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Two strategies to unravel gene expression responses of host–microbe interactions in cod (*Gadus morhua*) larvae

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Abstract

The commensal bacteria in the intestine play essential roles in the development and functionality of the host. To unravel the host–microbe interactions in Atlantic cod (*Gadus morhua* L.) larvae, we used two molecular approaches: (1) suppression subtractive hybridization-polymerase chain reaction (SSH-PCR) to identify host gene responses and (2) expression analysis of selected genes reported to be differentially expressed in gnotobiotic zebrafish in a previous study to determine whether these host responses are also conserved in cod. Suppression subtractive hybridization-PCR identified 156 transcripts putatively regulated by the presence of bacteria. However, out of 22 selected transcripts, only four were significantly differentially expressed when quantified using quantitative (real-time) PCR. Expression analysis of selected genes from zebrafish revealed possible conservation of host responses for three out of eight genes analysed. For most of the genes quantified, the gene expression pattern varied between two biological replicates. This may reflect differences in the bacterial composition in the rearing bottles, and denaturing gradient gel electrophoresis analysis confirmed significant differences between the two replicates with regard to bacterial diversity. The varying effects on gene expression caused by differences in the microbial composition show the necessity of further studies where axenic cod larvae are compared with larvae raised in defined and controlled (gnotobiotic) environments.

Keywords: bacteria-free, host response, gene expression, cod larvae, suppression subtractive hybridization PCR

Introduction

Successful aquaculture of Atlantic cod (*Gadus morhua* L.) is still hampered by low survival at the larval stage. Opportunistic bacteria are thought to be a major cause of these problems (Vadstein, Øie, Olsen, Skjermo, Salvesen & Skjåk-Brækg 1993). During intensive culture, the immature cod larvae are exposed to, and interact with, large numbers of bacteria. They actively drink water before yolk sac re-absorption, and the uptake of bacteria exceeds the drinking rate by two orders of magnitude (Reitan, Natvik & Vadstein 1998). As a consequence, the undifferentiated intestinal tract is exposed to a large number of bacteria, even before the start of exogenous feeding. The host–microbe interactions in the gut of the cod larvae can lead to the formation of a healthy stable intestinal microflora or to infection and disease (Hansen & Olafsen 1999; Olafsen 2001). Whether a bacterium will colonize the intestine is determined by interactions between the different bacteria present, nutrient availability, adhesion properties and cross talk with the host cells (Kelly, Conway & Aminov 2005; Corthesy, Gaskins & Mercenier 2007).

In intensive rearing of marine fish larvae, the research focus is now shifting from non-specific removal of bacteria in the rearing water to controlling and maintaining a beneficial microflora (Ringø & Birkbeck 1999; Skjermo & Vadstein 1999; Vine, Leukes & Kaiser 2006). However, there is still a lack of knowledge concerning the host–microbe interactions that take place during the first weeks of larval growth, and the subsequent formation of an intestinal microflora.

The use of gnotobiotic vertebrates (containing a known, defined microbial flora) has revealed that

microbial colonization directly affects a wide range of biological processes, including nutrient processing and adsorption, development of the mucosal immune system and epithelial proliferation (Rawls, Samuel & Gordon 2004; Smith, Mccoy & Macpherson 2006; Cheesman & Guillemin 2007). A gnotobiotic model used to investigate the gene responses to the microflora in zebra fish (*Danio rerio*) revealed 212 host genes whose expressions were regulated by bacteria (Rawls *et al.* 2004). However, zebrafish hatch at a fairly developed state and are phylogenetically distant from marine fish.

The aim of this study was to investigate the effect of bacterial presence on the differential gene expression patterns of cod larvae. We have established a protocol for bacteria-free rearing of cod larvae, making it possible to compare cod larvae grown without bacteria with those grown in a mixed bacterial community (T. Forberg, O. Vadstein & A. Arukwe, unpublished data). To investigate host gene expression responses, we chose two strategies: (1) suppression subtractive hybridization- polymerase chain reaction (SSH-PCR) to generate sequences of differentially expressed genes, as an unbiased approach to identify host responses, and (2) a biased approach, expression analysis of selected genes reported to be differentially expressed in gnotobiotic zebrafish (Rawls *et al.* 2004), to determine whether these host responses are also conserved in cod.

Materials and methods

Biological material and experiments

Cod eggs were disinfected twice with 400 ppm glutaraldehyde for 10 min (Salvesen & Vadstein 1995; Salvesen, Øie & Vadstein 1997), and hatched in filtered (0.22 μm Micropore®, Derbyshire, UK), autoclaved seawater (FASW), containing 10 ppm each of rifampicin and ampicillin (T. Forberg, O. Vadstein & A. Arukwe, unpublished data). The water temperature during disinfection was around 6 °C; during the experiment, this temperature was increased by 1 °day^{-1} up until 12 °C. All work was performed using sterile equipment under a laminar flow hood. After hatching, the cod larvae were transferred to (Nalgene®, Thermo Scientific, Rochester, NY, USA) rearing bottles, containing either 2 L bacteria-free (FASW) or bacteria-containing seawater. The bacteria-containing seawater used was aged seawater, generated by filtering seawater through a GF/F (Whatman®, GE Healthcare, Amersham, UK) filter to remove large particles, and stored for 2 weeks without aeration at room temperature (approximately 20 °C) before use. Aged seawater that had been UV treated for 5 min was also used, to achieve variation with regard to the bacteria present. K-selected bacteria will presumably dominate the aged seawater, while UV treatment will lead to a domination of *r*-strategists (Andrews & Harris 1986; Skjermo, Salvesen, Øie, Olsen & Vadstein 1997).

Bacteria-free rotifers to be used as feed were obtained according to the protocol of Tinh, Phuoc, Dierckens, Sorgeloos and Bossier (2006), with one modification: the rotifer eggs were left to hatch in 10 ppm of rifampicin and ampicillin. Bacteria-free rotifers were added to the cod rearing bottles from day 3 until day 17 post hatch. Axenic *Isochrysis* sp. was also added, in accordance with the green-water technique (Skjermo & Vadstein 1993). Dead larvae were removed and counted on days 4, 10, 12, 14 and 17. The cod larvae were reared until day 17 post hatch. On day 17, they were sacrificed using MS-222 (0.5 g L^{-1}, lethal dose), rinsed in MilliQ water and placed in RNA*later*® solution (Ambion®, Life Technologies, Carlsbad, CA, USA) for storage at − 20 °C.

Two separate start feeding experiments were performed: the first to generate cod samples for SSH-PCR and the second to generate samples for gene expression analysis of cod genes identified by SSH and cod homologues of genes selected from the zebrafish study. In the first experiment, three bacteria-free rearing bottles and four bacteria-exposed (two with aged seawater and two with UV-treated aged seawater) were stocked with 80 larvae L^{-1}. In the second experiment, two bacteria-free (BF1 and BF2) and two bacteria-exposed (M1 and M2) (containing aged seawater) rearing bottles were stocked with 30 larvae L^{-1} (a lower density of larvae was chosen to reduce the amounts of bacteria-free rotifers needed).

Evaluation of bacteria-free conditions, bacterial density and diversity

Samples from the cod rearing water and from rotifer and algae cultures were taken every other day of the experiments. Liquid and solid M65 media (consisting of 0.5 g peptone, 0.5 g tryptone and 0.5 g yeast extract, dissolved in 800 mL FASW and 200 mL MilliQ water) and Marine Broth (Difco™, BD, Franklin Lakes, NJ, USA) were used to check for bacterial contamination. Serial dilution plating was used to estimate the density of culturable bacteria in the rearing bottles containing aged seawater.

In the second start feeding experiment, flow cytometry was used to investigate the presence and density of bacteria in all cod rearing bottles. Briefly, SYBR

green (SYBR Green I, Molecular Probes) was added to water from the rearing bottles, and a FACSScan flow cytometer (Becton Dickinson, BD) was used to detect fluorescent particles (Marie, Brussaard, Thyrhaug, Bratbak & Vaulot 1999). Filtered autoclaved seawater was used to quantify the number of background particles. Flow cytometry counts were performed on days 6, 10 and 17 after hatching.

Denaturing gradient gel electrophoresis (DGGE) was used to investigate the diversity of the microbial community present in the two bacteria-containing cod rearing bottles (M1 and M2) in the second start feeding experiment. DNA was isolated from centrifuged 10 mL water samples taken on days 10 and 17 post hatch, using the Qiagen DNAeasy kit (Hilden, Germany) according to the manufacturer's protocol. PCR was performed using 16S rDNA primers 338f-GC and 517r (Muyzer, De Waal & Uitterlinden 1993), under the following conditions: initial denaturation at 95 °C for 4 min, followed by 40 cycles of 30 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C and a final elongation step for 30 min at 72 °C. A denaturing gradient of 35–60% was used, and the gel was run for 17 h at a voltage of 100 V (using the Ingeny phorU system). The DGGE gel was stained with SYBR Green SYBR Gold (Invitrogen™, Life Technologies, Carlsbad, CA, USA) for 30 min and photographed under UV light. Denaturing gradient gel electrophoresis gel images were analysed using the GEL2K software (provided by Svein Norland, Department of Biology, University of Bergen, Norway). Peak detection parameters were set to 2 for vertical and horizontal sensitivity and five-pixel smoothing was used. The relative bandwidth was set to 0.0003. The peak area matrix for the samples was exported and normalized to per cent of sum area. Pearson's correlation coefficients were calculated to compare the normalized band intensity profiles between samples. The Shannon index (Shannon 1948) and the relative diversity J' (evenness) were used as measures of diversity in the DGGE profiles.

Generation of subtracted library and sequence analysis

Suppression subtractive hybridization-PCR was performed under contract by EcoArray (Alachua, FL, USA), using polyA cDNA from pooled larvae samples ($n = 11$) from the bacteria-free replicates and the bacteria-exposed replicates ($n = 8$) from the first start feeding experiment. The experiment was performed in both forward and reverse directions to obtain two clone libraries containing up- and down-regulated genes respectively. Sequenced clones were analysed using Blastx against the GenBank protein database and Blastn against the GenBank nucleotide database. The *e*-value cut-off was set at 10^{-5} for blast searches. EST sequences were submitted to the NCBI GenBank EST database and assigned accession numbers GW574323–GW574464, while ribosomal and mitochondrial sequences were submitted to the GenBank nucleotide database (acc# GU931777–GU931790).

RNA isolation and cDNA synthesis

Cod larvae from the second start feeding experiment were placed in TRK lysis buffer (supplied with the E.Z.N.A® kit) and B-mercaptoethanol before homogenization with a rotor-stator. Total RNA was isolated using the E.Z.N.A® total RNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol, including on-membrane *DNase* I treatment. Larvae were pooled to reduce the effect of inter-individual variation on gene expression. RNA was isolated from two pools of five larvae for each of the bacteria-containing replicates (M1 and M2), and two pools of five, plus one with four larvae from the bacteria-free (BF1) rearing bottle. RNA concentration was measured using a NanoDrop® ND-1000 UV visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was confirmed by inspection of intact ribosomal 28S and 18S bands after denaturing gel electrophoresis.

Total cDNA for qPCR was generated from 1 μg total RNA for all samples, using a mixture of random and poly-T primers from the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. A control lacking reverse transcriptase enzyme was included in each run. The synthesized cDNA was diluted 1:6 before qPCR.

Primer design, amplification efficiency and quantitative PCR

Twenty-two sequences identified from the subtracted libraries (generated from the first start feeding experiment) were selected for qPCR. Specific primers were designed to verify the differential expression of these genes in cod larvae from experiment 2.

Based on the findings of Rawls *et al.* (2004) and highly similar sequences available from cod in GenBank, qPCR primers were designed to specifically amplify eight genes (Table 1). *Serum ameloid* A1 was one gene reported as regulated by bacteria in zebrafish, but as there was no similar sequence available

Table 1 Primer sequences, amplicon size and putative function of genes quantified by real-time PCR

Gene abbreviation	Primer sequence forward	Reverse	Amplicon size	GenBank accession no	Gene name*	Putative function
Selected genes from the forward subtraction library (bacteria-free)						
*Apo*b	ATGTCTGTTACCACAAATGGAAGAAT	CTTGCTGTCTGCAACTAGGCTTT	81	GW574446	*Apolipoprotein* B	Lipid transportation
*Spg*21	GCGGCACCTCCATTTTTAAC	ATAAAGGCTGGCATCAACCAA	64	GW574447	*Maspardin*	Immune response
*Prdx*6	CCATGGTGCGGTTGAACTTC	CCGATAGGCAAAATTGGACAA	72	GW574434	*Peroxiredoxin* 6	Antioxidant response
*Sam*hd1	GGAAGCACGAGACTGCATCA	CATCACCGGCATTAGGTCATT	71	GW574403	*SAM and HD domain*-containing p1	Immune response
Sbds	GGGAACTCCAGGTTTCTGACAA	CGTCGCTATGTCCCTGAACA	68	GW574408	*Ribosome maturation* protein	RNA processing
*Sep*X	GGACAGTGTGTCCAAGCACGTA	TCCCGCACTTCCCACAGA	65	GW574431	*Selenoprotein* X	Antioxidant response
*Chmp*6	CAAATCTGGAGCGCATGGT	CAAATCTGGAGCGCATGGT	71	GW574453	*Charged multivesicular body* p 6	Endosomal sorting
*Txn*dc15	CCCGGAAACAGAGCAGGTTA	TTCTGCTCCTCCGTCACCAT	78	GW574421	*Thioredoxin domain containing p*15	Antioxidant response
Selected genes from the reverse subtraction library (bacteria-exposed)						
Bcrp	GGCATCCTCACCCTGAGAAC	GTCGCCTTCAGACCGATCAT	77	GW574350	*Breast cancer resistance* protein	Transportation
Bty	CTGATGTCCGTCTCCCTCTGA	CGCTCATTCAAATCAACAAACAC	67	GW574388	*Bloodthirsty*	Erythropoiesis
*Capns*1	TGCCGCGATGCCGTATATA	CCTGCCCACATATTGGTTCAT	67	GW574328	*Calpain*, small subunit 1 b	Protein catabolism
*Cdc*45	CAATGTCGACCTTTTGGAGATG	GCCGGTGAGTGTCACAGATG	71	GW574383	*Cll division cycle* 45 like	Replication
*Dtn*bp1	GCAAGCCTGCTGTCCATTTC	ATTGGAGACGGCTTCAGGAA	69	GW574367	*Dystrobrevin binding protein* 1a	Coiled coil domain
*Eif*4a1a	CACTGTCCCCGAAAAAGCA	CCCTTTTACATATGGTGAGGTAGGTT	74	GW574399	*Eukaryotic translation init. f* 4 i1a	Translation
*Glut*1	CCAAAGAGCTTGTAATGGCTGAT	CCGTCCTTAAAGGGCTGACA	66	GW574375	*Glucose transporter* 1	Transportation
*Muc*5b	GCTGGAGCTGTTCAGACATTGAC	TGGACGGCTGTGGGTGTAC	68	GW574336	*Mucin*	Cell integrity
Prvb	CCGCAGGATTTGAAAAACTTTG	GCCCTGGAAGCCTGCAA	62	GW574356	*Parvalbumin*	Muscle relaxation
*Psa*2	GCCAGCCTCGTTGCAGAT	AGAGCTTTGAGGGCCAGATG	62	GW574379	*Proteasome* subunit alpha 2	Protein catabolism
*Rp*A1	GAGCTGAACGAACCCCTTGA	TGGTCACCATGCCAACGA	64	GW574332	*Replication* protein a	Replication
*Sf*3b1	TTGGCATGAAAAGACTTTGCA	AAAGGTGAGCCAGCCCTACA	66	GW574376	*Splicing factor* 3b	Post-translational modifications
Selected genes from zebrafish						
C3	GTGGGAAACTACGCACCTTCA	AGATAGCTCGCATTTGTTTTCAGA	76	AY739672	*Complement component* 3	Immune response
Cyp1a	CCAACGCCACCATGAAGAG	GTGACGATCTTCTGCACAAAGG	66	DQ270488	*Cytochrome P450* family 1 sub. A	Xenobiotic biotransformation
Fdps	CGGCTGCAGGAGCTCATT	TGGCAAAATTGAGGAACACTGA	67	EB677097	*Farnesyl diphosphate synthetase*	Nutrient metabolism
Fiaf	ACCTCAGTGCGGACGTCAA	TCCGCAACTGCTGAACCA	65	CO542358	*Fasting induced adipose factor*	Nutrient metabolism
Gpx	ATGACCCCAATTCCCTCATG	AGATGTCGTTCCGGCAGACT	69	CO542193	*Glutathione peroxidise*	Stress response
*Itg*b3	GATCACTGCGTCCACGAACTT	GGATCGTGAAAGGACCGAAA	74	CO542430	*Integrin* ß3	Cell-surface receptor
Mbl-1	CGTCCAAAGATTGGCAGTTGA	ATGGCACCGACTGCTACAAAT	78	EB677057	*Serum lectin* 1	Immune function
Wars	TCGTCACAGGCCATGCAT	TCGAACACATCCTGTAGCCATT	71	CO542316	*Tryptophanyl t-RNA synthetase*	Amino acid metabolism

*In the gene name column, the letter 'p' is an abbreviation for 'protein'.

for cod, qPCR primers were designed to amplify cod *serum lectin* (*mbl*-1). Both serum ameloid A1 and serum lectins are thought to play important roles in the innate immune system of vertebrates. Primers were designed using the PRIMEREXPRESS software (Applied Biosystems, Life Technologies). Primer sequences, expected amplicon sizes and accession number are shown in Table 1. All qPCR primers designed in this study had an annealing temperature of 60 °C, and yielded a single band of expected size after gel electrophoresis of qPCR products.

qPCR reactions were performed using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA). Each 25 μL reaction contained 12.5 μL iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 5 μL diluted cDNA, 6.5 μL dH_2O and 200 nM of both the forward and reverse primers. The PCR program consisted of an initial step at 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 20 s at 72 °C. All reactions were run in triplicate, and a non-template control, as well as the control sample from the reverse transcription was included for each gene. Standard curves for each gene were generated by using 10-fold dilutions of known concentrations of plasmids, containing the specific amplicon. Using the standard curves, all the C_t values obtained were converted into mRNA copy number. Data from triplicate runs were averaged, and the results were finally normalized to β-*actin* expression, serving as a housekeeping gene (Kortner, Overrein, Øie, Kjørsvik & Arukwe 2010). Ninety-five per cent confidence intervals were calculated and used to infer statistical significance (data in supporting information).

Results

Survival of cod larvae, bacterial densities and diversity

Mortality was high in all rearing bottles in the first experiment. On day 10, the majority of larvae had died, and on the final day, only 2.3% of the bacteria-free larvae and 1% of the bacteria-exposed larvae were alive. There was no bacterial growth on any media from water sampled from the four bacteria-free replicates, while the density of bacteria in the bacteria-exposed replicates was around 10^6–10^7 cells mL^{-1} both on the day of stocking (day 1) and at 17 days post hatch, as determined by dilution plating. As there was no bacterial growth observed from the bacteria-free replicates, and the disinfection success of the protocol used has been shown to be 100% in previous experiments (T. Forberg, O. Vadstein & A. Arukwe, unpublished data), the bacteria-free status was considered to be maintained throughout the experiment.

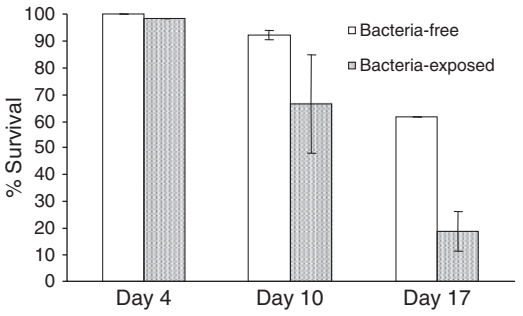

Figure 1 Percentage survival of cod larvae in experiment 2, with 95% confidence intervals ($n = 2$, except for day 17, where Bacteria-free $n = 1$).

Table 2 Flow cytometry results given as the number of gated events observed in samples from the different cod rearing bottles, on days 6, 10 and 17 after hatching

	Day 6	Day 10	Day 17
BF1	193	227	ND
BF2	176	238	42
M1	13 685	17 128	23 319
M2	13 486	17 154	32 341
FASW	123	17	23

The volume of sample counted was 47.31 μL. Three different batches of filtered autoclaved seawater (FASW) were used to indicate background 'noise'.
ND, not determined.

Survival in the second start feeding experiment was significantly higher than that in the first (Fig. 1). On day 10 of the experiment, survival was significantly higher in the bacteria-free replicates (BF1 and BF2) than that in the bacteria-exposed replicates (M1 and M2).

No bacterial contamination was detected in the rotifer or algae cultures throughout the experiment. For one of the two bacteria-free replicates, BF2, contamination was detected on day 12 (bacterial growth was discovered on a plate prepared on day 11), and the bottle was terminated. BF2 was considered to be bacteria-free up until day 10, as the flow cytometry results on that day did not indicate any bacterial growth. The remaining bacteria-free bottle (BF1) showed no bacterial growth on any media used, and the flow cytometry results (Table 2) also indicate that the bacteria-free condition was successfully maintained throughout the experiment. The density of culturable bacteria in M1 and M2 was 10^6–10^7 cells mL^{-1}, while the total bacterial count by flow cytometry (Table 2) was around 10^9 cells mL^{-1} on day 6, increasing to around 10^{10} cells mL^{-1} after

day 10 (numbers achieved by correcting the data in Table 2 with regard to dilutions and sample volume).

The number of bands in the DGGE profiles varied over time for both bacteria-exposed replicates M1 and M2 (Fig. 2). M1 showed an increase from seven to 18 bands from days 10 to 17 post hatch, while the opposite was true for M2, with a reduction from 23 to seven bands. A total of 32 unique bands were detected for the four samples, of which four were unique for M1 and 12 were unique for M2.

Pearson correlation coefficients calculated from the DGGE profiles showed a positive correlation in bacterial composition over time for both M1 (0.158) and M2 (0.260), while the correlations were negative when comparing M1 with M2 at the same sampling points (− 0.125 and − 0.133 respectively).

The Shannon diversity index calculated from the band pattern indices (Table 3) showed that the diversity of bands present almost doubled from 10 to 17 days post hatch in M1, while there was an almost threefold reduction in diversity in M2 during the same time span. Calculation of J', the relative diversity (evenness), showed the same trend.

Investigation of the individual peak area matrices (supporting information) from the M2b sample revealed that one of the seven bands represented 75% of the total band intensity. The distribution of DGGE bands was more even in the M1b sample, where the most dominating band at day 17 post hatch represented only 17% of the total band intensity. This is reflected in the differences in diversity and evenness (Table 3).

Suppression subtractive hybridization

Suppression subtractive hybridization PCR was used to analyse host gene expression responses. From each of the subtracted libraries generated, 96 randomly chosen clones were sequenced. After removing sequences that were too short or consisting only of the vector sequence, 156 remaining sequences were analysed using Blastx and Blastn.

From the reverse subtraction library, generated with bacteria-exposed cod (bacteria-exposed library), 87 putatively differentially expressed transcripts were identified. Among these were transcripts for proteins involved in cell adhesion, growth and transportation. A summary of the putative function of these sequences is shown in Fig. 3.

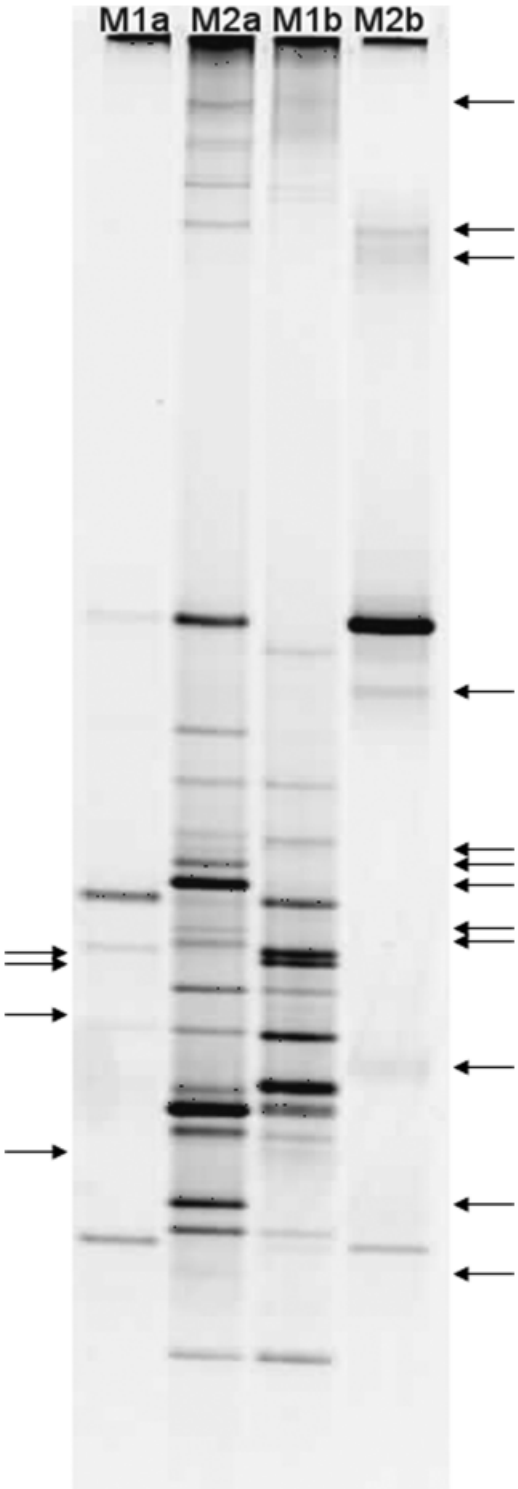

Figure 2 Denaturing gradient gel electrophoresis gel showing the separation of PCR products obtained by amplification of 16S rDNA genes (primers 338f-GC and 517r) from water sampled from M1 and M2 (the bacteria-exposed replicates) at day 10 (a) and 17 (b) post hatch. Arrows pointing to the left indicate bands unique for M1, while arrows pointing to the right indicate bands unique for M2.

Table 3 Species richness given as number of bands (*k*) in the DGGE profiles, Shannon diversity index (*H′*) and evenness (*J′*) calculated from DGGE results from water sampled from the bacteria-exposed replicates M1 and M2 at two different time points (a = 10 days post hatch, b = 17 days post hatch)

	M1a	M1b	M2a	M2b
k	7	18	23	7
H′	1.303	2.292	2.675	0.934
J′	0.670	0.793	0.853	0.480

The forward subtraction library, generated from the bacteria-free cod (bacteria-free library), produced 56 putatively differentially expressed transcripts, including transcripts for proteins involved in redox homeostasis and immune response (Fig. 3).

The majority of transcripts from the 'bacteria-exposed' and the 'bacteria-free libraries', 56% and 51%, respectively, had no significant similarity to sequences in GenBank. For clone IDs and Blastn/Blastx information of all transcripts, see Table S1 in supporting information.

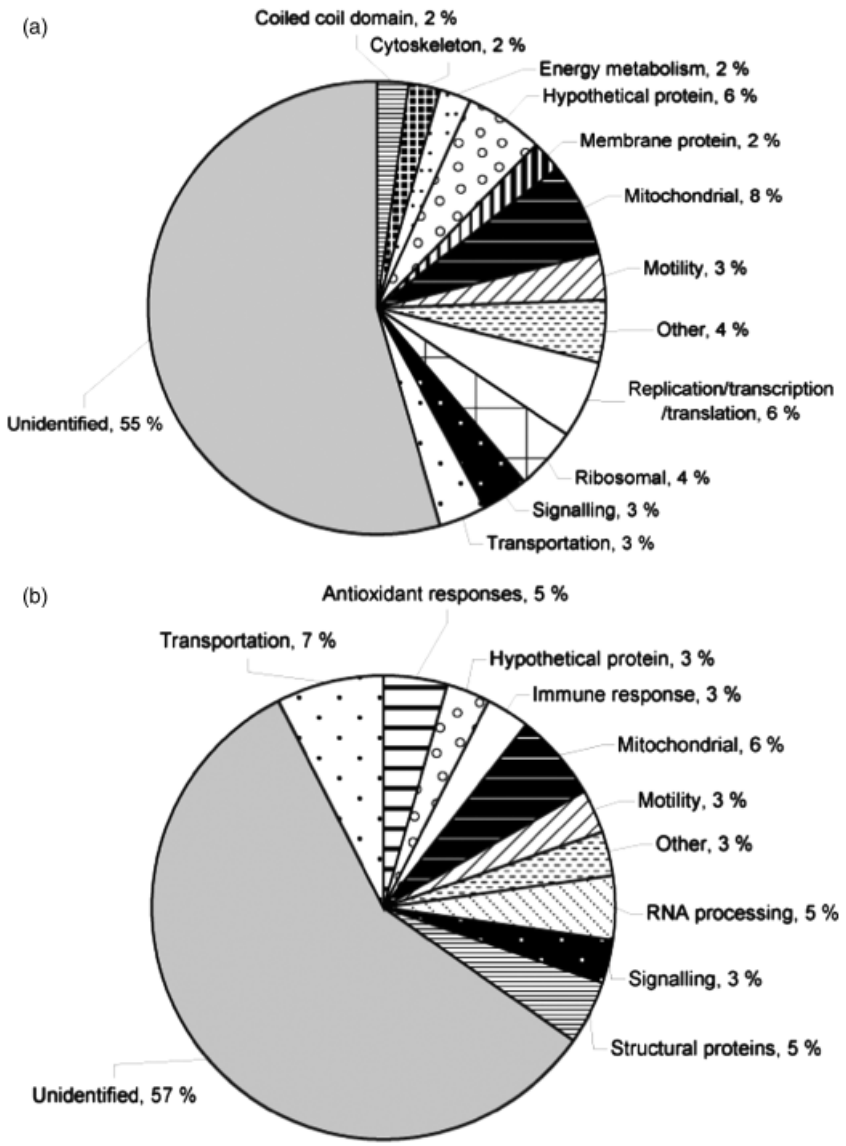

Figure 3 Distribution of sequenced clones from the; (a) 'bacteria-exposed' SSH library and (b) 'bacteria-free' SSH library, grouped by molecular function.

Quantitative expression of genes from the SSH libraries

To validate that the differential expression of transcripts identified by SSH was regulated either by the presence or absence of bacteria, gene-specific primers were designed to quantify expression in bacteria-free cod larvae vs. bacteria-exposed larvae (Table 1). The normalized expression data (see supporting information) for each gene were used to calculate the fold-change of expression between the bacteria-free larvae BF1 and the two bacteria-exposed biological replicates M1 and M2 (Fig. 4). Gene expression levels varied considerably between M1 and M2

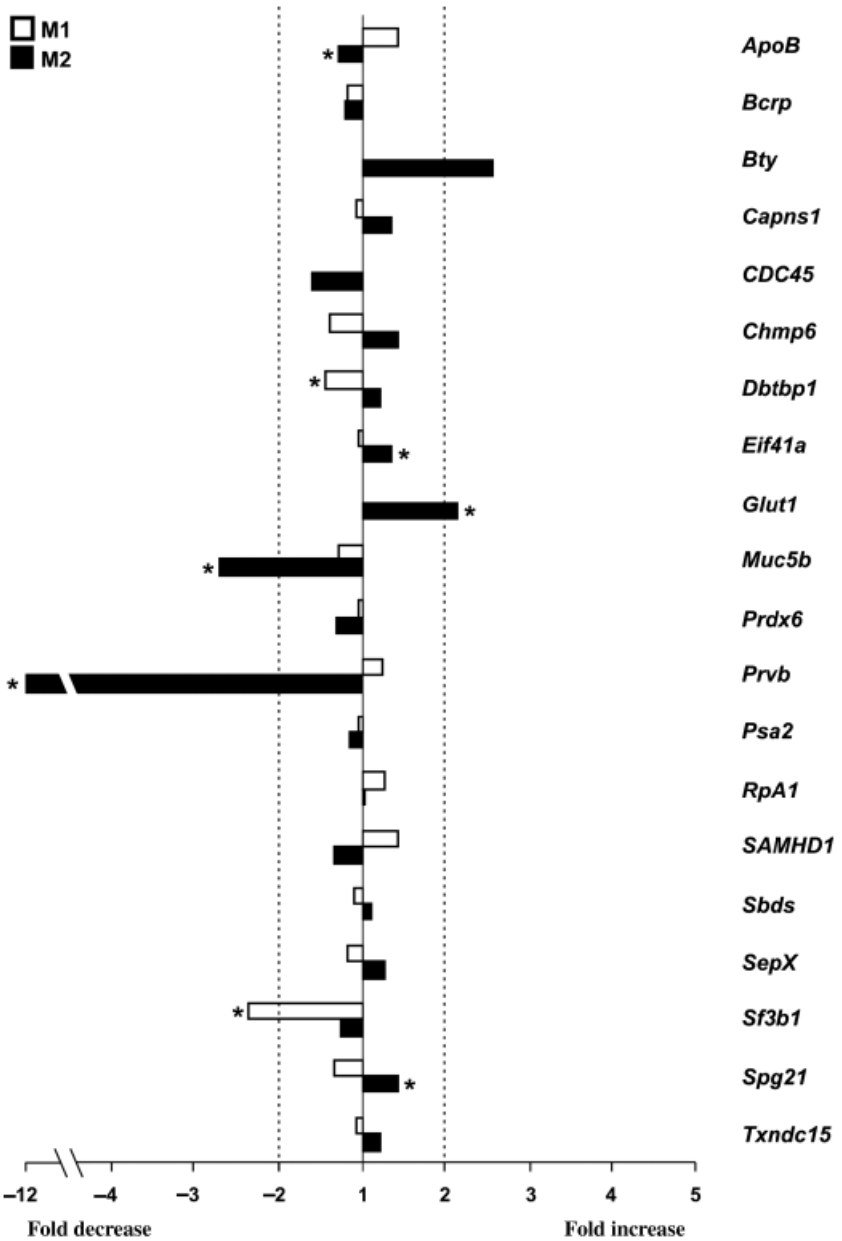

Figure 4 Fold change of gene expression levels between bacteria-free (set as 1) and the two bacteria-exposed cod larvae replicates M1 and M2. Positive values indicate up-regulation compared with the bacteria-free larvae, whereas negative values indicate down-regulation. Asterisks represent statistically significant differences ($P < 0.05$). The vertical dashed lines indicate fold changes > 2 (for gene names, see Table 1).

larvae. For 11 genes, the direction of fold-change was different (with regard to fold increase/decrease) in M1 and M2 larvae, while for five genes, the same fold-change trend was observed, but with differences in magnitude. A >2 fold-change was considered to be a biologically significant difference in expression pattern. Using these criteria, the expression of *splicing factor* 3b was significantly down-regulated in the M1 larvae. In the M2 larvae, the expression of *mucin* and *parvalbumin* was down-regulated, while *glut*1 and *bloodthirsty* was up-regulated, although the latter was not significant ($P>0.05$).

Quantitative expression of genes known to be regulated by bacteria in zebrafish

To investigate whether some host responses to bacteria are conserved between zebrafish and cod, eight genes were selected from Rawls *et al.* (2004) for quantitative expression analysis (Table 1). The bacteria-exposed cod replicates M1 and M2 varied with regard to the gene expression of all eight genes (Fig. 5). Above two fold change in gene expression, indicating biological significant differential expression was observed for four genes – *C*3 and *fiaf* were up-regulated in M2 larvae, while *mbl*-1 was down-regulated. For the M1 larvae, *cyp1a* was significantly down-regulated compared with the bacteria-free larvae.

Discussion

Strategies to unravel host gene expression responses to bacteria

Generally, there is a lack of knowledge on the molecular basis underlying host–bacteria interactions during an intensive culture of marine fish larvae, and relatively few studies have used molecular biological techniques to study these interactions in other fish species. Cod larvae are exposed to and interact with large numbers of bacteria during the larval stage, and their undifferentiated intestinal tract is exposed to large numbers of bacteria even before start feeding begins (Reitan *et al.* 1998). Understanding the molecular basis for interactions that may occur between the cod larvae and these bacteria could contribute to a more holistic understanding of host–microbe interactions. In this study, we reared bacteria-free cod larvae in order to unravel host responses to bacteria.

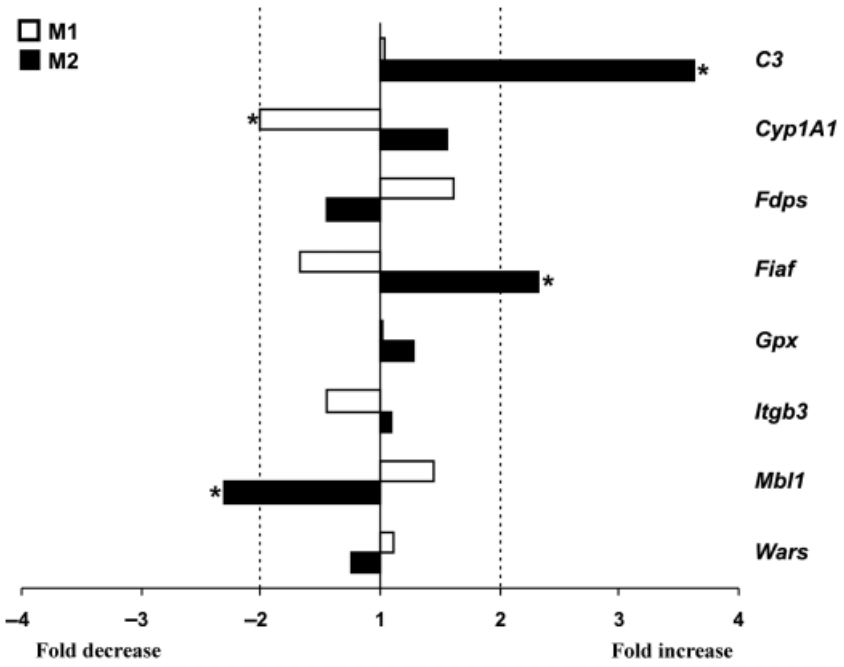

Figure 5 Fold change of gene expression levels between bacteria-free (set as 1) and the two bacteria-exposed cod larvae replicates M1 and M2. Positive values indicate up-regulation compared with the bacteria-free larvae, whereas negative values indicate down-regulation. Asterisks represent statistically significant differences ($P<0.05$). The vertical dashed lines indicate fold changes >2 (for gene names, see Table 1).

Two strategies were used to identify host gene expression responses in cod, namely SSH to identify potentially new host-response genes and analysis of selected host-response genes identified in zebrafish (Rawls *et al.* 2004) to investigate whether host responses are conserved between these phylogenetically distant fish species.

Suppression subtractive hybridization-PCR was chosen as an unbiased approach, because at the time when the experiments were performed, there was limited sequence information available for cod in the GenBank. Subtraction libraries were generated by hybridization between mRNA from bacteria-free and bacteria-exposed cod larvae, thereby enriching for genes that were responsive to these conditions. The SSH technique favours the enrichment of high-abundance transcripts, and is therefore susceptible to a high false-positive rate (Ji, Wright, Cai, Flament & Lindpaintner 2002; Mortensen & Arukwe 2007). We therefore performed the hybridization in both forward (up-regulated in bacteria-free cod) and reverse (up-regulated in bacteria-exposed cod) directions, to maximize the detection of host–microbe responsive genes. Sequencing of 192 clones revealed 143 putatively differentially expressed transcripts. A disadvantage of the SSH method is the generation of redundant clones, as was seen in this study. In addition, more than half of the clones sequenced had no significant hits in GenBank. These could represent important host-response genes, but were not investigated further. As can be seen in Fig. 3, the putatively differentially regulated genes identified represent wide ranges of molecular functions, indicating the complexity of host responses towards the bacteria.

To verify that the SSH transcripts represented host responses of cod to bacteria, 20 transcripts (Table 1) were selected for qPCR with mRNA isolated from a second start feeding experiment. The qPCR results showed that only five of the 20 genes had biologically significant differences in expression (i.e. a fold-change >2) when comparing bacteria-exposed M1 or M2 larvae with the bacteria-free cod larvae. *Bloodthirsty*, *glut* 1, *mucin*, *parvalbumin* and *splicing factor* 3b were all putatively up-regulated by bacteria according to our SSH results. However, the qPCR data showed significant down-regulation of *mucin*, *parvalbumin* and *splicing factor* 3b, while *glut*1 and *bloodthirsty* were up-regulated. This discrepancy suggests that the SSH library may represent genes that were randomly expressed differently between the pooled larvae used for the generation of the libraries, rather than genes regulated by the presence of bacteria. Despite the fact that the hybridization was performed in both directions to maximize the detection and identification of differentially expressed genes, it may still have omitted rare targets, suggesting that sequencing of more clones could have been necessary for the detection of differentially expressed genes. In accordance with our study, Ghorbel, Sharman, Hindmarch, Becker, Barrett and Murphy (2006) reported that only 459 out of 1152 sequenced SSH-PCR clones were actually differentially regulated using microarray analysis. The variable physiology of the cod larvae may have also caused the results to differ between the two experiments. For example the poor survival of cod larvae in the first experiment complicates the comparison of gene expression patterns. The composition of the bacteria present in the bacteria-exposed conditions in the two different experiments may have also been too different to produce comparable data between the qPCR and the SSH results. The successful use of SSH-PCR as a strategy to identify host responses to bacteria may be dependant on reduced biological variability in the experimental system. This could be achieved by running purely gnotobiotic studies, where bacteria-free cod larvae are compared against larvae reared with a known, defined microbiota.

As a biased approach, known zebrafish host responses to bacteria were analysed in order to evaluate whether these were also conserved in cod larvae. Quantitative PCR results showed that four out of the eight investigated genes produced biologically significant differences in expression pattern (i.e. fold-change >2) when comparing bacteria-exposed (M1 or M2) with the bacteria-free cod larvae. *Cyp1a* expression was significantly higher for the bacteria-free larvae, compared with M1 larvae. *Cyp1a* expression was also found to be higher in axenic zebra fish (Rawls *et al.* 2004), and in germ-free mice, the expression of several genes involved in xenobiotic metabolism has been shown to be up-regulated (Hooper, Wong, Thelin, Hansson, Falk & Gordon 2001). The hypothesis is that members of the normal intestinal microflora can assist the host in xenobiotic biotransformation (Hooper & Gordon 2001). A metagenomic analysis of the human microbiota also showed the enrichment of metabolic pathways for xenobiotic degradation (Gill, Pop, Deboy, Eckburg, Turnbaugh, Samuel, Gordon, Relman, Fraser-Liggett & Nelson 2006).

Fiaf is a circulating lipoprotein lipase inhibitor; its expression was suppressed in conventionalized (with a normal microflora) mice and zebrafish (Rawls *et al.* 2004), and this suppression is thought to be respon-

sible for microbiota-induced fat storage. Germ-free animals are known to have reduced fat storage, even if their food consumption equals that of conventionalized animals, and *fiaf* expression is increased in these animals (Backhed, Ding, Wang, Hooper, Koh, Nagy, Semenkovich & Gordon 2004; Rawls *et al.* 2004; Rakoff-Nahoum & Medzhitov 2006). In the present study, M2 larvae showed a significantly higher expression of *fiaf* than the bacteria-free larvae. The M1 larvae had lower expression levels than the bacteria-free larvae, albeit not significant. It could be argued that the observed absence of bacterial effects on *fiaf* suppression in M2 larvae may be due to the composition of bacteria present. In the study by Rawls *et al.* (2004), *fiaf* was suppressed by the presence of an unfractionated microbiota, but not suppressed in fish that were mono-associated with two different bacterial species.

As cod larvae have not yet developed an adaptive immune system, their only defence against bacteria involves the components of the innate immune system. Commensal bacteria are thought to play an important part in educating the immune system of the host (Rakoff-Nahoum & Medzhitov 2006). Previous studies have shown differential up-regulation of immune-related genes in cod head kidney cells exposed to different probiotic candidates (Caipang, Brinchmann & Kiron 2009; Lazado, Caipang, Gallage, Brinchmann & Kiron 2010). The expression levels of two innate immune system components, *C3* and *mbl*-1, were quantified in this study. *C3* was significantly up-regulated in M2 larvae, but not in M1 larvae. *C3* was also one of the genes identified by Rawls *et al.* (2004), where the expression was induced by unfractionated microflora, but not by the two mono-associations tested. The putative lectin sequence (*mbl*-1) we selected for qPCR encodes a C-type serum lectin with probable galactosyl-binding properties, and could be involved in binding carbohydrate structures present on bacteria. Our results indicate that its expression in cod larvae is probably not directly up-regulated by the presence of bacteria, as it was down-regulated in M2 larvae and no significant fold-change was observed for the M1 larvae.

By choosing a biased approach where only selected, characterized genes are investigated, the amount of information gained is limited, and previously unknown host responses cannot be identified. On the other hand, the unbiased approach we chose in this study (SSH) was limited by the amount of sequence information available for cod in GenBank, as more than half of the sequenced clones have yet to be assigned a function. Combining biased and unbiased approaches may be the best strategy to identify host-response genes.

Are host gene expression responses to bacteria conserved?

Out of the 212 host-response genes identified in zebrafish, 59 are also conserved in mouse intestinal cells (Rawls *et al.* 2004). There seems to be some conservation of host gene expression responses to bacteria also between cod and zebrafish, as three out of eight genes, *C3*, *cyp1a* and *fiaf*, had biologically significant differences in expression depending on the bacterial status of the cod larvae. The effect on the individual genes differed, however, especially for *fiaf*. This indicates that the type of response is not always conserved even if the bacteria present influence gene expression. Although some host responses are conserved between mice and zebrafish, this may not be valid for marine species such as cod. One of the major morphological differences observed in germ-free mice and zebrafish is reduced differentiation of epithelial cells in the digestive tract, which is also reflected in the gene expression responses (Guarner & Malagelada 2003; Rawls *et al.* 2004; Bates, Mittge, Kuhlman, Baden, Cheesman & Guillemin 2006). However, in a recent study by Rekecki, Dierckens, Laureau, Boon, Bossier and Van Den Broeck (2009), bacteria-free sea bass (*Dicentrarchus labrax*) larvae were significantly larger than those reared with bacteria, and also had more developed digestive tracts. This morphological difference probably also reflects differences in gene expression.

Effect of different bacterial content on host responses

There were significant differences in the expression between the two bacteria-containing replicates for most of the genes quantified in this study, which could be due to different bacteria present in the M1 and M2 rearing bottles. Bacterial densities in both bottles increased towards the end of the experiment. This could be due to the increased amounts of available nutrients, as more rotifer culture was added each day. The faecation of both rotifers and cod larvae contributed to the dissolved organic carbon in the rearing bottles. Denaturing gradient gel electrophoresis and subsequent profile analysis revealed significant differences between M1 and M2 with regard to the bacterial communities present.

In the M2b profile, there was an indication of dominance by one bacterial species, as one band represented 75% of the bacterial community. This could go a long way towards explaining why the gene expression results were so different between M1 and M2. Both *C3* and *fiaf* expression were differentially expressed (and significantly so) in zebrafish exposed to mono-associations of bacteria, compared with an unfractionated microflora. In our study, the fold-changes for M1 and M2 for these two genes were contradictory with regard to up- or down-regulation, and based on the DGGE analysis, it could be speculated that the M2 cod larvae were practically mono-associated at the time of sampling.

In conclusion, we have identified eight cod genes whose expressions were influenced by the bacterial content of the rearing water. The up- or down-regulation of these genes by bacteria should be confirmed by analysing gnotobiotic mono- and poly-associated cod larvae, as undefined bacterial communities clearly produce large differences with regard to gene expression. When undertaking host–microbe interaction studies, we recommend starting with as defined microbial conditions as possible, even if gnotobiotic conditions are very distant from the normally complex microbial communities present.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clone IDs, blastn/blastp hits, *e*-value, GenBank accession # and assigned function for all sequenced SSH clones.
Table S2. QPCR data of genes selected from the SSH libraries.
Table S3. Peak area matrices for DGGE profiles, samples M1 and M2 at day 10 (a) and day 17 (b) after hatching.

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