ABC transporters and xenobiotic defense systems in early life stages of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Embryos of oviparous fish, in contrast to (ovo)viviparous species, develop in the aquatic environment and therefore need solute transport systems at their body surfaces for maintaining internal homeostasis and defending against potentially harmful substances. We hypothesized that solute transporters undergo changes in tissue distribution from the embryo to the larval stage and therefore studied the mRNA profiles of eight ABC transporters (*abcb1a*, *abcb1b*, *abcc1*, *abcc2*, *abcc3*, *abcc4*, *abcc5*, *abcg2*) and three solute carriers (*oatp1d*, *putative oatp2*, *mate1*) in different body regions (head, yolk sac epithelium, abdominal viscera, skin/muscles) of developing rainbow trout. Additionally, we investigated mRNA levels of phase I (*cyp1a*, *cyp3a*) and phase II (*gstp*, *putative ugt1*, *putative ugt2*) biotransformation enzymes. The study covered the developmental period from the eleuthero-embryo stage to the first-feeding larval stage (1 - 20 days post-hatch, dph). At 1 dph, transcripts of *abcc2*, *abcc4*, *abcg2*, *cyp3a*, *gstp*, *mate1*, and *putative oatp2* occurred primarily in the yolk sac epithelium whereas at later stages expression of these genes was predominantly observed in the abdominal viscera. The functional activity of ABC transporters in fish early life stages was assessed by rhodamine B accumulation assays. Finally, we investigated the potential impact of xenobiotics (clotrimazole, clofibric acid) on the ABC and biotransformation systems of trout early life stages. While clofibric acid had no effect, clotrimazole lead to an increased rhodamine B accumulation. The results provide evidence that the transition from the eleuthero-embryo to the larval stage is accompanied by a major alteration in tissue expression of ABC transporters.

Key words: ABC transporter, biotransformation, yolk sac, solute carriers, early life stage, *Oncorhynchus mykiss*

1. Introduction

1
2 Aquatic organisms deploy a wide range of protective systems against harmful
3 substances originating from endogenous and exogenous sources occurring in water.
4 One of the mechanisms of particular interest is the transport system of ATP-binding
5 cassette transporter family (ABC transporters) responsible for cellular efflux of
6 endogenous substances, as well as xenobiotics and their metabolites. Membrane
7 transporters involved in detoxification are mainly expressed in organs and tissues with
8 barrier and excretion function such as blood-brain barrier, liver, and kidney (Leslie et al.,
9 2005; Pritchard and Miller, 1993; Schinkel, 1999).

10 ABC transporters play a significant role in the toxicokinetics of compounds.
11 These membrane proteins influence the accumulation of organic compounds in cells
12 and organisms and their activity can be inhibited by certain chemicals leading to altered
13 transport activity (Epel et al., 2008a). Such interfering substances can affect not only the
14 toxicokinetics, but also the toxicity of certain compounds (Zaja et al., 2008a). The
15 presence of ABC transporters, including P-glycoprotein (P-gp) and multidrug resistance-
16 associated proteins (MRP-type/ABCC proteins) transporters, have been shown in more
17 than 40 aquatic species (Luckenbach et al., 2014; Sturm and Segner, 2005). In rainbow
18 trout (*Oncorhynchus mykiss*), members of some ABC transporter families known to be
19 involved in xenobiotic transport have been sequenced and annotated (Zaja et al., 2007;
20 Zaja et al., 2008d; Zaja et al., 2008f). Research to date has mainly focused on ABC
21 transporters in the kidney and liver of fish (Miller, 2014; Sturm et al., 2001) and fish cell
22 lines (Caminada et al., 2006; Zaja et al., 2008a), while little attention has been given to
23 extra-hepatic tissues of fish. Except Lončar et al. (2010) presented the mRNA profiles of
24 eight ABC transporters in different organs of adult *O. mykiss* and found *abcb1* and
25 *abcg2* expressed in organs with barrier functions.

26 Beside the ABC transporter family, many other membrane transporters affect uptake,
27 distribution and efflux of xenobiotics and their metabolites in an organism. Out of the
28 solute carrier gene family, namely the gene subfamily SLC22A comprising the organic
29 anion transporters (OAT) and the organic cation transporters (OCT), the gene subfamily
30 of organic anion transporting polypeptides (Oatp, SLCO gene family, formerly *SLC21A*)
31 (Zair et al., 2008) as well as the multidrug and toxin extrusion proteins (MATE / SLC47)
32 (Staud et al., 2013), are especially involved in uptake and efflux of xenobiotics and
33 metabolites. The mRNA profile of Oatps has been characterized in zebrafish (Popovic
34 et al., 2010), but this transporter family is not yet investigated in other fish species. The

zebrafish Oatps have diverse functions including the transport of endogenous compounds such as bile salts and steroid hormones, but they also transport a large variety of contaminants found in the aquatic environment (Popovic et al., 2014).

Beside ABC and SLC transporters, also phase I and II metabolizing enzymes are important components of the detoxification system involved in the fate and metabolism of endo- and xenobiotics. In fish, still little is known about presence and function of membrane transporters, and there is scarce knowledge about the distribution of phase I and II enzymes in the body of early life stages of fish.

Surface epithelia, which regulate exchange of ions, solutes and respiratory gases between the organism and the environment, typically display ABC transporter proteins including those known to be involved in xenobiotic transport. For instance, in sea urchin embryos, the apical membranes possess numerous ABC transporters, which are able to prevent toxic molecules from entering the embryo (Gokirmak et al., 2012; Shipp and Hamdoun, 2012). In early life stages of teleost fish, the main sites of ion and gas exchange are the epithelia of the yolk sac and the skin; the gills develop only later and then become the main sites of ion and gas exchange (Fu et al., 2010; Rombough, 2002). Leading to the question of the location of ABC transporters in the surface epithelia of early life staged fish. Recently, it has been shown in whole body extracts from zebrafish and tilapia that ABC transporters are already present at the embryonic and larval stages (Costa et al., 2012; Fischer et al., 2013).

The presence of functional ABC transporters in the surface epithelia can be of major importance for the protection of the embryos against toxic compounds in the environment (Epel et al., 2008b). Environmental toxicants, which have been shown to act as ABC transporter inhibitors in mammals, might interfere with the ABC-mediated transport in fish early life stages. For instance, the anti-mycotic compound, clotrimazole, was shown in mammals to inhibit ABC transporter activity (Bain and LeBlanc, 1996; Yasuda et al., 2002) and to induce ABC transporters (Courtois et al., 1999), most probably via pregnane X receptor (PXR) agonism (Moore et al., 2002). Another compound which is widely found in the aquatic environment in the ng/L range (Corcoran et al., 2015; Fent et al., 2006) is clofibric acid, the active metabolite of the antilipidemic agent clofibrate (Castillo et al., 2000; Lindberg et al., 2010). Clofibric acid is a PPAR- α receptor agonist leading to increased beta-oxidation and decreased triglyceride secretion but about its potential effect on aquatic organisms little is known (Coimbra et al., 2015; Corcoran et al., 2015; Fent et al., 2006).

The present study aimed to examine presence, distribution and ontogenetic development of gene families known to be involved in xenobiotic metabolism and transport in eleuthero-embryos and early larval stages (1, 10, and 20 dph) of *Oncorhynchus mykiss*. In a first step, we measured mRNA levels of ABC transporters, phase I and II enzymes, and solute carriers in distinct body parts (head/gills, yolk sac epithelium, abdominal viscera (including the organs from the visceral cavity except the heart), skin/muscles) at 1, 10 and 20 days post-hatch (dph). This period includes the freshly hatched eleuthero-embryos up to the early larvae. Secondly, we assessed the functional capacity of the transporters using ABC transporter substrate and inhibitors. Finally, the influence of environmental contaminants – here: clofibric acid and clotrimazole – on ABC transporter activity and mRNA level changes was assessed.

2. Materials and Methods

2.1 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma (Buchs, Switzerland). Sterile and pyrogenic free water was used for buffer and reconstituted water preparations (B. Braun Medical AG, Sempach, Switzerland). Clofibric acid, clotrimazole, MK-571, cyclosporin A, reversin 205 were dissolved in dimethylsulfoxid (DMSO) and stored at -20°C.

2.2 *Oncorhynchus mykiss* early life stages

All-female rainbow trout eyed eggs were kindly provided by the Research Centre for Animal Nutrition and Health of the company DSM Nutritional Products in Village-Neuf (France). The all-female eggs were incubated in a standard trout breeding stand-up incubator (producer company EUVAG, vertical incubator system with trays for fish egg incubation) supplied with temperature controlled tap water of $8 \pm 1^\circ\text{C}$ until hatching. Hatched eleuthero-embryos were separated according to hatching day. At 11 dph eleuthero-embryos were transferred in cages (41x41x14 cm) placed in a fish breeding channel (210x41x16 cm). The laboratory-based system was supplied with flow-through tap water of $10 \pm 1^\circ\text{C}$ and with an aeration system. The fish were kept under natural photoperiod conditions. Standard trout food (Trout Start 0.4mm, Hokovit, Bützberg, Switzerland) for fry stage was distributed all over the channel twice a day (ad libitum

feeding, 7% food of body weight per day and changed accordingly to body weight every fourth day). Dissolved oxygen and temperature were routinely measured (Oxy Guard) and water quality was assessed by measuring temperature, pH, carbonate hardness, ammonium, nitrite, nitrate using commercial test kits (Aquamerck #1.11102.0001).

The development stages 1 and 10 dph were called eleuthero-embryos, because they are free living but still rely on endogenous energy supply (Balon, 1975). The 20 dph fish almost completely resorbed the yolk sac and took up exogenous food therefore we called them early larvae.

2.3 RNA sampling at different fish development stages:

Three random samplings of fish at each time point 1, 10, and 20 dph were done with five fish per sampling. For RNA isolation, tissues of five individuals was dissected under the stereo microscope and the different body parts (Fig. S2) were pooled. Extracted tissues were stored in RNeasy lysis buffer (Qiagen, Basel, Switzerland) on ice for 24h before storage at -80°C.

2.4 RNA extraction

RNA was extracted from eleuthero-embryos at 1 dph and 10 dph, and early larvae at 20 dph. Tissue samples stored in RNeasy lysis buffer were extracted using TRI Reagent following the manufacturer's instructions (Sigma, Buchs, Switzerland). The amount and purity of isolated RNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington DE, USA). The quality of some random selected samples was tested by the Agilent RNA 6000 Nano Kit, in combination with the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). Potential traces of genomic DNA contamination were removed using RQ1 RNase-Free DNase (Promega AG, Dübendorf, Switzerland).

2.5 Primer design and testing

Some of the primers were used previously (Table 1). Annotated sequences of *Salmo salar* and *Danio rerio* were used for homologous sequences search in *Oncorhynchus mykiss* expressed sequence tag databases (Salem et al., 2010) for the genes of interest: *ugt1*, *ugt2*, *oatp2*, and *mate1*.

The homologous expressed sequences tags found for *O. mykiss* (Table 1) were translated into amino acid sequences for the identification of protein family specific

domains (Marchler-Bauer et al., 2015). The protein domain information and the alignment to annotated sequences of other fish species were used to confirm association of the sequences to the protein superfamilies' of interest. Due to this assignment, the primers have the prefix "*putative*" to indicate that those primers were not designed based on annotated sequences.

Primers were designed based on Primer3 algorithms (Koressaar and Remm, 2007; Untergasser et al., 2012) and adjusted manually if necessary. The oligonucleotides were purchased (Microsynth, Basel, Switzerland) and *O. mykiss* cDNA from liver or kidney were used to test the designed primers. PCR products were resolved by 2% agarose gels along with a 100 bp step DNA marker, stained by SYBR green I (Invitrogen) and visualized by UV light. The size of product bands correlated with the expected amplicon size.

For the verification of specificity, the amplicon was purified using the High Pure PCR Product Purification Kit from Roche (# Cat. No. 11 732 668 001) followed by sequencing (Microsynth, Basel, Switzerland). The sequenced nucleotide information was used to perform nucleotide blasts for checking the specificity and the annotation of the primer pairs.

2.6 cDNA synthesis and quantitative real-time PCR

For each sample 1'000 ng of DNA-free RNA was used for cDNA synthesis with the iScriptTM cDNA synthesis kit using a mix of oligo(dT) and random hexamer primers following the manufacturer instructions (#170-8891, Bio Rad, Reinach, Switzerland). The total volume of the cDNA syntheses was 20 µl and cDNA was stored at -80°C.

The real time PCR quantification was performed on an Applied Biosystems 7300 analyzer (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 12.5 µl, containing 6.25 µl FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland), 1 µl of 3.75 µM primer stock (final concentration 300nM), and 5.25 µl Nuclease-Free water (Qiagen, Basel, Switzerland) containing 0.4 µl of the cDNA synthesis mix. Each sample was run in duplicates and repeated in case of a deviation higher than 0.5 ct value.

The real time qPCR was performed with the following conditions: 2 min 50°C, 10 min 95°C, followed by 40 cycles of amplification. Each cycle consisted of 8 seconds of denaturation at 95°C, annealing and elongation at 60°C for 40 seconds. The PCR was

always terminated with a melting curve analysis starting with a denaturation step of 95°C followed by the start ramping temperature of 60°C for 30 sec.

The amplification efficacy was determined for each primer pair using at least five different cDNA concentrations (average value of 3 independent experiments, Table 1).

The data were analyzed with the 7300 system sequence detection software version 1.3.1 of Applied Biosystems. The calculated ct values were exported to Microsoft Excel for further analysis. Several reference genes were tested and *ef1α* turned out to be most stable expressed in different tissues at different time points (supplementary material, Fig. S1) and was thus taken as reference to which all genes were related.

2.7 Rhodamine B accumulation assay using ABC transporter inhibitors

To assess the functional activity of the transporters *abcb1*, *abcc* and *abcg2*, the fluorescent transporter substrates rhodamine B (Fischer et al., 2013; Keiter et al., 2016; Smital and Sauerborn, 2002) was used in combination with pharmacologic transporter inhibitors. The fish were transferred to experimental vessel containing reconstituted deionized water (CaCl₂ 111 mg/l, KCl 2.9 mg/l, MgSO₄·7H₂O 61.6 mg/l, NaHCO₃ 32.4 mg/l) (Bluthgen et al., 2013) 45 min prior to the experiment. For each development time point (1, 10 and 20 dph) three independent trials were performed using five fish per condition. Eleuthero-embryos and early larvae were exposed to one of the following chemicals; ABC transporter inhibitors known from mammalian literature, such as cyclosporin A (inhibitor of ABCB, ABCC, and ABCG family members) (Germann et al., 1997; Rautio et al., 2006; Robey et al., 2004) at a concentration of 15 µM, reversin (marked as ABCB1 specific inhibitor) (Sharom et al., 1999) at 15 µM and MK-571 (ABCC family specific inhibitor) (Chen et al., 1999; Gekeler et al., 1995; Reid et al., 2003) at 15 µM. All the exposures lasted one hour followed by the addition of rhodamine B (with a final concentration of 1 µM) and a further exposure for two hours. Neither death, nor malformations were observed in the fish after exposure.

The experimental incubation temperature of 10°C was held constant by the use of an incubator connected to a cooling system. The fish were transferred to another beaker to be killed by an overdose of MS-222 (Tricaine methanesulfonate, concentration over 250 mg/l for 3 min), and embryo viability was assessed by observing respiration and fin movement. After confirmation of death, the individuals were quickly rinsed with water to remove MS-222 and unspecific adsorption of rhodamine B followed

by shock freezing in liquid nitrogen. The frozen eleuthero-embryos and early larvae were dissected under the stereo-microscope in three different body parts, such as head, dorsal body part (containing skin and muscle tissue) and abdominal viscera (organs of the visceral cavity) including the yolk sac and fry (supplementary information, Fig. S2). The five fish per condition in a trial were pooled for analyses.

Using tissue lyser (Qiagen), the three body parts were homogenized in phosphate-buffered saline (PBS) with 0.1% Triton in presence of 5 mm steel beads followed by a centrifugation step at 4°C 15'000 g for 10 min. For each tissue sample 100 µl supernatant was measured for rhodamine B fluorescence in duplicates in black 96 well plate (# 611F96BK, Sterilin Limited, Newport, U.K.) using the wavelength of 555 nm for excitation and 574 nm for emission. Fluorescence analysis took place in the EnSpire multimode plate reader (PerkinElmer, Waltham, MA, USA). Values were referred to mg tissue (wet weight).

2.8 Exposure of eleuthero-embryos to clotrimazole and clofibric acid

2.8.1 Effect on ABC transporter function

Analogous to the ABC transporter functional assay with the known pharmacological ABC transporter inhibitors (2.7), exposures were performed in the same way with the antifungal pharmaceutical clotrimazole at concentrations of 15 µM and 150 µM and the lipid lowering drug metabolite clofibric acid at 15 µM and 135 µM.

2.8.2 Effect on mRNA pattern

Eleuthero-embryos at development stages of 1 dph were randomly taken from the culturing system and placed in reconstituted deionized water (Bluthgen et al., 2013) for 12 h at a cultivation temperature of 10 °C for acclimation. The subsequent exposure of eleuthero-embryos was performed in reconstituted deionized water in Petri dishes (11 cm diameter; Semadeni, Ostermundigen, Switzerland). The experimental setup consisted of six groups (untreated control, solvent control (DMSO), 100 nM and 1 µM clotrimazole and 20 nM and 100 µM clofibric acid). The final concentration of DMSO was 0.1%, except in the untreated control. The experiment was performed for 18 h at a water temperature of 10°C. The eleuthero-embryos were regularly observed and abnormal behavior documented. The exposure was terminated by killing the eleuthero-embryos with an overdose of MS-222. The tissues (head, skin and muscles, yolk sac

epithelium and abdominal viscera) of five fish were pooled and stored in RNAlater overnight on ice before final storage at -80°C. The RNA isolations, reverse transcriptions and RT-qPCRs were done as mentioned above. The exposures were repeated independently at least three times and statistical analysis was based on delta ct values.

2.9 Data analyses

Gene and protein sequence manipulations, analysis and multiple alignments were done using BioEdit software (Hall, 1999) and BLAST services by NCBI. The data from gene transcripts of ABC transporters at the trout development stages 1, 10, and 20 dph were evaluated with the relative quantification method of Q-Gene (Muller et al., 2002; Simon, 2003) and multiplied by the factor of 10^6 for a clearer presentation of the values. Those qPCR data were also referred to time point 1 dph and summarized in heat maps using MEV (Multi Array Viewer) software (free download from <http://www.tm4.org>).

Data were statistically evaluated with NCSS and SigmaPlot (Systat Software, San Jose, CA). The non-parametric test of Kruskal-Wallis one-way analysis of variance on ranks was used for statistical analysis. Significant data sets were further analysed by the post hoc test of Dunn's. Data were considered to be significant at $p \leq 0.05$ and indicated graphically with an asterisk (*). Data are graphically presented with GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA).

3. Results

3.1 Gene transcript abundance of ABC transporters during development

At 1 dph (Fig. 1), there were clear differences between the ABC transporters in mRNA abundance and organ distribution. Gene transcript abundance of the transporters *abcb1a*, *abcb1b*, *abcc1*, and *abcc3* showed a rather homogenous distribution among the body parts. A second group of genes encoding ABC transporters including *abcc2*, *abcc4*, *abcc5*, and *abcg2* was characterized by a heterogeneous organ distribution at 1 dph: while *abcc4* (Fig. 1F) and *abcg2* (Fig. 1H) transcripts were elevated in the yolk sac epithelium, *abcc5* showed elevated gene transcript abundance in the head and *abcc2* in the yolk sac epithelium and in the abdominal viscera. The gene transcript levels of each

investigated gene is related to 1 dph and summarized in form of a heat maps for a better, more integrative overview about gene transcript changes (Fig. 2).

Distinct patterns were observed in ABC transporter mRNA abundance from 1 to 20 dph (Fig. 2). One group, consisting of gene transcripts of *abcb1a*, *abcb1b*, *abcc2* and *abcg2*, was characterized by a strong ontogenetic increase of mRNA levels, which took place in the abdominal viscera with the exception of *abcb1b* mRNA (Fig. 1B), where the increase took place in the head. In contrast to the ABC transporters, which ontogenetically increased, *abcc4* mRNA (Fig. 1F) experienced a decrease in all organs, including the yolk sac epithelium and the abdominal viscera, where it was particularly strongly expressed at 1 dph. For the remaining transporters - *abcc1*, *abcc3* – (Fig. 1C, 1E) no major ontogenetic changes of mRNA abundance were evident.

3.2 Gene transcript abundance of phase I and phase II enzymes

Gene transcripts of phase I and phase II enzymes (Fig. 3A-E) at 1 dph differed in organ distributions. While the *cyp1a*, *cyp3a* and *gstp* transcripts were predominantly in the yolk sac epithelium and in the abdominal viscera, mRNA levels of *putative ugt1* and *ugt2* were mainly prominent in the abdominal viscera.

From 1 to 20 dph, mainly three mRNA patterns were observed. *Cyp1a* transcripts (Fig. 3A) showed an increase in all body parts with a peak at 10 dph. *Ugt* transcripts (Fig. 3D-E) showed only a slight increase in the head and in the abdominal viscera from 1 to 20 dph. The *cyp3a* (Fig. 3A) and *gstp* mRNA (Fig. 3C) had high abundance in the yolk sac epithelium after hatching, followed by a decrease and concurrent rise in the abdominal viscera.

Overall, phase I and II enzymes peaked in the abdominal viscera at 20 dph, except the mRNA of *cyp1a* (Fig. 3A), which showed highest levels in the head.

3.3 Gene transcript abundance of solute carriers

At 1 dph, highest mRNA levels of *putative oatp2* (Fig. 3H) and *putative mate1* (Fig. 3F) were detected in the yolk sac epithelium, whereas *oatp1d* mRNA levels (Fig. 3G) were high in the abdominal viscera.

Following the eleuthero-embryo to early larvae development, two distinct mRNA patterns appeared. Firstly, *putative mate1* transcripts (Fig. 3F) had high mRNA levels in the yolk sac epithelium at 1 dph with a constant decrease, and stable levels in the head and the abdominal viscera. Secondly, the profile of *putative oatp2* had the same

constant decrease in the yolk sac epithelium but with an increase in the head and stable mRNA levels in the abdominal viscera.

3.4 Functional assays: Rhodamine B accumulation in eleuthero-embryos and early larvae in presence of ABC transporter inhibitors

To determine, whether the presence of ABC transporter mRNA correlates with the functional activity of ABC transporters in early life stages of *O. mykiss*, accumulation of rhodamine B was measured in the absence and presence of ABC transporter inhibitors at 1, 10 and 20 dph (Fig. 4).

The ABC transporter inhibitor reversin 205 (ABCB1 specific) showed non-significant trend of rhodamine B accumulation, especially at 1 and 10 dph (Fig. 4). No significant accumulation of the ABC transporter substrate was also detected for cyclosporine A exposed fish (inhibitor of ABCB1, ABCC and ABCG2 family members). MK-571 (an ABCC family specific inhibitor) led to significant accumulation in all tissues at 1 and 10 dph. At 20 dph, none of the inhibitors resulted in a significant increase of rhodamine B concentration in any body part.

3.5 Effect of environmental pharmaceuticals clotrimazole and clofibric acid on the accumulation of rhodamine B

To evaluate whether environmental contaminants may be able to disrupt ABC transporter-mediated transport in the trout eleuthero-embryos and early larvae, rhodamine B accumulation in embryos was measured in the presence of the pharmaceuticals clotrimazole and clofibric acid. Exposures to clotrimazole led in some cases to a concentration-dependent increase in rhodamine B accumulation (Fig. 5). Significantly elevated levels were observed in almost all body parts at any time point, especially at the higher clotrimazole concentration. Clofibric acid had no effects in any body part or at any time point.

3.6 Effects of clotrimazole and clofibric acid on gene transcript levels

In addition to functional investigations, we assessed the effects of the two pharmaceuticals on the abundance of ABC transporters mRNA and gene transcripts of xenobiotic metabolism in freshly hatched trout (1 dph) (Fig. 6). There occurred slight increases in mRNA levels of *cyp1a*, *abcb1a* and *abcc2* especially for fish treated with 1 μ M clotrimazole but the differences could not be proved to be significant.

4. Discussion

4.1 ABC transporters in developing (1-20 dph) *O. mykiss*

Two previous studies examined the presence of efflux transporters in fish early life stages. Costa *et al.* (2012) measured some ABC transporter gene transcripts (*abcb11*, *abcc1*, *abcc2*, *abcg2a*, *abcb1b*) as well as of metabolizing enzymes (*cyp1a*, *gsta*) in whole body RNA extracts at embryonic and larval development stage of Nile tilapia (*Oreochromis niloticus*). In zebrafish embryos, the presence of *abcb4* and *abcb5* transcripts over the first 48 hours post-fertilization was reported, and evidence for the functionality of the transporters was obtained by means of a morpholino knock-down approach which resulted in altered accumulation of ABC transporter substrates (Fischer *et al.*, 2013).

Compared to warm water fish, the early ontogenetic phases of rainbow trout last longer, even when accounting for the temperature effect. Importantly, the hatched rainbow trout go through an eleuthero-embryo phase specific for salmonidae where the fish do not feed on exogenous food, but live on the yolk reserve (Belanger *et al.*, 2010).

In our study, ABC transporters displayed differential transcription patterns in eleuthero-embryos and early larvae. Some ABC transporters like *abcc4* showed highest gene transcript levels at 1 dph, mostly in the yolk sac epithelium, while other ABC transporters like *abcb1a* and *abcb1b* experienced a clear developmental increase in gene transcripts. Still other ABC transporters like *abcc1*, *abcc3*, and *abcc5* showed no major developmental changes in abundance and organ distribution.

Compared to humans, there are two described subfamily members of ABCB1 in fish. Transcripts of *abcb1* increased during development and showed a completely different organ predominance at 20 dph. Whereas *abcb1a* transcript was prominent in the abdominal viscera, *abcb1b* transcripts were particularly prominent in the head. This is in agreement with the expression and function of *abcb1a* in intestine, liver and kidney of mammals and fish (Lončar *et al.*, 2010; Tutundjian *et al.*, 2002), and the strong presence of *abcb1b* transcripts in the interlamellar region of the gills of juvenile rainbow trout (Kropf *et al.*, in preparation). In the eleuthero-embryos, gills represent the largest

1 surface area for exchange of solutes with the environment, having an important barrier
2 function.

3 The ABCC4 transporter is known to have a dual localization in polarized cells, is
4 involved in renal excretion (Russel et al., 2008), has a broad range of substrates with a
5 cytoprotective function in the kidney and the brain (Imaoka et al., 2007; Nies et al.,
6 2004). The high abundance of *abcc4* transcripts in the yolk sac epithelium of eleuthero-
7 embryos therefore suggests the existence of an ABC-mediated transport function when
8 the detoxification via kidney has not reached full capacity (Varsamos et al., 2005).

9 In addition, the mRNA of *abcg2* and *abcc2* were also abundant in the yolk sac
10 epithelium at 1 dph. *Abcg2* is known to have a gate keeping function in the intestine,
11 blood-brain barrier, and placenta of mammals (Jonker et al., 2000; Maliepaard et al.,
12 2001) and often colocalizes with *abcc2*, another important transporter for the efflux of
13 conjugates (Nies and Keppler, 2007). The presence of these transporters in the yolk sac
14 epithelium suggests its role in solute transport, including excretion of waste products
15 and/or prevention of influx of toxic compounds, as found in sea urchin embryos
16 (Hamdoun et al., 2004).

17 During development from freshly hatched embryo to the early larvae, mRNA
18 abundance of most ABC genes decreased in the yolk sac epithelium, often
19 concomitantly with an increase in the abdominal viscera, including *abcc2* and *abcg2*.
20 Also the mRNA of *abcb1a* showed a rise in the abdominal viscera. All this is in
21 agreement with data from *abcg2*, *abcc2* and *abcb1a* transcripts found in gonads,
22 intestine, liver and kidneys of juvenile and adult fish (Lončar et al., 2010; Tutundjian et
23 al., 2002; Yuan et al., 2014). The change from the yolk sac epithelium as prominent
24 organ of ABC-mediated transport to the abdominal viscera reflects the transition from
25 the yolk-dependent early developmental stage to stages with a more adult-type
26 anatomy and physiology. The ontogenic transition of ABC transporter location further
27 supports the hypothesis that the dominating efflux site of endo- and/or exogenous
28 compounds in early eleuthero-embryo is the body surface, namely the yolk sac
29 epithelium.

30 Functional ABC transporter assays were performed to prove the presence of
31 active transport activity in early life stages using pharmacological transport inhibitors.
32 Cyclosporin A, as an inhibitor of the ABCB, ABCC and ABCG2 subfamilies (Germann et
33 al., 1997; Ozvegy et al., 2001; Rautio et al., 2006), was already used in fish (Fischer et
34 al., 2011; Zaja et al., 2008a). We found no significant rhodamine B accumulation in

presence of 15 μ M cyclosporin A, which might be due to the pharmacokinetics resulting in too low concentration at the sites of ABC transporters to compete with rhodamine B for ABC mediated transport. Reversin 205 (a rather ABCB1 specific inhibitor) had also no significant effect, similarly to the slight effects in fish cell lines (Zaja et al., 2007). MK-571 as known abcc-transporter specific inhibitor (Caminada et al., 2008; Fischer et al., 2011) resulted in rhodamine B accumulation in all body parts of eleuthero-embryos at 1 and 10 dph. In contrast, the early larvae at 20 dph showed no increased rhodamine B accumulation. The elevated presence detoxifying enzymes and membrane transporters at 20 dph might lead to changed toxicokinetics and metabolism of MK-571. Thus, early larvae were not susceptible to the abcc-specific inhibitor MK-571 in contrast to eleuthero-embryos. Therefore, no conclusion about the ABC transporter activity can be drawn from the early larvae life stage likewise not for the activity of *abcb1* and *abcg2* transporters in all the investigated trout early life stages. However, the exposures with MK-571 provide evidence for the activity of abcc transporters in the eleuthero-embryo and confirm mRNA data.

4.2 Phase I and II metabolizing enzymes and solute carriers

ABC transporters are involved in the active transport of unconjugated chemicals and products from phase I and II metabolism. In order to obtain a broader view of the xenobiotic defence capacities in the trout early life stages, the study not only determined the ABC transporters mRNA patterns, but included selected biotransformation enzymes as well as solute transporters.

Besides the prominent roles in the synthesis and metabolism of endogenous molecules, a number of cytochrome P450 enzymes like *cyp1a* and *cyp3a* are also involved in the metabolism of xenobiotics. In trout eleuthero-embryos, *cyp1a* transcripts showed a general increase from the eleuthero-embryo to the larval stage implying a minor role of *cyp1a* at 1 dph. Similar to *abcc2*, *abcc4* and *abcg2*, high mRNA levels of *cyp3a* and *gstp* were found in the yolk sac epithelium at 1 dph. This suggests a potential for oxidative metabolism and conjugation in the yolk sac epithelium directly after hatch. Furthermore, there might be a functional link between phase I and II metabolism enzymes and ABC transporters in the yolk sac epithelium.

The UDP-glucuronosyltransferases, *putative ugt1* and *putative ugt2*, showed tenfold higher mRNA levels in the abdominal viscera compared to the other body parts.

1 This is in accordance with high abundance of *ugt* transcripts in the liver and
2 gastrointestinal tract of zebrafish (Christen and Fent, 2014).

3 Another group with more than 300 membrane transport proteins are the solute
4 carriers, some of which proteins are known to be involved in xenobiotic transport. The
5 multidrug and toxin extrusion (MATE)-type transporter *slc47a* was most prominent in the
6 yolk sac epithelium after hatching with a strong decrease later in development similar to
7 the *abcc4* mRNA. SLC47A is involved in renal tubular secretion and transport of various
8 organic cations into bile (Omote et al., 2006; Yonezawa and Inui, 2011), which implies a
9 proton-dependent excretion potential for compounds in the yolk sac epithelium after
10 hatch. Oatps were also present in the yolk sac epithelium at 1 dph. The transcriptional
11 pattern in our study for *oatp1d* fits well with data from adult rainbow trout, where
12 expression occurred in the liver and moderately in the brain (Steiner et al., 2014). The
13 increase of *oatp2* transcripts in the head is in accordance with high *oatp2b1* mRNA
14 levels in *D. rerio* gill tissue (Muzzio et al., 2014; Popovic et al., 2010). Because oatps
15 are known to transport steroids like cortisol, which is crucial for glucocorticoid and
16 mineralocorticoid metabolism in fish, the observed shift of *oatp2* abundance might
17 document a physiological function transfer to the head (Rombough, 2002).

18 Collectively, the occurrence of phase I and II enzymes as well as of certain ABC
19 transporters in the yolk sac epithelium suggests that the yolk sac epithelium not only
20 serves as an important site of transport, but also as a site of metabolism for
21 endogenous and xenobiotic compounds in early eleuthero-embryos. The decrease in
22 gene transcripts in the yolk sac epithelium with a simultaneous increase in the
23 abdominal viscera documents the functional shift from the eleuthero-embryos to the
24 larval stage.

26 4.3 Effects of clotrimazole and clofibric acid

27 Clotrimazole led to an increased accumulation of rhodamine B, especially at 150 μ M, in
28 eleuthero-embryos and early larvae without affecting the viability of fish. ABC
29 transporter inhibition, as well as binding of clotrimazole was recently documented (Bain
30 and LeBlanc, 1996; Klokouzas et al., 2001). No significant changes in transcript levels
31 were found in presence of clotrimazole. Also clofibric acid had no inducing effect as well
32 as had no effect on rhodamine B accumulation, even after incubations in presence of
33 135 μ M clofibric acid. Overall, the results suggest that environmental toxicants can

1 interfere with xenobiotic transport systems of trout early life stages and principally may
2 influence gene expression.

5 **5. Conclusions**

6 In summary, this study demonstrates for the first time organ- and stage-specific mRNA
7 patterns of ABC transporters, solute carriers and phase I and II genes in eleuthero-
8 embryos and early larvae of *O. mykiss*. The presence of a set of xenobiotic
9 metabolising enzymes and ABC transporters after hatching suggests that eleuthero-
10 embryos of rainbow trout have a capacity to transport and metabolize endogenous as
11 well as exogenous compounds. After hatching, the yolk sac epithelium appears to be a
12 major site of the transport and metabolic capacities, but during development this is
13 increasingly taken over by the abdominal viscera, reflecting the anatomical and
14 physiological transition from the eleuthero-embryos to the early larval stage.

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Table

Table 1. Primers used for qPCR analysis in *O. mykiss*.

ABC transporter					
Gene	Genbank accession number	Primer sequence 5'-3'	PCR product size (bp)	Primer efficiency (%)	Ref.
<i>abcb1a</i>	AY863423	F) GGCCAGAACGTGGCTAAC R) CAGCCATGACAGGTACCACACA	101	107	2
<i>abcb1b</i>	BX861573.3	F) GGAGAATTCTTGGCTTGTGG R) CGCAAAGTGGATCCCTCG	113	107.8	1
<i>abcc1</i>	GQ166973	F) CATACAGAGTGTTCAGTGGCAACA R) CCCCTGGACCGTCTCATTG	100	100.4	2
<i>abcc2</i>	NM_001124655	F) CGCTTCCTCAAACACAACGAG R) GAACTCTAGACGGATGGCCAG	99	95.6	2
<i>abcc3</i>	GQ888533.1	F) GGTATGGAAGGCTCTGGAAGTGT R) CTGAACACTCCAGCTCCAGTTAG	80	95.6	2
<i>abcc4</i>	GU480583.1	F) GCATGTGTGCCGTTTTCTGT R) ACAACGCCAGGCCTACAGAT	90	103.6	2
<i>abcc5</i>	GU079635.1	F) CAATCCGCAATGCCTTTCA R) TGTCCCTGGTTGAGCACCAT	100	108.3	2
<i>abcg2</i>	EU163724.1	F) AGGCCTGCTGGTGAACCTG R) ACTCATTAAATTTGGAGAGCTGTTAGTCC	101	92.7	2

Phase I and II, solute carriers					
Gene	Genbank accession number	Primer sequence 5'-3'	PCR product size (bp)	Primer efficiency (%)	Ref.
<i>cyp1a</i>	AF015660.1	F) GCCGTTTCATCATCCACACTGCAC R) TTCCACAGCTCCGGGTCATGGT	120	101.0	1
<i>cyp3a</i>	U96077.1	F) GGTCCCAAACCCCTACCTTA R) CCCAGATTCTCCCGTACTT	101	100.4	1
<i>gstp</i>	BQ036247.1	F) ATTTTGGGACGGGCTGACA R) CCTGGTGCTCTGCTCCAGTT	81	95.1	4
<i>putative ugt1</i>	-	F) TCCCTGAGACATCCATCCTC R) TGCATGTCGGTGAAGTCAAT	187	107.0	3 (#TC189549)*
<i>putative ugt2</i>	-	F) CTGGGGATACCCCTGTTCTT R) CCTCTGCATGTTCTCCCTGT	163	103.5	3 (#02878)
<i>putative mate1</i>	gi 620601163**	F) TCTTCCACTGGGCATCTCTT R) GCTCGTTTCACAGCCTTCTC	159	102.9	3
<i>oatp1d</i>	KJ831065.1	F) GGGATTCCATTTGGTTTCCT	167	107.9	1

		R) GGTGCATCCTCCTCAGTGTT			
<i>putative oatp2</i>	-	F) TCCTTCCAAACACCCCTTGTC R) CGATTCCCAGGAGTAGCTGT	106	116.8	3 (#108159)

Reference genes					
Gene	Genbank accession number	Primer sequence 5'-3'	PCR product size (bp)	Primer efficiency (%)	Ref.
18s ribosomal RNA (18S)	AF308735.1	F) TGGAGCCTGCGGCTTAATTT Rev) ATGCCGGAGTTTCGTTTCGTT	172	85.1	5
elongation factor (<i>ef1α</i>)	AF498320.1	F) TGCCCCTGGACACAGAGATT R) CCCACACCACCAGCAACAA	90	105.3	5
beta-actin (β -actin)	NM_001124235.1	F) GGACAGGTCATCACCATCGG R) GTGGTCTCGTGGATACCGCA	101	108.6	6
glyceraldehyde -3- phosphate dehydrogenase (<i>gapdh</i>)	AF027130.1	F) GATGGACCCATGAAGGGAATT R) CAGCGCCAGCATCAAAGAT	100	104.3	5

References: 1) self-designed, 2) (Lončar et al., 2010) , 3) (Salem et al., 2010), 4) (Salaberria et al., 2009), 5) (Fischer et al., 2011), 6) (Zaja et al., 2008d)

*) TC189549 TC189549 TC144935, base positions 95-1750

**) gi|620601163:<1099-1211, 1369-1466, 1567-1680, 1799-1866, 2116-2224, 2378-2453, 2554-2623, 7040-7172, 7472-7566, 7750-7833, 7937-8239) *Oncorhynchus mykiss* genomic scaffold, scaffold_1488, whole genome shotgun sequence

Figure captions

Fig 1. Abundance of ABC transporter gene transcripts (mRNA) in different body parts of eleuthero-embryos at 1 and 10 dph, and of early larvae at 20 dph. 1A: *abcb1a*; 1B: *abcb1b*; 1C: *abcc1*; 1D: *abcc2*; 1E: *abcc3*; 1F: *abcc4*; 1G: *abcc5*; 1H: *abcg2*. The mRNA levels are given as mean normalized expression (MNE) $\pm$ standard deviation (SD) using *ef1a* as reference gene. Each time point consists of three samplings each containing five pooled fish (asterisks, significantly different within an organ, $p < 0.05$).

Fig 2. Heat maps of ABC transporter gene transcript (mRNA) abundance in different body parts of eleuthero-embryos at 1 and 10 dph, and early larvae at 20 dph. Gene transcript levels are related for each gene to its level at 1 dph, (A) head, (B) skin and muscles, (C) abdominal viscera, and (D) yolk sac epithelium. Upper color represents the relative mRNA level to 1 dph. Key: green color: lower gene transcript amount, red color: higher gene transcript amount. The clustering of the patterns is also shown.

Fig 3. Abundance of gene transcript (mRNA) levels of phase I and phase II enzymes and solute carriers in different body parts of eleuthero-embryos at 1 and 10 dph, and early larvae at 20 dph. 3A: *cyp1a*; 3B: *cyp3a*; 3C: *gstp*; 3D: *putative ugt1*; 3E: *putative ugt2*; 3F: *putative mate1*; 3G: *oatp1d*; 3H: *putative oatp2*. The mRNA levels are given as mean normalized expression (MNE) to the reference gene *ef1a* $\pm$ SD. Each experiment time point consists of three samplings with five pooled fish (asterisks, significantly different within an organ, $p < 0.05$). BDL: below detection level

Fig 4. Rhodamine B accumulation in different body parts of eleuthero-embryos at 1 and 10 dph, and early larvae at 20 dph in presence of ABC transporter inhibitors cyclosporin A, reversin 205 and MK-571 at a concentration of 15 μ M each. Rhodamine B concentrations are given as relative fluorescence units related to mg tissue; mean levels $\pm$ SD are shown. The setup was independently repeated three times and contains five pooled fish per condition (asterisks, significantly different to solvent control, $p < 0.05$).

Fig 5. Rhodamine B accumulation in different body parts of eleuthero-embryos at 1 and 10 dph, and early larvae at 20 dph in presence of clofibric acid (at 15 μ M and 135 μ M) or clotrimazole (15 μ M and 150 μ M). Rhodamine B concentrations are given as mean

values of relative fluorescence units per mg of tissue $\pm$ SD. The setup was independently repeated three times and contains five pooled fish per condition (asterisks, significantly different to solvent control, $p < 0.05$).

Fig 6. Gene transcript levels after exposure of 1 dph eleuthero-embryos to clofibric acid or clotrimazole for 18 hours. Gene transcript levels were normalized to *ef1 α* and compared to solvent control (DMSO). Data is given in log₂; the values represent means $\pm$ SD of three independent exposures each consisting of five pooled fish per condition.