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The effect of tert-butyl hydroperoxide on hepatic transcriptome expression patterns in the striped sea bream (Lithognathus mormyrus; *Teleostei*)

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Abstract

The study was aimed at examining the effects of tert-butyl hydroperoxide (tBHP) on hepatic transcriptome expression patterns of the teleost fish *Lithognathus mormyrus*. tBHP is an organic hydro-peroxide, widely used as a model pro-oxidant. It generates the reactive oxygen species (ROS) tert-butoxyl and tert-butylperoxyl. Complementary DNAs of tBHP-treated vs control fish were applied onto a previously produced cDNA microarray of ~1500 unique sequences. The effects of the tBHP application were demonstrated by leukocyte infiltration into the liver and by differential expression of various genes, some already known to be involved in ROS-related responses. Indicator genes of putative ROS effects were: aldehyde dehydrogenase 3A2, Heme oxygenase and the hemopexin-like protein. Putative indicators of transendothelial leukocyte migration and function were: p22phox, Rac1 and CD63-like genes. Interestingly, 7-dehydrocholesterol reductase was significantly down-regulated in response to all treatments. Several non-annotated genes revealed uniform directions of differential expression in response to all treatments.

Keywords: *Reactive oxygen species, aqueous medium, liver, fish, tert-butyl hydroperoxide, gene expression*

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Introduction

Concerted expression of genes underlies almost all biological processes. Consequently, transcriptome expression patterns are widely used as increasingly important research and diagnostic tools across biology and bio-medicine [1,2]. The present study was aimed at elucidating the effect of the model reactive oxygen species (ROS) precursor, tert-Butyl Hydro Peroxide (tBHP) $(CH_3)3COOH$, on hepatic transcriptome expression patterns in the teleost fish *Lithognathus mormyrus*.

ROS are chemically active species of oxygen, harmful to a variety of biological macromolecules and processes in living organisms. They are products of incomplete reduction which occurs during normal reduction of molecular oxygen. They are produced also through metabolism of xenobiotic ROS precursors. ROS interfere with cellular functions through impairing nucleic acids, proteins and lipids. Oxidative stress in response to exposure to ROS occurs when the adaptive detoxification capacity of the cellular anti-oxidizing mechanisms is insufficient [3,4]. Defense mechanisms against oxidative stress include up-regulation of anti-oxidation-related genes, neutralization of ROS by small anti-oxidant scavenger molecules [5] and elimination of damaged proteins through the ubiquitin-proteasome [3] and autophagy [6] pathways. ROS were shown also to be signalling molecules participating in the maintenance of their own homeostasis and in other regulatory pathways [7–11].

Several pollutant types can alter the equilibrium between pro- and anti-oxidants leading to oxidative stress [4,12]. Oxidative stress stimulated by pollutants has been shown to occur in fish [13]. Liver, gills and

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intestine are interesting organs in the environmental context of oxidative stress. The liver is a centre of xenobiotic metabolism and the other organs are located at the interface between the environment and the fish body. This study was concentrated on the liver.

tBHP is an organic hydro-peroxide widely used experimentally as a model pro-oxidant. It has been applied to mammals and mammalian cells [14–17] and also to fish, as a dissolved compound in the ambient water and as an injected compound [18–20]. tBHP metabolism in the liver includes several iron ion related reactions, involving both free ions and ions acting through the cytochrome P450 heme. Two free radical species are produced, tert-butylperoxyl and tert-butoxyl. The latter can initiate lipid peroxidation [21].

It is hypothesized that increased levels of ROS are produced as a result of tBHP exposure and may cause changes in the expression of specific genes. These changes are hypothesized to be related to defense mechanisms against free radicals or to toxic effects caused by the free radicals.

The utilization of the hepatic transcriptome expression profile of the fish *L. mormyrus* as an environmental biomarker is continuously studied in our laboratory [22] and an hepatic cDNA microarray of *L. mormyrus*, cloned from a multi-pollutant affected transcriptome, has served as an expression evaluation tool [22]. tBHP has not yet been identified in natural environments, in spite of its industrial utilization [23]. Therefore, it does not directly mimic environmental situations. However, being a pro-oxidant, it can produce at least part of the ROS-related cell response repertoire. Its experimental application was therefore expected to contribute to the broad perspective of ROS toxic effects on hepatic expression patterns in *L. Mormyrus*, hence, to be relevant to the potential utilization of this fish as an environmental sentinel.

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Materials and methods

Fish sampling and processing

L. mormyrus individuals were sampled alive by gillnetting in a clean habitat along the Mediterranean coast of Israel. Winter sampling was aimed at avoiding catching fish during their reproductive season. Two exposure experiments to tBHP were conducted. Fish were kept in the laboratory maintenance facilities 1 or 2 months prior to experiments 1 and 2, respectively. Flow through seawater at 20–21°C was supplied. Length and weight of the fish were measured at the termination of each experiment, followed by immediate sacrifice by decapitation and dissection of the liver and the gonads. Livers were divided into smaller aliquots, snap frozen in liquid nitrogen and stored at –80°C. Gonads were fixed in 4% formaldehyde. The fish reproductive activity was determined by microscopic observation of fixed gonads smeared between two slides, measuring the average ova diameter of 20 oocytes. Being a proteandrous hemaphrodite, *L. mormyrus* ovotestes always contained at least primordial oogonia [24]. Hence, individuals containing solely primordial oogonia were selected for further analysis.

Total hepatic RNAs were extracted from livers a few days after freezing using the EZ II kit (Biological Industries, Beit Haemek, Israel) according to the manufacturers' instructions. Messenger RNA was isolated from the total RNA preparations using the PolyATtract kit (Promega, Madison, WI, USA). The quality of produced RNAs was evaluated by electrophoresis on a 1% agarose-formaldehyde gel and their concentration was evaluated by spectrophotometry (Nanodrop, Nanodrop Technologies, Wilmington, DE).

Two experiments were conducted:

Experiment 1. Three groups with five fish in each of them were injected with three tBHP doses, 0.5, 5 and 50 mg/kg body weight/injection. Three injections were applied to each fish with 3-day intervals between consecutive injections. A control group of five fish was sham-injected with saline, the tBHP carrier. Both the control and the treated fish were sacrificed 3 days after the last injection. Hence, fish were exposed to tBHP during 9 days. The injected concentrations were aimed at applying a broad range of tBHP sub-lethal doses, initially determined in previous tBHP exposure studies cited in the introduction.

Experiment 2. Two groups with 18 fish in each of them were injected with two tBHP doses of 5 and 30 mg/kg body weight/injection. Five consecutive injections were applied daily to each fish. The first injection of the high dose contained 50 mg/kg body weight, but this was reduced to 30 mg/kg body weight due to mortality of two fish several hours after the first injection. A control group of 18 fish was sham-injected by similar volumes of saline. Six fish of each of the treatment and control groups were sacrificed at three time points: 2, 20 and 68 h after the last injection. Hence, fish were exposed to tBHP during 4–7 days.

Fish characteristics of both experiments are presented in Table I. They reveal no statistically significant differences in fish size among samples and an absence of reproductive activity, judged by their oocyte diameter, according to Funkenstein et al. [25].

Microarray hybridization and analytical procedures

The construction procedure of the utilized microarray followed Auslander et al. [22]. Briefly, cDNAs were

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Table I. Characteristics of sampled fish.

	Fish weight (g)	Fish length (mm)	Oocyte diameter (μm)
Experiment 1	34.6±5.9*	138.7±7.7	127.8±32.9
Experiment 2	39±7.9	143.5±9.7	86.5±29.1

*Average±standard deviation.

cloned from hepatic mRNA of multi-pollutant exposed fish. tBHP was not one of the inducing compounds. The cloned cDNAs were amplified by PCR and the cleaned PCR products were printed on a treated glass slide. In addition, the cDNAs were sequenced and annotated using the BLASTN and BLASTX programmes [26]. Additional functional information was provided by the Gene Ontology (GO) database [27] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [28] using the Blast2Go software [29] and the KEGG bio-informatics tools (http://www.genome.jp/kegg/). Functional characterization of each clone was summarized based on the combined information. DNA fragments which share sequences were assembled and the resulting sequence was designated here *unique clone*, representing the sequence of a specific transcript. The microarray platform characteristics were deposited in the Gene Expression Omnibus (GEO) database of the American National Center for Biotechnology Information (NCBI) (GEO platform accession number – GPL 5351).

The hybridization procedure is composed of the labelling protocol of the target RNAs extracted from the fish livers, their dual hybridization on the microarray slides and the imaging of the resulting labelled slides. The three procedures are described in details in Yudkovski et al. [30]. Briefly, labelling of hepatic mRNA populations with the fluorescent dyes Cy3 and Cy5 was performed using the amino-allyl indirect cDNA labelling method. Each target mRNA population was reverse transcribed in the presence of anchored oligo dT primer and dNTP mix in which the dTTP had been replace by an amino allyl-dUTP/dTTP mixture (4:1). The resulting cDNAs were chemically coupled to an aliquot of the Cy3- or Cy5-mono reactive N- hydroxyl succinimidyl ester-derivatized fluorescent dye via the reactive amino allyl groups. Labelling efficiency was evaluated by spectrophotometry at 260 nm for cDNA estimation and 550 nm (Cy3) and 650 nm (Cy5) for determining labelling intensity.

Microarray slides were incubated with a prehybridization solution [30] to block non-specific DNA interactions, thus reducing background fluorescence followed by washing. Dual-labelled solutions for binary comparisons between the two experimental conditions were prepared by mixing equal amounts of the two labelled cDNA populations to be compared, followed by a 1:1 dilution with a hybridization solution [30]. The heat denatured hybridization mixture was layered onto the microarray, covered and incubated overnight at 42°C in a humidified hybridization chamber. Following incubation, the slides were washed and dried.

GenePix Pro 6.1 software (MDS Analytical Technologies, Sunnyvale, CA) was used for slide imaging. Hepatic significant differentially expressed genes were determined by the LIMMA software [31,32] assisted by the LIMMA user guide. Two spot-related parameters were calculated: $M = \log_2$ (Cy3/Cy5); the $\log_2$ expression ratios for each dual labelled spot on each array, and $A = (\log_2 (Cy5^{*}Cy3))/2$; the average fluorescence intensity across the two dyes. Cy3 and Cy5 are the two normalized values of the fluorescence signals. LIMMA provided also the average A, calculated across each experiment. Only spots with $A > 8.5$ (362 arbitrary fluorescence units out of a maximum of 65 536) and which agreed with the GenePix Pro 6.1 eligibility criteria were included in the analysis. LIMMA analytical procedure was modified to conform to the background characteristics and the distribution of the differentially expressed genes. Hence, the Cy5 and Cy3 intensities within each slide were normalized using the print-tip LOWESS method. No background subtraction was applied by LIMMA, as the area under each spot was protected from a non-specific fluorescent signal, revealing lower signal than the background when not specifically hybridized. LIMMA was also used to statistically test the hepatic differentially expressed genes in each defined sample ($M \neq 0$; $p < 0.05$). *P*-values were adjusted for multiple testing corrections [33]. The differentially expressed clones were further selected by applying $M \geq 0.5$ threshold. This threshold was aimed at minimizing false-positive M values resulting from suspected weak differential expression, even if statistically valid, leaving only the more stringent differential expressions.

Experimental design of the hybridizations

RNA preparations resulting from the two exposure experiments were labelled and hybridized on the cDNA microarray. The hybridization designs of Experiments 1 and 2 are described in Figure 1. The design is composed of biological replicates. Each replicate is actually a complete experiment, including randomly selected individual fish from each of the treatments and controls. Hence, the number of the biological replicates in each experiment equals the number of fish in each experimental group, five replicates in Experiment 1 and six in Experiment 2. The design of Experiment 1 (Figure 1A) included intensive dye-swap which contain four technical replicates for each hybridized cDNA pair. The total number of slides in Experiment 1 comprised: 4 technical replicates of each slide × 3 hybridized fish pairs in each biological replicate × 5 biological replicates = 60 arrays. When examining the added information of

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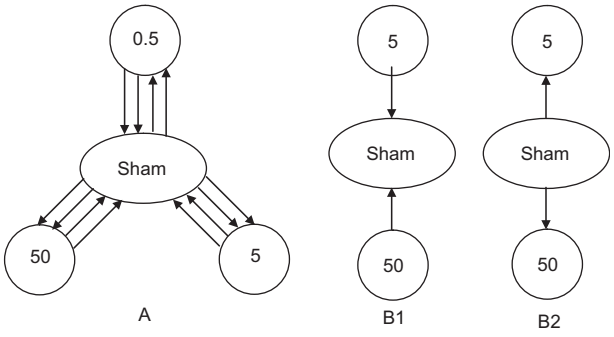

Figure 1. Hybridization designs of both experiments. Circles represent randomly selected fish from each of the treatments, designated by the tBHP concentrations in mg/kg body weight. Ellipses represent randomly selected sham-injected fish. Each arrow represents dual-labelled slide of cDNA preparations of a fish pair. Arrowhead represents Cy3 and arrow tail, Cy5. Each figure (A, B1 and B2) represents a biological replicate. (A) Experiment 1, included four dye-swapped technical replicates per hybridized labelled cDNAs of a fish pair. The total number of slides in Experiment 1 was: 4 technical replicates × 3 hybridized fish pairs in a biological replicate × 5 biological replicates = 60 arrays. (B1 and B2) Experiment 2, The total number of slides in Experiment 2 was: 2 hybridized fish pairs in a biological replicate × 6 biological replicates = 12 arrays. Alternate labelling of half of the biological replicates (B1 or B2) was used, partially compensating the dye effect.

each technical replicate of Experiment 1, it occurred to us that they did not provide additional information justifying the effort. Therefore, Experiment 2 was differently designed. No technical replicates were applied and potential dye effects were partially compensated by the alternate labelling of half of each biological replicate (Figures 1B1 and 1B2). The total number of slides in Experiment 2 was: 2 hybridized pairs in a biological replicate × 6 biological replicates = 12 arrays.

Relative qRT-PCR

Relative expression levels of target transcripts (Table II) between selected RNA populations of individual treated fish and their mutually hybridized individual controls were evaluated using reverse transcription coupled to relative real-time PCR, according to Pfaffl [34]. The used primer pairs are presented in Table II. Total RNAs of individual fish were serially diluted to 1, 0.5, 0.25 and 0.125 µg. Each of them was reverse transcribed at 42°C for 1 h using MMLV reverse transcriptase (Promega, Madison, WI, USA) and reverse primers, according to Sambrook and Russell [35]. PCR reactions were performed using the four reverse transcriptase solutions of the serially diluted RNAs as templates. Each reaction was performed in triplicate with the GeneAmp 5700 PCR thermocycler (Applied Biosystems, Foster City, CA) (one cycle at 50°C, 2 min; one cycle at 95°C, 10 min; 40 cycles at 95°C, 15 s and 60°C, 1 min). Each PCR reaction was conducted with a volume of 25 µl composed of 12.5 µl SYBR Green mix (Applied Biosystems), 0.2 µM of each of the PCR primers and a 2 µl aliquot sample from the reverse transcription mixture. A linear regression equation relating the crossing-point (CP) of the PCR cycle to the log of the four dilutions was calculated for each PCR-reacted RNA population. CP is the PCR cycle in which the fluorescent intensity crosses the threshold of detection. The equation slope was used to ensure a reasonably similar PCR efficiency of the two RNA populations compared. ΔCP is the difference between the CPs of two compared populations at identical dilution. Their average across the four dilutions was used to express the difference between the two compared beta-actin normalized RNA populations. Both M-values and ΔCPs represent $\log_2$ expression ratio between compared RNA extracts. Although evaluated by different methods, they can be qualitatively compared, demonstrating expression trends. The ΔCPs were compared to the corresponding M values taken from the microarray results (Figure 2).

Histology

Liver tissue pieces from all fish of Experiment 2 were fixed in Bouin's reagent, embedded and stained by haematoxylin and Eosin according to Stevens [36]. Pathological structures were identified according to Feist et al. [37].

Results

Analysis of hybridized slides

Experiment 1. All the three applied tBHP doses were sub-lethal. One hundred and thirteen annotated unique clones (Table III) and 56 non-annotated ones (not shown) fulfilled our criteria for differential expression with M≥0.5 in at least one of the treatment groups. M values of redundant clones sharing one unique sequence were averaged. Up-regulation of 28, 45 and 58 clones was observed in the 0.5, 5 and 50 mg tBHP/kg body weight/injection dosed groups, respectively, revealing dose dependency. No such dependency was observed for the down-regulated clones (34, 39, 24 clones in the 0.5, 5 and 50 mg tBHP/kg bwt/injection dosed groups, respectively). Nine days elapsed from the first injection to fish sacrifice. The results of Experiment 1 were deposited in the Gene expression omnibus (GEO) database maintained by the American National Center for Biotechnology Information (NCBI) in dataset accession number GSE19216, sample accession numbers GSM476020, GSM476029 and GSM476030.

Experiment 2. This was aimed at examining the gene expression patterns during shorter time periods than

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Table II. qRT-PCR of selected genes, carried out for microarray validation.

Accession number		Primer pairs	Microarray results (Experiment 2)	qRT-PCR results	Annotation
DQ849783	f	CAGAAGCACTCTGAGCAAATCC	Up-regulation	4.982	Haemoglobin beta-A chain
	r	GCACACACTGATGCATTCAGC			
DQ850833	f	AGACGTTCACCTTCTTACGCC	Up-regulation	2.245	Betaine-homocysteine methyltransferase
	r	AGAGACTGACCCCTGTTCTCC			
DQ849829	f	GTGTTACTCCTGCCCTGACG	Down-regulation	–1.458	Null
	r	TCTCACCACTTTTGAGTTTGAGG			
DQ849652	f	TGACTTTTGTGTCCCTGACTGG	Down-regulation	–1.957	Complement component C7
	r	CTGCGAAAAACTGACAAAATCC			
DQ850757	f	CTGTGCAAAATAGATCATTGGC	Down-regulation	–0.682	7-dehydrocholesterol reductase
	r	TGCCACTACAACTCATTCTACCTC			
DQ850885	f	TCAAGCAGTTCCACGACTCC	Down-regulation	3.247	Ribosomal protein L18a (fragment)
	r	CGACAAAATGCTTTCAGTAGAAGG			

f and r designate forward and reverse primers, respectively.

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those of Experiment 1. Only 4–7 days elapsed from the first injection to fish sacrifice. The 5 and 30 mg tBHP/kg bwt doses were applied in this experiment and only fish dosed with the higher amount of tBHP and sacrificed after 7 days from first injection elucidated differentially expressed clones (31 clones). All these clones were differentially expressed also in Experiment 1 and the annotated ones are included in Table III. The results of Experiment 2 were deposited in the Gene expression omnibus (GEO) database in dataset accession number GSE19216, sample accession numbers GSM476074 and GSM476112.

Prominent differential expression was revealed in Experiment 1, even after exposure to relatively low doses for 9 days. In contrast, milder changes of expression were detected in Experiment 2 and only at the highest tBHP dosage group after 7 days of exposure.

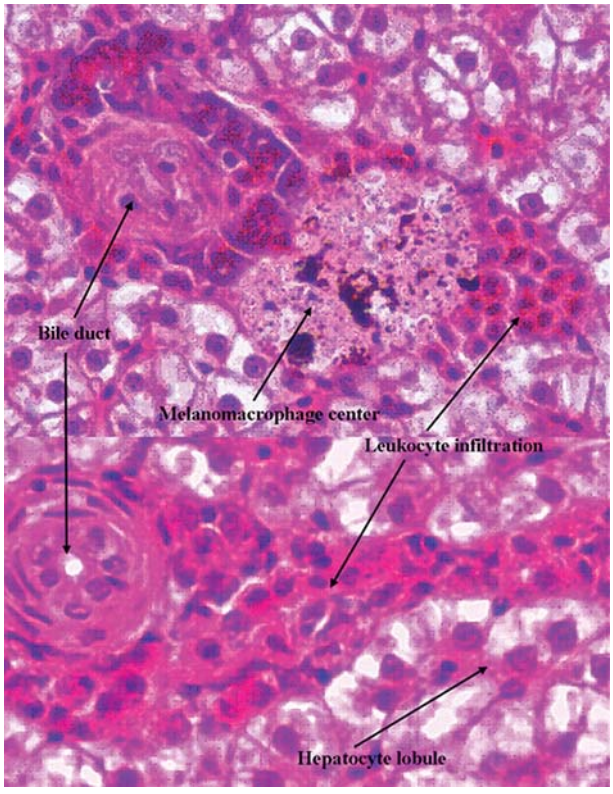

Figure 2. Histological appearance of leukocyte infiltration.

The differentially expressed genes were classified to functional groups (Table III). Certain functional groups were characterized by a uniform trend of differential expression. Hence, all the proteolytic enzymes, their inhibitors, the genes related to amino acid metabolism, genes related to energy provision and the signal transduction-related genes were up-regulated. The genes related to lipid and cholesterol metabolism were highly represented in the differentially expressed genes and most of them were up-regulated. Clones of the complement component of the innate immune system revealed both up- and down-regulation. C1q, C3 and C7 were down-regulated and Bf-1 and C9 were up-regulated. Most oxidation-reduction enzymes were up-regulated as well as proteins related to heme degradation. Other differentially expressed functional gene groups affected by exposure to tBHP were genes related to sugar metabolism and metal ion homeostasis. The majority of the non-annotated differentially expressed genes revealed constant expression direction across treatments.

Several of the differentially expressed genes can be assigned to ROS metabolism: aldehyde dehydrogenase 3A2, Cytochrome p4501A, warm temperature acclimation-related 65 kDa protein (Wap65)-Hemopexin, Heme oxygenase and Heme oxygenase 1, all of which showed up-regulation. Metallothionein genes revealed down-regulation. NADPH oxidase flavocytochrome b small sub-unit p22phox and Ras-related C3 botulinum toxin substrate 1 (Rac1) are known to participate in the regulation of endothelial intercellular adhesion, part of the process of leukocyte transendothelial migration and CD63-like gene participate in the leukocyte function at the inflammation site. For references to the above functions see the discussion section below.

Several other genes showed tBHP-related differential expression. These were the up-regulated betaine

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Table III. Short annotations and M values of genes differentially expressed after exposure to tBHP.

Accession number	Sequence description	M values			
		Exp. 2		Exp. 1	
		30	0.5	5	50
Immune response					
DQ849652	Complement protein component C7-1	**–1.07**	**–0.58**	**–0.92**	**–0.98**
DQ849772	Complement component C3		**–1.00**	**–1.43**	–0.12
DQ850938	Complement C1q-like factor, ovary-specific		**–3.37**		
DQ850276	Complement C1q-like		**0.58**	**0.74**	**0.58**
DQ849671	Complement component C9		0.48	**0.56**	0.48
DQ850243	Complement factor bf-1			**0.75**	
DQ850026	Fucose-binding lectin 32 precursor			**2.57**	
DQ850717	Fucose-binding lectin 32 precursor	**0.50**			
DQ849688	Fucose-binding lectin 32 precursor		–0.34	**1.11**	0.49
Lipid metabolism					
DQ849794	Anti-freeze protein LS-12		–0.37		**0.61**
DQ849747	Vitellogenin b, Lipid transporter to the ovary.		**–0.73**	**0.82**	**0.93**
DQ850184	Vitellogenin a. Lipid transporter to the ovary.		0.30	0.37	**0.50**
DQ850565	Serum amyloid A protein. Extracellular lipid transporter.			**0.56**	**0.52**
DQ850542	Intestinal fatty acid-binding protein. Fatty acid transporter. Function in digestion.			**0.78**	
DQ850124	Apolipoprotein C-II. Function in lipid catabolic process.		**0.51**	0.46	**0.61**
DQ850232	Fatty acid binding protein-like protein	**0.72**			
Cholesterol metabolism					
DQ850757	7-dehydrocholesterol reductase. Cholesterol biosynthesis.	**–0.97**	**–1.10**	**–1.44**	**–1.12**
DQ850201	Apolipoprotein C-I. Its main function is inhibition of Cholesteryl ester transfer protein (CETP).	**–0.90**			
DQ850382	Sterol carrier protein 2. Function in lipid transport and steroid biosynthetic process.		0.36	**0.52**	**0.59**
DQ850011	Sterol o-acyltransferase 2. Cholesterol absorption by the intestine and assembly and secretion of apolipoprotein B-containing lipoproteins.			**2.58**	
DQ850208	Carboxyl ester lipase. Function in lipid catabolic process.		**0.50**	0.42	0.36
DQ849686	Apolipoprotein E. Plasma protein that mediates the transport and uptake of cholesterol and lipid.			0.35	**0.76**
DQ850078	Apolipoprotein B. Carrier of cholesterol to tissues.			1.16	
Oxireductases					
DQ850458	Cytochrome P450		**–3.53**		
DQ850847	Cytochrome P450 1a		0.44	**0.53**	0.30
DQ850564	Cytochrome P450 1a		0.45	**0.62**	
DQ850233	Cytochrome P450 1a			**0.73**	
DQ850667	Warm temperature acclimation-related 65 Kda protein (Wap65) - Hemopexin		0.45	**0.87**	0.47
DQ849637	Warm temperature acclimation-related 65 Kda protein (Wap65) - Hemopexin		**0.55**		0.40
DQ849764	Warm temperature acclimation-related 65 kda protein (Wap65) - Hemopexin		0.43	**–0.57**	0.23
DQ850220	Heme oxygenase				**0.77**
DQ850976	Heme oxygenase 1				**0.61**
DQ850217	alpha-1-microglobulin bikunin precursor				**1.80**
DQ849680	NADPH oxidase flavocytochrome b small sub-unit p22phox	**0.85**			
DQ850646	Urate oxidase		**0.59**	**0.53**	**0.67**
DQ849916	Aldehyde dehydrogenase 3a2				**4.08**
DQ849769	Glutaredoxin-related protein		0.88		**0.57**
DQ850023	Carbonyl reductase		–0.33	**–0.53**	–0.40

(Continued)

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Table III. *(Continued)*

Accession number	Sequence description	M values			
		Exp. 2		Exp. 1	
		30	0.5	5	50
DQ850710	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide		**–0.58**	**–0.80**	**–0.61**
DQ849814	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide		**–2.65**		
Energy providing processes					
DQ849860	Cytochrome c oxidase sub-unit I		**0.53**	**0.57**	0.47
DQ850258	Cytochrome c oxidase sub-unit I		**0.51**	0.47	**0.77**
DQ849968	Cytochrome c oxidase sub-unit I	**0.55**			
DQ849783	Haemoglobin beta-a chain	**1.29**	**0.91**		**0.98**
DQ849795	ATPase family AAA domain containing 4				**1.22**
Translation					
DQ850804	60s ribosomal protein L35		**–3.53**		
DQ850885	Ribosomal protein L18a	**–1.25**	**–0.54**	**–1.02**	**–0.61**
Signal transduction					
DQ850697	Ras-related C3 botulinum toxin substrate 1 (Rac1)	**–1.00**		**–0.61**	**–0.48**
DQ850858	Membrane-spanning 4-domains sub-family a member 15		–4.08		
DQ849681	Serine/threonine-protein phosphatase PP1-gamma catalytic sub-unit			**–1.35**	
DQ849775	CD63-like protein transmembrane 4 superfamily, also known as the tetraspanin.			**–0.71**	**–0.54**
DQ850277	Basigin (CD147), immunoglobulin, enhances cellular adhesion		0.36	**0.53**	**0.56**
DQ890533	Chromatin modifying protein 2b		0.47	**0.51**	**0.57**
DQ850817	Stromal cell derived factor 4, chemokine		**0.56**	**1.19**	
DQ850044	Amine sulphotransferase		0.43	0.49	**0.55**
DQ850416	Ornithine decarboxylase antizyme small isoform				**0.71**
Amino acid metabolic process					
DQ850833	Betaine-homocysteine methyltransferase	**1.10**	0.47	0.45	**0.54**
DQ850165	Glutamate dehydrogenase 1				**1.88**
DQ850075	Glutamate dehydrogenase		**0.52**	0.35	0.28
DQ850777	Taurine transporter		0.36	0.33	**0.57**
DQ850604	Homogentisate -dioxygenase		0.31	0.37	**0.61**
DQ849752	Indoleamine 2,3-dioxygenase 1				**0.77**
Proteases					
DQ850449	Carboxypeptidase A1 precursor	**0.60**	0.43	**0.56**	
DQ850591	Carboxypeptidase B1		0.49	**0.68**	**0.53**
DQ850573	Elastase 2b		0.41	0.38	**0.52**
DQ850587	Elastase 4-like		**0.60**	**0.52**	0.43
DQ850215	Serine-type endopeptidase		**0.63**	**0.70**	**0.57**
DQ850609	Cathepsin C		**0.52**	0.40	
DQ850346	Chymotrypsin-like protease ctrl-1 precursor		0.38	**0.69**	
DQ849715	loc397853 protein		**0.55**	0.36	
DQ850245	Elastase 2b				**2.00**
Protease inhibitors					
DQ849836	Putative latexin				**–0.51**
DQ890529	loc567732 protein		0.44	**0.52**	0.40
DQ849714	C1 inhibitor		0.33	0.37	**0.53**
DQ849954	Alpha-2-macroglobulin		**0.54**	0.47	0.42
DQ850345	Inter-alpha-trypsin heavy chain 4		**0.60**	**0.64**	**0.67**
DQ850027	Inter-alpha inhibitor h2		0.39	0.46	**0.53**
DQ850475	Inter-alpha inhibitor h3		**0.55**	0.50	**0.51**
DQ850827	Kininogen-1 precursor		**0.57**	**0.58**	**0.57**
DQ850387	Serpin a1		0.47	**0.68**	**0.53**
DQ850763	Serpin peptidase clade d1 (heparin cofactor)		**0.82**	0.43	0.38
DQ849957	Serpin peptidase clade f			0.39	**0.79**

(Continued)

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Table III. *(Continued)*

Accession number	Sequence description	M values			
		Exp. 2		Exp. 1	
		30	0.5	5	50
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process					
DQ849825	Deoxyribonuclease II family protein		–0.38	**–0.59**	0.13
DQ850595	Histone cluster h2bb		**–2.28**		
DQ850297	Myst histone acetyltransferase 2		0.37	**0.50**	0.47
Cellular di-, trivalent inorganic cation homeostasis					
DQ850666	Metallothionein		–0.39	**–0.62**	–0.18
DQ850585	Hepcidin		–0.45	**–0.56**	**–0.88**
DQ850742	Hepcidin 1		**–0.60**	**–0.92**	**–0.71**
DQ849665	Hepcidin 3	**–0.64**	–0.40	**–0.59**	–0.38
DQ850860	Hepcidin-like precursor	**–1.14**	–0.48	**–0.57**	–0.49
DQ849830	Hepcidin 4 precursor	**–0.74**			
DQ849812	Hepcidin-like precursor	**–1.00**			
DQ849645	Hepcidin-like precursor	**–0.99**			
DQ849864	Ceruloplasmin		0.30	**0.76**	0.42
DQ849713	Ceruloplasmin				**0.70**
Regulation of biological processes					
DQ849872	Cytohesin 1		–0.48	**–0.57**	–0.47
DQ850360	Insulin-like growth factor 1		**–1.02**	**–1.29**	**–0.86**
DQ849684	4SNc-Tudor domain protein,		**–2.98**		
DQ850317	F11 receptor		**–3.71**	0.38	0.25
DQ850439	14 kDa apolipoprotein		0.34	0.43	**0.54**
DQ850362	Leptin receptor			**0.84**	**0.86**
Carbohydrate metabolism					
DQ849831	Rhamnose-binding lectin			**–0.51**	0.26
DQ850234	Serum amyloid P-component precursor			**–1.42**	
DQ850266	Serum amyloid p-component precursor		0.39	**–0.74**	**–1.31**
DQ850844	Fructose-bisphosphate aldolase		0.46	**–0.71**	
DQ850064	Fructose-bisphosphate aldolase B		**0.52**	–0.21	
DQ849771	Fructose-1,6-bisphosphate aldolase			**1.46**	
DQ850724	Resembles several lectins		**1.53**		**1.51**
DQ849855	Chitinase		**0.56**	0.67	
Blood coagulation					
DQ849770	Fibrinogen gamma polypeptide		**–0.42**	**–0.72**	**–0.53**
DQ850270	Fibrinogen beta chain precursor	**0.57**			
Defense response to bacteria					
DQ850160	Peptidoglycan recognition protein			**–0.76**	

Exp. 1 and 2 are the two performed experiments. The numbers 0.5, 5, 50 and 30 are injected tBHP doses in mg/kg body weight/injection. Only Experiment 2 data for the time point of 68 h after last injection is presented (see Materials and methods for details of the experiments). Positive M values designate up-regulation; negative M values designate down-regulation and blank cells designate no significant differential expression. Bold M values designate $M \geq |0.5|$.

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homocysteine methyltransferase (BHMT), Haemoglobin beta-a chain and ceruloplasmin and the down-regulated Ribosomal protein L18a, Hepcidin-like/Hepcidin precursor and insulin-like growth factor 1.

7-Dehydrocholesterol reductase (7-DHCR) (Accession number DQ850757) and two non-annotated genes (Accession numbers DQ849868 and DQ849829) were prominently attenuated in all the treatment groups in both experiments ($M < –0.9$).

Quantitative RT-PCR validation of microarray results

Relative qRT-PCR was performed with six selected genes using selected mRNAs from two pairs of mutually hybridized fish from Experiment 2, sacrificed 68 h after last injection. Each pair included a fish dosed by 30 mg/kg body weight and a sham-injected fish. The ΔCP between treated and sham-injected fish of each examined pair were compared to the microarray M values obtained for those fish pairs. The results are presented in Table II. Five out of six qRT-PCRs agreed with the microarray M values.

Liver histology

Several apparent pathological structures were observed in the hepatic histological sections taken from fish of Experiment 2. They included paracristal inclusions, vacuolization, spongiosis, foci of cellular alteration, necrosis, high level of macrophage aggregates, bile

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duct proliferation and leucocyte infiltration. The pathological appearances were defined according to Feist et al. [37]. Only one of them, leukocyte infiltration (Figure 2) was correlated to tBHP application (Table IV). This phenomenon was observed even after the minimal 4 days of exposure to the lowest applied dose.

Discussion

tBHP, a model pro-oxidant, was applied through peritoneal injection, a direct effective application route in comparison to exposure through the gills or the intestine. However, it took at least 7 days (high dose and longest exposure duration) in Experiment 2 to detect differentially expressed genes. The 7 days gap between application and its effect is probably composed of the transportation period from the peritoneum to the liver, the period required for the postulated production of ROS from tBHP and the period required for visualization of ROS effects on gene expression. The levels of the assumedly produced ROS was not directly measured.

It was mainly the more prominent differential expression results of Experiment 1 that were used to identify differentially expressed genes. Differential expression of 169 unique clones was observed in this experiment in response to all applied tBHP doses in comparison to the appropriate control, but only the numbers of up-regulated genes corresponded to the tBHP injection dose. It is postulated that this dose responsive up-regulation is related to specific regulative and defensive pathways activated directly or indirectly by elevated ROS levels. Down-regulation may be a result of specific regulative and defensive pathways as well. However, the lack of similar dose response in down-regulated genes indicates a mixture of putative specific ROS-related regulation pathways and non-specific toxicity, both affected by tBHP.

Fish liver contains several types of cells which facilitate regular liver and pancreatic functions. Other cell types such as leukocytes migrate to the liver in pathological situations. The pathological effect of the tBHP injections on the liver was demonstrated in Experiment 2 by leukocyte infiltration in all dose- and time-related treatment groups starting 4 days after treatment. Leukocyte infiltration is known as a general response to inflammation, but also to the presence of ROS [38,39].

Table IV. Fish from *Experiment 2* revealing hepatic leukocyte infiltration.

Sacrifice time (h)	tBHP dose (mg/kg bwt/injection)	# of fish†
2	0 (Sham injected)	–
	5	1
	50	3
20	0 (Sham injected)	–
	5	2
	50	3
68	0 (Sham injected)	–
	5	1
	50	2

†Out of six fish/group.

An attempt was made to find common denominators to certain groups of differentially-expressed genes in relation to their functions and in view of their expression upon tBHP application. Assignment of genes to a functional group does not necessitate functioning in the same pathway or activation by the same regulatory route. Nevertheless, several functional groups of genes elucidated a uniform trend of differential expression, which can be interpreted in the context of ROS effect. Hence, all the elucidated proteolytic enzymes, proteolysis inhibitors, amino acid metabolism-related enzymes and genes which are part of the energy providing system were solely up-regulated. This trend may indicate an activity aimed at preserving homeostasis of properly functioning protein molecules in the liver, while eliminating damaged ones.

Unsaturated lipids are sensitive to oxidative stress and lipid peroxidation causes damage to membranes, low density lipoproteins and free fatty acids. The lipid metabolism-related genes and specifically those which are related to cholesterol metabolism were a prominent component of the differentially-expressed genes. There was no uniform direction for the differential expression, but the majority of genes in this group were up-regulated. Again, broadly speaking, lipid homeostasis may be preserved by the differential expression of these genes, both synthetic and catabolic ones. Interestingly, the only prominently down-regulated lipid metabolism-related transcript in response to all tBHP exposures was 7-dehydrocholesterol reductase (7-DHCR). 7-DHCR catalyses the conversion of 7-dehydrocholesterol to cholesterol. We could not find a reasonable explanation for its down-regulation in response to tBHP exposure.

The differentially expressed genes which belong to the innate immune system elucidated a mixed trend of expression. It is possible that changes in the expression level of the innate immune system genes is related to the general inflammation response indicated by the leukocyte infiltration (Figure 2).

It was not possible to distinguish the exact cellular origin of differentially expressed genes using the present experimental procedure. However, the process of leukocyte transendothelial migration was intensively studied in mammals [40,41] and may assist in indicating differentially-expressed genes putatively participating in this process. Basigin (CD147) enhances leukocyte cellular adhesion to endothelial cells by its binding to integrins [42]. It was up-regulated here and may participate in the adhesion stage of leukocyte transendothelial migration. NADPH oxidase flavocytochrome b small sub-unit p22phox is a regulatory sub-unit of

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NADPH oxidase (Nox) enzymatic complex, a major ROS producer [43]. The ROS produced by Nox are used as part of anti-bacterial defense mechanisms [44]. However, Nox is also part of the complex regulatory pathway of leukocyte transendothelial migration. The Nox system, including p22phox, is induced to produce ROS by genes involved in leukocyte-endothel adhesion, which in turn signals dysfunction of endothelial cell-to-cell adhesion. The later decreased adhesion enables leukocyte transendothelial migration [11]. Alternatively, as shown by Djordjevic et al. [45] in human endothelial cells, p22phox levels were elevated by a direct redox-sensitive mechanism. Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small GTPase G signal protein belonging to the Rac sub-family of the Rho GTPases. It functions as a regulator of endothelial inter-cellular adhesion and of cyto-skeletal migration on the actin network, through its participation in the Nox pathway [11,46] Hence, it is involved in leukocyte migration. Its down-regulation here is, on one hand, incompatible with the p22phox up-regulation [11]. However, on the other hand its role in controlling endothelial permeability is still controversial [47].

The up-regulated CD63-like gene may be involved in another leukocyte-related function of protein catabolic processes at the hepatic inflammation sites, as it is involved in targeting mechanisms of pro-neutrophil elastase to its site of action [48].

Certain differentially expressed transcripts are known to be related to ROS metabolism and their patterns of expression further indicate a tBHP effect. Aaldehyde dehydrogenase 3A2, an NAD-dependent oxidoreductase, detoxifies aldehyde toxicity caused in the course of alcohol metabolism or lipid peroxidation. The enzyme was previously used as a biomarker of pre-neoplastic hepatocyte foci in fish [46,49].

Cytochrome p450 1A (CYP1A) is a monooxygenase, reducing molecular oxygen to water while transferring the other oxygen atom into a variety of organic compounds. CYP1A is induced by several groups of organic pollutants (e.g. dioxins, PCBs and PAHs) and its expression level is one of the most established environmental biomarkers in fish [50–52]. It is known to produce ROS in the course of its normal function. However, oxidative stress led to its inactivation [53–55]. This inactivation was interpreted as a defense mechanism against oxidative stress by the reduction of ROS producing enzymes. Increasing transcript levels of CYP1A in response to tBHP exposure may be explained by potential tBHP inducing activity of CYP1A that is not related to the inactivation of the enzyme or by a yet unknown compensating mechanism, increasing transcript level in response to reduced enzymatic activity.

The heme catabolytic enzymes heme oxygenase, alpha-1-microglobulin bikunin precursor and Wap65-Hemopexin were mostly up-regulated. Graça-Souza et al. [56] and Arruda et al. [57] showed that heme is a pro-inflammatory molecule when present in a suitable concentration, leading to Nox-dependent formation of ROS. Therefore, increasing heme degradation is a probable response to inflammation and the consequent increased ROS level. The fish warm temperature acclimation-related 65 kDa protein (Wap65) is a hemopexin-like gene, increasingly expressed in fish with the elevation of the ambient temperature [58]. Its resemblance to hemopexin indicates an additional function, trapping free heme and its transportation to the liver for its catabolism and iron recycling [59]. Gracey et al. [60] showed increased expression of hemopexin-like protein during fish hypoxia. Aliza et al. [59] suggested this phenomenon as an explanation for the increased hemopexin obtained upon exposure of the fish *Xiphophorus helleri* to copper. Increased level of hemopexin transcript is correlated to increased binding of its protein derivative to heme [61,62]. Binding of the heme–hemopexin complex to hemopexin receptor located on the cell membrane can initiate a sequence of cellular events [63,64]. Heme oxygenase (HO) is the first enzyme in the heme catabolic pathway, producing the catabolytes biliverdin, iron and carbon monoxide. HO is induced during oxidative stress [66] and it is assumed that its products biliverdin and bilirubin are potential antioxidants [66–68].

Metallothionein is a cysteine-rich protein involved in the homeostasis of essential metals, detoxification of toxic metals and free radical scavenging [69]. The transcript of this gene is dramatically induced in response to increased cadmium and zinc and also in response to oxidative and inflammatory factors [70,71]. An oxidative stress response and cadmium response sequence was located in the promoter of the mammalian MT-1 [72,73]. A specific antioxidant response element (ARE) was identified along this sequence [74]. However, an un-explained reduction of metallothionein expression was revealed in Experiment 1 here and by Williams et al. [18] upon fish exposure to tBHP.

Other differentially expressed transcripts which were elucidated only in some of the tBHP treatments included the up-regulated betaine homocysteine methyltransferase (BHMT) and haemoglobin beta-a chain and the down-regulated complement component C7, ribosomal protein L18a, hepcidin-like/hepcidin precursor and insulin-like growth factor 1.

Many of the differentially-expressed genes were non-annotated. However, the vast majority elucidated a uniform expression pattern across treatments, up- or down-regulation, indicating cause and effect relationships between the tBHP exposure and the expression pattern. These genes should be further studied.

The only other fish exposure study to injected tBHP was done by Williams et al. [18]. In that study, a single 5 mg tBHP/kg body weight intraperitoneal

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injection was applied to the European flounder, followed by evaluation of gene expression patterns 1, 2, 4, 8 and 16 days after injection. In addition to the different gene composition of their microarrays, different tBHP doses, injection schedules and sacrifice timing make the present results difficult to compare to Williams et al. [18]. Nevertheless, few genes, including haemoglobin beta-a chain, vitellogenin and Cytochrome P4501A, were up-regulated and Metallothionein was down-regulated throughout all treatment groups in both studies. Ribosomal protein L18a, NADPH oxidase flavocytochrome b small sub-unit p22phox and most of the hepcidin clones showed contradicting trends between the two studies.

Possible reasons for inaccuracies and false results in microarray assays have been thoroughly discussed in the literature. They are assigned mainly to cross-hybridizations of similar sequences belonging to different genes [75–77]. Real-time PCR, an accurate, sensitive and quantifiable method, is usually used to confirm microarray results. Despite its advantages it may also be biased due to accidental utilization of primers which are suitable to more than one transcript. Only one out of the six transcripts examined here by qRT-PCR showed trends which were not compatible with the microarray results.

The interest in hepatic expression patterns of *L. mormyrus* emerged from its potential utilization as an environmental sentinel. Albeit, tBPH, its application route and the concentrations used did not represent likely interactions with known environmental pollutants. Nevertheless, known environmental pollutants can affect cellular redox status [13] and tBHP injections could therefore mimic some of these redox responses.

In summary, this microarray study of liver cell responses to the pro-oxidant tBPH detected some previously observed ROS-related transcription patterns. It also detected novel differential expression of annotated and non-annotated genes that were not previously known to be associated with ROS challenge. These genes are promising candidates for further study of ROS-related metabolism in fish.

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References

[1] Kohlmann A, Kipps TJ, Rassenti LZ, Downing JR, Shurtleff SA, Mills KI, Gilkes AF, Hofmann W-K, Basso G, Campo Dell'Orto M, Foà R, Chiaretti S, De Vos J, Rauhut S, Papenhausen PR, Hernandez JM, Lumbreras E, Yeoh AE, Koay ES, Li R, Liu W-M, Williams PM, Wieczorek L, Haferlach T. An international standardization programme towards the application of gene expression profiling in routine leukaemia diagnostics: the Microarray Innovations in Leukemia study prophase. Br J Haematol 2008;142:802–807.

[2] Flanagan N. Microarray market is growing as applications for diagnostics and research are realized. Genet Eng Biotechnol News 2007;27:1.

[3] Grune T, Merker K, Sandig G, Davies KJ. Selective degradation of oxidatively modified protein substrates by the proteasome. Biochem Biophys Res Comm 2003;305:709–718.

[4] Vertuani S, Angusti A, Manfredini S. The antioxidants and pro-oxidants network: an overview. Curr Pharm Des 2004;10:1677–1694.

[5] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford: Clarendon Press; 1999. p. 936.

[6] Scherz-Shouval R, Elazar Z. ROS mitochondria and the regulation of autophagy. Trends Cell Biol 2007;17:422–427.

[7] Biswas S, Chida AS, Rahman I. Redox modifications of protein-thiols: emerging roles in cell signaling. Biochem Pharmacol 2006;71:551–564.

[8] Fruehauf JP, Meyskens FL. Reactive oxygen species: A breath of life or death? J Clin Cancer Res 2007;13:789.

[9] Thannical VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Cell Mol Physiol 2000; 279:L1005–L1028.

[10] D'Autréaux B, Toledano MB. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 2007;8:813–824.

[11] Fernandez-Borja M, van Buul JD, Hordijk PL. The regulation of leucocyte transendothelial migration by endothelial signalling events. Cardiovasc Res 2010:86:202–210.

[12] Regoli F, Pellegrini D, Winston GW, Gorbi S, Giuliani S, Virno-Lamberti C, Bompadre S. Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (*Mullus barbatus*). Mar Pollut Bull 2002;44:912–922.

[13] Livingstone DR. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 2001;42:656–666.

[14] Valentao P, Carvalho M, Carvalho F, Fernandes E, Pires das Neves N, Pereira ML, Andrade PB, Seabra RM, Bastos ML. *Hypericum androsaemum* infusion increases tert-butyl hydroperoxide-induced mice hepatotoxicity *in vivo*. J Ethnopharmacol 2004;94:345–351.

[15] Kaur P, Kaur G, Bansal MP. Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: role of transcription factor NF-κB and testicular antioxidant enzymes. Reprod Toxicol 2006;22:479–484.

[16] Kim JA, Kang YS, Kim YO, Lee SH, Lee YS. Role of Ca^{2+} influx in the tert-butyl hydroperoxide-induced apoptosis of HepG2 human hepatoblastoma cells. Exp Mol Med 1998; 30:137–44.

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[17] Unterluggauer H, Hampel B, Zwerschke W, Jansen-Dürr P. Senescence-associated cell death of human endothelial cells: the role of oxidative stress. Exp Gerontoly 2003;38:1149–1160.
[18] Williams TD, Diab A, Ortega F, Sabine VS, Godfrey RE, Falciani F, Chipman JK, George SG. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. Aquat Toxicol 2008;90:83–91.
[19] Samson SLA, Paramchuk WJ, Gedamu L. The rainbow trout metallothionein-B gene promoter: contributions of distal promoter elements to metal and oxidant regulation. Biochim Biophys Acta 2001;1517:202–211.
[20] Ploch SA, Lee Y, MacLean E, Di Giulio RT. Oxidative stress in liver of brown bullhead and channel catfish following exposure to tert-butyl hydroperoxide. Aquat Toxicol 1999;46: 231–240.
[21] Barton DHR, Gloahec VNL, Patin H, Launay F. Radical chemistry of tert-butyl hydroperoxide (TBHP). Part 1. Studies of the FeIII TBHP mechanism. New J Chem 1998;6:559–563.
[22] Auslander M, Yudkovski Y, Chalifa-Caspi V, Herut B, Ophir R, Reinhardt R, Neumann PM, Tom M. Pollution-affected fish hepatic transcriptome and its expression patterns upon exposure to cadmium. Mar Biotechnol 2008;10:250–261.
[23] OECD. t-butyl hydroperoxide CAS N°: 75-91-2. In: Chemicals screening information dataset for high volume chemicals. Available online at: http://www.chem.unep.ch/irptc/sids/OECDSIDS/75912.pdf 2007; UNEP publication: 368–396.
[24] Besseau L, Brusle-Sicard S. Plasticity of gonad development in hermaphroditic sparids – ovotestis ontogeny in a protandric species, *lithognathus-mormyrus*. Environ Biol Fishes 1995;43: 255–267.
[25] Funkenstein B, Dyman A, Levavi-Sivan B, Tom M. Application of real time PCR for quantitative determination of hepatic vitellogenin transcript levels in the striped seabream, *Lithognathus mormyrus*. Mar Environ Res 2004;58:659–663.
[26] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–3402.
[27] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. (The Gene Ontology Consortium) Gene ontology: tool for the unification of biology. Nat Genet 2000;25:25–29.
[28] Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG resource for deciphering the genome. Nucleic Acids Res 2004;32:D277–D280.
[29] Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. Blast2GO, a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 2005;21:3674–3676.
[30] Yudkovski Y, Shechter A, Chalifa-Caspi V, Auslander M, Ophir R, Dauphin-Villemant C, Waterman M, Sagi A, Tom M. Hepatopancreatic multi-transcript expression patterns in the crayfish *Cherax quadricarinatus* during the molt cycle. Insect Mol Biol 2007;16:661–674.
[31] Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Molec Biol 2004;3:Article 3. DOI: 10.2202/1544-6115.1027. Available at: http://www.bepress.com/sagmb/vol3/iss1/art3.
[32] Smyth GK. LIMMA: linear models for microarray data. In: Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, editors. Bioinformatics and computational biology solutions using R and bioconductor. Statistics for biology and health. New York, NY: Springer; 2005. p. 397–420.
[33] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc Ser B 1995;57:289–300.
[34] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29: 2003–2007.
[35] Sambrook J, Russell DW. Molecular cloning, a laboratory manual, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
[36] Stevens A. The haematoxylins. In: Bancroft JD, Stevens A, editors. Theory and practice of histological techniques. 3rd ed. Edinburgh: Churchill Livingstone; 1990.
[37] ICES. Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limandalimanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. By Feist SW, Lang T, Stentiford GD, Köhler A. ICES. Tech Mar Environ Sci 2004;No. 38: 42 pp.
[38] Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Lin WL, Chu CY. Inhibitory effect of berberine on tert-butyl hydroperoxide-induced oxidative damage in rat liver. Arch Toxicol 2002;76:664–70.
[39] Liu CL, Wang JM, Chu CY, Cheng MT, Tseng TH. *In vivo* protective effect of proto catechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity. Food Chem Toxicol 2002;40:635–641.
[40] Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nature Rev 2007;7:678–689.
[41] Barreiro O, Martin P, González-Amaro R, Sánchez-Madrid F. Molecular cues guiding inflammatory responses. Cardiovas Res 2010;86:174–182.
[42] Khunkaewla P, Schiller HB, Paster W, Leksa V, Čermák L, Anděra L, Hořejší V, Stockinger H. LFA-1-mediated leukocyte adhesion regulated by interaction of CD43 with LFA-1 and CD147. Molec Immunol 2008;45:703–1711.
[43] Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181–189.
[44] Rada B, Leto TL. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. Contrib Microbiol 2008; 15:164–187.
[45] Djordjevic T, Pogrebniak A, BelAiba SR, Bonello S, Wotzlaw C, Acker H, Hess J, Görlach A. The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells. Free Radical Biol Med 2005; 38:616–630.
[46] Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol 2006; 16:522–529.
[47] Aghajanian A, Wittchen ES, Allingham MJ, Garrett TA, Burridge K. Endothelial cell junctions and the regulation of vascular permeability and leukocyte transmigration. J Thromb Haemost 2008;6:1453–1460.
[48] Källquist L, Hansson M, Persson AM, Janssen H, Calafat J, Tapper H, Olsson I. The tetraspanin CD63 is involved in granule targeting of neutrophil elastase. Blood 2008;112: 3444–3454.
[49] Winzer K, Kohler A. Aldehyde dehydrogenase and glucose-6-phosphatase dehydrogenase as markers for enzyme-altered foci in the liver of dab (*Limanda limanda*). Mar Environ Res 1998:46:215–219.
[50] Stegeman JJ, Hahn M. Biochemistry and molecular biology of monoxygenases: current perspectives on form, functions and regulation of cytochrome P450 in aquatic species. In: Ostrander GK, Malins D, editors. Aquatic toxicology: molecular, biochemical and cellular perspectives. Boca Raton, FL: Lewis; 1994. p. 87–206.
[51] Bucheli TD, Fent K. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit Rev Environ Sci Technol 1995;25:201–268.
[52] Goksoyr A, Husoy A-M. Immunochemical approaches to studies of CYP1A localization and induction by xenobiotics in fish. In: Braunbeck T, Hinton DE, Streit B, editors. Fish

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ecotoxicology. Basel, Switzerland: Birkhauser Verlag; 1998. p. 165–202.
[53] Schlezinger JJ, White RD, Stegeman JJ. Oxidative inactivation of cytochrome P-450 1A (CYP1A) stimulated by 3,39,4,49-Tetrachlorobiphenyl: production of reactive oxygen by vertebrate CYP1As. Mol Pharmacol 1999;56:588–597.
[54] Morel Y, Barouki R. Repression of gene expression by oxidative stress. Biochem J 1999;342:481–496.
[55] Barker CW, Fagan JB, Pasco DS. Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress. J Biol Chem 1994;269:3985–3990.
[56] Graça-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. Blood 2002;99:4160–4165.
[57] Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C, Graça-Souza AV. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. J Immunol 2004;173:2023–2030.
[58] Choi CY, An KW, Choi YK, Jo PG, Min BH. Expression of warm temperature acclimation-related protein 65-kda (Wap65) mRNA, and physiological changes with increasing water temperature in black porgy, *Acanthopagrus schlegeli*. J Exp Zool 2008;309:206–214.
[59] Aliza D, Shazrina II, Kuah M-K, Shu-Chien AC, Muhammad TST. Identification of Wap65, a human homologue of hemopexin as a copper-inducible gene in swordtail fish, *Xiphophorus helleri*. Fish Physiol Biochem 2008;34:129–138.
[60] Gracey AY, Troll JV, Somero GN. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc Natl Acad Sci USA 2001;98:1993–1998.
[61] Altruda F, Poli V, Restagno G, Argos O, Cortese R, Silengo L. The primary structure of human hemopexin deduced from cDNA sequence: evidence for internal, repeating homology. Nucleic Acid Res 1985;13:3841–3859.
[62] Nikkila H, Gitlin JD, Muller-Eberhard U. Rat hemopexin. Molecular cloning, primary structural characterization, and analysis of gene expression. Biochemistry 1991;30:823–829.
[63] Smith A, Morgan WT. Hemopexin-mediated heme transport to liver: evidence for a heme-binding protein in liver plasma membranes. J Biol Chem 1985;260:8325–8329.
[64] Morgan WT, Muster P, Tatum F, McConnell J, Conway TP, Hensley P, Smith A. Use of hemopexin domains and monoclonal antibodies to hemopexin to probe the molecular determinants of hemopexin-mediated heme transport. J Biol Chem 1988;263:8220–8225.

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[65] Kikuchi G, Yoshida T, Noguchi M. Heme oxygenase and heme degradation. Biochem Biophys Res Comm 2005;338: 558–567.
[66] Stocker R, Glazer AN, Ames BN. Antioxidant activity of albumin-bound bilirubin. Proc Natl Acad Sci USA 1987;84:5918–5922.
[67] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043–1046.
[68] Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiological cytoprotectant. Proc Natl Acad Sci USA 2002;99:16093–16098.
[69] Buico A, Cassino C, Dondero F, Vergani L, Osella D. Radical scavenging abilities of fish MT-A and mussel MT-10 metallothionein isoforms: an ESR study. J inorg biochem 2008; 102:921–927.
[70] Borghesi LA, Lynes MA. Stress proteins as agents of immunological change: some lessons from metallothionein. Cell Stress Chaperones 1996;1:99–108.
[71] Andrews GK. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem Pharmacol 2000; 59:95–104.
[72] Dalton TP, Palmiter RD, Andrews GK. Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. Nucleic Acids Res 1994;22:5016–5023.
[73] Dalton TP, Li QW, Bittel D, Liang LC, Andrews GK. Oxidative stress activates metal-responsive transcription factor-1 binding activity. Occupancy *in vivo* of metal response elements in the metallothionein-I gene promoter. J Biol Chem 1996;271: 26233–26241.
[74] Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Characterization of a DNA–protein interaction at the antioxidant responsive element and induction by 12-O-tetradecanoylphorbol 13-acetate. J Biol Chem 1993;268:19875–19881.
[75] Wren J, Kulkarni A, Joslin J, Butow R, Garner H. Cross hybridization on PCR-spotted microarrays. IEEE Eng Med Biol Mag 2002;21:71–75.
[76] Chen Y, Chou C-C, Lu X, Slate E, Peck K, Xu W, Voit E, Almeida J. A multivariate prediction model for microarray cross hybridization. BMC Bioinformat 2006;7:101.
[77] Flikka K, Yadetie F, Laegreid A, Jonassen I. XHM: a system for detection of potential cross hybridizations in DNA microarrays. BMC Bioinformat 2004;5:117.

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