Effects of temperature decrease on feeding rates, immune indicators and histopathological changes of gilthead sea bream *Sparus aurata* fed with an experimental diet

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Abstract

Gilthead sea bream *Sparus aurata* were fed with either a commercial feed or a specifically prepared “winter” feed in order to assess whether fish showed signs similar to the so-called winter syndrome and whether the experimental diet showed protection against the syndrome. Fish were subjected to a ramp of temperature decrease and after 2 weeks, a further recovery of temperature. The temperature ramp produced changes on most immune indicators and feed intake was affected more severely than predicted by feeding tables. However, no generalised signs of the syndrome were detected. The experimental diet tested showed some degree of immune protection related to complement and phagocytosis activity and slight changes in the levels of leucocytic infiltration of the intestinal mucosa and submucosa.

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Keywords: Winter syndrome; Immune indicators; Histopathology; Temperature
1. Introduction

Sea bream culture has expanded in the Mediterranean area, especially in countries such as Spain, Greece and Italy. However, noticeable mortalities usually referred as “winter disease” or “winter syndrome” are recurrently recorded at specific periods in winter. Fish affected by this particular problem are characterised by altered swimming behaviour, abdominal distension and oedematous intestine containing transparent or yellowish fluid with mucous casts. Low but persistent mortalities are usually recorded, but in some cases, higher morbidity and peaks of heavier mortalities have also been noticed. Depending on the geographic area and the site of the farms, one of the two forms of the syndrome is predominant. Thus, in northern Mediterranean (northern Spain and Italy, Croatia), the disease is more severe during the coldest periods of winter (also called first winter), whereas in other parts such as south of Spain and south Greece and Portugal, the winter disease appears at the onset of spring or end of winter (also called second winter). In these cases, *Pseudomonas anguilliseptica* is frequently isolated. Other authors (Padró’s et al., 1996; Sarusic, 1999) have also noticed different periods in the development of the syndrome. Fish farmers observe the following in the first winter: (a) mortalities appeared mostly at temperatures below 11–12 °C; (b) if fish were disturbed or handled during this period, the mortality increased; and (c) when the syndrome appeared, affected fish stopped feeding and heavily affected fish started to swim side-up and shows a decrease of reactivity. Some of these observations were coincident with the data previously reported (Bovo et al., 1995; Le Breton, 1996; Bilei et al., 1996; Doimi, 1996; Bossu et al., 1997; Galeotti et al., 1998; Tort et al., 1998b).

Laboratory analysis on affected fish provided further data which described immunological suppression, physiological disturbances, histopathological alterations and microbiological implications. Thus, reductions in most immune indicators (30–50% with respect to normal values) and histopathological alterations in pancreatic, intestinal, muscular and hepatic tissues (Padró’s et al., 1996; Galeotti et al., 1998; Tort et al., 1998b) were described. The presence of certain pathogens, in particular *P. anguilliseptica*, has been reported in some occasions (Berthe et al., 1995; Domenech et al., 1997, 1999) but there is no evidence that this microorganism would be the only causative agent and, therefore, such bacteria act as opportunistic pathogens under specific conditions. In addition, several trials failed in reproducing the disease in laboratory conditions (personal observations). When looking at the energetic and metabolic status, affected fish showed a severe reduction in performance together with the sudden arrest of feeding and haematocrit decrease, and indications of cell dysfunction such as heavy decreases of membrane ATPase activity (Hernández et al., unpublished results; Tort et al., 1998a,b).

The present work was designed to test for a protective effect of an adapted diet that could counteract some of the consequences of the syndrome and help to prevent any possible nutritional causes of the winter syndrome by providing a supplementary dosage of vitamins (vitamins C and E, choline and inositol) and trace minerals to assist the immune system, a high palatability to maximize feed uptake during the low temperature period, a high nutrient density to maximize nutrient intake at lower feeding rate, a high digestible protein and fat, and high levels of highly unsaturated fatty acids and phospholipids. This feed was tested in an experimental temperature ramp.
2. Material and methods

2.1. Experimental groups and laboratory control

Fish were held in duplicate control and experimental tanks, i.e. fish fed with the respective control (C) and winter (W) feed (see composition in Table 1). They were fed to satiation during 2 weeks at 18 °C, prior to the experimental temperature change. After 2 weeks, a programmed decrease of temperature (1 °C per day) was performed reaching 11 °C. The temperature was maintained 13 days and then raised again up to 18 °C. The four sets of samples (see Table 2) were taken after the acclimation period before temperature change (set 1), immediately after the decrease of temperature (set 2), 2 weeks after the temperature reached 11 °C (set 3) and immediately after the final rise to 18 °C (set 4). This gave eight experimental groups, C1–C4 and W1–W4. The photoperiod was set as 11L/13D and decreased progressively to 10L/14D at the end of the experiment, thus simulating natural photoperiod.

Water and tank conditions were monitored during the experiment. The parameters of oxygen, salinity, pH and temperature were controlled daily. Flow rate, ammonia, nitrite and nitrate concentrations were monitored twice a week. Food intake was controlled daily for each tank.

2.2. Fish and blood sampling

After transport, fish were placed in the experimental tanks and left to recover for 2 days, and after that time they started feeding. A previous health control in these fish indicated

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis and vitamin specifications of a standard commercial ongrowing feed and an experimental winter diet for sea bream <em>S. aurata</em></td>
</tr>
<tr>
<td><strong>Analysis (% of the product)</strong></td>
</tr>
<tr>
<td>Moisture</td>
</tr>
<tr>
<td>Crude protein</td>
</tr>
<tr>
<td>Total lipid (after hydrolysis)</td>
</tr>
<tr>
<td>Total lipid (Folch)</td>
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<tr>
<td>Total polar lipids (column chromatography)</td>
</tr>
<tr>
<td>Ash</td>
</tr>
<tr>
<td>Starch</td>
</tr>
<tr>
<td>Fibre</td>
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<tr>
<td>Fatty acid composition of total lipids (mg/g DW)</td>
</tr>
<tr>
<td>EPA</td>
</tr>
<tr>
<td>DHA</td>
</tr>
<tr>
<td>Sum n-3 HUFA</td>
</tr>
<tr>
<td>Vitamin specifications</td>
</tr>
<tr>
<td>Vit A (IU/g)</td>
</tr>
<tr>
<td>Vit D (IU/g)</td>
</tr>
<tr>
<td>Vit E (mg/g)</td>
</tr>
<tr>
<td>Vit C (mg/g)</td>
</tr>
</tbody>
</table>
Table 2
Mean and standard errors for the immune and metabolic variables analysed in the four samples (S1–4) in control and winter feed groups

<table>
<thead>
<tr>
<th>Variable (temperature)</th>
<th>S1 (18 °C)</th>
<th>S2 (11 °C)</th>
<th>S3 (11 °C)</th>
<th>S4 (18 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme, kunits/ml</td>
<td>59.35 ± 17.2 A</td>
<td>47.86 ± 16.9 a</td>
<td>36.37 ± 12.1 A</td>
<td>20.82 ± 6.1 b</td>
</tr>
<tr>
<td>NBT, mmol rNBT</td>
<td>0.43 ± 0.03 A</td>
<td>0.46 ± 0.04 a</td>
<td>0.42 ± 0.02 A</td>
<td>0.51 ± 0.02*b</td>
</tr>
<tr>
<td>Lymphocytes, × 10^6 cells/ml</td>
<td>36.8 ± 4.1 A</td>
<td>38.8 ± 3.6 a</td>
<td>28.7 ± 2.5 B</td>
<td>29.0 ± 2.7 b</td>
</tr>
<tr>
<td>Complement, ach50 unit/ml</td>
<td>6.0 ± 0.29 A</td>
<td>5.6 ± 0.18 a</td>
<td>5.5 ± 0.2 A</td>
<td>5.6 ± 0.1 a</td>
</tr>
<tr>
<td>Osmolality, mosM/l</td>
<td>385 ± 3.2 A</td>
<td>400 ± 9.0 a</td>
<td>388 ± 2.7 A</td>
<td>399 ± 1.9*a</td>
</tr>
<tr>
<td>Glucose, g/dl</td>
<td>63.1 ± 4.58 A</td>
<td>63.1 ± 5.03 a</td>
<td>52.5 ± 2.49 A</td>
<td>39.8 ± 1.68 b</td>
</tr>
</tbody>
</table>

Different letters mean significant difference ($p < 0.05$) among controls (A, B, C) or winter feed (a, b, c) groups.

* Significant difference ($p < 0.05$) compared to the respective control group.
the presence of a mild infection by epitheliocystis and monogenan parasites, a usual finding in cultured sea bream. Due to the low level of the infection and in order to emulate the same health conditions of the fish in the farm, no previous bacterial or parasite treatment was carried out. The fish density along the experiment was 6.8 kg/m³ and water volume was adjusted accordingly. A total of 64 fish (85.66 ± 18.00) were sampled in this experiment. In order to sample the blood, fish were quickly caught, placed in anaesthetic (0.1 ml/l 2-phenoxyethanol) and sampled through caudal puncture with a syringe. Fresh blood was placed in heparinized tubes for NBT determination and lymphocyte counting. The rest of the blood was left to clot at 4 °C for 2 h, the clot removed after centrifugation, and the serum aliquoted and deep-frozen for lysozyme, complement, agglutination, glucose and osmolality.

2.3. Histopathological analysis and analytical determinations

After blood sampling, fish were killed immediately by a sharp cut on the head and processed for histopathology. Samples of gills, liver, digestive tract, kidney, spleen and musculature (including red and white muscle) were fixed in 10% phosphate-buffered formalin, and sections stained with haematoxylin and eosin.

Lymphocyte numbers were determined by direct counting under the microscope using a Neubauer chamber. Whereas granulocytes and monocytes can easily be differentiated, circulating lymphocytes of sea bream can be distinguished from erythrocytes and thrombocytes by means of a suspension based in methylene blue. Haemagglutination assays were performed as in Tort et al. (1998a). Haemolytic assays for the determination of the activity of alternative complement pathway were performed following the technique described by Sunyer and Tort (1995). The results are expressed in ACH50 units, as the titre at which 50% of the haemolysis is produced. Lysozyme activity assays were performed by a turbidimetric method that uses the lysis of Micrococcus luteus for determination of the enzymatic activity (Rotllant et al., 1997), using egg-white lysozyme as standard. The phagocytic activity was measured by the oxygen radical production which reduce nitroblue tetrazolium. Glucose levels in plasma were measured by means of a commercial kit based in the glucose-oxidase enzyme activity (Boehringer-Mannheim) and osmolality was measured directly in a freezing-point osmometer (Osmomat).

2.4. Statistics

Student’s t-test was used to check differences between control and winter fed groups for each sample. Regarding differences among either control or winter fed fish from the different samples, a one-way analysis of variance was used followed by a posteriori SNK test. In all cases, 0.05 was used as the level for accepted significance.

3. Results

No significant differences in any of the water quality parameters were recorded during the experiment and all of them were considered normal. Fig. 1 plots the food intake by fish
in tanks fed with control (C) and winter feed (W). These values were calculated as percentages of the expected feed intake given by the standard tables for sea bream at each temperature in commercial farms. Values for body weight gain did not show significant differences among all groups tested and ranged between 3.8 and 4.4 g. No significant differences were found regarding the type of food given but a noticeable decrease in food intake at low temperatures was observed, which was lower than the expected values from the feeding tables stated for the corresponding temperatures. No complete recovery of food intake was achieved during the temperature recovery period tested (C4 and W4),

![Graph showing percentage of food intake with respect to commercial tables.](image)

**Fig. 1.** Percentage of food intake with respect to commercial tables, which were considered as 100%, during the experimental time (x-axis). Closed circles are the mean of control fed groups. Open circles are the mean of winter fed groups. Triangles show the temperature values.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sample 1 (18 °C)</th>
<th>Sample 2 (11 °C)</th>
<th>Sample 3 (11 °C)</th>
<th>Sample 4 (18 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>W</td>
<td>C</td>
<td>W</td>
</tr>
<tr>
<td>Muscle</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Digestive tract</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Pancreas</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

Table 3
Histopathological features in control and winter fed fish

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sample 1 (18 °C)</th>
<th>Sample 2 (11 °C)</th>
<th>Sample 3 (11 °C)</th>
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<td></td>
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<td>–</td>
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<td>Pancreas</td>
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</table>

No lesions related to winter syndrome were found in muscle, digestive tract and pancreas. Levels of leucocytic infiltration in the digestive tract are qualified as not relevant (–), low (+), moderate (+) and intense (+++).
indicating that temperature drop also produced a delay in the complete recovery of the theoretically calculated intake. Table 2 shows the results of the two experimental groups and the four sampling times. Results on the immune indicators showed significant increases ($p < 0.05$) in phagocytic activity in W fish in samples 2 and 4 (asterisks) compared to the respective controls. Regarding the effect of temperature, results show significant changes after temperature drop (shown by different letters). Among the variables shown in Table 2, lysozyme values show the biggest changes at lower temperatures (samples 2 and 3) and recovery (sample 4). Lymphocytes show changes at sample 2

Fig. 2. Intestinal fold. S3, winter feed. Notice the light lymphocytic infiltration mainly in the epithelium. Scale bar: 100 μm.

Fig. 3. Intestinal fold with an intense lymphocytic infiltration in the epithelium and also in the intestinal submucosa.
and a recovery at sample 3, but a later decrease at sample 4. NBT show the latest dynamics with reductions at sample 4. Osmolality and glucose showed changes also after sample 2 and no changes in later samples.

No macroscopic lesions related to winter syndrome were observed in any of the samples (both control and winter feed). After histopathological examination, some fish in samples 3 and 4 that showed an unspecific slight mucous hyperplasia in the intestinal mucosa, both in control and winter fed fish (Table 3). One particular condition was observed from histological observations during the experience: A progressive increase in the infiltration of the intestinal mucosa, lamina propria and submucosa from leucocytic cells was noticed in the later groups (samples 3 and 4) and with some differences between “C” and “W” feed groups (the level of infiltration was tentatively qualified as (+) low (Fig. 2), (+) moderate and (++) intense (Fig. 3).

4. Discussion

The effects of the specific “W” feed on the fish performance did not show conclusive results, since the temperature change clearly affected both groups. Nevertheless, it appeared that fish fed with W feed showed some indications of better performance after temperature change. Thus, phagocytic activity measured by NBT showed higher levels in the W group after both decrease and recovery of temperatures and complement showed higher but not significant mean levels after temperature changes. Since the positive or negative effects of a particular diet formulation depend on multiple factors, it would be difficult to assess which component or components could be the responsible for the positive effects. Nevertheless, it has been shown that higher levels of vitamins E and C are favourable for the nonspecific immune defence in sea bream (Montero et al., 1999; Ortuño et al., 1999, 2001) and a similar conclusion was drawn by Kiron et al. (1995) in trout regarding the levels and quality of protein. In addition, positive effects of vitamins, as membrane-protecting agents, have been described since they may give more stability to the lipid bilayer (Hazel, 1995).

It must be noted that fish subjected to the temperature ramp showed a more severe reduction of feed intake than predicted in the feeding tables (Fig. 1), though available tables are based on daily feeding rates at a stable and narrow ranges of temperatures (Petridis and Rogdakis, 1996). The results also suggest that once the fish reach the novel stable temperature, the feeding behaviour is re-established and the expected intake is recovered though after certain delay.

Temperature has an effect on a number of the physiological and immune parameters analysed, i.e. plasma glucose osmolality, the NBT, lymphocyte number and lysozyme. Notably, lysozyme activity appears to be more affected after 1 week at low temperature. Nevertheless, the relevant comparisons between control and winter feed groups for each sample showed changes only in NBT and glucose. The significant changes observed in the immune parameters analysed were limited to lysozyme and phagocytic index. Complement and NBT showed transient changes. The dynamics found suggest a better performance of W fed fish in phagocytic activity when fish are facing the stressful stimuli of temperature shock (fall or rise) and less
difference during more stable thermal periods. It could be possible that the W feed was favourable to macrophage function rather than lymphocyte function and this can be related to the reactivity of intestinal mucosa to W feed. Thus, such a reactivity is coincident with better levels of macrophage activity in W feed groups. In addition, a decrease of the lymphocyte number is also observed, as found previously in similar situations (Tort et al., 1998a). Therefore, a lower degree of cell mitosis and proliferation could be induced, but it is also possible that this apparent decrease might be associated to a progressive cell migration. Histological results suggest a possible lymphocyte mobilisation from the blood to the intestinal submucosa that could result in a progressive increase in the inflammatory infiltration (groups 3 and 4). Previous evidence suggests the existence of a gut-associated lymphoid tissue (GALT) in teleosts with similar functions to the GALT of ectothermic vertebrates (Zapata and Cooper, 1990). The changes found in the number of inflammatory cells in the gut of fish fed with W feed could be associated to a certain activation of the GALT of these fish. Nevertheless, this hypothesis should be confirmed by further studies. No other specific signs of a severe winter syndrome were clearly generated, except for mild mucous cell hyperplasia recorded after temperature drop, which could be interpreted as a preliminary stage of the syndrome. As no lesions associated to virus, bacteria or parasites were observed, the possibility of a inflammatory local response induced by an specific pathological condition can be discounted. Regarding parasites, the reduced extension of lesions observed should not significantly affect the results obtained. Otherwise, gill lesions associated to the presence of monogenean parasites and epitheliocystis are currently found in sea bream fish farms (Papoutsoglou et al., 1996; Crespo et al., 1999).

In conclusion, the present results show that experimental temperature drop induces a number of changes in several physiological compartments that can also be observed in the fish affected by the winter syndrome, and also confirm that sea bream is a poikylothermic species that shows difficulties in adapting to low temperature gradients. Although the experimental setup is a laboratory trial without all the particular conditions of field aquaculture, indications that sea bream may be adaptable to low but stable temperatures, and less adaptable to decreasing temperature changes, may be of interest for fish farmers. The results also stress the importance of the feeding management when episodes of temperature drop occur, as it affects feeding behaviour in terms of reducing feed intake to lower levels than those expected following food charts and delaying recovery. Results also indicate that higher quality feed may help to counteract or reduce some of the effects generated in the syndrome. Therefore, additional work should be carried out regarding the eventual effects of such feeds delivered during longer periods, especially during the period previous to water temperature drops.

Acknowledgements

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