SUPPRESSION SUBTRACTION HYBRIDIZATION (SSH) AND MACROARRAY TECHNIQUES REVEAL DIFFERENTIAL GENE EXPRESSION PROFILES IN BRAIN OF SEA BREAM INFECTED WITH NODAVIRUS

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ABSTRACT

Despite of the impact that viruses have on aquatic organisms, relatively little is known on how fish fight against these infections. In this work, the brain gene expression pattern of sea bream (*Sparus aurata*) in response to nodavirus infection was investigated. We used the SSH (Supression Subtractive Hybridization) method to generate a subtracted cDNA library enriched with gene transcripts differentially expressed after one day post infection. Some of the ESTs from the infected tissues fell in gene categories related to stress and immune responses. For the reverse library (ESTs expressed in controls compared with infected tissues) the most abundant transcripts were of ribosomal and mitochondrial nature. Several ESTs potentially induced by virus exposure were selected for *in vivo* expression studies. We observed a clear difference in expression between infected and control samples for two candidate genes, ubiquitin conjugating enzyme 7 interacting protein, which seems to play an important role in apoptosis and the interferon induced protein with helicase C domain 1 (mda-5) that contributes to apoptosis and regulates the type I IFN production, a key molecule of the antiviral innate response in most organisms.

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1. INTRODUCTION

Viral infections are among the most destructive diseases that affect fish aquaculture. Nodavirus are responsible of Viral Encephalopathy and Retinopathy disease (VER disease), causing neurological disorders such as abnormal swimming behavior and lethargy. Histopathologically, the disease is characterized by vacuolization in the brain grey matter, spinal cord and granular layers of the retina (Comps et al., 1994). Nodavirus cause massive mortalities in a wide range of species of cultured marine fish (Frerichs et al., 1996). Sea bream (*Sparus aurata* L.), a high economic value species cultivated in the Mediterranean area, has been reported as a healthy asymptomatic carrier of the virus (Castric et al., 2001). However, Aranguren et al. (2002) demonstrated the susceptibility of juvenile sea bream to nodavirus by intra muscular (i.m.) injection, depending on age and temperature. Also, sea bream is often cultured in the Mediterranean in the vicinity of sea bass *Dicentrarchus labrax* L and other susceptible species, raising the possibility of cross infection. Despite of the impact that viruses have on aquatic organisms and the lack of treatments, relatively little is known how fish fight these infections.

Viral diseases in aquaculture generally cannot be controlled mainly due to the lack of commercial vaccines. The generation of vaccines might be helped by the understanding of the effect of the infection on the host gene expression. The study of the differentially expressed genes after a viral infection has been done using several approaches such as subtractive hybridization, polymerase chain reaction, analysis of expressed sequence tags (EST) and gene arrays (microarray) (Nam et al., 2000; Alonso and Leong, 2002; Ju et al., 2002; O'Farrell et al., 2002; Munir et al., 2004; Purcell et al., 2004; He et al., 2005; Ji et al., 2005). In this study, we have investigated the genes expressed in response to nodavirus infection in the brain of sea bream, the target organ of the virus, in order to identify genes involved in fish defense.

2. MATERIAL AND METHODS

2.1 Experimental viral infections

15 sea bream (mean weight of 49.5 g) from a commercial fish farm were anaesthetized and infected with 100 µl (10^6 $TCID_{50}$ ml^{-1}) of the nodavirus strain 475-9/99 by intra muscular injection. 15 control individuals were injected with 100 µl of Minimum Essential Medium (MEM). The fish were sacrificed by overexposure of anesthetic after 24 h post infection and brains removed aseptically and frozen for RNA isolation from both anaesthetized groups.

2.2 RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Organs were pooled and homogenized in 1 ml of Trizol and mixed with 200 µl of chloroform. The suspension was then centrifuged at 12000xg for 15 min. The clear upper phase was aspirated and placed in a clean tube. 500 µl of isopropanol were then added, and samples were again centrifuged at 12000xg for 10 min. The RNA pellet was washed with 75% ethanol, dissolved in diethylpyrocarbonate-treated water and stored at -80 °C.

2.3 SSH technique

The Suppression Subtractive Hybridization technique (SSH) (Diatchenko et al., 1996) was used to characterize new genes involved in anti-viral innate immunity in the disease model involving nodavirus and sea bream. Briefly, cDNA was synthesized from 1µg of each brain RNA (infected and control) using the SMART PCR cDNA Synthesis Kit

(Clontech), which allowed the full-length amplification of cDNA from mRNA transcripts. A SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech). The cDNA from the tester and from the driver were digested with *Rsa* I, and the tester cDNA was then ligated to either two different cDNA adaptors. During a first hybridization, excess driver was added to tester cDNA samples, which were then denatured and allowed to anneal. In the second hybridization, the two primary hybridization samples were mixed without denaturation. To further select for differentially expressed sequences, denatured driver cDNA was again added to these hybrid samples. As a result, the remaining subtracted, equalized single-stranded tester cDNA reassociated to form hybrids with a different adaptor on each end. This forward-subtracted sample (genes present or up-regulated in infected tissues compared with controls) was then used in PCR to amplify the differentially expressed sequences. PCR mixture was ligated using TOPO TA cloning kit (Invitrogen) and transformed in *E. coli* competent cells. A reverse subtracted library was also performed following the same protocol than the forward subtraction, in order to identify genes present or up-regulated in controls compared with infected tissues.

Selected colonies were amplified by PCR using Nested PCR primer 1 and 2R from PCR-Select cDNA Subtraction Kit. Agarose gel electrophoresis was performed to visualize the amplified fragments and to select by size the samples to be sequenced and arrayed. The PCR profile consisted of: initial denaturation for 5 min at 94 ºC; 35 cycles of 30 s denaturation at 94 ºC, 30 s annealing at 65 ºC and 1.5 min elongation at 72 ºC; final extension for 7 min at 72 ºC. Excess of primers and nucleotides was removed by enzymatic digestion using 10 U and 1 U of ExoI and SAP, respectively (Amersham Biosciences) at 37 ºC for 1 h followed by inactivation of the enzymes at 80 ºC for 15 min. DNA sequencing was performed using a BigDye terminator Cycle Sequencing Ready Reaction Kit and an automated DNA sequencer ABI PRISM^{TM} 377. Basic Local Alignment Search Tool (BLAST) analysis from the National Center for Biotechnology

Information was used to search GenBank for homologous nucleotide and protein sequences.

2.4 Macroarray construction

In order to confirm the expression of the generated ESTs by SSH and also to follow this expression in three additional sampling points (1, 3 and 7 days post infection), PCR fragments higher than 300 bp were spotted on macroarrays, which were constructed at DNA chips Service-SCSIE and Department of Biochemistry and Molecular Biology (University of Valencia, Spain). Briefly, BioGrid (BioRobotics, UK) was used as the spotting robot and macroarrays were made by printing the PCR products onto a positively charged nylon membrane (Amershan Hybond N+). Sea bream RNA extracted from newly infected fish used in the gene expression studies (see below) was labeled by reverse transcription with ^{33}P-α-dCTP. The hybridization protocol used was as follows: macroarrays were pre-treated for 30 min at 80 °C with 0.5% SDS to remove particles deposited during array printing and filters were pre-hybridized with 5 ml pre-hybridization solution (the same as used for hybridization but without the radioactive sample). The pre-hybridization solution was then replaced with 5 ml of the same solution containing the radioactive sample and allowed to hybridize. After the washing step, membranes were kept humid, sealed in Saran wrap and exposed to an imaging plate (BAS-MP, FujiFilm). For new hybridizations, filters were stripped by pouring 3x150 ml boiling stripping buffer over the membrane. For methodology details see Alberola et al. (2004). Images were acquired using a FujiFilm FLA3000 Phosphorimager. Spot intensities were measured as ARM density (artifact-removed density), background and sARM density (background-corrected ARM density) by using the Array Vision software (Imaging Research Canada).

2.5 Expression studies.

The original macroarray data set was prepared for clustering genes in order to group ESTs with similar expression pattern. Hybridization values were filtered using GEPAS software package (http://gepas.bioinfo.cnio.es), log2 transformed and used for cluster analysis. The TIGR (The Institute for Genome Research) MultiExperiment Viewer program http://www.tigr.org/software/tm4/ was used to derive the *k*-means and for hierarchical tree clustering analysis displaying 104 sequences for the TIGR analysis (Soukas et al., 2000).

Selected subtracted genes, based on macroarray fold change values, were evaluated *in vivo* by semiquantitative and quantitative Real Time PCR (qPCR). Eighteen fish were i.m. challenged with 100 µl of nodavirus (10^6 $TCID_{50}$ ml^{-1})/fish and eighteen fish were injected i.m. with 100 µl of cell culture medium as control. The same procedure was also carried out at the same time with intracranial (i.c.) injection. Sea bream β-actin expression (F-TCGGTCGCCCCAGGCATC; R-CTCCTTAATGTCACGCACGATTT) was used to normalize the amount of cDNA present in each PCR experiment. Fish were sacrificed 1, 3 and 7 days post challenge (three pools of two fish each one) and brain was removed aseptically and frozen for RNA isolation. RNA (5-10 µg) was extracted as described above and then treated with DNase I previously to reverse transcription (RT-PCR). RT-PCR was performed using SuperScript II RNase H-Reverse Transcriptase and oligo $(dT)_{12-18}$ (Invitrogen) following the manufacturer's instructions. For semiquantitative PCR, cDNA amplification was performed using specific primers designed by Primer3 software (Rozen and Skaletsky, 2000). All PCR reactions were carried out in a final volume of 25 µl containing 1 µl 10 mM dNTP mix, 0.125 µl Taq polymerase (5 units μl^{-1}), 2.5 µl Taq 10x buffer, 1.25 µl $MgCl_2$ 25 mM, 1.25 µl of each primer (10 µM) and 1 µl of cDNA. Reactions were conducted with a denaturing step of 94 ºC for 5 min followed by 30 cycles of 94 ºC 30 s, 55 ºC 30 s and 72 ºC 30 s, and 7

min at 72 °C for the final extension. Quantitative PCR assays were performed using the GeneAmp 5700 sequence detection system (Applied Biosystems). 0.5 µl of each primer (10 µM) were mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Fold units were calculated dividing the expression fold changes of the candidate genes by the expression fold changes of the housekeeping gene (β-actin).

3. RESULTS AND DISCUSSION

Nodavirus infection causes high losses to sea bass cultures in the Mediterranean (Le Breton et al., 1997). Sea bream can act as a carrier for the virus and transfer it to nearby sea bass cultures (Aranguren et al., 2002). In the present study we analyzed the effect of nodavirus on sea bream brain transcriptome. The brain was selected because is the main target of this virus. Studies on brain transcriptome against viral infections are not abundant in higher vertebrates (Gruslin et al., 2005; Paulus et al., 2006) and to our knowledge this is the first in vivo analysis of the transcriptional response of fish central nervous system (CNS) to a virus infection.

The SSH method was used to generate a subtracted cDNA library enriched in gene transcripts differentially expressed after 1 day post infection in brains of sea bream infected with nodavirus. Following cloning and transformations, 952 bacterial clones (476 forward and 476 reverse) were isolated and amplified by PCR. Among them, 280 forward and 85 reverse ESTs were sequenced. Sequence homology searching was conducted within GeneBank using BLAST, taking into account BlastX and tBlastX entries. For the forward ESTs, 41 out of 280 (14.64 %) showed significant similarities ($\leq 10^{-3}$) with mitochondrial or nuclear genes from different organisms; 184 out of 280 (65.71 %) showed e-values higher than 10^{-3} and 55 out of 280 (19.64 %) displayed no

similarity or repetitive sequences. With regard to the reverse ESTs, 47 out of 85 (55.29 %) showed significant similarities; 28 out of 85 (32.94 %) displayed expectation e-values higher than 10^{-3} and 10 out of 85 (11,76 %) showed no hits or repetitive sequences (Figure 1). These percentages were quite similar to other non-model organisms cDNA libraries or subtracted libraries obtained after infection or immunostimulation, as described for the Japanese flounder *Paralichthys olivaceus* (Inoue et al., 1997; Nam et al., 2000; Kono and Sakai, 2001); rainbow trout *Oncorhynchus mykiss* (Bayne et al., 2001; Liu et al., 2002); common carp *Cyprinus carpio* (Savan and Sakai, 2002; Kono et al., 2004) etc. The clones that showed no similarity, repetitive sequences or expected e-values higher than 10^{-3} revealed unknown sea bream genes that will be characterized in future studies. Several of the significant ESTs represented fragments of the same gene. Consequently, the total number of differential genes represented by these clones was 16 and 26 for genes up-regulated in infected and control tissues, respectively. The ESTs differentially expressed were deposited under GeneBank accession numbers from **EE191544** to **EE191791**.

Most of the ESTs differentially expressed in infected tissues fell in gene categories related to cell structure, transcription, cell signaling or different metabolic routes. Other interesting putative homologies corresponded to genes expressed in stress responses as heat shock proteins (Hsp-70) and to immune related genes such as the Fms interacting protein, TNF-α induced protein, interferon induced with helicase C domain protein (mda-5), etc. The best matching was found for Fms interacting protein (BlastX, expectation value 2e-65). A list of the significant genes obtained after Blast searching is included in Table 1. For the reverse library (genes up-regulated in normal tissues), there was mostly abundance of ribosomal and metabolic transcripts, revealing genes highly expressed for energy production and translation (Venier et al., 2003) (Table 2). The best

matching was found for adenin nucleotide translocator s6 (BlastX, expectation value 1e-78).

To validate the SSH library obtained 1 day post-infection and also to increase the number of sampling points, a large-scale mRNA analysis using macroarrays was conducted. 385 forward and 380 reverse PCR products were selected by size (300-800 bp) and printed onto a nylon membrane. Macroarray with these cDNA spots was hybridized with 1, 3 and 7 days p.i. cDNA obtained from the tissue expression experiment (Figure 2a, 2b and 2c, respectively). The statistical analysis of macroarrays displayed an over expression value (> 2) or suppression value (< 2) called fold change, which was used for TIGR analysis in order to group ESTs with similar expression pattern. Up to 12 groups with different expression patterns were constructed, although they could be finally grouped in five main clusters (Figure 3). The smaller cluster (4.80% of the ESTs) displayed one unique group with 5 genes up-regulated 1, 3 and 7 days post-infection. The larger cluster (36.54% of the genes) corresponded to four groups with ESTs suppressed in the three sampling points. 27.88% of the ESTs considered formed a cluster with light to intense up-regulation 1 day post-infection and then mid and late term suppression. A cluster (21.15% of the genes) showed up-regulation after 3 days and inhibition 1 and 7 days post-infection. Finally, 9.62% of the clones included genes with up-regulation after 1 and 7 days and suppression 3 days after nodavirus infection (Figure 4). These results showed that approximately half (42.31%) of the subtracted ESTs (groups 1, 3, 5) and therefore supposedly induced by the infection after one day, were actually over expressed *in vivo*. However, the remaining percentage (57.69%) corresponded to groups of genes (2, 4), which were suppressed 1 day post-infection.

Taking into account the clustering analysis and the hybridization screening procedure, the ESTs up-regulated (fold change > 2) with significant e-values were summarized in Table 3. Several ESTs potentially induced by virus exposure were selected for *in vivo*

expression studies, based on their homology to known genes known to be involved in immune function or cell signaling. Sequences were amplified by PCR using specific primers, to further confirm the differential expression of these candidate genes. We observed a clear difference between infected and control samples for two candidate genes, the ubiquitin conjugating enzyme 7 interacting protein (amplified by primers F-TCCCGACAACTGTAGAGCAA; R-GATCCAATCACAGCCGTCTT) and the mda-5 protein (amplified by primers F-CATCGAGATCATCGAGGACA; R-CATCATGGAACCCCATCTCT), where a basal expression was observed in the control samples, but an increase of expression was promoted by virus treatment in all pools studied. This result was observed both using semiquantitative (data not shown) and quantitative PCR. For the ubiquitin conjugating enzyme 7 interacting protein, an induction was observed after 1, 3 and 7 days p.i. both with i.m. injection as with i.c. injection (Figure 5a and 5c, respectively). The ubiquitin-like family proteins were also the most represented genes in a subtracted library from rainbow trout leukocytes subjected to VHSV infection. These genes were also up-regulated during experimental infection *in vivo* and likely play a role in the course of viral infection (O'Farrell et al., 2002). For the mda-5, the expression results were similar to the ones obtained for ubiquitin conjugating enzyme 7 interacting protein (Figure 5b and 5d, respectively). The differences of expression for both genes were much more obvious at day 1 by intracranial injection as brain is the main target of nodaviruses. Nevertheless, these differences were more marked 3 days after infection by intramuscular injection, which may suggest that immune responses seem to be activated when nodaviruses reach the brain and start replication.

Ubiquitination plays an important role in various cellular functions such as apoptosis, cell cycle progression, transcription and endocytosis (Hershko and Ciechanover, 1998). One of the principal roles is to regulate the half-life of proteins by targeting them for

26S proteasomal degradation. A cascade of enzymes (E1, E2 and the RING domain E3 ligases) transfers the free ubiquitin to a specific substrate that will be degraded by the proteasome (Ito et al., 2001; Pickart, 2001; Vaux and Silke, 2005). This RING finger motif, contained in our amplified EST, is a highly conserved module defined by cysteines and histidines that coordinates two zinc ions (Borden and Freemont, 1996; Lorick et al., 1999). Figure 6 shows an aligment of the sea bream domain with other representatives of this family. The IAPs proteins (inhibitor of apoptosis) also contain this RING domain, which might ubiquitinate proteins (including themselves) that interact with them directly through the RING domain such as caspases, and target them for degradation by the 26S proteasome. This could be a simple explanation for how IAPs prevent apoptosis. So far we do not know which is the function of the gene codifying the ubiquitin conjugating enzyme 7 interacting protein found in our SSH library. Since its expression increases after nodavirus infection, it may possible play a role in the chain of decisions that cells have to take to follow the cell death program, depending on the type and stage of infection. Moreover, the vacuolation observed in sea bream after nodavirus infection in brain, spinal cord and retina, could be the result of an apoptotic mechanism that progresses in neurodegeneration of these tissues. Indeed, this neurodegenerative and neuroinflammatory process of the brain has been already described in humans with different viral encephalitis. Several studies have shown that the neurodegenerative process is associated with up-regulation of apoptotic genes (that may have both pathogenic and protective effects), cytokines and chemokines and those involved in anti-viral responses such as the interferon-inducible molecules (Kim et al., 2004; Masliah et al., 2004; Everall et al., 2005; Venter et al., 2005; Sabri et al., 2006; Sutton et al., 2006). Further studies will be necessary to investigate the possible role of our protein as an apoptotic anti-viral mechanism during nodavirus infection.

The involvement of mda-5 or helicard in innate immune responses has been described in humans and mice (Kang et al., 2002, 2004; Andrejeva et al., 2004; Kawai et al., 2005; Meylan et al., 2005; Yoneyama et al., 2005; Hiscott et al., 2006; Seth et al., 2006), and it plays an important role in the synthesis and secretion of type I interferons (IFN) (Honda et al., 2005). Mda-5 is induced by IFN-β and participates in the cytoplasmic recognition of dsRNA through its helicase domain (Yoneyama et al., 2004; Kato et al., 2005; Meylan et al., 2005; Rothenfusser et al., 2005; Yoneyama et al., 2005; Meylan and Tschopp, 2006; Werts et al., 2006). Figure 7 shows an alignment of the conserved helicase sequences. Helicases bind dsRNA and then associate to another protein that functions downstream called MAVS (Mitochondrial Antiviral Signaling) (Kawai et al., 2005; Meylan et al., 2005; Xu et al., 2005; Seth et al., 2005, 2006). MAVS can signal the downstream kinase pathways that turn on the IFN-ß. Also, mda-5 may contribute to apoptosis induction through its CARD domain (Kang et al., 2002, 2004). Interestingly, ubiquitination plays a role during this cascade of events as well, allowing free NF-κB to activate the transcription of type I IFN (Silverman and Maniatis, 2001). This let us speculate about the over expression of the two candidate genes found in our sea bream SSH library.

Both ubiquitin conjugating enzymes and helicard functions have been described in mammals, but as far as we know, this is the first time that these genes have been identified in fish. If these genes are really involved in apoptosis and IFN regulation, as an innate response against nodavirus infection in teleost, should be further investigated in the future. Cells of the immune system are always present in the brain and they might be responsible for some of the gene expression related to immune response detected in the present study. One of the future steps in our research will be to identify which brain cells are responsible for the immune genes expressed. The role of these genes in the CNS response to infections remains to be understood but it may be possible that

differentially regulated genes reported here may include potential diagnostic and therapeutic targets for viral encephalitis and other neurodegenerative or neuroinflammatory diseases.

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FIGURE LEGENDS

Figure 1. Percentage of ESTs for forward and reverse libraries based on e-value obtained from GeneBank searching.

Figure 2. Membrane support array. a: 1 day post challenge RNA hybridization; b: 3 days post challenge RNA hybridization; c: 7 days post challenge RNA hybridization.

Figure 3. Groups constructed by cluster analysis (TIGR) using fold change value from macroarray statistical analysis. The percentages correspond to the total number of genes included in each cluster. (G= group).

Figure 4. Annotated genes for clusters with similar expression pattern in the three sampling points.

Figure 5. Real time PCR results for ubiquitin conjugating enzyme 7 interacting protein and interferon induced protein with helicase C domain protein 1 after 1, 3 and 7 days nodavirus infection. a) and b) intramuscular injection. c) and d) intracranial injection. Fold units were calculated dividing the expression fold changes of the candidate genes by the expression fold changes of the housekeeping gene (β-actin).

Figure 6. Predicted amino acid sequence of sea bream ubiquitin conjugating enzyme 7 interacting protein aligned with other species sequences. (`*´ : indicates positions which a single, fully conserved residue. `:´ : indicates that one of the following strong groups

is fully conserved. `.´ : indicates that one of the following weaker groups is fully conserved). Histidines (dark grey) and cysteines (light grey) involved in RING finger domain are highlighted.

Figure 7. Aligment of the amino acid sequence of sea bream helicase with other species helicases. (`*´ : indicates positions which a single, fully conserved residue. `:´ : indicates that one of the following strong groups is fully conserved. `.´ : indicates that one of the following weaker groups is fully conserved).

Table 1. Up-regulated ESTs (infected brain). Base pairs (Bp) are given for the longest fragment.

Clone number (bp)	Nº clones	Nº contigs	Homology	e-value (Blast identity)	Species
Mitochondrial					
S1400 (364)	1		Mitochondrial DNA complete genome	1e-35 (tBlastX)	*Pagrus major*
Metabolic					
S2255 (307)	1		Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	1e-06 (tBlastX)	*Danio rerio*
S2000 (545)	1		Ubiquitin specific protease 9	2e-04 (tBlastX)	*Gallus gallus*
Cell structure					
S2246 (178)	4	1	Formin binding protein 1-like	3e-11 (BlastX)	*Danio rerio*
S2105 (449)	11	1	H2A Histone family	8e-29 (BlastX)	*Danio rerio*
Immune related					
S2167 (553)	3	1	Fms interacting protein	2e-65 (BlastX)	*Rattus norvegicus*
S1819 (458)	1		OmpA-related protein	8e-15 (BlastX)	*Idiomarina baltica*
S2100 (341)	1		TNF alfa-induced protein 8	3e-11 (BlastX)	*Danio rerio*
S2250 (573)	3	1	Ubiquitin conjugating enzyme 7 interacting protein	8e-48 (Blastx)	*Bos taurus*
S2136 (365)	1		Interferon induced with helicase C domain protein 1	2e-27 (BlastX)	*Canis familiaris*
S1782 (167)	1		Hsp70-1 gene	4e-16 (tBlastX)	*Fugu rubripes*
Cell signaling					
S2078 (284)	5	1	Guanine nucleotide binding protein	3e-10 (tBlastX)	*Gallus gallus*
Transcription/translation					
S2111 (180)	3	1	Transducer of ERBB2	9e-07 (tBlastX)	*Danio rerio*
S2104 (638)	3	1	Zinc finger homeodomain	2e-31 (BlastX)	*Danio rerio*
Other					

S2183 (549)	1	Protein phosphatase 2, regulatory subunit	3e-07 (BlastX)	*Danio rerio*
S1407 (77)	1	GHRH gene for growth hormone releasing	1e-06 (tBlastX)	*Homo sapiens*

Table 2. Up-regulated ESTs (control brain). Base pairs (Bp) are given for the longest fragment.

Clone number (bp)	Nº clones	Nº contigs	Homology	e-value (Blast identity)	Species
Mitochondrial					
S2354 (279)	5	2	Mitochondrial DNA complete genome	2e-45 (tBlastX)	*Pagrus major*
Metabolic					
S2357 (598)	2	1	Cytochrome c oxidase subunit I	5e-61 (BlastX)	*Sparus aurata*
S2377 (324)	3	2	Cytochrome c oxidase subunit II	4e-29 (BlastX)	*Galaxias maculatus*
S2337 (502)	1		Adenin nucleotide translocator s6	1e-78 (BlastX)	*Takifugu rubripes*
S2387 (119)	1		Sarcoplasmic/endoplasmic reticulum calcium ATPase	3e-06 (tBlastX)	*Makaira nigricans*
S1565 (721)	1		Proteasome 26S non-ATPase subunit	1e-72 (BlastX)	*Danio rerio*
Cell structure					
S2304 (592)	1		Ependymin	1e-13 (BlastX)	*Chalceus sp.*
Immune related					
S2297 (306)	1		C-type natriuretic peptide	7e-11 (BlastX)	*Oreochromis mossambicus*
S2336 (594)	1		Ferritin heavy chain	2e-56 (BlastX)	*Pagrus major*
S1573 (122)	1		Septin 8	6e-13 (BlastX)	*Gallus gallus*
S2339 (340)	1		Hsp-90	2e-37 (BlastX)	*Dicentrarchus labrax*
Cell signaling					
S2330 (557)	2	1	Stathmin-like protein	6e-56 (BlastX)	*Danio rerio*
Transcription/translation					
S1607 (126)	1		Splicing factor PRP8	9e-09 (BlastX)	*Xenopus laevis*
Ribosomal					
S2341 (266)	11	2	18S ribosomal RNA gene	3e-45 (tBlastX)	*Perca fluviatilis*
S2327 (323)	2	1	60S Ribosomal protein L29	2e-21 (BlastX)	*Pan troglodytes*

S2358 (475)	1		60S Ribosomal protein L23	5e-53 (BlastX)	*Sparus aurata*
S2373 (225)	1		60S Ribosomal protein L11	2e-11 (BlastX)	Synthetic construct
S2375 (312)	1		60S Ribosomal protein L18	1e-41 (BlastX)	*Pagrus major*
S2379 (301)	1		60S Ribosomal protein L27a	4e-21 (BlastX)	*Sparus aurata*
S2383 (341)	1		40S Ribosomal protein S27a	3e-39 (Blastx)	*Pseudopleuronectes americanus*
S1568 (173)	3	1	40S Ribosomal protein S16	3e-17 (BlastX)	*Gallus gallus*
S1615 (157)	1		60S Ribosomal protein L24	9e-20 (tBlastX)	*Pagrus major*
S1566 (163)	1		28S rRNA	5e-25 (tBlastX)	*Gallus gallus*
Hormonal regulation					
S1560 (112)	1		Thyroid hormone receptor interactor TRIP12	3e-10 (BlastX)	*Danio rerio*
Other					
S2361 (290)	1		Male specific protein	6e-10 (BlastX)	*Sarotherodon galilaeus*
S2378 (362)	1		Nac-alfa mRNA	5e-09 (BlastX)	*Pagrus major*

Table 3. Up-regulated ESTs (fold change >2). The following sequences are represented in this table for more than one clone, but those with lower fold change were omitted for table clarifying. **Day 1 post-infection**: ubiquitin conjugating enzyme 7 interacting protein (2 clones); **Day 3 post-infection**: mitochondrial DNA (5 clones), stathmin-like protein (2 clones); **Day 7 post-infection**: formin binding protein 1-like (2 clones), fms interacting protein (2 clones), mitochondrial DNA (2 clones), 18s ribosomal gene (3 clones).

Clone number (bp)	Homology (Blast identity)	Fold change
Day 1 post-infection:		
S1782 (167)	Hsp70-1 gene (4e-16 tBlastX)	6.30
S2100 (341)	TNF alfa-induced protein 8 (3e-11 BlastX)	5.59
S2086 (672)	Ubiquitin conjugating enzyme 7 interacting protein (2e-63 BlastX)	3.99
Day 3 post-infection:		
S2361 (290)	Male specific protein (6e-10 BlastX)	5.27
S2354 (279)	Mitochondrial DNA complete genome (2e-45 tBlastX)	5.14
S2389 (515)	Stathmin-like protein (7e-55 BlastX)	3.73
S2375 (312)	60S Ribosomal protein L18 (1e-41 BlastX)	3.56
S2336 (594)	Ferritin heavy chain (2e-56 BlastX)	3.46
S2378 (362)	Nac-alfa mRNA (5e-09 BlastX)	2.80
S2385 (321)	Cytochrome c oxidase subunit II (2e-05 BlastX)	2.37
S2383 (341)	40S Ribosomal protein S27a (3e-39 Blastx)	2.17
S1824 (464)	H2A Histone family (9e-28 BlastX)	2.10
S2308 (265)	18S ribosomal RNA gene (2e-42 tBlastX)	2.07
Day 7 post-infection:		
S2096 (177)	Formin binding protein 1-like (3e-11 BlastX)	16,28
S1804 (524)	Fms interacting protein (2e-61 BlastX)	12,51
S2007 (465)	Ubiquitin conjugating enzyme 7 interacting protein (3e-21 BlastX)	12.32
S1782 (167)	Hsp70-1 gene (4e-16 tBlastX)	11.47
S2361 (290)	Male specific protein (6e-10 BlastX)	4.53
S1400 (364)	Mitochondrial DNA complete genome (1e-35 tBlastX)	3.96
S2136 (365)	Interferon induced with helicase C domain protein 1 (2e-27 BlastX)	2.56
S2389 (515)	Stathmin-like protein (7e-55 BlastX)	2.32
S2308 (265)	18S ribosomal RNA gene (2e-42 tBlastX)	2.25

Figure 1.

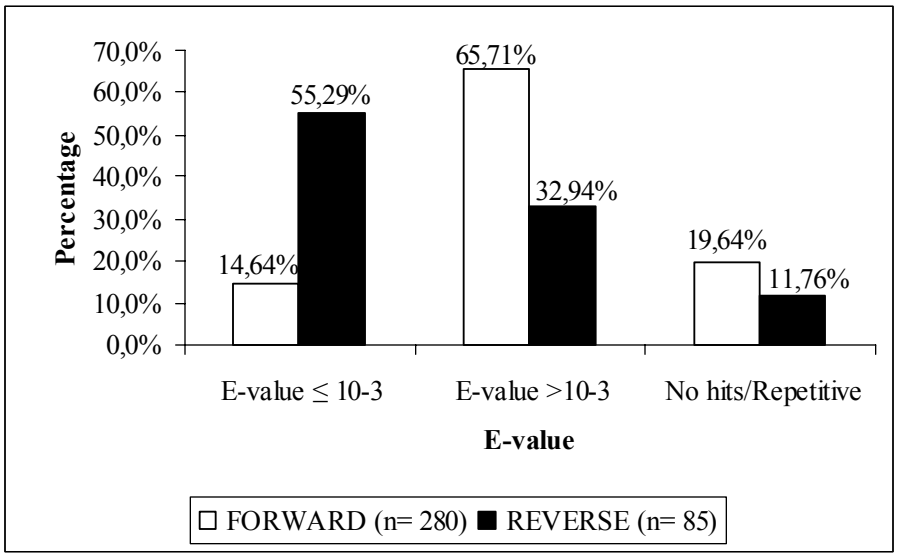

70,0%
60,0%
50,0%
40,0%
30,0%
20,0%
10,0%
0,0%
Percentage
14,64%
55,29%
65,71%
32,94%
19,64%
11,76%
E-value ≤ 10-3
E-value >10-3
No hits/Repetitive
E-value
FORWARD (n= 280)
REVERSE (n= 85)

Figure 2.

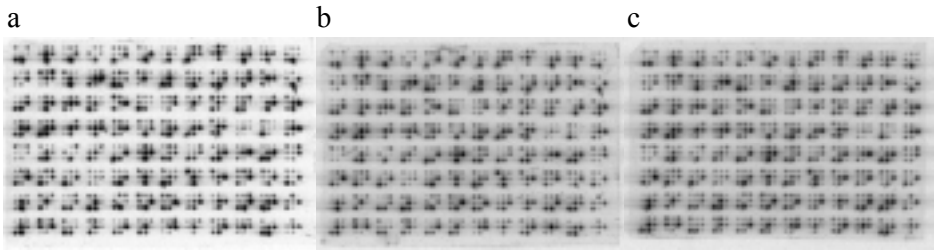


Figure 3.

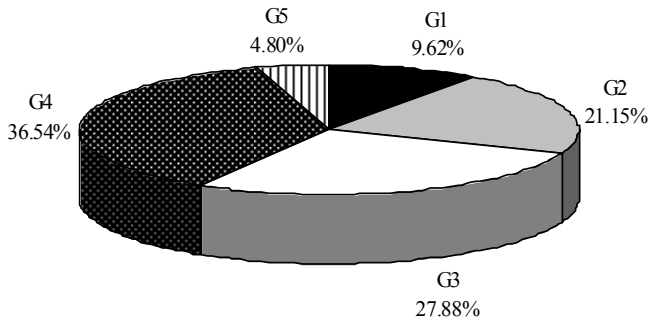
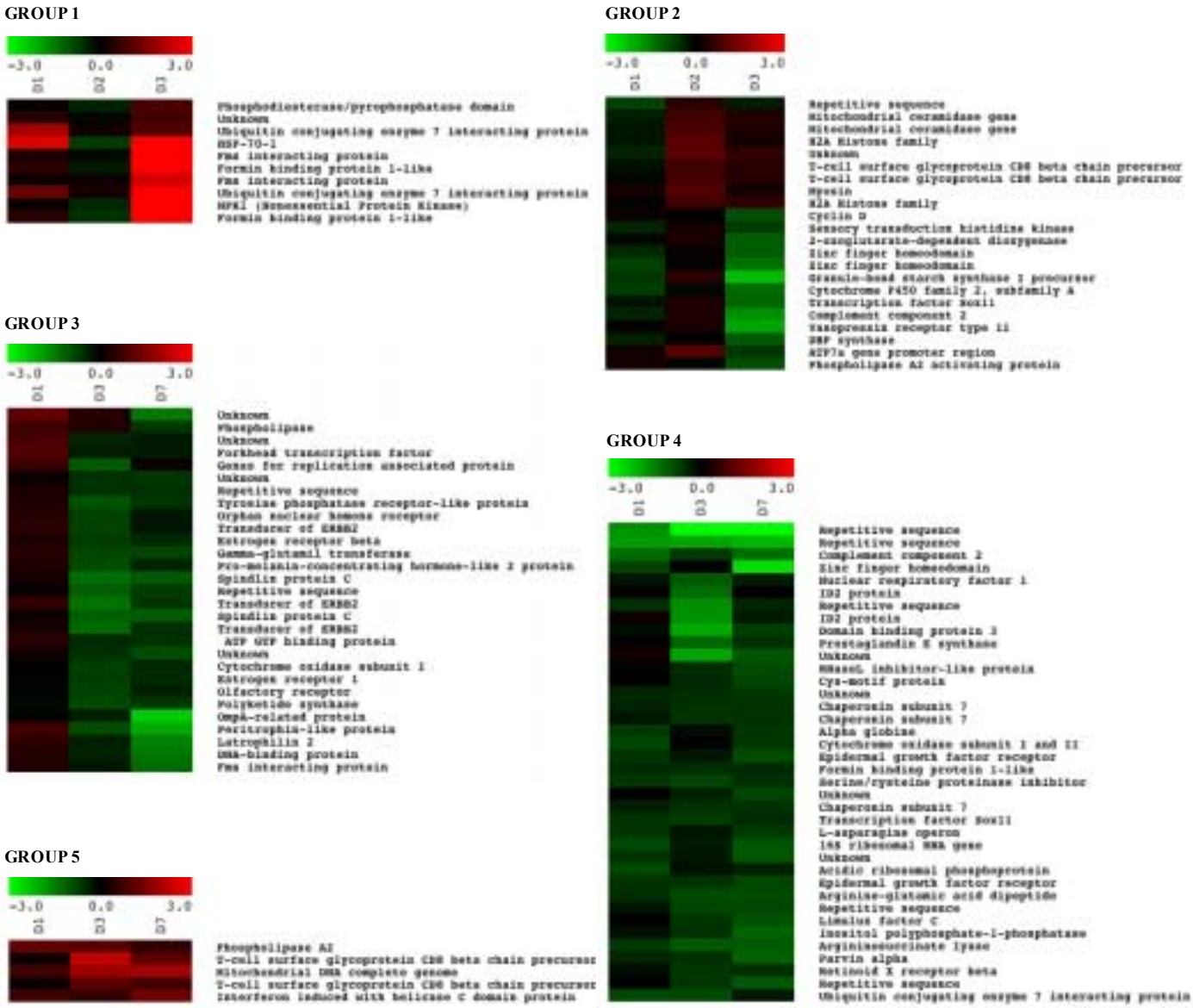


Figure 4.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D1 D2 D3
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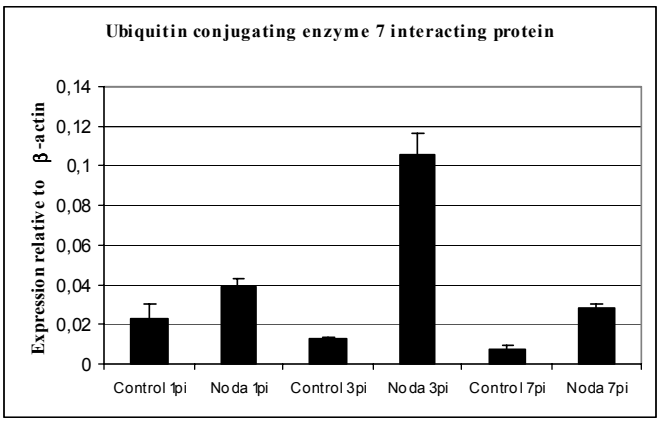
Ubiquitin conjugating enzyme 7 interacting protein
HSP-70-1
Fms interacting protein
Formin binding protein 1-like
Fms interacting protein
Ubiquitin conjugating enzyme 7 interacting protein
HPK1 (Nonessential Protein Kinase)
Formin binding protein 1-like
GROUP 2
-3.0 0.0 3.0
D1 D2 D3
Repetitive sequence
Mitochondrial ceramidase gene
Mitochondrial ceramidase gene
H2A Histone family
Unknown
T-cell surface glycoprotein CD8 beta chain precursor
T-cell surface glycoprotein CD8 beta chain precursor
Myosin
H2A Histone family
Cyclin D
Sensory transduction histidine kinase
2-oxoglutarate-dependent dioxygenase
Zinc finger homeodomain
Zinc finger homeodomain
Granule-bound starch synthase 1 precursor
Cytochrome P450 family 2, subfamily A
Transcription factor Sox11
Complement component 2
Vasopressin receptor type 1i
DHP synthase
ATP7a gene promoter region
Phospholipase A2 activating protein
GROUP 3
-3.0 0.0 3.0
D1 D3 D7
Unknown
Phospholipase
Unknown
Forkhead transcription factor
Genes for replication associated protein
Unknown
Repetitive sequence
Tyrosine phosphatase receptor-like protein
Orphan nuclear hormone receptor
Transducer of ERBB2
Estrogen receptor beta
Gamma-glutamil transferase
Pro-melanin-concentrating hormone-like 2 protein
Spindlin protein C
Repetitive sequence
Transducer of ERBB2
Spindlin protein C
Transducer of ERBB2
ATP UTP binding protein
Unknown
Cytochrome oxidase subunit 1
Estrogen receptor 1
Olfactory receptor
Polyketide synthase
Ompa-related protein
Peritrophin-like protein
Latrophilin 2
DNA-binding protein
Fms interacting protein
GROUP 4
-3.0 0.0 3.0
D1 D3 D7
Repetitive sequence
Repetitive sequence
Complement component 2
Zinc finger homeodomain
Nuclear respiratory factor 1
ID2 protein
Repetitive sequence
ID2 protein
Domain binding protein 3
Prostaglandin E synthase
Unknown
RNaseL inhibitor-like protein
Cys-motif protein
Unknown
Chaperonin subunit 7
Chaperonin subunit 7
Alpha globins
Cytochrome oxidase subunit I and II
Epidermal growth factor receptor
Formin binding protein 1-like
Serine/cysteine proteinase inhibitor
Unknown
Chaperonin subunit 7
Transcription factor Sox11
L-asparagine operon
18S ribosomal RNA gene
Unknown
Acidic ribosomal phosphoprotein
Epidermal growth factor receptor
Arginine-glutamic acid dipeptide
Repetitive sequence
Limulus factor C
Inositol polyphosphate-1-phosphatase
Argininesuccinate lyase
Parvin alpha
Retinoid X receptor beta
Repetitive sequence
Ubiquitin conjugating enzyme 7 interacting protein
GROUP 5
-3.0 0.0 3.0
D1 D3 D7
Phospholipase A2
T-cell surface glycoprotein CD8 beta chain precursor
Mitochondrial DNA complete genome
T-cell surface glycoprotein CD8 beta chain precursor
Interferon induced with helicase C domain protein

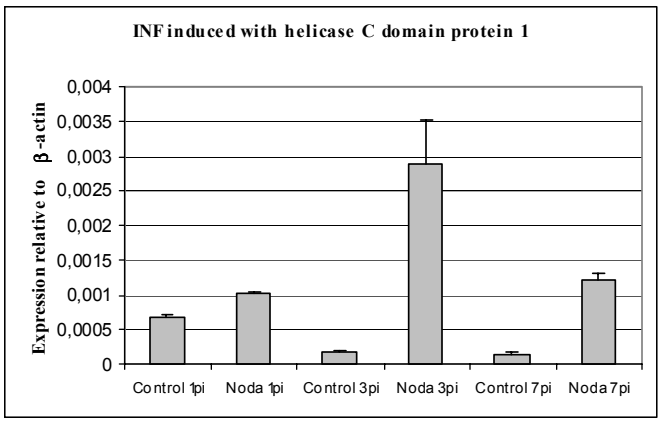
Figure 5.

Intramuscular injection

a

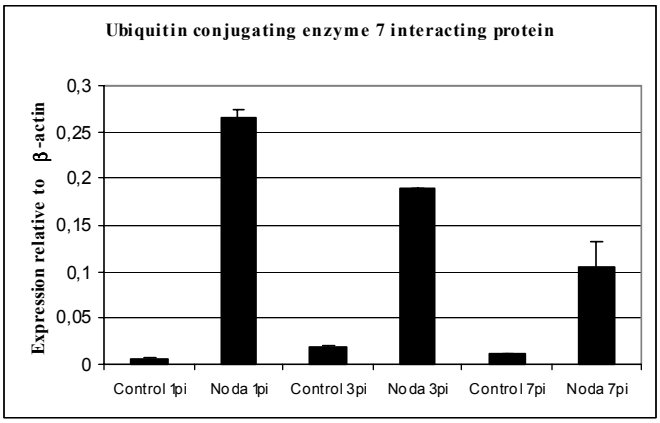


b



Intracranial injection

c



d

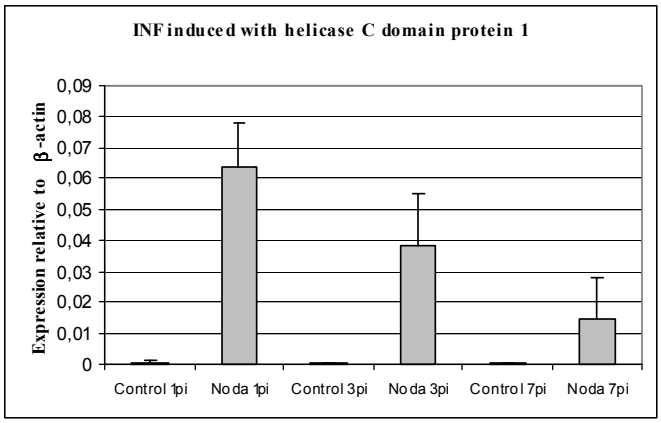


Figure 6.

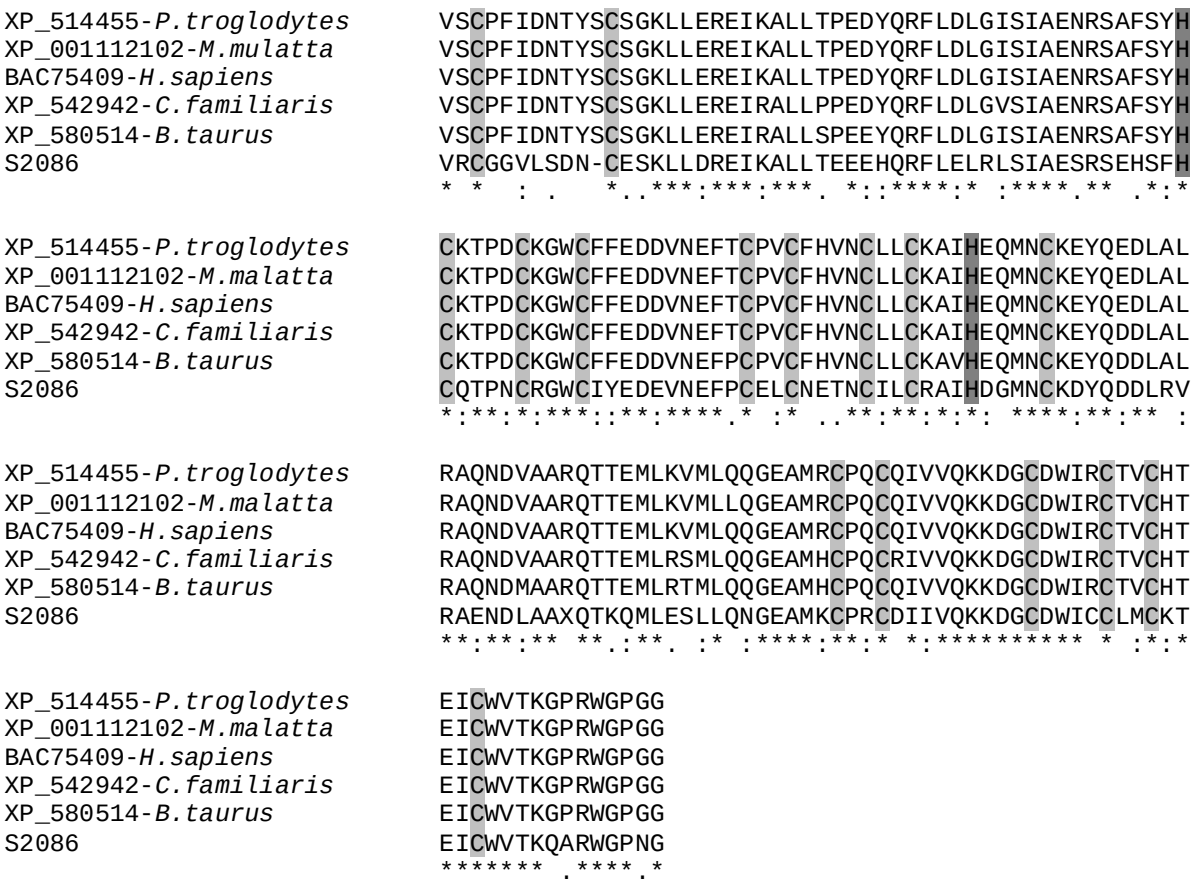

XP_514455-P.troglodytes VSCPFIDNTYSCSGKLLEREIKALLTPEDYQRFLDLGISIAENRSAFSYH
XP_001112102-M.mulatta VSCPFIDNTYSCSGKLLEREIKALLTPEDYQRFLDLGISIAENRSAFSYH
BAC75409-H.sapiens VSCPFIDNTYSCSGKLLEREIKALLTPEDYQRFLDLGISIAENRSAFSYH
XP_542942-C.familiaris VSCPFIDNTYSCSGKLLEREIRALLPPEDYQRFLDLGVSIAENRSAFSYH
XP_580514-B.taurus VSCPFIDNTYSCSGKLLEREIRALLSPEEYQRFLDLGISIAENRSAFSYH
S2086 VRCGGVLSDN-CESKLLDREIKALLTEEEHQRFLELRLSIAESRSEHSFH
XP_514455-P.troglodytes CKTPDCKGWCFFEDDVNEFTCPVCFHVNCLLCKAIHEQMNCKEYQEDLAL
XP_001112102-M.malatta CKTPDCKGWCFFEDDVNEFTCPVCFHVNCLLCKAIHEQMNCKEYQEDLAL
BAC75409-H.sapiens CKTPDCKGWCFFEDDVNEFTCPVCFHVNCLLCKAIHEQMNCKEYQEDLAL
XP_542942-C.familiaris CKTPDCKGWCFFEDDVNEFTCPVCFHVNCLLCKAIHEQMNCKEYQDDLAL
XP_580514-B.taurus CKTPDCKGWCFFEDDVNEFPCPVCFHVNCLLCKAVHEQMNCKEYQDDLAL
S2086 CQTPNCRGWCIYEDEVNEFPCELCNETNCILCRAIHDGMNCKDYQDDLRV
XP_514455-P.troglodytes RAQNDVAARQTTEMLKVMLQQGEAMRCPQCQIVVQKKDGCDWIRCTVCHT
XP_001112102-M.malatta RAQNDVAARQTTEMLKVMLLQGEAMRCPQCQIVVQKKDGCDWIRCTVCHT
BAC75409-H.sapiens RAQNDVAARQTTEMLKVMLQQGEAMRCPQCQIVVQKKDGCDWIRCTVCHT
XP_542942-C.familiaris RAQNDVAARQTTEMLRSMLQQGEAMHCPQCRIVVQKKDGCDWIRCTVCHT
XP_580514-B.taurus RAQNDMAARQTTEMLRTMLQQGEAMHCPQCQIVVQKKDGCDWIRCTVCHT
S2086 RAENDLAAXQTKQMLESLLQNGEAMKCPRCDIIVQKKDGCDWICCLMCKT
XP_514455-P.troglodytes EICWVTKGPRWGPGG
XP_001112102-M.malatta EICWVTKGPRWGPGG
BAC75409-H.sapiens EICWVTKGPRWGPGG
XP_542942-C.familiaris EICWVTKGPRWGPGG
XP_580514-B.taurus EICWVTKGPRWGPGG
S2086 EICWVTKQARWGPNG

Figure 7.

```
Q9BYX4-H.sapiens            VQNMKPEEYAHKILELQMQSIMEKKMKTKRNIAKHYKN-NPSLITFLCKN
XP_001097292-M.mulatta      VQNMKPEEYAHKILELQMQSIMEKKMKTKRSIAKHYKN-NPSLITFLCKN
Q8R5F7-M.musculus           VQNMKPEEYAHKILELQVQSILEKKMKVKRSIAKQYND-NPSLITLLCKN
XP_615590-B.taurus          VQNMKPEEYAHKILELQMQSIMEKKMKTKRSIAKQFKG-KPSLINFLCKN
XP_545493-C.familiaris      VQNMNPEEYAHKILELQMQSIMEKKMKIKRSAAKCYKE-NPSLINFLCKN
S2136                       -RALEQEEYDKRIMEYQMQAIMENRVLAKKKKQKGMKNENPSSVKFSCRG
                             : :: *** ::*:* *:*:*:*:::  *:.  *  :  :** :.: *:.

Q9BYX4-H.sapiens            CSVLACSGEDIHVIEKMHHVNMTPEFKELYIVRENKALQKKCADYQINGE
XP_001097292-M.mulatta      CSVLACSGEDIHVIEKMHHVNMTPEFKELYIVRENKALQKKCADYQTNGE
Q8R5F7-M.musculus           CSMLVCSGENIHVIEKMHHVNMTPEFKGLYIVRENKALQKKFADYQTNGE
XP_615590- B.taurus         CGVPACSGEDIHVIEKMHHVNMTPEFKKLYLVRGNKALQTMCVDYQTNGE
XP_545493-C.familiaris      CSVLACSGEDIHVIEKMHHVNMTPVFKELYIVRENKALRKKFIDYQTNGE
S2136                       CNEHACSGEDIEIIEDMHRVNLTPQFRELFIQRENTTLQERHLDYETNGY
                            *.  .****:*.:**.**:**:** *: *:: * *.:*:    **: **

Q9BYX4-H.sapiens            IIC-KCGQAWGTMMVHKGLDLPCLKIRNFVVVFKNNSTKKQYKKWVELPI
XP_001097292-M.mulatta      IIC-KCGQAWGTMMVHKGLDLPCLKIRNFVVVFKNNSTKKQYKKWVELPI
Q8R5F7-M.musculus           IIC-KCGQAWGTMMVHKGLDLPCLKIRNFVVNFKNNSPKKQYKKWVELPI
XP_615590-B.taurus          IICNKCGQAWGTMMVHKGLDLPCLKIKNFVVVFQNNLPKKQYKKWVELPI
XP_545493-C.familiaris      IICKMCGQAWGTMMVHKGLDLPCLKIKNFVVIFKNNTSKKQYKKWVELPI
S2136                       IACKKCGERWGSMMLYLGRRPR----------------------------
                            * *  **: **:**:: *
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Table 1

Table 1. Up-regulated ESTs (infected brain). Bp is given for the longest fragment.

Clone number (bp)	Nº clones	Nº contigs	Homology	e-value (Blast identity)	Species
Mitochondrial					
S1400 (364)	1		Mitochondrial DNA complete genome	1e-35 (tBlastX)	*Pagrus major*
Metabolic					
S2255 (307)	1		Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	1e-06 (tBlastX)	*Danio rerio*
S2000 (545)	1		Ubiquitin specific protease 9	2e-04 (tBlastX)	*Gallus gallus*
Cell structure					
S2246 (178)	4	1	Formin binding protein 1-like	3e-11 (BlastX)	*Danio rerio*
S2105 (449)	11	1	H2A Histone family	8e-29 (BlastX)	*Danio rerio*
Immune related					
S2167 (553)	3	1	Fms interacting protein	2e-65 (BlastX)	*Rattus norvegicus*
S1819 (458)	1		OmpA-related protein	8e-15 (BlastX)	*Idiomarina baltica*
S2100 (341)	1		TNF alfa-induced protein 8	3e-11 (BlastX)	*Danio rerio*
S2250 (573)	3	1	Ubiquitin conjugating enzyme 7 interacting protein	8e-48 (Blastx)	*Bos taurus*
S2136 (365)	1		Interferon induced with helicase C domain protein 1	2e-27 (BlastX)	*Canis familiaris*
S1782 (167)	1		Hsp70-1 gene	4e-16 (tBlastX)	*Fugu rubripes*
Cell signaling					
S2078 (284)	5	1	Guanine nucleotide binding protein	3e-10 (tBlastX)	*Gallus gallus*
Transcription/translation					
S2111 (180)	3	1	Transducer of ERBB2	9e-07 (tBlastX)	*Danio rerio*
S2104 (638)	3	1	Zinc finger homeodomain	2e-31 (BlastX)	*Danio rerio*
Other					
S2183 (549)	1		Protein phosphatase 2, regulatory subunit	3e-07 (BlastX)	*Danio rerio*
S1407 (77)	1		GHRH gene for growth hormone releasing	1e-06 (tBlastX)	*Homo sapiens*

Table 2. Up-regulated ESTs (control brain). Bp is given for the longest fragment.

Clone number (bp)	Nº clones	Nº contigs	Homology	e-value (Blast identity)	Species
Mitochondrial					
S2354 (279)	5	2	Mitochondrial DNA complete genome	2e-45 (tBlastX)	*Pagrus major*
Metabolic					
S2357 (598)	2	1	Cytochrome c oxidase subunit I	5e-61 (BlastX)	*Sparus aurata*
S2377 (324)	3	2	Cytochrome c oxidase subunit II	4e-29 (BlastX)	*Galaxias maculatus*
S2337 (502)	1		Adenin nucleotide translocator s6	1e-78 (BlastX)	*Takifugu rubripes*
S2387 (119)	1		Sarcoplasmic/endoplasmic reticulum calcium ATPase	3e-06 (tBlastX)	*Makaira nigricans*
S1565 (721)	1		Proteasome 26S non-ATPase subunit	1e-72 (BlastX)	*Danio rerio*
Cell structure					
S2304 (592)	1		Ependymin	1e-13 (BlastX)	*Chalceus sp.*
Immune related					
S2297 (306)	1		C-type natriuretic peptide	7e-11 (BlastX)	*Oreochromis mossambicus*
S2336 (594)	1		Ferritin heavy chain	2e-56 (BlastX)	*Pagrus major*
S1573 (122)	1		Septin 8	6e-13 (BlastX)	*Gallus gallus*
S2339 (340)	1		Hsp-90	2e-37 (BlastX)	*Dicentrarchus labrax*
Cell signaling					
S2330 (557)	2	1	Stathmin-like protein	6e-56 (BlastX)	*Danio rerio*
Transcription/translation					
S1607 (126)	1		Splicing factor PRP8	9e-09 (BlastX)	*Xenopus laevis*
Ribosomal					
S2341 (266)	11	2	18S ribosomal RNA gene	3e-45 (tBlastX)	*Perca fluviatilis*
S2327 (323)	2	1	60S Ribosomal protein L29	2e-21 (BlastX)	*Pan troglodytes*
S2358 (475)	1		60S Ribosomal protein L23	5e-53 (BlastX)	*Sparus aurata*
S2373 (225)	1		60S Ribosomal protein L11	2e-11 (BlastX)	Synthetic construct
S2375 (312)	1		60S Ribosomal protein L18	1e-41 (BlastX)	*Pagrus major*
S2379 (301)	1		60S Ribosomal protein L27a	4e-21 (BlastX)	*Sparus aurata*

S2383 (341)	1		40S Ribosomal protein S27a	3e-39 (Blastx)	*Pseudopleuronectes americanus*
S1568 (173)	3	1	40S Ribosomal protein S16	3e-17 (BlastX)	*Gallus gallus*
S1615 (157)	1		60S Ribosomal protein L24	9e-20 (tBlastX)	*Pagrus major*
S1566 (163)	1		28S rRNA	5e-25 (tBlastX)	*Gallus gallus*
Hormonal regulation					
S1560 (112)	1		Thyroid hormone receptor interactor TRIP12	3e-10 (BlastX)	*Danio rerio*
Other					
S2361 (290)	1		Male specific protein	6e-10 (BlastX)	*Sarotherodon galilaeus*
S2378 (362)	1		Nac-alfa mRNA	5e-09 (BlastX)	*Pagrus major*

Table 3

Table 3. Up-regulated ESTs (fold change >2). The following sequences are represented in this table for more than one clone, but those with lower fold change were omitted for table clarifying. **Day 1 post-infection**: ubiquitin conjugating enzyme 7 interacting protein (2 clones); **Day 3 post-infection**: mitochondrial DNA (5 clones), stathmin-like protein (2 clones); **Day 7 post-infection**: formin binding protein 1-like (2 clones), fms interacting protein (2 clones), mitochondrial DNA (2 clones), 18s ribosomal gene (3 clones).

Clone number (bp)	Homology (Blast identity)	Fold change
Day 1 post-infection:		
S1782 (167)	Hsp70-1 gene (4e-16 tBlastX)	6.30
S2100 (341)	TNF alfa-induced protein 8 (3e-11 BlastX)	5.59
S2086 (672)	Ubiquitin conjugating enzyme 7 interacting protein (2e-63 BlastX)	3.99
Day 3 post-infection:		
S2361 (290)	Male specific protein (6e-10 BlastX)	5.27
S2354 (279)	Mitochondrial DNA complete genome (2e-45 tBlastX)	5.14
S2389 (515)	Stathmin-like protein (7e-55 BlastX)	3.73
S2375 (312)	60S Ribosomal protein L18 (1e-41 BlastX)	3.56
S2336 (594)	Ferritin heavy chain (2e-56 BlastX)	3.46
S2378 (362)	Nac-alfa mRNA (5e-09 BlastX)	2.80
S2385 (321)	Cytochrome c oxidase subunit II (2e-05 BlastX)	2.37
S2383 (341)	40S Ribosomal protein S27a (3e-39 Blastx)	2.17
S1824 (464)	H2A Histone family (9e-28 BlastX)	2.10
S2308 (265)	18S ribosomal RNA gene (2e-42 tBlastX)	2.07
Day 7 post-infection:		
S2096 (177)	Formin binding protein 1-like (3e-11 BlastX)	16,28
S1804 (524)	Fms interacting protein (2e-61 BlastX)	12,51
S2007 (465)	Ubiquitin conjugating enzyme 7 interacting protein (3e-21 BlastX)	12.32
S1782 (167)	Hsp70-1 gene (4e-16 tBlastX)	11.47
S2361 (290)	Male specific protein (6e-10 BlastX)	4.53
S1400 (364)	Mitochondrial DNA complete genome (1e-35 tBlastX)	3.96
S2136 (365)	Interferon induced with helicase C domain protein 1 (2e-27 BlastX)	2.56
S2389 (515)	Stathmin-like protein (7e-55 BlastX)	2.32
S2308 (265)	18S ribosomal RNA gene (2e-42 tBlastX)	2.25