**The nutritional background of the host alters the disease course
in a fish-myxosporean system**

Itziar Estensoro^{1}, Laura Benedito-Palos^{2**}, Oswaldo Palenzuela¹, Sadasivam Kaushik³,
Ariadna Sitjà-Bobadilla^{1*} and Jaume Pérez-Sánchez²**

*¹Fish Pathology Group, ²Fish Nutrition and Growth Endocrinology Group, Department of Fish
Species Biology, Culture and Pathology, Instituto de Acuicultura de Torre de la Sal (CSIC), 12595
Ribera de Cabanes, Castellón, Spain*

*³UMR Nutrition, Aquaculture & Genomics, INRA, Unité-Mixte INRA-IFREMER-Univ. Bordeaux I,
64310 Saint-Pée-sur-Nivelle, France*

****These authors contributed equally to this article**

***Corresponding author: Dr. Ariadna Sitjà-Bobadilla**

Address: Instituto de Acuicultura de Torre de la Sal

Consejo Superior de Investigaciones Científicas, Torre de la Sal s/n,

12595 Ribera de Cabanes, Castellón, Spain

Phone: +34-964319500

Fax: +34-964319509

e-mail: ariadna@iats.csic.es

Note: Supplementary data associated with this article

ABSTRACT

The aim of the present work was to determine if a practical plant protein-based diet containing vegetable oils (VO) as the major lipid source could alter the disease course when challenged with the myxosporean *Enteromyxum leei*, a wide-spread parasite in the Mediterranean basin causing heavy economic losses. Gilthead sea bream (*Sparus aurata*) fed for 9 months either a fish oil (FO) diet or a blend of VOs at 66 % of replacement (66VO diet) were challenged by exposure to parasite-contaminated water effluent. All fish were periodically and non-lethally sampled to obtain biometrical data and to know their infection status. After 102 days of exposure, fish were euthanized and haematological, biometrical, histological, immunological, glutathione and anti-oxidant data were obtained from tissue, blood and serum samples. Anorexia appeared in both exposed groups, but feed intake reduction was higher in 66VO fish. The signs of disease (lower growth, condition factor, specific growth rate, haematocrit) as well as the disease course were worse in fish from 66VO group, with a higher prevalence and intensity of infection, a higher percentage of fish harbouring the parasite in the entire intestinal tract, and a faster establishment of the parasite. Parasite intensity of infection was negatively correlated with growth parameters and haematocrit in both groups, and with complement, lysozyme and hepatic total glutathione in 66VO fish.

Key words: Myxozoa; *Enteromyxum*; parasites; immune response; *Sparus aurata*; anorexia; vegetable oils

1. Introduction

Parasites are a major constraint on animal production through the world, and recent cases of massive losses in salmon culture due to sea lice are an outstanding example (Costello, 2009). Economic losses are due not only to mortality but also to poor growth performance, low reproduction efficacy, emaciation or other external signs that turn fish into unmarketable products (Sitjà-Bobadilla, 2004, 2009; Guo and Woo, 2009). Gilthead sea bream (*Sparus aurata*) is the main cultured fish species in the Mediterranean area, with a production of more than 150,000 tonnes in 2008 (APROMAR, 2009). Diseases and feed costs are the main limitations for enhancing the productivity. *Enteromyxum leei* is a widely spread myxosporean responsible for one of the most threatening parasitic diseases in Mediterranean fish cultures (Palenzuela, 2006). This parasite invades the intestine of gilthead sea bream producing a slow-progressing disease, which induces anorexia, cachexia and eventually the death of fish. Its impact is further enhanced due to its direct fish-to-fish transmission either by cohabitation with infected fish, by contact with contaminated effluent and *per os* (reviewed in Sitjà Bobadilla et al., 2007). Thus far, there are neither preventive nor curative treatments for enteromyxosis. Therefore, there is an urgent need in advancing our knowledge of the parasite itself and the host-parasite interaction.

Substitution of fish meal (FM) and fish oil (FO) by optimised levels of vegetable ingredients stands as one of the current strategies for reducing the cost of fish feeds and the dependency on fisheries to produce aquafeeds (Tacon and Metian, 2008). While using such alternative ingredients, not only the possible effects on growth performance, but also animal health and welfare should be analysed in an integrative manner. In this context, studies on the effect of FO substitution by vegetable oils (VO) on fish health and the possible nutritional modulation of resistance to infectious diseases are of major importance. In previous works in gilthead sea bream, it has been demonstrated that FO can be replaced in plant-protein based diets up to 66% without detrimental effects on growth, redox balance, immunocompetence or on the intestinal and hepatic architecture (Benedito-

Palos et al., 2007, 2008, 2009; Saera-Vila et al., 2009). Thus, a further step was to test whether plant proteins and VO optimised diets could alter the disease outcome when confronted with a pathogen. For this purpose, chronic exposure to *E. leei* by contact with parasite-containing water was chosen to mimic the natural infections. The present work aimed to determine the effects of dietary fat sources on infection levels, growth performance, host immune response and protection against oxidative stress.

2. Material and methods

2.1. Experimental design and sampling procedure

Naïve gilthead sea bream were obtained from a commercial fish farm and checked for the absence of the parasite (see below). Fish were divided into two experimental groups, which were fed two different diets (supplementary Table S1). After 9 months of feeding with the corresponding two diets, 60 fish from each diet were individually tagged with passive integrated transponders (PIT-tags) (Trovan, Spain), transferred to the Pathology unit of the Instituto de Acuicultura de Torre de la Sal (IATS) and acclimated for 2 weeks before the parasite challenge. Each diet group was divided again into two groups, control (C, n = 30) and recipient (R, n = 30) in 500-L fibre-glass tanks. The average initial weight before the challenge was 223.7 g. The two R-tanks (one for each dietary treatment) were exposed to *E. leei*-contaminated effluent as previously described (Sitjà-Bobadilla et al., 2007). Briefly, R tanks were set to receive exclusively the effluent water from another tank containing 30 infected fish (donors; average weight = 240.2 g; prevalence of infection = 100 %; fed a standard commercial diet). Control fish were kept under the same conditions, but without receiving *E. leei*-contaminated water.

Day length followed natural changes at IATS latitude (40°5'N, 0°10'E) and water temperature was kept always above 18 °C (average = 21.3 °C, range =18-26 °C, Sea water (37.5 ‰

salinity) was pumped from ashore (open system), 5 µm-filtered and UV irradiated. Each diet group was fed *ad libitum* with the same experimental diet it was receiving before the challenge, and daily food intake in each tank was registered. Disease signs and daily mortality were recorded.

The progression of the infection and the evaluation of growth performance were monitored by sampling both C and R groups at 32, 53 and 88 days post exposure (p.e.). At each sampling, all fish were sized and weighed and non-lethally (NL) sampled for parasite diagnosis with a PCR test (see below). An additional last sampling was performed at 102 days p.e. Then, all R remaining fish and 15 fish from each C group were killed by over-exposure to benzocaine (3-aminobenzoic acid ethyl ester, 100 mg l⁻¹, Sigma, St. Louis, MO, USA) and blood and tissue samples were taken for histological, immunological, and anti-oxidant analyses. Length and body weight, liver and spleen weights were measured and the condition factor ($CF = [\text{weight (g)} \times \text{length (cm)}^{-3}] \times 100$), and the hepatosomatic (HSI) and splenosomatic (SSI) indexes were calculated as the ratio between the organ weight and body weight. Specific growth rates (SGR) of all fish were calculated for the period ranging from one week previous to the challenge up to the end of the experiment (109 days) as follows: $SGR (\%) = 100 \times (\ln W_t - \ln W_0)/t$, where W_0 represents weight at the beginning of the period, W_t the weight at the end of the trial and t the number of growth days.

One heparinised blood aliquot was immediately used to measure the respiratory burst activity and another aliquot was drawn into heparinised capillary tubes, centrifuged at 1,500 g for 30 min, and the haematocrit measured. The remaining non-heparinised blood was allowed to clot overnight at 4 °C, centrifuged at 3,000 g for 20 min at 4 °C, and serum aliquots were stored at -80 °C until used in immunological, anti-oxidant and glutathione assays.

One replicate tank per group was established to minimize the number of experimental fish (a mandatory requirement of the ethical committee) and to guarantee an infective effluent to the R tanks compatible with a good water quality. According to our previous experience, no tank effect has been found when two tanks are infected from the same D tank under these experimental

conditions (Sitjà-Bobadilla et al., 2007, 2008). As all fish were individually monitored along the entire experimental period, each fish was considered the experimental unit (Fisher, 2000).

2.2. Parasite diagnosis

Parasite diagnosis was performed by histology (at 102 days p.e.) or by NL-PCR (at 32, 55 and 88 days p.e.). For histological examination, pieces of anterior, medium and posterior intestine were fixed in 10 % buffered formalin, embedded in paraffin, 3 μ m-sectioned and stained with haematoxylin and eosin. NL samples were obtained by probing the rectum with a cotton swab and PCR diagnosis was carried out as described in Palenzuela and Bartholomew (2002) with primers specific for *E. leei* rDNA. This procedure has been validated against a gold standard (histological observation of the whole digestive tract), resulting in a high sensitivity (0.96) and specificity (1) (O. Palenzuela, unpublished data). NL-PCR was also applied to evaluate the parasitic status of donors, and R fish upon arrival to the experimental facilities. The prevalence of infection at each sampling point was calculated considering positive fish detected by either PCR or histology. The intensity of infection was semiquantitatively evaluated in one histological cross section of each of the three intestinal portions (anterior, medium and posterior) and scored 1+ to 6+ according to the number of parasitic stages per microscope field at x120 with the range: 1+ = 1-5; 2+ = 6-10; 3+ = 11-25; 4+ = 26-50; 5+ = 51-100; 6+ > 100. For each intestinal portion, the mean intensity of infection was calculated. For each parasitized fish, the mean intensity of infection was calculated as an average of the intensity of infection in the parasitized portions.

Fish tagging and NL-diagnosis by PCR allowed individualized monitoring of the infection along the experimental period. Thus, for each R fish euthanized at 102 days p.e., the time at which the parasite was first detected was known. Therefore, each R fish was classified as being first-infected at one of the four sampling times, and four timing-classes were established: late (102 days), medium (88 days), early (53 days), and very early (32 days).

2.3. Immunological assays

Induction of the respiratory burst (RB) activity in blood leucocytes was measured directly from heparinised blood, as previously described (Saera-Vila et al., 2009). Briefly, blood was incubated with a luminol suspension containing PMA for 1 hour and the resulting integral chemiluminescence in relative light units (RLU) was calculated.

Total serum peroxidases (PO) were chosen as a measure of the serum oxidizing capacity. Serum lysozyme (LY) was measured by a turbimetric assay. The lytic capacity of the serum by the alternative complement pathway (ACP) was determined using sheep red blood cells (SRBC) as targets, and the dilution corresponding to 50 % haemolysis/ml was expressed as ACH₅₀. All these assays were performed following the procedure described in Sitjà-Bobadilla et al. (2005), but using 2.85×10^8 SRBC ml⁻¹ in ACP.

Total nitric oxide (NO) production in serum samples was determined as the sum of nitrite (NO₂) and nitrate (NO₃), using a fluorimetric assay kit, which detects up to 10 nM naphthotriazole (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 10 µl duplicated serum samples, previously centrifuged in Microcon-10 filter devices (cut-off value = 10KDa) (Millipore Corp., Madrid, Spain) at 12,000 g for 10 min at 4 °C, were incubated with enzyme cofactors and nitrate reductase for 2 h. 2, 3-diaminonaphthalene (DAN) and NaOH were sequentially added and the resulting fluorescent product (naphthotriazole) was measured using an excitation wave length of 360 nm and an emission wave length of 465 nm in a microplate reader.

2.4. Anti-oxidant and glutathione assays

The total antioxidant status (TAS) was measured in serum samples with a commercial kit (Cayman Chemical). Oxidized (GSSG) and total (tGSx) levels of glutathione in frozen liver and

intestine samples were determined enzymatically with a commercial kit (Cayman Chemical). The GSH/GSSG ratio, was calculated as the quotient of reduced GSH equivalent, as a measure of cellular toxicity. Both assays were performed as previously described (Saera-Vila et al., 2009).

2.5. Statistical analysis

The influence of the diet on the presence of *E. ictali* was statistically analyzed using a Chi-square test of independence with Yates correction for continuity, considering all the fish sampled at 102 days p.e. For all the data, differences between the four experimental groups at any sampling point were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. When the tests of normality or equal variance failed, a Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's method was applied instead. The same test was applied to determine possible differences in the mean intensity of infection between time-classes at 102 days. p.e. A Student-*t* test was used to analyze the differences between both R groups within each time-class, and within each intestinal portion.

The strength of the possible association of all the studied variables in individual fish was measured with a Spearman rank order correlation test, since the normality test failed for some of them. Thus, all the data of fish from the same diet obtained at 102 days p.e. were pooled and the Spearman correlation coefficients between all the variables (except those conceptually correlated) were calculated. In addition, for some infection parameters and the timing of the infection, a linear regression test was performed to further analyse the relationship between them.

All the statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA) and in all cases the significance level was set at $P < 0.05$.

3. Results

3.1. Progression of the infection

Fig. 1A shows the prevalence of infection during the exposure to the parasite. Although the final infection levels were high in both R-groups, the progression of the infection was higher and faster in 66VO-R fish than in FO-R ones. In fact, the relationship between time and prevalence of infection had a pattern of exponential rise to a maximum ($r^2 = 0.9169$, $P = 0.0424$) in the 66VO group, whereas it remained linear in the FO group ($r^2 = 0.953$, $P = 0.0238$). This differential progression was clearly outlined by the statistically significant relationship between the dietary treatment and the cumulative prevalence of infection, as analyzed with a Chi-square test of independence ($P = 0.032$). No C fish was found to be parasitized at any sampling point. Mortality along the experimental period was similar in both R-groups, 4 fish died in 66VO vs 5 in FO.

Fig. 1B shows the mean intensity of infection of infected fish when euthanized (102 days p.e.), considering the four different time-classes related to the first detection of the infection. No fish was detected as newly infected at the last sampling in the 66VO group. In general, the mean intensity of infection was higher in fish of the 66VO-R group than in FO-R fish, but there were no statistically significant differences between them at any time. In both R-groups, it was evident that the earlier the infection was established, the higher the intensity was at the final sampling. Furthermore, a strong positive correlation was found between the intensity of the infection and the number of days of establishment of the infection, in FO-R ($r_s = 0.944$, $P = 0.0000007$) and 66VO-R ($r_s = 0.783$, $P = 0.0000007$).

At the last sampling point, all the infected fish, regardless of the group, had the parasite established at the posterior intestine. However, the percentage of parasitized fish harbouring the myxosporean also at the anterior and medium sections was clearly higher in 66VO group (Fig. 1C), being always the medium intestine the less frequently affected part.

3.2. Biometrical data

No differences in growth performance were detected between control (C) groups fed the two different diets (Fig. 2A). As expected, both groups exposed to the parasite (R) exhibited the physiological effects of the disease, but they were more acute in 66VO-R fish. Growth performance was reduced in both R groups, with a significant lower weight at 88 and 102 days p.e. vs their corresponding C groups, and even between 66VO-R and FO-R fish at the last sampling (Fig. 2A). Similarly, body weight gain in the whole experimental period was markedly lower in both R groups, and again the percentage of reduction respect to the C group with the same diet was higher in fish fed 66VO than in those fed FO (54.2 % vs 29.4 %) (Table 1). At 102 days p.e., both R-groups also had significantly lower length and CF than their corresponding C groups (Table 1). When analysing in detail the decrease in SGR in R groups, all the 66VO-R infected fish, regardless of the time in which they acquired the infection, had significantly lower SGR values than 66VO-C fish. By contrast, only those FO-R fish with early acquisition of the infection had significantly lower values than their respective C group. SGR was lower in all 66VO-R time classes fish than in FO-R, though the difference was statistically significant only in the medium time-class (Fig. 2B). The lower increase of body weight of R fish was due in part to a reduced feed intake in R animals, which was 15 % and 22 % less than the corresponding C groups in FO and 66VO, respectively (Table1). Anorexia thus explained about 52 % of body weight losses in FO-R animals, but only 41 % in 66VO-R group. Anorexia was first detected between 32 and 53 days, increased progressively and was most evident in the last weeks of the experimental period (between 88 and 102 days) when feed intake was 30 % and 45 % lower in FO-R and 66VO-R than in the corresponding C groups, respectively. Haematocrit values were significantly lower in 66VO-R animals (Table 1). No significant differences were found in the SSI and HSS indexes between any groups (Table 1).

3.3. Immune factors (Table 1)

Both R groups, regardless of the diet, exhibited a significantly higher respiratory burst in circulating leucocytes than their corresponding C groups at 102 days p.e. Although the values obtained for 66VO-R fish were higher than those of FO-R animals, differences were not significant. Total serum peroxidases (PO), serum lysozyme (LY) and serum NO were lower in both R groups, but the decrease was only statistically significant for PO in 66VO-R and for LY and NO in FO-R. Both 66VO groups (R and C) had significantly lower values of LY and NO than those of FO-C group. The lytic capacity of the serum by the alternative complement pathway (ACH_{50}) was significantly higher in 66VO-C fish than in the remaining groups, and no differences were observed between both R groups.

3.4. Glutathione and antioxidant defence system (Table 1)

Fish from the 66VO-C group exhibited a significantly higher total antioxidant status (TAS) than the remaining groups and no differences were found between both R groups. Concerning the glutathione values, no significant differences were found in the intestinal values, whereas the hepatic synthesis of glutathione (tGSx) and the hepatic GSH/GSSH ratio were significantly decreased in 66VO-R fish with respect to FO-R and also respect to 66VO-C for the hepatic ratio.

3.5. Meta-analysis: correlation between all data and the disease course

Spearman's correlation coefficients between all the biometrical, immunological, antioxidant and glutathione factors for FO and 66VO groups are shown in supplementary Tables S2 and S3, respectively. The information derived from S2 and S3 is diagrammatically presented in Fig. 3, showing the main correlations found between *E. leei*-intensity of infection and the remaining measured factors. There was a strong negative correlation with biometrical parameters (length,

weight, SGR, CF) in both diet groups (Fig. 3, red boxes), also with Hc, HSI and some immunological (ACH_{50}) and glutathione (hepatic GSH/GSSG ratio, liver tGSx) factors only in the 66VO group, and with LY and NO only in the FO group (Fig. 3, red boxes). In addition, a positive correlation was found between the parasite and RB in 66VO fish (Fig. 3, green box). Similarly, a negative correlation was also found between the number of days of establishment of the infection and most biometrical values in both groups, and also with HSI, ACH_{50} , Hc, and hepatic glutathione (GSH/GSSG ratio, tGSx) factors in the 66VO group, and with SSI, PO and NO in the FO group (supplementary Tables S2, S3).

Hc was positively correlated with ACH_{50} , weight, length, CF and SGR in 66VO fish, and with PO, SGR, HSI and intestinal GSSG in FO fish. By contrast, Hc was negatively correlated with RB in 66VO. In both groups, PO correlated positively with HSI, and RB negatively with SGR. Other correlations were found only in 66VO animals: positive between ACH_{50} and several biometrical values (weight, length and SGR), and negative between RB and HSI, and between RB and ACH_{50} and PO.

From the statistical analysis we can conclude that in 66VO fish, the earlier the infection was established, the higher the intensity of infection was achieved and consequently the lower weight, length, CF, SGR, Hc, LY, ACH_{50} and hepatic glutathione ratio were exhibited.

4. Discussion

The present study has shown that feeding fish with a VO diet may increase the disease effects of enteromyxosis in gilthead sea bream. Although the final prevalence and intensity of infection were high in both dietary experimental groups and similar to other effluent-transmission studies (Sitjà-Bobadilla et al., 2007), the progression of the infection and the severity of the disease signs were higher in R fish fed the 66VO diet than in those fed the FO diet. Thus, the effect on weight, length, CF, SGR and Hc, which are the consequences of the emaciative disease provoked

by the parasite, were more pronounced in 66VO-R fish. In other fish-*Enteromyxum* systems in which the myxosporeans exhibit a higher pathogenicity, a significant reduction in weight and Hc was detected earlier (Sitjà-Bobadilla et al., 2006; Alvarez-Pellitero et al., 2008). By contrast, in other gilthead sea bream also exposed to *E. leei* by effluent transmission, but fed a standard commercial diet and reared under lower water temperature, no significant differences in weight were detected between C and R, probably due to the lower growth rates and the lower achieved infection levels (Sitjà-Bobadilla et al., 2008)

The 66VO-C fish did not exhibit any detrimental effect of the diet on their biometric and haematological values. Therefore, as already demonstrated in previous growth studies (Benedito-Palos et al., 2007, 2008, 2009), the nutritional background by itself did not produce any detrimental effect on the fish body mass, and it was not responsible alone for the severe disease, but a predisposing factor. Besides, for the first time in a fish-myxosporean model, we find evidence that the body mass loss of R fish is mainly due to a decrease of voluntary feed intake (anorexia). Parasite-induced anorexia is a prominent feature of several host-parasite systems in vertebrates, but the causes and mechanisms of anorexia remain unresolved (Kyriazakis et al., 1998; Colditz, 2008). In fish, anorexia has been scarcely documented with accuracy under cases of parasitic infections (Thomas and Woo, 1992; Dezfuli et al., 2003). Reduced nutrient availability due to decreased feed intake is the major contributor to reduced growth rates in parasitized animals (see Colditz, 2008). However, the decrease in weight gain in R fish was higher than what could be explained by feed intake decrease. Therefore, other factors such as parasite-derived intestinal damage could contribute to the impairment of intestinal absorption and therefore nutrient availability. Indeed, intestinal damage was higher in 66VO-R fish, as more intestinal portions were affected and with a higher intensity of infection, and the typical enteritis syndrome was observed in fish with high intensity of infection (data not shown). It has previously been shown that the massive invasion of the intestinal epithelium by *E. leei* stages produces enteritis with atrophy and blunting of villi which can lead with the detachment of the epithelium from the underlying lamina propria (Fleurance et al., 2008).

In addition, the intensity of the lesions produced by *E. scopththalmi* in the intestine of turbot was correlated with the progression of the infection (both prevalence and intensity) and the development of the parasite (Bermúdez et al., 2006, 2010). Finally, body weight loss could also be due to an osmoregulatory failure, as suggested by the pathophysiological evidences in *E. leei*-infected *Takifugu rubripes* (Ishimatsu et al., 2007). Therefore, we are clearly in front of a case of cachexia, in which weight is lost much more rapidly than would be expected from a decrease in feed intake of the same magnitude (Emery, 1999).

The host's immune response has a metabolic cost (Ots et al., 2001) and could also have adverse effects on growth and feed intake. In fact, the immune response is responsible for the production of several cachectic cytokines (TNF- α , IL-1 and IL-6) that induce cachexia (Cerami and Beutler, 1988; Argilés et al., 2006). In *E. leei*-infected gilthead sea bream, transcripts of IL-1 β and TNF- α were significantly decreased in the intestine at 113 days p.e. (Sitjà-Bobadilla et al., 2008), whereas IL-1 β expression was increased in head kidney shortly after exposure (10 days) (Cuesta et al., 2006). Thus, other anorexigenic factors, such as gastrointestinal peptides (Mendieta-Zerón et al., 2008) or growth factors (Carroll 2008) could be involved. In fact, the number of enteroendocrine cells positive for neuromodulators related with feed intake and digestion, such as neuropeptide Y and substance-P were lower in the intestine of R fish than in C gilthead sea bream (Estensoro et al., 2009).

The main difference between the two diets tested in the current study is the fatty acid (FA) profile, since 66VO diet has lower levels of n-3 long-chain polyunsaturated fatty acids (LC-PUFAs), though it meets the theoretical requirements of essential FAs. The complex relationship between nutrition, immune status and parasitic infection is well recognized (Fekete and Kellems, 2007), and dietary FAs are capable of modulating the immune system in mammals (De Pablo and De Cienfuegos, 2000) and n-3 LC-PUFAs present in FO in particular have well recognized anti-inflammatory properties (Calder, 2007). However, in fish, changing the dietary n-3 LC-PUFA levels can have both beneficial and, in some instances, detrimental effects on disease resistance and

immune status. Thus, the intake of high levels of dietary n-3 LC-PUFAs suppressed some immune functions and reduced survival after pathogen challenge (Erdal et al., 1991; Fracalossi and Lovell, 1994; Kiron et al., 1995; Misra et al., 2006). Conversely, in our study, the disease course was worsened in fish fed the 66VO diet (with lower n-3 LC-PUFAs), as it occurred in other studies in which significantly higher mortality rates were found in fish fed diets with different types of VOs (with lower n-3/n-6 PUFAs ratio, or lower n-3 LC PUFAs) compared to those fed FO when subsequently challenged with bacteria (Thompson et al., 1996; Bransden et al., 2003) or the ciliate parasite *Ichthyophthirius multifiliis* (Vargas et al., 2008).

The involvement of innate mechanisms in the fish immune response to parasitic infections has been documented in several host-parasite models (see Alvarez-Pellitero, 2008). In the current model, ACH₅₀ was the only enhanced innate factor in 66VO-C, which is in accordance with the increased ACH₅₀ reported in grouper (*Epinephelus malabaricus*) fed a diet partially replaced with corn oil (Lin and Shiau, 2007). In gilthead sea bream, a similar 66VO diet fed during 6 months also produced a slight increase in ACH₅₀ (Saera-Vila et al., 2009), but another diet with totally replaced FO by a single VO invoked a decrease in ACH₅₀ (Montero et al., 2003). By contrast, ACH₅₀ was not affected by feeding with different single or mixed VOs in other fish species (Kiron et al., 2004).

On the other hand, the worse disease outcome in fish fed the 66VO diet could be due to the significantly lower values of serum nitric oxide (NO) and lysozyme (LY). NO is an important molecule in regulating immune functions and has a direct antimicrobial effect. As in FO-R fish, nitrite production of head kidney leucocytes of sharpsnout sea bream (*Diplodus puntazzo*) infected with a myxosporean *Myxobolus* species was hampered (Karagouni et al., 2005). LY is a main defence molecule of fish innate immune system (Saurabh and Sahoo, 2008) and interestingly, in sharpsnout sea bream, a species highly susceptible to *E. leei* in which the infection progresses rapidly, LY was undetectable in either infected or healthy fish (Golomazou et al., 2006; Alvarez-Pellitero et al., 2008). By contrast, serum LY appears to be unaffected in most studies with fish fed diets rich in VOs (Bell et al., 1996; Montero et al., 2003; Kiron et al., 2004; Mourente et al., 2005).

These apparent contradictory effects on immune factors are probably due to different experimental conditions (fish meal inclusion, type of vegetable oil, PUFAs ratios, feeding time, etc.), type of immunocytes involved and species model. In most fish trials, the basal diet is replaced either by fish meal or FO, whereas a significant difference of the current trial is that high levels of substitution of fish meal by plant proteins was also achieved.

There are other hypothetical reasons for the worse disease signs in 66VO-R, related to the direct effect on the intestinal tract and mucosal immunity. Some dietary VOs and saturated FAs produce cellular damages in fish enterocytes due to the accumulation of lipid droplets (Olsen et al., 2000), but this damage was observed only with total FO replacement (Benedito-Palos et al., 2008). Other possible changes in the cellular architecture and composition of the digestive tract might favour parasite entrance and proliferation in the intestinal epithelium, through changes at the host-parasite interface (Yoshino et al., 2001), alterations of the gut barrier or the mucosal immunity (Kasper and Buzoni-Gatel, 2001), or alterations of the biochemical makeup of the cell membrane lipid rafts/caveolae microdomains (Chapkin et al., 2008). In fact, the effect of PLs, FAs and soybean meal on tight-junction permeability and therefore in bacterial translocation (Sawai et al., 2001), on fish intestinal mucus and bacterial adhesion (Bakke-McKellep et al., 2007; Schroers et al., 2008) have been reported. Thus, further studies are needed to determine the effect of vegetable diets in the distribution and composition of enzymes, other molecules of interest and leucocyte populations in the intestinal tract.

Normal metabolism and immune and detoxification systems generate reactive oxygen and nitrogen species that if not counterbalanced, lead to oxidative stress, host tissue damage and ageing. Oxidative stress is intimately linked to parasite resistance and fitness (Kurtz et al., 2006) and the removal of these free radicals is achieved through non-enzymatic and enzymatic reactions. The primary enzymatic antioxidant defence system is the glutathione redox system that reduces hydrogen peroxide and lipid hydroperoxides at the expense of oxidizing GSH to its disulfide form (GSSG). The antioxidant and anti-inflammatory effects of n-3 LC-PUFAs have been extensively

documented (Fang et al., 2002). Nevertheless, in the current study, 66VO-C fish not only had no oxidative/antioxidative imbalance, but also exhibited higher circulating antioxidant defences (TAS) than FO-C fish. This could be due, at least in part, to the presence of some substances, such as flavonoids, in VOs (Beecher 2003). Flavonoids are potent scavengers of free radicals such as hydroxyl and superoxide radicals, and also act as chelators of transient elements, having therefore biological effects against inflammatory and allergic disorders (Kim et al., 2004). Their presence in VOs could also explain the lower serum NO values than in FO fish, as they are also known to inhibit the production of NO (Pergola et al., 2006).

Parasite challenge increased reactive oxygen species derived for the immune response in both R fish, due to increased RB, as previously reported in different *Enteromyxum*-infected fish (Sitjà-Bobadilla et al., 2006; Alvarez-Pellitero et al., 2008; Sitjà-Bobadilla et al., 2008). However, TAS was significantly decreased only in 66VO-R animals with respect to 66VO-C group, as occurred in other host-parasite systems (Lightbody et al., 2001). This lower TAS of 66VO-R fish could endanger their health, since their hepatic GSH/GSSG ratio was also decreased, showing a higher risk of lipid peroxidation and oxidative damage. Similarly, in other intestinal inflammatory diseases induced by parasites, there is an imbalance in the oxidant/antioxidant defence mechanisms, which can increase the susceptibility of the intestine to inflammation (Sundaram et al., 2003).

In conclusion, this study has shown that 66VO replacement in gilthead sea bream diet is a predisposing cause that worsens the disease course when fish are experimentally exposed to *E. leei*, the precipitating cause. These results should be confirmed with other fish-pathogen models, as the effects of the diet may be beneficial or not, depending on the virulence factors of the pathogen and the site of infection. Further studies are underway to study the invasion strategy of this parasite and to determine which immune factors are key for controlling this disease.

All the experiments were carried out according to national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgements

This work was funded by EU (FOOD-CT-2006-16249; Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers, AQUAMAX) and the Spanish project AGL2006-13158-C03-01. Additional funding was obtained from the “Generalitat Valenciana” (research grant PROMETEO 2010/006). I. E. received a Spanish PhD fellowship (FPI) from the Spanish Ministry of Science and Innovation (MICINN). The authors thank V. Descalzo, C. Ferrer and M. A. González for assistance in fish maintenance and samplings, and J. Montfort and L. Rodríguez for histological processing.

References

- Alvarez-Pellitero, P., 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Vet. Immunol. Immunopathol.* 126, 171-198.
- Alvarez-Pellitero, P., Palenzuela, O., Sitjà-Bobadilla, A., 2008. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). *Parasitol. Int.* 57, 110-120.
- APROMAR. 2009. La acuicultura marina de peces en España. (www.apromar.es/informes.asp).

- 454 Argilés, J.M., Busquets, S., López-Soriano, F.J., 2006. Cytokines as mediators and targets for
455 cancer cachexia. *Canc. Treat.* 130, 199-217.
- 456 Bakke-McKellep, A.M., Penn, M.H., Salas, P.M., Refstie, S., Sperstad, S., Landsverk, T., Ringo,
457 E., Krogdahl, A., 2007. Effects of dietary soyabean meal, inulin and oxytetracycline on
458 intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost
459 Atlantic salmon (*Salmo salar* L.). *Brit. J. Nutr.* 97, 699-713.
- 460 Beecher, G.R., 2003. Overview of dietary flavonoids: Nomenclature, occurrence and intake. *J. Nutr.*
461 133, 3248s-3254s.
- 462 Bell, J.G., Ashton, I., Secombes, C.J., Weitzel, B.R., Dick, J.R., Sargent, J.R., 1996. Dietary lipid
463 affects phospholipid fatty acid compositions, eicosanoid production and immune function in
464 Atlantic salmon (*Salmo salar*). *Prostaglandins Leukot. Essent. Fatty Acids* 54, 173-182.
- 465 Benedito-Palos, L., Saera-Vila, A., Caldach-Giner, J.A., Kaushik, S., Pérez-Sánchez, J., 2007.
466 Combined replacement of fish meal and oil in practical diets for fast growing juveniles of
467 gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of
468 GH/IGF axis. *Aquaculture* 267, 199-212.
- 469 Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Bell, J.G., Kaushik, S., Pérez-Sánchez, J.,
470 2008. High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream
471 (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological
472 alterations of target tissues. *Brit. J. Nutr.* 100, 992-1003.
- 473 Benedito-Palos, L., Navarro, J.C., Bermejo-Nogales, A., Saera-Vila, A., Kaushik, S., Pérez-
474 Sánchez, J., 2009. The time course of fish oil wash-out follows a simple dilution model in
475 gilthead sea bream (*Sparus aurata* L.) fed graded levels of vegetable oils. *Aquaculture* 288,
476 98-105.
- 477 Bermúdez, R., Vigliano, F., Marcaccini, A., Sitjà-Bobadilla, A., Quiroga, M.I. and Nieto, J.M.,
478 2006. Response of Ig-positive cells to *Enteromyxum scophthalmi* (Myxozoa) experimental

- 479 infection in turbot, *Scophthalmus maximus* (L.): A histopathological and
480 immunohistochemical study. Fish & Shellfish Immunol. 21, 501-512.
- 481 Bermúdez, R., Losada, A.P, Vázquez, S., Redondo, M.J., Alvarez-Pellitero, P., Quiroga, M.I., 2010.
482 Light and electron microscopic studies on turbot *Psetta maxima* infected with *Enteromyxum*
483 *scophthalmi*: histopathology of turbot enteromyxosis. Dis. Aquat. Org. 89, 209-221.
- 484 Bransden, M.P., Carter, C.G., Nichols, P.D., 2003. Replacement of fish oil with sunflower oil in
485 feeds for Atlantic salmon (*Salmo salar* L.): effect on growth performance, tissue fatty acid
486 composition and disease resistance. Comp. Biochem. Phys. B 135, 611-625.
- 487 Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot. Essent.
488 Fatty Acids 77, 327-335.
- 489 Cerami, A., Beutler, B., 1988. The role of cachectin/TNF in endotoxic-shock and cachexia.
490 Immunol. Today 9, 28-31.
- 491 Carroll, J.A., 2008 Bidirectional communication: growth and immunity in domestic livestock. J.
492 Anim. Sci. 86, E126-E137.
- 493 Chapkin, R. S., Wang, N., Fan, Y. Y., Lupton, J. R., Prior, I. A., 2008. Docosahexaenoic acid alters
494 the size and distribution of cell surface microdomains. Biochim. Biophys. Acta 1778, 466-
495 471.
- 496 Colditz, I.G., 2008. Six costs of immunity to gastrointestinal nematode infections. Parasite
497 Immunol. 30, 63-70.
- 498 Costello, M.J., 2009. The global economic cost of sea lice to the salmonid farming industry. J. Fish
499 Dis. 32, 115-118.
- 500 Cuesta, A., Muñoz, P., Rodriguez, A., Salinas, I., Sitjà-Bobadilla, A., Alvarez-Pellitero, P., Esteban,
501 M.A., Meseguer, J., 2006. Gilthead sea bream (*Sparus aurata* L.) innate defence against the
502 parasite *Enteromyxum leei* (Myxozoa). Parasitology 132, 95-104.
- 503 De Pablo, M.A., De Cienfuegos, G.A., 2000. Modulatory effects of dietary lipids on immune
504 system functions. Immunol. Cell Biol. 78, 31-39.

- 505 Dezfuli, B.S., Giari, L., Arrighi, S., Domeneghini, C., Bosi, G., 2003. Influence of enteric helminths
506 on the distribution of intestinal endocrine cells belonging to the diffuse endocrine system in
507 brown trout, *Salmo trutta* L. J. Fish Dis. 26, 155-166.
- 508 Emery, P.W., 1999. Cachexia in experimental models. Nutrition 15, 600-603.
- 509 Erdal, J.I., Evensen, O., Kaurstad, O.K., Lillehaug, A., Solbakken, R., Thorud, K., 1991.
510 Relationship between diet and immune response in Atlantic salmon (*Salmo salar* L.) after
511 feeding various levels of ascorbic-acid and omega-3-fatty-acids. Aquaculture 98, 363-379.
- 512 Estensoro, I., Bermúdez, R., Losada, A.P., Quiroga, M.I., Pérez-Sánchez, J., Alvarez-Pellitero, P.,
513 Sitjà-Bobadilla, A., 2009. Effect of *Enteromyxum leei* (Myxozoa) on gastrointestinal
514 neuromodulators and cell apoptosis of gilthead sea bream (*Sparus aurata*). In: Diseases of
515 Fish and Shellfish, abstract book, 14th EAAP Int. Conf. Prague, Czech Republic, pp. 264,.
- 516 Fang, Y.Z., Yang, S., Wu, G.Y., 2002. Free radicals, antioxidants and nutrition. Nutrition 18, 872-
517 879.
- 518 Fekete, S.G., Kellems, R.O., 2007. Interrelationship of feeding with immunity and parasitic
519 infection: a review. Vet. Med. Czech. 52, 131-143.
- 520 Fisher, D.S., 2000. Defining the experimental unit in grazing trials. J. Anim. Sci. 77, 1E-5E.
- 521 Fleurance, R., Sauvegrain, C., Marques, A., Le Breton, A., Guereaud, C., Cherel, Y., Wyers, M.,
522 2008. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream
523 *Sparus aurata*. Dis. Aquat. Org. 79, 219-228.
- 524 Fracalossi, D.M., Lovell, R.T., 1994. Dietary lipid sources influence responses of channel catfish
525 (*Ictalurus punctatus*) to challenge with the pathogen *Edwardsiella ictaluri*. Aquaculture
526 119, 287-298.
- 527 Golomazou, E., Athanassopoulou, F., Karagouni, E., Tsagozis, P., Tsantilas, H., Vagianou, S.,
528 2006. Experimental transmission of *Enteromyxum leei* Diamant, Lom and Dyková, 1994 in
529 sharpsnout sea bream, *Diplodus puntazzo* C. and the effect on some innate immune
530 parameters. Aquaculture 260, 44-53.

- 531 Guo, F.C., Woo, P.T.K., 2009. Selected parasitosis in cultured and wild fish. Vet. Parasitol. 163,
532 207-216.
- 533 Ishimatsu, A., Hayashi, M., Nakane, M., Sameshima, M., 2007. Pathophysiology of cultured tiger
534 puffer *Takifugu rubripes* suffering from the myxosporean emaciation disease. Fish Pathol.
535 42, 211-217.
- 536 Karagouni, E., Athanassopoulou, F., Tsagozis, P., Ralli, E., Moustakareas, T., Lytra, K., Dotsika,
537 E., 2005. The impact of a successful anti-myxosporean treatment on the phagocyte functions
538 of juvenile and adult *Sparus aurata* L. Int. J. Immunopathol. Pharmacol. 18, 121-132.
- 539 Kasper, L.H., Buzoni-Gatel, D., 2001. Ups and downs of mucosal cellular immunity against
540 protozoan parasites. Infect. Immun. 69, 1-8.
- 541 Kim, H.P., Son, K.H., Chang, H.W., Kang, S.S., 2004. Anti-inflammatory plant flavonoids and
542 cellular action mechanisms. J. Pharmacol. Sci. 96, 229-245.
- 543 Kiron, V., Fukuda, H., Takeuchi, T., Watanabe, T., 1995. Essential fatty-acid nutrition and defense
544 mechanisms in rainbow trout *Oncorhynchus mykiss*. Comp. Biochem. Phys. A 111, 361-
545 367.
- 546 Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S., Watanabe, T., 2004. Antioxidant status and
547 nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of
548 vitamin E along with three lipid sources. Aquaculture 234, 361-379.
- 549 Kurtz, J., Wegner, K.M., Kalbe, M., Reusch, T.B.H., Schaschl, H., Hasselquist, D., Milinski, M.,
550 2006. MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach.
551 Proc. R. Soc. B 273, 1407-1414.
- 552 Kyriazakis, I., Tolkamp, B.J., Hutchings, M.R., 1998. Towards a functional explanation for the
553 occurrence of anorexia during parasitic infections. Anim. Behav. 56, 265-274.
- 554 Lightbody, J.H., Stevenson, L.M., Jackson, F., Donaldson, K., Jones, D.G., 2001. Comparative
555 aspects of plasma antioxidant status in sheep and goats, and the influence of experimental
556 abomasal nematode infection. J. Comp. Pathol. 124, 192-199.

- 557 Lin, Y.H., Shiau, S.Y., 2007. Effects of dietary blend of fish oil with corn oil on growth and non-
558 specific immune responses of grouper, *Epinephelus malabaricus*. Aquacult. Nutr. 13, 137-
559 144.
- 560 Mendieta-Zerón, H., López, M., Diéguez, C., 2008. Gastrointestinal peptides controlling body
561 weight homeostasis. Gen. Comp. Endocrinol. 155, 481-495.
- 562 Misra, S., Sahu, N.P., Pal, A.K., Xavier, B., Kumar, S., Mukherjee, S.C., 2006. Pre- and post-
563 challenge immuno-haematological changes in *Labeo rohita* juveniles fed gelatinised or non-
564 gelatinised carbohydrate with n-3 PUFA. Fish Shellfish Immunol. 21, 346-356.
- 565 Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M.J., Izquierdo, M.S.,
566 2003. Vegetable lipid sources for gilthead sea bream (*Sparus aurata*): effects on fish health.
567 Aquaculture 225, 353-370.
- 568 Mourente, G., Good, J.E., Bell, J.G., 2005. Partial substitution of fish oil with rapeseed, linseed and
569 olive oils in diets for European sea bass (*Dicentrarchus labrax* L.): effects on flesh fatty acid
570 composition, plasma prostaglandins and E₂ F_{2a}, immune function and effectiveness of a fish
571 oil finishing diet. Aquacult. Nutr. 11, 25-40.
- 572 Olsen, R.E., Myklebust, R., Ringø, E., Mayhew, T.M., 2000. The influences of dietary linseed oil
573 and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): a
574 quantitative ultrastructural study. Fish Physiol. Biochem. 22, 207-216.
- 575 Ots, I., Kerimov, A.B., Ivankina, E.V., Ilyina, T.A., Horak, P., 2001. Immune challenge affects
576 basal metabolic activity in wintering great tits. Proc. R. Soc. B 268, 1175-1181.
- 577 Palenzuela, O., 2006. Myxozoan infections in Mediterranean mariculture. Parassitologia 48, 27-29.
- 578 Palenzuela, O., Bartholomew, J.L., 2002. Molecular tools for the diagnosis of *Ceratomyxa shasta*
579 (Myxozoa). In: Cunningham, C.O., (Ed.), Molecular Diagnosis of Salmonid Diseases, The
580 Netherlands: Kluwer Academic Publishers., pp. 285-98.

- 581 Pergola, C., Rossi, A., Dugo, P., Cuzzocrea, S., Sautebin, L., 2006. Inhibition of nitric oxide
582 biosynthesis by anthocyanin fraction of blackberry extract. Nitric Oxide-Biol. Chem. 15, 30-
583 39.
- 584 Saera-Vila, A., Benedito-Palos, L., Sitjà-Bobadilla, A., Nácher-Mestre, J., Serrano, R., Kaushik, S.,
585 Pérez-Sánchez, J., 2009. Assessment of the health and antioxidant trade-off in gilthead sea
586 bream (*Sparus aurata* L.) fed alternative diets with low levels of contaminants. Aquaculture
587 296, 87-95.
- 588 Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune
589 system. Aquacult. Res. 39, 223-239.
- 590 Sawai, T., Drongowski, R.A., Lampman, R.W., Coran, A.G., Harmon, C.M., 2001. The effect of
591 phospholipids and fatty acids on tight-junction permeability and bacterial translocation.
592 Pediatr. Surg. Int. 17, 269-274.
- 593 Schroers, V., Van der Marel, M., Steinhagen, D., 2008. Influence of carp intestinal mucus
594 molecular size and glycosylation on bacterial adhesion. Dis Aquat. Org. 81, 135-142.
- 595 Sitjà-Bobadilla, A., 2004. Parasites in Mediterranean aquacultured fish: Current impact and future
596 research directions. In: Mas-Coma, S., (Ed.), Multidisciplinary for Parasites, Vectors and
597 Parasitic Diseases, Bologna, Italy: Medimond. pp. 301-311.
- 598 Sitjà-Bobadilla, A., 2009. Can Myxosporean parasites compromise fish and amphibian
599 reproduction? Proc. R. Soc. B 276, 2861-2870.
- 600 Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez,
601 J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence
602 mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249,
603 387-400.
- 604 Sitjà-Bobadilla, A., Redondo, M.J., Bermudez, R., Palenzuela, O., Ferreiro, I., Riaza, A., Quiroga,
605 I., Nieto, J.M., Alvarez-Pellitero, P., 2006. Innate and adaptive immune responses of turbot,

- 606 *Scophthalmus maximus* (L.), following experimental infection with *Enteromyxum*
 607 *scophthalmi* (Myxosporea: Myxozoa). Fish Shellfish Immunol. 21, 485-500.
- 608 Sitjà-Bobadilla, A., Diamant, A., Palenzuela, O., Alvarez-Pellitero, P., 2007. Effect of host factors
 609 and experimental conditions on the horizontal transmission of *Enteromyxum leei* (Myxozoa)
 610 to gilthead sea bream, *Sparus aurata* L. and European sea bass, *Dicentrarchus labrax* (L.).
 611 J. Fish Dis. 30, 243-250.
- 612 Sitjà-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Alvarez-Pellitero, P., Pérez-
 613 Sánchez, J., 2008. Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa:
 614 Myxosporea) modulates the immune response and the expression of growth, redox and
 615 immune relevant genes in gilthead sea bream, *Sparus aurata* L. Fish Shellfish Immunol. 24,
 616 610-619.
- 617 Sundaram, U., Hassanain, H., Suntres, Z., Yu, J.G., Cooke, H.J., Guzman, J., Christofi, F.L., 2003.
 618 Rabbit chronic ileitis leads to up-regulation of adenosine A1/A3 gene products, oxidative
 619 stress, and immune modulation. Biochem. Pharmacol. 65, 1529-1538.
- 620 Tacon, A.G. J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in
 621 industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285, 146-158.
- 622 Thomas, P.T., Woo, P.T.K., 1992. Anorexia in rainbow trout, *Oncorhynchus mykiss* (Walbaum),
 623 infected with *Cryptobia salmositica* (Sarcomastigophora: Kinetoplastida): its onset and
 624 contribution to the immunodepression. J. Fish Dis. 15, 443-447.
- 625 Thompson, K.D., Tatner, M.F., Henderson, R.J., 1996. Effects of dietary (n-3) and (n-6)
 626 polyunsaturated fatty acid ratio on the immune response of Atlantic salmon, *Salmo salar* L.
 627 Aquacult. Nutr. 2, 21-31.
- 628 Vargas, R.J., De Souza, S.N.G., Mabilia, R.G., Carlet, F., Baggio, S.R., 2008. Physiological
 629 response of experimental challenge of *Ichthyophthirius multifiliis* (Fouquet, 1876) in jundia
 630 (*Rhamdia quelen* Quoy and Gaimard, 1824) fingerlings rather fed with different lipid
 631 sources. Rev. Bras. Parasitol. Vet. 17, 81-86.

632 Yoshino, T.P., Boyle, J.P., Humphries, J.E., 2001. Receptor-ligand interactions and cellular
633 signalling at the host-parasite interface. *Parasitology* 123, S143-S157.

634

635

636

Accepted Manuscript

Legends to figures

Fig. 1. Infection levels of gilthead sea bream fed two different diets (66VO and FO) and exposed to *Enteromyxum leei* for 102 days. (A) Prevalence of infection at the different times post-exposure (p.e.). (B) Mean intensity of infection at day 102 p.e. in the different time-classes of fish according to the initial acquisition of the infection. There was a linear relationship between the two variables in the FO group ($r^2 = 0.953$, $P = 0.0238$) and an exponential progression in the 66VO group ($r^2 = 0.9169$, $P = 0.0424$). (C) Percentage of infected fish harbouring the parasite in each intestinal portion at day 102 p.e.

Fig. 2. Biometrical data of gilthead sea bream fed two different diets (66VO and FO) and not exposed (C) or exposed (R) to *Enteromyxum leei* for 102 days. (A) Progression of body weight along the experimental period. Different letters stand for statistically significant differences among the groups at each sampling time ($P < 0.05$). (B) Specific growth rate (SGR) at the end of the experimental period of C and R fish classified in the different time-classes according to the initial acquisition of the infection. Note that some R fish were never infected. Different capital letters stand for statistically significant differences among 66VO fish, and low case letters for FO fish. * indicates significant differences between 66VO and FO groups within a time-class. ($P < 0.05$).

Fig. 3. Diagrammatic representation of the main correlations found between the intensity of infection by *Enteromyxum leei* at the posterior intestine and the different biometrical, immunological and antioxidant factors (see the text for abbreviations) in the two gilthead sea bream diet groups (66VO and FO). Red and green boxes stand for negative and positive correlations, respectively, and empty boxes for no correlation ($P < 0.05$). The corresponding Spearman's correlation coefficients can be found in Tables S2 and S3 of supplementary electronic material.

SUPPLEMENTARY ELECTRONIC MATERIAL

Table S1. Ingredients and chemical composition of experimental diets.

Table S2. Spearman correlation coefficients resulting from the meta-analysis of all the measured variables in FO fish. Those coefficients of variables conceptually related have been omitted. The pair of variables with positive correlation coefficients (in green) tends to increase together, whereas negative correlation coefficients (in red) indicate that one variable tends to decrease while the other increases. To facilitate visualization significant *P* values (< 0.05) have been highlighted in yellow. *E. izei* = intensity of infection at the posterior intestine; days = number of days of establishment of the infection; H = hepatic; Int = intestinal. For the remaining abbreviations see the text.

Table S3. Spearman correlation coefficients resulting from the meta-analysis of all the measured variables in 66VO fish. Those coefficients of variables conceptually related have been omitted. The pair of variables with positive correlation coefficients (in green) tends to increase together, whereas negative correlation coefficients (in red) indicate that one variable tends to decrease while the other increases. To facilitate visualization significant *P* values (< 0.05) have been highlighted in yellow. *E. izei* = intensity of infection at the posterior intestine; days = number of days of establishment of the infection; H = hepatic; Int = intestinal. For the remaining abbreviations see the text.

Table 1. Biometric, antioxidant, redox and immunological values (mean $\pm$ SEM) of gilthead sea bream fed the two different diets (66VO, FO) and exposed for 102 days to *Enteromyxum leei* (R) or not (C). Different superscript letters stand for statistical differences between groups for each parameter ($P < 0.05$). *Total value accumulated during the experimental period. See abbreviations in the text.

	66VO-C	66VO-R	FO-C	FO-R
Final length (cm)	23.6 $\pm$ 0.21 ^a	22.5 $\pm$ 0.16 ^b	23.6 $\pm$ 0.17 ^a	22.9 $\pm$ 0.19 ^b
Weight gain (g/fish)	134.7 $\pm$ 4.03 ^a	61.7 $\pm$ 6.76 ^c	127.4 $\pm$ 3.48 ^a	89.9 $\pm$ 6.8 ^b
Feed intake (g/fish)*	180.2	140.3	177.1	149.6
SGR (%)	0.47 $\pm$ 0.015 ^a	0.25 $\pm$ 0.027 ^c	0.47 $\pm$ 0.015 ^a	0.36 $\pm$ 0.023 ^b
CF	2.69 $\pm$ 0.048 ^a	2.52 $\pm$ 0.035 ^b	2.69 $\pm$ 0.048 ^a	2.58 $\pm$ 0.028 ^b
HSI	0.920 $\pm$ 0.053	0.846 $\pm$ 0.049	0.961 $\pm$ 0.032	0.901 $\pm$ 0.029
SSI	0.102 $\pm$ 0.129	0.088 $\pm$ 0.005	0.119 $\pm$ 0.011	0.114 $\pm$ 0.012
Hc (%)	46.33 $\pm$ 1.64 ^a	33.96 $\pm$ 1.79 ^c	41.46 $\pm$ 0.97 ^{ab}	40.58 $\pm$ 1.77 ^b
RB (I. RLU)	9905.0 $\pm$ 3345.6 ^a	30333.7 $\pm$ 6020.2 ^b	10717.9 $\pm$ 1796.4 ^a	20617.2 $\pm$ 3633.8 ^b
PO (OD _{450 nm})	0.324 $\pm$ 0.0669 ^{ab}	0.208 $\pm$ 0.0254 ^b	0.361 $\pm$ 0.0337 ^a	0.293 $\pm$ 0.0358 ^{ab}
LY (units/ml)	242.82 $\pm$ 66.48 ^b	220.59 $\pm$ 10.40 ^{ab}	306.82 $\pm$ 14.75 ^c	200.56 $\pm$ 11.33 ^a
ACH ₅₀	130.36 $\pm$ 19.21 ^a	20.55 $\pm$ 1.86 ^b	42.08 $\pm$ 17.77 ^b	26.48 $\pm$ 4.36 ^b
NO (μ M)	4.16 $\pm$ 0.87 ^a	3.36 $\pm$ 0.38 ^a	8.12 $\pm$ 0.31 ^b	3.78 $\pm$ 0.89 ^a
TAS (mM Trolox)	0.467 $\pm$ 0.025 ^a	0.370 $\pm$ 0.009 ^b	0.384 $\pm$ 0.017 ^b	0.370 $\pm$ 0.009 ^b
Liver:				
tGSx (nmol/g)	3079.6 $\pm$ 178.6 ^{ab}	2179.2 $\pm$ 293.7 ^a	2841.7 $\pm$ 178.6 ^{ab}	3306.4 $\pm$ 306.7 ^b
GSSG (nmol/g)	137.9 $\pm$ 7.6	143.6 $\pm$ 9.7	156.0 $\pm$ 10.8	135.0 $\pm$ 9.4
GSH/GSSG	20.5 $\pm$ 1.7 ^a	13.6 $\pm$ 1.6 ^b	17.4 $\pm$ 2.4 ^{ab}	23.1 $\pm$ 2.1 ^a
Intestine:				
tGSx (nmol/g)	1894.9 $\pm$ 160.6	1874.2 $\pm$ 103.1	2076.1 $\pm$ 101.3	1914.6 $\pm$ 86.1
GSSG (nmol/g)	106.7 $\pm$ 18.4	93.6 $\pm$ 16.3	95.8 $\pm$ 22.6	102.4 $\pm$ 13.1
GSH/GSSG	23.5 $\pm$ 5.9	73.1 $\pm$ 24.9	39.2 $\pm$ 14.2	34.2 $\pm$ 10.9

Figure 3

Fig. 3