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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

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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

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