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Novel Potential Molecular Biomarkers of Aquatic Contamination in *Dicentrarchus labrax* and *Liza aurata*

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Abstract—The responses of *Dicentrarchus labrax* and *Liza aurata* to aquatic pollution were assessed in a contaminated coastal lagoon, using a combination of both traditional and novel biomarkers. DNA damage, assessed by comet assay, was higher in both fish species from the contaminated sites, whereas levels of *cytochrome P450 1A1* gene expression were not significantly altered. The liver histopathological analysis also revealed significant lesions in fish from contaminated sites. Alterations in *ras* and *xpf* genes were analysed and additional pollutant-responsive genes were identified. While no alterations were found in *ras* gene, a down-regulation of *xpf* gene was observed in *D. labrax* from a contaminated site. Suppression subtractive hybridization applied to *D. labrax* collected at a contaminated site, revealed altered expression in genes involved in energy metabolism, immune system activity and antioxidant response. The approach and results reported herein demonstrate the utility of anchoring traditional biomarker responses alongside novel biomarker responses.

Keywords: pollution, biomarker, toxicology, fish

Abbreviations: bp, base pairs; PCR, polymerase chain reaction; RT, reverse transcriptase

INTRODUCTION

Aquatic organisms are exposed to a wide variety of environmental contaminants and frequently a set of biomarkers is employed to assess the possible biological impacts. Such biomarkers act as an early warning of a specific detrimental biological endpoint. Histopathological analysis, DNA integrity and detoxification enzyme status in fish tissues have frequently been employed as such biomarkers in environmental monitoring. Nevertheless, the specific and ultimate impact is rarely established. In the present study, two molecular biomarkers were employed

to fill the gap in the current knowledge by determining whether damaged DNA occurs and if so investigate if it is repaired or leads to mutations, the first of many steps in the possible progression to cancer. Also an exploratory approach was adopted to search for complementary molecular biomarkers.

To retain genomic stability, cells have mechanisms to detect and repair damaged DNA (Bi *et al.*, 2006). Bulky adducts, such as those induced by polycyclic aromatic hydrocarbons (PAHs), are repaired by the nucleotide excision repair (NER) mechanism which involves numerous proteins (Notch *et al.*, 2007), including Xeroderma Pigmentosum group F (XPF). In fish, DNA repair mechanisms are not well studied, when compared to that of mammals. Any defect in DNA repair mechanisms leads to genomic instability, either as mutations or other genetic alterations. The *ras* gene, one of the most important genes involved in carcinogenesis, has been found to be mutated in fish from areas of high PAH contamination (Rotchell *et al.*, 2001). These mutations often occur at "hot spots" in codons 12, 13 and 61 producing altered proteins that lead to deregulated activation of the ras protein (Rotchell *et al.*, 2001).

The aim of this work is to identify molecular responses as potential biomarkers of environmental pollution. Here we combine traditional (histopathology, *cyp1A* expression and Comet assay) with further molecular biomarker responses (*xpf* and *ras*), in an integrated approach to monitor environmental biological effects, in two species of feral fish (*L. aurata* and *D. labrax*) living in a contaminated coastal lagoon, Ria de Aveiro. Both species have high economic importance and have demonstrated to be very sensitive species for assessing genotoxicity and biotransformation responses to several aquatic contaminants (Gravato and Santos 2003; Pacheco *et al.*, 2005). Ria de Aveiro is a shallow estuarine coastal lagoon that receives multiple anthropogenic discharges, PAHs, organometallic compounds and heavy metals (Pacheco *et al.*, 2005; Sousa *et al.*, 2007; Mieiro *et al.*, 2009). We also adopt an exploratory approach using suppression subtractive hybridization (SSH) to isolate differentially expressed transcripts (Diatchenko *et al.*, 1996). The aim is to identify novel molecular responses from yet uncharacterised complex changes that occur at the level of gene expression, either as a direct or compensatory response, to molecular damage or cellular dysfunction in the same fish samples, and in doing so, anchoring any newly characterised responses to traditional biomarker responses.

MATERIALS AND METHODS

Study area and sampling

Juvenile specimens ($n = 5$) of *D. labrax* of average length 15.4 ± 1.8 cm and weight 47.5 ± 14.8 g, and *L. aurata* with an average length of 13.1 ± 1.7 cm and a weight of 25.9 ± 12.7 g, were collected in May 2005 from Ria de Aveiro, Portugal. The sampling sites were located along the main channels and at the lagoon entrance, and included various types and sources of contamination: Laranjo (LAR) close to a chlor-alkali plant, an important source of mercury

Table 1. PCR primer sequences.

Gene	Primer	Sequence (5′–3′)	GenBank access. No.
ras	RasF1	ATGACNGAATATAAGCTGG	Various
	RasR1	GTTGATGGCGAAGACGCA	Various
	RACE3ras	CGGAGAGACGTGTCTGCTGGACATCC	FJ529376
	RACE5ras	GGATGTCCAGCAGACACGTCTCTCCG	FJ529376
	RasF2	ATGACCGAGTATAAGCTGGT	FJ529376
	RasR2	CCTGTCCTCATGTACTG	FJ529376
	RASmulF	CCCTCACCATCCAGCTCA	FJ529377
	RASsbF	GGCAGAGCGCACTTACTA	FJ529376
xpf	XpfF1	GTTTTGTYCGCCAGCTGG	Various
	XpfR1	TGACGGGCTCGATGTCCA	Various
	RACE3xpf	CGGAGGCTCGACAGAAGAACAGA	FJ438473
	RACE5xpf	AGGTCCAGGTTGGTGTCTTCTCGTC	FJ438473
28S	28SF	GCCTGAGTCCTTCTGATCGAG	AY141637
	28SR	TCCCAAACAACCCGACTCCGAG	AY141637
cyp1A	Cyp1AF	GTCTTCATCAATCAGTGGCAG	U78316
	Cyp1AR	CAGCGTTTGTGCTTCATTGTGA	U78316
Real-time PCR primer sequences			
18S		For: TGTGCCGCTAGAGGTGAAAT Rev: GCATCGTTTATGGTCGGAAC	AM419038
XPF		For: GGAAAGCCACTCAGGGTGTA Rev: CCTCCCTCTCCTCTGGTATG	FJ438473
A2MG		For: GCTGACCCCAGCTATCAAAG Rev: GTTTCCTGCTCCTGATCCAA	GH196448
HGF		For: GGACCAGACCATCCCAGTAA Rev: GGGTCTCCTTGGCACAGTAA	GH196449
ITIH2		For: TGAGTTCCGGGACGATGTAA Rev: GCTGGCAAAAGGACTACAGG	GH196468
BDH		For: CCTGACCCAGAACAGGCTTA Rev: CAGTCACATAGGCCGACTCA	GH196471

contamination; Rio Novo do Principe (RIO), 6 km from a pulp/paper mill effluent outlet; Gafanha (GAF) situated in the vicinity of a deep-sea fishing port and dry-docks, also receiving domestic discharges; and Vagos (VAG) receiving municipal and domestic effluents. S. Jacinto (SJA) located close to the lagoon entrance and far from the main pollution sources was used as reference site. Immediately after sampling, fish were sacrificed and blood was collected. Livers were removed and

a portion fixed in Bouin's solution for histopathological analysis. The remaining liver was immediately frozen in liquid nitrogen and stored at –70°C until further analysis.

Traditional biomarker responses

Liver histopathology

The liver samples fixed in Bouin's solution were embedded in paraffin for light microscopy. Sections of 3–5 μm thick were stained with hematoxylin and eosin (HE). Histopathological alterations of the liver were evaluated qualitatively and semi-quantitatively using a modified version of the protocol described by Bernet *et al.* (1999).

Evaluation of DNA integrity by Comet Assay

The alkaline version of comet assay was conducted with whole blood under yellow light to prevent UV-induced DNA damage as described in Nogueira *et al.* (2006). Slides were examined under a fluorescence microscope (Leica DMLS, Germany) and fifty randomly selected cells per slide (2 slides/animal) were analysed using the image analysis software Comet Assay IV (Perceptive Instruments, UK). DNA damage was expressed as the percentage of tail DNA (% tDNA) and presented as mean of medians ± standard error of the mean (SEM).

Determination of cyp1A1 gene expression levels

cyp1A1 gene expression levels were analysed in *D. labrax* and *L. aurata* liver samples by semiquantitative reverse transcriptase-PCR (sqRT-PCR). First strand cDNA was synthesised from 1 μg of total RNA in the presence of oligo d(T) primers using Superscript reagents (Invitrogen, UK). The cDNAs were amplified using *cyp1A1* gene specific primers (Cyp1AF/Cyp1AR, Table 1). To normalize for initial variations in sample concentration and differences in efficiency during amplification, 28S rRNA primers (Table 1, primers 28SF/28SR) were also included in each reaction. Ten microliters of each PCR product were used for agarose gel electrophoresis and cDNA amounts were quantified by densitometric analysis using Quantity One software (BioRad, USA). The results were expressed in arbitrary densitometric units after background subtraction. The ratio between the sample RNA to be determined and 28S was calculated, and the results presented as mean ± SEM for each sampling site.

Development of novel biomarkers of exposure in D. labrax and L. aurata

Analysis of ras gene mutations

The normal *D. labrax* and *L. aurata ras* gene sequences were isolated from the liver of animals collected at a relatively clean site (SJA). cDNA was the template for the amplification of *ras* gene fragments with the primer pairs RasF1/RasR1 (Table 1). The resulting nucleotide sequence acted as a starting point for gene specific primer design. The isolation of the complete 5′ and 3′ ends of *ras* genes was performed using liver total RNA (1 μg) and SMART^{TM} RACE cDNA amplification protocol (Clontech, France) and the gene specific primers RACE3ras (3′ end) and RACE5ras (5′ end) (Table 1). The RACE products sequence was

obtained using commercial sequencing (Eurofins MWG Operon, Germany).

The analysis of *ras* gene mutations was carried out for all *D. labrax* and *L. aurata* liver samples. cDNA was used as template to amplify exon 1 and part of exon 2 of *D. labrax* and *L. aurata ras* genes using specific primers (RasF2/RasR2, Table 1). Amplified fragments were directly sequenced (Eurofins MWG Operon, Germany) in both directions in order to identify and characterise any mutations present.

Determination of ras gene expression levels

The expression levels of *ras* gene were analysed for all liver samples using the sqRT-PCR method as described in Subsection "Determination of *cyp1A1* gene expression levels". Primers RASmulF/RasR2 for *L. aurata* samples and RASsbF/RasR2 for *D. labrax* (Table 1) were used in the PCR reaction. *28S rRNA* was used as an internal standard.

Determination of xpf gene expression levels

The isolation of *xpf* gene was carried out as described in Subsection "Analysis of *ras* gene mutations". The primers used in the amplification of the first *xpf* fragment were XpfF1/XpfR1 (Table 1). The sequenced *xpf* fragments for both species were the basis for the design of specific primers used in RACE amplification (RACE3xpf/RACE5xpf, Table 1).

The analysis of *xpf* expression levels was performed in *D. labrax* samples from the sampling sites SJA and GAF, using real-time quantitative PCR (qPCR). *D. labrax 18S rRNA (18S)* was used as an internal control. First-strand cDNA was synthesized using SuperScript® Vilo™ cDNA synthesis kit (Invitrogen, UK). qPCR reactions were performed using Express SYBR® GreenER™ qPCR SuperMix (Invitrogen, UK) and specific primers (Table 1). A control lacking cDNA template was included in qPCR analysis to determine the specificity of target cDNA amplification. Amplification was detected using the Mx3005P real time PCR system (Stratagene, UK). Melting curves were performed to identify the presence of primer dimers and analyse the specificity of the reaction. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA. The relative expression levels of target genes were calculated according to the efficiency-corrected method described by Pfaffl (2001). The reference site group (SJA) was used as calibrator.

SSH

SSH was employed to isolate and enrich for genes differentially-expressed between *D. labrax* captured at the reference site and at a contaminated site (GAF). For each group, equal amounts of RNA were pooled (5 fish in each group). cDNA was synthesised using SuperSMART PCR cDNA Synthesis kit (Clontech, France). The forward- and reverse-subtracted libraries were produced using PCR-Select cDNA Subtraction reagents (Clontech, France) according to the manufacturer's protocol. A total of 20 clones per library were randomly selected for sequencing (Eurofins MWG Operon, Germany). Sequence identities were obtained by BLAST searches against the NCBI nucleic acid and protein databases. Sequence reads with *E*-value $>10^{-5}$ were filtered out. The differential screening result was confirmed by qPCR as described in Subsection "Determination of *xpf* gene

expression levels", using four candidate genes (Table 1): alpha-2-macroglobulin (A2MG), HGF activator like protein (HGFA), inter-alpha globulin inhibitor H2 (ITIH2) and 3-hydroxybutyrate dehydrogenase (BDH).

Statistical analysis

For statistical analysis (SigmaStat 3.11 Software), all data were tested for normality and homogeneity of variances and, when these assumptions were met, differences between control and exposed fish were determined using analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

RESULTS

Traditional biomarkers

A macroscopic examination of liver samples was carried out *in situ*. The histopathological liver injury indices varied between 12.6 and 21.8 in *D. labrax*, and from 15.0 to 39.0 in *L. aurata*. A significant difference in liver injury index was observed in *L. aurata* from all the contaminated sites when compared to the reference site. Statistically different liver indices were also observed in *D. labrax* specimens from RIO and GAF. Both species from the reference site showed a normal liver architecture with hepatocytes presenting a homogeneous cytoplasm and a large spherical nucleus containing one nucleolus. The main alterations found in *L. aurata* liver were karyomegaly and pyknosis, leukocyte infiltration, haemorrhage and areas of necrosis. Melano-macrophages centres (MMC) were also more abundant in *L. aurata* collected at contaminated sites than in the reference site. Additionally, specimens from GAF and VAG showed alterations in the bile ducts, such as pyknosis, inflammation, presence of MMC, and, in the worst cases, destruction of some bile ducts. In general, the liver of *D. labrax* was less damaged than *L. aurata* liver. The main alterations in *D. labrax* included nuclear changes (karyomegaly and pyknosis), some haemorrhage and small areas of necrosis. Vacuolar degeneration was also observed in *D. labrax* from RIO and VAG, and, to a smaller extent, GAF. A strong leukocyte infiltration was additionally observed in specimens from RIO and GAF.

DNA damage levels measured by comet assay in blood cells of *L. aurata* and *D. labrax* collected from the GAF and RIO sampling sites showed significant increases when compared to those of the reference site. The levels measured in *L. aurata* from contaminated sites were higher than those observed in *D. labrax*. Nevertheless, the differences observed between contaminated and reference site animals were higher in *D. labrax*.

The analysis of *cyp1A1* gene expression levels in individual samples using sqRT-PCR revealed no significant variation between animals from the contaminated and reference sites. A high inter-individual variability was observed in both species.

Table 2. Genes differentially expressed in liver of *D. labrax* from contaminated (GAF) and reference areas (sequences showing no similarity to genes with known functions are not included). Asterisk denotes a statistically significant difference from the reference site group: $*P < 0.05$.

Clone access. No.	Species	Accession No.	Putative identity	Blast *E*-value	Relative expression
Upregulated genes					
GH196447	*Paralichthys olivaceus*	BAA88901	Complement component C3	6E-69	—
GH196448	*Sparus aurata*	AAR06589	Alpha-2-macroglobulin	6E-81	3.57*
GH196449	*Ornithorhynchus anatinus*	XP_001513341	Similar to HGF activator like protein	5E-38	4.39*
GH196450	*Oncorhynchus mykiss*	CAE45341	Secreted phosphoprotein 24	2E-29	—
GH196451	*Catostomus commersonii*	BAF41382	Cytochrome c oxidase subunit III	3E-51	—
GH196452	*Lepidocybium flavobrunneum*	ABY88956	Cytochrome c oxidase subunit I	9E-71	—
GH196454	*Danio rerio*	CAQ14687	Immune-related, lectin-like receptor 2	3E-10	—
GH196453	*Danio rerio*	AAH47845	Electron-transfer-flavoprotein alpha polypeptide	1E-64	—
GH196456	*Danio rerio*	AAH59638	Cytochrome c oxidase subunit VIIc	1E-27	—
GH196457	*Salmo salar*	X81856	Apolipoprotein B	6E-6	—
GH196458	*Zu cristatus*	BAB70118	NADH dehydrogenase subunit 1	2E-51	—
Downregulated genes					
GH196461	*Salmo salar*	ACI68200	Ribosome biogenesis protein RLP24	8E-70	—
GH196463	*Epinephelus coioides*	AAW29025	Copper/zinc superoxide dismutase	9E-78	—
GH196466	*Zebrasoma flavescens*	AP006032	Mitochondrial DNA	4E-138	—
GH196467	*Salmo salar*	AAO43606	Serum lectin isoform 2 precursor	3E-19	—
GH196460	*Oplegnathus fasciatus*	EU812516	Apolipoprotein A-I (apoA-I) mRNA	6E-178	—
GH196462	*Pagrus major*	AY335444	Transferrin mRNA	2E-59	—
GH196468	*Danio rerio*	BC045924	Inter-alpha globulin inhibitor H2 mRNA	6E-18	0.73*
GH196469	*Xenopus laevis*	BC080996	Stromal cell derived factor 4, mRNA	4E-10	—
GH196471	*Salmo salar*	ACI67111	3-hydroxybutyrate dehydrogenase, type 2	6E-29	0.85*

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Analysis of ras gene mutations and expression levels

The *D. labrax ras* cDNA contained a complete open reading frame (ORF) of 567 nucleotides (GenBank Accession No. **FJ529376**) encoding a 188 amino acid protein. For *L. aurata*, two *ras* cDNAs were isolated corresponding to the two alternative spliced *Ki-ras* genes: *K-ras-1* (GenBank Accession No. **FJ529377**) with an ORF of 567 nucleotides encoding 188 amino acids, and *K-ras-2* (GenBank Accession No. **FJ529378**) containing a complete ORF of 570 nucleotides encoding a 189 predicted amino acid sequence. No mutations were found in the *ras* fragments screened in either species though polymorphic variation was observed. No differences in *ras* gene expression levels were observed between fishes from different sites.

Determination of xpf gene expression levels

xpf genes from *D. labrax* and *L. aurata* contained an ORF of 2700 and 2694 nucleotides, respectively (GenBank Accession No. **FJ438473** and **FJ438474**), revealing a predicted amino acid sequence of 899 and 897 residues. Both genes showed a high homology between them (91%) and more than 75% identity with other fish sequences. *xpf* gene expression was studied in the liver samples of *D. labrax* from the sampling site GAF, where the most significant increase of DNA damage was observed as measured by comet assay. The qPCR method revealed a statistically significant ($P < 0.05$) downregulation of this gene (relative expression = 0.44) when compared to the expression levels in *D. labrax* from the reference site (SJA).

SSH

Eleven sequences from each forward (up-regulated genes) and reverse (down-regulated genes) libraries could be matched to genes from different organisms, mainly fish species (Table 2). Eighteen remaining sequenced clones showed homology to unidentified hypothetical or novel proteins or showed no homology with the sequences deposited in the database. The SSH results were validated by qPCR using two of the genes highlighted for each library (Table 2).

DISCUSSION

In summary, we have reported liver cellular damage and gross DNA damage in two species of fish from selected sites (RIO and GAF) with different levels of environmental contamination. Based on those traditional biomarker responses one may conclude that the fish at RIO and GAF sampling sites are impacted detrimentally by the contamination present. At the next level of biological effects, which probes the actual significance of this damage, no *ras* gene mutations or changes in *ras* gene expression were observed, yet a decrease in *xpf* gene expression was observed (at GAF). The exploratory global approach using SSH also highlights the complexity in response. Detoxification and cell growth genes were highlighted as differentially expressed in impacted fish, yet genes from other process, particularly the immune response, also appear to be important in considering the wider implications of contaminant exposure.

Traditional biomarker responses to contamination

Two of the main histopathological alterations observed in both species were necrotic foci and inflammatory processes, although changes such as nuclear alterations (karyomegaly and pyknosis) and increased number and size of MMCs were also present. These alterations have been described in other species of fish living in contaminated environments (Teh *et al*., 1997; Nero *et al*., 2006) suggesting that these alterations might be related to the exposure to environmental chemicals present. The presence of these changes was less pronounced in the liver of *D. labrax* than in *L. aurata*, which suggests that *D. labrax* liver is being less affected by aquatic contamination than *L. aurata*. While *D. labrax* feeds in the water column, *L. aurata* is in regular contact with the sediments where the majority of the contaminants accumulate. In this way, *L. aurata* may be in contact with higher concentrations of contaminants which may lead to an increased risk of damage to the liver as observed in this study.

No significant differences in *cyp1A* gene expression were found between the fish populations analysed. However, a high inter-individual variability was observed, which may be due to natural variation (Cajaraville *et al*., 2000). Another possible explanation is that, in the natural environment, a mixture of both inducers and inhibitors of cyp1A system may act simultaneously, and any cyp1A regulation (up or down) is likely to be the net result of additive, synergistic or antagonist chemical interactions (Hartl *et al*., 2007).

Both species of fish collected at GAF and RIO sampling sites showed significant increases of DNA damage when compared to the reference populations (SJA). One cause for the significant levels of DNA damage observed may be the action of highly reactive xenobiotic metabolites following liver metabolism. The higher levels of DNA damage found in *L. aurata* may again reflect its feeding behaviour and habitat.

Novel potential molecular biomarkers of exposure in D. labrax and L. aurata

The induction of DNA damage in fish from contaminated environments may lead to mutations, which contribute towards the multistage carcinogenesis process. Oncogenes, such as *ras*, activated by point mutations, have been identified as genetic targets both in feral fish from heavy contaminated areas as in laboratory-exposed fish (Rotchell *et al*., 2001). The *ras* sequences obtained in the present study displayed all the conserved structural domains of the gene and showed a high degree of similarity with other fish *ras* genes characterized to date. Screening for *ras* mutations in hot spots of the gene revealed no mutations in the fish collected from the contaminated sites and no statistically significant differences were detected in the expression of this gene. Nevertheless, neither of the species used in this study is prone to tumour formation and few studies have demonstrated *ras* mutations or altered gene expression in normal tissues from species that are not susceptible to neoplasia (Roy *et al*., 1999). *D. labrax* and *L. aurata* may have also efficiently repaired any mutation in the *ras* gene or in any other part of their genome, through mechanisms that did not involve the induction of *xpf* gene

expression.

Relevantly, a general lack of information on DNA repair mechanisms still exists for aquatic species. The *xpf* gene was isolated from *D. labrax* and *L. aurata* and the sequences obtained revealed a high homology with the human gene. Decreased *xpf* gene expression was observed in the fish sampled at the contaminated site (GAF) compared to those from the reference site (SJA), suggesting that contamination may reduce fish repair capacity and in this way increase the risk for genomic instability. Notch and colleagues (2007) also reported a significant decrease in gene expression in several hepatic NER components, including *xpf*, in zebrafish liver after exposure to environmentally relevant concentrations of 17α-ethinylestradiol. Combined, these findings seem to suggest that *xpf* gene may be a potential candidate for future biomarker development.

The SSH approach revealed differently expressed genes (Table 2) some of which share roles in common physiological processes. Genes related to energy production and ATP metabolism (cytochrome c oxidase, electron-transfer-flavoprotein, and NADH dehydrogenase genes) and genes involved in the inflammatory and innate immune response (e.g. complement component C3, alpha-2-macroglobulin and lectin-like receptor 2 genes) have been found to be upregulated in the liver of *D. labrax* from the contaminated sampling site (GAF). This result is consistent with the literature in that genes involved in these processes were also found induced in fish exposed to contamination (Williams *et al.*, 2003; Marchand *et al.*, 2006). The hepatocyte growth factor activator (HGFA) was also found upregulated. This gene is involved in the regeneration of injured liver tissue and therefore the upregulation of HGFA gene observed in *D. labrax* from the contaminated site suggests an injured liver associated with the exposure to contamination, a finding supported by the liver histopathology alterations observed in this study. HGFA gene has also been found induced in the liver of other fish species following contaminant-induced tissue injury (Roling *et al.*, 2004; Marchand *et al.*, 2006), and thus seems to be a good indicator of liver tissue injuries. Some of the genes found downregulated in the exposed *D. labrax* include transferrin and copper/zinc superoxide dismutase (SOD) genes which share a role in the antioxidant defence system. The downregulation of these genes suggest that the antioxidant defence system is being suppressed, possibly failing to protect the fish from the toxic effects caused by ROS.

These transcriptional changes are a response of the organism to environmental stress. In this respect, gene expression biomarkers may be used to indicate exposure to toxicants, thus acting as early warning sensors.

CONCLUSIONS

Here, novel early-warning molecular responses, alongside with traditional biomarker responses, were studied in *D. labrax* and *L. aurata* exposed to environmental contamination. The *xpf* and *ras* responses add important information regarding genome stability, while the SSH allowed a global approach showing that DNA repair capacity, immune response and antioxidant defence system seem compromised after the exposure to environmental contamination. Alterations in

these important systems can have potential future repercussions at higher levels of biological organisation. Alterations in the expression of *xpf* gene and some of the genes found by SSH, such as HGFA, are potential good biomarkers of environmental contamination in fish and should be the focus of future work.

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