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Normalization strategies for gene expression studies by real-time PCR in a marine fish species, *Scophthalmus maximus*

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ABSTRACT

Thorough evaluation of normalization approaches is a fundamental aspect in real-time quantitative RT-PCR experiments to avoid artificial introduced intergroup variations. In our study, we tested three normalization strategies in an experimental data set derived from a toxicological exposure of *Scophthalmus maximus* to the peroxisome proliferator-activated receptor alpha (PPARα) agonist WY-14643. Juvenile turbots were exposed by repeated injections to 5 mg or 50 mg WY-14643/kg, and liver samples were taken at day 1, 7 and 21. Specifically, the mRNA expression of peroxiredoxin 5 (*prdx5*) was normalized to the cDNA content, to the mRNA expression of single reference genes (b-actin, *b-act*; elongation factor 1 α, *ef1a*; glyceraldehyde-3-phosphate dehydrogenase, *gapdh*; ribosomal protein L8, *rpl8*; tata-box binding protein, *tbp*; tubulin beta 2C chain, *tubb2c*; ubiquitin-conjugating enzyme E2L 3, *ub2l3*) or to a combination of multiple reference genes using geNorm, BestKeeper or NormFinder algorithms.

Four single reference genes (*ef1a*, *rpl8*, *tubb2c*, *tbp*) did not show any significant differences between the treatment groups over time, while significant intergroup variations were observed for cDNA content, *gapdh*, *b-act* and *ub2l3*. The normalization of *prdx5* to the valid (not altered) single reference genes led to significant up-regulated (*prdx5*/*rpl8*), not-regulated (*prdx5*/*ef1a*; *prdx5*/*tbp*) or down-regulated (*prdx5*/*tubb2c*) mRNA expression pattern. The multiple reference gene approaches resulted in different rankings and combinations of the most stable expressed reference genes (geNorm: *ef1a* > *rpl8* > *b-act*; BestKeeper: *ub2l3* > *gapdh* > *ef1a*; NormFinder: *b-act* > *ef1a*). However, the normalization with the three multiple reference gene procedures demonstrated consistent expression pattern with a significant up-regulation of *prdx5* in response to the higher concentration after 21 days.

Concluding, even if not yet established as "gold" standard for expression profiling in environmental toxicology or physiology using freshwater or marine fish models, the multiple reference gene approach is recommended, since it eliminates any biased results, which represented the major flaw of single reference genes.

1. Introduction

Mechanistic studies that aim to analyze the effects of environmental compounds can be performed by expression profiling of target genes with real-time PCR to get insights into the transcriptional networks and affected signaling pathways, as it was the case with endocrine disrupter chemicals (Kloas et al., 2009; Larkin et al., 2003). Furthermore, genomics techniques like microarrays or next-generation sequencing rely on the confirmation of their results by real-time PCR.

Generally, reference genes are recommended for the use of real-time quantitative PCR quantification with the comparative C_T method (Bustin et al., 2005; Huggett et al., 2005; Pfaffl, 2001; Vandesompele et al., 2002), which have several advantages over the absolute quantification strategy using standard curves or other approaches using external standards (e.g. cDNA content, total RNA content per mg tissue, etc.). If a suitable reference gene has been found, it controls for many sources of variability in PCR reactions as RNA integrity, efficiency of RT reactions or cDNA sample load differences (Pfaffl, 2001) ensuring low sample-to-sample and low run-to-run variations. Main difficulties in accurate quantification of mRNA targets are based on the RNA integrity and the RT reactions assuming that all mRNAs of individual samples are transcribed with the same efficiencies (Bustin et al., 2005). Approaches using external standards (e.g. normalization to total RNA or absolute quantification) do not control for RNA integrity or RT efficiencies, while internal standards (e.g. reference genes) are amplified side by

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side with target genes and are part of the same procedural steps. Prerequisites for reference genes are that they are stable expressed between tissues, developmental stages, chemical exposures or other experimental situations. No ideal or universal reference genes exist, and hence, reference genes have to be evaluated in each experimental system (Andersen et al., 2004; Vandesompele et al., 2002; Zheng and Sun, 2011). If no suitable reference genes can be found in difficult experimental situations, the quantification of cDNA was proposed as an alternative (Libus and Storchova, 2006). However, this approach cannot control for sample load differences or different RNA integrity. Likewise, the normalization to ribosomal RNA (rRNA) is seen critically since 18S and 28S rRNAs compose the majority of the total RNA and are not reflective of changes on the mRNA fraction; imbalances have already been described between rRNA and mRNA fractions (Solanas et al., 2001; Vandesompele et al., 2002). Even if no perfect normalization strategy is present for the accurate quantification of mRNA expression, the reference gene approach offers some advantages, since it is seen as a 'full procedure control' from the sample storage conditions to the RNA integrity, RT efficiency and sample load (Bustin et al., 2005; Pfaffl et al., 2004; Vandesompele et al., 2002), which is especially important in the analysis from complex solid tissues that consist of different subpopulations or cell types (tissue heterogeneity). Thorough normalization strategies are needed for quantitative real-time PCR to avoid artificial introduced bias by the random selection of reference genes (Filby and Tyler, 2007; McCurley and Callard, 2008).

The aim of our study was to evaluate different normalization strategies for real-time PCR experiments on an experimental data set that was derived from a toxicological exposure of *Scophthalmus maximus* to the peroxisome proliferator activated receptor (PPAR) α agonist WY-14643. PPARs are involved in the regulation of lipid and carbohydrate metabolism. Natural ligands are poly- and monounsaturated fatty acids (FA) and FA derivates in mammals (Hihi et al., 2002) and teleosts (Colliar et al., 2011), but many synthetic compounds have been shown to activate PPARs including lipid lowering pharmaceuticals (Rosal et al., 2010), the phthalate mono-esters (Bility et al., 2004; Hurst and Waxman, 2003), or perfluorinated chemicals (Huang et al., 2011). Especially, the fact that many environmental chemicals have the potential to interfere with PPARs, has raised the concern that peroxisome proliferators may be involved in the worldwide epidemic of obesity and obesity-related disorders like diabetes, hypertension or cardiovascular disease (Grun and Blumberg, 2009). Lipid lowering agents, as bezafibrate, clofibric acid or fenofibric acid, are commonly detected in surface waters or sewage treatment plant effluents, and are of potential concern for aquatic vertebrates due to their large use, persistence and potential for bioaccumulation (Rosal et al., 2010). Turbot is a marine flatfish species that is distributed along the Northeast Atlantic, but which is also frequently available by fish farmers. Herewith, turbots are suitable as a sentinel species for environmental pollution or for laboratory experiments. Peroxiredoxin-5 (*prdx5*) was selected as target gene, which belongs to the peroxiredoxins, a family of peroxidases that reduces peroxides and organic hydroperoxides, and is involved in the antioxidant protective mechanisms (Banmeyer et al., 2004, 2005). Specifically, the mRNA expression of *prdx5* in the liver was normalized using different strategies including cDNA content (Libus and Storchova, 2006; Urbatzka et al., 2010), single reference genes (Pfaffl, 2001), or multiple reference gene approaches as geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004). Since the reference gene selection is regarded as critical point for each given experimental situation, a set of seven reference genes (b-actin, *b-act*; elongation factor 1 α, *ef1a*; glyceraldehyde-3-phosphate dehydrogenase, *gapdh*; ribosomal protein L8, *rpl8*; tata-box binding protein, *tbp*; tubulin beta 2C chain, *tubb2c*; ubiquitin-conjugating enzyme E2L 3, *ub2l3*) were tested either as single reference gene or combined to multiple reference genes, and three multiple reference gene approaches were compared for consistency. Results are discussed with respect to their validity and to the differences observed between normalization approaches. Future studies may benefit from this exhaustive comparison of normalization strategies in real-time PCR data. The recommended reference genes for liver toxicological studies with a PPARα agonist in turbot are likely to work in other similar, chemical exposures in marine flatfish species.

2. Material and methods

The present work was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.1. Experimental solutions

WY-14643 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid, dimethylsulfoxide (DMSO), potassium chloride (KCl) and sodium chloride (NaCl) were purchased from Sigma-Aldrich. Compounds were dissolved in 50% DMSO in saline solution (0.2 g KCl and 8.0 g NaCl in 1 L distilled H_2O) and the same concentration of vehicle was used in all treatment groups. Fish were exposed through intra-peritoneal injection and the following exposure treatments were considered: a positive control of DMSO (Ctrl), a low-dose of 5 mg WY-14643/kg of fish (lo-WY) and a high-dose of 50 mg WY-14643/kg of fish (hi-WY).

2.2. Exposure and sampling

Three independent systems were built alongside, one per treatment (DMSO, lo-WY, hi-WY). Each system consisted in three 60 L opaque tanks (three replicates per treatment) interconnected by flow-through (2 L per min.) artificial seawater (Tropic Marin Sea Salt, Tropical Marine Center, United Kingdom), kept at 35 psu and 18 ± 1 °C, continuously aerated and biologically filtered. Water turnover in each tank was two renovations per hour, since the water flow was 120 L/h. Every day feces and uneaten food were siphoned from the bottom of the tanks with 2–3 L of water from each subsystem, which was then replaced. The tank dimensions were 0.22 × 0.40 m (wide × length), which give a surface of 0.088 m^2/tank. The standing biomass was 9.96 kg/m^3 or 6.79 kg/m^2 (mean weight of fish = 0.0854 kg). The surface area is important for turbot, but only at very high densities or high tanks; at low or moderate densities and shallow tanks, the volume tends to be more important, because it grants water quality. Each tank had a volume of 60 L and the three tanks of the subsystem had 180 L. However, the total water volume of the subsystem was 360 L if taken into account the water volume in the filters, sedimentation tank and foamer (63 L + 112 L + 5 L, respectively). *S. maximus* juveniles were obtained from Aquacria Piscícolas, SA (Torreira, Portugal), and 21 animals were maintained in each system (i.e. 7 fish per tank * 3 replicates). The total length of the juvenile turbot was 13.7 ± 0.9 cm, and the total weight was 85.4 ± 18.3 g. Turbot were fed with a commercial diet (A. Coelho e Castro Lda., Póvoa de Varzim, Portugal) at 0.5% body weight per day. Fish were acclimatized for approximately one month (until stabilization of nitrogenous excretion products, monitored daily). The water parameters during the experimental period were assessed at least 2–3 times per week and are presented separately for each subsystem of the experimental setup (mean ± SD). The values were the following: pH (8.1 ± 0.2/8.2 ± 0.1/8.2 ± 0.1); NO_2 (0.3 ± 0.2/0.4 ± 0.2/0.4 ± 0.2 mg/L); $NH4^+$ (0.4 ± 0.3/0.3 ± 0.1/0.3 ± 0.1 mg/L); NO_3 (8.1 ± 6.7/7.4 ± 6.9/5.2 ± 4.7 mg/L); water temperature (18.3 ± 0.2/18.2 ± 0.2/18.0 ± 0.1 °C); salinity (33.4 ± 0.2/33.5 ± 0.3/33.5 ± 0.3‰). The oxygen content in the tanks was always higher than 90% saturation.

At day 0, fish were intra-peritoneally injected (480 μL) with the respective treatment using a 1 mL sterile syringe. A treatment re-boost was made at day 7 and day 14. Fish were dissected after 24 h from initial injection (day 1), and after 7 and 21 days. Fish were dissected under deep anesthesia (by immersion in 0.16 mL ethylene-glycol monophenyl ether per liter for three min) and after sacrifice by cutting the spinal