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Strong effect of long-term *Sparicotyle chrysophrii* infection on the cellular and innate immune responses of gilthead sea bream, *Sparus aurata*

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ABSTRACT

One thousand healthy recipient gilthead sea bream, *Sparus aurata*, cohabited with 250 donor fish parasitized by *Sparicotyle chrysophrii* (Van Beneden and Hesse, 1963) (Monogenea: Polyopisthocotylea), a common parasite of the gills of this fish species. Controls consisted of 1000 healthy fish kept in a separate tank. After 10 weeks, fish were weighed and parasite load, hemoglobin concentration and immunological parameters were assessed. Rather than the absence of parasite, hemoglobin concentration was a better marker of the health status of the fish, because *S. chrysophrii* had detached from the strongly anemic gills of some animals leaving fish with affected immune system but without parasites. The parasite infection seemed to trigger a cellular response of the fish immune system but to inhibit its humoral components. Thus, parasitized fish may control the parasite infection through the action of reactive oxygen species but they may become more sensitive to potential secondary bacterial or parasitical infections. This phenomenon was demonstrated not only through significant differences between recipient and control fish but also through strong correlations between those parameters and parasite load, fish weight and/or hemoglobin concentration.

1. Introduction

The intensification of the production of gilthead sea bream, *Sparus aurata*, in the Mediterranean sea has been accompanied by an increased occurrence of parasitic diseases in sea cages (Sitjà-Bobadilla, 2004) especially those caused by parasites with direct life cycles such as monogeneans (Nowak, 2007). In the last decades, the monogenean parasite *Sparicotyle* (previously named *Microcotyle*) *chrysophrii* (Van Beneden and Hesse, 1863) infection, favored by high fish densities and net biofouling, has been a real burden for fish farmers (Álvarez-Pellitero, 2004; Athanassopoulou et al., 2005; Sanz, 1992; Sitjà-Bobadilla and Alvarez-Pellitero, 2009). Therapeutic methods involving the removal of infected nets and the bath-treatments of infected fish are both time-consuming and labor-intensive (Sitjà-Bobadilla et al., 2006a). A recent publication suggested the use of dietary caprylic acid to alleviate the intensity of *S. chrysophrii* infection (Rigos et al., 2013) but no preventive or therapeutic agent has been successful so far in eradicating totally this parasite. Although mortalities are limited (10%) (Athanassopoulou et al., 2005; Faisal and Iman, 1990; Vagianou et al., 2004), *S. chrysophrii* infections provoke lethargy, histopathological damage, severe anemia and, as a result, it strongly impairs fish growth, thus reducing marketability of the fish (Sitjà-Bobadilla et al., 2006a).

The recent success in the transmission of *S. chrysophrii* in control experimental environment (Rigos et al., 2015; Sitjà-Bobadilla and Alvarez-Pellitero, 2009) has enabled the study of this economically important host–parasite model. A pattern of initial increase and then decline in *S. chrysophrii* infection of gilthead sea bream has been suggested to be due to the immune response and the development of resistance of the host (Sitjà-Bobadilla and Alvarez-Pellitero, 2009). Indeed, the interaction between the host immune system and the direct blood-feeding ectoparasite is an important and complex matter because the parasites need healthy fish gills to feed on but they also need to fight and/or evade the fish immune system. To survive, parasites may immune-suppress their host that may become more sensitive to secondary bacterial, viral or parasitic infections (Cusack and Cone, 1986; Sitjà-Bobadilla et al., 2006b). Most information are available on the fish response to infection deals with bacteria and virus (Álvarez-Pellitero, 2008). Recent reviews are available on the effect of parasites (ciliates, copepods and myxozoa) on the immune system of fish (Álvarez-Pellitero et al., 2008; Dickerson and Findly, 2014; Fast, 2014; Piazzon et al., 2013; Sitjà-Bobadilla

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et al., 2008) but the effects of monogeneans have been scarcely studied (Buchmann and Lindenstrøm, 2002) and no study has been done on the effect of *S. chrysophrii* on the fish immunity despite the importance of this infectious agent.

The aim of the present study was therefore to assess the effect of a *S. chrysophrii* infection, experimentally-induced by cohabitation with infected fish, on the immune system of gilthead sea bream in order to better understand how the parasite affects its host and the potential consequences it may have on the fish health.

2. Materials and methods

2.1. Fish and experimental design

Healthy gilthead sea bream were grown in land based tanks in the facilities of Andromeda (western Greece) to avoid any contact with sea cages where *S. chrysophrii* infections occur. Two thousand of these fish (16 ± 4 g) were transferred to the facilities of the Hellenic Centre for Marine Research in Athens (Greece) and were evenly distributed in two 6 m^3 nets stretched in 2 large cement tanks of 50 m^3. The physiologically red gills of 20 of these fish were examined under a stereoscope to ascertain the absence of *S. chrysophrii*. After 1 week, 250 donor fish (50 ± 13 g) naturally infected with *S. chrysophrii*, obtained from Andromeda S.A. fish farm, were added in one of the two cement tanks, and left to swim freely beneath the net containing the 1000 healthy fish. The infection of these donor fish was confirmed by stereoscopical observation of 10 fish. The other cement tank did receive neither donor fish nor infested nets and were used as control. The 2 cement tanks were supplied with well-oxygenated seawater through an open-circulation system and followed the natural photoperiod (salinity 32%, temperature decreasing gradually from 27 to 20 °C). Fish were fed with a commercial diet (Irida S.A.) at a daily rate of 2–2.5% B.W. for 10 weeks. Weekly examination of the gills of 5–10 recipient fish showed that parasitical infection of recipient fish initiated after 3 weeks of cohabitation with donor fish. Mortality of recipient fish started after 4 weeks and reached 9.4% of cumulative mortality after 10 weeks (Fig. 1).

2.2. Samplings

After 10 weeks of cohabitation, 24 control fish and 378 recipient fish were anaesthetized with clove oil and weighed (W, g). For each fish, the 2 external gill arches were sampled and blood was withdrawn from the caudal vein using a 1 ml syringe. Twenty microliters of blood of 187 recipient fish and 24 control fish were mixed with 4 units of heparin in a round bottom microplate. The heparinized blood was directly used to determine the respiratory burst activity and the hemoglobin concentration. The remaining blood of all 378 recipient fish and 24 control fish was left to coagulate overnight at 4 °C. The next day the coagulated blood samples were centrifuged at 9000 *g* for 10 min and serum was collected and frozen at –80 °C. The serum samples were used to determine the humoral immune parameters.

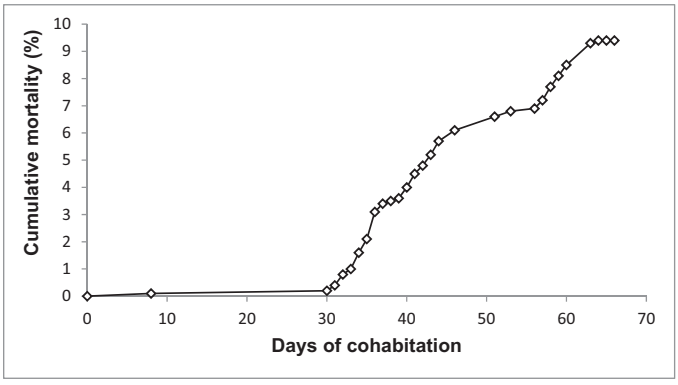

Fig. 1. Cumulative mortality (%) of Gilthead sea bream during the 10 weeks of cohabitation with donor fish parasitized with *Sparicotyle chrysophrii*. n = 1000.

2.3. Histological, hematological and immunological parameters

The 2 external gill arches of each fish were placed on a glass slide and histological examination under a stereoscope (×0.67, Olympus SZ61) enabled the determination of the total numbers of adult parasites for each sampled fish (parasite load).

The hemoglobin concentration was determined using a miniaturized version of the Drabkin method (Rigos et al., 2010).

The spontaneous and zymosan-triggered respiratory burst activities were assessed in the heparinized whole blood using the chemiluminescence assay described before (Henry et al., 2009). The serum lysozyme activity was assessed using the turbidimetric kinetic method (Cotou et al., 2013). The serum myeloperoxidase and the complement antibacterial activities in the fish serum were measured as described before (Kokou et al., 2012) with some modifications: The myeloperoxidase reaction was stopped by adding 50 µl of 1N H_2SO_4. The kinetic of the complement anti-*Escherichia coli* activity consisted in a rise of relative luminescent units corresponding to the increase of bacterial viability up to a maximum of relative luminescence unit (RLUmax) followed by a sigmoidal decrease of the bacterial viability corresponding to the killing of the luminescent strain of *E. coli*. Results were calculated using the Gaussian curve fit and the sigmoidal fit of the Origin software and expressed as (1) the percentage of bacterial killing (%) = 100 – (100 × RLUmax in serum/RLUmax in buffer control), (2) the time at RLUmax (min) corresponding to the time for assembly of the complement complex, (3) the time necessary to kill 50% of *E. coli* (min) = time at the change of curvature of the sigmoidal decrease of luminescence – time at RLUmax. The serum antiprotease and ceruloplasmin oxidase activities were measured following the methods described before (Henry and Fountoulaki, 2014). The nitric oxide concentration was measured in the fish serum using the Griess reagent described in Green et al. (1982). Twenty microliters of Griess reagent was added to 150 µl of fish serum or $NaNO_2$ standard and 120 µl of distilled water and left to react for 30 min at room temperature. Then the OD was measured at 550 nm. Results were expressed as NO_2 concentration in µM. The total antibody in fish serum samples diluted at 1:50 was determined using an ELISA kit containing monoclonal anti-sea bream IgM (at 6.25 µg/ml; 100 µl/well) and following manufacturer's instructions (Aquatic Diagnostics, Stirling, Scotland, UK). The blocking buffer used was bovine serum albumin (BSA), the secondary antibody used was a HRP-labeled goat anti-mouse immunoglobulin G diluted 1:2000 (100 µl/well) and the color substrate used was O-phenylenediamine (OPD). The reaction was stopped using 50 µl of 3N sulfuric acid and OD was measured at 492 nm.

2.4. Statistical analysis

Data were analyzed using the software SPSS at a confidence level of 95%. Bivariate parametric (Pearson r) and non-parametric (Kendall τ) correlations were determined for all growth, pathological, hematological and immunological factors. The non-parametric correlation does not rely on any assumptions on the distributions of the values of each parameter and compares their ranks whereas the parametric correlation can be applied to a linear relationship between two variables. The normality was assessed using the Kolmogorov–Smirnov test. Homogeneity of variances was checked