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ABSTRACT

The aim of the present study was to examine the molecular and organism reaction of rainbow trout, *Oncorhynchus mykiss*, to the combined impact of two environmental stressors. The two stressors were the myxozoan parasite, *Tetracapsuloides bryosalmonae*, which is the etiologic agent of proliferative kidney disease (PKD) and a natural stressor to salmonid populations, and 17 β -estradiol (E2) as prototype of estrogen-active chemical stressors in the aquatic environment. Both stressors, the parasite and estrogenic contaminants, co-exist in Swiss rivers and are discussed as factors contributing to the decline of Swiss brown trout populations over the last decades. Using a microarray approach contrasting parasite-infected and non-infected rainbow trout at low or high estrogen levels, it was observed that molecular response patterns under joint exposure differed from those to the single stressors. More specifically, three major response patterns were present: (i) expression responses of gene transcripts to one stressor are weakened by the presence of the second stressor; (ii) expression responses of gene transcripts to one stressor are enhanced by the presence of the second stressor; (iii) expression responses of gene transcripts at joint treatment are dominated by one of the two stressors. Organism-level responses to concurrent E2 and parasite treatment – assessed through measuring parasite loads in the fish host and cumulative mortalities of trout – were dominated by the pathogen, with no modulating influence of E2. The findings reveal function- and level-specific responses of rainbow trout to stressor combinations, which are only partly predictable from the response to the single stressors.

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1. Introduction

In nature, organisms are usually exposed to multiple stressors including biological, chemical and physical entities. In environmental sciences, much emphasis is given on the impact of single stressors, disregarding possible interactive effects arising from aggregate exposure to multiple stressors (Folt et al., 1999; Heugens et al., 2001; Crain et al., 2008; Sheras and Ross, 2010). For instance, natural stress situations such as poor food supply or infection with pathogens can change the response of organisms to toxic chemicals (Segner, 1987; Braunbeck and Segner, 1992; Jokela et al., 2005; Coors and De Meester, 2008). Similarly, exposure to toxic chemicals

can influence the ability of organisms to cope with natural stressors (Arkoosh et al., 1998; Kiesecker, 2002; Springman et al., 2005; Morley, 2010; Song et al., 2011). Several examples from field studies point to the importance of combined actions of anthropogenic and natural stressors on manifestation and dynamics of environmental disease (Kiesecker et al., 2001; Landis et al., 2004; Rohr et al., 2008), but currently we lack concepts for the risk assessment of multiple stressors (Callahan and Sexton, 2007; Altenburger and Greco, 2009). There is a strong need to better understand cumulative and interactive effects of chemical, biological and physical stressors, of strategies by which organisms deal with the demands placed on them by combined stressor exposure, how they prioritize stressors, and how their vulnerability to one stressor may be changed by exposure to a second stressor (Segner, 2007, 2011a; Couillard et al., 2008a,b; Sheras and Ross, 2010).

A pronounced decline of brown trout (*Salmo trutta*) has taken place in Switzerland during the last decades, and available evidence

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suggests that this is not caused by a single factor but rather by multiple factors (Burkhardt-Holm et al., 2005). One stressor that is suspected to play a key role in the brown trout decline is the proliferative kidney disease (PKD) which is caused by the myxozoan parasite *Tetracapsuloides bryosalmonae*. The disease is widespread in brown trout populations of Swiss rivers (Wahli et al., 2007) and can lead to high mortalities in infected populations, particularly in the young-of-the-year life stage. PKD-induced mortalities are temperature-dependent, increasing strongly if summer water temperatures are reaching levels of approximately 15 °C and higher (Bettge et al., 2009a,b). Such water temperatures occur mainly in rivers at low altitudes (<600–800 m above sea-level), which are present in the Swiss midland region (Wahli et al., 2008). However, the midland region is also strongly urbanized and industrialized, and therefore many midland rivers are impacted by chemical pollution (Burkhardt-Holm et al., 2005; Goetz et al., 2010). This situation leads to the question if the adverse impact of PKD on brown trout populations in Swiss midland rivers could be potentiated by simultaneous exposure of trout to chemical contaminants.

The aim of the present study is to examine in a laboratory environment the combined impact of *T. bryosalmonae*-infection and contaminant exposure on fish. The contaminant group selected for this purpose is estrogen-active compounds. These substances are present in Swiss midland rivers (Vermeirssen et al., 2005; Burki et al., 2006), and, in addition to their well-known effects on the reproductive physiology (Segner, 2011b), they can modulate immunocompetence of fishes and their susceptibility towards pathogens (Thilagam et al., 2009; Cabas et al., 2011; Casanova-Nakayama et al., 2011; Milla et al., 2011; Wenger et al., 2011). Experimentally, young-of-the-year rainbow trout, were (i) infected with the parasite, *T. bryosalmonae*, (ii) exposed to the prototype estrogenic substance 17 β -estradiol (E2), or (iii) treated with a combination of the parasite and E2. Treatment effects were compared at the levels of transcript expression of genes, intensity of parasite infection of the fish, and fish survival. Hepatic expression profiles of gene transcripts were measured using a trout cDNA microarray platform which has been extensively validated for assessing salmonid responses to stressors (Krasnov et al., 2005, 2007; Koskinen et al., 2004; MacKenzie et al., 2006). Liver was selected as target organ as the liver of fish integrates responses to diverse types of environmental stressors and as it plays an important role in immunity (Rice, 2001).

2. Materials and methods

2.1. Exposure of rainbow trout to estrogen and *T. bryosalmonae* (PKD)

Two batches of juvenile (young-of-the-year) rainbow trout (age 0+, weight 1.5–4 g) of the same genetic origin were obtained from a fish farm. The two batches did not differ in terms of body weight and body length, but one batch was infected with *T. bryosalmonae*, while the other batch consisted of healthy, non-infected fishes. Representative samples of both batches were analysed for *T. bryosalmonae* infection status by means of PCR. The results showed 100% prevalence in the batch with infected fishes, while in the control batch, no single fish was positive for the parasite. Overt disease signs such as swollen excretory kidneys or anemia were not present in the infected fishes at the beginning of the experiment. The fishes were kept in 100 L flow-through aquaria, with PKD-positive and -negative rainbow trout in the same aquaria since the disease is not transmitted from fish to fish (D'Silva et al., 1984). To distinguish non-infected from infected fishes, the latter were marked by a cut of the adipose fin. After an acclimation period of 1 week at a water temperature of 14 °C, an initial sampling for biometric,

microarray and PCR analyses was done. Afterwards, water temperature was slowly elevated to 18 °C as this temperature promotes the clinical outbreak of PKD (Bettge et al., 2009a,b). The fish were fed with (i) feed without hormone addition, (ii) feed supplemented with a low amount of E2 (0.5 mg E2/kg food) and (iii) feed supplemented with a high amount of E2 (20 mg E2/kg food). E2 (Sigma, Buchs, Switzerland) was incorporated into pelleted trout dry food (Hokovit, Bützberg, Switzerland) using the alcohol evaporation method (Guerrero, 1975). Actual E2 concentrations in the diet were confirmed by HPLC analysis, and the efficiency of the dietary E2 treatment to induce an estrogenic response was assessed by measuring hepatic mRNA expression of the estrogenic biomarker, vitellogenin (VTG).

The experimental treatments were as follows:

- (1) temperature control (18 °C, no PKD, no E2) (T); this group was included in order to control for the effect of the temperature increase from 14 to 18 °C on the hepatic transcriptome
- (2) low amount of E2 (LE), no PKD
- (3) high amount of E2 (HE), no PKD
- (4) PKD infection (I), no E2
- (5) PKD infection plus low amount of E2 (ILE)
- (6) PKD infection plus high amount of E2 (IHE).

Fish were kept under the experimental conditions for 25 days. All treatments were run in duplicate ($n=80$ per replicate; $n=160$ per treatment). Mortalities were recorded daily and used for calculation of cumulative mortalities. Since the main interest in this study was to assess stress responses of trout transcriptome under combined PKD and E2 impact, we decided to perform no sampling shortly before the onset of disease-induced mortality, but only during the late disease stage, after the level of maximum mortality had been reached, which was the case at day 20. Afterwards, mortality rates decreased drastically and sampling for expression analysis of gene transcripts was then done at day 25. In this way, we examined fish that are likely to survive the treatments and largely excluded moribund fish which could confound the transcript expression results. For sampling, fish were anaesthetized with buffered 3-aminobenzoic acid ethyl ester methanesulphonate (Argent Chemical Laboratories, Redmont, CA, USA). Livers and kidneys were removed and stored either in RNAlater (Ambion, Austin, USA) (livers) for later expression analysis or in liquid nitrogen (kidneys) for later analysis of parasite DNA.

All animals used in this study have been treated humanely according to the Swiss regulations for animal Welfare with regard for alleviation of suffering.

3. Microarray analysis

We used high density salmonid fish microarray SFA3, an extended version of SFA2, which was described in detail elsewhere (Gene Expression Omnibus GPL1212; 34). This platform includes 1766 genes printed in 6 replicates each. In comparison with SFA2 it is substantially enriched in genes implicated in responses to pathogens and toxicity. The genes were annotated by the functional classes of Gene Ontology, GO (Ashburner et al., 2005).

Total RNA was extracted from the rainbow trout livers with Trizol (Invitrogen, Paisley, UK) and four individuals were pooled in each sample. Labeling and hybridization was performed as previously described in detail (Krasnov et al., 2007). The microarray hybridizations followed a common reference design with dye swap. The start sample (water temperature 14 °C, no PKD, no estrogen) was used as a control for all treatment groups ("common negative control"). Each pooled sample was hybridized to two microarrays. For the first slide, test and control cDNA were labeled with Cy5

and Cy3, respectively, and for the second array, dye assignments were reversed. Since this experiment was performed with six different treatment groups (T, LE, HE, I, ILE and IHE), a total of twelve microarrays were used. Scanning was performed with ScanArray 5000. The quantification of the spots was done with TIGR Spotfinder (Institute of Genomic Research, MD, USA). The measurements were filtered by criterion $(I - S_I) > (B + S_B)$, where I and B are the mean signal and background intensities and S_I , S_B are the standard deviations. After subtraction of mean background, Lowess normalization was performed separately with each slide. Using the dye swap design of hybridization, each gene was analyzed in 12 spot replicates. Data for two slides were consolidated and technical accuracy of differential transcript expression was assessed by the difference of $\log_2(\text{Expression ratios})$ at the reverse labeling (Student's t -test, $p < 0.01$).

The statistical analyses were conducted with 388 genes that showed differential transcript expression in at least two treatment groups. Hierarchical clustering of $\log_2(\text{Expression Ratios})$ values by Euclidian distances with Ward's method was used for classification of samples and preliminary search for the groups of genes with similar transcript expression profiles. To define more exactly composition of the gene groups, Pearson correlation to the mean expression profile was determined for each member of cluster and genes with $r < 0.74$ were discarded. The resulting groups are referred to as transcription modules (TM). Genes were assigned to the TMs exclusively by their expression profiles. A complementary approach was applied to include the functional annotations into statistical analyses. Genes were grouped by the GO categories and the mean $\log_2(\text{Expression Ratios})$ were calculated. The effects of treatments were analyzed with multiple regression. For multiple linear regression analyses we assumed $\log_2(\text{Expression Ratio}) = b_1 I + b_2 L_E + I_n$, where I and L_E denote respectively the levels of infection and E2. The effects of treatments were assessed by significance of b_i , i.e. beta weights (standardized coefficients of multiple regression), while significant deviation of intercept (I_n) from zero indicated to possibility of joint effects. Complete expression data of gene transcripts and full composition of TMs are available in the on-line supplemental material.

3.1. Real-time PCR for determination of intensity of infection by *T. bryosalmonae*

Real-time PCR was used to quantify *T. bryosalmonae* DNA in trout kidneys. The method was performed according to the protocol of Bettge et al. (2009b), which is routinely used in the Berne laboratory.

4. Results

4.1. Cumulative mortality

Cumulative mortality was measured over the experimental period of 25 days. In the control group, i.e. non-infected and non E2-exposed fishes kept at 18 °C (T), mortality during the 25-day-period was negligible (only 1 fish died out of 160). Very low cumulative mortalities (2.5%) were observed also in the two non-infected estradiol groups (LE, HE) (Fig. 1). In contrast, very high cumulative mortalities of almost 90% were recorded in *T. bryosalmonae*-infected group. Additional exposure of *T. bryosalmonae*-infected fish to low (ILE) or high (IHE) levels of E2 did not significantly change cumulative mortalities compared to the "infection only" group, neither the absolute level of mortality nor the rate of mortality increase (Fig. 1). In order to evaluate if the dietary E2 was indeed accumulated and effective in the exposed fishes, we measured hepatic expression levels of vitellogenin (VTG) mRNA. Both

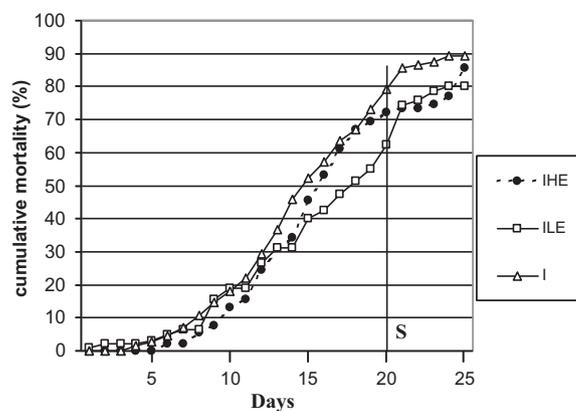


Fig. 1. Cumulative mortalities of the experimental groups: *T. bryosalmonae* infection (I), *Tetracapsuloides bryosalmonae* infection plus exposure to the low dose of E2 (ILE), or *T. bryosalmonae* infection plus exposure to the high dose of E2 (IHE). The line marked with S represents the time point (day 20) when rainbow trout were sampled for microarray analysis and determination of parasite density.

in the non-infected and in the infected groups, E2 feeding resulted in a significant and dose-dependent elevation (data not shown).

4.2. Prevalence and intensity of parasite infection

The prevalence of infection was 100% in all groups with *T. bryosalmonae* infection (I, ILE, IHE), regardless whether the fishes were treated with E2 or not. Also parasite loads, estimated by measuring concentrations of parasite DNA in the infected kidneys, did not show significant differences between the infected group (I) and groups exposed to both the parasite and E2 (ILE, IHE) (Fig. 2). The same individuals used for determining parasite loads were also used for the microarray analyses (see below).

4.3. Microarray analysis

The transcriptional profile of rainbow trout liver in response to the presence of the two stressors, E2 and PKD, alone or in combination was investigated using a trout cDNA microarray. Hierarchical clustering of the transcript expression profiles of hepatic genes confirmed compliance between the treatments and transcriptomic responses. Main findings of the cluster analysis (Fig. 3) were that (a) the low E2 treatment (LE) was not clearly different from that of the 18 °C control group (T) whereas the high level E2 treatment

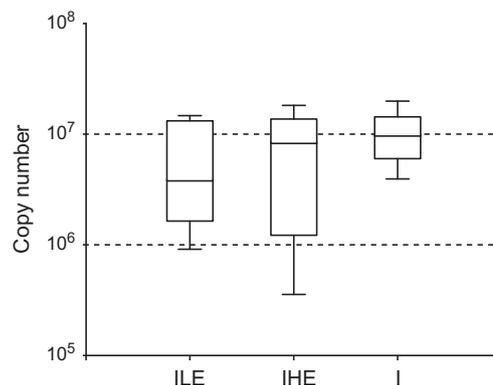


Fig. 2. Copy numbers of the 18S rDNA gene of the parasite *Tetracapsuloides bryosalmonae* in excretory kidneys of infected rainbow trout (I), infected rainbow trout additionally treated with the low dose of E2 (ILE), or infected rainbow trout additionally treated with the high dose of E2 (IHE). The 18S rDNA copy numbers were determined by means of real time PCR. The results are presented as box plots indicating median, 75th and 95th percentiles. $N = 10$ per treatment.

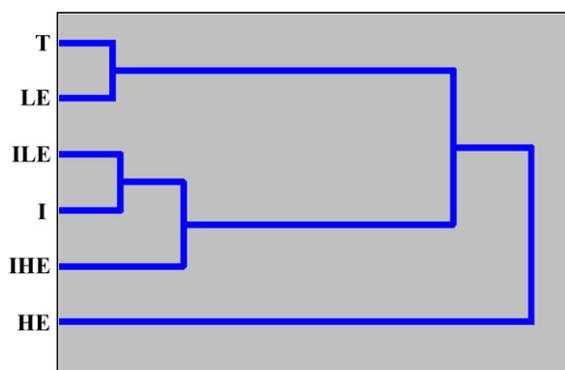


Fig. 3. Responses of the transcriptional expression of hepatic genes in rainbow trout to control conditions at 18 °C (T), feeding with low (LE) and high (HE) levels of E2, *T. bryosalmonae* infection (I) or combinations of these treatments (ILE, IHE) (all at 18 °C). The log (expression ratios) values of 388 transcriptionally differentially expressed genes were clustered by Euclidian distances using Ward's method. For details see Material and Methods.

(HE) yielded a distinct expression signature that differed from T and LE, and (b) all *T. bryosalmonae*-infected groups separated from the non-infected groups, with I and ILE being very close to each other, and IHE being more distant. These findings point to a prevailing effect of infection over estrogen on transcript expression of rainbow trout hepatic genes.

To evaluate the effects of PKD and E2 and their interactions, multiple regression was applied to genes grouped according to either similarity of transcript expression profiles (transcription modules, TMs) or by Gene Ontology (GO) classes. Use of TMs instead of individual genes smoothes random fluctuations in expression of gene transcripts thereby increasing accuracy and robustness of statistical analyses. We successfully used this approach previously to dissect the effects of aquatic contaminants applied singly or in combinations (Krasnov et al., 2007). Though genes were selected exclusively by correlation of transcript expression changes regardless of their functional roles, the identified TMs included functionally related genes indicating that they possess biological relevance. Fig. 4 presents selected genes from the four identified TMs. GO categories with significant responses to the treatments are presented in Table 1. The two independent approaches produced concordant results.

TM1 is characterized by stimulation by E2, particularly by the high levels of E2 (HE) (Fig. 4A). Infection significantly reduces the stimulatory effect of the hormone. Being heterogeneous by composition, TM1 nonetheless includes several groups of functionally

Table 1
Examples of gene ontology GO categories with responses to PKD and/or E2. The table reports the beta weights. Significant effects and interactions ($p < 0.05$) are indicated.

GO functional category	Number of genes	Beta weight estrogen	Beta weight PKD
Immune response ^{a,b,c}	93	-0.138	0.194
Humoral immune response ^{a,b}	32	-0.149	0.307
Complement activation ^b	20	-0.136	0.379
Chemokine activity ^b	5	-0.163	0.504
Cell-cell signaling ^b	8	-0.189	0.401
Primary active transporter activity ^a	16	0.330	-0.156
ATP biosynthesis ^a	4	0.680	-0.114
Protein biosynthesis ^{a,b}	63	0.292	-0.597
Superoxide metabolism ^{a,b}	3	0.596	-0.735
Hemoglobin complex ^b	7	-0.186	-0.735

^a Significant response to estrogen.

^b Significant response to PKD.

^c Significant interaction of treatments.

related genes. A large fraction (23 of 68 genes) is represented by genes encoding proteins involved in ATP synthesis (e.g., ATP synthase) and protein biosynthesis (e.g., 13 ribosomal proteins). Analyses by GO classes were in line with this finding, as hormonal treatment markedly stimulated the GO categories "ATP biosynthesis" and "protein biosynthesis" (Table 1). The observed stimulation of superoxide dismutase and peroxiredoxin transcripts (Fig. 4A) and transcripts associated with superoxide metabolism (Table 1) might be related to an estrogen-induced production of reactive oxygen species, as has been demonstrated in a number of studies (e.g., Felty, 2006). Estrogens are known to affect the coagulation cascade at the transcriptional level, with tissue factor pathway inhibitor being one of the targets (Jayachandran et al., 2005); this gene is also represented in TM1. The up-regulation of cyclin A2 transcript, polyposis locus protein transcript and transcripts of several genes involved in modification of chromosomes (e.g., transcripts of histone 3A-ATP synthase and histone deacetylase 9 genes) are indicative of the stimulatory influence of estradiol on cell proliferation (Sutherland et al., 1998). Further, we observed transcriptional up-regulation of cathepsin D gene, which is responsible for the specific proteolysis of the major egg yolk protein vitellogenin (Nakamura et al., 1996). It is noteworthy that a group of genes involved in lipid metabolism (genes encoding delta-6 fatty acid desaturase, fatty acid-binding protein, high density lipoprotein-binding protein, sulfotransferase) were also significantly transcriptionally responsive to estrogen treatment. TM1 also included several immune system-related genes, e.g., those encoding leukocyte antigen CD37 and eosinophil chemotactic cytokine.

TM2 (Fig. 4B) includes genes that are transcriptionally down-regulated with both experimental factors, though the effect of the parasite is markedly greater than that of E2 (the beta weights are -0.64 and -0.31, respectively). An especially strong inhibitory response was seen in the hemoglobin complex (Table 1), a finding that is in concordance with the fact that anemia is a major clinical sign associated with the *T. bryosalmonae*-induced disease, PKD (Hedrick et al., 1993).

TM3 and TM4 are enriched in genes implicated in immune responses. TM3 (Fig. 4C) includes mainly genes involved in the innate immune response with predominance of plasma proteins (components of the complement system including properdin, acute phase protein and ceruloplasmin). TM4 (Fig. 4D) contains genes involved in communication between immune cells, i.e. those encoding cytokines and chemokines (CC chemokine SCYA110-1), their receptors (C3a chemotactic receptor) and downstream regulated genes (Ig kappa chain V-I region). Both TMs are significantly down-regulated by E2 (beta weights -0.199 and -0.741), pointing to a potential immunosuppressive action of E2 in rainbow trout. The response to *T. bryosalmonae*, however, differed between TM3 and TM4. TM3 showed a strong induction of transcript expression in response to *T. bryosalmonae* infection (beta weight 0.63) and this induction completely overrules the down-regulating effect of the high E2 (HE) treatment (Fig. 4C). In TM4 (Fig. 4D), in contrast to TM3, parasite-related induction of transcript expression (I) was modulated by the presence of low or high E2 concentrations (ILE, IHE). Analysis of transcriptionally differentially expressed genes grouped by GO classes are in line with the above findings since the predicted humoral immune response and complement activation showed opposite responses to PKD and E2 with no significant interactions between these stressors (Table 1).

Non-immune genes in TM3 which were transcriptionally stimulated by parasite infection and down-regulated by E2 included metallothioneins and cell cycle regulators. Transcriptional up-regulation of metallothionein genes may indicate changes in sequestration of zinc, which is characteristic for various acute conditions. However, transcriptional metallothionein gene up-regulation may also be indicative for oxidative stress (e.g., Kling

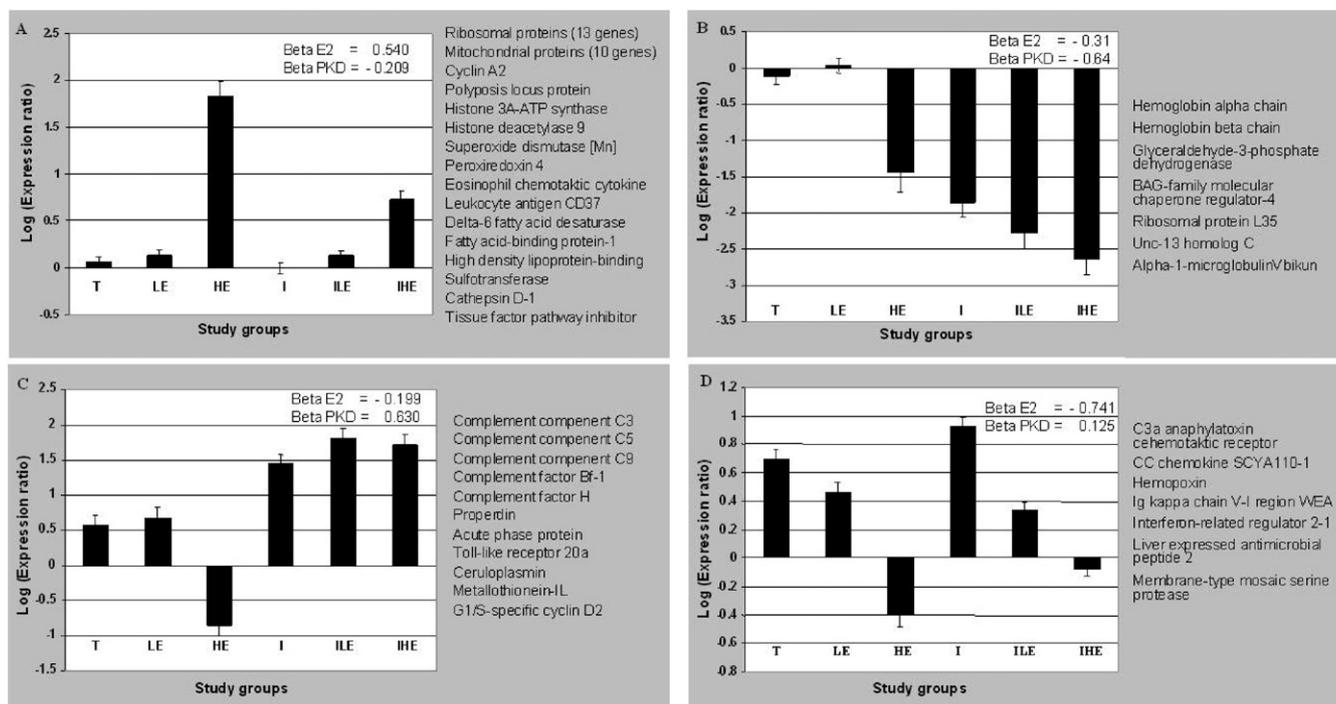


Fig. 4. Effects of *T. bryosalmonae* and E2 on transcript expression of hepatic genes in rainbow trout. The groups of genes with tightly correlated transcriptional expression profiles (transcription modules or TM) were identified as described in Section 2. The data in left parts of panels are mean $\log_2(\text{expression ratio}) \pm \text{SE}$, the names of genes discussed in text are shown on the right. The numbers of genes and Pearson r (mean \pm SD) are indicated for each TM. A: TM1 includes 68 genes, $r = 0.91 \pm 0.05$. B: TM2 includes 21 genes, $r = 0.88 \pm 0.08$. C: TM3 includes 35 genes, $r = 0.88 \pm 0.07$. D: TM4 includes 34 genes, $r = 0.89 \pm 0.05$.

and Olsson, 2000). Presence of G1/S-specific cyclin D2 and growth factors in TM3 may suggest tissue repair processes in the infected fish.

5. Discussion

This study examined whether exposure to a combination of two environmental agents alters the reaction of rainbow trout in comparison to the single agent at the molecular level, and if this interaction relates to effects observed at the organism level. As experimental stressors we used the myxozoan parasite, *T. bryosalmonae*, which is the etiological agent of PKD, and E2 as a prototypic estrogen-active compound. Both stressors, the parasite and estrogenic contaminants, co-exist in Swiss rivers and are discussed as factors contributing to the drastic decline of Swiss brown trout populations over the last decades (Burkhardt-Holm et al., 2005). The results from the present study which point to diverse interactions of both stressors at the molecular level, but the absence of a combination effect on fish mortality does not provide evidence that the PKD impact on Swiss trout populations is enhanced by the presence of estrogen-active contaminants, although these laboratory findings would need further evaluation under field conditions to be conclusive.

In the transcriptomic part of this study, we focused on principal response patterns rather than on individual genes or pathways, as our aim was to learn how the patterns to the joint stressor impact differ from the pattern to the individual stressors. Microarray analysis of the hepatic transcriptome revealed three major response patterns to combined stressor action: (i) Transcriptional gene expression responses to one stressor are weakened by the presence of the second stressor (TM1 and TM4). In TM1, E2 upregulates transcriptional gene expression in non-infected fish, while under parasite infection, the upregulation is reduced. In TM 4, E2 alone downregulates transcriptional expression levels of the genes, whereas *T. bryosalmonae* infection upregulates the genes

transcriptionally; under combined exposure, the parasite-mediated induction of transcriptional gene expression as well as E2-induced downregulation are diminished. (ii) Transcriptional gene expression responses to one stressor are enhanced by the presence of the second stressor. This is the case with TM2, where genes that were transcriptionally downregulated both by E2 alone and by the parasite alone, showed an even stronger downregulation under joint exposure. (iii) Transcriptional gene expression responses at joint treatment are dominated by one of the two stressors. This is the case with TM3, where E2 alone decreases transcriptional expression of the genes, parasite infection alone increases transcriptional gene expression, and fishes exposed to both stressors exhibit transcript expression levels comparable to fishes exposed to the parasite only. The demonstration of such principal response patterns of transcriptional gene expression highlights the value of -omic approaches in studying and classifying stressor interactions.

Stressor combination effects as described above might be described in terms of antagonism, synergism or additivity. However, given the problems in aligning dose metrics of a pathogen response with dose metrics of an estrogen response, and given the fact that we have only rather few data points instead of full dose-response curves, it is difficult to interpret the results of this study under these terms. Difficulties to evaluate additive, synergistic or antagonistic effects from gene array data were also pointed out by Vinuela et al. (2011). Therefore, we avoid the use of these terms for describing stressor interactions observed in the present study.

Genes of TM3 appeared to show no combination effect but to be exclusively responsive to parasite infection, irrespective of the simultaneous presence of E2. TM 3 contains mainly genes of the innate immune system which has a primary function in combating invading pathogens, and, therefore, genes of this system may give priority to the pathogen signal. It is tempting to speculate that the prevailing effect of the pathogenic over the estrogenic

stressor is related to their “severity” (protection against lethal challenge dominates over sublethal damage), although this may be a rather anthropomorphic view and the question would be how the organism can sense the severity. Regardless of the responsible mechanism, TM3 points to an obvious ability of the organism for priority setting in a mixed exposure situation. This may bear implications for the risk assessment of multiple stressors, and highlights the urgent need to improve our understanding of organism’s responses to combinations of chemical, physical and biological stressors.

The genes contained in TM1 encode proteins with roles in cell proliferation and DNA replication, energy metabolism, protein synthesis and secretion, lipid metabolism and responses to oxidative stress. These cellular pathways and functions are known to be estrogen-sensitive, both in mammals (e.g., Moggs, 2005) and fish (e.g., Hoffmann et al., 2006; Williams et al., 2007). In contrast to TM1, E2 alone had a transcriptional downregulating effect on genes of TM3 and TM4. As both TMs contain many immune genes, this observation points to a possible immunosuppressive activity of E2 in rainbow trout. Immunomodulating actions of estrogens are well documented for mammals (e.g., Martin, 2000; Straub, 2007) and increasingly also for teleost fish (e.g., Thilagam et al., 2009; Milla et al., 2011; Wenger et al., 2011). A potentially immunomodulatory role of estrogens in fish is corroborated by recent findings that estrogen receptors are expressed in piscine immune cells (Casanova-Nakayama et al., 2011). From eco-immunological research, the existence of resource competition and trade-offs between immune functions and steroid-regulated reproductive functions is well documented (French et al., 2009), and the transcriptomic changes observed in the present study may provide initial insight into the molecular processes and mechanisms underlying such physiological trade-offs.

Transcriptional downregulation of immune genes by E2, both when administered alone (TM3) or in combination with the parasite (TM4), leads to the question if this can be translated into altered disease manifestation and *T. bryosalmonae*-induced mortality of trout. This was not the case in the present study, neither with respect to parasite levels in the fish host (Fig. 2) nor with respect to mortality (Fig. 1). This finding is in contrast to other studies which reported increased susceptibility of fish to immunogenic agents in the presence of estrogen-active compounds (Cabas et al., 2011; Wenger et al., 2011). To understand the absence of an E2 effect on pathogen-induced mortality in the present study, two factors need to be considered. The first factor is the hierarchy of gene responsiveness observed at the transcriptome level: although a number of immune genes was transcriptionally downregulated by E2 in the presence of parasite infection (TM4), many other immune genes (those in TM3) showed full transcriptional activation in the presence of parasite infection, up to the level as observed in the “infection only” group (genes of TM3). This implicates that under combined E2/parasite exposure, the fish organism was able to activate at least part of its immune defense, despite some immunosuppressive activities of E2, and this hierarchy of gene regulation may have translated into the absence of increased parasite susceptibility at the organism level. Also, it must not be overlooked that activation of immune responses can also have negative impacts on the host (e.g., Skugor et al., 2009), and thus, the assumption that an E2-mediated transcriptional downregulation of immune genes implicates increased pathogen susceptibility may be oversimplified. A second factor that might explain why trout exposed to both the parasite and E2 did not show increased mortalities and parasite loads is the fact that the pathogen alone resulted already in 90% cumulative mortality. Thus, there was little room left for a further increase. Wenger et al. (2011), when studying the combined impact of E2 and a bacterial pathogen on rainbow trout, used bacterial concentrations that per se caused low or

moderate cumulative mortalities, and under these conditions obtained significantly increased cumulative mortalities for the E2/pathogen combinations, what was paralleled by a significantly suppressed immune response to the bacterial infection.

In conclusion, this study analyzed transcriptional responses to the joint impact of a chemical and biological stressor, and the relation of the molecular changes to organism-level responses. The findings demonstrate the existence of distinct molecular response patterns (“transcription modules”, TM) to the joint impact of a chemical and biological agent. Importantly, many of the joint effects of the two stressors on transcriptional gene expression were not anticipated, i.e. could not be deduced from simply combining the results of the single treatments. In view of the diversity of the molecular response patterns, the question is which of them may be a main driver of the organism response to the stressor combination. In this study, particularly TM3, which was characterized by a dominance of the pathogenic over the estrogenic impact on gene transcription, appeared to be relevant for organism fitness – although this is a correlative and not a mechanistic statement. Finally, the question on the relation between the molecular and organism response has to be brought back into the ecological context in order to better understand how organisms handle multiple stressors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2012.02.026.

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