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ORIGINAL ARTICLE

Estrogen Modulates Hepatic Gene Expression and Survival of Rainbow Trout Infected with Pathogenic Bacteria *Yersinia ruckeri*

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Abstract In the aquatic environment, fish are exposed to various stimuli at once and have developed different response mechanisms to deal with these multiple stimuli. The current study assessed the combined impacts of estrogens and bacterial infection on the physiological status of fish. Juvenile rainbow trout were exposed to two different concentrations of 17β-estradiol (E2) (2 or 20 mg/kg feed) and then infected with three concentrations of *Yersinia ruckeri*, a bacterial pathogen causing massive losses in wild and farmed salmonid populations. Organism-level endpoints to assess the impact of the single and combined treatments included hepatic vitellogenin transcript expression to evaluate the E2 exposure efficiency and survival rate of pathogen-challenged fish. The two E2 doses increased vitellogenin levels within the physiological range. Infection with *Y. ruckeri* caused mortality of trout, and this effect was significantly enhanced by a simultaneous exposure to high E2 dose. The hormone reduced survival at intermediate and high (10^4 and 10^6 colony forming units, cfu) bacterial concentrations, but not for a low one (10^2 cfu). Analysis of hepatic gene expression profiles by a salmonid 2 k cDNA microarray chip revealed complex regulations of pathways involved in immune responses, stress responses, and detoxification pathways. E2 markedly reduced the expression of several genes implicated in xenobiotic metabolism. The results suggest that the interaction between pathogen and E2 interfered with the fish's capability of clearing toxic compounds. The findings of the current study add to our understanding of multiple exposure responses in fish.

Keywords Disease resistance · 17β-Estradiol · Immunity · Xenobiotics metabolism · Rainbow trout · *Yersinia ruckeri*

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Introduction

In their natural and farm environments, fish are exposed to multiple external and internal stimuli, including endocrine perturbations, pollutants, and pathogens. In the past, studies of "single stimulus, single effect" prevailed. Limitations of this research strategy are obvious in light of the growing body of evidence that combined exposures lead to alterations that can be markedly different from the effects of single stressors (e.g., Kiesecker 2002; Sexton and Hattis 2007; Crain et al. 2008; Wenger et al. 2011). Therefore, interest for a better understanding of interactive and

cumulative effects of joint exposures is increasingly growing. Recent advances of high-throughput analytical techniques enable researchers to combine targeted experimental studies on multiple stressors with hypotheses-free screening of molecular responses that may reveal biological effects, which are hard to predict based on existing knowledge on the response to single exposures.

Sex steroid hormones are known as immunomodulators in mammals and may act in a similar way in fish (Casanova-Nakayama et al. 2011; Milla et al. 2011; Liarte et al. 2011). The potential role of sex steroids for disease resistance of fish is of substantial interest and importance for aquaculture as well as in environmental research. Effects of androgens on various components of fish immune system have been addressed in studies with different species and isolated cells (reviewed in Milla et al. (2011)). While the action of androgens appears to be predominantly suppressive, estrogens have been reported to both stimulate and inhibit immune processes (Straub 2007; Milla et al. 2011). Estrogen effects on the fish immune system were found to be primarily suppressive, be it at the level of immune gene expression (e.g., Tilton et al. 2006), soluble immune factors such as lysozyme or immunoglobulins (e.g., Thilagam et al. 2009), immune cell functions such as phagocytosis activity (Watanuki et al. 2002), or overall immunocompetence against pathogens (Wenger et al. 2011). The suppressive effect might be explained by competition between the immune system and the reproductive system for energy resources. Thus, E2 would mediate a trade-off between two energy-costly processes, immune defense and reproduction, as it has been shown for other vertebrates (French et al. 2009; Barber et al. 2011). There exists circumstantial evidence that the immunocompetence of reproducing fish is compromised (Rice 2001; Yada and Nakanishi 2002). Currently, however, we lack understanding of the drivers and mechanisms behind this phenomenon. Also, environmental substances with sex steroid-like activities may be able to modulate the immune system of exposed fish (Casanova-Nakayama et al. 2011; Milla et al. 2011). The aquatic environment receives a variety of hormonally active compounds from sewage treatment plants, industry, animal farms, etc. (Sumpter and Johnson 2005). Many of these so-called endocrine disruptors are able to bind to estrogen receptors (ER), thereby mimicking the activity of endogenous ER ligands; they include both natural estrogens such as 17 β-estradiol (E2) and synthetic chemicals such as ethinylestradiol. To date, studies on the effects of environmental estrogen-active compounds on fish focused on disturbances of reproduction and sexual development, for example, feminization of male fish (e.g., Jobling et al. 1998; Fenske et al. 2005). More recently, the potential of estrogen-active environmental contaminants to modulate immunocompetence of exposed fish has attracted attention (Shved et al. 2009; Thilagam et al. 2009; Jin et al. 2010; Cabas et al. 2012). However, the ability of environmental estrogen-active compounds to modulate the immune parameters of exposed fish does not necessarily implicate an influence on disease resistance. To assess the potential impact of environmental estrogens on the immunocompetence of fish, challenge experiments, in which fish are exposed to pathogens, are needed. Strong evidence for the reduced capability of estrogen-exposed fish to combat a bacterial pathogen was recently provided by Wenger et al. (2011). Juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to E2 showed significantly reduced survival after infection with *Yersinia ruckeri* compared to a non-E2-exposed control group challenged with the pathogen. The effect of the hormone on pathogen resistance coincided with a reduced ability to up-regulate several complement genes in response to the bacterial infection (Wenger et al. 2011).

The present study aims to gain a broader understanding of how environmental estrogens influence the physiological status of rainbow trout under bacterial pathogen challenge. To this end, gene expression profiles in the liver of bacteria-infected fish kept under control conditions or under E2 exposure were assessed using a salmonid cDNA microarray chip designed for studies of responses to stressors and pathogens (Krasnov et al. 2005; Jorgensen et al. 2008). The liver was selected as a target organ due to its important role in the (innate) immunity of fish (Rice 2001) and, on the other hand, because it is the central metabolic organ of the vertebrate body executing a broad range of metabolic processes and functions (Hinton et al. 2008). Experimentally, we applied a multi-factorial design using different levels of E2 and bacteria in various combinations. The efficiency of the E2 treatment was assessed by measuring the hepatic mRNA levels of vitellogenin (*vtg*), which is under direct control of the estrogen receptor being the most widely used biomarker for an estrogenic exposure.

Materials and Methods

Experimental Setup

Juvenile rainbow trout (average weight, 1.15 g; average length, 5 cm) were obtained from a local trout farm (Pisciculture de la Gruyère, Neirivue, Switzerland) and kept in aerated glass tanks with a flow-through of 1 l/s of normal tap water (municipally non-chlorinated, but UV-treated water, which is a mix of groundwater and spring water with an average water temperature of 13.5±0.9 °C) until 3 days before the challenge experiment. The fish were divided into three groups with two replicates per group to obtain three different 17beta-estradiol (E2) treatments (no E2−control feed=NE2, low-dose E2=2 mg E2/kg food=LE2, high-dose E2=20 mg E2/kg food=HE2; Fig. 1). Five individuals per treatment group were examined parasitologically and

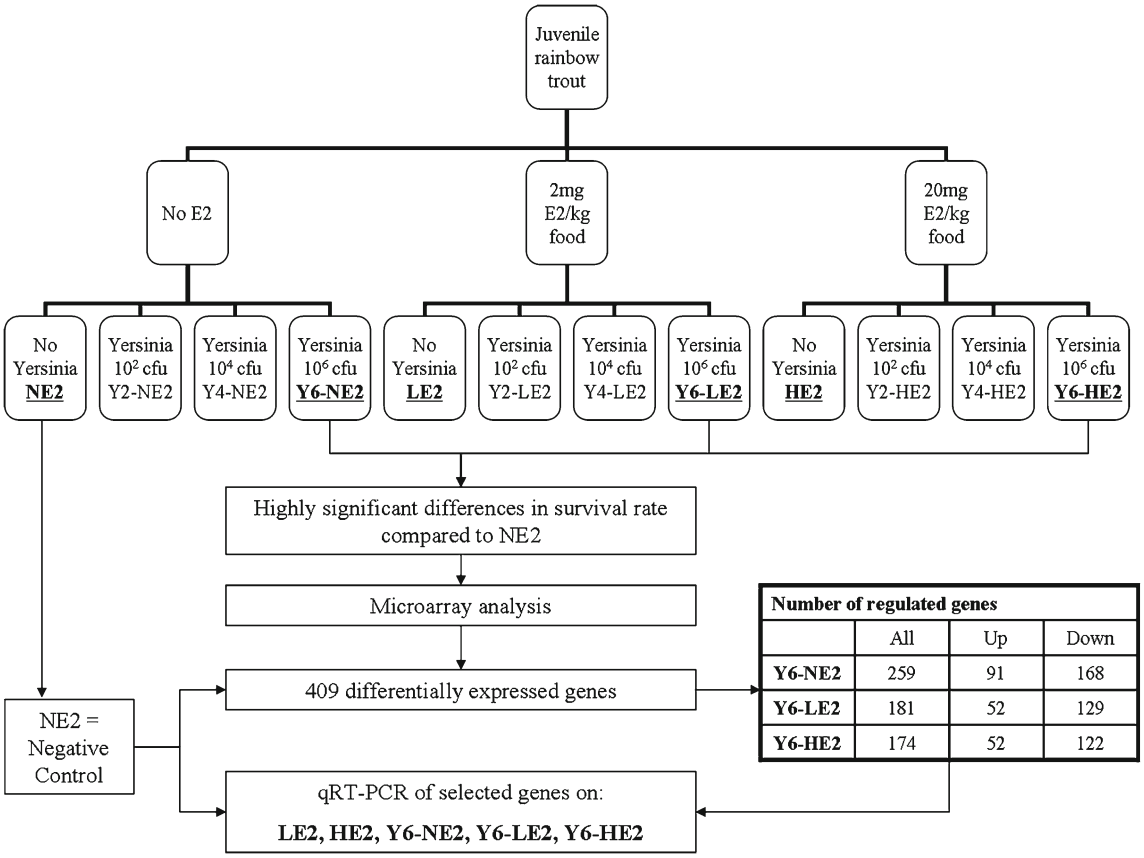

Fig. 1 Experimental design with treatment groups and respective nomenclature. All measurements and results were compared to the negative control group NE2. The design of microarray and qPCR analyses and numbers of differentially regulated genes are shown (treatment groups included in the analyses are in *bold*). More details are presented in "Materials and Methods". *E2* 17β-estradiol, *cfu* colony forming units/milliliter

bacteriologically before the start of the treatment. Feeds were prepared as described in Wenger et al. (2011). Shortly, commercially available dry trout food (Biomar, Brande, Denmark) was spiked with E2 (Sigma, Buchs, Switzerland) and then processed according to the alcohol evaporation method as described by Guerrero (1975). The initial feeding level was at 3 % of body weight for 5 weeks and was adjusted accordingly every 2 weeks. During the experiment, feeding level was reduced to 1.5 % of body weight per day until the end of the experiment and included the E2-enriched diet. Three days before the challenge experiment, the three groups were sub-divided into 12 challenge groups with two replicates per group and 75 fish/replicate. Each replicate was kept in a separate aerated glass tank (volume, 120 l; flow-through, 0.8 l/s; average temperature, 13.8±0.1 °C) for the duration of the experiment.

Infection with *Y. ruckeri*

Y. ruckeri strain JF3685 was grown overnight in Luria Bertani broth at 25 °C with aeration. Cell suspensions were then prepared in sterile phosphate-buffered saline (pH 7.4). The bacterial concentration was adjusted according to the optical density at 600 nm, confirmed by plate count from serial dilutions, and indicated as colony forming units (cfu)/ml solution. Infection with *Y. ruckeri* was conducted as previously described in Wenger et al. (2011). For infection, fish (n=75/treatment group) were transferred and kept in aerated 30-l tanks/replicate filled with 5 l of normal tap water (non-chlorinated but UV-treated municipal water, temperature 13.8±0.7 °C) for 1 h with four different bacterial concentrations (no *Yersinia*=negative control, 10^2 colony forming units/ml (cfu)=Y2, 10^4 cfu=Y4, 10^6 cfu=Y6; Fig. 1) and then re-transferred to their original tanks. Next, fish were sampled at day 0 (before infection), day 3, and day 10 post-infection (p.i.). At each sampling, ten fish/replicate (total, 20 fish/treatment group) were sacrificed using MS-222 (ready-to-use, buffered 3-aminobezoic acid ethyl ester methanesulphonate, Argent Chemical Laboratories, Redmont, CA, USA, dose 150 mg/L). Fish length and weight were first measured and then liver, head kidney, and spleen were carefully dissected and placed in 1 ml RNAlater© (Ambion, Austin, TX, USA) at 4 °C for 24 h and afterwards stored at −20 °C until further processing. Ten liver samples

per treatment group were used for vitellogenin transcript measurements and five samples (three plus two per replicate) were used for microarray analysis. The experiment ended at day 15 p.i. when no more mortality occurred.

To confirm the *Yersinia* infection as the cause of mortality, a re-isolation of *Y. ruckeri* from infected fish was performed after every sampling as described in Wenger et al. (2011). Samples were taken freshly from the liver (before storing), spleen, and head kidney of five sampled fish, plated on blood agar, and incubated for 48 h at 22 °C. Re-isolated bacteria were identified phenotypically using the API20E® system (BioMérieux (Suisse) SA, Geneva, Switzerland). The identity was then confirmed by sequencing of the 16 S rRNA gene according to Kuhnert et al. (2002). Survival rate analysis was performed using Mantel–Haenszel log-rank test to detect differences ($p<0.05$ for significant and $p<0.001$ for highly significant) between negative control and treatment groups as well as within and between different treatment groups. The software used to perform this statistical analysis was NCSS 2004 (NCSS, Kaysville, UT, USA).

Microarray Analyses

Microarray analyses were performed in three treatment E2 groups (Y6-NE2, Y6-LE2, and Y6-HE2; $n=5$ fish (three plus two per replicate) randomly picked and analyzed per treatment group; see Fig. 1) infected with a high concentration of bacteria, totalling in 15 microarrays, and compared to a pool of five fish from negative control (NE2=no E2, no *Y. ruckeri*). Total RNA was extracted and purified from the sampled livers by using a combined Trizol Plus/PureLink RNA Mini Kit (Invitrogen, Basel, Switzerland) extraction method according to the manufacturer's protocol. Yield and purity of extracted RNA were tested on a NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). The salmonid fish cDNA microarray platform SFA2.0 (GEO Omnibus GPL6154) includes 1,800 genes, each printed in six spot replicates. Synthesis of cDNA and hybridization were carried out as described in Koskinen et al. (2004). Shortly, samples with 10 μg RNA in each were labeled with Cy3-dUTP (pooled sample of untreated fish used as reference) and Cy5-dUTP (fish from the exposed groups) (Amersham Biosciences, UK); labels were introduced during cDNA synthesis using SuperScript III RT (Invitrogen, Basel, Switzerland). The cDNA synthesis was performed at 46 °C for 3 h in 25-μl reaction volumes. RNA was degraded by adding 2.5 M NaOH at 37 °C for 15 min and alkaline was neutralized with 2 M HEPES buffer. Targets were then combined and purified using Microcon YM-30 filter (Millipore, Billerica, USA). The microarray slides were pretreated with blocking solution (1 % BSA fraction V, 20× SSC, and 5 % SDS) at 50 °C for 30 min, then washed with 2× SSC (3 min) and 0.2× SSC (3 min). For hybridization, Lifter Slips (Erie Scientific, Portsmouth, NH, USA) were placed on the slides. Labels were adjusted to 80-μl volumes and contained 1.3× Denhardt's, 3× SSC, 0.3 % SDS, 0.67 μg/μl polyadenylate, and 1.4 μg/μl yeast tRNA. Hybridization was carried out overnight at 60 °C in a water bath; ArrayIt® Hybridization Chamber was used. Next, slides were washed in 0.5× SSC/0.1 % SDS (15 min), 0.5× SSC/0.01 % SDS (15 min), and twice in 0.06× SSC (3 min each) at room temperature in dim lighting with gentle agitation. Slides were dried using ArrayIt® Microarray High-Speed Centrifuge. Scanning was performed on a GenePix Personal 4100A microarray scanner (Molecular Devices, CA, USA) and images were processed with GenePix Pro 6.0. The spots were filtered by criterion $(I-B)/(S_I+S_B)\geq 0.6$, where I and B are the mean signal and background intensities and S_I and S_B are the standard deviations, respectively. The low-quality spots were excluded from analyses and the genes presenting with less than three high-quality spots on a slide were discarded. After subtraction of median background from median signal intensities, the $\log_2$-ER (test to control expression ratios) was calculated. Lowess normalization was performed first for the whole slide and next for 12 metarows and four metacolumns per slide. The data were submitted to GEO Omnibus (GSE38763).

Statistical analyses were performed in three stages: first, technical accuracy was assessed by difference of the mean $\log_2$-ER from zero (six spot replicates per each gene; Student's t-test, $p<0.01$); second, the genes with technically significant changes in at least three of five samples in at least one study group were selected and difference from uninfected control ($\log_2$-ER equal to zero) was assessed (one sample t-test, $p<0.05$). The differentially expressed genes that responded to *Yersinia* were selected at this stage. Finally, effects of E2 were assessed with one-way ANOVA followed with Newman–Keuls test ($p<0.05$). All study groups analyzed with microarray were infected with the pathogen and E2 was the only differential factor. Hierarchical clustering of samples was performed by profiles of differentially expressed genes. Pearson's r was used as a distance metric and a tree was constructed with Ward's method.

Quantitative Real-Time RT-PCR

The qPCR analyses were carried out with six treatment groups and five fish/replicate of treatment group (total of ten fish/treatment group). Total RNA was extracted from liver tissue using RNEasy Mini Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Possible genomic DNA contamination was removed using TURBO DNA-free™ (Ambion, Austin, TX, USA). Yield and purity of extracted RNA were tested on a

NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA) according to the manufacturer's protocol. Measurements of 260/280 ratio and 260/230 ratio were made in triplicate and only samples with a range of 1.8–2.1 (260/280 ratio) and 1.7–2.0 (260/230 ratio) were used for subsequent steps. For cDNA synthesis, total RNA amount was adjusted to 1 μg per 8-μl solution and then reverse-transcribed using the following reagents per 8 μl sample: 80 U RNAse inhibitor, 10 mM of each dNTP, 200 U M-MLV Reverse Transcriptase, 500 ng Random Primers (all Promega AG, Wallisellen, Switzerland), and 1 μg total RNA.

Primers and probes (Table 1) were designed using Primer Express Version 3.0 software (Applied Biosystems, Rotkreuz, Switzerland) produced by Microsynth (Balgach, Switzerland) and spanned intron/exon boundaries when appropriate information was accessible. Internal probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' with 6-carboxytetramethylrhodamine (TAMRA). Primer and probe efficiencies were assessed by generating a dilution curve representing tenfold dilution steps with at least five measurement points in triplicate and calculated according to the equation $E=10^{(-1/\text{slope})}-1$ where $E=2$ equals 100 % efficiency corresponding to a slope of −3.32. Quantification was performed using the TaqMan® real-time RT-PCR assay (Applied Biosystems). All analyses were conducted on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) in triplicate with the following steps: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C (point of data collection). Each sample contained 12.5 μl Taqman® PCR Universal Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 500 nM of forward primer and reverse primer, respectively, 160 nM internal probe, 6 μl DEPC-treated water (Ambion Inc.), and 2 μl of template to a final volume of 25 μl. DEPC-treated water (Ambion Inc.) was used as a negative control template in each analysis. Expression levels of all target transcripts were normalized against 18 S rRNA, which was used as a reference gene and measured using the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems) according to the manufacturer's protocol. 18 S rRNA expression did not show a significant variation between samples and was therefore considered suitable as a reference gene. The efficiency of the supplied primers for the reference gene was tested by generating a dilution curve representing tenfold dilution steps as previously described. ER of the different treatment groups were calculated using REST 2009—Relative Expression Software Tools, version 2.0.13 (Pfaffl et al. 2002). Differences between the expression ratios of the treatment groups were determined using Pair Wise Fixed Reallocation Randomisation Test© (Pfaffl et al. 2002). To test for significant differences between replicates within the treatment groups at each sampling point, one-way analysis of variance (ANOVA) for normally distributed data and Kruskal–Wallis ANOVA for unequal distributed data were used. These statistical analyses were performed using NCSS 2004 (NCSS, Kaysville, UT, USA). All differences with $p<0.05$ were considered as statistically significant and $p<0.001$ as highly significant.

Results

Hepatic Vitellogenin Expression

The efficiency of exposure to E2 was tested by measuring hepatic *vtg* mRNA expression using quantitative real-time RT-PCR. Samples ($n=15$) were taken before the start of E2 treatments and again immediately before the onset of the challenge experiment (after 5 weeks of exposure). By this time, significant up-regulation of *vtg* was detected in those groups receiving E2-enriched food, with expression ratios ER (to control group) of 4,213±542 in LE2 and 32,036±3,719 in HE2 ($p<0.001$ in both groups), while no significant elevation was observed in group NE2 (ER=2.063±0.241). Also, the difference between LE2 and HE2 was significant ($p<0.001$). No significant differences in *vtg* were detected in the replicate groups.

Survival Analysis

The survival rates were determined in control and E2-treated rainbow trout exposed to four concentrations of *Y. ruckeri*. No significant differences in mortalities were detected between the replicates ($p>0.05$), and therefore results of the replicates were combined. Also, no mortalities occurred in the negative control group (no bacteria, no estrogen), which was evidence for *Yersinia* infection-related mortalities in bacteria-infected groups Y4 and Y6 (Fig. 2). No mortalities were recorded in the groups exposed to E2 exposure only (NE2, LE2, HE2) and the lowest dose of bacteria —10^2 cfu (data not shown). In the groups infected with 10^4 cfu (Y4), survival rates were equal to 99.1 % (Y4-NE2); co-treatment with E2 did reduce survival rates (Y4-LE2, 98.3 %; Y4-HE2, 92.6 %), but only the difference between groups Y4-NE2 and Y4-HE2 was significant ($p<0.05$). Treatment with 10^6 cfu of *Y. ruckeri* (Y6) markedly reduced survival rates ($p<0.001$) in comparison with 10^4 cfu and reached 61.1 % in Y6-NE2. Again, E2 co-treatment led to a further reduction of survival rates, with 53.2 % in Y6-LE2 and 34.8 % in Y6-HE2. The differences of survival rates were highly significant between group Y6-NE2 and group Y6-HE2 ($p<0.001$) but not between groups Y6-NE2 and Y6-LE2. Also, comparing groups Y6-LE2 and Y6-HE2 showed highly significant different survival rates ($p<0.001$).

Table 1 Primers and probes used for qPCR

Gene name	Accession EMBL	Short name		Sequence	Amplicon length	Efficiency, %
Vitellogenin	X92804	Vtg	Forward primer	TCTGAAGTCAACGCAGTTAAATGT	90 bp	99.85
			Reverse primer	AGAGGCATGTTGATCTTGTACTT		
			Probe	CATGGTCAGAGACACATTGACAACATTTA		
NF-kappaB inhibitor alpha-1	CA343143	Nfkbia	Forward primer	AGCAGACGACAGTGGGAACAC	78 bp	98.47
			Reverse primer	TGGGTGATCACACTGAAGGAGAT		
			Probe	TCTCCACATCGCCTGTAAGAAGGGCTCT		
Cathepsin D-1	CA347041	Ctsd1	Forward primer	ATCTGCCTGAGTGGCTTCATG	78 bp	104.60
			Reverse primer	AATGAATACATCTCCCAGGATCCA		
			Probe	TGGATATCCCCGCCCCCGC		
UDP-glucuronosyltransferase 1-8	CA349227	Ugt8	Forward primer	ACTCACTATAGTTCTCATTGTTGTCATCGT	93 bp	107.63
			Reverse primer	TTCTTCATAGACCTCTTCGTTCCA		
			Probe	CGTTAAATGCTGCATAGTCTGCTTCCGC		
Mitogen-activated protein kinase 9-1	CA373029	mapk9	Probe	TTCTGTATTTCGATCCAACGCCGCTTAATC	77 bp	100.77
			Forward primer	TGGCCTCCGACACTGACA		
			Reverse primer	CCTGGTGCGATCTCATTGG		
			Probe	AGCAGCATTGACACACTCACTGGACACCT		
Saxitoxin and tetrodotoxin binding protein 1	CA374637	psbp1	Forward primer	GGACTGTGGTAAAATGGTCAAACC	74 bp	100.94
			Reverse primer	CGATGAGAATCCATTCACCAAA		
			Probe	CCTGCCGACAAGCTCAACACGGT		
Phosphotyrosine independent ligand for the Lck SH2 domain p62	CA375694	sqstm1	Forward primer	GCCCTGGACACCATCCACTA	70 bp	101.41
			Reverse primer	TCCCTCAGGGTACGGTGTTG		
			Probe	TCCAAACCCGCCAAGAAGTAACGGG		
CREB-binding protein	CA385588	crebbp	Forward primer	GCAGGCTTAGCAATGACTTGAAT	79 bp	97.96
			Reverse primer	TGCTACAATCCTTCAACAAACTTCTC		
			Probe	TGGGCGACACCACAGGAGACACACT		
Beta-2-microglobulin-2 JB1	CX026208	b2m	Forward primer	TGTCACGTGAGTGGCTTCCA	67 bp	101.67
			Reverse primer	GGATCTCCACGCCGTTCTT		
			Probe	CCCCCTGACATCAGCATCCAGCTC		
Glutathione S-transferase, mitochondrial	BX074823	gst	Forward primer	GCTGAAACTGTCCACCTCTAAGG	76 bp	102.18
			Reverse primer	GGCCCTATGGTCCAGTGCTT		
			Probe	AATCAAAGACAAGCTGAAGAGATCCACCCA		
Transcription factor jun-B-1	CF752495	junb	Forward primer	GCATACACGCACAGCGATATTC	72 bp	101.98
			Reverse primer	CGTCGCTGCTCTGCATGT		
			Probe	TGAAAGAGGAGCCCCAGACCGTTCC		

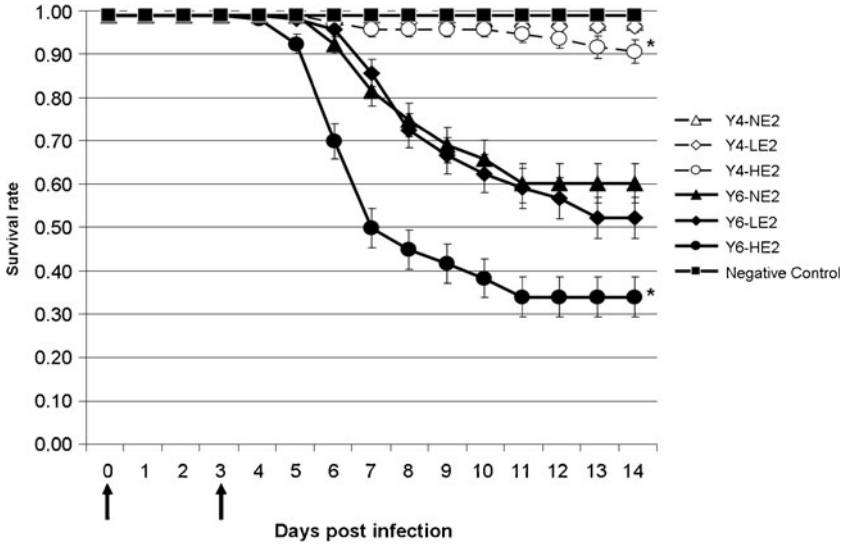

Fig. 2 Survival time plot in days showing the rate of survival (*y-axis*) of different estradiol-treated groups after infection at day 0 with different concentrations of *Y. ruckeri* (*x-axis*). The *asterisks* indicate significant difference to the corresponding no E2 treatment group (*NE2*) of the same *Yersinia* treatment group. Sample size, n=140 fish/treatment group. *NE2* no estradiol (E2) treatment, *LE2* 2 mg E2/kg food, *HE2* 20 mg E2/kg food

Microarray and qPCR Analysis of Hepatic Gene Expression

To assess how differences of survival rates associate with gene expression changes in the liver, analyses were performed using microarray and qPCR. We focused on the 10^6 cfu *Yersinia* treatment groups as treatment-related differences in survival were greatest at the maximum dose of bacteria tested in this experiment (Fig. 2). Samples were collected at 3 days p.i., shortly before the onset of mortality. Therefore, the observed expression changes are considered to be associated with infection and hormonal treatment rather than with the moribund condition of the fish.

At total, 409 genes showed expression differences compared to the negative control NE2/no *Yersinia*. Hierarchical clustering of expression profiles of 409 genes relatively well separated the experimental groups (Fig. 3). Results suggested a closer relationship between the Y6-NE2 and the Y6-LE2 groups compared to the Y6-HE2 group, which was in line with the survival analysis. The highest number of genes that were differentially regulated was found in the Y6-NE2 group (259 genes), pointing to a strong influence of the pathogen exposure, while in the combined exposure groups 181 genes (Y6-LE2) and 174 genes (Y6-HE2) were differentially regulated (Fig. 1). A greater number of genes were down-regulated, both in the pathogen-only treatment (Y6-NE2) and in the combined treatment (Y6-LE2, Y6-HE2; Fig. 1).

By expression profiles determined with microarray analyses, genes were divided in three groups: (a) response to only bacterial infection (no significant difference between Y6-NE2, Y6-LE2, Y6-HE2), (b) induced with combined exposure to estrogen and pathogen (up-regulation in Y6-HE2 or in both E2-exposed groups but not in Y6-NE2), and (c) repressed with combined treatment (reduced expression in Y6-HE2). Examples of genes from these groups are presented in Tables 2, 3, and 4; results for all differentially expressed genes are found in the "Electronic Supplementary Materials". Expression changes of several genes were validated with qPCR (Figs. 4 and 5) and the results of two independent methods were in concordance (Pearson's r= 0.77, data not shown). It is important to note that all microarray hybridizations were performed with pathogen-challenged rainbow trout, while qPCR analyses included uninfected fish, thus making finding of the E2-only effects impossible.

A suite of genes involved in immune and stress responses changed expression with no difference between the treatment groups (Table 2; Fig. 4). *Sqstm1* and *nfkbia*, which are inhibitor components of the pathway that plays an essential part in signaling downstream from pathogen recognition and cytokine receptors, were significantly up-regulated. A similar result was produced for *junb*, a transcription factor involved in inflammation (Wagner 2010). Co-exposure to E2 did not alter the bacteria-induced regulation of this gene (Fig. 4). *Hamp* and *aim2* were also significantly up-regulated, irrespective of the presence or absence of E2 (Table 2). The former combines the properties of a hormone regulating iron metabolism and antibacterial protein and commonly shows strong pathogen responses in fish (Shi and Camus 2006), while the latter belongs to a group of small interferon-inducible transmembrane proteins that are activated with bacterial and viral infections (Siegrist et al. 2011). Further, bacterial infection, but not E2 exposure, suppressed the expression of genes involved in the metabolism of eicosanoids, inflammatory regulators of lipid origin (*alox5r* and *cyp2k4*; Table 2). Also, *leap2b*, a peptide with predicted antibacterial properties and a sensitive stress marker that decreases expression under various adverse conditions, was significantly down-regulated under *Yersinia* challenge. The *Yersinia* infection also caused expression

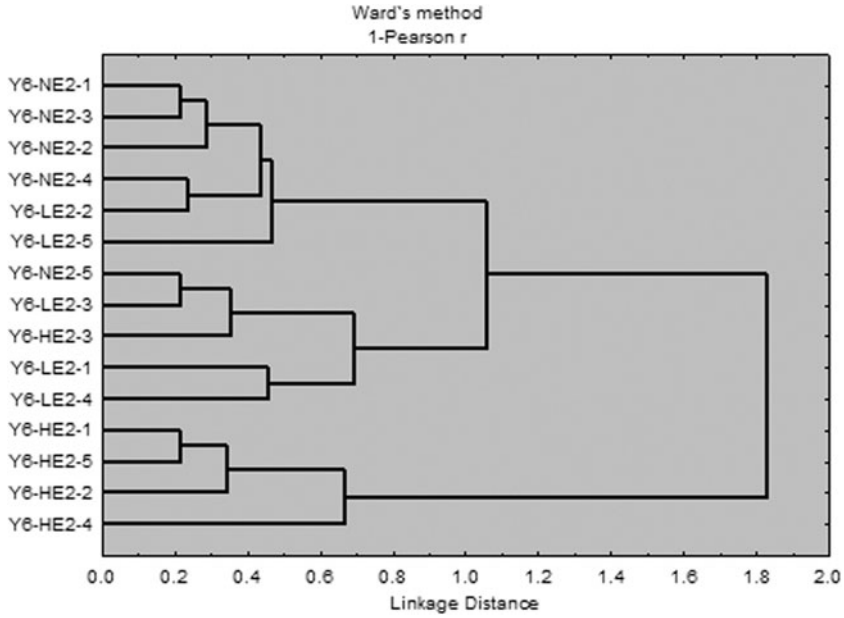

Fig. 3 Hierarchical clustering of samples analyzed with microarray (log_2-ER of 409 differentially expressed genes, Pearson *r*, Ward's method). *NE2* no E2 exposure, *LE2* low (2 mg/kg) E2 exposure, *HE2* high (20 mg/kg) exposure

changes of genes involved in protection against reactive oxygen species (*gsr, gsp, txn*, and *txndc4*) and endosomal chaperones *hspa5* and *hsp90b1*, markers of endoplasmic reticulum stress (Malhotra and Kaufman 2007). All of these genes are known to be highly sensitive to pathogens and stressors in salmonid fish (Skugor et al. 2008; Skugor et al. 2009).

Table 3 includes examples of genes that were up-regulated by E2 exposure as shown by a significant difference of expression ratios between Y6-NE2 on one hand and one or both E2-treated groups on the other hand. Functionally, these genes are associated with metal ion regulation, lysosome activity, hemostasis, and metabolism. The small metal binding proteins metallothioneins (*mta*, *mtb*, and *mt1l*) are known to be important prognostic markers in estrogen-dependent cancers (Goulding et al. 1995; Bay et al. 2006). The lysosomal protease *ctsd1*, which is known to be estrogen-inducible (Rochefort, 1990; Ravdin 1993), showed response to E2 exposure in qPCR analyses (Fig. 5). Strong up-regulation was seen in *tfpi2*, an anticoagulant protein, and similar expression changes were found in *vps52* (Table 3). Both genes are known to be induced by estrogens (Jayachandran et al. 2005; Burki et al. 2007).

Exposure to E2 reduced the expression of a panel of genes that were either up-regulated in Y6-NE2 or did not respond to the pathogen (Table 4). This group included genes associated with metabolism, immune responses, and stress. Two immune genes—*b2m*, a component of MHC1, and *crebbp*, a transcriptional coactivator involved in diverse immune and metabolism pathways—showed significant and dose-dependent down-regulation responses under E2 co-exposure, while bacterial infection alone had no effect (Table 4). However, qPCR found only a trend to E2 suppression of these genes with no difference between bacteria-infected and uninfected groups (Fig. 5). Two more immune-related genes, *ambp* and *hpx*, were down-regulated at combined treatment (Table 4). E2 exposure also down-regulated genes involved in lipid and steroid metabolism (*vlacs* and *akr1d1*). Similar changes were found in genes encoding proteins with important roles in the metabolism of endogenous insoluble substrates and biotransformation of xenobiotics (Table 4; Fig. 5). *Gst*, *ugt8*, and *ugt2b15* play an essential part in phase II by conjugating polar functional groups that enhance the solubility and facilitate the removal of toxins. *Ephx1* (Decker et al. 2009) and *chst4* (Jancova et al. 2010), which are also among the key enzymes of xenobiotics metabolism, were all down-regulated at exposure to combined stressors. Two more genes, *sepp1*and *prdx1*, which are responsive to oxidative stress, showed similar changes. *Timp3* and a gene with unknown functions denoted as an *acute phase protein*, a marker of chemical stress characterized by high sensitivity to diverse contaminants in salmonid fish (Burki et al. 2007), were up-regulated by bacterial infection and suppressed with E2 treatment. Finally, the gene *psb1*, which showed the greatest response to E2 exposure in both microarray and qPCR analyses (Table 4; Fig. 5), is a homolog of the puffer fish saxitoxin and tetrodotoxin-binding protein characterized by high affinity to several toxic compounds (Yotsu-Yamashita et al. 2001).

Discussion

This study investigated the combined effects of estrogen (E2) treatment and pathogen infection on disease susceptibility and hepatic transcriptome of rainbow trout. The native

Table 2 Examples of genes with responses to *Y. ruckeri* infection that were not affected with E2 (microarray results) (difference from uninfected control, no differences between the study groups). Data are $\log_2$-ER±SE. Asterisks mark genes that were analyzed with qPCR. Genes were assigned to functional categories by annotations in databases and information from publications

Genbank	Probe	Y6-NE2	Y6-LE2	Y6-HE2
Immune				
CA362766	Interferon inducible protein (*aim2*)	1.72 ± 0.45	1.11 ± 0.38	1.69 ± 0.39
CA375694	Phosphotyrosine independent ligand for the Lck SH2 domain p62 (*sqstm1*) *	1.69 ± 0.51	1.13 ± 0.54	1.85 ± 0.66
CA343143	NF-kappaB inhibitor alpha-1 (*nfkbia*)*	1.31 ± 0.58	0.72 ± 0.55	1.37 ± 0.64
CA348284	CCAAT/enhancer binding protein beta (*cebpb*)	1.43 ± 0.39	0.70 ± 0.51	1.26 ± 0.47
CA353501	C type lectin receptor B (*clec1b*)	1.57 ± 0.46	1.77 ± 0.68	1.88 ± 0.41
BI468191	Hepcidin 1 (*hamp)*	1.26 ± 0.51	1.38 ± 0.69	1.39 ± 0.51
CA345780	Lysozyme g-2 (*lyg2*)	−0.20 ± 0.49	−0.72 ± 0.11	−0.88 ± 0.32
CA362179	Interleukin-1 receptor-like protein 2 (*il1rl2*)	−0.65 ± 0.03	−0.76 ± 0.14	−0.42 ± 0.07
CA376350	Serum amyloid P-component-1 (*apcs*)	−1.07 ± 0.06	−0.89 ± 0.40	−1.28 ± 0.51
CA377504	Cold autoinflammatory syndrome 1 protein (*nalp3*)	−0.99 ± 0.11	−0.82 ± 0.10	−0.82 ± 0.10
CA387966	Liver-expressed antimicrobial peptide 2B (*leap2b*)	−1.52 ± 0.12	−2.77 ± 0.56	−3.36 ± 0.45
CA349943	C-type mannose-binding lectin (*mbl-1*)	−0.81 ± 0.37	−0.48 ± 0.47	−0.65 ± 0.64
Signal transduction				
CA343700	CXC chemokine receptor transcript variant B (*cxcr*)	−0.10 ± 0.16	−0.80 ± 0.26	−0.66 ± 0.27
CA372428	Leukotriene B4 receptor 1 (*ltb4r*)	−0.53 ± 0.12	−0.76 ± 0.17	−0.51 ± 0.21
Stress				
CA352456	Glutathione reductase, mitochondrial-2 (*gsr*)	0.75 ± 0.16	0.73 ± 0.06	0.48 ± 0.16
CF753103	Glutathione peroxidase-gastrointestinal (*gsp*)	0.65 ± 0.23	0.70 ± 0.39	0.72 ± 0.27
CA354578	Thioredoxin domain containing protein 4 (*txndc4*)	0.84 ± 0.37	1.12 ± 0.14	0.69 ±0.38
CX153146	Thioredoxin *(txn*)	1.32 ± 0.35	0.96 ± 0.41	0.32 ± 0.22
CA362998	DnaJ homolog, subfamily C, member 3 (*dnajc3*)	1.04 ± 0.52	1.28 ± 0.73	1.28 ± 0.59
CA368961	78-kDa glucose-regulated protein (*hspa5*)	2.33 ± 0.80	2.23 ± 1.07	0.78 ± 0.83
CA369202	Growth arrest and DNA-damage-inducible GADD45 beta (*gadd45b*)	0.72 ± 0.26	0.48 ± 0.37	0.82 ± 0.46
CA381199	94-kDa glucose-regulated protein (*hsp90b1*)	0.56 ± 0.36	0.67 ± 0.35	1.01 ± 0.43
CA368739	BCL2-associated athanogene 1	−1.02 ± 0.10	−0.83 ± 0.14	−0.79 ± 0.18
Various				
CF752495	Transcription factor junB * (*junb*)	1.38 ± 0.45	0.77 ± 0.46	1.45 ± 0.42
EV384586	Cytochrome P450 2 K4-2 (*cyp2k4*)	−0.71 ± 0.17	−0.49 ± 0.11	−0.79 ± 0.16
CA387866	Arachidonate 5-lipoxygenase-2 (*alox5*)	−0.68 ± 0.20	−0.95 ± 0.28	−0.19 ± 0.09

form of the female sex hormone was chosen due to its relevance for both aquaculture and environmental research. Endogenous production of E2 increases during puberty and sexual maturation of fish. Furthermore, this hormone serves as a model for assessing the effects of environmental estrogen-active contaminants. Efficiency of the E2 treatment in this study was assessed by measuring hepatic *vtg* transcription, and the results showed a significant, dose-dependent up-regulation of the E2 target gene. In a previous study (Wenger et al. 2011), we had shown that the circulating E2 levels resulting from feeding high E2 diets, as used in the current study, remained within the physiological range. As a pathogen, the bacterium *Y. ruckeri*, the etiological agent of the redmouth disease, was included in the study because of its economical and environmental importance. Outbreaks of enteric redmouth disease usually lead to hemorrhages in various organs and can cause high mortalities in aquaculture and in wild fish populations (Horne and Barnes 1999; Tobback et al. 2007). The availability of information on immunological response makes this bacterium an appropriate model to investigate host–pathogen interactions (MacDonald et al. 2007). The efficiency of *Y. ruckeri* infection in this study was assessed by re-isolating and genotyping the pathogen from moribund fish.

Survival rate of rainbow trout infected with *Y. ruckeri* was reduced by concomitant exposure to E2 in a dose-dependent manner. An interaction between *Yersinia* infection and E2 exposure had been evident already in a previous study (Wenger et al. 2011). However, in the present study that used a dose-dependent multi-factorial experimental

Table 3 Examples of genes induced by E2 at combined exposure (no difference from uninfected control; differences between the study groups are denoted with lowercase letters (a, b, c)). Data are $\log_2$-ER±SE. Asterisks mark genes that were analyzed with qPCR

Genbank	Probe	Y6-NE2	Y6-LE2	Y6-HE2
Lysosome				
CA347041	Cathepsin D-1 * (*ctsd1*)	−0.18 a ± 0.24	−0.11 a ± 0.25	1.77 b ± 0.25
CA365458	Cathepsin D-2 (*ctsd2*)	−0.08 a ± 0.20	0.11 a ± 0.23	1.69 b ± 0.18
Metal ion regulation				
CF752699	Metallothionein A (*mta*)	0.01 a ± 0.22	0.49 a ± 0.21	1.13 b ± 0.26
CA359170	Metallothionein B (*mtb*)	−0.15 a ± 0.24	0.01 a ± 0.23	1.21 b ± 0.22
CB507951	Metallothionein-IL (*mt1l)*	0.38 a ± 0.30	0.64 a ± 0.34	1.86 b ± 0.40
Hemostasis				
EL553001	Tissue factor pathway inhibitor 2 precursor (*tfpi2*)	0.37 a ± 0.25	1.14 a ± 0.41	4.77 b ± 0.11
Metabolism				
EG938935	Vacuolar sorting protein 52 (*vps52*)	−0.78 a ± 0.19	1.27 b ± 0.69	4.51 b ± 0.33

design, the results clearly demonstrated the dose–response relationship of this interaction. With respect to E2, an interactive effect with pathogen infection was observed for the high E2 dose. With respect to the bacterial pathogen, the high E2 dose tended to increase pathogen-induced mortality at 10^4 cfu and significantly enhanced mortality at a high pathogen load (10^6 cfu). At a bacterial concentration of 10^2 cfu, which alone did not cause mortality, the combined E2 pathogen treatment also remained without effect on survival.

To gain insight into processes underlying the interaction between the two stressors addressed in this study, we performed microarray analyses of the hepatic transcriptome. The focus was laid on the interaction of E2 with the highest

Table 4 Examples of genes down-regulated by combined exposure to E2 and *Y. ruckeri* (microarray results) (differences between the E2 exposed study groups are denoted with superscript letters (a, b)). Data are $\log_2$-ER±SE. Asterisks mark genes that were analyzed with qPCR

Genbank	Gene	Y6-NE2	Y6-LE2	Y6-HE2
Xenobiotic metabolism				
CA371001	Very-long-chain acyl-CoA synthetases (*vlacs*)	−0.09 a ± 0.19	−0.91 a, b ± 0.38	−1.73 b ± 0.29
CA377953	3-oxo-5-beta-steroid 4-dehydrogenase (*akr1d1*)	−0.22 a ± 0.15	−0.88 a, b ± 0.49	−0.84 b ± 0.16
CA378723	Sulfotransferase 4 (*chst4*)	1.04 a ± 0.15	0.49 a, b ± 0.33	−0.26 b ± 0.12
CA358621	Microsomal glutathione S-transferase 3 (*mgst3*) *	1.06 ± 0.19	0.34 a, b ± 0.28	−0.01 b ± 0.19
CA349227	UDP-glucuronosyltransferase 1-8 (UGT8) *	0.12 a ± 0.37	−0.70 a, b ± 0.29	−0.84 b ± 0.14
CA376450	Epoxide hydrolase 1 (EPHX1)	0.21 a ± 0.06	−0.52 a, b ± 0.37	−0.84 b ± 0.16
BX074823	Glutathione S-transferase, mitochondrial (GST) *	0.68 a ± 0.13	−0.23 b ± 0.34	−0.65 b ± 0.17
CA342060	UDP-glucuronosyltransferase 2B15 (*ugt2b15*)	0.23 a ± 0.12	−0.46 b ± 0.17	−0.93 b ± 0.31
Immune				
CX026208	Beta-2-microglobulin *(b2m)* *	0.03 a ± 0.09	−0.66 b ± 0.18	−1.15 b ± 0.31
EG920678	Alpha-1-microglobulin/bikunin-2 (*ambp*)	0.02 a ± 0.09	−0.41 b ± 0.11	−0.73 b ± 0.12
Metabolism, immune				
CA363230	Hemopexin (*hpx*)	0.13 a ± 0.20	0.32 a ± 0.18	−0.86 b ± 0.23
CA385588	CREB-binding protein (*crebbp*) *	0.49 a ± 0.12	−0.15 b ± 0.21	−0.66 b ± 0.37
Stress				
CA373506	Selenoprotein P (*sepp1*)	0.02 a ± 0.05	−0.57 a, b ± 0.44	−0.91 b ± 0.24
BX085117	Peroxiredoxin 1-2 (*prdx1*)	0.36 a ± 0.08	0.03 a ± 0.23	−0.75 b ± 0.08
CX141783	Acute phase protein	1.14 a ± 0.51	0.31 a ± 0.69	−1.88 b ± 0.24
CA371538	Metalloproteinase inhibitor 3 (*timp3*)	0.60 a ± 0.28	−0.45 b ± 0.28	−1.02 b ± 0.16
Various				
CA388340	Beta-2-glycoprotein I (*b2gp1*)	−0.10 a ± 0.13	−0.01 a ± 0.21	−0.79 b ± 0.17
CA374637	Saxitoxin and tetrodotoxin binding protein 1 * (*psbp1*)	0.31 a ± 0.30	−0.44 a ± 0.21	−2.02 b ± 0.34

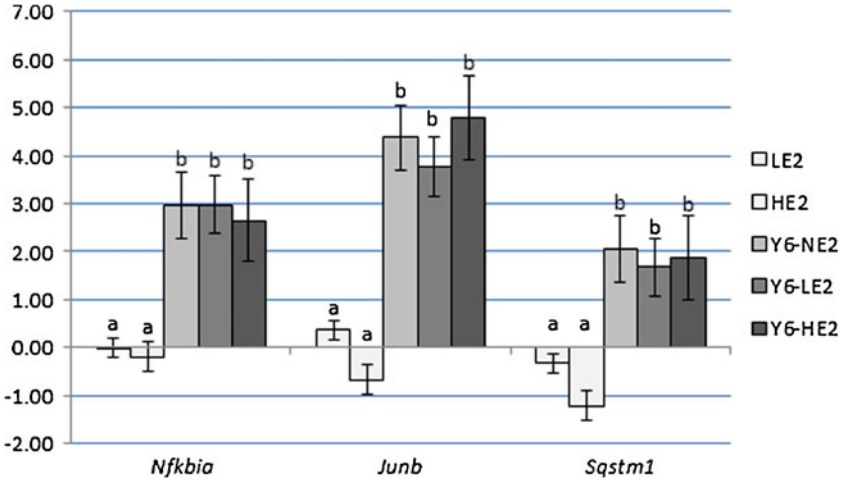

Fig. 4 Pathogen-induced genes (qPCR). Results are presented as $\log_2$-ER±SE (n=8) compared to negative control group (no E2, no *Yersinia* exposure) and normalized against reference gene 18 S rRNA. For names of genes, see Table 1. *NE2* no estradiol (E2) treatment, *LE2* 2 mg E2/kg food, *HE2* 20 mg E2/kg food. *Different letters* (*a*, *b*) indicate a significant difference (p<0.05)

dose of bacteria as this treatment evoked the most prominent effects at the level of fish mortality. The clustering of experimental groups on the basis of gene expression shows that an interactive effect between bacterial infection and E2 appears at the Y6-HE2 treatment as it was separated from the other treatment groups. This finding was in agreement with the survival data.

In a previous study (Wenger et al. 2011), we investigated hepatic complement gene expression in rainbow trout exposed to E2 and *Y. ruckeri* and observed that E2-treated fish were not able to up-regulate complement gene expression in response to bacterial challenge—a finding that was in line with the supposed immunosuppressive effect of estrogens in fish (Milla et al. 2011). The present miocroarray study, however, shows that a variety of immune genes can still be up-regulated in the presence of E2 (Table 2). This finding indicates that, in E2-exposed fish, the immunosuppressive activities of estrogens were not the only cause for reduced survival. A similar conclusion was obtained in the study with Atlantic salmon (*Salmo salar* L.) challenged with *Aeromonas salmonicida* (Skugor et al. 2009). Microarray analyses in the liver of individuals with high and low resistance found minor differences in the expression of immune genes. Highly resistant salmon was characterized with a greater expression of genes involved in tissue protection and metabolism of xenobiotics. The results suggested that, in this case, it is the ability to avoid tissue damage arising from the inflammatory response to the bacterial infection rather than the up-regulation of specific immune genes that may be of importance for the pathogen resistance of the

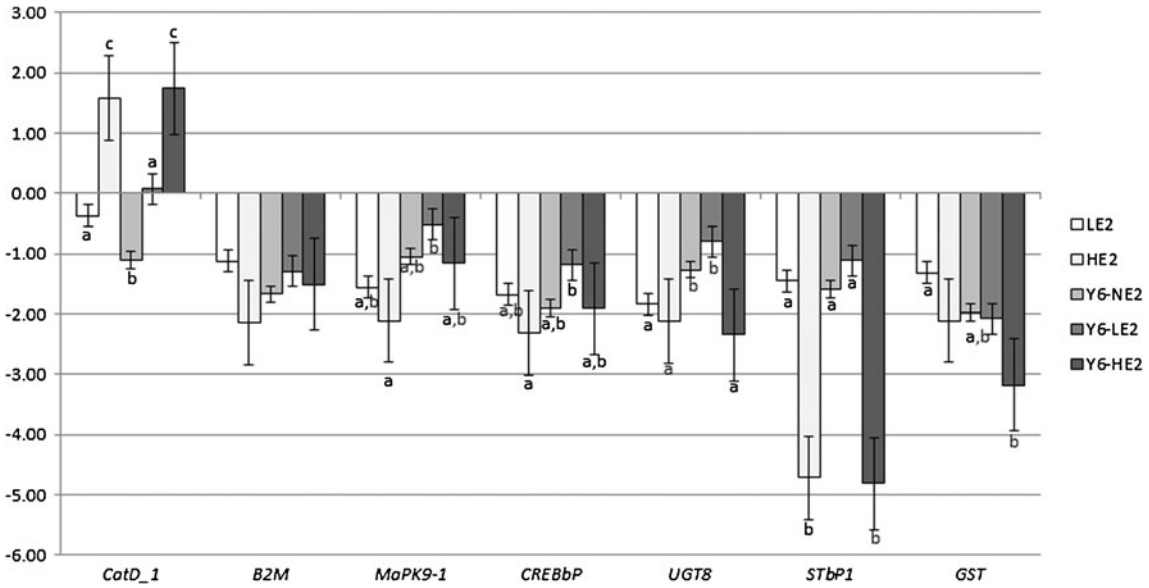

Fig. 5 E2-responsive genes (qPCR). Results are presented as Log_2-ER ±SE (n=8) compared to negative control group (no E2, no *Yersinia* exposure) and normalized against reference gene 18 S rRNA. For names of genes, see Table 1. *NE2* no estradiol (E2) treatment, *LE2* 2 mg E2/kg food, *HE2* 20 mg E2/kg food. *Different letters* (*a*, *b*) indicate a significant difference (p<0.05)

host. It is possible, however, that the immunosuppressive activities of estrogens are manifested in other organs involved in innate immune responses to bacteria. We also need to note that the current study was implemented with juvenile rainbow trout and immune responses could be different at other life stages. Recently, Chettri et al. (2012) reported significant differences in mortality and immune-related gene expression between rainbow trout larvae and fry.

One group of genes that responded prominently to the combined *Yersinia*/E2 exposure (in particular, in the HE2 group) contained genes with a dual role in the metabolism of both lipophilic xenobiotics and lipophilic endogenous compounds (Table 4) (Monostory and Dvorak 2011). The expression of these genes can be up-regulated by their endogenous and exogenous substrates, but at the same time they are under complex control by hormonal and immune factors (Vrzal et al. 2004; Xie and Tian 2006). Estrogens influence xenobiotic metabolism and biotransformation pathways in mammals and fish (Vodicnik and Lech 1983; Arukwe and Goksøyr 1997; Navas and Segner 2001; Vaccaro et al. 2005; Carrera et al. 2007; Monostory and Dvorak 2011). Likewise, immune mediators such as cytokines or nitric oxide are also able to regulate the expression and activity of biotransformation proteins (Aitken et al. 2008; Lee et al. 2009). Under conditions of bacterial infection, these genes can play a role in tissue protection and clearance by metabolizing toxic products released from the bacteria or from cell damage in the host. Interestingly, Skugor et al. (2009) observed that Atlantic salmon with high resistance against *A. salmonicida* infection showed elevated expression of biotransformation genes such *ugt8*, *ephx1*, and *gst*—pointing to their possible function in tissue protection and toxic metabolite clearance. In the present study, the expression of these genes was down-regulated by the combined E2/pathogen treatment. Likewise, *b2gp1*—a precursor of antibacterial peptides—and *psbp1*—a toxin-binding protein—were up-regulated in *A. salmonicida*-resistant Atlantic salmon, whereas they were down-regulated in rainbow trout exposed to both E2 and *Y. ruckeri*. These findings suggest that the interaction of the estrogenic and pathogen exposure compromised the capacity of infected rainbow trout to clear toxic products and metabolites and to protect tissues from the damaging impact of such compounds. Also here, as discussed earlier with respect to the immune processes, linear relationships of the molecular effect to the compromised defense capacity of the organism are unlikely, but it is probably more the interplay of an array of protective processes that eventually translates into an organism-level consequence.

In conclusion, the results of the current study clearly demonstrate that a combination of pathogen- and chemical-induced disturbance leads to a significantly higher mortality in juvenile rainbow trout and that this happens in a dose-dependent manner. The results of the study further provide evidence that the organism's attempt to adapt to different stressor impacts involves a complex interplay of multiple pathways. Microarray analyses provide a sound possibility to develop a better understanding of the individual pathways and processes in driving the organism's stress response under multiple-stressor exposure.

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