RESEARCH NOTE

Identification of genes involved in cold-shock response in rainbow trout (Oncorhynchus mykiss)

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Running title: Cold-shock in rainbow trout

Introduction

A rapid decline in temperature poses a major challenge for poikilothermic fish, as their entire metabolism depends on ambient temperature. We compared the gene expression of rainbow trout (*Oncorhynchus mykiss*) having undergone such a cold shock (0 °C) to a control (5 °C) using microarrays and quantitative real-time PCR. The number of genes found to be regulated at 0 °C was surprisingly low. Instead of classical genes involved in temperature shock, the three genes encoding fibroblast growth factor 1 (*fgf1*), growth arrest and DNA-damage-inducible, alpha (*gadd45a*) and sclerostin domain-containing protein 1 (*sostdc1*) were upregulated in the liver upon cold shock in two different rainbow trout strains, suggesting that these genes may be involvedin the response to cold shock in rainbow trout.

Fluctuating biotic and abiotic factors have a profound effect on the well-being of animals. One abiotic key factor is the ambient temperature, which is especially important for ectothermic animals, because it sets the rates for metabolic processes and also affects oxygen availability. Most fishes are ectothermic animals adapting to changes in ambient temperature due to seasonal, sometimes sudden weather events, without the possibility of maintaining a preferred body temperature. Effects of heat shock including the induction of heat-shock

proteins (reviewed in Tutar and Tutar, 2010)have extensively been studied in a great variety of species (Feder and Hofmann, 1999)including fishes(Basu*et al.*, 2002; Roberts *et al.*, 2010).Knowledge about molecular responses of teleosts to cold-exposure is less extensive. However, it is known that cold shock in fish can lead to changes in hormone balance, metabolism, immune system and behaviour (reviewed in Donaldson *et al.*, 2008).Changes in gene expression due to cold were for example recently analysed holistically employing a transcriptome-wide RNA-Seq approach in zebrafish (Long *et al.*, 2013) and more gene-specific in the large yellow croaker (Xu *et al.*, 2015).

Cold-shock is an event strongly impacting fish in natural bodies of water as well as in aquaculture. In particular, open and semi-open aquaculture facilities are prone to such changes in temperature, as it is not technically controlled. Rainbow trout is one of the most economically important aquaculture species, with lethal temperatures below 0 °C and above 29.4 °C (Bell, 1990). To our knowledge the cold-shock response of this species has not been examined before, though a drop to temperatures around 0 °C occurs regularly in semi-open aquaculture systems in countries with cold winters like Germany (Figure 1(a)).

The main objective of this study was thus the identification of genes that are regulated upon an acute cold shock from 5 °C to 0 °C in rainbow trout. We focussed on kidney, liver and gills. Liver (Mininni*et al.*, 2014) and kidney (Verleih*et al.*, 2015)are known to be involved in long-term cold acclimation, whereas gills are in direct contact with the water.

Materials and methods

To analyse the effects of cold shock on *O. mykiss,* fish of two different strains - BORN, Research Centre for Agriculture & Fishery, Born, DE and TACOMA/TCO, Troutlodge, Tacoma, US - were kept under comparable conditions in lanes of a semi-open aquaculture facility. Strain BORN is bred and selected for survival under local conditions since 1975 (Anders, 1986), whereas strain TCO is selected for growth and is genetically adapted to water conditions of the North American west coast.

We started the experiment, when the temperature declined naturally to 5 °C in November.Using a water chiller and crushed ice, two basins filled with seawater were cooled down to 0 °C with a layer of ice on top. Twenty fish per strain were exposed to this ice water for at least 30 minutes. The fish were 224 (TCO) or 239 (BORN) days old, had a total length

of 18.1 ± 1.5 (TCO) or 18.4 ± 1.3cm (BORN) and a weight of 76.4 ± 15.2 (TCO) or 81.6 ± 16.7 (BORN) g. After the incubation period, four fish of the first strain were subjected at the same time to an overdose of phenoxyethanol. Animals were dissected and tissue samples of gills, liver and kidney, were taken and flash-frozen in liquid nitrogen. Parallel to the tissue extraction the next four fish of the other strain were sacrificed. In this way all animals were analogously sampled. Due to the duration of sampling the minimal cold-shock exposure was 30 minutes and the maximal exposure was 130 minutes, with similar exposure times for BORN as well as TCO trout. Average exposure length was 1h and 20 minutes. As control, tissue samples from 5 °C acclimated animals (10 animals per strain) were taken.

RNA was extracted using Trizol and the RNeasy Mini Kit (Qiagen, Hilden, DE) following a standard protocol(cf. Borchel *et al.*, 2014). Five individual RNA samples from the same tissue, temperature and strain were pooled in equal amounts. This was done twice per condition, resulting in a total of 24 pools. All pools showed RIN values between 8.5 and 10 on the Agilent 2100 Bioanalyzer platform, indicating high-quality RNA. Using the Agilent Low Input Quick Amp Labelling Kit, 100 ng of total RNA were employed in linear T7-based amplification and labelled with Cyanine-3. Agilent Whole Salmon Genome Oligo Microarrays 8×60K (GEO Platform GPL21057; MiltenyiBiotec GmbH, Germany) were hybridized with 600 ng labelled cRNA for 17 h at 65 °C utilizing the Agilent Gene Expression Hybridization Kit. The experiment yielded 24 datasets (3 tissues x 2 conditions x 2 strains x 2 duplicates;see GEO accession GSE75563).

Agilent's Microarray Scanner System detected the fluorescence signals of the hybridized microarrays. The Agilent Feature Extraction Software (FES) read out and processed the microarray image files. For the interpretation of the spot intensities, the limma package of the R/Bioconductor suite was used (Smyth, 2005). For each tissue and strain, a comparison between intensities at 0 °C (cold-shock treatment) and 5 °C (control) was performed employing control of the false discovery rate (Benjamini and Hochberg, 1995). Differences between 0 °C-shocked and 5 °C-acclimated trout were considered significant in the case of fold-changes larger than two and an adjusted p-value smaller than 0.05.Gene annotation was based on blasting the probe sequences against the NCBI nucleotide collection. In case of high identity with already annotated genes, we then used the recommended gene symbols approved by the HUGO Gene Nomenclature Committee.

To verify the results of the microarray experiment, quantitative real-time PCR was conducted for selected genes from liver that were found to be differently expressed in the present microarray study. 1.5 µg RNA of individual samples were deployed in cDNA synthesis using Superscript II (Invitrogen) and a mixture of Oligo-d$(T)_{24}$ and random hexamer primers. The cDNA was purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, DE) and diluted in 100 µl nuclease-free water.

Quantitative real-time PCR was conducted on a LightCyler 96 (Roche) following a standard approach (cf. Borchel *et al.*, 2014)using the primers given in Table 1. For each gene 20 (BORN) or 19 (TCO) samples of cold-shocked fish and 10 samples of control fish were measured individually. Copy numbers were calculated relative to external standards and normalized by the geometric mean of the copy numbers of the reference genes *eef1a1*, *rps5* and *actb*. Significance levels of observed differences were calculated using Mann−Whitney Rank Sum Testsperformed by SigmaPlot 11, considering p-values < 0.05 significant.

Results and discussion

Only a comparatively small number of 29 annotated genes were found to be differentially expressed in the three analysed tissues − liver, kidney, and gill − of trout having been incubated between 0 and 5 °C, as determined with microarray technology (Figure 1(b)). Further 35 differentially expressed features could not be annotated by blasting against GenBank entries and were therefore excluded from subsequent analyses. Twenty-one differently expressed annotated genes were found in the liver, whereas in the kidney and gills, a lower number of six and two genes, respectively, showed significantly modulated mRNA levels. Surprisingly, there was little concordance regarding the modulation of gene expression between TCO (Troutlodge strain) and BORN (regional strain) rainbow trout. A statistically significant regulation was monitored for most of the genes only in one of the twostrains. This strain specificity in gene expression might correlate with the assumption that both strains are somehow genetically adapted to their original water bodies used for breeding. Water temperatures of lakes in State Washington (USA) rarely drop below 5 °C, compared to Germany where water temperatures of 0 °C or slightly below occur regularly in winter in brackish water (cf. Figure 1(a) and http://green2.kingcounty.gov/lakes).

Quantitative RT-PCR was used to verify whether the microarray analysis reliably reflected the cold-shock-induced patterns of transcriptional changes in rainbow trout (Figure 1(c)). For more than half of the identified genes the correlation (coefficient of determination, $R^2 = 0.80$;

Pearson's correlation significance, $p < 0.001$) between the fold-change values obtained *via* microarray as well as qRT-PCR was assessed. Apart from*ube2a*all analysed genes showed the same direction of regulation according to both techniques and a high correlation, indicating microarray validation.

The comparison of both *O. mykiss* strains revealed that only *sostdc1* gene was significantly upregulated around twofold in strain BORN as well as strain TCO with adjusted p-values lower 0.05. The qRT-PCR-obtained expression values for this gene as well as *fgf1* and *gadd45a*, which showed fold changes higher than two in both strains and a p-value lower 0.05 in still one strain, are shown in Figure 1(d). Significant upregulations based on temperature effects were found for the genes *gadd45a, fgf1* and *sostdc1* (Mann−Whitney Rank Sum Test; $p < 0.05$).

Classical genes directly linked to temperature stress like heat-shock proteins (HSPs) or the cold-inducible RNA-binding protein CIRBP (Gracey*et al.*, 2004, Rebl *et al.*, 2013)were not found to be differently expressed between cold-shocked and control fish. Possibly, a prolonged cold-shock may lead to a detectable induction of expression of these genes at the RNA level. It is on the other hand possible that a lot of the classical genes related to lowtemperatures like *cirbp* are already strongly expressed at 5 °C. A further decline of temperature to 0 °C may not increase their transcription levels any further. Fish that have been acclimated for weeks to declining temperatures may be prepared for such a rapid temperature shock, which may naturally occur, for instance while swimming through different water layers or water bodies. It has been shown that the *cirbp* expression of *O. mykiss* is already increased at a temperature of 8 °C in kidney (Verleih*et al.*, 2015)

Though we found a high variation in gene expression of the 0 °C-exposed fish, the genes *gadd45a*, *fgf1* and *sostdc1* were found to be clearly upregulated in both examined fish strains after a cold shock and may thus be involved in the response to stress caused bylow temperatures. The tasks and functions of these genes are quite diverse. *Gadd45a* belongs to the 'growth arrest and DNA damage-inducible protein' (GADD45) family. The members of this family encode small, highly acidic proteins, which are localized in the nucleus where they act as stress sensors (Liebermann and Hoffman, 2008). An involvement of the GADD45 proteins in resistance to thermal stress has been described for fruit fly *Drosophila melanogaster*. Its orthologue*D-GADD45* gene was stronger expressed after hyperthermia and contributed to elevated survival rates (Moskalev*et al.*, 2012). Moreover,the GADD45 family

members interact with partner proteins involved in cell cycle arrest and DNA repair, thereby promoting cell survival. GADD45 family members are also tasked with oxidative stress, heat shock response, starvation and inflammation (Moskalev*et al.*, 2012).

The gene *fgf1* encodes a fibroblast growth factor, which is also known to be connected to temperature response. It has been demonstrated in 3T3 cells that upon heat shock *fgf1* is secreted from the cytosol into the extracellular space (Jackson *et al.*, 1992). Once excreted, it induces growth and differentiation in various cell types (Mizukoshi*et al.*, 1999).

The gene *sostdc1* (sclerostin domain-containing protein 1) is known to be involved in cell signalling and plays a role in various developmental processes (Clausen *et al.*, 2011). It regulates bone morphogenetic proteins that are involved in cell proliferation and differentiation and also inhibits the Wnt pathway(Gopal *et al.*, 2013). Thus, *sostdc1* influences various cellular processes.

Overall, a rapid cold shock of a temperature decrease from 5 °C to 0 °C had only slight effects on gene expression in *O. mykiss*. Of the three examined tissues, the liver was the most affected one. In this tissue the three genes *gadd45a*, *fgf1* and *sostdc1* were found to be involved in cold shock response in salmonid liver. More work is needed to elucidate their exact role in cold shock.

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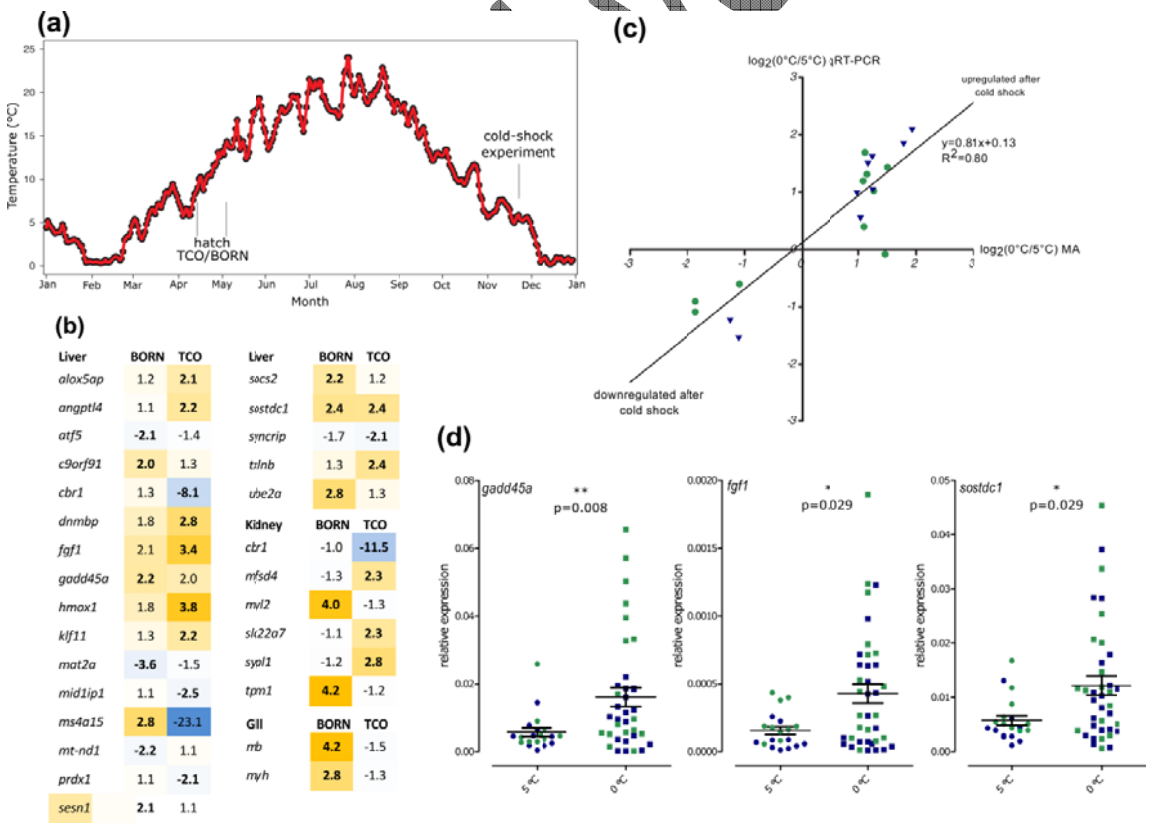
Captions:

Figure1:Critical temperatures during the husbandry of trout (a) and profiling of gene expression during cold shock (b-d). (a) Temperature profile of the brackish water in the aquaculture facility, where BORN trout werebred and where the experiment took place, as measured over a period of 13 months. Hatching of the trout as well as the date of the cold-shock experiment are labelled. (b) Overview of genes regulated by cold-shock according to

microarray analysis in in liver, kidney and gills of*O. mykiss* strains BORN and TCO. Fold changes (0 °C/5 °C) printed bold are statistically significant (adjusted $p < 0.05$). Orange boxes indicate upregulation at 0 °C, blue boxes downregulation at 0 °C, compared with the control group at 5°C. (c) Correlational analysis of microarray (MA) and qRT-PCR results based on binary logarithm (log2n), measured for regulated genes in liver from BORN (green circles), and TCO (blue triangles) strains. A regression line is shown with the corresponding formula and coefficient of determination. (d) Results of qRT-PCR for *gadd45a*, *fgf1* and *sostdc1* at 5 °C and 0 °C in TCO (blue) and BORN (green) strain. Each dot represents one individual measurement, normalized against three reference genes. Lines represent means ± SEM of all measurements. Statistical significance was determined by Mann-Whitney Rank Sum Test.



Liver	BORN	TCO
alox5ap	1.2	**2.1**
angptl4	1.1	**2.2**
atf5	**-2.1**	-1.4
c9orf91	**2.0**	1.3
cbr1	1.3	**-8.1**
dnmbp	1.8	**2.8**
fgf1	2.1	**3.4**
gadd45a	**2.2**	2.0
hmox1	1.8	**3.8**
klf11	1.3	**2.2**
mat2a	**-3.6**	-1.5
mid1ip1	1.1	**-2.5**
ms4a15	**2.8**	**-23.1**
mt-nd1	**-2.2**	1.1
prdx1	1.1	**-2.1**
sesn1	**2.1**	1.1

Liver	BORN	TCO
sacs2	**2.2**	1.2
sostdc1	**2.4**	**2.4**
syncrip	-1.7	**-2.1**
tilnb	1.3	**2.4**
ube2a	**2.8**	1.3
Kidney	**BORN**	**TCO**
cbr1	-1.0	**-11.5**
mfsd4	-1.3	**2.3**
myl2	**4.0**	-1.3
slc22a7	-1.1	**2.3**
syal1	-1.2	**2.8**
tpm1	**4.2**	-1.2
Gill	**BORN**	**TCO**
mb	**4.2**	-1.5
myh	**2.8**	-1.3

Table 1: Primers used in this study

Gene symbol	Forward primer	Reverse primer	Gene name
Target genes			
alox5ap	GTGTCCTGCGCCAATCGTAAC	TCTGACCCATGTACCCGACAAA	arachidonate 5-lipoxygenase-activating protein
angptl4	CCGACATGAATGCAGGCAACTA	TTGTCAGCTGAATCTGGTTTTGC	angiopoietin-like 4
atf5	AAACAGCTGCTCACAGGTATCG	GCCTTGTAGACCTCGATGAGTA	activating transcription factor 5
fgf1	GGGGCCAACGACACAGCAAAA	CCAGGCACTCACTGCTTTAACCT	fibroblast growth factor 1
gadd45a	GTACTAGTTACTAACCCCCAGTT	TGCAGTGTGCGATGTTTCCATAT	growth arrest and DNA-damage-inducible, alpha
hmox1	CACGTCGGACACATTCCAAACT	AGCATAGATTCCCATGCCAACC	heme oxygenase (decycling) 1
mat2a_A	GTAGCCCATCCCCTCTCAATC	TAGGCAGCGGTCCTCTGATAC	methionine adenosyl-transferase II, alpha A
mat2a_B	TTAAGGCAGAGCTGTGCAAGAG	CACCAGGCCGAAGGTCAAAGT	methionine adenosyl-transferase II, alpha B
mid1ip1	AGAGGAGCAGCAATCGATAACC	TTTCCCTCTTGTAGCGGTTGGT	MID1 interacting protein 1
ms4a15	AACATATGCACCAGTTGATCAGC	TTTCTTTATTCAGTCTGGTGATGG	membrane-spanning 4-domains, subfamily A, member 15
sesn1	AGCCAAAATGGACTTTGGAGTTC	CCCATCACCAAGGAGATGTTGT	sestrin 1
socs2	CGGGTTGTAGATTCCGACGAG	AGTCCCTCTGGGAGCTATCTC	suppressor of cytokine signaling 2
sostdc1	TGATGCTACGGAGATCCTATACT	TGGTGGACCTCAGCTCTCTAC	sclerostin domain containing 1

syncrip	ATCAAAGCCCTGCTGGAGAGAA AGGTACCAGCTCATCCTCAAATA	synaptotagmin binding, cytoplasmic RNA interacting protein
txlnb	AGCGAGAGAGGGATCATTTGATA taxilin beta	CACTCCGTTGCTCTTCGACAC
ube2a	CAAGAAGACGATTGATGAGAGATT TAGGGTATTCTTCTGTGAACTCAA	ubiquitin-conjugating enzyme E2A

Reference genes

actb	ACCCAGCTTCTCAGTCTCATT actin, beta	TCAGCTGCATGATAGAATCTC
eef1a1	TGATCTACAAGTGCGGAGGCA eukaryotic translation elongation factor 1 alpha 1	CAGCACCCAGGCATACTTGAA
rps5	ATGACATCTCACTGCAGGATTAC ribosomal protein S5	ATCAGCTTCTTGCCGTTGTTGC