Journal of Genetics, Vol. 96, No. 4, September 2017, pp. 701–706
DOI 10.1007/s12041-017-0811-x

RESEARCH NOTE

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

ANDREAS BORCHEL[1,2], MARIEKE VERLEIH[1], ALEXANDER REBL[1] and TOM GOLDAMMER[1]*

[1] *Leibniz Institute for Farm Animal Biology (FBN), Institute of Genome Biology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany*
[2] *SLCR-Sea Lice Research Centre, Department of Biology, University of Bergen, Thormøhlensgate 55, 5008 Bergen, Norway*
*For correspondence. E-mail: tom.goldammer@uni-rostock.de.

Received 21 November 2016; revised 22 December 2016; accepted 2 January 2017; published online 30 August 2017

Abstract. A rapid decline in temperature poses a major challenge for poikilothermic fish, as their entire metabolism depends on ambient temperature. The gene expression of rainbow trout *Oncorhynchus mykiss* having undergone such a cold shock (0°C) was compared to a control (5°C) in a microarray and quantitative real-time PCR based study. The tissues of gill, kidney and liver were examined. The most differently expressed genes were found in liver, many of them contributing to the network 'cellular compromise, cellular growth and proliferation'. However, 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 considered as general biomarkers for cold shock in rainbow trout.

Keywords. stress response; fibroblast growth factor 1; growth arrest and DNA-damage-inducible alpha; microarray; sclerostin domain-containing protein 1; temperature.

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*) that undergone such a cold shock (0°C) to a control (5°C) using microarrays and quantitative real-time PCR (qRT-PCR). The number of genes found to be regulated at 0°C was surprisingly low. Instead of classical genes involved in the 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 involved in 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) are extensively 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 recently analysed holistically, for example, by 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

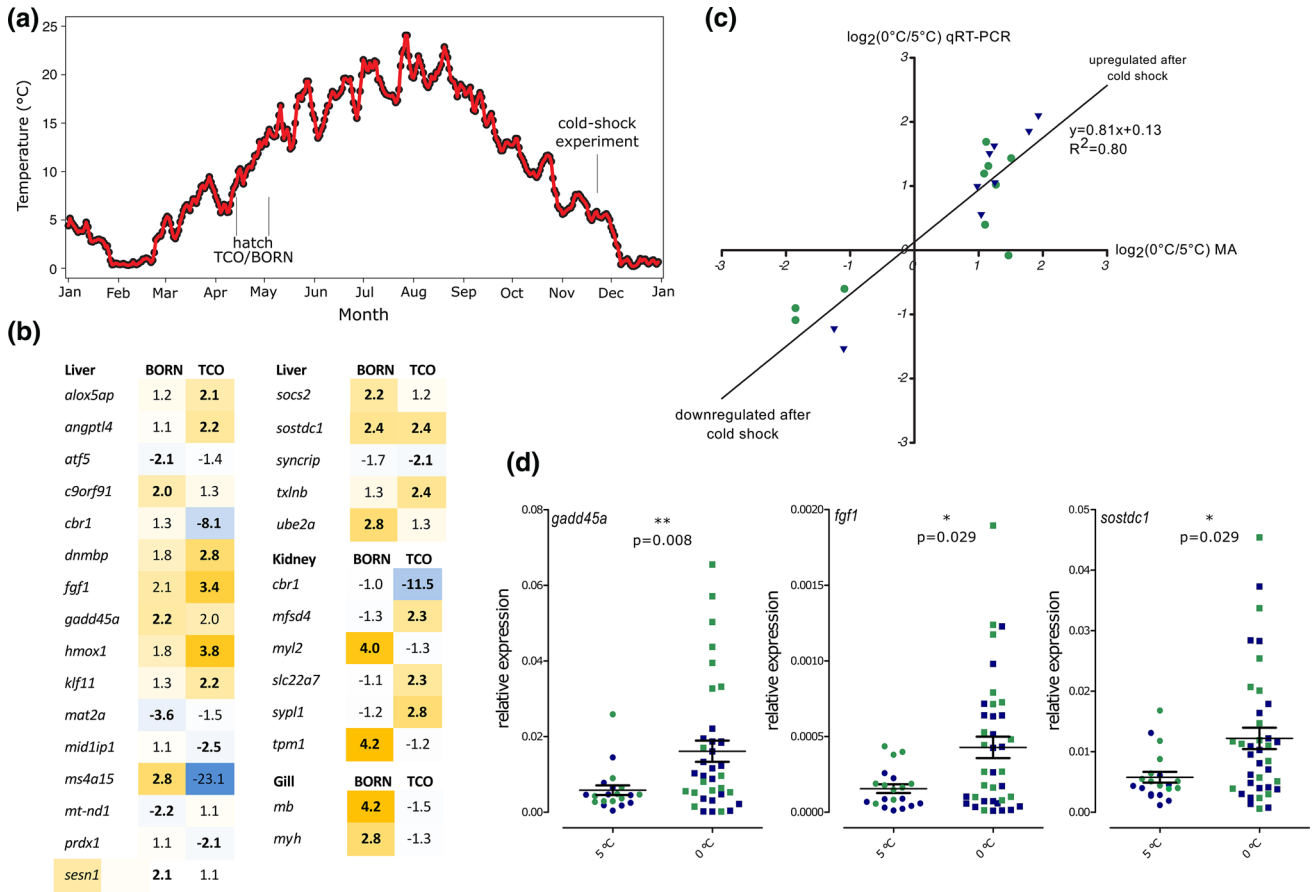

Figure 1. (a) Critical temperatures during the husbandry of trout and (b–d) profiling of gene expression during cold shock. (a) Temperature profile of the brackish water in the aquaculture facility, where BORN trout were bred and where the experiment took place, as measured over a period of 13 months. 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 liver, kidney and gills of *O. mykiss* strains, BORN and TCO. Fold changes (0°C/5°C) printed in bold are statistically significant (adjusted $P < 0.05$). Orange boxes indicate upregulation at 0°C, and blue boxes indicate downregulation at 0°C when compared with the control group at 5°C. (c) Correlational analysis of microarray (MA) and qRT-PCR results based on binary logarithm (log 2*n*), 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 and 0°C in TCO (blue) and BORN (green) strains. Each dot represents one individual measurement, normalized against three reference genes. Lines represent means ± SEM of all the measurements. Statistical significance was determined by Mann–Whitney rank sum test.

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 is not examined before, though, a drop in temperatures around 0°C occurs regularly in semi-open aquaculture systems in countries with cold winters like Germany (figure 1a).

The main objective of this study was thus, the identification of genes that are regulated upon an acute cold shock from 5 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 and Fishery, Born, Germany and TACOMA/TCO, Troutlodge, Tacoma, USA) were kept under comparable conditions in lanes of a semi-open aquaculture facility. BORN strain 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 min. The fish were 224 (TCO) and 239 (BORN) days old, had a total length of 18.1 ± 1.5 (TCO) and 18.4 ± 1.3 cm (BORN), and a weight of 76.4 ± 15.2 (TCO) and 81.6 ± 16.7 g (BORN). 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. Owing to the duration of sampling, the minimal cold-shock exposure was 30 min and the maximal exposure was 130 min with similar exposure times for BORN as well as TCO trout. Average exposure length was 1 h 20 min. As a 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, Germany) 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 was employed in linear T7-based amplification and labelled with Cyanine-3. Agilent whole salmon genome oligo microarrays 8 × 60K (GEO Platform GPL21057; Miltenyi Biotec 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 × 2 conditions × 2 strains × 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) was 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 (cold-shock treatment) and 5°C (control) was performed by 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 >2 and an adjusted P value <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, qRT-PCR was conducted for selected genes from liver, which were found to be differently expressed in the present microarray study. Individual samples of 1.5 μg RNA were deployed in cDNA synthesis using Superscript II (Invitrogen, Carlsbad, USA) 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, USA) and diluted in 100 μL nuclease-free water.

qRT-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*. Significant levels of observed differences were calculated using Mann–Whitney rank sum tests performed 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 1b). 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 in one of the two strains alone. This strain specificity in gene expression might correlate with the assumption that both the strains are somehow genetically adapted to their original water bodies used for breeding. Water temperatures of lakes in Washington state (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 1a and http://green2.kingcounty.gov/lakes).

qRT-PCR was used to verify whether the microarray analysis reliably reflected the cold-shock-induced patterns of transcriptional changes in rainbow trout (figure 1c). 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 through microarray as well as qRT-PCR was assessed. Apart from *ube2a*, all analysed

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	CCAGCACTCACTGCTTTAACCT	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	CACTCCGTTGCTCTTCGACAC	Taxilin beta
ube2a	CAAGAAGACGATTGATGAGAGATT	TAGGGTATTCTTCTGTGAACTCAA	Ubiquitin-conjugating enzyme E2A
Reference genes			
actb	ACCCAGCTTCTCAGTCTCATT	TCAGCTGCATGATAGAATCTC	Actin, beta
eef1a1	TGATCTACAAGTGCGGAGGCA	CAGCACCCAGGCATACTTGAA	Eukaryotic translation elongation factor 1 alpha 1
rps5	ATGACATCTCACTGCAGGATTAC	ATCAGCTTCTTGCCGTTGTTGC	Ribosomal protein S5

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 two-fold in BORN strain as well as TCO strain with adjusted *P* values <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 <0.05 still in one strain, are shown in figure 1d. Significant upregulations based on temperature effects were found in 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 low temperatures 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 are 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 is shown that the *cirbp* expression of *O. mykiss* is already increased at a temperature of 8°C in kidney (Verleih *et al.* 2015).

Although, 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 fish strains that were examined after a cold shock and may thus be involved in the response to stress caused by low 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 and 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 is described for fruit fly, *Drosophila melanogaster*. Its orthologue *D-GADD45* gene was strongly 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 *fgf1* gene encodes a fibroblast growth factor, which is also known to be connected to the temperature response. It is 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 *sostdc1* gene 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* gene influences various cellular processes.

Overall, a rapid cold shock of a temperature decrease from 5 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.

Acknowledgements

This project was funded by the European Fisheries Fund (EFF) and the Ministry of Agriculture, the Environment and Consumer Protection Mecklenburg-Western Pomerania (pilot project: Rainbow trout BORN; VI-560/7308-4). Carsten Kühn is gratefully acknowledged for keeping the fish. Ingrid Hennings, Brigitte Schöpel, Luisa Falkenthal and Marlies Fuchs are acknowledged for expert technical assistance.

References

Anders E. 1986 Stand der Züchtung und reproduktion brackwasseradaptierter Regenbogenforellenbestände im Küstenbereich der DDR. *Fischerei-Forsch.* **24**.

Basu N., Todgham A. E., Ackerman P. A., Bibeau M. R., Nakano K., Schulte P. M. and Iwama G. K. 2002 Heat shock protein genes and their functional significance in fish. *Gene* **295**, 173–183.

Bell M. 1990 *Fisheries handbook of engineering requirements and biological criteria.* Corps of Engineers, North Pacific Division, Portland, USA.

Benjamini Y. and Hochberg Y. 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* **57**, 289–300.

Borchel A., Verleih M., Rebl A., Kühn C. and Goldammer T. 2014 Creatine metabolism differs between mammals and rainbow trout (*Oncorhynchus mykiss*). *Springer Plus* **3**, 510.

Clausen K. A., Blish K. R., Birse C. E., Triplette M. A., Kute T. E., Russell G. B. *et al*. 2011 SOSTDC1 differentially modulates Smad and beta-catenin activation and is down-regulated in breast cancer. *Breast Cancer Res. Treat.* **129**, 737–746.

Donaldson M. R., Cooke S. J., Patterson D. A. and Macdonald J. S. 2008 Cold shock and fish. *J. Fish Biol.* **73**, 1491–1530.

Feder M. E. and Hofmann G. E. 1999 Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243–282.

Gopal G., Raja U. M., Shirley S., Rajalekshmi K. R. and Rajkumar T. 2013 SOSTDC1 down-regulation of expression involves CpG methylation and is a potential prognostic marker in gastric cancer. *Cancer Genet.* **206**, 174–182.

Gracey A. Y., Fraser E. J., Li W., Fang Y., Taylor R. R., Rogers J. *et al*. 2004 Coping with cold: an integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proc. Natl. Acad. Sci. USA* **101**, 16970–16975.

Jackson A., Friedman S., Zhan X., Engleka K. A., Forough R. and Maciag T. 1992 Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* **89**, 10691–10695.

Liebermann D. A. and Hoffman B. 2008 Gadd45 in stress signaling. *J. Mol. Signal.* **3**, 15.

Long Y., Song G., Yan J., He X., Li Q. and Cui Z. 2013 Transcriptomic characterization of cold acclimation in larval zebrafish. *BMC Genomics* **14**, 612.

Mininni A. N., Milan M., Ferraresso S., Petochi T., Di Marco P., Marino G. *et al.* 2014 Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. *BMC Genomics* **15**, 765.

Mizukoshi E., Suzuki M., Loupatov A., Uruno T., Hayashi H., Misono T. *et al.* 1999 Fibroblast growth factor-1 interacts with the glucose-regulated protein GRP75/mortalin. *Biochem. J.* **343**, 461–466.

Moskalev A., Plyusnina E., Shaposhnikov M., Shilova L., Kazachenok A. and Zhavoronkov A. 2012 The role of D-GADD45 in oxidative, thermal and genotoxic stress resistance. *Cell Cycle* **11**, 4222–4241.

Rebl A., Verleih M., Köbis J. M., Kühn C., Wimmers K., Köllner B. and Goldammer T. 2013 Transcriptome profiling of gill tissue in regionally bred and globally farmed rainbow trout strains reveals different strategies for coping with thermal stress. *Mar. Biotechnol.* **15**, 445–460.

Roberts R. J., Agius C., Saliba C., Bossier P. and Sung Y. Y. 2010 Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *J. Fish Dis.* **33**, 789–801.

Smyth G. K. 2005 Limma: linear models for microarray data. In *Bioinformatics and computational biology solutions using R and Bioconductor* (ed. R. Gentleman, V. Carey, W. Huber, R. Irizarry and S. Dudoit), pp. 397–420. Springer, New York, USA.

Tutar L. and Tutar Y. 2010 Heat shock proteins: an overview. *Curr. Pharm. Biotechnol.* **11**, 216–222.

Verleih M., Borchel A., Krasnov A., Rebl A., Korytář T., Kühn C. and Goldammer T. 2015 Impact of thermal stress on kidney-specific gene expression in farmed regional and imported rainbow trout. *Mar. Biotechnol.* **17**, 576–592.

Xu H., Zhang D. L., Yu D. H., Lv C. H., Luo H. Y. and Wang Z. Y. 2015 Molecular cloning and expression analysis of scd1 gene from large yellow croaker *Larimichthys crocea* under cold stress. *Gene* **568**, 100–108.

Corresponding editor: Silvia Garagna