SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BROODSTOCK NUTRITION: ARACHIDONIC ACID (20:4n-6, ARA) AND REPRODUCTIVE PHYSIOLOGY

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Thesis

Submitted in fulfilment of the requirements for the degree of doctor at Autonomous University of Barcelona by the authority of the rector Ana Ripoll Aracil in the presence of the Thesis Committee appointed by the Academic Board

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Abstract

Considering the complete reproductive failure to spawn viable eggs from G1 cultured Senegalese sole (*Solea senegalensis*) broodstock compared to successful spawning from wild caught captive broodstock, this thesis was conducted with the aim to determine the importance of dietary essential fatty acids, specifically arachidonic acid (20:4n-6, ARA) in the reproductive dysfunction exhibited by G1 Senegalese sole. Lipids and fatty acid (FA) composition, cyclooxygenase (COX-2) activity and prostaglandins production (PGE2, PGE3 PGF2α and PGF3α) of recently caught mature wild verses mature G1 fish were studied. The total lipid of G1 fish was higher compared to wild fish in liver, and wild fish showed higher levels of cholesterol (CHOL). Moreover, differences were found in fatty acids, particularly in ARA, which was lower in G1 fish (i.e., liver, testis and muscle). The observed differences in lipids and fatty acids between wild and G1 fish were a reflection of the extruded diet used to feed the fish. Transcripts of COX-2 were significantly up regulated in sperm-duct, oviduct and gills of males wild fish compared to G1 fish. Furthermore, wild fish showed higher levels of total 2-series PGs and lower levels of 3-series. To determine optimal dietary levels of ARA and the effect of ARA in the reproductive physiology of G1 Senegalese sole two studies were conducted: 1) The effects of six different ARA-enriched diet (A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA) on blood lipid and fatty acid composition, prostaglandins, blood steroid levels (11-ketotestosterone, 11-KT, testosterone, T and estradiol, E2) and fatty acid tissue levels (gonads, liver and muscle) was studied. Additionally, the expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts was assessed in the liver of fish (groups A, C and F) to examine the effects of ARA on the regulation of these genes. 2) The preferred dietary ARA level of G1 Senegalese sole was studied using self-feeders. In the dose/response experiment ARA and CHOL relative content increased in an ARA dose related manner whereas EPA and EPA/ARA ratio were reduced. Furthermore, PGs 3-serie were reduced in parallel to increased blood ARA levels, and the steroids (11-KT and T) levels increased significantly with ARA-enriched diet in males. The expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts also increased in response to dietary ARA content in males, showing a pattern of up-regulation in both transcripts, with a subsequent increase in adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) acids. Arachidonic acid (ARA) was rapidly transferred and conserved in testis and ovary, followed by liver and muscle. Thus, fish

fed 2.3 and 3.2% ARA showed no differences in the composition of testis, ovary and liver compared with the same tissues of wild fish. Moreover the results obtained by the self-fed study showed that Senegalese sole changed preference for ARA during the year and preferred ARA levels were correlated with the seasonal water temperature changes (r^2=0.65). The average ARA level demanded was 3.0% TFA for all the experimental period (16 months) and dietary levels of 3.2% ARA can be suggested as optimal and possibly a lower level of 2.3% over extended feeding periods (> 9 months). In conclusion, this thesis demonstrated that G1 Senegalese sole had an imbalance in lipids and fatty acid nutritional status compared to wild sole that contributed to significant differences in reproductive physiology. These differences were discussed in relation to the reproductive dysfunction that has been described in G1 Senegalese sole.

Chapter I

General introduction and objectives

GENERAL INTRODUCTION

1. Lipids and fatty acids in fish

Lipids and fatty acids, along with proteins, are the major constituents of fish, with carbohydrates being quantitatively lower (Halver *et al.*, 2002). Lipids play an important role in fish physiology as sources of metabolic energy for growth as well as for reproduction and movement (swimming), including migration (Tocher, 2003). Nutritional requirements of fish for lipids have been broadly studied and summarized (Sargent *et al.*, 2002), however, information about the specific requirements during reproduction and larval development of most marine fish species is still missing or insufficient (Fernández-Palacios *et al.*, 1995, Izquierdo *et al.*, 2001, Tocher, 2003, Watanabe *et al.*, 2003, Pickova *et al.*, 2007).

2. Essential fatty acid (EFA) and polyunsaturated fatty acids (PUFA)

All vertebrate species have a requirement for essential fatty acids (EFAs), which they cannot synthesize *de novo* and must be supplied in the diet (Sargent *et al.*, 1999). All species have a requirement for polyunsaturated fatty acid (PUFA) n-3 and n-6 series, although the exact level of each fatty acid or the ratio between both series vary and is species specific (Sargent *et al.*, 1995). Marine organisms, specially algae, contain a plethora of PUFAs, with either sixteen carbons chain length (C16) and two to four double bonds, or eighteen (C18) and twenty carbons (C20) with two to five double bonds (Sargent *et al.*, 2002). These PUFA belong mainly to n-3 series like eicosapentaenoic acid (20:5 *n*-3, EPA), docosahexaenoic acid (22:6 *n*-3, DHA) and their metabolic precursor linoleic acid (18:3 *n*-3). However, n-6 series fatty acids are also important, being arachidonic acid (20:4 n-6, ARA) one of the main PUFAs to be considered in fish nutrition together with its precursor linolenic acid (18:2 *n*-6) (Sargent *et al.*, 2002). All these fatty acids are constituents of tissues in which membrane fluidity is of paramount importance (i.e., neural-brain and retina and reproductive tissues-gonads), and play important metabolic roles such as the production of eicosanoids (Tocher, 1995, Sargent *et al.*, 2002, Tocher, 2003).

3. Biosynthesis of fatty acids

Natural marine phyto- and zoo-plankton are very rich in n-3 fatty acids, thus marine fish larvae, juveniles and adults usually feed on n-3 rich-diets (EPA and DHA), showing low activity of the enzymes that elongate or desaturate the n-3 precursor (18:3 *n*-3), whereas in fresh water plankton

are characterized by higher levels of 18:2n-6 and 18:3n-3 (Sargent *et al.*, 1997, Kainz *et al.*, 2004). Therefore, in marine fish hatcheries it is important to ensure that fish have enough quantity of n-3 and n-6 EFA-enriched diets (Sargent *et al.*, 2002). All vertebrates, including fish, have a dietary requirement of 18:2n-6 and 18:3n-3 that can be further desaturated and elongated to form the physiologically essential C20 and C22 PUFA (i.e., 20:4n-6, 20:5n-3, and 22:6n-3) (Fig. 1) (Sprecher, 2000, Tocher, 2003). The degree to which an animal can perform these conversions is dependent on the relative activities of fatty acid elongase and desaturase (i.e., Δ6, Δ5 and Δ4) in the organism and these activities in turn are dependent on the extent to which the species can or cannot obtain the end products 20:4n-6, 20:5n-3, and 22:6n-3 preformed from their natural diets (Rivers *et al.*, 1975, Sprecher, 2000, Cook *et al.*, 2004, Tocher, 2010). Moreover, the same enzymes act on the n-3 and the n-6 fatty acid series and the affinity of the enzymes, especially the desaturase, is higher for the n-3 than for the n-6 series (Tocher, 2003) (Fig. 1). Some animals, the extreme carnivores have very limited ability to synthesize fatty acids having carbon chain lengths of ≥ C20 and with ≥ 3 double bonds, commonly called highly unsaturated fatty acids (HUFA) and consequently have a high requirement for preformed C20 and C22 PUFA in their diets (Tocher, 2010, Rivers *et al.*, 1975, Tocher, 2003). The dietary requirement of EFA needs to be considered not only in terms of absolute amounts of individual fatty acids but that also in terms of their relative quantity, and also by the ability of the animal to metabolize these fatty acids (Sargent *et al.*, 2002). In the case of protein and essential amino acids (EAA), the requirement of one amino acid is not changed by an excess of another EAA in the diet, but in the case of fatty acids, the excess of one EFA, like ARA, alters the dietary requirement for another, like EPA, because both fatty acids are precursors of the same biological substances, for this reason ratios between of two EFAs are commonly used in the design of marine fish diets (Bell *et al.*, 1994a, Bell *et al.*, 1996b). Additionally, the final levels of these fatty acids in fish tissues will be determined by the innate ability of the fish to biosynthesize that fatty acid from a shorter chain precursor and the ability to catabolize the precursor or the end product (i.e., 18:3 *n*-3 to 20:5 *n*-3) (Sargent *et al.*, 2002, Tocher, 2003).

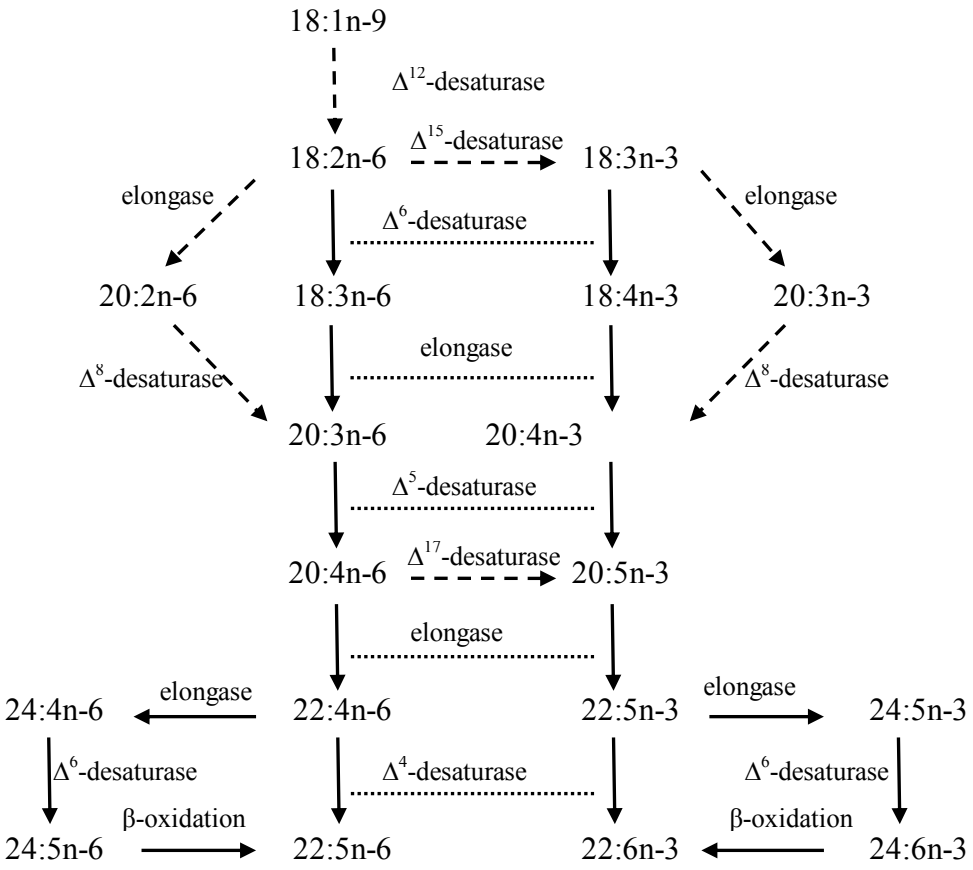

Fig. 1 *Pathway of biosynthesis confirmed in teleost of C20, C22 and C24 from n-9, n-6 and n-3 C18 precursor (solid lines). Pathways shown in other organisms but unconfirmed in teleost are also shown (broken lines) (Li et al., 2010).*

4. Senegalese sole and aquaculture

Senegalese sole (*Solea senegalensis*) is a common flatfish highly regarded in the south of Europe and industrially cultured in the Mediterranean and Atlantic coasts, with most farms located along the Atlantic coast in Portugal, Northern Spain, France and the Canary Islands. The estimated production for 2011 in Europe is 2200 Tons (Howell *et al.*, 2011), being the production in 2008 600 Tons with a value of €13.2 kg^{-1} (FAO) and an estimated production cost of €8-9 kg^{-1} (Howell *et al.*, 2011). Sole (Senegalese and common soles) fishery in Catalonia (Spain) has decreased by 30% during the period 2000-2008 with 118 tons captured in 2008 and a market price of €16 kg^{-1} (Departament d'Agricultura, Alimentació i Acció Rural, Catalunya) (DAR, 2010). Several problems for the intensive culture of Senegalese sole have been identified: (1)

control of the reproduction process especially in the case of captive bred broodstock, (2) incidence of pathologies and (3) development of sustainable feeds for use in open and recirculation systems (Howell *et al.*, 2011). The main pathological issues detected for Senegalese sole are Tenacibaculosis (*Tenacibaculum ssp*) and Vibriosis (*Vibrios ssp*) rather than Pasteurellosis induced by *Photobacterium damselae ssp piscicida* (Zarza, 2006, Zarza *et al.*, 2008), which was more associated with warm water, Mediterranean farms, in which gilthead sea bream and Senegalese sole were cultured together and that are no longer in production. Disease outbreaks in Senegalese sole develop rapidly and often result in high mortalities, the need for effective (autologous) vaccines and vaccination protocols has been already emphasized (Howell *et al.*, 2011). However, the main industrial issue remains the failure of G1 fish to produce naturally fertilized eggs in captivity.

5. Senegalese sole reproduction

The reproduction of Senegalese sole in captivity has been studied since the early 1980s (Rodriguez *et al.*, 1982), and evidence suggests that the poor spawning in G1 broodstock cannot be attributed to any hormonal dysfunction (Agulleiro *et al.*, 2006, Bertotto *et al.*, 2006, Agulleiro, 2007, Agulleiro *et al.*, 2007, Guzmán *et al.*, 2008). However, failure of G1 males to display normal courtship behaviour has been demonstrated (Carazo *et al.*, 2009, Carazo *et al.*, 2011a). G1 females were shown to be able to produce fertilized eggs from natural spawning as long as they were accompanied by wild males (Mañanos, 2011, Carazo *et al.*, 2011b) Senegalese sole is a seasonal spawner, with full gonadal development and maximum peaking levels of plasma steroids at the beginning of the spawning season (April-June) being higher in wild fish than in G1 fish (García-López *et al.*, 2006, García-López *et al.*, 2007, Guzmán *et al.*, 2008, Guzmán *et al.*, 2009). The reason for the failure of courtship and spawning remains unclear and consequently, the reproduction of cultured Senegalese sole remains a bottleneck to establish a sustainable industrial production of the species, being one of its priorities (Howell *et al.*, 2011). This reproductive problem of G1 broodstock might in part be determined by one or a combination of the following factors: the culture environment during early developmental stages, effects of broodstock selection on the genetic composition of the broodstock, or broodstock nutrition (Bromage, 1995, Mañanos *et al.*, 2008, Howell *et al.*, 2011).

6. Nutrition and reproductive physiology

Egg quality and spawning performance are related to the nutritional state of the broodstock, especially important biochemical constituents, i.e., lipids, proteins, EFAs, vitamins, minerals and trace elements are required for the gametogenesis of oocytes and milt (Luquet *et al.*, 1986, Bromage *et al.*, 1992, Izquierdo *et al.*, 2001, Hardy *et al.*, 2002, Howell *et al.*, 2003). Commercial extruded diets contain a significant proportion of fish meal as the main source of protein, minerals and vitamins and supplementation of fish meal-based diet with certain elements has been considered essential in practical broodstock diets. Thus, producers often supplement extruded diets with fresh squid, muscles and polychaetes to ensure the nutritional demands of broodstock are covered (Fernández-Palacios *et al.*, 1997, Izquierdo *et al.*, 2001). Total energy spent during reproduction exceeds that obtained from the diet during the period of gametogenesis and spawning and the required surplus is mobilized from body tissue reserves (Almansa *et al.*, 1999, Bureau *et al.*, 2002, Halver *et al.*, 2002). The major dietary sources of energy that are utilised by broodstock are protein, lipids and fatty acids, and carbohydrates, however, the nutrient requirements and mobilisation of body reserves may vary according to the species (Watanabe *et al.*, 2003). Studies have shown that Senegalese sole require high protein (>50%) and low fat diets (<8%) (Rodiles *et al.*, 2011) and were more efficient at metabolising carbohydrate than other farmed marine fish (Clark *et al.*, 1983). The formulated diets used for broodstock contain fish and plant meals, and fish and vegetable oils (Skretting, Stavanger, Norway), which are not considered part of the diet of wild sole (Garcia *et al.*, 1996). Stomach and gut contents of wild sole indicated that the natural diet consisted mainly of polychaetes, crustaceans, and molluscs (Garcia, 1996, Garcia *et al.*, 1996, Cabral, 2000, Bertotto *et al.*, 2006). In the diet of Senegalese sole the most important preys were polychaetes Glyceridae and the crustaceans Callianassidae and small fishes were less important, all these prey are rich in proteins, essential aminoacids, and fatty acids (Molinero *et al.*, 1994, Garcia, 1996).

The fatty acid composition of the diet is essential for successful reproduction and offspring survival (Izquierdo *et al.*, 2001), and in particular the importance in the reproduction of fish of PUFA ARA, EPA and DHA, as well as other fatty acids such as adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) acids, is well documented (MacDonald *et al.*, 1984, Bell *et al.*, 1997, Bell *et al.*, 1996b, Vassallo-Agius *et al.*, 2001, Izquierdo *et al.*, 2001, Mazorra *et al.*, 2003,

Watanabe *et al.*, 2003, Alorend, 2004, Cejas *et al.*, 2004, Meunpol *et al.*, 2005, Pickova *et al.*, 2007, Pérez *et al.*, 2007, Lund *et al.*, 2008, Sawanboonchun, 2009). Although, the exact physiological function of all these PUFAS in fish reproduction is not well know, essential fatty acids (EFAs) are required for a number of functions that are important for both reproductive control and embryo- larval development of fish (Sargent *et al.*, 1995, Izquierdo *et al.*, 2001). EFAs are considered to be a source of metabolic energy (Henderson *et al.*, 1989), important for a proper embryonic and yolk-sac larval development (Tocher, 2003), involved in the eicosanoid and steroid production (Stacey *et al.*, 1982, Wade *et al.*, 1994) and sperm transportation (Leat *et al.*, 1983). Moreover, EFAs are required for the membrane permeability (Bell *et al.*, 1996a, Brodtkorb *et al.*, 1997, Furland *et al.*, 2007) and are involved in neural function and development, being constituents of brain cells and eyes (Bell *et al.*, 1995b). Perhaps one of the best studied EFA in broodstock fish is ARA, which is considered an important nutritional factor for a successful fish reproduction (Falk-Petersen *et al.*, 1989, Sargent *et al.*, 1999, Izquierdo *et al.*, 2001, Mazorra *et al.*, 2003, Furuita *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Perez *et al.*, 2007, Tocher, 2010). ARA is the main precursor of eicosanoids that include among others prostaglandins (PGs) and thromboxanes (TXA2) (Smith, 1989, Smith *et al.*, 2002, Tocher, 2003). Eicosanoids are essential in physiological processes such as ovulation (PGE2), implantation of embryo (PGI2), inflammation resolution (PGD2), T cell development (PGE2), perinatal kidney development (not determinate), ductus arteriosus remodelling (TXA2 and PGH2), intestinal cancer (PGE2) and ulcer healing (not determinate) (Smith *et al.*, 2001). In fish, PGs have been showed to stimulate ovarian and testicular steroidogenesis and follicle maturation and act hormonally to trigger female sexual behaviour and milt increase in males (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001, Kobayashi *et al.*, 2002, Sorensen *et al.*, 2004). The enzymatic pathway involved in eicosanoid production is often called the 'arachidonic cascade', which includes the action of cyclooxygenase (COX) (Smith, 1989). Prostaglandins can also be produced from other fatty acids such as EPA, being converted to 3-series PGs, biologically less active than the corresponding 2-series produced from ARA (Tocher, 2003). Both fatty acids (ARA and EPA) compete for prostaglandin production (Tocher, 2003) and the different actions of the different series of PGs (ARA and 2 series PGs versus EPA and 3 series PGs) might affect the physiology of the fish (Wade *et al.*, 1994, Bell *et al.*, 1994b, Mercure *et al.*, 1995). Previous studies

demonstrated that changes in dietary fatty acids resulted in extensive alteration in the profile of PGs in heart, brain, gills and kidney of fish (Bell *et al.*, 1995a), with the concentration and magnitude being different in all the tissues analysed. In reproductive physiology of mammals, ARA and its metabolites regulate the steroidogenic acute regulatory protein (StAR protein) which in turn transfers cholesterol from outer to inner mitochondrial membrane and initiate steroid hormone synthesis (Wang *et al.*, 2005, Hu *et al.*, 2010). However, ARA might have differential effects on teleost fish steroid biosynthesis, it stimulates testosterone production by elevating cAMP levels in a dose dependent manner, but at higher doses ARA might inhibit steroidogenesis, in spite of elevated cAMP, by affecting the availability of cholesterol (Mercure *et al.*, 1995, Mercure *et al.*, 1996).

Several studies on fish composition have shown that ARA content is higher in the tissues of wild fish compared with cultured fish (Bell *et al.*, 1996b, Cejas *et al.*, 2004, Cejas *et al.*, 2003, Harrell *et al.*, 1995, Lund *et al.*, 2008, Aslan *et al.*, 2007). Additionally, other 22C n-6 fatty acids such as adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) (Bell *et al.*, 1996b), produced through elongation and desaturation of ARA, were also observed in higher quantities in wild fish (Linares *et al.*, 1991). These 22C n-6 fatty acids are present in reproductive tissue (i.e., sperm plasma membranes) as well as in the membranes of neural cells in much larger quantities than those reported in any other tissue (Ahaluwalia *et al.*, 1969, Tinoco, 1982, Picardo *et al.*, 1990, Lenzi *et al.*, 1996) although the physiological function of these PUFAs in sperm is not well know. In mammals C22 (n-3 and n-6) are involved in sperm transportation specially when spermatozoa pass from the caput to the cauda of the epididymis and in the fertilization process (Leat *et al.*, 1983). Desaturation of 22:4n-6 to 22:5n-6, and 22:5n-3 to 22:6n-3 might be carried out by a pathway in which desaturase Δ4 is involved (Fig. 1) (Li *et al.*, 2010), shorter than the 'Sprecher pathway' (Sprecher, 2000). Thus, more than one possible pathway for the synthesis of DPA n-6 and DHA in vertebrates is possible, being *Fad* with Δ4 activity a new functional characterization in fish (Li *et al.*, 2010).

7. Hypothesis and aims

Considering the above statements and the failure of G1 males to display normal courtship behavior the hypothesis of this thesis was: 'Differences in lipids and fatty acid profiles between wild and G1 Senegalese sole cause differences in the reproductive physiology of sole'. Thus, five objectives were established to determine the importance of dietary EFAs in the reproductive dysfunction exhibited by G1 Senegalese sole.

1. To characterize the proximal composition and fatty acid content of several tissues (muscle, liver, and gonads) of wild *versus* cultured Senegalese sole (S*olea senegalensis*) broodstock and the diets (extruded as well as fresh supplements) used for captive sole feeding (**Chapter II**).

2. To study the prostaglandins (F and E, 2- and 3-series) production and cyclooxygenase (COX-2) gene expression of wild and cultured Senegalese sole (*Solea senegalensis*) broodstock (**Chapter III**).

3. To study the effect of graded levels of ARA on the reproductive physiology of Senegalese sole (*Solea senegalensis*): fatty acid composition and prostaglandin and steroid levels in the blood of cultured fish (**Chapter IV**).

4. To determine dietary self selection of ARA by Senegalese sole (*Solea senegalensis*) broodstock (**Chapter V**).

5. To determine dietary levels of ARA and its effect on the fatty acid profile of tissues (liver, gonads and muscle) of Senegalese sole (*Solea senegalensis*) **(Chapter IV).**

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Chapter II

Lipids and fatty acid in *S. Sole*

PROXIMATE AND FATTY ACID COMPOSITIONS IN MUSCLE, LIVER AND GONADS OF WILD *VERSUS* CULTURED BROODSTOCK OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

Abstract

A complete reproductive failure to spawn viable eggs from G1 cultured Senegalese sole (*Solea senegalensis*) broodstock compared to successful spawning from wild caught captive broodstock has been described previously. The present study aimed to compare the proximate and fatty acid composition of recently caught mature wild verses mature G1 fish, to identify possible factors that may contribute to this reproductive problem. The proximate and fatty acid composition was compared between 22 wild fish, captured in the Ebro Delta (Tarragona, Spain), and 25 cultured fish raised under natural captive conditions. The results showed significantly higher levels of total lipid, in male liver in G1 fish compared to wild fish (3.0- and 2.5-fold higher in female and male respectively). The neutral lipid class, was also significantly higher in liver (88 and 90% total lipids (TL) in female and male, respectively) and male muscle (64% TL) from cultured fish. The relative value of linoleic acid (18:2n-6) was significantly higher in all tissues from cultured fish (approximately 11, 9 and 7.4% total fatty acid (TFA) in liver, gonads and muscle respectively). However, significantly lower levels were observed in cultured fish for cholesterol (CHOL) in liver of female (22% in wild and 7.8% in G1) and male (20 in wild and 7.0% in G1) and the essential fatty acid, like arachidonic (20:4n-6, ARA) in the liver (4.6-fold down male and 2.4-fold in female), testis (1.4-fold down) and muscle (2.9- and 2.0-fold down in male and female respectively); docosahexaenoic (22:6n-3, DHA) in liver (1.6- and 1.3 fold down in G1 female and male) and male muscle (1.3-fold down); eicosapentaenoic (20:5n-3, EPA) in muscle of males (1.4-fold down). These differences resulted in significant higher ratios of EPA/ARA in the liver and muscle of cultured fish. This imbalance in lipids and essential fatty acids (EFAs) of G1 fish were a reflection of the extruded diet used to fed the fish. Future research should consider the effect of this nutritional imbalance on reproductive physiology of G1 fish, specially the effects of ARA and CHOL on prostaglandins (2- and 3-series) and steroid production.

1. Introduction

Senegalese sole (*Solea senegalensis*) has good aquaculture potential, is highly regarded in the south of Europe and achieved a high market price of €13.2 kg^{-1} during 2008 (FAO, 2010). The Spanish landings from the fishery of Soleidae species has declined from an average during 1991-1999 of 4580 ± 816 t to 1271 ± 106 t during the period 2000-2008 (FAO, 2010). Industrial culture of the Senegalese sole began in 2005 and raised a production of 60 tons in 2008 (FAO, 2010). The principal problems limiting the expansion of Senegalese sole aquaculture were identified to be the incidence of pathologies (Pasteurellosis and Tenacibaculosis infections), the weaning of juveniles, the individual differences in growth during the ongrowing phase and the control of reproduction (Howell *et al.*, 2008). The main problem for the control of reproduction was that Senegalese sole bred and reared in captivity (G1 generation, cultured broodstock) completed the processes of spermatogenesis, vitellogenesis and final oocyte maturation, but spawn irregularly and the egg quality was poor, with low fertilization rates and/or arrested embryonic development (Agulleiro *et al.*, 2006, Howell *et al.*, 2008). Therefore, the industry is currently unsustainable, relies on the successful spawning of wild caught broodstock and cannot develop any kind of genetic selection program. Studies on the reproductive behaviour of captive wild and G1 cultured broodstock have identified that G1 cultured broodstock do not complete courtship to fertilise the liberated ova (Carazo *et al.*, 2009, Carazo *et al.*, 2011). The use of hormone therapies increased the number and volume of spawns, but not the reproductive behaviour (Carazo *et al.*, 2011) or quality of the eggs (Agulleiro *et al.*, 2007, Guzmán *et al.*, 2009). This reproductive behavioural problem or dysfunction of cultured broodstock might be determined by one or a combination of the following factors: the culture environment during early developmental stages, effects of broodstock selection on the genetic composition of the broodstock, or broodstock nutrition (Bromage, 1995, Mañanos *et al.*, 2008, Howell *et al.*, 2011).

Egg quality and spawning performance were related to the nutritional state of the broodstock and suboptimal nutrition effects the availability of essential biochemical constituents required for gametogenesis and reproductive control (Izquierdo *et al.*, 2001). The fatty acid composition of the diet was essential for successful reproduction and

offspring survival (Mazorra *et al.*, 2003, Furuita *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Perez *et al.*, 2007, Tocher, 2010) and in particular the importance of the long-chain essential fatty acids (EFAs), arachidonic (20:4n-6, ARA), eicosapentaenoic (20:5n-3. EPA) and docosahexaenoic (22:6n-3, DHA), in the reproduction of fish is well documented (Izquierdo *et al.*, 2001, Mazorra *et al.*, 2003, Watanabe *et al.*, 2003, Pickova *et al.*, 2007, Sawanboonchun, 2009). Evidence for the essential nature of fatty acids has been demonstrated as deficiencies in EFAs lead to observations of poor fecundity, fertilization and hatching success of eggs whereas the inclusion of higher levels of essential fatty acids in broodstock diets increased fecundity, fertilization, egg quality, hatching success, survival of offspring and reproductive behaviour (Stacey *et al.*, 1982, Luquet *et al.*, 1986, Mercure *et al.*, 1995, Fernández-Palacios *et al.*, 1995, Sorbera *et al.*, 2001, Cejas *et al.*, 2003, Mazorra *et al.*, 2003, Furuita *et al.*, 2003, Meunpol *et al.*, 2005, Pickova *et al.*, 2007).

The EFAs are required for a number of functions that are important for both reproductive control and embryo/larval development of fish (Sargent *et al.*, 1995, Izquierdo *et al.*, 2001) and include a source of stored metabolic energy (Henderson *et al.*, 1989), embryonic and yolk-sac larval development (Tocher, 2003) membrane functions, brain, eye (Bell *et al.*, 1996a, Brodtkorb *et al.*, 1997) and neural function and development (Bell *et al.*, 1995b) and eicosanoid and steroid production (Stacey *et al.*, 1982, Wade *et al.*, 1994b).

Lipid was mainly stored in liver and muscle of fish and transported together with protein to the ovaries during maturation of the gonad (Jobling *et al.*, 1998, Almansa *et al.*, 2001, Cejas *et al.*, 2004). Unbalanced lipid class compositions or essential fatty acid profiles that were probably a consequence of the diet have been observed in cultured fish such as rainbow trout (Aslan *et al.*, 2007), black sea bream (Rodríguez *et al.*, 2004) European seabass (Bell *et al.*, 1996a), white sea bream (Perez *et al.*, 2007, Cejas *et al.*, 2004), Japanese flounder (Furuita *et al.*, 2003), Japanese eel (Furuita *et al.*, 2007) and cod (Salze *et al.*, 2005), which reduced gamete quality and reproductive success (Izquierdo *et al.*, 2001). Studies of the stomach and gut content of adult Senegelese sole (*Solea senegalensis*) (Garcia *et al.*, 1996, Cabral, 2000) and common sole (*Solea solea*) (Molinero *et al.*, 1994, Cabral, 2000) found that small crustaceans and polychaetes were the two dominant food items, followed by molluscs, however, small fish were also founded but with low

frequency. In general as described in the literature, G1 broodstock reared in captivity were fed exclusively extruded diets (García-López *et al.*, 2006, Guzmán *et al.*, 2009) that may be supplemented with varying combinations of squid, polychaetes and mussels prior and during the spawning season (Agulleiro *et al.*, 2006, Guzmán *et al.*, 2008). The extruded diets used for broodstock contain fish and plant meals, and fish and vegetable oils (Skretting, Stavanger, Norway), none of which were principal parts of the diet of wild sole (Garcia *et al.*, 1996). Studies on common sole have found that eggs from G1 broodstock had different fatty acid profiles compared to eggs from wild broodstock (Lund *et al.*, 2008). It would appear that the broodstock diets used for G1 Senegalese sole may not provide the fatty acid profile required as has been observed in the common sole (Lund *et al.*, 2008) and other species (Rodríguez *et al.*, 2004, Aslan *et al.*, 2007, Furuita *et al.*, 2007, Perez *et al.*, 2007).

The present study aimed to compare the lipid nutrition of recently caught mature wild which hypothetically contains the desirable composition for the nutrition verses mature G1 captivity reared Senegalese sole fed with commercial diet. The wild broodstock were caught with procedures similar or identical to those used to set up wild spawning broodstock. The objective was to identify differences in proximate, lipid and fatty acid composition, into which future research could be directed to determine the importance of dietary EFAs in the reproductive dysfunction exhibited by G1 Senegalese sole. The proximate and fatty acid composition of two commercial broodstock diets used to fed G1 fish, and four fresh (frozen) diet commonly used as supplement in broodstock fish were also analysed.

2. Materials and methods

Twenty two wild fish (12 males and 10 females), captured in the Ebro Delta during May 2008 (between 40º 37' and 40º 48' N and 0º 21' and 0º 40' E., Tarragona, Spain), and 25 cultured fish (4-5 years old, 13 males and 12 females) were euthanized with an overdose of 2-phenoxyethanol (0.3 mL L^{-1}) followed by pithing of the spinal cord during the spawning season of 2008 (May). Gonads, liver and muscle were dissected, weighed and stored at -80ºC until biochemical analysis. Wild fish weighed 345 ± 45g (mean ± SEM) and measured 30 ± 1.5 cm length and cultured fish were 540 ± 48g and 33 ± 1.3 cm, respectively. Wild

fish were sacrificed on the same day as capture. The age of the wild fish was estimated to be 5-6 years old, by a growth function (Von Bertalanffy, 1938) and the length and age data of *Solea senegalensis* cited by Garcia (1996). Cultured fish were reared under natural conditions (photoperiod and temperature) and for one year before sampling were fed with two commercial broodstock diets (Vitalis Repro® and Elite®, Skretting, Burgos, Spain). The daily extruded feed supply was 0.5-0.7% the body weight (BW) per day and feeding was maintained close to satiation to ensure all feed was eaten. Additionally frozen diets (-20ºC) commonly used as a supplements of broodstock fish: mussels (*Mytilus galloprovincialis* from Galicia, Spain (Congelados Marcos, Tarragona), squid (*Loligo gahi*, from Falkland island, Congelados Marcos, Tarragona) and polycheates (two sources of the polychaete *Nereis virens*, Seabait®, Northumberland, UK and Topsy Baits®, Zeeland, Netherlands), were purchases at the start of the trial for biochemical analysis. The broodstock were fed seven days per week, four days with extruded diets and one day with each of the frozen diets, mussels, squid and polycheates. Duplicate samples (100g) of extruded diet stored at 4^{o}C were collected from three different bags (25 kg) and ground every fourth months and stored at -80ºC until biochemical analysis, meanwhile frozen diets commonly used as a supplements of broodstock fish, were purchases at the start of the trial and stored at -20^{o}C and samples were collected for duplicate (100g) from three different batches mixed ground and analysed every six months.

2.1. Determination of sexual maturation

Gonadosomatic (GSI: gonad weight x body weight^{-1} x100), and hepatosomatic (HSI: Liver weight x body weight^{-1} x 100) indices were calculated. During the dissection of each fish, samples of testis and ovaries were collected and fixed in Bouin solution for 24 h. The samples were then transferred and stored in 70% ethanol until histological processing. Samples were dehydrated, embedded in paraffin (Paraplast®, Sigma-Aldrich, Madrid, Spain), sectioned at 4 μm (Microm HM323) and stained with hematoxylin -eosin. Sexual maturity was determined by examining sections and classifying 50 randomly selected oocytes or seminiferous tubules under a microscope (40-100X) into different maturation stages (Bromage *et al.*, 1988, Grier *et al.*, 1998). Females not showing oocytes in

vitellogenesis and male with less than 10% spermatozoids in the seminiferous tubules were not used in the biochemical studies.

2.2. Proximate composition

Protein content of all the 'fresh' food items and tissues, except testis, was analysed by Lowry *et al.* (1951), with prior overnight hydrolysis in sodium-hydroxide solution, carbohydrates by Dubois *et al*. (1956), and lipids extracted by Folch *et al*. (1957) and quantified gravimetrically. Protein content in extruded diets was carried out by the Dumas method using a protein determinator (LECO FP-528)(AOAC, 2000). Water content was calculated by drying the samples at 105ºC until a constant weight was obtained (AOAC, 2000), whereas ash content was determined by ashing the samples in a muffle furnace for 5 h at 550ºC. To avoid variability associated to the water content in tissue, the results are presented as percentage (%) of the dry matter (DM) as mean ± standard error of mean (SEM).

2.3. Lipid Analysis

Total lipids were extracted from samples by the Folch method as described above. Fatty acid methyl esters prepared by acid-catalysed transmethylation (Christie, 1982), and extracted and purified using thin-layer chromatography (Tocher *et al.*, 1988). Fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using Helium as carrier gas (1.2 mL min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well characterised fish oil, quantified by the response factor to the internal standard, 21:0. The results are presented as percentage of the total fatty acids (% TFA) as mean ± SEM.

Lipid class composition was determined by high-performance thin layer chromatography (HPTLC) using 10×10 cm HPTLC plates (Macherey-Nagel gMBh & Co, Düren, Germany). Approximately 10 μg of total lipid was applied as 2 mm streaks, 1 cm from the bottom, and the plates developed in methyl acetate/isopropanol/ choloform/methanol/0.25% aqueous KCl (25: 25: 25: 10: 9, by vol) to two-thirds up the plate. After desiccation for 20

min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol) and placed in a vacuum desiccator for 20 min. The lipid classes were visualized by charring at 160 ºC for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a BIO-RAD GS-800 calibrated densitometer (BIO-RAD, Spain) and WINCATS software (Henderson *et al.*, 1992). The identities of individual lipid classes were confirmed by comparison with authentic standards. The results are presented as percentage (%) of the total lipids (TL) as mean ± SEM.

2.4. Statistic analysis

Statistical differences of fish morphometry, proximate composition and fatty acid and lipid composition between wild and cultured groups and males and females were analysed by two-way ANOVA and nutritional differences amongst diets determined with a one-way ANOVA. All ANOVAs were followed by the post-hoc multiple comparison by Tukey's HSD for unequal N test with a significance level of $P<0.05$. The compliance of data with normality and homogeneity of variance was tested by the Kolmogorov-Smirnov and Bartlett (Chi-Sqr) methods and, when necessary, log-transformation was carried out. The data are expressed as mean of percentage ± SEM whereas the results of diet composition are presented in relative percentage. The statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA). Pearson correlations among fatty acids in different tissues were made, using JMP®, statistical discovery software, with a significance level of $P<0.05$ (SAS Campus Drive, Building S, Cary, NC, USA).

3. Results

3.1. Fish morphometry and maturation stage

Weight of cultured male was significantly higher than wild male ($P<0.05$) and no significant differences were found between cultured and wild females in terms of weight and length, while cultured female fish had a significantly ($P<0.05$) higher weight than the wild male fish (Table 1). The mean GSI of females was significantly ($P<0.05$) higher than males and GSI of cultured females was significantly ($P<0.05$) higher than wild females and

no differences in GSI were found between wild and cultured males (Table 1). The mean HSI from wild females was significantly higher than in males (wild and cultured), while the HSI of cultured females was intermediate not significantly different from any group. All broodstock used in the study were in advanced stages of maturation with vitellogenic oocyte or spermatozoids present in the gonads. There was no significant difference in the percentage of vitellogenic oocyte or percentage of tubules with spermatozoids between wild and cultured groups (Table 1).

Table 1 *Morphometry and maturation stage of wild and cultured female and male Senegalese sole (Solea senegalensis)*

	Females		Males	
	Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
Weight (g)	415 ± 77 ^{ab}	566 ± 11 ^{a}	276 ± 20 ^{b}	513 ± 79 ^{a}
Length (cm)	32.6 ± 2.3 ^{a}	34.1 ± 0.4 ^{a}	28.1 ± 0.8 ^{a}	30.2 ± 2.2 ^{a}
GSI (%)	2.2 ± 0.3 ^{b}	5.6 ± 0.5 ^{a}	0.4 ± 0.1 ^{c}	0.3 ± 0.1 ^{c}
HSI (%)	1.1 ± 0.1 ^{a}	0.8 ± 0.1 ^{ab}	0.6 ± 0.1 ^{b}	0.7 ± 0.1 ^{b}
VtO (%)	49.8 ± 7.8 ^{a}	54.5 ± 4.7 ^{a}		
T Spz (%)			51.6 4.9 ^{a}	42.6 5.4 ^{a}
Age (years)	5-6 *	4-5	5-6 *	4-5

Within a row, means without a common superscript letter differ significantly (ANOVA, $P<0.05$), N = number of samples, GSI: gonadosomatic index (gonad weight x body weight^{-1} x100), HSI: hepatosomatic index (liver weight x body weight^{-1} x 100). VtO = vitellogenic oocytes, T Spz = tubules with at least 10-30% spermatozoids, * estimated age using Von Bertalanffy (1938) growth functions and the length and age data of *S. Sole* shows by Garcia (1996).

3.2. Proximate composition of tissues

Lipid content and dry matter were significantly higher ($P<0.05$) in the liver of cultured fish compared to the wild fish, while protein and ash contents were significantly ($P<0.05$) lower in the liver of cultured fish compared to the wild fish (Table 2). Muscle and gonads did not show differences between wild and cultured fish in lipid, protein and ash content and dry matter. No significant differences were observed in carbohydrate content between tissues from wild and cultured fish (Table 2).

Table 2 *Proximate composition of wild and cultured female Senegalese sole (Solea senegalensis) (lipid, protein, carbohydrate, dry matter and ash) content in muscle, liver and ovaries or testis (%)*

		Female		Male	
		Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
Lipid (% DM)	Liver	18.0 ± 1.0 ^{a}	55.0 ± 4.0 ^{b}	21.1 ± 1.3 ^{a}	52.3 ± 2.8 ^{b}
	Gonad	13.9 ± 0.9 ^{a}	20.0 ± 1.1 ^{a}	15.5 ± 1.2 ^{a}	17.5 ± 1.7 ^{a}
	Muscle	3.3 ± 0.3 ^{a}	4.6 ± 0.9 ^{a}	3.2 ± 0.2 ^{a}	6.1 ± 0.7 ^{a}
Protein (% DM)	Liver	47.8 ± 0.8 ^{a}	33.3 ± 2.0 ^{b}	47.0 ± 1.5 ^{a}	24.7 ± 1.4 ^{b}
	Gonad	59.9 ± 2.1 ^{a}	55.4 ± 1.3 ^{a}	** **	** **
	Muscle	74.1 ± 3.1 ^{a}	74.9 ± 2.0 ^{a}	76.0 ± 2.0 ^{a}	75.3 ± 2.8 ^{a}
CHO (% DM)	Liver	14.0 ± 2.6 ^{a}	11.0 ± 1.6 ^{b}	19.9 ± 4.2 ^{a}	16.6 ± 3.2 ^{a}
	Gonad	9.8 ± 0.8 ^{a}	7.9 ± 0.6 ^{a}	** **	** **
	Muscle	3.4 ± 0.4 ^{a}	4.0 ± 0.4 ^{a}	3.5 ± 0.3 ^{a}	3.6 ± 0.3 ^{a}
D M (% FW)	Liver	27.7 ± 0.6 ^{a}	41.2 ± 2.8 ^{b}	29.3 ± 1.0 ^{a}	45.6 ± 1.4 ^{b}
	Gonad	23.8 ± 0.9 ^{ab}	26.1 ± 1.5 ^{a}	17.7 ± 0.8 ^{b}	15.5 ± 0.4 ^{c}
	Muscle	22.7 ± 0.6 ^{a}	24.1 ± 0.4 ^{a}	23.5 ± 0.4 ^{a}	23.8 ± 0.4 ^{a}
Ash (% DM)	Liver	2.0 ± 0.1 ^{a}	0.9 ± 0.1 ^{b}	1.6 ± 0.2 ^{a}	0.9 ± 0.1 ^{b}
	Gonad	2.0 ± 0.1 ^{a}	2.0 ± 0.1 ^{a}	1.2 ± 0.3 ^{b}	1.2 ± 0.2 ^{b}
	Muscle	1.5 ± 0.0 ^{a}	1.6 ± 0.1 ^{a}	1.5 ± 0.1 ^{a}	1.5 ± 0.0 ^{a}

Within a row, means without a common superscript letter differ significantly (ANOVA, $P<0.05$), N = number of samples, DM = dry matter, FW = fresh weight, ** protein and carbohydrate content was not analysed in testis.

3.3. Proximate composition of diets

Extruded feed Elite® had a significantly ($P<0.05$) higher protein content than any other analysed diet (Fig. 1) and the diets were ranked as significantly different in protein content with the following order: Vitalis® and squid > mussel > the two polychaetes (UK and Netherlands). Elite® also had a significantly ($P<0.05$) higher lipid content than any other diet and the diets were ranked as significantly different in the following order: Vitalis® > polychaetes from UK > polychaetes from Netherlands > mussels. Lipid levels in squid were significantly lower than polychaetes from UK, but not different from levels in polychaetes from Netherlands and mussels. Mussels had a significantly ($P<0.05$) higher carbohydrate content than any other diet and there were no differences among any other diet. Polychaetes either from the Netherlands or UK showed the highest ash content.

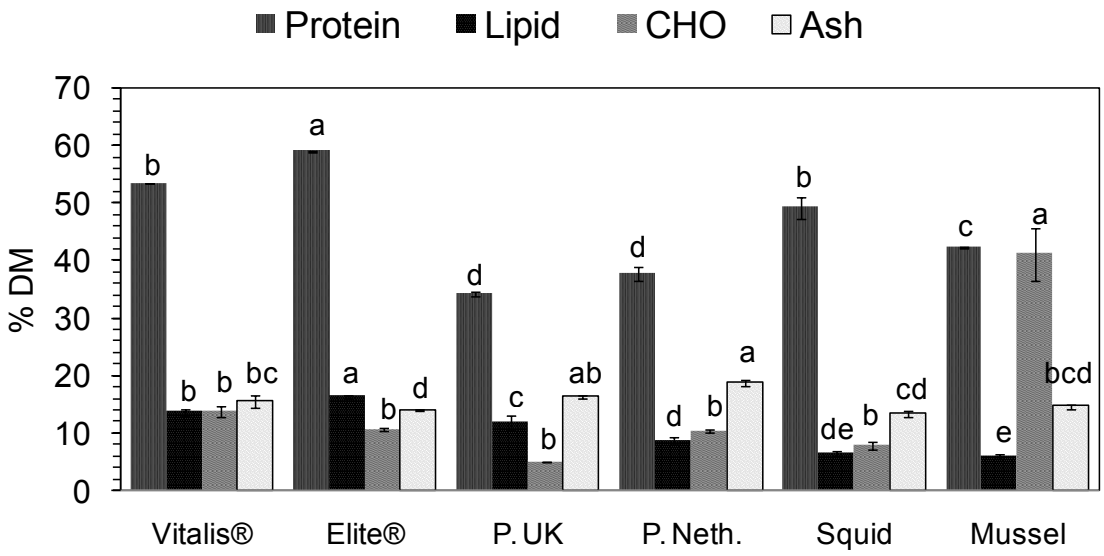

Fig. 1 *Proximate composition of six diets used for cultured broodstock feeding of Senegalese sole (Solea senegalensis): lipid, protein, carbohydrate and ash (% DM ± SEM). Columns assigned different letters for the same proximate component analysis were significantly different (ANOVA, P<0.05). Extruded feed Vitalis Repro (Vitalis®), Elite (Elite®), polychaete Nereis from England (P.UK), polychaete Nereis from Netherlands (P. Nth.), squid (Squid), and mussel (Mussel).*

3.4. Fatty acid composition

Significant (P<0.05) differences between wild and cultured fish were found in the fatty acid composition of liver, ovary, testis and muscle (Tables 3, 4 and 5). The relative fatty acid composition of total lipids extracted from liver, gonad and muscle of wild and cultured fish was dominated by polyunsaturated fatty acids of the n-3 series, predominantly by DHA, and saturated fatty acids, especially 16:0. Among the monounsaturated fatty acids, oleic acid (18:1n-9) and vaccenic acid (18:1n-7) were the highest. The principal differences between wild and cultured fish fatty acid profiles were in levels of n-6 polyunsaturated fatty acid (PUFA) including linoleic acid (18:2n-6), ARA, adrenic acid (22:4n-6) and docosapentaenoic acid (22:5n-6) and the essential n-3 PUFAs, EPA and DHA (Tables 3, 4 and 5).

The levels of n-6 PUFA were significantly (P<0.05) higher in the liver and gonads of cultured fish and these differences gave a concomitant significantly (P<0.05) higher n-3/n-6 ratio in wild fish (Tables 3 and 4). The higher n-6 PUFA levels in cultured fish were primarily the result of significantly (P<0.05) higher levels of 18:2n-6 acid in the liver (6.5- to 5.2-fold higher), gonads and muscle of cultured females and males. Wild fish showed a significantly (P<0.05) higher accumulation of ARA in the liver, testis and muscle, compared with cultured counterparts (Tables 3, 4 and 5). Levels in gonads of males were significantly higher than in the females, ARA=7.8% TFA in the testis. In the liver, wild male fish showed almost a 5-fold higher ARA content than in cultured counterparts, whereas in wild females ARA was only 2.4-fold higher, which was similar to differences in testis and muscle (Tables 1, 2 and 3).

The 22:4n-6 and 22:5n-6 acid were significantly higher (P<0.05) in male liver, muscle and testis of wild fish compared to cultured fish (Tables 3, 4 and 5). The differences were particularly pronounced for 22:4n-6 acid in testis (3.9-fold higher in wild fish, Table 4) and male muscle (8.9-fold higher in wild fish, Table 5). In wild fish, the content of these two fatty acids was significantly (P<0.05) and positively correlated with the tissue content of ARA. In wild males the correlation coefficient between ARA and 22:4n-6 acid was r^2=0.89 in liver-muscle and r^2=0.57 in testis, and in wild females r^2=0.4 in liver-muscle and r^2=0.6 in ovary. Similar correlations were found between ARA and 22:5n-6 acid with r^2=0.89 in liver-muscle of males and r^2=0.79 in testis and in females r^2=0.6 liver-muscle and r^2=0.5 in ovary. No correlation between the two fatty acids (22:4n-6 and 22:5n-6) and ARA was observed in cultured fish (data not shown).

In general the two essential n-3 PUFAs, DHA and EPA were higher in wild fish, but without significant differences and with the exception of EPA in liver that was non-significantly lower in wild fish. DHA content was significantly (P<0.05) higher in liver from wild fish and muscle from wild males, compared with the cultured counterparts (Tables 3 and 5), while EPA was significantly (P<0.05) higher in muscle from wild male (Table 5). Thus, the differences observed in DHA, EPA and particularly ARA levels between captive and wild fish produced significant differences in the ratios of the essential

fatty acids. The EPA/ARA ratio was significantly (P<0.05) higher in the liver and muscle of cultured fish (Fig. 2, Tables 4 and 5). The same trend was observed in ovaries and testis, although the differences were not significant. The DHA/ARA ratio was significantly (P<0.05) higher in male liver and ovaries from cultured fish and the EPA/DHA ratio was significantly (P<0.05) higher in female liver and testis from cultured fish (Fig. 2, Tables 3 and 4).

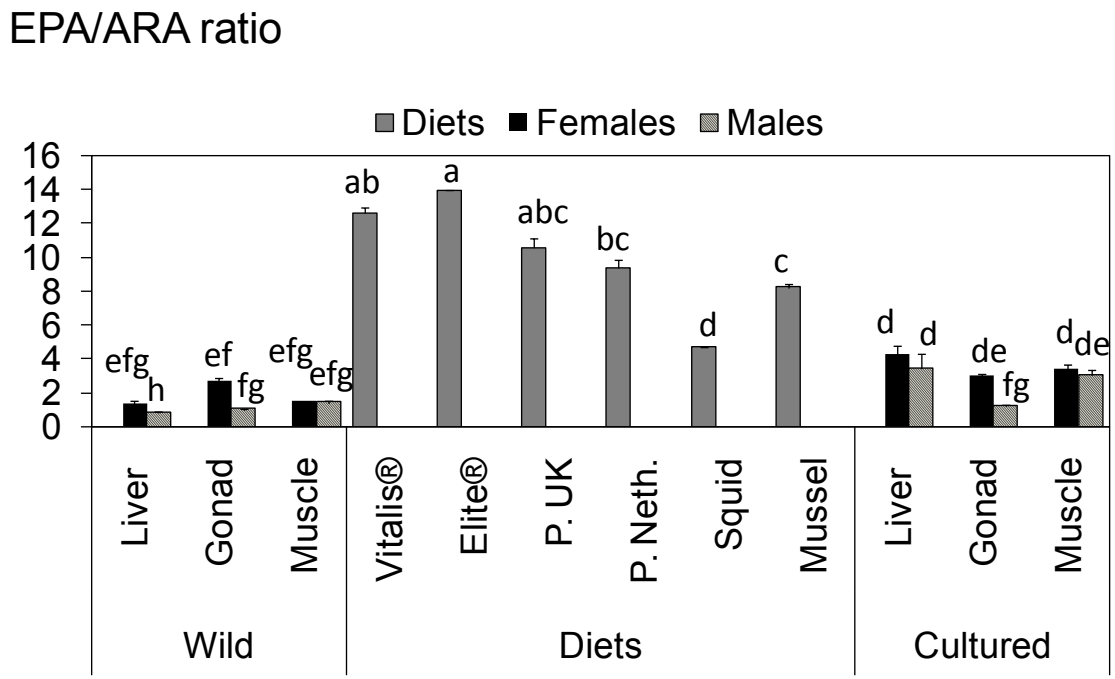

Fig. 2 *Eicosapentanoic acid and arachidonic acid ratios (EPA/ARA) of liver, gonads and muscle from wild and cultured female and male Senegalese sole (Solea senegalensis) and two commercial diet used to feed the fish (extruded feed Vitalis® Repro and Elite®, Skretting, Norway) and four wet diets commonly used to fed cultured broodtock of Senegalese sole (Solea senegalensis). Polychaete Nereis virens from England (P.UK), polychaete Nereis virens from Netherlands (P. Neth.), squid Loligo gahi (Squid), and mussel Mytilus galloprovincialis (Mussel). Columns assigned different letters were significantly different (ANOVA, P<0.05).*

Table 3 *Fatty acid content and fatty acid composition (% TFA ± SEM) of the liver of wild and cultured female and male Senegalese sole (Solea senegalensis)*

	Female		Male	
	Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
TFA(μg mg^{-1} L)	561.1 ± 36.4 ^{a}	642.2 ± 55.3 ^{a}	526.6 ± 31.1 ^{a}	646.1 ± 28.8 ^{a}
14:0	5.06 ± 0.44 ^{a}	3.73 ± 0.38 ^{a}	4.44 ± 0.27 ^{a}	3.95 ± 0.25 ^{a}
16:0	19.64 ± 0.90 ^{a}	17.76 ± 0.95 ^{a}	20.07 ± 1.41 ^{a}	18.74 ± 0.74 ^{a}
18:0	6.84 ± 0.45 ^{a}	2.13 ± 0.33 ^{be}	4.97 ± 0.38 ^{c}	2.53 ± 0.31 ^{de}
Total SFA	34.95 ± 1.36 ^{a}	26.33 ± 1.85 ^{b}	33.89 ± 1.81 ^{a}	28.49 ± 1.38 ^{ab}
16:1n-7	9.93 ± 0.71 ^{a}	7.41 ± 0.33 ^{b}	8.43 ± 0.48 ^{ab}	7.44 ± 0.45 ^{b}
18:1n-7	3.92 ± 0.76 ^{a}	1.59 ± 0.61 ^{a}	2.26 ± 0.77 ^{a}	1.32 ± 0.55 ^{a}
18:1n-9	10.41 ± 1.19 ^{a}	17.85 ± 0.94 ^{b}	12.07 ± 0.66 ^{a}	17.50 ± 0.94 ^{b}
Total MUFA	24.69 ± 1.15 ^{a}	28.29 ± 1.42 ^{a}	24.14 ± 0.62 ^{a}	30.44 ± 1.46 ^{a}
18:2n-6	1.83 ± 0.17 ^{a}	11.94 ± 0.64 ^{b}	1.95 ± 0.16 ^{a}	10.25 ± 0.62 ^{b}
18:3n-6	0.46 ± 0.07 ^{a}	0.43 ± 0.12 ^{a}	0.39 ± 0.06 ^{a}	0.38 ± 0.03 ^{a}
20:3n-6	0.20 ± 0.08 ^{a}	0.15 ± 0.08 ^{a}	0.13 ± 0.08 ^{a}	0.15 ± 0.07 ^{a}
20:4n-6, ARA	2.63 ± 0.24 ^{a}	1.08 ± 0.10 ^{b}	2.50 ± 0.29 ^{a}	0.74 ± 0.06 ^{b}
22:4n-6	0.39 ± 0.08 ^{a}	0.22 ± 0.03 ^{a}	0.96 ± 0.13 ^{b}	0.30 ± 0.03 ^{a}
22:5n-6	0.57 ± 0.05 ^{a}	0.38 ± 0.02 ^{a}	1.06 ± 0.09 ^{b}	0.40 ± 0.03 ^{a}
Total n-6 PUFA	6.09 ± 0.38 ^{a}	14.21 ± 0.71 ^{b}	7.91 ± 0.53 ^{a}	12.23 ± 0.67 ^{b}
18:3n-3	1.00 ± 0.10 ^{a}	1.52 ± 0.18 ^{a}	1.02 ± 0.19 ^{a}	1.09 ± 0.09 ^{a}
18:4n-3	1.85 ± 0.24 ^{a}	0.89 ± 0.13 ^{b}	0.78 ± 0.14 ^{b}	0.62 ± 0.06 ^{b}
20:4n-3	0.85 ± 0.12 ^{a}	0.40 ± 0.09 ^{a}	0.62 ± 0.15 ^{a}	0.66 ± 0.11 ^{a}
20:5n-3, EPA	3.32 ± 0.40 ^{ab}	4.89 ± 0.73 ^{b}	2.56 ± 0.26 ^{a}	2.90 ± 0.65 ^{ab}
22:5n-3	4.00 ± 0.16 ^{a}	7.46 ± 0.78 ^{b}	6.07 ± 0.48 ^{ab}	7.53 ± 0.71 ^{b}
22:6n-3, DHA	24.75 ± 1.15 ^{a}	15.72 ± 0.94 ^{b}	23.91 ± 1.34 ^{a}	18.63 ± 0.97 ^{b}
Total n-3 PUFA	35.77 ± 1.58 ^{a}	30.91 ± 2.46 ^{a}	35.06 ± 2.03 ^{a}	31.49 ± 2.06 ^{a}
Total PUFA	41.86 ± 1.58 ^{a}	45.12 ± 2.98 ^{a}	43.19 ± 2.08 ^{a}	43.73 ± 2.28 ^{a}
Total others	2.91 ± 0.04 ^{a}	3.16 ± 0.04 ^{a}	3.17 ± 0.05 ^{a}	2.52 ± 0.03 ^{a}
EPA/ARA	1.33 ± 0.17 ^{ab}	4.25 ± 0.54 ^{c}	0.79 ± 0.11 ^{a}	3.41 ± 0.83 ^{b}
EPA/DHA	0.13 ± 0.06 ^{a}	0.31 ± 0.04 ^{b}	0.11 ± 0.01 ^{a}	0.15 ± 0.03 ^{a}
DHA/ARA	10.27 ± 1.23 ^{ab}	15.96 ± 1.76 ^{b}	7.54 ± 0.89 ^{a}	23.22 ± 1.35 ^{d}
n-3/n-6	6.16 ± 0.57 ^{a}	2.17 ± 0.12 ^{b}	4.71 ± 0.54 ^{a}	2.63 ± 0.19 ^{b}

Within a row, means without a common superscript letter differ significantly (ANOVA, $P<0.05$), N = number of samples, L: Lipids, SFA: total saturated fatty acids, MUFA: total mono saturated acids, ARA: arachidonic acid, PUFA: total polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Table 4 *Fatty acid content and fatty acid composition (% TFA ± SEM) of gonads from wild and cultured female and male Senegalese sole (Solea senegalensis)*

	Female		Male	
	Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
TFA(μg mg^{-1} L)	526.5 ± 34.3 ^{ab}	591.1 ± 39.5 ^{a}	405.3 ± 25.1 ^{b}	410.0 ± 20.4 ^{b}
14:0	3.75 ± 0.16 ^{a}	2.68 ± 0.16 ^{b}	1.67 ± 0.16 ^{c}	1.42 ± 0.21 ^{c}
16:0	19.45 ± 0.54 ^{a}	19.44 ± 0.51 ^{a}	18.64 ± 0.64 ^{a}	22.00 ± 0.60 ^{b}
18:0	5.50 ± 0.35 ^{a}	3.56 ± 0.18 ^{b}	7.24 ± 0.38 ^{c}	7.98 ± 0.45 ^{c}
Total SFA	30.67 ± 1.01 ^{ab}	27.85 ± 0.63 ^{a}	30.00 ± 1.08 ^{a}	34.00 ± 1.11 ^{b}
16:1n-7	9.04 ± 0.37 ^{a}	5.01 ± 0.26 ^{b}	3.95 ± 0.33 ^{b}	1.89 ± 0.22 ^{c}
18:1n-7	4.16 ± 0.27 ^{a}	4.16 ± 0.66 ^{a}	9.43 ± 0.91 ^{b}	9.26 ± 0.70 ^{b}
18:1n-9	9.52 ± 0.34 ^{ac}	13.04 ± 0.50 ^{b}	11.12 ± 0.57 ^{abc}	9.83 ± 0.55 ^{c}
Total MUFA	23.96 ± 0.44 ^{ab}	23.72 ± 0.58 ^{ab}	25.20 ± 0.82 ^{a}	22.16 ± 0.81 ^{b}
18:2n-6	2.27 ± 0.23 ^{a}	9.37 ± 0.33 ^{b}	1.73 ± 0.22 ^{a}	8.67 ± 0.56 ^{b}
18:3n-6	0.48 ± 0.05 ^{a}	0.27 ± 0.03 ^{b}	0.48 ± 0.03 ^{a}	0.36 ± 0.06 ^{ab}
20:3n-6	0.06 ± 0.05 ^{a}	0.04 ± 0.28 ^{a}	0.04 ± 0.03 ^{a}	0.02 ± 0.11 ^{a}
20:4n-6, ARA	2.55 ± 0.14 ^{a}	1.97 ± 0.11 ^{a}	7.81 ± 0.39 ^{b}	5.46 ± 0.34 ^{c}
22:4n-6	0.51 ± 0.09 ^{a}	0.15 ± 0.06 ^{b}	1.22 ± 0.14 ^{c}	0.31 ± 0.03 ^{ab}
22:5n-6	0.55 ± 0.05 ^{a}	0.33 ± 0.04 ^{a}	1.01 ± 0.10 ^{b}	0.35 ± 0.04 ^{a}
Total n-6 PUFA	6.42 ± 0.30 ^{a}	12.51 ± 0.37 ^{b}	12.14 ± 0.53 ^{b}	16.04 ± 0.47 ^{c}
18:3n-3	1.67 ± 0.26 ^{a}	1.23 ± 0.09 ^{ab}	0.74 ± 0.25 ^{cb}	0.54 ± 0.11 ^{c}
18:4n-3	2.41 ± 0.28 ^{a}	0.80 ± 0.03 ^{b}	0.82 ± 0.20 ^{b}	0.50 ± 0.09 ^{b}
20:4n-3	0.84 ± 0.10 ^{a}	0.46 ± 0.04 ^{b}	0.36 ± 0.05 ^{bc}	0.21 ± 0.03 ^{c}
20:5n-3, EPA	6.62 ± 0.39 ^{ab}	5.71 ± 0.38 ^{b}	7.46 ± 0.34 ^{a}	6.11 ± 0.36 ^{ba}
22:5n-3	6.21 ± 0.34 ^{a}	6.40 ± 0.28 ^{a}	4.79 ± 0.33 ^{b}	3.75 ± 0.24 ^{b}
22:6n-3, DHA	20.28 ± 0.66 ^{ab}	21.08 ± 1.18 ^{a}	16.86 ± 0.54 ^{ab}	15.65 ± 0.66 ^{b}
Total n-3 PUFA	38.23 ± 1.08 ^{a}	35.82 ± 0.85 ^{a}	31.08 ± 0.73 ^{c}	26.77 ± 0.93 ^{b}
Total PUFA	44.65 ± 0.96 ^{ab}	48.34 ± 0.90 ^{a}	43.44 ± 0.98 ^{b}	42.81 ± 1.25 ^{b}
Total others	4.58 ± 0.08 ^{a}	2.94 ± 0.06 ^{b}	4.08 ± 0.10 ^{a}	3.92 ± 0.08 ^{b}
EPA/ARA	2.68 ± 0.22 ^{a}	2.95 ± 0.20 ^{a}	0.99 ± 0.06 ^{b}	1.17 ± 0.12 ^{b}
EPA/DHA	0.33 ± 0.06 ^{ab}	0.29 ± 0.03 ^{a}	0.44 ± 0.06 ^{c}	0.40 ± 0.03 ^{bc}
DHA/ARA	8.19 ± 0.53 ^{a}	9.63 ± 0.41 ^{b}	2.25 ± 0.12 ^{c}	2.86 ± 0.16 ^{c}
n-3/n-6	6.11 ± 0.40 ^{a}	2.89 ± 0.11 ^{b}	2.61 ± 0.12 ^{b}	1.67 ± 0.05 ^{c}

Abbreviation as in the table 3.

Table 5 *Fatty acid content and fatty acid composition (% TFA ± SEM) of muscle from wild and cultured female and male Senegalese sole (Solea senegalensis)*

	Female		Male	
	Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
TFA(μg mg^{-1} L)	587.9 ± 34.7 ^{a}	611.8 ± 48.0 ^{a}	538.0 ± 23.4 ^{a}	673.2 ± 48.1 ^{a}
14:0	2.39 ± 0.50 ^{ab}	2.39 ± 0.42 ^{ab}	1.12 ± 0.25 ^{a}	3.21 ± 0.33 ^{b}
16:0	17.02 ± 1.37 ^{a}	17.73 ± 1.14 ^{a}	14.80 ± 1.14 ^{a}	18.73 ± 0.63 ^{a}
18:0	6.76 ± 0.50 ^{a}	6.08 ± 0.45 ^{ab}	7.58 ± 0.21 ^{a}	4.63 ± 0.41 ^{b}
Total SFA	30.97 ± 1.67 ^{a}	30.04 ± 1.45 ^{a}	27.40 ± 1.55 ^{a}	31.20 ± 1.08 ^{a}
16:1n-7	4.99 ± 0.85 ^{a}	2.62 ± 0.47 ^{a}	2.77 ± 0.52 ^{a}	3.54 ± 0.56 ^{a}
18:1n-7	1.86 ± 0.22 ^{a}	2.26 ± 0.27 ^{a}	1.86 ± 0.30 ^{a}	2.31 ± 0.26 ^{a}
18:1n-9	8.94 ± 0.46 ^{ac}	11.54 ± 0.49 ^{b}	8.32 ± 0.45 ^{c}	11.92 ± 0.56 ^{b}
Total MUFA	16.64 ± 1.33 ^{ab}	17.39 ± 0.75 ^{ab}	13.94 ± 1.05 ^{a}	19.13 ± 0.83 ^{b}
18:2n-6	1.26 ± 0.09 ^{a}	6.98 ± 0.38 ^{b}	1.04 ± 0.06 ^{a}	7.78 ± 0.44 ^{b}
18:3n-6	0.26 ± 0.08 ^{a}	0.21 ± 0.03 ^{a}	0.24 ± 0.05 ^{a}	0.49 ± 0.19 ^{a}
20:3n-6	0.18 ± 0.04 ^{a}	0.16 ± 0.39 ^{a}	0.14 ± 0.12 ^{a}	0.14 ± 0.15 ^{a}
20:4n-6, ARA	4.01 ± 0.25 ^{a}	2.03 ± 0.16 ^{b}	4.93 ± 0.34 ^{c}	1.68 ± 0.16 ^{b}
22:4n-6	1.29 ± 0.11 ^{a}	0.25 ± 0.02 ^{c}	2.24 ± 0.20 ^{b}	0.25 ± 0.06 ^{c}
22:5n-6	1.58 ± 0.10 ^{a}	0.60 ± 0.05 ^{b}	2.35 ± 0.19 ^{c}	0.60 ± 0.05 ^{b}
Total n-6 PUFA	8.41 ± 0.43 ^{a}	10.63 ± 0.53 ^{ab}	11.13 ± 0.70 ^{b}	11.16 ± 0.55 ^{b}
18:3n-3	0.44 ± 0.14 ^{a}	0.82 ± 0.04 ^{a}	0.33 ± 0.07 ^{a}	1.18 ± 0.49 ^{a}
18:4n-3	0.47 ± 0.26 ^{a}	0.52 ± 0.13 ^{a}	0.32 ± 0.12 ^{a}	0.70 ± 0.21 ^{a}
20:4n-3	0.22 ± 0.09 ^{a}	0.28 ± 0.07 ^{a}	0.21 ± 0.08 ^{a}	0.32 ± 0.06 ^{a}
20:5n-3, EPA	5.95 ± 0.42 ^{ab}	6.24 ± 0.52 ^{b}	6.46 ± 0.19 ^{b}	4.51 ± 0.37 ^{a}
22:5n-3	7.64 ± 0.38 ^{a}	7.53 ± 0.50 ^{a}	8.23 ± 0.20 ^{a}	7.04 ± 0.40 ^{a}
22:6n-3, DHA	29.55 ± 1.93 ^{ab}	27.91 ± 1.32 ^{ab}	32.50 ± 1.47 ^{a}	25.99 ± 1.59 ^{b}
Total n-3 PUFA	44.28 ± 2.48 ^{ab}	43.29 ± 2.00 ^{ab}	48.06 ± 1.68 ^{a}	39.74 ± 1.76 ^{b}
Total PUFA	52.69 ± 2.78 ^{ab}	53.92 ± 1.89 ^{ab}	59.20 ± 1.99 ^{a}	50.90 ± 1.66 ^{b}
Total others	1.33 ± 0.18 ^{a}	0.30 ± 0.11 ^{b}	0.61 ± 0.10 ^{b}	0.42 ± 0.07 ^{b}
EPA/ARA	1.42 ± 0.03 ^{a}	3.37 ± 0.28 ^{b}	1.38 ± 0.10 ^{a}	3.03 ± 0.3 ^{b}
EPA/DHA	0.21 ± 0.06 ^{a}	0.23 ± 0.06 ^{a}	0.20 ± 0.05 ^{a}	0.18 ± 0.1 ^{a}
DHA/ARA	7.63 ± 0.31 ^{a}	14.40 ± 0.88 ^{b}	6.83 ± 0.43 ^{a}	16.40 ± 1.1 ^{b}
n-3/n-6	5.31 ± 0.25 ^{a}	4.20 ± 0.29 ^{a}	4.47 ± 0.28 ^{a}	3.70 ± 0.3 ^{b}

Abbreviation as in the table 3.

3.5. Fatty acids in the diets

3.5.1. PUFAs in Senegalese sole diet

The relative fatty acid composition of total lipid extracted from extruded feed (Vitalis® and Elite®, Skretting, Norway) was dominated by n-3 PUFAs (32% TFA, predominantly EPA), saturated fatty acids (26-27% TFA, predominantly 16:0), and monoenoic fatty acids (23-24% TFA, predominantly 18:1n-9) (Table 6). The n-6 PUFA (12-15% TFA was dominated by 18:2n-6 acid. Concerning the wet diets, n-3 PUFAs (18-28%) were predominant in all the diets used except in the polychaetes from UK (9%), which showed the lowest DHA levels, whereas the highest DHA content was observed in Squid, followed by polychaetes from Netherlands, and mussels. EPA content was significantly (P<0.05) higher in mussels followed by polychaetes from Netherlands, squid, and polychaetes UK. ARA relative content was significantly (P<0.05) higher in squid, mussels and polychaetes from Netherlands compared to the extruded feeds and polychaetes from UK, which had a similar ARA content (Table 6).

All the diets studied showed an EPA/ARA ratio significantly (P<0.05) higher than the ratio found in liver, gonads and muscle of wild fish and gonad from cultured fish (Fig. 2). Both extruded diets (Vitalis® and Elite®) showed a 9.3-fold higher EPA/ARA ratio than wild fish, followed by polychaetes from UK (7.3-fold), polychaetes from Netherlands (6.5-fold), mussels (5.8-fold) and squid (3.3-fold) (Fig. 2). The ratio in squid was similar to the ratio in liver, muscle and ovary from cultured fish.

Table 6 *Fatty acid content and fatty acid composition (% TFA ± SEM) of two commercial diets used to feed the fish (extruded feed Vitalis® Repro and Elite®, Skretting, Norway) and four wet diet commonly used to fed cultured broodstock of Senegalese sole (Solea senegalensis). Polychaete Nereis virens from England (P.UK), polychaete Nereis virens from Netherlands (P. Neth.), squid Loligo gahi, and mussels Mytilus galloprovincialis*

	Vitalis® (N=4)	Elite® (N=4)	P. UK (N=3)	P. Neth. (N=3)	Squid (N=3)	Mussel (N=3)
TFA(μg mg^{-1} L)	654 ± 76.8 ^{a}	684 ± 22.9 ^{a}	404 ± 43.4 ^{a}	378 ± 18.1 ^{a}	381 ± 31.2 ^{a}	431 ± 9.9 ^{a}
14:0	4.73 ± 0.14 ^{ab}	5.72 ± 0.32 ^{b}	3.00 ± 0.22 ^{ac}	0.62 ± 0.62 ^{c}	2.81 ± 0.11 ^{ac}	2.84 ± 0.76 ^{ac}
16:0	16.79 ± 2.31 ^{a}	17.23 ± 1.78 ^{a}	17.72 ± 0.20 ^{a}	12.46 ± 2.23	20.29 ± 0.41 ^{a}	15.46 ± 2.61 ^{a}
18:0	4.06 ± 0.59 ^{a}	3.88 ± 0.40 ^{a}	3.54 ± 0.15 ^{a}	6.81 ± 0.17 ^{b}	4.23 ± 0.27 ^{a}	3.89 ± 0.78 ^{a}
Total SFA	25.90 ± 3.07 ^{a}	27.13 ± 2.55 ^{a}	24.65 ± 0.56 ^{a}	20.00 ± 1.34 ^{a}	27.57 ± 0.73 ^{a}	22.46 ± 4.14 ^{a}
16:1n-7	7.75 ± 1.00 ^{ac}	8.45 ± 0.68 ^{ac}	5.48 ± 0.50 ^{cb}	3.08 ± 1.27 ^{b}	2.50 ± 0.73 ^{b}	10.12 ± 0.37 ^{a}
18:1n-9	11.26 ± 1.09 ^{ac}	10.39 ± 0.82 ^{ac}	6.49 ± 0.16 ^{ab}	13.69 ± 2.12 ^{c}	2.55 ± 0.14 ^{b}	1.48 ± 0.35 ^{b}
18:1n-7	2.72 ± 0.25 ^{a}	3.15 ± 0.33 ^{a}	2.70 ± 0.06 ^{a}	2.16 ± 2.16 ^{a}	0.82 ± 0.06 ^{a}	1.61 ± 0.14 ^{a}
20:1n-9	1.62 ± 0.23 ^{a}	1.61 ± 0.17 ^{a}	9.22 ± 0.34 ^{b}	2.73 ± 1.14 ^{a}	2.92 ± 0.50 ^{a}	1.33 ± 0.19 ^{a}
Total MUFA	23.35 ± 0.58 ^{a}	23.60 ± 1.95 ^{a}	23.89 ± 0.03 ^{a}	21.66 ± 1.44 ^{a}	8.79 ± 0.10 ^{b}	14.54 ± 1.01 ^{b}
18:2n-6	14.13 ± 1.49 ^{a}	10.75 ± 0.83 ^{a}	5.96 ± 0.35 ^{b}	5.00 ± 0.44 ^{bd}	0.42 ± 0.04 ^{c}	1.35 ± 0.10 ^{cd}
18:3n-6	0.15 ± 0.03 ^{a}	0.12 ± 0.03 ^{a}	0.05 ± 0.05 ^{a}	0.78 ± 0.51 ^{a}	0.18 ± 0.07 ^{a}	0.07 ± 0.00 ^{a}
20:4n-6, ARA	1.16 ± 0.14 ^{a}	1.12 ± 0.09 ^{a}	0.90 ± 0.08 ^{a}	2.02 ± 0.11 ^{b}	3.90 ± 0.08 ^{c}	3.40 ± 0.24 ^{c}
22:4n-6	0.10 ± 0.01 ^{a}	0.12 ± 0.01 ^{a}	0.84 ± 0.12 ^{ab}	1.08 ± 0.31 ^{b}	0.22 ± 0.02 ^{a}	0.46 ± 0.06 ^{ab}
22:5n-6	0.24 ± 0.04 ^{ab}	0.19 ± 0.03 ^{a}	0.06 ± 0.05 ^{a}	0.86 ± 0.27 ^{b}	0.44 ± 0.02 ^{ab}	0.19 ± 0.01 ^{a}
Total n-6	15.77 ± 1.64 ^{a}	12.29 ± 0.97 ^{ac}	7.77 ± 0.55 ^{bc}	9.74 ± 0.26 ^{bc}	5.16 ± 0.20 ^{b}	5.47 ± 0.20 ^{b}
18:3n-3	2.00 ± 0.25 ^{a}	1.46 ± 0.19 ^{ab}	1.01 ± 0.20 ^{ab}	1.61 ± 0.52 ^{ab}	0.16 ± 0.04 ^{b}	0.33 ± 0.05 ^{b}
18:4n-3	1.36 ± 0.29 ^{a}	1.47 ± 0.00 ^{a}	0.12 ± 0.12 ^{a}	1.38 ± 0.45 ^{a}	0.07 ± 0.00 ^{a}	1.24 ± 0.43 ^{a}
20:4n-3	0.54 ± 0.06 ^{a}	0.50 ± 0.05 ^{a}	0.36 ± 0.06 ^{a}	0.46 ± 0.09 ^{a}	0.14 ± 0.05 ^{a}	0.31 ± 0.03 ^{a}
20:5n-3, EPA	14.51 ± 0.95 ^{ac}	15.64 ± 1.21 ^{ac}	9.38 ± 0.24 ^{a}	18.79 ± 1.02 ^{c}	18.32 ± 0.47 ^{c}	28.09 ± 3.28 ^{b}
21:5n-3	1.03 ± 0.25 ^{a}	0.98 ± 0.07 ^{a}	0.62 ± 0.22 ^{a}	0.32 ± 0.12 ^{a}	0.78 ± 0.18 ^{a}	1.09 ± 0.36 ^{a}
22:5n-3	1.81 ± 0.15 ^{ab}	1.84 ± 0.11 ^{ab}	1.39 ± 0.05 ^{ab}	3.35 ± 0.82 ^{a}	1.02 ± 0.01 ^{b}	2.21 ± 0.38 ^{ab}
22:6n-3, DHA	10.95 ± 0.73 ^{a}	10.72 ± 0.94 ^{a}	4.78 ± 0.06 ^{b}	18.66 ± 1.26 ^{c}	35.46 ± 0.39 ^{d}	11.18 ± 1.10 ^{a}
Total n-3	32.20 ± 2.18 ^{ab}	32.61 ± 2.56 ^{ab}	17.67 ± 0.16 ^{a}	44.57 ± 0.12 ^{cb}	55.96 ± 1.10 ^{cd}	44.44 ± 5.59 ^{bd}
Total PUFA	47.97 ± 3.82 ^{a}	44.90 ± 3.53 ^{a}	25.45 ± 0.38 ^{b}	54.31 ± 0.14 ^{a}	61.11 ± 1.30 ^{a}	49.91 ± 5.79 ^{a}
Total others	2.68 ± 0.17 ^{a}	4.24 ± 0.96 ^{a}	23.89 ± 0.21 ^{b}	1.84 ± 0.40 ^{a}	2.38 ± 0.67 ^{a}	12.52 ± 0.64 ^{c}
EPA/ARA	12.65 ± 0.75 ^{ac}	13.99 ± 0.09 ^{c}	10.56 ± 1.21 ^{ad}	9.37 ± 1.01 ^{ad}	4.70 ± 0.02 ^{b}	8.24 ± 0.39 ^{bd}
EPA/DHA	1.33 ± 0.00 ^{ac}	1.46 ± 0.06 ^{a}	1.96 ± 0.03 ^{b}	1.02 ± 0.12 ^{c}	0.52 ± 0.01 ^{d}	2.51 ± 0.05 ^{e}
DHA/ARA	9.54 ± 0.55 ^{a}	9.58 ± 0.04 ^{a}	5.38 ± 0.54 ^{b}	9.24 ± 0.12 ^{a}	9.10 ± 0.09 ^{a}	3.28 ± 0.09 ^{c}
n-3/n-6	2.05 ± 0.07 ^{a}	2.65 ± 0.00 ^{a}	2.29 ± 0.18 ^{a}	4.58 ± 0.13 ^{b}	10.86 ± 0.21 ^{c}	8.10 ± 0.72 ^{d}

Abbreviation as in the table 3.

3.6. Lipid class composition of Senegalese sole tissues

In the liver, lipid class composition varied according to the origin of the fish, thus in wild females and males contained significantly (P<0.05) higher levels of total polar lipids, phosphatidylcholine, pospatidylserine plus phosphatidylinositol, phosphatidylethanolamine, cholesterol and free fatty acids compared to the liver of cultured fish (Table 7). Total

neutral lipids (NL), triacylglycerides in both sexes and sterol ester and wax in females showed the opposite trend, with significantly (P<0.05) higher levels found in the liver of cultured fish. In the case of male muscle similar trends were observed, with significantly (P<0.05) higher values in total polar lipids, phosphatidylcholine, posphatidylserine plus phosphatidylinositol, phosphatidylethanolamine and cholesterol in wild fish compared with the cultured counterparts and total NL and triacylglycerides were higher in cultured fish (Table 7). No differences were found in the lipid class composition of the female muscle and gonads between wild and cultured fish (Table 7), with the exception of free fatty acids that were significantly higher in testis from wild fish. Cholesterol (CHOL) content was high in the testis of both fish (42% in wild and 45% TL in cultured) without any significant difference.

***Table** 7 Lipid class composition (% ± SEM) of liver, gonads and muscle from wild and cultured female and male Senegalese sole (Solea senegalensis)*

	Female		Male	
Liver	Wild (N=9)	Cultured (N=12)	Wild (N=11)	Cultured (N=13)
PC	25.1 ± 1.3 ^{a}	6.5 ± 0.7 ^{b}	12.9 ± 1.1 ^{c}	4.9 ± 0.4 ^{b}
PS+PI	6.0 ± 0.3 ^{a}	1.4 ± 0.2 ^{b}	3.8 ± 0.4 ^{c}	1.5 ± 0.2 ^{b}
PE	14.8 ± 1.0 ^{a}	3.5 ± 0.5 ^{b}	9.8 ± 0.7 ^{c}	3.1 ± 0.4 ^{b}
Total PL*	47.0 ± 2.5 ^{a}	11.8 ± 1.2 ^{b}	27.2 ± 2.3 ^{c}	9.7 ± 0.9 ^{b}
CHOL	21.5 ± 1.6 ^{a}	7.8 ± 0.7 ^{b}	19.6 ± 1.5 ^{a}	7.0 ± 0.9 ^{b}
FFA	5.3 ± 0.7 ^{abc}	2.9 ± 0.7 ^{b}	7.8 ± 1.0 ^{a}	3.5 ± 1.0 ^{c}
TAG	20.7 ± 4.1 ^{a}	41.0 ± 2.7 ^{b}	22.3 ± 3.6 ^{a}	59.1 ± 2.1 ^{c}
SE+W	4.9 ± 3.3 ^{a}	35.1 ± 3.3 ^{b}	22.1 ± 4.0 ^{c}	18.0 ± 3.0 ^{ac}
Total NL**	53.0 ± 2.5 ^{a}	88.2 ± 1.2 ^{b}	72.8 ± 2.3 ^{c}	90.3 ± 0.9 ^{b}
Gonad				
PC	16.3 ± 0.4 ^{a}	11.8 ± 1.7 ^{a}	12.5 ± 2.8 ^{a}	16.7 ± 0.4 ^{a}
PS+PI	2.9 ± 0.4 ^{a}	2.6 ± 0.5 ^{a}	3.3 ± 0.3 ^{a}	5.4 ± 0.3 ^{b}
PE	9.5 ± 2.0 ^{a}	6.0 ± 1.8 ^{a}	20.0 ± 2.2 ^{b}	22.3 ± 1.0 ^{b}
Total PL*	29.9 ± 2.4 ^{a}	22.3 ± 2.6 ^{a}	41.1 ± 2.7 ^{b}	45.4 ± 1.7 ^{b}
CHOL	21.9 ± 3.5 ^{a}	27.3 ± 4.0 ^{a}	41.7 ± 4.4 ^{b}	45.1 ± 1.7 ^{b}
FFA	2.7 ± 0.6 ^{a}	2.4 ± 0.8 ^{a}	5.9 ± 0.7 ^{b}	4.6 ± 0.4 ^{a}
TAG	18.8 ± 2.3 ^{a}	24.3 ± 2.3 ^{a}	5.5 ± 3.3 ^{b}	3.2 ± 0.6 ^{b}
SE+W	26.8 ± 4.1 ^{a}	26.7 ± 3.6 ^{a}	5.8 ± 4.1 ^{b}	1.2 ± 0.6 ^{b}
Total NL**	70.3 ± 2.4 ^{a}	77.7 ± 2.6 ^{a}	58.9 ± 2.7 ^{b}	54.6 ± 1.7 ^{b}
Muscle				
PC	34.8 ± 2.9 ^{a}	28.5 ± 2.3 ^{ab}	34.2 ± 2.2 ^{a}	22.2 ± 3.3 ^{b}
PS+PI	7.7 ± 0.5 ^{a}	5.9 ± 0.7 ^{ab}	7.6 ± 0.3 ^{a}	4.8 ± 0.8 ^{b}
PE	16.1 ± 0.8 ^{a}	12.0 ± 1.1 ^{ab}	15.7 ± 0.6 ^{a}	8.8 ± 1.6 ^{b}
Total PL*	59.8 ± 3.7 ^{a}	46.7 ± 4.1 ^{ab}	58.5 ± 2.5 ^{a}	36.0 ± 5.6 ^{b}
CHOL	21.0 ± 1.4 ^{ab}	19.4 ± 1.0 ^{ab}	23.5 ± 1.9 ^{a}	15.0 ± 1.9 ^{b}
FFA	2.7 ± 0.4 ^{a}	1.7 ± 0.1 ^{a}	2.8 ± 0.3 ^{a}	2.5 ± 0.5 ^{a}
TAG	16.5 ± 4.3 ^{a}	31.4 ± 4.8 ^{ab}	13.5 ± 4.1 ^{a}	45.5 ± 6.8 ^{b}
SE+W	0.0 ± 0.0 ^{a}	1.4 ± 1.5 ^{a}	1.8 ± 1.5 ^{a}	2.7 ± 1.9 ^{a}
Total NL**	40.2 ± 3.7 ^{a}	53.3 ± 4.1 ^{ab}	41.5 ± 2.5 ^{a}	64.0 ± 5.6 ^{b}

PC: phosphatidylcholine, PS+PI: posphatidylserine and phosphatidylinositol, PE: phosphatidylethanolamine, PL: polar lipids, CHOL: cholesterol, FFA: free fatty acids, TAG: triacylglycerides, SE+W: sterol ester and wax and NL: neutral lipids, * include lysophosphatidylcholine, sphingomyelin, sphingolipidoses, phosphatidylinositol and phosphatidylserine. ** include monoacylglycerols. Within a row, means without a common superscript letter differ significantly (ANOVA, $P<0.05$), N = number of samples.

3.7. Lipid classes in Senegalese sole diets

In all the diets examined, lipid class composition was dominated by NL, with the highest (P<0.05) values obtained in the case of extruded feed and polychaetes, 90-92 and 81-90% TL of the total lipids respectively (Table 8). Triacylglycerides was the most abundant NL in extruded feed with 61 and 63% in Vitalis® and Elite®, respectively significantly (P<0.05) higher than in the others diets (P<0.05). Polychaete Netherlands showed the highest values in CHOL (46.6% TL) (P<0.05) followed by squid (36% TL) and polychaete UK (28% TL). Total polar lipids were significantly (P<0.05) higher in squid and muscle with 37 and 40%, respectively, compared to the rest of the diets (P<0.05), with phosphatidylethanolamine and phosphatidylcholine as the significantly higher polar lipid in squid and mussels (P<0.05) (Table 8).

Table 8 *Lipid class composition (% ± SEM) of two commercial diet used to feed the fish (extruded feed Vitalis® Repro and Elite®, Skretting, Norway) and four wet diet commonly used to fed cultured broodstock of Senegalese sole (Solea senegalensis)(polychaete Nereis virens from England (P.UK), polychaete Nereis virens from Netherlands (P. Neth.), squid Loligo gahi, and mussels Mytilus galloprovincialis)*

	Vitalis® (N=4)	Elite® (N=4)	P. UK (N=3)	P. Neth. (N=3)	Squid (N=3)	Mussel (N=3)
PC	5.6 ± 0.0 ^{c}	5.3 ± 0.0 ^{c}	3.3 ± 0.3 ^{d}	6.8 ± 0.3 ^{c}	17.5 ± 0.2 ^{a}	11.7 ± 0.8 ^{b}
PS/PI	1.0 ± 0.1 ^{bc}	0.0 ± 0.0 ^{c}	1.9 ± 0.1 ^{bc}	1.3 ± 0.2 ^{bc}	2.6 ± 0.0 ^{b}	10.9 ± 1.0 ^{a}
PE	3.1 ± 0.1 ^{d}	2.8 ± 0.6 ^{d}	4.4 ± 0.4 ^{d}	8.2 ± 0.2 ^{c}	15.4 ± 0.0 ^{b}	17.2 ± 0.4 ^{a}
Total PL*	9.6 ± 0.1 ^{c}	8.1 ± 0.6 ^{c}	9.5 ± 0.1 ^{c}	18.8 ± 0.5 ^{bc}	37.2 ± 0.8 ^{a}	40.3 ± 2.3 ^{a}
CHOL	13.6 ± 0.8 ^{e}	12.5 ± 0.0 ^{e}	28.1 ± 0.0 ^{c}	46.6 ± 0.1 ^{a}	36.3 ± 0.2 ^{b}	22.4 ± 0.8 ^{d}
FFA	9.1 ± 0.4 ^{c}	9.4 ± 0.0 ^{c}	38.7 ± 0.5 ^{a}	19.0 ± 1.6 ^{bc}	19.1 ± 1.0 ^{b}	4.1 ± 0.1 ^{c}
TAG	61.1 ± 0.3 ^{b}	63.2 ± 0.2 ^{a}	12.6 ± 0.1 ^{d}	7.1 ± 0.2 ^{e}	7.5 ± 0.4 ^{e}	24.3 ± 0.4 ^{c}
SE+W	6.6 ± 0.6 ^{a}	6.9 ± 0.4 ^{a}	11.3 ± 0.4 ^{a}	8.6 ± 0.9 ^{a}	0.0 ± 0.0 ^{b}	9.2 ± 1.9 ^{a}
Total NL**	90.3 ± 0.1 ^{a}	92.0 ± 0.7 ^{a}	90.6 ± 0.2 ^{a}	81.2 ± 0.5 ^{b}	62.9 ± 0.8 ^{c}	59.9 ± 2.3 ^{c}

Abbreviation as in the table 7.

4. Discussion

In the present study, cultured Senegalese sole exhibited higher levels of total lipid and particularly neutral lipids compared to wild sole, as has been observed in similar wild versus captive comparisons in white seabream (Cejas *et al*., 2004) and black seabream (Rodríguez *et al.*, 2004). The high levels of lipid in cultured sole appeared to be a reflection of high levels of lipid in the diet and an imbalanced diet as was also suggested for similar

observations in black seabream (Rodríguez *et al.*, 2004). In trout, excess dietary energy, was mainly stored in the form of neutral lipids, triacylglycerides deposited in muscle and adipose tissue (Kiessling *et al.*, 2001). In European seabass (Sorbera *et al.*, 1998) and gilthead seabream (Almansa *et al.*, 2001), neutral lipids stored in liver and muscle were mobilized to contribute to the egg reserves and or energy substrate for specific lipoprotein synthesis, which transports lipids and protein from liver and muscle to ovaries. Almansa *et al.* (2001) have shown that before the beginning of the spawning period, gilthead seabream female showed a high neutral lipid content (above 88% TL) in liver and muscle, and during the spawning period these values decrease to 20 and 40% respectively to demonstrate the importance of neutral lipid reserves for reproduction. However, the present study found that the fatty acid profile of the stored lipid was different compared to wild sole. Cultured fish generally had lower percentage levels of polar lipids, ARA, 22:4n-6 and 22:5n-6 and higher levels of neutral lipids, EPA/ARA ratios and linoleic acids.

Higher levels of ARA in wild fish compared to those in cultured fish have also been reported in eggs and larvae of common sole (Lund *et al.*, 2008), sperm and eggs of European seabass (Bell *et al.*, 1996b, Bruce *et al.*, 1999), ovaries, eggs, muscle and liver of white seabream (Cejas *et al.*, 2003, Cejas *et al.*, 2004) and muscle of black seabream (Rodríguez *et al.*, 2004). In Pacific herring, spawning fish had a 3.7-fold higher ARA liver content than non-spawning fish (Huynh *et al.*, 2007). Further Studies with captive fish have established that a dietary increase in ARA levels improved significantly the gamete quality in European seabass (Thrush *et al.*, 1993, Bruce *et al.*, 1999), Atlantic halibut (Bromage *et al.*, 2001, Mazorra *et al.*, 2003, Alorend, 2004) and common sole (Lund *et al.*, 2008). Atlantic halibut fed higher levels of dietary ARA exhibited higher fertilisation and hatching rate (Bromage *et al.*, 2001, Mazorra *et al.*, 2003) and increased the quality of eggs, sperm and fecundity (Alorend, 2004), while European seabass exhibited increased egg viability, survival and hatching rate (Bruce *et al.* 1999). Lund *et al.* (2008) found better fertilization and hatching rates in wild common sole eggs and larvae with 3.1 and 3.3% ARA respectively. Low levels of ARA, in the diet and tissues of cultured Senegalese sole that do not spawn regularly were identified in the present study. Similar observations and comparisons can be made for EPA and DHA, cultured common sole that had lower EPA values also had lower fertilization and hatching rates compared with wild (Lund *et al.*,

2008) and in Pacific herring, higher DHA content was found in spawning compared to non-spawning fish (Huynh *et al.*, 2007). Again, in the present study, low levels of EPA and DHA were observed in some tissues of cultured Senegalese sole that do not spawn regularly. However, it should be noted that despite of the low relative levels, cultured fish had large amounts of ARA and other essential fatty acids stored, and at the time of sampling ARA did not appear to be a limiting essential fatty acid, for example in the liver, cultured male fish had 0.74 ± 0.06 % ARA of the 52.3 ± 2.8% of total lipid compared to wild male with 2.50 ± 0.29% ARA of the 21.1 ± 1.3% of total lipid.

Another important aspect of essential fatty acids is the ratios and in the present study cultured fish exhibited high EPA/ARA and DHA/ARA ratios compared wild fish. Both ARA and EPA are precursors of prostaglandins (Tocher, 2003, Smith, 1989) which have an important role in fish reproduction (Smith, 1989, Zhang *et al.*, 1992, Tocher, 2003, Alorend, 2004), ARA form 2-series prostaglandins and EPA 3-series prostaglandins, being the latter biologically less active than the corresponding 2-series and possess antagonistic functions (Smith, 1989, Tocher, 2003). This is a competitive reaction, which EPA modulates by inhibiting formation of high biologically active 2-series prostaglandins from ARA (Sargent, 1995). The importance of 2-series prostaglandins in reproduction and in particular reproductive behaviour has been identified in some fish species (Stacey and Goetz, 1982, Stacey *et al*., 1994, Sorensen and Stacey, 2004). Additionally to the roles as precursors of eicosanoids, ARA modulates the production of steroid hormones (Lin, 1985, Van Der Kraak *et al.*, 1990) to induce oocyte maturation (Mustafa *et al.*, 1989, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Wade *et al.*, 1994b, Sorbera *et al.*, 2001). Studies carried out in vitro on testosterone production, using ARA and its cyclooxygenase metabolites, have demonstrated that prostaglandins 2-series stimulate testosterone production in testis and ovaries of carp and lead to steroidogenesis of ovarian follicles (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995) and the action of ARA on fish steroidogenesis and oocyte maturation has been blocked by treatments with EPA or DHA due to the inhibition of prostaglandins 2-series formation from ARA (Wade *et al.*, 1994b, Mercure *et al.*, 1995). PUFAs (n-3 and n-6) can be potent modulators of both prostaglandin production and the interaction between steroid hormone and the steroid binding protein in fish plasma (Mercure *et al.*, 1995, Van Der Kraak *et al.*, 1999).

Therefore, the EPA/ARA ratio modulates steroidogenesis in testis increasing testosterone production and any change in the ratio or in the levels of ARA, EPA and DHA in the tissues may influence the prostaglandins and steroid production (Wade *et al.*, 1994a, Stacey *et al.*, 2003, Tocher, 2003). The high EPA/ARA ratio observed in cultured fish were concomitant with the higher ratios observed in the extruded diets. Differences in fatty acid composition between captive and wild fish have been attributed to broodstock diets, this is the case for gilthead seabream (Mourente *et al.*, 1989), striped bass (Harrell *et al.*, 1995), turbot (Silversand *et al.*, 1996), European seabass (Bell *et al.*, 1997) and striped trumpeter (Morehead *et al.*, 2001).

The C22 n-6 fatty acids, 22:4n-6 and 22:5n-6 acid which are a consequence of the elongation of ARA and desaturation of 22:4n-6, respectively (Sprecher, 2000), were positively correlated to the levels of ARA in wild fish. To study the capacity of Senegalese sole to desaturate and elongate in response to changes in dietary ARA levels is a future need. The n-6 fatty acids and consequently n-3/n-6 ratio were different for cultured and wild fish. This increase in total n-6 PUFA in cultured fish was a consequence of the high levels of 18:2n-6 acid in the commercial extruded diet, as has also been reported in white seabream and common sole (Cejas *et al.*, 2004, Lund *et al.*, 2008). Bell *et al*. (1994) studied the effect of 18:2n-6 acid in growth, fatty acid composition and eicosanoid production in turbot fish deficient in Δ5 desaturase, and concluded that 18:2n-6 acid, might affect the prostaglandins production but did not affect the growth performance. As observed in the present study, 18:2n-6 acid was accumulated by turbot in the liver with the elongation product 20:2n-6 (not analysed in present study). However, the level of ARA was significantly reduced in fish fed with 18:2n-6 acid with a concomitant reduction in prostaglandins (Bell *et al.*, 1995a). The 18:2n-6 acid is found in most vegetable oils including soybean, safflower and sunflower used in the extruded diets fed to the cultured sole and is not a normal component of the sole diet. The 18:2n-6 acid are preferentially added on triacylglycerides (Pérez *et al.*, 1999) and Bell *et al.* (1997) suggested that exclusion of 18:2n-6 acid rich vegetable oils in broodstock diet should be beneficial in terms of larval quality and survivability, however there is a lack of information related to this.

The cholesterol (CHOL) was significantly lower in cultured fish in comparison with wild counterparts, as it has been observed in other marine fish species like white Sea bream (Cejas *et al.*, 2003, Cejas *et al.*, 2004), in which differences were detected in gonads and muscle. CHOL levels in the extruded feeds used for Senegalese sole were significantly lower than in the 'fresh' feeds analysed especially in polychaetes and squid. Considering the relatively high amounts of vegetable derived ingredients (either meals or oils) included in extruded diets, dietary CHOL, and consequently the levels of CHOL in fish tissues were reduced (Tocher *et al.*, 2008). CHOL is the predominant sterol precursor to fat-soluble vitamins and steroid hormones in animals thus, changes in dietary CHOL could be affecting the steroid production in broodstock fish (Baron *et al.*, 1997). Leaver *et al.* (2008) showed that Atlantic salmon increased CHOL biosynthesis after dietary substitution of fish oil with vegetable oil. Further researches are needed to elucidate the Senegalese sole capacity to synthesise CHOL and the effect of extruded diet in steroid production.

5. Conclusion

The present study showed that cultured broodstock fish had lower levels of ARA, CHOL polar lipid, 22:4n-6 and 22:5n-6 acid, and higher levels of total lipid, NL, 18:2n-6 acid and ratios of EPA/ARA and DHA/ARA compare with the wild counterpart. These differences were mainly reflection of extruded diet used to fed G1 fish, with high content of total lipid, 18:2n-6 acid, and EPA and deficiencies of ARA, polar lipid and CHOL. Therefore, the present result suggest that the lipid composition of the commercial diets supplied to G1 sole differed greatly from that of the diet consumed by the fish in the wild. The imbalance of essential fatty acids in tissues, together with the absence of spawning of G1 fish suggests that, future research should consider the importance of ARA, EPA and CHOL on the production of prostaglandins (2- and 3-series) and steroids that are involved in the control of reproductive physiology and behaviour.

6. Acknowledgments

We gratefully thank Alicia Estevez, Gordon Bell and Ignacio Carazo for the scientific help. Thanks are also given to Karl Andree for the English help and Noelia Gras, Pili Gonzalvo and Marta Sastre for sample collection and technical support. Thanks to Olvido

Cherenguini from Instituto Español de Oceanografía, Santander for supplying the cultured fish. This research was funded by two projects: 1) Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project Lenguado coordinated nationally by P. Cañavate and in Catalonia by N. Duncan and 2) Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by N. Duncan and the PhD grant awarded to F. Norambuena by Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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Chapter III

PGs and COX-2 in *S. Sole*

PROSTAGLANDIN (F AND E, 2- AND 3 -SERIES) PRODUCTION AND CYCLOOXYGENASE (COX-2) GENE EXPRESSION OF WILD AND CULTURED BROODSTOCK OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

Abstract

Prostaglandin levels and cyclooxygenase (COX-2) gene expression that characterise the prostaglandin pathway were compared between wild and cultured Senegalese sole (*Solea senegalensis*) broodstock that were known to have significantly different fatty acid profiles, particularly higher tissue levels of arachidonic acid (20:4n-6, ARA) and lower levels of eicosapentanoic acid (20:5n-6, EPA) in the wild fish compared to cultured. This is the first report of the COX-2 mRNA expression in Senegalese sole. Cyclooxygenase (COX-2) mRNA expression and prostaglandin (2- and 3- series) levels were determined in tissues from 32 broodstock fish, 16 (8 males and 8 females) from each origin wild and G1 cultured. Transcripts of COX-2 were highly expressed in gills, sperm-duct, testis, oviduct and spleen compared to liver, kidney and muscle. Differences in transcripts COX-2 expression were found in response to the origin of the fish and expression was significantly up regulated in sperm-duct and gills from wild fish compared to cultured. Wild fish showed significantly higher levels of total 2-series PGs and lower levels of 3-series compared to cultured fish. The significance of COX-2 down-regulation and low production of PG 2-series in some of the tissues of cultured fish was discussed in relation to lower levels of ARA, higher levels of EPA and previously described reproductive failure to spawn viable eggs from G1 cultured Senegalese sole.

1. Introduction

The prostaglandin (PGs) 2-series were first discovered in seminal fluid involved in the muscle contraction and ejaculation (Euler, 1936). Studies carried out in vitro with cell testis and follicle, using ARA and its metabolites, have shown that PGE2 stimulates testosterone production in testis and ovaries of teleost fish (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995, Sorbera *et al.*, 2001). Moreover PGF2α acts hormonally to trigger female sexual behaviour, as a postovulatory circulating PG to trigger courtship and to stimulate increased milt production in males (Kobayashi *et al.*, 2002, Sorensen *et al.*,

2004, Laberge *et al.*, 2003). In mammals PGE2 is involved in the physiological process of ovulation, T cell development and crypt stem cell survival, while PGF2α is involved in labour and birth (Smith *et al.*, 2001). The enzymatic pathway involved in eicosanoid production is often called the 'arachidonic cascade', since arachidonic acid (20:4n-6, ARA) is the major eicosanoid precursor. The eicosanoids are metabolites of ARA, which include the cyclooxygenase-derived (COX) products prostaglandins (PGs) and thromboxanes (TXA2) (Smith, 1989, Smith *et al.*, 2002). Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthases (PGHS), catalyze the conversion of ARA to PGs. Two different COX isoenzymes, COX-1 and COX-2, encoded by separate genes, have been identified (Smith *et al.*, 2001). Studies of COX-2 regulation in fish, showed that COX-2 was primarily responsible for the production of PGs either to maintain homeostatic function or involved in fish reproduction and inflammatory response (Patiño *et al.*, 2003, Choe *et al.*, 2006, Sirois *et al.*, 2004). Smith and Langenbach (2001), summarized the role of these two isoforms in mammals and showed that COX-1 was mainly a constitutive form that maintained normal cell functions while COX-2 was the enzyme that produced the PGs (PGE2, PGF2α, PGI2, PGD2, TXA2 and PGH2) that are essential in physiological processes such as ovulation (PGE2), implantation (PGI2), inflammation resolution (PGD2), T cell development (PGE2), perinatal kidney development (not determinate), ductus arteriosus remodelling (TXA2 and PGH2), ulcer healing (not determinate) and intestinal cancer PGE2). The prostaglandin metabolites can also be produced from fatty acids such as eicosapentaenoic acid (20:5n-3, EPA), being converted to 3-series PGs, however these metabolites are biologically less active than the corresponding 2-series PGs produced from ARA (Tocher, 2003). Thus, EPA competes with ARA in PGs production. In vitro studies in cell testis and follicles of teleost fish, shows that EPA blocked steroid production induced by ARA and cAMP formation due to interference with 2- and 3-series eicosanoid production (Wade *et al.*, 1994, Bell *et al.*, 1994, Mercure *et al.*, 1995). Previous studies (Bell *et al.*, 1995, Bell *et al.*, 1994) demonstrated that changes in dietary fatty acids resulted in extensive alteration in the profile of PGs in heart, brain, gills and kidney of fish, with the concentration and magnitude being different in all tissues analysed.

Reproductive failure to spawn viable eggs from G1 cultured Senegalese sole (*Solea senegalensis*) has been shown (Carazo *et al.*, 2009, Carazo *et al.*, 2011a). Wild

counterparts, held in captivity, spawn regularly producing eggs of sufficient quality and quantity. As previously reported (Carazo *et al.*, 2009, Carazo *et al.*, 2008, Carazo *et al.*, 2011a, Carazo *et al.*, 2011b), studies on reproductive behaviour indicate that although cultured females release eggs, the courtship between males and females was not completed and the eggs were not fertilised. Moreover significant differences in the fatty acid profiles in the liver, gonads and muscle from wild and G1 cultured broodstock were described (Norambuena *et al.*, 2011b, Norambuena *et al.*, 2008, Norambuena *et al.*, 2009) (chapter II). In particular, wild Senegalese sole broodstock showed a significantly higher accumulation of arachidonic acid (ARA) in the liver, testis and muscle compared to cultured fish with these differences being particularly marked in testis (Norambuena *et al.*, 2011b, Norambuena *et al.*, 2008, Norambuena *et al.*, 2009) (chapter II). Conversely EPA and EPA/ARA ratios levels were significantly higher in G1 fish compared with wild counterparts. The fatty acid composition and particularly ARA is considered one of the most important factors for successful fish reproduction (Izquierdo *et al.*, 2001, Falk-Petersen *et al.*, 1989, Mazorra *et al.*, 2003, Furuita *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Perez *et al.*, 2007, Tocher, 2010) and play an important role in production of PGs (Tocher, 2003, Smith, 1989, Smith *et al.*, 2002) which also have significant roles in the success of fish reproduction (Van Der Kraak *et al.*, 1990, Sorensen *et al.*, 1993, Wade *et al.*, 1993, Mercure *et al.*, 1995, Sorbera *et al.*, 2001, Stacey *et al.*, 2003, Sorensen *et al.*, 2004). Cultured Senegalese sole had lower values of ARA and higher EPA/ARA ratio in their tissues compared with wild fish, and the actions of PGs is determined by the ratio of EPA/ARA in cellular membranes and tissues (Bell *et al.*, 1995, Tocher *et al.*, 2008, Norambuena *et al.*, 2009) (chapter II). This apparent imbalance of EPA and ARA in cultured fish may be interfering in regulation of COX-2 transcripts and the production of 2-series PGs.

In order to elucidate if there is an alteration in the pathway of PG production, we present a study of COX-2 gene expression and prostaglandin (PGE2, PGF2α, PGE3 and PGF3α) production in wild and G1 broodstock fish of Senegalese sole. The present study aimed to measure COX-2 gene expression in Senegalese sole, for the first time, and describe the distribution of COX-2 gene expression in different Senegalese sole tissues. The second aim was to study prostaglandin 2-series production produced from ARA and involved in fish

reproduction (PGE2 and PGF2α) and the 3-series metabolites produced from EPA (PGE3 and PGF3α) in wild and G1 broodstock of Senegalese sole that were sub-samples of the same fish that had been determined previously to have significantly different levels of ARA, EPA and ratios of EPA/ARA (Norambuena *et al.*, 2011b) (chapter II) .

2. Material and methods

Sixteen wild fish (8 males and 8 females), captured in the Ebro estuary (between 40º 37' and 40º 48' N and 0º 21' and 0º 40' E., Tarragona, Spain), and sixteen cultured fish (9 years old, 8 males and 8 females) reared at IRTA were sacrificed. Wild fish weighed 380 ± 40g (mean ± SEM) and measured 30 ± 5.4 cm length whereas cultured fish were 442 ± 43g and 31 ± 6.3 mm, respectively. Cultured fish were fed every day with commercial broodstock diets (Skretting, Vitalis Repro® and Elite®), with a daily food supply of 0.5 -0.8% body weight (BW). Wild fish were captured, anesthetised, weighed and sampled. Wild and cultured fish were euthanized with an overdose of 2-phenoxyethanol (0.3 ml l^{-1}) (Norambuena *et al.*, 2011a), followed by pithing of the spinal cord during the spawning season of 2008 (May). Blood was taken from the caudal vein using heparinized syringes and placed in ice-cold heparinized tubes. Plasma was obtained by centrifugation (3000 g, 15 min., 4 ºC) and stored at -20 ºC for pheromone analysis. Gonads, gill, and head kidney were dissected, weighed (approximately 2 g) and homogenized in 4 ml Hank`s balanced salt solution containing 0.6 ml of absolute ethanol and 0.2 ml of 2M formic acid and the homogenate frozen at -20 ºC. Protein content of gonads, gill and kidney was analysed (Lowry *et al.*, 1951), with prior overnight hydrolysis in sodium-hydroxide solution. For study of COX gene expression, ten wild and ten cultured fish were sampled (5 males and 5 females), and liver, brain, kidney, gill, oviduct, sperm duct (s-duct), testis, ovary, spleen, and white muscle tissue were collected, weighed frozen immediately in liquid nitrogen, and subsequently stored at −70°C pending RNA extraction. The 16 wild and 16 cultured fish used in this study were also analysed to determine the proximate composition, fatty acid profile and stage of maturity and this has been showed previously (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011b) (chapter II). From this work the wild fish used in this study had significantly (P<0.05) higher levels of ARA in testis, liver and muscle, while EPA/ARA ratios were significantly higher in G1 (Table 1) and all the

fish used were in advanced stages of maturation, females with vitellogenic oocytes without ovulation and males with spermatozoids in seminiferous tubules.

Table 1 *Total lipid (TL), fatty acid content (TFA), arachidonic acid (20:4n-6, ARA), eicosapentanoic acid (20:5n-3, EPA) content (% total fatty acids) and ARA/EPA ratio in the tissues muscle, gonad and liver from wild males, wild females, cultured males and cultured females (N=8 per group) of Senegalese sole (Solea senegalensis) that were used in the present study of prostaglandin levels and COX-2 expression. Columns assigned different letters were significantly different (ANOVA, P<0.05)*

	Female		Male	
	Wild	Cultured	Wild	Cultured
Liver				
TL (mg g^{-1} DW)	181.1 ± 10.2 ^{b}	564.4 ± 43.4 ^{a}	224.6 ± 11.2 ^{b}	578.0 ± 14.1 ^{a}
TFA(µg mg^{-1} L)	528.7 ± 20.7 ^{a}	698.8 ± 49.5 ^{a}	497.0 ± 27.4 ^{a}	663.5 ± 10.6 ^{a}
20:4n-6, ARA	2.66 ± 0.26 ^{b}	1.21 ± 0.06 ^{c}	3.52 ± 0.31 ^{a}	0.72 ± 0.07 ^{c}
20:5n-3, EPA	3.56 ± 0.34 ^{b}	6.20 ± 0.31 ^{a}	2.81 ± 0.23 ^{b}	3.70 ± 0.67 ^{b}
EPA/ARA	1.42 ± 0.15 ^{b}	5.19 ± 0.27 ^{a}	0.86 ± 0.11 ^{b}	4.37 ± 0.90 ^{a}
Gonads				
TL (mg g^{-1} DW)	143.3 ± 8.96 ^{a}	200.7 ± 12.2 ^{b}	169.0 ± 7.2 ^{ab}	194.8 ± 17.6 ^{ab}
TFA(µg mg^{-1} L)	496.9 ± 21.1 ^{a}	522.3 ± 14.2 ^{a}	389.5 ± 24.1 ^{a}	373.5 ± 10.9 ^{a}
20:4n-6, ARA	2.47 ± 0.13 ^{c}	2.02 ± 0.09 ^{c}	7.59 ± 0.41 ^{a}	4.96 ± 0.29 ^{b}
20:5n-3, EPA	6.87 ± 0.33 ^{a}	6.39 ± 0.18 ^{a}	7.29 ± 0.34 ^{a}	6.57 ± 0.12 ^{a}
EPA/ARA	2.83 ± 0.17 ^{a}	3.21 ± 0.14 ^{a}	1.03 ± 0.06 ^{b}	1.36 ± 0.10 ^{b}
Muscle				
TL (mg g^{-1} DW)	33.3 ± 3.0 ^{a}	39.5 ± 3.9 ^{a}	33.2 ± 1.8 ^{a}	56.4 ± 7.5 ^{a}
TFA(µg mg^{-1} L)	566.1 ± 29.8 ^{a}	592.5 ± 53.7 ^{a}	555.3 ± 22.6 ^{a}	650.8 ± 38.7 ^{a}
20:4n-6, ARA	4.24 ± 0.16 ^{a}	2.19 ± 0.15 ^{b}	5.03 ± 0.35 ^{a}	1.82 ± 0.17 ^{b}
20:5n-3, EPA	6.31 ± 0.27 ^{b}	7.09 ± 0.29 ^{a}	6.51 ± 0.21 ^{b}	4.78 ± 0.41 ^{b}
EPA/ARA	1.44 ± 0.02 ^{b}	3.41 ± 0.27 ^{a}	1.37 ± 0.11 ^{b}	3.06 ± 0.34 ^{a}

DW= dry matter, L=lipids.

2.1. RNA extraction and cDNA synthesis

Samples stored at -80ºC were melted on ice, and 100 mg of each tissue were weighed and rapidly disrupted in 1ml of Tri-reagent (Sigma-Aldrich) using an Ultra-Turrax homogenizer (Fisher Scientific, Loughborough, UK). Total RNA was extracted from the homogenate following the Tri-reagent manufacturer's instructions (GibcoBRL, Grand Island, NY), and

the pellet was dissolved in DEPC water. RNA quantification was carried out with nanodrop1000 (Thermo scientific) and RNA integrity was checked with an experion bioanalyzer (Agilent technologies). Samples were pooled depending on tissue, sex and wild/reared condition, each sample contributing the same amount of RNA, only five samples of eight from each group with RIN over eight were chosen for pooling. A sample of 3µg of total RNA from the different pools were used for cDNA synthesis with Superscript III™ transcriptase (Invitrogen™) and oligo-dT_{15} primer (Promega™), obtaining a final volume of 20µl of cDNA for each tissue and fish group (wild and cultured males and females).

2.2. Primer design

Specific primers for COX-2 were designed. Four different fish species phylogenetically close to *Solea senegalensis* superorder acanthopterygii (Nelson, 1984): *Oplegnathus fasciatus, Myoxocephalus octodecemspinosus, Micropogonias undulatus, Gasterosteus aculeatus* were used to carry out the alignment. Most conserved regions were chosen for primer design. Two different pairs of specific primers for COX-2 transcript were designed. For semi quantitative RT-PCR analysis a 644 base pairs primers was used and for quantitative real-time**,** qrtPCR a primer with 135 bases. Those were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and OlygoAnalyzer (IDT SciTools) for deep primer analysis. Amplicons were purified and cloned into a P-GEM easy vector (Promega) and transformed with JM109 competent cells (Promega) for sequencing and identity confirmation.

2.3. Polymerase chain reaction (RT-PCR) and real time PCR (qrtPCR)

To develop COX-2 expression pattern in sole tissues, cDNA of pooled samples were used as a template for RT-PCR analysis, using a G-storm thermocycler. Amplification of a specific COX-2 644 base pair fragments were carried out by one step of 95ºC for 5 min., 30 cycles of three steps, 94ºC 45 sec., 64ºC for 45 sec and 72ºC for one min, followed by a final step of 72ºC for seven min. The specific primers used were 5'-GTGCTGACATCTCGATCCCAC-3' forward primer, and 5'-AGCCAGATGGTGGCGTACATCATCAG-3' reverse primer. As a control, 18S ribosomal

RNA (18S rRNA) with 148 base pair fragment was amplified from the same cDNA samples (forwards 5'- GAATTGACGGAAGGGCACCACCAG and reverse 5'- ACTAAGAACGGCCATGCACCACCAC) (GenBank ID: AM882675), the same housekeeping gene was used for qrtPCR normalization (Infante *et al.*, 2008). Products were separated into a 1.5% agarose gel electrophoresis containing GelGreen^{TM} (Biotium) and visualized under a UV light in AlphaImager 2200 (Alpha Innotech).

For quantitative gene expression analysis, a specific COX-2 pair of primers was used, which amplified a shorter fragment of 135 base pairs. Forward primer was 5'- CGACCTGGAGAGGCAACACA-3' and, 5'- TCGGGGACATGAGGAGGGTA-3' for reverse. Three females and 3 males fish cDNA samples were used for qrtPCR. cDNA was diluted 1:100 for COX-2 and 1:1000 for reference gene (18S rRNA) and used as a template for the reaction. Reactions were performed using a MyiQ instrument (BioRad) following the protocol: 5 min initial denaturation at 95ºC, followed by 40 cycles of 10 s at 95ºC and 30 s at 64ºC for COX-2 and 18SrRNA. After the amplification phase, a final melting curve of 81 cycles was performed with a melt curve of 0.5°C increments from 55°C to 90°C, enabling confirmation of the amplification of a single product in each reaction. Non-template controls and RNA controls were added in the analysis and all samples were run in triplicate, fluorescence was measured at the end of every extension step threshold cycle (Ct) and quantification was done according to the Pfaffl method corrected for efficiency for each primer set (Pfaffl, 2001).

2.4. Extraction, separation and enzyme immunoassay of PG, E and F isomers

The frozen tissue homogenates for prostaglandin analysis were thawed and centrifuged at 3000 g for five min to precipitate cell debris. The supernatant was extracted using octadecylsilyl (C18) 'Sep-Pak' minicolumns (Milipore, UK) by the method of Powell (Powell, 1982) as previously described (Bell *et al.*, 1995). The final extract was redissolved in 200 µl of methanol prior to high-performance liquid chromatography (HPLC). The isomers of PGE and PGF (2 and 3) were separated by reverse-phase HPLC (Bell *et al.*, 1995). An isocratic solvent system was employed containing 17 mM phosphoric acid/acetonitrile (70/30 v/v) at a flow rate of one ml/min. Before collecting the PG fractions the elution time of the two PGE standards (2- and 3-series) was determined by UV

detection at 205 nm using a ThermoFisher Spectrasystem UV 3000 detector (ThermoFisher, Hemel Hempstead, UK), (10 to 16 min for fraction 3 and 16 to 22 min for fraction 2). A sample of 100 µl of eicosanoids extract was injected into the column (25 cm x 4.6 mm, ODS-SB5-21987) and fractions of 6 ml were collected using a Waters Fraction collector. The fractions 2 and 3 were extracted separately as follows: The fraction was applied to a C18 'Sep-Pak' previously washed with 5 ml methanol and 10 ml distilled water and the PG fraction eluted in 5 ml ethyl acetate. Samples were dried under nitrogen and redissolved in immunoassay buffer. Measurement of PGs, was performed using two enzyme immunoassay (EIA) kits, PGs of series E2 and E3 with a PGE2 kit and PGs, of series F2α, and F3α with a PGF2α kit in accordance to the protocols of the manufacturer (Cayman®, USA).

2.5. Statistical analysis

Statistical differences in the expression of the transcript COX-2 in different tissues (qrtPCR) and in reproductive prostaglandins between wild and cultured groups of males and females were analysed by two-way ANOVA followed by the post-hoc multiple comparison by Tukey's HSD for equal N test with a significance level (*P*) of 0.05. Moreover, correlations among ARA, COX-2 and PGs were made, using *P* of 0.05. The compliance of data with normality and homogeneity of variance was tested by the Kolmogorov–Smirnov and Bartlett (Chi-Sqr) methods and, when necessary, log-transformation was carried out. In homogenate tissues (gills, gonads and kidney), the prostaglandins were expressed in pg μg^{-1} protein and in blood in pg ml^{-1} of plasma as mean of percentage ± standard error of mean (SEM). Statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA).

3. Results

3.1. Semi-quantitative expression of COX-2 (RT-PCR) in Senegalese sole

The expression of the transcript COX-2 in different tissues was normalized by the expression of the 18S ribosomal RNA gene (Fig. 1). The RT-PCR tissue distribution showed that the most robust COX-2 signal in females was detected in the oviduct (3.9-fold), gills (3.9-fold), liver (1.6-fold), brain (1.2-fold) and lower in spleen (0.8 fold), muscle

and kidney (0.1-fold) (Fig 1 a). In the males the COX-2 transcripts were expressed more in gills (5.8-fold), testis (4.5-fold), liver (2.3-fold), spleen (1.8-fold), kidney (1.7-fold) and s-duct (1.4-fold) and lower in brain (0.3-fold) and muscle (0.02-fold) (Fig. 1 b).

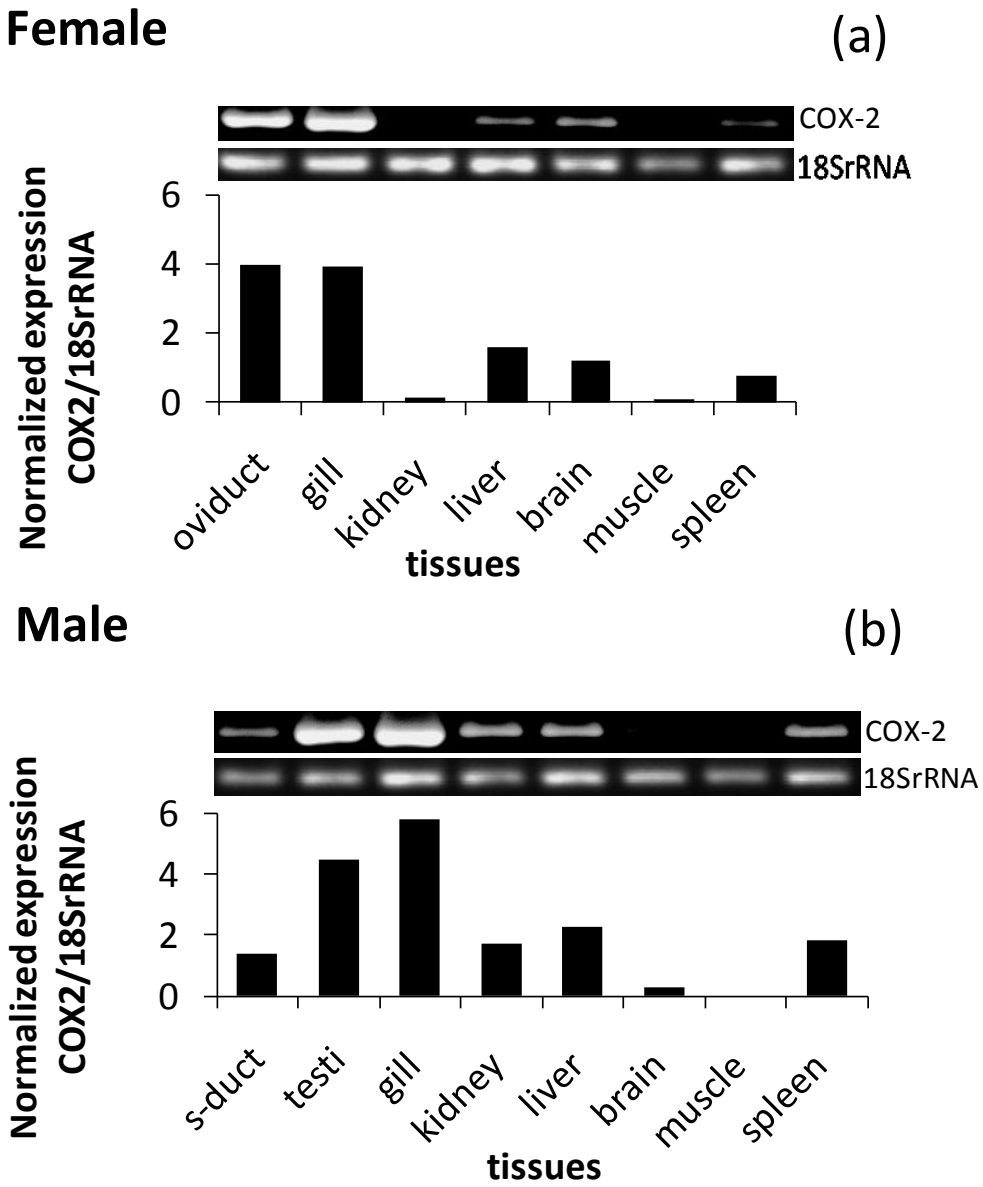

Fig. 1 *Results of semi-quantitative PCR in different tissues from wild fish (5 fish per pool). PCRs with primer pairs for COX-2, 644 base pair fragment was amplified in decreasing order from oviduct gill, kidney, liver, brain, muscle, and spleen in a) wild female, and sperm duct (s-duct), testis, gill, kidney, liver, brain, muscle and spleen in b) wild male. 18SrRNA was amplified approximately equally from the cDNA of all tissues. PCR performance was visualized and semi quantified the normalized expression (18S rRNA) with the software Quantity One®.*

3.2. Transcript (COX-2) expression ratio determined by quantitative real-time PCR (qrtPCR)

Differences in transcripts expression (qrtPCR) were found in response to the origin of the fish, wild and cultured (P<0.05). The expression of COX-2 was significantly down regulated in gill and s-duct of cultured males and oviduct of cultured females (P<0.05), while up-regulation of COX-2 was observed in gills of cultured females (P<0.05), no significant differences in expression were found in testis, liver and kidney (Fig. 2). Similar differences in COX-2 gene expression between tissues were observed with the two methods RT-PCR and the quantitative real time q-PCR assay. The q-PCR assay showed that COX-2 transcripts were expressed significantly higher in gills, s-duct, testis and oviduct (from cultured fish) compared to oviduct (from wild fish), liver and kidney (P<0.05) (Fig. 2).

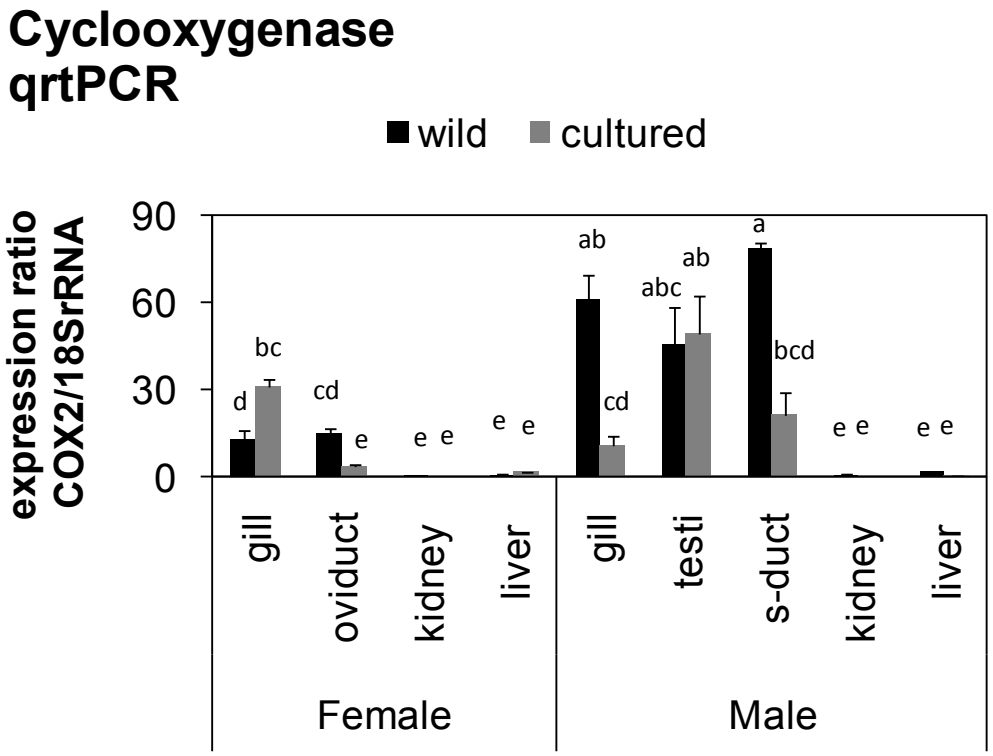

Fig. 2 *Tissue distribution of COX-2 in Senegalese sole, wild (black bars) and cultured (grey bars) females and males. Transcript (mRNA) expression ratio was determined by quantitative real-time PCR (qrtPCR) as described in the materials and methods section. Results are expressed as expression of COX-2 standardized by 18S rRNA, means + SEM (N= 5). Significant differences between tissues were determinate by one-way ANOVA and are indicated by different superscript letters within individual bars (P<0.05).*

3.3. Prostaglandins

Total PGs were significantly different between tissues from males and females and between tissues from wild and cultured fish (Fig. 3). Levels in the testis were significantly higher (P<0.05) compared to the ovaries for both wild and cultured fish. Total PGs were significantly higher (P<0.05) in testis from wild fish compared to all other tissues including testis from cultured fish. In females total PGs in gills from cultured fish were significantly higher than in other female tissues (P<0.05) (Fig. 3).

All significant differences (P<0.05) observed in the levels of 2 series PGs within a tissue type exhibited significantly higher (P<0.05) levels in wild fish compared to cultured fish (Table 2). The following were significantly the highest (P<0.05) within a tissue type: PGE2 in wild testis, PGE2 in wild female blood, PGF2α in wild female blood, PGF2α in wild male gills and PGF2α in wild testis (Table 2). Levels of PGF2α were significantly higher (P<0.05) in testis than ovaries such that wild testis > cultured testis > ovaries. Within a tissue type all other comparisons were not significantly different and no tissue from a cultured fish exhibited significantly higher levels of 2 series PGs compared to the same tissue from a wild fish.

The opposite was observed with 3 series PGs and all significant differences (P<0.05) observed in levels of 3 series PGs within a tissue type exhibited significantly higher (P<0.05) levels in cultured fish compared to wild fish (Table 2). The following were significantly the highest (P<0.05) within a tissue type: PGE3 in cultured male gills, PGE3 in cultured fish testis, PGE3 in cultured male kidneys, PGF3α in cultured male and female gills, PGF3α in wild and cultured fish testis, PGF3α in cultured male kidneys and PGF3α in cultured female and male blood (Table 2). Levels of PGE3 and PGF3α were significantly higher (P<0.05) in testis than ovaries such that for PGF3 levels in cultured fish testis > wild fish testis > ovaries. Within a tissue type all other comparisons were not significantly different and no tissue from a wild fish exhibited higher levels of 3 series PGs compared to the same tissue from a cultured fish. The ratio between F prostaglandins (PGF3α/PGF2α) in blood and tissues was always higher in cultured females and males than in their wild counterparts (P<0.5) (table 3). The ratio PGE3/PGE2 in tissue homogenates (gills, gonads and kidney) and blood were always higher in cultured fish.

Generally, COX2 expression and levels of total PGs had similar patterns across the different tissues and tissues that exhibited a high expression of COX2 also had high levels of total PGs and conversely tissues with low levels of COX2 expression had low levels of total PGs. The variation in the two variables, COX2 expression and levels of total PGs, was described with an exponential regression equation $y = 12.7e^{0.04x}$ and $r^2 = 0.53$.

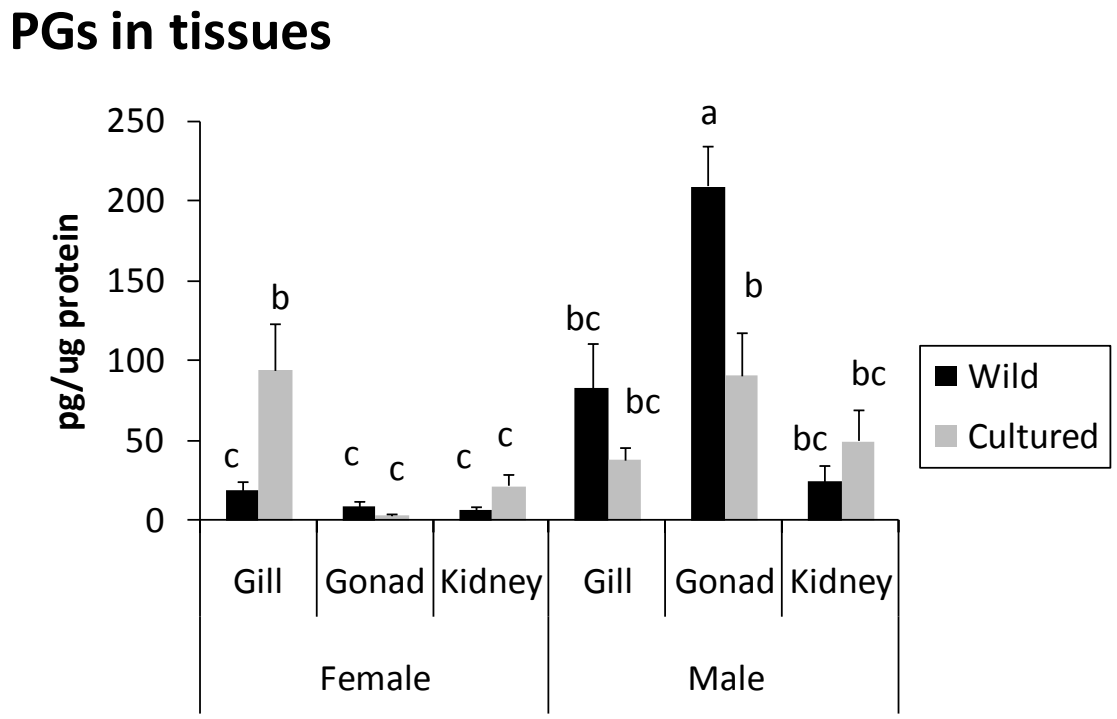

Fig. 3 *Total prostaglandin (PGE2 + PGF2α + PGE3 + PGF3α) in the homogenates of gills, gonads and kidney of wild and cultured females and males. Values are mean + SEM of eight samples and are expressed as pg/µg protein, significant differences between tissues were established by one-way ANOVA and are indicated by different superscript letters within individual bars (P<0.05).*

Table 2 *Concentration of prostaglandins E and F of the 2- and 3- series (PGE2, PGF2α, PGE3 and PGF3α) in the homogenates of gill, gonad, kidney and blood of wild and cultured females and males of Senegalese sole. Values are mean ± SEM of eight samples, and were expressed as pg/μg protein in the homogenates and as pg/ml plasma in the blood. Significant differences were established by one-way ANOVA and are indicated by different superscript letters within individual rows (P<0.05)*

		Female				Male			
	Tissues	Wild		Cultured		Wild		Cultured	
	Gills	0.31 ± 0.05	a	2.16 ± 0.54	a	2.10 ± 0.59	a	0.94 ± 0.27	a
	Gonads	0.13 ± 0.03	a	0.10 ± 0.02	a	6.47 ± 0.75	b	1.17 ± 0.19	a
	Kidney	0.28 ± 0.13	a	0.33 ± 0.05	a	0.95 ± 0.31	a	1.13 ± 0.39	a
PGE2	Blood	833 ± 205	a	217 ± 49	b	1074 ± 151	a	565 ± 69	ab
	Gills	2.35 ± 0.38	a	30.53 ± 8.5	a	50.11 ± 9.88	b	8.82 ± 2.10	a
	Gonads	2.26 ± 0.46	a	1.84 ± 0.39	a	162.3 ± 15.2	b	71.98 ± 11.4	c
	Kidney	2.73 ± 1.09	a	3.66 ± 0.93	a	6.32 ± 1.75	a	9.51 ± 3.70	a
PGF2α	Blood	5793 ± 1492	a	1534 ± 297	b	6383 ± 1294	a	4291 ± 461	ab
	Gills	0.78 ± 0.17	a	2.87 ± 0.43	ab	1.91 ± 0.38	a	4.43 ± 1.07	b
	Gonads	0.36 ± 0.08	a	0.22 ± 0.03	a	8.61 ± 0.71	b	16.08 ± 3.81	c
	Kidney	0.37 ± 0.11	a	1.78 ± 0.51	a	0.67 ± 0.24	a	5.29 ± 1.00	b
PGE3	Blood	2171 ± 322	a	1077 ± 328	a	2003 ± 317	a	1652 ± 272	a
	Gills	15.68 ± 3.49	a	57.23 ± 11.4	b	29.52 ± 6.09	a	65.72 ± 14.8	b
	Gonads	2.51 ± 0.47	a	1.75 ± 0.26	a	55.84 ± 3.23	b	78.04 ± 13.6	b
	Kidney	4.08 ± 1.02	a	24.85 ± 5.67	a	22.05 ± 6.35	a	55.65 ± 13.2	b
PGF3α	Blood	20677 ± 4891	a	32223 ± 4577	b	28910 ± 2223	ab	39483 ± 4244	b

Table 3 *Ratios of concentration of prostaglandins E (PGE3/PGE2) and F (PGF3α /PGF2α) in the homogenates of the gill, gonad, kidney and blood of wild and cultured females and males of Senegalese sole. Values are mean ± SEM of eight samples (ratios). Significant differences were established by one-way ANOVA and are indicated by different superscript letters within individual rows (P<0.05)*

		Female		Male	
	Tissues	Wild	Cultured	Wild	Cultured
	Gills	1.65 ± 0.27 ^{ab}	2.22 ± 0.31 ^{ab}	1.10 ± 0.15 ^{a}	3.16 ± 1.09 ^{b}
	Gonads	2.33 ± 0.35 ^{a}	3.56 ± 0.67 ^{a}	1.91 ± 0.23 ^{a}	2.99 ± 0.54 ^{a}
	Kidney	1.33 ± 0.24 ^{a}	4.33 ± 1.49 ^{b}	1.33 ± 0.22 ^{a}	2.34 ± 0.36 ^{ab}
PGE3/PGE2	Blood	1.71 ± 0.51 ^{a}	3.49 ± 0.51 ^{b}	2.54 ± 0.44 ^{ab}	3.04 ± 0.36 ^{b}
	Gills	3.57 ± 0.68 ^{a}	4.26 ± 0.93 ^{a}	1.34 ± 0.16 ^{b}	4.93 ± 1.30 ^{a}
	Gonads	1.08 ± 0.12 ^{a}	1.43 ± 0.13 ^{a}	0.40 ± 0.53 ^{b}	1.00 ± 0.22 ^{a}
	Kidney	1.55 ± 0.23 ^{a}	5.99 ± 1.81 ^{b}	2.56 ± 0.54 ^{ab}	4.45 ± 1.01 ^{ab}
PGF3/PGF2	Blood	4.24 ± 0.80 ^{a}	10.3 ± 1.73 ^{b}	6.46 ± 0.82 ^{ab}	8.06 ± 1.11 ^{b}

4. Discussion

This study has for the first time described COX-2 mRNA expression in gills, s-duct, testis, oviduct, spleen, liver, kidney and muscle in Senegalese sole. Transcripts of COX-2 in both wild and cultured broodstock of Senegalese sole were highly expressed in gills, s-duct, testis, oviduct and spleen compared to liver, kidney and muscle. The expression of COX-2 was significantly down regulated in male gills, sperm duct and oviduct from cultured fish compared to wild, while up-regulation of COX-2 was observed in the gills of cultured females, with a high PGF-isomer production. Similarly, significantly higher levels of total PGs were observed in testis of wild fish compared to all other tissues and gills of cultured female fish compared to all other tissues from female fish. The pattern in COX 2 expression was similar to the pattern in levels of total PGs and an exponential regression (r^2= 0.53) partially described how as COX 2 expression increased while levels of total PGs also increased.

Previous studies of expression in fish tissues of COX-2 showed that transcripts were most abundant in the gills, opercular epithelium and kidney and much less in liver, ovary, heart, brain, intestine and stomach (Choe *et al.*, 2006, Havird *et al.*, 2008, Fujimori *et al.*, 2010). COX-2 plays several roles in chordates and because of this the expression could vary

according to the different fish species, being mainly related with inflammation and prostaglandin production. It has been shown that COX-2 is induced during inflammation and steroid hormone production concomitant with a higher expression in brain, kidney and reproductive organs (Balaji *et al.*, 2007, Sirois *et al.*, 2004). The present study exhibited similar patterns with high COX-2 expression in testis, s-duct and gills. However a down regulation of COX-2 in reproductive organs (oviduct and s-duct) of cultured fish was observed, possibly leading to reduced production in total PGs in these tissues, although not evaluated in the present study. COX has been shown to play a role in reproduction in fish and by using the non-specific COX inhibitor indomethacin, it was shown in the Atlantic croaker (*Micropogonias undulatus*) and European seabass (*Dicentrarchus labrax*) that the COX pathway plays a role in the maturation of ovarian follicles and ovulation through prostaglandin formation (Sorbera *et al.*, 2001, Patiño *et al.*, 2003). In the Japanese medaka, it was shown that low chronic levels, comparable to those found in waste water (Metcalfe *et al.*, 2003), of the non-specific COX inhibitor ibuprofen had effects on reproduction causing a decrease in the number of spawning events, but increased the number of eggs per spawning event. Therefore, the down-regulation of COX-2 transcripts in the reproductive organs of Senegalese sole G1 fish might indicate a reproductive problem in cultured fish since this enzyme and its metabolites are considered relevant in ovulation and spermiation of fish (Stacey *et al.*, 1982, Sorensen *et al.*, 1988, Van Der Kraak *et al.*, 1990, Zhang *et al.*, 1992, Wade *et al.*, 1993, Kobayashi *et al.*, 2002, Fujimori *et al.*, 2010). However, further studies are required to elucidate the reproductive effect that might have influenced the down regulation of COX-2 in G1 fish.

A study on the expression of COX-2 in the gills of killifish (*Fundulus heteroclitus*) (Choe *et al.*, 2006) concluded that COX-2 increased following salinity transfers of the fish, and increased PG production which plays an important role in ion transport. Moreover a elevation of PGF in isolated gill cells following seawater transfer (*Salmo salar*) smolt, was demonstrated (Tocher *et al.*, 2000), suggesting that PGs are involved in fish osmoregulation. This might be an explanation for the high regulation (compared to kidney and liver) of COX-2 and PGs in Senegalese sole gills observed in the present study. The fish were sampled in spring with increasing water temperatures and slightly higher salinity levels (Mariotti *et al.*, 2002) that might have induced a response in the gills. Other

possibilities in the up regulation of COX-2 of wild males might be the migration in spring from (estuaries) with low salinity to high salinity areas (deep water) with lower water temperatures to where this species is normally found. The effect of salinity in the growth of *Solea solea* was studied (Fonds, 1979), without detecting any difference when the fish were reared at salinities between 10 g L^{-1} and 40 g L^{-1}. These might suggest that changes in COX expression and PG production in gills can be due to differences in the environmental conditions between wild and cultured fish.

In the present study the isomers of PGF and PGE, 2- and 3-series produced by ARA and EPA (Tocher, 2003), were also measured. The levels of 2-series PGs (PGF2α and PGE2) in blood and tissue homogenates of wild fish (particularly in testis and gills) were always the same or significantly higher compared with cultured counterparts. Conversely, cultured fish showed the same or significantly higher values in 3-series PGs (PGF3α and PGE3), with these differences being largest in gills and testis of males and in the blood of females. Thus, the ratios PGF3α/PGF2α and PGE3/PGE2 were always higher in cultured fish and in most tissues significantly higher. Previous studies have demonstrated that the production of 2- and 3-series PGs in fish tissues is proportional to the EPA/ARA ratio found in the tissues (Bell *et al.*, 1994) and fish with a low EPA/ARA ratio produced more 2- series PGs, while fish with a higher EPA/ARA ratio produced more 3-series PGs. In the present study the wild fish had low EPA/ARA ratios (high ARA concentration) and the cultured fish had high EPA/ARA ratios (low ARA concentration). Recent comprehensive studies on the fatty acid profile of Senegalese sole have shown that the EPA/ARA ratio was significantly increased in cultured compared to wild fish (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011b, Norambuena *et al.*, 2011c) (chapter II) since the levels of ARA were higher in the tissues of wild than in cultured Senegalese sole. Furthermore the high EPA/ARA ratio and low levels of ARA in cultured fish appeared to be a reflection of the diet of the cultured fish (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011b, Norambuena *et al.*, 2011c) (chapter II). Thus, it is not surprising that in the results of the present study the blood and tissues of wild fish showed higher concentration of PGF2α and PGE2 whereas in cultured fish blood and tissues, with less ARA content, PGF2α and PGE2 were less concentrated than the 3-series. It has been explained that 3-series PGs, produced by EPA, are biologically less active than the

corresponding 2-series produced from ARA (Tocher, 2003), and was shown that PG3-series produce no effect on oocyte maturation (Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). Previous studies in fish reproduction behaviour have demonstrated that PGE2 and PGF2α are important during pre- and postovulatory stages, with PGE2 being important in the steroidogenesis, induction of follicle maturation and ovulation and stimulation of testosterone production in testis and ovaries (Stacey *et al.*, 1982, Sorensen *et al.*, 1988, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Zhang *et al.*, 1992, Espey *et al.*, 2006, Fujimori *et al.*, 2010). Whilst, PGF2α was involved in the synchronization of courtship between male and female fish during spawning (Sorensen *et al.*, 1993, Kobayashi *et al.*, 2002, Stacey *et al.*, 2003, Sorensen *et al.*, 2004). Therefore, the reduction of 2-series PGs in G1 fish blood and tissues might be triggering a problem in the final maturation, ovulation and spawning synchronization of G1 Senegalese sole and a reproductive failure/dysfunction, especially in male.

The present result showed that cultured female gill tissue homogenates contained significantly higher levels of 2-series PGs than wild fish (12.9- fold up). Previously was explained that PGs are released across the gills or in controlled urinary pulses (Kobayashi *et al.*, 2002), then males are attracted to the odour of females in vitellogenic phase, associated with a circulating PGs just before ovulation, which enhances male fertilization (Kobayashi *et al.*, 2002). The high values of 2-series PG in gills of G1 female fish might indicate that females were sending cues about their reproductive stage through the gills to the males. However, males were producing only low concentrations of the corresponding 2-series PGs. Previous studies showed the absence of reproductive behaviour in G1 Senegalese sole (Carazo *et al.*, 2008, Carazo *et al.*, 2011a), indicating that these chemical cues released by the females into the water, might not be received by the males and/or were so intense, saturating the water, that they prevented the communication and synchronization between G1 fish. However, these might be investigated further, focusing on breeding behaviour and/or female and male fish communication.

5. Conclusion

This study showed that COX-2 in wild and cultured Senegalese sole was highly expressed in gills, oviduct and testis. Moreover it was demonstrated that there was a COX-2 down-regulation and low production of PG 2-series in some of the tissues of cultured fish, which might have important consequences in the reproductive dysfunction observed in G1 Senegalese sole. The present study supports the competitive nature of EPA and ARA in the production of respective 3-series and 2-series PGs and that changes in ARA and EPA content in the tissues of cultured Senegalese sole previously shown (Norambuena *et al.*, 2011b) (chapter II), may have important effects on reproductive physiology, COX-2 expression and reproductive prostaglandin production.

6. Acknowledgments

We gratefully thank to Simon Mackenzie, Gordon Bell, Agnès Callol and Alicia Estévez for the scientific help. Thanks are also given to Karl Andree for the English help and James R. Dick and Fiona Strachan for technical support. This research was funded by two projects: 1) Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project and 2) Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by N. Duncan and the PhD grant awarded to F. Norambuena by Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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Chapter IV

ARA in blood and *S. Sole* physiology

EFFECTS OF GRADED LEVELS OF ARACHIDONIC ACID ON THE REPRODUCTIVE PHYSIOLOGY OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*): FATTY ACID COMPOSITION AND PROSTAGLANDIN AND STEROID LEVELS IN THE BLOOD OF G1 FISH

Abstract

Previous studies on Senegalese sole indicate that cultured broodstock fish (G1) have less arachidonic acid (20:4n-6, ARA) accumulation in their tissues than wild counterparts. ARA is metabolized to form prostaglandins (PGs) that are involved in steroid production and follicle maturation in fish, whereas ARA might affect the transfer of CHOL through the mitochondrial membrane to initiate steroidogenesis. In the present study the effects of different dietary levels of ARA on blood lipid and fatty acid composition, prostaglandin (PGF2α, PGF3α, PGE2 and PGE3) levels and plasmatic steroid levels (11-ketotestosterone, 11-KT, testosterone, T and estradiol, E2) in G1 Senegalese sole was studied. For this purpose, six groups of fish were fed six dietary ARA levels during nine months (diets A=0.7, B=1.6, C=2.3, D=3.2, E=5.0, F=6.0% ARA). The ARA and CHOL relative content in blood showed a significant increase in an ARA dose related manner (P<0.05) whereas EPA and EPA/ARA ratio were reduced. In males, the steroid (11-KT and T) levels increased significantly with increasing dietary ARA in a dose dependent response, whereas in females estradiol (E2) did not show any change related to dietary ARA content. Furthermore, plasma concentration of PGE3 and PGF3α were reduced in parallel to increased blood ARA levels (P<0.05). However, prostaglandins 3-series (PGE3 and PGF3α) was always higher than 2-series prostaglandins (PGE2 and PGF2α) (males and females). Further studies are required to understand the interactions among ARA ingestion, PGs and CHOL production and the endocrine control of reproduction in Senegalese sole.

1. Introduction

The spawning performance of cultured Senegalese sole broodstock (G1 generation) was poor compared to wild counterparts. Cultured females released eggs, but no courtship between males and females was observed and eggs that were collected were not fertilised (Carazo *et al.*, 2011). Fatty acids and in particular polyunsaturated fatty acids including

ARA and products formed from ARA affect the reproduction by modulating various aspects of endocrine function (Sorbera *et al.*, 2001, Silversand *et al.*, 1996, Sorbera *et al.*, 1998, Izquierdo *et al.*, 2001, Mazorra *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Furuita *et al.*, 2007, Huynh *et al.*, 2007, Pickova *et al.*, 2007). The metabolites of ARA act as mediators or regulators in the cardiovascular, immune and nervous system (Smith *et al.*, 2001) and particularly the reproductive system (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a, Wade *et al.*, 1993b, Sorbera *et al.*, 1998, Wang *et al.*, 1999, Smith *et al.*, 2001, Sorbera *et al.*, 2001, Alorend, 2004). Thus, ARA has been shown to stimulate ovarian and testicular steroid production in goldfish and trout, as steroid production was blocked by inhibitors of cyclooxygenase (COX-2) (i.e., indomethacin) but not lipoxygenase, suggesting that ARA exerts its effects by its conversion to prostaglandins (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a, Wade *et al.*, 1994, Mercure *et al.*, 1995, Mercure *et al.*, 1996, Van Der Kraak *et al.*, 1999). Evidence for a role of PG in mediating maturation or ovulation has been generated from studies of several different species of fish including goldfish (*Carassius auratus*)(Stacey *et al.*, 1975), brook trout (*Salvelinus fontinalis*) (Goetz *et al.*, 1983), yellow perch (*Perca flavescens*) (Goetz *et al.*, 1989), European sea bass (*Dicentrarchus labrax*) (Sorbera *et al.*, 2001) and Atlantic croaker (*Micropogonias undulatus*) (Patiño *et al.*, 2003). In vitro studies in teleost fish examining effects of ARA and its COX-2 metabolites on testosterone production have shown that PGE2 is synthesized within the gonads where it stimulates ovarian and testicular steroidogenesis and follicle maturation (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a, Mercure *et al.*, 1995, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). In addition, ARA and its metabolites have been shown to regulate the cholesterol transfer from outer to inner mitochondrial membrane where the P450 enzyme initiates steroid hormone synthesis, using cholesterol as a precursor (Hu *et al.*, 2010), studies in gold fish ovaries showed that steroids ovarian level significantly increases in fish prior to the increase in transcript COX-2 (Lister *et al.*, 2009). However, ARA has differential effects on steroid biosynthesis, as ARA is involved in testosterone production by elevating the cyclic adenosine monophosphate (cAMP) levels in a dose dependent manner, but at high doses ARA might inhibit the biosynthesis of steroid hormones, in spite of elevated cAMP, by affecting the availability of cholesterol (Mercure *et al.*, 1995, Mercure *et al.*, 1996) and the steroidogenic acute regulatory protein expression

(StAR protein) that regulates cholesterol transfer within the mitochondria (Wang *et al.*, 2005, Hu *et al.*, 2010).

Although ARA is the main precursor of PGs, these metabolites can also be produced from eicosapentaenoic acid (20:5 n-3, EPA), which is a substrate for biosynthesis of 3-series PGs (Smith, 1989, Tocher, 2003). Metabolites, produced from EPA, have modest effects on basal testosterone production in the goldfish testis but blocked the ARA-derived steroid production and cAMP formation due to competition for the same enzyme (Wade *et al.*, 1994, Bell *et al.*, 1994b, Mercure *et al.*, 1995). The production of 2- or 3-series PGs is determined by the EPA/ARA ratio in cellular membranes that is dependent on the dietary intake of each fatty acid ARA and/or EPA (Bell *et al.*, 1994a, Bell *et al.*, 1995, Bell *et al.*, 1996, Tocher *et al.*, 2008, Norambuena *et al.*, 2009).

Recent studies on the composition of Senegalese sole broodstock have shown that G1 fish had significant lower accumulation of ARA in their tissues compared to wild counterparts, both in females and males (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011a) (chapter II). Low levels of CHOL were also found in the liver of G1 fish (chapter II), being CHOL the precursor of fat-soluble vitamins and steroid hormones in vertebrates (Baron *et al.*, 1997, Hu *et al.*, 2010) and deficiencies might have a consequence in reproduction. On the other hand, PGs 2-series (F- and E-isomers) were always produced in higher amounts in wild fish compared to cultured G1fish, in both sexes, being PGs 3-series higher in G1fish. Studies in ARA dietary self-selection by G1 Senegalese sole have also shown changes in ARA preferences along the year, with periods of high (winter and summer) and low (spring and autumn) ARA demand (Norambuena *et al.*, 2011b, Norambuena *et al.*, 2011e) (Chapter V). Senegalese sole is a seasonal spawner, with full gonadal development in early spring (April-May), and maximum levels of plasma steroids peaking at the beginning of the spawning season (García-López *et al.*, 2006a, Guzmán *et al.*, 2008, Guzmán *et al.*, 2009). In wild fish peak levels of steroids were always higher than those reported in G1 fish (García-López *et al.*, 2006a, García-López *et al.*, 2006b, García-López *et al.*, 2007). Thus, there is a reproductive dysfunction of Senegalese sole with (1) G1 fish showing a complete absence of reproductive behaviour and synchronization of the spawning, (2) G1 fish having lower ARA and CHOL content in

their tissues, (3) lower production of PGs 2-series compared to wild fish and (4) lower levels of circulating testosterone.

Considering the above statements, a study of the effect of graded levels of dietary ARA on blood lipid and fatty acid composition, prostaglandin and steroid production of G1 Senegalese sole was conducted. For this, six groups of fish were fed six different diets with graded ARA levels for nine months. Total lipid content, fatty acid composition and steroid (11-KT, T and E2) production was analyzed monthly in the blood, and in May, during the spawning season, fish were also sampled to analyze PGs levels (PGF2α, PGF3α, PGE2 and PGE3).

2. Materials and methods

2.1. Fish

One hundred and twenty, 4 year-old (524 ± 11 g) fish reared in captivity were tagged with passive integrated transponders (PIT tags, AVID, UK) and sexed by using a heterologous vitellogenin ELISA for European seabass (*Dicentranchus labrax)* (Mañanós *et al.*, 1994). The fish were distributed among six experimental tanks (10 fish per tank) and fed six experimental diets (54% dietary protein and 12% of total lipids) with different ARA content: diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0, F= 6.0% of total fatty acids (TFA) (Table 1), the six groups were duplicated. The fish were held in a recirculation system with a simulated natural photoperiod and temperature (40º 37' and 40º 48' N and between 0º 21' and 0º 40' E., Tarragona, Spain), with minimal temperature in January – February (13ºC) and maximal in June - September (20°C). The fish were fed six day per week a daily ration of 0.15- 0.3% body weight. The fish received the diets during ten months, from September 2009 until June 2010. Specific growth rate SGR= $100 \times [(\ln wt_2 - \ln wt_1) \times (t_2 - t_1)^{-1}]$) was calculated where wt_2= final weight, wt_1= initial weight, and $(t_2 - t_1)$= days of experiment.

Table 1 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of the diets used (A, B, C, D, E and F) to feed G1 Senegalese sole (Solea senegalensis). Rows assigned different letters were significantly different (ANOVA, P<0.05, N=3)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	121 ± 2.9	122 ± 2.2	130 ± 2.2	128 ± 4.9	124 ± 1.5	120 ± 2.9
TFA(μg mg^{-1} L)	880 ± 87	702 ± 34	820 ± 74	861 ± 82	961 ± 19	911 ± 94
Fatty acid composition (%TFA)						
14:0	2.5 ± 1.2	3.6 ± 2.8	3.8 ± 2.1	3.6 ± 2.3	3.7 ± 2.8	4.0 ± 2.9
16:0	14.9 ± 1.1	15.3 ± 3.7	17.6 ± 2.6	15.8 ± 1.8	15.0 ± 2.8	16.1 ± 2.7
18:0	1.8 ± 0.9	2.4 ± 0.9	2.6 ± 0.7	2.6 ± 0.6	3.0 ± 0.7	3.2 ± 0.4
Total SFA	19.4 ± 1.5	21.7 ± 5.7	24.4 ± 4.2	22.3 ± 3.6	21.9 ± 4.8	23.4 ± 5.2
16:1n-7	4.9 ± 0.8	4.8 ± 2.0	5.0 ± 0.8	4.9 ± 0.8	4.3 ± 1.8	4.4 ± 1.2
18:1n-9	15.0 ± 1.3	15.7 ± 2.4	16.9 ± 1.9	15.9 ± 2.0	15.1 ± 2.7	15.7 ± 0.6
18:1n-7	1.2 ± 2.0	0.9 ± 1.6	1.0 ± 1.7	0.8 ± 1.4	0.8 ± 1.4	1.0 ± 1.7
20:1n-9	7.2 ± 1.5	7.1 ± 0.3	7.2 ± 1.1	6.3 ± 0.5	6.9 ± 0.7	6.6 ± 0.4
22:1n-9	3.5 ± 6.0	3.9 ± 6.7	2.9 ± 5.0	3.0 ± 5.2	3.9 ± 6.7	2.9 ± 5.1
Total MUFA	32.3 ± 11	32.7 ± 4.7	33.4 ± 4.4	31.3 ± 4.9	31.4 ± 5.5	31.1 ± 6.0
18:2n-6	5.9 ± 0.7	6.4 ± 0.7	6.0 ± 0.9	6.6 ± 0.4	5.9 ± 0.1	7.2 ± 0.7
20:4n-6, ARA	0.7 ± 0.3 ^{c}	1.6 ± 0.6 ^{c}	2.3 ± 0.8 ^{bc}	3.2 ± 0.7 ^{b}	5.0 ± 0.6 ^{a}	6.0 ± 0.1 ^{a}
22:4n-6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-6	0.3 ± 0.6	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.2	0.3 ± 0.4
Total n-6 PUFA	9.3 ± 3.4	8.4 ± 1.3	8.9 ± 1.5	10.6 ± 0.8	12.4 ± 1.0	14.0 ± 1.6
18:3n-3	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
18:4n-3	2.2 ± 0.3	2.0 ± 0.1	1.8 ± 0.3	2.0 ± 0.1	1.8 ± 0.2	1.7 ± 0.2
20:4n-3	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.1
20:5n-3, EPA	13.0 ± 8.4	16.8 ± 5.3	15.9 ± 5.4	16.4 ± 5.4	14.8 ± 6.0	14.7 ± 4.9
22:5n-3	1.6 ± 0.4	3.0 ± 2.3	2.0 ± 1.0	2.3 ± 1.3	4.6 ± 5.7	2.0 ± 0.9
22:6n-3, DHA	14.4 ± 2.0	13.0 ± 2.1	11.3 ± 2.5	13.0 ± 1.6	11.1 ± 0.6	11.3 ± 2.3
Total n-3 PUFA	39.0 ± 11	37.1 ± 0.7	33.4 ± 2.9	35.8 ± 2.1	34.3 ± 1.9	31.5 ± 1.4
Total PUFA	48.3 ± 11	45.5 ± 1.4	42.3 ± 2.6	46.4 ± 1.4	46.6 ± 2.0	45.5 ± 1.7
EPA/ARA	23.6 ± 18 ^{a}	12.4 ± 6.6 ^{a}	7.8 ± 4.2 ^{ab}	5.5 ± 2.5 ^{ab}	3.0 ± 1.3 ^{b}	2.4 ± 0.8 ^{b}
EPA/DHA	0.9 ± 0.5	1.3 ± 0.6	1.5 ± 0.8	1.3 ± 0.5	1.3 ± 0.6	1.4 ± 0.7
DHA/ARA	23.5 ± 9.0 ^{a}	8.8 ± 1.6 ^{b}	5.1 ± 1.1 ^{b}	4.1 ± 0.5 ^{b}	2.2 ± 0.3 ^{b}	1.9 ± 0.4 ^{b}
n-3/n-6	4.6 ± 2.3	4.5 ± 0.7	3.8 ± 0.8	3.4 ± 0.4	2.8 ± 0.3	2.3 ± 0.3

TL: total lipids, L: lipids, TFA: total fatty acids, DW: dry weigh, SFA: total saturated fatty acids, MUFA: total monounsaturated fatty acids, PUFA: total polyunsaturated fatty acids, ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, SEM: standard error of the mean.

Table 2 *Lipid class composition (% ± SEM) of the diets used (A, B, C, D, E and F) to feed G1 Senegalese sole (Solea senegalensis). Rows assigned different letters were significantly different (ANOVA, P<0.05, N=3)*

	A	B	C	D	E	F
PC	11.0 ± 0.4	12.2 ± 0.1	11.6 ± 0.3	11.1 ± 0.3	10.2 ± 0.4	10.6 ± 0.0
PS/PI	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PE	3.4 ± 0.1	3.2 ± 0.0	3.3 ± 0.2	3.3 ± 0.2	5.2 ± 0.0	4.2 ± 0.6
Total PL	14.3 ± 0.1	15.4 ± 0.2	14.9 ± 0.1	14.3 ± 0.3	15.4 ± 0.3	14.8 ± 0.6
CHOL	11.9 ± 0.8 [a]	12.3 ± 0.9 [a]	12.5 ± 0.8 [a]	11.1 ± 0.7 [a]	13.3 ± 0.6 [a]	11.2 ± 0.1 [a]
FFA	2.9 ± 0.4	3.8 ± 0.5	3.3 ± 0.6	3.6 ± 0.3	4.3 ± 0.5	4.0 ± 0.1
TAG	61.5 ± 0.3	60.2 ± 0.4	60.8 ± 0.5	62 ± 0.7	59 ± 0.6	60 ± 0.2
SE+W	10.4 ± 0.6	9.32 ± 0.6	9.9 ± 0.6	9.2 ± 0.9	9.8 ± 0.4	9.5 ± 0.4
Total NL	86.7 ± 0.5	85.6 ± 0.2	86.5 ± 0.2	85.7 ± 0.2	86.6 ± 0.2	85.2 ± 0.7

PC: phosphatidylcholine, PS+PI: posphatidylserine plus phosphatidylinositol, PE: phosphatidylethanolamine, PL: polar lipids, CHOL: cholesterol, FFA: free fatty acids, TAG: triacylglycerides, SE+W: sterol ester plus wax and NL: neutral lipids.

2.2. Blood samples

In September 2009 and then on a monthly basis from December 2009 until the end of the experiment in June 2010 a blood sample of 2.0 mL was collected using cold heparinized syringes. The fish were not fed the previous day and blood samples collected from the caudal vein of the fish three hours after the light of the tank was switched on. The erythrocyte volume fraction was calculated and the samples centrifuged at 3000 x g during 15 minutes at 4^{o}C to separate plasma and red cells in eppendorf tubes. From plasma a fraction 150 µL was separated for posterior steroid analysis (from December 2009 until June 2010) and a further 550 µL used for prostaglandins analysis in May 2010. Fatty acid profile was determined from erythrocyte fraction at the beginning of the experiment and in September and December 2009 and March and May 2010. All the samples were stored at -80^{o}C, until analysis.

2.3. Lipids and fatty acids

Total lipids were extracted from red blood samples and diets (Folch *et al.*, 1957) fatty acid methyl esters (FAME) prepared by acid-catalysed transmethylation (Christie, 1982) and FAME were then extracted and purified (Tocher *et al.*, 1988). FAME were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan,

Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using Helium as carrier gas (1.2 mL min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well characterized fish oil, and quantified by the response factor to the internal standard, 21:0. The results were presented as percentage (%) of the total fatty acids (TFA) as mean ± standard error of the mean (SEM).

Lipid class composition in blood and diets was determined by high-performance thin layer chromatography (HPTLC) using 10×10 cm HPTLC plates (Macherey-Nagel gMBh & Co, Düren, Germany). Approximately 10 µg of total lipid were applied as 2 mm streaks, 1 cm from the bottom, and the plates developed in methyl acetate/ isopropanol/ chloroform/methanol/0.25% aqueous KCl (25: 25: 25: 10: 9, by vol.) to two-thirds up the plate. After desiccation for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.) and placed in a vacuum desiccator for 20 min. The lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a BIO-RAD GS-800 calibrated densitometer (BIO-RAD, Spain) and WINCATS software (Henderson *et al.*, 1992). The identities of individual lipid classes were confirmed by comparison with authentic standards, however, only cholesterol was presented. The results of cholesterol were presented as percentage (%) of total lipids (TL) as mean ± SEM.

2.4. Eicosanoids extraction

The samples of frozen acidified plasma were thawed and centrifuged at 12000 x g for 2 min to remove precipitates. The supernatants were extracted using octadecyl silyl (ODS, C18) Sep-Pak mini-columns (Millipore, Watford, UK) by the method of Powell (1982) and as described in detail by Bell *et al.* (1994b). The Sep-Pak cartridges were then washed with 10 mL water and 5 mL methanol and the plasma samples loaded onto the cartridge with a flow rate of 2 mL min^{-1}. The cartridge was washed with 5 mL 15% ethanol, to remove polar lipids, 10 mL distilled water, to restore the initial polarity of the cartridge and 5 mL hexane/chloroform (65:35 v/v). Eicosanoids were then eluted with 10 mL ethyl acetate and solvent evaporated with N_2. The final extract was re-dissolved in 100 µl methanol prior to separation with HPLC.

2.4.1. Separation by HPLC and quantitation of prostaglandins

The homologues of PGs 2- and 3-series were separated by reverse phase HPLC using a column ODS2-SB5 and the methodology previously described (Bell *et al.*, 1994b). An isocratic solvent system was employed containing 17 mM phosphoric acid/acetonitrile (70/30, v/v at a flow rate of 1ml min-1). The elution times of the PGs standards (PGF2α, PGF3α, PGE2 and PGE3) were determined by UV detection at 205 nm using a Pye-Unicam LC-UV detector. A total of 100 ml of the eicosanoid plasma extracts were injected into the column and 6 ml fractions were collected using an LKB 2112 Redirac. Fractions corresponding to 2- and 3-series were extracted as follows: the pooled fractions were applied to a C18 Sep-Pak, which had been pre-washed with 5 ml methanol and 10 ml distilled water. The column was then washed with a further 10 ml of distilled water and the PGs eluted in 5 ml of ethyl acetate. Samples were dried under nitrogen and re-dissolved in immunoassay buffer. Quantification of PGs homologues was performed using enzyme immunoassay (EIA) kits for PGF and PGE prostaglandins according to the manufacturers protocol (Cayman®). The cross-reactivity of the PGF2α and PGE2 antibody with PGF3α and PGE3 was 80% and 65% respectively.

2.5. Steroids

Analysis of 11-ketotestosterone (11-KT) and testosterone (T) were performed in the plasma of males and estradiol (E2) in that of females. Plasma samples were first extracted with alcohol, then ice-cold methanol was added (6:1 v/v), shaken and centrifuged (3000g, 15 min, 4 ^{o}C). The pellet was re-extracted twice with 200 µl methanol. Supernatants were pooled, dried and reconstituted in 0.1 M potassium buffer (pH 7.4), then stored at -20^{o}C until analysis. Levels of 11-ketotestosterone, testosterone and estradiol were quantified by ELISA, using a protocol previously validated for Senegalese sole (Guzmán *et al.*, 2009).

2.6. Statistics analysis

Statistical differences in lipid, fatty acids, lipid classes, prostaglandins, and steroids between different fish groups of males and females were analysed by one-way ANOVA followed by the post-hoc multiple comparison by Tukey's HSD for equal N test with a significance level of $P<0.05$. In the case of steroids the group fed diet A was used as

control. Moreover, correlations among ARA, steroids, cholesterol and PGs were made, using *P* of 0.05. The compliance of data with normality and homogeneity of variance was tested by the Kolmogorov–Smirnov and Bartlett (Chi-Sqr) methods and, when necessary, log-transformation was carried out. Fatty acid content was expressed as % TFA, and lipid class as % TL. The prostaglandins were expressed in pg ml^{-1} of plasma and steroids as ng ml^{-1} of plasma, both as mean of percentage ± standard error of the mean (SEM). The statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA).

3. Results

Groups A (control), B, C, D, E and F were fed diets with increasing levels of ARA (Table 1) and no changes in total lipids and cholesterol were observed in all diets used (Table 2). After the nine month feeding period, females increased their weight from 533 ± 13g to 950 ± 25g, with a specific growth rate (SGR) of 0.20% dia^{-1} and males from 515 ± 16g to 755 ± 24g, with a SGR of 0.13% day^{-1}. The fed conversion rate was 1.3 ± 0.04, and no significant differences were observed among the six groups, either in growth or fed conversion. Total mortality during the experimental period was 0.8%.

3.1. Monthly changes in ARA content in blood

Blood fatty acid composition was analyzed from the samples taken in September, December 2009, March and May 2010. ARA content was 3.6 ± 0.3% in males and 3.4 ± 0.2% TFA in females at the beginning of the feeding period (September 2009) and during the experiment the content of ARA increased significantly ($P<0.05$) on blood in all the groups except group A (control) and females from group B (Figs. 1 and 2). In December, ARA in both males and females showed significant differences between groups ($P<0.05$), which were maintained until the end of the experiment (Fig. 1). Thus, male fish from group F showed 8.7 ± 0.8% ARA in the blood, 3.1-fold higher than the control group A (2.8 ± 0.2% ARA) ($P<0.05$) whereas the fish from groups E, D and C had 7.3 ± 0.4%, 6.7 ± 0.8% and 5.5 ± 0.6% ARA, respectively all significantly higher than the control ($P<0.05$). Fish from group B also showed an increment in blood ARA levels, but levels were not significantly different from the control. In the case of females, in December the fish from groups F (8.6 ± 0.4% ARA, 2.5-fold higher than the control), E (8.4 ± 0.8% ARA) and D

(7.4 ± 0.7% ARA) also showed a significant (P<0.05) increase in ARA levels compared to the control A. Fish from the groups C and B also exhibited an increase in levels of ARA in blood, but levels were not significantly higher than control A. After this initial increase in ARA content found in December ARA remained stable within each group throughout the following months being the values recorded in March very similar to those of December with a slightly increment in the fish from groups E (males) and F (both females and males). In March 2010 the males of all the groups showed ARA values significantly higher than the control (P<0.05) whereas only the females from groups F, E, D and C showed this same trend, and those from B and control groups had lower ARA levels. In May 2010 significantly higher ARA levels were found in groups F, E, D and C in both sexes (P<0.05) compared to groups A and B (Fig. 1). Male fish fed diet F showed a slight ARA reduction compared to the levels found in March, while female from groups C, B and control showed a slight increment in ARA compared to March.

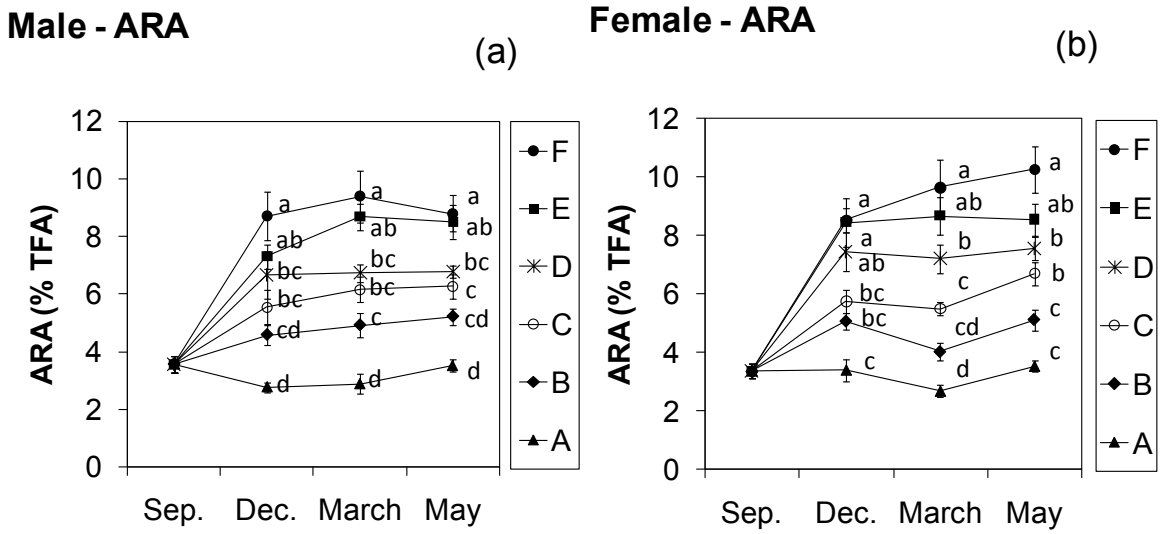

Fig. 1 *ARA levels in red blood cells (% TFA) of six (A, B, C, D, E F) fish groups of Senegalese sole (Solea senegalensis) fed different dietary ARA levels during nine months. Fish are grouped by sex in Fig 1a) males and Fig 1 b) females. Different letters on the same month indicate significant differences (ANOVA, P<0.05) amongst groups. ARA content, diet A = 0.7, B = 1.6, C = 2.3, D = 3.2, E= 5.0 and F= 6.0 % TFA.*

3.2. Monthly changes in blood EPA/ARA ratio

All the fish showed a similar EPA/ARA ratio (2.3 ± 0.2) in September, at the beginning of the experiment (Fig. 2). In December the ratio was significantly reduced in both males and females from F, E, and D and C groups, wich were maintained until May. In the case of males, those from groups F (0.8 ± 0.1), E (1.1 ± 0.1), D (1.2 ± 0.2), C (1.7 ± 0.4) and B (2.1 ± 0.2) showed a significantly (P<0.05) lower ratio than the control A (3.5 ± 0.4), similarly to what was observed in females from groups F (1.1 ± 0.1), E (1.2 ± 0.2), D (1.4 ± 0.1), C (1.7 ± 0.4) and B (2.1 ± 0.2). In females, all except B, having significantly lower ratios than group A (3.5 ± 0.1), that showed an increase in the ratio in both sexes (Fig. 2). In March fish from groups A and B increased their ratio whereas those from F, E, D, and C continued with decreasing values. In May all the groups, including B and in both sexes showed lower EPA/ARA ratio than the control A (P<0.05).

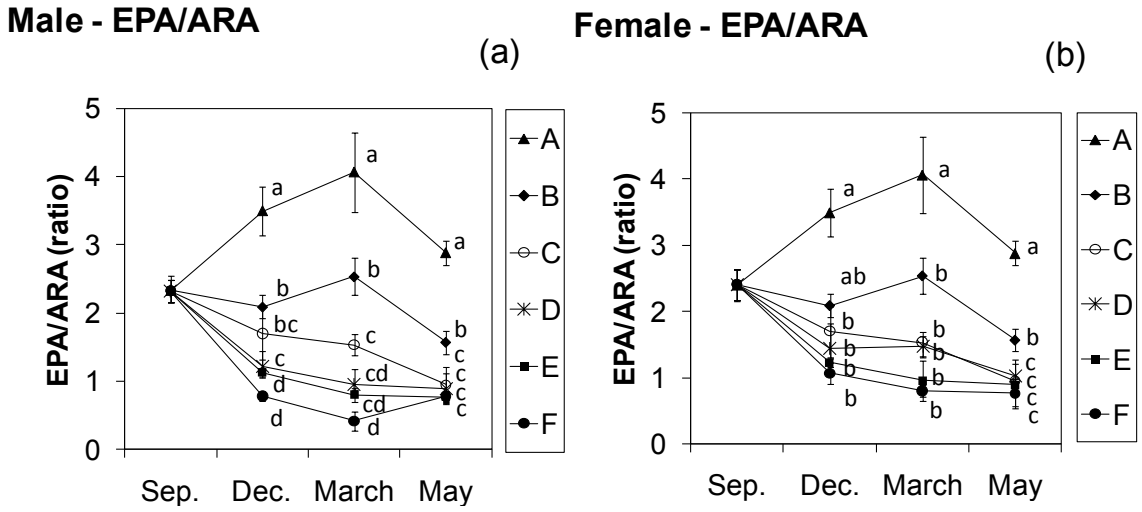

Fig. 2 *EPA/ARA ratio in red blood cells of six groups (A, B, C, D, E, F) of Senegalese sole (Solea senegalensis) fed different dietary ARA levels during nine months. The fish were sampled in September (Sep.), December (Dec.), March and May. Fish are grouped by sex in Fig 2a) males and Fig 2b) females. Values are mean ± SEM significant differences between groups were established by one-way ANOVA and are indicated by different superscripts for each month (P<0.05) (N=6). ARA content, diet A = 0.7, B = 1.6, C = 2.3, D = 3.2, E= 5.0 and F= 6.0% TFA.*

3.3. Fatty acid composition of the blood at the end of the feeding period (May 2010)

Compared to initial values in September at the start of the experiment (3.6 ± 0.3 in males and 3.4 ± 0.2% TFA in females), ARA levels in May were significantly higher ($P<0.01$) in group F, E, D, C and B in male and the same was observed in female for group F, E, D and C. The ARA content in blood varied in a range from 5.2 ± 0.3 from group B to 10.3 ± 0.3% in group F females (Tables 3 and 4). In May, group A had ARA levels similar to the values in September with 3.5 ± 0.2 and 3.4 ± 0.2% in males and females, respectively.

In May, EPA levels in the blood of group A were 9.4 ± 0.3% in male and 9.0 ± 0.5% in females, both values significantly higher ($P<0.05$) than the obtained in September in both males and females and higher than the values of the fish groups F, E, D, C and B in May for males and D, E and F (Tables 3 and 4). Consequently, these differences also attributed to differences in EPA/ARA ratios (see above).

Adrenic (22:4n-6) acid were significantly higher ($P<0.05$) in the blood of the males of groups F, E, D and C and in the blood of females of groups F, E and D. Similarly docosapentaenoic (22:5n-6) acid was higher in the blood of fish from group F compared to the control A ($P<0.05$), in both males and females. Total lipid and total fatty acid content were similar and not significantly different in all the groups (from A to F), either compared to the beginning or among groups at the end of the feeding period and in both sexes.

Table 3 *Lipid, fatty acid content and fatty acid composition (% TFA) of Senegalese sole male blood sampled in September 2009 (Initial), and in May 2010 (Final) and fed 6 different diets (A, B, C, D, E, F). Rows assigned different letters were significantly different (ANOVA, P<0.05) (N=10). ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA*

	Initial Sept.	Final A	Final B	Final C	Final D	Final E	Final F
TL (mg mL^{-1})	8.0 ± 1.6 ^{a}	8.6 ± 0.5 ^{a}	8.0 ± 0.5 ^{a}	8.9 ± 1.1 ^{a}	7.8 ± 1.2 ^{a}	8.4 ± 0.6 ^{a}	8.7 ± 0.9 ^{a}
TFA(µg mg^{-1} L)	398 ± 11 ^{a}	408 ± 21 ^{a}	387 ± 13 ^{a}	359 ± 23 ^{a}	374 ± 23 ^{a}	399 ± 47 ^{a}	323 ± 19 ^{a}
Fatty acid composition (%TFA)							
14:0	1.4 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	1.0 ± 0.1 ^{a}	1.1 ± 0.2 ^{a}	0.7 ± 0.1 ^{a}	1.0 ± 0.1 ^{a}	1.1 ± 0.1 ^{b}
16:0	15.0 ± 0.3 ^{a}	14.7 ± 0.4 ^{a}	14.1 ± 0.7 ^{a}	14.9 ± 0.5 ^{a}	14.8 ± 0.6 ^{a}	13.6 ± 0.5 ^{a}	14.9 ± 0.5 ^{a}
18:0	8.7 ± 0.4 ^{a}	8.1 ± 0.4 ^{a}	8.1 ± 0.2 ^{a}	8.4 ± 0.7 ^{a}	8.9 ± 0.5 ^{a}	9.0 ± 0.4 ^{a}	9.7 ± 0.5 ^{a}
Total SFA	28.0 ± 0.6 ^{a}	24.4 ± 0.8 ^{b}	23.5 ± 0.9 ^{b}	24.6 ± 0.5 ^{b}	24.6 ± 0.7 ^{b}	24.2 ± 0.6 ^{b}	26.1 ± 0.9 ^{a}
16:1n-7	2.1 ± 0.2 ^{a}	2.1 ± 0.3 ^{a}	2.2 ± 0.1 ^{a}	2.1 ± 0.4 ^{a}	1.8 ± 0.3 ^{a}	1.9 ± 0.2 ^{a}	2.5 ± 0.2 ^{a}
18:1n-9	16.2 ± 0.7 ^{a}	12.6 ± 1.5 ^{b}	12.5 ± 0.5 ^{b}	13.5 ± 0.6 ^{ab}	14.2 ± 0.5 ^{ab}	11.9 ± 0.5 ^{b}	13.6 ± 0.9 ^{ab}
18:1n-7	0.5 ± 0.5 ^{b}	3.6 ± 1.1 ^{a}	2.4 ± 0.1 ^{ab}	2.1 ± 0.3 ^{ab}	1.2 ± 0.4 ^{b}	1.7 ± 0.3 ^{ab}	1.5 ± 0.3 ^{ab}
20:1n-9	1.4 ± 0.2 ^{b}	4.2 ± 0.3 ^{a}	4.0 ± 0.2 ^{a}	3.8 ± 0.5 ^{a}	3.9 ± 0.4 ^{a}	4.0 ± 0.4 ^{a}	3.4 ± 0.5 ^{a}
22:1n-9	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}
Total MUFA	20.2 ± 0.6 ^{a}	22.7 ± 1.0 ^{a}	21.3 ± 0.8 ^{a}	21.5 ± 1.4 ^{a}	20.7 ± 0.7 ^{a}	19.5 ± 0.8 ^{a}	21.5 ± 0.9 ^{a}
18:2n-6	7.5 ± 0.2 ^{a}	5.8 ± 0.2 ^{b}	5.1 ± 0.3 ^{b}	5.6 ± 0.4 ^{b}	5.7 ± 0.2 ^{b}	4.7 ± 0.2 ^{b}	5.3 ± 0.3 ^{b}
20:4n-6, ARA	3.6 ± 0.3 ^{c}	3.5 ± 0.2 ^{c}	5.2 ± 0.3 ^{b}	6.3 ± 0.4 ^{b}	6.8 ± 0.2 ^{b}	8.5 ± 0.6 ^{a}	8.7 ± 0.6 ^{a}
22:4n-6	0.2 ± 0.0 ^{f}	0.4 ± 0.0 ^{ef}	0.6 ± 0.0 ^{de}	0.9 ± 0.1 ^{cd}	1.0 ± 0.1 ^{c}	1.4 ± 0.1 ^{b}	1.6 ± 0.1 ^{a}
22:5n-6	0.4 ± 0.0 ^{c}	0.6 ± 0.1 ^{bc}	0.7 ± 0.1 ^{abc}	0.7 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	0.9 ± 0.0 ^{a}
Total n-6 PUFA	12.1 ± 0.4 ^{cd}	10.3 ± 0.3 ^{d}	11.7 ± 0.4 ^{cd}	13.9 ± 0.4 ^{bc}	14.8 ± 0.4 ^{ab}	16.7 ± 0.6 ^{a}	16.7 ± 0.9 ^{a}
18:3n-3	0.4 ± 0.0 ^{a}	0.2 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}
18:4n-3	0.7 ± 0.0 ^{a}	0.6 ± 0.2 ^{ab}	0.2 ± 0.0 ^{b}	0.6 ± 0.1 ^{ab}	0.6 ± 0.1 ^{ab}	0.2 ± 0.1 ^{b}	0.3 ± 0.2 ^{ab}
20:4n-3	0.3 ± 0.0 ^{a}	0.2 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	0.4 ± 0.1 ^{a}	0.4 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}
20:5n-3, EPA	7.8 ± 0.2 ^{b}	9.4 ± 0.3 ^{a}	8.6 ± 0.3 ^{b}	7.9 ± 0.2 ^{b}	7.7 ± 0.3 ^{b}	7.4 ± 0.4 ^{b}	7.3 ± 0.6 ^{b}
22:5n-3	6.2 ± 0.3 ^{a}	6.2 ± 0.3 ^{ab}	6.6 ± 0.4 ^{a}	5.5 ± 0.4 ^{a}	5.5 ± 0.4 ^{a}	5.3 ± 0.2 ^{a}	4.6 ± 0.5 ^{b}
22:6n-3, DHA	21.3 ± 0.7 ^{a}	23.9 ± 1.1 ^{a}	22.3 ± 1.2 ^{a}	21.0 ± 1.1 ^{a}	22.2 ± 1.0 ^{a}	21.1 ± 0.6 ^{a}	21.0 ± 0.9 ^{a}
Total n-3 PUFA	37.1 ± 0.9 ^{ab}	40.6 ± 1.4 ^{a}	38.0 ± 1.6 ^{ab}	35.4 ± 1.6 ^{abc}	36.6 ± 1.4 ^{abc}	34.3 ± 0.6 ^{bc}	31.3 ± 1.1 ^{c}
Total PUFA	49.2 ± 0.8 ^{a}	50.9 ± 1.4 ^{a}	49.6 ± 1.7 ^{a}	49.3 ± 1.8 ^{a}	51.5 ± 1.4 ^{a}	51.0 ± 0.8 ^{a}	48.0 ± 1.5 ^{a}
EPA/ARA	2.3 ± 0.2 ^{a}	2.8 ± 0.2 ^{a}	1.7 ± 0.1 ^{b}	1.3 ± 0.1 ^{b}	1.2 ± 0.0 ^{b}	0.8 ± 0.1 ^{b}	0.9 ± 0.1 ^{b}
EPA/DHA	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}
DHA/ARA	6.4 ± 0.6 ^{a}	7.0 ± 0.4 ^{a}	4.3 ± 0.2 ^{b}	3.4 ± 0.2 ^{b}	3.3 ± 0.2 ^{b}	2.4 ± 0.2 ^{b}	2.2 ± 0.2 ^{b}
n-3/n-6	3.1 ± 0.1 ^{bc}	4.0 ± 0.2 ^{a}	3.2 ± 0.2 ^{b}	2.6 ± 0.1 ^{cd}	2.5 ± 0.1 ^{cd}	2.1 ± 0.1 ^{d}	1.9 ± 0.1 ^{d}

Abbreviation as in the table 1.

Table 4 *Lipid, fatty acid content and fatty acid composition (% TFA) of Senegalese sole female blood sampled in September 2009 (Initial), and in May 2010 (Final) and fed 6 different diets (A, B, C, D, E, F). Rows assigned different letters were significantly different (ANOVA, P<0.05) (N=10). ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA*

	Initial Sept.	Final A	Final B	Final C	Final D	Final E	Final F
TL (mg mL^{-1})	8.0 ± 1.6 ^{a}	8.6 ± 0.5 ^{a}	8.0 ± 0.5 ^{a}	8.9 ± 1.1 ^{a}	7.8 ± 1.2 ^{a}	8.4 ± 0.6 ^{a}	8.7 ± 0.9 ^{a}
TFA(μg mg^{-1} L)	398 ± 11 ^{a}	408 ± 21 ^{a}	387 ± 13 ^{a}	359 ± 23 ^{a}	374 ± 23 ^{a}	399 ± 47 ^{a}	323 ± 19 ^{a}
Fatty acid composition (%TFA)							
14:0	1.4 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	1.0 ± 0.1 ^{a}	1.1 ± 0.2 ^{a}	0.7 ± 0.1 ^{a}	1.0 ± 0.1 ^{a}	1.1 ± 0.1 ^{b}
16:0	15.0 ± 0.3 ^{a}	14.7 ± 0.4 ^{a}	14.1 ± 0.7 ^{a}	14.9 ± 0.5 ^{a}	14.8 ± 0.6 ^{a}	13.6 ± 0.5 ^{a}	14.9 ± 0.5 ^{a}
18:0	8.7 ± 0.4 ^{a}	8.1 ± 0.4 ^{a}	8.1 ± 0.2 ^{a}	8.4 ± 0.7 ^{a}	8.9 ± 0.5 ^{a}	9.0 ± 0.4 ^{a}	9.7 ± 0.5 ^{a}
Total SFA	28.0 ± 0.6 ^{a}	24.4 ± 0.8 ^{b}	23.5 ± 0.9 ^{b}	24.6 ± 0.5 ^{b}	24.6 ± 0.7 ^{b}	24.2 ± 0.6 ^{b}	26.1 ± 0.9 ^{a}
16:1n-7	2.1 ± 0.2 ^{a}	2.1 ± 0.3 ^{a}	2.2 ± 0.1 ^{a}	2.1 ± 0.4 ^{a}	1.8 ± 0.3 ^{a}	1.9 ± 0.2 ^{a}	2.5 ± 0.2 ^{a}
18:1n-9	16.2 ± 0.7 ^{a}	12.6 ± 1.5 ^{b}	12.5 ± 0.5 ^{b}	13.5 ± 0.6 ^{ab}	14.2 ± 0.5 ^{ab}	11.9 ± 0.5 ^{b}	13.6 ± 0.9 ^{ab}
18:1n-7	0.5 ± 0.5 ^{b}	3.6 ± 1.1 ^{a}	2.4 ± 0.1 ^{ab}	2.1 ± 0.3 ^{ab}	1.2 ± 0.4 ^{b}	1.7 ± 0.3 ^{ab}	1.5 ± 0.3 ^{ab}
20:1n-9	1.4 ± 0.2 ^{b}	4.2 ± 0.3 ^{a}	4.0 ± 0.2 ^{a}	3.8 ± 0.5 ^{a}	3.9 ± 0.4 ^{a}	4.0 ± 0.4 ^{a}	3.4 ± 0.5 ^{a}
22:1n-9	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}	0.0 ± 0.0 ^{a}
Total MUFA	20.2 ± 0.6 ^{a}	22.7 ± 1.0 ^{a}	21.3 ± 0.8 ^{a}	21.5 ± 1.4 ^{a}	20.7 ± 0.7 ^{a}	19.5 ± 0.8 ^{a}	21.5 ± 0.9 ^{a}
18:2n-6	7.5 ± 0.2 ^{a}	5.8 ± 0.2 ^{b}	5.1 ± 0.3 ^{b}	5.6 ± 0.4 ^{b}	5.7 ± 0.2 ^{b}	4.7 ± 0.2 ^{b}	5.3 ± 0.3 ^{b}
20:4n-6, ARA	3.6 ± 0.3 ^{c}	3.5 ± 0.2 ^{c}	5.2 ± 0.3 ^{b}	6.3 ± 0.4 ^{b}	6.8 ± 0.2 ^{b}	8.5 ± 0.6 ^{a}	8.7 ± 0.6 ^{a}
22:4n-6	0.2 ± 0.0 ^{f}	0.4 ± 0.0 ^{ef}	0.6 ± 0.0 ^{de}	0.9 ± 0.1 ^{cd}	1.0 ± 0.1 ^{c}	1.4 ± 0.1 ^{b}	1.6 ± 0.1 ^{a}
22:5n-6	0.4 ± 0.0 ^{c}	0.6 ± 0.1 ^{bc}	0.7 ± 0.1 ^{abc}	0.7 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	0.9 ± 0.0 ^{a}
Total n-6 PUFA	12.1 ± 0.4 ^{cd}	10.3 ± 0.3 ^{d}	11.7 ± 0.4 ^{cd}	13.9 ± 0.4 ^{bc}	14.8 ± 0.4 ^{ab}	16.7 ± 0.6 ^{a}	16.7 ± 0.9 ^{a}
18:3n-3	0.4 ± 0.0 ^{a}	0.2 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}
18:4n-3	0.7 ± 0.0 ^{a}	0.6 ± 0.2 ^{ab}	0.2 ± 0.0 ^{b}	0.6 ± 0.1 ^{ab}	0.6 ± 0.1 ^{ab}	0.2 ± 0.1 ^{b}	0.3 ± 0.2 ^{ab}
20:4n-3	0.3 ± 0.0 ^{a}	0.2 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	0.4 ± 0.1 ^{a}	0.4 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}
20:5n-3, EPA	7.8 ± 0.2 ^{b}	9.4 ± 0.3 ^{a}	8.6 ± 0.3 ^{b}	7.9 ± 0.2 ^{b}	7.7 ± 0.3 ^{b}	7.4 ± 0.4 ^{b}	7.3 ± 0.6 ^{b}
22:5n-3	6.2 ± 0.3 ^{a}	6.2 ± 0.3 ^{ab}	6.6 ± 0.4 ^{a}	5.5 ± 0.4 ^{a}	5.5 ± 0.4 ^{a}	5.3 ± 0.2 ^{a}	4.6 ± 0.5 ^{b}
22:6n-3, DHA	21.3 ± 0.7 ^{a}	23.9 ± 1.1 ^{a}	22.3 ± 1.2 ^{a}	21.0 ± 1.1 ^{a}	22.2 ± 1.0 ^{a}	21.1 ± 0.6 ^{a}	21.0 ± 0.9 ^{a}
Total n-3 PUFA	37.1 ± 0.9 ^{ab}	40.6 ± 1.4 ^{a}	38.0 ± 1.6 ^{ab}	35.4 ± 1.6 ^{abc}	36.6 ± 1.4 ^{abc}	34.3 ± 0.6 ^{bc}	31.3 ± 1.1 ^{c}
Total PUFA	49.2 ± 0.8 ^{a}	50.9 ± 1.4 ^{a}	49.6 ± 1.7 ^{a}	49.3 ± 1.8 ^{a}	51.5 ± 1.4 ^{a}	51.0 ± 0.8 ^{a}	48.0 ± 1.5 ^{a}
EPA/ARA	2.3 ± 0.2 ^{a}	2.8 ± 0.2 ^{a}	1.7 ± 0.1 ^{b}	1.3 ± 0.1 ^{b}	1.2 ± 0.0 ^{b}	0.8 ± 0.1 ^{b}	0.9 ± 0.1 ^{b}
EPA/DHA	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}
DHA/ARA	6.4 ± 0.6 ^{a}	7.0 ± 0.4 ^{a}	4.3 ± 0.2 ^{b}	3.4 ± 0.2 ^{b}	3.3 ± 0.2 ^{b}	2.4 ± 0.2 ^{b}	2.2 ± 0.2 ^{b}
n-3/n-6	3.1 ± 0.1 ^{bc}	4.0 ± 0.2 ^{a}	3.2 ± 0.2 ^{b}	2.6 ± 0.1 ^{cd}	2.5 ± 0.1 ^{cd}	2.1 ± 0.1 ^{d}	1.9 ± 0.1 ^{d}

Abbreviation as in table 1

3.4. Cholesterol in blood

At the end of the experiment in May cholesterol (CHOL) levels exhibited a positive correlation increase in males and females (r^2=0.95), in relation to the level of dietary ARA (Fig. 3). In male fish from groups F and E, CHOL levels in the blood were 32 ± 1.6 and 30 ± 1.9% TL, which was significantly higher (P<0.05) than the levels found in the males from groups C, B and A that exhibited levels of 23 ± 1.1%, 24 ± 0.8% and 22 ± 0.9% TL respectively (P<0.05). Males from group D had an intermediate value, 26 ± 0.6%, which was not different from the other groups. In the case of female fish from groups F (35 ± 1.3), E (33 ± 1.5) and D (30 ± 1.8) showed a significant increase in CHOL compared with control A (26 ± 0.7) (P<0.05). The CHOL in diets was 12 ± 0.8% CHOL, and no differences were found between A, B, C, D, E and F (Table 2).

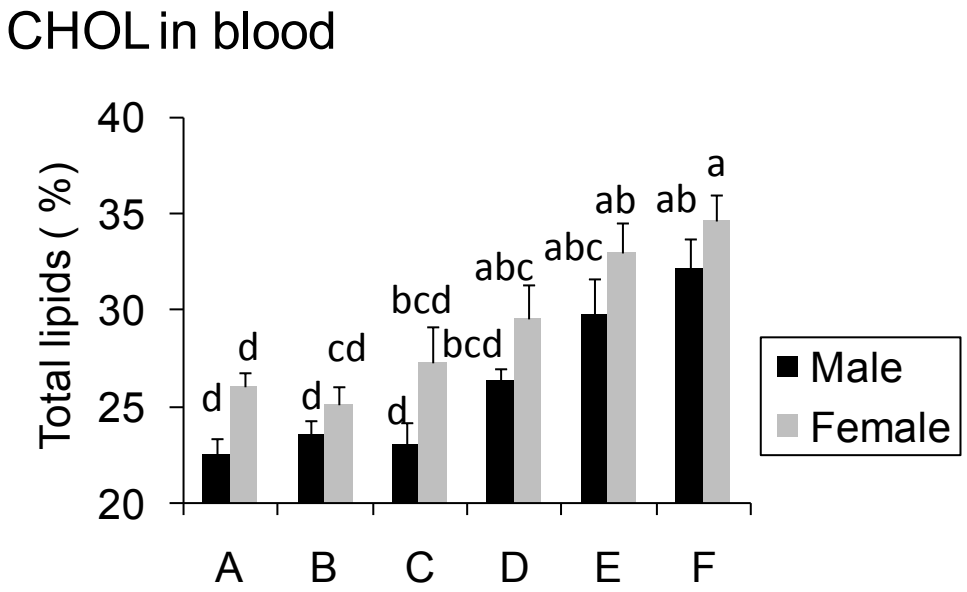

Fig. 3 *Cholesterol (CHOL) levels in the blood of six fish groups of Senegalese sole, males and females fed different dietary ARA levels (A, B, C, D, E and F), during nine month (September – May). Values are mean + SEM and are expressed as % of total lipids. Columns assigned different letters were significantly different (ANOVA, P<0.05) (N=10). ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA.*

3.5. Prostaglandins in blood

Prostaglandins were measured at the end of the experiment, in May. Plasma concentration of PGs 3-series was always higher than PGs 2-series in all the fish groups studied (Figs. 4

and 5). Thus, PGF3α was significantly (P<0.05) higher than PGF2α in both females and males of the control and females in group B. Similarly higher PGE3 levels were found in both sexes compared to PGE2, although in this case the differences were not significant. The concentration of PGs 3-series showed a negative correlation with ARA in blood of males and females (r^2=0.45), thus was reduced concomitant to the increased levels of ARA in blood (P<0.05) (Fig. 5) and consequently no statistical differences were found between PGs 2- and 3- series in groups C, D, E and F and levels in groups, D, E and F were similar in concentration and between sexes. In the case of PGs 2-series, the levels found in the plasma of males and females did not change with the diet, although PGs F2-isomers was always found in higher concentration than PG E2-isomer in both sexes.

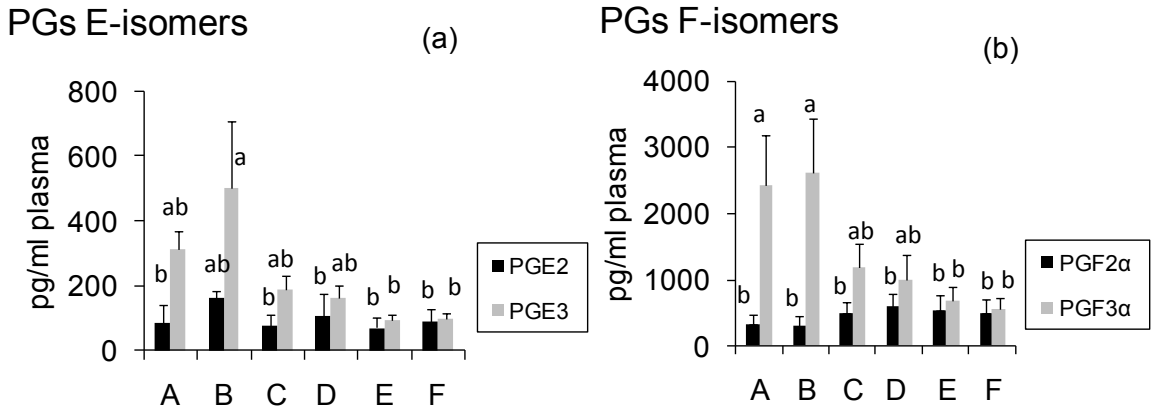

Fig. 4 *Concentration of plasma prostaglandins a) E-isomers, PGE2 and PGE3 and b) F-isomers, PGF2α and PGF3α, of six groups of Senegalese sole females fed during nine month with different dietary ARA levels (A, B, C,D, E and F). Values are mean + SEM and are expressed as pg ml^{-1} plasma, significant differences between groups were established by one-way ANOVA and are indicated by different superscript letters within individual bars (P<0.05) (N=6). ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA.*

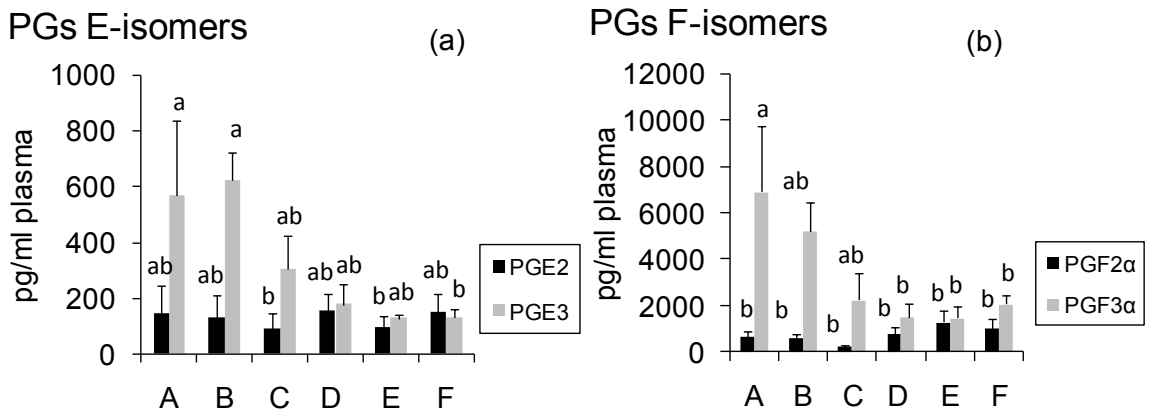

Fig. 5 *Concentration of plasma prostaglandins a) E-isomers, PGE2 and PGE3 and b) F-isomers, PGF2α and PGF3α, of six groups of Senegalese sole males, fed during nine months with different dietary ARA levels (A, B, C,D, E and F). Values are mean + SEM are expressed as pg ml^{-1} plasma, significant differences between groups were established by one-way ANOVA and are indicated by different superscript letters within individual bars (P<0.05) (N=6). ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA.*

3.6. Plasma steroids levels

The sex hormone, 11-ketotestosterone (11-KT) was the most concentrated steroid in males with plasma levels varying between 2.2 and 16.4 ng ml^{-1}, compared to testosterone (T) with 0.8- 2.2 ng ml^{-1} (Fig. 6). The levels of both esteroid hormones were significantly (P<0.05) affected by the diet in the month of April in group E and F for 11-KT (Figs. 6d and 6f) and D for T (Fig. 6c). Although, generally the levels were not significantly different from the control group A, during the months February, March, April and May, the fish fed diets D, E and F exhibited higher amounts of steroid levels in the plasma. Plasma estradiol (E2) levels in females were similar between the different diets, E2 levels increased in group B compared to the control and the other groups, but without significant differences (Fig. 7a).

Circulating 11-KT, T, and E2, followed a seasonal trend in plasma concentration (Figs. 6 and 7). In males, the first increase in the titre of steroid levels was detected between January and February with plasma concentrations of both androgens increasing progressively to a significantly (P<0.05) higher peak found in April. Then steroid levels gradually declined until reaching the minimal initial values in June 2010. In females an increase in E2 levels was detected between January and February with plasma

concentrations increasing progressively afterwards to reach a peak in March, one month earlier than in males, in all the groups except for the control which peaked in April. Then, plasma levels gradually declined until reaching the initial values in June 2010.

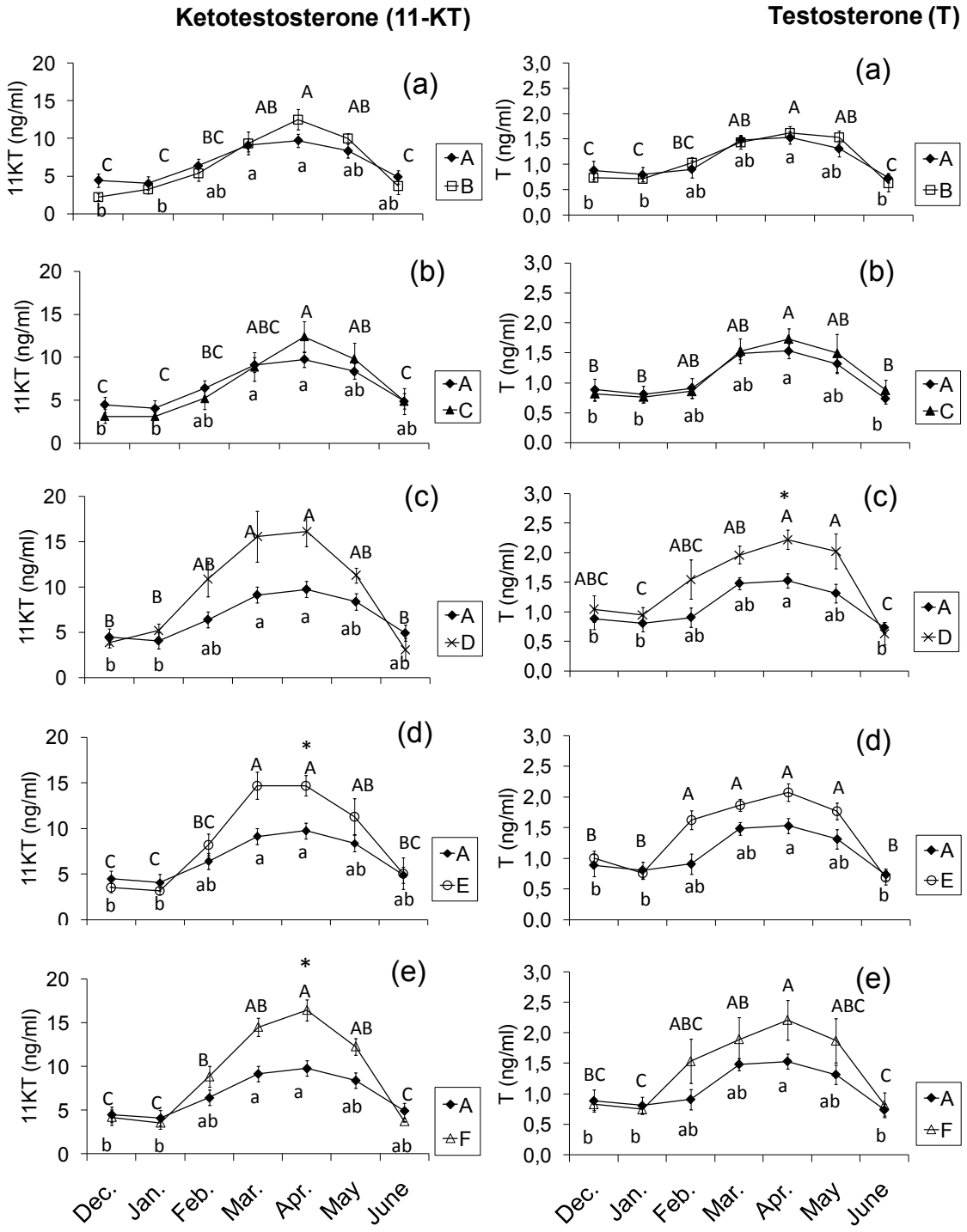

Fig. 6 *Changes in plasma levels of 11-ketotestosterone (11-KT) and testosterone (T) (mean ± SEM) of Senegalese sole males (N=6) measured in December 2009 (Dec.), January (Jan.), February (Feb.), March (Mar.), April (Apr.), May and June 2010 and fed different dietary ARA levels. a) Fish fed diets A and B, b) fish fed diets A and C, c) fish fed diets A and D, d) fish fed diets A and E, e) fish fed diets A and F. Different letters indicate significant differences (ANOVA, P<0.05) between sampling months and within treatments.*

Small letters are used for diet A used as a control and capital letters for dietary treatments B, C, D, E and F. (*) indicate significant differences (P<0.05) with respect to the control group (A) and the dietary treatment. ARA content, diet A= 0.7 (control), B= 1.6, C= 2.3, D= 3.2, E= 5.0, F= 6.0% TFA.

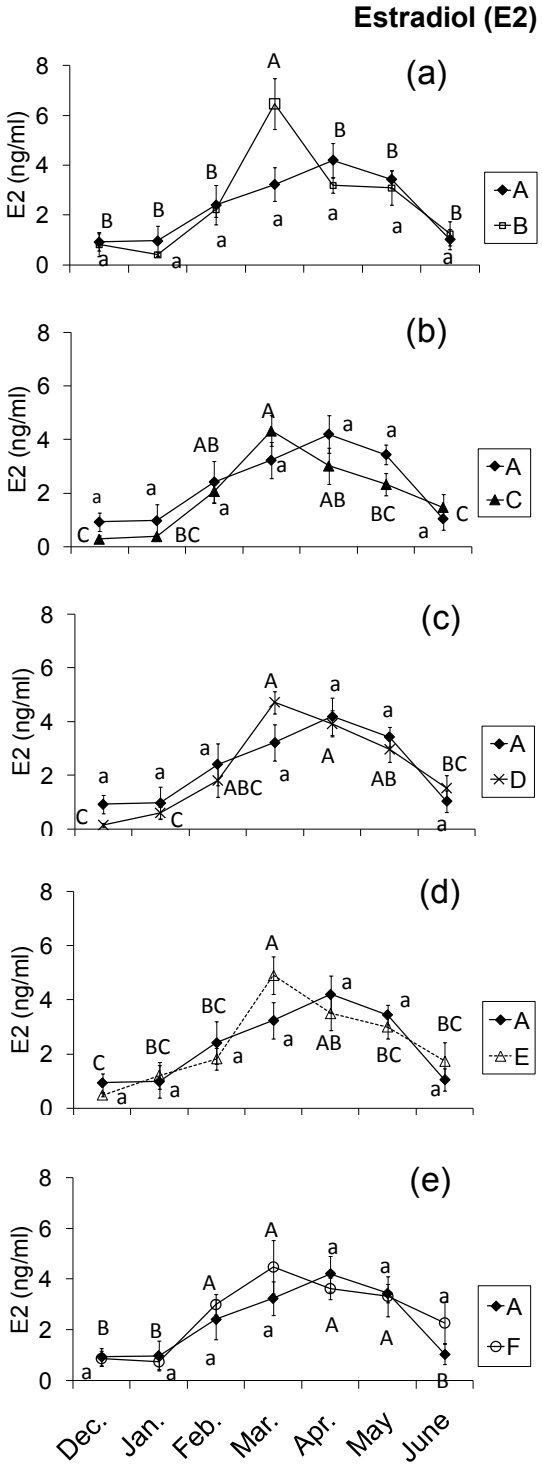

Fig. 7 *Changes in plasma levels of estradiol (E2) (mean ± SEM) of Senegalese sole females (N=6) measured in December 2009 (Dec.), January (Jan.), February (Feb.), March (Mar.), April (Apr.), May and June 2010 and fed different dietary ARA levels. a) Fish fed diets A and B, b) fish fed diets A and C, c) fish fed diets A and D, d) fish fed diets A and E, e) fish fed diets A and F. Different letters indicate significant differences (ANOVA, P<0.05) between sampling months and within treatments. Small letters are used for diet A used as a control and capital letters for dietary treatments B, C, D, E and F. (*) indicate significant differences (P<0.05) with respect to the control group (A) and the dietary treatment. ARA content, diet A= 0.7 (control), B= 1.6, C= 2.3, D= 3.2, E= 5.0, F= 6.0% TFA.*

4. Discussion

This study was conducted to analyse the effects of six dietary ARA levels on the reproductive physiology of G1 Senegalese sole. Blood and plasma analyses of the lipid and fatty acid composition, cholesterol levels, prostaglandins and steroid production were carried out. ARA levels increased in a dose dependent manner during the first period of feeding (September to December 2010) remaining stable throughout the rest of the experiment, and in both sexes, to reach levels of around 8.5% TFA (groups E and F). In parallel to ARA increase, EPA levels remained almost the same and EPA/ARA ratio decreased. Thus, dietary fatty acid composition showed a direct effect on blood composition. Increasing ARA levels in blood had as a consequence an increase in 22:4n-6 and 22:5n-6 acids especially in males and which are a consequence of elongation of ARA and desaturation of 22:4n-6 acid (Linares *et al.*, 1991). Long chain PUFAs (n-3 and n-6) have been detected in the spermatozoa of mammals (Bridges *et al.*, 1970, Lenzi *et al.*, 1996, Lenzi *et al.*, 2002, Furland *et al.*, 2007, Tam *et al.*, 2008) and in the gonads of male marine fish (i.e., wild Senegalese sole, chapter II) and related to the fluidity of the sperm plasma membrane needed to participate in the membrane fusion events associated with fertilization (Lenzi *et al.*, 1996). The diets used in the experiment were formulated using high quality northern fish oil (G. Rosenlund, pers. Comm. 2011, Skretting, Norway) with similar levels of EPA (approx., 15% TFA) and DHA (approx., 13% TFA) (see Table 1) being the variations in ARA dietary content a consequence of the addition of a specialist ARA-rich oil. Thus, EPA content in the blood of the fish was very similar for all the groups and the variations in the ratio EPA/ARA were derived mostly due to the graded levels of dietary ARA.

Both ARA and EPA fatty acids are precursors of PGs, 2- and 3-series respectively (Smith *et al.*, 2002, Tocher, 2003). In the fish of the present experiment, PG 3-series were always higher than PG 2-series in spite of the increase in dietary ARA content. PGs F-isomer PGF2α, is responsible for the synchronization of courtship between male and female fish during spawning (Stacey *et al.*, 1982, Golubev, 1984, Sorensen *et al.*, 1995, Moore, 1996, Sorensen *et al.*, 2004, Stacey *et al.*, 2003) whereas PGE2 is important for the follicle maturation and steroid production in ovaries and testis (Van Der Kraak *et al.*, 1990, Wade

et al., 1993a, Sorensen *et al.*, 1995, Kobayashi *et al.*, 2002, Stacey *et al.*, 2003, Sorensen *et al.*, 2004, Espey *et al.*, 2006, Fujimori *et al.*, 2010). Thus, the low concentrations in PGs 2-series in G1 Senegalese sole found in this study, compared to those of PGs 3-series, might have a detrimental effect in fish reproduction. In previous studies the production of 2- and 3-series PGs was proportional to EPA/ARA ratio found in fish tissues, having the ARA dietary content a direct effect on the production of PG 2-series, (Norambuena *et al.*, 2011d, Bell *et al.*, 1994b, Bell *et al.*, 1995, Norambuena *et al.*, 2011c) (chapter III). In the present experiment although PGs 3-series were always higher than 2-series in both F- and E-isomers, both metabolites PGE3 and PGF3α were reduced concomitant with the increase in ARA content in the blood. The high values of PGs 3-series might be explained by the high dietary EPA content and the high EPA/ARA ratio found in the diet. Although, the ratio was progressively reduced from diet A (24) to diet F (2.4). Consequently with the increase of dietary content of ARA a significant reduction of 3-series PGs was obtained, although the effects on fish reproduction (i.e., ovulation, spermiation or courtship behaviour) could not be established. Further studies are required to clarify the effects of EPA dietary content in COX pathway, since the optimal dietary ARA content will be strongly dependent on the EPA content.

Steroid production (11-KT and T) in males was higher with higher ARA content and increased significantly with certain diets in April at the peak of maturation, whereas E2 in females did not show any change. ARA and cholesterol levels in blood also showed a significant increase in ARA dose dependent manner. In vitro studies with goldfish and trout have shown an ARA-stimulated testosterone production via COX pathway, being the ARA effect mediated through its conversion to PGs (Mustafa *et al.*, 1989, Wade *et al.*, 1994, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a). According to these studies, EPA exerted an opposite effect inhibiting testosterone production via the inhibition of cAMP production, with ARA-induced maturation depressed. Thus, an increased in the production of EPA-derived PGE3 had no effect on fish maturation (Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). Any change in dietary EPA/ARA ratio or in the levels of these two fatty acids in fish tissues will influence the production of PGs and steroids. The results obtained in vivo in the present experiment are consistent with previous in vitro studies showing a significant correlation (r^2=0.7, P<0.05) increase of plasma steroid levels derived from increasing

dietary levels of ARA. Thus, 11-KT and T showed a clear dietary response in males with blood ARA levels increasing and EPA/ARA ratio reduced, and with steroid levels increasing progressively to peak in April in all the groups. However, steroid levels found were lower than those reported in wild males of Senegalese sole (i.e., 11-KT peak of ≈33 ng ml^{-1}) (García-López *et al.*, 2006a). In femaless Estradiol (E2) did not show any increase in plasma levels derived from increasing ARA dietary content, only group B showed a higher peak of E2 similar to the levels found by Garcia-López *et al* in wild female (6.5 ng ml^{-1}) (García-López *et al.*, 2006a). The peak in E2 levels was in March, one month earlier than in males, in all the groups except the control A. Similar results have been found in Atlantic cod and Atlantic halibut in response to graded levels of dietary ARA content in which plasma vitellogenin concentration peaked one month earlier in cod fish fed with increasing levels of ARA (Norberg *et al.*, 2009) and a two week delay in the spawning season of halibut (Alorend, 2004). However, more studies are required to clarify the effects of dietary ARA levels in the steroid production in females. Changes in dietary CHOL could be affecting the steroid production in broodstock fish (Baron *et al.*, 1997). The results presented here show that blood ARA and CHOL levels increased in parallel (r^2=0.95) the dietary content of ARA, in spite of CHOL levels being the same in all the diets used (i.e., 12% TL, see Table 2). Thus, dietary ARA increased the mobilization of CHOL in the blood by means of cAMP formation (Mercure *et al.*, 1996). Wang and Stocco (2005) showed the interaction between ARA metabolism and CHOL during steroidogenesis biosynthesis in Leydig cells of rat, indicating that mobilization of CHOL regulated by StAR protein in turn affected by the ARA-metabolites and lypoxygenase, epoxygenase and cyclooxygenase activity. Mercury and Kraak (1995) demonstrated that EPA blocks cAMP formation and consequently StAR protein and the transport of CHOL in steroidogenic tissues. The results of the present experiment showed that EPA in blood was reduced in a dietary ARA dose related manner (r^2=0.6), therefore availability of ARA in blood (tissues) might be inducing StAR regulation and CHOL circulation, however StAR protein, lypooxygenase, epoxygenase and clooxygenase gene expression were not studied in the present experiment. Another explanation of CHOL incorporation in blood, is the biosynthesis by the fish, Leaver *et al.* (2008) showed that Atlantic salmon increased CHOL biosynthesis after dietary substitution of fish oil with vegetable oil (Leaver *et al.*, 2008), however, in

Senegalese sole there is no information regarding to the capacity of the fish for CHOL synthesis.

5. Conclusion

There is a clear and statistically significant effect of dietary ARA levels on steroid production in G1 Senegalese sole males, which might have important consequences in the reproduction of cultured fish although not such an effect was observed in females. Changes in dietary ARA content modified the fatty acid profile of the fish blood, with a concomitant increase in ARA and a reduction of EPA levels and of the EPA/ARA ratio. The increased ARA levels in blood resulted in an increase CHOL mobilization on blood and a reduction in the production of 3-series prostaglandins. However more studies are needed to understand the relationship between ARA dietary levels, ARA accumulation in fish blood and tissues, CHOL production and hormone function in Senegalese sole.

6. Acknowledgments

We gratefully thank to Gordon Bell, Evaristo Mañanos, Alicia Estevez, James R. Dick Pepa, and Juan C. Navarro for the scientific help and Grethe Rosenlund from Skretting ARC, Norway for diets manufacturing. Thanks are also given to Noelia Gras, Marta Sastre, Ignacio Carazo and Vanessa for sample collection and technical support. This research was funded by two projects: 1) Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project Lenguado and 2) Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by N. Duncan and the PhD grant awarded to F. Norambuena by Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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Chapter V

ARA Self-selection in *S. Sole*

SELF-SELECTION OF DIETS WITH DIFERENT CONTENT OF ARACHIDONIC ACID (20:4n-6, ARA) BY SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BROODSTOCK

Abstract

Reproductive failure to spawn viable eggs from G1 cultured Senegalese sole (*Solea senegalensis*) has been shown. Cultured fish, showed a significantly lower accumulation of arachidonic fatty acid (20:4n-6, ARA) in reproductive tissues compared to their wild counterparts. However, there is no information about ARA requirements in *S. sole*. The aim of the present study was to determine dietary ARA selection of Senegalese sole G1 by the use of self-feeders. For this, groups of G1 fish were offered the choice between two diets, one with high and another with low dietary ARA content (6.0 and 0.7% TFA), by means of two self-feeders per tank. The experiment was conducted during 16 months. The results obtained shows that Senegalese sole changed their preference for ARA during the year and level demanded was correlated with the seasonal water temperature changes (r^2=0.65), with periods of high and low demand of ARA and food ingestion. Thus high ARA selection in late-summer, early-autumn with 3.9% TFA and low selection in winter with 2.2% TFA was shown. The average ARA demand was 3.0% TFA for all the experiment and there was high accumulation of ARA in male testis (8.8% TFA).

1. Introduction

Previous studies on reproductive behaviour and broodstock nutrition of Senegalese sole (*Solea senegalensis*) indicate that although cultured females release eggs the courtship between males and females was not completed, such behavioural dysfunction might be caused by physiological and/or nutritional deficiencies (Norambuena *et al.*, 2011d, Carazo *et al.*, 2008, Norambuena *et al.*, 2009, Carazo *et al.*, 2011, Norambuena *et al.*, 2011b, Norambuena *et al.*, 2011a, Norambuena *et al.*, 2011e) (chapters II, III, IV). Inclusion of essential fatty acids (EFAs) in broodstock diets increases sex steroid production, fecundity, fertilization, embryogenesis, egg quality, hatching rate, survival of the offspring and reproductive behaviour (Wade *et al.*, 1994b, Silversand *et al.*, 1996, Sorbera *et al.*, 1998, Van Der Kraak *et al.*, 1999, Izquierdo *et al.*, 2001, Sorbera *et al.*, 2001, Mazorra *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Furuita *et al.*,

2007, Huynh *et al.*, 2007, Pickova *et al.*, 2007). Arachidonic acid (20:4n-6, ARA) plays an important role as a source for metabolic energy and as a precursor of eicosanoids by cyclooxygenase pathway (COX), particularly 2-series prostaglandins (PGF2α, PGE2) and thromboxanes (Smith, 1989). Studies carried out in-vitro using ARA and its COX metabolites have shown that PGE2 stimulates testosterone production in testis and ovaries of teleost fish (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). Moreover the prostaglandin PGF2α acts hormonally to trigger female sexual behaviour, as a postovulatory circulating PG to trigger courtship and milt increase in males (Kobayashi *et al.*, 2002, Sorensen *et al.*, 2004). Although ARA is the main chief precursor of PGs, these metabolites can also be produced from eicosapentaenoic acid (20:5n-3, EPA). Thus EPA competes in the COX pathway with ARA in PGs production being converted to 3-series, PGs that are biologically less active than the corresponding 2-series produced from ARA (Tocher, 2003). Previous studies in G1 Senegalese sole showed a significantly lower accumulation of ARA in liver, gonads and muscle compared to their wild counterpart (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011b), moreover G1 showed high values of EPA and EPA/ARA ratio in liver and muscle of G1 Senegalese (chapter II). Recently, it was shown that PGs 2-series (PGF2α and PGE2) were higher in wild Senegelese sole broodstock fish compare with G1, while the opposite was observed with PGs 3-series, (Norambuena *et al.*, 2011e, Norambuena *et al.*, 2011c, Norambuena *et al.*, 2011d) (chapter III). These low levels in ARA and imbalances in the EFAs, particularly EPA/ARA ratio in cultured brood fish might have a detrimental effect on reproduction, as has been suggested in other teleosts (Wade *et al.*, 1994a, Bell *et al.*, 1996, Stacey *et al.*, 2003, Tocher, 2003). Low levels in ARA and imbalances in the EFAs, such as EPA/ARA ratio appeared to be a consequence of the dietary intake (Bell *et al.*, 1994, Bell *et al.*, 1997, Mazorra *et al.*, 2003, Alorend, 2004, Norberg *et al.*, 2009, Norambuena *et al.*, 2011b).

Senegalese sole shows different maturation stages along the year, with minimum steroid levels observed in winter and maximum in spring at pre-spawning (Guzmán *et al.*, 2009). Throughout the year, wild Senegalese sole are subjected to environmental changes (i.e., temperature, salinity) and fluctuations in the availability and composition of food, showing seasonal changes in terms of feeding habits and nutritional requirements (Garcia, 1996, Garcia *et al.*, 1996); changes that affect the biochemical composition of sole tissues, including the fatty acid profile. Studies in

common sole (*Solea solea*), capelin (*Mallotus villosus)* and white seabream (*Diplodus sargus*) have demonstrated that there is a seasonal variation in the fatty acid composition of fish tissues (Henderson *et al.*, 1984, Gökçe *et al.*, 2004, Pérez *et al.*, 2007) and probably in their nutritional requirements. Female fish develop large gonads that store relatively high amounts of HUFAs needed for maturation, steroidogenesis and reproduction (Izquierdo *et al.*, 2001). Moreover, somatic and gonadal growth accelerates until the fish spawns, with a decrease of feeding during part, or all, of the maturation process (Volkoff *et al.*, 2005, Leal *et al.*, 2009).

There is no information about ARA requirements in Senegalese sole broodstock, and in the use of self-feeders to determine their macronutrient or micronutrient requirements. Several self-feeder studies have been carried out to show the ability of fish to discriminate between diets which differ in nutritional content and to detect small differences in micro-nutrients, demonstrating the capacity to select from among different food sources to compose a balanced diet that meets their nutritional requirements (Garcia *et al.*, 1996, Sánchez-Vázquez *et al.*, 1999). Studies on macronutrient selection (protein, lipid and carbohydrate) were conducted in rainbow trout (Brannas *et al.*, 1994) (Sánchez-Vázquez *et al.*, 1999, Shima *et al.*, 2003), European seabass (Madrid *et al.*, 1997, Aranda *et al.*, 2000, Paspatis *et al.*, 2000, Aranda *et al.*, 2001, Paspatis *et al.*, 2003, Madrid *et al.*, 2004, Rubio *et al.*, 2004, Coves *et al.*, 2006, Vivas *et al.*, 2006), seabream (Paspatis *et al.*, 2000, Vivas *et al.*, 2006), golfish (Sánchez-Vázquez *et al.*, 1998), common carp (Yamamoto *et al.*, 2003), and juveniles of Senegalese sole (Rubio *et al.*, 2009). In rainbow trout, small differences in dietary composition were detected by fish that selected amongst diets with vegetable and fish oil (Geurden *et al.*, 2005), diets with different amino acid profile (Yamamoto *et al.*, 2001), different types of fluoroquinolone (Boujard *et al.*, 1997), and zinc (Cuenca *et al.*, 1993). In the case of European seabass the fish were able to select individual amino acids (Hidalgo *et al.*, 1988, Brotons-Martinez *et al.*, 2004), as were Nile tilapia that selected between diets with three essential amino acids (L-tryptophane, L-methionine, L-threonine) (Fortes-Silva *et al.*, 2011) Seabream, selected between diets with different vitamin C (Paspatis *et al.*, 1997), Senegalese sole were able to discriminate diets with or without commercial flavor additives and betaine, showing changes on it feeding behavior with clear preferences for the commercial flavor (Reig *et al.*, 2003). Therefore, self feeding devices proved useful to investigate the levels and composition of micro-nutrients needed in fish diets, as the fish were able to detect small differences in the levels of several micro-nutrients.

Thus, the aim of the present study was to determine dietary ARA preference of Senegalese sole G1, using two diets one with high dietary ARA (6.0% total fatty acid content, TFA) and another with low ARA content (0.7% TFA). For this, groups of Senegalese sole were offered the choice between these two diets by means of two self-feeders per tank. The use of self-feeders in preference tests imposes precautionary measures in this respect, every four or more weeks the diets placed in a feeder were switched over. The experiment was conducted during 16 months.

2. Material and methods

2.1. Fish disposal and self-feeding system

Forty eight fishes hatched and reared in captivity were randomly distributed in three circular tanks (2.1 m diameter, 2000 L), 16 fish per tank, eight females and eight males. The female fish weighed 716 ± 106g (mean ± SEM) and measured 35 ± 1 cm length and male fish were 974 ± 61g and 38 ± 1 cm length. The fish were held in a recirculation system with simulated natural fluctuations of the temperature and photoperiod for the region (40º 37' and 40º 48' N and between 0º 21' and 0º 40' E., Tarragona, Spain), reaching the maximum temperature during late summer (21 ^{o}C) and the minimum in winter (13 ^{o}C) (Fig. 1). Sixteen fish were sacrificed at the end of the experiment (8 males and 8 females) for lipids and fatty acid (FA) analysis.

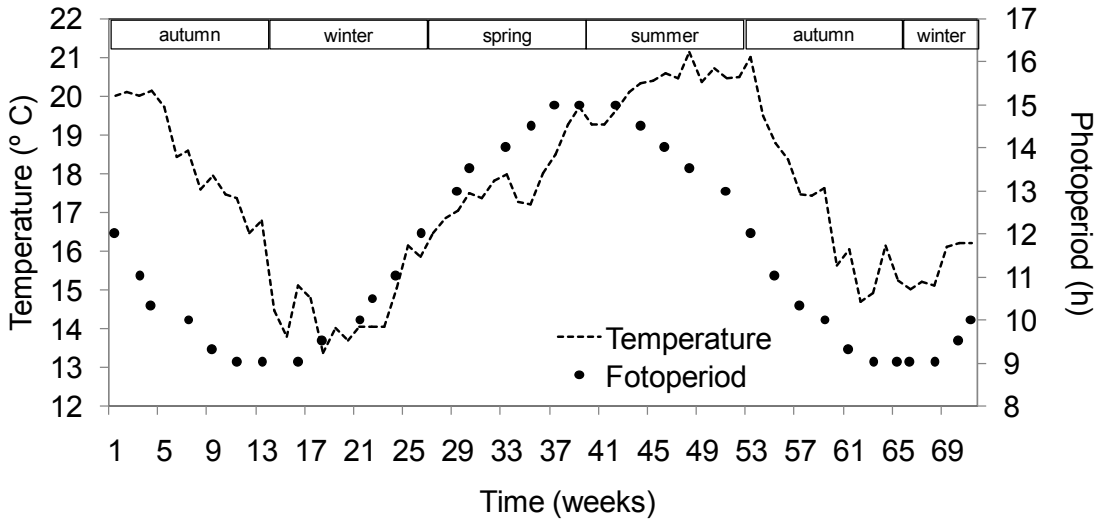

Fig. 1 *Seasonal changes of weekly water temperature fluctuations (dash line, primary axis) and photoperiod of fish tank (full circles, secondary axis). Weekly values are the mean of daily values of three tanks.*

2.1.1. Self-feeder systems

Each tank had two automated demand feeders (Fig. 2), connected to a programmable logic controller (PLC) and a computer. The PLC controller and the computer registered the demand activity of the fish (pull of the trigger) and the information was recorded in the hard disc of the computer. Prior to the experiment the fish were feed with extruded commercial diet and trained during some weeks to learn how to operate the self demand feeding system. The self demand feeding system was composed by a small hopper, engine motor, a pocket roll chamber, a distribution tube, a spring contact sensor (to avoid accidental demand signals), and an attractant 'bait' with a synthetic polychaete. The bait was placed 6 cm above the tank bottom where the feed was supplied by the distribution tube. The feed reward from each pull of the bait (trigger) was of 5-6 g of feed (Fig. 2).

The feeders were loaded every week with two diets supplied by Skretting®, Norway, with the same energy and proximate composition, i.e., 53 % of protein 12% of fat and 13% carbohydrate. Diet A, with high level of ARA, 6.0% total fatty acids (TFA) and diet B with low level of ARA, 0.7% TFA. The fatty acid profile (Table 1) of the diets was analyzed every four months (duplicate). Every week, the feed was weighed, the demanded feed was calculated and the feeders were emptied and reloaded with new feed. In order to avoid that the fish selected a feeder position rather than the diet, the feeds were switched between feeders six times during the 70 weeks of experiment, after periods of 7, 9, 12 10, 5 and 16 weeks. Changes in diet selection had no effect on total feed or energy intakes. Percentage of ARA preferred or selected in the demanded diet (*Y)* was calculated with the equation $Y= ((A \times 6.0) + (B \times \% 0.7))*100^{-1}$, where A= proportion of diet A selected multiplied by 6.0 (ARA dietary level in diet A) and B= proportion of diet B selected multiplied by 0.7 (ARA dietary level in diet B). The final feed conversion rate (FCR) was calculated with equation $FCR= F*(W_f – W_i)^{-1}$, where F is feed supply (g), W_i and W_f is the initial and final biomass (g).

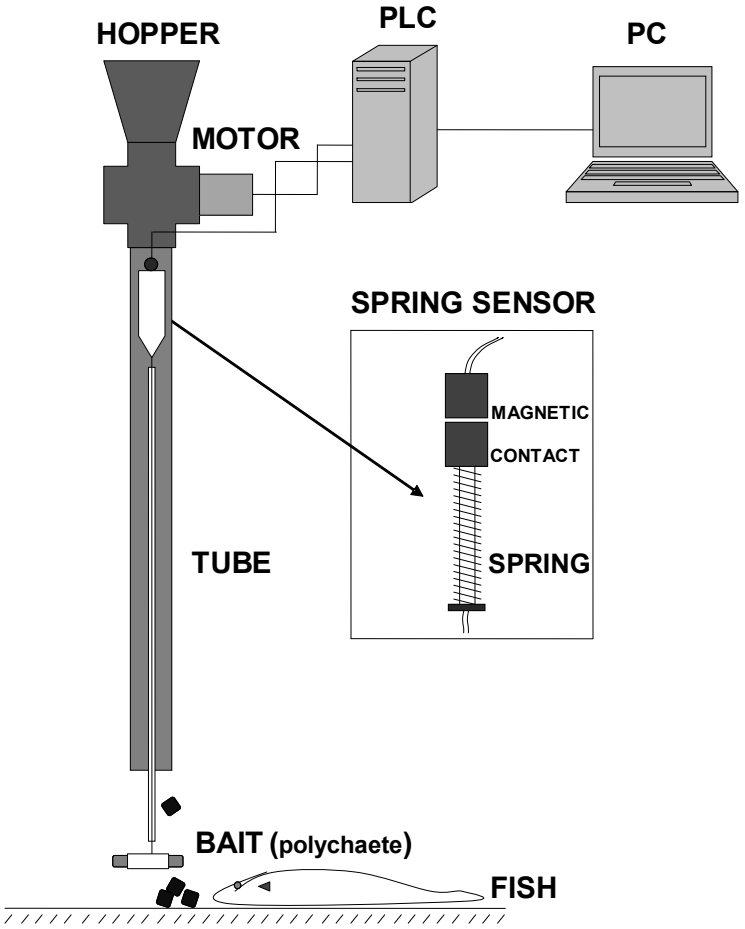

Fig. 2 *Schematic illustration of the self feeding system. The system comprises three parts. 1) The feeder (hopper, feed chambers, motor, distribution tube and bait), 2) the programmable logging controller (PLC) and the 3) computer (PC). Each time fish operate the sensor, an output signal reaches the PLC and the computer through an interface giving an order to deliver feed by the feeder. The feed demand sensor consists of a string sensor connected with bait placed at 5 cm above the bottom of the tank, through a fishing line covered by a methacrylate tube.*

Table 1 *Lipid and fatty acid content and fatty acid composition (% TFA ± SEM) of the diets with high (A) and low (B) dietary ARA content. ** Significant differences between diet A and B (ANOVA, P<0.05, N=8)*

	A	B	
TL (mg g^{-1} DW)	125.1 ± 2.9	124.2 ± 2.9	
TFA(µg mg^{-1} L)	944 ± 48	904 ± 50	
Fatty acid composition (%TFA)			
14:0	4.0 ± 1.7	2.5 ± 0.7	
16:0	16.1 ± 1.6	14.9 ± 0.6	
18:0	3.2 ± 0.2	1.8 ± 0.5	
Total SFA	23.4 ± 3.0	19.4 ± 0.9	
16:1n-7	4.4 ± 0.7	4.9 ± 0.5	
18:1n-9	15.7 ± 0.3	15.0 ± 0.8	
20:1n-9	6.6 ± 0.2	7.2 ± 0.9	
22:1n-9	2.9 ± 2.9	3.5 ± 3.5	
Total MUFA	31.1 ± 3.5	32.3 ± 6.1	
18:2n-6	7.2 ± 0.4	5.9 ± 0.4	
18:3n-6	0.6 ± 0.0	0.2 ± 0.2	
20:4n-6, ARA	6.0 ± 0.5	0.7 ± 0.2	**
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	
22:5n-6	0.0 ± 0.0	0.0 ± 0.0	
Total n-6 PUFA	14.0 ± 0.9	9.3 ± 2.0	
18:3n-3	1.2 ± 0.1	1.3 ± 0.1	
18:4n-3	1.7 ± 0.1	2.2 ± 0.2	
20:5n-3, EPA	14.7 ± 2.8	16.2 ± 4.9	
22:5n-3	2.0 ± 0.5	1.6 ± 0.2	
22:6n-3, DHA	11.3 ± 1.4	14.4 ± 1.1	
Total n-3 PUFA	31.5 ± 0.8	39.0 ± 6.3	
Total PUFA	45.5 ± 1.0	48.3 ± 6.5	
EPA/ARA	2.8 ± 0.7	32.2 ± 11.6	**
EPA/DHA	1.4 ± 0.4	1.2 ± 0.3	
DHA/ARA	2.1 ± 0.2	23.5 ± 5.2	**
n-3/n-6	2.3 ± 0.2	4.6 ± 1.3	**

TL: total lipids, L: lipids, DW: dry weigh, SFA: saturated fatty acids, MUFA: mono saturated acids, ARA: arachidonic acid, PUFA: polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

2.2. Fish sampling and lipid analysis

Sixteen fish were sacrificed after anesthesia with 0.3 ml L^{-1} Aqui-S®, iso-eugenol (50%) (Scan Aqua A.S, Årnes, Norway) by pitting. Samples of liver and gonads were collected weighted and stored at -20 °C for posterior analysis. Samples of tissues and feed were homogenized and total lipids were extracted (Folch *et al.,* 1957) and quantified by gravimetry. Fatty acid methyl esters

(FAME) prepared by acid-catalysed transmethylation (Christie 1982), and FAME extracted and purified (Tocher *et al.*, 1988). FAME were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using Helium as carrier gas (1.2 mL min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well characterised fish oil, and quantified by the response factor to the internal standard, 21:0. The results are presented as percentage of the total fatty acids (% TFA) as mean ± standard error of the mean.

2.3. Statistic analysis

Statistical differences in selection diet and fatty acid composition in tissues of males and females were analyzed by one-way ANOVA, in case of significant differences between means, it was followed by the post-hoc multiple comparison Tukey's HSD for unequal N test with a significance level (*P*) of 0.05. Moreover, correlations among ARA, feed demand and temperature were made, using *P* of 0.05. The compliance of data with normality and homogeneity of variance was tested by the Kolmogorov–Smirnov and Bartlett (Chi-Sqr) methods. Statistical effect of feeder position and diet, in the ARA selection was analyzed by two-way ANOVA. The data are expressed as mean of percentage ± standard error of mean (SEM). The statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA).

3. Results

3.1. Fish and diet performance

Once the feeders were set up as described before, the G1 Senegalese sole learned to operate the self feeding system after four weeks of training. The fish demanded a total of 39.8 ± 1.8 g day^{-1} (mean ± SEM, each tank), approx. 0.12 ± 0.01% BW day^{-1}, however periods with increase and reduction in demand were observed (Fig. 3). The demand increased in late-summer, earlier-autumn (i.e., years 2009 and 2010) followed by a period with reduced demand in late-winter and spring, which was correlated with the water temperature change (r^2=0.5) (Fig. 3). There was also an increase in fed demand in late-winter and early- spring just before the reproduction period, which was not correlated with water temperature (Fig. 3). The final FCR was 1.6 ± 0.2 and

cumulative mortality was 15%. The fish did not spawn, however swollen or highly swollen abdomen (state II and III) (Guzmán *et al.*, 2008) was observed in 60% of females.

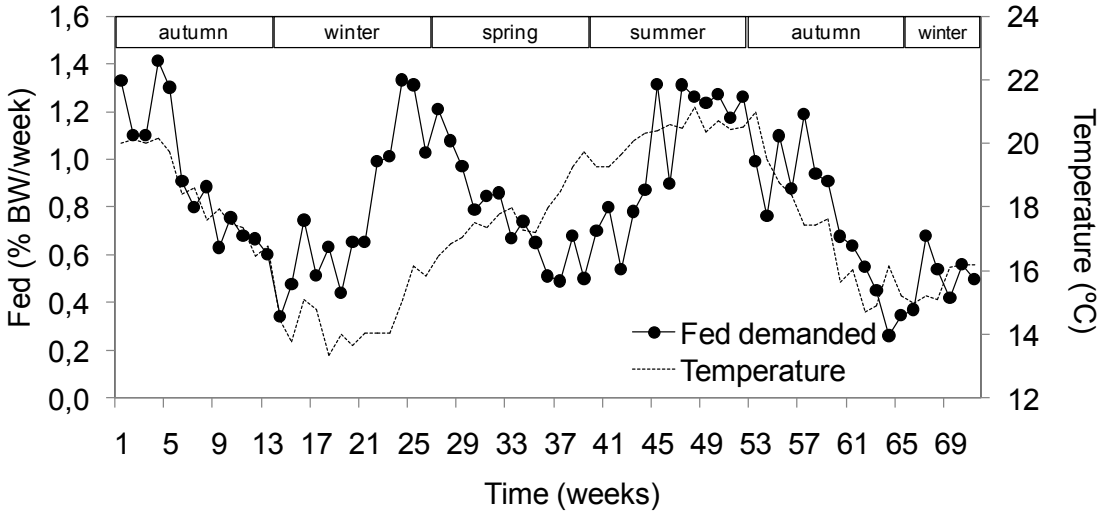

Fig. 3 *Feed demanded per week (full circles, primary axis) (mean, from three replicates) in percentage of body weight (% BW), by Senegalese sole and water temperature (dash line, secondary axis) over 70 weeks (N=3, experimental tanks).*

3.2. Diet selection

The diets placed in a feeder were reversed six times during the experiment (date of diet change between feeder is indicated by the vertical arrow above Fig. 4) to force the fish to select a diet rather than a feeder and no significant (P>0.05) effect of the feeder position in dietary ARA selection was found, thus fish selected the diet A or B rather than the feeder position.

During the 70 weeks experiment the fish changed diet preferences (high ARA and low ARA) (Figs. 4 and 5), that allowed the identification of two periods of high demand (periods A and C in Fig. 5) follow with a drastic reduction and low demand (period B and D in Fig. 5) of ARA. With all these changes in ARA demand, being correlated (r^2=0.6) with the water temperature (Fig. 5). Thus, at the beginning of the experiment (16 weeks, from autumn and earlier-winter, period A) the fish showed preference for the diet high in ARA (70% preference), demanding in average 3.9% ARA (Fig. 4 and 5). After this in winter with the temperature reduction a drastic change was observed in the fish preferences, with a high demand of low ARA diet, in winter. Although the preference for the diet high in ARA started to increase gradually when temperature started to increase in later winter, the selected level of ARA for the period later-winter and spring, B was

2.3% ARA. The water temperature progressively increase follow with a preference for the diet high in ARA and a concomitantly reduction of the diet low ARA, with a final selection of 3.9% ARA in the period summer and earlier-autumn (period C). In autumn, together with the water temperature decrease, the preference for the high ARA diet was once more drastically reduced and demand remained low until the end of the experiment, with a demand of 1.9% ARA during the final period late-autumn and winter (period D, Fig. 5). The selected level of ARA for all the experimental period was 3% TFA. Periods A and C were significantly higher than periods B and D in % ARA demanded (P<0.05).

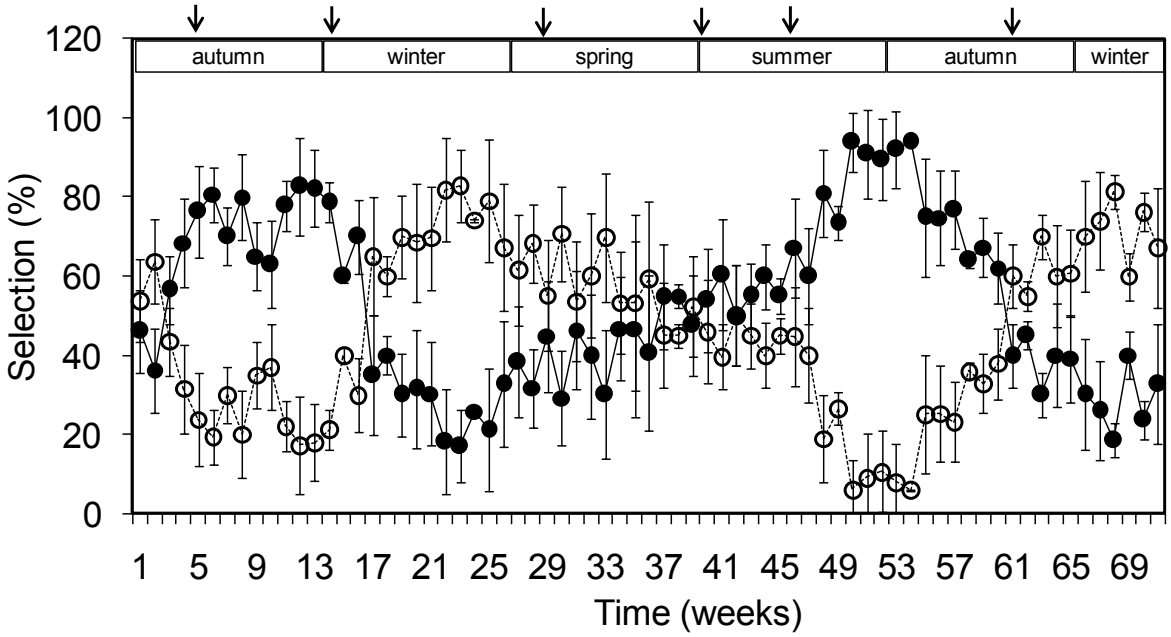

Fig. 4 *Feed demanded per week (mean ± SEM, from three replicates) for two diets (high and low in ARA) in percentage of total diet (%) consumed, by Senegalese sole over 70 weeks. Solid line represents the mean (full circles) of the diet with 6.0% ARA and dash line shows the mean (open circles) of the diet with 0.7% ARA. Vertical arrows* (↓) *show the date when the content of the feeder was switched over.*

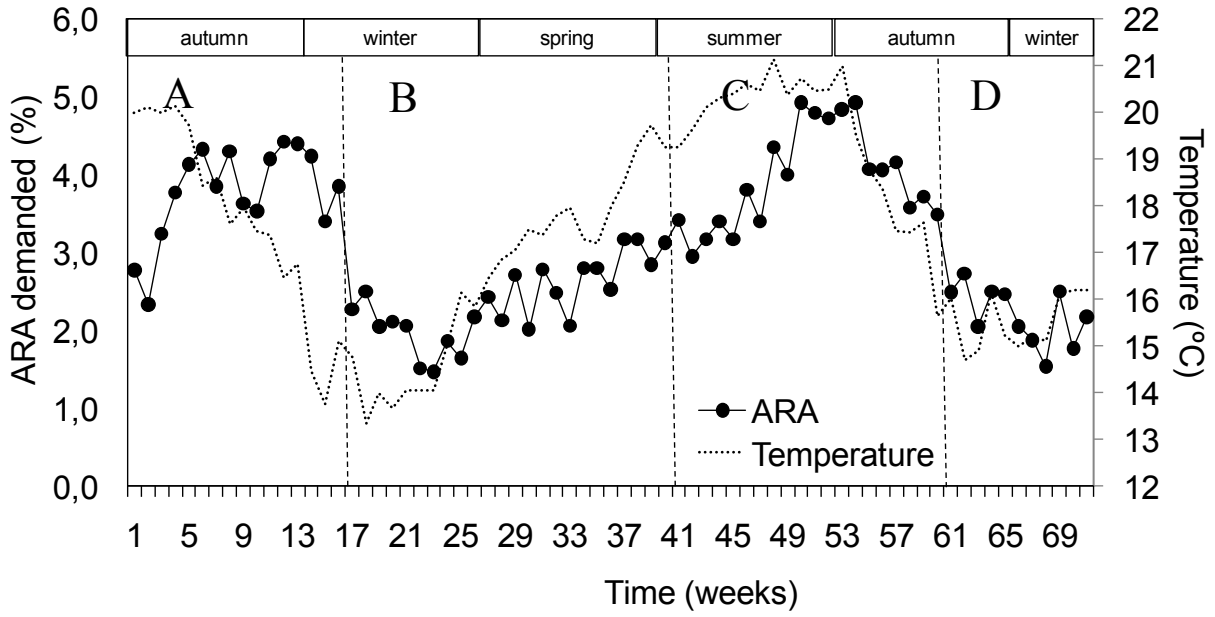

Fig. 5 *Total ARA demanded as % Total Fatty Acids in the diet per week (mean, from three replicates) by Senegelese sole over 70 weeks (full circles) and water temperature profile (dash line). Four different periods (A, B, C, D) were determined based on the relative changes in ARA demanded along the 70 weeks experiment. A and C periods with high ARA demand and B and D periods with low ARA demand. Vertical dash lines show the periods in which ARA demanded crossed the mean (3.0% ARA).*

3.3. ARA content in fish tissues

Significant (P<0.05) differences in the fatty acid composition of liver and gonads of females and males were found (Table 2), especially in the levels of n-6 PUFA. Thus, the levels of ARA were significantly higher in testis (8.75% TFA) than in the ovary (3.92%), similarly was showed in 20:4n-6 in gonad and 22:5n-6 in gonad and liver, with significantly (P<0.05) higher values in males. In liver ARA did not show differences among males and females. The differences in ARA gave a significantly (P<0.05) lower DHA/ARA ratio in the gonads of males (Table 2). Additionally, the levels of EPA in testis (4.11%) were significantly (P<0.05) higher than in the ovary (2.52%) and the liver of males (1.58%) and females (1.15%) and no differences in EPA/ARA ratios between males and females were observed. Total lipid content (mg g^{-1} DW) was significantly higher (P<0.05) in the liver compared to the gonads (P<0.05) and total fatty acid content (μg mg^{-1} lipids) was similar in liver and gonads between females and males.

Table 2 *Lipid and fatty acid content and fatty acid composition (% TFA ± SEM) of the liver and gonads of Senegalese sole (Solea senegalensis) females and males fed with self-feeder systems. Columns assigned different letters were significantly different (ANOVA, P<0.05, N=8)*

	Liver		Gonad	
	Female	Male	Female	Male
TL (mg g^{-1} DW)	282.4 ± 3.12 ^{a}	287.1 ± 41.2 ^{a}	118.0 ± 2.7 ^{b}	106.3 ± 4.2 ^{b}
TFA(µg mg^{-1} L)	685.5 ± 66.9	641.1 ± 45.8	514.3 ± 43.8	531.5 ± 86.8
Fatty acid composition (%TFA)				
14:0	1.98 ± 0.50	1.80 ± 0.28	1.40 ± 0.32	0.59 ± 0.15
16:0	17.43 ± 0.83	15.82 ± 1.01	16.49 ± 2.74	17.68 ± 0.86
18:0	3.07 ± 0.08 ^{a}	3.21 ± 0.33 ^{a}	4.34 ± 0.14 ^{a}	6.70 ± 0.46 ^{b}
Total SFA	22.88 ± 1.15	21.21 ± 1.26	24.53 ± 1.21	25.10 ± 1.17
16:1	6.70 ± 0.49 ^{a}	4.78 ± 0.39 ^{a}	4.01 ± 0.32 ^{ab}	2.62 ± 0.11 ^{b}
18:1n-9	19.76 ± 1.59 ^{a}	14.62 ± 1.09 ^{b}	15.17 ± 0.58 ^{ab}	13.26 ± 0.45 ^{b}
18:1n-7	4.39 ± 0.22 ^{a}	3.74 ± 0.32 ^{a}	3.30 ± 0.06 ^{a}	6.24 ± 0.42 ^{b}
20:1n-9	2.83 ± 0.14 ^{a}	3.08 ± 0.36 ^{a}	3.29 ± 0.20 ^{a}	1.49 ± 0.36 ^{b}
22:1	1.44 ± 0.30 ^{ab}	2.82 ± 0.43 ^{a}	1.76 ± 0.20 ^{ab}	0.57 ± 0.23 ^{b}
Total MUFA	35.12 ± 1.70 ^{a}	27.98 ± 2.73 ^{ab}	27.53 ± 0.68 ^{b}	23.27 ± 1.50 ^{b}
18:2n-6	9.38 ± 0.50 ^{a}	7.61 ± 0.63 ^{ab}	6.74 ± 0.18 ^{b}	6.90 ± 0.63 ^{b}
20:4n-6, ARA	2.54 ± 0.31 ^{a}	2.41 ± 0.35 ^{a}	3.92 ± 0.34 ^{a}	8.75 ± 0.89 ^{b}
22:4n-6	0.71 ± 0.04 ^{ab}	0.77 ± 0.06 ^{ab}	0.49 ± 0.05 ^{a}	0.89 ± 0.10 ^{b}
22:5n-6	0.23 ± 0.03 ^{b}	0.51 ± 0.07 ^{a}	0.10 ± 0.05 ^{c}	0.22 ± 0.07 ^{b}
Total n-6 PUFA	13.42 ± 0.87 ^{a}	11.76 ± 0.80 ^{a}	11.74 ± 0.25 ^{a}	17.17 ± 1.13 ^{b}
18:3n-3	1.01 ± 0.10	0.74 ± 0.10	0.94 ± 0.06	0.60 ± 0.17
18:4n-3	0.91 ± 0.11 ^{a}	0.50 ± 0.08 ^{b}	0.71 ± 0.04 ^{a}	0.16 ± 0.07 ^{b}
20:4n-3	0.76 ± 0.08	0.74 ± 0.20	0.71 ± 0.04	0.36 ± 0.05
20:5n-3, EPA	1.15 ± 0.26 ^{a}	1.58 ± 0.41 ^{a}	2.52 ± 0.35 ^{a}	4.11 ± 0.43 ^{b}
22:5n-3	5.32 ± 0.56	6.80 ± 0.65	5.04 ± 0.36	4.76 ± 0.40
22:6n-3, DHA	18.28 ± 1.31 ^{a}	23.01 ± 1.44 ^{ab}	25.24 ± 1.02 ^{b}	23.02 ± 1.70 ^{ab}
Total n-3 PUFA	27.68 ± 1.90	33.72 ± 2.02	35.33 ± 1.68	33.08 ± 2.29
Total PUFA	41.10 ± 2.55	46.23 ± 2.15	47.07 ± 1.76	50.24 ± 1.93
EPA/ARA	0.41 ± 0.09	0.73 ± 0.23	0.65 ± 0.08	0.50 ± 0.08
EPA/DHA	0.06 ± 0.01	0.05 ± 0.01	0.10 ± 0.01	0.18 ± 0.01
DHA/ARA	7.79 ± 1.04 ^{a}	8.22 ± 1.77 ^{a}	6.61 ± 0.43 ^{a}	2.76 ± 0.30 ^{b}
n-3/n-6	2.07 ± 0.10	2.26 ± 0.45	3.01 ± 0.14	1.99 ± 0.23

Abbreviation as in the table 1.

4. Discussion

The results obtained in the present study clearly showed that Senegalese sole had significant different ARA preference during the year, well correlated with the seasonal water temperature changes (r^2=0.65). Thus, periods of high and low demand of ARA and periods of high and low food ingestion, were showed without any correlation between both ARA and total fed demand (r^2=0.07). With a mean ARA demand of 3.0% TFA for the entire experimental period of 16 months. Moreover ARA as well as EPA was significantly higher in the gonads of male compared to females, without changes in EPA/ARA ratios. Senegalese sole is a seasonal spawner which reaches sexual maturity at 3-4 years of age in both males and females. During early spring (April-May) gonad maturation occurs in parallel to the increase in plasma sex steroids and luteinizing hormone with maximum levels reached just before spawning (Guzmán *et al.*, 2008, Guzmán *et al.*, 2009, García-López *et al.*, 2007). The results of the present study showed that the preference of Senegalese sole for ARA increased gradually, in parallel to the water temperature increase, starting in late winter (12ºC) and reaching the maximum demand in late-summer to early-autumn (21ºC) with a posterior drastic diminution of ARA demand in early winter, together with the drop in water temperature. These seasonal variations in ARA demanded by Senegalese sole are in agreement with the fatty acid composition of the tissues of wild common sole (*Solea solea*). Gökçe *et al.* (2004), studied the seasonal variation in muscle fatty acid composition of wild common sole and observed a pronounced seasonal fluctuation in ARA with the highest values found in the fish of winter (12.3% TFA) and a significant reduction in ARA levels in the fish sampled in spring (6.0% TFA) just after the reproduction period which commonly occurs in winter (Mediterranean sea), for this species (Ramos, 1986, Imsland *et al.*, 2003). Previous studies showed that fish stored ARA first in gonad and liver and then in muscle (Pérez *et al.*, 2007, Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Huang *et al.*, 2010) (chapter II), therefore, the seasonal variation showed in muscle of common sole (Gökçe *et al.*, 2004) might be indication a previous FA storage in gonad and liver (before autumn). Studies on white seabream (*Diplodus sargus*) broodstock showed that ARA was stored in the gonad and liver, during the summer with the lowest accumulation in the spring (Pérez *et al.*, 2007), while in muscle the highest ARA storage was observed in late-autumn and the lowest in summer (Özyurt *et al.*, 2005). Although, studies on anchovy (*Engraulis encrasicholus*) showed that ARA was stored during spring in gonad, then in liver in later summer, and in muscle in winter (Tufan

et al., 2010). These studies showed that seasonal FA variation was different between tissues and fish species, as has been shown in species such as horse-mackerel (*Trachurus trachurus*) (Bandarra *et al.*, 2001), anchovy (*Engraulis encrasicolus*) (Zlatanos *et al.*, 2007, Tufan *et al.*, 2010), gilthead seabream (*Sparus aurata*), white seabream (*Diplodus sargus*) (Özyurt *et al.*, 2005), sardine (*Sardina pilchardus*) (Bandarra *et al.*, 1997, Zlatanos *et al.*, 2007), picarel (*Spicara smaris*) (Zlatanos *et al.*, 2007) and vimba (*Vimba vimba*) (Kalyoncu *et al.*, 2009). Further studies are required to elucidate the seasonal fatty acid content in wild Senegalese sole fish tissues and the relationship between this seasonal changes and the food intake or food composition.

The present study found a period of maximum food intake in G1 is in late-summer and autumn in years, 2009 and 2010, with a gradually posterior decline during winter, with water temperature correlated with these periods of food intake (r^2=0.7). However, before spring there was a peak in food intake with a posterior decline, which was not correlated with water temperature (r^2=0.2). The spring peak in food intake, seems to be related to the increase of energetic reserves required before spawning, that has been observed in April-May, for *S. sole* (Guzmán *et al.*, 2009), however, after this peak, during the spawning period the fish reduced food intake and probably essential fatty acids and other nutrients needed for ovarian growth were taken from stored reserves. The results showed a daily feed intake of 0.12% BW day^{-1}, whereas in broodstock management 0.7-1.0% BW day^{-1} is commonly used. However *S. sole* reduce significantly the fed demand (0.07% BW day^{-1}) during the breeding period, therefore, gonadal lipid content and composition was probably derived from the endogenous reserves of the liver and less from the diet. Similar observations were found for gilthead seabream broodstock (Martín *et al.*, 2009) and female rainbow trout, that mobilized mainly carcass and visceral lipid reserves (Nassour *et al.*, 1989). Freshwater catfish use abdominal fat as the major energy source for sexual maturation (Lal *et al.*, 1987), while farmed Atlantic salmon use both muscle lipid and protein (Aksnes et al., 1986) and Atlantic cod use lipid reserves accumulated in the liver during the autumn and early winter (Tocher *et al.*, 1988). Moreover, the reduction in food intake during sexual maturation coincided with increase in plasma sex steroids previously showed in *S. sole* (Guzmán *et al.*, 2009, Norambuena *et al.*, 2011a) (chapter IV), that have been shown to affect the food intake, feed efficiency and growth rates (Volkoff *et al.*, 2005, Leal *et al.*, 2009). According to the observations in wild Senegalese sole seasonal fluctuations also exist in food intake, in the winter

(pre-spawning period), full stomachs and intestines were recorded (Garcia *et al.*, 1996, Castelo-Branco *et al.*, 2010). Previous studies carried out in *S. sole* showed a higher lipid accumulation in the liver and muscle and an increase in the hepatosomatic index of G1 fish compared to wild counterparts (Norambuena *et al.*, 2011b) (chapter II), which suggested either a dietary imbalance and/or overfeeding as the causative factors for this high lipid accumulation in cultured fish (Norambuena *et al.*, 2011b). The essential fatty acid content of the diet has been shown to affect the reproduction of both male and female marine fishes (Izquierdo *et al.*, 2001). ARA is the main precursor of reproductive PGs involved in testosterone production and the synchronization of courtship during spawning (Sorensen *et al.*, 2004, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995, Kobayashi *et al.*, 2002). Therefore, the need and utilization of ARA might change during the year and especially during maturation and spawning of broodstock fish. However, the current experiment was not evaluating PGs production and more studies are needed to determine possible seasonal changes in PGs profiles along the year.

Female Senegalese sole developing larger gonads as compared to males with 5.7% and 0.3% body weight respectively (chapter II). Further, there is a requirement for ARA in larval offspring which is mainly supplied in the egg (Villalta *et al.*, 2005). The present results showed that ARA was significantly higher in testis than ovary. However, considering the large weight of ovary compared with testis (i.e., ovary 27 g and testis 0.25 g, dry weight), the total ARA content in ovaries was approximately 70 mg, meanwhile in testis only 1.2 mg. This suggests that females need more ARA than males, although more studies will be required to clarify this assessment. The EPA content in both experimental diets was 14 and 16% in high and low ARA, respectively. Therefore, the EPA/ARA ratio observed in the diets was 32 in the diet low in ARA and 2.8 in the diet high in ARA, however, in fish tissues was not higher than 0.57 (i.e., 56- and 4.8-fold less than the ratios observed in the diets). These indicate that the ratios EPA/ARA used in the diet were well above fish requirement. EPA dietary level was approximately 15%, while in the fish the levels observed were 1.2-4.1% TFA. These observations are in agreement with other results, showing high EPA/ARA ratios in cultured fish compared to wild ones (Cejas *et al.*, 2003, Lund *et al.*, 2008, Norambuena *et al.*, 2009). Thus, the results suggest that beside the dietary requirement of ARA it is necessary to reduce the levels of dietary EPA in order to improve the nutritional state of the G1 Senegalese sole. However, more studies will be required to elucidate the optimal dietary level of EPA during the reproduction cycle of Senegalese sole.

5. Conclusion

By using a self-feeder the dietary ARA preference for Senegalese sole G1 fish was determined. A seasonal fluctuation in the selection of ARA that was correlated (r^2=0.65) with water temperature was observed, with an average demand of 3.0% TFA during the 16 months of the experiment. Content of ARA was significantly higher in testis (8.8% TFA) compared with ovaries (3.9% TFA). Therefore, the dietary preference may be different between males and females, considering the different tissue levels and the large differences in weight between ovaries and testis. Further studies are needed to clarify these differences in ARA demand according to the sex of the fish and dietary EPA.

6. Acknowledgments

We gratefully thank Francisco Javier Sánchez-Vásquez and Alicia Estevez for scientific help and Grethe Rosenlund from Skretting ARC, Norway for diets manufacturing. Thanks are also given to Karl Andree for English help and Gloria Macia and Noelia Gras for technical support. This research was funded by two projects: 1) Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project Lenguado and 2) Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by N. Duncan and the PhD grant awarded to F. Norambuena by Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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Chapter VI

Dietary ARA level in *S. Sole*

DIETARY LEVELS, STORAGE AND METABOLISM OF ARACHIDONIC ACID (20:4n-6, ARA) IN G1 BROODSTOCK OF SENEGALESE SOLE (*SOLEA SENEGALENSIS*)

Abstract

Previous studies on Senegalese sole showed a significantly higher accumulation of arachidonic (20:4n-6, ARA) acid in testis, liver, and muscle of wild fish compared to cultured G1 fish. ARA is the main chief precursor for production of 2-series prostaglandins (PGs), which are involved in testicular steroidogenesis and follicle maturation in fish and act hormonally to trigger female sexual behaviour. The present study was conducted to establish the optimal dietary ARA levels for G1 Senegalese sole broodstock. The fish were fed six experimental diets with graded ARA levels for nine months. Diets A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA. The expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts was assessed in the liver of fish from groups A, C and F to examine the effects of ARA on the elongation and desaturation capacity of the fish. At the end of the feeding period fish were sacrificed and gonad, liver and muscle analysed and the ARA content compared with that of wild fish. The result showed that the relative ARA levels in G1 tissues, increased in a dose dependent manner in both sexes. The ARA was rapidly transferred and conserved in testis and ovary, followed by liver and muscle. Thus, fish fed 2.3% and 3.2% ARA showed no differences in the composition of testis, ovary and liver with wild fish. The expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts also increased in response to dietary ARA content in males, showing a pattern of up-regulation in both transcripts. Thus, the levels of *d4fad* transcripts were significantly higher in the liver of males from groups C and F and *elovl5* in group F with a subsequent accumulation of 22:4n-6 and 22:5n-6 acids as a consequence of the ARA elongation and 22:4n-6 desaturation. Most of the other PUFA did not show changes in tissue content, although in the case of EPA it was significantly lower in the tissues (2-4% TFA) than in the diets (13-16% TFA). In conclusion in feeding period shorter than nine months could be suggested the use of 3.2% ARA enriched diets.

1. Introduction

One of the most important nutritional factors for successful fish reproduction and among the best studied fatty acids is arachidonic acid (20:4n-6, ARA) (Sargent *et al.*, 1999, Izquierdo *et al.*, 2001, Falk-Petersen *et al.*, 1989, Mazorra *et al.*, 2003, Furuita *et al.*, 2003, Alorend, 2004, Meunpol *et al.*, 2005, Pérez *et al.*, 2007, Tocher, 2010). The ARA is the main chief precursor for production of 2-series prostaglandins (PGs) (Smith, 1989, Smith *et al.*, 2002, Tocher, 2003), which stimulate ovarian and testicular steroidogenesis and follicle maturation in teleost fish and act hormonally to trigger female sexual behaviour and milt production in males (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993, Mercure *et al.*, 1995, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001, Kobayashi *et al.*, 2002, Sorensen *et al.*, 2004). Although ARA is the main precursor of PGs, these metabolites can also be produced from eicosapentaenoic acid (20:5 n–3, EPA) (Smith, 1989, Tocher, 2003). The EPA-metabolites had modest effects on basal testosterone production in gold fish testis but blocked the ARA-derived steroid production and cAMP formation due to competition for the same enzyme (Wade *et al.*, 1994, Bell *et al.*, 1994, Mercure *et al.*, 1995). Arachidonic acid itself and its metabolites also regulated cholesterol (CHOL) transfer from outer to inner mitochondrial membrane where the P450 enzyme resides to initiate steroid hormone synthesis (Mercure *et al.*, 1995). Moreover, ARA had differential effects on steroid biosynthesis, it stimulates testosterone production by elevating cAMP levels in a dose dependent manner, but at high doses ARA can inhibit steroidogenesis, in spite of elevated cAMP, by affecting the availability of CHOL (Mercure *et al.*, 1995, Mercure *et al.*, 1996). Studies on Senegalese sole showed a significantly higher accumulation of ARA in testis (7.8% TFA), liver (2.6-3.0% TFA), and muscle (4.0-4.9% TFA) of wild fish compared to those obtained from aquaculture (G1), with differences in the testis and male liver possibly having particular relevance to reproduction (Norambuena *et al.*, 2008, Norambuena *et al.*, 2009, Norambuena *et al.*, 2011c) (chapter II). Similar ARA and ARA-derived fatty acids accumulation in wild fish tissues compared with cultured fish has been observed in other fish species like common sole (*Sole solea*) (Lund *et al.*, 2008), Atlantic turbot (*Scophthalmus maximus*) (Silversand *et al.*, 1996), white seabream (*Diplodus sargus*) (Cejas *et al.*, 2003, Cejas *et al.*, 2004), black seabream (*Dicentrarchus labrax*) (Rodríguez *et al.*, 2004), striped bass (*Morone saxatilis*) (Harrell *et al.*, 1995), macquarie perch

(*Macquaria australasica*) (Sheikh-Eldin *et al.*, 1996), Atlantic cod (*Gadus morhua*) (Salze *et al.*, 2005) and rainbow trout (*Onchorhynchus mykis*) (Blanchet *et al.*, 2005, Aslan *et al.*, 2007). Furthermore, high accumulation of ARA in fish sperm has also been reported, sperm from rainbow trout (*Oncorhynchus mykiss*) fed diets low in 22:6n-3 (DHA) and ARA had 10% ARA of total fatty acids (Vassallo-Agius *et al.*, 2001), and in seabass, which showed significantly higher accumulation of ARA in wild fish sperm (5.2%) compared with sperm from G1 fish (2.3%) (Bell *et al.*, 1996). Previous studies in Senegalese sole showed that differences in the content of ARA in fish tissues gave differences in cyclooxygenase gene expression (COX-2), which was significantly up regulated in sperm duct and oviduct and gills of males from wild fish compared to cultured G1 fish (Norambuena *et al.*, 2011d) (chapter III). Thus, wild fish showed significantly higher levels of 2-series PGs compared to cultured fish (i.e., testis), whereas G1 Senegalese sole that had lower ARA content in some tissues exhibited significantly higher levels of 3-series PGs (Norambuena *et al.*, 2011d) (chapter III) and lower levels of CHOL in the liver (Norambuena *et al.*, 2011c) (chapter II), being CHOL the precursor of steroid hormones in vertebrates (Baron *et al.*, 1997). Studies on reproduction have shown that G1 cultured Senegalese sole failed to spawn viable eggs, contrary to wild counterparts held in captivity which spawn regularly producing eggs of sufficient quality and quantity (Carazo *et al.*, 2009, Carazo *et al.*, 2011). Putting together the low ARA accumulation in fish tissues, the changes in COX-2 expression, and the lower production of PGs and CHOL a cumulative effect might be responsible of the reproductive failure of G1 fish. On the other hand, it has been shown that Senegalese sole fed artificial diets formulated with graded ARA levels showed an increase in blood ARA concentration, which in turn induced an increase in PGs, CHOL and steroid production especially in males (Norambuena *et al.*, 2011a) (chapter IV). Higher levels of ARA in the tissues of wild fish and increased levels in the blood of G1 fish fed high dietary ARA levels had as a consequence an increase in ARA-derived fatty acids, adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) acids (Norambuena *et al.*, 2011a) (chapter IV). In the case of wild males compared to G1 fish, 22:4n-6 was 3.9 and 8.9-fold higher respectively in testis and muscle and 22:5n-6 was aprox. 3.5-fold higher in both testis and muscle (Norambuena *et al.*, 2011c) (chapter II). A similar effect was observed in the blood of G1 Senegalese sole fed high dietary ARA levels (Norambuena *et al.*, 2011a) (chapter IV)

and in the sperm of wild sea bass (Bell *et al.*, 1996). These polyunsaturated fatty acids (PUFAs) are present in the cells of the reproductive (i.e., seminiferous tubules, sperm) and nervous tissues in larger quantities than those reported in other tissues of fish (Ahaluwalia *et al.*, 1969, Tinoco, 1982, Picardo *et al.*, 1990, Lenzi *et al.*, 1996) and mammals (Bridges *et al.*, 1970, Ayala *et al.*, 1973). Although the physiological function of these PUFAs in sperm is not well known, in mammals they are considered indicators of normal testicular development, spermatogenesis, germ cells population and fertility, especially 22:5n-6 acid (Leat *et al.*, 1983, MacDonald *et al.*, 1984, Lenzi *et al.*, 1996, Lenzi *et al.*, 2000, Furland *et al.*, 2007). It was suggested that C22 and C24 PUFAs are using as a dynamic chemical repository sources of major PUFA required in germ cells (Furland *et al.*, 2007). However, was also showed that C22 PUFA were involved in sperm formation and transportation in rat testicle (Ayala *et al.*, 1973), being required when spermatozoa pass from the caput epididymis to the caudal region and in the fertilization process (Lenzi *et al.*, 1996).

The ARA and 22:5n-6 synthesis in marine fish occurs through elongation and desaturation of shorter precursors at very low levels (Linares *et al.*, 1991) and wild fish obtain these n-6 PUFAs from the diet. Most biologically active essential fatty acids (EFAs) such as 20:4n-6, 20:5n-3 and 22:6n-3 acids can be synthesized by mammals and some freshwater fishes; however, carnivores and marine fish have very limited ability to synthesize PUFA from their precursors 18:2n-6 and 18:3n-3 acids (Rivers *et al.*, 1975, Sprecher, 2000, Tocher, 2010). Synthesis of ARA is achieved by delta Δ6 desaturation of 18:2n-6 to produce 18:3n-6 that is elongated to 20:3n-6 followed by Δ5 desaturation to get 20:4n-6 and then it is elongated by *Elovl5* to produce 22:4n-6. However, to obtain 22:5n-6, two further elongations to 24:4n-6, a second Δ6 desaturation to 24:5n-6 and a peroxisomal chain shortening step to 22:5-6 is required similarly to DHA synthesis (Cook *et al.*, 2004). Interaction of n-3 and n-6 fatty acid desaturation occurs among the fatty acids with the same carbon length (Cook *et al.*, 2004), with the affinity of the enzymes, especially the desaturases higher for the n-3 than for the n-6 series (Tocher, 2003). Marine fish have a dietary requirement for preformed C20 and C22 PUFA (Rivers *et al.*, 1975, Sprecher, 2000, Tocher, 2010). Recently, it has been demonstrated that desaturation of 22:4n-6 to 22:5n-6, and 22:5n-3 to 22:6n-3, may be carried out by a shorter pathway in which desaturase Δ4 is involved (Li *et al.*, 2010). This represents a new functional characterization of a vertebrate

Fad with Δ4 activity and demonstrates that there is more than one possible pathway for the synthesis of 22:5n-6 and 22:6n-3 in vertebrates (i.e., see 'sprecher pathway' in Sprecher, 2000). Recently Morais *et al.* (2011, unpublished) have cloned and characterized the enzymes fatty acyl desaturase and elongase corresponding to a *Δ4fad* and *elovl5* with the potential to catalyze the final steps of 22:5n-6 and 22:6n-3 acids synthesis in Senegalese sole (Morais *et al.*, 2011).

Considering all the observations cited above, a study using six groups of Senegalese sole fed a standard commercial diet with six graded ARA levels during nine months (A=0.7, B=1.6, C=2.3, D=3.2, E=5.0 and F=6.0% total fatty acid) was conducted in order to:

1) Determine the optimal dietary ARA level for G1 Senegalese sole, by comparing the ARA content in gonads, liver and muscle obtained after feeding the fish with six experimental diets with the fatty acid profile of tissues obtained from wild fish (Norambuena *et al.*, 2011c) (chapter II, Table 1).

2) To examine a possible differential nutritional regulation in the expression of fatty acyl desaturase (*Δ4fad*) and elongase (*elovl5*) in the liver of G1 Senegalese sole fed with three dietary ARA levels (diets A, C and F, cited above) and study the accumulation of 22:4n-6 and 22:5n-6 acids in the tissues.

2. Materials and methods

2.1. Fish and diets

One hundred and twenty Senegalese sole (four years old and mean 524 ± 11 g), that had been reared in captivity were PIT tagged (AVID, UK) and sexed using a heterologous vitellogenin ELISA for European seabass (*Dicentranchus labrax*) and validated for Senegaleses sole (Mañanós *et al.*, 1994). The fish were distributed among six experimental tanks (10 fish per tank) and fed during nine months, from September 2009 until May 2010, six experimental diets (54% dietary protein and 12% of total lipids) with different ARA content: diet A= 0.7 (control), B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% of total fatty acids (Table 2), the six groups were duplicated. The fish were held in a recirculation system with a simulated natural photoperiod and temperature (40º 37' and 40º 48' N and between

0º 21' and 0º 40' E., Tarragona, Spain), with minimal temperature during two weeks in January - February (13ºC) and twelve weeks maximal during June - September (21°C). The fish were fed six day per week a daily ration of 0.15- 0.3% body weight.

Table 1 *Total lipid (TL) and essential fatty acid composition (% TFA ± SEM) of gonads, liver and muscle of wild Senegalese sole (Solea senegalensis) males and females*

	Gonad		Liver		Muscle	
	Male (N=11)	Female (N=9)	Male (N=11)	Female (N=9)	Male (N=11)	Female (N=9)
TL (mg g^{-1} DW)	155 ± 12	139 ± 10	211 ± 13	180 ± 9.6	32.1 ± 2.0	32.9 ± 2.8
TFA (µg mg^{-1} L)	405 ± 25	527 ± 34	527 ± 31	561 ± 36	538 ± 23	588 ± 35
Fatty acid composition (%TFA)						
20:4n-6, ARA	7.8 ± 0.4	2.6 ± 0.1	3.0 ± 0.2	2.6 ± 0.2	4.9 ± 0.3	4.0 ± 0.2
22:4n-6	1.2 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	2.2 ± 0.2	1.3 ± 0.1
22:5n-6, DPA	1.0 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	2.4 ± 0.2	1.6 ± 0.1
20:5n-3, EPA	7.5 ± 0.3	6.6 ± 0.4	2.6 ± 0.3	3.3 ± 0.4	6.5 ± 0.2	5.9 ± 0.4
22:6n-3, DHA	16.9 ± 0.5	20.3 ± 0.7	23.9 ± 1.3	24.8 ± 1.1	32.5 ± 1.5	29.5 ± 1.9
EPA/ARA	1.0 ± 0.1	2.7 ± 0.2	0.8 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.0
EPA/DHA	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
DHA/ARA	2.3 ± 0.1	8.2 ± 0.5	7.5 ± 0.9	10.3 ± 1.2	6.8 ± 0.4	7.6 ± 0.3
n-3/n-6	2.6 ± 0.1	6.1 ± 0.4	4.7 ± 0.5	6.2 ± 0.6	4.5 ± 0.3	5.3 ± 0.2

TL: Total lipids, L: lipids, DW: dry weight, TFA: total fatty acids, ARA: arachidonic acid, DPA: docosapentaenoic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Table 2 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of the diets used (A, B, C, D, E and F) for feeding G1 Senegalese sole (Solea senegalensis). Columns assigned different letters were significantly different (ANOVA, P<0.05, N=3)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	121 ± 2.9	122 ± 2.2	130 ± 2.2	128 ± 4.9	124 ± 1.5	120 ± 2.9
TFA(μg mg^{-1} L)	880 ± 87	702 ± 34	820 ± 74	861 ± 82	961 ± 19	911 ± 94
Fatty acid composition (%TFA)						
14:0	2.5 ± 1.2	3.6 ± 2.8	3.8 ± 2.1	3.6 ± 2.3	3.7 ± 2.8	4.0 ± 2.9
16:0	14.9 ± 1.1	15.3 ± 3.7	17.6 ± 2.6	15.8 ± 1.8	15.0 ± 2.8	16.1 ± 2.7
18:0	1.8 ± 0.9	2.4 ± 0.9	2.6 ± 0.7	2.6 ± 0.6	3.0 ± 0.7	3.2 ± 0.4
Total SFA	19.4 ± 1.5	21.7 ± 5.7	24.4 ± 4.2	22.3 ± 3.6	21.9 ± 4.8	23.4 ± 5.2
16:1n-7	4.9 ± 0.8	4.8 ± 2.0	5.0 ± 0.8	4.9 ± 0.8	4.3 ± 1.8	4.4 ± 1.2
18:1n-9	15.0 ± 1.3	15.7 ± 2.4	16.9 ± 1.9	15.9 ± 2.0	15.1 ± 2.7	15.7 ± 0.6
18:1n-7	1.2 ± 2.0	0.9 ± 1.6	1.0 ± 1.7	0.8 ± 1.4	0.8 ± 1.4	1.0 ± 1.7
20:1n-9	7.2 ± 1.5	7.1 ± 0.3	7.2 ± 1.1	6.3 ± 0.5	6.9 ± 0.7	6.6 ± 0.4
22:1n-9	3.5 ± 6.0	3.9 ± 6.7	2.9 ± 5.0	3.0 ± 5.2	3.9 ± 6.7	2.9 ± 5.1
Total MUFA	32.3 ± 11	32.7 ± 4.7	33.4 ± 4.4	31.3 ± 4.9	31.4 ± 5.5	31.1 ± 6.0
18:2n-6	5.9 ± 0.7	6.4 ± 0.7	6.0 ± 0.9	6.6 ± 0.4	5.9 ± 0.1	7.2 ± 0.7
20:4n-6, ARA	0.7 ± 0.3 ^{c}	1.6 ± 0.6 ^{c}	2.3 ± 0.8 ^{bc}	3.2 ± 0.7 ^{b}	5.0 ± 0.6 ^{a}	6.0 ± 0.1 ^{a}
22:4n-6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-6	0.3 ± 0.6	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.2	0.3 ± 0.4
Total n-6 PUFA	9.3 ± 3.4	8.4 ± 1.3	8.9 ± 1.5	10.6 ± 0.8	12.4 ± 1.0	14.0 ± 1.6
18:3n-3	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
18:4n-3	2.2 ± 0.3	2.0 ± 0.1	1.8 ± 0.3	2.0 ± 0.1	1.8 ± 0.2	1.7 ± 0.2
20:4n-3	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.1
20:5n-3, EPA	13.0 ± 8.4	16.8 ± 5.3	15.9 ± 5.4	16.4 ± 5.4	14.8 ± 6.0	14.7 ± 4.9
22:5n-3	1.6 ± 0.4	3.0 ± 2.3	2.0 ± 1.0	2.3 ± 1.3	4.6 ± 5.7	2.0 ± 0.9
22:6n-3, DHA	14.4 ± 2.0	13.0 ± 2.1	11.3 ± 2.5	13.0 ± 1.6	11.1 ± 0.6	11.3 ± 2.3
Total n-3 PUFA	39.0 ± 11	37.1 ± 0.7	33.4 ± 2.9	35.8 ± 2.1	34.3 ± 1.9	31.5 ± 1.4
Total PUFA	48.3 ± 11	45.5 ± 1.4	42.3 ± 2.6	46.4 ± 1.4	46.6 ± 2.0	45.5 ± 1.7
EPA/ARA	23.6 ± 18 ^{a}	12.4 ± 6.6 ^{a}	7.8 ± 4.2 ^{ab}	5.5 ± 2.5 ^{ab}	3.0 ± 1.3 ^{b}	2.4 ± 0.8 ^{b}
EPA/DHA	0.9 ± 0.5	1.3 ± 0.6	1.5 ± 0.8	1.3 ± 0.5	1.3 ± 0.6	1.4 ± 0.7
DHA/ARA	23.5 ± 9.0 ^{a}	8.8 ± 1.6 ^{b}	5.1 ± 1.1 ^{b}	4.1 ± 0.5 ^{b}	2.2 ± 0.3 ^{b}	1.9 ± 0.4 ^{b}
n-3/n-6	4.6 ± 2.3	4.5 ± 0.7	3.8 ± 0.8	3.4 ± 0.4	2.8 ± 0.3	2.3 ± 0.3

Abbreviation as in the table 1.

2.2. Fish sampling

In May (2010), seventy two fish were sacrificed (twelve fish for each dietary treatments, six males and six females) after anesthesia with 0.3 ml L^{-1} Aqui-S® (Scan Aqua A.S, Årnes, Norway) (Norambuena *et al.*, 2011b) by pithing of the spinal cord. Gonads, liver and muscle were dissected, weighed and 2 g were collected and frozen immediately in liquid nitrogen, and subsequently stored at −70°C pending RNA extraction. The rest of the tissues were stored at -20 ^{o}C to determine the lipid and fatty acid profile. All the fish used in this study were also analysed to determine the steroid levels in blood (Norambuena *et al.*, 2011a) (chapter IV) showing a peak in April. The females used were in an advanced state of maturation II-B (Guzmán *et al.*, 2008).

2.3. Lipid Analysis

Samples of tissues and diet were homogenized and total lipids extracted (Folch *et al.*, 1957) and quantified by gravimetry. Tissue samples were measured in five males and five females for each diet treatment and diets were analyzed in triplicate every three months during the experiment. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transmethylation (Christie, 1982), and extracted and purified following (Tocher *et al.*, 1988). FAME were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using helium as carrier gas (1.2 mL min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well characterized fish oil, and quantified by the response factor to the internal standard, 21:0. The results are presented as percentage of the total fatty acids (% TFA) as mean ± standard error of the mean (SEM). Water content was calculated by drying samples at 105ºC until a constant weigh was obtained (AOAC, 2000).

2.4. Tissue RNA Extraction and Quantitative Real-Time (qrt-PCR)

Total RNA was extracted by organic solvent (Tri-reagent), according to manufacturer's instructions (Ambion, Applied Biosystems), and the quality and quantity was assessed by electrophoresis (Bioanalyzer 2100, Agilent Technologies, Santa Clara, USA) and

spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA), respectively. One microgram of total RNA per sample was reverse-transcribed into cDNA using a Verso™ cDNA kit (ABgene, Surrey, UK), following manufacturer's instructions. Briefly, each 20-µl reaction contained one µg of total RNA, 300 ng of random hexamers and 125 ng of anchored oligo-dT, dNTP mix (500 µMeach), 1× cDNA synthesis buffer, RTenhancer, and Verso enzyme mix. Following cDNA synthesis at 42°C for 1 h, reactions were stopped by heating at 95°C for 2 min and cDNA was diluted tenfold with water. Expression of the desaturase (*Δ4fad*) and elongase (*elovl5*) transcripts was studied by quantitative real-time PCR (qrt-PCR) (Table 3). The qPCR primers were designed in regions corresponding to the 3′ untrans or ORF-lated region of *Δ4fad* and *elovl5* cDNAs. In addition, amplification of two reference genes ubiquitin (*UBQ*) and the ribosomal proteins S4 (*RPS4*) (Infante *et al.*, 2008) (Table 4). Primers for desaturase (*Δ4fad*) and elongase (*elovl5*) transcripts were designed with specific sizes and annealing temperatures using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 3) To determine the effect of the experimental diets, qrt-PCR analysis used relative quantification with *UBQ* and *RPS4* as reference genes, and the amplification efficiency of the primer pairs was assessed by serial dilutions of cDNA pooled from the samples being quantified. qPCR amplifications were carried out in duplicate using a quantica machine (Eppendorf: QPCR Cycler, Stanford) in a final volume of 20 µl containing 2 µl (for reference genes) or 5 µl (for tissue distribution) diluted (1/50) cDNA, 1 µMof each primer, and 10 µl AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (non-template control (NTC), containing no cDNA). The qrt-PCR profiles contained an initial activation step at 95°C for 15 min, followed by 30 to 35 cycles: 15 s at 95°C, 15 s at the specific primer pair annealing Tm (Tables 3 and 4), and 30 s at 72°C. After the amplification phase, a melt curve of 0.5°C increments from 75°C to 90°C was performed, enabling confirmation of the amplification of a single product in each reaction. The qPCR product sizes were checked by agarose gel electrophoresis and their identity was confirmed by sequencing. No primer–dimer formation occurred in the NTC.

Table 3. *Sequences of PCR primers utilized in this study*

Transcript	Primer name	Sequence	Amplicon	Tm
Δ4fad	Δ4fad -Solea-F8	AAGCCTCTGCTGATTGGAGA	131 bp	60
	Δ4fad-Solea-R5	GGCTGAGCTTGAAACAGACC		
Elovl5	Elovl5-Solea-F3	TTTCATGTTTTTGCACACTGC	161 bp	60
	Elovl5-Solea-R3	GACACCTTTAGGCTCGGTTTT		

bp: base pairs, Tm: annealing temperature ^{o}C.

Table 4. *Reference genes used in this study* (Infante *et al.*, 2008)

Transcript	Primer name	Sequence	Amplicon	Tm	Accession no.
UBQ	qUBQ-F	AGCTGGCCCAGAAATATAACTGCGACA	93 bp	70	AB291588
	qUBQ-R	ACTTCTTCTTGCGGCAGTTGACAGCAC			
RPS4	qRPS4-F	GTGAAGAAGCTCCTTGTCGGCACCA	83 bp	70	AB291557
	qRPS4-R	AGGGGGTCGGGGTAGCGGATG			

UBQ: Ubiquitin, RPS4: 40S ribosomal protein S4, bp: base pairs, Tm: annealing temperature ^{o}C.

2.5. Statistical Analysis

Statistical differences in lipid and fatty acid composition among the fish groups A (control), B, C, D, E and F and in the ARA content observed in wild fish were analysed by one-way ANOVA followed by the post-hoc multiple comparison by Tukey's HSD for equal N test with a significance level (*P*) of 0.05. Moreover, correlations among ARA, and 22 Cn-6 acid were made, using *P* of 0.05. The compliance of data with normality and homogeneity of variance was tested by the Kolmogorov–Smirnov and Bartlett (Chi-Sqr) methods and, when necessary, log-transformation was carried out. Fatty acid content was expressed as % TFA ± SEM. The statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA).

The relative expression of elongase and desaturase of the fish from groups A (control), C and F was normalized by the expression of *UBQ* and *RPS4* (reference genes) and carried out using geNorm© Software version 3.5. The value of fractional cycle at which the fluorescence intensity equal the threshold fluorescence (Cts values) was analyzed for statistical significance using the relative expression software tool (REST-MCS©, version 2, http://www.gene-quantification.info/), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction (Pfaffl *et al.*, 2004).

3. Results

After 9 months feeding the experimental diets the females increased weight by 1.8-fold (from 533 ± 13g to 950 ± 25g - SGR=0.20% day^{-1}) whereas males increased by 1.5-fold, (from 515 ± 16g to 755 ± 25g - SGR=0.13% day^{-1}) with a feed conversion ratio of 1.3 ± 0.04, without any significant difference among the six groups (A, B, C, D, E and F), neither in growth nor in feed conversion. Total mortality during the experimental period was 0.8%.

3.1. Fatty acid composition of tissues

All the tissues analyzed, testis (Table 5), ovaries (Table 6), liver (Table 7 and 8) and muscle (Tables 9 and 10) showed a significant ARA accumulation in a dose dependent manner (P<0.05). Thus in testis, the fish in groups C, D, E and F showed a significantly (P<0.05) higher ARA content compared to the control group A, especially in the case of group F which had 13% ARA, 2.7-fold higher (P<0.05) than group A with 5% ARA. In the ovary, the fish from group F showed a 3.2-fold higher (P<0.05) ARA content than group A, whereas the groups B, C, D and E did not show significant differences compared to group A. The liver of the fish from groups C, D, E and F had higher (P<0.05) ARA level than the control A, both in males and females. Males from group B also had a significantly (P<0.05) higher ARA values than group A males, while females from groups and B had similar ARA levels. The muscle of male fish from groups C, D, E and F showed significantly (P<0.05) higher ARA values than from A group, whereas in females only those from E and F groups had significantly (P<0.05) higher ARA levels than those from A. EPA/ARA ratio reduced significantly (P<0.05) in an ARA dose dependent manner in all the fish tissues (gonads, liver and muscle) of both males and females. The increase of ARA in gonad, liver and muscle of males gave a concomitant significant (P<0.05) increase in total n-6 PUFA and a significant (P<0.05) reduction in total n-3 PUFA, in the case of testis.

Table 5 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of testis of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	78 ± 7	68 ± 22	66 ± 20	81 ± 3	76 ± 22	81 ± 17
TFA(µg mg^{-1} L)	503 ± 46	533 ± 52	546 ± 39	561 ± 56	538 ± 34	520 ± 13
Fatty acid composition (%TFA)						
14:0	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
16:0	17.8 ± 0.9	17.8 ± 0.6	18.9 ± 0.6	19.8 ± 0.4	17.7 ± 1.1	19.0 ± 0.9
18:0	7.0 ± 0.5	6.4 ± 0.4	6.3 ± 0.8	6.7 ± 0.7	6.8 ± 0.6	6.4 ± 0.3
Total SFA	26.3 ± 0.8	26.3 ± 1.7	26.9 ± 1.2	28.0 ± 0.8	25.4 ± 1.7	26.5 ± 0.9
16:1n-7	3.3 ± 0.4	2.6 ± 0.1	3.2 ± 0.4	3.2 ± 0.3	2.4 ± 0.3	3.6 ± 0.3
18:1n-9	15.8 ± 1.4	14.2 ± 1.6	15.1 ± 1.8	14.9 ± 0.6	13.0 ± 1.0	17.3 ± 1.2
18:1n-7	6.1 ± 1.7	6.5 ± 0.6	6.7 ± 0.7	5.2 ± 1.5	7.4 ± 0.6	6.0 ± 1.6
20:1n-9	1.9 ± 0.3	1.7 ± 0.4	1.8 ± 0.2	1.3 ± 0.2	1.8 ± 0.2	1.5 ± 0.1
22:1n-9	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Total MUFA	27.8 ± 1.8	24.9 ± 2.5	26.1 ± 1.1	24.2 ± 1.3	24.6 ± 0.9	28.9 ± 1.1
18:2n-6	8.5 ± 0.5	8.0 ± 0.2	6.9 ± 0.2	8.1 ± 0.3	7.0 ± 0.4	7.0 ± 0.4
20:4n-6, ARA	5.0 ± 0.5 ^{d}	7.1 ± 0.8 ^{cd}	8.6 ± 1.4 ^{c}	9.5 ± 0.3 ^{bc}	12.5 ± 1.0 ^{ab}	13.4 ± 0.5 ^{a}
22:4n-6	0.2 ± 0.1 ^{d}	0.4 ± 0.1 ^{c}	0.8 ± 0.1 ^{bc}	0.8 ± 0.1 ^{b}	1.1 ± 0.1 ^{ab}	1.5 ± 0.1 ^{a}
22:5n-6, DPA	0.4 ± 0.1 ^{d}	0.5 ± 0.1 ^{cd}	0.6 ± 0.0 ^{bcd}	0.7 ± 0.1 ^{abc}	0.9 ± 0.1 ^{ab}	1.0 ± 0.1 ^{a}
Total n-6 PUFA	16.1 ± 0.5 ^{c}	16.6 ± 0.8 ^{c}	18.8 ± 1.5 ^{bc}	19.6 ± 0.5 ^{ab}	22.2 ± 0.6 ^{ab}	22.3 ± 0.2 ^{a}
18:3n-3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
18:4n-3	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	1.5 ± 0.7	0.8 ± 0.3	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0
20:5n-3, EPA	4.1 ± 0.5	5.1 ± 0.6	3.6 ± 0.2	3.4 ± 0.8	3.9 ± 0.4	2.9 ± 0.3
22:5n-3	3.6 ± 0.3	4.4 ± 0.5	3.7 ± 0.4	3.9 ± 0.4	3.5 ± 0.3	2.9 ± 0.2
22:6n-3, DHA	16.5 ± 1.2	18.8 ± 0.5	18.9 ± 1.7	19.2 ± 0.5	17.8 ± 0.5	15.1 ± 0.8
Total n-3 PUFA	27.4 ± 1.7 ^{a}	30.7 ± 1.6 ^{a}	27.3 ± 2.0 ^{a}	27.4 ± 0.4 ^{a}	26.1 ± 0.6 ^{a}	20.7 ± 0.8 ^{b}
Total PUFA	43.5 ± 1.5 ^{ab}	47.3 ± 2.3 ^{ab}	46.1 ± 0.7 ^{ab}	46.9 ± 0.6 ^{ab}	48.4 ± 0.6 ^{a}	43.0 ± 0.8 ^{b}
EPA/ARA	0.8 ± 0.1 ^{a}	0.7 ± 0.1 ^{ab}	0.5 ± 0.1 ^{bc}	0.4 ± 0.1 ^{c}	0.3 ± 0.1 ^{c}	0.2 ± 0.0 ^{c}
EPA/DHA	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
DHA/ARA	3.5 ± 0.5	2.3 ± 0.6	3.0 ± 1.3	2.0 ± 0.1	1.5 ± 0.1	0.9 ± 0.2
n-3/n-6	1.7 ± 0.1 ^{a}	1.8 ± 0.1 ^{a}	1.5 ± 0.3 ^{ab}	1.4 ± 0.0 ^{ab}	1.2 ± 0.1 ^{b}	0.9 ± 0.0 ^{c}

TL: Total lipids, L: lipids, DW: dry weight, TFA: total fatty acids, ARA: arachidonic acid, DPA: docosapentaenoic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. Diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F= 6.0% TFA. Columns assigned different letters were significantly different (ANOVA, $P<0.05$, N=6).

Table 6 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of ovary of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	108 ± 20	100 ± 22	125 ± 10	127 ± 6	131 ± 9	130 ± 8
TFA(µg mg^{-1} L)	503 ± 46	533 ± 52	546 ± 39	561 ± 56	538 ± 34	520 ± 13
Fatty acid composition (%TFA)						
14:0	2.0 ± 0.2	1.8 ± 0.3	1.7 ± 0.4	2.0 ± 0.2	2.0 ± 0.1	1.9 ± 0.4
16:0	19.1 ± 1.0	19.8 ± 1.5	18.2 ± 0.9	19.0 ± 1.0	18.7 ± 0.7	19.6 ± 0.5
18:0	4.2 ± 0.4	4.3 ± 1.4	4.4 ± 0.8	3.2 ± 0.3	3.5 ± 0.1	4.8 ± 0.6
Total SFA	25.5 ± 0.9	28.1 ± 2.9	25.3 ± 1.1	24.5 ± 1.4	24.9 ± 1.0	27.4 ± 0.2
16:1n-7	5.3 ± 0.5	4.8 ± 0.7	4.6 ± 0.6	6.2 ± 0.4	5.0 ± 0.2	4.6 ± 0.7
18:1n-9	16.5 ± 1.1	14.9 ± 1.0	15.7 ± 1.3	13.3 ± 2.8	14.9 ± 0.8	16.2 ± 0.8
18:1n-7	3.8 ± 0.4	3.8 ± 0.3	4.4 ± 0.7	3.1 ± 0.1	3.8 ± 0.4	3.3 ± 1.1
20:1n-9	2.9 ± 0.3	2.5 ± 0.5	2.6 ± 0.5	2.9 ± 0.2	3.1 ± 0.3	2.6 ± 0.4
22:1n-9	1.6 ± 0.3	1.8 ± 0.2	1.8 ± 0.6	1.6 ± 0.2	1.4 ± 0.5	1.4 ± 0.4
Total MUFA	30.7 ± 1.4	26.6 ± 2.1	28.9 ± 1.8	25.1 ± 2.7	28.4 ± 1.1	27.4 ± 1.2
18:2n-6	7.8 ± 0.4	8.4 ± 0.4	7.1 ± 0.7	7.9 ± 0.5	7.1 ± 0.3	7.5 ± 0.5
20:4n-6, ARA	2.0 ± 0.9 ^{b}	2.9 ± 1.0 ^{b}	3.8 ± 1.3 ^{ab}	4.1 ± 0.1 ^{ab}	5.1 ± 0.2 ^{ab}	6.4 ± 1.6 ^{a}
22:4n-6	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.2
22:5n-6, DPA	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
Total n-6 PUFA	11.3 ± 1.3	12.9 ± 1.1	14.1 ± 2.0	13.2 ± 0.8	14.1 ± 0.5	15.9 ± 1.8
18:3n-3	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	1.0 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
18:4n-3	0.6 ± 0.1	0.9 ± 0.4	0.5 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.1
20:4n-3	1.4 ± 0.7	1.1 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:5n-3, EPA	2.8 ± 0.5	2.0 ± 0.8	2.9 ± 0.7	2.8 ± 0.2	2.3 ± 0.0	2.7 ± 0.4
22:5n-3	4.1 ± 0.4	4.3 ± 0.6	3.9 ± 0.3	4.3 ± 0.4	4.3 ± 0.1	4.0 ± 0.3
22:6n-3, DHA	20.5 ± 1.5	21.2 ± 3.4	22.8 ± 2.9	23.3 ± 1.3	22.7 ± 0.8	20.0 ± 0.8
Total n-3 PUFA	31.0 ± 1.5	30.8 ± 2.0	31.1 ± 2.0	35.8 ± 1.4	31.6 ± 0.8	28.0 ± 1.2
Total PUFA	42.4 ± 1.6	43.6 ± 1.7	45.2 ± 1.9	49.0 ± 1.4	45.7 ± 0.9	43.9 ± 1.3
EPA/ARA	1.9 ± 0.0 ^{a}	0.9 ± 0.2 ^{b}	0.8 ± 0.1 ^{b}	0.7 ± 0.0 ^{b}	0.4 ± 0.0 ^{b}	0.5 ± 0.0 ^{b}
EPA/DHA	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DHA/ARA	17.3 ± 0.2	12.3 ± 5.1	12.0 ± 5.8	6.0 ± 0.2	4.5 ± 0.3	3.4 ± 0.9
n-3/n-6	2.9 ± 0.2	2.5 ± 0.3	2.5 ± 0.4	2.8 ± 0.2	2.3 ± 0.1	1.9 ± 0.3

Abbreviation as in the table 5.

Table 7 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of male liver of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	485 ± 39	472 ± 41	477 ± 29	455 ± 43	457 ± 36	450 ± 22
TFA(µg mg^{-1} L)	562 ± 60	538 ± 109	555 ± 59	566 ± 66	504 ± 69	539 ± 50
Fatty acid composition (%TFA)						
14:0	3.9 ± 0.7	2.8 ± 0.8	3.8 ± 0.5	2.2 ± 1.1	2.9 ± 0.7	3.4 ± 0.7
16:0	19.9 ± 0.4	17.8 ± 0.8	18.6 ± 0.5	19.4 ± 1.2	16.6 ± 0.9	16.7 ± 1.5
18:0	3.7 ± 0.7	3.5 ± 0.5	2.7 ± 0.2	2.5 ± 0.4	2.5 ± 0.3	3.0 ± 0.3
Total SFA	28.8 ± 1.1	24.8 ± 1.0	25.7 ± 1.1	24.4 ± 1.7	22.5 ± 1.1	23.5 ± 2.3
16:1n-7	7.3 ± 0.6	6.5 ± 1.0	8.8 ± 0.5	7.4 ± 0.4	6.2 ± 0.7	6.1 ± 1.7
18:1n-9	20.9 ± 1.3	17.1 ± 2.2	21.9 ± 1.3	21.1 ± 1.4	18.3 ± 1.0	20.4 ± 1.2
18:1n-7	3.7 ± 0.0	3.3 ± 0.3	3.6 ± 0.3	3.8 ± 0.1	3.3 ± 0.2	3.5 ± 0.9
20:1n-9	3.5 ± 0.6	3.2 ± 0.7	3.7 ± 0.4	3.8 ± 0.6	3.8 ± 0.4	2.7 ± 0.3
22:1n-9	3.0 ± 0.4	2.9 ± 0.4	2.9 ± 0.5	3.1 ± 0.8	3.4 ± 0.6	2.8 ± 0.4
Total MUFA	29.9 ± 5.8	30.7 ± 3.8	40.5 ± 2.6	45.8 ± 4.5	33.7 ± 2.1	33.7 ± 2.0
18:2n-6	6.7 ± 0.5	6.7 ± 1.0	6.4 ± 0.5	6.8 ± 0.7	7.9 ± 0.4	7.5 ± 0.4
20:4n-6, ARA	0.5 ± 0.1 ^{d}	1.2 ± 0.2 ^{c}	1.5 ± 0.1 ^{c}	1.8 ± 0.1 ^{bc}	2.4 ± 0.2 ^{ab}	2.8 ± 0.4 ^{a}
22:4n-6	0.1 ± 0.0 ^{c}	0.1 ± 0.1 ^{c}	0.5 ± 0.0 ^{bc}	0.5 ± 0.2 ^{bc}	0.9 ± 0.1 ^{ab}	1.2 ± 0.1 ^{a}
22:5n-6, DPA	0.2 ± 0.1 ^{b}	0.3 ± 0.1 ^{b}	0.6 ± 0.1 ^{ab}	0.6 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	1.1 ± 0.1 ^{a}
Total n-6 PUFA	7.8 ± 0.6	8.4 ± 1.1	9.0 ± 0.6	9.0 ± 1.1	12.6 ± 0.6	12.8 ± 0.8
18:3n-3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:4n-3	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.3 ± 0.1
20:4n-3	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:5n-3, EPA	2.7 ± 0.7	2.2 ± 0.8	2.7 ± 0.8	1.7 ± 0.8	2.3 ± 0.6	3.2 ± 0.7
22:5n-3	4.3 ± 0.4	4.8 ± 0.8	4.2 ± 0.7	4.0 ± 0.8	5.2 ± 0.2	4.7 ± 0.4
22:6n-3, DHA	20.2 ± 3.7	23.8 ± 4.1	18.8 ± 1.9	17.3 ± 2.5	22.0 ± 2.0	23.0 ± 4.8
Total n-3 PUFA	29.3 ± 3.3	33.5 ± 3.3	24.2 ± 2.2	24.4 ± 4.3	30.7 ± 2.1	29.6 ± 3.8
Total PUFA	39.4 ± 3.0	41.7 ± 3.6	34.8 ± 2.4	29.9 ± 2.6	42.5 ± 1.8	42.5 ± 3.9
EPA/ARA	7.4 ± 0.9 ^{a}	2.6 ± 0.9 ^{b}	1.7 ± 0.7 ^{b}	1.4 ± 0.7 ^{b}	1.3 ± 0.2 ^{b}	1.4 ± 0.1 ^{b}
EPA/DHA	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
DHA/ARA	32.9 ± 2.5 ^{a}	20.0 ± 5.6 ^{b}	7.1 ± 2.9 ^{b}	5.8 ± 2.3 ^{b}	6.2 ± 1.7 ^{b}	6.0 ± 1.9 ^{b}
n-3/n-6	3.8 ± 0.5	3.5 ± 1.1	2.7 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	2.3 ± 0.4

Abbreviation as in the table 5.

Table 8 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of female liver of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	442 ± 83	436 ± 35	419 ± 27	365 ± 30	361 ± 40	406 ± 33
TFA(µg mg^{-1} L)	482 ± 94	417 ± 85	542 ± 20	534 ± 27	456 ± 25	427 ± 94
Fatty acid composition (%TFA)						
14:0	4.3 ± 0.6	4.2 ± 0.7	3.7 ± 0.7	4.3 ± 0.7	3.9 ± 0.6	3.9 ± 1.0
16:0	21.9 ± 0.7	19.6 ± 1.4	18.5 ± 1.3	21.0 ± 0.9	21.5 ± 0.6	19.8 ± 0.8
18:0	3.7 ± 1.0	2.8 ± 0.2	1.9 ± 0.1	2.1 ± 0.2	2.9 ± 0.2	3.2 ± 0.5
Total SFA	31.1 ± 1.5	26.9 ± 2.0	24.2 ± 1.7	28.3 ± 1.3	28.8 ± 1.0	27.0 ± 2.3
16:1n-7	9.5 ± 1.7	7.8 ± 0.6	7.3 ± 1.6	9.8 ± 0.4	7.8 ± 0.4	7.3 ± 1.3
18:1n-9	18.2 ± 4.4	19.4 ± 2.0	21.0 ± 2.0	21.4 ± 0.6	22.5 ± 0.1	20.2 ± 1.8
18:1n-7	4.2 ± 0.4	4.1 ± 0.3	4.7 ± 0.2	4.7 ± 0.4	5.1 ± 0.2	4.9 ± 0.2
20:1n-9	2.9 ± 0.5	3.5 ± 0.8	3.2 ± 0.6	2.5 ± 0.2	3.8 ± 0.4	2.6 ± 0.5
22:1n-9	2.2 ± 0.7	2.3 ± 0.3	2.4 ± 0.6	2.0 ± 0.7	2.2 ± 0.5	2.5 ± 0.3
Total MUFA	32.2 ± 5.9	35.2 ± 3.4	39.8 ± 2.8	38.8 ± 1.1	39.7 ± 0.2	33.7 ± 3.7
18:2n-6	8.0 ± 1.1	8.0 ± 0.1	9.6 ± 0.5	9.7 ± 0.6	9.1 ± 0.2	8.7 ± 0.6
20:4n-6, ARA	0.6 ± 0.3 ^{c}	1.1 ± 0.2 ^{c}	1.7 ± 0.1 ^{b}	1.8 ± 0.2 ^{b}	2.3 ± 0.1 ^{ab}	3.0 ± 0.1 ^{a}
22:4n-6	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1
22:5n-6, DPA	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Total n-6 PUFA	9.0 ± 0.9 ^{b}	9.6 ± 0.4 ^{b}	12.6 ± 0.4 ^{a}	12.6 ± 0.6 ^{a}	13.9 ± 0.4 ^{a}	13.0 ± 0.6 ^{a}
18:3n-3	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:4n-3	0.8 ± 0.2	0.5 ± 0.0	0.6 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
20:4n-3	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
20:5n-3, EPA	1.2 ± 0.3	1.9 ± 0.4	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.3	1.1 ± 0.1
22:5n-3	2.8 ± 0.4	3.8 ± 0.4	3.7 ± 0.5	3.0 ± 0.7	3.5 ± 0.4	3.7 ± 0.7
22:6n-3, DHA	16.9 ± 3.9	14.4 ± 1.4	15.7 ± 2.3	13.4 ± 1.9	12.0 ± 1.3	16.4 ± 6.0
Total n-3 PUFA	22.6 ± 4.0	22.9 ± 1.7	23.1 ± 3.0	19.5 ± 1.9	17.2 ± 0.8	22.3 ± 5.8
Total PUFA	31.7 ± 4.6	37.3 ± 5.4	35.7 ± 3.3	32.1 ± 2.3	31.1 ± 1.2	38.9 ± 5.9
EPA/ARA	3.3 ± 1.0 ^{a}	2.0 ± 0.6 ^{ab}	0.7 ± 0.2 ^{b}	0.6 ± 0.1 ^{b}	0.4 ± 0.1 ^{b}	0.3 ± 0.0 ^{b}
EPA/DHA	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DHA/ARA	45.1 ± 17 ^{a}	13.4 ± 3.6 ^{ab}	9.6 ± 2.0 ^{b}	7.5 ± 1.3 ^{b}	5.4 ± 1.1 ^{b}	4.5 ± 2.1 ^{b}
n-3/n-6	2.5 ± 0.3	2.0 ± 0.5	1.8 ± 0.2	1.5 ± 0.1	1.2 ± 0.0	0.9 ± 0.3

Abbreviation as in the table 5.

Table 9 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of male muscle of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	44 ± 7	39 ± 6	36 ± 7	43 ± 4	41 ± 7	47 ± 5
TFA(µg mg^{-1} L)	542 ± 115	584 ± 98	421 ± 50	491 ± 28	478 ± 52	426 ± 99
Fatty acid composition (%TFA)						
14:0	1.7 ± 0.1	1.7 ± 0.3	1.6 ± 0.5	1.5 ± 0.1	1.6 ± 0.3	1.8 ± 0.2
16:0	17.6 ± 0.7	16.4 ± 1.2	17.1 ± 1.5	17.2 ± 0.4	15.3 ± 1.6	17.4 ± 0.7
18:0	4.9 ± 0.6	5.0 ± 0.9	5.2 ± 0.5	5.2 ± 0.5	5.4 ± 1.0	4.9 ± 0.4
Total SFA	24.8 ± 1.3	20.5 ± 3.7	24.5 ± 2.2	24.5 ± 0.8	23.2 ± 2.2	24.9 ± 0.9
16:1n-7	3.9 ± 0.3	4.0 ± 0.8	3.1 ± 0.8	3.5 ± 0.3	3.2 ± 0.6	4.3 ± 0.3
18:1n-9	13.4 ± 0.6	13.5 ± 1.8	13.9 ± 1.4	13.7 ± 0.6	11.5 ± 0.4	14.5 ± 0.7
18:1n-7	2.3 ± 0.6	2.9 ± 0.3	2.5 ± 0.3	2.7 ± 0.6	2.9 ± 0.5	2.8 ± 0.3
20:1n-9	2.2 ± 0.2	2.4 ± 0.3	2.3 ± 0.3	2.6 ± 0.2	2.5 ± 0.5	2.4 ± 0.1
22:1n-9	1.0 ± 0.3	1.1 ± 0.6	1.3 ± 0.5	1.3 ± 0.5	1.0 ± 0.3	1.4 ± 0.4
Total MUFA	23.1 ± 0.6	24.2 ± 3.1	23.2 ± 2.9	24.0 ± 1.0	20.7 ± 1.4	25.8 ± 1.1
18:2n-6	6.4 ± 0.4	6.6 ± 0.6	6.0 ± 0.4	6.6 ± 0.4	6.6 ± 0.6	6.2 ± 1.4
20:4n-6, ARA	1.4 ± 0.1 ^{c}	2.2 ± 0.2 ^{bc}	2.7 ± 0.4 ^{ab}	2.7 ± 0.2 ^{ab}	3.2 ± 0.3 ^{a}	3.2 ± 0.2 ^{a}
22:4n-6	0.2 ± 0.0 ^{c}	0.3 ± 0.1 ^{bc}	0.6 ± 0.0 ^{ab}	0.6 ± 0.1 ^{ab}	0.5 ± 0.0 ^{ab}	0.7 ± 0.1 ^{a}
22:5n-6, DPA	0.4 ± 0.1 ^{a}	0.6 ± 0.1 ^{ab}	0.7 ± 0.1 ^{ab}	0.7 ± 0.0 ^{ab}	1.2 ± 0.5 ^{a}	0.7 ± 0.1 ^{ab}
Total n-6 PUFA	9.3 ± 0.4 ^{b}	12.3 ± 0.3 ^{ab}	11.4 ± 0.9 ^{ab}	12.0 ± 0.5 ^{ab}	14.4 ± 1.7 ^{a}	13.3 ± 0.5 ^{ab}
18:3n-3	0.7 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.1
18:4n-3	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
20:4n-3	1.1 ± 0.2	0.9 ± 0.3	0.5 ± 0.4	0.7 ± 0.2	1.7 ± 0.9	1.3 ± 0.4
20:5n-3, EPA	3.6 ± 0.2	3.9 ± 0.3	3.5 ± 0.2	4.0 ± 0.2	3.5 ± 0.4	3.4 ± 0.4
22:5n-3	6.7 ± 0.6	7.6 ± 0.8	6.4 ± 0.5	7.4 ± 0.4	6.4 ± 0.3	6.4 ± 0.8
22:6n-3, DHA	27.2 ± 1.4	27.4 ± 1.4	27.9 ± 3.2	25.0 ± 0.8	21.8 ± 5.1	21.1 ± 2.1
Total n-3 PUFA	41.4 ± 1.7	41.3 ± 0.9	39.8 ± 4.1	38.0 ± 0.9	39.3 ± 1.5	34.3 ± 2.5
Total PUFA	50.7 ± 1.6	53.6 ± 0.9	51.2 ± 4.8	50.1 ± 1.0	53.7 ± 1.8	45.1 ± 1.7
EPA/ARA	2.6 ± 0.2 ^{a}	1.9 ± 0.3 ^{ab}	1.3 ± 0.1 ^{b}	1.5 ± 0.1 ^{b}	1.1 ± 0.2 ^{b}	1.2 ± 0.1 ^{b}
EPA/DHA	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
DHA/ARA	16.0 ± 4.2	12.8 ± 0.4	9.7 ± 0.8	9.5 ± 0.4	7.7 ± 1.0	7.2 ± 0.4
n-3/n-6	4.5 ± 0.3 ^{a}	3.4 ± 0.1 ^{ab}	3.5 ± 0.3 ^{ab}	3.2 ± 0.2 ^{b}	2.9 ± 0.4 ^{b}	2.5 ± 0.3 ^{b}

Abbreviation as in the table 5.

Table 10 *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of female muscle of Senegalese sole fed with six different diets (A, B, C, D, E and F) during nine months*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	47 ± 8	48 ± 9	44 ± 9	41 ± 5	57 ± 14	45 ± 9
TFA(µg mg^{-1} L)	519 ± 75	528 ± 72	506 ± 61	541 ± 70	518 ± 50	495 ± 51
Fatty acid composition (%TFA)						
14:0	1.7 ± 0.3	1.9 ± 0.3	2.2 ± 0.4	1.5 ± 0.2	1.9 ± 0.2	1.9 ± 0.4
16:0	16.2 ± 0.8	16.5 ± 1.6	16.1 ± 0.6	15.6 ± 1.4	17.6 ± 1.1	17.1 ± 0.7
18:0	4.3 ± 0.6	4.5 ± 0.8	4.3 ± 0.6	4.9 ± 0.3	4.7 ± 0.5	4.9 ± 0.6
Total SFA	22.6 ± 1.1	24.0 ± 2.0	24.2 ± 0.4	21.1 ± 2.4	25.3 ± 1.4	24.6 ± 1.0
16:1n-7	4.4 ± 0.7	3.8 ± 0.7	5.0 ± 0.6	3.8 ± 0.4	4.4 ± 0.5	4.0 ± 0.7
18:1n-9	14.2 ± 0.9	13.2 ± 1.5	14.7 ± 0.2	14.0 ± 1.4	16.6 ± 0.4	13.7 ± 0.7
18:1n-7	2.9 ± 0.2	2.5 ± 0.2	2.8 ± 0.2	2.2 ± 0.3	2.6 ± 0.2	2.8 ± 0.3
20:1n-9	2.0 ± 0.2	1.9 ± 0.4	1.9 ± 0.4	1.8 ± 0.4	2.3 ± 0.1	2.2 ± 0.3
22:1n-9	1.1 ± 0.5	1.1 ± 0.4	1.0 ± 0.5	1.3 ± 0.3	1.6 ± 0.4	1.0 ± 0.4
Total MUFA	24.0 ± 1.9	22.9 ± 1.9	26.6 ± 1.0	21.4 ± 3.2	28.5 ± 0.2	23.2 ± 1.6
18:2n-6	6.9 ± 0.8	6.0 ± 0.8	6.6 ± 0.2	5.7 ± 0.2	5.7 ± 1.4	6.8 ± 0.7
20:4n-6, ARA	1.5 ± 0.2 ^{b}	1.5 ± 0.2 ^{b}	1.7 ± 0.2 ^{b}	2.2 ± 0.3 ^{ab}	3.3 ± 0.5 ^{a}	3.4 ± 0.3 ^{a}
22:4n-6	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
22:5n-6, DPA	0.5 ± 0.0	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Total n-6 PUFA	10.2 ± 1.1	10.6 ± 0.7	11.5 ± 0.5	13.9 ± 1.8	11.3 ± 1.0	12.2 ± 0.2
18:3n-3	0.8 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
18:4n-3	0.7 ± 0.1	0.3 ± 0.1	0.9 ± 0.2	0.5 ± 0.0	0.9 ± 0.1	0.6 ± 0.1
20:4n-3	0.8 ± 0.3	1.6 ± 1.1	1.0 ± 0.5	3.0 ± 1.1	0.6 ± 0.3	1.7 ± 0.6
20:5n-3, EPA	3.9 ± 0.3	3.1 ± 0.6	3.4 ± 0.6	2.7 ± 0.3	3.1 ± 0.3	3.6 ± 0.1
22:5n-3	6.9 ± 0.8	5.6 ± 1.1	5.6 ± 0.7	5.1 ± 0.6	5.6 ± 0.7	6.5 ± 0.2
22:6n-3, DHA	25.9 ± 2.4	27.5 ± 1.7	23.5 ± 2.9	23.5 ± 1.3	21.2 ± 0.8	23.5 ± 2.3
Total n-3 PUFA	41.6 ± 3.0	39.9 ± 2.6	35.6 ± 1.2	37.8 ± 1.8	32.8 ± 0.7	38.1 ± 2.0
Total PUFA	51.8 ± 2.1	50.5 ± 3.1	47.2 ± 1.3	51.7 ± 3.3	44.1 ± 0.9	50.3 ± 1.9
EPA/ARA	2.8 ± 0.4 ^{a}	1.9 ± 0.3 ^{ab}	1.7 ± 0.2 ^{ab}	1.3 ± 0.1 ^{b}	1.1 ± 0.3 ^{b}	1.1 ± 0.1 ^{b}
EPA/DHA	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
DHA/ARA	13.3 ± 3.6	12.0 ± 3.8	15.3 ± 4.0	11.6 ± 1.3	6.9 ± 1.0	6.0 ± 1.6
n-3/n-6	4.4 ± 0.7	3.8 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	3.0 ± 0.4	3.1 ± 0.2

Abbreviation as in the table 5.

3.2. Optimal dietary ARA level

The dose dependent response in the accumulation of ARA from the experimental diets into the different tissues was different in each tissue when compared to levels observed in wild fish (Fig. 1). The levels of ARA in the ovary of fish from group A were similar and not significantly different to those from the wild fish. However, ARA content in the testis, liver and muscle (female and male) was significantly (P<0.05) lower than the wild group. In the fish from group B, ARA lvevels in testis, ovary and liver of female fish were similar to the wild group whereas in the liver of males and in the muscle of males and females ARA content was significantly (P<0.05) lower than in the wild group. The testis, ovary and liver of fish from groups C and D showed similar ARA values than those from the wild group and only the muscle showed significant (P<0.05) differences. Fish from group C and males from group D had significantly (P<0.05) lower levels in muscle, while levels in muscle from females in group D were not significantly different from wild fish. The testis and ovary of fish from groups E and F showed significantly (P<0.05) higher accumulation of ARA compared with the wild group. In the liver and muscle from group F, ARA was similar to that found in wild fish, same was found in group E, except in muscle of males, with ARA levels significantly (P<0.05) lower than in the wild group.

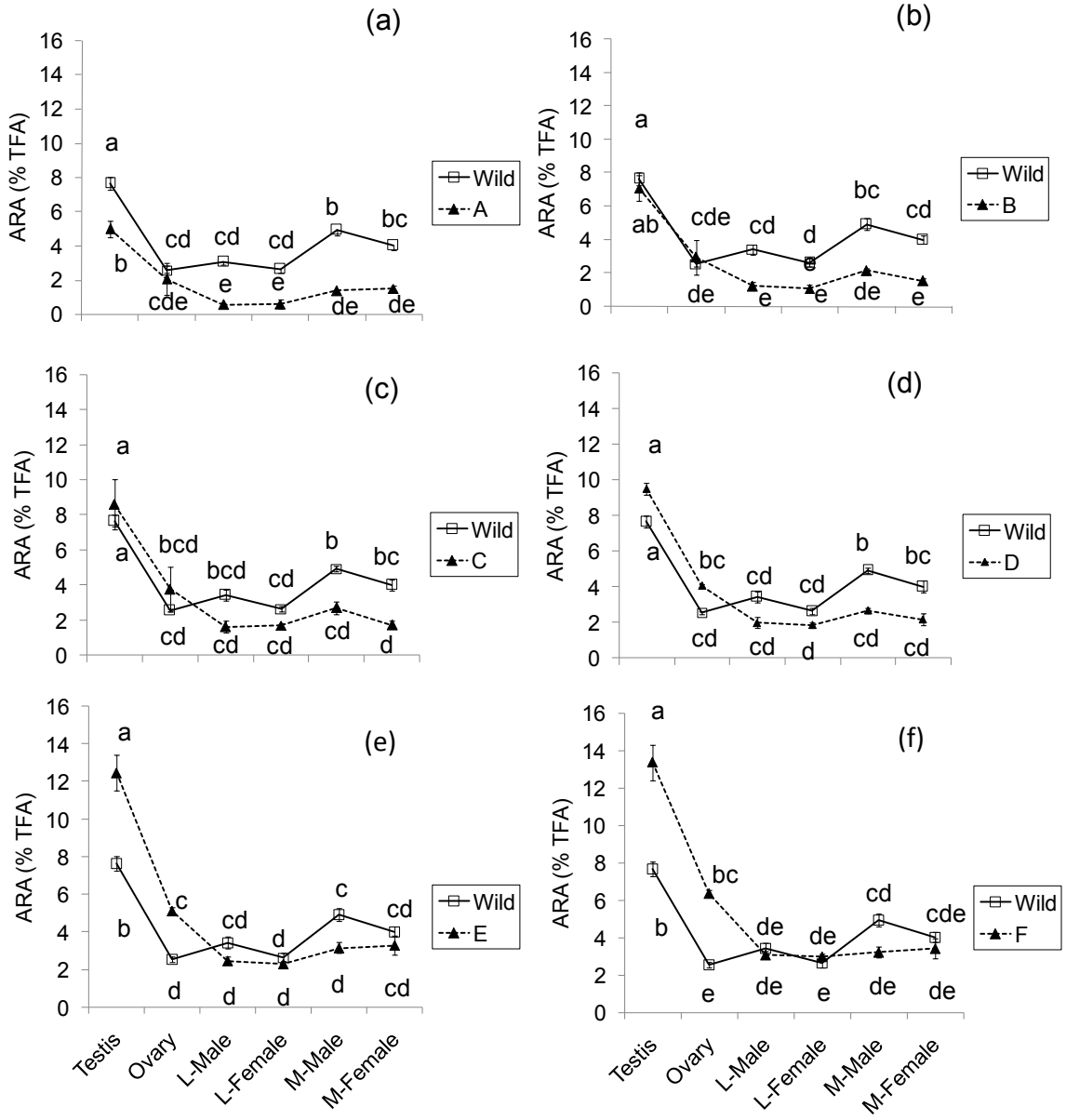

Fig. 1 *Results of arachidonic acid, ARA (% TFA ± SEM) content in the tissues, testis, ovary, male liver (L-male), female liver (L-female), male muscle (M-male) and female muscle (M-female) of wild (solid line, open square) and cultured (G1) fish (dash line, full triangle) fed during nine months with fed different dietary ARA levels (N=6). a) Fish fed diets A, 0.7% ARA, b) fish fed diets B, 1.6% ARA c) fish fed diets C, 2.3% ARA, d) fish fed diets D, 3.2% ARA, e) fish fed diets E, 5.0% ARA and f) fish fed diets F, 6.0% ARA. Different letters indicate significant differences (ANOVA, P<0.05, N=6) between tissues of G1 and wild fish (control).*

3.3. The adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) acids

As in the case of ARA content in the tissues, fish fed the six experimental diets showed a dose dependent and significant (P<0.05) increase of 22:4n-6 and 22:5n-6 in testis and liver of males, whereas in females it was not significant (Fig. 2). The relative level of 22:4n-6 acid in the testis was significantly (P<0.05) higher in group B (2.2-fold), C (4.2-fold), D (4.5-fold), E (6.1-fold) and F (8.1-fold) compared with the lowest levels found in the control group A (0.2% TFA). The 22:5n-6 content in the testis was also higher in group C (1.5-fold), D (1.8-fold), E (2.2-fold) and F (2.4-fold) compared with the control group A (0.4% TFA). The 22:4n-6 levels in the liver of males were also significantly higher in E (7.1-fold) and F (9.5-fold) compare to control group A (0.1% TFA), while 22:5n-6 was 5-fold higher in group F compare with group A (0.2% TFA) (Fig. 2).

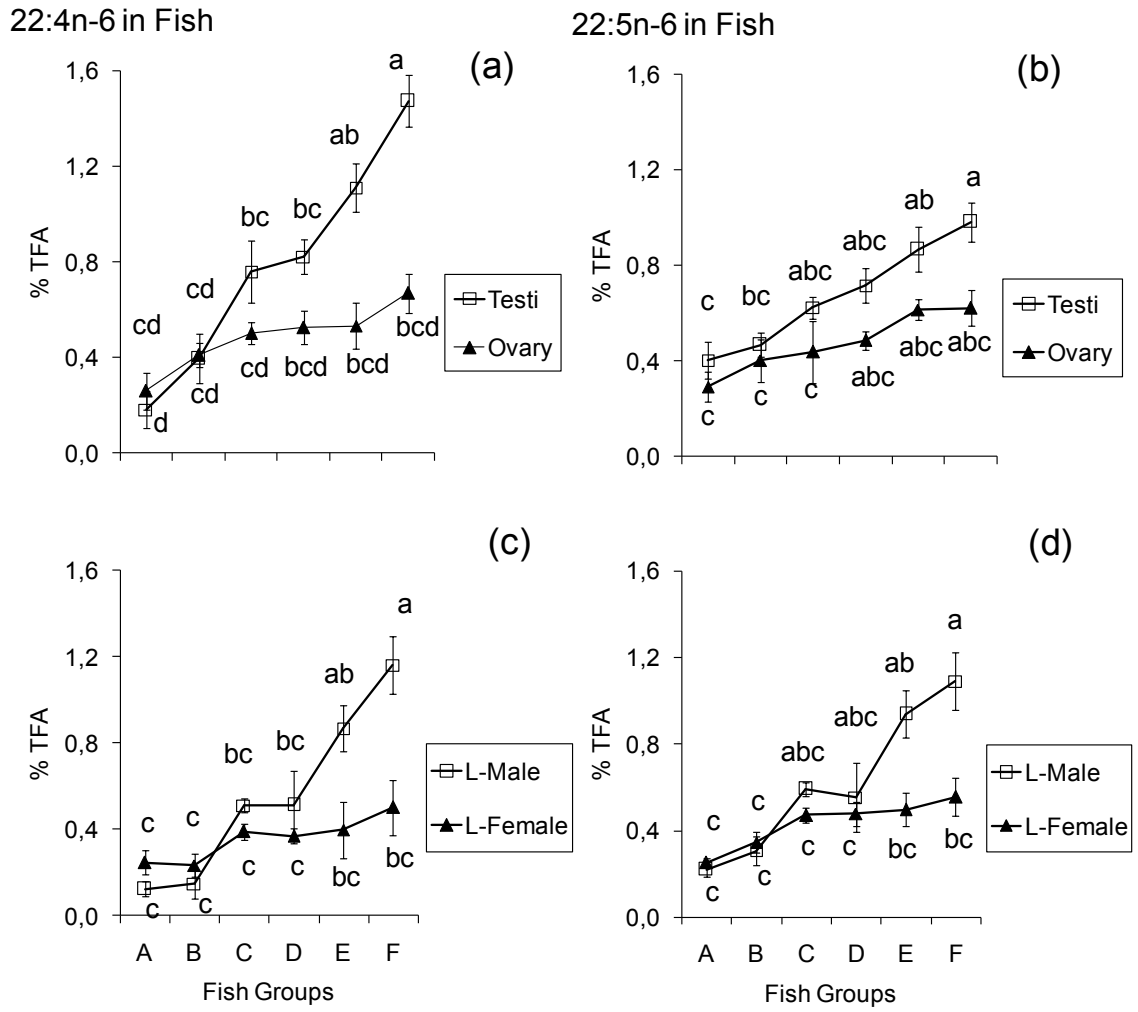

Fig. 2 *Results of adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) acids content (% TFA ± SEM) in tissues of cultured fish fed with different dietary ARA levels (N=6). a) Adrenic (22:4n-6) acid in testis (square) and ovary (triangle), b) docosapentaenoic (22:5n-6) acids in testis (square) and ovary (triangle), c) Adrenic (22:4n-6) acid in liver of male, L-male (square) and female, L-female (triangle) fish and d) docosapentaenoic (22:5n-6) acids in liver of male, L-male (square) and female, L-female (triangle). Different letters indicate significant differences (ANOVA, P<0.05) between fish groups in 22:4n-6 and 22:5n-6 fatty acids content. ARA content, diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0, F= 6.0% TFA.*

3.4. Elongase and desaturase gene expression (qrt-PCR)

The expression of *Δ4Fad* and *elovl5* increased in a dose dependent way in relation to the increasing ARA content in the diets feed to the fish in groups A (control), C and F (Fig. 3). Levels of *Δ4Fad* transcripts were significantly (P<0.05) higher in the liver of male fish from groups C and F compared with group A. Liver of male fish fed a diet high in ARA

(6.0%) showed a significant (P<0.05) 6.8-fold higher in *Δ4Fad* expression, compared with the control (A) whereas in the fish from group C a significant (P<0.05) 3.2-fold increase was observed. Expression of *elovl5* increased significantly (P<0.05) in group F with 5.3-fold higher than in the control group (A), but not in group C (1.8-fold increase). Thus, all the differences in *Δ4Fad* and *elovl5* gene expression were observed only in the males (Fig. 3).

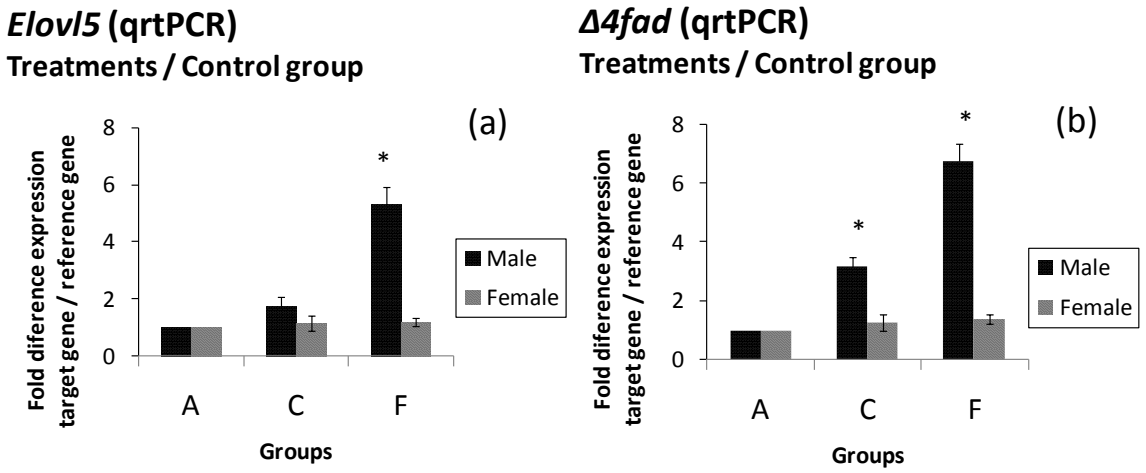

Fig. 3 *Relative expression (qrtPCR) of fatty acyl a) elongase (elovl5) and b) desaturase (Δ4fad) in liver of female and male Senegalese sole in response to changes in dietary arachidonic composition (group A (control) diet A= 0.7, group C diet C= 2.3 and group F diet F= 6.0% TFA), normalized by the expression of UBQ and RPS4 (reference genes). * denote significant differences of groups C and F with respect to group control (A), calculated by REST (P<0.05, N=6).*

4. Discussion

The relative ARA levels in G1 tissues, increased in a dose dependent manner in both sexes. Thus, fish fed 2.3% and 3.2% ARA showed no differences in the composition of testis, ovary and liver with wild fish. The expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts also increased in response to dietary ARA content in males, showing a pattern of up-regulation in both transcripts. Thus, the levels of *d4fad* transcripts were significantly higher in the liver of males from groups C and F and *elovl5* in group F with a subsequent accumulation of 22:4n-6 and 22:5n-6 acids, especially in the testis. Most of the other PUFA did not show changes in tissue content, although in the case of EPA it was significantly lower in the tissues (2 - 4% TFA) than in the diets (13 - 16% TFA).

Muscle of Senegalese sole did not accumulate ARA at the same rate of other tissues (liver and gonads) and only the fish fed 5% or higher dietary ARA had the same ARA level in muscle compared to wild fish. Norambuena *et al*. (2011) studied the ARA preference of *S. Sole* broodstock using a self-feeding system and found that fish regulate the ingestion of ARA to around 3.0% (Norambuena *et al.*, 2011e) (chapter V). The analysis of the tissues of fish from the self-feeding experiment showed an ARA content of 8.9% TFA in testis, 4% in ovary and 2.5% in liver (Norambuena *et al.*, 2011e) (chapter V). All these values were similar to those found in wild *S. Sole* fish (Norambuena *et al.*, 2011c) (chapter II) and to those obtained in the present study in G1 fish testis and ovaries from groups C and D. Thus, the ARA accumulation in the liver was lower than in the self-feeding, except for groups D (3.2% ARA in diet) and E (5.0% ARA in diet). Considering that the self-feeding experiment was conducted for 16 months and the present lasted only nine months, optimal dietary ARA level seems to be dependent on the feeding period. If fish are fed during a period shorter than nine months, it might be appropriate to use at least 3.2% ARA in the diet. For longer feeding periods 2.3% ARA in the diet might be sufficient for Senegalese sole G1, having in account that no differences in ARA content could be found in the testis and ovary of the fish compared to wild fish or fish used in the self-feeding experiment. Alorend *et al*. (2004), studied ARA requirements for G1 Atlantic halibut during three years and suggested 2.3% as the optimal dietary content as this level achieved the longest milt production period and highest fecundity, being ARA rapidly incorporated into the reproductive tissues (after only three months feeding) (Alorend, 2004). Previous studies on ARA requirements for broodstock fish show that optimal dietary level is species-specific, with higher or lower levels of ARA producing detrimental effects in reproductive physiology (Mercure *et al.*, 1995, Mercure *et al.*, 1996, Norberg *et al.*, 2009, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001, Furuita *et al.*, 2003, Mazorra *et al.*, 2003, Alorend, 2004, Norambuena *et al.*, 2011c). A significant increase in the production of steroids was observed in Senegalese sole males fed 3.2% ARA (Norambuena *et al.*, 2011a) (chapter IV), and the highest egg production was obtained using diets with 3.6% ARA for Japanese flounder (*Paralichthys olivaceus*) (Furuita *et al.*, 2003). A study with Atlantic cod (*Gadus morhua*) found that a diet with 4% ARA, increased the production of estradiol and extended the length of the spawning season (Norberg *et al.*, 2009). However, negative

effects in spawning, egg and larval quality and steroid production have been observed when fish were fed high ARA levels. Japanese flounder fed 7.3% ARA exhibited a significant reduction in egg and larval quality (Furuita *et al.*, 2003) and in Atlantic halibut fed 3.2% ARA a delay in spawning season was observed (Alorend, 2004). Other effects such as an earlier estradiol peak in Senegalese sole (Norambuena *et al.*, 2011a) (chapter IV), and an earlier peak in estradiol and vitellogenesis in Atlantic halibut (Norberg *et al.*, 2009) have been suggested to be negative consequences of dietary levels of ARA. Thus, more research is needed to establish the dietary ARA effects on sperm and oocyte quality in *S. Sole* and the feeding time required to incorporate dietary ARA into reproductive organs.

The results obtained in the present study showed that dietary ARA was transferred and conserved in testis and ovary, followed by liver and muscle, which was similar to what was previously observed in Atlantic halibut, white seabream, black seabream and silver pomfret (Alorend, 2004, Cejas *et al.*, 2004, Rodríguez *et al.*, 2004, Pérez *et al.*, 2007, Huang *et al.*, 2010). A positive correlation among the levels of ARA and the concentration of 22:4n-6 and 22:5n-6 acids in testis, liver and muscle of wild fish was shown (Norambuena *et al.*, 2011c) (chapter II) as a consequence of ARA metabolism. Similar pattern of 22:4n-6 and 22:5n-6 acids concentration in the tissues after ARA feeding was observed in the present study. Arachidonate acids (i.e., 20:4n-6, 22:4n-6 and 22:5n-6) accumulation in the muscle of wild black seabream (Rodríguez *et al.*, 2004) and Senegalese sole (Norambuena *et al.*, 2011c) (chapter II), in the gonads of seabass (Bell *et al.*, 1997), seabream (Cejas *et al.*, 2003), silver pomfret (Huang *et al.*, 2010) and the sperm of seabass (Bell *et al.*, 1996) and rainbow trout (Bell *et al.*, 1996, Vassallo-Agius *et al.*, 2001) have been cited previously being the accumulation of 22:5n-6 acids a consequence of local production and uptake from the circulation system (Rosenthal *et al.*, 1991, Tam *et al.*, 2000, Tam *et al.*, 2008). These fatty acids are found in storage lipids but they are also present in testis in mammals (Ayala *et al.*, 1973) being considered indicators of normal testicular development, spermatogenesis and fertility (Bridges *et al.*, 1970, Ayala *et al.*, 1973, Leat *et al.*, 1983, MacDonald *et al.*, 1984, Lenzi *et al.*, 1996, Lenzi *et al.*, 2000, Furland *et al.*, 2007). Although, the physiological function of these PUFAs in sperm is not well know, it is believed they are involved in sperm formation and fertilization (Lenzi *et al.*, 1996), increasing the degree of fatty acid metabolism and desaturation either during spermatogenesis and sperm maturation

in mammals (Furland *et al.*, 2007). However the actual function of 22:4n-6 and 22:5n-6 in fish and its effects on reproduction and spermatogenesis has not been established.

It has been suggested that 22:5n-6 is accumulated in the testis as an ARA reservoir, being retro-converted into ARA by hydrogenation and subsequent oxidation (Bridges *et al.*, 1970, Kunau, 1971, Rosenthal *et al.*, 1991, Tam *et al.*, 2000). Cultured fish fed commercial extruded diets show a significant lower accumulation of 22:5n-6 and 22:4n-6 compared to wild fish (Norambuena *et al.*, 2011c) (chapter II), although if the fish were fed increasing ARA levels a parallel increase in these two n-6 fatty acids was observed, especially in the liver and the testis (present experiment). Therefore, the expression of elongase (*elovl5*) and desaturase (*Δ4fad*) transcripts showed that ARA induced a marked dose dependent up-regulation for both transcripts, with *elovl5* increasing 5.3-fold in males fed 6.0% ARA, indicating the elongation of 20:4n-6 into 22:4n-6 in fish liver and testis and *Δ4fad* being up regulated in response to 2.3% and 6.0% ARA in the diet (groups C and F), indicating the desaturation of 22:4n-6 into 22:5n-6 in the same tissues. This is in agreement with previous studies that show the relevance of *Δ4fad* pathway in Senegalese sole fed short chain PUFAs, demonstrating the functional characterization of *Δ4fad* in the production of PUFA (Li *et al.*, 2010, Morais *et al.*, 2011). However, the up-regulation of these two enzymes was only observed in males. Gender differences in liver desaturase expression was previously observed in Wistar rats, fed n-3 (18:3n-3) enriched diet (Extier *et al.*, 2010). In that case desaturation activity was increased significantly in the females compare with males in order to produce DHA (Extier *et al.*, 2010), moreover it was shown that female rats have higher plasma DHA concentrations than males (Childs *et al.*, 2008) and in the present experiment desaturase expression was associated to the conversion of 22:4n-6 into 22:5n-6 in G1 male fish, both are fatty acids important in testis and sperm composition (Leat *et al.*, 1983, MacDonald *et al.*, 1984, Lenzi *et al.*, 1996, Lenzi *et al.*, 2000, Furland *et al.*, 2007). More studies are necessary to understand the importance of these two fatty acids and any physiological function in male fish reproduction.

5. Conclusion

Based on previous results of ARA content in wild Senegalese sole (Norambuena *et al.*, 2011c) (chapter II), and considering the results of the experiment presented here, we found

that fish fed either 2.3% or 3.2% ARA enriched diets did not show any difference in testis, ovary and liver fatty acid composition compared to wild fish. Thus, could be suggested to use 3.2% ARA enriched diets if the feeding period is shorter than nine months (i.e., this chapter) and 2.3% dietary ARA for prolonged feeding periods (i.e., this chapter and chapter V). The ARA was transferred and conserved in testis and ovary, followed by liver and muscle. The increase in the expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts in response to dietary ARA content revealed a marked up-regulation in both transcripts in males with a parallel increase in 22:4n-6 and 22:5n-6 acids as a consequence of the elongation of ARA and 22:4n-6 desaturation. Further studies are needed to establish the dietary ARA effect on reproductive performance of Senegalese sole and the time required for an effective incorporation of ARA into the reproductive organs.

6. Acknowledgments

We gratefully give thanks to Gordon Bell, Sofia Morais, Alicia Estevez and Douglas Tocher for the scientific help and Grethe Rosenlund from Skretting ARC, Norway for diets manufacturing. Thanks to Joan Cerdà from IRTA for providing gene sequences for the determination of expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts. Thanks are also given to Juan Carlos Navarro from IATS-CSIC for help with the separation and quantification by gas-liquid chromatography of fatty acid. Thanks to Noelia Gras, Marta Sastre and Ignacio Carazo for sample collection and technical support. This research was funded by two projects: 1) Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project Lenguado and 2) Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by N. Duncan and the PhD grant awarded to F. Norambuena by Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR).

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Chapter VII

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSION

1. Wild and cultured fish: ARA, COX and PGs

In chapter II, proximate, lipid class and fatty acid composition of several tissues of wild and cultured broodstock fish (G1) raised under natural captive conditions was studied together with the diets commonly used to feed Senegalese sole. G1 fish had a higher content of lipid reserves, exhibited a significantly lower accumulation of ARA and CHOL and higher levels of EPA in some tissues compared to wild fish and these differences were a consequence of the diet used for feeding G1 fish. In chapter III, for the same groups of wild and cultured fish, cyclooxygenase (COX-2) mRNA expression and prostaglandin (2- and 3- series) levels in tissues and blood were examined. The expression of COX-2 was significantly down regulated in male gills and sperm-duct and in female oviduct of cultured fish compared to wild fish, with the pattern in COX-2 expression, which showed a similar trend to that observed for total PGs ($r^2 = 0.53$). Wild fish showed significantly higher levels of total 2-series PGs and lower levels of 3-series compared to cultured fish, especially in blood and testis. Studies that compared lipid profiles from wild and cultured fish found similar differences in respective lipid profiles and particularly higher levels of ARA and ARA-derived fatty acids in wild versus cultured fish for common sole (*Sole solea*) (Lund *et al.*, 2008), turbot (*Scophthalmus maximus*) (Silversand *et al.*, 1996) white seabream (*Diplodus sargus*) (Cejas *et al.*, 2003, Cejas *et al.*, 2004), black seabream (*Dicentrarchus labrax*) (Rodríguez *et al.*, 2004), striped bass (*Morone saxatilis*) (Harrell *et al.*, 1995), macquarie perch, (*Macquaria australasica*) (Sheikh-Eldin *et al.*, 1996) Atlantic cod (*Gadus morhua*) (Salze *et al.*, 2005), and rainbow trout (*Onchorhynchus mykis*) (Blanchet *et al.*, 2005, Aslan *et al.*, 2007). Previous studies on the reproductive behaviour of captive wild and G1 cultured broodstock have shown that G1 cultured males do not complete courtship to fertilise the ova released by females (Carazo *et al.*, 2009, Carazo *et al.*, 2011). ARA, which was found to be at higher levels in wild Senegalese sole, is the main precursor of 2-series PGs which have an important role in fish reproduction (Smith, 1989, Zhang *et al.*, 1992, Tocher, 2003, Alorend, 2004). In teleosts PGs have been shown to be involved in follicle maturation (Kobayashi *et al.*, 2002, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001), ovulation (Sorensen *et al.*, 1993, Sorbera *et al.*, 2001, Fujimori *et al.*, 2010) spermiation

(Zheng *et al.*, 1996), stimulation of steroidogenesis (Wade *et al.*, 1993b, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1994, Mercure *et al.*, 1995) and to act hormonally to trigger female sexual behaviour (Laberge *et al.*, 2003, Moore, 1996, Sorensen *et al.*, 1995, Sorensen *et al.*, 2004, Sveinsson *et al.*, 1995, Kobayashi *et al.*, 2002), acting as a pheromones as well as increase milt production in males. Although these COX-derived metabolites can be produced from EPA in the form of 3-series PGs (Smith, 1989, Wade *et al.*, 1994, Bell *et al.*, 1994, Mercure *et al.*, 1995, Tocher, 2003), the 3-series PGs did not produce the same effects on oocyte maturation (Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). The results presented in chapter III show a down-regulation of COX-2 transcripts in the sperm- and ovi-duct and low concentration of total PGs in testis of G1 fish that might indicate a reproductive problem since this enzyme and its metabolites are considered, as stated previously, relevant in ovulation and spermiation of fish. Moreover, the 2-series PGs (PGF2α and PGE2) were found to be in higher concentration in blood and some tissues (i.e., testis and gills of male fish), of wild fish whereas in cultured fish, which also exhibited an overall lower ARA content had 2-series PGs levels that were less concentrated than 3-series PGs. PGF2α is responsible for the synchronization of courtship between male and female fish during spawning (Stacey *et al.*, 1982, Golubev, 1984, Sorensen *et al.*, 1995, Moore, 1996, Sorensen *et al.*, 2004, Stacey *et al.*, 2003) whereas PGE2 is important for the follicle maturation and steroid production in ovaries and testis (Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a, Sorensen *et al.*, 1995, Kobayashi *et al.*, 2002, Stacey *et al.*, 2003, Sorensen *et al.*, 2004, Espey *et al.*, 2006, Fujimori *et al.*, 2010). Therefore, the reduction of PGs 2-series observed in the blood and some tissues, particularly testis, of G1 fish might be triggering problems in the final maturation, ovulation, spawning synchronization and reproductive behaviour of G1 Senegalese sole and, as a consequence, a reproductive dysfunction especially important in the case of males.

2. Dietary ARA levels of cultured fish, and effects on reproductive physiology

Taking into account the results obtained in previous chapters II and III, a study of the effects of graded dietary levels of ARA on blood lipid and fatty acid composition, as well as on PGs and steroid hormones production of G1 Senegalese sole was conducted (chapter IV). Six replicated groups of equal number of fish (groups A to F) were fed six different

diets with graded levels of ARA (0.7% to 6.0% total fatty acids) for nine months. The graded levels of dietary ARA gave a dose dependant response in ARA in blood and tissues in the fish in the different experimental groups. The EPA/ARA ratio consequentially decreased with increasing dietary ARA. The levels of PGs 3-series were always higher than 2-series in both F- and E- isomers while levels of 2-series PGs were similar. However, 3-series PGs were reduced concomitant with the increase in ARA content in the blood as a result of increasing ARA levels in the diet. The results in chapter III indicated that the production of 2- and 3-series PGs was proportional to the EPA/ARA ratio found in fish blood and tissues which was also a consequence of the diet fed to fish. Thus, ARA dietary content had a direct effect on the production of PG 2-series (Bell *et al.*, 1994, Bell *et al.*, 1995) whereas 3-series PGs were a consequence of the high EPA content in all the experimental diets (approx 13% TFA).

Steroid production of 11-KT and T in the males increased significantly in parallel to dietary ARA content, whereas estradiol (E2) produced by females did not change. Cholesterol levels in blood also showed a significant increase in an ARA dose dependent manner. In vitro studies with goldfish and trout have shown an ARA-stimulated testosterone production via COX pathway, being the ARA effect mediated through its conversion to PGs (Mustafa *et al.*, 1989, Wade *et al.*, 1994, Van Der Kraak *et al.*, 1990, Wade *et al.*, 1993a). According to these studies, EPA exerted an opposite effect inhibiting testosterone production via the inhibition of cAMP production, with ARA-induced maturation depressed. Thus, an increase in the production of EPA-derived PGE3 had no effect on fish maturation (Sorbera *et al.*, 1998, Sorbera *et al.*, 2001). Any change in dietary EPA/ARA ratio or in the levels of these two fatty acids in fish tissues will influence the production of PGs and steroids. The results obtained in vivo in chapter IV were consistent with previous in vitro studies showing a significant increase of plasma steroid levels derived from increasing dietary levels of ARA. 11-KT and T showed a clear dietary response in males with blood ARA levels increasing and EPA/ARA ratio being reduced, whereas steroid (11-KT and T) levels increased progressively to peak in May in all the groups. Although the steroid levels found in chapter IV were lower than those reported previously in wild males of Senegalese sole (García-López *et al.*, 2006). In females E2 did not show any increase in plasma levels derived from increasing ARA content. However, E2 levels peaked in March,

one month earlier than in males, in all the groups except the control A which was fed the lowest ARA content. Similar results have been reported in Atlantic cod and Atlantic halibut in response to graded levels of dietary ARA. In Atlantic cod plasma vitellogenin concentration peaked one month earlier (Norberg *et al.*, 2009) whereas in halibut a two week delay of vitellogenin production was observed (Alorend, 2004). Although a certain regulation of steroid production by ARA was observed, more studies are required to clarify the effects. Changes in dietary CHOL might also be affecting the steroid production in broodstock fish (Baron *et al.*, 1997). The results presented in chapter IV showed that blood ARA and CHOL levels increased in parallel to the dietary content of ARA, in spite of CHOL levels being the same in all the diets used. Similar results were observed in chapter II with wild Senegalese sole showing significantly higher CHOL levels in the liver compared with cultured G1 fish. One explanation might be that dietary ARA induced the mobilization of CHOL in the blood by means of cAMP formation (Mercure *et al.*, 1996). It has been shown that EPA blocks cAMP formation and consequently the transport of CHOL in steroidogenic tissues (Mercure *et al.*, 1995). The results presented in chapter IV showed that EPA in blood, despite of all diets containing the same level of EPA, was reduced in an ARA dose related manner, therefore, the lower EPA levels in combination with the higher ARA levels in blood might be inducing cAMP formation and CHOL circulation. Another explanation of the high CHOL levels in blood might be the biosynthesis by the fish. Leaver *et al*. (2008) showed that Atlantic salmon increased CHOL biosynthesis after dietary substitution of fish oil with vegetable oil, however, for Senegalese sole no information is available regarding the capacity of the fish to synthesis CHOL .

In summary, feeding the correct dietary ARA levels improved the physiological state of broodstock fish, reducing the levels of 3-series PGs, increasing the steroid levels in males (11-KT and T) and CHOL production in both males and females. However, further studies are required to clarify the effects of EPA dietary content in COX pathway, since the optimal dietary ARA content will be strongly dependent on the EPA/ARA ratio.

3. Requirement of dietary ARA of G1 fish, and tissue composition

Two experiments were carried out to determine the ARA requirement of G1 Senegalese sole and the results presented in chapters V and VI. In chapter V, ARA preference of G1

fish were estimated by the use of self-feeders. For this purpose, three groups of G1 fish were offered the choice between two diets, one with high (6.0% TFA) and another with low (0.7% TFA) dietary ARA content by means of two self- feeders per tank during 16 months. The second experiment was the dose-response experiment (also reported in chapter IV), which was performed using six different graded levels of ARA (0.7 to 6.0% TFA) during nine months before the fish sampled and the ARA content of G1 fish tissues examined and compared with those obtained from wild fish already presented in chapter II. Fatty acyl desaturase (*Δ4fad*) and elongase (*elovl5*) expression in the liver of the fish fed the three ARA-enriched diets (0.7%, 2.3% and 6.0% TFA) as well as the increase of adrenic (22:4n-6) and docosapentaenoic (22:5n-6, DPA) in fish tissues were analysed in order to examine ARA nutritional regulation in Senegalese sole.

The results of the self-feeding experiment showed that Senegalese sole have a significantly different ARA preference during the year, with periods of high (≈ 4% TFA) and low (≈2% TFA) demand for ARA. The average ARA demanded for the whole 16 month period was 3.05% TFA and the fish at the end of the feeding period had no differences in tissue (i.e., gonads and liver) ARA content compared with wild fish (chapter II). Additionally, the dose-response experiment showed that ARA levels in G1 fish tissues increased in a dose dependent manner in both sexes. Fish fed 2.3% and 3.2% ARA showed no differences in ARA content in their gonads and liver compared with wild fish. The expression of elongase (*elovl5*) and desaturase (*d4fad*) transcripts in response to dietary ARA content revealed a pattern of up-regulation in both transcripts with a corresponding increase of 22:4n-6 and 22:5n-6 acids especially in testis, as this up-regulation was only observed in males.

Senegalese sole is a seasonal spawner, and gonad maturation occurs during early spring (April-May) in parallel to the increase in plasma sex steroids and luteinizing hormone with maximum levels reached just before spawning (Guzmán *et al.*, 2008, Guzmán *et al.*, 2009, García-López *et al.*, 2007). The results of the ARA self-feeding experiment presented in chapter V shows that the selection of Senegalese sole for ARA change with the water temperature (r^2=0.65). ARA preference increases gradually in February reaching the maximum demand at late summer-autumn with a posterior drastic diminution, with the water temperature reduction. These seasonal variations in ARA demanded by Senegalese

sole are in agreement with the fatty acid composition of the tissues of wild common sole (*Solea solea*). Gökçe *et al.*, (2004), studied the seasonal variation in muscle fatty acid composition of wild common sole and observed a pronounced seasonal fluctuation with significantly lower ARA levels in the fish sampled during the summer (4.7% TFA) and the highest values found in the fish of late winter (12.3% TFA), just after the reproduction period which commonly occurs in early winter for this species (Ramos, 1986, Imsland *et al.*, 2003). Similar fluctuations were observed in white seabream (*Diplodus sargus*) (Özyurt *et al.*, 2005, Pérez *et al.*, 2007), with the highest ARA accumulation in gonads (≈ 4%) in post-spawning (June) stages (Pérez *et al.*, 2007). This seasonal variation in ARA content has been also reported in horse-mackerel (*Trachurus trachurus*) (Bandarra *et al.*, 2001), anchovy (*Engraulis encrasicolus*) (Zlatanos *et al.*, 2007, Tufan *et al.*, 2010), sardine (*Sardina pilchardus*) (Bandarra *et al.*, 1997, Zlatanos *et al.*, 2007) picarel (*Spicara smaris*) (Zlatanos *et al.*, 2007) and vimba (*Vimba vimba*) (Kalyoncu *et al.*, 2009).

The analysis of the tissues of fish fed by self-feeding showed ARA content in gonads and liver similar to that found in wild fish, and presented in chapter II. The same was also observed in the case of testis and ovary of fish fed 2.3% and 3.2 % ARA diets C and D, respectively of the dose-response experiment (result showed on chapter VI). Thus, fish fed diets C and D showed no differences in tissue composition when compared to wild fish. The self-feeding experiment lasted for 16 months, seven more than the dose-response experiment, suggesting that the ARA accumulation in the tissues is dependent not only on the dietary level but also on the feeding period. Therefore, if fish are fed with ARA-enriched diets for a period shorter than 9 months, it might be appropriate to use at least 3.2% ARA in the diet. However for prolonged feeding periods, longer than nine months, a 2.3% ARA in the diet might be enough for Senegalese sole G1. The ARA requirements for G1 Atlantic halibut were studied during three years and Alorend (2004) suggested that 2.3% ARA was an optimal dietary content to obtain a longer milt production period and the higher fecundity, being this fatty acid (ARA) rapidly incorporated into reproductive fish tissues (i.e., 3 months feeding). ARA requirement of broodstock fish is species-specific with a precise optimal dietary level. Higher or lower levels than the optimal have shown a detrimental effect in the reproductive physiology (Mercure *et al.*, 1995, Mercure *et al.*,

1996, Norberg *et al.*, 2009, Sorbera *et al.*, 1998, Sorbera *et al.*, 2001, Furuita *et al.*, 2003, Mazorra *et al.*, 2003, Alorend, 2004, Norambuena *et al.*, 2011).

In chapter IV, a significant increase in male steroids (11-KT and T) was observed in the fish fed a diet above 3.2% ARA, with females showing a peak in E2 one month earlier. Similar results were observed in female Atlantic cod, with one month earlier production of E2 and vitellogenin (Norberg *et al.*, 2009) although, the effect of ARA on hormonal production is species-specific. It has been shown in Japanese flounder (*Paralichthys olivaceus*) that a 3.6% ARA dietary content induced the highest egg production (Furuita *et al.*, 2003), whereas a negative effect on steroid production and egg and larval quality were observed when the fish were fed 7.3% ARA. Norberg *et al.* (2009) observed that 4% dietary ARA increased E2 production and vitellogenesis, which was advanced by one month, and extended the spawning season in Atlantic cod (*Gadus morhua*). Similar findings were obtained in Atlantic halibut fed with 3.2% ARA, in this case with a delay in the spawning season (Alorend, 2004). Considering all these results, one of the conclusions is that more research is needed to establish the dietary ARA effects on reproductive performance of Senegalese sole, and the minimal feeding time required in order to incorporate dietary ARA in fish tissues and among them the gonads.

A positive correlation between the levels of ARA in the diet and the concentration of 22:4n-6 and 22:5n-6 acids in testis, liver and muscle of wild fish and produced as a consequence of ARA elongation and 22:4n-6 desaturation, was found (chapter II) (Linares *et al.*, 1991). Thus, cultured fish showed a significantly lower accumulation of C22 n-6 compared to wild fish. In the dose-response experiments presented in chapters IV and VI both fatty acids were observed to increase significantly in blood, testis and liver in an ARA dose dependent manner. Similar accumulation of ARA and C22 n-6 was observed in muscle, testis, ovary, and sperm of different species of wild fish (Bell *et al.*, 1997, Vassallo-Agius *et al.*, 2001, Cejas *et al.*, 2003, Rodríguez *et al.*, 2004, Huang *et al.*, 2010, Norambuena *et al.*, 2011, Bell *et al.*, 1996) as well as in testis and sperm of mammals, being the accumulation of 22:5n-6 in testis supported by local production and uptake from the circulation system, after its synthesis in the liver (Rosenthal *et al.*, 1991, Tam *et al.*, 2000, Tam *et al.*, 2008). Although, the physiological function of these PUFAS is not well

known, it is believed that they are involved in sperm formation and membrane fluidity as a prerequisite for normal cell fusion in the fertilization process (Lenzi *et al.*, 1996), increasing the degree of fatty acid metabolism and desaturation either during spermatogenesis and sperm maturation (Lenzi *et al.*, 2000, Furland *et al.*, 2007).

The level of expression of elongase (*elovl5*) and desaturase (*Δ4fad*) transcripts in the liver of G1 fish was assessed in response to dietary ARA content in chapter VI. ARA induced a marked dose-dependent up-regulation for both transcripts, with *elovl5* increasing 5.3-fold in males fed 6.0% ARA (Group F), indicating the elongation of 20:4n-6 and subsequently an increase of 22:4n-6 in fish tissues, mainly in gonad and liver of males. Meanwhile, *Δ4fad* was significantly up-regulated in response to 2.3% and 6.0% ARA in the diet (groups C and F), which indicates the desaturation of 22:4n-6 to 22:5n-6 which showed a clear increase in the liver and testis of male fish. These results are in agreement with previous studies that show the relevance of *Δ4fad* pathway in Senegalese sole fed short chain PUFAs, demonstrating the functional characterization of *Δ4fad* in production of LC-PUFA (Li *et al.*, 2010, Morais *et al.,* (2011, unpublished). However, this was not observed in the females.

Gender differences in liver desaturase expression were observed in rats, showing n-3 (18:3n-3) fatty acid depletion in parallel to an significantly increase in desaturation activity in females compare with males, in order to produce DHA, moreover it was shown that female rats have higher plasma DHA concentrations than males (Childs *et al.*, 2008). In the present experiment with fish, desaturase expression was associated to the production of 22:5n-6 from ARA both important fatty acids in testis and sperm composition. More studies are necessary to elucidate the purpose of this increase of 22:4n-6 and 22:5n-6 acid on male tissues and its effects on spermatogenesis and reproduction.

4. Significant results

- Cultured fish showed significantly higher levels of total lipid, in male liver, and in neutral lipid class, and linoleic 18:2n-6 acid in gonads, liver and muscle compare with wild fish.

- Cultured fish compare with wild had lower levels of ARA acid in the liver, testis and muscle. Moreover CHOL and DHA, in the liver of male and female G1 fish was lower compared with wild. These differences resulted in significantly higher ratios of EPA/ARA in the liver and muscle of cultured fish.
- The observed imbalance in lipids and essential fatty acids (EFAs) in G1 fish were mainly reflection of the extruded diet used to feed the fish. Thus commercial extruded feed, commonly used to fed broodstock fish, had a deficiency of ARA, polar lipid and CHOL and supplied an excess of total lipid, 18:2n-6, and EPA.
- Down-regulation of cyclooxygenase gene expression in oviduct, sperm duct and gills of male (highly expressed in oviduct and testis) and low production of PGs 2-series (PGE2 and PGF2α) in blood and testis of G1 fish was found compared to wild fish.
- In male fish, the steroid (11-KT and T) levels increased significantly with increasing dietary ARA in a dose dependent response. Whereas in females estradiol (E2) did not show any change related to dietary ARA content.
- Plasma concentration of PGs 3-series (PGE3 and PGF3α) were reduced in parallel to increased blood ARA levels. However plasma concentration of PGs 3-series (PGE3 and PGF3α) was always higher than 2-series prostaglandins (PGE2 and PGF2α) (males and females).
- The increased ARA levels in blood resulted in an increase CHOL mobilization on blood. Therefore was suggested an interactions among ARA ingestion and tissue accumulation, PGs and CHOL production and the endocrine control of reproduction in Senegalese sole.
- In-vivo study supported the competitive nature of EPA and ARA acid content in the production of respective 3- and 2-series PGs that was previously described in-vitro studies.
- Senegalese sole using self-feeders showed a seasonal fluctuation in selection of dietary ARA, which was correlated with water temperature (r^2=0.65), with maxim demand in late-summer, early-autumn.
- Senegalese sole showed a dietary ARA preference of 3.0% TFA, after 16 month self-selection.

- *S. Sole* fish fed either 2.3 or 3.2% ARA enriched diets during nine months, did not show any differences in testis, ovary and liver ARA composition compared to wild fish.
- Increase of dietary ARA content, revealed a marked up-regulation in elongase (*elovl5*) and desaturase (*d4fad*) transcripts in liver of males, with a parallel increase in 22C n-6 acids (especially in testis) as a consequence of the elongation and desaturation of ARA.
- Based on these results, was suggested to use 3.2% ARA enriched diets if the feeding period is shorter than nine months and 2.3% dietary ARA for prolonged feeding periods.

5. Conclusion

The hypothesis tested here was: 'there are differences in lipids and fatty acid profiles between wild and G1 Senegalese sole cause differences in the reproductive physiology of sole'. The hypothesis is fully accepted, as it was demonstrated that G1 fish compared with wild fish had lower levels of cholesterol and arachidonate acids and changes in EPA/ARA ratios (mainly in liver). Consequently these nutritional changes were shown to affect the reproductive physiology of G1 fish, thus down-regulation of cyclooxygenase gene expression (i.e., oviduct and sperm duct) and low production of PGs 2-series in blood and testis of G1 fish was shown. Moreover, it was demonstrated that the reproductive physiology of G1 fish was affected by dietary ARA content in extruded fed and increasing ARA dietary levels partially restored aspects of the G1 fish physiology to that of wild fish. Increasing dietary ARA increased availability of cholesterol in blood, reduced 3-series PGs, and markedly increased steroid production (11-KT and T) in G1 male fish. Furthermore, up-regulation in elongase (*elovl5*) and desaturase (*d4fad*) transcripts in liver of male fish, with a parallel increase of 22C n-6 acids that might be involved in fish reproduction were demonstrated. All this might have important consequences in the reproductive dysfunction previously observed in G1 Senegalese sole.

6. Further studies

- The effects of the 'optimal' levels of ARA on Senegalese sole gamete production and spawning i.e., ovulation, spermiation or courtship behaviour need to be established. Therefore, more research effort is needed to establish the dietary ARA effect on reproductive performance of Senegalese sole and minimal times required to incorporate ARA in reproductive organs.
- Further studies are necessary to examine the mechanism for arachidonic acid regulation of steroidogenic acute regulatory (StAR) protein expression in fish and in Senegalese sole and clarify the relationship between, ARA-release and ARA-metabolites, StAR protein, cholesterol and steroids production.
- Studies are required to elucidate the seasonal fatty acid content in wild Senegalese sole fish tissues and the relationship between these seasonal changes and utilization of ARA during the year, evaluating CHOL and PGs production along the year.
- Considering the high ARA concentration on gonads and the large differences in weight between ovaries and testis of Senegalese sole, studies are required to elucidate if the ARA dietary requirement between males and females are different.
- Studies are necessary to understand the purpose of adrenic (22:4n-6) and DPA (22:5n-6) acids observed on male tissues (i.e., especially on testis) and its possible effects on spermatogenesis and male reproduction.
- High levels of PGs 2-series were observed in gills of cultured female sole. Considering the described reproductive behavioural problems between cultured male and female broodstock, the significance of these high levels should be investigated.

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Abbreviation list

11-KT	Ketotestosterone
18S rRNA	18S ribosomal RNA
ANOVA	Analysis of variance
ARA	Adrenic acid, 22:4n-6 Arachidonic acid, 20:4n-6
CHOL	Cholesterol
COX	Cyclooxygenase
COX-1	Cyclooxygenase isoenzymes one
COX-2	Cyclooxygenase isoenzymes two
Ct	Threshold cycle
DHA	Docosahexaenoic acid, 22:6n-3
DM	Dry matter
DPA n-6	Docosapentaenoic, 22:5n-6
E2	Estradiol
EAA	Essential amino acids
EFA	Essential fatty acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immuno sorbent assay
Elite®	Elite repro, extruded fed
elovl5	Fatty acid elongase
EPA	Eicosapentaenoic acid, 20:5n-3
FAME	Fatty acid methyl esters
FCR	Feed conversion rate
FFA	Free fatty acids
G1	Cultured broodstock fish
GSI	Gonad somatic index
HIS	Hepatosomatic index
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
HUFA	Highly unsaturated fatty acids
MUFA	Mono saturated acids
L-male	Liver male
L-female	Liver female
M-male	Muscle male
M-female	Muscle female
NL	Neutral lipids
P. Neth.	Polychaetes from Netherlands
P. UK	Polychaetes from England
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGs	Prostaglandins
PGE	Prostaglandin E-isomer
PGF	Prostaglandin F-isomer
PI	Phosphatidylinositol
PIT tags	Passive integrated transponders
PL	Polar lipids
PLC	Programmable logging controller
PS	Phatidylserine
PUFA	Polyunsaturated fatty acid
qrtPCR	Quantitative real-time
RT-PCR	Semi quantitative real-time
S. Sole	Senegalese sole (*Solea senegalensis*)
S-duct	Spermi duct
SE	Sterol
SEM	Stanadar error of the mean
SFA	Total saturated fatty acids
SGR	Specific growth rate
Spz	Spermatozoids
T	Testosterone
TAG	Triacylglycerides
TFA	Total fatty acids
TL	Total lipids
TW	Total wet weight
Vitalis®	Vitalis repro, extruded fed
Δ4fad	Delta 4 desaturase

Acknowledgements

I would like to thank everyone who has contributed to this thesis. Thank you to my supervisor Neil Duncan for the opportunity to work in fish reproduction and complete this thesis project. Special thank also to Alicia Estevez, for her constant help in fish nutrition and advice during the entire study. Thanks are also given to Simon Mackenzie from UAB-IBB for the thesis guide and help and bimolecular training and study of cyclooxygenase gene expression. Thanks to Gordon Bell from Stirling University for his continuous help in the studies of fatty acids, prostaglandins analysis and training during my stays in Stirling. Thank you also to Douglas Tocher, for his help in the studies of metabolism of fatty acids and the elaboration of inform for the European doctorate mention. Thank to Agnes Callol from UAB-IBB and Sofia Morais from Stirling University for help in the studies of gene expression. Thanks to Evaristo Mañanós, Maria Jose Bayarri y Vanesa Piquer from IATS-CSIC for help in hormone analysis. Thanks are given to Birgitta Norberg from Institute of Marine Research, Norway for her inform for the European doctorate mention. Thanks to Grethe Rosenlund from Skretting ARC, Norway for diets manufacturing free of charge and help in experimental design. Thank also to Juan Carlos Navarro from IATS-CSIC for help with the separation and quantification by gas-liquid chromatography of fatty acid and Francisco Javier Sánchez-Vázquez from University of Murcia for helps in the study of self-feeding and experimental design. Thanks to Joan Cerdà from IRTA for providing gene sequences. Special thank to Karl Andree who was helping me with the English and Mike Hites for technical support in the self feeder's designs. Thanks are also to Ignacio Carazo for help with the sampling procedures and in general things. Special thank are given to Noelia Gras and Marta Sastre and Pili Gonzalvo for help in biochemical analysis and Gloria Macia, Josep Lluis Celades, Nuria Soler, Sandra Molas, Esteban Hernandez and Magda Monllaò for looking after the fish and helping with sampling and general things related with the hatchery, thanks also to Pietro Vidossich and Francesco Bucci for help in sampling collection. Special thank to Lluis Torres, (who sadly passed away before the completion of this thesis) for help with the statistical analysis. Thank are also given to Olvido Chereguini who provide the cultured fish and Angel the fisherman from cooperative of Sant Carles de la Ràpita, for provide the wild fish. Thanks to the Agència de Gestió d'Ajuts Universitaris i de Recerca, Catalonia (AGAUR) for the grand award (FI) during four years and IRTA for

the grand award during the first year year. The thesis was developed and most of the work completed in IRTA-SCR and thanks are given for the institutional support that IRTA provided. Thank also for the help provided by the Catalan Network for Aquaculture Research (XRAq). This research was funded by two projects of the Ministerio de Agricultura, Pesca y Alimentación (MAPA)-JACUMAR Project Lenguado coordinated nationally by Pedro Cañavate and in Catalonia by Neil Duncan and proyect of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentarías (INIA)-FEDER project RTA2005-00113-00-00 coordinated by Neil Duncan.

Agraïments

Arribats a n'aquest punt puc dir que aquesta tesi doctoral ha estat per mi com una ascensió a un difícil tres mil dels Pirineus, durant la qual he gaudit i he aprés moltíssim. Tot i que de vegades ha estat molt difícil portar-ho endavant i hagi necessitat d'una gran motivació interior per a poder continuar en direcció al cim. Ha sigut molt important el recolzament de persones properes que van saber entendre les motivacions e inquietuds que m'han dut a seguir aquest camí. Gràcies a n'aquestes persones i a la n'aquesta inquietud interior per buscar respostes als problemes relacionats amb la nutrició i reproducció dels llenguados, va sorgir aquesta tesi doctoral que des d'un principi va tenir un propòsit, una pregunta, una hipòtesis però només després de cinc anys ha arribat a tenir cos i nom. He d'agrair totes aquestes experiències personals, converses anònimes, discussions de passadís, a tants articles científics que em van il·lusionar amb els seus resultats i discussions, als investigadors que he pogut conèixer durant aquests anys, i en especial al reconeixement humil de la meva pròpia ignorància i de les limitacions que m'han acompanyat des de sempre. Tots aquests elements han aportat en la seva mesura a l'elaboració d'aquest treball final.

Aquests capítols de tesi s'han escrit gràcies a una beca atorgada per l'AGAUR i a projectes d'investigació conduïts pel meu supervisor Neil Duncan. A més de la confiança i del criteri científic de la investigadora Alicia Estévez i de la bona voluntat d'investigadors com Gordon Bell, Simon Mackenzie i Evaristo Mananos, els quals em van rebre amb un gran somriure i una forta encaixada de mans, als seus laboratoris, per a intentar aprendre a trobar respostes i a superar les dificultats que qualssevol treball científic presenta. Conjuntament he d'agrair a Noelia Gras i a Marta Sastre pel seu gran treball professional, així mateix també a Glòria Macias per la seva entrega i dedicació en l'atenció dels peixos i a Karl Andree per la seva desinteressada ajuda, en les sempre complicades redaccions d'anglès. També vull agrair de forma especial a Dolors Furones, directora de l'IRTA-SCR per la seva confiança dipositada en mi quan encara estava a Noruega i es gestava en mi la il·lusió de venir a treballar aquí a Catalunya. Potser en el moment que més necessitava de la confiança d'una institució com l'IRTA, li agraeixo a ella i a Neil, per l'oportunitat que em van donar d'iniciar un treball de tesi doctoral.

Per acabar vull agrair les oportunitats que m'ha donat la vida. Com em va recordar un dia Josep M. Monfort (director de l'IRTA) durant una curta conversa quan recent començava aquesta tesi, "La sort s'ha de sortir a buscar-la" fent al·lusió a la meva partida des de Xile... fa uns anys vaig arribar a l'IRTA amb la il·lusió de treballar en aquesta tesi. No obstant això, durant aquest trajecte la vida em va voler donar una sorpresa encara més gran i vaig trobar aquí, entre peixos, àcids grassos i prostaglandines a Pili. Una catalana que m'ha ensenyat a estimar i a formar part d'aquesta bonica Catalunya, per mi fins llavors desconeguda i distant. Gràcies al seu suport, les seves abraçades, somriures i paciència en els moments d'angoixa i desesperació, he pogut acabar aquesta tesi, i amb això traient el millor de mi. Gràcies per regalar-me aquesta il·lusió de creure i de voler recórrer aquest camí al meu costat. Gràcies també a Mikel, Pili i Josep i al meu amic i company d'escalada Jordi. Tots junts heu aconseguit que durant aquest viatge em senti com a casa....moltes gràcies!


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