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

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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, 2011). 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, 2011).

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 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, 2006b; Sitjà-Bobadilla et al.,

2007; Sitjà-Bobadilla et al., 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, 2008b). 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 analyzed 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 ^{o}C. Subsequently, tissue samples were thawed overnight in RNAlater-ICE (Applied Biosystems) and stored at -20 ^{o}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 at 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 intothree groups; CTRL, (non-exposed), R-PAR (recipient fish exposed to the parasite and parasitized), and R-NonPAR (recipient fish exposed to theparasite, 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 and 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 μg 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 Genetics (MPI, Berlin, Germany). All sequence reactions were carried out using ABI BigDye terminator version 3.1. An initial analysis of 192 clones from each library indicated a low level of sequence redundancy (Table 1). Additional sequencing was performed for each library to give a total of 3072 pathogen exposure cDNA clones sent to MPI for commercial sequencing. Nucleotide sequences from SSH libraries were then combined with other available gilthead sea bream EST collections (Calduch-Giner et al., 2010; Louro et al., 2010). Sequences were edited to remove vector and adaptor sequences and were then cleaned and filtered before clustering and annotation by the SIGENAE information system (INRA, Toulouse, France). Contigs were annotated by comparison to the UniProt database using the Blast X program (Altschul et al., 1990) for gene identity assignment.

2.6. Microarray construction and hybridization

After clustering and assembly purified PCR products from non–redundant cDNA clones were printed on epoxy-coated Nexterion Slide E slides (Schott, Germany) by MPI. Each spotted cDNA microarray consisted of PCR products from 18,490 cDNA clones printed in duplicate, to

give a total (along with positive cDNA and negative buffer controls) of 38,976 spots printed at 5 µm resolution. This platform has been assigned the Gene Expression Omnibus (GEO) accession number GPL8467. For the *E. leei* microarray analysis a reference design was used, where both experimental and control RNA were compared to a common reference RNA for each tissue. The two reference RNAs consisted of aliquots of total RNA from HK or INT from CTRL, R-NonPAR and R-PAR fish. Microarray analysis involved the comparison of gene expression profiles of five individuals from the CTRL, R-NonPAR and R-PAR cohorts for both HK and INT. Each hybridization included a Cy3/Cy5 dye swap thus 60 microarray hybridizations were performed in total. Ten micrograms of total RNA per fish and ten micrograms of reference RNA per slide were indirectly labelled following an 'in house' protocol with either Cy3 or Cy5 fluorescent dyes (GE Healthcare). In brief, cDNA was synthesised using Superscript III MMLV reverse transcriptase (Invitrogen) incorporating aminoallyl dUTP (Sigma Aldrich). Synthesis took place at 42 ^{o}C for 2 hours. RNA was hydrolysed with 1M NaOH/0.5M EDTA for 15 min and the reaction was then neutralised with 1M HEPES. Reverse transcription reactions were purified using Microcon 30 columns (Millipore). After vacuum drying, purified cDNA's were were resuspended in 0.1M sodium bicarbonate buffer. Cy dyes were resuspended in the same buffer and coupling to the Cy dye ester took place in the dark for 2 hours at room temperature. The removal of unincorporated dye was performed using the Illustra CyScribe GFX Purification Kit (GE Healthcare). Successful dye incorporation was confirmed by means of a Nanodrop spectrophotometer (Nanodrop Technologies). The two cDNA samples per slide were combined together with appropriate blockers (sheared salmon sperm genomic DNA, poly dA), concentrated and resuspended in 70 µl slide hyb buffer #1 (Ambion). After denaturation (95 ^{o}C for 2 min), the hybridization mixture was added to the microarray slide, sealed using a coverslip (Erie Scientific) and hybridizations were performed in a hybridization chamber (Genetix) at 42 ^{o}C for 16 hours. Following hybridization the slides were washed (2 x 5 min washes using 0.2 X

SSC/0.1 % SDS and 2 x 5 min washes using 0.2 X SSC) using an Advawash automatic washing station (Advalytix). Microarray slides were scanned in two channels 543 nm (Cy3) and 633 nm(Cy5) at 5 µm resolution using a confocal laser scanner (ScanArray Express, Perkin Elmer).

2.7. Data acquisition and statistical analysis

The two channel TIFF image files were imported into the GenePix Pro 6 software program (Molecular Devices Corporation) for feature (spot) finding and alignment using a batch alignment process, and pixel intensities for feature and background were quantified. Output GenePix results (GPR) files were imported into the GeneSpring GX 7.3 software program (Agilent Technologies). Data were normalised for signal, dye swap and intensity (intensity dependent lowess normalisation) and normalised data was filtered on confidence and signal strength. A Welch t-test was performed to select those genes differentially expressed ($p < 0.05$) between the CTRL, R-PAR and R-NonPAR conditions. Data was further analysed using fold change filters and K means clustering. Raw and normalized data was submitted to the SIGENAE version of Bioarray Software Environment (BASE) database. All data was submitted to GEO with accession numbers GSE18219 and GSE19646. Genes were functionally annotated by means of the AmiGO annotation tool (Carbon et al., 2009). When a direct link was not observed between UniProt accession and Gene Ontology (GO) accession, the equivalent human or mouse GO accession for that gene was taken with the assumption that functional classification would not vary too widely between species. Multilevel GO term analysis was performed by the Blast2GO program (Conesa et al., 2005; Götz et al., 2008) under default settings (minimum cutoff 1.0E-04 Blast X) and were filtered by annotation score.

2.8. Quantitative PCR validation

A selection of genes potentially differentially expressed between CTRL/R-PAR/R-NonPAR fish were chosen for confirmation by quantitative real-time PCR (qPCR). PCR primers to these candidate genes were designed using Vector NTI suite 6 (Table 2) and synthesized by MWG Biotech. qPCR confirmation was carried out using an MX3000P Real-time PCR System (Stratagene). Reverse transcription of 2.5 µg aliquots of total RNA from each individual were performed using Superscript III reverse transcriptase (Invitrogen) at 50 ^{o}C for 1 hour in a final volume of 20 µl. Following an enzyme inactivation step of 70 ^{o}C for 15 min, the cDNA was diluted to 10 ng/µl and qPCR assays were performed using the Quantitech SYBR Green PCR Kit (Qiagen). PCR reactions were carried out in a 20 µl total reaction volume consisting of 10 µl of 2X QuantiTect® SYBR Green RT-PCR Master Mix, 0.5 µM sense and anti-sense gene-specific primers and 50 ng of cDNA template. The thermal profile was set up to include a initial denaturation step of 95 ^{o}C for 15 min, followed by 40 cycles of 94 ^{o}C for 15 s, 58-60 ^{o}C (primer-dependent) for 30 s and 72 ^{o}C for 30 s. In order to detect the presence of non-specific amplifications, control reactions containing all the reaction components except for the template were included for each primer set. DNA melt curve analysis was performed using a ramping rate of 1 ^{o}C / min over a temperature range of 60-95 ^{o}C and the specificity of the PCR product was confirmed by a single peak. Amplification efficiencies of qPCR primers (for candidate and reference genes) were routinely calculated by means of a standard curve of serial dilutions of cDNA and these figures were incorporated into comparisons of gene expression data. PCR efficiencies of qPCR primers in this study were greater than 95%. All genes were normalised to the reference genes, β-actin, ribosomal protein S18 and elongation factor 1α using the GENorm method (Vandesompele et al., 2002). Statistical analysis of qPCR results was performed in SPSS V17.

3. Results

3.1. Intestine gene expression

The global profile of the INT cDNA obtained by microarray analysis is provided in Fig. 1A. The comparison of CTRL *vs*. R-PAR fish resulted in the identification of 371 genes significantly differentially regulated (mainly down-regulated) with a fold change >1.5 whereas a lower amount of genes (175) appeared to be differentially regulated (mostly overexpressed) between CTRL and R-NonPAR fish. Of these significant genes 349 genes (133 annotated) were exclusive to R-PAR fish, 153 (50 annotated) genes were exclusive to the R-NonPAR fish and only 22 (1 annotated) genes were common to both fish groups (Fig. 2A). K-means clustering of differentially expressed genes indicated four major sets of genes and a complete list of these genes and summary GO pie charts for each set are provided in Supplementary files S1 and S2. Set 1 (30 genes) was composed of genes up-regulated in R-PAR fish, which were related to cell cycle (both pro and anti-apoptotic) and cell proliferation, cell transcription and translation, and ROS scavenging to prevent oxidative damage (e.g. glutathione S-transferase 3, phospholipid hydroperoxide glutathione perioxidase). Set 2 (104 genes) was composed of genes down-regulated in R-PAR fish, particularly lysosomal and digestive proteases (e.g. cathepsins B, L, S, trypsins, chymotrypsins, choriolysins) and genes involved in carbohydrate and lipid metabolism. In addition, there was down-regulation of genes related to complement activation, acute phase response (APR) and cell adhesion (e.g. mannose binding lectin 2, complement C3-1, complement C1s, fibronectin, adhesive plaque matrix protein). Set 3 (38 genes) clustered those up-regulated genes in R-NonPAR fish involved in the activation of the immune response via interferon signalling and antigen processing and presentation (e.g. interferon induced protein 44, interferon regulatory factor 1, H-2, RLA and SLA class II histocompatability antigens). Set 4

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was the smallest group (13 genes) and contained genes down-regulated in R-NonPAR fish including those that were involved in apoptosis (STE20-like serine/threonine protein kinase), immune response (interleukin-6 receptor alpha chain, complement factor 1) or fatty acid metabolism (diacylglycerol O acyltransferase 2, elongation of very long chain fatty acids protein 1).

3.2. Head kidney gene expression

The global profile of the HK cDNA obtained by microarray analysis is provided in Fig. 1B. A total of 373 genes were significantly differentially regulated between CTRL and R-PAR fish at the 1.5 fold level whereas a higher number of genes (501) were identified when comparing CTRL *vs.* R-NonPAR. In both cases, the percentage of genes down-regulated was higher than that of those up-regulated. A further analysis revealed that 225 (79 annotated) genes were only present in R-PAR fish, 353 genes (181 annotated) were exclusively expressed R-NonPAR fish, and 148 genes (14 annotated) were common to exposed fish regardless of their infection status (Fig. 2B). K-means clustering of differentially expressed genes indicated four major sets of genes (sets 5-8 in Supplementary file S3 and summary GO pie charts for each set provided in Supplementary file S4). Set 5 included 27 genes up-regulated in R-PAR fish, which were involved in various aspects of the cell cycle from DNA replication to cytokinesis and cell division. There was also up-regulation of genes involved in protease inhibition and protein synthesis. Set 6 included 66 genes down-regulated in R-PAR fish, particularly those involved in complement activation, APR, innate and humoural immune responses, proteolysis, lipid metabolism and transport, and serine protease inhibition. Set 7 consisted of 31 genes activated in R-NonPAR fish, and was enriched in genes involved in nucleic acid metabolism and regulation of transcription. Set 8 (164 genes down-regulated in R-NonPAR fish) was the largest group and

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included genes related to complement activation and immune response, proteolysis, protease inhibition, lipid metabolism and transport, oxidation reduction, translation and APR/iron homeostasis genes.

3.3. Real time qPCR validation

Confirmation of microarray results was performed by means of real time qPCR for a selection of candidate genes from INT and HK (Fig. 3A). For all genes the pattern of expression was consistent between the two methods. Statistical analysis (t test $p<0.05$) was performed to confirm differential expression between CTRL, R-PAR and R-NonPAR groups and this analysis indicated significant differences in expression for seven if the 10 genes analysed. For a further 2 genes (MBL2 and CPI1), 2 genes with the largest fold change values there was more inter individual variation so group values were outside of significance ($p<0.09$ and $p<0.08$ respectively). Pearson correlation analysis of individual microarray and qPCR values for all 10 genes indicated that all showed a positive correlation between the 2 methods, with 7 genes achieving significance at either a $p<0.05$ or $p<0.01$ level (Fig. 3B).

4. Discussion

The current results are based on a previous experimental infection, from which several humoral and cellular immune factors and the expression of a few immune and antioxidant genes were analyzed (Sitjà-Bobadilla et al., 2008). From that experiment, four SSH libraries were constructed to preselect those cDNAs differentially regulated between CTRL and R-PAR fish. In combination with cDNA clones from a parallel study on confinement stress (Calduch-Giner et al., 2010), a cDNA microarray was constructed, which allowed a more extensive and global evaluation of the molecular host response against *E. leei* that would help to identify potential candidate gene markers for resistance.

Microarray analysis indicated that many of the differentially regulated genes are immune response genes (e.g. MHC genes, interferon-induced proteins, complement components, antioxidants, lectins), with clear differences between those fish that were infected (R-PAR) and those which were not (R-NonPAR). In the INT of R-PAR fish there was a marked general down-regulation of the complement system, cell adhesion and proliferation, carbohydrate and lipid metabolism and lysosomal and digestive protease genes. In the HK of R-PAR fish down-regulation affected mainly digestive and lysosomal proteases, complements, lectins, and immune response genes. This marked down-regulation of the host immune system after infection could be considered a mechanism of immune evasion, as described for other fish and mammalian parasites (see Sitjà-Bobadilla, 2008). By contrast, at the local level (INT) R-NonPAR fish appeared to have far less repression of transcription, as only 13 down-regulated genes passed a 1.5 fold filter. The marked immunodepression in R-PAR fish is in accordance with the depletion of immune factors such as total serum peroxidases and lysozyme in fish exposed to *E. leei* (Sitjà-Bobadilla et al., 2008), or the alternative complement pathway exhaustion observed in different species after chronic exposure to *Enteromyxum* spp. (Cuesta et al., 2006b; Sitjà-Bobadilla et al.,

2006; Álvarez-Pellitero et al., 2008). In a subsequent study investigating the effects of gilthead sea bream nutritional status on *E. leei* disease progression, total serum peroxidases, lysozyme and nitric oxide were also significantly reduced in exposed fish fed either fish oil or a vegetable oil-based diet (Estensoro et al., 2011). Other immune parameters such as the phagocytic activity of macrophages and/or respiratory burst of leukocytes were also decreased in different hosts infected with other parasites (Mustafa et al., 2000; Fast et al., 2002; Scharsack et al., 2004; Karagouni et al., 2005). At the transcriptional level, the parasite-mediated amoebic gill disease (AGD) in Atlantic salmon produced a marked up-regulation of gene expression in early stages (4-8 days) of infection (Morrison et al., 2006), whereas at medium (19 days) (Wynne et al., 2008a) and long term exposure (36 days) (Young et al., 2008), a strong overall gene suppression was evident in the immune response, transport, translation and catalytic activity functional categories. This late suppression is in accordance with that observed in the present model of long term exposure to the myxosporean. Transcriptional studies have also demonstrated this gene suppression effect in other species, as in molluscs infected by the metazoan parasite *Echinostoma paraensei*, which had many down-regulated transcripts with potential immune functions (Adema et al., 2010), or in oysters chronically infected by the protozoan parasite *Perkinus marinus* (Wang et al., 2010).

Mannose binding lectin 2 (MBL2) stands out among the down-regulated genes (almost 4 fold) in the INT of R-PAR individuals. In mammals, MBL functions as part of the innate immune system through the lectin complement pathway, and bacteria, yeast, parasites, mycobacteria and viruses are recognised by MBL (Crouch and Wright, 2001; Holmskov et al., 2003; Turner, 2003). MBL levels contribute to the severity of illness in several bacterial infections (Hibberd et al., 1999; Roy et al., 2002; Kars et al., 2005) and low MBL levels are associated with increased risk, severity and frequency of some infections (Botto et al., 2009). In fish species MBL have been reported in trout, salmon, carp, rohu fish, channel catfish, blue

catfish and sea lamprey (Jensen et al., 1997; Ewart et al., 1999; Vitved et al., 2000; Mitra and Das, 2002; Ourth et al., 2007, 2008) and functional binding studies to fish pathogens have suggested a similar lectin complement pathway for these species (Ewart et al., 1999; Ourth et al., 2007, 2008). Analysis by qPCR confirmed the down-regulated expression pattern of MBL2 in the INT of R-PAR fish (148-fold) whereas in R-NonPAR fish down-regulation was less apparent (1.7-fold). Ourth et al. (2007) have suggested that MBL could be used as a genetic marker for disease resistance in the different strains of cultured catfish, as those more resistant to *Edwardsiella ictaluri* had nearly three fold higher levels of serum MBL. In other infection studies the transcriptomic responses of MBL were dependent on the pathogen involved, as demonstrated by the 4.2-fold down-regulation of the MBL gene in the gills of AGD-affected Atlantic salmon (Morrison et al., 2006), and the 2-fold up-regulation in juvenile Atlantic salmon infected with saprolegniasis, a fungal infection (Roberge et al., 2007).

Parasite-derived proteases play a variety of roles in establishing, maintaining and exacerbating an infection (McKerrow et al., 2006) and their presence in several myxosporeans has been demonstrated (Martone et al., 1999; Dörfler and El-Matbouli, 2007). It is hypothesised that proteases are also involved in *E. leei* host invasion as the parasite breaches the epithelium and infiltrates between enterocytes in the paracellular space. The modulation of serum levels of antiproteases by *Enteromyxum* infections has been demonstrated in sharpsnout sea bream (Muñoz et al., 2007) and turbot (Sitjà-Bobadilla et al., 2006). The results of the microarray revealed a complex interplay between parasitic and/or host derived proteases and protease inhibitors. The INT of R-PAR fish showed up-regulation of two serine protease inhibitors (leukocyte elastase inhibitor and neuroserpin), the antiprotease α2-macroglobulin (α2-M) at 1.4-fold, and a marked down-regulation of a variety of proteases (e.g. cathepsins B/L/L2/S, carboxypeptidases A/B, choriolysins H2/L, elastases 1/2A, trypsins 1/2). In a previous study

α2-M was also up-regulated in the INT of R-PAR gilthead sea bream (Sitjà-Bobadilla et al., 2008). Similarly, this gene was overtranscribed in grass carp parasitized by the copepod *Sinergasilus major* (Chang et al., 2005) and in carp intraperitoneally injected with *Trypanoplasma borelli* (Saeij et al., 2003). In the HK of R-PAR fish a marked up-regulation of two protease inhibitors (stefins A1 and D1) with concomitant downregulation of several proteases (e.g. chymotrypsin C, trypsin 1, carboxpeptidases A1/B and elastase 1) was detected. However there was also downregulation of α2-M (>3-fold), alpha-1- inhibitor 3, inter-alpha-trypsin inhibitor and AMBP protein. Increased expression of protease inhibitors in the INT and HK of R-PAR fish may be an attempt by the host to inhibit parasite-derived proteases or indeed inhibit host proteases released from injured tissues. The possibility also exists that this downregulation of proteases/protease inhibitors is a result of parasite induced gene depression or a result of the host reaction to extended immune stimulus.

Iron is central to the host-pathogen interaction and during the APR a systemic depletion of iron is induced as a host defence mechanism against a variety of infectious agents (Marx, 2002; Schaible and Kaufmann, 2004; Drakesmith and Prentice, 2008). In fact the APR includes changes in the plasma concentrations of a variety of proteins including members of the complement system, coagulation factors, antiproteases, transport proteins and major acute phase proteins such as C-reactive protein and serum amyloid A (Gabay and Kushner, 1999). Several genes encoding APR proteins (mainly complement components) were down-regulated in the INT and HK of R-PAR fish. More notable, however, was the downregulation of many APR genes in the HK of R-NonPAR fish, with many genes involved in iron transport and/or homeostasis (hemopexin, hepcidin, ceruloplasmin, serotransferrins I and II, haptoglobin, heme oxygenase, fibronectin, fibrinogen). The depression of all these genes could be a rebound effect after an initial increase shortly after exposure, which could reflect the host's attempt to return to a condition of iron and tissue homeostasis. Further kinetic studies with samples taken at different

times are needed to corroborate this hypothesis. This time effect can be observed in other pathogen models, as several APR genes were also downregulated in the gills of AGD-resistant Atlantic salmon after chronic exposure to the parasite *Neoparamoeba perurans* (Wynne et al., 2008b). This is in contrast with a marked upregulation of APR transcripts which included many genes involved in iron homeostasis in both channel and blue catfish shortly after infection with the Gram-negative bacterium *Edwardsiella ictaluri* (Peatmann et al., 2007, 2008).

The production of reactive oxygen species (ROS) is induced by infection with a variety of microorganisms (Pacelli et al., 1995; Miyagi et al., 1997; Darrah et al., 2000). However, ROS are cytotoxic not only to pathogens but are also extremely damaging at high levels to host tissues, thus cells have evolved powerful defence mechanisms and antioxidant enzymes to counteract their deleterious effects (reviewed by Schrader and Fahimi, 2006; Valko et al., 2007). In the present study with a chronic pathogen challenge, the potential tissue damage by ROS can be substantial and thus induction of genes that may protect from ROS is essential. This would explain the up-regulation of several antioxidants (glutathione S-transferase 3, phospholipid hydroperoxide glutathione peroxidase, epoxide hydrolase 1) in the INT of R-PAR fish. In fact the respiratory burst of blood leukocytes was significantly higher in R-PAR than in R-NonPAR fish (Sitjà-Bobadilla et al., 2008). Conversely, there was down-regulation of thioredoxin reductase 1 in the INT and HK of R-PAR fish, which is in accordance with the down-regulation of glutathione peroxidase-1 in the INT of R-PAR fish in a previous study (Sitjà-Bobadilla et al., 2008). In the HK of R-NonPAR individuals several members of the cytochrome P450 superfamily were down-regulated. These enzymes, which participate in a wide range of biochemical pathways in different organisms (McLean et al., 2005), are hemeproteins and therefore their down-regulation is consistent with the previously cited depression of other heme-APR proteins in this group of fish. All these results possibly reflect the complex role of antioxidants in both normal physiological functions and disease conditions (Valko et al., 2007).

Apoptosis plays a crucial role in normal tissue development and organogenesis but it also has an important role in the pathogenesis of different diseases (Thompson, 1995; White, 1996). Apoptosis can be either initiated or down-regulated by parasitic infections, contributing to dissemination within the host, inhibiting or modulating host immune responses or facilitating the intracellular survival of the pathogen (Lüder et al., 2001; James and Green, 2004). Differential apoptotic responses have been demonstrated in parasitic infections of mammals, sometimes with a biphasic modulation (early down-regulation and late up-regulation) (Lüder et al., 2001; Liu et al., 2009). In the INT and HK of R-PAR fish and the INT of R-NonPAR fish there was up-regulation of several genes with pro-apoptotic functions and depression of others with anti-apoptotic function. Other pro-apoptotic genes in the INT of R-PAR fish and the HK of R-NonPAR fish were also decreased. Complex adjustments of apoptotic signals in these fish may reflect the parasite attempts at dissemination within the host or the host's immune response to eliminate the invading parasite, as observed in other fish-parasite models. The ciliate *Philasterides dicentrarchi* induces apoptosis of turbot pronephric leucocytes (Paramá et al., 2007), and severely *E. scophthalmi*-infected turbot have increased apoptotic rates in the digestive epithelium, which could facilitate the spreading of the parasite (Bermúdez et al., 2010). The skin culture fluid from channel catfish resistant to the ciliate *Ichthyophthirius multifiliis* induces apoptosis of parasite theronts (Xu et al., 2005). Furthermore apoptosis-inducing protein, a protein purified from chub mackerel infected with the nematode *Anisakis simplex*, provokes apoptosis in various mammalian cell lines (Murakawa et al., 2001). These apoptotic adjustments may also reflect the host's attempt to reduce immunopathology associated with unwanted activity of effector leukocytes and return to a status of cell/tissue homeostasis. An overcompensated host immune response which includes elevated apoptotic transcriptomic signals has been implicated in the immunopathology associated with infection with the malarial parasite *Plasmodium falciparum* (Lovegrove et al., 2007).

Interferons (IFNs) induce a state of antiviral activity by transcriptional regulation of several hundred IFN-stimulated genes and play a major role in the defence against virus infection in vertebrates, including fish (Samuel, 2001; Alonso and Leong, 2002; Shultz et al., 2004; Robertsen, 2006; Ellis et al., 2010). They are also known to play a role in some parasitic infections, including resistance (Lillehoj and Choi, 1998; Pollock et al., 2001; McCall and Sauerwein, 2010). R-NonPAR fish exhibited significant up-regulation of several IFN-stimulated genes in the INT (interferon-induced protein 44, interferon regulatory factor 1 and also interferon-induced 35kDa protein at a 1.47-fold level), and HK (interferon regulatory factor 1, interferon related developmental regulator 1, albeit at less than 1.5-fold). Together with other immune response genes which at the local level were up-regulated (lipopolysaccharide-binding protein, H-2 class II, RLA class II and SLA class II MHC antigens), the IFN-induced genes may represent a global anti-parasitic response to counteract parasite invasion with the effect of resistance to infection or enhanced clearance of parasite. Since only MHC class II genes were up-regulated, it is suggested that IFN-γ could be responsible for the stimulation, as type I IFNs only up-regulate MHC class I genes, whereas IFN-γ promotes antigen presentation by up-regulating the expression of both MHC class I and MHC class II molecules (Boehm et al., 1997). This hypothesis is also in accordance with the chronic nature of the current infection model, as IFN-γ has a key role in adaptive cell mediated immunity. These genes may thus be considered as potential candidate markers for pathogen resistance especially at the local level (INT), as they were not evident in R-PAR fish. Similarly, interferon regulating factor 1 and interferon-induced 35kDa protein were also found to be upregulated 7- and 2-fold respectively, in resistant rainbow trout Hofer strains 24 hours after exposure to the myxozoan parasite *Myxobolus cerebralis* (Baerwald et al., 2008), and AGD-resistant Atlantic salmon had higher expression of several cDNA transcripts involved in adaptive immunity supporting the hypothesis that AGD resistance may be associated with an adaptive immune response (Wynne et al., 2008b).

5. Conclusions

The INT and HK transcriptome response after chronic exposure to *E. leei* was analyzed with a cDNA microarray enriched in immunorelevant genes, and preliminary insights into to the molecular processes at play during infection and resistance were gained. A global strong immunosuppressive effect was apparent after infection. Complex adjustments of apoptotic signals, proteolytic and antioxidant defence genes were demonstrated. A marked depression of APR genes in the HK of R-NonPAR fish was noted. At the local level (INT), R-NonPAR fish exhibited far less repression of transcription and upregulation of genes involved in interferon signalling and antigen processing, indicative of the involvement of intestinal cells in the host's adaptive immune response and the potential of these processes as candidate markers for resistance to *E. leei*.

Acknowledgements

Appendix A. Supplementary data

Supplementary file S1: K-means clustering of differentially expressed genes in the intestine of gilthead sea bream. Up-regulated and down-regulated genes (>1.5-fold) in R-PAR (Sets 1 and 2) and R-NonPAR fish (Sets 3 and 4), respectively.

Supplementary file S2: Summary Pie charts of Blast2GO analysis performed for intestine K-means Sets 1-4. Main GO categories observed are provided along with their relative abundance (in parenthesis).

Supplementary file S3: K-means clustering of differentially expressed genes in the head kidney of gilthead sea bream. Up-regulated and down-regulated genes (>1.5-fold) in R-PAR (Sets 5 and 6) and R-NonPAR fish (Sets 7 and 8), respectively.

Supplementary file S4: Summary Pie charts of Blast2GO analysis performed for head kidney K-means Sets 5-8. Main GO categories observed are provided along with their relative abundance (in parenthesis).

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Figure Captions

Fig. 1. Total number of differentially expressed genes from intestine (A) and head kidney (B) microarray experiments, identified at $p<0.05$ with a ≥ 1.5 fold filter. Control (CTRL) fish were compared with R-PAR and R-NonPAR fish. Green bars indicate up-regulated genes and red bars indicate down-regulated genes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

Fig. 2. Venn diagram representation of the CTRL *vs.* R-PAR and CTRL *vs.* R-NonPAR gene lists for intestine (A) and head kidney (B) of gilthead sea bream. Total numbers of genes in each group are indicated along with number of annotated, non-redundant genes in parenthesis.

Fig. 3. qPCR validation of microarray data. (A) qPCR expression profiles (normalized mean fold change relative to CTRL) of 10 selected genes in intestine or head kidney of R-PAR and R-NonPAR populations. Full gene names are provided in Table 2. Bars indicate mean ± standard deviation. Up-regulation of gene expression is indicated by a positive fold change and down-regulation by a negative fold change. For the purposes of illustration, MBL2 values have been converted to a log10 scale. Groups with statistically significant differences (p<0.05) to one or both of the other groups are indicated by different letters. *For 2 genes MBL2 and CPI1 group values were outside significance (p<0.09 and p<0.08 respectively) due to larger inter-individual variability. (B) Correlation of individual qPCR and microarray expression data for the 10 selected genes in intestine (horizontal lines) and head kidney (vertical lines). * indicates significance at the p<0.05 level, ** indicates significance at the p<0.01 level.
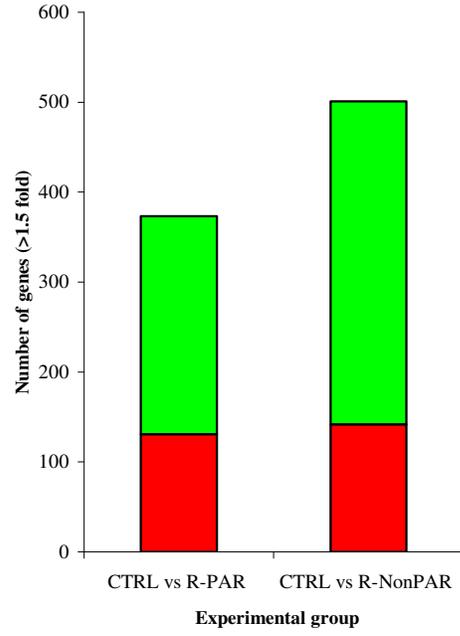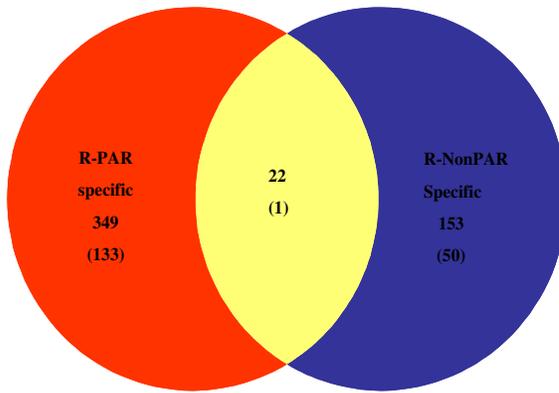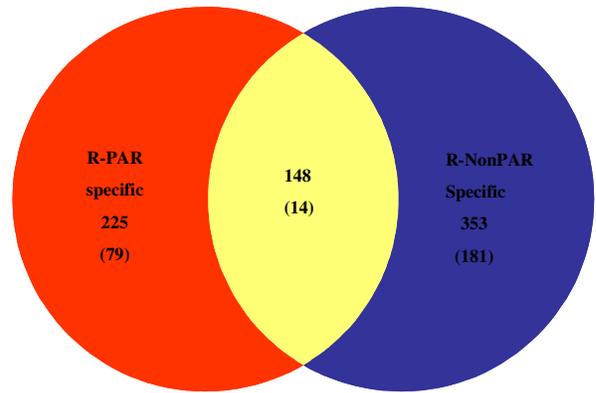
A
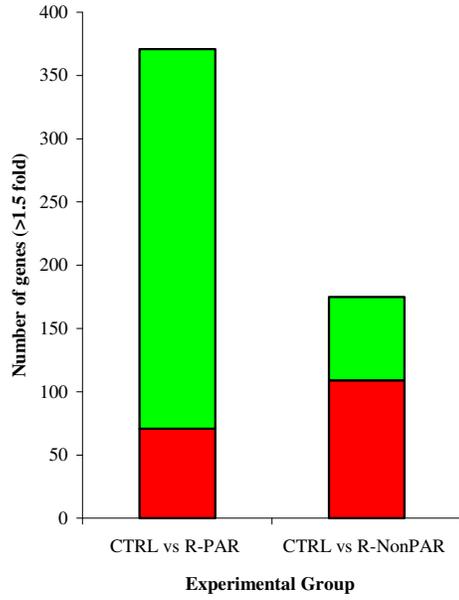
B

Figure 1

A   **B**
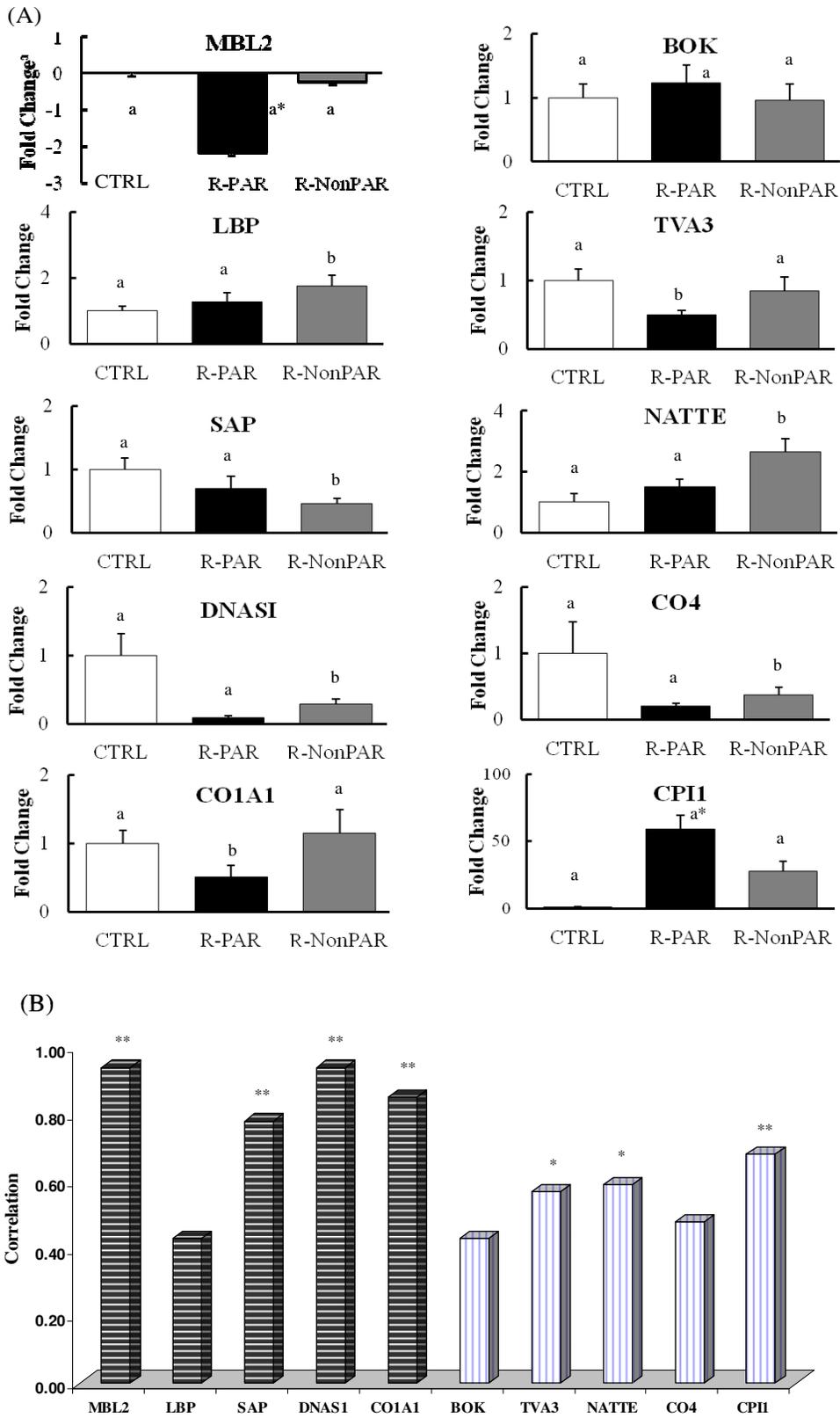

Figure 2

(A)

(B)

Figure 3

Table 1 Initial analysis of sequence redundancy in the SSH cDNA libraries

Library Name[a]	Tissue	Number of cDNA clones sequenced initially	Number of valid sequences	Number of different contigs	% sequence redundancy	Total Number of cDNA clones sequenced	Number of ESTs in dbEST
gsgi07	intestine	192	172	135	21.5	768	701
gsgi08	intestine	192	170	144	15.3	768	655
gsgy07	head kidney	192	175	158	9.7	768	672
gsgy08	head kidney	192	174	159	8.6	768	697
Total		768	691			3072	2725

[a] gsgi07 and gsgy07 are SSH cDNA libraries subtracted in the forward direction so should be enriched in genes upregulated in infection, gsgi08 and gsgy08 are their reverse subtractions so should be enriched in genes downregulated in infection.

Table 2 Primer sequences used for qPCR confirmation

Gene Name (Best Blast X match)	Blast match symbol	Uniprot accession number	Forward primer	Reverse primer
Mannose binding lectin 2	MBL2	Q66S54	AAAGGAGCCCAACAACGCAGG	CTTGATTTCTTCCTGGAGCAGACG
Lipopolysaccharide binding protein	LBP	P18428	GGCCGTACGTTCCTCAGCTTCCTA	AAGCCTTGACAGCGCCCTTCAG
Saposin	SAP	P07602	GTTGCTCTT TGCTTCCGCTTGAG	GGCTCCGTTCATCATCAATGTGC
Collagen 1A1	CO1A1	P02457	GAGGCACAGCCGCTTCCATACA	GGAGCAATGTCAATGATGGGCA
DNase 1	DNAS1	O42446	AGTCTCCCAGCAACACGATGTCG	CGGGGAAGAACTTTGTTCTGATCC
Bcl2-related ovarian killer protein	BOK	Q9I8I2	AAACAGAATGCAGGGAAACTTCAG	TGCTGCTGAATGCCATAAGCTAGTT
T-cell receptor alpha chain V region	TVA3	P06323	TGTTGTAGAGGTGGAACATTGGCT G	TGGTCTCAAATGTGCAGTCCAGTGT
Nattectin	NATTE	Q66S03	CGATGGCGTCAGCTCTTCCTCT	CGCTCTGCATCAGCATCGT
Complement C4	CO4	P01030	CAA ACAGAGCGTACCAATAGGCCTC	TTGGCACACATCCTCTGAACACAG
Leukocyte cysteine proteinase inhibitor 1	CPI1	P35479	CCTCCATAACAGGGAAGTGCTTGG	TGTGCAGGGAAAGACGAACAAGAT
Beta actin	ACTB	X89920	CTGGAGAAGAGCTATGAGCTGCCC	GGTGGTCTCATGGATTCCGCAG
Ribosomal protein S18	RPS18	AM490061	CCA TGT TGT CCT GAG GAA GGC C	CGT CCT TGA CGT CCT TCT GCC T
Elongation factor 1- alpha	EF1α	AF184170	GTATTGGAACTGTACCCGTCGGC	TTGTCACCAGGGACAGCCTCT G