Original article

Assessing feeding history and health status through analysis of fatty acids and fat content in golden mullet *Liza aurata*

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The aim of this study was to check the suitability of using fatty acids of vegetable origin as biomarkers of aquafeed consumption in fish that aggregate around off-shore fish farms, analysing their different accumulation patterns and their persistence in different tissues in juveniles of *Liza aurata* (Risso, 1810). Their natural diet was replaced by a commercial feed, followed by a return to the natural diet (wash-out period). The fatty acid profiles of flesh, liver, and brain were modified after 2 months of commercial feed consumption, while 2 months of the wash-out period were not sufficient to return to original values, the brain being particularly resilient in this respect. Histological examination of the liver showed no alterations of the lipid droplet distribution or fat content. The combined use of flesh and brain for fatty acid analysis can be recommended for tracking aquaculture waste intake in the form of lost pellets by wild fish.

Keywords: aquaculture wastes, biomarker, fatty acids, linoleic acid, teleost.

Introduction

During recent decades, the aquaculture industry has had to face the challenge of providing adequate and sustainable fish feeds that ensure optimal growth without compromising fish health and welfare, while keeping production costs and potential ecolog- ical impact to a minimum (Naylor *et al.*, 2000; Pauly *et al.*, 2002, Shepherd *et al.*, 2005). The current composition of aquafeeds reflects how fish oil has been partially substituted by vegetable oils (Dalsgaard *et al.*, 2003; Hardy, 2010; Turchini *et al.*, 2010). In general terms, fish oils are characterized by high levels of long-chain polyunsaturated fatty acids (LC-PUFA), mainly of the n—3 series, such as docosahexaenoic acid (22:6n—3, DHA) and eicosapentaenoic acid (20:5n—3, EPA) (Ackman, 1989). The vegetable oils used in aquafeeds include soybean, sunflower, rapeseed, or linseed oils among others. Such oils are rich in short/medium-chain polyunsaturated fatty acids, including linoleic acid (18:2n—6, LA) and a-linolenic acid (18:3n—3, LNA), besides monounsaturated fatty acids like oleic acid (18:1n—9, OA) and saturated fatty acids (Watanabe, 2002). The high amounts of these fatty acids in fish feeds entail low levels of DHA and EPA, which are essential for marine fish (Sargent *et al.*, 2002; Glencross, 2009; Tocher, 2010), and also arachidonic acid (20:4n—6, ARA), a fatty acid usually present in low levels in fish tissues but which plays an important role in reproduction and regulation of the immune functions (Rowley *et al.*, 1995; Koven *et al.*, 2001; Bell and Sargent, 2003).

It is well known that off-shore aquaculture facilities may act as Fish Aggregation Devices (Dempster *et al.*, 2002, 2010; Vita *et al.*,

2004; Sanchez-Jerez *et al.*, 2011), where wild fish and macroinvertebrate species can benefit from the protection provided by the submerged structure and the excess feed (Fernandez-Jover *et al.*, 2008; Gonzalez-Silvera *et al.*, 2015). It has been demonstrated that wild fish can consume up to 80% of fish feed in the form of lost pellets (Vita *et al.*, 2004), which differ from a natural diet in their higher lipid content and the fatty acid profile. Such uncontrolled consumption of excess feed by the aggregated wild fish can lead to modifications in total lipid percentages and fatty acid composition (Skog *et al.*, 2003; Fernandez-Jover *et al.*, 2011; Abaad *et al.*, 2016), due to the limited capacity of marine fish to metabolize the excess of both LA and LNA through the LC-PUFA biosynthesis pathway (Cook and McMaster, 2002; Dalsgaard *et al.*, 2003; Glencross, 2009; Tocher, 2015). Consequently, the accumulation of those fatty acids in different tissues, when the natural diet of wild fish is changed through the consumption of lost pellets in the vicinity of fish farms should be considered, as well as the effect of a subsequent return to their natural diets when aggregated wild fish later migrate to areas far from the fish farm sea-cages. Although this has been investigated widely in flesh because of the importance of its fatty acid profile in terms of human consumption (Steffens, 1997; Rosenlund *et al.*, 2001; Kris-Etherton *et al.*, 2002; Özogul *et al.*, 2007), less attention have been paid to other tissues. Most research has focused on carnivorous species, as they are the most commonly cultivated fish and less attention has been paid to omnivorous or herbivorous species. Therefore, golden mullet *Liza aurata* (Risso, 1810) was chosen for this study because of its omnivorous feeding behaviour and its capacity to move to freshwater areas, as well as for its prevalence around sea-cage fish farms (Fernandez-Jover *et al.*, 2009; Arechavala-Lopez *et al.*, 2010).

The aim of this study was to evaluate, under laboratory conditions, the use of fatty acids as temporal biomarkers (Dalsgaard *et al.*, 2003; Elsdon, 2010), to assess the extent to which this unnatural feeding behaviour can affect the fatty acid profile of three different tissues in wild-caught juvenile fish. We also tested whether the levels of fatty acid markers change after a wash-out period consisting of a return to a natural diet, as well as the speed and the magnitude of any changes. The following tissues were analysed: Flesh, which is known to reflect changes in the fatty acid profile following a shift in diet in a short period of time; brain, a tissue with a more conservative fatty acid profile due to the selective transport of fatty acids through the brain blood barrier and the active biosynthesis of certain fatty acids needed for its natural function; and liver, which initially receives and transforms the dietary fatty acids from the gut. Hepatocytes are responsible for the metabolism of endogenous and exogenous compounds, besides the synthesis and storage of products such as glycogen, amino acids, and fat. So their morphology and vacuole formation in the cytoplasm are related to the fish nutritional status (Sargent *et al.*, 2002). Structural changes in the hepatic tissue and modifications in the distribution pattern of the lipid droplets in the hepatocytes were also assessed in this study.

Material and methods

Animals and diets

Ninety wild juvenile mugilids (*L. aurata*) captured from the Mediterranean Sea, off the south-east coast of Spain, were kept in a re-circulating seawater system at the Faculty of Biology of the University of Murcia. The photoperiod was 12 h light:12 h dark, and the water was maintained at a temperature of 19 ± 2°C, with a water salinity of 27 and flow rate of 700 l h^{-1}. Fish were fed with an omnivore natural diet until the beginning of the experiment. This natural diet was prepared according to the indications of Goemans and Ichinotsubo (2008) for omnivore diets, with some modifications made by the staff from the Aquarium of the University of Murcia. Both natural and commercial feed were mashed and mixed with gelatine, and then frozen at −20°C. Fish (average weight 7.23 g and length 7.74 cm) were starved for 24 h prior to sampling. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

Experimental design

Fish were randomly distributed into six identical tanks (250 l) with 15 fish each, and were fed with a natural omnivore diet for 4 months to ensure their fully adaptation to captivity. After the acclimation period, the fish in half of the tanks were fed with the same natural diet (control) for the whole experiment (4 months), and 45 fish were fed with a commercial diet (CD group) rich in vegetable oils for 2 months and afterwards, with the control diet for the last 2 months of the experiment (wash-out period). Samples of flesh, brain, and liver were taken at 0, 30, 60, 90, and 120 days. Both groups were fed at a rate of 3% body weight day^{-1}. Three fish per tank were sampled at each sampling point (one fish per tank at day 0), and samples were stored at −80°C until they were analysed.

Sample collection

Fish were sampled under sterile conditions and total weight and length were recorded for all fish at each sampling point. Brain, liver fragments, and an anterior-dorsal white muscle portions were sampled and frozen at −80°C. Liver fragments were also sampled for histology.

Growth performance

The body weight and length of each fish were measured before the trial. Growth was monitored by obtaining the initial weight (Wi), final weight (Wf), weight gain (%WG), specific growth rate (SGR), and condition factor (CF), which were calculated for each group according to Silva-Carrillo *et al.* (2012): CF = (weight $length^{-3}$) × 100; SGR = [(Ln final weight − Ln initial weight) number of $days^{-1}$] × 100; and %WG = ((Wf − Wi) Wi^{-1}) × 100.

Determination of the tissue fatty acid profiles

Samples were analysed in pools of three fish per sampling point and tank. Fatty acids were extracted from 0.5 to 1.5 g of sample, by homogenising in 20 ml of chloroform/methanol (2:1 v/v) in an Ultra Turrax tissue disrupter (IKA ULTRA-TURRAX T 25 digital, IKA-WERKE). Total lipids were prepared according to the method of Folch *et al.* (1957) and non-lipid impurities were removed by washing with 0.88% (w/v) KCl. The weight of lipids was determined gravimetrically after evaporation of the solvent and overnight desiccation in vacuum. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method described by Christie (2003). Heptadecanoic acid (17:0) was added to the samples as internal standard (at 10% of the total lipid mass), and the total lipid samples were transmethylated overnight in 2 ml of 1% sulphuric acid in methanol (plus 1 ml of toluene to dissolve neutral lipids) at

50°C. The methyl esters were extracted twice in 5 ml hexane-diethyl ether (1:1, v/v) after neutralization with 2 ml of 2% $KHCO_3$, dried under nitrogen and redissolved in 0.1 ml of iso- hexane. Methyl esters were purified by TLC (thin layer chromatography) using iso-hexane:diethyl-ether:acetic acid (90:10:1 v/v/ v). FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m × 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, UK) and on-column injection at 50°C. Hydrogen was used as carrier gas and temperature was programmed from 50 to 150°C at a rate of 40°C/min, further increased to 230°C at a rate of 2°C/min. Individual methyl esters were identified by compar- ing their retention times with known standards. Data were col- lected and processed using Chromcard for Windows (version 1.19). Individual fatty acid concentrations were expressed as percentages of the total content.

Liver histology

Liver samples were processed histologically to estimate the hepatocyte lipid distribution. For this, the samples were fixed in 4% (v/v) buffered formaldehyde for 12 h, and then washed and kept in 70% ethanol. Fixed pieces were processed in an automatic tis- sue processor (MYR, Spain), and embedded in paraffin wax. For histological observations, dewaxed sections (5 μm) were rehydrated, routinely stained with haematoxylin-eosin and with periodic acid-Schiff (PAS) to verify the presence of glycogen and rule out vacuole formation as a cause of glycogen storage. Slices were documented photographically with an Axioskop Zeiss microscope.

Stereological analyses

The image analysis system consisted of a light microscopy (Zeiss Axioskop, Madrid) connected to a video camera (Sony 151-AP). An average of 16 different fields at 40× original magnification were examined for the presence of vesicles in hepatocytes of a selected number of slices (n¼6) from each dietary treatment group, at the beginning of the experiment, after 2 months of consuming the CD and after 2 months of the wash-out period (day 0, 60, and 120). The photographs were converted into black and white 2 bit images, where white corresponds to the area occupied by lipid droplets. The percentage of the area covered by them was measured using the MIP 4 advanced v. 5.01.02 software (Microm, Image Processing Software, Consulting Image Digital, Barcelona).

Statistical analysis

The results are expressed as mean 6 s.e. Individual fatty acids data were statistically analysed by two-way analysis of variance (ANOVA) to determine differences between groups. The same statistical method was applied to the area percentage data of the histological samples. Principal component analysis (PCA), SIMPER (similarity percentages) procedure (Warwick *et al.*, 1990; Clarke, 1993), and a permutational multivariate ANOVA (PERMANOVA) test (Clarke, 1993; Anderson *et al.*, 2008) were carried out to assess the difference in the overall fatty acid composition of the groups. SIMPER analyses allow us to identify the fatty acids that are most important in creating the observed pat- tern of dissimilarity between the experimental groups (Henderson and Seaby, 2014), and they were performed using the Bray–Curtis dissimilarity index (Bray and Curtis, 1957). PERMANOVA allow us to compare fatty acid signatures between treatments, calculating *p*-values using permutations, and the analysis was performed comprising 4 999 permutations. Statistical analyses were conducted using SPSS Statistical Software System version 15.0 (SPSS Inc., Chicago, IL) and Primer (Plymouth Routines in Multivariate Ecological Research; v.6.1.13) and its complementary statistical package PERMANOVAþ (v.1.0.3). As the data were in the form of percentages, they were transformed with arcsin (x þ 1), and all statistical tests were performed with a significance level of a¼0.05.

Results

Growth performance

Golden mullet specimens showed the same initial weight at the beginning of the experiment. The CF showed significant differences between groups during the period of commercial feed consumption (sampling point at 30 and 60 days). Significant differences were found in growth at 120 days, when it was seen that the fish from the natural feed group had gained more weight than the fish from the CD group (Table 1).

Composition of feeds

When the natural and CDs were analysed for their macronutrients and fatty acid profile (Table 2), the natural diet showed a higher percentage of ARA, EPA, and DHA than the commercial feed, and a lower percentage of OA and LA. Therefore, the natural diet can be said to be rich in n—3 fatty acids, whereas the CD is rich in n—6 fatty acids.

Fatty acid levels in flesh, brain, and liver reflected dietary changes

The number of fatty acids showingsignificant differences between groups was high in flesh 1 month after the change in diet, the differences between the two groups remained after 2 months of the wash-out period. Inter-group differences were found in all three tissues studied, regarding the percentages of LA and LNA, whose levels were higher in the CD group than in the control group. The DHA percentage in flesh showed a significant decrease in the CD group at each sampling time. Brain DHA showed a decrease only during the wash-out period, while no differences were found in liver. The EPA percentage remained without change in flesh and brain, whereas in liver a significant increase was detected in the CD group after 1 month of consuming a commercial feed and after 1 month of returning to the natural diet (wash-out period). ARA showed a significant decrease in the flesh of the CD group at

Table 1. Growth performance of golden mullet specimens fed with natural diet or commercial feed after 120 days of the experiment

	Natural feed	Commercial feed
Wi	7.0860.03	7.3060.09
Wf	14.3760.28	11.1060.57*
SGR	0.5760.02	0.3460.04*
WG%	103.564.49	52.0467.01*
CF (day 0)	1.5260.02	1.5260.02
CF (day 30)	1.6060.02	1.5460.02*
CF (day 60)	1.5460.02	1.6560.03*
CF (day 90)	1.5160.04	1.5260.02
CF (day 120)	1.6060.04	1.6460.04

Asterisks denote significant differences between natural and commercial feed groups ($p \leq 0.05$). Values are expressed as mean 6 s.e.

Table 2. Composition (g kg^{-1}) of the natural feed and proximate composition (% of dry weight) and main fatty acid levels (mean values 6 s.e.) of natural and CDs.

Components of natural feed		
Fishmeal (g kg^{-1})	241.99	
Shrimp (g kg^{-1})	323.13	
Squid (g kg^{-1})	81.14	
Freeze-dried algae (g kg^{-1})	10.96	
Vitamin supplement (g kg^{-1})	14.23	
Red plankton (g kg^{-1})	47.40	
Gelatin (g kg^{-1})	14.23	
Water (g kg^{-1})	266.90	
Composition (dry weight)	Natural feed	Commercial feed
Ash (%)	11.36	6.07
Protein (%)	72.64	58.54
Fat (%)	10.41	16.77
Nitrogen-free extract (%)	5.60	18.62
Fatty acids (%)	Natural diet	Commercial feed
Total saturated	31.63 6 0.39	26.06 6 0.45
18:1n—9	9.41 6 0.09	20.12 60.04
Total monounsaturated 20:4n—	20.28 6 0.22	30.92 60.07
18:2n—6 (LA)	1.74 60.05	18.74 60.10
6 (ARA)	2.46 60.03	0.62 6 0.02
Total n—6 PUFA	5.95 60.07	20.52 6 0.09
18:3n—3 (LNA)	0.69 60.01	2.99 6 0.04
20:5n—3 (EPA)	14.89 60.16	10.49 6 0.20
22:6n-3 (DHA)	22.64 60.39	6.58 6 0.15
Total n-3 PUFA	42.14 60.55	22.49 6 0.42
Total PUFA	48.09 60.62	43.02 60.51

Fatty acid percentages showed significant differences between diets ($p < 0.05$). The commercial feed used to prepare the CD was provided by Skreeting.

all sampling times, whereas the brain just showed a decrease in the second month of commercial feed consumption and the sec- ond month of the wash-out period. Liver ARA percentage did not change. The n—3/n—6 ratio was lower in the flesh and the liver from fish of the commercial feed group throughout the experi- ment, while this effect was only evident in the brain at the end of the second month of consuming a CD (Figures 1–3).

The PCA results for flesh, liver, and brain fatty acid profiles are given in Figure 4. The flesh samples (Figure 4a) can be divided into two groups, one composed of fish from the control group, and the other composed of fish from the CD group, including those during the wash-out period (90 and 120 days). A similar trend was found in liver samples (Figure 4b), where fish sampled at day 0 and fish from the natural feed group (30 and 60 days) were represented together, while some (but not all) fish from commercial feed group are represented separately. Brain samples (Figure 4c) showed a more homogeneous distribution.

Fatty acid profile distribution

The one-way PERMANOVA analysis for the commercial feed consumption period (1 and 2 months after the beginning of the experiment) and the wash-out period (3 and 4 months after the beginning of the experiment) showed significant differences in the three studied tissues (Table 3). SIMPER analysis showed that the contribution to the dissimilarities between both commercial feed and natural feed groups were mainly due to LA, the main fatty acid in the commercial feed, its contribution being between 14 and 23% (Table 4). The average dissimilarity between the groups was higher during the period of commercial feed consumption than the wash-out period in flesh and liver, whereas brain showed a higher average dissimilarity during the wash-out period.

Liver histology

Under the light microscope (Figure 5) with the HE reaction, a normal structure of the liver parenchyma was observed. Hepatocytes containing numerous lipid droplets were mainly observed in the surroundings of the bile duct (periductular) areas, where they were faintly PAS stained compared with the hepatocytes of the non-periductular areas, which contained higher inclusions of glycogen. The storage of lipids in the liver parenchyma was homogeneous between the two experimental groups, and no significant differences were found between experimental groups in both the periductular and non-periductular areas (Data shown in supplementary material, Figure S1).

Discussion

Fish fatty acid composition may not be the same in all tissues, and a comparison of organs with different degrees of exposure to dietary fatty acids, and therefore dissimilarities in their storage and usage, may be of interest. For this reason the response to a change in diets with different fatty acids contents, simulating the arrival of golden mullet to the vicinity of fish farms, was assessed in the flesh, liver, and brain.

Fatty acids have been used as biomarkers in trophic transfer studies in aquatic ecosystems, but focusing in DHA, EPA, and ARA (Parrish, 2009). Contributing to this, our results confirmed the usefulness of LNA, but especially of LA, as markers to track the consumption of aquafeeds by aggregated wild fish, due to their rapid accumulation in flesh in only 1 month of commercial feed consumption, and to their persistence in the three tissues studied after a 2 month wash-out period.

Both, LA and LNA showed high percentages in flesh and liver after 1 month of commercial feed consumption, compared with the percentages measured in fish fed the natural diet. More importantly, these percentages did not return to their original values at the end of the experiment after 2 months of a wash-out period with the same natural diet. The percentages of the essential fatty acids ARA and DHA in flesh samples were lower than in fish from the natural feed group, even at the end of the wash-out period. However, this effect was slightly reduced in brain, where ARA levels were significantly different after 60 days of commercial feed consumption and after the 60 day wash-out period with the natural feed.

These results confirm the idea of a rapid change in flesh fatty acid percentages, while the number of fatty acids in which there were significant changes was lower in brain, where changes were of a lower magnitude than in flesh, suggesting that the incorporation of dietary fatty acids in brain tissue is a slow process, probably due to their selective transport through the blood brain barrier (Spector, 1988; Moore *et al.*, 1990). Unlike in flesh and brain, the percentages of the essential fatty acids ARA and DHA in liver, did not differ between groups.

Our results are similar to those found by Almaida-Pagán *et al.* (2007) in another omnivorous marine fish, sharpsnout seabream (*Diplodus puntazzo*), which showed high proportions of LA and LNA in fish fed diets rich in vegetable oils rather than fish oils. These authors also observed a reduction in the percentages of

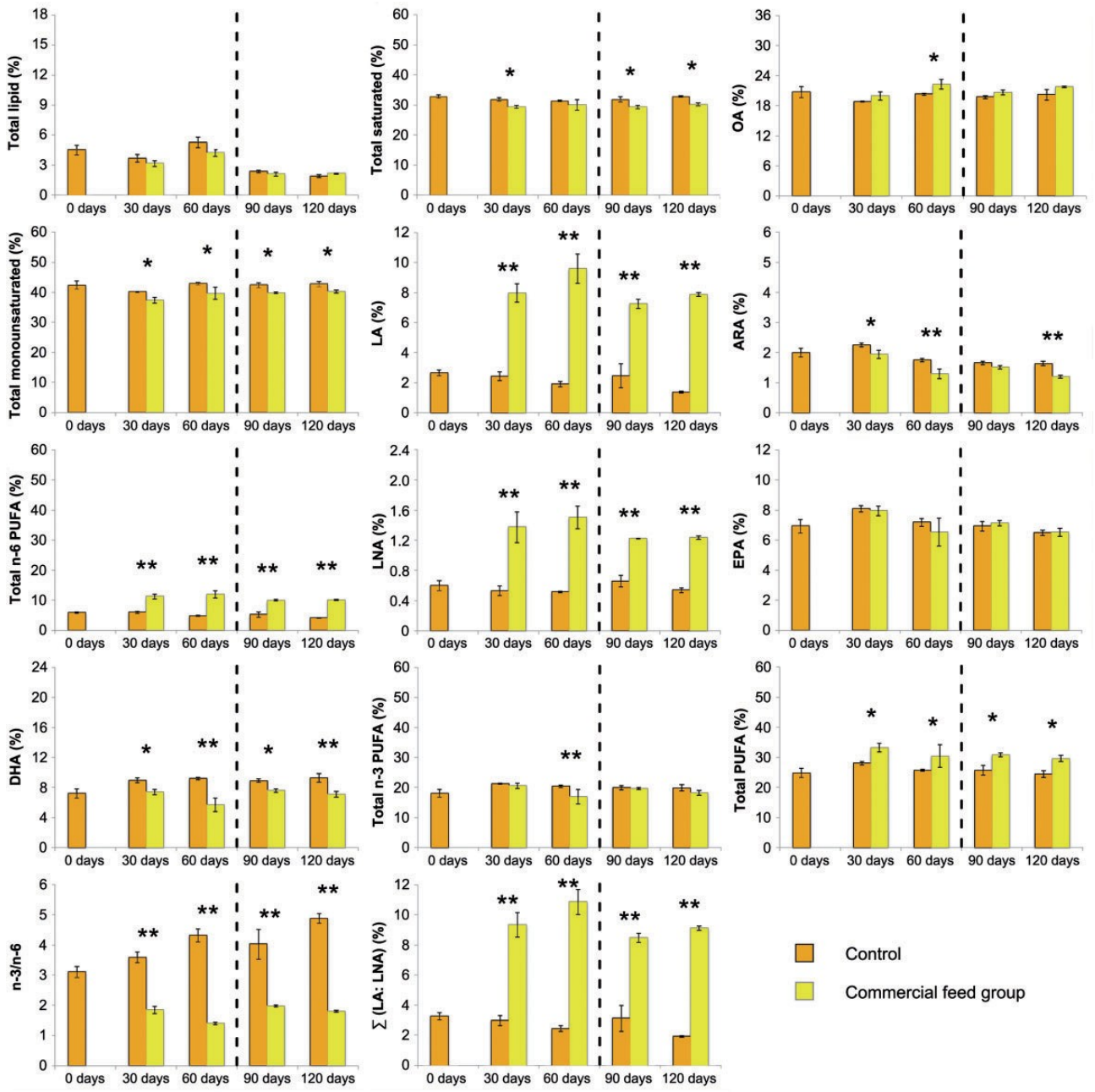

Figure 1. Fatty acid, total lipid percentages and n-3/n-6 index (mean values ± s.e.) in flesh samples of golden mullet. Dotted line points to the beginning of the wash-out period. One and two asterisks denote significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively) between natural and commercial feed groups at each sampling time.

EPA and DHA in liver, which was not observed in the liver of our golden mullet specimens, which showed higher liver EPA levels than control levels at 60 and 120 days, and showing the DHA higher percentages in flesh and brain tissues than controls.

Bell *et al*. (2003) showed that flesh EPA and DHA percentages were reduced in Atlantic salmon (*Salmo salar*) fed a diet rich in vegetable oils when compared with specimens fed a fish oil diet. Similarly to our results, they found that the levels of these two fatty acids were partially restored when the fish were transferred to a 100% fish oil finishing diet for 20 weeks, although the percentages were always significantly lower than in fish fed with a fish oil diet for 70 weeks. A similar study in European sea bass (*Dicentrarchus labrax*) showed the same trends: lower percentages of EPA and DHA and higher percentages of OA, LA, and LNA in fish fed diets rich in vegetable oils (Mourente *et al.*, 2005). In the same study, fish fed a finishing diet rich in fish oil for 14 weeks recovered their original DHA and total n—3 percentages although EPA remained at lower levels. In our study, an eight week wash-out period with the natural diet was not sufficient to return DHA levels to control values in any of the tissues studied. Nevertheless, the above studies focus on recovering levels of n—3 PUFA while less attention was paid to the percentages of LA, which as in this work, accumulated in flesh and liver but also in brain to a lower extent, and remained almost unaltered during the 60 days of the

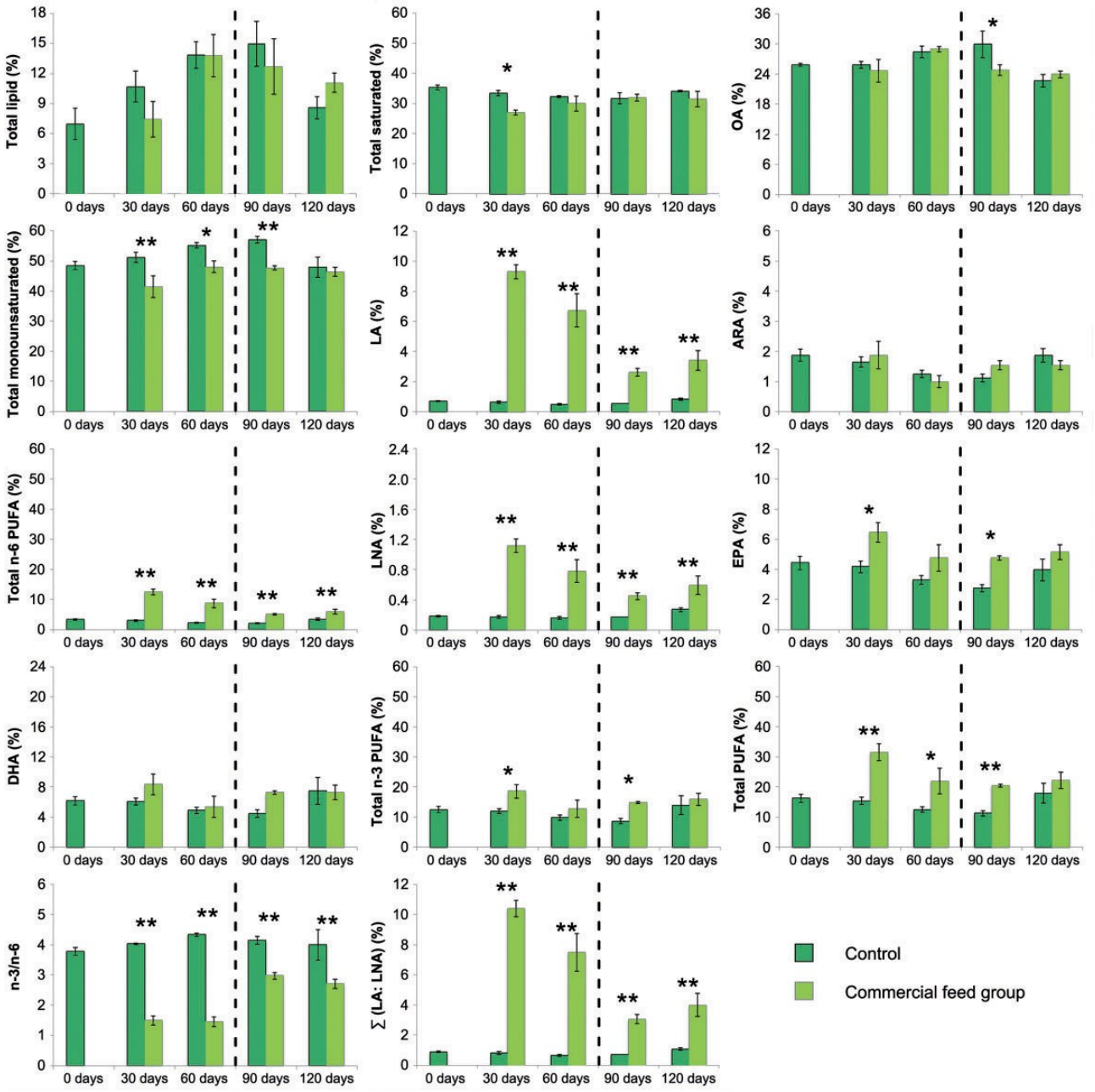

Figure 2. Fatty acid, total lipid percentages, and n–3/n 6 index (mean values 6 s.e.) in liver samples of golden mullet. Dotted line points to the beginning of the wash-out period. One and two asterisks denote significant differences ($p \leq 0.05$ and ≤ 0.01, respectively) between natural and commercial feed groups at each sampling time

wash-out period. Similar results were obtained by Regost *et al.* (2003) in turbot (*Psetta maxima*), using a 60 days fish oil finishing diet. Our results agree with the apparent incapacity of golden mullet (Mourente and Tocher, 1993) and generally of marine fish to elongate and desaturate LA, as mentioned above.

The use of flesh to track the incorporation of vegetable oils in wild fish consuming lost pellets may be the first option. Flesh responds rapidly, reflecting in just 1 month (and probably less in golden mullet) the fatty acid composition of the aquafeeds. In this respect, brain has been demonstrated to be a more conserva- tive tissue (Moore, 1990; Brodtkorb *et al.*, 1997; Soengas and Aldegunde, 2002) although it, too, reflects changes related to the fatty acid composition of the diet. These changes were slight and occurred slowly, 2 months being necessary for them to be reflected. The same can be said when fish were returned to their natural diet, the brain once again being more resilient to dietary changes than flesh or liver. This property makes brain a useful tool for tracking alterations in the diet of wild fish through fatty acid analyses, especially in conjunction with flesh. While the flesh shows changes that may occur on a short time scale, the brain is capable of showing the accumulation of fatty acids of vegetable origin on a long time scale. Even if fish revert to a natural diet

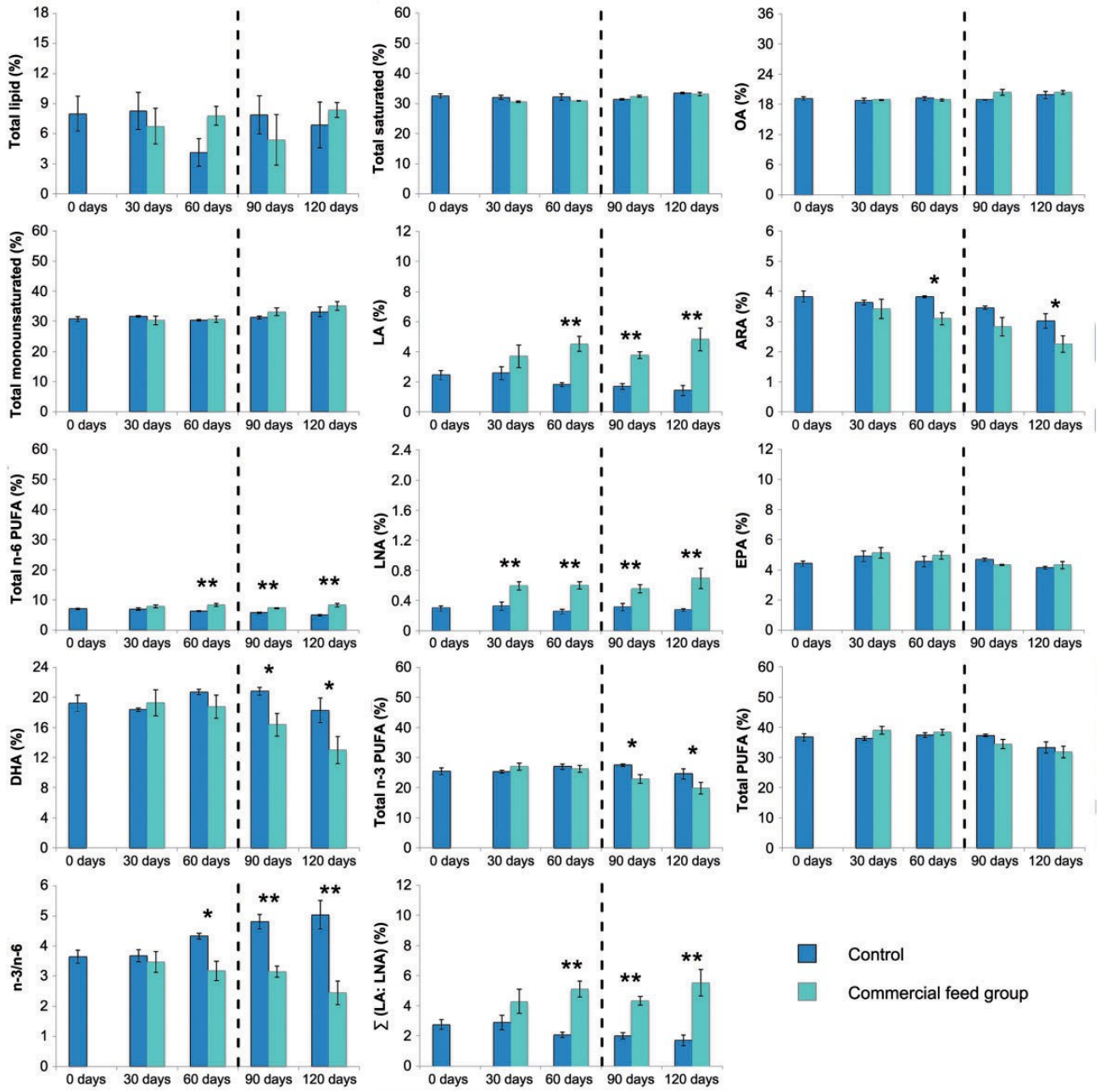

Figure 3. Fatty acid, total lipid percentages, and n-3/n-6 index (mean values ± s.e.) in brain samples of golden mullet. Dotted line points to the beginning of the wash-out period. One and two asterisks denote significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively) between natural and commercial feed groups at each sampling time.

consumption for a sufficiently long time to allow the flesh to reflect this diet, changes may still be detectable in brain, so the combined use of both flesh and brain tissues is highly encouraged. Nevertheless, this is not the case with the hepatic tissue, whose fatty acid profile also reflected that of the diet although showing higher variability than flesh or brain. Besides its function of storing lipids, liver has high metabolic activity, especially with dietary lipids, which are metabolized and incorporated into lipoproteins before being transported to the organism (Sheridan, 1988; Tocher, 2003). This metabolic activity entails mobilization and transformation of the dietary and stored lipids, depending on different factors such as sexual maturity and molt stage (Greene and Selivonchick, 1987; Almansa *et al.*, 2001), infection events (Calder, 2007; Trichet, 2010) or nutrition status. Therefore, the liver fatty acid profile may vary more with time, making it less suitable for tracking vegetable fatty acid consumption. For example, liver LA and LNA levels from fish of the CD group were closer to the control values than flesh samples after 60 days of wash-out, although this time was not sufficient to reach the original values. Despite the high levels of LA and LNA in liver, DHA and ARA levels did not show changes between groups, which could be related to the lack of any change in the pattern of lipid

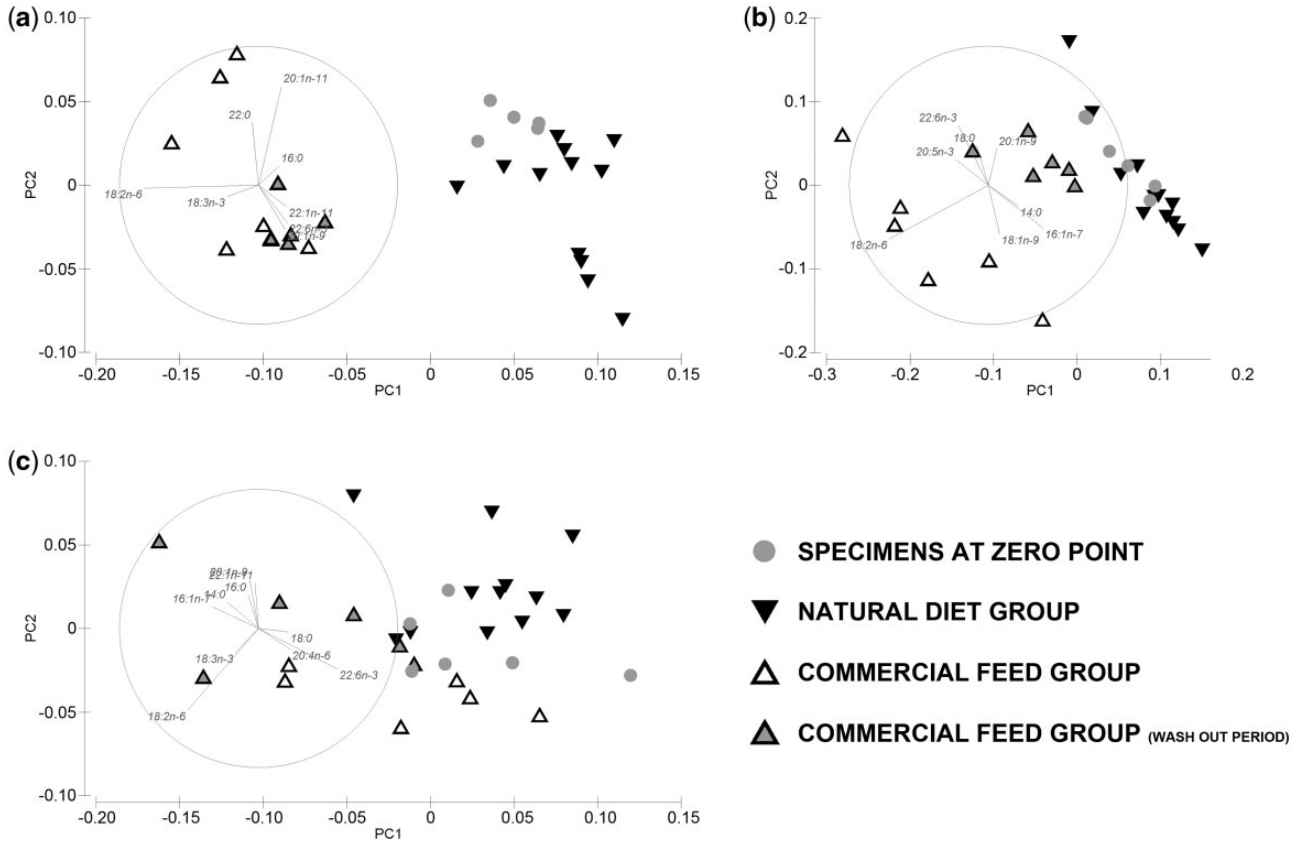

Figure 4. PCA of the fatty acids in each tissue. The total explained variance in flesh was 82.8% (a), in liver was 78.6% (b), and in brain was 74.9% (c).

Table 3. PERMANOVA results for the period of commercial feed consumption (sampling points at 30 and 60 days) and the wash-out period (sampling points at 90 and 120 days)

	Flesh					
30 þ 60 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	172.25	172.25	21.67	0.0016
	Res	10	79.486	79.486		
	Total	11	251.73			
90 þ 120 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	129.9	129.9	21.746	0.0022
	Res	10	59.735	59.735		
	Total	11	189.63			
	Liver					
30 þ 60 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	442.88	442.88	16.811	0.0024
	Res	10	263.45	26.345		
	Total	11	706.33			
90 þ 120 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	128.72	128.72	4.225	0.0264
	Res	10	304.67	30.467		
	Total	11	433.4			
	Brain					
30 þ 60 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	29.794	29.794	31.051	0.0754
	Res	10	95.954	95.954		
	Total	11	125.75			
90 þ 120 days	Source	d.f.	SS	MS	Pseudo-F	P(perm)
	Fatty acid profile	1	86.552	86.552	71.558	0.0074
	Res	10	120.95	12.095		
	Total	11	207.51			

Significant *p*-values are shown in both bold and italics.

Table 4. Similarity percentages (SIMPER), showing the three fatty acids which contributed most to the dissimilarities between, natural and commercial feed groups, for the period of commercial feed consumption (sampling points at 30 and 60 days) and the wash-out period (sampling points at 90 and 120 days)

Flesh		Liver		Brain	
30 þ 60 days		30 þ 60 days		30 þ 60 days	
18:2n–6	21.40	18:2n–6	19.77	18:2n–6	14.17
22:6n–3	6.95	16:1n–7	9.14	22:6n–3	7.27
18:3n–3	6.54	18:3n–3	5.23	16:1n–7	6.52
Cum.%	34.88	Cum.%	34.15	Cum.%	27.96
Av. Dis.	8.30	Av. Dis.	13.56	Av. Dis.	4.82
90 1 120 days		90 1 120 days		90 1 120 days	
18:2n–6	23.71	18:2n–6	12.13	18:2n–6	15.44
20:1n–9	6.19	16:1n–7	8.10	22:6n–3	13.03
22:1n–11	6.04	22:6n–3	7.22	16:1n–7	5.25
Cum.%	35.94	Cum.%	27.45	Cum.%	33.71
Av. Dis.	7.19	Av. Dis.	9.36	Av. Dis.	6.61

The cumulative percentage (Cum.%) and the average dissimilarity (Av. Dis.) between groups are also shown.

droplet accumulation in the hepatocytes, as some fatty acids like ARA may play a role in the formation of cell vesicles (Schmidt *et al*., 1999).

Every tissue in the body is able to synthesize eicosanoids from ARA, which present an important function modulating the inflammation process and the immune response in mammals, but also in fish (Rowley *et al*., 1995; Tocher, 2003; Russo, 2009).

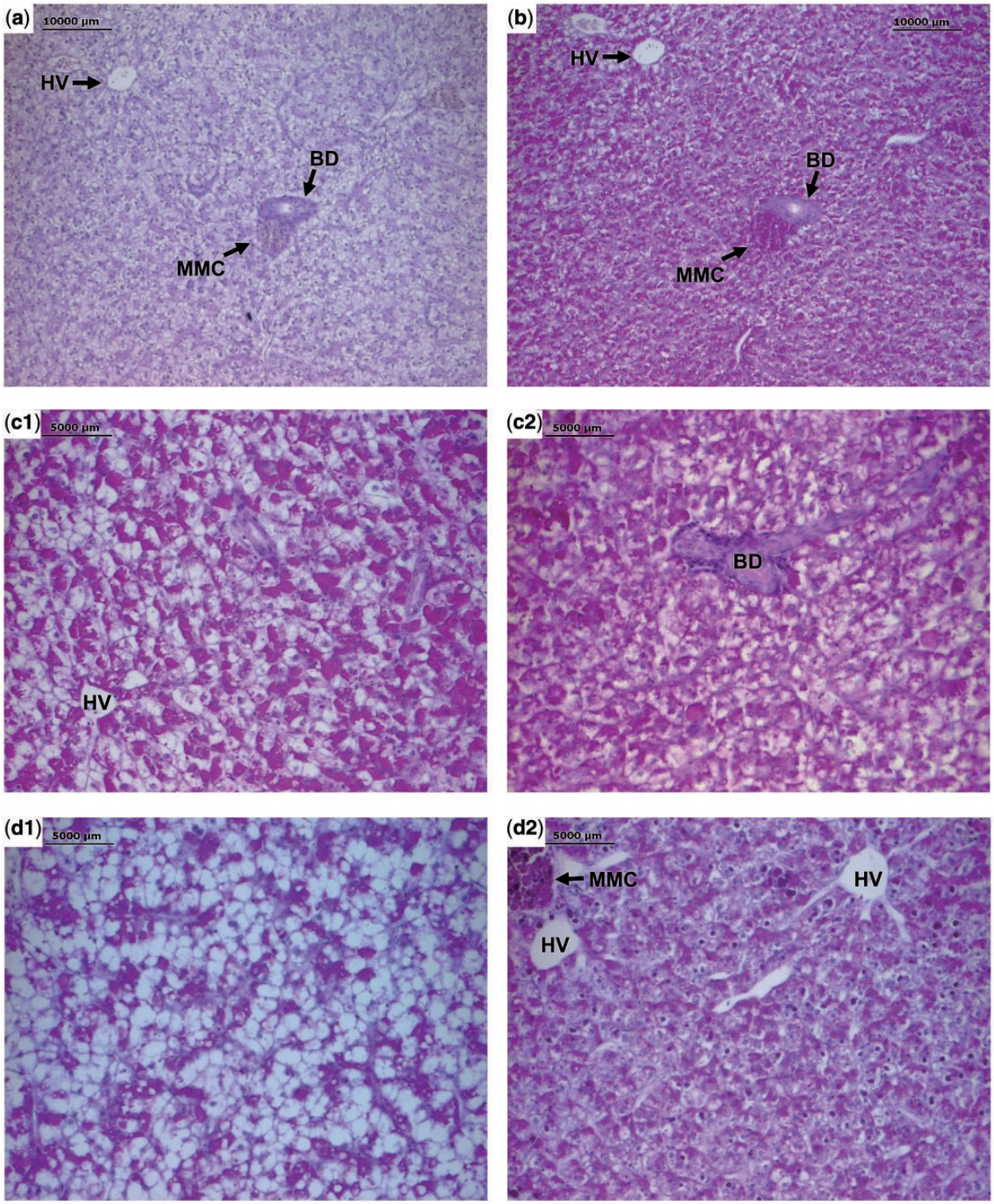

Figure 5. Light micrographs of liver sections in *Liza aurata* with HE reaction (a) and with PAS reaction (b). PAS reaction in control liver at 60 days (c1) and 120 days (c2); PAS reaction in liver of commercial feed group at 60 days (d1) and 120 days (wash-out period) (d2). HV: Hepatic venule; BD: Bile duct; MMC: Melano-macrophage centre.

Eicosanoids are also implicated in a wide range of physiological actions including neural function (Tocher, 2003, Tassoni *et al.*, 2008). It may be noted that the brain ARA percentage in both groups throughout the trial remained at higher levels than the percentages of this fatty acid in the natural feed or the commercial feed. Our data suggest that ARA plays an important role in the brain of golden mullet, where it may be regulated to reach the necessary minimum level to carry out its function, although how

the level of ARA is regulated remains unknown. Although it is known that marine fish have a limited capacity to convert LA and LNA into LC-PUFA (Tocher, 2003; Glencross, 2009), we can only speculate on a possible tissue-specific desaturation/elongation process (Bell and Tocher, 2009), the brain possibly being able to produce ARA from LA when the dietary input is not sufficient to fulfil the brains requirements (Moore *et al.*, 1990; Zheng *et al.*, 2009). As an euryhaline fish, *L. aurata* can be found not only in seawater but also in freshwater systems and, as consequence, HUFA biosynthesis may be possible in this species (Koussoroplis *et al.*, 2011). However, such thoughts should be taken with caution, as marine fish D6 desaturase (an enzyme responsible for the desaturation of certain fatty acids) has been suggested as being involved in the maintenance of optimal brain DHA levels (Bell and Tocher, 2009), rather than ARA levels. A further possibility is specific ARA retention by the brain. Such retention was noticed in *L. aurata* and other mugilids by Koussoroplis *et al.* (2011), who found evidence of ARA retention in flesh samples. That observation could explain the ARA percentage found in brain, although our results in laboratory conditions found no evidence of its retention in flesh or liver samples.

Generally, histological examination of the liver showed no alterations in the normal tissue structure. Nevertheless, some specimens from the commercial feed group had livers with a high inclusion of lipid vacuoles in both periductular and non-periductular zones, giving an appearance of hepatic steatosis to those samples (after 60 days of commercial feed consumption). In any case, this group showed no significant differences in lipid area percentage compared with the control group. This result agrees with the absence of significant differences in the total lipid content measured in the fatty acid analyses. Our results are similar to those found by Mourente *et al.* (2005) in European sea bass, who reported no changes in the hepatic structure following ingestion of a diet made with linseed oil, rich in LNA and LA. However, fish fed a diet with rapeseed oil, rich in OA and LA, showed a high variability in the size and number of the lipid droplets in the hepatocytes, with no associated pathology. In the same way, another study carried out in red hybrid tilapia (*Oreochromis* sp.) pointed to no histological alterations of the liver due to the ingestion of vegetable oils (Ng *et al.*, 2013).

Conclusion

The results indicated that golden mullet juveniles whose natural diet is changed to a commercial feed show significant differences in the fatty acid profile of the three studied tissues, although the magnitude and the time necessary to show changes in individual fatty acids differ among them. The flesh reflected more quickly (30 days) changes in the fatty acid percentages, followed by the liver, while differences were detected in brain after 60 days of commercial feed consumption. LA was the main fatty acid contributing to the differences between groups, and this fatty acid did not return to the values of the control group, even after a 2 month wash-out period consuming the natural diet. The speed of change in flesh and the resilience of the brain suggest that both tissues could be used for fatty acid analysis, representing an important ecological tool for tracing the temporal consumption of feeds rich in vegetable fatty acids. Liver function does not seemto be affected by the changes in its fatty acid profile due to the shift in diet. Wild juveniles of golden mullet may adapt their physiology to the new dietary conditions when they first arrive in the vicinity of fish farms. Nevertheless, migrating to distant areas is no guarantee of recovering the natural fatty acid profiles. There may exist long-term effects derived from this situation, and the better control of fish feed spill is encouraged to ensure the adequate growth and physiology of the wild fish populations.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Acknowledgements

AQ6
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