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Molecular profiling of the gilthead sea bream (*Sparus aurata* L.) response to chronic exposure to the myxosporean parasite *Enteromyxum leei*

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ABSTRACT

The aim of the present work was to investigate the transcriptome response of gilthead sea bream (*Sparus aurata*) after challenge with the myxosporean *Enteromyxum leei*, a wide-spread enteric parasite causing heavy economic losses in Mediterranean sparid farms. This parasite causes severe desquamative enteritis which usually leads to death of the fish, and there are no preventative or curative treatments for this enteromyxosis. After 113 days of exposure to parasite-contaminated effluent, fish were classified into three cohorts: control fish not exposed to parasite, those that were exposed and infected, and those that were exposed but not infected. In order to detect target genes that may be candidates for infective status or resistance, a cDNA microarray containing 18,490 cDNA clones enriched in genes differentially expressed after infection was hybridised with head kidney and intestine samples. In infected fish, 371 and 373 genes were differentially regulated at the >1.5-fold level in intestine and head kidney respectively, whereas in non-infected fish 175 and 501 genes were differentially regulated in these tissues, respectively. A global marked gene down-regulation was evident in infected fish, mainly in genes involved in the immune and acute phase response particularly complement and mannose binding lectin. Microarray analysis demonstrated a complex interplay between host and/or parasite derived proteases and protease inhibitors, apoptosis, cell proliferation and antioxidant defence genes in exposed fish. In the head kidney of non-infected fish a marked depression of genes involved in the acute phase response was evident. By contrast, in the intestine of non-infected fish, interferon-stimulated and MHC class II genes involved in antigen processing and presentation were up-regulated, possibly indicating that an active immune response at the local level is important to avoid infection with or proliferation of the parasite.

1. Introduction

Sparids are important fish species for Mediterranean aquaculture, and the gilthead sea bream has become one of the most important, with annual production levels of more than 160,000 tonnes in 2009 (APROMAR, 2010). However, the increased culture density, the international trading of fish stocks and the lack of approved drugs have increased the impact of parasitic diseases (Sitjà-Bobadilla, 2009; Rigos and Katharios, 2010). *Enteromyxum leei* is a myxosporean enteric parasite seriously affecting Mediterranean sparid cultures (Palenzuela, 2006; Sitjà-Bobadilla and Palenzuela, in press). The parasite has been experimentally transmitted by cohabitation with infected fish, oral intubation with infected intestinal scrapings, exposure to water from infected tanks (effluent transmission) and by anal intubation (Diamant, 1997; Diamant and Wajsbrot, 1997; Sitjà-Bobadilla et al., 2007; Estensoro et al., 2010). It was first described in cultured gilthead sea bream (Diamant, 1992) but it has since been detected in many ornamental and cultured fish in the Canary Islands, the Mediterranean and Red Sea and in Western Japan (Sitjà-Bobadilla and Palenzuela, in press).

The impact of the disease is not limited to direct mortality, but also to weight loss, poor conversion efficiency, delayed growth and reduced marketability (Golomazou et al., 2004; Palenzuela, 2006; Rigos and Katharios, 2010). In addition no prophylactic or palliative treatments are available. There are clear differences in susceptibility to and progress of infection in different hosts (Padrós et al., 2001; Sitjà-Bobadilla et al., 2007), with some gilthead sea bream strains seemingly resistant to infection (Jublanc et al., 2005; Sitjà-Bobadilla et al., 2007; Fleurance et al., 2008). In spite of the importance of this enteromyxosis, to date little is known about the mechanisms

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of infection, the parasite life-cycle, and some aspects of the host-parasite relationship, including the host immune response. Thus far, the humoral and cellular innate immune responses of gilthead sea bream (Cuesta et al., 2006a,b; Sitjà-Bobadilla et al., 2007, 2008) and sharpsnout sea bream (Golomazou et al., 2006; Muñoz et al., 2007; Álvarez-Pellitero et al., 2008) have been studied. Furthermore, the expression level of a small number of immunorelevant genes has been investigated (Cuesta et al., 2006b; Sitjà-Bobadilla et al., 2008) and clear differences have been observed in the expression profile of some of these genes between infected and non-infected fish. However, the exact mechanisms underlying these differences remain to be established.

In recent years genome resources for different fish species have been developed and a parallel increase in the use of microarrays in fish physiology has occurred, using model species such as zebrafish (Sreenivasan et al., 2008) or medaka (Kishi et al., 2008) and commercially important species such as salmonids (MacKenzie et al., 2008; Taggart et al., 2008; Guiry et al., 2010), catfish (Peatman et al., 2008), carp (Williams et al., 2008) and flatfish (Cerdá et al., 2008). Thus, microarray technology has emerged as a key tool for understanding developmental processes, basic physiology or the response to environmental stressors from toxic pollutants (Williams et al., 2006) to cold and confinement stress (Gracey et al., 2004; Cairns et al., 2008). For gilthead sea bream, microarrays have been constructed to study early development and response to cortisol injection (Sarropoulou et al., 2005), other developmental processes (Ferraresso et al., 2008) and the effect of confinement exposure (Calduch-Giner et al., 2010). The molecular basis of infection and disease resistance has also been investigated in Atlantic salmon, rainbow trout, Japanese flounder and channel/blue catfish. In salmonids, several studies have focused on the identification of molecular biomarkers in response to bacterial (Rise et al., 2004; Ewart et al., 2005; Baerwald et al., 2008), viral (Jorgensen et al., 2008; MacKenzie et al., 2008), fungal (Roberge et al., 2007) or parasitic infections (Morrison et al., 2006; Skugor et al., 2008; Wynne et al., 2008a,b). The immune response of channel and blue catfish to bacterial (Peatman et al., 2007, 2008) and that of Japanese flounder to bacterial (Matsuyama et al., 2007a), viral (Byon et al., 2005, 2006), and parasitic (Matsuyama et al., 2007b) infections have also been studied.

In the current study, a cDNA microarray enriched by suppression subtractive hybridization (SSH) with immunorelevant genes was used to investigate changes in gene expression in gilthead sea bream after chronic exposure to *E. leei*. Transcriptomic changes were analysed at the site of infection (intestine) and also in the head kidney, the most important hemopoietic and immune organ in fish (Tort et al., 2003). The aim of this work was to investigate differences in the gene expression profile between control fish not exposed to parasite (CTRL fish), fish which were exposed and infected (R-PAR fish), and those which demonstrated resistance to this important pathogen (R-NonPAR fish).

2. Materials and methods

2.1. Experimental design and fish sampling

Details of the experimental design and sampling procedure have been provided previously (Sitjà-Bobadilla et al., 2008). Briefly, naïve gilthead sea bream ($n = 132$) were divided into two replicated groups, control (CTRL) and recipient (R). R fish were exposed to *E. leei*-contaminated effluent whereas CTRL fish received parasite-free water. At 113 days after exposure fish were euthanized and both head kidney (HK) and posterior intestine (INT) were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. Subsequently, tissue samples were thawed overnight in RNAlater-ICE (Applied Biosystems) and stored at −20 °C until RNA extraction and gene expression analysis. Portions of INT were fixed in 10% buffered formalin for diagnosis of parasitic infection.

2.2. Parasite diagnosis

Diagnosis of parasite infection was performed histologically from INT samples embedded in Technovit resin (Kulzer), 1-μm sectioned and stained with toluidine blue. From each fish, four transverse sections of the posterior intestine (the target site) were completely observed by light microscope. A fish was considered infected when at least one parasite stage was found. Parasite intensity of infection was evaluated semi-quantitatively. The resulting prevalence of infection was 67.8%, mean intensity of infection was high, and fish were classified into three groups: CTRL (non-exposed), R-PAR (recipient fish exposed to the parasite and parasitized), and R-NonPAR (recipient fish exposed to the parasite, but not parasitized). For more details see Sitjà-Bobadilla et al. (2008).

2.3. RNA preparation and mRNA enrichment

Total RNA was prepared from HK and INT from eight fish from each of the three groups classified above using a Qiazol and RNeasy Maxi combination protocol (Qiagen). An on-column DNase treatment step was included to yield RNA samples predominately free of contaminating DNA. Total RNAs were quantified by spectrophotometric measurement at 260 nm and were analysed for quality by the Agilent 2100 bioanalyser (Agilent Technologies). The average yield of total RNA was 1.6 mg/g for INT and 3.1 mg/g for HK. Poly A^+ RNA was enriched from pools of HK or INT total RNA from CTRL and R-PAR fish using an Oligotex mRNA Midi Kit (Qiagen). mRNA was quantified by spectrophotometric measurement at 260 nm and analysed for quality by northern blot and hybridization to an elongation factor 1 alpha (EF1α) gene probe. The average yield of mRNA from total RNA was 1.7% for INT and 1.3% for HK.

2.4. SSH library construction

Two micrograms of mRNA from each population were used as template for dscDNA synthesis and SSH library construction using Clontech's PCR Select cDNA Subtraction Kit (BD Biosciences). This kit provides a cDNA hybridization protocol that allows both normalisation (of high abundance cDNAs) and subtraction (of common sequences) between a tester (population of interest) and driver cDNA populations. SSH library construction was performed following the manufacturer's protocol. This protocol included generation of dscDNA, *RsaI* digestion of the cDNA, ligation of adaptors to the tester cDNA, two rounds of hybridization of tester and driver cDNA and two PCR amplifications (primary and nested PCR). Four SSH libraries were constructed corresponding to forward and reverse subtractions for both HK and INT of CTRL and R-PAR cDNAs. All four libraries were cloned using the TA Cloning Kit (Invitrogen). PCR analysis using nested primers (BD Biosciences) was performed on 96 cDNA clones from each of the four libraries to assess the size range and proportion of cDNA clones with insert. The high quality of the constructed SSH libraries was confirmed by Southern blot and hybridization to several housekeeping genes with a reduction in their abundance in subtracted cDNA populations.

2.5. cDNA sequencing of subtracted cDNA products and sequence analysis

Sequencing of SSH libraries was carried out using ABI 3730XL (Applied Biosystems) and MegaBACE 4500 (GE Healthcare) capillary sequencing systems at the Max Plank Institute of Molecular