1 **Transcriptomic analysis of the impacts of ethinylestradiol (EE2) and its**

2 **consequences for proliferative kidney disease outcome in rainbow trout**

3 **(*Oncorhynchus mykiss*)**

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6 Christyn Bailey*, Elena Wernicke von Siebenthal, Kristina Rehberger and Helmut

7 Segner

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12 All authors are from the University of Bern, Vetsuisse Faculty, Centre for Fish and

13 Wildlife Health, Länggassstrasse 122, CH-3012 Bern, Switzerland

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*Corresponding author: Christyn Bailey

Telephone number: +41 31 631 2461

E-mail : christyn.bailey@vetsuisse.unibe.ch

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source: https://doi.org/10.7892/boris.141733 | downloaded: 26.4.2024

Abstract

Freshwater fish are threatened by the cumulative impact of multiple stressors. The purpose of this study was to unravel the molecular and organism level reactions of rainbow trout, *Oncorhynchus mykiss*, to the combined impact of two such stressors that occur in the natural habitat of salmonids. Fish were infected with either the myxozoan parasite, *Tetracapsuloides bryosalmonae*, which causes proliferative kidney disease (PKD), or exposed to ethinylestradiol (EE2) an estrogenic endocrine disrupting compound, or to a combination of both (PKD x EE2). PKD is a slow progressive chronic disease here we focused on a later time point (130-day post-infection (d.p.i)) when parasite intensity in the fish kidney has already started to decrease. At 130 d.p.i, RNA-seq technology was applied to the posterior kidney, the main target organ for parasite development. This resulted with 280 (PKD), 14 (EE2) and 444 (PKD x EE2) differentially expressed genes (DEGs) observed in the experimental groups. In fish exposed to the combination of stressors (PKD x EE2), a number of pathways were regulated that were neither observed in the single stressor groups. Parasite infection, alone and in combination with EE2, only resulted in a low intensity immune response that negatively correlated with an upregulation of genes involved in a variety of metabolic and inflammation resolution processes. This could indicate a trade-off whereby the host increases investment in recovery/resolution processes over immune responses at a later stage of disease. When PKD infection took place under simultaneous exposure to EE2 (PKD x EE2), parasite intensity decreased and pathological alterations in the posterior kidney were reduced in comparison to the PKD only condition. These findings suggest that EE2 modulated these response profiles in PKD infected fish, attenuating the disease impact on the fish.

Key words: RNA-seq; Transcriptome; Rainbow trout; *Tetracapsuloides bryosalmonae*; Proliferative kidney disease; Estrogen; Anthropogenic pollution; Multiple stressor; Immune response; Metabolic processes

64 **1. Introduction**

65 A known biological stressor of freshwater salmonids significantly affecting both the
66 aquaculture industry and wild fish populations globally is the extracellular myxozoan
67 parasite *Tetracapsuloides bryosalmonae,* the etiological agent of proliferative kidney
68 disease (PKD). In rainbow trout, *Oncorhynchus mykiss*, PKD causes an
69 immunopathological condition mediated by an increasing number of lymphocytes
70 and a decreasing number of myeloid cells, leading to a dysregulated B cell response
71 and intricate interplay between t-helper th1 and th2-like cytokines (Abos et al., 2018;
72 Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). Fish can become
73 infected at lower temperatures (<10 °C) but increasing water temperature has been
74 demonstrated to increase disease prevalence, incidence and, severity and elevate
75 mortalities (Bailey et al., 2017a; Burkhardt-Holm et al., 2005; Bettge. et al., 2009a;
76 Bettge. et al., 2009b). Thus, under the growing influence of environmental change
77 there is evidence that PKD is linked to the long-term decline of trout populations in
78 central European countries, such as the UK and Switzerland, but also from more
79 Nordic countries (Dash and Vasemägi, 2014; Gorgoglione et al., 2016;
80 Kristmundsson et al., 2010; Mo and Jørgensen, 2017; Okamura et al., 2011; Wahli
81 et al., 2002).
82
83 Given the growing impact of human activities upon aquatic ecosystems, freshwater
84 fish suffer from chemical stressors as well as biological ones (Carpenter et al., 2011;
85 Johnson and Sumpter, 2014). A number of pollutants are potential
86 immunomodulators in fish. Well-known environmental contaminants with
87 immunomodulating activity in fish include polycyclic aromatic hydrocarbons (PAHs)
88 (Billiard et al., 2002) or polybrominated diphenyl ethers (PBDEs) (Arkoosh et al.,
89 2015). There is now also growing evidence that estrogenic endocrine disrupting
90 compounds (EEDCs) can have immunomodulatory effects in fish (Casanova-
91 Nakayama et al., 2011; Milla et al., 2011). EEDCs have been reported to modulate
92 the innate (Cabas et al., 2012; Milla et al., 2011; Seemann et al., 2016; Seeman et
93 al., 2013) and adaptive immune response (Casanova-Nakayama et al., 2011; Cuesta
94 et al., 2007; Rodenas et al., 2015; Saha et al., 2004; Seemann et al., 2015; Thilagam
95 et al., 2009) in various fish species. For example, concerning the innate immune
96 response in sea bass, *Dicentrarchus labrax*, an EEDC, 17α-Ethinylestradiol (EE2)

97 was shown to downregulated pro-inflammatory cytokines il1β and tnfα (Seeman et
98 al., 2013). Regarding the adaptive immune response, EE2 exposure was shown to
99 decrease plasma IgM levels in gilthead seabream, *Sparus aurata* (Cuesta et al.,
100 2007). Thus, the published studies laboratory invetsigations provide evidence for
101 EEDCs acting as immunomodulators in fish.
102
103 In view of the abovementioned information on the influence EEDCs may have on the
104 immune system, exposure to these contaminants may modulate the response of fish
105 exposed to the PKD causing parasite. In fact, both *T. bryosalmonae* and EE2 are
106 considered potential factors contributing to the decline of Swiss brown trout
107 populations over the last decades. The two stressors co-exist in Swiss rivers (Burki
108 et al., 2006; Wahli et al., 2008, Vermeirssen et al., 2005)), what leads to the question
109 whether their combination may lead to cumulative effect on fish populations.
110 Nevertheless, to date only one study has examined the possible combination effects
111 of EEDCs and *T. bryosalmonae* infection on the health status of salmonids. In an
112 explorative study, Burki et al. (2013) exposed rainbow trout to EE2 and *T.*
113 *bryosalmonae* infection and observed a dominating influence of the infection over
114 estrogen on transcript expression of rainbow trout hepatic genes as well as parasite
115 loads in the fish host (Burki et al., 2013). However, this study investigated an
116 extreme disease scenario as the fish were infected at a water temperature of 18 °C,
117 resulting in too strong pathogenesis and disease-induced mortality. This approach
118 may have obscured possible EE2 modulation of the fish response to the parasite.
119 EE2 was administered to fish via feeding which does not allow the exposure to relate
120 to environmental EE2 concentrations as found in the water body. While this report
121 provided information on the transcriptome response in the liver, it did not investigate
122 the posterior kidney - the target organ for *T. bryosalmonae* infection.
123
124 The present study unravelled the transcriptomic response in the posterior kidney
125 using rainbow trout exposed to either the parasite, environmentally relevant EE2
126 concentrations or a combination of both. Here we focused on the question whether
127 the presence of EE2 administered in environmentally relevant concentrations via the
128 water influences infected fish under conditions where the parasite infection still
129 results in clinical PKD, but no elevated mortality. To this end, we performed the
130 experiment at a temperature <15 °C, as it occurs during summer in many river

systems in Switzerland. In using this approach, we could specifically pinpoint the response the host uses to manage the parasitic infection and how it may be modulated by EE2.

We studied the EE2 modulation on PKD at a disease stage, when the parasite load of the fish kidney has gone through its maximum and started to decline (130 days post parasite infection (d.p.i)), and the fish starts to recover from infection. During the PKD recovery/resolution process the presence of the parasite will disappear from the tissue and homeostatic recovery processes will start (Bailey et al., 2019; Schmidt-Posthaus et al., 2012). In mammals estrogen can promote anti-inflammatory responses and wound healing, hence these processes may be enhanced in infected fish exposed to EE2 (Ashcroft and Ashworth, 2003; Ashcroft et al., 1997; Ashcroft et al., 2003). Therefore we hypothesised that EE2 exposure will enhance rather than supress the host response to parasite infection. More specifically, the aims of this study were to 1) obtain a broader overview on the host response to *T. bryosalmonae* infection (using RNA-seq in contrast to pre-selected genes via RT-qPCR), and 2) to greater understand how the combined impact of an estrogenic contaminant (EE2) modified the host response to *T. bryosalmonae* infection.

2. Materials and Methods

2.1. Experimental design and fish exposures

The experimental design, exposures and fish sampling are as previously described (Wernicke von Siebenthal et al., 2018). Rainbow trout were obtained from the cantonal fish farm of Rodi, Switzerland (Piscicoltura Cantonale Rodi, 6772 Rodi, Switzerland). Upon arrival, fish were examined for the presence of pathogens, including *T. bryosalmonae*, and they were found to be free of infectious agents. Rainbow trout were transferred to flow-through 35 l glass tanks, supplied with tap-water (at approx. 1 l/m), with constant and 12 h light/12 h dark photoperiod. Water temperature was kept at 12.9 °C (±1.22°C).

After a 28-day acclimation fish were separated into four experimental groups (run in duplicate tanks, 122 fish per tank at the start of the experiment): two experimental groups were exposed to environmentally relevant concentrations of water-borne 17α-ethinylestradiol (EE2 purity ≥98%) (Sigma-Aldrich, Buchs, Switzerland) exposure

165 (5.5 ng/l EE2) and two were not. Estradiol in Swiss midland rivers were previously
166 assessed to range from 0.3 to 7.0 ng/l (Vermeirssen et al., 2005), thus the applied
167 concentration can be considered environmentally relevant.
168
169 EE2 was dissolved in molecular biology purpose ethanol (purity ≥ 99.8%) (Merck,
170 Darmstadt, Germany), and then diluted in the appropriate volume of water in a 20-l
171 glass beaker with a magnetic rotor on the bottom to mix the solution. The solution
172 was then pumped into the fish tanks through inert tubes (Flow Tubing, Pharamed,
173 4.0 mm ID, Gilson AG, Mettmenstetten, Switzerland) using peristaltic pumps
174 (Minipuls3®, Gilson AG, Mettmenstetten, Switzerland). The flow rate was adjusted to
175 achieve the targeted EE2 concentration in the fish tanks. The actual water inflow was
176 controlled using Flow Rotameters (Rota Yokogawa, Wehr, Germany). The
177 concentration of EE2 in the tanks was confirmed via water samples throughout the
178 duration of the experiment using a competitive EE2 Elisa Kit (Ecologiena, Tokyo,
179 Japan) (Wernicke von Siebenthal et al., 2018).
180
181 After two weeks of EE2 treatment, carried out to ensure the fish were in an
182 estrogenic condition before parasitic exposure, one of the experimental groups
183 exposed to EE2 and one not were then exposed to the parasite *T. bryosalmonae*.
184 Parasite exposures were performed as previously reported in our lab (Bailey et al.,
185 2017a). This parasite exposure still ensues in clinical PKD, but through eliminating
186 mass mortality and continuous infection, we can precisely evaluate the physiological
187 response the host uses to manage infection.
188
189 Consequently, the present study consists of the following four experimental groups
190 1) CTRL (no EE2, no parasite); (2) PKD (exposed to parasite only); (3) EE2
191 (exposed to 5.5 ng/L of EE2 only); (4) PKD x EE2 (exposed to 5.5 ng/L EE2 and the
192 parasite). All treatments were performed in duplicate. All procedures were carried out
193 according to the Swiss legislation for animal experimentation guidelines under
194 license number BE102/16.
195
196 **2.2. Fish sampling**
197 For the present study, we used fish sampled at 130 d.p.i. Fish were euthanized using
198 MS222 (150 mg/l buffered 3-aminobenzoic acid ethyl ester, Argent Chemical

Laboratories). Three fish (biological replicates) were sampled from each experimental group and used for all procedures. The length and weight of each fish was recorded and after blood collection, the posterior kidney was removed and weighed. The posterior kidney was then cut lengthwise. One half of the tissue was placed in a tube containing 1.5 ml TRI Reagent (Sigma-Aldrich, Switzerland) lysed and stored at -80 °C for future DNA / RNA extraction. The other half of the kidney was fixed in HistoChoice (Sigma-Aldrich, Switzerland) for 3 h at RT (room temperature) and then subsequently transferred to an ascending series of EtOH concentrations prior to paraffin embedding for histological procedures.

2.3. Pathological examination

After routine processing and paraffin embedding, kidney sections of 3–5 μm thickness was prepared on SuperFrost Plus positively charged glass slides (Thermo Fisher Scientific, Basel, Switzerland). The slides were then stained with haematoxylin and eosin (H&E) for histological assessment (Luna 1968). Histopathological alterations throughout the posterior kidney were examined, these included infection degree (presence and distribution of parasites), tissue proliferation and presence of fibrous tissue using an index previously described Bailey et al., (2018) modified from Schmidt-Posthaus et al., (2012) when assesing PKD histopathology. All parameters were scored 0–6. The degree of tissue was scored as: 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) as. This scoring system was used to compare all experimental groups. Pertaining to infection degree: 0 indicated no parasites, whereby a 6 specified an abundance of parasites throughout the field of view. Relating to the presence of fibrous tissue: fibrous tissue is an indicator of the tissue regeneration process that is stimulated to recover/regenerate organ structure and function during the resolution of infection. Its presence has been reported in rainbow trout recovering from PKD (Schmidt-Posthaus et al., 2012). Owing to our late stage of infection analysis, we expected fibrous tissue to be observed in infected samples; hence, presence of fibrous tissue was also scored from 0–6 using the same index as described above for tissue proliferation (Schmidt-Posthaus et al., 2012). In addition, a more general toxicology pathological assessment was performed for all groups to assess any adverse impacts of the EE2 exposure. One slide was assessed per fish. To further understand the impact of the different experimental treatments' posterior

233 kidney somatic index (posterior kidney weight/by total body weight x 100), Fulton's
234 condition factor (K = W× L^{-3} X 100) and body weight were compared between all
235 groups.
236

237 **2.4. DNA isolation and qPCR for determination of parasite intensity**

238 To determine the parasite intensity of infected fish genomic DNA was isolated from
239 the homogenized rainbow trout posterior kidney fraction as previously described
240 (Harun et al., 2011). DNA was eluted in 30 µl of EB buffer (Qiagen, Basel,
241 Switzerland) and stored at −20 °C until qPCR was carried out. qPCR was performed
242 targeting *T. bryosalmonae* 18 rDNA (Acc. N.: AF190669) as previously described
243 (Bettge et al., 2009a), using an Applied Biosystems 7500 analyser (Applied
244 Biosystems, Rotkreuz, Switzerland). qPCR was performed using PKD primers and
245 the TaqMan probe as earlier described in our lab for the rainbow trout–*T.*
246 *bryosalmonae* model using an Applied Biosystems 7500 analyser (Applied
247 Biosystems, Rotkreuz, Switzerland) (Bettge. et al., 2009a). The qPCR was carried
248 out in a final volume of 20 µl containing 1X TaqMan universal Master Mix (Applied
249 Biosystems, Switzerland), 0.5 µM of each primer (PKDtaqf1: 5′-
250 GCGAGATTTGTTGCATTTAAAAAG-3′ and PKDtaqr1: 5′-
251 GCACATGCAGTGTCCAATCG-3′), 0.2 µM of the probe PKD (5'-
252 CAAAATTGTGGAACCGTCCGACTACGA-3′) (18S rDNA gene of *Tetracapsuloides*
253 *bryosalmonae*: Genbank Accession No. **AF190669**) labelled with FAM-TAMRA, 1X
254 of IC DNA (TaqMan Univ. MMix w Exog IntPostC, Applied Biosystems), and 2 µl of
255 template DNA. Standard curves were formed for each qPCR cycle
256 using plasmids encompassing the amplified fragment. For every plate, five logs of
257 plasmid dilution standards were amplified (from 10^6 - 10^2 copies). Parasite intensity
258 in the posterior kidney (DNA copy number/g of kidney) was determined as the
259 average parasite quantification (over the duplicate qPCR reactions) in the DNA
260 elution, divided by the extracted kidney weight in grams (g).
261

262 **2.5. RNA isolation and sequencing**

263 RNA was isolated from the posterior kidney of three independent biological
264 replicates from each experimental group taken at 130 d.p.i. RNA isolation and
265 sequencing were carried out by ZF genomics. RNA was isolated using the Trizol
266 method. The quality of all biological replicates was tested by the Agilent RNA 6000

267 Nano Kit, using the Agilent 2100 Bioanalyzer System (Agilent Technologies,
268 Waldbronn, Germany) according to the manufacture's guidelines. To proceed to
269 sequencing a sample had to exceed the quality control (QC) requirements: RNA
270 yield >2 ug, A260/280≥1.8, and RNA Integrity Number (RIN) ≥7.0. RNA libraries
271 were constructed and sequenced on the Illumina HiSeq 2500 (Illumina, San Diego,
272 CA, USA). Raw reads were stored as FastQ files. FastQ files analysis and all
273 ensuing bioinformatics were performed by the Interfaculty Bioinformatics unit at the
274 University of Bern, CH.
275
276 **2.6. Bioinformatics, gene ontology of differential gene expression analysis**
277 The quality of the RNA-seq data was assessed using fastqc v. 0.11.5
278 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and RSeQC v. 2.6.4 8
279 (Wang et al., 2012). Sequencing reads were aligned to the available rainbow trout
280 genome (Berthelot et al., 2014) using HiSat2 (Kim et al., 2015) and quantification
281 was performed using featureCounts (Liao et al., 2013). Differential expressed gene
282 (DEGs) analysis between samples exposed to the different stressors (PKD, EE2 and
283 PKD x EE2) and the control samples was completed using the bioconducter package
284 DESeq2 (Love et al., 2014). The Benjamini Hochberg false discovery rate (FDR)
285 correction was applied with the criteria for identifying DEGs an adjusted p value
286 <0.05 (Benjamini and Hochberg, 1995). No threshold of log-2-fold changes was
287 applied due to low amount of DEGs in EE2 only group.
288
289 Gene ontology analysis for the list of DEGs was performed to identify their
290 prevalence in biological processes (BPs) with Enrichr
291 (http://amp.pharm.mssm.edu/Enrichr/), with the Fisher exact test P-value set to
292 <0.01. Enrichr, as per available all pathway analysis tools, is a database based on
293 human genes and pathways and does not contain rainbow trout information. This
294 analysis was therefore performed after converting the rainbow trout genes into their
295 human equivalents. BPs were visually summarized using REVIGO
296 (http://revigo.irb.hr/). For the interpretation of biologically relevant genes, we
297 individually inspected the DEGs and placed genes into three key broad functional
298 categories of interest using the PANTHER (protein annotation through evolutionary
299 relationship – using the model organism zebrafish *Danio rerio*) classification

300 system (http://www.pantherdb.org/) and current literature. The categories were 1)
301 Immune response related 2) Inflammation associated i.e. genes involved in
302 apoptosis, autophagy and extra cellular matrix (ECM) components and 3) Metabolic
303 processes. Genes were found with functions outside of these categories as well as
304 the entire full datasets of DEGs are available in the supplementary material
305 (Supplementary Table S1 A-C).
306
307 **2.7. Statistical analysis**
308 Here we report on and test statistically the samples used for sequencing, these were
309 the same samples that were used for the determination of pathological assessment,
310 posterior kidney somatic index, body weight, condition index and parasite intensity
311 this resulted in a sample size of n=3 per condition. The rationale for this was to
312 enable a comparison between the results of these proxies with the transcriptomic
313 data allowing us to provide both a molecular and organism-based assessment of the
314 host responses to the stressors. Hence, if we included extra samples in the proxy
315 assessment, we would not have the sequencing data to make the comparison. The
316 differences between experimental groups were tested for using a one-way ANOVA
317 and significant differences revealed with the post-hoc test of Dunnett's. Data failing
318 normality tests and displaying heterogeneity of variance was tested statistically
319 applying the non-parametric Kruskal Wallis ANOVA on ranks, and Dunn's non-
320 parametric multiple comparison tests to reveal differences. The differences between
321 infection groups in parasite intensity (PKD vs PKD x EE2) were tested for significant
322 differences using a t-test. Statistical analyses were performed using GraphPad Prism
323 5 (GraphPad Software, CA, USA) or in SigmaPlot 12.0 (Systat Software, San Jose,
324 CA, USA) and graphically presented with GraphPad Prism 5. Significance was set at
325 $P< 0.05$. Heatmaps were created using R (Team, 2013).
326
327 **3. Results**
328 **3.1. Organism-level assessment**
329 None of the treatments significantly increased mortality in comparison to controls
330 (experiment average - 6 ± 2.7% (Mean± S.E)). The EE2 exposure did not induce
331 any pathological changes, whereas the parasite induced alterations consistent with
332 PKD infection. Semi-quantitative scoring of the histological responses confirmed that
333 the PKD only treatment had a higher score in both presence and distribution of

334 parasites and tissue proliferation than the PKD x EE2 treatment (Fig 1a). One of the
335 histopathological features assessed in the trout kidney was the presence of fibrous
336 tissue, which is generally associated with the recovery phase towards PKD
337 resolution. No fish in the PKD-only group was found with increased fibrous tissue
338 (score 0). In the PKD x EE2 group, only one individual displayed increased presence
339 of fibrous tissue (score 2). In the uninfected control groups, *T. bryosalmonae* was
340 undetectable (neither by histology or qPCR), thus neither tissue proliferation nor
341 fibrotic changes were observed (Fig. 1a-b). No significant differences were found
342 between the experimental groups regarding the posterior kidney somatic index and
343 Fulton's condition factor. The weight of fish in parasite exposed groups was
344 markedly lower than those not exposed to *T. bryosalmonae* with a significant
345 decrease in body weight in the PKD-only group in comparison to the respective
346 CTRL group (Fig. 2a-c). Parasite intensity was lower in the PKD x EE2 treatment,
347 but no significant differences were found between the infection groups (Fig. 1b).
348

349 **3.2. Numbers of differentially expressed genes**

350 A number of uncharacterised DEGs in line with the study criteria but with no
351 published symbol or determined orthologue, i.e. genes that are constructed as LOC
352 were identified. However, as no functional information exists on these genes, they
353 are not further discussed. This resulted with 280 (PKD), 14 (EE2) or 444 (PKD x
354 EE2) DEGs significantly up or downregulated. Of these, the infection groups (PKD
355 and PKD x EE2) shared 187 DEGs (Fig 3). However, the PKD and EE2 groups
356 shared no DEGs. The number of DEGs was much greater in the multiple stressor
357 group, although the ratio of upregulated DEGs to downregulated DEGs was
358 comparable in the PKD and PKD x EE2 groups.
359

360 **3.3. Gene ontology enrichment analysis of biological processes**

361 The ten most significantly upregulated and downregulated gene ontology (GO)
362 classes are shown for each exposure group (Fig. 4). In both infection groups (PKD-
363 only and PKD x EE2) the top two BPs observed from the significantly upregulated
364 and downregulated DEGs relative to the unexposed controls were the same
365 (upregulated - fatty acid oxidation and fatty acid catabolic processes, downregulated
366 - rRNA processing and ribosome biogenesis) (Fig. 4). Concerning GO classes
367 enriched with downregulated DEGs in the PKD group, the major pathways were

associated with RNA processing and/or synthesis and chromatin organisation (Fig. 4). In the PKD x EE2 the BPs identified from the downregulated DEGs included those involved in RNA processing and/or synthesis but also several immune regulation pathways such as positive regulation of leukocyte cytotoxic cell processes and various natural killer cell positive regulation pathways. Collectively, in the infection groups many key BPs were shared, however, there seemed to be a greater amount of immune response pathways regulated in the PKD x EE2 condition. Concerning the EE2 group, surprisingly, the most significant downregulated BP was cellular response to organic substance (any process that causes a change in state or activity of a cell in terms of movement, secretion, enzyme production, gene expression, etc. as a result of an organic substance stimulus). Moreover, in this group the top 10 BPs seemed to have a greater diversity in contrast to the other experimental groups, probably due to the low amount of DEGs identified in this group.

The DEG enrichment analysis was supplemented using REVIGO (Fig. 5). The subsequent scatter plots show the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities (Supek et al., 2011). This analysis further confirms that metabolic processes in both infection groups were the most dominant BPs (Fig. 5). Concerning the EE2 condition owing to the low amount of DEGs (14) and their diversity after removing redundant GO terms the REVIGO enrichment analysis resulted in no cluster representatives.

3.4. Biological interpretation of transcriptomic changes

Here is a summary of the significant up or downregulated DEGs found in the present study system. The list of DEGs was checked for biological interpretation focusing on three broad categories of interest based on GO BPs from web-based tools (PANTHER) and current literature. The chosen categories were: 1) Immune response related 2) Inflammation associated, i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components and 3) Metabolic processes. In the following sub-sections, we work through each experimental group reporting the DEGs identified using the reported experimental criteria. A full list of all the genes

401 identified in this study and supporting data is available in the supplementary material
402 (Supplementary Table S1 A-C).
403

404 **3.5. PKD group**

405 **3.5.1. Immune response related**

406 Overall, 36 of the DEGs were linked directly to the immune response. Of these 36,
407 intriguingly only one was upregulated (sigirr) while the other 35 were downregulated.
408 Concerning the innate immune response, no markers of myeloid lineage were
409 among the DEGs. Several chemokines, ccl4 (chemokine (c-c motif) ligand 4), cxcf1a
410 and ccl13 (Small inducible cytokine A13) were downregulated. Regarding cytokine
411 gene expression modulation, the signal transducer and activator of transcription 3
412 (stat3), which acts as a key regulator of cytokine signalling pathways, was
413 significantly downregulated, as were several interleukins involved in a broad range of
414 host processes. These included il-1ra (interleukin-1 receptor antagonist) and il-1rii
415 (interleukin-1 -receptor two). il-1ra inhibits il-1, and its pro-inflammatory activities
416 (Zou and Secombes, 2016). However, not just pro-inflammatory cytokines
417 associated genes were downregulated compared to the control group, but also il-6rα
418 (interleukin-6 receptor alpha. High level expression of il-6ra is critical for il-6
419 responsiveness, which has both pro and anti-inflammatory properties (Costa et al.,
420 2011; Zou and Secombes, 2016). In addition, il4/13a, a th2-like signature cytokine
421 that has a role in increasing the amount of IgM secreting B cells was downregulated
422 (Wang et al., 2016) (Fig. 6b). This expression pattern corresponds with the
423 downregulation of all the T and B cell associated DEGs of the PKD only group in this
424 study (Fig. 6c). The only significantly upregulated immune response related gene
425 was sigirr, which encodes for the single Ig il-1-related receptor and is suggested to
426 acts as a negative regulator of immune processes in mammals (Campesato et al.,
427 2017).
428

429 **3.5.2. Inflammation associated: Autophagy, Apoptosis and ECM components**

430 pink1 (PTEN-induced kinase 1) a molecule involved autophagy processes in fish
431 (Zhang et al., 2017) was significantly upregulated. Whereas birc5 (baculoviral
432 inhibitor of apoptosis repeat-containing 5) and cflar (CASP8 and FADD like
433 apoptosis regulator), genes involved in aspects of apoptosis, were significantly
434 downregulated (Fig. 7). Regarding molecules linked to ECM components, three

435 DEGs were found; only one of these, mmp16 (Matrix Metallopeptidase 16) - was
436 significantly upregulated (Fig. 7). MMPs are a major group of proteases that are
437 important for ECM degradation in fish (Pedersen, et al., 2015).
438
439 cd63 and cd44, which were considered as ECM markers were both downregulated.
440 cd63 is a known tetraspanin family member in rainbow trout (Castro et al., 2015) and
441 cd44 has been reported as a tetraspanin associated protein in mammals (Zou et al.,
442 2018) (Fig. 7). Tetraspanins in mammals modulate the function of proteins involved
443 in all determining factors of cell migration including cell–cell adhesion, cell–ECM
444 adhesion, cytoskeletal protrusion/contraction, and proteolytic ECM remodelling
445 (Jiang et al., 2015).
446
447 **3.5.3. Metabolic processes**
448 In stark contrast to the DEGs reported in the immune response section, we observed
449 significant upregulation of all the genes associated with metabolic linked function in
450 the PKD only group. Functions of these genes include metabolic generalists (dcxr-
451 dicarbonyl and l-xylulose reductase), molecules with roles in the fatty acid
452 metabolism (acsf2 - Acyl-CoA synthetase family member 2, mitochondrial, pecr-
453 peroxisomal trans-2-enoyl-coa reductase), histidine catabolism (amdhd1-
454 amidohydrolase domain containing 1) and gluconeogenesis (pck1-
455 phosphoenolpyruvate carboxykinase 1). Pck1 was the most strongly upregulated
456 gene which is a key regulator of gluconeogenesis (Fig. 8).
457
458 **3.6. EE2 group**
459 Only 14 DEGs were found in the EE2-only exposed group, when compared to the
460 untreated control, thus results were analysed as a single entity (Fig. 9). Concerning
461 immune genes il-21r (interleukin 21 receptor) was downregulated. ddit4 (DNA-
462 damage-inducible transcript 4), a molecule that in mammals is involved in varied
463 biological processes such as antiviral activities and apoptosis was upregulated.
464 cyp1b1 (Cytochrome P450 1B1), which belongs to the Cytochrome P450 superfamily
465 of enzymes and functions to metabolize potentially toxic compounds was
466 downregulated (Uno et al., 2012). sult1a4 (sulfotransferase family 1a member 4) and
467 prodh2 (proline dehydrogenase 2) genes, which have metabolic functions were
468 significantly upregulated. Other upregulated genes included amh (muellerian-

469 inhibiting factor), which has a role in the fish gonadotropin-releasing hormone
470 receptor pathway and oplah (5-oxoprolinase, atp-hydrolysing), which has a role in
471 ATP binding. Other downregulated genes were fd6d (putative delta 6-desaturase-),
472 and genes involved in diverse pathways, from transmembrane trafficking (pik3r4 -
473 phosphoinositide-3-kinase regulatory subunit 4), transcriptional regulation (jun- jun
474 proto-oncogene), cytoskeleton junctions (ptpn4-protein tyrosine phosphatase non-
475 receptor type 4), biomineralization (fam20a- golgi associated secretory pathway
476 pseudokinase), RNA binding (dhx30- dexh-box helicase 30), and protein
477 ubiquitination in mammals (kbtbd3- Kelch repeat and BTB (POZ) domain-containing
478 3).
479
480 **3.7.PKD x EE2**
481 **3.7.1. Immune response related**
482 In the stressor combination group, 62 DEGs were associated with the immune
483 response. Out of these 7 were upregulated and 55 downregulated. Regarding the
484 innate immune response, markers of the myeloid cellular lineage; slc11a1 (formerly
485 known as natural resistance-associated macrophage protein1, nrampα) and mcsf1
486 (macrophage colony stimulating factor precursor) were significantly upregulated.
487 Likewise, kit (kit proto-oncogene receptor tyrosine kinase), the mast/stem cell growth
488 factor receptor was also significantly upregulated. However, several chemokines
489 including ccl4, cxcf1a and ccl13 were downregulated (Fig. 10a). Regarding genes
490 associated with the complement system, cfi (complement factor 1) was upregulated
491 and actually had the strongest expression of any of the immune genes in this
492 experimental group, although other molecules involved in the complement masp1
493 (mannan binding lectin serine peptidase 1) which functions in the lectin pathway of
494 complement and c4 (Complement component 4) were downregulated (Fig. 10b).
495
496 Regarding cytokine gene expression modulation, there were 15 DEGs identified all
497 of which were downregulated. In this group, we also included nmi (N-Myc (And
498 STAT) Interactor) as it closely interacts with the cytokine mediator stat1 (also
499 grouped here). Aside from nmi, 13 interleukins involved in a wide range of innate and
500 adaptive processes were significantly downregulated (Fig 10c). In addition, several
501 genes involved in a diverse array of immune processes such as interferons (irf8 and
502 irf-1) and toll-like-receptors (tlr7 and tlr22) were significantly downregulated.

503 Concerning the expression patterns of genes involved in adaptive immune
504 processes, for the B cell lineage only bcl6b (B cell CLL/lymphoma 6B) was
505 significantly upregulated. In fact, no other genes that were considered solely B cell
506 specific markers i.e. only molecules, which could be considered to be associated
507 with general lymphocyte processes (both B and T cells related) were differentially
508 expressed in this group (Fig. 11).
509
510 A large number of genes (17) encoding with diverse aspects of T cells functional
511 activity were observed in this study. Out of these genes, only notch was upregulated.
512 Notch is part of the notch-signalling pathway; a highly conserved cell signalling
513 system that in mammals is involved in multiple processes and has been suggested
514 to be is essential for initial commitment to the T cell lineage and may function
515 together with signals from the pre-tcr and the tcr to regulate subsequent steps of T
516 cell development (Deftos and Bevan, 2000; Guidos, 2006). The genes
517 downregulated that were markers of different T cell processes included many well-
518 studied molecules in fish immunology: tbet, cd8a, foxp3-1, foxp3-2, cd3e and mhcII.
519 A gene not grouped to any of the mentioned immune response pathways here: sigirr,
520 as in the PKD only group, was also significantly upregulated (0.8 in PKD x EE2 vs
521 0.8 in PKD only - log 2fold change).
522

523 **3.7.2. Inflammation associated: Autophagy, Apoptosis and ECM components**

524 DEGs involved in the autophagic processes in the combined stressor group included
525 ddit4l (DNA Damage Inducible Transcript 4 Like) and ddit4 which had different
526 expression profiles, with ddit4l downregulated and ddit4 upregulated. While all of the
527 transcripts associated with apoptosis activity were downregulated (cflar, birc5, casp8
528 - - caspase 8, sap30bp - sap30 binding protein). The transcriptional signatures of the
529 ECM component linked DEGs in this experimental group were similar to the PKD
530 only group in that cd63 (-0.69 in PKD x EE2 vs -0.74 in PKD only - log 2fold change)
531 was downregulated and mmp16 was upregulated (0.78 in PKD x EE2 vs 0.7 in PKD
532 only- log 2fold change. However, additional DEGs that encode for ECM constitutes
533 were also identified in the PKD x EE2 group such as (pxdn- peroxidasin, fgfr2-
534 fibroblast growth factor receptor 2, igtb1bp1- integrin beta-1-binding protein 1 and vit-
535 vitrin) with vit, pxdn and fgfr2 significantly upregulated. Indicating a stronger

536 presence of ECM components in this infection group in contrast to the other
537 conditions (Fig.11).
538
539 **3.7.3. Metabolic processes**
540 Similar to the PKD only group, almost all of the DEGs involved in metabolic functions
541 were upregulated. The only gene downregulated was npc2 (NPC intracellular
542 cholesterol transporter 2) which is involved in many different biological processes of
543 the metabolism including the cholesterol metabolism, lipid metabolism, lipid
544 transport, steroid metabolism and the sterol metabolism. DEGs with metabolic
545 functions that were upregulated-included pck2, which is, involved in the biological
546 process gluconeogenesis, as well acsf2 and acaa1 (acetyl-CoA acyltransferase 1)
547 which are involved in the fatty acid metabolism and amdhd1, which is involved in the
548 histidine catabolism (Fig. 12).
549
550 **4. Discussion**
551 In the present study, transcriptome profiling of the rainbow trout posterior kidney was
552 undertaken at 130 d.p.i to 1) obtain a broader overview on the host response to *T.*
553 *bryosalmonae* infection (using RNA-seq in contrast to pre-selected genes via RT-
554 qPCR), and 2) to greater understand how the combined impact of an estrogenic
555 contaminant (EE2) modified the host response to *T. bryosalmonae* infection. We
556 hypothesised that EE2 exposure will enhance rather than supress the host response
557 to parasite infection. In the *T. bryosalmonae* infected fish we found a less intense
558 immunological response in contrast to the published literature (see Abos et al., 2018;
559 Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). However, these
560 studies used either functional or RT-qPCR approaches in contrast to RNA-seq in the
561 present study. In the PKD x EE2 treated fish we found a more intense transcriptomic
562 response in comparison to the other conditions in terms of the amount of DEGs
563 upregulated in key physiological responses during *T. bryosalmonae* infection,
564 additionally both parasite intensity and pathology were reduced in comparison to the
565 PKD treatment. While posterior kidney somatic index, body weight and condition
566 factor were comparable in the two infection groups. Furthermore, concerning both
567 infection groups, many of the major BPs identified from upregulated and
568 downregulated DEGs were shared. However, there was a greater amount of immune
569 response related and inflammation associated genes upregulated in the PKD x EE2

570 group, although the regulation of metabolic genes was comparable between both
571 infection groups. While in the EE2 group, we found a very low amount of DEGs with
572 high diversity of BPs. Overall, in our study system the estrogen appeared to have no
573 negative suppressive action, but a partly enhancing action on *T. bryosalmonae*
574 infected fish.
575
576 The present study is the first of our knowledge to investigate the host response at a
577 late disease phase during *T. bryosalmonae* infection. Here we identified genes
578 involved in a variety of metabolic functions and tissue inflammation resolution and
579 repair processes, not just in the PKD group but in the PKD x EE2 group as well that
580 suggest the fish downscales its investment in the immune response and triggers the
581 recovery/resolution process. We show in both infection groups a negative
582 correlation: an upregulation in metabolic genes and a downregulation in immune
583 genes. This negative correlation could indicate a trade-off of priorities at the late
584 stage of infection whereby the host increases investments in one function (energy
585 and intermediary metabolism) leading to decreased investments in another function
586 (immunity). Life-history theory predicts trade-offs occur between different traits such
587 as growth and maintenance, reproduction and immunity (Zera and Harshman, 2001).
588 In the infection groups fish had a lower weight when compared to those not infected,
589 thus it could be speculated that this occurred due to an investment in the immune
590 response. At the investigated stage with parasite burden decreasing there may be a
591 change in host priorities to address this.
592
593 Previous immunological investigations of *T. bryosalmonae* infection in salmonids has
594 led to a description of the disease as an immunopathological condition mediated by
595 decreasing myeloid cells and increasing lymphocytes, in addition to complex back
596 and forth interplay between th-like subsets (Abos et al., 2018; Bailey et al., 2017a;
597 Bailey et al., 2017b; Bailey et al., 2019; Gorgoglione et al., 2013). In the present
598 study, relating to the PKD only treatment, our investigation performed at a late stage
599 of infection using an RNA-seq approach resulted in a different outcome concerning
600 the immune repertoire used by rainbow trout. The outcome was different in two
601 dimensions, in terms of intensity, and, in terms of the mechanisms of the immune
602 response. Many pathways previously reported to play a role in the PKD
603 pathogenesis were downregulated or unresponsive in the present study. The less

604 intense immune response observed in the infection groups might indicate that the
605 host never had to use this immune response, as the disease was not severe enough
606 or that the reduction in infection pressure at the later time point allowed the host to
607 shift priorities away from immunity and reallocate resources to other physiological
608 functions. For instance, the regulation of genes that are involved infection resolution
609 and recovery or metabolic processes. In the study by Wernicke von Siebenthal and
610 colleagues (2018), using data from the same experiment collected at an earlier time
611 point (90 d.p.i) demonstrated strong upregulation of il-10, blimp1 and IgM sec in both
612 infection groups. This would confirm that a more intense immune response did occur
613 at an earlier PKD phase. This response was consistent to previous studies of PKD
614 as per (Bailey et al., 2017a; Bailey et al., 2017b; Gorgoglione et al., 2013). This
615 suggests that the host switched priorities away from the immune response in the
616 present study.
617
618 Surprisingly, in the PKD-only group there was only one immune gene significantly
619 upregulated relative to the control, the single Ig and tir domain containing (sigirr).
620 Concerning studies of this gene in fish, sigirr was not found in the green spotted
621 pufferfish, *Tetraodon nigroviridis*, three-spined stickleback, *and Gasterosteus*
622 *aculeatus*, or Japanese puffer, *Takifugu rubripes* genomes. Instead, a double ig il-1r
623 related molecule (digirr) was found. In the same study phylogenetic analysis
624 revealed that digirr was homologous to sigirr, but distinct in both protein structure
625 and subcellular localization. *In vivo* and *in vitro* functional characterization indicated
626 that digirr acted as a negative regulator of il-1 mediated signalling (Gu et al., 2011).
627 In mammals sigirr is (known as il-1r8) acts as a negative regulator of toll-like and il-1
628 receptor family signalling, with its upregulation suggested to contribute to an
629 impaired innate immune sensing and the development of an antitumor immune
630 response (Campesato et al., 2017). However, as the expression of pro-
631 inflammatory cytokines in previous PKD studies has been shown to be only
632 transient or downregulated (Bailey et al., 2017a; Gorgoglione et al., 2013; Holland
633 et al., 2003), sigirr expression is probably not unique to this disease stage studied
634 here. Moreover, it could be plausible that sigirr may even be a target for parasite
635 manipulation in early stage infection as an immune evasion tactic, given its
636 negative modulation of the innate response and its reported role in innate immune
637 sensing.

638 Concerning the lack of a stereotypical PKD dominant B cell response, at 130 d.p.i;
639 this raises the question in the present study that the time point investigated was too
640 late and we missed this response. Corresponding to this, parasite intensity had
641 already plateaued at 90 d.p.i, thus the lack of a B cell response correlates to the
642 overall reduced intensity of infection from both a host and parasite perspective.
643 Notwithstanding, if we did not see a dominant lymphoid driven response it could be
644 speculated that there may be some indication or homeostatic rebalancing of the
645 myeloid arm of the immune response, given its described unresponsiveness during
646 clinical infection, but we did not see this here. It could be that this transpired at an
647 earlier time point or that it occurred via the downregulation or unresponsiveness of
648 the B and T mechanisms reported here. To really address such a question would
649 require a longitudinal approach with a greater amount of sequential data as well as
650 the inclusion of functional data. We only found upregulation of one specific B cell
651 marker; bclb6 occurring in the PKD x EE2 condition. bclb6 and both blimp1 are key
652 regulators of B cell terminal differentiation in mammals. Functional characterization
653 of these genes in fugu showed that they also regulate B-cell terminal differentiation
654 also in fish (Ohtani and Miyadai, 2011). Taking this into account and given the
655 reported role of blimp1 in PKD pathogenesis of both rainbow trout and brown trout in
656 the studies by Bailey et al., in 2017 and 2019, this molecule (bclb6) may be of
657 interest to future studies of B cell differentiation in fish.
658
659 While concerning the T cell response of interest in the PKD x EE2 was the
660 downregulation of tbet, cd8a, foxp3-1, foxp3-2, cd3e and mhcII. While PKD
661 mediated suppression of innate immune aspects has been reported (Chilmonczyk et
662 al., 2002). All of these genes (apart from cd3e) have been shown to be upregulated
663 in the rainbow trout immune response to *T. bryosalmonae* (Bailey et al., 2017a;
664 Gorgoglione et al., 2013). Thus, the downregulation of these genes might suggest
665 that PKD x EE2 is having a stronger immunosuppressant role than in PKD alone.
666 This would corroborate the study by Burki et al., 2013 who found many immune
667 genes downregulated in the liver after combined *T. bryosalmonae* infection and EE2
668 exposure and suggested a potential immunosuppressive action of EE2 in rainbow
669 trout. However, a greater number of upregulated genes found in our study in the
670 PKD x EE2 treatment suggests the fish can still mount an immune response even in
671 the face of EE2-induced immunosuppression and as also suggested by Burki et al.,

672 2013 this may have actually translated into the decreased parasite intensity and
673 reduced pathological alterations.
674

675 It might be expected that fish exposed to multiple stressors will have a more intense
676 transcriptomic response owing to increased physiological demands in managing the
677 combination of stressors resulting in increased pathogen burden and more severe
678 disease. However, owing to the previous study by Burki et al (2013) that EE2
679 exposure of *T. bryosalmonae* infected fish resulted in decreased parasite burden we
680 predicted that EE2 exposure will enhance rather than supress the host response to
681 parasite infection. The results obtained here would support our prediction as the
682 increased amount and presence of specific DEGs in the PKD x EE2 group correlated
683 with decreased parasite intensity and reduced pathology. Though it must be pointed
684 out, as a possible limitation of this study is that we only used three fish per
685 experimental group in our investigation. Our results raise the question: did EE2
686 enhance the immune response in the PKD x EE2 group? While, it cannot be ruled
687 out that there was a negative impact on the parasite in the present study as both
688 positive and negative impacts of environmental contaminants on the parasite have
689 been noted in other host-parasite systems (Krasnov et al., 2015; Romano et al.,
690 2015). We did have more immune response genes upregulated in the PKD x EE2
691 group at the time point investigated. Nevertheless, given the immunopathological
692 nature of PKD it could be disputed whether such “immunoenhancement” would
693 benefit the host. Consequently, we do not know from the present study if an
694 immunosuppressive response occurred at an earlier time point and if this is what
695 caused the lower pathology in the PKD x EE2 treatment, as decreasing resistance
696 mechanisms through immunosuppression might reduce immunopathology benefiting
697 the host.

698 Several lines of evidence in our study suggest that in the PKD x EE2 condition there
699 is a greater host investment in infection resolution/recovery. In this group, there was
700 an increased amount of DEGs linked to ECM processes relative to the PKD-only
701 group. This might indicate an increased investment in tissue reorganisation in the
702 kidney, through either immunopathological or parasite-induced damages. The
703 expression of genes involved in apoptosis was indeed seen downregulated in both
704 infection groups. It has been well documented that programme cell death plays an

705 important role in inflammation resolution via clearance of immune cells. For instance,
706 failure of apoptosis to clear such cells is a major driver of chronic inflammation (Yang
707 et al., 2015). However, the lack of a strong B or T cell response in the infection
708 groups might indicate that the host may have already undergone a major immune
709 cell clearance event, which would correspond with the observed downregulated of
710 expression patterns of these pathways in our study.

711 Additionally, in the PKD x EE2 group concerning the immune response related
712 genes there was also some indication that the host has further progressed along in
713 the recovery process, for instance through expression of M2 macrophage
714 phenotypes that have 'anti-inflammatory', or 'pro-healing' phenotypes (Grayfer et al.,
715 2018; Martinez and Gordon, 2014; Wiegertjes et al., 2016). In this perspective,
716 slc11a1 (formerly known as nrampα - natural resistance associated macrophage
717 protein alpha) and mcsf-1 were both upregulated in the PKD x EE2 infection group,
718 thus we questioned if these molecules were playing a role in tissue resolution.
719 slc11a1 in mammals is associated with the ability of macrophages to destroy
720 bacterial pathogens (Vidal et al., 1993), however in fish there is no functional data
721 concerning this gene. While mcsf-1 in fish has been associated with classical pro-
722 inflammatory actions, it has also been shown to play a role in macrophage
723 differentiation (Grayfer et al., 2009). Although il4/13a and il10, which are usually
724 suggested to be M2 polarizing agents, were both downregulated in this group which
725 would suggest the contrary (Grayfer et al., 2018; Wiegertjes et al., 2016).
726 Interestingly in the PKD x EE2 mcsf-1 (upregulated), il4/13a and il10
727 (downregulated) expression patterns contrasted to those previously reported in PKD
728 gene screenings (Bailey et al., 2017a; Gorgoglione et al., 2013). In addition, in this
729 group kit (kit proto-oncogene receptor tyrosine kinase) also known as a mast/stem
730 cell growth factor receptor was significantly upregulated. The presence of kit (then
731 called c-kit) on zebrafish mast cells was shown using immunohistochemistry by
732 Dobson et al., 2008. Therefore, expression of this molecule might indicate some
733 involvement of these cells. In fact, fish mast cells are also suggested to play a role in
734 wound healing in fish (Sfacteria et al., 2015). Thus, when piecing together the
735 histology data, the reduced parasite intensity and the transcriptomic results there is a
736 general indication that there is a greater investment in resolution/recovery processes
737 from infection in the PKD x EE2 group.

738 We did not find a strong impact of EE2 on the fish not exposed to the parasite, either
739 from the transcriptomic data or toxicologically. This response may be a tissue
740 specific issue as it has been previously reported that EE2 has major effects on gene
741 regulation in the liver and gonads at the selected concentrations (Shved et al., 2007).
742 In this group, there were only 14 DEGs and only one immune gene was identified il-
743 21r. il-21r transduces the growth-promoting signal of il-21. il-21 is a th17 cytokine
744 and suggested to be a likely regulator of T and B cell functions. The gene has been
745 described as an important player in the rainbow trout immune response against
746 bacteria and viruses as well correlated with increasing parasite intensity in PKD
747 pathogenesis (Gorgoglione et al., 2013; Wang et al., 2011). However, considering
748 the evidence of EE2 in the literature as an immunosuppressor (Cabas et al., 2012;
749 Cuesta et al., 2007; Milla et al., 2011; Rodenas et al., 2015; Saha et al., 2004;
750 Seemann et al., 2016; Seemann et al., 2015; Thilagam et al., 2009) the
751 downregulation of only one immune gene hardly accounts for immunomodulation.
752 Surprisingly, the most significant downregulated BP was cellular response to organic
753 substance. In line with this, cyp1b1 was also downregulated; cy1b1 is part of the
754 cytochrome p450 (CYP450) group of genes that play an important role in catalysing
755 oxidative metabolism of toxicants. Moreover, cyp genes are used as biomarkers of
756 exposure to several organic contaminates found in the aquatic environment (i.e.
757 PAHs and PCBs) (Nebert and Karp, 2008; Pinto et al., 2015; Zanette et al., 2009). In
758 addition, it has also been suggested that estrogens appear to have a modulating
759 effect on the expression of cytochrome P450 genes (Navas and Segner, 2000).
760 Therefore, we would have expected that this gene or other cytochrome p450 family
761 members might be upregulated. However, this could be explained by the fact that the
762 fish may have adapted to chronic EE2 exposure in the present study, as ectotherms
763 have been shown to have great plasticity to maintain key physiological processes
764 under these stressors (Wernicke von Siebenthal et al., 2018), or on the other hand
765 that the gene or one its isoforms are stronger expressed in other tissues as such
766 expression site exclusivity exists for this family of genes in fish (Zanette et al., 2009).
767

768 **3. Conclusion**

769 In this study, we investigated the rainbow trout transcriptome and its relevance for
770 the organism outcome when confronted by both a biological and chemical stressor.
771 We demonstrated that the cumulative impact of EE2 and *T. bryosalmonae* infection

772 as multiple stressors resulted in pleiotropic effects cascading multiple physiological
773 systems not observed in the other experimental groups. We observed post clinical
774 fish show a different molecular signature concerning the immune response, and EE2
775 appears to enhance aspects of the PKD recovery process. We provided evidence for
776 a different molecular signature of the host response during the PKD resolution stage
777 in rainbow trout. In doing this, we were able to build on the existing knowledge
778 concerning the fish response to *T. bryosalmonae* infection. Our results supported
779 earlier studies, that while EE2 could modulate the fish physiology, it might not
780 necessarily act as a stronger immune-suppressor when in concomitance with a
781 chronic immunosuppressant disease.

782 **Funding**

783 This research was funded by the Swiss National Funding, number 31003A_153427
784 awarded to HS.

785 **Acknowledgments**

786 The authors would like to acknowledge Valerie Alexander for improving the English,
787 Hanna Hartikainen for providing the parasites, Johanna Croton for assistance with
788 the graphical abstract and Heidi Tschanz-Lischer for support with the bioinformatics.

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Figures

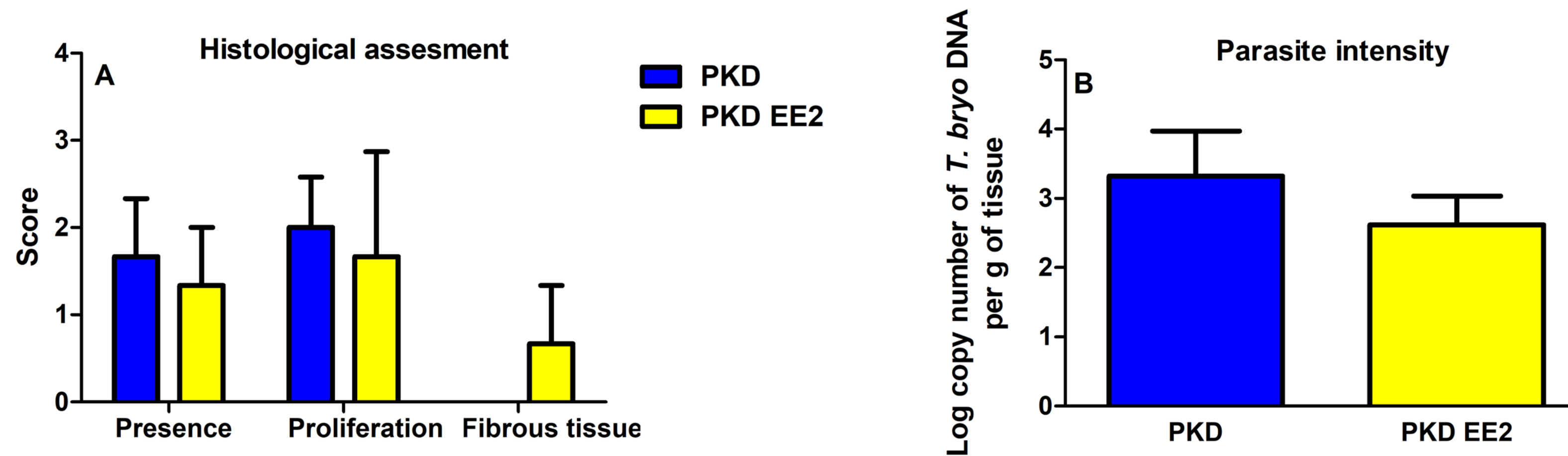

Fig 1. (A) Pathological assessment of the posterior kidney compromising of histopathological scores for presence of parasites, tissue proliferation and fibrous tissue. Different colour bars denote experimental groups. (B) Parasite intensity in PKD and PKD x EE2 conditions. Parasite intensity was determined using copy numbers of parasite DNA per fish standardised using the individual fish kidney weights. No pathological changes or presence of parasites was found in EE2 or CTRL groups. No significant differences were found concerning either parameter $P < 0.05$. N =3.

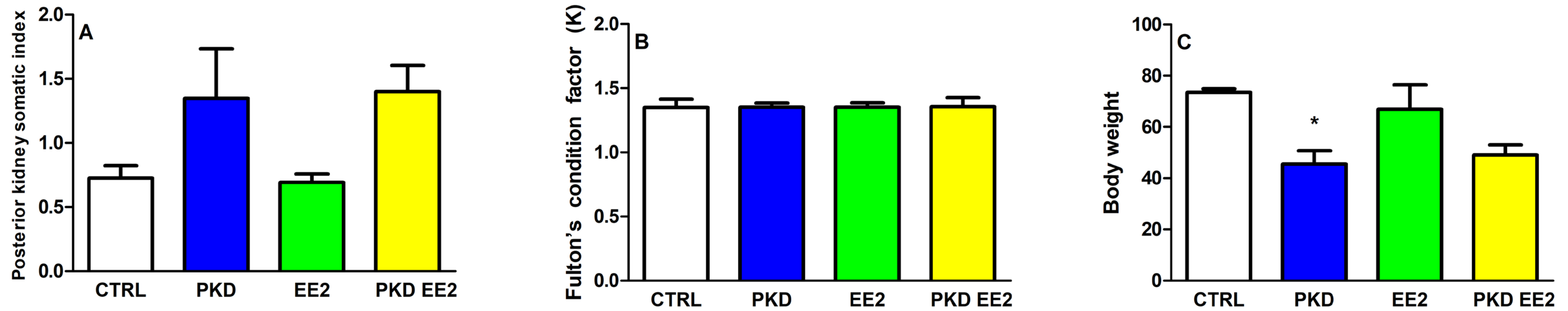

Fig 2. Bar charts showing (A) Posterior kidney somatic index scores (B) Fulton's condition factor (K) and (C) Body weight of all experimental groups. Different colour bars denote different experimental groups. CTRL = white bars, PKD = parasite exposure only (blue bars), EE2 = chemical exposure only (green bars) and PKD x EE2 = parasite and chemical exposure (yellow bars). P < 0.05. N =3.

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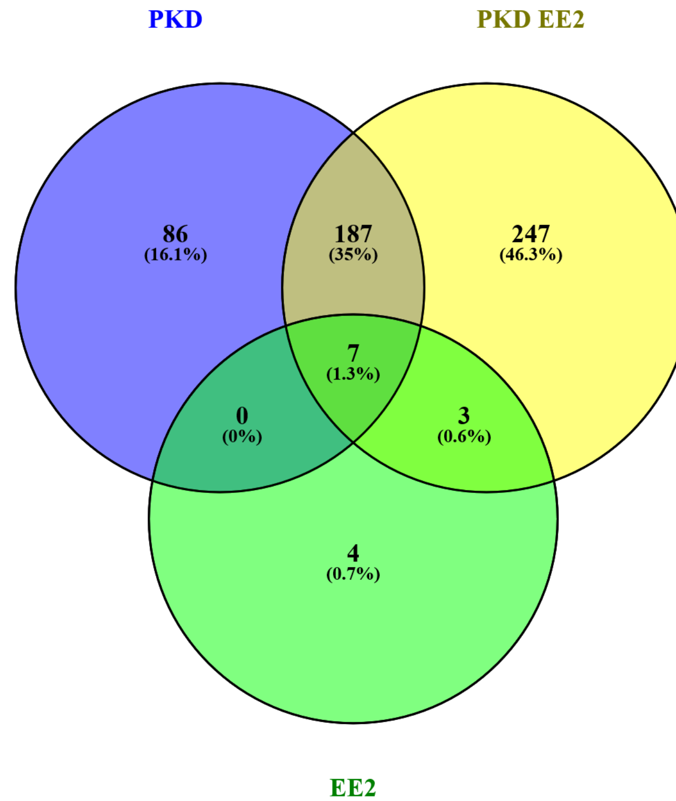
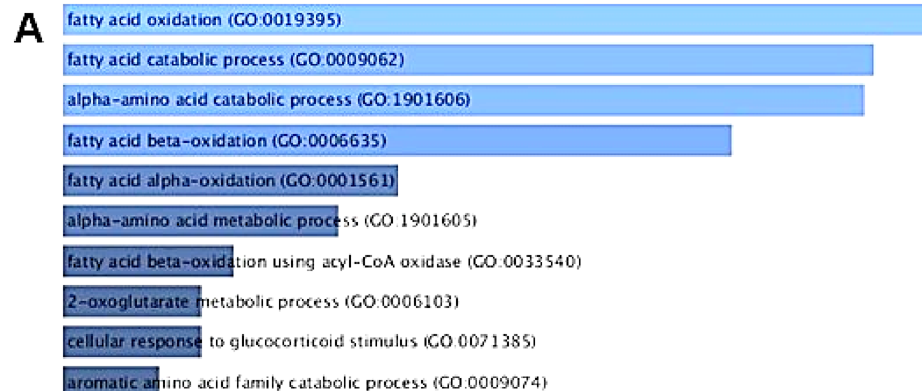
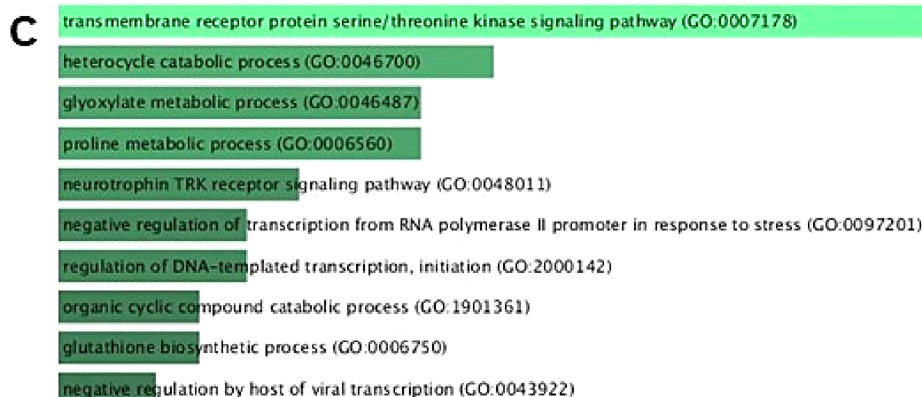
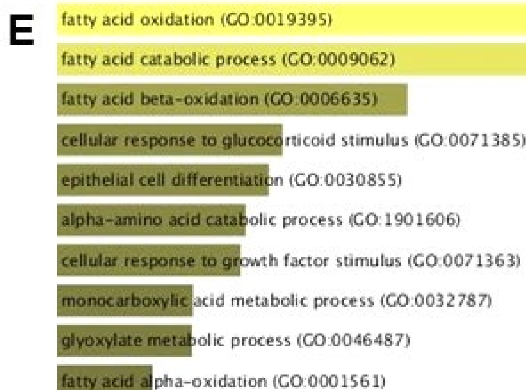


Fig 3. Venn diagram showing the number of differentially expressed genes in the different treatments and intersects between them. PKD = parasite exposure only (blue circle), EE2 = chemical exposure only (green circle) and PKD x EE2 = parasite and chemical exposure (yellow circle).

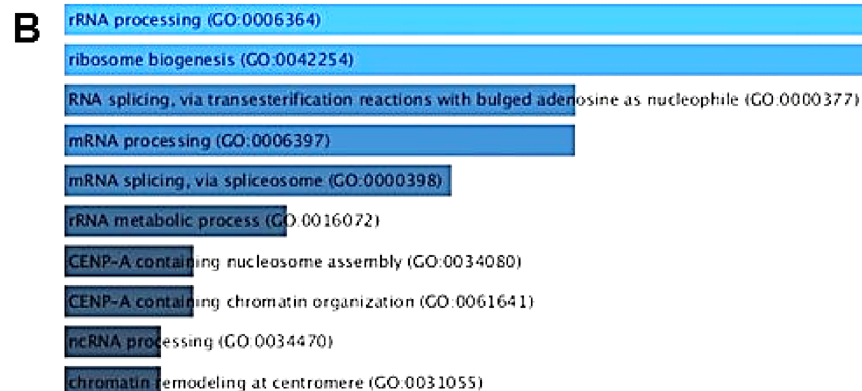
Upregulated GO classes

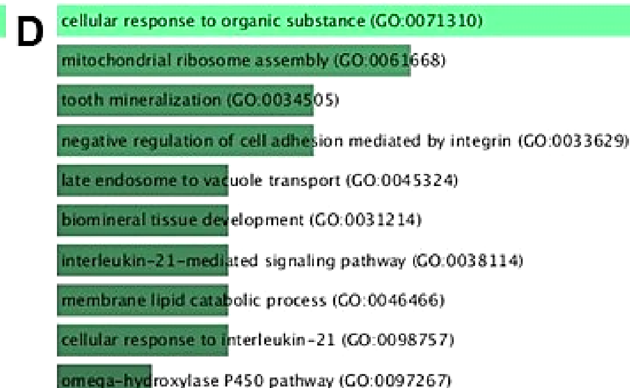






Downregulated GO classes





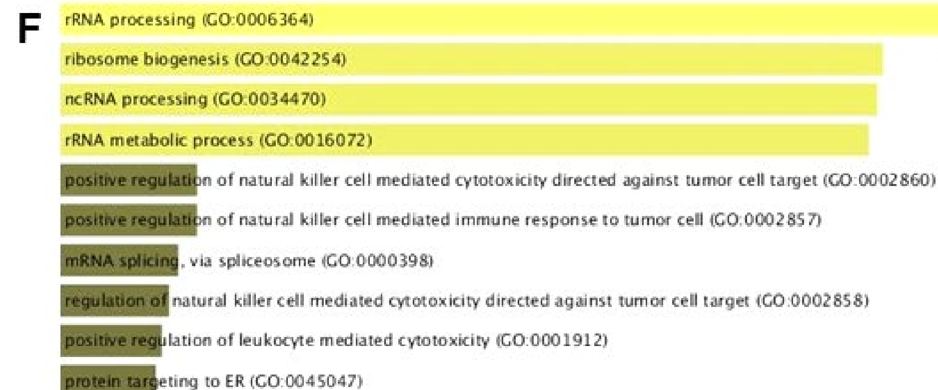
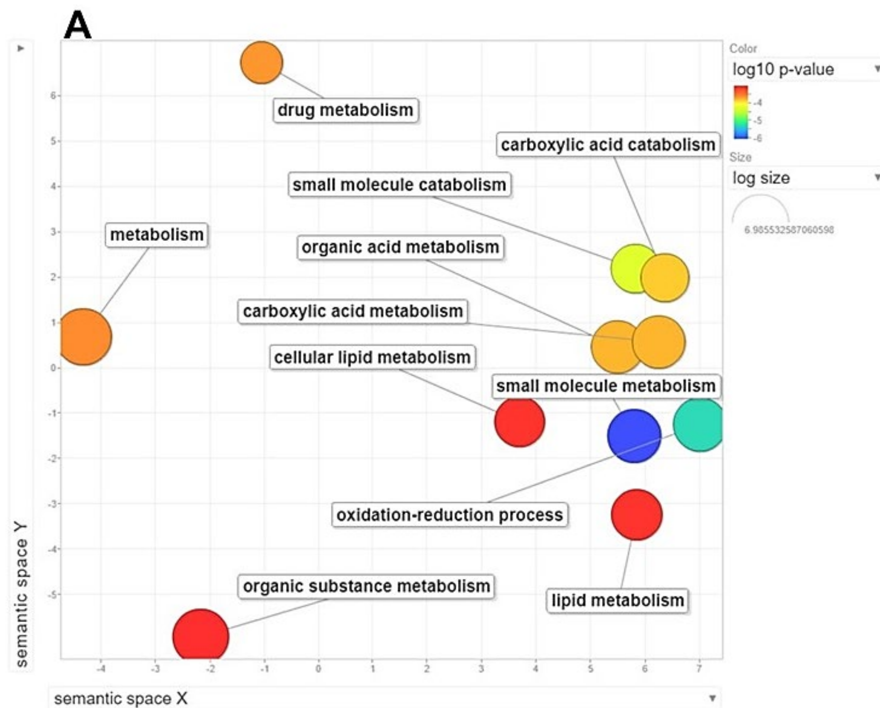


Fig 4. The ten most significantly upregulated and downregulated GO classes were classified according to their involvement in biological processes. All DEGs were subjected to an enrichment analysis via the online gene list analysis tool, *Enrichr*, are in the order of their P-value ranking. PKD = parasite exposure only (blue bars: A – upregulated, B - downregulated), EE2 = chemical exposure only (green bars: C – upregulated, D - downregulated) and PKD x EE2 = parasite and chemical exposure (yellow bars: E – upregulated, F - downregulated).



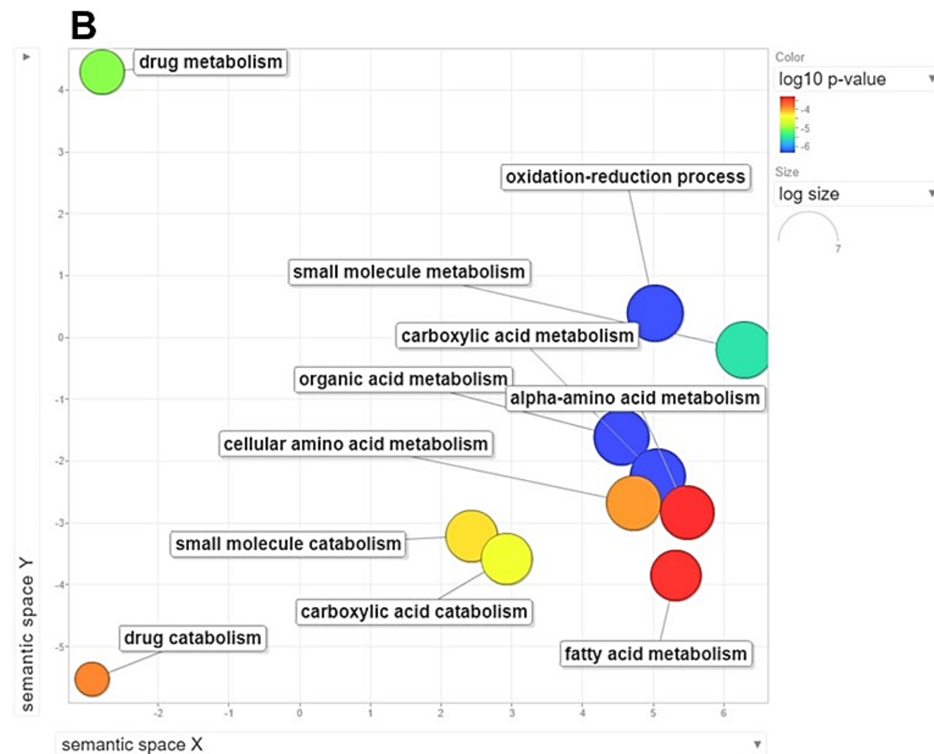
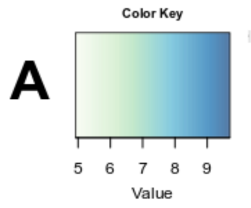
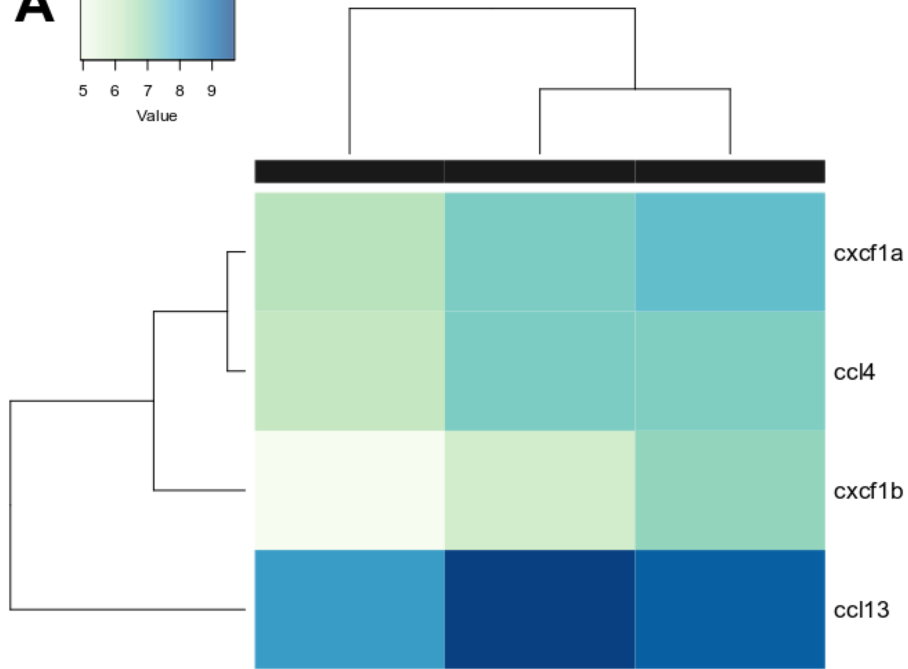


Fig 5. Results of REViGO semantic analysis of GO biological process of A) *T. bryosalmonae* infected fish and B) PKD x EE2 exposed fish. The principle is that semantically similar GO biological processes should remain close together in the plot, but the semantic space units have no intrinsic meaning. Circle colour indicates the p-value for the false discovery rates; circle size indicates the frequency of the GO term in the underlying GO database (circles of more general terms are larger; http://revigo.irb.hr/).

A





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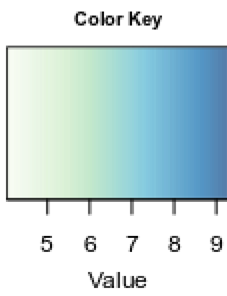
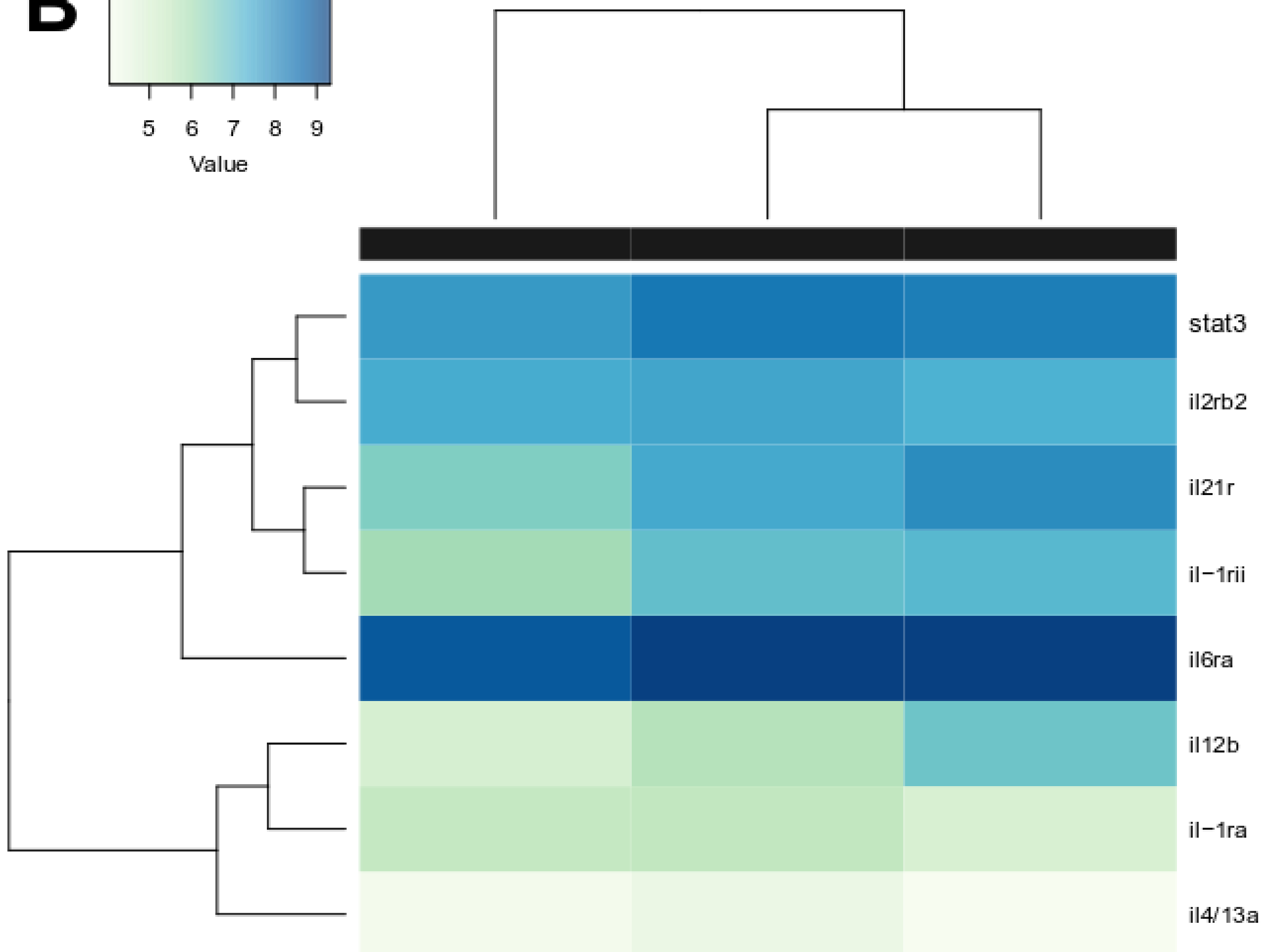




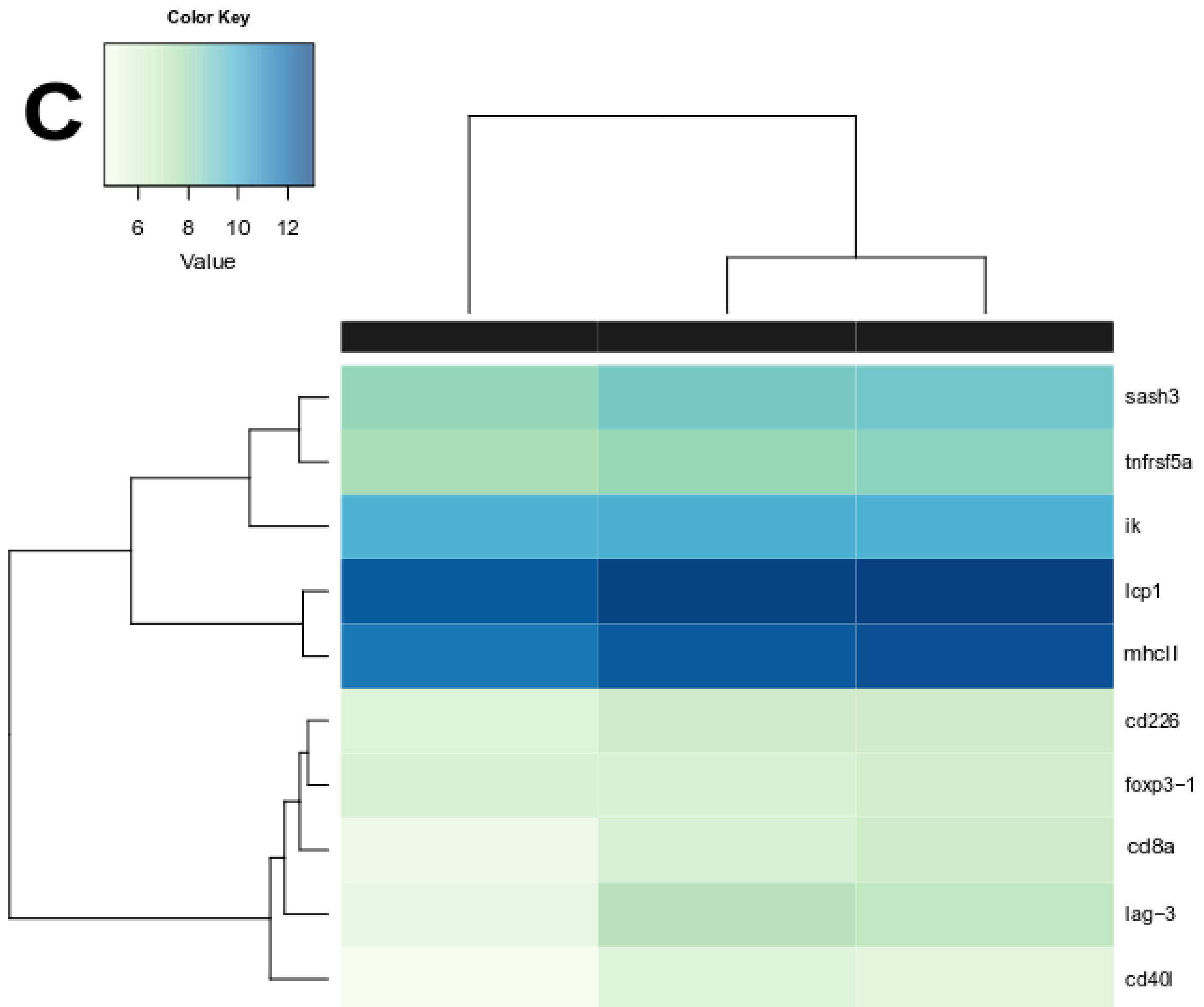


Fig 6. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of A) chemokines; B) cytokines; C) B cell and T cell related genes in rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Some genes in C) exclusive i.e. involved in both B and T cell mechanisms. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

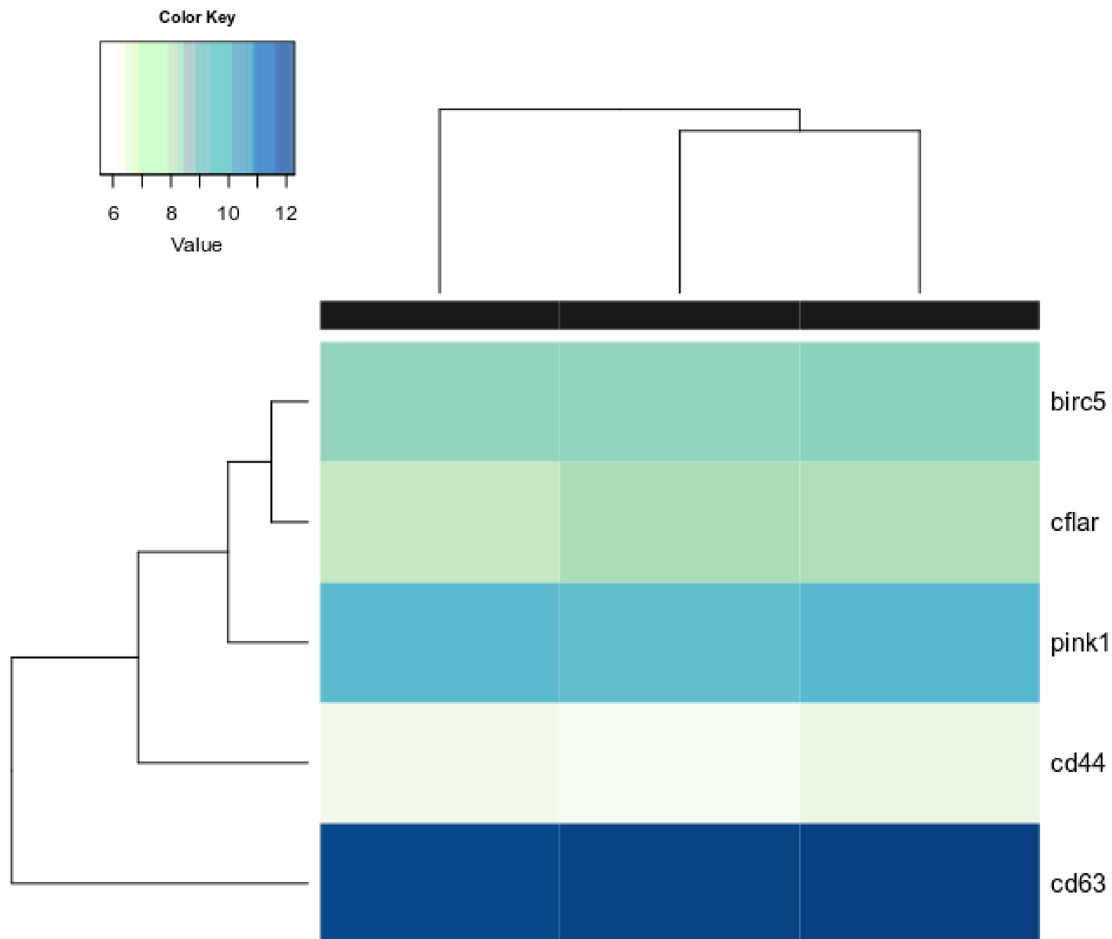


Fig 7. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of inflammation associated genes i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components in the rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

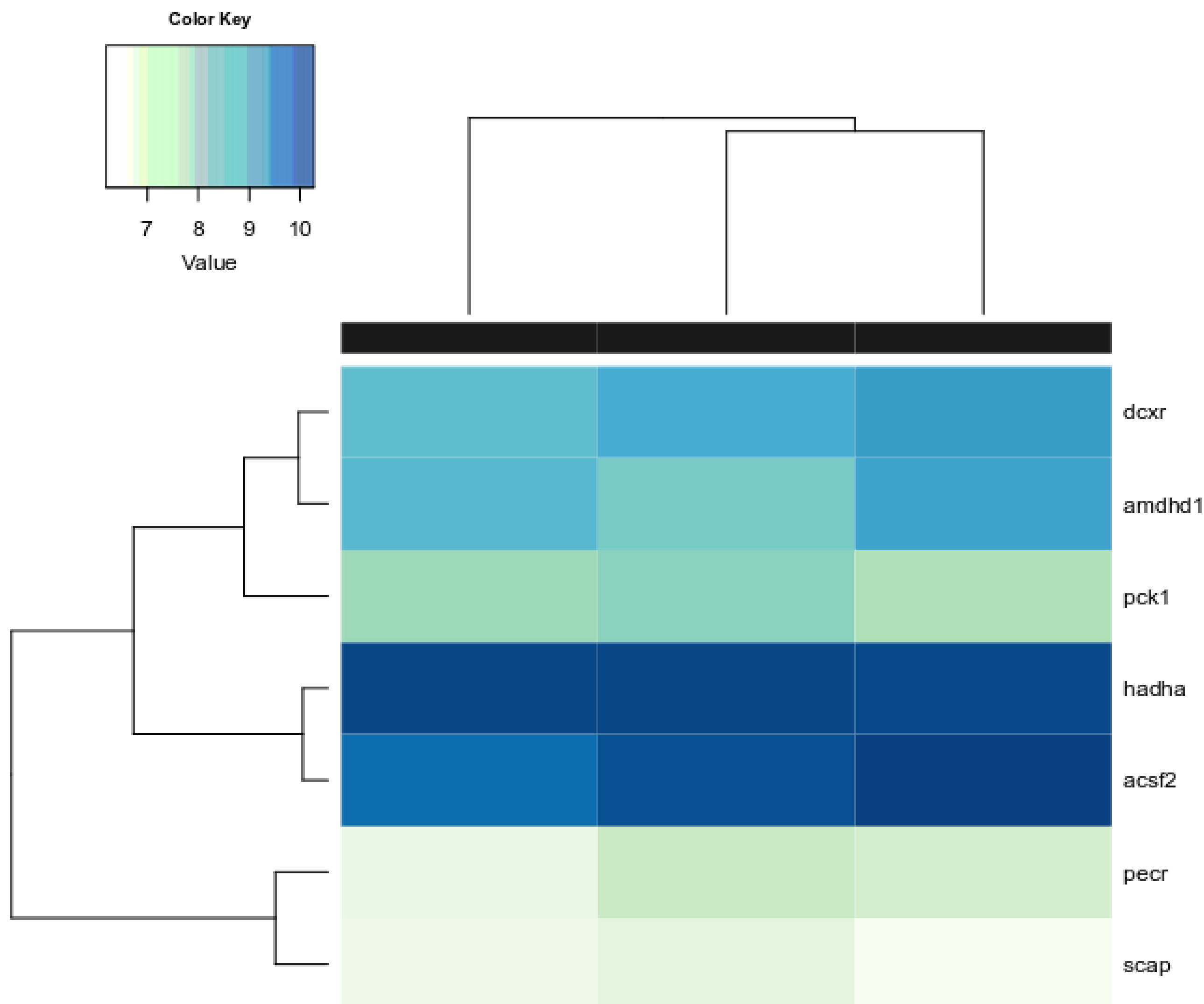


Fig 8. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of genes involved metabolic functions in the rainbow trout posterior kidney of *T. bryosalmonae* infected fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

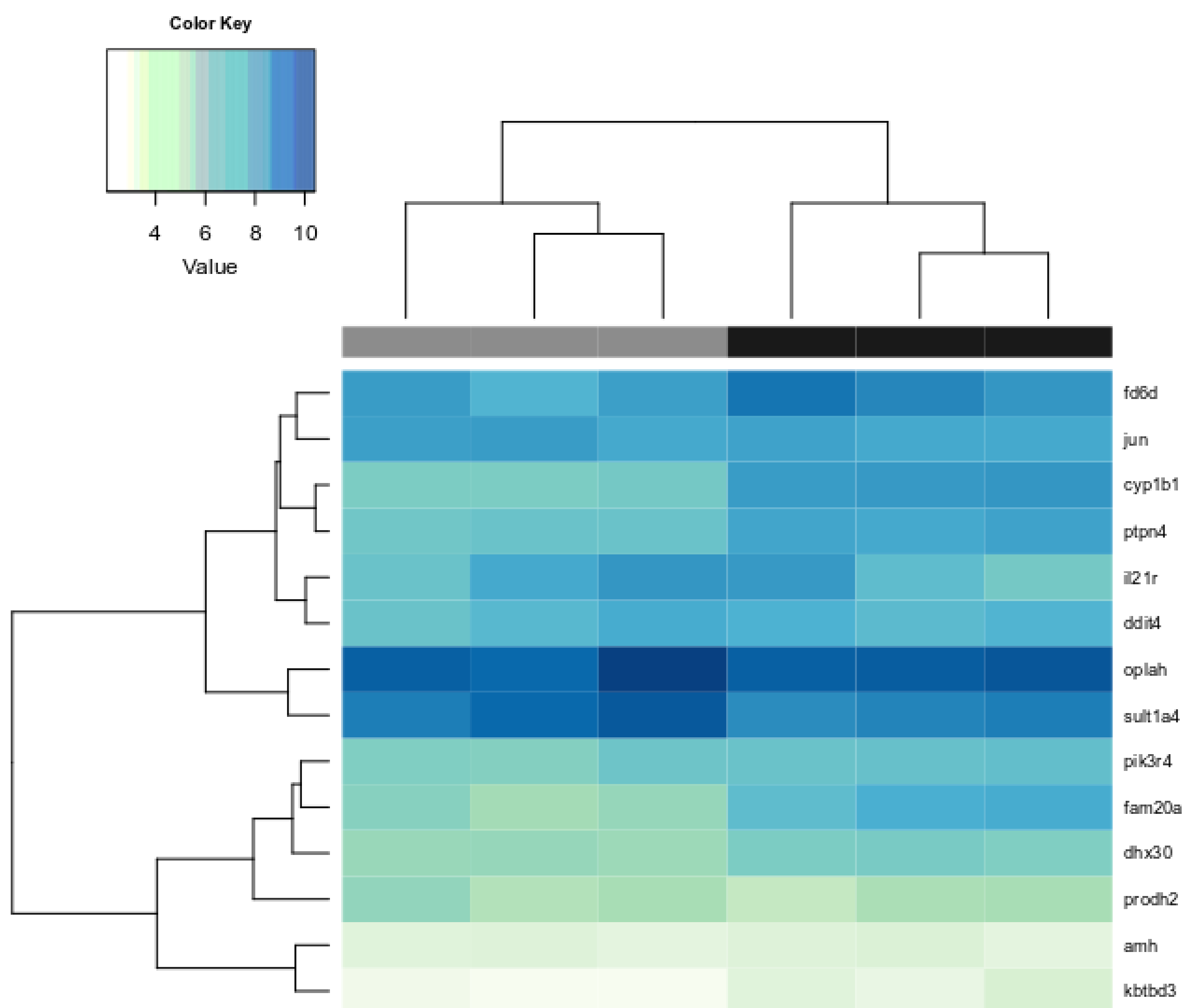
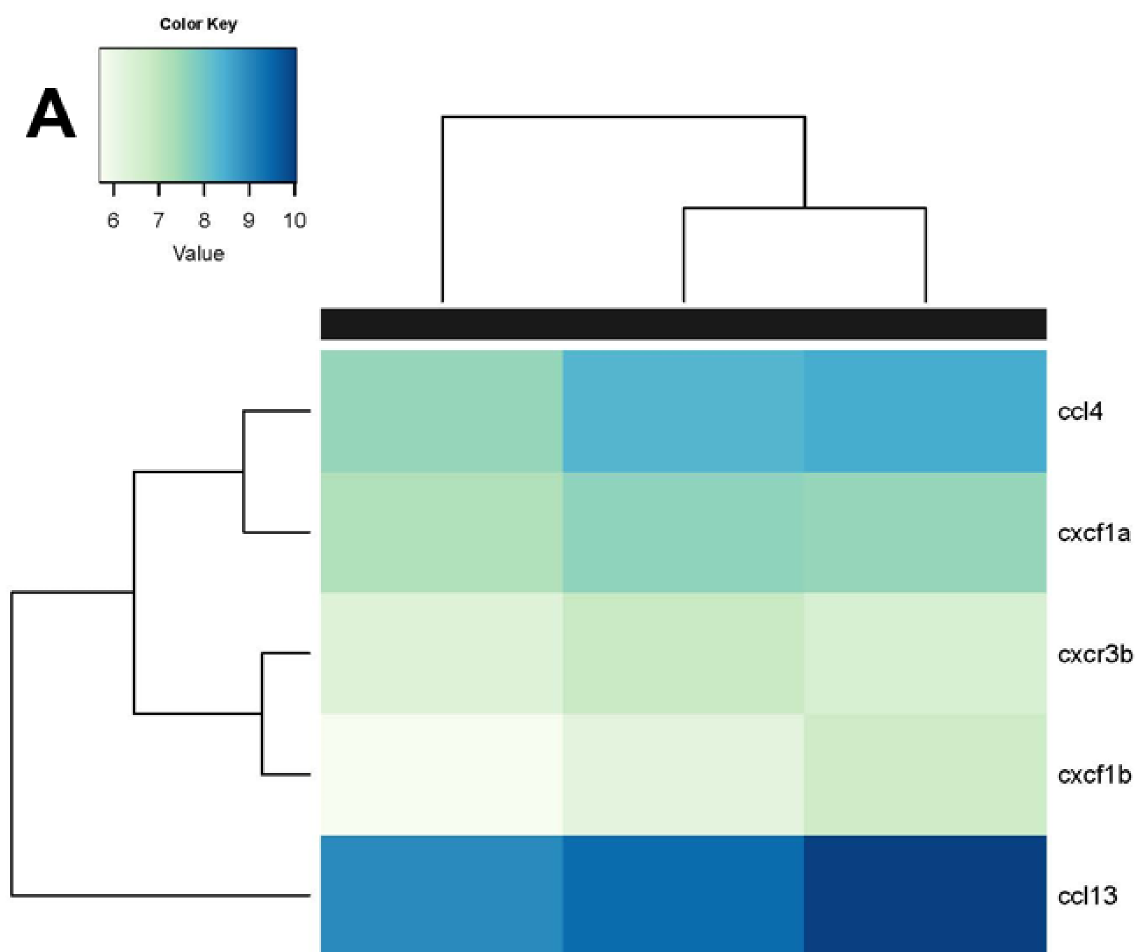


Fig 9. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of all genes identified in rainbow trout posterior kidney of EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.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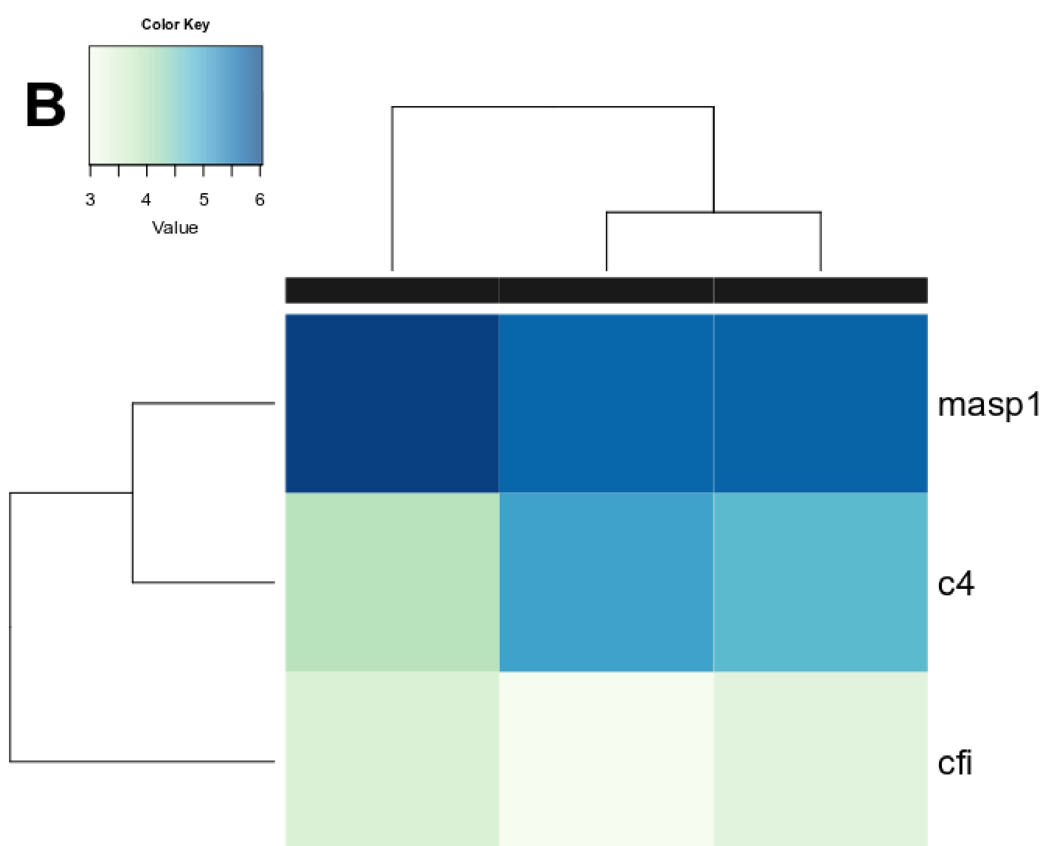
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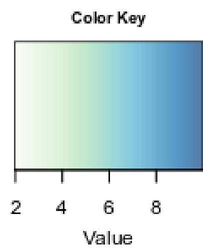

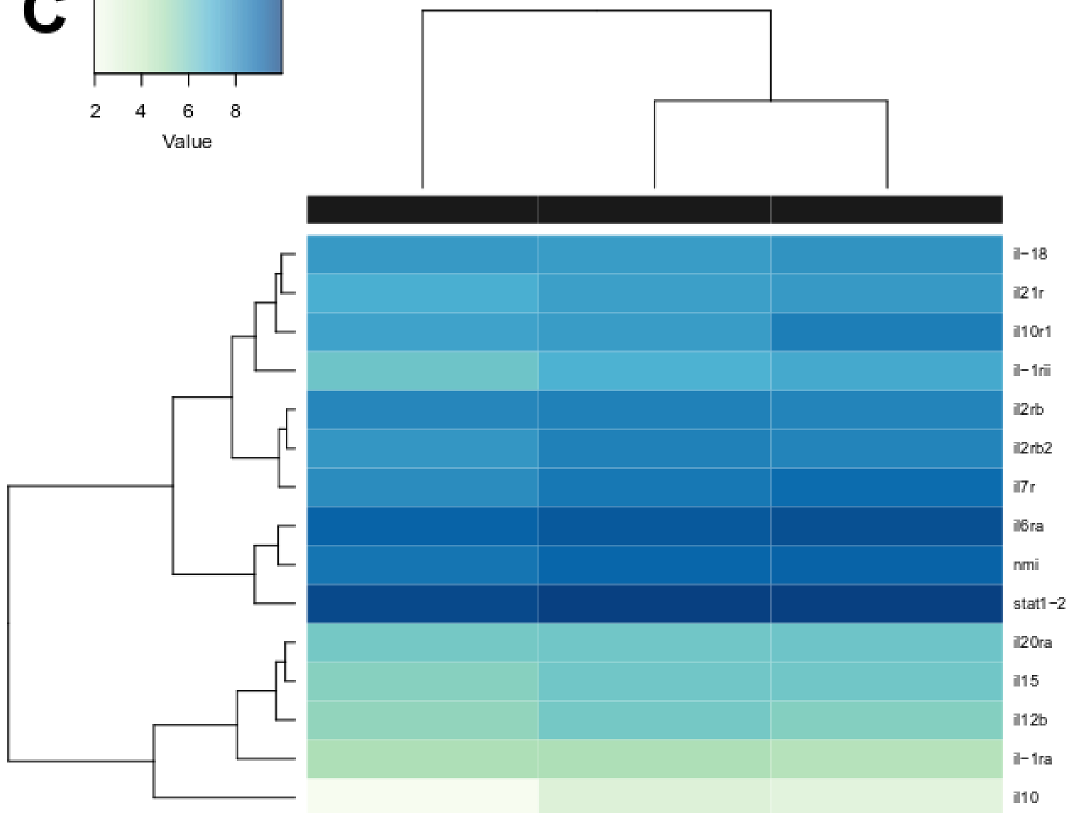

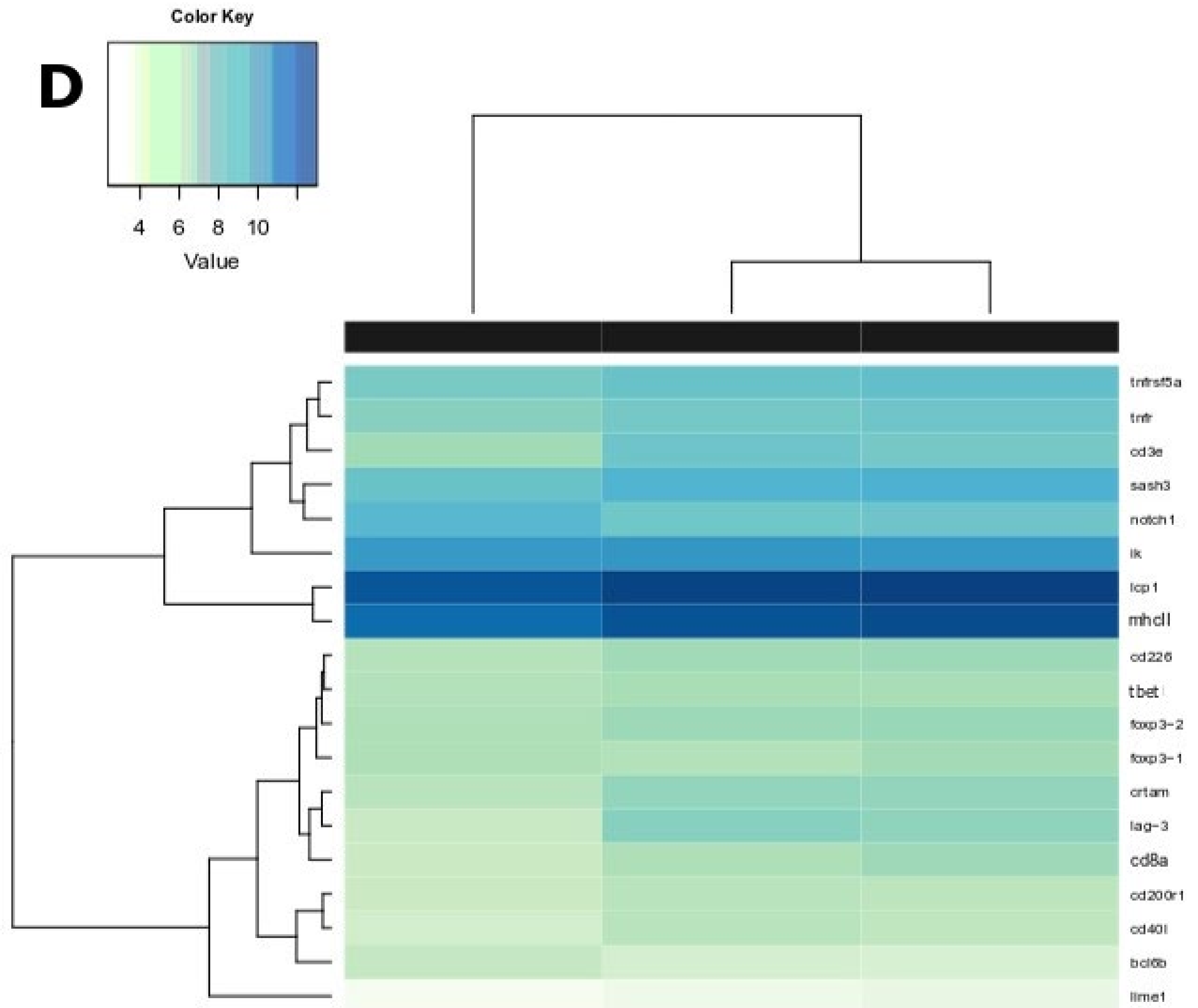

Fig 10. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of A) chemokines; B) complement system; C) cytokines and D) B cell and T cell related genes in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

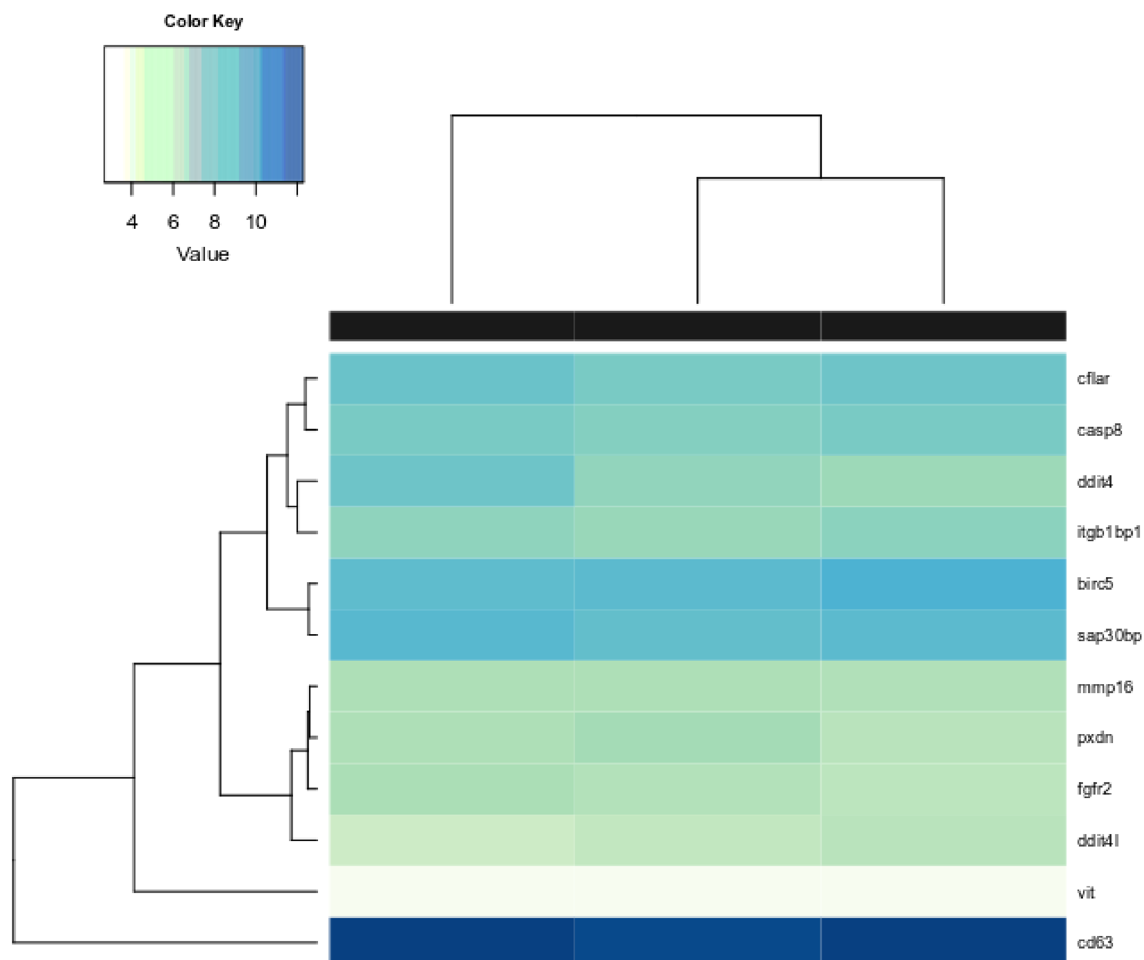

Fig 11. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of inflammation associated genes i.e. genes involved in apoptosis, autophagy and extra cellular matrix (ECM) components in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.

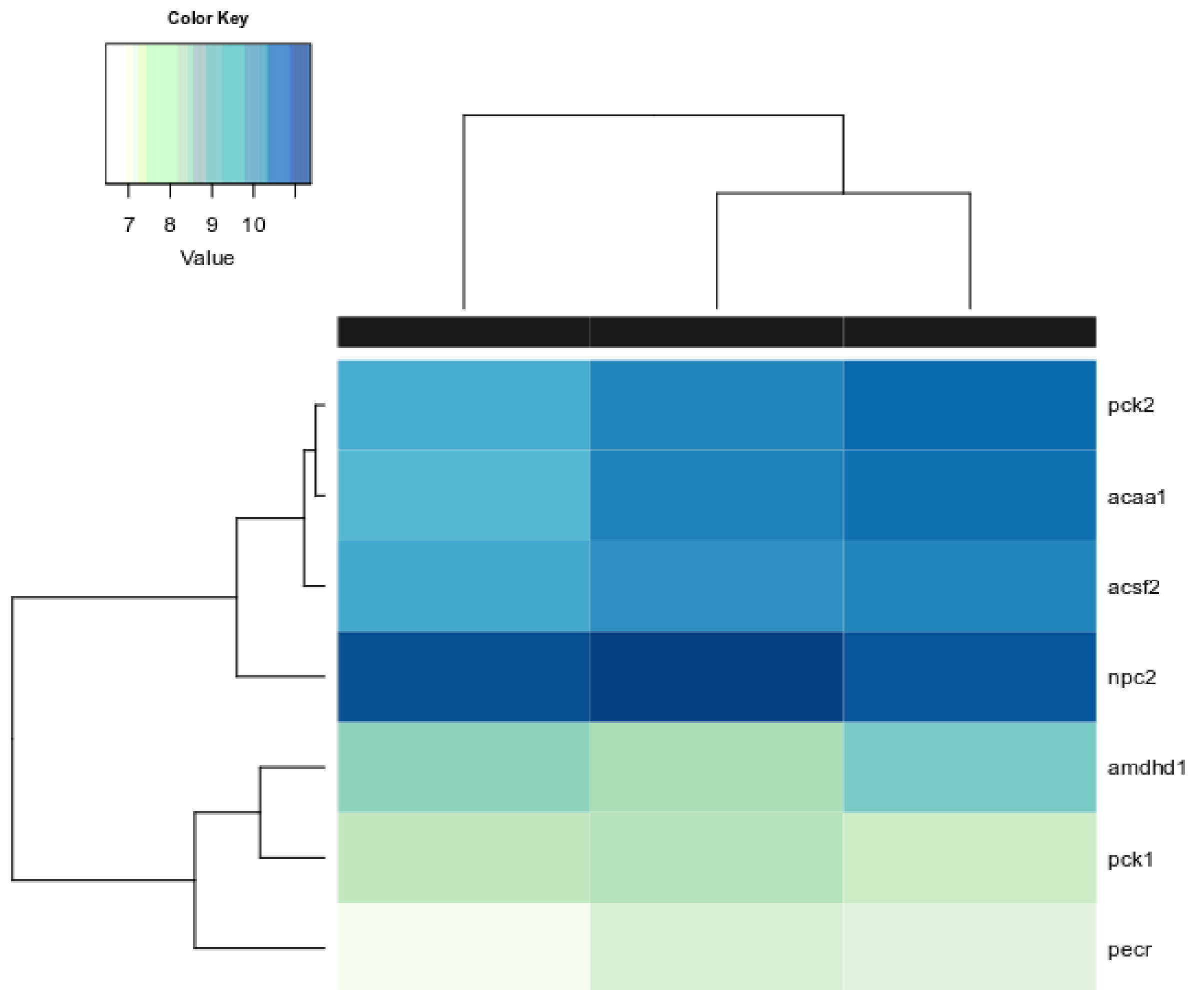

Fig 12. Hierarchical clustering and heatmap visualisation of significant gene expression (padj of 0.05) showing log2-fold changes of genes involved metabolic functions in the rainbow trout posterior kidney of PKD x EE2 exposed fish. Expression levels are normalized by log2 transformation. The colour scale represents log2-transformed values. Columns represent individual samples (x-axis), while rows represent differentially expressed genes (y-axis). This heatmap was built using DESeq2 on normalized gene read counts.