INTRODUCTION

The egg yolk precursor vitellogenin (VTG) in male or juvenile fish is a well-established biomarker for assessing exposure to estrogenic endocrine disruptors [1,2]. Biomarkers can be broadly defined as (sub)organismic) measures reflecting an interaction between a biological system and a chemical, biological, or physical environmental agent that is indicative of exposure to or adverse effects of the agent [3,4]. The utility of VTG as a biomarker of estrogenic exposure is based on its specific, estrogen-dependent induction mechanism, its rapid and concentration-dependent induction response, and the availability of sensitive and specific assays, including enzyme-linked immunosorbent assays (ELISA) for VTG protein and reverse transcriptase-polymerase chain reaction (RT-PCR) determination of VTG mRNA. To understand how the VTG gene through ligand activation of estrogen receptors [8]. Vitellogenin synthesis, however, can also be induced in immature and male fish if they are exposed to environmental estrogen receptor ligands. It is this abnormal VTG induction that is used as a biomarker of estrogen exposure.

In an environmental context, fish are exposed to multiple stressors, and the question arises of whether the VTG biomarker is able to indicate estrogen exposure reliably against the cumulative impact of other stressors. Particularly under conditions of low contaminant exposure, the biomarker response may be weak and thus easily confounded by concurrent exposure to additional stressors. For example, Kirby et al. [9] showed that exposure of fish to combinations of estrogen receptor-activating compounds and aryl hydrocarbon receptor-activating chemicals modulates the responses of the biomarkers VTG and 7-ethoxyresorufin-O-deethylase activity, respectively, which might lead to an underestimation of actual exposure in the field. A physical factor known to be able to alter the VTG biomarker response is water temperature [10,11]. Virtually nothing is known of the possible influence of biological stressors such as pathogens on the VTG biomarker. For the antiandrogenic biomarker spiggin, Rushbrook et al. [12] found that male three-spined sticklebacks infected with the cestode Schistocephalus solidus displayed reduced induction levels compared with healthy conspecifics.

The present study investigates the response of the VTG biomarker to a combination of a chemical stressor (environmental estrogen) and a biological stressor (a pathogenic parasite). Juvenile rainbow trout, Oncorhynchus mykiss, were exposed to the following: (1) exogenous 17β-estradiol (E2) as a prototypic estrogen-active compound; (2) the myxozoan parasite Tetracapsuloides bryosalmonae, which is the causative agent of proliferative kidney disease (PKD); or (3) a combination of E2 and the parasite. Proliferative kidney disease is a major threat to salmonid populations in the Northern Hemisphere [13,14]. In Switzerland, the parasite has been shown to occur in combination with estrogenic contamination of rivers [15]. The hepatic VTG response under treatment with the single stressors or their combination was assessed by real-time RT-PCR determination of VTG mRNA. To understand how the VTG biomarker response relates to the response of other estrogen-sensitive genes and pathways, as well as to the overall physiological status of the liver, we additionally examined hepatic transcriptome changes by means of microarray technology.

MATERIALS AND METHODS

Exposure of rainbow trout to estrogen and PKD

Two batches of juvenile rainbow trout (age 0+, 1.5–4 g) of the same genetic origin, one infected with T. bryosalmonae and
the other not, were transferred from a fish farm to the fish facility of the Centre for Fish and Wildlife Health in Bern, Switzerland. Immediately after transfer, samples were collected from both batches to check for infection with the PKD-causing parasite using the previously described PCR method [16] to determine the amount of parasite DNA in trout kidneys. Severity of infection was estimated from macroscopic inspection of kidney swelling [16]. Afterward, the fish were acclimated for one week to the water temperature (14°C) in the experimental facilities at Bern before initial samples were taken to check for hepatic VTG mRNA expression, microarray analysis, and infection status. Then, the fish were distributed to aquaria of 100 L volume and were subjected to an elevation of water temperature to 18°C, because this temperature promotes the clinical outbreak of PKD [16]. The elevation of the water temperature was done gradually over 1 d. Positive and negative rainbow trout were placed into the same aquaria, because the disease is not transmitted from fish to fish [17]. To distinguish noninfected from infected fish, the latter were marked by a cut on the adipose fin. Before exposing the fish to the diets containing E2, further samples were taken from the infected (I) and noninfected (T) groups for VTG mRNA and microarray analysis. The fish were fed with one of three different diets. The first diet was a commercial trout pellet feed (Hokovit) without hormone addition. The second diet was the same feed but with a low dose of E2 added (0.5 mg E2/kg food), and the third diet had a high dose of E2 added (20 mg E2/kg food). The hormone E2 (Sigma) was incorporated into the feed pellets using the alcohol evaporation method of Guerrero [18]. Actual E2 concentrations in the diet were confirmed by high-performance liquid chromatography analysis. Overall, the experiment included the following treatments: fish with treatment T received the control diet, and they were not infected by T. bryosalmonae. The low-dose group (LE) consisted of noninfected fish fed with the low-dose E2 diet (0.5 mg E2/kg). The high-dose group (HE) consisted of noninfected fish fed with the high dose of E2 (20 mg E2/kg). The “infection” group (I) was composed of fish infected with the parasite and fed with the control diet. Fish of the ILE treatment were parasite-infected and received the low-dose diet (0.5 mg E2/kg). Fish of the IHE treatment were parasite-infected and received the high-dose diet (20 mg E2/kg). All groups were maintained at 18°C water temperature.

The fish were kept under the described exposure conditions for three weeks. After 7, 14, and 20 d, 20 fish were sampled from each treatment and anesthetized with buffered 3-aminobenzoic acid ethyl ester methanesulphonate (Argent Chemical Laboratories). Livers were removed and stored in RNAlater (Ambion). All treatments were run in duplicate (n = 80 per replicate).

Real-time RT-PCR for determining absolute levels of VTG expression

Total RNA was extracted from the rainbow trout livers with Trizol (Invitrogen) according to the manufacturer’s protocol. Absolute quantification of VTG mRNA levels was achieved by means of real-time RT-PCR using the protocol described by Burki et al. [7] for brown trout, but with primers adapted for rainbow trout. Primers and probes were designed on the basis of the published rainbow trout vtg gene sequence (X92804): primer forward (5’→3’): TCTGAAGTCAACGCGTTTAAATGAT-, primer reversed (5’→3’): AGAGGCTAATGGATCTTGTGACTCTT-, probe (5’→3’): CATGTTGACAGACATTGACAAATTTA-. A standard curve was generated by performing a one-step, real-time RT-PCR on a 10-fold dilution series of in-vitro-transcribed VTG cRNA [7]. This standard curve was used to calculate the absolute amounts of VTG RNA in total RNA of unknown samples. Mean values of VTG expression levels of trout exposed only to E2 and trout coexposed to PKD and E2 were compared for significance (p < 0.05) by using the Mann–Whitney test.

Multiple gene expression analyses

Microarray analysis was performed with liver samples of fish sampled at day 20 of the experiment. We used high-density salmonid fish microarray SFA3, an extended version of SFA2, which has been described in detail elsewhere (Gene Expression Omnibus GPL1212) [19]. This platform includes 1,766 genes printed in six 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).

Total RNA was extracted from the rainbow trout livers with Trizol (Invitrogen), and four individuals were pooled in each sample. Labeling and hybridization was performed as previously described in detail [20]. In brief, fluorescent labels were incorporated at cDNA synthesis using a mixture (20 μl volume) that contained 20 μg RNA; buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl2); 5 mM DTT; 0.05 mM Cy3- or Cy5-dATP (Amersham Pharmacia); 0.5 mM each dCTP, dGTP, and dTTP; 0.25 mM dATP; 0.5 μg oligo(dT); 20 U RNase inhibitor (MBI Fermentas); and 200 U SuperScript III reverse transcriptase (Invitrogen). Reaction continued for 2h at 50°C. The RNA was degraded by an addition of 2 μl 2.5 M NaOH, and, after incubation at 37°C for 15 min, alkalinity was neutralized with 10 μl of 2 M 2-hydroxyethyl-1-piperazineethanesulfonic acid buffer. After labeling, the cDNA was purified with Microcon YM30 (Millipore). The microarray hybridizations followed a common reference design with dye swap. The start sample (T group, water temperature 14°C, no PKD, no estrogen) was used as a control for all treatment groups. Afterward, each purified sample was hybridized to two microarrays. On the first slide, test and control cDNA were labeled with Cy5 and Cy3, respectively, on the second array, dye assignments were reversed. The pretreatment of the slides was carried out with 1% bovine serum albumin, fraction V, 5× saline sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulfate (SDS) (30 min at 50°C). Thereafter, a washing step with 2× SSC (3 min) and 0.2× SSC (3 min) followed. Slides were covered with Lifter Slips (Erie Scientific) and placed into ArrayIt hybridization cassettes (TeleChem International). A mixture of 80 μl containing label, 1.3× Denhardt’s, 3× SSC, 0.3% SDS, 0.67 μg/μl polyadenylate, and 1.4 μg/μl yeast torula RNA was prepared, and hybridization was performed in a water bath for 16 h at 60°C. After the hybridization, the slides were washed in 0.5× SSC, 0.1% SDS (5 min twice), 0.5× SSC, 0.01% SDS (5 min), 0.06× SSC (3 min twice), and dried using a microarray centrifuge (TeleChem International). Scanning was performed with ScanArray 5000. The quantification of the spots was performed with TIGR SpotFinder. The measurements were filtered by criterion (I – SI) > (B + SB), where I and B are the mean signal and background intensities and SI and SB 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 differential expression was assessed by the difference of expression ratios at the reverse labeling (Student’s t test, p < 0.01). Microarray data were analyzed with proprietary software developed by one of the authors (S. Afanasyev).
RESULTS

Hepatic VTG mRNA response

The induction of VTG mRNA as a marker of the diet-borne E2 exposure was assessed by means of real-time RT-PCR. In healthy, nonparasite-infected fish, the 20-d treatment with E2 resulted in a significant ($p < 0.05$) induction of VTG, both for the low (LE) and for the high (HE) estrogen exposure levels (Fig. 1A). The two E2 concentrations differed with respect to the amplitude of VTG induction; exposure to the high E2 concentration led to an approximately 200 times higher induction level of VTG mRNA than LE. Also, the individual variability and time course of VTG induction differed between HE and LE, whereas with the high E2 dose, mean VTG mRNA levels showed a coefficient of variability (CV) below 12%, regardless of whether the fish were infected; the CV was above 20% in the groups receiving the low E2 dose. With regard to the time course of VTG induction, VTG mRNA levels had reached a plateau already after 7 d of treatment in the HE group, whereas, in fish receiving the low E2 dose, the increase of hepatic VTG mRNA levels slowed only after 14 d of E2 exposure (Fig. 1B).

In fish infected with *T. bryosalmonae* (treatments ILE, IHE), concurrent E2 exposure resulted in a significantly lower ($p < 0.05$) magnitude of the VTG induction response than in the corresponding groups of noninfected fish (Fig. 1A). The levels of VTG mRNA in PKD-infected fish were below those of nondiseased fish over the whole time course of the experiment, for both the high- and the low-E2 doses (Fig. 1B). Interestingly, presence of the parasite also affected the levels of hepatic VTG mRNA in non-E2-exposed fish at the end of the experiment (day 20), as indicated by the significant ($p < 0.05$) difference between healthy (treatment T) and diseased (treatment I) fish (Fig. 1A,B). At day 0, VTG mRNA levels did not differ significantly between groups T and I (Fig. 1B), but at the end of the experimental period, hepatic VTG expression levels had undergone an increase in the T group, whereas they remained unchanged with treatment I.

Hepatic transcriptome response

To determine whether the difference in the VTG response between healthy and diseased fish is restricted to the VTG biomarker response or represents a more general pattern of liver physiological pathways, we examined the transcriptional profile of trout liver in the presence of E2 and PKD (alone and in combination) using the rainbow trout cDNA microarray of Krasnov et al. [19]. Hepatic transcriptome analysis of all six study groups (T, I, LE, HE, ILE, IHE) at day 20 of the experiment identified 792 genes as being significantly altered by the experimental treatments. Estrogen administration had a significant influence on 314 of the 792 hepatic genes, with 142 genes of the 314 regulated genes upregulated and 172 genes downregulated. Significant effects of estrogenic treatment were present primarily in the HE group. Table 1 shows a selection of E2-responsive genes and their classification in GO. Genes of many different physiological processes such as the cell cycle, electron transport, lipid metabolism, protein biosynthesis, antioxidant activity, and apoptosis were stimulated by E2 exposure (Table 1). Some genes were downregulated with E2 treatment; these genes belonged primarily to functional groups involved in immune functions, defense pathways, and general stress responses (data not shown).

The expression levels of the vast majority of E2-inducible genes were altered when the fish were concurrently infected with *T. bryosalmonae* (Table 1). Among the 142 genes that were upregulated by E2 exposure in noninfected fish, 136 showed a reduced expression level in the additional presence of parasite infection. The mRNA expression levels of six genes (*actin cytoplasmic 2, complement factor B/C2-B, cyclophilin-3, nonhistone chromosomal protein HMG-17, ribosomal protein S12, thymosin beta 4-1*) were not reduced by concurrent parasite infection. Figure 2 exemplifies the combined impact of “E2 × parasite” on gene expression for three functional classes, protein biosynthesis, apoptosis, and electron transport. Overall, the described response pattern agrees well with the response pattern of the VTG biomarker under combined exposure (see above).

DISCUSSION

The present study investigates the response of the estrogenic biomarker VTG to combined estrogen–pathogen exposure. The
intention was not to reiterate that VTG can be induced by estrogenic exposure but to evaluate how and strongly the induction response might be confounded by a biological stressor. The VTG biomarker has been selected because it is a well-studied, robust biomarker, with a clear mechanistic basis and is widely used as a biomarker of estrogenic exposure. However, we have no indication of a clearcut difference in disease-wide presence of salmonin populations [13,14] and is well characterized with respect to pathogenesis and driving factors [22,23]. Finally, coexposure of salmonids to PKD and environmental estrogens represents a realistic concern [15].

The results of the study show that estrogen-induced induction of hepatic VTG in rainbow trout is significantly reduced in fish infected with T. bryosalmonae. A possible explanation is that the diseased fish show reduced food intake. From observations on the feeding behavior of the experimental fish, however, we have no indication of a clearcut difference in feeding activity between healthy and pathogen-infected fish. An alternative explanation would be that the biological stress situation, infection with a pathogenic parasite, impaired the ability of the fish to perform VTG synthesis, which might be explained on the basis of energetic constraints. The results from microarray analysis seem to support this hypothesis; the array data show that E2 exposure upregulated genes primarily involved in protein biosynthesis, electron transport, cell cycle, apoptosis, or antioxidative activity, an observation that is in good agreement with published data [24,25]. Processes such as VTG synthesis, or, more broadly speaking, protein synthesis are energetically costly, so their induction leads to resource competition and tradeoffs with other energy-consuming processes [26–28]. Key among such competition is the maintenance of health on the one side, which involves activation of immune pathways and other defense systems, and the production of offspring on the other side, which involves increased synthesis of proteins and lipoproteins such as VTG. Indeed, it has been shown repeatedly that investments in immunological defenses are associated with impaired reproductive functions and, vice versa, that reproductive activities can greatly influence an organism’s immunocompetence [28–30]. With respect to the present experiment, this would mean that the reduced induction...
of VTG in parasite-infected trout could result from the increased energy demands of those fish for immune defense. The same explanation would apply for fish from the control (non-E2-exposed) treatments (T, I), in which noninfected individuals increased their VTG mRNA levels during the experimental period, whereas infected fish showed no increase. The interpretation is supported by the transcriptomics finding that all 142 E2-inducible genes, except for six, showed reduced responsiveness to E2 in diseased fish; that is, they behaved in the same way as did VTG. Overall, the findings from the transcriptomic analysis suggest that the combined stressor impact on the VTG biomarker could be explained in terms of energy constraints, primarily by competition between energy-intensive immune and energy-intensive reproductive processes. Understanding the mode of stressor interaction in the biological target, together with information on the dose and time dependence of the interaction, provides the basis for developing prediction models of combination effects of multiple stressors.

Free-ranging fish populations frequently suffer from pathogenic infections, which leads to the question of whether the use of the VTG biomarker in biomonitoring for estrogen exposure could be compromised in fish populations suffering from overt or latent disease. The results from the present study suggest that under conditions of high-dose exposure, the VTG biomarker still would signal the estrogenic exposure, because E2-related VTG induction in infected fish, although significantly reduced compared to healthy fish, is still highly significant. The situation may be more critical in scenarios characterized by low-dose exposures, which are typical for many river systems [31,32]. For example, Burki et al. [7] analyzed VTG in rainbow trout from a river receiving low estrogenic input from a sewage treatment plant effluent. Still, estrogenic contamination in the river stretch downstream of the effluent (as determined by bioanalytical methods) was significantly higher than upstream. Rainbow trout collected downstream displayed hepatic VTG mRNA levels between 0.08 and 0.18 pg/10 ng total RNA, whereas trout collected upstream had hepatic VTG mRNA levels of 0.04 to 0.09 pg/10 ng total RNA. The difference in VTG biomarker expression between the two sites, although small, was significant. With the assumption that the downstream fish would have been diseased, their VTG mRNA levels could decline by 50% or more, as was the case for diseased fish in the present study. Then, however, VTG biomarker expression between upstream and downstream would no longer have been unequivocal, and the VTG biomarker would have failed to detect the difference in estrogenic contamination between the downstream and the upstream river stretches. This example illustrates that, in field situations with low estrogenic exposure, and consequently, low induction of the VTG biomarker, the reduced capacity for VTG synthesis of parasitized fish might obscure the biomarker signal.

Acknowledgement—The financial support of Swiss Nationalfonds (project 4050-066568) is gratefully acknowledged. We thank the Turku Centre for Biotechnology, Finland, for preparation of microarrays, and R. Schönenberger, Eawag, Dübendorf, Switzerland, for performing the analytical determination of E2 concentrations in the experimental diets.

REFERENCES


