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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.

1. Introduction

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

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

Beside the ABC transporter family, many other membrane transporters affect uptake, distribution and efflux of xenobiotics and their metabolites in an organism. Out of the solute carrier gene family, namely the gene subfamiliy SLC22A comprising the organic anion transporters (OAT) and the organic cation transporters (OCT), the gene subfamily of organic anion transporting polypeptides (Oatp, SLCO gene family, formerly *SLC21A*) (Zair et al., 2008) as well as the multidrug

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and toxin extrusion proteins (MATE/SLC47) (Staud et al., 2013), are especially involved in uptake and efflux of xenobiotics and metabolites. The mRNA profile of Oatps has been characterized in zebrafish (Popovic 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 *O. 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. O. 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 ± 1 °C until hatching. Hatched eleuthero-embryos were separated according to hatching day. At 11 dph eleuthero-embryos were transferred in cages (41 × 41 × 14 cm) placed in a fish breeding channel (210 × 41 × 16 cm). The laboratory-based system was supplied with flow-through tap water of 10 ± 1 °C and with an aeration system. The fish were kept under natural photoperiod conditions. Standard trout food (Trout Start 0.4 mm, 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 were dissected under the stereo microscope and the different body parts (Fig. S2) were pooled. Extracted tissues were stored in RNAlater (Qiagen, Basel, Switzerland) on ice for 24 h 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 RNAlater were extracted using TRI Reagent following the manufacturer's instructions (Sigma, Buchs, Switzerland). The amount and purity of isolated RNA were 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 *O. mykiss* expressed sequence tag databases (Salem et al., 2010) for the genes of interest: *ugt1*, *ugt2*, *oatp2*, and *mate1*.

The homologous expressed sequence 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

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