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Long-term exposure to low 17α-ethinylestradiol (EE2) concentrations disrupts both the reproductive and the immune system of juvenile rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Estrogenic endocrine disrupting compounds (EEDCs), such as ethinylestradiol (EE2), are well studied for their impact on the reproductive system of fish. EEDCs may also impact the immune system and, as a consequence, the disease susceptibility of fish. It is currently not yet known whether the low concentrations of EEDCs that are able to disrupt the reproductive system of trout are effective in disrupting the immune system and the fish host resistance towards pathogens, too, or whether such immunodisruptive effects would occur only at higher EEDC concentrations. Therefore, in the present study we compare the effect thresholds of low 17α-ethinylestradiol concentrations (1.5 and 5.5 EE2 ng/L) on the reproductive system, the immune system, the energy expenditures and the resistance of juvenile rainbow trout (*Oncorhynchus mykiss*) against the parasite *Tetracapsuloides bryosalmonae* – the etiological agent of proliferative kidney disease (PKD) of salmonids. The parasite infection was conducted without injection and under low pathogen exposure concentrations. The disease development was followed over 130 days post infection – in the presence or absence of EE2 exposure. The results show that the long-term EE2 exposure affected, at both concentrations, reproductive parameters like the mRNA levels of hepatic vitellogenin and estrogen receptors. At the same concentrations, EE2 exposure modulated the immune parameters: mRNA levels of several immune genes were altered and the parasite intensity as well as the disease severity (histopathology) were significantly reduced in EE2-exposed fish compared to infected control fish. The combination of EE2 exposure and parasite infection was energetically costly, as indicated by the decreased values of the swim tunnel respirometry. Although further substantiation is needed, our findings suggest that EE2 exerts endocrine disruptive and immunomodulating activities at comparable effect thresholds, since reproductive and immune parameters were affected by the same, low EE2 concentrations.

1. Introduction

The aquatic environment represents a sink for pollutants, including endocrine disrupting compounds (EDCs) (Sumpter 1998, Sumpter and Johnson 2005). Among those are estrogenic EDCs (EEDCs) that mimic natural estrogens by interacting with estrogen receptors (ERs). A prototypic EEDC is the synthetic estrogen ethinylestradiol (EE2), a constituent of contraceptive pills, which reaches the aquatic environment mainly *via* effluents from sewage treatment plants. In aquatic environments, EE2 is found ubiquitously and is present at concentrations ranging from "not detected" to 34 ng/L (Aris et al., 2014). Johnson et al. (2013) calculated that 12% by lengths of Europe's rivers would exceed the environmental quality standard for EE2 (0.035 ng/L). Compared to the naturally and endogenously produced estradiol (E2), EE2 was shown to possess higher persistence (cf. Fent et al., 2006, Juergens et al., 2002). It was reported that EE2 is more potent than E2 in the binding affinity to the ER (Legler et al., 2002) and in inducing hepatic vitellogenin (vtg) synthesis (Thorpe et al., 2003, cf. Burgos-Aceves et al., 2016) in fish. Both, laboratory and field studies provide evidence that the low concentrations of EE2 as present in the aquatic environment are able to interfere with the reproductive system of fish (e.g. Kidd et al., 2007, Länge et al., 2001, Schäfers et al., 2007). Research on EEDCs has focused mostly on such disruptive effects on the reproductive system. However, estrogens have pleiotropic actions and

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interact with numerous physiological systems other than the reproductive system (Segner et al., 2013). Therefore, disruptive effects of EEDs may reach well beyond disturbances of fish sexual development or reproductive performance.

One potential target of EEDC action that has attracted growing attention over recent years is the immune system of fish (Chaves-Pozo et al., 2012, Milla et al., 2011, Segner et al., 2017). Immunocompetence is a critical fitness determinant enabling the organism to survive, grow and reproduce well, but also to minimize the fitness costs of infections (French et al., 2009, Graham et al., 2010, Graham et al., 2011, Lochmiller and Deerenberg 2000, Owens and Wilson 1999). It was shown that fish immune cells possess estrogen receptors (Casanova-Nakayama et al., 2018, Paiola et al., 2017) and that EEDCs can indeed modulate the immune system of fish (e.g. Burgos-Aceves et al., 2016, Cabas et al., 2012, Casanova-Nakayama et al., 2011, Massart et al., 2014, Milla et al., 2011, Paiola et al., 2017, Seemann et al., 2016, Shelley et al., 2013, Thilagam et al., 2009, Wenger et al., 2011, Ye et al., 2018).

What is currently still uncertain is whether the low concentrations of EE2, as they occur in aquatic environments and which are able to disrupt the reproductive system of fish, are also effective in disrupting the immune system – or whether it would need higher EEDC effect concentrations for this physiological target system. The question is important since from an ecotoxicological perspective, the key question is not whether a compound can interfere with a specific vital function of the organisms, but which vital function is most sensitive to the adverse action of the EEDCs and thus, is most likely to compromise survival and reproduction of the population.

Organisms possess phenotypic plasticity that enables them to tolerate a certain level of environmentally induced stress. However, exposure to one stressor may compromise the capacity to respond to another stressor, either directly in that the two stressors influence the same physiological targets, or indirectly, for instance, due to resource trade-offs (Burki et al., 2013; Marteinson et al., 2017; Prosser et al., 2011; Seebacher and Franklin 2012; Todgham and Stillman 2013; Wernicke von Siebenthal et al., 2018). Such interactions that are shaped by the physiological and ecological traits of the exposed species can lead to cumulative effects of stressor combinations which differ markedly from the effects of the single stressors (Christensen et al., 2006, Segner et al., 2014, Shears and Ross 2010). An example for such interactive effects of environmental stressors is the growing evidence that numerous environmental contaminants are able to modulate disease susceptibility and epidemics in fish and wildlife populations (Acevedo-Whitehouse and Duffus 2009, Arkoosh et al., 1998, Blaustein and Kiesecker 2002, Burnett 2005, Clifford et al., 2005, Desforges et al., 2017, Morley et al., 2006, Rohr et al., 2008). The underlying mechanisms are diverse but include the alteration of ecological (e.g. abundance, density, distribution) and physiological traits (e.g. immunocompetence) of the host species as well as the alteration of traits of the pathogen species (e.g. virulence, survival, proliferation). *Vice versa*, pathogen infection can modify the response of the host to pollutants (Marcogliese et al., 2009, Prosser et al., 2011, Sures 2008).

In the present study, we compared the response of reproductive and immune parameters as well as pathogen resistance and energy expenditures of juvenile rainbow trout (*Oncorhynchus mykiss)* under identical EE2 exposure conditions. Three considerations were essential for the present study: firstly, we used low EE2 exposure concentrations (1.5 and 5.5 ng/L), as they can occur in aquatic environments (Aris et al., 2014, Vermeirssen et al., 2005). Secondly, at the current state of knowledge in fish immunotoxicology, a possible effect of EEDCs on the immunocapacity of fish cannot be fully assessed by measuring molecular or cellular responses only, but it needs to include the assessment of the host resistance towards pathogens (Segner et al., 2012, Rehberger et al., 2017). Therefore, we assessed the immune response at different levels of biological organization – including the susceptibility of the fish host towards a parasite infection – and we evaluated whether the treatments were energetically costly by means of swim tunnel respirometry. Host resistance studies with fish often rely on the injection of a pathogen, representing an artificial infection pathway, and high pathogen concentrations that lead to acute mortalities. Instead, we used an infection pathway without injection (juvenile rainbow trout were exposed to infectious spores in the water), low pathogen concentrations and chronic disease development associated with negligible mortality. As pathogen, we selected the parasite *Tetracapsuloides bryosalmonae*. *T. bryosalmonae* is the etiological agent of the emerging proliferative kidney disease (PKD), which is considered to be a main factor contributing to the decline of wild salmonid populations in Switzerland (Burkhardt-Holm et al., 2005). The parasite has a life cycle including two host: salmonids as vertebrate host and freshwater bryozoan as invertebrate host. Parasite spores released by the bryozoan infect the fish *via* the gills. There is no fish-to-fish transmission.

Overall, the aspects investigated in this study with juvenile rainbow trout included (i) the use of low intensities of both a non-infectious chemical stressor (EE2) and an infectious stressor (*T. bryosalmonae*) over a long-term period (about five month), (ii) the comparative evaluation of the responses of both the reproductive and the immune system under the same treatment conditions, (iii) the analysis of the single and combined stressor exposure at different levels of organization, including the (iv) assessment of the organism level responses by measuring the stressor-induced energetic costs to the fish (by means of swim tunnel respirometry), and by measuring the resistance of the fish towards the parasite infection under the different exposure scenarios.

2. Material and methods

2.1. Experimental animals

We used six-month-old rainbow trout (*Oncorhynchus mykiss*) obtained from a cantonal fish farm (Piscicoltura Cantonale Rodi, 6772 Rodi, Switzerland). Upon arrival, trout were about 10 g in weight. They were diagnosed negative for the presence of pathogens, including *T. bryosalmonae*. The fish were kept for four weeks in quarantine at the facilities of the Centre for Fish and Wildlife Health (University of Bern, Switzerland). After quarantine, trout were transferred into 35 L glass aquaria at a density of 35 g fish per liter water (122 fish per aquarium). The flow through water supply, at a rate of 60 L tap water per hour, was adjusted using flow rotameters (Rota Yokogawa, Germany). The day-night rhythm was set at 12 h light and 12 h darkness. Water temperature was 12.9 (± 1.22) °C on average. Trout were acclimated for 28 days in the glass aquaria before the EE2-exposure was initiated. This acclimation period included the adjustment of feeding to 1.5% of body weight per day (Hokovit Trout Start, Hokovit, Switzerland) 14 days prior EE2-exposure. Controlling of the behavior and appearance of the fish as well as cleaning of the aquaria was carried out on a daily basis. All procedures were performed in compliance with the Swiss legislation for animal experimentation guidelines under license number BE102/16.

2.2. Time course of the experiment, treatment groups and n-numbers

An overview about the time course of this study is shown in Fig. 1. After the 28-day acclimation period (see 2.1) water-borne EE2-exposure was initiated (day 0 of the present experiment) and continued until termination of the experiment. After 2 weeks (day 15 of the experiment), half of the fish were infected with *T. bryosalmonae* (see 2.5). Consequently, from day 15 until termination of the experiment, we had the following six treatment groups: (1) Control = no EE2, no parasite; (2) 1.5 ng/L EE2, no parasite; (3) 5.5 ng/L EE2, no parasite; (4) Infected control = no EE2, infected; (5) 1.5 ng/L EE2, infected; (6) 5.5 ng/L EE2, infected. Treatments were performed in two individual aquaria each (hence, 12 aquaria were used). For the initial sampling at day 0, in total 24 fish were sampled. At the subsequent samplings, 16 fish per treatment were sampled. Finally, for the swim tunnel respirometry on

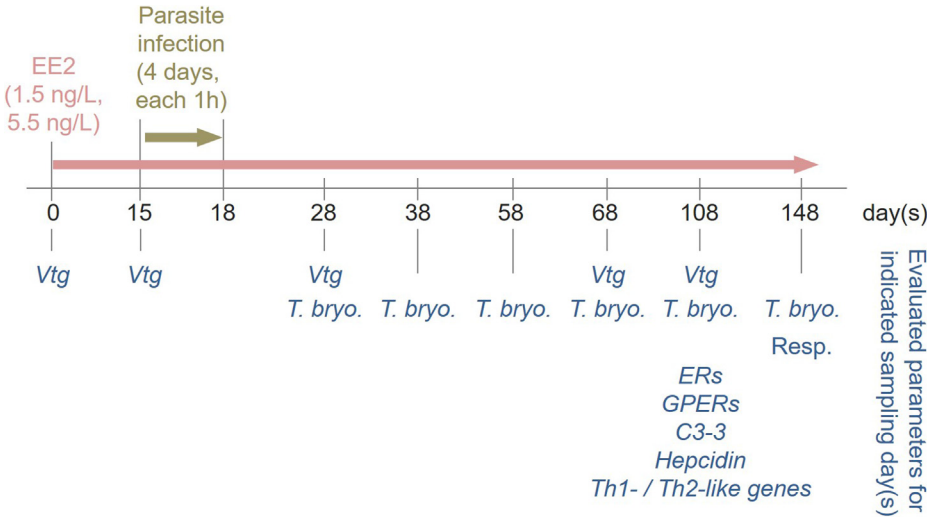

Fig. 1. Time course of the experiment. In this graph the timeline of the experiment is displayed, including the onset of chemical exposure (reddish), the timeframe for parasite infection (brownish) as well as the information which parameters were analyzed for which sampling day(s) (blue). Day 148 of the experiment equals 130 days post infection. Abbreviations: EE2 = Ethinylestradiol; Vtg = Vitellogenin; T. bryo. = *Tetracapsuloides bryosalmonae* (=the health / disease status); ERs = estrogen receptors (nuclear); GPER = G-protein coupled estrogen receptor; C3-3 = complement component 3, isoform 3; Th1- and Th2-like genes include T-bet = T box transcription factor, TNFα = tumor necrosis factor alpha, IFNγ = Interferon gamma, GATA-3 = Trans-acting T-cell-specific transcription factor, IL-10 = Interleukin 10, IL-4/13A = Interleukin 4/13A; Resp. = Swim tunnel respirometry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

day 148, eight fish were analyzed per treatment (but not the 1.5 ng/L EE2 treatments). Day 148 of the experiment equals 130 days post infection.

2.3. 17α-Ethinylestradiol (EE2) exposure

The EEDC used in the present study was EE2 (purity ≥ 98%, E4876-10G; Sigma-Aldrich, Switzerland). The compound was dissolved in EtOH (purity ≥ 99.8%; Merck, Germany) and further diluted with tap water. To pump this stock solution into the aquaria, inert tubing (Flow Tubing 4.0 mm ID, Pharamed, Gilson AG, Switzerland) and peristaltic pumps (Minipuls3, Gilson AG, Switzerland) were used. Water- and EE2-flow rates as well as EE2 water concentrations were controlled routinely. Wherever possible, glass equipment was used.

2.4. Analytical determination of EE2 concentrations in the aquarium water

For aquarium water samples, a solid phase extraction (SPE) using C18 filter cartridges (Grogg Chemie, Switzerland) was conducted. The EE2 concentration was determined by means of a competitive EE2 Elisa Kit (Ecologiena, Japan) and measured using an EnSpire plate reader (PerkinElmer, Waltham, MA, USA). For details, see Wernicke von Siebenthal et al. (2018).

2.5. Infection of juvenile rainbow trout with Tetracapsuloides bryosalmonae

Infection with the parasite *T. bryosalmonae* was achieved by bath-immersion as described in Wernicke von Siebenthal et al. (2018). Bryozoans, the invertebrate host, were collected from Swiss rivers by Dr. H. Hartikainen and her team (Eawag, Switzerland). The bryozoan samples were screened by optical inspection for the presence of *T. bryosalmonae* and were then transferred into clean water to release the infectious parasite spores. For all aquaria, regardless whether the fish were to be infected or not, water inflow was stopped, and water levels were lowered. Aquaria containing fish that should be infected received equal aliquots of water containing infective spores. Parasite spore density in the aquaria water was estimated by means of qPCR, detecting 1. 25E + 5 ± 38% parasite DNA copies per liter water on average. After one hour, the water flow-through was re-started for all aquaria to remove the parasite spores. The infection procedure was repeated on four consecutive days (day 15–18 of the experiment). Importantly, with this approach, infection of the fish was possible only during the 1-hour-exposure windows, and continued new infections were excluded (transfer of parasites form fish to fish is not possible). This means that a change of infection prevalence was no longer possible after the four 1-h-exposures, thus any change in parasite intensity during the subsequent experimental period is due to the proliferation of the parasite within the host but not to re-infection.

2.6. Sampling procedure and sample preparation

Trout were euthanized using an overdose of MS222® (150 mg/l buffered 3-aminobenzoic acid ethyl ester, Argent Chemical Laboratories). The length and weight of each fish was recorded, blood was collected, and organs were dissected and weighted. Blood was subsequently centrifuged (13300 U/min for 8 min) in micro-hematocrit-tubes to determine the hematocrit levels as volume percentage of blood cells in the blood (only at day 158). The trunk kidney (TK) was cut longitudinally. One half of the TK was sampled for TaqMan-qPCR and, thus, placed in a tube containing 1.5 ml TRI Reagent (Sigma-Aldrich, Switzerland) and a 5 mm stainless steel bead (Qiagen, Switzerland). The tissue was lysed (Tissue lyser, Qiagen, Switzerland) for 3 min and subsequently stored at minus 80 °C until further proceeding. Head kidney (HK) and liver were lysed in TRI Reagent for RT-qPCR, too. The other half of the TK and the gonads were placed into a histology-cassette and transferred into HistoChoice fixative (Sigma-Aldrich, Switzerland; see 2.10). Fig. 1 depicts which parameters were analyzed for which sampling day.

2.7. TaqMan-qPCR for parasite prevalence and intensity

For DNA extraction, 150 µl of the TK-homogenate and 900 µl of extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris; pH 8.0) were mixed for 2 h on ice. Afterwards, 375 µl of chloroform was added, well mixed and tubes were centrifuged (13.000 rcf, 15 min, 4 °C). Next, 150 µl from the top phase was mixed with 120 µl of isopropanol and centrifuged as before. Supernatant was discharged and the pellet was washed twice with 70% EtOH before air-dried and re-suspended. The parasite intensity was determined by TaqMan-qPCR according to Bailey et al. (2017a) and Strepparava et al. (2018). The primers and probe sequences are listed in supplement S1. The qPCR was performed on Applied Biosystems 7500 fast device (Applied Biosystems, Foster City, CA, USA) and data were analyzed using “system sequence detection software version 1.3.1”. Results are expressed as “log-copy number of parasite DNA per gram TK tissue”. The parasite prevalence represents the percentage of detectably infected rainbow trout among the infected fish.

2.8. qRT-PCR for mRNA transcript levels

The RNA of the tissue (HK, L) homogenates was isolated using TRI Reagent (guanidinium thiocyanate isolation) and the RNA concentration was measured (NanoDrop, ND-1000 Spectrophotometer, Witec AG). For details, see Wernicke von Siebenthal et al. (2018). The cDNA synthesis was conducted using the GoScript™ Reverse Transcription Mix (Random Primer, Promega, Switzerland). Samples were processed according to manufacturer's instructions, utilizing 1 µg RNA. For qRT-PCR the SYBR-green-type GoTaq® qPCR Master Mix (Promega, Switzerland) was applied. Modifications from the protocol were a hot start activation for 5 min and 45 amplification cycles. The primer sequences are listed in supplement S1. Samples were measured in technical duplicates using 96-well reaction plates (fast optical plates, Thermofisher Scientific, Switzerland), the Applied Biosystems 7500 fast device and corresponding software (see 2.8). Blanks (no cDNA) and melting curves were carried out to exclude contamination or false amplification. Data was normalized to the reference gene *EF-1α* and calculations were based on the $2^{-\Delta\Delta Ct}$ method.

2.9. Histological examination of trunk kidney (TK) and gonads

After 3 h of fixation, the tissues were embedded in paraffin, sectioned (3 µm thickness) and stained with hematoxylin-eosin.

The gonads were examined for sex determination by light microscopy. Female trout at this life stage (sampling day 108) usually have already differentiated ovaries, while males predominantly still display undifferentiated gonads (Burki and Segner, unpublished). Thus, trout that displayed no ovaries were considered as males.

The TK was subjected to semi-quantitative, histological examination to assess the severity of tissue pathological changes (Schmidt-Posthaus et al., 2012). Scoring of the pathological alterations ranged from 0 (no alterations) up to 6 (severe pathology). The following parameters were considered for the scoring: vasculitis, vascular necrosis, thrombosis, interstitial necrosis, multifocal, macrophage infiltration in the interstitium.

2.10. Swim tunnel respirometer

Swim tunnel respirometry was performed to assess the energy cost of the fish's handling of EE2 exposure, the parasite infection and their combination. Two days before the respiroemtric measurements, trout were no longer fed (Lauff and Wood, 1996). A swim tunnel respirometer (5 L, SW10050, Loligo Systems) and the corresponding software (AutoResp™, Loligo Systems) were used. For more details regarding settings and calibration see supplement S2. The length, width, depth and weight of fish was measured. Afterwards, the fish were acclimated in the swim tunnel at a velocity of 10 cm/s (approximately 1 BL/s on average; Liew et al., 2013, Pane et al., 2004). Fish were considered to be acclimated when basal oxygen consumption levels were reached (about 150 mg O2 / kg / h, based on pilot-experiments).

Exhaustive swimming speed (U_{max}) was determined by increasing water velocity for 10 cm/s (Farrell 2008) every 40 min interval (ramp function) until the fish fatigued. Fatigue was considered as the time point when the fish was no longer able to swim against the water current and was swept downstream against the posterior grid, not able to move forward anymore (Claireaux et al., 2007, Plaut 2001). The water speed was adjusted *via* the software using a SEW motor control unit regulated by the DAQ-M device.

Oxygen consumption (MO_2) measurements were performed in parallel to the swimming performance. MO_2 was recorded using fiber optic oxygen instrument (Witrox 4 connecting oxygen dipping probe mini senor and temperature sensor to the computer). Intervals were set as follows: flush 290 s, wait 10 s, measure 900 s (=20 min intervals; intermittent respirometry).

The fractional error (solid blocking correction; Bell and Terhune 1970) was automatically incorporated in the calculations by the software. MO_2 was thereafter manually corrected for the background MO_2 value. Finally, the aerobic scope was calculated as the difference between MO_2 at U_{max} and at the lowest swimming speed ($= MO_{2,\ Umax} - MO_{2,\ Umin}$; and factorial aerobic scope $= MO_{2,Umax} / MO_{2,Umin}$; Clark et al., 2013, Pane et al., 2004).

2.11. Statistical data analysis

The Shapiro-Wilk test was used to test for normality followed by one-way analysis of variance (ANOVA). If the data was normally distributed, the Tukey-Kramer post hoc test was carried out; otherwise the Kruskal-Wallis in combination with the Dunn's multiple comparison test was used to reveal differences between the treatments. Significance was set at $p < 0.05$. The results of all statistical comparisons are shown in supplement S3. Data analysis and graphical representation was carried out using NCSS 10 Statistical Software (2016).

3. Results

For the results concerning the effects of EE2-exposure, parasite infection and their combination, we focus on the data from sampling day 108 (unless alternative sampling days are stated in the text). At this time point, the parasite intensity in non-exposed juvenile rainbow trout had reached a maximum level (Fig. 8a) and the difference in parasite intensity between non-exposed and EE2-exposed fish was greatest at day 108. At the same time, differences in immune gene expression were most pronounced, as exemplified for *IL-10* in supplement S4a, and the impact of EE2 on the reproductive system was strongest at day 108, too, indicated by the transcript levels of the estrogenic biomarker *vtg* (supplement S4b).

3.1. Juvenile rainbow trout growth and survival

Trout at day 0 were on average 11.91 (± 4.57) g in weight and 10.33 (± 1.49) cm in length. At sampling day 108 the juvenile rainbow trout were on average 39.72 (± 1.62) g in weight and 14.50 (± 0.20) cm in length, and there were no statistically significant differences between the treatment groups. Over the time course of the experiment, mortality was on average 6 (± 2.7) % with no statistically significant differences between the six treatment conditions.

3.2. EE2-exposure

During the time course of the experiment, the EE2 concentrations in the water of control aquaria (no EE2) remained below 0.2 ng/L – if detectable at all. In the aquaria dosed with EE2-contaning water, EE2 concentrations were 5.5 ng/L (nominal 10 ng/L) and 1.5 ng/L (nominal 3 ng/L).

3.3. Parasite infection

The parasite infection was accomplished between day 15 to day 18 in a controlled manner (1 h on four consecutive days each; see 2.5) with *T. bryosalmonae*. Infection prevalence, parasite intensity and host immune responses - all develop from the baseline of infection achieved during this initial 4 × 1 h-exposures. The exposure density for the groups being infected was on average 1.25E + 5 ± 38% parasite DNA copies per liter water. As shown in previous studies (Bailey et al., 2017a, Strepparava et al., 2018), a low parasite density together with a water temperature of < 15 °C does not result in disease-induced mortalities. In this way, we avoided that the fish immune response is confounded by differences between moribund and surviving individuals.

3.4. EE2, parasite and EE2 × parasite impact on trout reproductive parameters

The impact of the treatments (EE2, parasite infection, EE2 × parasite infection) on the reproductive system of the trout was assessed by means of gonad histology for sex determination and the mRNA levels of hepatic *vtg* and the four *ER*-isoforms.

The sex ratio, based on gonad histology, was about half / half for all treatments. Since the response of fish to toxicants as well as pathogens may differ between sexes (Koehler 2004, Le Manach et al., 2016, Oertelt-Prigione 2012, Winzer et al., 2002a, Winzer et al., 2002b), we analyzed the data for male and female fish separately. No obvious differences were found in the responses between the sexes. Therefore, all data displayed represents the combined results from both, males and females.

To confirm that the EE2-exposure effectively interacted with the reproductive system of the fish, we used the well-established biomarker vtg. After the initial two weeks of EE2-exposure, hepatic *vtg* mRNA transcript levels were moderately higher in fish exposed to 1.5 ng/L EE2 and significantly higher in fish exposed to 5.5 ng/L EE2, when compared to the control (day 14). During continuation of the experiment, the induction of hepatic *vtg* mRNA levels by EE2 was increased concentration- and time-dependently (supplement S4b). This indicates that the estrogenic conditioning of the fish was maintained over the entire experimental period. The highest *vtg* mRNA levels were reached at day 108. At this time point, hepatic *vtg* transcript levels were significantly higher in both 1.5 and 5.5 ng/L EE2-exposed fish compared to the controls at day 108 (Fig. 2). The parasite infection had no significant influence on *vtg* transcription.

In addition to hepatic *vtg*, the transcript levels of the four hepatic *ERs* were analyzed (Fig. 3). EE2-exposure significantly and concentration-dependently increased the transcription of hepatic *ERα1* and, even more pronounced, of hepatic *ERα2*. In contrast, the transcription of hepatic *ERβ2* was significantly decreased under EE2-exposure. No significant changes were detected for hepatic *ERβ1*. The parasite infection had no effect on the mRNA levels of the hepatic *ERs* isoforms; they, were neither significantly affected by the parasite infection alone nor did the parasite infection alter the response to EE2.

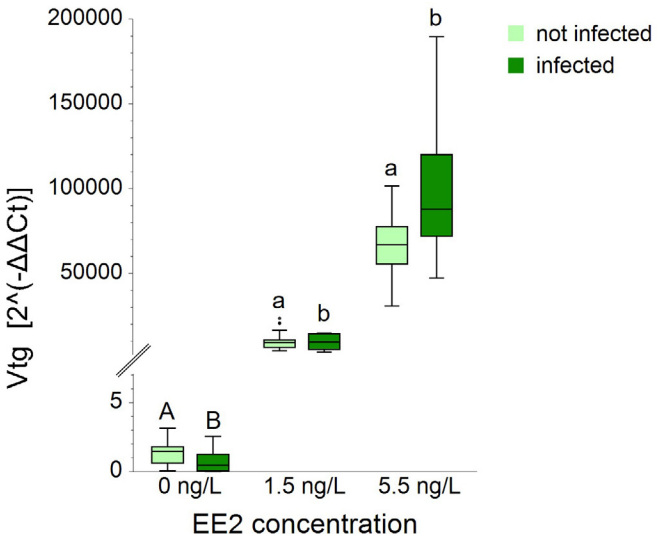

Fig. 2. Gene transcription of hepatic vitellogenin (vtg) at day 108. The mRNA levels of *vtg* in the liver are displayed as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$. Statistically significant differences ("A" to "a" and "B" to "b", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs*. 1.5 ng/L and 0 ng/L *vs*. 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs*. infected control (0 ng/L; to account for parasite-caused effect).

3.5. EE2, parasite and EE2 × parasite impact on the trout immune system

The treatment impact on the immune system of experimental trout was assessed at the molecular level by analyzing the mRNA levels of the four *ER*-isoforms (HK), the *GPER* (HK and liver), three Th1- and three Th2-like immune genes (HK) and of the hepatic *C3-3* and *hepcidin*.

In the HK, a key fish immune organ, the transcription of the four *ERs* was analyzed at day 108 of the experiment (Fig. 4). EE2-exposure alone significantly increased the transcription of *ERα2* in the HK of non-infected fish compared to the control. No significant EE2-caused effects were observed for the other *ERs* of non-infected, EE2-exposed fish. The parasite infection alone significantly increased the HK transcription of *ERα2* but significantly decreased the transcription of *ERβ1*. The combination of parasite infection and EE2 significantly decreased the transcription of *ERα1* and *ERα2* in the HK whereas it significantly increased for *ERβ1* and *ERβ2*, compared to the infected control.

In addition to the nuclear *ERs*, we analyzed the *GPER* in the HK (Fig. 5a) and liver (Fig. 5b). EE2-exposure alone significantly increased the transcription of *GPER* in the HK of non-infected trout compared to the control. The parasite infection, alone and in combination with EE2-expsoure, resulted in no significant changes in the HK. In the liver, EE2-exposure alone decreased the *GPER* mRNA level of non-infected fish compared to the control only slightly (not significant). Although the parasite infection alone had no significant effect on the transcript level, the EE2-exposure significantly decreased the *GPER* mRNA level in the liver of infected fish compared to the infected control.

In mammals, T-bet, TNFα and IFNγ are categorized as Th1-related cytokines, whereas GATA-3, IL-10 and IL-4/13A are part of the Th2 pathway. While it is not fully demonstrated that Th-like functionality exists in fish, many studies consider that these markers may have homologous physiological functions in fish to those they have in mammals (e.g. Yamaguchi et al., 2015). Consequently, in the frame of this article we talk of Th1-like and Th2-like immune genes. The immune genes were selected based on previous reports on the rainbow trout immune response to *T. bryosalmonae* infection (Bailey et al., 2017a, Bailey et al., 2017b, Gorgoglione et al., 2013, Wang et al., 2010). EE2-exposure alone had no effect on the mRNA transcript levels of the Th1-like genes (Fig. 6a–c). The parasite infection alone significantly increased the transcription of *TNFα* but had no significant effect on *T-bet* and *IFNγ*. In infected fish, the EE2-exposure resulted in significantly lower *TNFα* mRNA transcript levels than in the infected control – but had no significant effects on *T-bet* and *IFNγ*. Regarding the Th2-like genes (Fig. 6d–f), EE2-exposure had no significant effect on the transcription of the selected immune genes in the HK of both non-infected and infected fish compared to the related controls. The parasite infection alone significantly increased the transcription of all three genes (*GATA-3, IL-10* and *IL-4/13A*).

In the liver, we analyzed transcript levels of two immune factors being involved in general anti-parasite defense: the complement factor *C3-3* (Fig. 7a) and the acute phase protein, *hepcidin* (Fig. 7b). EE2-exposure alone significantly decreased *hepcidin* transcription in non-infected fish, while *C3-3* transcript levels were not altered compared to the control. The parasite infection alone significantly increased the transcription of hepatic *C3-3*, but not of *hepcidin*. The combined EE2 × parasite treatment resulted in a significantly decreased transcription of both *C3-3* and *hepcidin* compared to the infected control.

3.6. EE2 impact on the disease manifestation in T. bryosalmonae-infected trout

The exogenous EE2-exposure was initiated 14 days prior to the parasitic infection (see 2.2) to induce an estrogenic condition of the exposed fish. The development and manifestation of the parasite infection and disease in infected control and EE2-epxosed trout was evaluated by analyzing the parasite prevalence, parasite intensity and the degree of histopathological changes in the parasite target organ, the

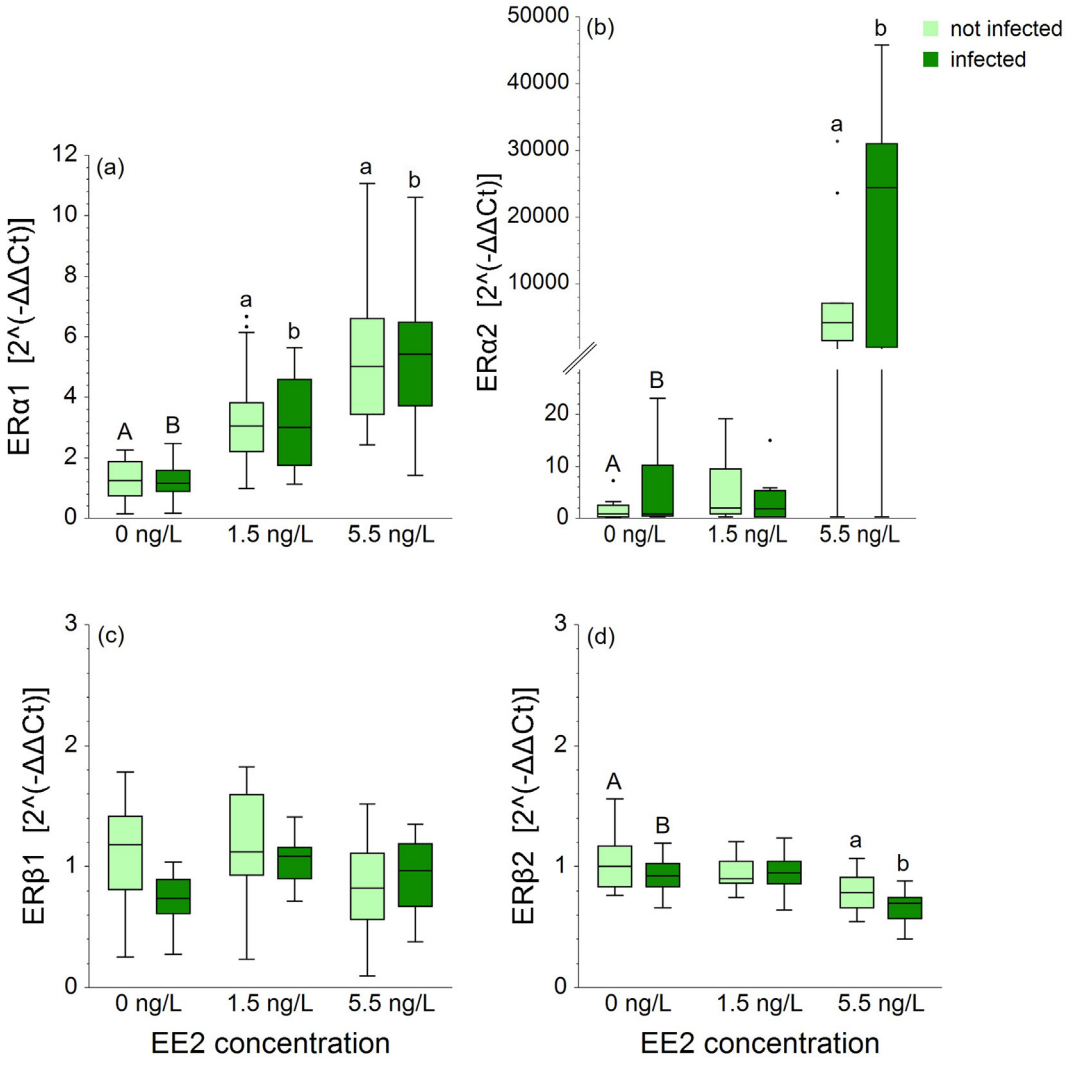

Fig. 3. Gene transcription of four hepatic estrogen receptor (ER) isoforms at day 108. The mRNA levels for *ERα1, ERα2, ERβ1, ERβ2* (a - d) in the liver are displayed as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$. Statistically significant differences ("A" to "a" and "B" to "b", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs.* 1.5 ng/L and 0 ng/L *vs.* 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs.* infected control (0 ng/L; to account for parasite-caused effect). The scale of axes was adjusted individually.

trunk kidney.

Non-infected trout were screened for the presence of *T. bryosalmonae* and found to be negative. All infected control fish were indeed infected with the parasite *T. bryosalmonae* (prevalence 100%). EE2-exposed fish showed a lower infection prevalence (87.5% and 93.75% for 1.5 and 5.5 ng/L EE2, respectively; day 108), which, however, did not significantly differ to the infected control group.

The parasite intensity in the trout TK was quantified as copy number of parasite DNA per gram tissue. The time course of parasite intensity is shown in Fig. 8a (no statistics). The parasite intensity was significantly lower in EE2-exposed fish compared to infected control fish at day 108 (Fig. 8b). We analyzed the correlations between the transcript levels of genes belonging to the immune and reproductive system *versus* the parasite intensity; the results are presented in supplement S5. For infected fish without EE2, significant negative correlations were detected for hepatic *ERα2* and *ERβ2* (HK), significant positive correlations for *vtg* and *C3-3*. No significant correlations were detected for infected fish exposed to 1.5 ng/L EE2. For infected trout exposed to 5.5 ng/L EE2, significant negative correlations were detected for *vtg*, hepatic *ERα1* and hepatic *ERα2*, TNF*α* and IFNγ.

To assess possible parasite-related lesions in the TK, a semi-quantitative histopathological scoring was performed (Fig. 8c). Infected trout exposed to EE2 displayed significantly less severe alterations than the infected control. The non-infected fish were all scored with zero.

3.7. Costs of EE2, parasite and EE2 × parasite treatment for the exposed fish trout

The energetic costs of the experimental treatments were evaluated by swim tunnel respirometry. EE2-exposure alone decreased the swim tunnel respirometry parameters by trend in non-infected fish compared to the control (Fig. 9, day 148). The parasite infection significantly lowered MO_2 when compared to the control; U_{max} and the aerobic scope were lower only by trend. In infected fish, EE2-exposure lowered all three parameters, being significantly decreased for U_{max} and MO_2 if compared to the non-infected control. No obvious differences between the absolute terms and the factorial aerobic scope were observed, consequently, only the absolute terms are graphically shown in the main text. For statistical evaluation, including the factorial aerobic scope, see supplement S3.

Hematocrit levels (day 158) of control fish were 43.09 ± 0.72 (mean ± Std. Error). The EE2-exposure significantly lowered the hematocrit levels to 40.95 ± 0.72 (5.5 ng/L EE2), whereas the parasite infection reduced the levels to 41.22 ± 0.96 in a non-significant

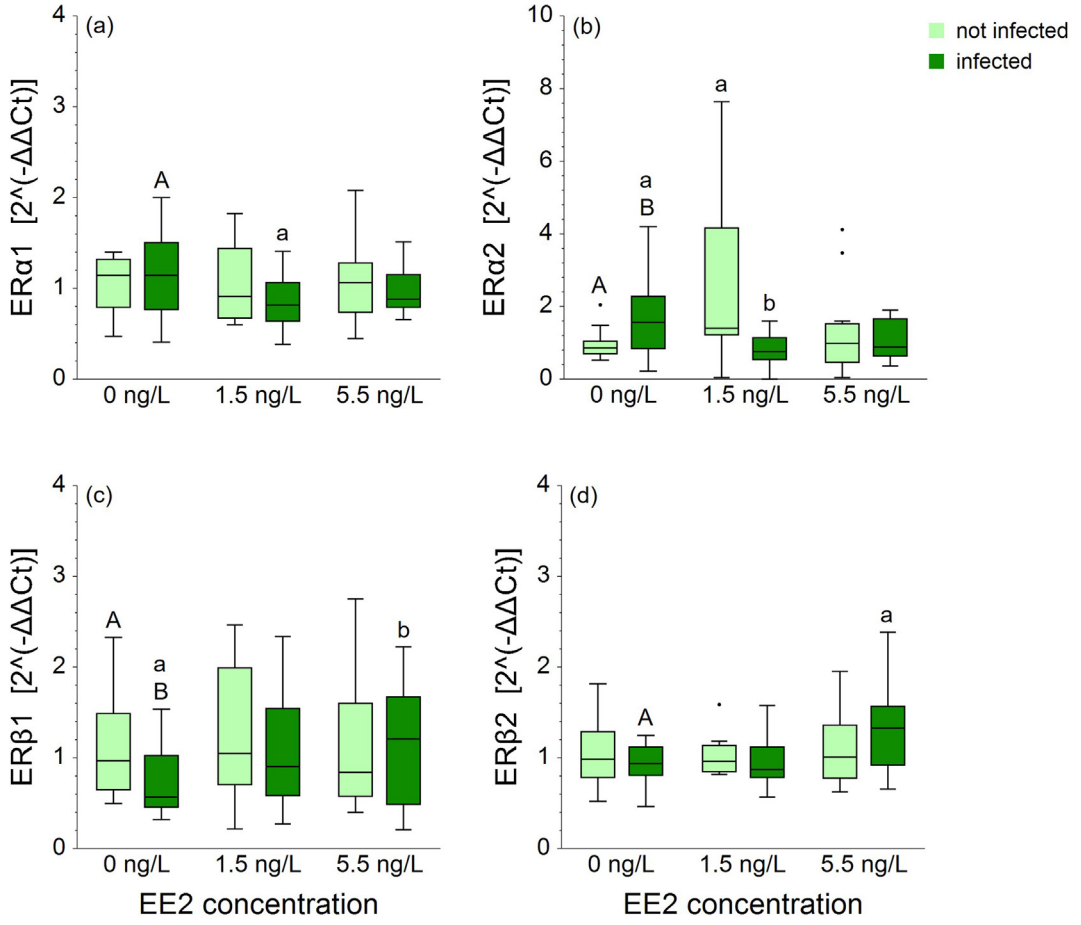

Fig. 4. Gene transcription of four estrogen receptor (ER) isoforms in the head kidney (HK) at day 108. The mRNA levels for *ERα1, ERα2, ERβ1, ERβ2* (a - d) in the HK are displayed as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$. Statistically significant differences ("A" to "a" and "B" to "b", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs*. 1.5 ng/L and 0 ng/L *vs*. 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs*. infected control (0 ng/L; to account for parasite-caused effect). The scale of axes was adjusted individually.

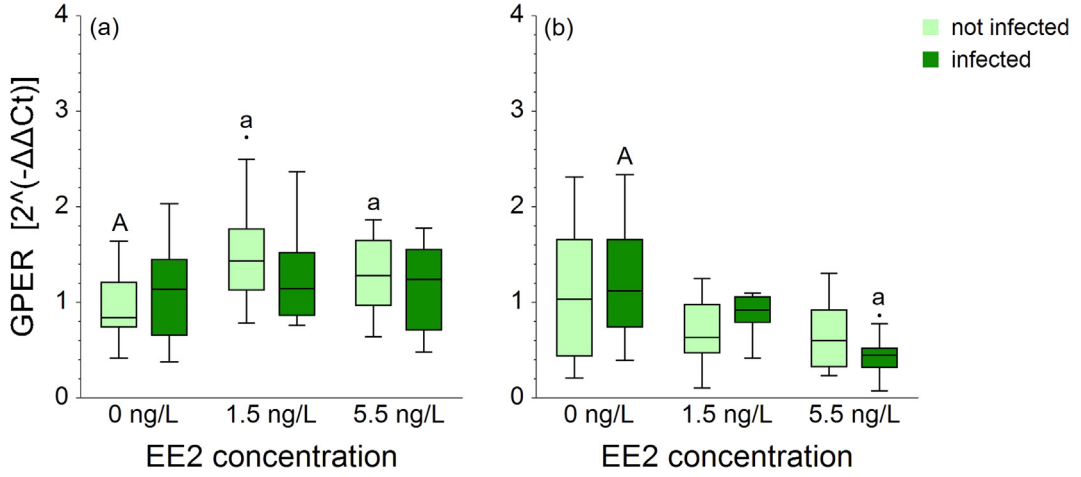

Fig. 5. Gene transcription of the G-protein coupled estrogen receptor (GPER). The mRNA levels of the GPER are displayed for the head kidney (HK, a) and the liver (b) at day 108 as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$. Statistically significant differences ("A" to "a", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs*. 1.5 ng/L and 0 ng/L *vs*. 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs*. infected control (0 ng/L; to account for parasite-caused effect).

manner. For infected trout exposed to 5.5 ng/L EE2 the hematocrit level was 43.17 ± 0.69.

4. Discussion

We investigated whether exposure to EE2, a prototypic estrogenic EDC, alters simultaneously selected parameters of the reproductive and immune system – including the response to a parasite infection – of

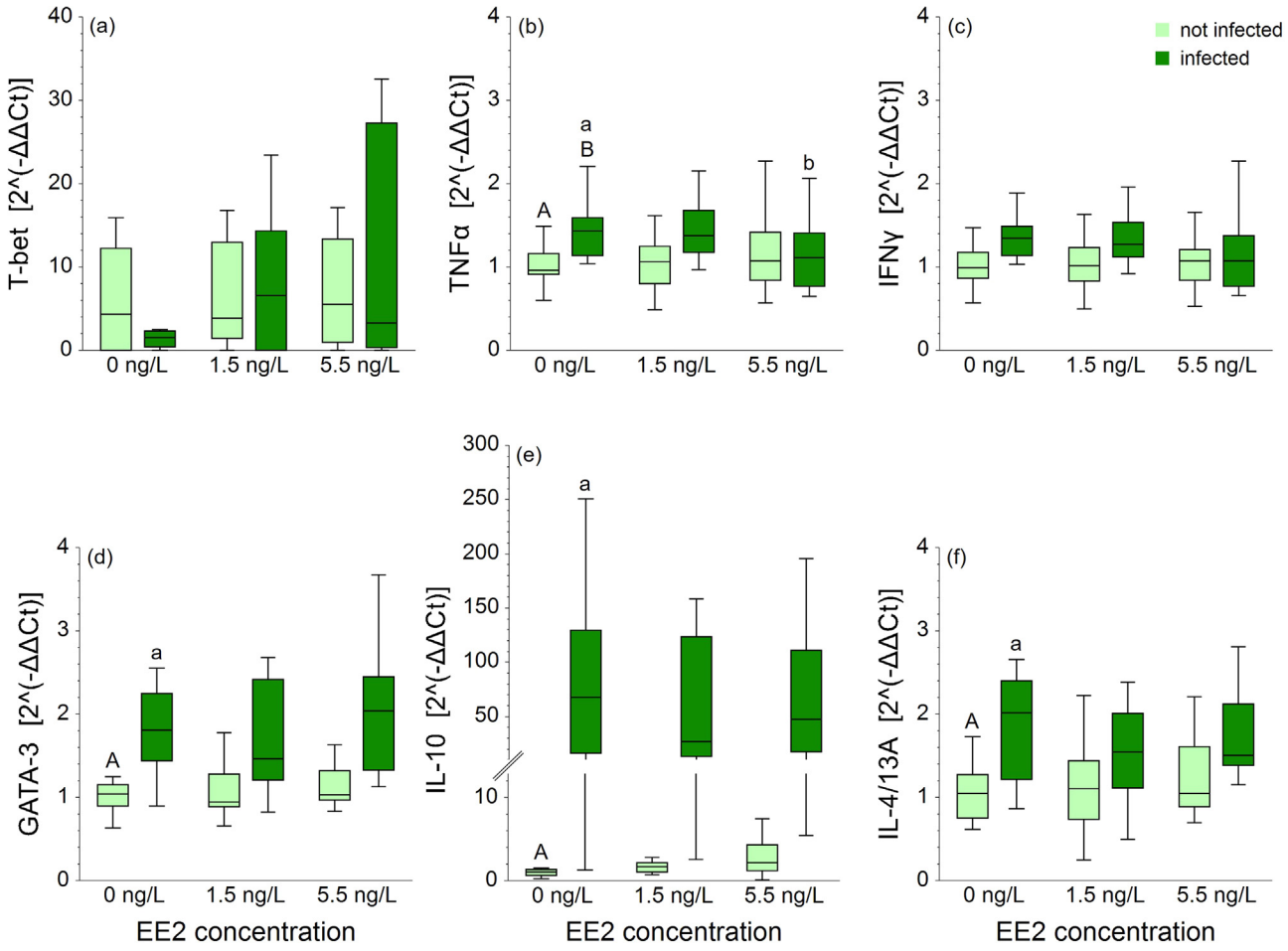

Fig. 6. Gene transcription of Th1- and Th2-like immune genes in the head kidney (HK) at day 108. The mRNA levels in the HK are displayed as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$ for *T box transcription factor* (*T-bet*, a), *Tumor necrosis factor alpha* (*TNFα*, b), *Interferon gamma* (*IFNγ*, c) as well as *Trans-acting T-cell-specific transcription factor* (*GATA-3*, d), *Interleukin 10* (*IL-10*, e) and *Interleukin 4/13A* (*IL-4/13A*, f). Statistically significant differences ("A" to "a" and "B" to "b", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs*. 1.5 ng/L and 0 ng/L *vs*. 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs*. infected control (0 ng/L; to account for parasite-caused effect). The scale of axis was adjusted individually.

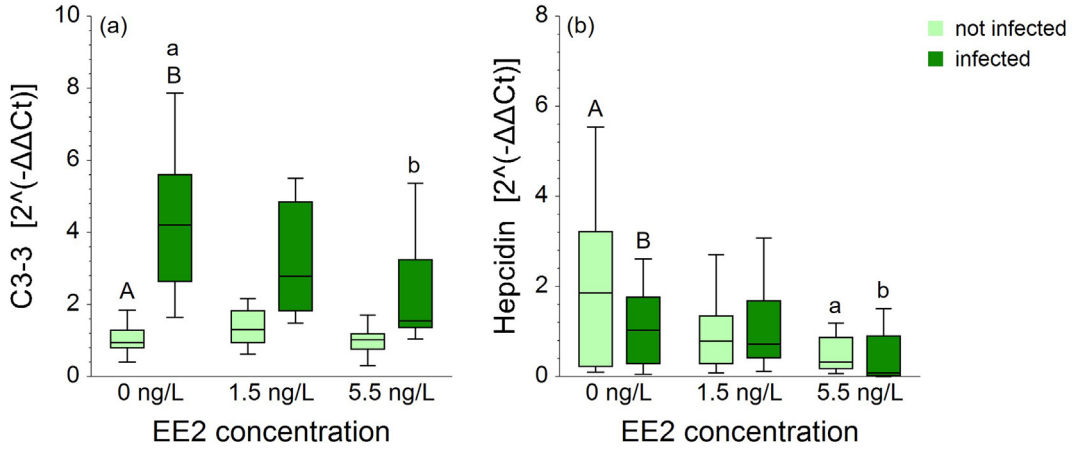

Fig. 7. Gene transcription of hepatic complement component 3, isoform 3 (C3-3) and hepcidin. The mRNA levels in the liver at day 108 are displayed as fold change of the control, expressed as $2^{-\Delta\Delta Ct}$ for *C3-3* (a) and *hepcidin* (b). Statistically significant differences ("A" to "a" and "B" to "b", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs*. 1.5 ng/L and 0 ng/L *vs*. 5.5 ng/L ethinylestradiol (EE2) for both, not infected and infected fish (to account for the EE2-caused effect), and additionally for the not infected *vs*. infected control (0 ng/L; to account for parasite-caused effect). The scale of axes was adjusted individually.

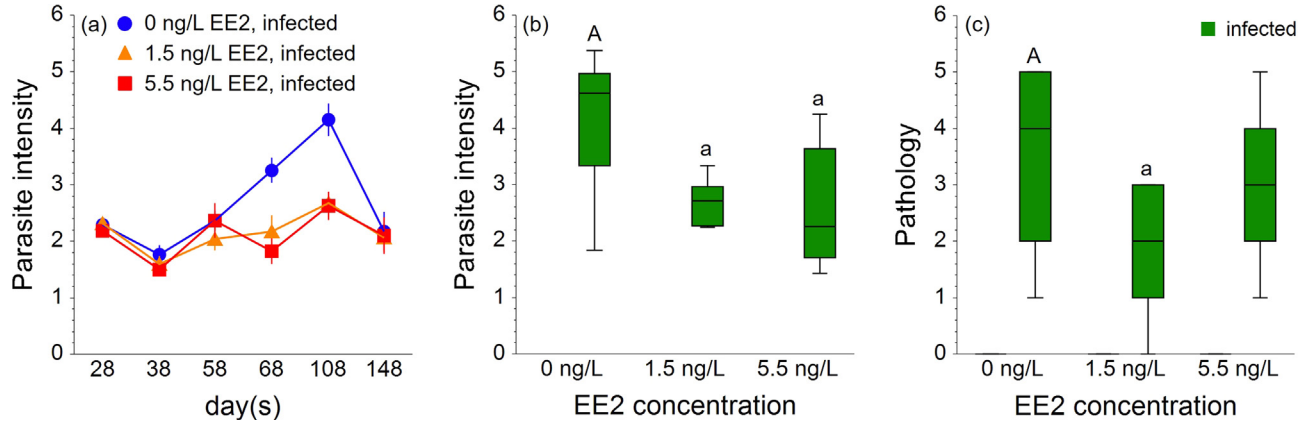

Fig. 8. Parasite intensity and histopathological evaluation. The results of infected fish at day 108 are displayed as log-copy number of parasite DNA per gram tissue for the parasite intensity (a – mean +/- SE, b), and based on a scoring for the parasite pathology (c). Statistically significant differences ("A" to "a", $p < 0.05$) are indicated for the following treatment comparisons: 0 ng/L *vs.* 1.5 ng/L and 0 ng/L *vs.* 5.5 ng/L ethinylestradiol (EE2) for infected fish (b, c).

juvenile rainbow trout. It is well established that low concentrations of EE2, as they typically occur in aquatic environments, are able to disrupt sexual development and reproductive performance of fish, at least under long-term exposure (e.g. Länge et al., 2001, Schäfers et al., 2007) and that this can translate into a population collapse (Kidd et al., 2007, Palace et al., 2002). The disruptive mechanism caused by EEDCs on the reproductive system was successfully implemented in the adverse outcome pathway (AOP) framework of Ankley et al. (2010). Beyond the adverse impact of EEDCs on the reproductive system of fish, growing evidence suggests that these chemicals also interfere with the fish immune system (e.g. Cabas et al., 2011, Milla et al., 2011, Seemann et al., 2016, Segner et al., 2013, Shelley et al., 2013, Ye et al., 2018) and modulate pathogen resistance (Krasnov et al., 2015, Wang and Belosevic 1994, Wenger et al., 2011). The question not fully answered yet is, whether the immunodisruptive actions of EEDCs are effective at low concentrations that disrupt sexual development and reproduction of fish, or whether the immunodisruptive effects are expressed only at comparatively high concentrations, which might not be relevant for most environmental situations. Thus, the present study aimed to compare the effect concentrations for the reproductive and immune effects of EE2. To this end we performed a long-term exposure (148 days) to low concentrations of EE2 (1.5 or 5.5 ng/L). Such concentrations have been detected in aquatic habitats and were in the sub-lethal range, as for rainbow trout a LC50 of 1.6 mg/L EE2 was reported (Schweinfurth et al., 1997). As the response of immune parameters can differ between the resting and the activated immune system, we examined the EE2 impact on immune parameters for both, the resting immune system of not infected fish and for the activated immune system of parasite-infected fish. Besides, the internal homeostasis of exposed organisms can be disrupt by environmental stressors, what may result in reductions of performance and fitness (Schulte 2014). To consider such aspects, we examined not only the mRNA levels of selected genes but also prevalence and intensity of the parasite infection, and we performed swim tunnel respirometry measurements.

4.1. Do low concentrations of EE2 impact reproductive system parameters of juvenile rainbow trout?

The relatively low and environmentally occurring concentrations of EE2 (1.5 and 5.5 ng/L) selected for our experiment indeed affected reproductive parameters of the juvenile rainbow trout. We assessed the mRNA transcript levels of the yolk-protein *vtg*, which is sensitive to EEDCs and serves as biomarker for the detection of estrogenic contamination (Garcia-Reyero et al., 2004, Hutchinson et al., 2006, Kime et al., 1999, Sumpter and Jobling 1995). Only limited amounts of hepatic *vtg* mRNA are produced by juvenile fish under natural conditions, as confirmed in the present study. The EE2-exposure up-regulated the *vtg* mRNA levels time- and concentration-dependently, thus, our findings are in agreement with previously published data on *vtg* induction in E2- / EE2-exposed rainbow trout (e.g. Boyce-Derricott et al., 2009, Casanova-Nakayama et al., 2018, Hook et al., 2006, Van den Belt et al., 2003).

As a second indicator for the EE2-caused effects on the reproductive system, we analyzed the mRNA level of the hepatic *ERs*. In trout, four

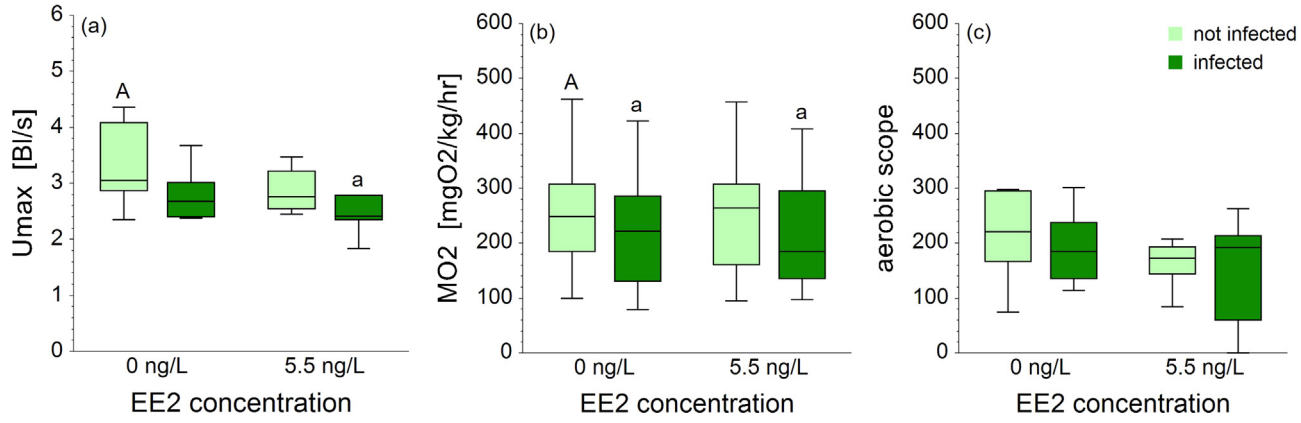

Fig. 9. Results of the respiratory swim tunnel measurements. Results are shown for the exhaustive swimming speed (U_{max}, a), the oxygen consumption (MO_2, b) and the aerobic scope (c) at day 148. Statistically significant differences ("A" to "a", $p < 0.05$) are indicated for comparing the control (0 ng/L ethinylestradiol (EE2), not infected) to the other, displayed treatments. The scale of axes was adjusted individually.

receptor isoforms are described: *ERα1, ERα2, ERβ1, ERβ2* (Hawkins et al., 2000, Nagler et al., 2007). *ERα1*, but even more so *ERα2* transcription was strongly induced by EE2, whereas *ERβ2* transcription was suppressed. Casanova-Nakayama et al. (2018) observed similar effects in 6-month old female rainbow trout fed 5 days with E2-containing food. Wenger et al. (2014) observed, as well, similar effects after juvenile rainbow trout received E2-enriched food over 5 weeks. Boyce-Derricott et al. (2009) found significant up-regulation of *ERα1* but not of *ERα2* in male rainbow trout injected intra-peritoneal with E2 or EE2. Overall, our findings are well in agreement with the known disruptive effect of environmental concentrations of EE2 on the reproductive system of trout. Interestingly, parasite intensity showed a negative correlation with the transcript levels of the reproductive parameters. This may point to trade-offs between the immune and the reproductive system, i.e. at increased infection intensity the fish downregulate reproductive parameters in order to allocate resources to the immune system. This effect is more pronounced in EE2-exposed fish with their intensified *vtg* synthesis than in control fish producing only limited amounts of *vtg*. The interpretation is in line with the findings of Wernicke von Siebenthal et al. (2018) of negative correlation between vitellogenesis and immune parameters (immune organ indices and immune gene expression) in juvenile rainbow trout.

4.2. Do low concentrations of EE2 impact immune system parameters of juvenile rainbow trout?

In a first step, we analyzed the response of the *ERs* transcript levels in the head kidney (HK). The evaluation of *ERs* was conducted because estrogenic actions in a target organ are mediated through the respective receptors and the HK represents an important fish immune organ. There are two key findings: firstly, in control fish EE2-exposure resulted in a significantly increased transcription of *ERα2* but remained without effect on the other isoforms. This observation agrees partly with the findings of Shelley et al. (2013) who observed an E2-induced up-regulation of *ERα1* and *ERα2* in HK leukocytes of rainbow trout and with the findings of Rastgar et al. (2019) on an estrogen-induced increased expression of *ERα* in isolated goldfish leukocytes. The result is, however, in contrast to Casanova-Nakayama et al. (2018) who reported no modulation of the *ERs* mRNA level in the HK of rainbow trout after they were fed 5 days with E2-containing food. Secondly, in parasite-infected fish EE2 caused a significant decrease of the HK transcript levels of both *ERα*-isoforms and a significant increase of both *ERβ*-isoforms. Interestingly, the EE2 treatment led to opposite effects on the *GPER* in HK and liver: while *GPER* mRNA levels increased in the HK, they decreased in the liver. Again, an influence of the infection status was indicated. *GPER* mRNA levels in the HK showed a significant increase under EE2 in the non-infected fish but not in infected fish and hepatic *GPER* transcripts lack a significant effect in non-infected fish but showed a significant decrease under EE2-exposure in infected fish. The detected EE2-induced changes in *GPER* mRNA levels supports the suggested involvement of the GPER in immune reactions of fish (Cabas et al., 2018). In previous studies the GPER expression and modulation of this signaling pathway in immune tissue and immune cells was demonstrated for gilthead seabream (*Sparus aurata*) and common carp (*Cyprinus carpio*; Cabas et al., 2013, Szwejser et al., 2017). The human GPER (and its correlated estrogen-signaling pathway) is considered to influence physiological functions including those of the immune system (Prossnitz and Barton 2011). In addition, the estrogen binding and signaling of the GPER seems to be conserved between mammals and fish – what might be indicative for substantial function(s) of the immune organ-GPER in those organisms (Thomas et al., 2010).

In a next step, we examined the response of hepatic immune genes involved in the defense against infectious agents in general. The two hepatic immune genes were the anti-microbial *hepcidin* (Alvarez et al., 2014) and the complement factor *C3-3*. Previous studies with rainbow trout reported regulation of the *hepcidin* gene expression under EE2- and estrone-exposure (e.g. Massart et al., 2014, Osachoff et al., 2016, Robertson et al., 2009). This agrees with the findings of the present study: *hepcidin* was not responsive to the parasite infection, however, it was responsive to EE2. The complement system component C3 is an essential part of the teleost innate immune defense against pathogens including parasites (Holland and Lambris 2002). For instance, Hook et al. (2006) and Massart et al. (2014), showed alterations of *C3* mRNA levels after EE2-exposure in rainbow trout. In the present study, EE2 alone had no significant effect on the transcription of *C3-3* but the parasite-triggered induction of *C3-3* was EE2-concentration-dependently inhibited. This is in line with Wenger et al. (2011) who observed that E2 treatment did not affect basal *C3-3* expression in rainbow trout liver but suppressed its upregulation after bacterial infection. In line with this finding, the authors observed higher mortality for E2-exposed, infected trout. Taken together, these findings point to crosstalk between estrogens and infectious agents in regulating genes which are involved in the immune reaction against invading pathogens – although the underlying mechanisms remains unclear. An interesting hint in this context might be the finding of Szwejser et al. (2017) that physiological stress alters the expression of *ERs* in HK phagocytes of carp, *Cyprinus carpio*, and that this may be relevant for leukocyte differentiation, maturation and migration, i.e. the estrogen effects may be effected through alterations in leukocyte proliferation rather than through the functional modulation of the already existing leukocytes.

Finally, we analyzed HK immune genes that are involved in the immune response against *T. bryosalmonae*. The immune response of rainbow trout to *T. bryosalmonae* involves an imbalance in the Th-like pathways in that there is not a mode of complete Th1- or Th2-like polarization (Bailey et al., 2017a, Gorgoglione et al., 2013, Wang et al., 2010). Therefore, we focused on genes related to the Th1- and Th2-like pathways. A further motivation to study Th-like genes was that from mammalian studies there exists evidence that they are sensitive to estrogens (Javadian et al., 2014, Lambert et al., 2005, Salem 2004). Our results confirm the responsiveness particularly of the Th2-like cytokines to *T. bryosalmonae* (here: *GATA-3*, *IL-10* and *IL-4/13A*), but we found almost no indication of an estrogen modulation of these genes. Only the Th1-like cytokine *TNFα* was significantly inhibited by EE2 in infected fish.

Taken together, our findings suggest that the low EE2 concentrations used in this study did not only affect parameters of the reproductive system (see 4.1) but at the same time they affected parameters of the resting and activated immune system of juvenile rainbow trout. These findings are in line with the recent report of Ye et al. (2018) that exposure of the marine medaka (*Oryzias melastigma*) to 33 and 113 ng/L EE2 concurrently disrupted the reproductive and the immune system.

4.3. How do low EE2 concentrations impact the severity of parasite infection in juvenile rainbow trout?

Manifestation of disease at the organisms' level is not a simple linear function of any changes in molecular or cellular immune processes, but depends on further physiological processes (for instance, the competition for resources). Therefore, in the present study we evaluated, in addition to the changes at molecular level, whether the EE2-exposure modified the severity of the parasite infection by measuring the parasite prevalence, parasite intensity and histopathology.

The disease metrics pointed to a reduced manifestation of the parasite infection in EE2-exposed trout. This is in contrast with most other studies on chemical-induced immunotoxic effects in fish, which reported rather an increase in pathogen infection and associated mortality. The question is why EE2-treated fish show reduced disease metrics compared to infected fish that were not exposed to EE2. A possible explanation could be a control over the infection-associated immune pathology in EE2-exposed fish. Transcript levels of the Th1- and Th2-like immune genes that are directly involved in the defense against *T.*

bryosalmonae were not affected by EE2. However, EE2-caused effects on the immune capacity of trout is supported by data from mammals, for which immunoenhancing effects of estrogens are well documented (e.g. Bouman et al., 2005, Kovats 2015, Sakiani et al., 2013). They have been discussed as one mechanism contributing to the generally higher resistance of females to parasite infections (Klein, 2004, Klein and Flanagan, 2016, Nava-Castro et al., 2012). The estrogen actions in the human immune system appear to be concentration-dependent, with low concentrations tend to be immunoenhancing while high concentrations are immunosuppressive (Straub, 2007). These dichotomic actions are of biological importance, for instance during pregnancy when the suppressive actions of high estrogen levels prevent the immunological rejection of the fetus (Beagley and Gockel, 2003, Schaefer et al., 2005, Straub, 2007). An important facet of the immunoenhancing activity of estrogens in humans is that they attenuate pro-inflammatory responses and stimulate inflammation resolution processes, thereby limiting inflammation-induced pathologies (e.g. Nadkarni and McArthur, 2013). The finding of our study that transcript levels of *hepcidin* and *C3* were suppressed by EE2 may point to a similar attenuating effect of low estrogen concentrations on inflammatory reactions in fish. In line with this are the significant negative correlations of parasite intensity with the transcript levels of the pro-inflammatory cytokines, *TNFα* and *IFN*γ in infected fish (supplement S5). It seems that the increase in the transcription of *TNFα* and *IFN*γ under parasite infection is attenuated in the presence of EE2, what may explain why the TK immunopathology of infected fish is significantly reduced under EE2 exposure. The altered response of the inflammasome may underlie significantly reduced parasite intensity and reduced parasite prevalence (although not significant) of EE2-exposed infected trout compared to infected control fish. Together, our findings point to an attenuating effect of EE2 on the inflammatory response of trout to the parasite infection. For mammals, it is well established that estrogens can reduce the inflammatory response to infections and the associated immunopathology (e.g. Straub, 2007). Further support to an attenuating effect of EE2 on the trout immune response comes from a transcriptomic analysis reporting an upregulation of inflammation resolution processes in the TK of *T. bryosalmonae*-infected, EE2-exposed rainbow trout (Bailey et al., 2019). However, to reveal the exact mechanism(s) through which EE2 reduced the parasite intensity in EE2-exposed fish, further studies are required. Our observation that EE2-exposed trout displayed milder parasite infection than non-exposed fish is supported further by additional studies on combined estrogen-parasite treatments of fish, though. Burki et al. (2013) described a trend of decreased mortality in PKD-positive trout when exposed to E2. Although this trend was not significant, the E2 exposure was associated with significant changes of the immune response at the transcriptome level. Krasnov et al. (2015) found increased resistance of Atlantic salmon, *Salmo salar,* towards the salmon louse (*Lepeophtheirus salmonis*) when treated with E2. However, there are also contradictory findings. For instance, Wang and Belosevic (1994) found that E2-treated goldfish were more susceptible to *Trypansoma danilwesky*. From mammals, it is known that the immunomodulatory effects of estrogens depend on their concentration, with high estrogen levels tending to be immunosuppressive and low levels having an enhancing effect (Straub, 2007). Possibly this duality applies for fish as well.

As an alternative to effects of EE2 on the host, chemical-induced alterations in the pathogen may be considered as well. At least some parasites are known to be affected by sexual steroids (such as E2) and some are known to express human ER-like proteins (Escobedo et al., 2005, Ibarra-Coronado et al., 2011, Snider et al., 2009). Estrogens can modulate the intracellular calcium metabolism of parasites what may affect their pathogenicity (Zhang et al., 2018). Estrogens typically induce mitogenic, proliferative effects, and in fact, there are indications that estrogens can promote parasite proliferation in hosts, including fish (Macnab et al., 2016, Romano et al., 2015). Nevertheless, such a proliferative effect of the EE2-exposure would not be in line with the observed reduction of parasite intensity in EE2-exposed fish of the present study.

4.4. Do low EE2 concentrations impact energy metabolism-related parameters in juvenile rainbow trout?

The results of the swim tunnel respirometry provide an additional perspective on the organismic outcome of the EE2 × parasite combination. The value of respirometry measurements in ecotoxicological studies comes from the fact that they provide information on the organism-level about the cost of pollutant exposure and they can provide a link to the population-level consequences (e.g. Calow and Sibly, 1990, Handy and Depledge, 1999, Wilson et al., 1994).

The data of the present study on exhaustive swimming speed U_{max}, oxygen consumption MO_2 and aerobic scope showed that (i) EE2-exposure alone led to a decrease, which was not significant, (ii) parasite infection significantly lowered MO_2, while U_{max} and aerobic scope were lower only by trend, and (iii) the combined EE2 × parasite treatment significantly lowered U_{max} and the MO_2. Osachoff et al. (2014) measured lower burst-swimming speed for EE2-exposed (0.5 and 1 μg/L) rainbow trout. Oxygen-carrying capacity ([erythrocytes]) and osmoregulatory-related variable ([Cl −]) were considered as possible underlying mechanisms. Estrogenic compounds are known to alter mitochondrial function and energy metabolism on the gene expression level of fish (Hook et al., 2007, Osachoff et al., 2014, Santos et al., 2007, Williams et al., 2007). Our findings of a significantly reduced hematocrit level in EE2-exposed fish compared to control fish would be in agreement with the suggestion of Osachoff et al. (2014) that the reduced aerobic performance of E2-exposed trout is due to lowered oxygen transport capacity of the blood. Concerning PKD, Bruneaux et al. (2017) described a negative correlation between PKD disease severity and the aerobic scope of brown trout. In our study we found only a trend but no significant effect of the parasite on the aerobic scope, but a significant effect on MO_2. This again is in line with the reduced hematocrit values (although not significant) of this treatment group. In contrast, we observed no relation between the significant reduction of U_{max} and MO_2 in fish exposed to both stressors (EE2-exposure and parasite infection) and lowered hematocrit levels. The relation between swimming performance and hematocrit levels appear to be complex. While some studies find a positive correlation between reduced swimming performance and reduced hematocrit levels (e.g. Jones, 1971), other studies found no such correlation (e.g. Gallaugher et al., 1992). Moreover, the findings concerning the PKD effects on swimming performance are not consistent. For instance, Foott et al. (2005) found no PKD effect on the time to exhaustion in a swimming challenge, whereas Bruneaux et al. (2017) observed reduced maximum metabolic rates in *T. bryosalmonae* infected fish. It is likely that differences in infection intensity and disease severity are at least partly responsible for these controversial findings. The rainbow trout of the present study, despite the reduction in the respirometry measurements, obviously was still able to maintain an effective immune response against the parasite infection (lower parasite intensity in EE2-exposed fish), probably because the defense against the invading pathogen is prioritized over other physiological functions (Wernicke von Siebenthal et al., 2018). In fact, functions other than the immune responses can be negatively affected by a reduced aerobic scope in PKD-positive fish. This is indicated from Bruneaux et al. (2017) who found a negative correlation between PKD severity and the aerobic scope and this in turn correlated with a reduced upper thermal limit.

5. Conclusions

The findings from the present study provide strong evidence that, the reproductive and immune system of juvenile rainbow trout are sensitive to similar EE2 concentrations. Although further substantiation is needed, our results suggest that EE2 has comparably low effect

thresholds for reproductive and immune hazards. Our results highlight a well-known but often neglected fact, i.e. that one and the same stressor can influence different target systems (physiologically as well as ecologically). Finally, the findings emphasize, how important it is to consider stressor interactions and trade-offs between stressor responses (Segner et al., 2014, Wernicke von Siebenthal et al., 2018). It is a matter of fact that organisms in their environment are rarely exposed to single stressors but are usually exposed to multiple stressors. While to date, the majority of environmental studies tended to examine one environmental stressor at a time, in many cases it might be the cumulative impact of stressor combinations that is responsible for an observed ecological outcome (e.g., Burkhardt-Holm et al., 2005, Crain et al., 2008, Rohr et al., 2008). Importantly, stressor interactions may be not just additive, but may lead to unexpected and surprising outcomes (Christensen et al., 2006), as exemplified also in the present study by the attenuating effect of the EE2-exposure on the fish parasite infection.

CRediT authorship contribution statement

Kristina Rehberger: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Elena Wernicke von Siebenthal:** Project administration, Writing - review & editing, Data curation, Investigation, Methodology, Conceptualization. **Christyn Bailey:** Data curation, Writing - review & editing. **Patrick Bregy:** Data curation. **Melanie Fasel:** Writing - review & editing, Data curation. **Elio L. Herzog:** Data curation, Writing - review & editing. **Silvia Neumann:** Data curation. **Heike Schmidt-Posthaus:** Writing - review & editing, Data curation. **Helmut Segner:** Funding acquisition, Project administration, Supervision, Writing - review & editing, Resources, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105836.

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