The short-term effects of farmed fish food consumed by wild fish congregating outside the farms

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ABSTRACT

We simulated in the laboratory the possible effects on fatty acids and immune status of wild fish arriving for the first time in the vicinity of a sea-cage fish farm, shifting their natural diet to commercial feed consumption, rich in fatty acids of vegetable origin. The flesh fatty acid profile of golden mullet specimens was altered after 2 weeks of commercial feed consumption, showing an increase in fatty acids of vegetable origin. The serum peroxidase and bactericidal activities, and head-kidney leucocyte phagocytic capacity, increased after eight weeks of the new diet, while the respiratory burst activity decreased. The extent of these changes cannot be considered large enough to regard them as compromising the health status of fish. More research is needed in order to elucidate whether the rapid assimilation of the dietary fatty acids could harm the immune status of fish when feeding for longer periods than two months.

1. Introduction

Aquaculture activities have increased in magnitude during recent decades and are expected to continue doing so in the future. The growth of this industry has led to a corresponding increase in the number of offshore fish farms. These act as FADs (Fish Aggregation Devices), attracting a large number of wild fish and macro-invertebrate species, which take advantage of the protection provided by the sea-cage structure, as well as of the availability of high-energy food through lost pellets (Dempster et al., 2002; Vita et al., 2004; Sanchez-Jerez et al., 2011; Gonzalez-Silvera et al., 2015). Consequently, there are fears that this could change animal behaviour, including nutrition.

In recent years, cultured fish have been fed using feeds rich in vegetable oils, an economic and sustainable alternative to fish oils. Vegetable oils are rich in fatty acids belonging to the n-6 series, such as linoleic acid (18:2n-6, LA), although α-linolenic acid (18:3n-3, LNA), from the n-3 family, is also abundant in some vegetables. Nevertheless, vegetable oils present a deficiency of polyunsaturated fatty acids (PUFA) like arachidonic acid (20:4n-6, ARA), and those belonging to the n-3 family, eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), which are abundant in fish oils (Turchini et al., 2010). ARA, EPA and DHA are essential fatty acids for marine fish and therefore they have to form part of the diet due to the limited capacity of the marine fish to elongate and desaturate their precursors, LA and LNA (Sargent et al., 2002; Tocher, 2003). Consequently, a shift from a natural diet to a commercial feed consumption through lost pellets could have an effect on the wild fish living close to fish farms, due to retention of fatty acids of vegetable origin in fish cell membranes at the expense of long chain polyunsaturated fatty acids like DHA or EPA. This fact may lead to changes in different physiological processes that are influenced by the percentages of fatty acids.

Modern aquaculture practices tend to replace fish oils by vegetable oils in feeds, the level of fish oil replaced being specific of every species, as it has been observed that an excessive substitution may result in adverse effects for reared fish. To example, some studies have found that complete substitution decreases fish growth in juveniles (Montero et al., 2003; Peng et al., 2008) though most reports confirm that partial replacement, around 60–80%, does not significantly affect fish growth (Mourente et al., 2005; Hernández et al., 2007; Peng et al., 2008; Fountoulaki et al., 2009). However, it is also known that fish nutritional status is a major aspect that influences the immune response, modulating the resistance to pathogens (Oliva-Teles, 2012; Zhang and Gui, 2015). Overall, increase in the concentration of n-3 PUFA in the diet of fish can have beneficial or detrimental effects but the balance between dietary n-3 and n-6 HUFA seems to be the most important factor regarding the immune responses and disease resistance (Kiron, 2012). For example, European sea bass (*Dicentrarchus labrax*) fed with diets that included vegetable oils showed a significant reduction in the total number of circulating leucocytes and a reduction in macrophage respiratory

burst activity (Mourente et al., 2005), while in the gilthead seabream (*Sparus aurata*), a similar diet did not affect the haematocrit or haemoglobin contents, serum lysozyme and macrophage respiratory burst activities, reduced the number of circulating red blood cells, phagocytic, serum alternative complement and bactericidal activities and increased the expression of gut and head-kidney pro-inflammatory cytokines and liver Mx genes (Montero et al., 2003, 2008, 2010). In this respect, the connection between dietary fatty acids and the immune response is mainly mediated by the eicosanoids, highly active molecules with a wide range of physiological actions, including regulation of lipid metabolism and mediation of the inflammation process and immune response (Calder, 2007; Trichet, 2010). Not only eicosanoids but also the effect of fatty acids on cell membrane fluidity and permeability (Leray et al., 1986) can be related to the immune function, modifying the expression and distribution of cell surface proteins and receptors, and altering the degree of interaction between leucocytes and endothelial cells (Jenski and Stilwell, 2001; Calder, 2006) and the phagocytic capacity (Calder et al., 1990).

This study was performed with the knowledge that fatty acids of vegetable origin might find their way into the diet of wild fish attracted to fish farms through lost pellets and faeces consumption (Fernandez-Jover et al., 2007, 2008), and that the n-3/n-6 ratio might be altered and affect the immune status. This possible scenario (Dempster et al., 2009; Uglem et al., 2009; Sanchez-Jerez et al., 2011; Izquierdo-Gómez et al., 2015) deserves further consideration. Our aim was to study, under controlled conditions, the possible effect of a change from a natural diet to a commercial feed on both the fatty acids profile and the innate immune parameters of golden mullet *L. aurata* (Risso, 1810) juveniles - a fish species that is well appreciated by consumers in the Mediterranean area, and which is known congregate around Mediterranean fish farm facilities (Fernandez-Jover et al., 2009; Arechavala-Lopez et al., 2010).

2. Material and methods

2.1. Fish care and maintenance

Wild juvenile specimens of the seawater teleost mugilid *L. aurata*, captured in the Mar. Menor lagoon (Murcia, south-east coast of Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water temperature was maintained at 19 ± 2 °C with a flow rate of 700 L h^{-1} and 33‰ salinity according to southwest Mediterranean salinity conditions. The photoperiod was 12 h light: 12 h dark and fish ingested an omnivorous fish diet (included in Supplementary material, Table S1) until the beginning of the experiment (3 months acclimation period). This natural diet was prepared according to the indications of Goemans and Ichinotsubo (2008) for omnivorous 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.

2.2. Experimental design

Forty-two fish were randomly distributed into two different tanks as described in previous studies (Diaz-Rosales et al., 2007; Salinas et al., 2008). Fish from one tank received the same omnivorous natural diet (natural diet group) while fish of the other tank (commercial feed group) were fed a commercial feed (D4 protec, Skreeting). This commercial diet was chosen due to their utilization in previous field studies with aggregated wild fish (Fernandez-Jover et al., 2009). Both groups were fed once at midday at a rate of 3% body weight day^{-1}. Nine fish per tank were sampled after 2 and 8 weeks, in order to check for changes in immune parameters. Fish were starved for 24 h prior to sampling. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.3. Sample collection

Fish were sampled under sterile conditions and total weight and length were recorded. Blood samples were obtained from the caudal vein of each specimen with a 27-gauge needle and 1 mL syringe. After clotting at 4 °C, each sample was centrifuged and the serum removed and frozen at −80 °C until use. Head-kidney fragments were cut into small fragments and transferred to 8 mL of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride, 2% foetal calf serum (FCS, Gibco), 100 i.u. mL^{-1} penicillin (Flow) and 100 mg mL^{-1} streptomycin (Flow)] for leucocyte isolation, as described by Esteban et al. (1998). Leucocyte suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 mm), washed twice (400 g, 10 min), counted and adjusted to 10^7 cells mL^{-1} in sRPMI. Cell viability was determined by the trypan blue exclusion test. Flesh samples were taken from the anterior-dorsal white muscle portion and frozen at −80 °C.

2.4. 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): $CF = (\text{weight length}^{-3}) \times 100$; $SGR = [(\text{Ln final weight} - \text{Ln initial weight})\ \text{number of days}^{-1}] \times 100$; and $\%WG = ((Wf - Wi)\ Wi^{-1}) \times 100$.

2.5. Determination of the fatty acid profile of flesh

Fatty acids were extracted from 0.5–1.0 g flesh samples by homogenization 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 of Christie (2003), 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 isohexane. Methyl esters were purified by TLC (thin layer chromatography) using isohexane:diethyl-ether:acetic acid (90:10:1 v/v/v). FAME were separated and quantified by gas-liquid chromatography using an SP™ 2560 flexible fused silica capillary column (100 m long, internal diameter of 0.25 mm and film thickness of 0.20 mm; SUPELCO) in a Hewlett-Packard 5890 gas chromatograph. The oven temperature of the gas chromatograph was programmed for 5 min at an initial temperature of 140 °C, and increased at a rate of 3 °C/min to 230 °C, further increased at a rate of 2 °C/min to 240 °C and then held at that temperature for 12 min. The injector and flame ionization detector were set at 260 °C. Helium was used as the carrier gas at a pressure of 300 kPa, and peaks were identified by comparing their retention times with appropriate FAME standards (Sigma). Individual fatty acid concentrations were expressed as percentages of the total content.

2.6. Immune parameters

2.6.1. Serum and leucocyte peroxidase activity

The peroxidase activity in serum or leucocytes was measured according to Quade and Roth (1997). Briefly, 15 μL of serum were diluted with 135 μL of Hank's balanced salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Samples of 50 μL of 20 mM

3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added. To determine the leucocyte peroxidase content, 10^6 head-kidney leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 g, 10 min), 150 μL of the supernatants were transferred to a fresh 96-well plate containing 25 μL of 10 mM TMB and 5 mM H_2O_2. In both cases, the colour-change reaction was stopped after 2 min by adding 50 mL of 2 M sulphuric acid and the optical density was read at 450 nm in a plate reader (BMG labtech-Fluostar galaxy). Standard samples without serum or leucocytes, respectively, were used as blanks.

2.6.2. Bactericidal activity

Two opportunist marine pathogenic (*Vibrio harveyi* and *Photobacterium damselae* subsp. *piscicida*) (Austin and Zhang, 2006; Austin, 2010) and one non-pathogenic bacteria (*Escherichia coli*) were used. Bacteria were grown in agar plates at 25 °C in tryptic soy (TSB, Sigma) for *V. harveyi* and *P. damselae* and in Luria (LB, Sigma) for *E. coli*. Then, fresh single colonies of 1–2 mm were diluted in 5 mL of liquid culture medium and cultured for 16 h at 25 °C on an orbital incubator at 200–250 rpm.

The serum antimicrobial activity was determined by evaluating their effects on the bacterial growth curves using the method of Sunyer and Tort (1995) with some modifications. Aliquots (100) μL of each of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of golden mullet serum. The OD of the samples was measured at 620 nm at 30 min intervals over a 24 h period at 25 °C. Samples without bacteria were used as blanks (negative control). Samples without serum were used as positive control (100% growth or 0% bactericidal activity).

2.6.3. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by *L. aurata* head-kidney leucocytes was studied by flow cytometry as described by Rodríguez et al. (2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5×10^7 cells mL^{-1} of sRPMI. Phagocytosis samples consisted of 125 μL of labelled-yeast cells and 100 μL of head-kidney leucocytes in sRPMI (6 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 g, 5 min, 22 °C), resuspended and incubated at 22 °C for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 μL ice-cold phosphate buffer (PBS) was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 μL ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or head-kidney leucocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerized system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC fluorescent cells) within the phagocytic cell population while the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometry results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

2.6.4. Respiratory burst activity

The respiratory burst activity of *L. aurata* head-kidney leucocytes was studied by a chemiluminescence method, as described by Bayne and Levy (1991). Briefly, samples of 10^6 leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 μL of HBSS containing 1 μg mL^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and luminescence was immediately read in a plate reader for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

2.7. Statistical analysis

The results are expressed as mean ± standard error. Immunological parameters and individual fatty acids data were statistically analysed by t-Student test to determine differences between groups. The normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Fatty acid percentages were transformed with arcsine. Principal component analysis (PCA), SIMPER (similarity percentages) procedure (Warwick et al., 1990; Clarke, 1993) and a permutation test (PERMANOVA) (Clarke, 1993; Anderson, 2004) comprising 4999 permutations was carried out to assess the significance of the overall fatty acid composition among groups. SIMPER analysis was performed using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957). Statistical analyses were conducted using SPSS 19.0 and Primer (Plymouth Routines In Multivariate Ecological Research; v.6.1.13) and its complementary statistical package PERMANOVA + (v.1.0.3). Differences were considered statistically significant when $p \leq 0.05$. Correlations between each fatty acid and the immune parameters are represented using CIRCOS software (Krzywinski et al., 2009).

3. Results

3.1. Fatty acid profile of feeds

The natural and commercial diets were analysed, with emphasis on the fatty acid profile. The natural diet showed a higher percentage of ARA, EPA and DHA than the commercial feed, as well as a lower percentage of OA and LA. Therefore, the natural diet can be said to be rich in n-3 fatty acids while the commercial diet is rich in n-6 fatty acids (Supplementary material, Table S2).

3.2. Growth performance

Golden mullet specimens fed with natural or commercial diets showed no statistically significant variations in growth rates (Table 1).

3.3. Fatty acid percentages in flesh reflected dietary changes

Individual fatty acid percentages in flesh reflected the respective dietary fatty acid compositions. Thus, flesh from fish receiving the commercial feed showed significant differences in individual fatty acids compared with specimens from the natural diet group as early as 2 weeks following the diet change, while the differences were slightly higher after 8 weeks (Table 2). Markers of commercial feed consumption (OA, LA and LNA) were higher in the fish from the commercial feed group, and concomitant with lower percentages of ARA, 20:3n-6 (dihomo-γ-linoleic acid, DGLA) and DHA, while EPA remained without change. Therefore, the total percentage of n-6 fatty acids was significantly higher, and n-3 fatty acids lower in fish receiving the commercial feed, resulting in an altered n-3/n-6 ratio (Table 2).

Further analysis of the results using PERMANOVA also pointed to significant differences between fish from the natural diet and commercial feed groups at both 2 and 8 weeks. The SIMPER analysis showed that the contribution to the dissimilarity between groups was mainly due to

Table 1
Growth performance of golden mullet specimens fed with natural diet or commercial feed after 8 weeks of experiment. Values are expressed as mean ± SE ($n = 9$).

Experimental groups	Wi	Wf	SGR	CF	WG%
Natural diet	8.68 ± 0.48	9.80 ± 1.05	0.21 ± 0.04	2.20 ± 0.04	12.91 ± 3.11
Commercial feed	8.58 ± 0.71	9.69 ± 0.84	0.21 ± 0.07	1.84 ± 0.09	12.88 ± 2.41

Wi: initial weight; Wf: final weight; SGR: specific growth rate; CF: condition factor; WG (%): percentage gain weight.

LA, with a contribution of 26% at 2 weeks and 23% at 8 weeks. Changes in LA, DHA, LNA and OA percentages were responsible, to a great extent, for the dissimilarity between the groups, contributing a percentage of 52% at 2 weeks and, together with the 16:0 fatty acid, 49% at 8 weeks.

The PCA results for flesh fatty acid profiles are given in Fig. 1, with a total explained variance of 97.8% at 2 weeks (Fig. 1A) and 98.3% at 8 weeks (Fig. 1B). The representation of samples based on their fatty acid percentages at both 2 and 8 weeks showed two groups, one composed of fish fed the natural diet, and the other composed of fish fed the commercial feed, characterized by higher concentrations of LA and LNA at both sampling times.

3.4. Humoral innate immune parameters

The serum peroxidase activity of golden mullet specimens was differently affected by the type of feed administered (natural or commercial) (Fig. 2). Serum peroxidase activity in fish from the two groups showed no significant differences 2 weeks after diet substitution, but was significantly higher in fish fed the commercial feed at 8 weeks (Fig. 2).

One of the most practical immune functions is the direct ability to kill bacteria. The fish fed the commercial feed showed a statistically significant increase at 2 and 8 weeks of serum bactericidal activity against *V. harveyi* and *E. coli*, while a statistically significant decrease in this activity was observed after 8 weeks of commercial feed consumption against *P. damselae* compared with the values obtained in the serum from fish fed the natural diet (Fig. 3).

3.5. Cellular innate immune parameters

The cellular innate immune parameters of juveniles of golden mullet were affected in different ways. Phagocytic (Fig. 4), respiratory burst (Fig. 5) and peroxidase (Fig. 6) activities were never affected significantly by the change to a commercial diet. However, after 8 weeks of diet shift, the phagocytic capacity (Fig. 4B) significantly increased while the respiratory burst (Fig. 5) decreased.

3.6. Correlation between fatty acids and immune parameters

The correlation factors between the fatty acid percentages and the humoral and cellular immune parameters are provided in the Supplementary material (Table S3). The results showed that the respiratory burst of head-kidney leucocytes presented the highest number of correlations (both positive and negative) with fatty acids, followed by

Table 2
Fatty acid percentages (mean values ± S.E.) of flesh of golden mullet fed with natural diet and commercial feed diet over 2 and 8 weeks. Asterisks denote significant differences between natural and commercial feed groups ($p \leq 0.05$) at each sampling time.

	Experimental time			
	2 weeks		8 weeks	
	Natural diet	Commercial feed	Natural diet	Commercial feed
14:0	5.28 ± 0.20	4.85 ± 0.19	5.63 ± 0.56	4.84 ± 0.13
15:0	0.37 ± 0.02	0.24 ± 0.01*	0.27 ± 0.03	0.19 ± 0.02*
16:0	26.56 ± 0.62	23.23 ± 0.25*	27.35 ± 0.99	23.57 ± 0.47*
18:0	3.72 ± 0.23	2.78 ± 0.10	3.55 ± 0.68	2.82 ± 0.09
20:0	0.31 ± 0.01	0.27 ± 0.01	0.28 ± 0.04	0.27 ± 0.01
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total saturated	36.25 ± 0.63	31.37 ± 0.41*	37.08 ± 0.97	31.68 ± 0.59*
16:1n-7	9.17 ± 0.47	9.42 ± 0.30	12.06 ± 1.28	8.67 ± 0.22*
18:1n-9	15.42 ± 0.42	19.94 ± 0.24*	17.85 ± 0.61	22.77 ± 0.45*
18:1n-7	3.45 ± 0.10	3.26 ± 0.04	3.38 ± 0.09	3.23 ± 0.07
20:1n-9	3.63 ± 0.11	1.97 ± 0.09*	3.09 ± 0.17	1.33 ± 0.11*
22:1n-9	0.38 ± 0.01	0.25 ± 0.00*	0.31 ± 0.03	0.23 ± 0.01
24:1n-9	0.46 ± 0.02	0.31 ± 0.01*	0.45 ± 0.07	0.26 ± 0.01*
Total monounsaturated	32.51 ± 0.86	35.16 ± 0.27	37.14 ± 1.56	36.47 ± 0.57
18:2n-6 (LA)	1.29 ± 0.04	9.29 ± 0.27*	1.31 ± 0.18	12.81 ± 0.36*
18:3n-6	0.06 ± 0.04	0.16 ± 0.03*	0.00 ± 0.00	0.23 ± 0.01*
20:2n-6	0.34 ± 0.01	0.35 ± 0.01	0.29 ± 0.02	0.40 ± 0.01*
20:3n-6	0.33 ± 0.02	0.21 ± 0.01*	0.27 ± 0.03	0.03 ± 0.02*
20:4n-6 (ARA)	2.12 ± 0.13	1.20 ± 0.06*	2.16 ± 0.61	0.61 ± 0.05*
22:2n-6	0.44 ± 0.01	0.49 ± 0.01	0.39 ± 0.02	0.51 ± 0.02*
22:4n-6	0.26 ± 0.01	0.19 ± 0.01	0.25 ± 0.03	0.02 ± 0.02*
Total n-6 PUFA	4.84 ± 0.17	11.89 ± 0.25*	4.67 ± 0.88	14.61 ± 0.39*
18:3n-3 (LNA)	0.48 ± 0.02	1.54 ± 0.04*	0.45 ± 0.02	1.95 ± 0.07*
18:4n-3	0.95 ± 0.03	0.90 ± 0.03	0.83 ± 0.07	0.78 ± 0.03
20:3n-3	0.00 ± 0.00	0.10 ± 0.03*	0.00 ± 0.00	0.17 ± 0.03*
20:5n-3 (EPA)	8.18 ± 0.33	7.48 ± 0.19	7.61 ± 0.79	6.65 ± 0.29
22:5n-3	1.99 ± 0.09	1.77 ± 0.06	1.63 ± 0.11	1.50 ± 0.07
22:6n-3 (DHA)	14.80 ± 1.01	9.79 ± 0.49*	10.59 ± 0.71	6.17 ± 0.46*
Total n-3 PUFA	26.40 ± 1.17	21.59 ± 0.67*	21.12 ± 1.56	17.23 ± 0.82*
Total PUFA	31.24 ± 1.31	33.48 ± 0.62	25.79 ± 2.40	31.84 ± 1.12*
n-3/n-6	5.45 ± 0.15	1.83 ± 0.08*	5.15 ± 0.35	1.18 ± 0.04*
EPA/DHA	0.58 ± 0.05	0.77 ± 0.02*	0.71 ± 0.03	1.11 ± 0.05*
Total lipids	2.24 ± 0.25	3.42 ± 0.24*	5.48 ± 0.76	7.63 ± 0.96

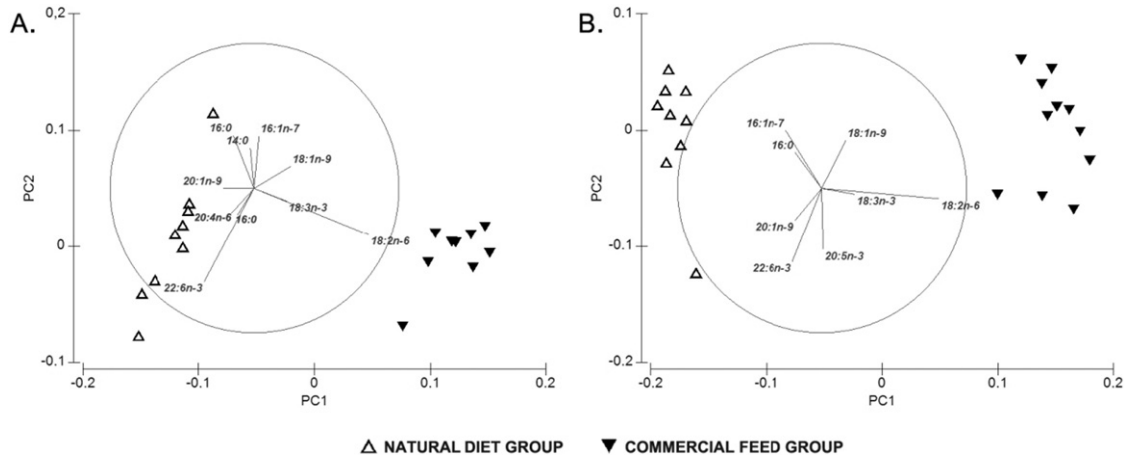

Fig. 1. PCA of the flesh fatty acids of fish after two (A) and eight weeks (B) of shift in diet.

peroxidase activity and the phagocytic capacity of head-kidney leucocytes of fish fed the experimental diets (Fig. 7).

4. Discussion

Most marine reared fish are piscivorous species that require long-chain n-3 fatty acids in their diet, as they have a limited capacity to synthesise them from their precursor, LNA. Golden mullet *L. aurata* is an eurihaline warm water fish, which aggregates around the sea-cage structures of fish farms, taking advantage of lost pellets (Fernandez-Jover et al., 2009). At the same time, it is well known that aggregated wild fish can transmit parasites or diseases to other wild fish stocks or reared fish when moving from one farm to another (Dempster et al., 2009; Arechavala-Lopez et al., 2010), which may be particularly important if the immune system of wild fish is altered due to consumption of waste feed. Fish tissues reflect the fatty acid composition of the diet (Farndale et al., 1999; Fountoulaki et al., 2009; Izquierdo-Gómez et al., 2015). Compared with the natural diet, the commercial feed used in this study presented low concentrations of the detected n-3 fatty acids, with the exception of LNA, which was relatively abundant (3%), while LA concentrations were 19%, and OA concentrations were 20%. Most studies in the literature show changes in the fatty acid profile of fish after several months of feeding on a new diet. Changes in the fatty acid profiles in several tissues of *L. aurata* specimens fed with the same diets were reported previously by our group (Gonzalez-Silvera et al., 2016). In the present work we found that just two weeks of feeding on the new diet were sufficient for the whole flesh fatty acid profile to be modified. Individual fatty acid percentages showed a profile that reflected the consumed diet, whereby the accumulation of LA, OA and LNA, and the decrease of DHA concentrations in commercial feed group were mainly responsible for the observed differences. These changes in fatty acid profiles agree with those of other authors working in wild mullet feeding on lost pellets from sea-cages (Fernandez-Jover et al., 2009) as well as mullet and other species in laboratory conditions, where similar trends were found when feeding on diets rich in vegetable oils (Argyropoulou et al., 1992; Ashton et al., 1994; Mourente et al., 2007). Due to limitations of the sample size, we could not analyse the fatty acid profile of leucocytes, but other studies have demonstrated that tissues like flesh tend to reflect the fatty acid composition of the diet (Farndale et al., 1999; Kiron et al., 2011). Changes in flesh may be accompanied by changes in the membranes of the immune cells. Therefore, the present results suggest a possible fatty acid modification in several tissues, including the head-kidney, which is the main haematopoietic organ in fish.

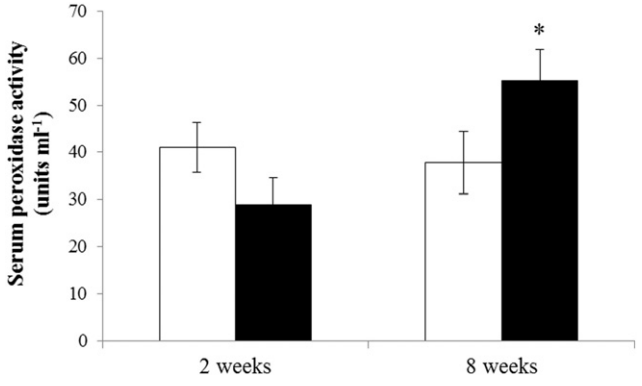

Fig. 2. Serum peroxidase activity (units mL serum^{-1}) of golden mullet fed with natural diet (white bars) and commercial feed (black bars) for 2 and 8 weeks. Bars represent the mean ± S.E. (n = 6). Asterisk denotes significant differences between natural and commercial feed groups ($p \leq 0.05$).

The influence of the relative consumption of n-3 and n-6 fatty acids have been extensively studied (Calder, 2007; Russo, 2009) and it is clear that the consumption of high amounts of n-3 fatty acids displaces n-6 fatty acids such as ARA in cell membrane phospholipids, diminishing the production of ARA-derived eicosanoids (Calder, 2006), which are more biologically potent than EPA-derived eicosanoids (e.g. leukotriene B5, derived from EPA, shows a 10- to 100-fold less biological effect than leukotriene B4, derived from ARA) (Kelley and Rudolph, 2000; Calder, 2006), and inhibiting the ARA metabolism. Despite some differences between fish and mammals as regards immune cell membrane composition (where EPA and ARA are the predominant fatty acids, respectively) fish leucocytes predominantly use ARA as substrate of 2-series and 4-series eicosanoids. When the dietary intake of n-3 fatty acids is sufficiently high (as occurs when feeding on natural diets), they compete as substrate for the cyclooxygenases and lipoxygenases, giving rise to 3-series and 5-series eicosanoids (Rowley et al., 1995; Tocher, 2003). Russo (2009) highlighted the greater importance of the relative amounts of n-3 and n-6 fatty acids than absolute amounts, a low n-3/n-6 ratio being considered deleterious for human cardiovascular health, and this also applies to fish (Martinez-Rubio et al., 2012). Unfortunately, information about the effects of aquaculture activities on wild fish immune status is scarce, but it can be extrapolated from the numerous studies carried out with reared fish and in laboratory facilities. In our research, fish fed on the commercial diet showed a decrease in the n-3/n-6 ratio at both sampling times. Changes in fatty acid profiles were accompanied by modifications of some immune paramaters, which may be indicative of an immunomodulatory effect of such a shift in diet.

We first evaluated whether the substitution of the natural diet by a commercial one affects the humoral innate parameters. The bactericidal activity of serum was tested against two fish pathogenic bacteria

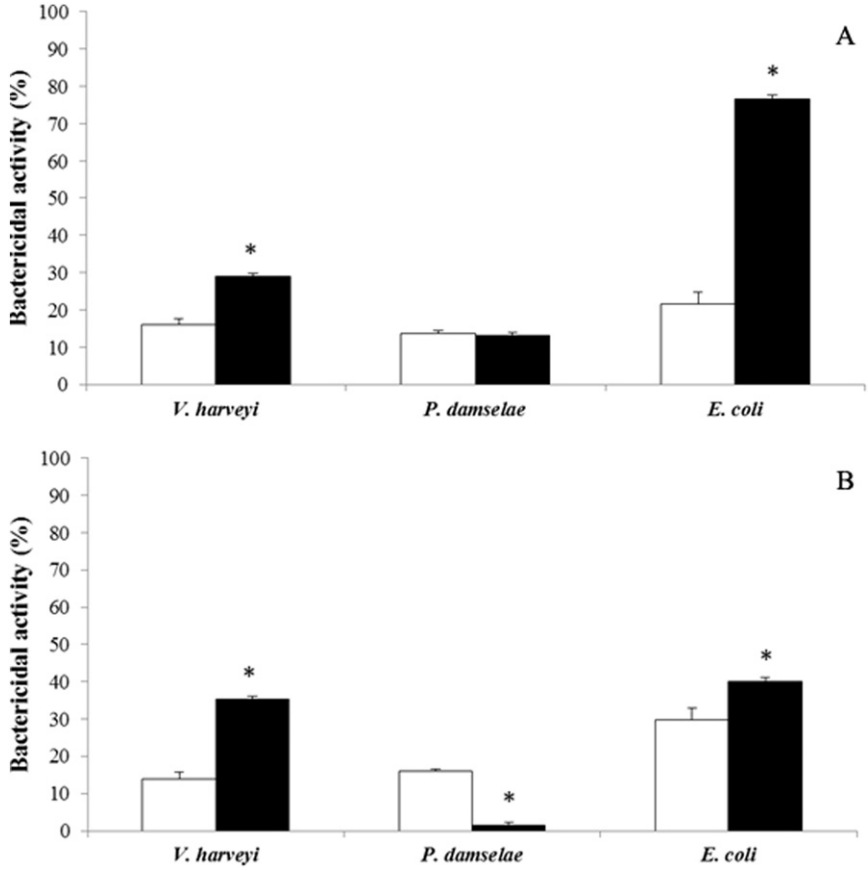

Fig. 3. Bactericidal activity present in serum samples of golden mullet specimens fed with natural diet (white bars) or commercial feed (black bars) for 2 (A) or 8 (B) weeks. Bars represent the mean ± S.E. ($n = 6$). Asterisks denote significant differences between natural and commercial feed groups ($p \leq 0.05$).

(*V. harveyi* and *P. damselae*), and one non-pathogenic bacterium (*E. coli*). The bactericidal activity of serum from the commercial feed group had a significantly lower effect against *P. damselae* at 8 weeks, while the same group presented a higher bactericidal activity towards *V. harveyi* and *E. coli* at both 2 and 8 weeks. Our results for *P. damselae* agree with those of Montero et al. (2010) in gilthead seabream where they concluded that the bactericidal activity against this pathogen was better when fish were fed with diets low in n-6. These results suggest that the shift from natural diet to commercial feed decreased the bactericidal properties of serum of golden mullet against *P. damselae* after 8 weeks. In contrast, other authors (Bell et al., 1996; Mourente et al., 2005; Montero et al., 2008) observed no differences in the plasmatic bactericidal activity of fish, measured as lysozyme activity (the most studied bacteriolytic enzyme in fish) (Minagawa et al., 2001) when fed with diets rich in vegetable oils. These apparently contradictory results suggest that more research involving a higher number of pathogenic organisms is needed.

With regard to cellular innate immunity, similarly to the results found by Bell et al. (1996) using fish and vegetable oils in different diet compositions, phagocytic activity (as phagocytic cell percentage) showed no significant difference between groups, although the leucocytes from the commercial diet group showed higher phagocytic capacity after 8 weeks of diet shifting. Wu et al. (2003) found that, in general, the immune responses, including phagocytosis, of juvenile grouper (*Epinephelus malabaricus*) were enhanced to a great extent by DHA, but also by EPA. This resulted in higher phagocytic values at low EPA/DHA ratios in the diet, which is not consistent with our results in golden mullet juveniles, in which the phagocytic capacity was significantly higher after 8 weeks of commercial diet consumption, with a higher EPA/DHA ratio in this group than fish fed on the natural diet. Kiron et al. (2011) compared the use of diets rich in safflower oil (rich in n-6, predominantly LA) or linseed oil (rich in n-3, predominantly LNA) in rainbow trout, finding no significant differences in phagocytic activity. Gjøen et al. (2004) observed no effect of dietary fatty acids on phagocytic activity in Atlantic salmon (*Salmo salar*) despite the fact that the ARA percentage increased 3-fold in head-kidney leucocytes when fish were fed a diet rich in soybean oil. These data could be extrapolated to our results concerning phagocytic ability (percentage of phagocytic cells), but not to phagocytic capacity (the relative number of ingested yeasts per cell). In light of the different data provided by the literature, more research seems to be necessary before firm conclusions can be reached about the effects of dietary fatty acids on fish phagocytic cells.

Once foreign particles such as bacteria have been phagocytized by macrophages and granulocytes, they are kept in a vacuole where the phagocytes release the killing machinery to destroy the pathogen. One way, and probably the most important and most widely studied, is the production of reactive oxygen species (ROS) by the process known as respiratory burst. In the present study, fish fed the commercial feed showed a decrease in respiratory burst activity, which became statistically significant after 8 weeks of diet shifting. These results agree with those of Mourente et al. (2005) in European sea bass. They showed a decrease in the respiratory burst activity in fish fed diets rich in vegetable oils compared to fish fed diets rich in fish oils. In contrast to our results, Montero et al. (2003) found no differences in respiratory burst activity when juveniles of gilthead seabream were fed with experimental diets containing different vegetable or fish oils. Similarly, another study carried out in Atlantic salmon showed that respiratory burst did not differ as a function of the type of lipids (marine or vegetable origin) in diets (Bell et al., 1996). Another study in Atlantic salmon fed fish oil and rapeseed oil (high in n-6 fatty acids), using an ex vivo plasma incubation

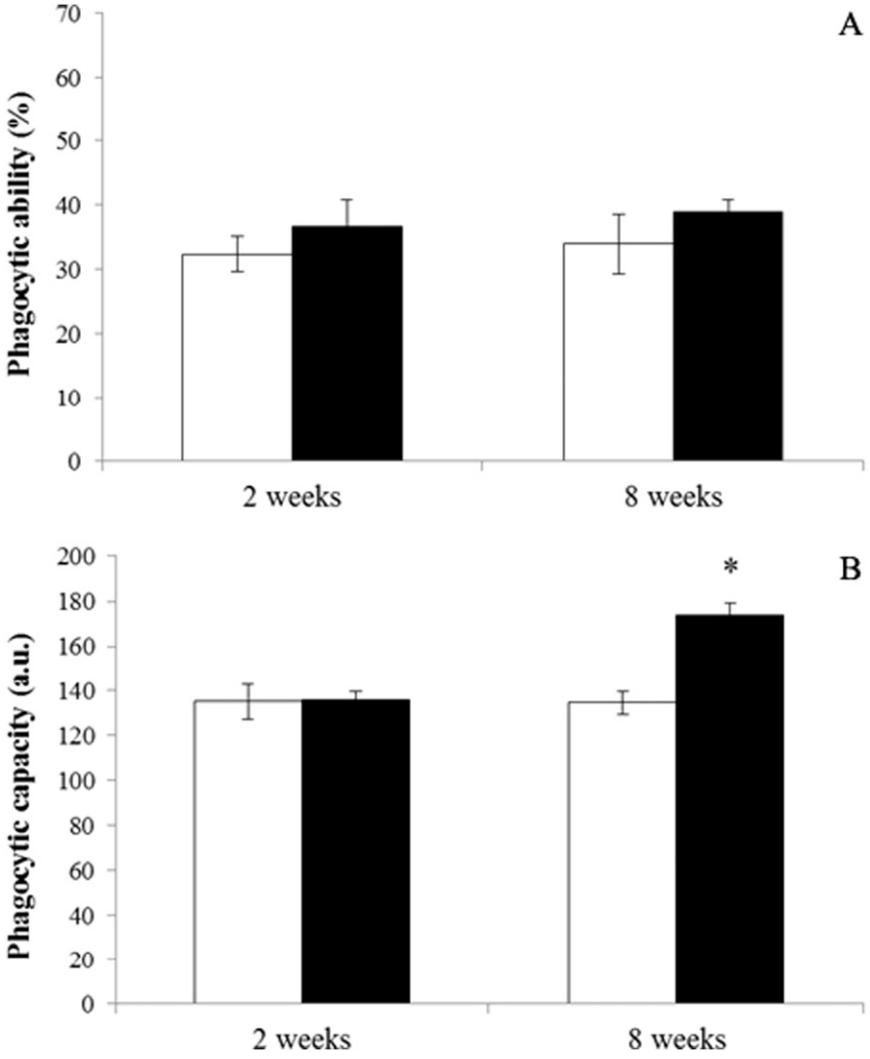

Fig. 4. Phagocytic ability (%) (A) and capacity (a.u.) (B) of head-kidney leucocytes of golden mullet fed with natural diet (white bars) or commercial feed (black bars) for 2 or 8 weeks. Bars represent the mean ± S.E. (n = 6). Asterisk denotes significant differences between natural and commercial feed groups ($p \leq 0.05$).

model to study the fatty acid composition of head-kidney leucocyte membranes, inferred that there were no differences in the respiratory burst activity, even when leucocytes presented 5-fold reduction in the n-3/n-6 ratio compared to the control (Seierstad et al., 2009). Regarding in vitro experiments comparing the different effect of individual fatty acids on the respiratory burst activity, Li et al. (2013) suggested that DHA had an inhibitory effect on the production of the superoxide anion in macrophages isolated from large yellow croaker (*Larimichthys crocea*), while production was enhanced by EPA. Although the literature is contradictory, we consider that any increase in the capacity of *L. aurata* macrophages to phagocytize

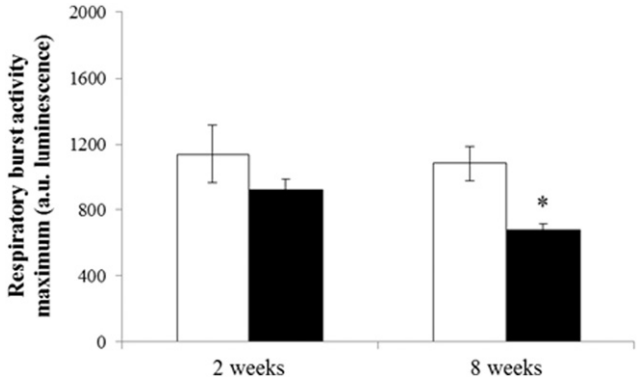

Fig. 5. Respiratory burst activity (slope min^{-1}) of head-kidney leucocytes of golden mullet fed with natural diet (white bars) or commercial feed (black bars) for 2 and 8 weeks. Bars represent the mean ± S.E. (n = 6). Asterisk denotes significant differences between natural and commercial feed groups ($p \leq 0.05$).

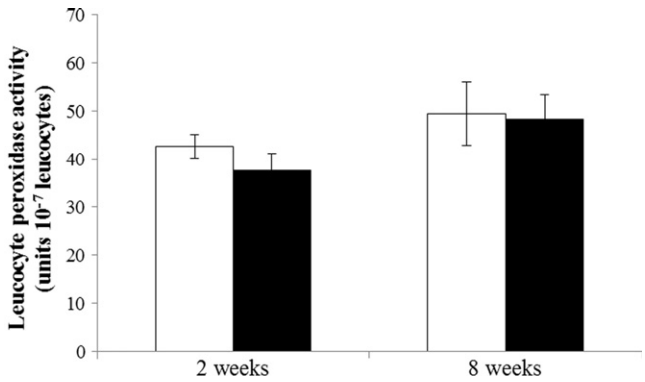

Fig. 6. Head-kidney leucocyte peroxidase activity (units 10^{-7} leucocytes) from golden mullet specimens fed with natural diet (white bars) or commercial feed (black bars) for 2 and 8 weeks. Bars represent the mean ± S.E. (n = 6).

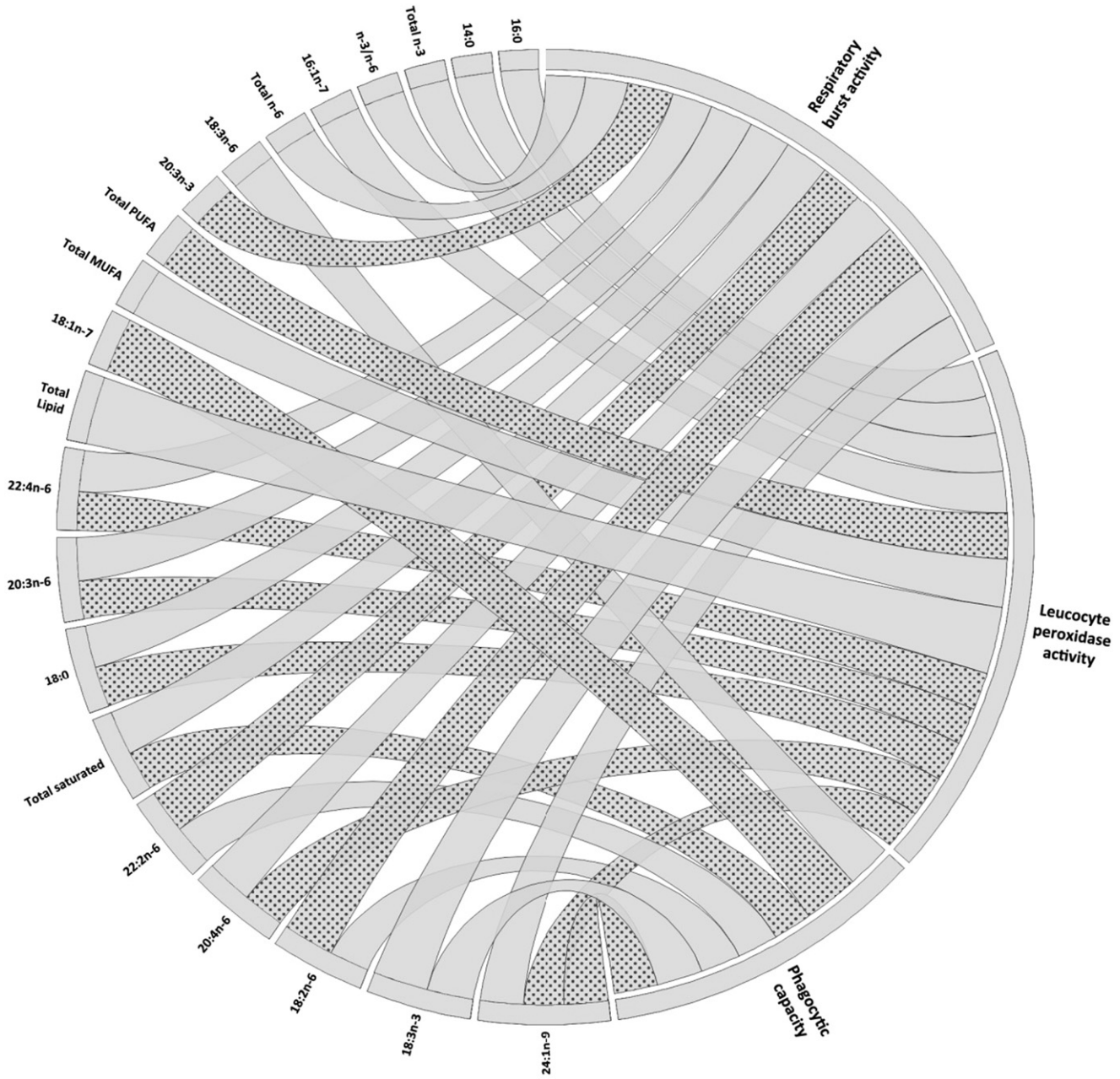

Fig. 7. Circular diagram representing correlations among fatty acids, including n-3/n-6 ratio and total lipid values, and immune parameters. Only significant results are represented ($p \leq 0.05$). Dotted and non-dotted ribbons indicate negative and positive correlations, respectively.

particles is not accompanied by a subsequent increase in their capacity to destroy the phagocytized particles using toxic chemicals. Indeed, this capacity was significantly diminished at 8 weeks after feeding changed from a natural diet to a commercial feed. The intake of feed rich in vegetable fatty acids resulted in an unchanged peroxidase activity in head-kidney leucocytes but increased activity in the serum of fish from the commercial diet group at 8 weeks, suggesting that this enzyme is secreted and not stored in the leucocytes. These results suggest that a short-term modulation of the serum peroxidase activity could take place in response to changes in the diet composition. These findings could point to the potential role of fatty acids in peroxidase activity and deserve further analysis.

Our results with a number of different fatty acids revealed a high number of significant correlations of cellular immune parameters in the following decreasing order: respiratory burst activity > peroxidase activity > phagocytic capacity of head-kidney leucocytes. Nevertheless, the values of the correlations are, in general, not high enough to identify a single fatty acid as being responsible for the differences in the cellular immune parameters, at least within the time period established in this experiment after changing diet.

This work demonstrates in fish the existence of an immunomodulation by the shift from a natural diet to a commercial feed consumption. Nevertheless, the real effect in the wild may differ in some way, as fish are under the influence of other factors like temperature changes, exposition to parasites and diseases, as well as consumption of other foods than lost pellets. Other studies have been pointed out a modification of the fatty acid composition of different tissues in wild fish aggregated to fish farms (Skog et al., 2003; Fernandez-Jover et al., 2009; Izquierdo-Gómez et al., 2015). The total or partial substitution of a natural feeding regime by commercial feeds consumption could also affects the immune response in aggregated wild fish, and this fact deserves to be thoroughly investigated.

5. Conclusions

In conclusion, the observations made of the flesh fatty acid profile after two weeks of shift in diet suggest that wild fish aggregated around fish farms present modifications in their natural fatty acids profile and their n-3/n-6 ratio after a short period of commercial feed consumption. Some immune parameters may change in the event of wild fish reaching a fish farm sea-cage and feeding on the excess of lost pellets rich in vegetable oils. The extent of these changes cannot be considered large enough to regard them as compromising the health status of fish. However, more research is needed in order to elucidate whether the rapid assimilation of the dietary fatty acids could harm the immune status of juveniles of golden mullet when feeding for longer periods than two months. Furthermore, more research is needed relating these results to field conditions before more rigorous conclusions can be drawn of their impact on wild mullet populations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.marpolbul.2016.10.055.

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