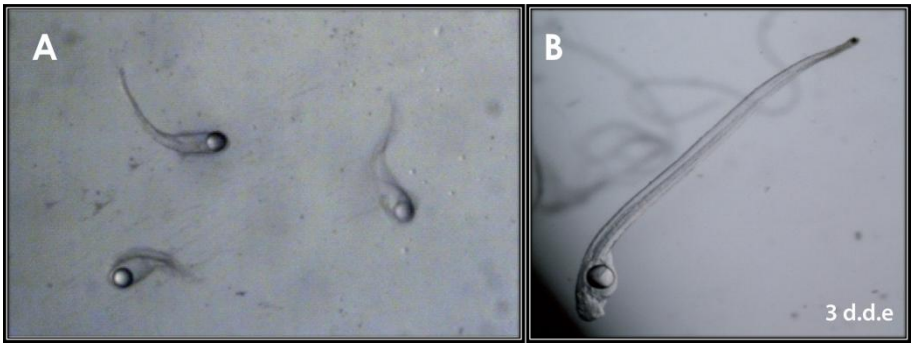

Figure 9. Larvae of *Anguilla anguilla* newly hatched (A) and 3 days after hatching (B) by Pérez et al. (2012).

3.4 The European eel and fatty acids

3.4.1 Energy requirements: migration and fasting.

Lipids are the most energy dense form of energy storage, having a twofold greater energy than protein and carbohydrates. Long distance migrations are energically demanding and feeding during long-distance migrations is rare. When the dietary energy intake is insufficient, fish prioritize gonad production at the expense of somatic growth, and then extract energy from their body reserves. In these fish species that do not feed during migration and reproduction, the energy management is a decisive factor for reproductive success (Binder et al., 2011).

In fish, the energetic requirement during fasting is between 10 to 30 times lower than in mammals, mainly to be ectothermic and ammonotelic, but also due to flotation. One of the more extreme cases is the European eel in which mortality sets in at from three years of fasting (Boëtius & Boëtius, 1967). In this concrete species, reproduction is not limited to a simple production of gametes. In the eel, the development of sexual characters (morphological or color changes), mucus production and the reproductive behavior involve extra energy requirements.

The metabolic response to the fluctuations in food availability varies depending on numerous factors, such as age, size and especially the

species. Following a nutritional approach (defined in section 2), two main patterns in reserve mobilization in fish can be distinguished:

- In "blue fish" in which muscle lipids are the first to be mobilized, causing tissue hydration. Inverse dynamics between the lipid content and the water content in muscle are stablished.

- In "white fish" hepatic or mesenteric lipids (visceral fat) are initially mobilized, and after having been reduced strongly, muscle proteins are used. In these species, protein mobilization from the muscle causes significant tissue hydration, thus an inverse relationship between both parameters occurs, not unlike the relationship between water-lipids in the previous group (Shulman, 1974).

However, reserve mobilization in fasting fish does not always define the previous general classification and, large discrepancies exist. In the case of the eel (following the previous classification, a blue fish) an initial decrease of hepatic lipids is produced and then muscle lipids are mobilized, despite being latter on, the main lipid storage organ of this species (Larsson and Lewander, 1973). In contrast, Moon (1983) observed in one species of eel, an important mobilization in muscle protein without observing changes in hepatic or muscular lipids. Earlier studies and in strong disagreement with the results have coincided in show that reserve mobilization depends on numerous factors and until now, has not been fully elucidated.

However, , something in which the authors coinciding is that the consequences of fasting on the metabolism are more pronounced in larvae being in the early stages than in adult fish, probably due to a smaller amount of energy reserves (Izquierdo, 2006).

3.4.2 Adaptation to environmental conditions

From a physiological point of view, the eel is a particularly popular experimental animal. This is due to many distinctive characteristics: its euryhaline osmoregulation capacities, its homeoviscous membrane adaptation to different temperature rates, its phases of differential activity and behavior patterns, its multistage metamorphosis during

ontogeny, its great endurance and ability to navigate during migration, and lastly, even its unusual body shape.

Consequently, extrinsic causes such as variable temperatures and salinities during migration (see section 3.3), may have an incidence especially in lipid metabolism.

In fish, fatty acids play an important role in maintaining adequate fluidity of the membranes in response to temperature changes, a reaction known as homeoviscous adaptation (Sinensky, 1974). When the temperature increases, membranes tend to be more fluid (PUFAs increase) and when the temperature decreases, the membranes tend to be more rigid (PUFAs decrease). This complex phenomenon is not tissue-specific (Hazel and Williams, 1990).

In addition to this change related to temperature variation, it is known that changes in water salinity affect the length of fatty acid chains and the degree of unsaturation of membrane lipids (Halver, 1980). Ghazali et al. (2013) studying changes in fatty acids during water salinity adaptation in the yellow eel under fasting found that the combined effect of starvation and salinity slightly affected the fatty acid composition in muscle, and concluded that yellow eels regulate energy use in order to establish a constant fatty acid profile in muscle. Finally they suggested that the eel has developed some sort of metabolic independence to cope with salinity changes due to its peculiar life cycle and adaptability.

Both lipid membrane changes should be studied in depth and considered in eel reproduction.

3.4.3 Quality gamete

One of the most important aspects to improve eel reproduction in captivity is obtaining quality gametes (eggs and sperm), and it is therefore essential to consider broodstock feeding given its direct effect on gamete composition.

The importance of lipid composition and fatty acids in eggs of the Japanese eel was evidenced by Furuita et al. (2006), who found that

high-quality eggs presented a lower content of polar lipids, a lower content of ARA and a higher level of DHA. Moreover, eels fattened in captivity presented a higher HUFA content in the liver and ovary, while wild eels presented a higher content of n6-HUFAs in muscle, liver and ovary (Ozaky et al., 2008).

The few works focused on males have conducted that, at least in the European sea bass, that sperm quality is influenced by the PUFA diet profile, through its effect on the synthesis of steroids in the testes (Asturiano, 1999; Asturiano et al., 2000, 2001). However, in the effect of feeding on the sperm quality of males has not been studied neither in the European nor Japanese eel, a relevant issue to achieve larval survival in these species.

3.5 Projects, grants and companies involved in this Thesis

Finally, it is noteworthy that all the studies carried out in this thesis were funded by the PRO-EEL project (Reproduction of European eel towards self-sustained aquaculture) funded by the European Community's 7th Framework Programme under Theme 2 "Food, Agriculture and Fisheries, and Biotechnology" (grant agreement n°245257).

The fish farm Valenciana de Acuicultura, S.A. (Puzol, Spain) supplied all the male eels used in the Chapters I, II and II that were transported to Universitat Politècnica de València. The eels mentioned in the Chapter IV were supplied by Stensgård Eel Farm, A.S. (Denmark) and were transported to an experimental facility of the Technical University of Denmark.

OBJECTIVES

The overall aims of this thesis were to shed light on several unknown aspects relate to the role of fatty acids in male eel reproduction and to apply them practically to improve gamete quality through broodstock diets. We assumed that fatty acids play a crucial role during European eel spermatogenesis and established a series of specific objectives:

- ✓ To study the changes in fat and fatty acids and try to elucidate their dynamics and roles during reproduction (Chapter I).

- ✓ To explore the relationship between fatty acids and steroid hormones to gain new insights into their functions and interactions in fish spermatogenesis (Chapter II).

- ✓ To investigate the effect of fatty acids on sperm quality parameters in order to know which are able to improve gamete quality (Chapter III).

- ✓ To design optimized broodstock diets and study their effect on sperm quality parameters (Chapter IV).

CHAPTER I

Effect of thermal regime on fatty acid dynamics 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 research 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 during hormonally induced sexual maturation. Three different thermal regimes were used (two variable: T10 and T15; and one constant: T20) to replicate the changes in temperature that these fish experience during their transoceanic reproductive migration. Spermatogenesis was reached earlier in treatment T20, suggesting that spermatogenesis in the European eel is closely regulated by water temperature. Although eels lose body mass due to the period of fasting that accompanies gonadal growth, no significant changes were found in the fat and fatty acid content of the muscle during the experimental period.

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

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

1. Introduction

European eel (*Anguilla anguilla* L.) populations have declined steadily. After the high levels of the late 1950s, there was a rapid decrease that continues to the present day (ICES 2011). The causes

for the decline in the eel population include 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 key to their sexual maturation. During their migration they swim in depths of between 200-1000 m (Aarestrup et al., 2009, Tesch, 2003), and thus experience great variations 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 that their migration takes several months, it seems likely that gonadal development happens during the journey, at low temperatures, whereas the spawning takes place at high temperatures. In an attempt to closely imitate what happens in the wild 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) regime, which is the usual method used with eel males. Gonadal maturation and reproduction in many teleost fish, including the European eel, are accompanied by a starvation period in which they carry out long and exhausting migrations. The eel´s fat stores, which can be as much as 30% of their body weight at the silver stage, are essential in order to complete their long reproductive journey to the Sargasso Sea. 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 responsible for regulating membrane fluidity and, temperature may have an effect on its biosynthesis (Dey et al., 1993; Farkas and Csengeri, 1976; Farkas et al., 1994). Fatty acids are also important in the reproduction of male and female fish. In females, the lipids are mainly stored in the muscle and the 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, the composition of the fatty acids provides the sperm cell membrane with the necessary fluidity, conditioning the functionality of the membrane which is linked to the fertilization process (Wathes et al., 2007). Polyunsaturated fatty acids are precursors of eicosanoids, whose metabolites (e.g., prostaglandins) play 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 Labbé, 2010; Glencross, 2009; Heinsbroek et al., 2013; Izquierdo et al., 2001; Watanabe and Vasallo-Agius, 2003). The development of optimum diets for European eel broodstocks to ensure high quality gametes is key if we want to improve the chances of reproduction in captivity. The importance of dietary lipids in reproduction has been extensively described in literature (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, ignoring the effects of diet on sperm and male reproductive performance. Studies of 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 have suggested that fertilization could be affected as a consequence. Asturiano et al. (2001) showed that the diet of the European sea bass male affected the survival of the 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 the European eel. From the different environmental factors which we could mimic in order to gain a deeper understanding of eel reproduction, we decided to focus this study on temperature, as it plays an important role in the 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). The aim of this study therefore was to quantify the fatty acid levels in the muscle, liver and testes during induced sexual maturation of eel males, under different thermal regimes, in order to determine the dynamics of fat and fatty acids under different temperatures and as such which fatty acids are the most important in testis development and spermatogenesis. This information could be used for the development of suitable diets to improve sperm quality, which would in turn improve fertilization success and 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), which were 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 for the duration of 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 carried out in the first two weeks. Three g L^{-1} of commercial aquarium salt per day was added 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 °C first 6 weeks, 15 °C next 3 weeks and 20 °C last 6 weeks); T15, (15 °C first 6 weeks and 20 °C last 9 weeks); and T20, (20 °C throughout the whole experimental period; Fig. 1).

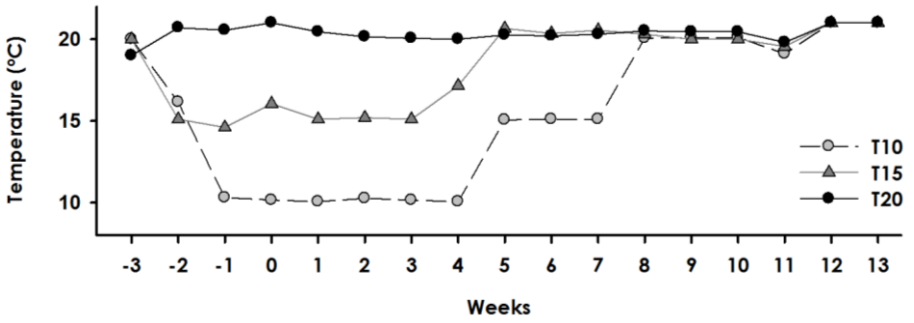

Figure 1. Water temperature treatments (T10, T15 and T20) during induced sexual maturation in male eels. Arrows indicate the first injection with human chorionic gonadotropin (hCG).

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

2.3 Measurements and sampling

When the animals arrived at our facilities, and before starting any treatment, eight specimens were sacrificed to serve as freshwater

controls. Eight fish per treatment were sacrificed weekly during the first eight weeks of the experiment, and 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 7th week, GSI=0.28); T15 (the 4th week, GSI=0.34); and T20 (the 3rd 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 °C 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 an 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 carried out, the results were classified into the different development stages of the testis which had previously been determined by the histology processing.

2.5 Lipid extraction

In order to achieve optimum homogenization, the muscle samples were first lyophilized. Crude fat was extracted using 0.3 g of lyophilized muscle. Fat extraction was carried out using 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. Once the extraction was completed the solvent was condensed in a Soxtec extraction unit (1043, Tecator). The remaining solvent was then evaporated and the recovered fat was left to dry for 2 h at 110 ºC. The water content of the muscle samples was determined in triplicate, by weighing 0.5 g (w/w) of the sample and maintaining this at 110 ºC for 24 h. After this period the samples were weighed again and the weight difference enabled us to calculate the moisture level.

Because of the small size of the testis and liver, a different method for lipid extraction was used and adapted. The total lipids were extracted from the testis and the liver using a modified version of the 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 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^{-1} ultrapure water) to separate it into two layers: one with lipids and another with the non-lipid substances, and the glass tube was preserved in the refrigerator. Between 8 to 48 h later, once the two layers had formed, the upper layer containing the 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 and maintained at -80 °C until synthesis of the fatty acids methyl esters (FAME) was carried out.

2.6 Fatty acid quantification

The fatty acids 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 using 20-30 mg of freeze-dried sample and analysis of the testis and liver using 10-30 mg of extracted oil. First, 1 mL of tridecanoic acid (C13:0) was used as an internal standard (0.5 mg of C13:0 mL^{-1} of methanol). We also added, 0.7 ml of KOH 10 N and 5.3 mL of HPLC quality methanol (High Performance Liquid Chromatography). The tubes were incubated at 55 °C 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 incubated again at 55 °C 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 maintained at -80 °C 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 a flow rate of 20 cm s^{-1}. Samples were injected with a split ratio of 1/100.

The initial oven temperature, set to 140 °C, was held for 5 min and was then increased to 240 by 4 °C min^{-1} before finally being maintained at

that temperature for 30 min. The fatty acids were identified by comparing their retention times with the standards supplied by Supelco. The fatty acid levels are reported as percentages in 100 g of fat. Only those fatty acids which were present at minimum levels of 0.1% in each tissue were considered. In order to quantify the fatty acids, we used the sample weight data from the analysis to calculate the g of fatty acids per 100 g of sample. Subsequently, using the fat content of the sample we transformed this 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. Comparisons of means were carried out using Newman-Keuls 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 thermal 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 treatment over the course of the different stages. These statistical analyses were carried out using Statgraphics Plus® 5.1.

A principal component analysis (PCA) was carried out. First, to estimate the fatty acid distribution in the three tissues and later, to determine the contribution of the individual fatty acids to the different development stages in each tissue. Score plots illustrate the relationship between individual cases (stage groups) and the variables (fatty acids), and help in the analysis of the data by showing graphical associations. Factor scores were analyzed by one-way ANOVA and PCA was performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Analysis of morphometric parameters and testis development stages

With regards to the morphometric parameters, only under the highest temperature regime, T20, did the body weight of the males decrease significantly during the treatment period, from 108.9±5.6 to 77.1±7.1 g (data not shown). In parallel, the weight of the testis increased (Fig. 2) in all the treatments. The mean GSI of fish from treatment T20 increased gradually over the course of the weeks, reaching the highest value (11.3±0.9%) in the 11th week. In treatment T15, the maximum GSI (8.1±0.7%) appeared in the 10th week, while T10 males showed the slowest increase, with GSI levels not becoming significantly higher than the basal levels until week 8.

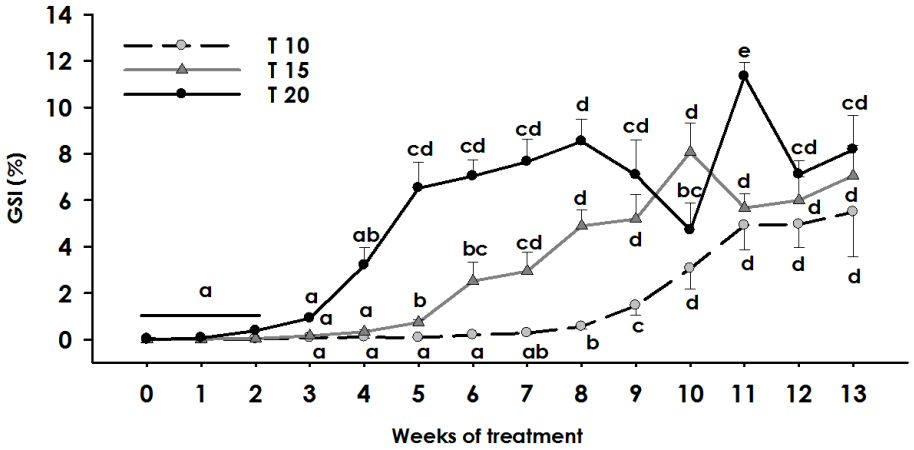

Figure 2. Gonadosomatic index (GSI) throughout hormonal treatment under three thermal regimes (T10, T15, and T20). Number of samples: T10, n0-7 = 8; n8-12 = 5; n13 = 4; T15, n0-7 = 8; n8-13 = 5; T20, n0-7 = 8; n8-13 = 5. Different letters mean significant statistical differences between weeks in each treatment.

There were no significant differences in HSI over the course of the weeks in treatments T10 and T15, and only under the highest temperature regime did HSI increase.

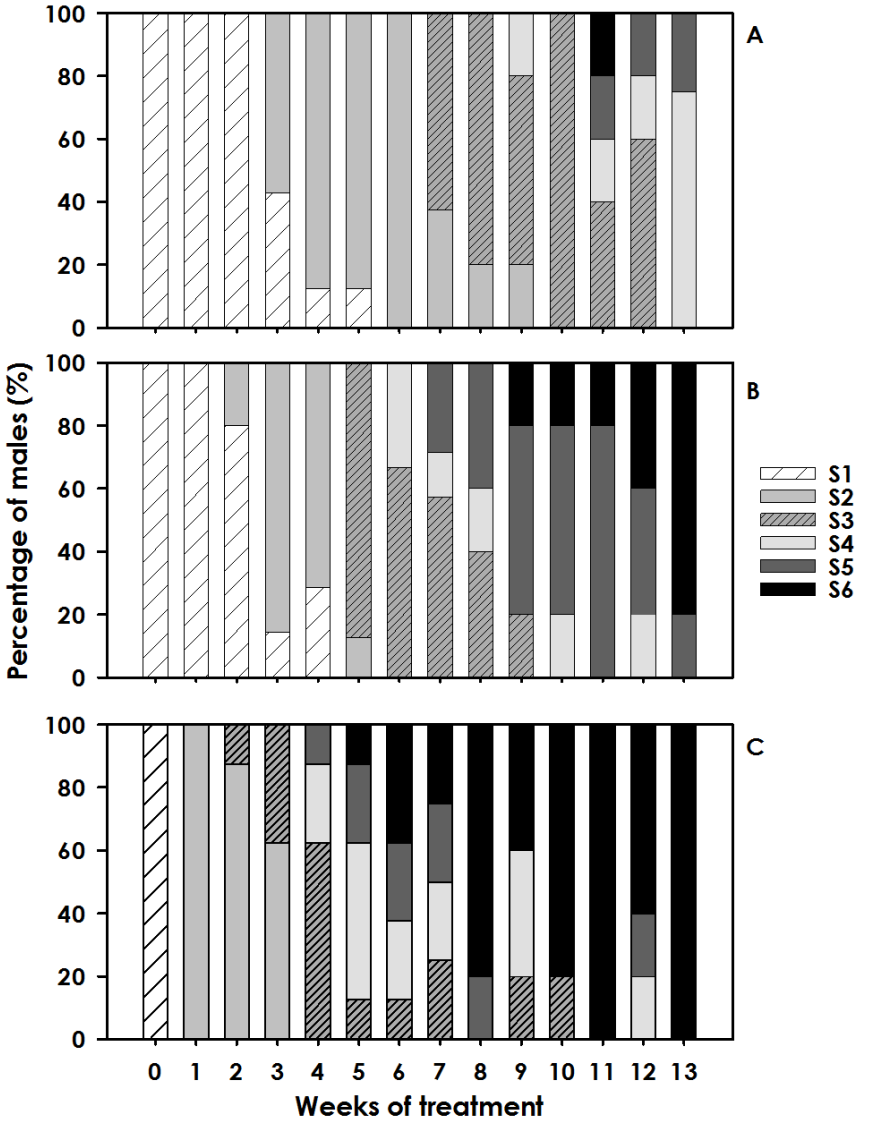

Figure 3. Percentage of fish reaching the different stages of testis development throughout the hormonal treatment in each thermal regime: A) T10; B) T15; C) T20. Number of samples in each stage under different thermal regimes: T10, nS1= 28; nS2= 29; nS3= 22; nS4 = 6; nS5= 3; nS6 = 1; T15, nS1= 19; nS2= 14; nS3= 18; nS4 = 6; nS5= 17; nS6 = 9; T20, nS1= 8; nS2= 19; nS3= 15; nS4 = 13; nS5= 9; nS6 = 28. Stages S1-S6 were described by Peñaranda et al. (2010). Temperature changes are described in Figure 1.

Regarding the evolution of the testis maturation stages (Fig. 3), T10 males reached stage S1 in the first three weeks, with this treatment being the slowest in terms of gonad development. Treatment T15 eels reached S2 from the third week, and spermatozoa (S4) were not found until the 6th week. The treatment with the highest temperature (T20) resulted in the fastest gonad development. In the last week of the test (13th week), all T20 males in the testis were at stage S6.

3.2. Changes in total fat content of the tissues

Treatment T20 induced a sudden and significant (from S2) increase in fat content in the muscle (Fig. 4A1), whereas the differences were not significant in the other treatments.

The evolution of the fat content of the liver throughout all the testis development stages varied greatly in each thermal 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 in fat content (from 14.0±1.4 to 5.0±1.8%) was found from the 7th to 8th week. Between these weeks (7-8th) 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 T10 and T20 fish, while, at S5, T20-treated fish showed higher fat values than T15 treated fish. When we analyzed the differences in the fat content in testis which were at the same stage (S2), we found that the fat content of T15 samples was significantly higher than that of T20 (Figure 4C1).

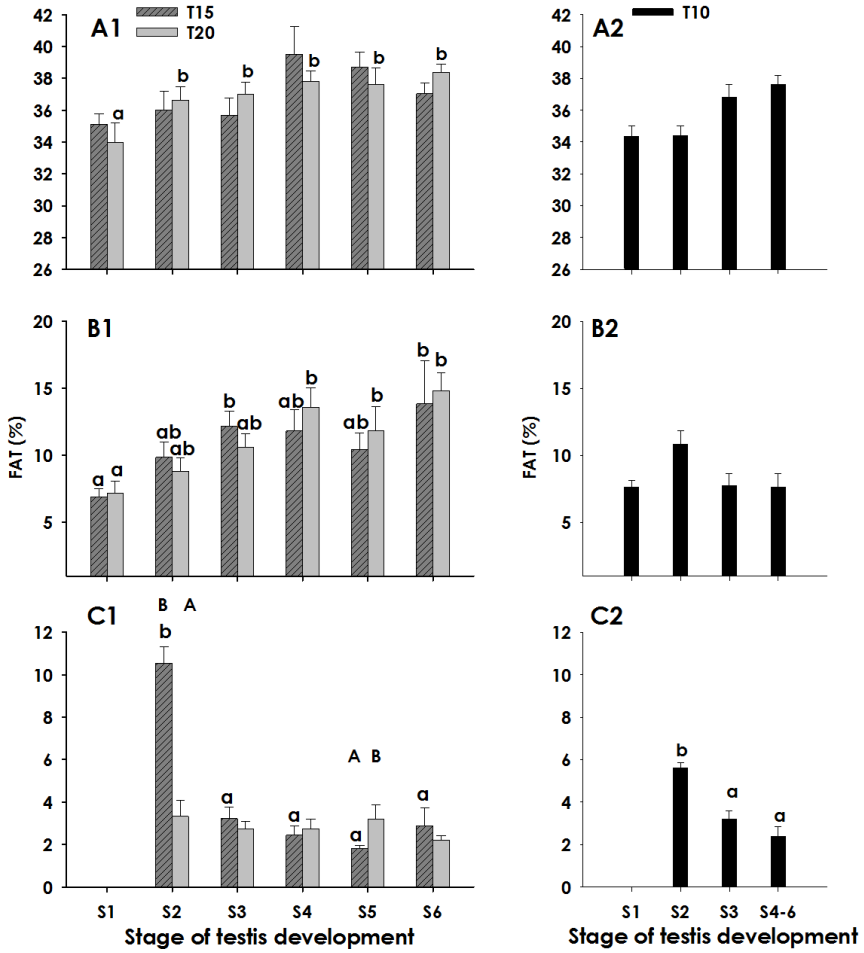

Figure 4. Fat content in percentage of wet weight (% w w) in A1) muscle, B1) liver and C1) testis of T15 and T20 treatment (A2, B2 and C2 shows the same but in T10 treatment with S4-S6 stages grouped as S4-6) shown in relation to the different testis development stages. Small letters show significant differences in the same treatment between the different development stages; capital letters show significant differences in the same stage between different thermal treatments. Results show as mean ± SEM ($P < 0.05$). Number of samples in each tissue under different thermal regimes: Muscle T10, nS1= 28; nS2= 29; nS3= 22; nS4-6 = 10; Muscle T15, nS1= 19; nS2= 14; nS3= 18; nS4 = 6; nS5= 17; nS6 = 9; Muscle T20, nS1= 8; nS2= 19; nS3= 15; nS4 = 13; nS5= 9; nS6 = 28; Liver T10, nS1= 28; nS2= 28; nS3= 22; nS4-6 = 8; Liver T15, nS1= 18; nS2= 14; nS3= 18; nS4 = 6; nS5= 17; nS6 = 8; Liver T20, nS1= 8; nS2= 19; nS3= 15; nS4 = 13; nS5= 9; nS6 = 28; Gonad T10, nS2= 4; nS3= 13; nS4-6= 10; Gonad T15, nS2= 3; nS3= 14; nS4 = 6; nS5= 17; nS6 = 9; Gonad T20, nS2= 5; nS3= 15; nS4 = 10; nS5= 7; nS6 = 26.

3.3 Muscle

Neither significant increases nor decreases in the total amounts of SFA, MUFA and PUFA between the different testis development stages were noticed in any of 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) at approximately 16, 15 and 12%, respectively.

The levels of SFA in the liver increased notably over the course 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.

Figures 5B1 and 5B2 show 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 treatment T10 the highest PUFA values were found in S2 (Fig. 5C2).

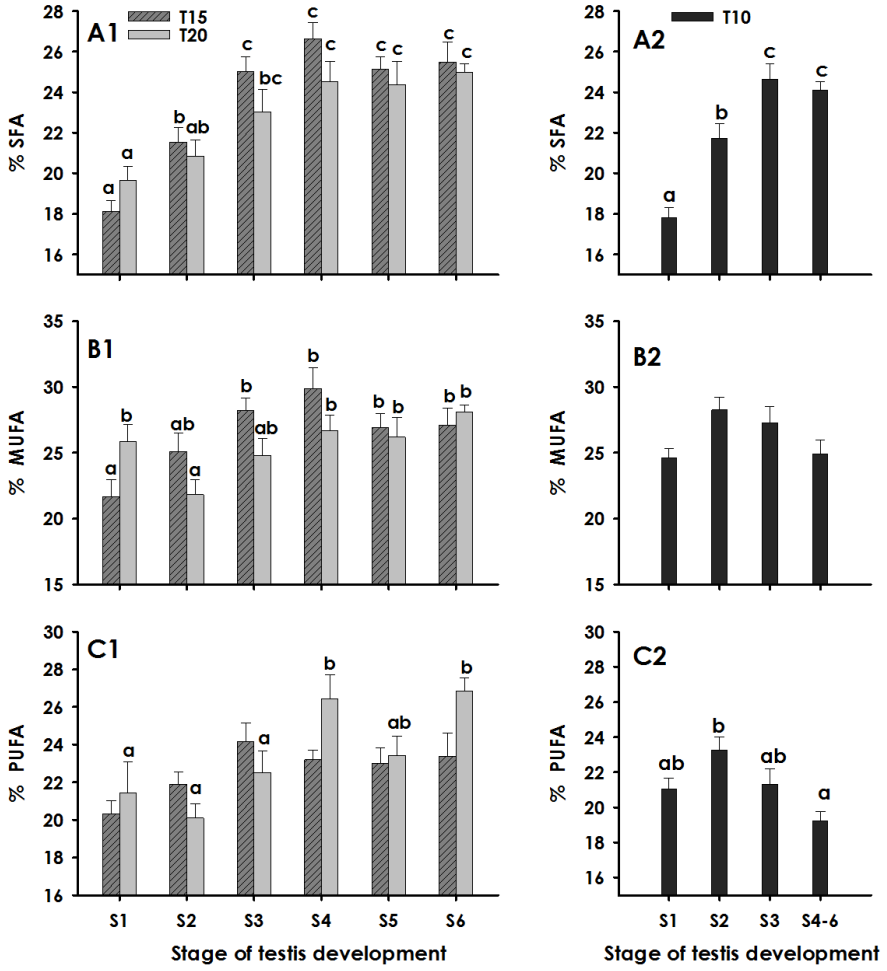

Figure 5. Classes of fatty acids in the liver during the development stages: A1) Saturated fatty acids (SFA); B1) Monounsaturated fatty acids (MUFA) and C1) Polyunsaturated fatty acids (PUFA) in T15 and T20 treatment. A2, B2 and C2 Shows the same but in T10 treatment with S4-S6 grouped as S4-6. Small 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_{S1}= 28; n_{S2}= 28; n_{S3}= 22; $n_{S4\text{-}6}$= 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.

3.5. Testis

Figure 6 shows the evolution of the different fatty acid groups through the testis development stages. 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 stage. 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 latter). T10 treatments showed a significant decrease in all fatty acid classes in the testis over the course of the development stages (Fig 6A2, 6B2 and 6C2). With regards to SFA (Fig 6A1), treatment T20 resulted in a significant decrease coinciding with stages S5 and S6. When we analyzed the differences between treatments at the same development stage, we observed significant differences in PUFA in S3, with treatment T15 levels being higher than those of T20 ($p<0.001$).

3.6. Principal component analyses

The PCA demonstrated different patterns of fatty acid distribution in the testis, liver and muscle. In the first component of the score plot (Fig. 7A), all the muscle samples are located to the right of 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, correspond to the muscle samples.

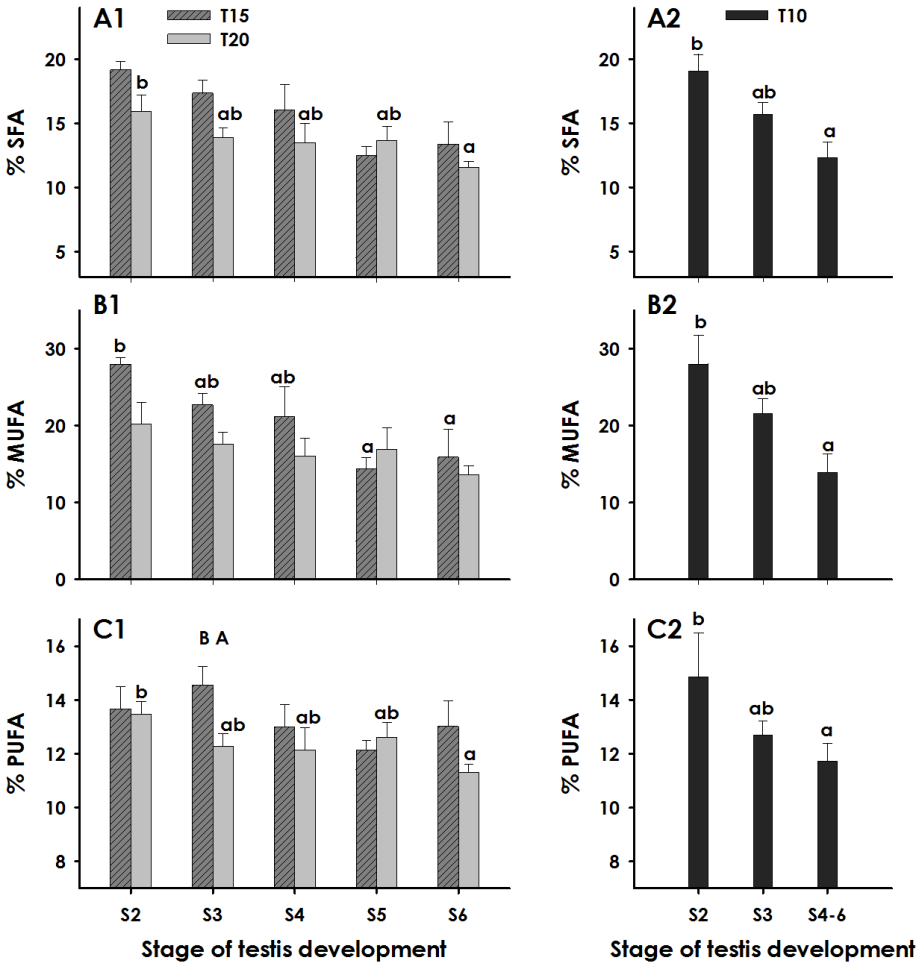

Figure 6. Classes of fatty acids in the gonad during the development stages: A1) Saturated fatty acids (SFA); B1) Monounsaturated fatty acids (MUFA) and C1) Polyunsaturated fatty acids (PUFA) in T15 and T20 treatment. A2, B2 and C2 Shows the same but in T10 treatment with S4-S6 grouped as S4-6. Small 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, $n_{S2}= 4$; $n_{S3}= 13$; $n_{S4\text{-}6} = 10$; T15, $n_{S2}= 3$; $n_{S3}= 14$; $n_{S4} = 6$; $n_{S5}= 17$; $n_{S6} = 9$; T20, $n_{S2}= 5$; $n_{S3}= 15$; $n_{S4} = 10$; $n_{S5}= 7$; $n_{S6} = 26$.

Furthermore, in the score plot (Fig. 7A) all the liver samples are located on the positive axis of the second component meaning 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 correspond to the liver samples.

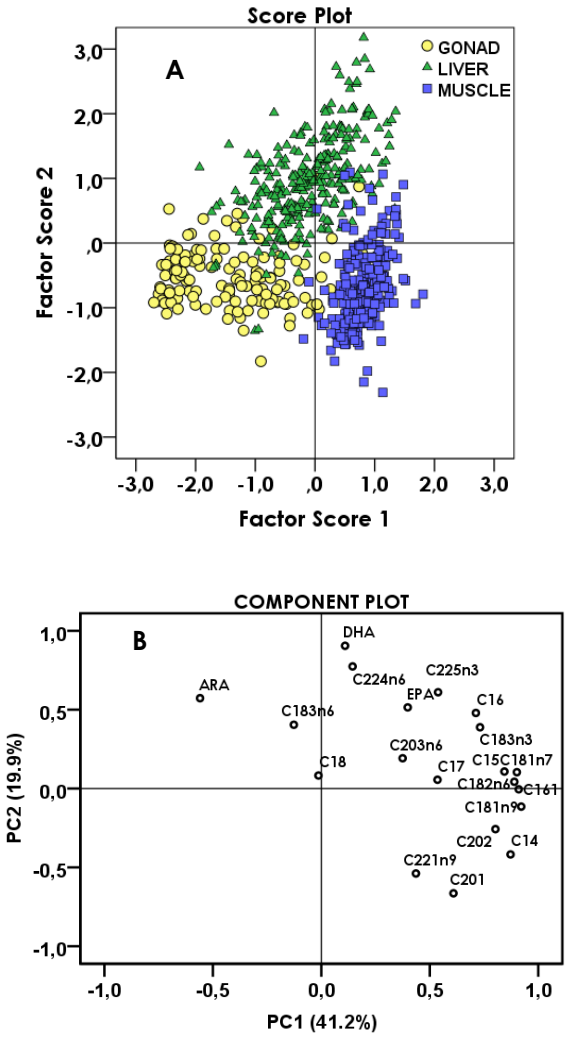

Figure 7. Factor score plot (A) and component plot (B) from principal component analysis on the most abundant fatty acid composition from the muscle, liver and gonad of European eel. n= 699.

The variables found between the positive axis of the first and the second component show a similar pattern in both the liver and muscle. The values corresponding to stearic acid (18:0) are near the origin in the component plot graph, indicating that it follows a similar pattern in the three tissues.

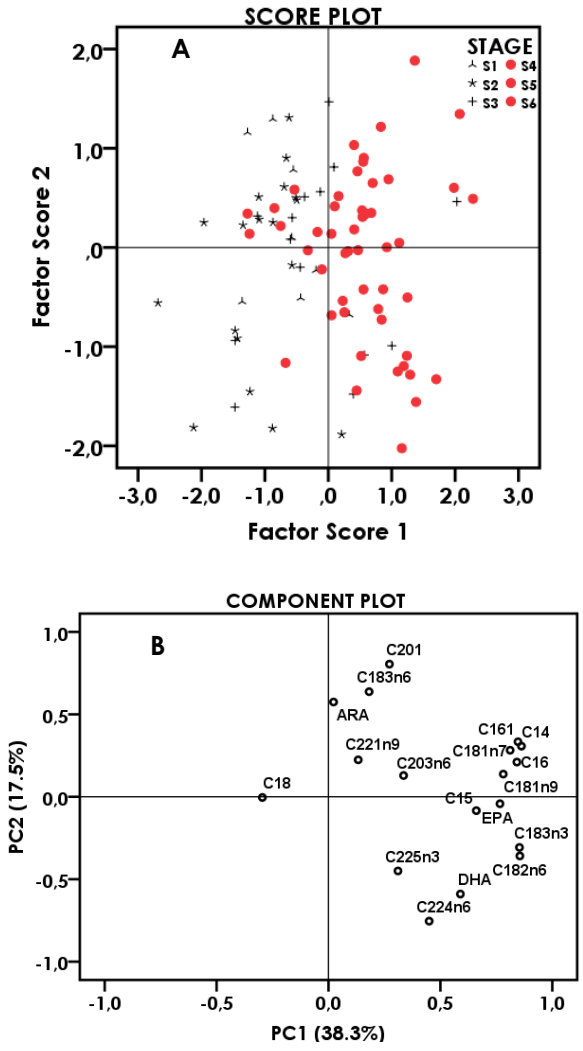

Figure 8. Factor score plot (a) and component plot (b) from principal component analysis on fatty acid composition of the liver of European eel in T20 treatment, at each development stage. n= 93.

The PCA of the liver fatty acids in the three treatments resulted in similar plots, and only the results from treatment T20 are shown (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 correspond to more advanced development stages (S4-S6) in the liver. The results obtained from the PCA of the liver agree with Table 1 which shows the fatty acids in the liver over the development stages.

Table 1 shows that, palmitic acid (16:0) as well as EPA (both on the right of the first component, Fig. 8B) 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, considering all the collected samples independently of the thermal regime (Figure 9 and Table 2). This was because of the lower quantity of analyzed testis samples (T10, n=27; T15, n=48, T20, n=63) in comparison to the rest of tissues considered in the experiment.

When the samples were 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).

Table 1 . 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 legend: 14:0, Myristic; 15:0, Pentadecanoic; 16:0, Palmitic; 17:0, Heptadecanoic; 18:0, Stearic; 16:1, Palmitoleic; 18:1n-7, Cis-vaccenic; 18:1n-9, Oleic; 20:1, Eicosenoic; 22:1n-9, Erucic; 18:2n-6, Linoleic; 18:3n-6, γ –Linolenic; 18:3n-3 Linolenic; 20:2, Eicosadienoic; 20:3n-3, Eicosatrienoic; 20:4n-6,Arachidonic (ARA) 20:5n-3, Eicosapentaenoic (EPA); 22:4n-6, Docosateraenoic; 22:5n-3, Docosapentaenoic; 22:6n-3, Docosahexaenoic (DHA).

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

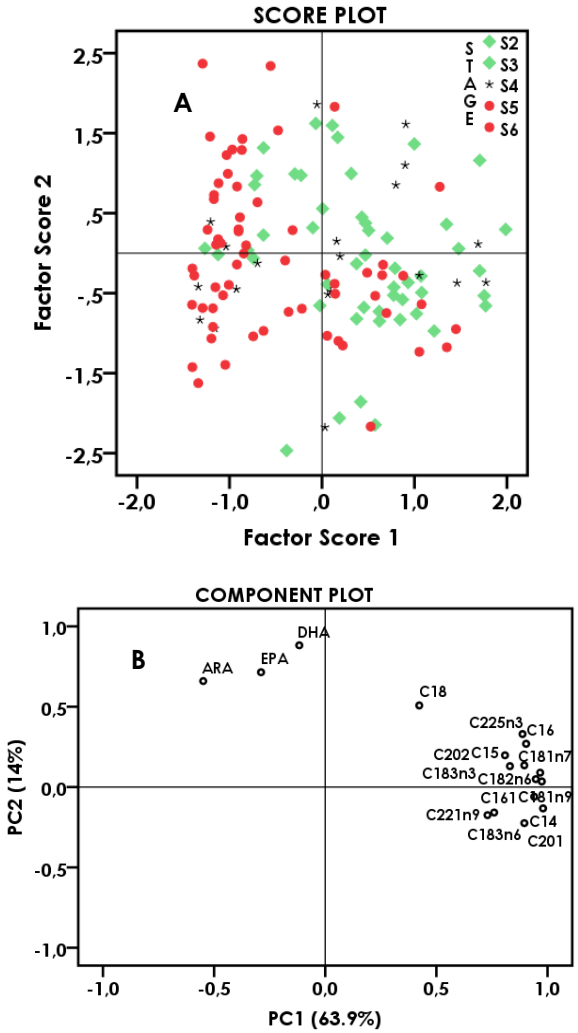

Figure 9. Factor score plot (a) and component plot (b) from principal component analysis on fatty acid composition of the gonad of European eel (independently of the thermal regime), at each development stage. n=139.

In the scores plot for the testis (Fig. 9A), the first component from the PCA of the testis fatty acids shows that the samples from S5-S6 stages are located to the left of the diagram, while the rest of the samples (S2-S3) are located to the right. The corresponding component plot for the testis fatty acids (Fig. 9B) suggests that all the fatty acids located to the right of the diagram correspond to stages S2-S3. Furthermore, EPA, ARA and DHA, located on the positive axis of the

second component of the component plot suggest that these fatty acids could explain different causes of the variations in the analysis. Regarding these results, Table 2 details the levels of fatty acids in the testis over the development stages, and suggests they are used in differing amounts throughout the spermatogenesis process. Table 2 shows a general decrease in 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 sperm quality obtained under the same three thermal regimes. It was observed that T20 males began spermiating earlier and had a higher percentage 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 °C, proving the importance of temperature in the final stages of the eel male maturation process.

Moreover, in terms of the use of lipid reserves, it´s known that in European eels the fat in the muscle increases 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.

Table 2. 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. Table legend: 14:0, Myristic; 15:0, Pentadecanoic; 16:0, Palmitic; 17:0, Heptadecanoic; 18:0, Stearic; 16:1, Palmitoleic; 18:1n-7, Cis-vaccenic; 18:1n-9, Oleic; 20:1, Eicosenoic; 22:1n-9, Erucic; 18:2n-6, Linoleic; 18:3n-6, γ –Linolenic; 18:3n-3 Linolenic; 20:2, Eicosadienoic; 20:3n-3, Eicosatrienoic; 20:4n-6,Arachidonic (ARA)20:5n-3, Eicosapentaenoic (EPA);22:4n-6, Docosateraenoic;22:5n-3, Docosapentaenoic;22:6n-3,Docosahexaenoic (DHA).

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

Besides, under fasting conditions eels usually go through a body (muscle) mass decrease but, although this was also observed in this study, the fat content in the muscle increased throughout maturation. There are two possible explanations for this: firstly, as Lovern (1940) demonstrated, eels lose weight mainly due to the use of protein for energy during fasting, whereas 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 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 fasting fish (Godavarthy and Kumari, 2012). The second explanation 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 the liver and the 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 throughout the course of the treatments.

Regarding the distribution of fatty acids in these three different tissues, through the PCA (Fig. 7) we could interpret that samples of muscle (on the right of the first component, Fig. 7A) were associated with 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 proportion in the muscle (data not shown). In particular, the high quantity of MUFA in the 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 are on left of the first component (Fig. 7A). This could be explained by the fact that the testis has a similar fatty acid profile to the muscle but in lower quantities. In terms of the liver fatty acid composition, the PCA (Fig. 7) shows that the liver samples fall on the positive axis of second component (Fig 7A), demonstrating that liver fatty acids have a different composition to the muscle and testis. These variations could be explained by the fact that the liver has higher amounts of DHA (falling on the positive axis of the second component, Fig.7B), than the muscle and testis.

In terms of the variations in the fatty acids found in the three different tissues during spermatogenesis, as Mazzeo et al. (2010) previously reported, no fatty acid content variations in the muscle was seen in European eel males induced to maturation at 20 °C, 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 the sum of SFA, MUFA and PUFA equated to 100% of detected fatty acids. In our study quantification is expressed as g of fatty acid 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 1). 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 they would most likely 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 we do know 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). 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 perform this conversion with differing levels of efficiency. 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 conversion ability (from 18:2n-6 and 18:3n-3 to LC-PUFA) to have good ARA, EPA and DHA levels (Bell and Tocher, 2009), as the freshwater environment is not so rich in LC-PUFA. Different fish species have different ways of increasing PUFA to maintain the structural and functional integrity of their cellular membranes (Farkas et al., 2001). 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 observing 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 should be an important focus of future research. Finally, a decrease in fatty acids in the eel testis coinciding with the most advanced developmental stages was witnessed (Fig. 6).

Among the quantified fatty acids, a decrease could be also observed during the most advanced development stages. The classes of fatty acids that decreased in particular in all the thermal treatments were SFA and MUFA.

Only EPA, DHA and ARA remained constant, while the rest of the fatty acids detected in the gonad decreased when males began to produce sperm (Table 2). These differences can be explained by the selective use of fatty acids by 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 has an important structural function. DHA maximizes transport of electrons in the mitochondria leading to increased energy output (Valentine and Valentine, 2004). On the other hand, ARA and EPA are the major eicosanoid precursors in fish cells, including prostaglandins, thromboxans and leukotrienes. EPA is known to be precursor of 3-series prostaglandins (PGE_3) and ARA forms 2-series prostaglandins (PGE_2) (Sargent et al., 2002; Tocher, 2003). Therefore, the EPA/ARA ratio modulates steroidogenesis in the testis increasing testosterone production. Any change in the ratio or in the levels of ARA and EPA in the gonad may influence the prostaglandins and steroid production. In vitro ARA stimulates the production of testicular testosterone in goldfish through its conversion to PGE_2, whereas EPA may function as an inhibitory regulator (Wade et al., 1994; Wade & Van Der Kraak, 1993). Asturiano et al. (2000) found similar results in European sea bass males, indicating that PUFA are capable of regulating prostaglandin and androgen production. Mercure and Van Der Kraak (1995), in their studies of in vitro ovarian follicles in goldfish and rainbow trout showed that EPA inhibits gonadotrophin-stimulated testosterone production, whereas ARA was only slightly inhibitory. Sorbera et al. (2001) showed that ARA and its metabolites, PGE_2, stimulate European sea bass oocyte maturation.

PUFA conservation in European eel testis during spermiation suggests that the spermatozoa membrane contains a high concentration of PUFA. Our results are corroborated by Mazzeo et al. (2010) who reported that PUFA are the main components of European eel milt and PUFA are also found in other fish species such as rainbow trout (Labbé et al., 1993), European sea bass (Asturiano et al., 2001; Bell et al., 1996) and herring (Huynh et al., 2007).

The fatty acids in fish sperm are affected by diet. Studies of goldfish (Wade et al., 1994), rainbow trout (Labbé et al., 1993, 1995; Pustowka et al., 2000; Vassallo-Agius et al., 2001) and European sea bass (Asturiano et al., 1999; 2001; Bell et al., 1996) have shown the relationship between the fatty acid content of the broodstock diet and the fatty acid composition of the sperm and suggest that consequently, fertilization could be affected. The preliminary results of the fatty acid composition of European eel testis during sexual maturation have demonstrated that the use of diets with appropriate fatty acid profiles might improve sperm quantity and quality (Butts and Baeza et al., unpublished results; Chapter IV).

In summary, the results have shown that temperature affects the sexual maturation of the male eel, with sperm production occurring earlier at the highest temperature (20 °C). The achieved results cohere with the importance of PUFA in teleost reproduction due to the role they play in male maturation and sperm composition. The increase of EPA observed in the liver when the eels produce sperm, possibly due to their biosynthesis, probably occurred in anticipation of their subsequent mobilization to the testis where they play an important role during steroidogenesis. In the testis, there may be a physiological significance for the fact that ARA and EPA levels remain constant, whereas the reason for the maintenance of the DHA levels may be structural. Further research on the relationship between fatty acids and sperm quality should be considered. Additionally, because eels fast 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 improve sperm quality.

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CHAPTER II

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

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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.

1. 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 a 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 °C for the first 6 weeks, 15 °C for the next 3 weeks and 20 °C for the last 6 weeks); T15 (15 °C for the first 6 weeks and 20 °C for the last 9 weeks) and T20 (20 °C 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 °C 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 °C 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; Chapter I). The fatty acid quantification was carried out by gas chromatography as described by Baeza et al. (2014; Chapter I).

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).

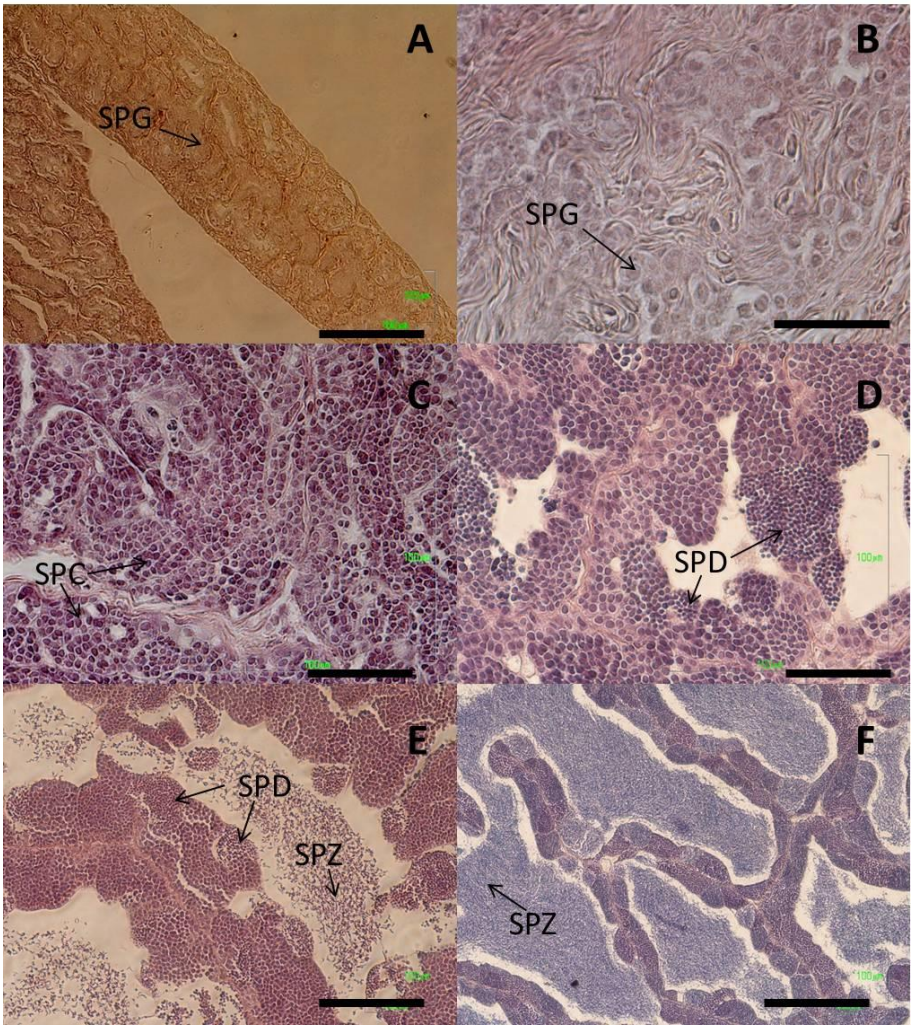

Figure 1. Histological sections of testis in different development stages. A, B: testis at stage 1; C: testis at stage 2; D: testis at stage 3, E: testis at stage 4; F: testis at stage 5. Scale bar: A, E= 100 µm; B, C, D= 50 µm; F= 200 µm. SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: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

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 a-Linolenic acid (18:3-n3, ALA) at S1.

Table 1. Plasma levels (in ng/mL) of different hormones throughout the different eel testis development stages.

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-KT	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}

Testosterone (nS1= 94; nS2= 43; nS3= 26; nS4 = 24; nS5= 39); 11-Ketotestosterone (nS1= 81; nS2= 43; nS3= 25; nS4 = 23; nS5= 36); 17β-Estradiol (nS1= 82; nS2= 45; nS3= 25; nS4 = 23; nS5= 40); DHP (nS1= 95; nS2= 44; nS3= 28; nS4 = 25; nS5= 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 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.

Table 2. Correlations between liver fatty acids and steroidogenic hormones during different testis development stages.

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*	

Asterisks indicate significant correlations (*, P<0.05; **, P<0.01). Number of samples correlated in each stage, n_{S1}= 80-95; n_{S2}= 44.

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).

Table 3. Correlations between testis fatty and steroidogenic hormones during different testis development stages.

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**

Asterisks indicate significant correlations (*, P<0.05; **, P<0.01). Number of samples correlated in each stage: n_{S3}= 24; n_{S5}= 39.

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).

Table 4. Correlations between liver fatty acids in different stages of testis development.

Liver F.A	S1	S2	S3	S4	S5
16:0 – 16:1	.498**	.457**	.877**	.529**	.665**
16:0 – 18:0	.234*		.587**	.407*	
16:0 – 18:1n9	.594**	.447**	.813**	.684**	.556**
16:0 – 18:2n6	.577**			.469*	.344*
16:0 – 20:1	.225*	.382*	.771**		.511**
16:0 – 18:3n3	.435**			.529**	.328*
16:0 – ARA	-.145				
16:0 – EPA	.387**				.578**
16:0 – 22:5n3	.407**			.455*	
16:0 - DHA	.205*				.408**
16:1 – 18:0	-.416**	-.353*			
16:1 – 18:1n9	.772**	.673**	.851**	.602**	.638**
16:1 – 18:2n6	.476**	.448**	.416*	.502*	.462**
16:1 – 20:1	.475**	.427**	.823**	.645**	.729**
16:1 – 18:3n3	.392**	.477**		.597**	.474**
16:1 – ARA	-.401**	-.460**	-.472*		-.508**
16:1 – EPA	.244*			.583**	.547**
16:1 – 22:5n3	.522**	.335*		.418*	.418**
16:1 - DHA	.254*				.353*

Liver F.A	S1	S2	S3	S4	S5
18:0 – 18:1n9			.477*		
18:0 – 18:2n6		-.375*			
18:0 – 20:1		.322*	.602**		
18:0 – 18:3n3		-.429**			
18:0 – ARA	.421**				.572**
18:0 – EPA		-.393**			
18:0 – 22:5n3	-.301**	-.425**	-.404*		-.323*
18:0 - DHA	-.229*	-.449**	-.467*		
18:1n9 – 18:2n6	.640**	.338*	.433*	.794**	.577**
18:1-n9 – 20:1	.444**	.494**	.743**		
18:1n9 – 18:3n3	.560**	.345*		.623**	.531**
18:1-n9 – ARA	-.465**	-.506**	-.384*		-.332*
18:1-n9 – EPA					.410**
18:1n9-22:5n3	.569**			.648**	.356*
18:1n9 - DHA					.420**
18:2n6 – 18:3n3	.834**	.879**	.911**	.877**	.761**
18:2-n6 – ARA					
18:2-n6 – EPA	.427**			.446*	
18:2n6 – 22:5n3	.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**			
18:3-n3 – ARA	-.017				
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 – 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**

*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.

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).

Table 5. Correlations between testis fatty acids in different stages of testis development.

Testis F.A	S2	S3	S4	S5
16:0 – 16:1	.775**	.856**	.960**	.926**
16:0 – 18:0	.794**	.556**	.690**	.518**
16:0 – 18:1n9	.827**	.687**	.949**	.933**
16:0 – 18:2n6	.677**	.548**	.908**	.905**
16:0 – 20:1	.640**		.902**	.829**
16:0 – 18:3n3		.666**	.914**	.896**
16:0 – ARA				-.388**
16:0 – EPA				
16:0 – 22:5n3	.696**	.843**	.940**	.923**
16:0 - DHA		.578**		
16:1 – 18:0	.602*		.620**	.382*
16:1 – 18:1n9	.768**	.842**	.953**	.977**
16:1 – 18:2n6	.689**	.739**	.909**	.937**
16:1 – 20:1	.674**	.668**	.956**	.935**
16:1 – 18:3n3	.609**	.761**	.920**	.876**
16:1 – ARA				-.639**
16:1 – EPA				-.422**
16:1 – 22:5n3		.741**	.912**	.886**
16:1 - DHA				-.360*

Testis F.A	S2	S3	S4	S5
18:0 – 18:1n9	.918**	.485*	.720**	.427**
18:0 – 18:2n6	.854**	.433*	.731**	.441**
18:0 – 20:1			.557**	.358*
18:0 – 18:3n3	.589*		.656**	.428**
18:0 – ARA				
18:0 – EPA				.315*
18:0 – 22:5n3	.783**	.477*	.725**	.519**
18:0 - DHA				.321*
18:1n9 – 18:2n6	.942**	.943**	.983**	.977**
18:1-n9 – 20:1		.769**	.896**	.898**
18:1n9 – 18:3n3	.663**	.793**	.952**	.916**
18:1-n9 – ARA		-.411*		-.578**
18:1-n9 – EPA				-.429**
18:1n9-22:5n3	.831**	.708**	.952**	.917**
18:1n9 - DHA				-.330*
18:2n6 – 20:1		.655**	.832**	.843**
18:2n6 – 18:3n3	.813**	.832**	.964**	.946**
18:2-n6 – ARA				-.514**
18:2-n6 – EPA				-.446**
18:2n6 – 22:5n3	.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**
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**		

*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.

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 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. (20142014; Chapter I) 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. (20142014; Chapter I). 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.

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SUPPLEMENTARY TABLES

Supplementary Table 1. P-values from fatty acids quantified in liver samples of fish treated with different thermal regimes, when samples were classified in different testis stages of development.

Liver fatty acids	Stages				
	S1	S2	S3	S4	S5
14:0	0.278	0.007*	0.003*	0.644	0.798
16:0	0.984	0.346	0.063	0.392	0.366
16:1	0.502	0.366	0.176	0.809	0.267
18:0	0.025*	0.620	0.108	0.809	0.286
18:1n-9	0.107	0.175	0.055	0.594	0.172
18:1n-7	0.125	0.093	0.094	0.764	0.598
18:2n-6	0.094	0.378	0.357	0.341	0.102
18:3n-6	0.954	0.215	0.080	0.275	0.288
20:1	0.279	0.422	0.107	0.814	0.943
18:3n-3	0.085	0.337	0.205	0.313	0.667
20:2	0.337	0.116	0.229	0.324	0.915
22:1n-9	0.069	0.509	0.094	0.361	0.370
20:4n-6 (ARA)	0.061	0.970	0.646	0.758	0.724
20:5n-3 (EPA)	0.129	0.605	0.090	0.054	0.355
22:5n-3	0.064	0.680	0.063	0.349	0.011*
22:6n-3 (DHA)	0.363	0.345	0.060	0.168	0.155

* Differences were considered significant when $P<0.05$. Number of samples in each stage of development and from each thermal regime: Stage 1 (S1): T10n=49; T15n=29; T20n=17, Stage 2 (S2): T10n=15; T15n=14; T20n=14; Stage 3 (S3): T10n=10; T15n=7; T20n=9; Stage 4 (S4): T10n=4; T15n=10; T20n=10; Stage 5 (S5): T10n=0; T15n=16; T20n=22.

Supplementary Table 2. P-values from fatty acids quantified in testis samples of fish treated with different thermal regimes, when samples were classified in different testis stages of development.

Testis	Stages			
Fatty acids	S2	S3	S4	S5
14:0	0.726	0.174	0.345	0.872
16:0	0.656	0.061	0.176	0.669
16:1	0.736	0.162	0.257	0.865
18:0	0.699	0.311	0.357	0.009*
18:1n-9	0.509	0.208	0.377	0.864
18:1n-7	0.805	0.076	0.155	0.850
18:2n-6	0.601	0.415	0.433	0.801
18:3n-6	0.883	0.229	0.103	0.436
C20:1	0.845	0.165	0.494	0.896
18:3n-3	0.716	0.295	0.279	0.656
20:2	0.236	0.463	0.242	0.816
22:1n-9	0.482	0.216	0.312	0.536
20:4n-6 (ARA)	0.609	0.941	0.395	0.814
20:5n-3 (EPA)	0.918	0.225	0.192	0.219
22:5n-3	0.253	0.308	0.182	0.724
22:6n-3 (DHA)	0.996	0.600	0.364	0.106

* Differences were considered significant when $P<0.05$. Number of samples in each stage of development and from each thermal regime: Stage 1 (S1): T10n=8; T15n=5; T20n=4; Stage 3 (S3): T10n=9; T15n=7; T20n=8; Stage 4 (S4): T10n=4; T15n=10; T20n=10; Stage 5 (S5): T10n=0; T15n=16; T20n=22.

Supplementary Table 3. P-values from sex steroids plasma levels of fish treated with different thermal regimes, when samples were classified in different testis stages of development.

Steroids	Stages				
	S1	S2	S3	S4	S5
T	0.081	0.227	0.671	0.311	0.086
11-KT	0.477	0.849	0.523	0.609	0.559
DHP	0.517	0.447	0.438	0.211	0.303
E2	0.101	0.355	0.189	0.710	0.550

* Differences were considered significant when P<0.05. Testosterone (T); 11-ketotestosterone (11KT); 17a-20β-dihydroxy-4-pregnen-3-one (DHP); 17β-Estradiol (E2). Number of samples in each stage and from each thermal regime: Stage 1 (S1): T10n=49; T15n=29; T20n=17, Stage 2 (S2): T10n=15; T15n=14; T20n=14; Stage 3 (S3): T10n=10; T15n=7; T20n=9; Stage 4 (S4): T10n=4; T15n=10; T20n=10; Stage 5 (S5): T10n=0; T15n=16; T20n=22.

CHAPTER III

Relationship between sperm quality parameters and the fatty acid composition of the muscle, liver and testis of European eel

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Abstract

This study looks at the correlations that fatty acids have with different tissues in the European eel (*Anguilla anguilla*, L.) during hormonally-induced sexual maturation, with different sperm quality parameters. In order to evaluate the different dynamics of the use of fatty acids, a categorization of the results from each sperm quality parameter (volume, concentration, motility and velocity) was performed.

Low and moderate correlations were observed between muscle tissue and some sperm quality parameters but no high correlations were found. Eicosapentaenoic acid (20:5n3, EPA) in the liver seems to have a role in determining the volume of sperm produced. This can be explained by the fact that EPA is a major requirement in the early phases of sperm production (probably as a component of the spermatozoal membrane). In addition, the levels of a-linolenic acid (18:3-n3, ALA) and linoleic acid (18:2-n6, LA) in the liver decreased when sperm motility increased. In all the tissues, a negative correlation was observed between arachidonic acid (20:4n-6, ARA) and the different sperm velocity parameters. The fact that an increase in the consumption of ARA coincides with an increase in the speed of spermatozoa, highlights the important role that this fatty acid plays not only in sperm production, but also in sperm velocity.

All this information could prove useful in the development of suitable broodstock diets to improve sperm quality and subsequently, the larval development of this species.

1. Introduction

Over the past 25 years European eel populations have been declining. Several factors such as infections, pollution, overfishing and habitat destruction, mean that the stock is now considered outside of safe biological limits and immediate protection measures have been recommended (van den Thillart and Dufour, 2009; ICES, 2011).It is known that in the autumn eels begin their maturation to the silver eel stage, when they descend from the rivers and migrate to the sea. Spawning occurs between April and June, between 200 and 600 m, in

the Sargasso Sea (Aarestrup et al., 2009), although many details of the migration still remain unknown.

A key factor in the success of eel reproduction in captivity is good quality gametes (both eggs and sperm), and therefore it is important to consider different hormonal induction treatments. The sexual maturation of males can be induced by using long-term hormonal treatments (Ohta et al., 1997; Pérez et al., 2000; Asturiano et al., 2005; Huang et al., 2009; Gallego et al., 2012). The effect of different hormonal treatments and environmental parameters on gamete quality has been studied in both Japanese and European eels (Miura et al., 1991; Asturiano et al., 2005; Gallego et al., 2012; Mazzeo et al., 2012) but the effect of broodstock feeding on gamete quality has been investigated in female eels (Furuita et al., 2006, 2007; Ozaki et al., 2008; Oku et al., 2009; Støttrup et al., 2012), not in males.

An assessment of sperm quality is important in order to ensure the success of the reproduction process. It is clear that broodstock nutritional requirements have to be met in order to achieve reproductive performance, and several studies indicate that the composition of dietary lipids affects male reproductive performance in different teleosts, including the European sea bass (*Dicentrarchus labrax*; (Bell et al., 1996; Asturiano et al., 2001), Eurasian perch (*Perca fluviatilis*; (Henrotte et al., 2010)), rainbow trout (*Oncorhynchus mykiss*; (Labbé et al., 1995; Pustowka et al., 2000)), Indian major carp (*Catla catla*; (Nandi et al., 2007)) and African catfish (*Clarias gariepinus*; (Nyina-Wamwiza et al., 2012))

The effect of male broodstock feed on sperm quality has not been studied in either European eel or Japanese eel. Mazzeo et al. (2010) studied the changes in fat and fatty acid levels in the muscle, liver and testis of European eel throughout spermatogenesis. More recently, the variations in the levels of fatty acids in different tissues of males undergoing hormonal induction at different thermal regimes were studied (Baeza et al., 2014; Chapter I).

Little is known about the effect of fatty acids on the sperm quality of eels. Although eels cease to feed from the onset of sexual maturation (Tesch, 2003), the body composition at the time of sexual maturation

is fundamental, and developing suitable diets appears to be essential for reproductive success. The aim of this research was to clarify the variations in the fatty acid composition of different tissues and to determine whether there is any link with the changes in sperm quality parameters. The knowledge generated will be implemented in broodstock diets to potentially improve sperm quality.

2. Material and methods

2.1 Fish maintenance and hormonal treatment

Three hundred and seventeen male eels (mean body weight 100 ± 2 g) were moved from the Valenciana de Acuicultura, S.A. fish farm (Puzol, Valencia; East coast of Spain) to the facilities of the Universitat Politècnica de València (Spain). The animals were placed in six 200 L aquaria equipped with separated recirculation systems, thermostats and coolers and covered to maintain constant shade. The fish were gradually acclimatized to sea water (salinity 37 ± 0.3 g L^{-1}) over the course of a week and were fasted during both the acclimatization and the experimental periods.

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

For 13 weeks, the males were hormonally treated to induce maturation and spermiation through weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Argent Chemical Laboratories. USA) as previously described by Pérez et al. (2000).

Different spermiation patterns were observed depending on the initial water temperature. At the sampling time, all the fish were at 20 °C because this is the water temperature needed in order for the eels to produce sperm. The samples used to determine the relationship between the fatty acid levels and the sperm quality parameters were collected once sperm production had been achieved, independently of the initial temperature. Samples from T20 fish were

collected at the 5th week, T15 samples were collected at the 7th week and T10 samples were collected at the 10th week.

2.2 Samplings and sperm collection

Between 5 and 8 fish per thermal regime were sacrificed each week by decapitation, after having previously been anesthetized with benzocaine (60 ppm). Only spermiating males were sampled and fatty acids from the muscle, liver and testis were correlated with the sperm parameters. A different number of samples was obtained from each group, depending on the length of the spermiation period: in the case of T10, a total of 12 fish were sampled from the 10th week; in the case of T15, a total of 32 fish were sampled from the 7th week; in the case of T20, a total of 47 males were sampled from the 5th week.

The sperm was collected by applying gentle abdominal pressure to previously anesthetized males after cleaning the genital area with distilled water to avoid contamination with faeces, urine or seawater. A small aquarium air pump was modified to obtain a vacuum suction force, and the sperm was collected in a tube. Sperm samples were collected 24 h after the administration of the hormone because previous studies (Pérez et al., 2000) have demonstrated that this is when sperm quality is higher.

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

2.3 Lipid and fatty acid analysis of tissues

Total lipids from muscle were extracted in a Soxtec extraction unit (1043, Tecator). The total lipids from the testis and the liver were extracted using a modified version of the Folch method (Folch et al., 1956). A direct method of FAME synthesis was performed according to O´Fallon et al. (2007). Fatty acid quantification was carried out by gas chromatography. All the methodologies used were carried out and described deeply in Baeza et al. (2014; Chapter I).

2.4 Determination of sperm concentration and volume

Dilutions to measure the sperm concentration were done according to Asturiano et al. (2004). Sperm volume (mL) and concentration were carried out following the methodologies specified in Gallego et al. (2012).

2.5 Evaluation of sperm motility and velocity parameters

A standardized methodology was used for the motility analysis (MOT) (Gallego et al., 2013). Cells were considered to be "Progressive motile cells" when they swim forward in 80% of a straight Line (P-MOT). Different velocity parameters were assessed, including: curvilinear velocity (VCL, μm/s), defined as the time/average velocity of a sperm head along its actual curvilinear trajectory; straight line velocity (VSL, μm/s), defined as the time/average velocity of a sperm head along the straight line between its first detected position and its last position; and average path velocity (VAP, μm/s), defined as the time/average of a sperm head along its spatial average trajectory. All motility and velocity analyses were performed by Gallego et al. (2012).

2.6 Parameter categorization and statistical analysis

The fatty acids were quantified in each tissue (muscle, liver and testis) to estimate the possible correlation with the sperm quality parameters. First, linear correlations between sperm quality parameters and fatty acids were performed for each tissue, with the data obtained from the different thermal regimes considered separately. No linear correlations were however appreciated. Then, nonlinear regressions (which could be considered more powerful in the evaluation of these kinds of parameters) were carried out. Several significant but low correlations were found when the whole data range from each thermal regime was considered. This could be due to the fact that fatty acids have different roles throughout the final sperm maturation process. We therefore decided to analyze the data in an alternative way. The results of each sperm quality parameter were categorized in order to better analyze the data and try to find all the meaning from the results. The established parameter ranges and categories are shown in table 1.

Table 1. Categorization of sperm parameters.

Parameter	Category	Range
Volume (mL 100 g^{-1} fish)	VOL1	0-0.5
	VOL2	0.5-1
	VOL3	1-3
	VOL4	>3
Concentration (10^9 cells mL^{-1})	CON1	0-5
	CON2	5-10
	CON3	10-15
	CON4	>15
Total Motility (% motile cells)	MOT1	0-25
	MOT2	25-50
	MOT3	>50
Progressive Motility (% progressive motile cells)	P-MOT1	0-5
	P-MOT2	5-15
	P-MOT3	15-25
	P-MOT4	>25
Curvilinear Velocity (µm/s)	VCL1	0-50
	VCL2	50-100
	VCL3	100-130
	VCL4	>130
Straight Line Velocity (µm/s)	VSL1	0-30
	VSL2	30-50
	VSL3	50-80
	VSL4	>80
Average Path Velocity (µm/s)	VAP1	0-30
	VAP2	30-50
	VAP3	50-80
	VAP4	>80

The number of samples from each category was very low when the thermal treatments (T10, T15 and T20) were considered separately, but all the samples were obtained from fish producing sperm once over a threshold temperature of 20 °C. The absence of statistical differences was checked by one way ANOVA comparing the means of the fatty acids (the thermal regimes were considered separately) in relation to each sperm quality parameter categorized. Prior to this, data normality had been checked using the asymmetry standard coefficient and Kurtosis coefficient. For example, P-value results of

categorized VCL showed no significant differences in any tissue (Supplementary material Tables 1-3). So, in order to get a higher number of samples all the data were considered together and correlations were made independently of the thermal regime.

Pearson's correlation, coefficient of determination and linear regression analyses ($P<0.05$) were used to determine the relationship between each fatty acid and the different categorized parameters. All the statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Muscle

The correlations found between the sperm quality parameters and fatty acids in the muscle are shown in Table 2. The low sperm concentration categories (CON1 and CON2; 0-10 x 10^9 cells mL^{-1}) showed significant moderate negative correlations ($P<0.05$) with several fatty acids: palmitic acid (16:0), stearic acid (18:0)and EPA with CON1, and oleic acid (18:1-n9) with CON2.

No significant correlation between motility and any fatty acid in particular was found, but a positive correlation between the n-3/n-6 ratio and MOT1 was registered, with a greater proportion of n-3 series fatty acids present when motility was between 0 and 25%. The progressive motile spermatozoa (P-MOT), specifically P-MOT2, were negatively correlated ($P<0.05$) with monounsaturated fatty acids (MUFA) including palmitoleic acid (16:1) and vaccenic acid (18:1-n7), and consequently with total MUFA ($P<0.05$). Regarding the sperm velocity parameters, only VSL3 and VAP3 showed significant correlations ($P<0.05$) with several fatty acids in the muscle. VSL3 was negatively correlated with DHA, ARA and their precursors ALA and LA. On the other hand, VAP3 showed significant positive correlations (being $P<0.05$ in all the cases) with the following fatty acids: myristic acid (14:0), total SFA and n-9 fatty acids such as eicosenoic acid (20:1-n9) and erucic acid (22:1-n9).

Table 2. Correlations between muscle fatty acids and sperm quality parameters are shown. Asterisks indicate significant correlations between parameters and fatty acids.

Fatty acid	VOL3 n=29	CON1 n=25	CON2 n=28	MOT1 n=31	P-MOT2 n=16	VSL3 n=30	VAP3 n=28
14:0							0.410*
16:0		-0.498*					
16:1					-0.559*		
18:0	-0.388*	-0.462*					
18:1-n7					-0.585*		
18:1-n9			-0.422*				
20:1-n9							0.381*
22:1-n9							0.531*
18:2-n6						-0.542*	
18:3-n3						-0.498*	
EPA		-0.435*					
22:5-n3					0.504*		
DHA						-0.390*	
ARA						-0.402*	
SFA		-0.504*			-0.502*		0.415*
MUFA					-0.516*		
PUFA						-0.510**	
Total n-3						-0.453*	
Total n-6						-0.587**	
n-3/n-6		-0.481*		0.369*			

VOL: Sperm volume; CON: Sperm concentration; MOT: Sperm motility; PMOT: Progressive motile sperm; VSL: Straight line velocity of sperm; VAP: Average path velocity of sperm. (*, p-*value* < 0.05; **, p-*value* < 0.01).

3.2 Liver

The correlations found between the sperm quality parameters and liver fatty acids are shown in Table 3. All the sperm volume categories showed significant correlations with different fatty acids, in most cases, from the n-3 series. A negative correlation at P<0.01 level between VOL2 and EPA (Fig. 1) was found.

Table 3. Correlations between liver fatty acids and sperm quality parameters are shown. Asterisks indicate significant correlations between parameters and fatty acids.

Fatty acid	VOL1 n= 25	VOL2 n= 15	VOL3 n=29	CON2 n=28	CON3 n=19	MOT2 n=24	MOT3 n=29	P-MOT3 n=18	P-MOT4 n=18	VAP2 n=23	VAP3 n=26
14:0											0.447*
16:0							-0.408*		-0.503*		
16:1											
18:0						-0.451*		0.537*			
18:1-n9							-0.403*		-0.502*		
18:2-n6									-0.604**	0.496*	
18:3-n3			-0.428*		-0.472*				-0.613**	0.540**	
EPA		-0.691**				-0.431*					
22:5-n3	-0.397*			-0.442*	-0.465*						
DHA										0.415*	
ARA											-0.424*
SFA							-0.371*		-0.492*		
MUFA			-0.369*				-0.370*		-0.473*		
PUFA						-0.406*					
Total n-3		-0.515*				-0.413*					
Total n-6									-0.570*	0.426*	
ARA/EPA		0.544*									
EPA/DHA										-0.425*	

). VOL: Sperm volume; CON: Sperm concentration; MOT: Sperm motility; PMOT: Progressive motile sperm; VAP: Average path velocity of sperm. (*, p-*value* < 0.05; **, p-*value* < 0.01

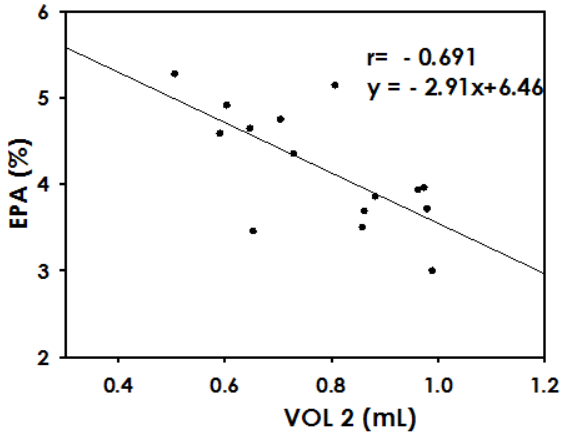

Figure 1. Relationship between VOL2 (Sperm volume) and EPA in the liver (n=15). Linear regression equation was calculated for each parameter.

A moderate negative correlation was registered between sperm motility in the different categories (P<0.05) and several fatty acids: 18:0, EPA, total PUFA and total n-3 series fatty acids with MOT2, and 16:0, 18:1-n9, total SFA and total MUFA with MOT3.

In terms of progressive motile spermatozoa, Figure 2 (A and B) shows the high negative correlations (P<0.01) between P-MOT4 and ALA and LA fatty acids, respectively.

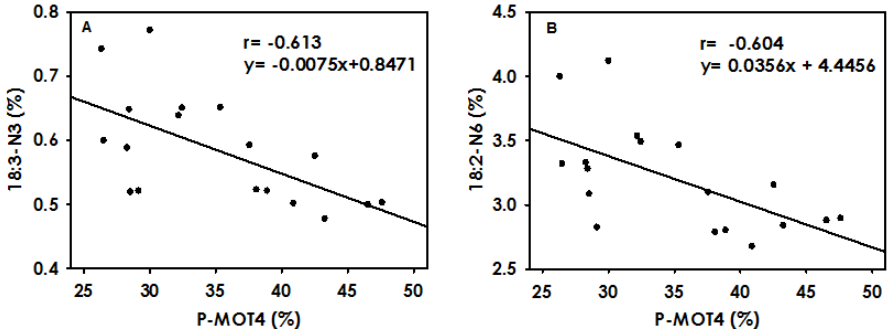

Figure 2. Relationship between: A) P-MOT4 (progressive motile sperm) and 18:3-n3 and; B) P-MOT4 (progressive motile sperm) and 18:2-n6 in the liver (n=18). Linear regression equations were calculated for each parameter.

Regarding the sperm velocity parameters, only VAP (but not VCL and VSL) was positively correlated with some liver fatty acids: ALA, LA, DHA and total n-6 series fatty acids. On the other hand, a positive correlation was registered between VAP3 and 14:0 and a negative correlation with ARA in the liver ($P<0.05$).

3.3 Testis

The correlations between the sperm quality parameters and testis fatty acids are shown in Table 4. The ARA/EPA ratio was negatively correlated with concentration when this was higher than 15 x 10^9 cells mL^{-1}.

With regards to P-MOT, 22:1-n9 was positively correlated in the testis when the percentage of progressive spermatozoa was 5-15% (P-MOT2, $P<0.05$). P-MOT3 showed significant negative correlations ($P<0.05$) with the following fatty acids: 18:0 and n3-series fatty acids such as EPA, 22:5-n3 and DHA.

When considering the sperm velocity parameters, many testis fatty acids were positively correlated with the highest curvilinear velocities (VCL4, >130 µm/s): 14:0, 16:0, 18:1-n9, 18:1-n7, ALA, LA, and total SFA (with $P<0.05$) and 16:1, 20:1-n9, 22:1-n9, and total MUFA (with $P<0.01$). There was also a significant high negative correlation at the $P<0.01$ level between VCL4 and ARA and the ARA/EPA ratio in the testis (Fig. 3), suggesting a reduction of ARA at the end of the sperm maturation process.

In terms of the VSL values, 18:0 ($P<0.01$), EPA, total PUFA and total n-3 series fatty acids ($P<0.05$), were negatively correlated with VSL3.

A relationship between VAP and fatty acids in the testis, with positive and negative correlations was found. A significant positive correlation was registered between VAP3 and EPA and a negative correlation between VAP3 and the EPA/DHA ratio ($P<0.05$). Moderate negative correlations ($P<0.01$) were also found between VAP4 and ARA and the ARA/EPA ratio.

Table 4. Correlations between testis fatty acids and sperm quality parameters are shown. Asterisks indicate significant correlations between parameters and fatty acids.

Fatty acid	CON4 n=21	MOT1 n=32	PMOT2 n=16	PMOT3 n=21	VCL4 n=33	VSL3 n=31	VAP3 n=30	VAP4 n=32
14:0					0.432*			
16:0		-0.361*			0.419*			
16:1					0.452**			
18:0		-0.388*		-0.493*		-0.484**		
18:1-n7					0.415*			
18:1-n9					0.427*			
20:1-n9					0.474**			0.381*
22:1-n9			0.499*		0.465**			
18:2-n6					0.390*			
18:3-n3					0.365*			
EPA				-0.454*		-0.418*	0.381*	
22:5-n3		-0.329*		-0.483*				
DHA		-0.370*		-0.438*				
ARA					-0.614**			-0.492**
SFA					0.412*			
MUFA					0.446**			
PUFA		-0.519**		-0.595**		-0.448*		
Total n-3		-0.465**		-0.566**		-0.433*		
ARA/EPA	-0.507**				-0.608*			-0.477**
EPA/DHA							-0.382*	

CON: Sperm concentration; MOT: Sperm motility; P-MOT: Progressive motile sperm; VCL: Curvilinear velocity of sperm; VSL: Straight line velocity of sperm; VAP: average path velocity of sperm. (*, p-*value* < 0.05; **, p-*value* < 0.01)

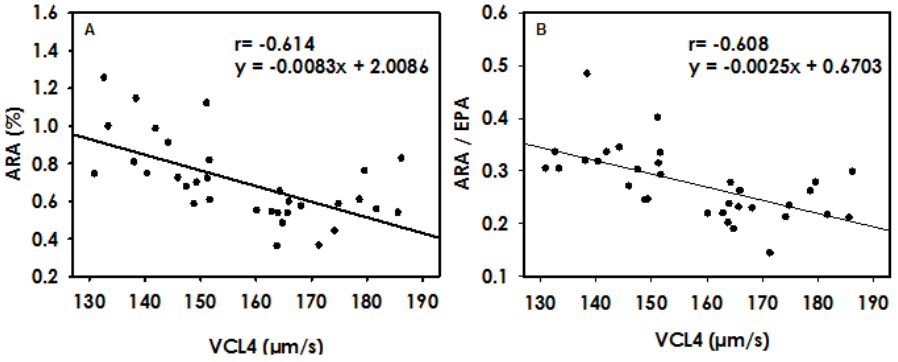

Figure 3. Relationship between: A) VCL4 (curvilinear velocity of sperm) and ARA and; B) VCL4 (curvilinear velocity of sperm) and ARA/EPA in the testis (n=33). Linear regression equation was calculated for each parameter.

4. Discussion

We used data from Gallego et al. (2012), who registered sperm volumes similar to those described by other authors (1–4 mL 100 g fish^{-1}; (Pérez et al., 2000; Asturiano et al., 2005) and, an increasing trend in sperm volume over the weeks of spermiation. Regarding the correlations found between sperm volume and the fatty acids present in the different tissues, significant correlations with the liver fatty acids were found. Levels of EPA, n-3 series fatty acids and total MUFA decreased in the liver when sperm volume was between 0 and 3 mL. This decrease in EPA meant that the ARA/EPA ratio was positively correlated with sperm volume. Our hypothesis suggests that, when the volume of sperm being produced is low, EPA could be synthesized in the liver (negative correlation with sperm found in the liver 0.5-1 mL volumes) and sent to the testis, which requires EPA for the production ofsperm cell membranes (Lenzi et al., 2000). Figure 4A shows the main fatty acid mobilization from the liver to the gonad during eel spermatogenesis (described by Baeza et al. (2014; Chapter I). Figure 4B shows (in green) the correlations between EPA from the liver and sperm volume.

Pérez et al. (2000), in their analysis of European eel sperm fatty acids, found significant negative linear correlations between sperm volume

and total n-3 fatty acids, EPA and DHA. Our results also show negative correlations between sperm volume and different n-3 series fatty acids but in our case from the liver, supporting the important role of this tissue (especially when eels produce sperm) highlighted by Baeza et al. (2014; Chapter I). Similar results were found by Pérez et al. (2000) in the sperm, suggesting a connection. In our opinion, the decrease of n3 fatty acids in the liver coinciding with the sperm volume increase could be due to their mobilization to the gonad, where spermatozoa use them, with the consequent reduction of n3 fatty acids also in sperm. In fish species the influence of dietary fatty acids on sperm concentration has been demonstrated. Nandi et al. (2007) showed that spermatozoal concentration and spermatocrit in Indian major carp (*C. catla*) were significantly higher in fish fed PUFA enriched test diets than fish fed control diet. Furthermore, fatty acid supplementation in male European sea bass induced a longer spermiation period and higher milt spermatozoal concentrations (Asturiano et al., 2001). Recently, a higher sperm concentration was found in rats fed a diet with a high n-3/n-6 fatty acid ratio (Yan et al., 2013). Moreover, in humans, sperm concentration has been positively correlated with DHA levels (Nissen and Kreysel, 1983).

All of these results highlight the influence of fatty acids in relation to sperm concentration and, in the present study, although several negative correlations between fatty acids and different concentrations were found especially in muscle, we only can propose a hypothesis. The decrease found in these fatty acids in the muscle can be explained by their mobilization to other tissues, where local consumption might occur, explaining why no increases were registered in the other tissues. In the testis, when the highest concentrations were registered, there was a negative correlation with ARA/EPA, due to an increase in ARA. Figure 4B shows (in orange) the most important correlations between the fatty acids and the sperm concentration in the three tissues.

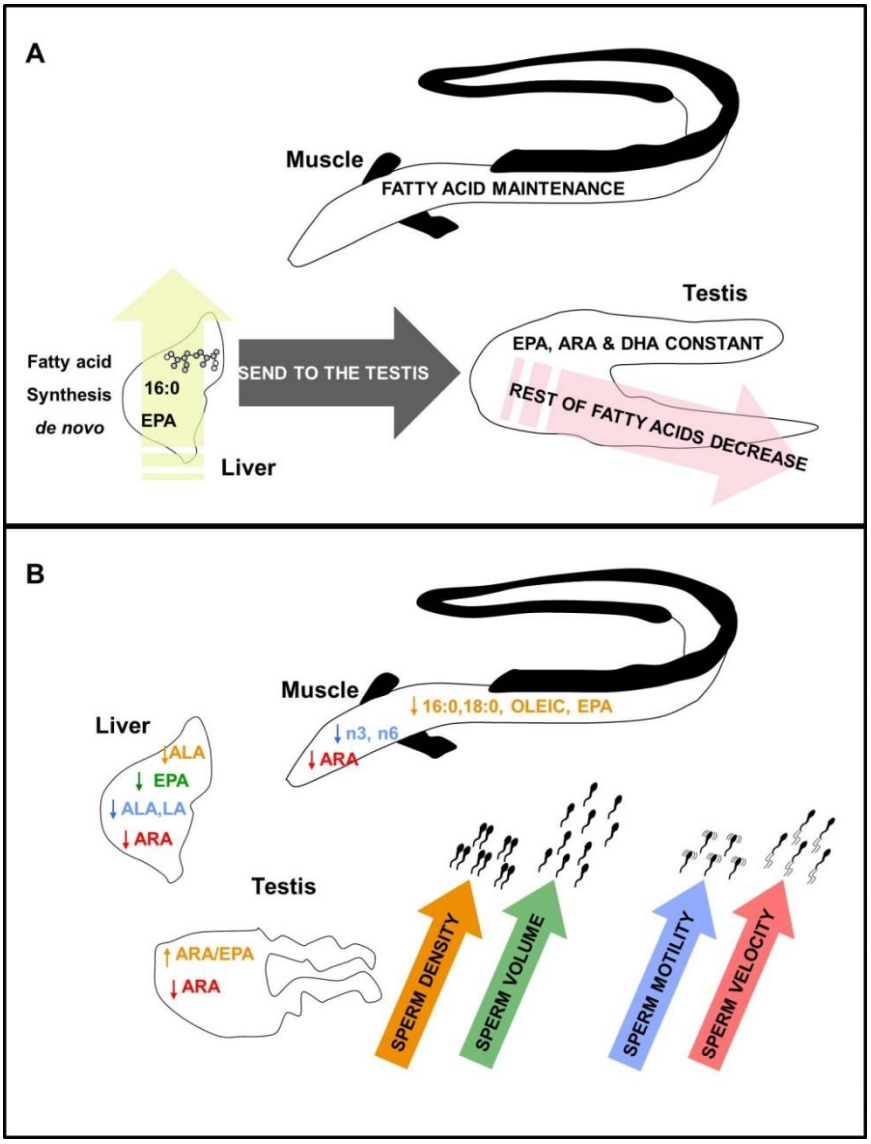

Figure 4. A) Fatty acid dynamics during eel spermatogenesis. Fatty acid content did not change in muscle. Liver highlighted as the main site of synthesis and in gonad EPA, ARA and DHA remained constant while the rest decrease. B) Summary of the main correlations between the most important fatty acids and sperm quality parameters in different tissues. The three main conclusions were: 1-EPA in liver decreased when sperm volume increased; 2-ALA and LA (PUFA precursors) decreased in liver wen motility increased; 3-ARA levels decreased in all tissues when sperm velocity increased.

Beirão et al. (2012a) studied the lipid content of the sperm flagella and head membrane of gilthead seabream (*Sparus aurata*) and suggest that fatty acid composition differs depending on their function and their effect on sperm motility and viability. Vassallo-Agius et al. (2001) showed that motility was lower in rainbow trout (*O. mykiss*) fed an n-3 essential fatty acid deficient diet compared to a control group fed a commercial diet, highlighting the importance of PUFA in sperm motility, just as in humans (Lenzi et al., 2000). Recently, Butts et al. (2011) in studies of Atlantic cod (*Gadus morhua*) suggested that differences observed in fatty acid composition between wild and cultivated cod sperm were derived from their diets and influenced sperm activity. In the present study, several correlations were found between MOT and P-MOT and fatty acids in all the tissues analysed, highlighting again, as previously reported in other publications, the relationship between fatty acids and sperm motility. This suggests that these fatty acids are probably used as an energy source to increase sperm energy for motility requirements. Recently, Mehdinejad et al. (2013) highlighted the importance of fatty acids for sperm movement in Iranian sturgeon (Acipenser persicus).Furthermore, there was a high negative correlation between LA and ALA in the liver and progressive motile cells (Fig. 2). An explanation for all of the obtained results could be that the liver synthesizes PUFA from their precursors (LA and ALA, also PUFAs) and sends them to the testis, where they are used to increase sperm motility. The importance of fatty acids, especially PUFA, in sperm motility has been demonstrated in other animal species. For example, in boars fed a diet supplemented with shark oil, rich in PUFA, an improvement in sperm motility was found (Mitre et al., 2004). Again in boars, DHA and n-3 series fatty acids in sperm were positively correlated with motility (Am-in et al., 2011). Furthermore, in rats sperm motility was found to be positively correlated with the n-3/n-6 fatty acids ratio in the diet (Yan et al., 2013). Additionally, in humans sperm motility was negatively correlated to the seminal plasma concentration of n-6 series fatty acids (Safarinejad et al., 2010) and also in humans, motility has been positively correlated with the DHA levels in sperm (Nissen and Kreysel, 1983) although DHA supplementation does not affect human sperm motility (Conquer et al., 2000). All the studies listed above, have highlighted the main role of PUFA on sperm motility, and concur with the results of the present

study into eel, where correlations between motile cells with ALA and LA (both PUFAs) from the liver were found. Figure 4B summarizes (in blue) the most important correlations between fatty acids from the liver and the muscle and sperm motility.

Sperm speed improved in African catfish (*C. gariepinus*) fed a diet in which fishmeal was completely substituted by agricultural products and consequently had high levels of n-6 series fatty acids (Nyina-Wamwiza et al., 2012). Martínez-Páramo et al. (2012) in a study where they evaluated the correlation between sperm lipid peroxidation and the sperm quality of precocious European sea bass (*D. labrax*), found a positive correlation between VSL and the amount of ARA in the sperm, as well as a negative correlation with the DHA/EPA ratio.

The principal results from the present study have shown a negative correlation between ARA in all the tissues and the different categorized velocities and in particular, a high negative correlation in the testis between ARA and the highest VCL (Fig. 3). Therefore, this may indicate that ARA is metabolized to form prostaglandins which are involved in steroid production (Wade and Van der Kraak, 1993), which may help increase the speed of spermatozoa, thus highlighting the importance of this fatty acid. Eels do not feed during the maturation and spermiation period and, their energy reserves are consumed to maintain their metabolism and also to carry out several processes as gonad formation (Baeza et al., 2014; Chapter I) or, as described by the present study, energy from fatty acids could be used to increase the motility and velocity of sperm. Recently, tests were carried out on Senegalese sole (*Solea senegalensis*), using diets with different contents of ARA along the reproductive cycle, and it was found that the presence of ARA in tissues differs depending on the sex (Norambuena et al., 2012). In the present study, negative and positive correlations were found (but lower than in the case of ARA) between EPA in the testis and the highest registered velocities. Recently, EPA seems to have a modulatory effect on the synthesis of androgens in eels with mature sperm (Baeza et al., 2015; Chapter II) so, both fatty acids (ARA and EPA) play important roles in male reproduction. ARA has been shown to be the main precursor for the production of series-2 prostaglandins, whereas it has been reported

that EPA functions as an inhibitory regulator (Asturiano et al., 2000, Sargent et al., 2002) and in our study, both ARA and EPA appear to have an important function with regard to spermatozoal velocity. Baeza et al. (2014; Chapter I) also highlighted the importance of these fatty acids (ARA and EPA) in male European eel reproduction, stating that EPA seems to be mobilized from the liver, where it has previously been synthesized. Figure 4B shows (in red) the most important correlations between ARA from the three tissues and sperm velocity.

Another important result from the present study is that, in different tissues (muscle and testis), a positive correlation between several n-9 series fatty acids (20:1-n9 and 22:1-n9) and higher sperm velocities was found. The eels used in this study came from a fish farm and these fatty acids are not usually present when they feed in the wild. Positive correlations were found between these n-9 series fatty acids and velocity parameters, which could prove important in the design of broodstock diets for male European eel and in the improvement of sperm quality.

5. Conclusions

Overall our results suggest that, in the European eel, fatty acids, in particular ARA, EPA, ALA and LA are linked to sperm quality parameters. All of this information, together with the conclusions made by Baeza et al. (2014, 2015; Chapters I-II), could prove useful in the development of enriched diets that may improve sperm quality, which in turn, could have an impact on the reproductive abilities of European eel males, thus improving fertilization success and embryo development. With the importance of PUFA in mind we propose further research aimed at improving the reproductive performance of eels by manipulating dietary requirements. The first step for further investigations might be to try to find the optimum inclusion levels of these fatty acids for commercial diets.

Acknowledgements

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SUPPLEMENTARY TABLES

Supplementary Table 1. P values evidencing the general lack of significant differences between fatty acids data obtained from **muscle** samples of fish treated with different thermal regimes, when samples were classified into several sperm Curvilinear Velocity (VCL) categories. Comparisons were made by one way ANOVA. All ANOVA were followed by post-hoc multiple comparison by Newman-Keuls with a significance level of $P<0.05$.
No differences were found in this case.

Muscle fatty acid	PARAMETER			
	VCL1	VCL2	VCL3	VCL4
14:0	0.935	0.507	0.629	0.717
16:0	0.961	0.069	0.673	0.383
18:0	0.495	0.720	0.326	0.365
16:1	0.911	0.951	0.910	0.886
18:1n-7	0.378	0.395	0.665	0.916
18:1n-9	0.212	0.951	0.472	0.620
20:1	0.726	0.905	0.335	0.580
22:1n-9	0.149	0.710	0.225	0.241
18:2n-6	0.492	0.887	0.463	0.729
18:3n-3	0.939	0.983	0.793	0.725
20:4n-6 (ARA)	0.298	0.587	0.235	0.714
20:5n-3 (EPA)	0.475	0.322	0.144	0.926
22:5n-3	0.546	0.386	0.418	0.431
22:6n-3 (DHA)	0.249	0.352	0.060	0.759

Number of samples in each category and from each thermal regime: VCL1, T10n=3; T15n=7; T20n=3; VCL2, T10n=3; T15n=7; T20n=18; VCL3, T10n=5; T15n=5; T20n=10; VCL4, T10n=4; T15n=13; T20n=16.

Supplementary Table 2. P values evidencing the general lack of significant differences between fatty acids data obtained from **liver** samples of fish treated with different thermal regimes, when samples were classified into several sperm Curvilinear Velocity (VCL) categories. Comparisons were made by one way ANOVA. All ANOVA were followed by post-hoc multiple comparison by Newman-Keuls with a significance level of P<0.05.
* Differences were considered significant when P values <0.05.

Liver fatty acid	PARAMETER			
	VCL1	VCL2	VCL3	VCL4
14:0	0.431	0.828	0.903	0.741
16:0	0.867	0.825	0.927	0.560
18:0	0.577	0.644	0.036*	0.475
16:1	0.805	0.433	0.508	0.983
18:1n-7	0.701	0.302	0.914	0.613
18:1n-9	0.839	0.531	0.868	0.857
20:1	0.660	0.311	0.449	0.984
22:1n-9	0.021*	0.247	0.594	0.127
18:2n-6	0.685	0.201	0.272	0.103
18:3n-3	0.495	0.122	0.530	0.195
20:4n-6 (ARA)	0.833	0.451	0.132	0.720
20:5n-3 (EPA)	0.071	0.065	0.271	0.076
22:5n-3	0.360	0.098	0.338	0.032*
22:6n-3 (DHA)	0.268	0.465	0.170	0.139

Number of samples in each category and from each thermal regime: VCL1, T10n=3; T15n=7; T20n=3; VCL2, T10n=3; T15n=7; T20n=18; VCL3, T10n=4; T15n=5; T20n=10; T10n=4; T15n=12; T20n=16.

Supplementary Table 3. P values evidencing the general lack of significant differences between fatty acids data obtained from testis samples of fish treated with different thermal regimes, when samples were classified into several VCL sperm categories. Comparisons were made by one way ANOVA. All ANOVA were followed by post-hoc multiple comparison by Newman-Keuls with a significance level of P<0.05.
* Differences were considered significant when P values <0.05.

Testis fatty acid	PARAMETER			
	VCL1	VCL2	VCL3	VCL4
14:0	0.760	0.227	0.125	0.766
16:0	0.929	0.517	0.148	0.168
18:0	0.949	0.159	0.145	0.584
16:1	0.711	0.208	0.110	0.545
18:1n-7	0.883	0.379	0.187	0.325
18:1n-9	0.673	0.317	0.198	0.504
20:1	0.862	0.166	0.025*	0.828
22:1n-9	0.905	0.525	0.176	0.904
18:2n-6	0.501	0.333	0.216	0.587
18:3n-3	0.619	0.589	0.145	0.250
20:4n-6 (ARA)	0.507	0.899	0.105	0.518
20:5n-3 (EPA)	0.189	0.774	0.639	0.070
22:5n-3	0.991	0.776	0.442	0.291
22:6n-3 (DHA)	0.131	0.310	0.973	0.264

Number of samples in each category and from each thermal regime: VCL1, T10n=3; T15n=7; T20n=3; VCL2, T10n=3; T15n=7; T20n=18; VCL3, T10n=5; T15n=5; T20n=10; VCL4, T10n=4; T15n=13; T20n=16.

CHAPTER IV

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

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Abstract

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

1. Introduction

Proper nutrition is essential for rearing healthy broodstock and for the production of high-quality gametes and viable offspring (reviewed in Izquierdo et al., 2001). Most of the work presented in the literature on dietary impacts has focused on female rather than male broodstock. However, when dietary effects are tested in properly designed experiments, they have been shown to account for a significant portion of variation in phenotypic expression and reproductive traits in males (Asturiano et al., 2001; Alavi et al., 2009; Henrotte et al., 2010; Nyina-Wamwizaet et al., 2012; Norambuena et al., 2013). For instance,

dietary fatty acids affected sperm morphology and sperm velocity in common barbel, *Barbus barbus* (Alavi et al., 2009); biochemical composition of sperm in Eurasian perch, *Perca fluviatilis* (Henrotte et al., 2010) was altered by dietary components; Nyina-Wamwiza et al. (2012) found higher milt volumes and improved sperm velocity in African catfish, *Clarias gariepinus*, fed a diet where fishmeal was completely substituted by agricultural products and consequently had higher levels of n-6 series fatty acids; and there was an effect of dietary arachidonic acid [ARA; C20:4(n-6)], levels on steroid production in Senegalense sole, Solea senegalensis (Norambuena et al., 2013). Furthermore, European sea bass, *Dicentrarchus labrax*, exhibited enhanced reproductive performance (i.e. increased sperm quality and fertilization success) when males were fed polyunsaturated fatty acids (PUFA)-enriched diets (Asturiano et al., 2001). Together, these studies (among others) demonstrate that broodstock diet is an important factor for male reproductive performance, thus dietary influences on male as well as female broodstock gamete production and quality need attention.

European eel, *Anguilla anguilla*, has long been a highly valued species targeted for aquaculture production (Ottolenghi et al., 2004; Nielsen and Prouzet, 2008). In order for eel aquaculture to be sustainable, the life cycle should be completed in captivity. In this regard, the development of 'optimal' broodstock diets may improve the species' reproductive success in captivity, through the production of high-quality gametes and viable offspring from farmed fish (Heinsbroek et al., 2013; Støttrup et al., 2013). After studying sperm quality parameters and fatty acid composition during hormone-induced sexual maturation, Baeza et al. (2013; Chapter III) found significant correlations between several fatty acids and sperm quality parameters in male European eel. They mainly found a relationship between eicosapentaenoic acid [EPA; C20:5(n-3)] and sperm volume; sperm motility and PUFA precursors; and ARA and sperm velocity parameters.

In this study, male European eel were reared on three "enhanced" diets and one commercial diet, each with different levels of dietary fatty acids, ARA, EPA and docosahexaenoic acid [DHA; C22:6(n-3)].

Here, our aim was to evaluate the influence of these diets on muscle composition, and liver lipids prior to induced maturation, and the resulting sperm composition and performance (i.e. total volume and motility). Together, this information will prove useful for the development of sustainable aquaculture for European eel, through development of diets that enhance sperm quality, fertilization success, and larval production.

2. Materials and methods

2.1. Fish husbandry, treatments, and experimental procedures

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

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

After acclimatization, 32 males (8 randomly selected per diet) were sacrificed for morphometric records, histology, and lipid analyses. The

remaining male eels (n = 39) received weekly injections of human chorionic gonadotropin at 1.5 IU/g fish (Sigma Aldrich Denmark A/S).

Table 1. Composition of the diets (% of total fatty acids) used to examine the impact of dietary fatty acids on muscle, liver and milt lipid composition, and sperm performance in male European eel, *Anguilla anguilla*.

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

Prior to hormonal treatment males were anaesthetized (Benzocaine, 60 mg/L) and tagged with a passive integrated transponder in the dorsal muscle. During maturation, male eels were kept at a density of ≤ 30 kg per m3. Salinity and temperature ranged from 36.7 to 37.3% and 19.5 to 20.5 °C, respectively.

After the 11th injection, milt was sampled for analyses of lipid composition as well as sperm performance and males were subsequently sacrificed for analyses of morphology and histological testes development.

2.2. Data collection

2.2.1. Body morphology

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

For the hormonally treated males, total body mass and length of each fish was recorded at the time of first injection (22 September 2011) and then at weekly intervals for 10 weeks (last injection was 28 November 2011). On 29 November 2011, i.e. ~24 h after the last treatment, the males were stripped to obtain all available milt. Sperm analyses were performed (see below) and subsamples frozen as above for lipid analysis. The fish were subsequently sacrificed and dissected. Total body mass and testes weight was recorded and testes preserved for histological analysis of reproductive development (see above). Gonadosomatic index (GSI = (testes mass/total body mass) × 100) was later calculated and used as the proxy for male reproductive investment.

2.2.2. Sperm quality analyses

Milt was collected from 27 males 24 h after administration of the 11th hormone injection, as previous studies (i.e. Pérez et al., 2000) showed that this is the time when the highest sperm quality is found. Prior to harvest, males were anaesthetized with benzocaine (60 mg/L) and the genital pore was wiped dry after cleaning with deionized water. Milt was collected by applying light pressure on the abdomen, and

stored at 4 °C. Milt was filled into graduated tubes to calculate the total volume stripped per male (expressed as mL 100 g/fish).

Sperm motility was assessed according to Gallego et al. (2013). In brief, individual sperm samples were evaluated by checking the percentage of motile spermatozoa. The sperm cells were activated by mixing 2 μL of milt with 200 μL of artificial seawater [Aqua Medic Meersalz, 37 g/L, with 2% BSA (w/v), pH adjusted to 8.2; Peñaranda et al., 2010]. The sperm suspension (2 μL) was then pipetted into a Makler reusable chamber (10 μm deep; Sefi Medical Instruments, Haifa, Israel) and motility was observed between 15 and 30 s after activation using a Nikon Eclipse 55i microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon DS-Fi1 camera head and and 40× objective lens. All equipment was maintained at room temperature (~20 °C). Samples were performed in triplicate and analyzed by the same trained observer to avoid subjective differences in the motility evaluation.

2.2.3. Lipid analyses

Lipid extraction and determination of lipid content

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

Lipid class separation

Lipids were separated into neutral lipids (NL) and phospholipids (PL) by chromatography on a solid phase consisting of aminopropyl modified silica. Solvents with increasing polarity were used to separate the lipid classes. A lipid extract corresponding to 10 to 100 mg lipid was used

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

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

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

2.2.4. Histological analyses

For the assessment of testes development, lobes sampled from the middle part of testes were dehydrated and embedded in paraffin and sectioned at 5 µm. The sections were stained with haematoxylin and eosin (H & E, VWR - Bie & Berntsen A/S, Denmark). The histological sections were photographed (Olympus DP 71 digital camera) at 200× magnification for identification of gamete development stages and tissue types. Testes tissues were categorized according to cell types and their relative area fraction (F) estimated (Tomkiewicz et al., 2011). Cell types included testicular somatic cells (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz) (Fig. 1).

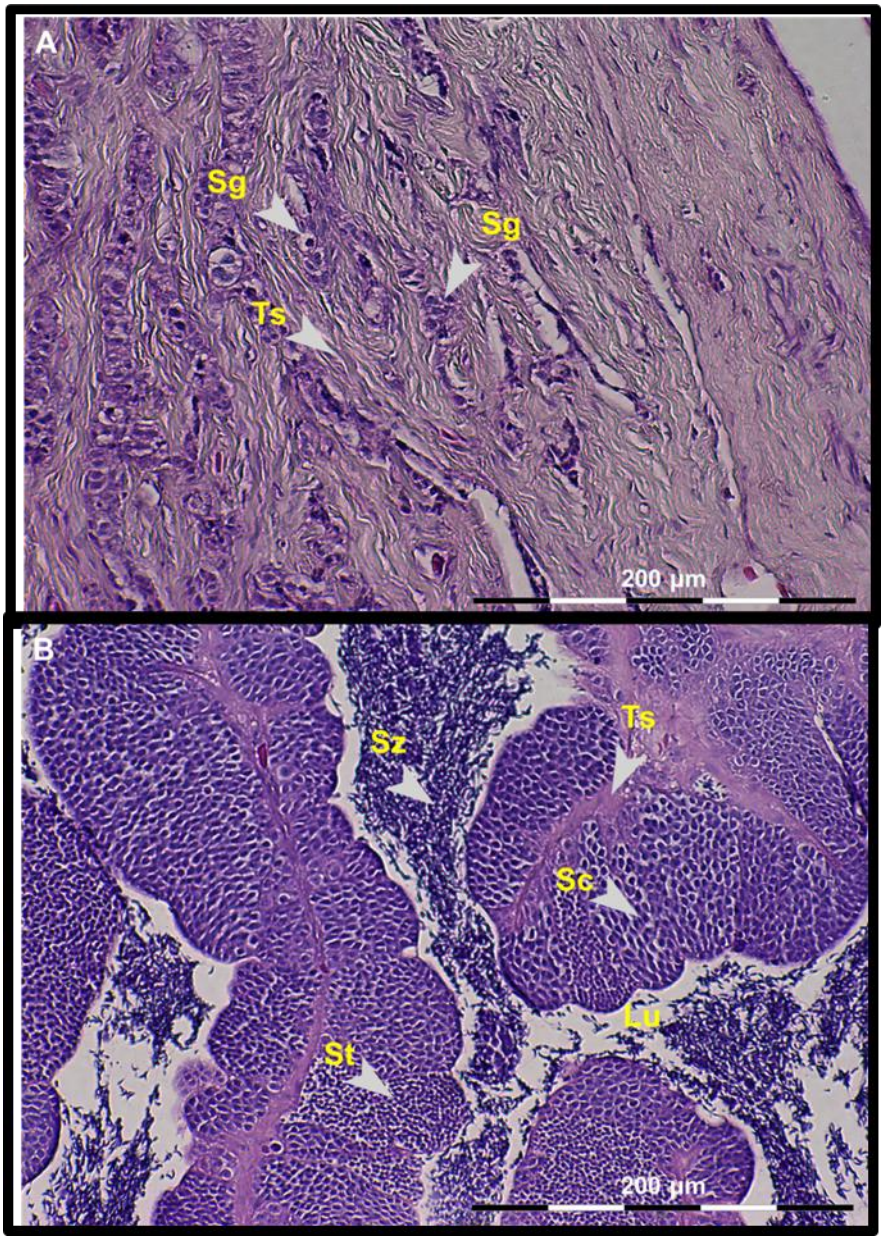

Figure 1. Photomicrographs of histological testes tissue sections of European eel, *Anguilla anguilla*, illustrating testes development and cell types after 38 weeks of feeding, prior to hormonal treatment (A) and after 11 weeks of hormonal treatment (B). Ts = testicular somatic cells, Sg = spermatogonia,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

Excluded areas included areas with no tissue and lumen (Lu). The area fractions (F) of the different tissue categories were estimated by placing a point grid (48 points) on the images (photomicrographs) using the software ImageJ plugin Analyze. The categories were marked and counted using Cell Counter.

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

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

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

2.3. Statistical analyses

Data were analysed using SAS statistical analysis software (v.9.1; SAS Institute Inc., Cary, NC, USA) and the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA). Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs. predicted values; PROC GPLOT). Data were transformed to meet assumptions of normality, and homoscedasticity when necessary. Alpha was set at 0.05 for main effects and interactions. Treatment means were contrasted using the Tukey's test.

2.3.1. Body morphology

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

$$Yipn = \mu + Mi + Ap + MAip + \varepsilon n(ip)$$

where μ is the true mean; Mi is the diet effect (where p = PRO-EEL1, PRO-EEL2, PRO-EEL3, and the DAN-EX diet); Ap is the effect of sampling week (where i = number of weeks); MAip is the diet × sampling week interaction; and εn(ip) is the residual error. When a non-significant first-order diet × sampling week interaction was detected, main effects were interpreted. Diet and sampling week were fixed factors, while female was random and included as the subject in the REPEATED statement in SAS PROC MIXED (Littell et al., 1996; SAS, 2003). The Kenward-Roger procedure was used to approximate the denominator degrees of freedom for all F-tests (Spilke et al., 2005). The repeated statement was used to model the covariance structure within subjects (Littell et al., 1996). Three covariance structures were modeled: compound symmetry (type = cs), autoregressive order one (type = ar(1)), and "unstructured" (type = un). Akaike's (AIC) and Bayesian (BIC) information model-fit criterion were used to assist in final model inference determination (Littell et al., 1996). One-way ANOVA was used to compare GSI between the dietary groups.

2.3.2. Lipid analyses

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

2.3.3. Sperm quality and histological analyses

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

3. Results

3.1. Body morphology

Prior to the onset of hormonal treatment, total body mass for the 8 fish sacrificed per diet (these fish were used for lipid analyses) ranged from 86 to 174 cm and there was no significant difference in body mass between the fish fed the four diets ($P > 0.05$). For the hormonally treated males, body mass ranged from 83 to 187 g, 87 to 159 g, 90 to 156 g, and 92 to 143 g for the dietary groups (Fig. 2). The broodstock diet × sampling week interaction ($P > 0.05$) and broodstock diet main effect ($P > 0.05$) both had no significant impact on total body mass of the fish throughout the experimental sampling period (Fig. 2A). On the contrary, the sampling week main effect was significant ($P < 0.0001$; Fig. 2B), such that total mass of the fish declined over the sampling period.

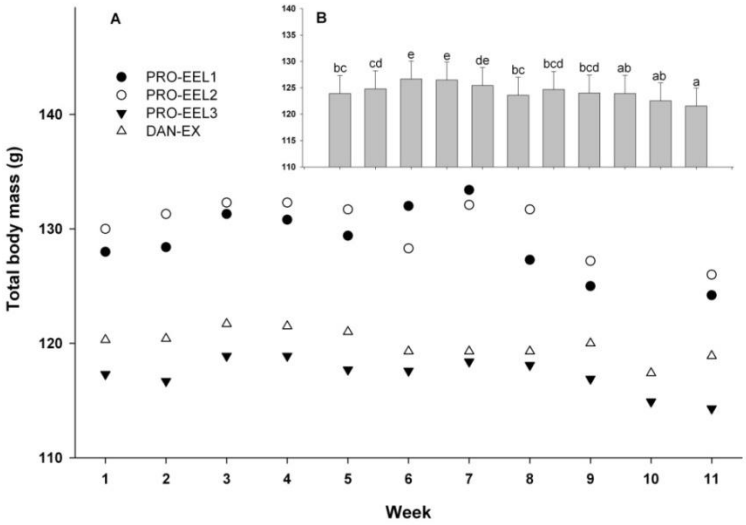

Figure 2. Effect of diet (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet) on total body mass of hormonally treated European eel, *Anguilla anguilla,* broodstock throughout the sampling period (A). Data were analyzed using a repeated measures ANOVA model. The broodstock diet × sampling week interaction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

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

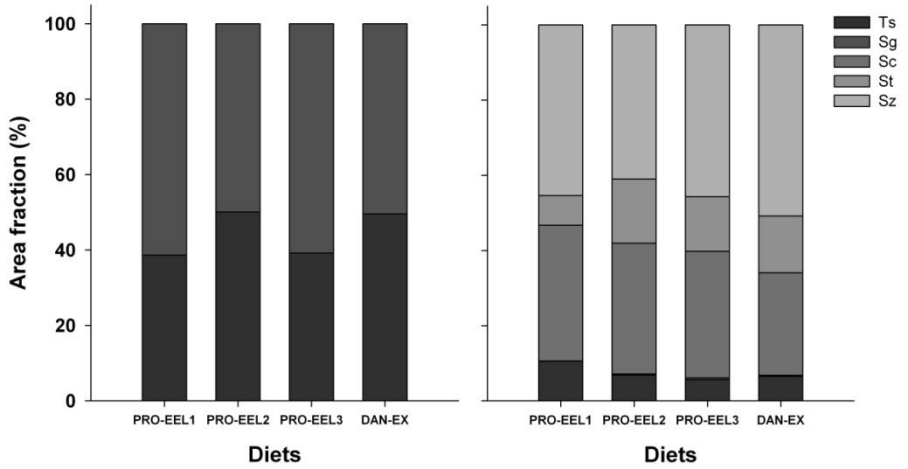

Figure 3. Testes development of European eel, *Anguilla anguilla* males after 38 weeks of feeding on different diets (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet) illustrated as the average area fractions, *F*, of different tissue types in males sacrificed prior to hormonal treatment (A) or after 11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

3.2. Lipid analyses

3.2.1. Muscle composition

After 38 weeks of feeding on the various diets and prior to maturation, MUFA represented 47.0 to 51.8% of the NL fraction of the muscle tissue, whereas SFA and PUFA were found in quantities of 19.8 to 25.8% (Table 2). The most abundant fatty acids in the NL fraction of muscle

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

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

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

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

Fatty acids	Dietary treatment			
	PRO-EEL1	PRO-EEL2	PRO-EEL3	DAN-EX
C14:0	4.07±0.10	4.02±0.38	3.43±0.20	3.80±0.16
C16:00	16.73±0.42	17.16±0.25	16.28±0.26	16.09±0.32
C16:1(n-7)	1.68±0.14 a	1.96±0.11 a	1.57±0.08 a	2.74±0.35 b
C18:00	4.20±0.29	4.57±0.24	3.82±0.16	3.85±0.63
C18:1(n-9)	12.06±0.25 b	9.40±0.26 a	12.34±0.24 b	9.13±0.21 a
C18:1(n-7)	2.40±0.08 ab	2.17±0.06 a	2.53±0.10 b	2.39±0.08 ab
C18:2(n-6)	2.20±0.11 b	1.01±0.06 a	2.52±0.21 b	1.39±0.05 a
C18:3(n-3)	0.34±0.03 b	0.16±0.01 a	0.40±0.07 b	0.26±0.03 ab
C20:1(n-7)	0.06±0.01 ab	0.05±0.01 ab	0.01±0.01 a	0.07±0.02 b
C20:4(n-6)	7.97±0.58 d	6.37±0.31 c	3.98±0.16 b	2.38±0.10 a
C20:5(n-3)	9.56±0.43 a	14.11±0.38 c	11.45±0.30 b	15.85±0.29 d
C22:1(n11)	0.58±0.06 b	0.24±0.02 a	0.20±0.02 a	0.70 ±0.15 b
C22:5(n-3)	1.68±0.03 a	2.69±0.12 c	1.92±0.09 ab	2.04±0.10 b
C22:6(n-3)	22.73±0.62 a	24.42±0.45 ab	26.13±0.44 bc	26.77±0.74 c
total n-3	34.97±0.84 a	42.07±0.63 b	40.50±0.43 b	45.94±0.67 c
total n-6	10.88±0.55 c	7.74±0.25 b	7.16±0.34 b	4.3±0.12 a
n-3/n-6	3.28±0.21 a	5.47±0.15 ab	5.76±0.32 ab	10.74±0.25 b
total SFA	25.52±0.45 ab	26.27±0.36 b	24.20±0.42 a	24.21±0.70 a
total MUFA	22.02±0.63 b	17.08±1.48 a	20.32±0.40 b	19.67±1.01 ab
total PUFA	46.50±0.98 a	50.77±0.82 b	48.42±0.52 ab	51.00±0.72 b

The dominant classes of fatty acids in the PL fraction of the muscle were PUFA (46.5 to 51.0%), followed by SFA (24.2 to 26.3%) and MUFA (17.1 to 22.0%; Table 3). The most abundant fatty acids were DHA, representing 22.7 to 26.8%, and palmitic acid, representing 16.1 to 17.2% (Table 3). Diet had a significant effect on the majority of fatty acids in the PL fraction of muscle (P < 0.05; Table 3). Here, the essential fatty acids, ARA, EPA, and DHA were impacted by diet (ARA: P < 0.0001; EPA: P < 0.0001; DHA: P < 0.0001). PRO-EEL1 had the highest levels of ARA (8.0%), the DAN-EX diet had the highest levels of EPA

(15.9%), and PRO-EEL3 as well as the DAN-EX diet had the highest level of DHA (26.1 and 26.8%, respectively) in the PL fraction of muscle tissue (Table 3).

3.2.2. Liver lipid

After 38 weeks of feeding on the various diets and prior to maturation, diet had no impact on levels of total SFA in liver NL. On the contrary, PRO-EEL1 had higher levels of MUFA than PRO-EEL2 or the DAN-EX diet, while PRO-EEL2 had the highest level of PUFA ($P < 0.05$; Table 4) in liver NL. Palmitic acid and oleic acid were the most abundant fatty acids in the NL fraction of liver. In particular, for oleic acid the percentage of total fatty acids was highest in PRO-EEL1 and PRO-EEL3 ($P < 0.01$), while no significant difference was detected for palmitic acid in NL fraction of the liver (Table 4). Diet had a significant effect on ARA, EPA and DHA levels in liver NL (ARA: $P < 0.0001$; EPA: $P < 0.0001$; DHA: $P < 0.0001$), where the PRO-EEL2 diet typically had the highest level of these fatty acids (Table 4).

DHA and palmitic acid were the most abundant fatty acids in the PL fraction of the liver (Table 5). The dominant classes of fatty acids in the PL fraction of the liver were PUFA (48.3 to 53.8%), followed by SFA (27.6 to 31.2%) and MUFA (8.8 to 11.5%; Table 5). Diet had a significant effect on ARA, EPA and DHA levels in liver PL (ARA: $P < 0.0001$; EPA: $P < 0.0001$; DHA: $P < 0.0001$; Table 5). Here, PRO-EEL1 had the highest ARA, DAN-EX had the highest EPA, and PRO-EEL3 had the highest level of DHA (Table 5).

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

Fatty acids	Dietary treatment			
	PRO-EEL1	PRO-EEL2	PRO-EEL3	DAN-EX
C14:0	3.95±0.18a	3.68±0.11a	3.94±0.17b	4.83±0.33b
C16:00	19.07±0.54	18.83±0.28	18.48±0.64	18.67±0.81
C16:1(n-7)	7.59±0.32b	7.67±0.24b	6.54±0.19a	7.47 ±0.19b
C18:00	2.38±0.34	2.79±0.37	2.15±0.12	4.86±2.26
C18:1(n-9)	29.29±1.08c	20.09±1.05a	27.21±0.70bc	21.58±2.57ab
C18:1(n-7)	3.42±0.14	3.15±0.19	3.82±0.08	3.39±0.48
C18:2(n-6)	4.14±0.13b	2.48±0.10a	5.49±0.23c	2.72±0.09a
C18:3(n-3)	0.81±0.04b	0.57±0.03ab	1.22±0.07c	0.44±0.10a
C20:1(n-7)	0.26±0.03bc	0.17±0.02ab	0.13±0.02a	0.33±0.06c
C20:4(n-6)	1.12±0.09b	1.38±0.09b	0.76±0.02a	0.53 ±0.07a
C20:5(n-3)	3.20±0.39a	8.05±0.35b	4.03±0.29a	2.87±0.19a
C22:1(n11)	0.95±0.07b	0.47±0.06a	0.54±0.04ab	2.20 ±0.21c
C22:5(n-3)	1.80±0.17a	4.36±0.17c	2.81±0.11b	1.88±0.15a
C22:6(n-3)	5.85±0.69a	12.76±0.60c	10.18±0.57b	6.65±0.64a
total n-3	11.66±1.25a	25.74±0.83c	18.23±0.93b	11.82±0.50a
total n-6	5.26±0.20b	3.86±0.11a	6.25±0.24c	3.25±0.13a
n-3/n-6	2.19±0.18a	6.69±0.24c	2.95±0.18ab	3.68±0.23b
total SFA	25.40±0.69	25.29±0.49	24.57±0.74	28.36±3.06
total MUFA	41.50±0.89c	31.54±0.78a	38.24±0.67bc	34.96±3.10ab
total PUFA	16.92±1.42a	29.60±0.88c	24.48±1.01b	15.07±0.50a

3.2.3. Sperm composition

DHA and palmitic acid were the most abundant fatty acids in eel sperm, followed by oleic acid and EPA (Table 6). PRO-EEL2 had higher levels of SFA than the DAN-EX diet, while MUFA levels were higher in PRO-EEL1 than PRO-EEL2; no other significant differences were observed between the other diets for total SFA and total MUFA (Table 6). Dietary effects had an influence on the relative content of the majority of the sperm fatty acids ($P < 0.05$). Here, PRO-EEL1 and PRO-EEL2 had the highest levels of ARA, while PRO-EEL1 had the lowest level of EPA (Table 6).

3.3. Sperm quality analyses

Mean ± SEM total extractable milt volume from the males stripped in week 11 was 3.69 ± 0.4 mL/100 g fish. Diet had a significant influence on sperm volume ($P < 0.05$), such that fish reared on the DAN-EX diet produced significantly more milt volume than fish reared on PRO-EEL1, while PRO-EEL 2 and PRO-EEL 3 showed intermediate values (Fig. 4A). Mean sperm motility for the diets ranged from 42.8 ± 8.1 to 72.5 ± 7.2%. Diet had a significant effect on sperm motility ($P < 0.05$; Fig. 4B), such that PRO-EEL2 had significantly higher motility than PRO-EEL1; no significant differences in motility were observed between the other diets.

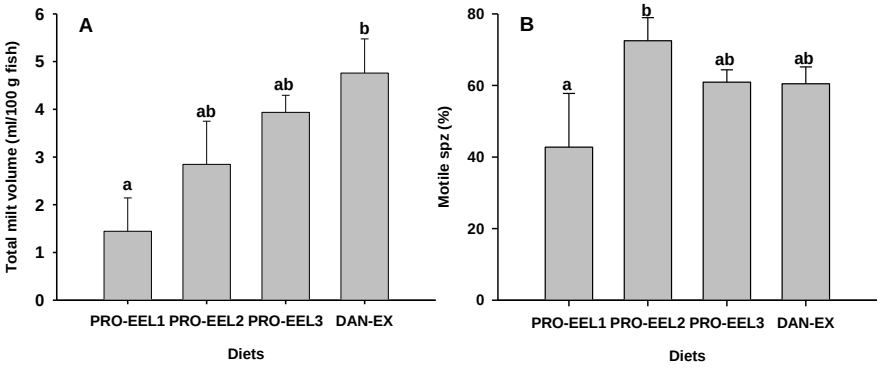

Figure 4. Effect of diet (PRO-EEL1, PRO-EEL2, PRO-EEL3, and DAN-EX diet) on total milt volume (A) and percent motile sperm (B) of European eel, *Anguilla anguilla*. Error bars represent standard errors. Bars without a common superscript differed significantly ($P < 0.05$).

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

Fatty acids	Dietary treatment			
	PRO-EEL1	PRO-EEL2	PRO-EEL3	DAN-EX
C14:0	1.34±0.08b	1.43±0.04b	1.05±0.05a	1.51±0.03b
C16:00	20.54±0.38ab	21.81±0.45b	19.66±0.63a	20.90±0.47ab
C16:1(n-7)	1.53±0.11ab	1.67±0.07bc	1.25±0.03a	1.91±0.08c
C18:00	7.64±0.31	7.97±0.42	6.93±0.99	6.23±0.27
C18:1(n-9)	7.47±0.27b	5.25±0.12a	7.38±0.16b	6.75±0.17b
C18:1(n-7)	1.90±0.04	1.80±0.04	2.40±0.73	1.88±0.05
C18:2(n-6)	1.07±0.06b	0.48±0.02a	1.28±0.08c	0.95±0.03b
C18:3(n-3)	0.16±0.02a	0.08±0.00a	0.27±0.05b	0.13±0.01a
C20:1(n-7)	0.12±0.01b	0.07±0.01a	0.07±0.01a	0.11±0.01b
C20:4(n-6)	6.56±0.54c	4.65±0.19b	3.68±0.18ab	2.89 ±0.12a
C20:5(n-3)	6.97±0.26a	10.27±0.50b	9.44±0.23b	12.40±0.53c
C22:1(n11)	0.49±0.30	0.06±0.02	0.05±0.01	0.31±0.02
C22:5(n-3)	2.48±0.47	2.23±0.15	2.14±0.11	2.55±0.11
C22:6(n-3)	31.05±1.04a	34.32±0.49b	36.96±0.80c	31.91±0.64ab
total n-3	40.65±0.63a	46.90±0.58b	48.81±0.90b	46.99±0.64b
total n-6	7.63±0.58c	5.13±0.17b	4.96±0.24ab	3.84±0.14a
n-3/n-6	5.62±0.55a	9.22±0.36b	10.04±0.59b	12.34±0.45c
total SFA	29.51±0.56ab	31.20±0.45b	27.64±1.46a	28.64±0.62ab
total MUFA	11.51±0.26b	8.84±0.16a	11.14±0.81b	10.97±0.23b
total PUFA	48.28±0.83a	52.02±0.59bc	53.76±0.89c	50.83±0.67ab

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

Fatty acids	Dietary treatment			
	PRO-EEL1	PRO-EEL2	PRO-EEL3	DAN-EX
C14:0	0.83±0.21 b	0.73±0.12 ab	0.63±0.12 a	0.64±0.07 ab
C16:00	18.89±1.23	19.39±0.85	18.69±1.09	18.38±0.72
C16:1(n-7)	1.55±0.15	1.71±0.21	1.53±0.16	1.64±0.12
C18:00	5.65±0.30 a	6.35±0.27 b	6.08±0.34 ab	5.79±0.43 a
C18:1(n-9)	12.89±2.12 ab	10.92±0.86 ab	12.90±2.26 b	10.65±0.84 a
C18:1(n-7)	3.25±0.08	3.52±0.22	3.33±0.19	3.28±0.13
C18:2(n-6)	2.70±0.60 bc	1.58±0.32 a	2.73±0.44 bc	2.05±0.27 ab
C18:3(n-3)	0.12±0.04	0.10±0.03	0.08±0.02	0.08±0.02
C20:1(n-7)	5.94±1.11 b	3.98±0.61 a	4.26±0.65 a	5.74±0.43 b
C20:4(n-6)	7.01±0.55 c	7.13±0.72 c	5.29±0.53 b	4.03±0.43 a
C20:5(n-3)	13.18±1.50 a	18.21±1.79 b	16.66±2.02 b	18.25±1.43 b
C22:1(n11)	0.35±0.19 b	0.21±0.03 ab	0.18±0.04 a	0.24±0.03 ab
C22:5(n-3)	1.60±0.23 a	2.02±0.28 b	1.73±0.17 ab	1.64±0.21 a
C22:6(n-3)	18.92±2.62 ab	17.04±2.67 a	19.01±0.91 ab	20.59±1.55 b
total n-3	34.64±1.76 a	38.00±1.76 b	38.13±2.23 b	41.21±0.75 c
total n-6	10.52±0.47 c	9.32±0.94 bc	8.90±0.65 b	6.92±0.73 a
n-3/n-6	3.30±0.21	4.12±0.49	4.3±0.40	6.01±0.61
total SFA	25.56±1.20 ab	26.71±0.97 b	25.61±1.09 ab	24.99±0.88 a
total MUFA	24.79±1.64 b	20.75±1.68 a	22.49±2.26 ab	22.04±0.73 ab
total PUFA	45.78±1.80	47.70±1.63	47.54±2.29	48.57±0.95

4. Discussion

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

Males reared on the PRO-EEL1 diet had "sub-optimal" sperm quality parameters; i.e. lowest mean milt volume and sperm motility. This diet also had the lowest n-3/n-6 ratio (1.04), due to a low percentage of n-3 series fatty acids and a high percentage of n-6 series fatty acids [consequently, high linoleic acid, (LA, 18:2(n-6)), and ARA levels]. Nowadays, the global shortages in fish oil are forcing the aquaculture feed industry to use alternative oil sources. Such dietary supplementations with vegetable oils contain large amounts of n-6 series fatty acids, such as LA. Thus, our findings further exemplify the need to control and optimize the ratio of n-3/n-6 fatty acids in formulated diets to improve reproductive performance in eel. Moreover, it has been demonstrated that EPA and DHA are major components of membrane phospholipids and their presence helps to facilitate the quantity and mobility of ejaculated sperm (Sargent et al., 2002; Henrotte et al., 2010). Thus, the low sperm motility reported for males reared on PRO-EEL1 could be linked to the lower levels of n-3 series fatty acids in this diet (mainly EPA), when compared to the other treatments. Similarly, Vassallo-Agius et al. (2001) found that rainbow trout fed with deficient n-3 series fatty acids, showed lower sperm motility.

The PRO-EEL2 diet, with the highest level of ARA, induced medium milt volumes but had the highest mean sperm motility. This coincides with the previously proposed role of ARA metabolites during the sperm maturation process; this includes prostaglandins, which essentially modulate the sperm maturation (Asturiano et al., 2000; Baeza et al., 2014a; Norambuena et al., 2013). Furthermore, PRO-EEL2 had the highest percentage of n-3 fatty acids levels, especially with regard to

EPA content. In this regard, PRO-EEL2 diet had a 2-fold higher EPA content in comparison to PRO-EEL3 and the DAN-EX diet, however the milt samples of these three experimental groups after induced maturation did not show any differences in EPA percentage. Recently, studies have suggested a preferential utilization of dietary EPA as an energy source for sperm (Senadheera et al., 2011; Al-Souti et al., 2012; Wing-Keong et al., 2013); thus, it could be related to higher sperm motility for the fish fed diets with a high percentage of this fatty acid and producing sperm with higher EPA levels. Baeza et al. (2014a) suggested the existence of a synthesis of PUFA in the liver (especially EPA) from their precursors to be sent to the testis to increase sperm energy for motility and highlighted the importance of EPA during spermatogenesis. Baeza et al. (2014b) found a negative correlation between EPA from the liver and sperm volume, suggesting its synthesis in the liver and its subsequent mobilization to the testis to be used to produce sperm cell membranes. Finally, correlations between EPA and androgens during the final phase of sperm maturation, exemplifies the modulatory effect of EPA on the synthesis of androgens (Baeza et al., 2015). Together, these studies highlight the importance of EPA in male eel reproduction and compliment our findings; our study shows that PRO-EEL2, PRO-EEL3 and DAN-EX diet, having the highest percentage of EPA in sperm, showed the highest sperm motility. Thus, EPA seems to be important for European eel with respect to male reproductive performance.

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

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

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

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

species, thus this focus area should also be explored for the European eel.

The PRO-EEL3 diet (higher LA and ALA) induced higher levels of n-3 series fatty acids in liver NL (especially DHA content) than the DAN-EX diet. Thus, the results from the present study, suggest an effect of high percentages of LA and ALA due to different fatty acid elongation activity in the PRO-EEL3 diet, as explained above.

The allocation of resources from the muscle to the testes was evident from an increase in GSI from week 0 to week 11, which was accompanied by spermatogenesis induced by weekly hormonal treatment. The diets did not influence the progression of spermatogenesis among groups, and all males were still actively producing sperm and milt at the end of the experiment. Muscle and liver fatty acids were analyzed prior to the fasting period, but eel have the ability to reallocate and synthesize lipids from their endogenous sources, even after a prolonged period of fasting (Gnoni et al., 1990). Therefore, although in our experiment the eels were fasted for >70 days, the analyses of sperm fatty acid after the hormonal treatment still reflected the effect of diet previously supplied. But, despite the differences between the compositions of diets, the sperm fatty acids did not show differences in total PUFA. Long chain PUFAs give the sperm plasma membranes the fluidity it needs to participate in membrane fusion events associated with fertilization (Whates et al., 2007). Recently, in gilthead seabream it was reported that membrane unsaturated fatty acids from the sperm head and flagella were positively correlated with motility parameters (Beirao et al., 2012b). Here, our results did not show a direct correlation between unsaturated fatty acids and sperm quality parameters, but independent of the diet supplied, all experimental groups registered a high content of unsaturated fatty acids in the sperm; highlighting their importance in normal sperm function. Independent of diet, eels reached the same PUFA levels probably due to their mobilization from other tissues. Baeza et al. (2014a) found in European eel that stored fatty acids in the liver can be transferred to the gonad, contributing to sperm membrane formation. Thus, defining the adequate PUFA percentage in diets is an important issue to improve sperm quality,

since several studies have highlighted the negative impacts that occur if inadequate PUFA percentages are supplied in diets for both males and female reproductive performance. Accordingly, an excessive n-3 PUFA supplementation in rats decreased both sperm density and motility (Yan et al., 2013). Furuita et al. (2002) also found higher n-3 series PUFA in broodstock diets impaired reproduction in female Japanese flounder, *Paralichthys olivaceus*.

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

5. Conclusions

Overall, our results suggest that an optimal feed for eel should contain high levels of DHA to induce the production of high sperm volumes, high EPA levels (even >7%) to induce high sperm motility, medium levels of ARA, and should have n-3/n-6 ratio close to or >2. We also conclude that LA and ALA levels have importance in diets and their inclusion can fundamentally influence liver lipid metabolism. Taken together, this information will be useful to develop broodstock diets to improve the sperm quality and subsequently, larval production for this species.

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GENERAL DISCUSSION

1. Main issues to discuss

The number of European eels has been declining and even the possible disappearance of populations is very serious indeed, making captive breeding the only sustainable solution to supply young fish to fattening farms and also to recover populations by means of stocking programs.

The complex life cycle of the European eel (explained in detail in the General Introduction) makes it accumulate large lipid reserves mainly to achieve reproductive success when they swim across the Atlantic Ocean within 5-6 months. Migrating eels do not feed, therefore they depend on the energy accumulated in fat stores, which can be as much as 30% of their body weight (Tesch, 2003). Eels have a great amount of fat as energy stores, which they use for gonad development (60%) as well as for migration (40%) (van Ginneken and van den Thillart, 2000). This highlights the high energetic requirements represented by gonad development and gamete formation.

All this has motivated the interest in shedding light on several unknown aspects related to the composition and functions of male energy reserves. The questions raised were:

- Which lipid reserves are used to fuel migration and which to provide offspring with sufficient energy?
- What is the role of these reserves during the spermatogenesis and steroidogenesis?
- Which lipids could influence sperm quality?
- How to apply all the previous findings in broodstock diets to improve sperm quality?

2. Lipid functions in the sexual maturation of eels

The energy required for migration and gonadal development thus depends totally on a large amount of fat accumulation in the body. In Chapter I, we shown which fatty acids were selectively consumed and which fatty acids were maintained, as well as how lipid reserves were mobilized in order to achieve a successful gonad development.

Figure 1 summarizes the main results obtained in Chapter I, in which increases and decreases of fatty acid in muscle, liver and testes in each stage of testis development were studied. Maintenance of muscle fatty acids and increase in eicosapentaenoic acid (20:5-n3, EPA) and palmitic acid from the liver when eels began to produce sperm were found. It was hypothesized that liver fatty acids were increased to be successively sent to the testes. In testes, maintenance of EPA, docosahexae-noic acid (22:6-n3, DHA) and arachidonic acid (20:4-n6, ARA) was found, while the rest of fatty acids decreased.

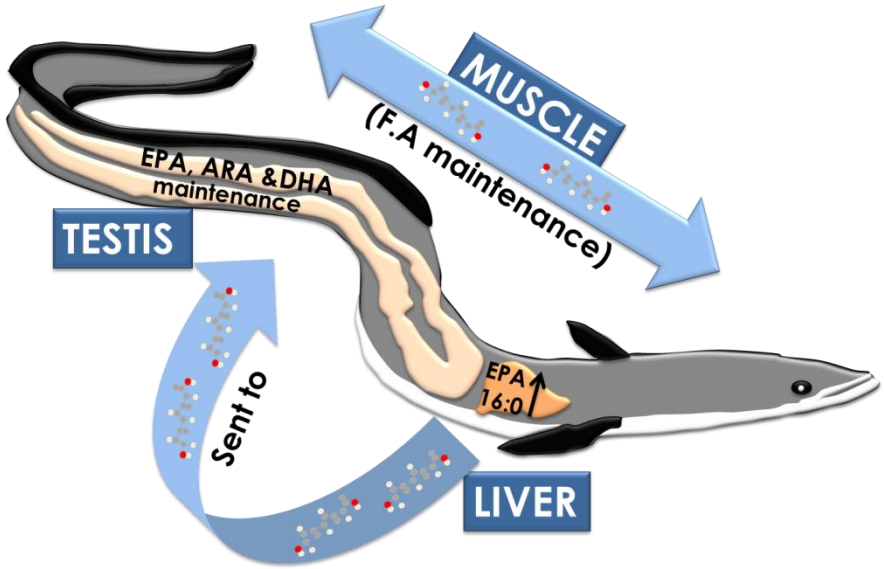

Figure 1. Fatty acid dynamics during testes development in the eel. EPA: Eicosapentaenoic acid; ARA: Arachidonic acid; DHA: Docosahexaenoic acid; F.A: Fatty acid.

The maintenance of polyunsaturated fatty acids (PUFAs) in the testis during sperm maturation while the rest of fatty acids decreased, as explained above, leads to the conclusion that PUFAs are very important in eel reproduction. This was confirmed with the results obtained in Chapter IV, in which independently of the considerable differences between the diets supplied, and after 70 days of fasting, eels showed no differences in the total percentage of PUFAs in the sperm. Both chapters (I and IV) highlighted the idea of the important role of PUFAs in adequate male reproductive performance in terms of sperm production and quality. The spermatozoa membrane contains a high concentration of PUFAs (Whates et al., 2007) which mainly have a structural as well as energetic function.

Regarding the structural function of fatty acids in sperm cells, Beirão et al. (2012a) demonstrated that a sperm head and flagella have a different fatty acid composition in gilthead seabream (*Sparus aurata*). Unsaturated fatty acids in the flagella were higher than in the spermatozoa head membrane, and they attributed this higher fluidity of the flagella membrane to the capacity of movement that flagella give to sperm.

On the other hand, several works highlighted the importance of fatty acids as an energy source for spermatozoa movement. Lahnsteiner et al. (2010) proposed that PUFAs could be used as energy substrate for the spermatozoa. Moreover, Mehdinejad et al. (2013) found that fatty acid oxidation in sperm cell membranes produces ATP, which is subsequently used for spermatozoa movement. Recently, Beirão et al. (2015) described an increment in free fatty acids in semen of sole (*Solea senegalensis*) and they suggested that they could be used as energy source to increase sperm velocity. In Chapter III, we also found a relationship between fatty acids and spermatozoa kinetic parameters such as velocity and motility, and we found a decrease in PUFA precursors in the liver when sperm motility increased and a decrease in ARA in all tissues when sperm velocity increased. These results are in accordance with the works cited above about the use of PUFAs as an energy source.

Regarding the structural function of fatty acids in sperm cells, DHA, EPA and ARA were maintained constant in the testis during sperm

maturation (Chapter I) and it is well known that they are vital in maintaining cell membrane functions (Sargent et al., 1999). ARA has a minor contribution to the fish sperm membrane but small variations may affect its function (Beirão et al., 2015). In Chapter I the main structural role was conferred to DHA. The structure of DHA, with six double bounds, provides a structure that is strong but flexible enough to undergo conformational transitions, making a rapid and successive reorganization of the membrane possible (Glencross et al., 2009). DHA is the most abundant fatty acid present in eel sperm (Chapter IV), as reported by other authors in several fish species (Asturiano et al., 2001, *Dicentrarchus labrax;* Butts et al., 2011*, Gadus morhua*; Beirão et al., 2015, *Solea senegalensis;* Pustowa et al., 2000, *Oncorhynchus mykiss*).

In addition to energy and structural functions that PUFAs have in spermatozoa, as explained above, they may also help spermatozoa at the time of fertilization. In humans, where sperm has acrosome, it has been demonstrated that fatty acids confer to the sperm cell membrane the appropriate fluidity and flexibility to enable membrane fusion (Lenzi et al., 2000). Furthermore, sperm in teleost fish lacks acrosome (Bobe and Labeé, 2010), thus our results suggest that sperm membrane components (such as fatty acids) could be key constituents for a normal function and also for gamete fusion.

Fatty acids affect male gametogenesis as they ensure appropriate sperm membrane composition, as explained above. Moreover, Dupont et al. (2014) recently reviewed the important function of fatty acids also regulating steroid production.

In Chapter II, the main changes in steroid plasma levels during different stages of testis development were showed and related to the amounts of fatty acids (studied in Chapter I). All of the main changes registered in Chapter I-II are represented in Figure 2.

Recently, Peñaranda et al. (submitted) studied in depth all of the changes in hormone levels occurring in the different stages of testis development. Summarizing all of these findings, in the proliferation phase (S1, Figure 2) spermatogonia are replicated by mitosis. During this stage, gonadotropins stimulate the production of 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT), registered to be

at their peak levels at this stage. One of the functions of androgens (T, 11KT) is the induction of germ cell proliferation (Miura and Miura, 2003). As has been observed in other fish species, androgens maintain high levels just before the spermiation period, suggesting a role of androgen in spermatozoa viability (Baynes et al., 1985; Malison et al., 1994).

During the growth phase (S2, Figure 2), 17α-20β-dihydroxy-4-pregnen-3-one (DHP) induces meiosis I occurred. At that stage, spermatogonia become to spermatocytes. During these two first stages (S1-2), fatty acids changes were not observed in any tissue.

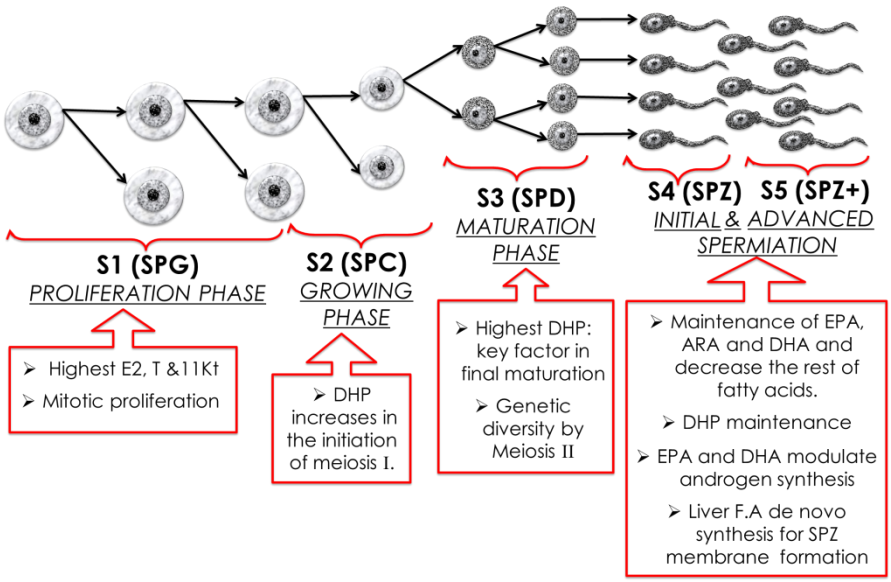

Figure 2. Stages of testis development in the European eel (S1-5) and related changes in fatty acids and hormones. SPG: Spermatogonia; SPC: Spermatocyte; SPD: Spermatid; SPZ: Spermatozoa; E2: 17β-estradiol; T: Testosterone; 11KT: 11-Ketotestosterone; DHP: 17α-20β-dihydroxy-4-pregnen-3-one.

DHP reaches its peak level in the maturation phase (S3, Figure 2) when spermatocytes become spermatids by meiosis II and a great deal of cellular division and multiplication take place. In S3, a lot of new cell membranes must be formed, which could be related to the increase

of fatty acid synthesis in the liver, observed in Chapter I, due to their subsequent use for spermatozoa membrane formation in the testis.

DHP remains constant during initial and advanced spermiation (S4-5, Figure 2). Several authors highlighted DHP as the essential steroid for final spermatogenesis, the meiosis process and also for the acquisition of motility by spermatozoa (Miura et al., 2006; Miura and Miura, 2003; Ohta et al., 1997). The maintenance of EPA and DHA in both spermiation stages observed in Chapter I could be linked to their actions as modulators of androgen synthesis observed in these spermiation stages (Chapter II), when androgen does no longer play a major role.

It is noteworthy that the correlations between fatty acids and steroid hormones (Chapter II) were always below 0.6 ($r \geq 0.6$). This fact invites a reflection. When the correlation coefficient is very high ($r \geq 0.8$), it is considered as satisfactory for researchers. Very low coefficients ($r \leq 0.2$) simply express a relationship and are useless from a practical perspective. However, low and moderate correlations ($r = 0.2$-0.5), like those found in Chapter II, when the fatty acids in different tissues were correlated with steroid hormones, have a very important significance and must be interpreted. The reason why low and moderate correlations between fatty acid and steroid hormones were found is not that they were not related. The explanation is that the steroid hormones are not exclusively determined by fatty acids, but also by other parameters such as animal status, metabolite concentrations or endocrine disruptors which act synergistically and contribute to sperm functionality and viability. Therefore, the correlations found have a profound and important significance and deserve to be thoroughly explained in the next section.

3. New approaches towards elucidating the role of fatty acids in steroidogenesis

The effects of steroid hormones on the fatty acids metabolism, and also the effect of fatty acids on steroid hormones production is a field scarcely studied in fish, other animals and even in humans. Table 1 summarizes the works either in fish, other animals or humans mentioned in Chapter II.

Table 1. Summary of highlighted results explained in Chapter II regarding the main steroids effects on fatty acids and the main fatty acids effects on steroids.

Steroids effect on fatty acids	Fatty acids effect on steroids
T alters desaturase activity in theliver of rats (Marra and Alaniz, 1989)	ARA-stimulates/EPA-inhibits T production in goldfish (Wade et al., 1994)
Progesterone induces lipogenesis in adipose tissue of rats (Lacasa et al., 2001)	-n3 Dietary deficiences affect T and E2 levels in ♀ sea bass(Cerdà et al., 1995)
E2 increases deasturase activity in the liver of tilapia (Hsieh et al., 2004)	-n3 Dietary deficiences affect 11KT levels in ♂ sea bass(Cerdà et al., 1997)
T modulates desaturase activity in the testis of rats (Hurtado and Gómez, 2005)	-n3 enriched diet inhibits T production in men (Nagata et al., 2000)
Estrogen-stimulates/ T-inhibits F.A elongation in the liver of rats (Decsi and Kennedy, 2011)	ARA in testis stimulate sprostaglandin which could affect steroid production in sea bass (Asturiano et al., 2000)
Progesterone stimulates lipogenesis adipose tissue of rats (Stelmanska and Aswierczynski 2013)	DHA enriched diet inhibits T and E2 levels in pigs (Castellano et al., 2011)

New insights into these interactions are found in Chapter II:

DHP modulates the lipid metabolism→ The results presented in Chapter II regarding the effect of fatty acids on steroid hormones production showed that during S1 and S2 a positive correlation between several liver fatty acids and DHP was found. Palmitic acid was the fatty acid correlating at both stages with DHP. The main product of fatty acid biosynthesis is palmitic acid (Cook and Mc Master, 2002). We suggested that DHP is able to stimulate fatty acid synthesis controlling the amount of fatty acid production which later will be used in spermatozoa membrane formation.

Fatty acid oxidation promotes E2 production→ Regarding the effect of steroid hormones on fatty acid production, the results presented in Chapter II showed negative correlations between several fatty acids and E2. This fact lead us suggest that ATP resulting from fatty acid oxidation could be used as an energy source for E2 production. In rats the secretion of E2 promoted by ATP (Rossato et al., 2001) was demonstrated, which could be occurring in S1 when the highest E2 level was registered.

EPA and DHA regulate androgen production → Finally, the last effect of fatty acids on the steroid hormones presented in Chapter II was the positive correlation between EPA and DHA with androgens registered during the spermiation period (S4-S5). The results suggest these fatty acids could modulate the androgen synthesis during the spermiation period.

4. The liver: a key organ in lipid metabolism during eel reproduction

The liver, one of the tissues analyzed in the tests, has been highlighted in all the chapters due to its high lipid metabolism. In Chapter I, EPA in the liver of spermating males was higher than in non-spermating males. Based on our quantification method (as a percentage of the fat in the tissue), the EPA increase does not occur because there is a decrease of other fatty acids, and this fact led us to suggest that EPA is synthesized *de novo* in the liver to be sent to the testis. We considered this increase of EPA in the liver as an essential step for male maturation. Moreover, fatty acids in the liver have been correlated with steroid hormones (Chapter II) as well as sperm quality parameters (Chapter III). And finally, also in the last chapter, the liver seems to metabolize lipids in a different way depending on the n3/n6 ratio supplied in the diet (Chapter IV).

All these findings summarized above, highlight the important role of the liver in the lipid metabolism of the eel. Manor et al. (2012) referred to the liver as an important organ as it is the site of fatty acid synthesis and oxidation. Abraham et al. (1984) highlighted the eel's ability to synthesize lipids from endogenous sources, even during a prolonged period of fasting. An active pathway for fatty acid elongation in eel liver was clarified by Kissil et al. (1987). A few years later, Gnoni and Mucci (1990) indicated an active *de novo* fatty acid synthesis, in eel liver. Only two years after, the characteristics of elongation pathways in eel liver microsomes were elucidated (Muci et al., 1992). Finally, Giudetti et al. (2001) highlighted that fatty acid chain elongation in eel liver mitochondria is more than twice as high and shows characteristics in some way different from those reported in rats (in rats, acetyl CoA is the precursor of elongation, while eel liver

mitochondria can use malonyl-CoA). Few, not very recent works have focused on eel liver lipid metabolism in detail.

Now, knowing the important role of this tissue in eel reproduction and its relation with the lipid metabolism, further studies should be conducted to elucidate how the enzymatic pack present in the liver acts in order to provide the fatty acid levels required, depending on the reproductive stage of the eel.

5. Fatty acids in broodstock diets

The first step in the culture of any species is to achieve captive breeding, thus reaching a massive larvae and juvenile production of good quality with low mortality and better growth, particularly based on the nutritional requirements of that species. Lipids are transferred from gonads to gametes and broodstock nutrition directly affects larval quality. Lipids are used during reproduction and development, serving as energy source and as structural components (Sargent, 1995).

The sperm composition in fatty acids is highly affected by diets and is highly species-specific, as evidenced studies on several species (Asturiano et al., 2001, *Dicentrarchus labrax*; Beirão et al., 2015, *Solea senegalensis;* Henrotte et al., 2010, *Perca fluviatis*; Nandi et al., 2007, *Catla catla;* Vassallo-Agius et al., 2001, *Oncorhynchus mykiss*).

Taking into account the results achieved in Chapters I-III, a study was carried out in Chapter IV, testing broodstock diets with different fatty acid levels, and the effects on tissue composition as well as on sperm quality parameters were evaluated.

As explained above, independently of the different diets supplied, no differences in the total PUFA percentage were registered in the sperm after 70 days of fasting. Labbé et al. (1995) also observed the maintenance of a specific level of some PUFAs in sperm membranes of trout. In our opinion, supplying an adequate diet is vital for the animal, as this will allow making the metabolic effort required to obtain the essential PUFA proportion needed in the sperm. If the diet is unbalanced or inadequate, eels may make endogenous metabolic

efforts to reach this required PUFA levels and spend the energy which could be useful to satisfy other needs. Hence, it is crucial to center efforts on finding an appropriate fatty acid profile in broodstock diets.

With all the findings achieved in Chapter IV, it can be concluded that there are numerous aspects to consider when designing diets and to highlight the importance of adequate fatty acid proportions in diets especially composed according to fatty acid classes. For example, the proportion of n3 series fatty acids with respect to n6 series fatty acids, or between saturated fatty acids (SFA) and unsaturated fatty acids (UFA) must be considered. A bad balance of any of these percentages can greatly influence the use of fatty acids by the animal as well as their endogenous metabolism.

It is widely known that the ability to convert α-linolenic acid (18:3-n3, ALA) and linoleic acid (18:2-n6, LA) in PUFAs is different between freshwater and seawater fish species. Seawater fish species live in PUFA-rich marine ecosystem, thus the conversion is less advantageous and they partly lost this ability, while freshwater fish are capable of converting these fatty acids in a much more efficient way (Bell and Tocher, 2009). The eel is born in seawater but spends most of its life and development in freshwater. This fact may be the cause for the eel's different behavior when compared to other fish species with regard to fatty acid elongation and desaturation capacity, as it remains unclear if the eel's fatty acid metabolism follows a freshwater or seawater pattern. Recently, Wang et al. (2014) highlighted that the Japanese eel showed a freshwater fish pattern in metabolic aspects, and findings from that study open the door to future studies focusing on clarifying the endogenous lipid metabolism of the European eel as crucial issue, in order to cast high on the question which enzymes are responsible for fatty acid desaturation and elongation.

The importance of PUFAs in European eel reproduction has been widely highlighted in all the Chapters, but it is important to keep in mind that high PUFA inclusion in the diet, may lead to a high sperm membrane oxidation rate (Whates et al., 2007). Beirão et al. (2015) have recently demonstrated that the introduction of antioxidant compounds may maximize the PUFA effect included in diets, improving subsequently, sperm quality.

Finally, the major conclusions in Chapter IV were that DHA-rich diets induced higher sperm volumes and EPA-rich diets induced higher sperm motility, thus motility and volume values were different depending on the diet supplied showing that the improvement of European eel's sperm cab be achieved through the diet.

6. New challenges in eel reproduction

The results obtained in this thesis lead us to think that new study areas could be addressed.

After noting the deep changes of the lipid content that the eel suffer during the spermatogenesis, and also the different use of dietary fatty acids depending on the proportions included in the diet, a depth study of the enzymes involved in the eel lipid metabolism should be carried out, to clarify how its complex desaturases and elongases enzyme system works.

Moreover, our results on the role of specific fatty acids improving the sperm quality can be very helpful for the design of broodstock diets for the improvement of sperm quality parameters.

Finally, it would be interesting to study how the eel can maintain a constant profile of muscle fatty acids after a period of food deprivation and even, after the gonad formation. Would be helpful to know how the eel organism can regulate their energy use in order to maintain this constant fatty acid profile.

CONCLUSIONS

i. A specific use of fatty acids depending on the tissue and on the stage of testis development has been demonstrated.

ii. EPA is synthetized *de novo* in the liver, probably to be sent to the testis, which seems to be a crucial step for sperm maturation.

iii. PUFA maintenance in the testis during the eel spermiation period has been related to their physiological and structural functions.

iv. DHA have been the fatty acid found in greater proportion in eel testis and sperm, indicating a structural role in sperm membranes.

v. New approaches towards the role of fatty acids in eel steroidogenesis have been proposed, highlighting the possible role of EPA and DHA as modulators of androgen synthesis.

vi. The liver, as the main site of lipid metabolism, has shown its importance in the spermatogenetic process in the eel.

vii. Fatty acids are used as a source of energy and can influence several kinetic parameters of sperm quality such us velocity and motility.

viii. Independently on diet supplied, a specific PUFA percentage is found in the European eel sperm.

ix. EPA-rich diets induce higher sperm motility, DHA-rich diets induce higher sperm volumes.

x. Diets with n3/n6 ratio close to or >2 improve sperm quality parameters.

xi. European eel sperm improvement is possible using a balanced diet.

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