Profiling of infection specific mRNA transcripts of the European seabass *Dicentrarchus labrax*

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Abstract

**Background:** The European seabass (*Dicentrarchus labrax*), one of the most extensively cultured species in European aquaculture productions, is, along with the gilthead seabream (*Sparus aurata*), a prospective model species for the Perciformes which includes several other commercially important species. Massive mortalities may be caused by bacterial or viral infections in intensive aquaculture production. Revealing transcripts involved in immune response and studying their relative expression enhances the understanding of the immune response mechanism and consequently also the creation of vaccines. The analysis of expressed sequence tags (EST) is an efficient and easy approach for gene discovery, comparative genomics and for examining gene expression in specific tissues in a qualitative and quantitative way.

**Results:** Here we describe the construction, analysis and comparison of a total of ten cDNA libraries, six from different tissues infected with *V. anguillarum* (liver, spleen, head kidney, gill, peritoneal exudates and intestine) and four cDNA libraries from different tissues infected with Nodavirus (liver, spleen, head kidney and brain). In total 9605 sequences representing 3075 (32%) unique sequences (set of sequences obtained after clustering) were obtained and analysed. Among the sequences several immune-related proteins were identified for the first time in the order of Perciformes as well as in Teleostei.

**Conclusion:** The present study provides new information to the Gene Index of seabass. It gives a unigene set that will make a significant contribution to functional genomic studies and to studies of differential gene expression in relation to the immune system. In addition some of the potentially interesting genes identified by *in silico* analysis and confirmed by real-time PCR are putative biomarkers for bacterial and viral infections in fish.
Background

The European seabass *Dicentrarchus labrax* is one of the most extensively aquacultured fish species in the Mediterranean, resulting in steadily increasing pressure on producers. Consequently, it is important to acquire new techniques and knowledge in order to improve aquaculture practices. Detailed information concerning growth, health, disease resistance and flesh quality benefit from the molecular as well as from the physiological point of view can provide illuminating new findings leading to improved aquaculture techniques. Several efforts have been made up till now to enrich the genomic resources in aquaculture production in the Mediterranean (chiefly for the gilt-head sea bream *Sparus aurata* and for the European seabass *Dicentrarchus labrax*), e.g. Marine Genomics Europe (Network of Excellence) (CT2003-505403), [1-5] as well as in the Atlantic (e.g. Atlantic halibut *Hippoglossus hippoglossus*, Salmon *Salmo salar* e.g. [6-13]. These studies focused mainly on non-challenged tissues in order to obtain a first unigene catalogue. Aquaculture production however is affected by viral and pathogenic bacteria, particularly in respect of *D. labrax* which has been shown to be the species most sensitive to pathogenic bacteria such as *Vibrio anguillarum* [14] and to viral infections such as Nodavirus [15,16]. There are several commercial vaccines which provide protection against infection from *V. anguillarum* although the mechanism of immune response still remains unknown. Nodavirus can cause massive mortalities [17] and cannot be controlled so far because the production of commercial vaccines here is still in its infancy. In the present study we have generated a collection of EST sequences from tissues of European seabass infected with *V. anguillarum* and Nodavirus. Within this collection we were able to isolate immune relevant genes, and have gone on to compare gene expression in different tissues after viral and pathogenic bacteria infection. Additionally we determined *in silico* differential expression between the two infections. In this context the construction and analysis of a total of ten cDNA libraries are described; six cDNA libraries were from tissues of the European seabass infected with *V. anguillarum* (liver, spleen, head kidney, peritoneal exudate, gill and intestine) with peritoneal exudate, gill and intestine as target organs for *V. anguillarum* infections, and four cDNA libraries were from tissues of the European seabass infected with Nodavirus (liver, spleen, head kidney and brain) with the brain as target organ of the virus. Comparisons between the predicted European seabass peptide data set and the zebrafish, medaka, stickleback, tetraodon and human proteomes were performed. Genes showing *in silico* differential expression between Nodavirus infection and *V. anguillarum* infection were further analysed by real-time PCR.

Results

Summary of ESTs from the cDNA libraries infected with Nodavirus and *V. anguillarum*

The amplified libraries contained insert size from approximately 0.5 to 2.0 kb. Single pass sequencing was performed resulting in 9605 high quality sequences. All sequences were submitted to the EST database (dbEST [http://www.ncbi.nlm.nih.gov/projects/dbEST/] with the accession numbers FK939975 – FK944381, FL484477 – FL488763 and FL501471 – FL502381. A set of 3075 unique sequences was generated. Among the unique sequences (3075) [see Additional file 1] 371 [12%, see Additional file 2] sequences contained Simple Sequence Repeats (SSR). Cluster analyses performed for each library separately (Table 1a and Table 1b) revealed redundancy rates which varied from 72% (28% unique sequences) in intestine cDNA library infected with *V. anguillarum* to 37% (63% unique sequences) in spleen cDNA library infected with *V. anguillarum* (Table 1a). The set of unique EST sequences was annotated with Blast2GO which carries out BLASTX searches and attempts to assign function and GO classification. Out of the 3075 unique EST sequences submitted to GO2Blast for annotation and GO classification, 1521 sequences fell into 14 categories of biological process function at GO annotation level 2 (Fig. 1), where two categories, cellular process and metabolic process, were predominant. The category "immune system process" was represented by 79 transcripts.

EST matches with known function

Out of the 3075 EST sequences, 1246 (~ 41%) had a positive hit after submission to BLASTX database search. Among those EST sequences with a known function, 128 homologues were found to be involved in the immune response and 79 of these were grouped into the GO category "immune system process". The remaining 49 transcripts were manually determined to be involved in the immune response (see Additional file 3). Immune related transcripts isolated for the first time for seabass amounted to 115 (Table 2). Among transcripts of interest, the transcript encoding for an important antimicrobial protein, hepcidin, was isolated. Aligning EST sequences grouped into one contig can provide additional data. In the case of hepcidin it is probable that different isoforms are grouped together. Alignments of other cDNA sequences either showed alternative polyadenylation or they showed *in silico* polymorphism of microsatellite DNA as for instance the transcript coding for cysteine-rich protein 1-I (see Additional file 4).

Similarity relationships

Figs. 2 and 3 show SimiTri representation of predicted seabass transcripts compared to *Danio rerio*, *Homo sapiens*, *Onchorhynchus mykiss*, *Gasterosteus aculeatus* and *Tetraodon nigroviridis* proteomes. Of 3075 isolated unique transcripts 1040, 1051, 1122 1159, 1103 had Blast hits with a score > 50 against the *H. sapiens*, *D. rerio*, *O. mykiss*, *G. aculeatus* and *T. nigroviridis* protein databases respectively.

Expression analysis

The test to compare multiple cDNA libraries with each other [22] revealed that the genes with the value > 6 of the
test statistic R can be confidently considered as genes with true variation with the slope of 1.081 and are therefore not significantly different from -1 at the 5% level (see Additional file 6). The hits with R > 6 are in total 109 out of 2234 contigs resulting from EST sequences of liver, spleen and head kidney infected with Nodavirus and \textit{V. anguillarum}. The list of the 109 transcripts with R > 6 and their putative homologues are shown in Additional file 5. It is interesting to note that although most transcripts were abundantly expressed in both bacterial and viral infected tissues, not all of them could be considered as specific markers of a specific infection. For example, fructose-1,6-biphosphate aldolase A, hepcidin, apolipoprotein A1 precursor, ferritin heavy chain and chemokine receptor 4 transcripts were found in \textit{V. anguillarum}-infected tissues, though rarely in Nodavirus-infected tissues (see Additional file 5). Conversely, fructose-1,6-biphosphate aldolase B and 14 kDa apolipoprotein tran-

### Table 1: Summary of sequences derived of cDNA libraries of \textit{D. labrax} tissue infected with \textit{V. anguillarum} (a) and Nodavirus (b)

| Tissue             | singletons | contigs | unique | total sequences | % of unique sequences |
|--------------------|------------|---------|--------|-----------------|----------------------|
| Liver              | 503        | 190     | 693    | 2140            | 32.38                |
| Spleen             | 326        | 38      | 364    | 651             | 63.14                |
| Kidney             | 266        | 66      | 332    | 911             | 36.24                |
| Peritoneal exudate | 386        | 103     | 489    | 827             | 59.13                |
| Gill               | 92         | 15      | 107    | 343             | 31.19                |
| Intestine          | 88         | 4       | 92     | 326             | 28.22                |

| Tissue             | singletons | contigs | unique | total sequences | % of unique sequences |
|--------------------|------------|---------|--------|-----------------|----------------------|
| Liver              | 253        | 127     | 380    | 1126            | 33.75                |
| Spleen             | 698        | 118     | 816    | 1284            | 63.55                |
| Brain              | 617        | 41      | 658    | 1099            | 59.87                |
| Kidney             | 321        | 55      | 376    | 934             | 40.26                |

Figure 1
Summary of GO category Biological process of unique ESTs obtained from seabass cDNA libraries infected with Nodavirus and \textit{Vibrio anguillarum}. 

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Table 2: Transcripts isolated for the first time in *D. labrax* and grouped to the GO category "immune system process"

| Contig ID | BLASTX Hit | Accession number |
|-----------|------------|------------------|
| Contig 265, Contig 3 | alpha globin | Q9PV4M BAA86218 |
| Contig 681 | alpha-1-microglobulin bikunin precursor | CAA45294 |
| Contig 3061 | b-cell leukemia lymphoma 6 | XP_001340785 |
| Contig 1838 | bcl2 adenovirus e1 b 19 kda interacting protein 3 | NP_001012245, AAR83676 |
| Contig 276 | beta-2 microglobulin | ABB60035, ABB60037 |
| Contig 273 | beta-2 microglobulin precursor | AAC64994 |
| Contig 2731 | blood thirsty | AAIX12162 |
| Contig 2210 | blood thirsty | XP_699830 |
| Contig 1617 | c1 inhibitor | NP_001117851, CAD58653 |
| Contig 936 | cathepsin s | AAOQ01147 |
| Contig 2779 | cc chemokine | AAY79324 |
| Contig 416 | ccaat enhancer binding protein (c ebp)beta | BAB40971 |
| Contig 1810 | cd59-like protein 2 | NP_001117969, AAT94063 |
| Contig 1441 | cell division cycle 42 | NP_956159, AAH48035, AAH75761, AAX20139, CAM56524, AAIX64988 |
| Contig 2676 | chemokine (c-c motif) ligand 13 | BAC20610 |
| Contig 2058 | chemokine (c-c motif) ligand 21b | AATS2146, ABA54959 |
| Contig 241 | chemokine (c-c motif) ligand 25 | ABC69050 |
| Contig 2858 | chemokine | NP_840092, AAN64414, AAS92649, AAI09418 |
| Contig 2859 | chemokine cxc-like protein | ABC69049 |
| Contig 524 | chemokine (c-c motif) ligand 9 | ABC69049 |
| Contig 627 | chemokine (c-c motif) receptor 4 | ABB48751 |
| Contig 525, Contig 1672 | chemokine cxc-like protein | ABC69049 |
| Contig 983 | complement c4-2 | CAD45003 |
| Contig 2306 | complement component 1 q subcomponent | ABV57766 |
| Contig 513 | complement component 1 qb chain | XP_001110783 |
| Contig 986 | complement component 5 | BAC23058 |
| Contig 1044 | complement component 7 | BAAX88999 |
| Contig 2558 | complement component alpha polypeptide | NP_001118096, CAIX6548 |
| Contig 1413 | complement component c3 | BAA89901 |
| Contig 1114 | complement component c4 | CAD45003 |
| Contig 2843 | complement component c5-l | BAC23057 |
| Contig 2534, Contig 2600 | complement component c9 | P79755, AAX60288 |
| Contig 2604 | complement component factor h | NP_001117882, CAF25505 |
| Contig 1496 | complement component gamma polypeptide | NP_001117880, CAF22027 |
| Contig 927 | complement component 1,1 gamma polypeptide | XP_544508 |
| Contig 2432 | complement component beta subunit | Q9PV4W7, BAA86877 |
| Contig 2605 | complement component q subcomponent binding protein | EDM05067 |
| Contig 1536 | complement component r subcomponent | AAR20889 |
| Contig 868 | complement factor b | CAD21938 |
| Contig 1607 | complement factor d preproprotein | XP_001117186 |
| Contig 2013 | complement factor h | NP_001117876, CAF05664, CAF05665 |
| Contig 1481 | complement factor h-related 1 | AA92556 |
| Contig 1375 | cornichon homolog | O35372, AAC5528 |
| Contig 1198 | c-reactive protein | NP_999009, O19062 BAA21473 |
| Contig 1657 | c-type lectin | BAE45333 |
| Contig 1596 | cu zn superoxide dismutase | AAV29025 |
| Contig 395 | deah (asp-glu-ala-his) box polypeptide 16 | NP_956318, AAH45393, AAI65206 |
| Contig 2814 | ets-1 transcript variant ets-1 delta(iii-vi) | AAY19514 |
| Contig 626 | ferritin heavy chain | NP_001117129, P49946, AAB34575 |
| Contig 280 | fth1 protein | CAL2185 |
| Contig 2392 | g-protein coupleded6 | NP_00112363 |
| Contig 2662 | heat shock 10 kda protein 1 (chaperonin 10) | AAV37068 |
| Contig 2975 | heat shock 70 kda protein 4 | AAH65970 |
| Contig 2939 | heme oxygenase1 | ABL47051 |
| Contig 275 | hemoglobin alpha chain | CAP69820 |
| Contig 731 | Hephaestin | NP_57938 |
| Contig 1713 | hypoxanthine phosphoribosyltransferase 1 | NP_001002056, AAH71336 |
scripts were frequently observed in Nodavirus infected tissues compared with *V. anguillarum*-infected tissues. The above results were further validated by determining the expression of putative markers for each infection in key tissues using real-time PCR. Here also control tissues were included in order to determine the expression of untreated fish. The real-time PCR confirmed the results obtained with the *in silico* analysis for selected genes. Taking into account the fold inductions of the real-time PCR experiments the correlations between *in silico* and qPCR are uniform. For instance the transcript for hepcidin precursor revealed *in silico* (R = 298.16) high expression only in liver tissues infected with *V. anguillarum*. The real-time PCR results show higher expression in all three tissues infected with *V. anguillarum*. However fold induction in liver is 20,000 times more than in spleen tissue, therefore theoretically 20,000 more cDNA clones had to be sequenced to obtain the sequence for hepcidin precursor in spleen infected with *V. anguillarum*. This correlation of high fold induction with *in silico* results can be observed for each transcript examined in this study. Thus, while the mRNA levels of hepcidin were found to increase considerably 24 h post-infection in the liver, spleen and head kidney of *V. anguillarum*-infected fish, they increased only slightly in Nodavirus-infected fish (Fig. 4). Notably, although the mRNA levels of transferrin and ferritin, both involved in iron metabolism with spleen and liver as the two main organs, increased in the liver after infection with both pathogens, they increased only in the spleen of *V. anguillarum*-infected animals (Figs. 5a and 5b). The mRNA levels of the chemokine receptor 4 were not affected or were slightly reduced in the head kidney and spleen of Nodavirus-infected fish but at 4 h and 24 h post-infection in the case of the Nodavirus and at 4 h post-infection in the case of *V. anguillarum* (Fig. 6). Here the expression in the liver is studied, as the liver is the major organ in the production of apoliprotein. Finally, although the mRNA lev-

Table 2: Transcripts isolated for the first time in *D. labrax* and grouped to the GO category “immune system process” (Continued)

| Contig | Name | Description |
|--------|------|-------------|
| Contig_1745, Contig_1876 | integrin beta 2 | BAB39130, NP_990582, CAA30671 |
| Contig_2452 | interleukin 1 receptor accessory protein | XP_001371334 |
| Contig_2261 | interleukin 1 type I | XP_416914 |
| Contig_1718 | interleukin 1 type II | NP_00105713, AAH89644 |
| Contig_1506 | interleukin 2 receptor gamma chain | CAJ38407 |
| Contig_1835 | interleukin enhancer binding factor 3 | AAH47175 |
| Contig_966 | interleukin-1 receptor type II | ABP99035 |
| Contig_852 | interleukin-1 receptor type II | CAL30143 |
| Contig_2196 | loc559360 protein | AAJ51869 |
| Contig_2044 | macrophage migration inhibitory factor | ABG54276 |
| Contig_1348 | major histocompatibility complex class I chain | BAD3369 |
| Contig_17 | mlf00348 protein | BAD90390 |
| Contig_889 | mhc class I alpha antigen | ABB04088 |
| Contig_1349 | mhc class I antigen | BAD13366 |
| Contig_2797 | mitochondrial ribosomal protein s18b | NP_00106610, AAJ52129, AAJ55448 |
| Contig_3008 | natural resistance-associated macrophage protein | AAG31225 |
| Contig_576 | neurofibrilobatosis 1 | AAD13839 |
| Contig_1716 | novel protein vertebrate complement component 3 | NP_001116778, CAQ13357 |
| Contig_1327 | otoquibiquitin aldehyde binding I | NP_001002500, AAH76301 |
| Contig_2661 | Proteasome activator subunit 1 (pa28 alpha) | ABE60902, ABK41199 |
| Contig_1948 | protein kinase alpha | AAJ51472 |
| Contig_2319 | protein tyrosine receptor | XP_547374 |
| Contig_2009 | purinergic receptor-protein13 | XP_00156794 |
| Contig_1715 | rhamnose binding lectin | NP_00117668, BAA92256 |
| Contig_394 | ribosomal protein s19 | P61155, AAP20214 |
| Contig_2680 | sam domain- and hd domain-containing protein 1 | XP_001097562 |
| Contig_684 | serum amyloid P-component | P12246 AAA40093, CAA34774, AAH61125, AAY88178, BAE25796, BAE38344, EDL39002 |
| Contig_2165 | sfv proviral integration 1 | NP_035485 |
| Contig_1473 | sh2 containing inositol-5-phosphatase | XP_687502 |
| Contig_2675 | skin mucus lectin | BAD90686 |
| Contig_840 | strawberry notch homolog 2 | EDL31603 |
| Contig_1540 | superfamily member 14 | NP_00118039, ABC84585 |
| Contig_469 | transcription factor 3 isomorf cRA_b | NP_571169, CAA54305 |
| Contig_2299 | transforming growth beta receptor ii (70 80 kda) | XP_534237 |
| Contig_2692 | trypsin 10 | BAF76146 |
| Contig_1814 | vascular endothelial growth factor | NP_001038320, AAY89335 |
| Contig_1660 | x-box binding protein 1 | AAQ08005 |
Similarity of *D. labrax* ESTs to the proteomes of *Homo sapiens* (H), *Danio rerio* (D), *Oryzias latipes* (M), *Gasterosteus aculateus* (ST) and *Tetraodon nigroviridis* (T). SimiTri plots show the graphical similarity i) between putative *D. labrax* peptides and *H. sapiens*, *O. latipes* and *T. nigroviridis*, cytochrome b is circled in red ii) between putative *D. labrax* peptides and *H. sapiens*, *O. latipes* and *G. aculateus*, iii) between putative *D. labrax* peptides and *H. sapiens*, *D. rerio* and *T. nigroviridis*, iv) between putative *D. labrax* peptides and *H. sapiens*, *D. rerio*, *G. aculateus*, v) between putative *D. labrax* peptides and *H. sapiens*, *D. rerio*, *O. latipes*, as well as vi) between putative *D. labrax* peptides and *H. sapiens*, *D. rerio*, *T. nigroviridis*.
els of fructose-1,6-bisphosphate aldolase B decreased in the liver and head kidney following infection with both pathogens, they increased at 24 h post-infection in the spleen of *V. anguillarum*-infected fish (Fig. 8).

**Discussion**

Although viral and bacterial infections are among the key challenges in fish aquaculture, nevertheless today the immune response of fish against *V. anguillarum* and Nodavirus remains largely unknown. Identification of genes involved in the immune response as well as the detection of differentially expressed genes between the two infections can make a significant contribution to future research leading to a better understanding of the biological system of immune response after fish infection. In the present study ten cDNA libraries, six from tissues infected with *V. anguillarum* and four from tissues infected with Nodavirus were analysed. Analysis of EST sequences coming from infected tissues will enhance the construction of an immune specific microarray chip containing already known transcripts involved in immune-related biological processes, such as the immune response as well as transcripts for which no annotation is available so far. Furthermore, transcripts indicating a higher expression level in one of the infections can be taken for future functional studies at RNA or DNA level as well as at protein level.

Over the past 30 years cDNA cloning for gene discovery and transcriptome analysis has become a very important molecular technique. Various techniques have been developed to address several scientific issues such as the cloning of rare transcripts, the construction of libraries with a wider cloning range, etc. (for review [26]). Construction of non-normalized libraries in the present study gave a first insight into the tissue-specific manner of transcript abundance according to their origin. Besides the possibility of identifying higher expressed transcripts, the percentages of unique sequences can also be assessed. In this study the redundancy of the cDNA libraries of liver, spleen and kidney infected with Nodavirus and *V. anguillarum* was in agreement with all three tissues (~33%, ~63% and ~38% respectively). This result is in line with other cDNA libraries of various fish species where the redundancy ranges between 40% and 60% depending on the tissues of origin [e.g. [12,13]]. Besides the identification and characterization of ESTs for components of the immune system, detection of microsatellite sequences will help in the completion of quantitative trait locus (QTL) scans currently being performed. Microsatellite sequences, also called Simple Sequence Repeats (SSR), are frequent in non-coding regions and are used as molecular markers. Detection of SSR within ESTs (exonic microsatellites or EST-SSRs) presents a shortcut to obtaining microsatellite markers. Since EST-SSRs are exonic they have two advantages over

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**Figure 3**

*Similarity of D. labrax ESTs to the proteomes of Danio rerio (D), Oryzias latipes (M), Gasterosteus aculateus (ST) and Tetraodon nigroviridis (T)*. SimiTri plots show the graphical similarity **A**: between putative *D. labrax* peptides and *G. aculeateus*, *O. latipes* and *T. nigroviridis*, **B**: between putative *D. labrax* peptides and *G. aculeateus*, *O. latipes* and *D. rerio*.
Figure 4

Hepcidin precursor expression index of liver, spleen, and head kidney infected with Nodavirus or with V. anguillarum for 4 h and 24 h. Infection for 4 h and 24 h of head kidney with V. anguillarum is pooled as not enough material was available. Each bar represents the mean of two technical duplicates of cDNA originating out of three individuals pooled prior to RNA extraction.
Figure 5

(A) Ferritin expression index of liver and spleen infected with Nodavirus or with V. anguillarum for 4 h and 24 h.

(B) Transferrin expression index of liver and spleen infected with Nodavirus or with V. anguillarum for 4 h and 24 h. Each bar represents the mean of two technical duplicates of cDNA originating out of three individuals pooled prior to RNA extraction.
Figure 6
Chemokine (c-x-c motif) receptor 4 (Cxcr 4) expression index of liver, spleen, and head kidney infected with Nodavirus or with V. anguillarum for 4 h and 24 h. Infection for 4 h and 24 h of head kidney with V. anguillarum is pooled as not enough material was available. Each bar represents the mean of two technical duplicates of cDNA originating out of three individuals pooled prior to RNA extraction. Between the two technical replicates of Noda 4 h and of VA 24 h a greater variation was detected. The values of the two replicates of Noda 4 h are 0.014 and 0.00095 and the values of the two replicates of VA 24 h are 0.03 and 0.009.
Figure 7

14 KDa apolipoprotein expression index of liver infected with Nodavirus or with V. anguillarum for 4 h and 24 h. Each bar represents the mean of two technical duplicates of cDNA originating out of three individuals pooled prior to RNA extraction.
Fructose-1,6-bisphosphatase aldolase B expression index of liver, spleen, and head kidney infected with Nodavirus or with *V. anguillarum* for 4 h and 24 h

Figure 8
Fructose-1,6-bisphosphatase aldolase B expression index of liver, spleen, and head kidney infected with Nodavirus or with *V. anguillarum* for 4 h and 24 h. Infection for 4 h and 24 h of head kidney with *V. anguillarum* is pooled as not enough material was available. Each bar represents the mean of two technical of cDNA originating out of three individuals pooled prior to RNA extraction.
intergenic microsatellites. First, it is expected that their flanking regions are more conserved, so that the primers can be used even in related species, and second, it is assumed that they are in strong linkage disequilibrium with functionally important sites. Therefore they are frequently used in population genomics or in mapping of genes of economic significance identified as candidate markers for QTL and/or quantitative trait nucleotide (QTN). For EST similarity search in the present study a homologue of a known gene is defined as a cDNA whose similarity to a gene of any other organism in the database exceeds a certain fixed threshold. The identification of orthologues is outside the scope of this study. In total 1246 (41%) were assigned to a known transcript, with 79 (6%) categorized to the GO category "immune system process". Separate examination of these 79 transcripts by GO annotation reveals their involvement in 11 other categories of biological function (Fig. 9), with three dominant categories of response to stimulus, cellular process, and biological regulation. This collection should provide the base material for further research into understanding the immune response of European seabass as well as for the isolation of putative biomarkers.

**Similarity relationships**

Comparison of predicted seabass genes compared to the genomes of zebrafish, medaka, tetraodon, stickleback and human (Fig. 2) showed that the majority of putative proteins were located in the centre. From separate examination of the different triads a bias towards the top and right sections is revealed. This bias is not unexpected as seabass is more closely related to medaka, zebrafish, stickleback and tetraodon. However it is worth noting, that the seabass cytochrome b seems to be more similar to human cytochrome b than to the tetraodon and medaka cytochrome b as shown in Fig. 2. This was not the case with stickleback and zebrafish cytochrome b. Interestingly, results from comparisons of putative proteins of the Atlantic halibut (*Hippoglossus hippoglossus*) with the human, zebrafish and tetraodon protein database showed that the halibut cytochrome c oxidase subunit 3 (Cox3) is more similar to human COX3 than the zebrafish and tetraodon Cox3 [13]. Comparison of predicted proteins with only the protein database of fish genomes shows a slight bias towards medaka and stickleback looking at the triad medaka, stickleback and tetraodon (Fig. 3A) and again a slight bias towards medaka and stickleback looking at the triad stickleback, medaka and zebrafish (Fig. 3B). These results give a first insight towards the evolution of immune related genes as the relatively equal distribution indicate that sequence variation between the clade Percomorpha is comparable to that between the clade Percomorpha and Ostariophysi.

**Expression analysis**

For in silico expression analysis transcript appearing more than once in the cDNA libraries were selected and their relative abundance were submitted to expression analysis.
after Stekel et al. [22]. Validation of *in silico* analysis was performed by qPCR. Individual variation may be masked in this approach as pooling strategy was chosen for qPCR experiments. The differential expressed transcripts detected in the present study can be further put forward for analysis of individual expression pattern. Nonetheless in order to study individual expression pattern the sampling frame has to be extended. In the present study the pooling strategy for qPCR was chosen in order to show cross-method consistency. However since results are consistent between the two approaches, influence of between individuals variability in response to infection has been addressed to some extent. In addition total RNA for qPCR analysis was extracted out of different individuals than the once used for cDNA library construction and patterns appear to be consistent between the different samples for all the selected candidate genes, which reflect the robustness of the approach and the small, if any bias, contributed by individual outliers. In *silico* expression analysis revealed a number of genes for R > 6 that are considerably above the exponential curve (see Additional file 6). Genes with R > 6 can be considered as significant and thus are candidate genes for further studies. Several of those transcripts including transcripts involved in iron metabolism such as ferritin and transferrin are also reported as differential expressed genes in the catfish *Ictalurus punctatus* and *Ictalurus furcatus* infected with the gram negative bacteria *Edwardsiella ictaluri* [27,28]. One of the main mechanisms whereby gram-negative bacteria pathogens like *V. anguillarum* obtain iron is the use of free heme or heme proteins from the host tissues [29]. The heme uptake mechanisms are considered to contribute to *V. anguillarum* virulence in fish [29]. However, it is surprising that Nodavirus infection also resulted in the up-regulation of transferrin and ferritin expression, especially within 24 h of infection. The abundance of transferrin transcripts in Nodavirus-infected tissues may not be related to the alteration of the iron metabolism by the pathogen but rather to the ability of enzymatically cleaved forms of this protein to activate fish macrophages [30]. The specific alteration of iron metabolism by *V. anguillarum* infection is also supported by the higher abundance of transcripts coding for hepcidin, a major homeostatic regulator of iron metabolism [31], and for α and β chains of hemoglobin in *V. anguillarum*- than Nodavirus-infected livers (255 clones vs. 1 clone, respectively). The expression of Hepcidin after bacterial infection has been shown in seabass [32] as well as in several other fish species like the striped bass [33], the red sea bream [34], the catfish [35,36], the Atlantic halibut [37], the zebrafish [38], the rainbow trout [39] and the perch [40]. In this study the qPCR experiments confirmed the up-regulation of hepcidin in *D. labrax* after infection with *V. anguillarum* and showed in addition to this, that the expression of hepcidin might be considered as an excellent marker of bacterial infections, since it was up-regulated in all examined tissues of *V. anguillarum*-infected fish but unaffected in Nodavirus-infected tissues. Another interesting observation of the *in silico* gene expression analysis is the differential abundance of transcripts encoding the isoforms A and B glycolytic/glucoseogenic enzyme fructose-1,6-biphosphate aldolase in bacterial and viral infected tissues. Although the role played by this enzyme in the outcome of these infections is difficult to anticipate due to its dual role in glucose metabolism, these results suggest that the expression ratio between the two enzyme isoforms may be used as a good indicator of the type of infection in the European seabass. Thus, the up-regulation of the B isoform in the spleen exclusively by *V. anguillarum* might be considered another potential marker for this bacterial infection. Similarly, apolipoprotein A1 and 14 kDa apolipoprotein, two major components of high density lipoproteins (HDL) and synthesized in the fish liver [41], also show a differential expression in the liver of fish infected with *V. anguillarum* and Nodavirus following the time course and, therefore, they also may be good candidate indicators of the fish health status and/or the type of infection. The real-time PCR confirmed observations of *in silico* expression analysis and also revealed that the expression of the 14 kDa apolipoprotein and aldolase B in the spleen is an appropriate marker of Nodavirus and *V. anguillarum* infections, respectively. Previous studies in carp and medaka have also shown the involvement of apolipoproteins in the immune response [42,43]. Finally, the differential expression of one of the clear immune-related genes, the chemokine receptor 4, was also found to be a good putative marker for *V. anguillarum* infection. For assessment of variability of putative markers further studies looking at individuals, exposed to other environmental or pathogenic conditions are needed to exclude possible biological variability caused by infections.

**Conclusion**

In this study we generated a collection of EST sequences from tissues of the European seabass infected with *V.
anguillarum and Nodavirus. We compared gene expression of different tissues after viral and pathogenic bacteria infection. A collection of 3075 unigenes was generated and candidate microsatellite sequences detected. Furthermore, comparisons of D. labrax transcripts with zebrafish, human, tetraodon, medaka and stickleback were performed. The majority of putative proteins were located in the centre with a bias towards the right sections, with D. labrax as expected being more closely related to the other fish species than to human. Comparison of putative D. labrax proteins was also performed among fish species. In this case a slight bias towards stickleback and medaka was observed when comparing medaka, stickleback and tetraodon and a slight bias towards stickleback and medaka was observed when comparing medaka, stickleback and zebrafish. Furthermore, in silico analysis of differential gene expression between the two infections based on EST sequences suggests a list of genes with a presumed function in the immune response of D. labrax revealing also the importance of looking at "non-classical" immune host proteins and emphasizing the significance of EST sequences generated from cDNA libraries of infected fish tissues. In addition, we show the power of sequencing cDNA sequences for expression analysis by performing real-time PCR experiments for transcripts with high, medium and low R-value. In view of new and high throughput sequence techniques detection of differential expression by measuring in silico the abundance of each transcript will enhance significantly the era of functional genomics. Furthermore in silico analysis in this study, followed by the confirmation with real-time PCR of potentially interested genes, has revealed some of them as potential biomarkers for bacterial and viral infections in fish.

Methods

Experimental condition and tissues collection

Two infections, one with Nodavirus strain 475-9/99 isolated from diseased sea bass [from the Instituto Zooprofilattico Sperimentale delle Venezie (Italy) [16]] and one with V. anguillarum strain R-82 (serogroup 01) [from the University of Santiago (Spain) [14]] were performed with seabass as previously described [14,16]. Tissues were taken 4 and 24 h post-infection. Three tissue types (spleen, liver and head kidney) of each experimental condition as well as peritoneal exudate, gill, intestine from V. anguillarum infection and brain from Nodavirus infection were selected and immediately frozen with liquid nitrogen. The experiments described comply with the guidelines of the European Union Council (86/609/EEU) for the use of laboratory animals and have been approved by the Bioethical Committee of the University of Murcia (Spain) and the CSIC National Committee on Bioethics.

In brief: For Nodavirus infection fish were injected intramuscularly with 100 μl of nodavirus suspension in Minimum Essential Medium (MEM) (5.9 × 10^6 TCID_{50} ml⁻¹) and placed at 25°C. Mock-infected control fish were injected with the medium alone, and maintained under the same experimental conditions. Three fish from each experimental and control groups were sampled 4 and 24 hours post-infection. Animals were sacrificed by anesthetic (MS-222) overdose and dissected. For the present study brain, spleen, head kidney and liver were sampled. For V. anguillarum infection fish were injected intraperitoneally (i.p.) with 1 ml of phosphate-buffered saline (PBS) alone or containing either 2 × 10⁸ live or 10⁸ formalin-killed V. anguillarum R82 cells (serogroup 01). Under these experimental conditions, about half of the fish were moribund at 24 h post-infection and all of them died within 48 h post-infection. Head kidney (bone marrow equivalent of fish) and peritoneal exudate cells were obtained 4 h and 24 h after bacterial challenge.

RNA extraction

Total RNA was extracted using the NucleoSpin RNA II extraction kit (Machinery Nagel, Dueren, Germany). RNA quantity was checked on EtBr stained agarose gels and RNA concentrations and purity were measured using a Nano-Drop spectrophotometer. For library construction equal amounts of total RNA extracted out of infected tissues (4 h and 24 h) were pooled. For qPCR experiments total RNA was freshly extracted out of infected tissues originating from three different individuals pooled prior to RNA extraction (liver, spleen and head kidney) with 4 h and 24 h post-infection.

cDNA library construction

All libraries were constructed from total RNA using the Creator SMART cDNA library construction kit (BD Bioscience-Clontech, Mountain View, Canada) using the LD PCR based method. Between 20 and 22 PCR cycles were performed before size separation of inserts. cDNA fragments > 600 bp were selected and directionally ligated at the restriction site 5' of the pDNR-lib vector (BD Clontech) or the pal 32 vector. Plasmids were transformed into E. coli strain DH10B (Invitrogen) by electroporation. The libraries were tested for the presence and the size of insert by PCR using two primer pairs. For the libraries constructed with pal 32 vector, the primer pair pal 32 F: 5’-CTCGGGAAAGGCGCCATT-3’ and pal 32, REV: 5’-TAATACGACTCTATAGGC-3’ were used. For the libraries constructed with pDNR-lib vector pDNR FOR: 5’-TAAACGACGGCCATGTC-3’ pDNR REV: 5’-GAAAGCTATGCACCATTGTC-3’ were used. The products were run on an EtBr stained agarose gel.

DNA sequencing

After plasmid preparation, dideoxy-termination DNA cycle sequencing was performed using the BigDye 3.1 sequencing method and the pDNR FOR (5’-TAAACGACGC-
The sequences were run on an ABI 3730 XL sequencer at MPI Molecular Genetics, Berlin.

**Sequence analysis**
The raw sequence reads were quality-trimmed and vector- and poly-A-clipped using PREGAP4 [18]. Clustering (grouping of clones related to one another by sequence homology) was performed using the software SeqManII (DNASTar Inc.). After clustering the term 'contig' is used to describe the sequence obtained from one cluster (the sequences of a cluster can be collapsed into a single, non-redundant sequence) and the term 'singleton' describes sequences appearing only once in the entire dataset. The set of sequences obtained by merging contigs and singletons are named as unique sequences.

**Simple Sequence Repeats (SSR) in EST sequences**
*In silico* mining for repeat motifs within the obtained unique sequences was performed with the programme Msatfinder [19].

**Homology search and GO annotation**
Gene Ontology (GO) category (Biological process) was assigned after BLASTX search of 3075 unique EST sequences using BLAST2GO. Threshold cutoff was at E-value 1e-3 and the alignment length of 33 amino acids (aa).

**Similarity relationships**
The unique sequences from all seabass libraries were submitted to BLASTX similarity searches [20] against the zebrafish, tetraodon, stickleback, medaka and human predicted proteomes (downloadable from [http://www.ensembl.org/index.html](http://www.ensembl.org/index.html)). For each database the highest BLAST scores (bit score values) in excess of 50 were retained. Relative similarities between triads were visualized as a triangular plot generated by the SimiTri software [21].

**Expression analysis**
*In silico*
All sequences of each cDNA library were submitted to BLASTX and BLASTN searches [20]. Transcripts appearing more than once in the cDNA libraries were selected for *in silico* expression analysis after Stekel et al. [22]. In brief, this method allows the comparison of gene expression in any number of libraries in order to identify differential expressed genes. The method uses a single statistical test to describe the extent to which a gene is differentially expressed between libraries by a log likelihood ratio statistic and tends asymptotically to a χ² distribution [22]. For real-time PCR experiments transcripts with high, medium and low R-value were selected.

**Real-time PCR**
Gene expression was assessed by real-time PCR (qPCR) in spleen, head kidney and liver at 4 h and 24 h post-infection. RNAs out of three animals pooled prior to RNA extraction were isolated as described above and were used to obtain cDNA by the Superscript II Reverse Transcriptase and oligo (dT)12–18 primer (Invitrogen) following the manufacturer’s instructions. Quantitative PCR assays were performed using the 7300 real-time PCR System (Applied Biosystems) with specific primers (Table 3). Each primer (0.5 μl; 10 μM) and the cDNA template (1 μl) 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°C for 10 min. followed by 40 cycles of 95°C 15 s and 60°C 1 min. For all reactions two technical duplicates were performed. The comparative CT method (2ΔΔCT method) was used to determine the expression level of analysed genes [23]. After evaluation of β-actin as a suitable reference gene for this study in seabass (data not shown) the expression of the candidate genes was normalized. The use of β-actin as a suitable reference gene was also shown in other fish studies [e.g. [24,25]].

**Authors’ contributions**
ES contributed to EST production, performed analysis and the conception, design and manuscript writing. PS performed infections with VA and tissue sampling and contributed to RNA extractions. LP performed qPCR experiments. VM conceived the study, contributed to the conception and design of the project and also to the manuscript writing. JM contributed to the conception and design of the project and helped to draft the manuscript. BN conceived qPCR experiments, Nodavirus infections and tissue sampling and contributed to conception and design of the project and helped to draft the manuscript. VT and RR created the cDNA libraries and performed the sequencing of the clones. AM contributed to conception and design of the project and helped to draft the manuscript. GK conceived the study, contributed to the conception and design of the project and also to the manuscript writing.

**Additional material**

**Appendix 1**
Catalogue of all unique sequences obtained from all constructed cDNA libraries in this study.

Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-10-157-S1.zip](http://www.biomedcentral.com/content/supplementary/1471-2164-10-157-S1.zip)
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References
1. Chistiakov DA, Helleman B, Haley CS, Tsigenopoulos CS, Kotoulas G, Bertotto D, Liberti A, Volckaert FA: A microsatellite linkage map of the European sea bass Dicentrarchus labrax L. Genetics 2005, 170:1821-1826.
2. Chistiakov DA, Helleman B, Tsigenopoulos CS, Law AS, Bartley N, Bertotto D, Liberti A, Kotoulas G, Haley CS, Volckaert FA: Development and linkage relationships for new microsatellite markers of the sea bass (Dicentrarchus labrax L.). Anim Genet 2004, 35:53-57.
3. Chini V, Rimoldi S, Terova G, Saroglia M, Rossi F, Bernardini G, Gornati R: EST-based identification of genes expressed in the liver of adult seabass (Dicentrarchus labrax, L.). Gene 2006, 376:102-106.
4. Sarropoulou E, Power DM, Magoulas A, Geisler R, Kotoulas G: Comparative analysis and characterization of expressed sequence tags in gilthead sea bream (Sparus aurata) liver and embryos. Aquaculture 2005, 243:69-81.
5. Sarropoulou E, Franch R, Louro B, Power DM, Bargelloni L, Magoulas A, Senger F, Talavoua M, Patarnello T, Galibert F, Kotoulas G, Geisler R: A gene-based radiation hybrid map of the gilthead sea bream Sparus aurata refines and exploits conserved synteny with Tetraodon nigroviridis. BMC Genomics 2007, 8:44.
6. Martin SA, Capece NC, Davie GC, Powell R: EST-based identification of genes expressed in the liver of adult Atlantic salmon (Salmo salar). Biochem Biophys Res Commun 2002, 293:578-585.
7. Hau gland O, Torgersen J, Syed M, Eversen O: Expression profiles of inflammatory and immune-related genes in Atlantic salmon (Salmo salar L.) at early time post vaccination. Vaccine 2005, 23:5488-5499.
8. Moriyasu AA, Vlasov AV, Hagen-Larsen H, Ruden TA, Laerdahl JK, Hoyheim B: Annotated expressed sequence tags (EST’s) from pre-smolt Atlantic salmon (Salmo salar) in a searchable data resource. BMC Genomics 2007, 8:209.
9. Hildahl J, Galay-Burgos M, Sweeney G, Einarsdottir IE, Bjornsson BT: Identification of two isoforms of Atlantic halibut insulin-like growth factor-I receptor genes and quantitative gene expression during metamorphosis. Comp Biochem Physiol B Biochem Mol Biol 2007, 147:395-401.
10. Hildahl J, Sweeney G, Galay-Burgos M, Einarsdottir IE, Bjornsson BT: Cloning of Atlantic halibut growth hormone receptor genes and quantitative gene expression during metamorphosis. Gen Comp Endocrinol 2007, 151:143-152.
11. Douglas SE, Knickle LC, Kimball J, Reith ME: Comprehensive EST analysis of Atlantic halibut (Hippoglossus hippoglossus), a commercially relevant aquaculture species. BMC Genomics 2007, 8:144.
12. Douglas SE, Gallant JW, Bullerwell CE, Wolff C, Munholdt J, Reith ME: Winter Flounder Expressed Sequence Tags: Establishment of an EST Database and Identification of Novel Fish Genes. Mar Biotechnol (NY) 1999, 1:458-0464.
13. Bai J, Solberg C, Fernandes JM, Johnston IA: Profiling of maternal and developmental-stage specific mRNA transcripts in Atlantic halibut Hippoglossus hippoglossus. Gene 2007, 386:202-210.
14. Sepulcre MP, Sarropoulou E, Kotoulas G, Meseguer J, Mulerov V: Vibrio anguillarum evades the immune response of the bony fish sea bass (Dicentrarchus labrax L.) through the inhibition of leukocyte respiratory burst and down-regulation of apoptotic caspases. Mol Immunol 2007, 44:3751-3757.
15. Ucko M, Colorni A, Diamant A: Nodavirus infections in Israeli mariculture. J Fish Dis 2004, 27:459-469.
16. Poisa-Beiro L, Dios S, Montes A, Aranguren R, Figueras A, Novoa B: Nodavirus increases the expression of Mx and inflammatory cytokines in fish brains. Mol Immunol 2008, 45:218-225.
17. Frerichs GN, Rodger HD, Peric Z: Cell culture isolation of piscine neuropathic nodavirus from juvenile sea bass, Dicentrarchus labrax. J Gen Virol 1996, 77(Pt 9):2067-2071.
18. Staden R, Beal KF, Bonfield JK: The Staden package, 1998. Methods Mol Biol 2000, 132:115-130.
19. Thurston MIFD: Msatfinder: detection and characterization of microsatellites. 2005 [http://www.genomics.ceb.ac.uk/msatfinder/].
20. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25:3389-3402.
21. Parkinson J, Blaxter M: SimiTri – visualizing similarity relationships for groups of sequences. Bioinformatics 2003, 19:390-392.
22. Stoeckl DJ, Gr Y, Falciani F: The comparison of gene expression from multiple cDNA libraries. Genome Res 2000, 10:2055-2061.
23. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25:402-408.
24. Tang R, Dodd A, Lai D, McNabb WC, Love DR: Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin (Shanghai) 2007, 39:384-390.
25. Ilyasiev HC, Petersen EF, Jakobsen RA, Petersen CB, Wergeland H: Expression profiling and validation of reference gene can-
didates in immune relevant tissues and cells from Atlantic salmon (Salmo salar L.). Mol Immunol 2006, 43:194-1201.

26. Harbers M: The current status of cDNA cloning. Genomics 2008, 91:232-242.

27. Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, Li P, Wang S, Somrithivej B, Dunham R, Liu Z: Expression analysis of the acute phase response in channel catfish (Ictalurus punctatus) after infection with a Gram-negative bacterium. Dev Comp Immunol 2007, 31:1183-1196.

28. Peatman E, Terhune J, Baoprasertkul P, Xu P, Nandi S, Wang S, Somrithivej B, Kucuktas H, Li P, Dunham R, Liu Z: Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with Edwardsiella ictaluri. Mol Immunol 2008, 45:553-566.

29. Stork M, Di Lorenzo M, Welch TJ, Cross LM, Cross JH: Plasmid-mediated iron uptake and virulence in Vibrio anguillarum. Plasmid 2002, 48:222-228.

30. Sjostrom JL, Belosevic M: Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev Comp Immunol 2003, 27:539-554.

31. Ganz T, Nemeth E: Regulation of iron acquisition and iron distribution in mammals. Biochim Biophys Acta 2006, 1763:690-699.

32. Rodrigues PN, Vazquez-Dorado S, Neves JV, Wilson JM: Dual function of fish hepcidin: response to experimental iron overload and bacterial infection in sea bass (Dicentrarchus labrax). Dev Comp Immunol 2006, 30:1156-1167.

33. Lauth X, Babon JJ, Stannard JA, Singh S, Nizet V, Carlberg JM, Ostland VE, Pennington MW, Norton RS, Westerman ME: Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infection. J Biol Chem 2005, 280:9272-9282.

34. Chen SL, Xu MY, Ji XS, Yu GC, Liu Y: Cloning, characterization, and expression analysis of hepcidin gene from red sea bream (Chrysophrys major). Antimicrob Agents Chemother 2005, 49:1608-1612.

35. Bao B, Peatman E, Li P, He C, Liu Z: Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. Dev Comp Immunol 2005, 29:939-950.

36. Hu X, Camus AC, Aono S, Morrison EE, Dennis J, Nusbaum RS, Judd RL, Shi J: Channel catfish hepcidin expression in infection and disease. Comp Immunol Microbial Infect Dis 2007, 30:55-69.

37. Park KC, Osborne JA, Tsoi SC, Brown LL, Johnson SC: Expressed sequence tags analysis of Atlantic halibut (Hippoglossus hippoglossus) liver, kidney and spleen tissues following vaccination against Vibrio anguillarum and Aeromonas salmonicida. Fish Shellfish Immunol 2005, 18:393-415.

38. Lin B, Chen S, Cao Z, Lin Y, Mo D, Zhang H, Gu J, Dong M, Liu Z, Xu A: Acute phase response in zebrafish upon Aeromonas salmonicida and Staphylococcus aureus infection: striking similarities and obvious differences with mammals. Mol Immunol 2007, 44:295-301.

39. Gerwick L, Corley-Smith G, Bayne CJ: Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish Shellfish Immunol 2007, 22:137-171.

40. Rossi F, Chini Y, Cattaneo AG, Bernardini G, Terova G, Saroglou M, Gornati R: EST-based identification of genes expressed in perch (Perca fluviatilis, L.). Gene Exp 2007, 14:117-127.

41. Kondo H, Kawazoe I, Nakaya M, Kikuchi K, Aida K, Watabe S: The novel sequences of major plasma apolipoproteins in the eel Anguilla japonica. Biochim Biophys Acta 2001, 1531:132-142.

42. Concha Mi, Smith VL, Castro K, Bastias A, Romero A, Asthauer RJ: Apolipoproteins A-I and A-II are potentially important effectors of innate immunity in the teleost fish Cyprinus carpio. Eur J Biochem 2004, 271:2984-2990.

43. Villarroel F, Bastias A, Casado A, Asthauer R, Concha Mi: Apolipoprotein A-I, an antimicrobial protein in Oncorhynchus mykiss: evaluation of its expression in primary defence barriers and plasma levels in sick and healthy fish. Fish Shellfish Immunol 2007, 23:197-209.