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Expression stability and selection of optimal reference genes for gene expression normalization in early life stage rainbow trout exposed to cadmium and copper

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ABSTRACT

Gene expression analysis represents a powerful approach to characterize the specific mechanisms by which contaminants interact with organisms. One of the key considerations when conducting gene expression analyses using quantitative real-time reverse transcription-polymerase chain reaction (qPCR) is the selection of appropriate reference genes, which is often overlooked. Specifically, to reach meaningful conclusions when using relative quantification approaches, expression levels of reference genes must be highly stable and cannot vary as a function of experimental conditions. However, to date, information on the stability of commonly used reference genes across developmental stages, tissues and after exposure to contaminants such as metals is lacking for many vertebrate species including teleost fish. Therefore, in this study, we assessed the stability of expression of 8 reference gene candidates in the gills and skin of three different early life-stages of rainbow trout after acute exposure (24 h) to two metals, cadmium (Cd) and copper (Cu) using qPCR. Candidate housekeeping genes were: beta actin (*b-actin*), DNA directed RNA polymerase II subunit I (*DRP2*), elongation factor-1 alpha (*EF1a*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), glucose-6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyltransferase (*HPRT*), ribosomal protein L8 (*RPL8*), and *18S* ribosomal RNA (*18S*). Four algorithms, geNorm, NormFinder, BestKeeper, and the comparative ΔCt method were employed to systematically evaluate the expression stability of these candidate genes under control and exposed conditions as well as across three different life-stages. Finally, stability of genes was ranked by taking geometric means of the ranks established by the different methods. Stability of reference genes was ranked in the following order (from lower to higher stability): *HPRT* < *GAPDH* < *EF1a* < *G6PD* < *RPL8* < *DRP2* < *b-actin* in gills of fish exposed to Cd; *b-actin* < *GAPDH* < *G6PD* < *DRP2* < *RPL8* < *HPRT* < *EF1a* in gills of fish exposed to Cu; *RPL8* < *HPRT* < *GAPDH* < *G6PD* < *EF1a* < *DRP2* < *b-actin* in the skin of fish exposed to Cd; and *EF1a* < *GAPDH* < *RPL8* < *HPRT* < *G6PD* < *b-actin* < *DRP2* in the skin of fish exposed to Cu. The results demonstrated that the stability of reference genes depended on the metal, life-stage and/or organ in question. Thus, attention should be paid to these factors before selection of reference gene for relative quantification of the gene expressions.

1. Introduction

Gene expression analysis provides critical information regarding the molecular mode of toxic action of contaminants in organisms (Hahn and Stegeman, 2000). Quantitative real-time reverse transcription-polymerase chain reaction (qPCR) is one of the most commonly used molecular biology techniques for measuring gene expression. The popularity of qPCR can be attributed to its simplicity, sensitivity, and specificity. The use of qPCR has increased tremendously in nearly all branches of biology including ecotoxicology. There are two major methods for the quantification of gene expression in a biological sample by qPCR: absolute and relative (Bustin, 2005). Absolute quantification is performed by generating a standard curve from serially diluted standards of known concentrations of genetic material. These standards

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are usually DNA fragments containing the target sequence (Wong and Medrano, 2005). Because of the standard curve, absolute quantification does not require normalization of target gene against a reference gene. However, absolute quantification is far more labour intensive than relative quantification and it is particularly difficult when quantification of a large number of genes is required. In relative quantification, the result for the gene of interest (GOI) is normalised against a reference gene in the same sample (Livak and Schmittgen, 2001). The normalised numbers of GOIs are then compared among treatment groups and control in order to obtain a fold change difference in the expression of GOIs as a result of the treatment. Genes that are expressed constitutively in a given organism are usually used as reference genes (Li et al., 2010). In order to reach meaningful conclusions using relative quantification, expression levels of reference genes must not undergo changes in response to the stressor of interest and should be constant among replicates (Gilliland et al., 1990). Because of the ease of performance and consistent results, relative quantification is more widely used method in qPCR experiments as compared to absolute quantification. However, there is increasing evidence that expression of assumed reference genes can vary significantly with experimental conditions such as developmental stage and chemical treatment, which can significantly affect the interpretation of relative quantification qPCR results (McCurley and Callard, 2008).

Metals are considered to be among the most toxic substances to aquatic organisms (Govind, 2014), and understanding the mechanisms that drive the sensitivity of aquatic organisms such as fish to metals is critical for conducting objective ecological risk assessments. Gene expression analyses have played a substantial role in furthering our understanding of several aspects of metal toxicity, such as metal induced oxidative stress, toxic effects of metals to the nervous system, and identification of biomarkers of exposure for metals in fish and other organisms (Gonzalez et al., 2006; Lu et al., 2005; Misra et al., 1989; Morcillo et al., 2016; Price-Haughey et al., 1986). Gene expression analyses have also helped in advancing our knowledge of metal uptake pathways in fish (Komjarova and Bury, 2014). A large number of mechanistic studies with metals revolve around measuring expression of genes such as metallothionein (*MT*), oxidative stress pathways, metal transporters, etc. (Doering et al., 2015; Komjarova and Bury, 2014; Tang et al., 2016).

Fish are considered to be most sensitive to metals during their early life-stages (Calfee et al., 2014; Grosell et al., 2002; Tang et al., 2016). Hence, many studies use early life-stages of fish as model test organisms. However, it is important to note that significant differences in metal sensitivity have been reported among different early life-stages of selected fish species (Vardy et al., 2013). Moreover, large differences in the sensitivity to metals are commonly observed among different fish species (Besser et al., 2007; Marr et al., 1995). At present, the mechanistic knowledge regarding species and life-stage specific differences in sensitivity of fish to metals is very limited at best. However, it is generally believed that differences in metal sensitivity occur due to differences in toxicokinetics and ionoregulatory physiology among different life-stages and species of fish, which in turn may arise due to the differences in the expression patterns of the genes encoding proteins involved in regulating metal and ion transport and homeostasis, as indicated recently by global transcriptome profiling of metal exposed populations of brown trout (*Salmo trutta*) (Uren Webster et al., 2013). Differences in oxidative stress and *MT* responses are also important candidates for the possible roles in species and life-stage specific differences in metal sensitivity (Tang et al., 2016). Hence, there is an increasing focus on the quantification of the expression patterns of genes involved in metal/ion-homeostasis and other molecular pathways (Tang et al., 2016; Uren Webster et al., 2013).

As discussed above, meaningful and reliable interpretation of changes in gene expression as a function of exposure to metals or other contaminants relies on reference genes that show high stability among treatment and control conditions. However, there is a lack of information regarding the stability of reference genes in fish species after exposure to metals or as a function of developmental stages during early life-stages. Therefore, in this study, we have evaluated the most suitable genes from a set of 8 most commonly used reference genes in three different life-stages of rainbow trout as a function of short term metal exposure by using four different mathematical algorithms: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and comparative ΔCt method (Chen et al., 2011). Four different algorithms were used because each of them is based on slightly different principles and sometimes produce slightly different results. Results obtained with four algorithms were then used to prepare a final ranking of the most suitable reference genes as described previously (Chen et al., 2011). Rainbow trout was chosen as the model organism in this study because it is known to be one of the most sensitive fish species to the exposure with metals and because it is widely used for regulatory testing procedures (Calfee et al., 2014). Copper (Cu) and Cadmium (Cd) were used as model metals as they are among the most studied metals in aquatic toxicology and are of high concern worldwide (Wood, 2011). Further, three distinct early life-stages viz. larval, swim up and juvenile were selected in order to determine the most suitable reference genes during critical developmental stages of rainbow trout. Skin and gills are the major ionoregulatory organs in fish, which play a critical role in metal uptake and elimination (Fu et al., 2010; Grosell and Wood, 2002). Hence, skin and gills were selected as model organs in this study.

2. Materials and methods

2.1. Test organisms

Eyed eggs of rainbow trout were obtained from Troutlodge Inc. (Washington, United States) and were reared in the Aquatic Toxicology Research Facility at the University of Saskatchewan in a flow through system maintained at approximately 12–13 °C. Fish were fed commercial trout food (Bio Vita Starter #0 Crumble, Bio-Oregon, British Columbia, Canada). Food was first introduced to fish at 12–13 days post hatch (dph). All procedures were approved by the University of Saskatchewan's University Council on Animal Care and Supply (Protocol 20140079).

2.2. Test chemicals and exposure protocol

Copper (II) sulfate pentahydrate (Chemical Abstracts Service [CAS] 7758-99-8; purity 99.995%) and cadmium chloride hemi-pentahydrate (CAS 7790-78-5; purity 99.999%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in laboratory reverse osmosis water. All exposure waters were prepared by mixing reverse osmosis water and dechlorinated city of Saskatoon municipal tap water at a ratio of 3:1 in order to adjust hardness and alkalinity to a moderate level (~65 and 40 mg/L as $CaCO_3$, respectively). Stock solutions of Cu and Cd were prepared in this water and stored at 13 °C for at least 24 h before exposure. Exposures were conducted for 24 h with each of three different life-stages: larval (5 dph), swim-up (15 dph), and juvenile (45 dph). Fish were exposed to 40 μg/L Cu (625 nM) and 5 μg/L Cd (45 nM), in addition to the control group (water), under static renewal conditions. The concentrations were selected on the basis of previous studies which demonstrated that similar ranges of concentrations were capable of inducing specific biological effects without causing mortality (Lim et al., 2015; McGeer et al., 2000; unpublished data). Four fish were used per group based on other studies (Dos Santos et al., 2015; McCurley and Callard, 2008; Zhang et al., 2012). Individual 500 mL high density polyethylene cups were used for exposing each fish. Half of the exposure media was replaced after every 12 h. The volume of exposure media in each cup was 250 mL (5 and 15 dph) or 500 mL (45 dph). Exposures were conducted under a 16:8 h light/dark cycle and at a temperature of 13 ± 1 °C. Fish were acclimated in exposure