1  **Sex-specific changes in gene expression and delayed sex differentiation in response to**
2  **estrogen pollution in grayling (Salmonidae)**
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26  **Abstract**
27  The synthetic 17α-ethinylestradiol (EE2) is an estrogenic compound of oral contraceptives
28  and therefore a common pollutant that has been suspected to affect the demography of river-
29  dwelling salmonids. We study a population of European grayling (*Thymallus thymallus*) that
30  suffers from sex ratio distortions. Here we test how ecologically relevant concentrations of
31  EE2 affect sex-specific gene expression around early stages of sex differentiation. We
32  collected gametes from F1s of wild spawners, used them for *in vitro* fertilizations, and raised
33  the resulting embryos singly under experimentally controlled conditions. Embryos were either
34  exposed to 1ng/L EE2 or sham-exposed. RNA was collected from samples taken 10 days
35  before hatching, at the day of hatching, and towards the end of the yolk-sac stage, to study
36  gene expression and relate it to genetic sex (sdY genotype). We found that EE2 affects gene
37  expression of a very large number of genes especially at the day of hatching. The effects of
38  EE2 on gene expression is strongly sex-specific. At the day of hatching, EE2 affected about
39  twice as many genes in females than in males, and towards the end of the yolk-sac larval
40  stage, EE2 effects were nearly exclusively observed in females. Among the many effects was,
41  for example, a surprising EE2-induced molecular masculinization in the females' heads.
42  Histological examination of gonadal development of EE2-treated or sham-exposed juveniles
43  during the first 4.5 months after hatching revealed a delaying effect of EE2 on sex
44  differentiation. Because grayling sex determination goes through an all-male stage (a rare
45  case of undifferentiated gonochorism), the rate of EE2-induced sex reversal could not be
46  unequivocally determined during the observational period. However, two EE2-treated genetic
47  males had ovarian tissues at the end of the study. We conclude that common levels of EE2
48  pollution affect grayling from very early stages on by interfering with male and female gene
49  expression around the onset of sex differentiation, by delaying sex differentiation, and by
50  feminizing some males.
51

**Author contribution**

MRR and CW initiated the project. OS, DM, LW, LMC, and CW sampled the adult fish, did the experimental *in vitro* fertilizations, and prepared the embryos for experimental rearing in the laboratory. All further manipulations on the embryos and the larvae were done by OS, DM, LW, and LMC. The RNA-seq data were analyzed by OS, JR, and MRR, the histological analyses were done by DM, supervised by SK, the molecular genetic sexing was performed by OS and DM, and EV supervised the EE2 analytics. OS and CW performed the remaining statistical analyses and wrote the first version of the manuscript that was then critically revised by all other authors.

## 1. Introduction

Endocrine-disrupting chemicals are common pollutants that typically enter the environment after wastewater treatment. One of the most potent of these micropollutants is the synthetic 17-alpha-ethinylestradiol (EE2) that is used in oral contraceptives and hormone replacement therapies, and that is more stable and persistent than the natural estrogen it mimics [1]. EE2 concentrations of 1ng/L and higher have been found in river or lake surface waters [2] in lake sediments [3], and even in groundwater [4].

Exposure to 1 or few ng/L EE2 can be damaging to fish at various developmental stages. Embryos and early larvae can suffer from increased mortality, reduced growth, or malformations when exposed to EE2 [5, 6]. In juveniles and adults, exposure to EE2 can affect the response to infection [7], increase the susceptibility to other micropollutants [8], generally reduces growth and fertility [8, 9], and can even induce transgenerational effects on behavior and fertility in F1 [10] and F2 progeny [11]. Studies with experimental populations kept in 1,100 L ponds revealed population declines at concentrations of 1ng/L EE2 [12], and long-term, whole-lake experiments revealed significant ecosystem changes after experimental addition of 5-6 ng/L EE2: local populations of small fish declined (one species nearly got extinct), average body conditions of other fish, including top predators, declined significantly, and the prevalence of some zooplankton and insect species seemed to increase [13].

Experimental exposure to EE2, for example of juvenile sticklebacks (*Gasterosteus aculeatus*) to 35-40 ng/L, or of juvenile coho salmon (*Oncorhynchus kisutch*) to 2 or 10 ng/L, are associated with significant down- and up-regulations of various physiological pathways [14, 15]. Some of these effects on gene expression may be linked to the toxic effects of EE2 observed in juveniles and adults. However, it is likely that EE2 effects on gene expression depend on life history and on the developmental stage of an individual, i.e. on the timing of some physiological pathways in the organism. One important physiological pathway in this context is sex determination and gonad formation.

Sex determination is probably best seen as a threshold trait, with processes that occur early in development determining later processes [16]. In amphibians and fishes, these early processes can be very labile, i.e. potentially modifiable by external factors, even if they often have a clear genetic basis [17, 18]. Among these external factors that interfere with these early steps of sex determination are temperature or endocrine disrupting chemicals such as EE2 [17, 19]. When applied at the beginning of sex determination, they can tip the balance and cause a sex reversal, i.e. a mismatch between genotypic and phenotypic sex. Such EE2-induced sex reversals are sometimes but not always observed. If they are observed, the timing of exposure seems more relevant than the concentration: Orn et al. [20] found, for example, nearly complete sex reversal in zebrafish exposure to 5 ng EE2/L during embryo and larval stages, while exposure to 5-20 ng EE2/L at later stages did not seem to induce sex reversal in the same species [21].

Apart from gonad development, there can be many other fundamental differences between male and female development. Males and females may differ, for example, in average growth, timing of maturation, habitat use, or susceptibility to various stressors

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103     including infections [22]. It is therefore possible that effects of EE2 on the organism crucially
104     depend on whether or not the organism is exposed to EE2 during the early steps of the sex
105     determination cascade, when sex reversal is still possible [23].
106          Exposure to EE2 has been found to induce sex-specific effects in juvenile and adult
107     fish [23]. These sex-specific effects can be large, as expected by the significant differences in
108     male and female physiology. It remains unclear whether large differences should also be
109     expected between the genetic sexes if their gonadal development may have been influenced
110     by the sex-reversing effects of EE2 [24, 25]. Such questions can be studied if reliable sex-
111     linked genetic markers are available for a given study species.
112          Here we study the sex-specific effects of ecologically relevant concentrations of EE2
113     on gene expression and gonad development in grayling (*Thymallus thymallus*), a river-
114     dwelling salmonid fish that may often be exposed to EE2 pollution. Yano et al. [26]
115     established sex-linked genetic markers that could be used to determine the genetic sex of
116     many salmonids, including their sample of grayling taken from a fish farm in France. These
117     markers could be successfully verified in over 100 phenotypically sexed adult grayling
118     sampled from our study population [27]. We therefore use them here to separate effects of
119     EE2 on gene expression in genetic males and females. Maitre *et al*. [27] found large effects of
120     genetic sex on gene expression around the time of hatching from eggs, while gene expression
121     did not seem to differ significantly at late embryogenesis. Their findings thus suggest that the
122     physiological cascade of sex determination starts during embryogenesis and before hatching.
123     We therefore study the interaction between EE2 and genetic sex on gene expression in
124     embryos and larvae. Within-family comparisons are used to minimize potential effects of
125     genetic variation. Possible interactions between EE2 and genetic sex on gonad development
126     are studied histologically on samples taken over a period of several months.
127
128     **2. Methods**
129     *2.1 Experimental breeding and raising*
130     Mature males and females were sampled from a captive breeding stock and stripped for their
131     gametes. These fish are F1 of the natural population described in Wedekind et al. [28]. Their
132     gametes were used in two full-factorial breeding blocks. For each breeding block, 4 females
133     were crossed *in vitro* with 5 males, i.e. 40 (2x4x5) different sibgroups were produced. After
134     egg hardening for 2 hours, the fertilized eggs were transported to the laboratory where they
135     were washed and distributed singly to wells of 24-well plates (Falcon, Becton-Dickinson),
136     following the methods of von Siebenthal et al. [29]. The wells had been filled with 1.8 mL
137     chemically standardized water [30] that had been oxygenated and temperated before use.
138          The embryos were incubated at 7°C and left undisturbed until 14 days post
139     fertilization (dpf), when embryos were exposed either to 1 ng/L EE2 (by adding 0.2 mL water
140     with a concentration of 10 ng/L EE2, see Brazzola *et al*. [6] for details), a strain of
141     *Pseudomonas fluorescens* ("PF"; $10^6$ bacterial cells per well, the low-virulence strain PF1 in
142     Clark et al. [31]), simultaneously to EE2 and *P. fluorescens* (EPF), or sham-treated
143     ("control", i.e. only adding 0.2 mL standardized water). A sample of 250 live embryos (EE2-
144     treated or sham-treated offspring of one haphazardly chosen female that had been crossed
145     with five males) was separated for analyses of gene expression, such that we had 25 embryos
146     for each of the five sibgroups and the two treatments "EE2" and "controls". The remaining
147     embryos were raised until 40 dpf, i.e. until several days after hatching, when a subset of each
148     treatment group was distributed to two 200 L tanks each filled with lake water (pumped from
149     Lake Geneva at 40 m depth), fed with live *Artemia* and copepods and later dry food, and
150     sampled in 5 monthly intervals for histological examination of the gonads (see Maitre *et al*.
151     [27] for a description of sex differentiation). For the EE2-treated groups, 200 ng EE2 were
152     dissolved in 200 L tanks each to reach a starting concentration of 1 ng/L. Every 7 days from
153     then on, 40 L (i.e. 20%) were replaced with fresh lake water spiked with 40 mL of a 1 µg/L

154      EE2 stock solution (i.e. 40 L at 1 ng/L EE2). Water samples (100 mL each) were then taken

155      from each of the 4 EE2-treated tanks 1 hour after this weekly water exchange ($T_0$) and 7 days

156      later, just before the next water exchange ($T_7$). These water samples were immediately frozen

157      and stored at -20°C protected from light. Four consecutive $T_0$ and 4 consecutive $T_7$ samples

158      were each pooled per tank for later determination of EE2 concentrations, i.e. EE2

159      concentrations were determined for the 4-week intervals these pooled samples covered,

160      starting 47 dpf, 75 dpf, 103 dpf, and 131 dpf, respectively.

161          To quantify EE2, the water samples were thawed and filtered over glass fibre filters,

162      their volume was set to 250 mL and the pH to 3. Four ng/L of EE2 D4 was added as internal

163      standard and samples were enriched on LiChrolut EN / LiChrolut RP-C18 cartridges that had

164      been conditioned with hexane, acetone, methanol and finally water (pH 3) [32]. After sample

165      enrichment, cartridges were dried with nitrogen and eluted with acetone and methanol.

166      Subsequently, solvents were changed to hexane/acetone 65:35 and extracts were passed over

167      Chromabond Silica columns [33] and set to a volume of 0.25 mL. LC-MS/MS analysis was

168      performed on an Agilent6495 Triple Quadrupole. An XBridge BEH C18 XP Column, 2.5 µm,

169      2.1 mm X 75 mm and an acetonitrile / water gradient was used for liquid chromatography

170      followed with post-column addition of ammonium fluoride solution. EE2 was quantified by

171      monitoring the mass transition of 295 to 269, the transition of 295 to 199 served as a qualifier

172      (internal standard was quantified at the following transitions: 299 to 273 and 299 to 147).

173          EE2 concentrations in 24-well plates and uptake by embryos are described in Marques

174      da Cunha et al. (unpublished manuscript). Briefly, plates with newly fertilized embryos were

175      spiked with EE2 to a concentration of 1 ng/L of EE2 in the plate wells. Analogously, plates

176      without embryos were spiked to the same concentration. EE2 concentrations in the 24-well

177      plates with and without embryos were measured in 5 time points across embryo development

178      (i.e. from 1 dpf until shortly before embryo hatching). While EE2 concentrations have

179      remained around 1 ng/L in plates without embryos throughout the 5 time points (indicating no

180      apparent degradation or sorption of EE2 in 24-well plates), in plates with embryos,

181      concentrations fell to below detection limit (<0.05 ng/L) before the first half of developmental

182      time (indicating rapid embryo uptake).

183          In the 200 L tanks, median EE2 concentration were 0.33 ng/L at $T_0$ and 0.11 ng/L at

184      $T_7$, corresponding to a median reduction of 66% of the EE2 dissolved in water over 7 days

185      (see Supplementary Figure S1). We found no significant effects of sampling period on the

186      EE2 measures at $T_0$ (ANOVA, $F_3 = 1.20$, $p = 0.35$) nor on the weekly reduction of EE2 in the

187      tanks ($F_3 = 1.88$, $p = 0.19$; excluding an unexplained outlier, see Figure S1 for discussion).

188      The median EE2 concentration across $T_0$ and $T_7$ samples in the EE2-treated tanks was 0.2

189      ng/L from the first water exchange on, i.e. from 47 dpf on (EE2 was also detected in samples

190      from control aquaria, see Figure S1 for discussion).

191          For the gene expression analyses, the first sampling of 12 embryos per family and

192      treatment took place at 21 dpf, i.e. well before hatching could be expected. Embryos were

193      immediately transferred to RNAlater (Thermo Scientific, Reinach, Switzerland). At 27 and 28

194      dpf, the incubation temperature was raised to 10°C and 11.5°C, respectively, in order to

195      induce and synchronize hatching. The second sampling took place at day of peak hatching,

196      i.e. 31 dpf (8 embryos per family and treatment). The third sampling took place at 52 dpf (5

197      yolk-sac larvae per family and treatment). Hatchlings and yolk-sac larvae were narcotized

198      with 0.5mL/L KoiMed (fishmed GmbH, Galmiz, CH) for five minutes and then decapitated.

199      The heads were immediately transferred to RNAlater. All samples were stored at -80°C.

200          RNA was extracted using the QIAgen 96 RNeasy Universal Tissue Kit (QIAGEN,

201      Hombrechtikon, Switzerland). Manufacturer instructions were followed except that

202      centrifugation (Eppendorf 5804 R centrifuge with an A-2-DWP rotor; Eppendorf,

203      Schönenbuch, Switzerland) was done twice as long at half the speed. Because the RNA

204      extraction protocol did not include a DNase treatment, DNA traces inside the RNA samples

4

205   were amplified to determine the sdY genotype [26] of each individual, using the 18S gene as
206   PCR internal control, either in a multiplex reaction used for samples with high amount of
207   DNA, or after a second PCR amplification in single reactions with half the amounts of the
208   respective primers each for samples with low DNA content (see Maitre *et al.* [27] for a more
209   detailed protocol). Based on the sdY genotype, one female and one male per family and
210   treatment group was haphazardly chosen for further analyses (in 2 of in total 30 combinations
211   of family x treatment x time point, two females or two males, respectively, were used because
212   only one sex could be found in the sample).
213       The RNA extracts were provided for library preparation in a equimolar concentration
214   of 6 ng/µL in 100 µL of volume. 50 µL (*i.e.* 300 ng of RNA) were each used for library
215   preparation on a robot using the Truseq Stranded RNA protocol (Illumina, Part# 15026495
216   Rev. A), then introduced in the Illumina sequencing platform (HiSeq 2500) for 100 cycles of
217   multiplexed paired-end reads sequencing. The total 60 samples were sequenced in ten lanes
218   (six samples per lane).
219
220   *2.2 Bioinformatics pipeline*
221   The RNA-seq analysis steps are described in Maitre *et al.* [27]. To summarize, reads were
222   quality trimmed or filtered, and PCR duplicates were removed, resulting in a set of 60 RNA
223   libraries with, on average, 2*40 millions of 80 bp reads each (standard deviation of six
224   million reads). Reads from 24 libraries were assembled using Trinity (version 2.0.3; [34]).
225   The resulting assembly was filtered to remove sequences that did not have a significant match
226   against the UniprotKB/Swiss-Prot database (version of October the 16th 2015) [35] using
227   blastx (version 2.2.26; [36]). Reads from all libraries were pseudo-mapped onto the
228   assembled transcriptome using Kallisto (version 0.42) [37], and the read counts of the
229   different isoforms of each gene were summed up for principal component analysis (PCA) and
230   differential expression analysis at the gene level. PCA was performed on TMM-normalized
231   [38] log2(count-per-million) values (CPM). Differential expression analysis was performed
232   using the limma-voom Bioconductor package (version 3.26.3) [39, 40], with samples quality
233   weights [41], on CPM values that were additionally cyclic loess normalized. In the linear
234   model we considered developmental stage, sex and treatment as a combined variable (with
235   twelve possible levels) and sib-group as an independent variable. A linear model was then fit
236   for each gene, coefficients and standard errors were computed for all the contrasts of interest.
237   Q-values [42] were calculated for each gene, and a threshold of q = 0.15 was used to call
238   differentially expressed genes. Transcripts were annotated by referring to the Gene Ontology
239   (GO) terms (Biological Function) [43] associated to similar genes in zebrafish. An enrichment
240   analysis of GO terms was performed on differentially expressed genes using the goseq
241   Bioconductor package (version 1.22.0; [44])
242       RNA extraction quality, male sex associated locus PCR, RNA-sequencing reads quality,
243   transcriptome assembly, gene expression principal component and differential gene
244   expression within control individuals are shown in Maitre *et al.* [27].
245
246   **3. Results**
247
248   *3.1 Differential gene expression*
249   In order to test for sex-specific effects, we compared the changes in gene expression under
250   EE2 treatment for individuals of the same sex at the same developmental stage (Table 1).
251   Under EE2 treatment, at the embryo stage there was an altered expression of several hundred
252   genes in males (Table 1a, Figures S2a, S3, S4, Table S1), but only of a few genes in females
253   (Table 1a, Figure S2b). At hatching day males showed an alteration in the expression of
254   nearly 12,000 genes (Table 1b, Figure S2c, S5, S6, Table S2), while females at the same time
255   point displayed an alteration in the expression of over 24,000 genes (Table 1b, Figure S2d,

256 S7, S8, Table S3). At the first feeding stage only very few genes appeared altered in males
257 (Table 1c, Figure S2e), whereas in female still around 14,000 genes were affected (Table 1c,
258 Figure S2f, S9, S10, Table S4).
259     In Table 2, the sex-specific alterations in gene expression are split according to the
260 direction of the changes. Around hatching, about 5,000 genes were up-regulated in EE2-
261 treated males while down-regulated in EE2-treated females, and about 4,000 were down-
262 regulated in EE2-treated males while up-regulated in EE2-treated females (Table 2). The
263 remaining sex-specific reactions to the EE2 treatment were mainly up- or down-regulation in
264 one sex while there was apparently no change in the other sex (Table 2). See Figures S11 and
265 S12 and Table S5 for EE2 effects on gene expression in both, males and females.
266
267 *3.2 Does EE2 treatment feminize males and masculinize females?*
268 After focusing on sex-specific gene expression changes induced by EE2 treatment, we
269 compared control males against EE2-treated females and control females against EE2 treated
270 males (Table 1). The aim of this analysis was to investigate whether the EE2 treatment would
271 feminize males, masculinize females, or increase the differences in gene expression between
272 sexes. At embryo stage, we found three genes differentially expressed between EE2 treated
273 males and control females (Table 1a) and 863 genes between control males and EE2 treated
274 females (Table 1a). On hatching day, we found no differences in gene expression levels
275 between control females and males treated with EE2 (Table 1b) and only two genes differing
276 between control males and EE2 treated females (Table 1b). At first feeding stage, EE2 treated
277 males expressed 5,533 genes differently in comparison to control females (Table 1c), while
278 gene expression in control males differed in 42 genes only from the gene expression of EE2-
279 treated females (Table 1c). Overall, there does appear to be transcriptome evidence of
280 feminization of males and of masculinization of females.
281
282 *3.3 Gene ontology enrichment analysis*
283 Tables S1-S5 show the top 25 Gene Ontology terms enriched among genes differentially
284 expressed in the above-described contrasts. Figures S3-S12 illustrate the Gene Ontology
285 terms significantly enriched, grouped by relatedness. Table 3 shows a summary take-home
286 from these Gene Ontology enrichments. Interestingly, many differences affect developmental
287 processes. In male embryos, we also notice response processes, whether endocrine, xenobiotic
288 or immunological. Finally, in female larvae a wide range of processes are affected, including
289 behaviour and digestion.
290
291 *3.4 Sex differentiation*
292 Exposure to EE2 delayed the onset of morphological sex differentiation while exposure to *P.*
293 *fluorescens* (PF) showed no effects (Table 4). Figure 1 shows the rates of juveniles without
294 any testis or ovarian tissues over the five monthly samples, separately for fish that were or
295 were not exposed to EE2, to illustrate the delaying effects of EE2. First signs of
296 morphological sex differentiation could be observed at the $2^{nd}$ sample, but still more than 30%
297 of the fish showed no sign of morphological sex differentiation at the end of the observation
298 period (Figure 1).
299     Among those fish where first stages of sex differentiation could be identified, only
300 testis tissue could be observed at the $2^{nd}$ sampling (79 dpf), while the rate of ovarian tissue
301 rose quickly to 70.8%, 75.9%, then 79.3% over the $3^{rd}$, $4^{th}$, and $5^{th}$ sampling periods,
302 respectively. The rates of ovarian versus testis tissue did not differ between EE2-treated and
303 sham-treated grayling ($\chi^2 = 0.26$, p = 0.61).
304     Genetic sexing of all 103 individuals of the $4^{th}$ and $5^{th}$ sample (135 dpf and 159-163
305 dpf) revealed a genetic sex ratio of 55.3% males that did not deviate from equal sex ratio ($\chi^2$
306 = 1.2, d.f. = 1, p = 0.28). Equal sex ratios can therefore be assumed for all earlier samples. At

6

307     these two last sampling days, all females except three showed ovarian tissue (ovaries or testis-
308     to-ovaries). The three exceptions were from the sham-treated controls and showed testis
309     tissue, i.e. no genetic female was undifferentiated at that these last sampling dates. In contrast,
310     45 of a total of 57 genetic males were still undifferentiated at that time, 10 showed testis
311     tissue, one showed the testis-to-ovaries phenotype, and one had ovaries. The latter two males
312     with ovarian tissues had both been EE2-treated.
313
314     **4. Discussion**
315     We tested and described the effects of exposure to low, ecologically relevant, concentrations
316     of EE2 on sex-specific gene expression in embryos and larvae of grayling, a river-dwelling
317     salmonid that is often exposed to this type of pollution. From what is known about possible
318     EE2 effects on fish in general, we expected that this common micropollutant may (i) affect
319     sex determination of grayling by influencing the few initial triggers that start the canalized
320     developmental process of gonad formation, and (ii) be toxic to the embryos and larvae
321     because it interferes with different types of physiological processes, especially those that are
322     endocrinologically regulated (see references cited in the Introduction). We therefore expected
323     EE2 to have significant effects on gene expression at various developmental levels, and we
324     indeed found such effects at all the developmental stages we studied here. However, we had
325     no clear *a priori* expectancy about whether EE2 would also affect the genetic males and
326     genetic females differently at any of these stages.
327             We started from the premise that sex in gonochoristic species is a threshold trait, i.e. a
328     canalized developmental process that has one or few initial triggers [16]. In grayling, the
329     initial trigger (or triggers) that determine phenotypic sex happen during embryogenesis well
330     before hatching, because over 25,000 genes are already differentially expressed between
331     genetic males and females at the day of hatching [27]. The 15 genes that Maitre et al. [27]
332     found to be differentially expressed in genetic males and females at the embryo stage 10 days
333     before hatching suggest that sex determination starts around then, i.e. at a time when the
334     embryos had already been exposed to EE2 for several days in the present study.
335             One possible scenario is hence that EE2 could tip the balance at the early steps of sex
336     determination so that all individuals follow the developmental process that leads to the female
337     phenotype regardless of their sdY genotype (i.e. sex reversal of genetic males). If so, EE2
338     would not be expected to show sex-genotype specific effects on gene expression during later
339     stages of sex differentiation. However, we found strong interactions between genetic sex and
340     EE2 on gene expression. These sex-specific reactions to EE2 also depended on the
341     developmental stages we studied. At embryo stage, expression of only few genes seemed
342     biased in genetic females, but gene expression in genetic males was already significantly
343     affected, with about 700 genes up- or down-regulated under the influence of EE2. The
344     outcome was somewhat reverse at the larval stage: now only few genes of genetic males
345     seemed to be affected by EE2, while over 14,000 genes were differentially expressed in
346     genetic females. An even more pronounced effect of EE2 could be seen at the day of
347     hatching: far over 20,000 genes showed differential expression, and about 9,000 of them were
348     either upregulated in genetic females and down-regulated in genetic males or down-regulated
349     in genetic females and upregulated in genetic males.
350             The strong sex-specific responses to EE2 suggest that exposure to ecologically
351     relevant concentrations of EE2 during embryogenesis did not simply tip the balance at early
352     steps of sex determination so that all individuals would become phenotypic females and
353     would show similar patterns of gene expression from then on. Instead, our observations
354     suggest that genetic sex largely determined phenotypic sex, and that EE2 then interfered with
355     sex-specific gene expression, creating the strong sex-specific reactions to EE2. This
356     conclusion is supported by the observation that the low concentrations of EE2 we used are
357     commonly observed in natural rivers and streams while there is little evidence for complete

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358    and population-wide sex reversal, even if natural populations sometimes show distorted sex
359    ratios [45].
360         While the interaction between EE2 and genetic sex on gene expression suggested that
361    exposure to EE2 is mostly interfering with the development of a phenotype that would
362    correspond to the genotypic sex, we nevertheless found two genetic males that had developed
363    ovarian tissues when exposed to EE2. It is possible that we missed some sex-reversal and the
364    rate of sex reversal is higher, because we learned only during the course of the study that the
365    grayling is a rare example (and probably even the only one so far) of an undifferentiated
366    gonochorist that goes through an all-male stage before gonads differentiate into testes and
367    ovaries [27]. Testis tissue in early juveniles can therefore not be interpreted as evidence for
368    normal development of a male phenotype, and we probably stopped the study too early to get
369    a reliable estimate of the rate of sex reversals in grayling exposed to EE2. However, by the
370    end of the study, nearly all genetic females had developed ovarian tissue. This suggests that
371    the rate of sex reversals is either low indeed, or that sex reversal would slow down gonad
372    development so much that we would have missed many sex-reversed individuals within our
373    observational window. We know of no examples or arguments in the literature that would
374    support the latter possibility. However, we found that ecologically relevant concentrations of
375    EE2 could affect sex determination in at least some individuals. It would therefore be
376    interesting to learn what makes an early embryo susceptible or resistant against chemically
377    induced sex reversal.
378         Our gene expression analysis suggested that exposure to EE2 induces effects in the
379    transcriptomes of the brain that could be interpreted as partial sex reversal, especially around
380    hatching time when many genes were differentially upregulated in genetic females and
381    downregulated in genetic male, and vice versa, under the influence of EE2. At the day of
382    hatching, genetic males appeared to have a somewhat feminized transcriptome when treated
383    with EE2. This effect seemed to cease before the first feeding stage. Genetic females seemed
384    to experience some form of masculinization of the transcriptome of the brain, at least from
385    hatching day to first feeding stage.
386         Estrogens are known to affect functions of the nervous system, including synapsis
387    homeostasis [46], neurogenesis [47], and sexual differentiation [47-49]. The latter association
388    is particularly interesting here because estrogens have repeatedly been shown to induce, to
389    some degree, a masculinization of the brain [47-49]. This is because androgenic hormones,
390    produced mainly in the male gonads, are transformed by brain cells into estrogen that
391    subsequently act locally via specific receptors to induce typical male development [48].
392         In salmonids, gonadal precursor cells typically differentiate during the weeks that
393    follow hatching [17, 50]. During this period, the emergence of an endogenous synthesis of
394    sexual hormones could explain why we observe a divergent response between sexes,
395    especially if we consider that endocrine active compounds often elicits non-monotonic dose-
396    responses [51, 52]. Such dose-effects could explain why we observed strong sex-specific
397    responses to EE2 at hatching day and why these responses partly declined towards first
398    feeding stage. Apart from the likely effects of EE2 on normal development of male and
399    female phenotypes, exposure to EE2 also affected the expression of genes linked to many
400    other physiological systems, including, for example, various aspects of the immune system,
401    the endocrine system, the development of skeletal muscles, or of the digestive system.
402         In conclusion, exposure to ecologically relevant concentrations of EE2 during early
403    embryogenesis may potentially tip the balance at early steps of sex determination so that all
404    individuals follow the developmental process that leads to the female phenotype regardless of
405    their sdY genotype (i.e. sex reversal of genetic males). If so, and if gene expression is then
406    mainly determined by this gonad development, EE2 would probably not be expected to show
407    strong sex-genotype specific effects on gene expression during later stages of sex
408    differentiation. However, we found strong effects of both, EE2 and genetic sex, on gene

409  expression at three different developmental stages shortly after the onset of sex determination.
410  Such sex-specific effects of EE2 suggest that sex reversal has mostly not happened, although
411  we found two genetic males with gonadal tissues at the end of the study. Our observations
412  support earlier conclusions that ecologically relevant concentrations of EE2 act mostly toxic
413  (i.e. affect growth and viability) but have little effect on sex determination in grayling [53],
414  and that the observed sex ratio distortion may in the population [45] may be due to sex-
415  specific survival instead rather than induced sex reversal.
416
417  **5. Acknowledgements**

430
431  **6. References**

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582　　　2013; 83(2):404-411.
583
584

585 **Table 1.** Number of genes that are differentially expressed (q<0.15) in males and females
586 tested at (a) embryo stage, (b) hatchling stage, and (c) larval stage at the onset of exogenous
587 feeding, each after they had been exposed to EE2 or sham exposed.
588
589

| | | sham-exposed females | EE2-exposed males | EE2-exposed females |
|---|---|---|---|---|
| a) Embryos | | | | |
| | Sham-exposed males | $15^1$ | 704 | 863 |
| | Sham-exposed females | | 3 | 32 |
| b) Hatchlings | | | | |
| | Sham-exposed males | $25{,}372^1$ | 11,619 | 2 |
| | Sham-exposed females | | 0 | 24,728 |
| c) Larvae | | | | |
| | Sham-exposed males | $1{,}110^1$ | 4 | 41 |
| | Sham-exposed females | | 5,533 | 14,395 |

590 [1] reported in Maitre *et al*. [27]
591

592  Table 2. Number of genes that were upregulated, i.e. had a positive log fold change of
593  expression with q > 0.15 (UP), experienced no significant change in expression (NO), or were
594  downregulated (DO) under exposure to EE2.
595
596  .
597

|  | | Males UP | Males NO | Males DO | Total |
|---|---|---|---|---|---|
| **a) Embryos** | | | | | |
| | Females UP | 0 | 12 | 0 | 12 |
| | Females NO | 245 | 34,613 | 457 | 35,315 |
| | Females DO | 1 | 18 | 1 | 20 |
| | Total | 246 | 34,643 | 458 | 35,347 |
| **b) Hatchlings** | | | | | |
| | Females UP | 147 | 7,959 | 4,043 | 12,149 |
| | Females NO | 1,341 | 8,267 | 1,009 | 10,617 |
| | Females DO | 4,962 | 7,495 | 117 | 12,574 |
| | Total | 6,450 | 23,721 | 5,169 | 35,340 |
| **c) Larvae** | | | | | |
| | Females UP | 3 | 8,589 | 0 | 8,592 |
| | Females NO | 1 | 20,949 | 0 | 20,950 |
| | Females DO | 0 | 5,803 | 0 | 5,803 |
| | Total | 4 | 35,341 | 0 | 35,345 |

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14

600 Table 3. Summary interpretation of the differential gene expression analysis. The
601 characterization of the biological processes relies on the gene ontology enrichment analysis of
602 differentially expressed genes. Feminization and masculinization represent the situation where
603 few genes (<100) are detected as differentially expressed, under EE2 treatment, in
604 comparison to control female or control male, respectively. See Supplementary Figures S2-
605 S13 and Tables S1-S5 for more detailed information.
606
607

| Developmental stage | Sex differences in gene expression [1] | EE2-effects in males | EE2- effects in females |
|---|---|---|---|
| Embryos | Weak | On genes associated with central nervous system development, immune response development, endocrine system and response to xenobiotic stimuli. | Unclear. |
| Hatchlings | Strong | On genes associated with skeletal muscle function and locomotion, metabolism of fatty acids and carbohydrates, DNA-repair and hindbrain morphogenesis. Possible feminization. | On genes associated with immune system activation, DNA-repair, axonal growth and synaptic transmission. Possible masculinization. |
| Larvae | Strong | A few genes only. | On genes associated with DNA-repair, signal transduction, immune system activation, carbohydrate metabolism, mechanosensory behavior and digestion. Possible masculinization. |

608 [1] Results from Maitre *et al*. [27]
609

15

610 Table 4. Likelihood ratio test on rate of undifferentiated gonads explained by time point of
611 sampling and exposure to ethinylestradiol (EE2), the bacterium *P. fluorescens* (PF), or both
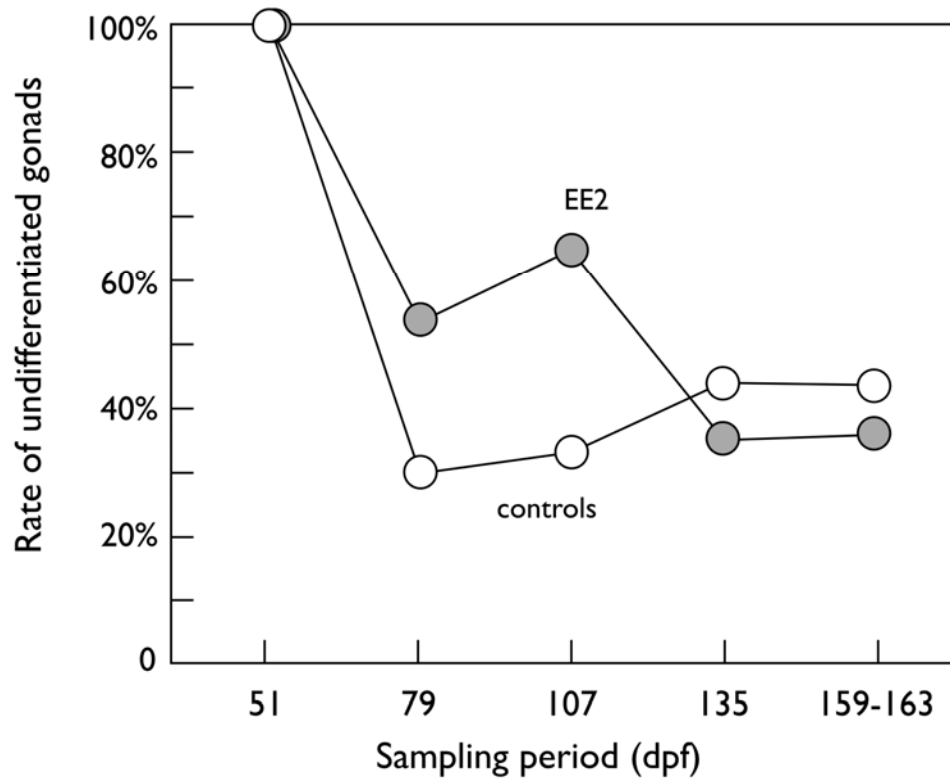612 (EE2 x PF). $N_{total} = 251$.
613
614

| Effect | $\chi^2$ | d.f. | P |
|---|---|---|---|
| **Time** | **72.3** | **4** | **<0.0001** |
| EE2 | <0.1 | 1 | 1.0 |
| PF | <0.1 | 1 | 1.0 |
| **Time x EE2** | **9.5** | **4** | **0.05** |
| Time x PF | 0.9 | 4 | 0.92 |
| EE2 x PF | <0.1 | 1 | 1.0 |
| Time EE2 x PF | 4.7 | 4 | 0.32 |

615
616
617

618 **Figure 1**
619 Rates of undifferentiated males and females (i.e. no testis nor ovarian tissues) when exposed
620 to EE2 (closed symbols) or sham exposed (controls, open symbols), with or without
621 additional exposure to *P. fluorescens* during embryogenesis each (exposure to the microbe
622 showed no significant effects on sex differentiation). See text and Table 4 for statistics.
623



624