

Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (*Sparus aurata*): Insights from fish exposed to dietary, pathogen and confinement stressors

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ABSTRACT. The aim of this work was to underline the physiological role of the antioxidant peroxiredoxin (PRDX) family in gilthead sea bream (*Sparus aurata* L.), a perciform fish extensively cultured in the Mediterranean area. First, extensive BLAST searches were done on the gilthead sea bream cDNA database of the AQUAMAX European Project (www.sigenae.org/iats), and six contigs were unequivocally identified as PRDX1-6 after sequence completion by RT-PCR. The phylogenetic analysis evidenced three major clades corresponding to PRDX1-4 (true 2-Cyst PRDX subclass), PRDX5 (atypical 2-Cys PRDX subclass) and PRDX6 (1-Cys PRDX subclass) that reflected the present hierarchy of vertebrates. However, the PRDX2 branch of modern fish including gilthead sea bream was related to the monophyletic PRDX1 node rather than to PRDX2 cluster of mammals and primitive fish, which probably denotes the acquisition of novel functions through vertebrate evolution. Transcriptional studies by means of quantitative real-time PCR evidenced a ubiquitous PRDX gene expression that was tissue-specific for each PRDX isoform. In a second set of transcriptional studies, liver and head kidney were chosen as target tissues in fish challenged with i) the intestinal parasite *Enteromyxum leei*, ii) a plant oil (VO) diet with deficiencies in essential fatty acids and iii) prolonged exposure to high rearing densities. These studies showed that PRDX genes were highly and mostly constitutively expressed in the liver and were not affected by dietary intervention or high density. In contrast, head kidney was highly sensitive to the different experimental challenges: significantly lower values were found for PRDX5 in the three trials, for PRDX6 in parasitized and high density fish and for PRDX1 in parasitized and VO fish. PRDX2, 3 and 5 were decreased only in VO, high density and parasitized animals, respectively. These findings would highlight the role of PRDXs as integrative and highly predictive biomarkers of health and welfare in fish and gilthead sea bream in particular.

Key words: *Sparus aurata*; antioxidant enzymes; Myxozoa; essential fatty acids; stress; immune response.

1. Introduction

Peroxiredoxins (PRDXs) are the most recently discovered family of antioxidant enzymes that catalyze the reduction of peroxides and alkyl peroxides using thioredoxin as the immediate reducing-cofactor [1,2]. PRDXs are non-selenium dependent enzymes initially identified in yeast [3], but they have been discovered in all kingdoms of life. The presence of multiple genes, encoding each one for a different isoform, was first reported in mammals in which six PRDXs have been identified to date. Five of them have two conserved and catalytically active Cys residues (PRDX1 to 5), though only that of the N-terminal region is directly involved in peroxidatic activity. PRDX6 only has the catalytic Cys residue of the N-terminal region and thus belongs to 1-Cys PRDX subclass with both glutathione peroxidase and phospholipase A activities leading to reduced membrane phospholipid peroxidation [4,5].

PRDX1 and 2, also referred as natural killer enhancing factors (NKEF-A, NKEF-B), are localized in the cytosol [6,7], whereas the other members of the 2-Cys PRDX subclass are widely distributed with PRDX3 in mitochondria [8], PRDX4 in endoplasmic reticulum and extracellular space [9] and PRDX5 in cytosol, mitochondria and peroxisomes [10]. In contrast, PRDX6 is restricted to cytosol [11], and the catalytic efficiency of all PRDXs is less than that of catalase or glutathione peroxidases by one or three orders of magnitude [12]. However, PRDXs are abundant proteins, typically constituting 0.1-0.8% of total soluble protein, and the overexpression of NKEF-A and B can decrease efficiently the cytosol level of H_2O_2 [13,14], which is now considered an ubiquitous intracellular messenger at subtoxic concentrations [15]. On the other hand, reduction of mitochondrial H_2O_2 by overexpressed PRDX3 is a key regulator of apoptosis [16,17]. Aberrant expression of most PRDX family members has

also been reported in various kinds of cancers, and their silencing is currently tested to enhance radiotherapy effects [18]. Thus, PRDXs can represent a first line of defense against oxidative stress, but they are also crucial to turn off the inflammatory and immune response mediated by H₂O₂ [15,19,20].

In fish, NKEF-A and B have been characterized in a wide range of teleosts including rainbow trout (*Oncorhynchus mykiss*) [21], carp (*Cyprinus carpio*) [22,23], channel catfish (*Ictalurus punctatus*) [24], Japanese flounder (*Paralichthys olivaceus*) [25], turbot (*Psetta maxima*) [26], pufferfish (*Tetraodon nigroviridis*) [27], ayu (*Plecoglossus altivelis*) [28], bluefin tuna (*Thunnus maccoyii*) [29] and lamprey (*Lampetra japonica*) [30]. Recently, PRDX4 has been cloned and characterized in yellowtail kingfish (*Seriola lalandi*) [31]. PRDX6 has also been cloned and characterized in catfish [32], Atlantic salmon (*Salmo salar*) [33] and turbot [34]. Additional fish PRDX sequences, like sablefish (*Anoplopoma fimbria*) PRDX3 (GenBank accession number BT083182) and PRDX5 (GenBank accession number BT082491) are available as direct submissions on public databases. However, complete and integrative studies are still lacking in fish, and the aim of the present study was to identify and address the tissue-specific regulation of all the members of PRDX family in gilthead sea bream, *Sparus aurata*, a successfully cultured fish in the Mediterranean area. To pursue this issue, extensive BLAST searches for PRDXs were done on the gilthead sea bream cDNA database of the AQUAMAX European Project (www.siginae.org/iats), and six contigs were unequivocally identified as PRDX1 to 6 after sequence completion by RT-PCR. The expression analysis of PRDX isoforms was then addressed by quantitative real-time PCR (qPCR) with special emphasis on liver and head kidney as target tissues in fish challenged with three different aquaculture stressors i) an intestinal parasite, ii) nutrient deficient diets and iii) high rearing

densities. The chronic exposure to *Enteromyxum leei*, a commonly occurring intestinal parasite in gilthead sea bream [35], was chosen as infection model. The total replacement of fish oil with a blend of vegetable oils in plant protein-based diets was chosen as a model of nutrient deficiencies in essential fatty acids (EFA) with detrimental effects on growth [36,37] and antioxidant capacity [38]. High rearing density was also selected as a common aquaculture stressor to assess the stress-mediated response in a pair-fed model [39].

2. Materials and methods

2.1. Animal care and sampling

Juveniles of gilthead sea bream were reared in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS). Day length and temperature followed natural changes at our latitude (40°5'N; 0°10'E), although water was heated in the infection trial to keep temperature always above 18 °C. The oxygen content of water was always higher than 85% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/l). Except when indicated, fish were fed a commercial diet (Proaqua, Palencia, Spain) containing 47% protein and 21% lipid. At the sampling time, fish were overnight fasted and decapitated under anesthesia (3-aminobenzoic acid ethyl ester, 100 mg/l). Target tissues were rapidly excised, frozen in liquid nitrogen, and stored at -80°C until RNA extraction and analysis. All procedures were carried out according to the national and institutional regulations on animal experimental handling (IATS-CSIC Review Board).

2.2. Experimental setup

Tissue screening for PRDX gene expression was carried out in two year-old fish. Two randomly selected fish from an IATS stock were sampled and target tissues (intestine, head kidney, spleen, brain, eyes, gills, heart, white skeletal muscle, liver, adipose tissue and testis) were excised and deep frozen in liquid nitrogen in less than 10 min.

To analyze the effect of three types of challenging experimental conditions on PRDX gene expression, samples were obtained from earlier studies already published [36,39,40]. The infection trial was performed by exposure of juvenile fish (134 g initial body weight) to *E. leei*-contaminated effluent [40]. After 113 days of exposure, two categories of recipient fish were obtained: non-parasitized (exposed but not infected, R-NON PAR) and parasitized (exposed and infected, R-PAR) fish, which were compared with control animals (not exposed to the parasite, CTRL). Head kidney samples were obtained from fish of the three categories.

The effect of the nutritional background (dietary EFA level) was analyzed from head kidney and liver samples obtained from a previous dietary trial [36]. Briefly, juvenile fish of 16 g initial body weight were fed to visual satiety from May to mid-August with plant protein diets containing either fish oil (FO diet) or a blend of vegetable oils at the 100% of FO replacement (VO diet). At the end of the trial, weight gain of VO fish was significantly reduced and 8 randomly selected fish per dietary treatment were sampled for collection of tissue samples.

The effects of prolonged confinement exposure were analyzed from head kidney and liver samples obtained from a previous experiment in which the effect of acute and prolonged crowding stresses on mitochondrial chaperones was also assessed [39].

Briefly, fish were reared at high (HD, 45–50 kg/m³) and low (LD-PF, 10 kg/m³) densities in a pair-fed study with a standard fish diet. Over the course of the 3 weeks-trial at midsummer, body weight of juvenile fish (18 g initial body weight) increased more than two-fold in the two experimental groups. Parallel decreases (10% reduction) in growth rates ($P < 0.09$) and feed efficiency ($P < 0.10$) were found in the HD group in comparison to LD-PF fish.

2.3. RNA extraction and RT procedure

Total RNA from target tissues was extracted using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA) with a DNase step. The RNA yield was 30-50 µg with absorbance measures ($A_{260/280}$) of 1.9-2.1. Reverse transcription (RT) of 500 ng total RNA with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). Negative control reactions were run without reverse transcriptase.

2.4. Completion of PRDX sequences by RT-PCR

Degenerated and specific primers for the completion of PRDX1 sequence between two non-overlapping contigs were designed after initial searches in the gilthead sea bream cDNA database. Forward primer (5′- CAG CCA AAG CWG TKA TGC C) and reverse (5′- TAA TCG GGA GAG GTG TCT TTG G) primer were located 47 nucleotides downstream the start codon and 287 nucleotides downstream the stop codon, respectively. PCR amplification was made with 2 µl of liver RT reactions and 2 units of Platinum Taq DNA polymerase (Invitrogen, Gaithersburg, MD, USA). Thirty

five cycles were carried out with denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s, and extension at 72 °C for 90 s.

2.5. Gene expression analyses

qPCR was performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). Briefly, diluted RT reactions were conveniently used for PCR reactions in 25- μ l volume in combination with a SYBR Green Master Mix (Bio-Rad) and specific primers for PRDX1-6 and housekeeping genes (β -actin, elongation factor 1, 18S rRNA and α -tubulin) at a final concentration of 0.9 μ M (Table 1). The efficiency of PCR reactions for target and reference genes varied between 95% and 98%. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and the fluorescence data acquired during the extension phase were ultimately normalized to β -actin by the delta-delta method [41].

2.6. Sequence and phylogenetic analyses

All amplified PCR-products were gel-extracted and sequenced by the deoxy chain termination method (ABI PRISM dRhodamine terminator cycle sequencing kit, Perkin-Elmer, Wellesley, MA, USA). A BLAST-X search strategy was used to corroborate the identity of amplified products. Multiple sequence alignments were carried out with

ClustalW. A phylogenetic tree was constructed on the basis of amino acid differences (poisson correction) with the Neighbor Joining (NJ) algorithm (complete deletion) in MEGA version 4.0 [42]. A total of 38 additional PRDX sequences from 16 species were used in the analysis. Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

2.7. Statistics

Data on gene expression are represented as the mean \pm SEM of 6-8 fish. For each PRDX isoform, the specific effects of pathogen exposure, dietary treatment and stocking density on transcript levels were analyzed by Student t-test (when two groups were compared) or by One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. The significance level was set at $P < 0.05$. All the statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA).

3. Results

As shown in Table 2, searches in the AQUAMAX gilthead sea bream database recognized (E-value $\leq 3e-92$) five contigs of 2-6 clones in depth as complete codifying sequences of 197 (NKEF-B), 247 (PRDX3), 263 (PRDX4), 190 (PRDX5) and 221 (PRDX6) amino acids in length. Two additional non-overlapping contigs of 314 and 667 bp in length were identified as NKEF-A (E-value $1e-105$) after gap sequence completion (81 amino acids) by RT-PCR. All these gilthead sea bream PRDX sequences were introduced in GenBank with accession numbers GQ252679 (NKEF-A), GQ252680 (NKEF-B), GQ252681 (PRDX3), GQ252682 (PRDX4), GQ252683

(PRDX5) and GQ252684 (PRDX6). Amino acid sequence alignments evidenced that gilthead sea bream PRDXs present a minimum of 72 % similarity (63 % identity) with human orthologous genes.

As depicted in Figure 1, PRDX1-4 sequences of gilthead sea bream share the characteristic signatures of the true 2-Cys PRDX subclass with a strict conservation of N- and C-terminal catalytically active Cys residues. The sequence recognized as the gilthead sea bream PRDX5 shows the alternative C-terminal Cys residue as a member of the atypical 2-Cyst PRDX subclass. In contrast, the sequence named as gilthead sea bream PRDX6 has only the N-terminal Cys residue and it was recognized as an antioxidant enzyme of the 1-Cys PRDX subclass. Within the gilthead sea bream PRDX family, the highest amino acid identity (83%) and similarity (91%) was found between NKEFs (PRDX1 and 2) decreasing up to 48-55% and 59-66% when PRDX3 and 4 were included in the analysis. Amino acid identity and similarity between PRDX5 and PRDX1-4 were reduced up to 12-15% and 24-28%, respectively. A reduced but higher degree of amino acid identity (15-23%) and similarity (30-41%) was found when PRDX6 and family members of the PRDX1-4 subclass were compared.

The phylogenetic tree undertaken in the present study evidenced three major clades corresponding to PRDX1-4 (true 2-Cyst PRDX subclass), PRDX5 (atypical 2-Cys PRDX subclass) and PRDX6 (1-Cys PRDX subclass) according to the present hierarchy of vertebrates (Figure 2). Of note, within the long-branch covering the true 2-Cys PRDX subclass, PRDX1, 3 and 4 were recognized as monophyletic clusters. This, however, is not the case of PRDX2 node and, interestingly, the PRDX2 branch of modern fish (pufferfish, turbot, gilthead sea bream, tuna) was related to neighboring PRDX1 node rather than to PRDX2 cluster of mammals and primitive fish (lamprey).

Primers for qPCR amplification of PRDX isoforms were designed within codifying and non-codifying sequences (reverse primers of NKEFs) to assure the specificity of the amplified product, confirmed by curve melting analysis and DNA sequencing. Up to four housekeeping genes (β -actin, α -tubulin, elongation factor I, 18S rRNA) were used on gene expression analysis and given the expression profile within and among tissues, β -actin was finally chosen as the most reliable reference gene in the normalization procedure. On this basis, a representative tissue-specific profile of gilthead sea bream PRDX isoforms is shown in Figure 3. PRDX genes were ubiquitously expressed in gilthead sea bream, although most PRDX isoforms were mainly found in liver and secondly in skeletal muscle, heart and brain. Overall, the abundance of PRDX mRNAs was low in gills, adipose tissue, intestine and immunorelevant tissues (head kidney and spleen), but the relative mRNA expression of NKEF-B in comparison to other PRDXs genes was 10-20 higher in head kidney than in other tissues as inferred from this and the more specific transcriptional studies described below.

In head kidney, the tissue-specific expression profile of PRDX isoforms was altered by pathogen exposure (Figure 4A). Thus, transcript levels of NKEF-A, PRDX3, 5 and 6 were decreased significantly in the head kidney of R-PAR fish. By contrast, in R-NON PAR fish, the expression pattern of PRDX isoforms remained almost unaltered (PRDX4-6) or increased (NKEFs and PRDX3) in comparison to CTRL and R-PAR fish. Fish fed VO (that developed essential fatty acid deficiencies) also showed an altered expression profile of PRDXs in head kidney, and transcript levels of NKEFs and PRDX5 were significantly lower (Figure 4B). Prolonged exposure to high stocking densities also decreased the overall expression of PRDXs in the head kidney of on-

growing gilthead sea bream, becoming transcript levels of PRDX4, 5 and 6 significantly lower (Figure 4C).

The specific effects of dietary treatment and confinement exposure upon hepatic transcript levels of PRDXs are shown in Figure 5. In this case, we failed to detect any significant effect of either dietary treatment or high density rearing upon PRDX mRNA levels, which evidences not only a high but also more constitutive expression of PRDXs in the hepatic tissue.

4. Discussion

PRDXs constitute a class of ubiquitous enzymatic antioxidants that have been identified in various organisms ranging from prokaryotic bacteria to eukaryotic organisms including yeast, plants and animals [3,19]. In particular, up to six PRDXs have been described in mammals, and the present study highlights their conservation in a modern fish that belongs to family Sparidae, order Perciformes. This is, thereby, the first study, indentifying unequivocally six mammalian PRDX counterparts in a teleost fish. Importantly, the concomitant gene expression of mitochondrial PRDX3 and 5 was also addressed for the first time in fish, and this will serve to match the primary antioxidant defense of mitochondrial respiratory chain as evidenced in mice during the experimentally-induced myocardial infarction [43].

The mapping of gilthead sea bream PRDX sequences also contributes to highlight invariant amino acids as well as the shared-derived amino acids within each cluster and vertebrate PRDX node. Hence, from already available sequences in fish, chicken and mammals it becomes conclusive that the major divergence among vertebrate PRDXs lies at the N-terminus with a strict conservation of amino acid residues surrounding the

N- and C-terminal catalytic Cys residues of PRDX1-4. Similar to mammals, the C-terminal region of the gilthead sea bream PRDX5 is smaller than those of true 2-Cys PRDX enzymes, and interestingly both in gilthead sea bream and sablefish the sequences identified as PRDX5 conserve the alternative Cys residue at the C-terminus belonging thus members of the atypical 2-Cys PRDX subclass. On the other hand, in a wide range of fish species, including gilthead sea bream, PRDX6 has only the N-terminal Cys residue and this PRDX isoform was unequivocally recognized as a member of 1-Cys PRDX subclass. As indicated by several authors in fish [27,34,44] and mammals [19,45], the PRDX family can be viewed through vertebrate evolution as a highly conserved family of antioxidant enzymes, though the phylogenetic tree with the long NKEF-B branch within the true 2-Cys PRDX subclass might reflect the acquisitions of novel PRDX functions just after the split of tetrapods and modern fish.

The expression of the six PRDX genes in the 11 gilthead sea bream tissues examined in this study confirms their ubiquity. Even though there might be a discrepancy between expression levels of the transcripts and the proteins, it still illustrates the importance of such enzymes in the basal defense metabolism of these tissues. This idea is reinforced by recent studies on knock-out or transgenic PRDX mice, which suggest that PRDX enzymes play a key role in several metabolic processes though they are not mandatory for life [17,46-48]. For instance, aberrant patterns of PRDX expression have been reported in the central nervous system of patients affected by neurodegenerative disorders [20], and reduced expression of PRDXs is a major factor in the etiology of cataracts [49]. Accordingly, a high expression level of NKEF-B and mitochondrial PRDXs was found in brain and eyes of gilthead sea bream. Since these organs and tissues are extremely vulnerable to oxidative damage, this PRDX expression pattern would contribute to protect them against oxidative insults. Liver is

also prone to be exposed to oxidative by-products of metabolic activity and it is remarkable the high constitutive expression of all the members of the PRDX family in the hepatic tissue of gilthead sea bream. Likewise, the intestinal mucosa is vulnerable to oxidative damage and NKEF-B was highly expressed in the intestine of gilthead sea bream, as reported for NKEF-A and B in pufferfish [27]. The tissue specific-distribution of PRDX6 has also been documented in fish, and both this and a previous study in turbot [34] support an important role of this particular PRDX isoform in liver, skeletal muscle and heart.

Modulation of PRDX expression by various stress signals has been observed by many research groups in both vertebrate and invertebrate species. Thus, the transcription of a Pacific oyster PRDX6 was increased by pollution [50], whereas a 1-Cys PRDX was down regulated in *Pseudopleuronectes americanus* injected with hexavalent chromium [51], but a PRDX was up-regulated in *Platichthys flesus* injected with cadmium [52]. The expression of a 2-Cys PRDX gene was enhanced by acute hypo-osmotic stress in the crustacean *Eurypanopeus depressus* [53], whereas the oyster *Saccostrea glomerata* exposed to reduced salinity had a lower PRDX6 mRNA expression [54]. Thermal stress increased transcripts levels of PRDX4 and PRDX5 in a bivalve [55] and protein levels of a non-specified NKEF in gilthead sea bream [56]. All this draw a complex regulatory balance, and interestingly in the present study, the expression of the six PRDX genes remained unaltered in the hepatic tissue of gilthead sea bream challenged with either VO or high-rearing densities. In contrast, PRDX transcription in the head kidney was especially sensitive to aquaculture stressors, including a parasite challenge, which supports a role of PRDXs on the host immune defense of gilthead sea bream. This concept is not new and several authors have proposed that PRDX transcription is regulated with the dual purpose to attenuate

mitochondrial reactive oxygen species (ROS) production and turn-off the inflammatory and immune response for the prevention of the excessive host response. Thus, PRDX3 knockout mice were more susceptible to LPS-induced oxidative stress than the wild-type [48,57]. Also, transplantation of PRDX2^{-/-} bone marrow cells into wild type mice increased the number of peripheral blood mononuclear cells and bone-marrow derived dendritic cells [58]. At the same time, PRDXs in malaria parasites participate in the defense against the host attacks becoming these antioxidant enzymes good targets for chemotherapy [59].

Available literature data show that most infection models of aquatic organisms with bacteria or virus or vaccination procedures mediate an increase in gene or protein expression of PRDXs [28,44,60,61], but some viral and bacterial models [62,63] induce a decrease in the expression of some PRDXs. The observed differences might be due to differences in the challenging agent, the tissue type and the kinetics of the infection making comparative analysis difficult. Furthermore, the information on the effect of parasite infection on PRDXs expression is very scarce. Hamsters infected with *Opisthorchis viverrini* showed up-regulation of PRDX6 at 30 days post infection [64], but there are no data concerning fish models. In our model of chronic parasite infection in which samples were taken in a time much longer than all the pathogen models thus far studied, it was noteworthy that the host expression of most PRDX isoforms was significantly down-regulated in head kidney. This fact can be viewed as a disease outcome at the later stages of the infection, in which an anti-inflammatory stage is promoted by the host to avoid the excessive immune reaction, as suggested by the lower IL-1 β and TNF- α observed in the intestine of R-PAR fish [40]. In contrast and importantly, the transcription rate of NKEFs and PRDX3 was significantly up-regulated in R-NON PAR fish. This observation would be indicative of an enhanced immune

response, in agreement with the also increased expression of growth factors with immune-stimulatory properties [40].

Oxidative damage can be treated nutritionally in different mammalian models [65]. In fish, nutritional intervention is also capable of modifying the expression of PRDXs. A low phosphorous diet decreased the expression of a NKEF in the pyloric caeca of rainbow trout in only 20 days [66], whereas kidney was marginally responsive to dietary phosphorus levels [67]. In *Gadus morhua*, substitution of fish meal by soybean meal produced no effect on the expression of PRDX-4 in the intestine [68]. In *Sander lucioperca* larvae, high levels of phospholipids increased the expression of hepatic PRDX1, whereas low levels decreased it [69]. In the present model, which induced a deficiency in EFAs, though dietary intervention did not alter the hepatic expression, it produced a marked down regulation in the head kidney. These fish also showed a lower plasma antioxidant capacity and depleted glutathione levels in liver, but the concurrent increase in the reduced/oxidized glutathione (GSH/GSSG) ratio was interpreted as an index of reduced oxidative stress that was coincident with an enhanced respiratory burst of blood leukocytes after PMA stimulation [38]. Given the inhibitory role of PRDXs on immune response, these observations were consistent with a significant down-regulated expression of NKEFs. Also, the results of the present study evidenced a significant down-regulated expression of PRDX5 that agrees with a reduced risk of oxidative stress in fish fed vegetal oils due to the low levels of tissue polyunsaturated fatty acids [37,70]. However, at the same time, these animals exhibited a lower buffer antioxidant capacity to face stressful oxidative challenges and this paradoxical dualism might explain, at least in part, why dietary requirements for optimal growth do not necessarily coincide with those for optimal functioning of the immune and antioxidant system. In particular, this was inferred from a recent study with

gilthead sea bream where enteromyxosis signs (lower growth, condition factor, specific growth rate, haematocrit) as well as *E. leei* infection course were worse in fish fed diets with high levels of vegetable oils [71].

Several studies have addressed the effects of handling and crowding conditions on oxidative stress in fish. For instance, a microarray analysis of the time-course stress response of gilthead sea bream after acute confinement exposure (100 Kg/m³) highlighted a vast array of metabolic adjustments with an increased ROS scavenging accompanied by a general decline of ROS production [72]. Less evident are transcriptional changes after prolonged exposure to intermediate high- stocking densities, but some of them are persistent over time. For instance, the induced expression of mitochondrial chaperones of the heat shock protein 70 family (GRP75 or mortalin) was persistent in liver but not in head kidney after prolonged exposure to intermediate-high stock densities [39]. Despite this, we observed here that the head kidney expression of PRDX4-6 was significantly down-regulated by prolonged exposure to high-rearing densities, which might be indicative of the well known immunosuppressive effects of stressors related to rearing conditions [73, 74], regardless of changes of feed intake in a pair-fed experimental model.

In summary, six gilthead sea bream PRDX isoforms were unequivocally recognized as PRDX mammalian counterparts with a ubiquitous expression that was tissue-specific for each PRDX isoform. Correlative evidence in challenged fish also supported a role on antioxidant defense and intracellular signaling, emerging the PRDX family as an integrative biomarker of fish health and welfare. However, further studies are needed to undertake specific effects and compensatory mechanisms in an oxidative milieu of intensive aquaculture conditions and epizootic outbreaks of different etiology.

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Figure Legends

Figure 1. (A) Amino acid alignment of gilthead sea bream PRDX. Black squares mark the position of catalytically active cysteine residues, and the alternative C-terminal cysteine of PRDX5 is marked with a white square. Conserved regions surrounding those cysteines are shaded in black, and conserved residues among most PRDX sequences are shaded in grey. Asterisks indicate identities among all six sequences. (B) Percentages of identity and similarity (in parentheses) between gilthead sea bream PRDXs.

Figure 2. Phylogenetic tree of the six members of the PRDX family. GenBank accession numbers are provided in parentheses for each sequence.

Figure 3. Tissue specific expression of the transcripts coding for the six gilthead sea bream PRDXs. Each value is the mean \pm SEM of 2 stock animals. β -actin was used as housekeeping gene in the normalization procedure.

Figure 4. Effect of three challenging experimental trials on the relative quantification of the transcripts coding for the six gilthead sea bream PRDXs in head kidney. β -actin was used as housekeeping gene in the normalization procedure. Each value is the mean \pm SEM of 6-8 animals. Statistically significant changes among experimental conditions for a given transcript are marked with different letters (ANOVA-I followed by Student-Newman-Keuls test, $P < 0.05$) or asterisks (Student t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (A) Infection trial: CTRL = animals not exposed to parasite; R-NON PAR = exposed but not infected; R-PAR = exposed and infected. (B) Nutritional stress trial: FO = animals fed plant protein diets with fish oil; VO = animals fed plant protein diets with a blend of vegetable oils. (C)

Confinement exposure trial: LD-PF = low culture density (10 kg/m³); HD = high culture density (40-50 kg/m³).

Figure 5. Effect of two challenging experimental trials on the relative quantification of the transcripts coding for the six gilthead sea bream PRDXs in liver. β -actin was used as housekeeping gene in the normalization procedure. Each value is the mean \pm SEM of 6-8 animals. (A) Nutritional stress trial: FO = animals fed plant protein diets with fish oil; VO = animals fed plant protein diets with a blend of vegetable oils. (B) Confinement exposure trial: LD-PF = low culture density (10 kg/m³); HD = high culture density (40-50 kg/m³).

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Table 1. Forward and reverse primers for quantitative real-time PCR.

Gene	Accession number	Primer sequence	Position
PRDX1 (NKEF-A)	GQ252679	F CTC CAA GCA ATA ATA AGC CCA AAG R TCA CTC TAC AGA CAA CAG AAC AC	605-628 779-757
PRDX2 (NKEF-B)	GQ252680	F CAA GCA GTA AAT GTG AAG GTC R GAT TGG ACG CCA TGA GAT AC	676-696 774-755
PRDX3	GQ252681	F ATC AAC ACC CCA CGC AAG ACT G R ACC GTT TGG ATC AAT GAG GAA CAG ACC	430-451 579-553
PRDX4	GQ252682	F TCT TCC TCC TCA CCA CAC TCA CTT CTG R CGG GCT TTG AAA TCT TGG CTT TGC	79-105 240-217
PRDX5	GQ252683	F GAG CAC GGA ACA GAT GGC AAG G R TCC ACA TTG ATC TTC TTC ACG ACT CC	590-611 762-737
PRDX6	GQ252684	F AGA GAC AAG GAC GGA ATG C R TGT GGC GAC CTT CTT CTG	418-436 585-568
β -Actin	X89920	F TCC TGC GGA ATC CAT GAG A R GAC GTC GCA CTT CAT GAT GCT	811-829 861-841
Elongation Factor 1	AF184170	F CCC GCC TCT GTT GCC TTC G R CAG CAG TGT GGT TCC GTT AGC	560-578 694-674
α -tubulin	AY326430	F GAC ATC ACC AAT GCC TGC TTC R GTG GCG ATG GCG GAG TTC	514-534 647-630
18S rRNA	AY993930	F GCA TTT ATC AGA CCC AAA ACC R AGT TGA TAG GGC AGA CAT TCG	182-202 316-296

Table 2. Classification of identified genes according to BLAST searches.

Contig(s)	F ^a	Contig size (nt)	Annotation ^b	Best match ^c	E ^d	Identity / similarity ^e
iats013b06 ^f	4	1017	PRDX1 (NKEF-A)	ACQ58049	1e-105	82 (88)
iats104d11	4	838	PRDX2 (NKEF-B)	ACQ58196	6e-105	77 (86)
iats013f22	4	1039	PRDX3	ACQ58889	4e-128	74 (81)
iats08b25g20	6	874	PRDX4	ACM47312	1e-130	79 (84)
iats016020	3	1092	PRDX5	ACQ58198	3e-92	63 (72)
iats08b05j10	2	757	PRDX6	ADJ57694	3e-103	75 (82)

^aNumber of sequences

^bGene identity determined through BLAST searches

^cBest BLAST-X protein sequence match (lowest E value)

^dExpectation value

^ePercentage of identity with human PRDX amino acid sequence. Number in parentheses is referred to amino acid similarity

^fNucleotide sequence between the two non-overlapping clones for PRDX1 was completed using a PCR approach with degenerated (F: 5'-CAG CCA AAG CWG TKA TGC C) and specific (R: 5'-TAA TCG GGA GAG GTG TCT TTG G) primers

(A)

NKEF-A	-----MAAGNAQIGKLPAD	14
NKEF-B	-----MSAGNARIG	9
PRDX3	-----MAATIGRLLRTSARVAAGGLKVAACQHGASGAARAFTGPALQRACFSTSTSRWAPAVT	58
PRDX4	MESVVHTNQKKELCRPRRAALFFLLTTLTFAEEGAQVKNSQCHNYAGGHVYPGEAFRVPVSDHSLHLKAKIS	74
PRDX5	-----MLSITGSLIKNTRVVQCVRLHLHTSPIARMPIQVG	34
PRDX6	-----MPGILLG	7
	* * * * *	
NKEF-A	FTAKAVMPDQGFRRTVTKDLKMSDYRG-KYVVFFFYPLDFTFVCP-TEIIAFSDAADDFFKKIGCEVIAAS-VDSH	81
NKEF-B	QPAPDFSATAVVNGQFKDIKLSDYKG-KYVVFFFYPLDFTFVCP-TEIVAFSDRADEFRSAGCEVIGCS-VDSH	80
PRDX3	QPAPDFKATAVLNGEFKEMSLADFKG-KYLVLFFYPLDFTFVCP-TEIISFSDKASEFHDVNCCEVVGVS-VDSH	129
PRDX4	KPAPHFEGTAVINGEFKELKLSDYKG-KYIVVFFYPLDFTFVCP-TEIIAFSDRVHEFQAINTEVVACS-VDSQ	145
PRDX5	EQLPAVEVQEGEPG--NKVAMDQLFKGKKGVLFAVPGAFTPGCSTHLPGFVEQASELKGKGIQEVACISVND	106
PRDX6	DEFPNFEADTTVG---RIKFHDFLGSSWRILFESHPRDFTPVCT-TELAACAALTDFFKRGVKMIALS-IDSV	75
	* * * * *	
NKEF-A	FSHLAWINTPRKQGGLG--TMKIPLVSDTRRTISTDYGVLKEDDG-----IAYRGLFIIDDKGILRQITINDL	147
NKEF-B	FSHLAWINTPRKQGGLG--PMKIPLVADLTKTISKDYGVLKEDDG-----IAYRGLFVIDDKGILRQITINDL	146
PRDX3	FTHLAWINTPRKTGGLG--HIHIIPLLSDLNKKQISRDIYGVLLGPG-----IALRGLFLIDPNGVVRHMSVNDL	195
PRDX4	FTHLAWINTPRKQGGLG--PMKIPLLSDLTHQISKDYGVYLEDQG-----HTLRGLFIIDDKGILRQITMNDL	211
PRDX5	FVMAAWGKEHGTGKVR-----MLADPTGAFTKAVDLLLDSQ-----IVQVLGNKRKSKRYSMLVED	163
PRDX6	EDHKAWSKDVMSVSSAADKDLFPPIIADDKRELSVKLGMLDPPERDKDGMPLTARCVFVVGPDKKLKLSILYPA	149
	** * *	
NKEF-A	PVGRSVEETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKDFFSKQ-----	198
NKEF-B	PVGRSVDETLSLVQAFQHTDKHGEVCPAGWKPGSDTIIPDVEKSKTFFSKQ-----	197
PRDX3	PVGRSVEETLRLVKAFQFVETHGEVCPASWTPESPTIKPTPEGSKEYFEKVN-----	247
PRDX4	PVGRSVDETLRLVQAFQYTDKHGEVCPAGWKPGSDTIIPDPSGKLKYFDKLN-----	263
PRDX5	GUVKKINVEPDGTGLTCSLASSILSDL-----	190
PRDX6	TTGRNFDLRLRVIDSLQLTAQKQVATEVDWKPGDKVMVIPSLSAEAAANLFPNGVTTKEVPSGKKYLRYTQP	221
	* *	

(B)

	NKEF-B	PRDX3	PRDX4	PRDX5	PRDX6
NKEF-A	83 (91)	48 (61)	52 (59)	15 (27)	23 (41)
NKEF-B		51 (62)	55 (62)	15 (28)	23 (40)
PRDX3			52 (66)	12 (26)	15 (30)
PRDX4				12 (24)	16 (30)
PRDX5					11 (26)

FIGURE 1

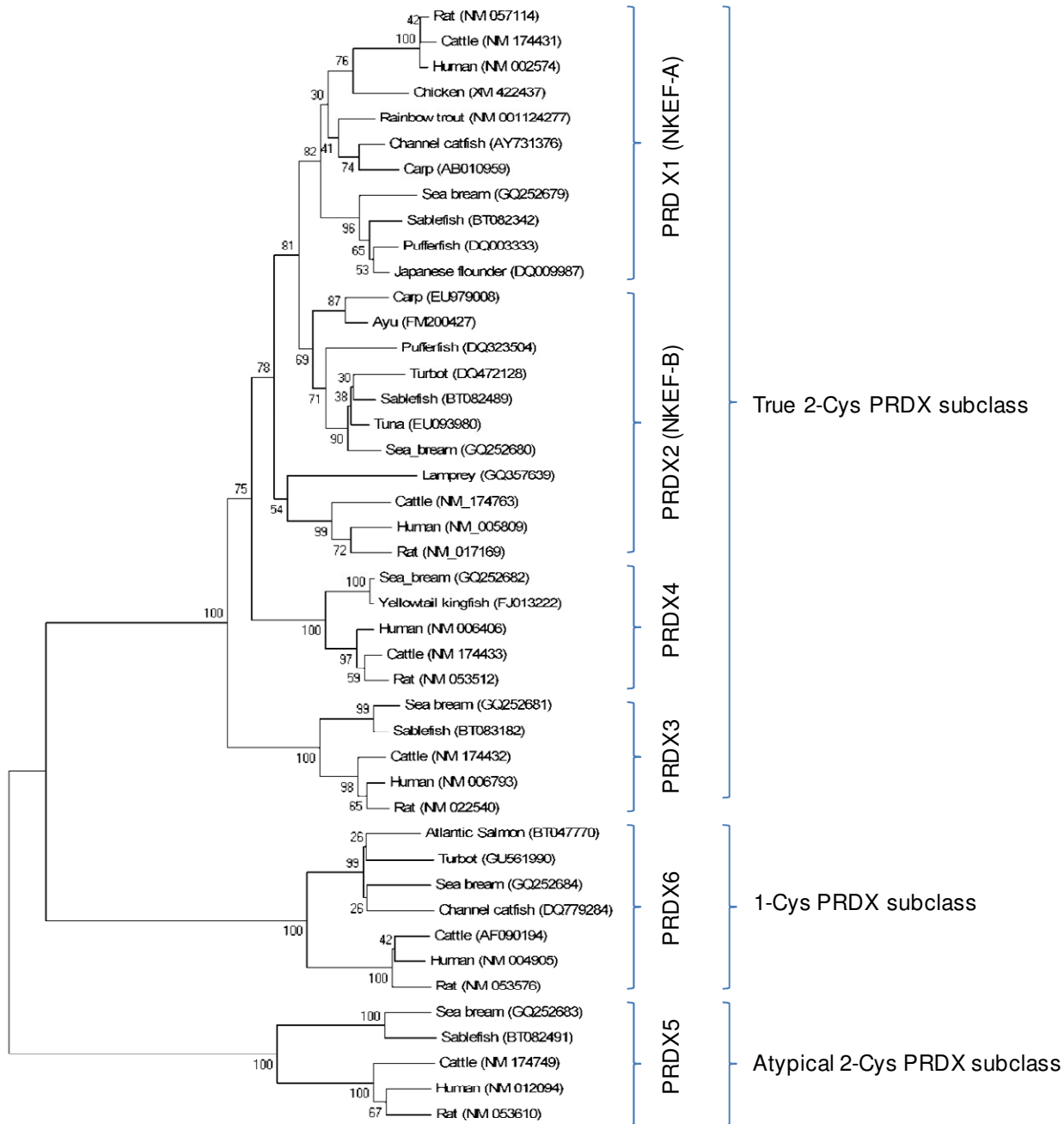


FIGURE 2

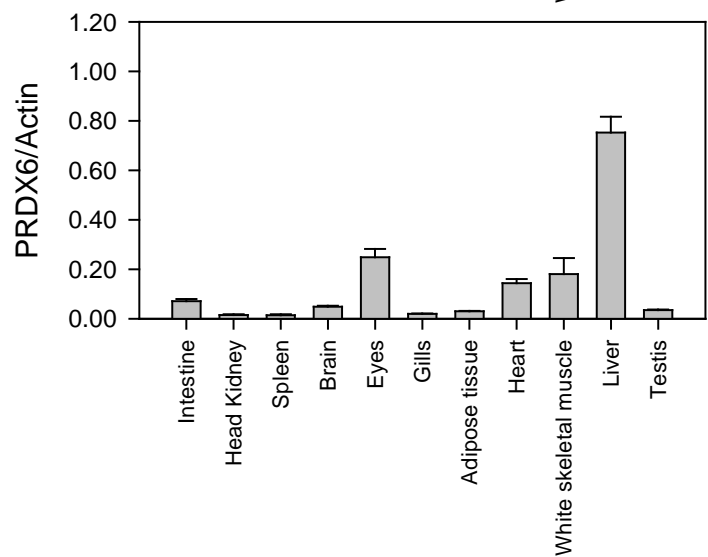
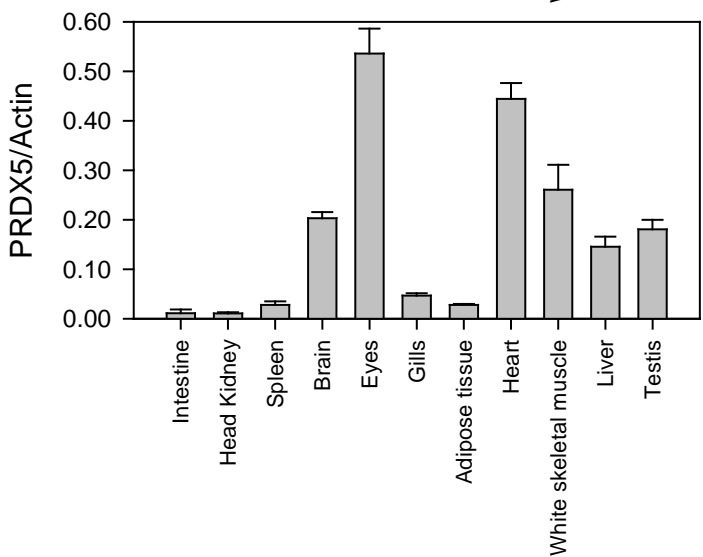
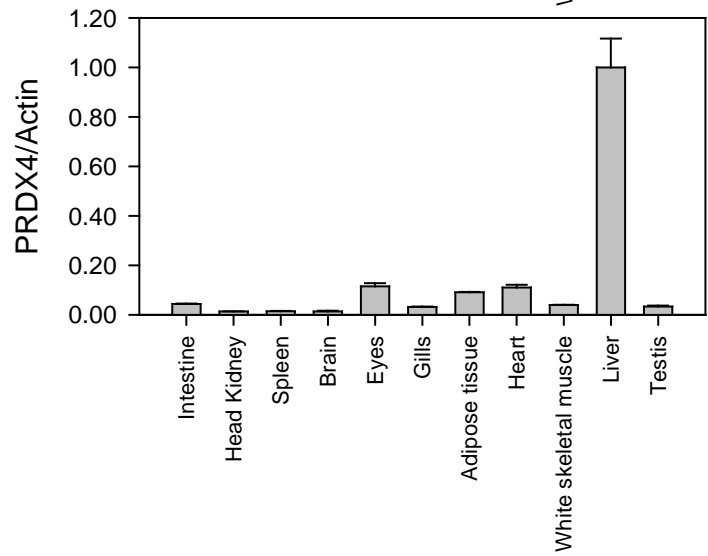
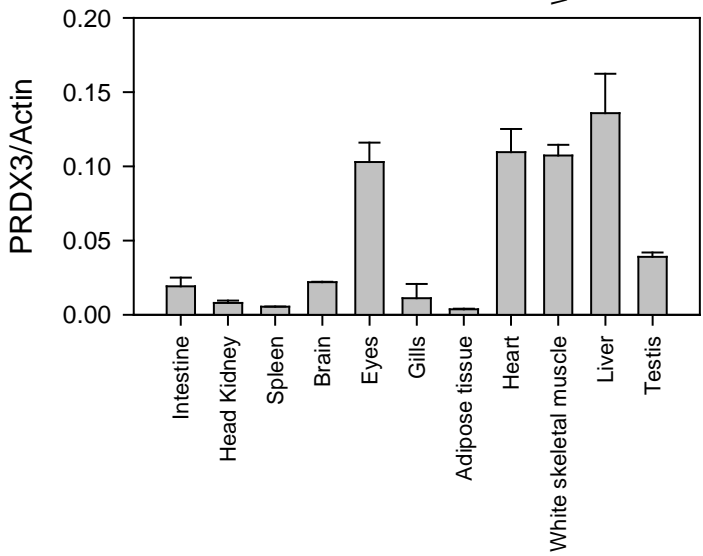
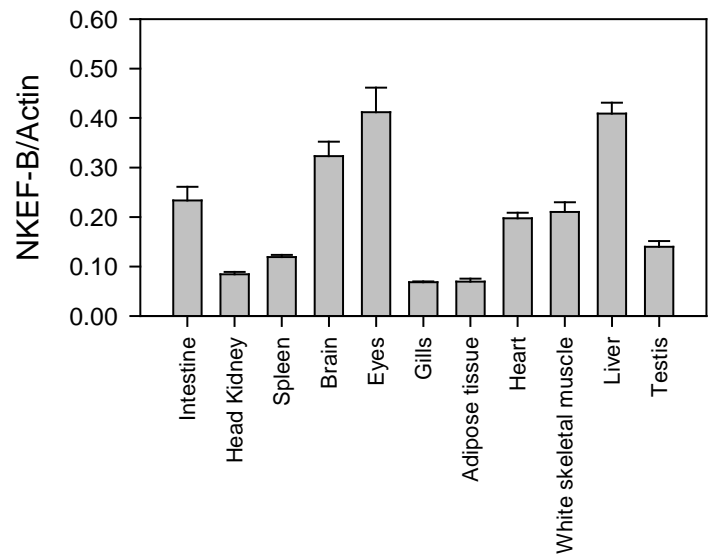
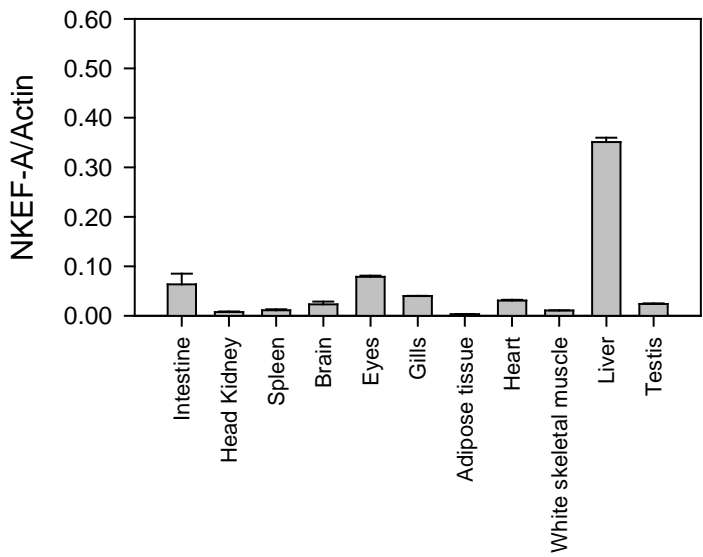


FIGURE 3

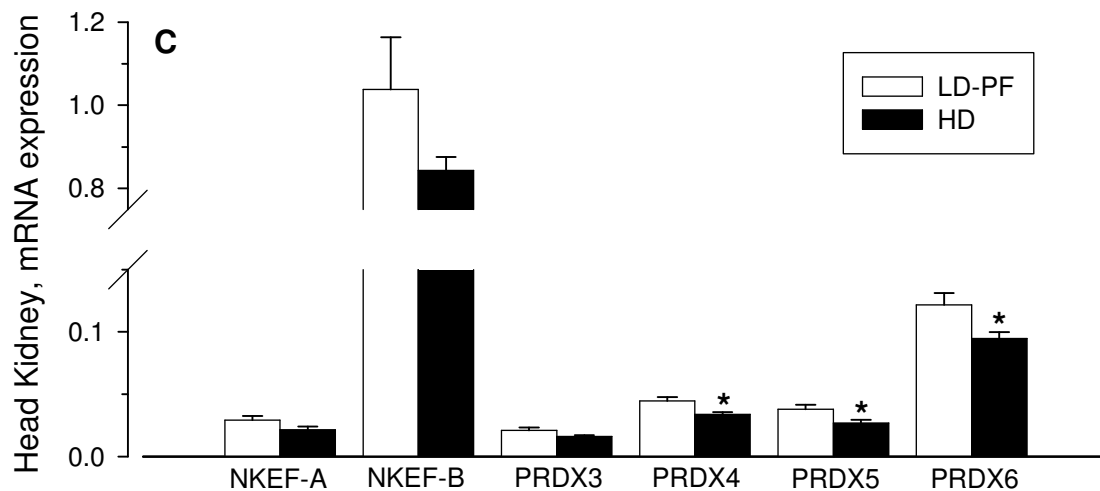
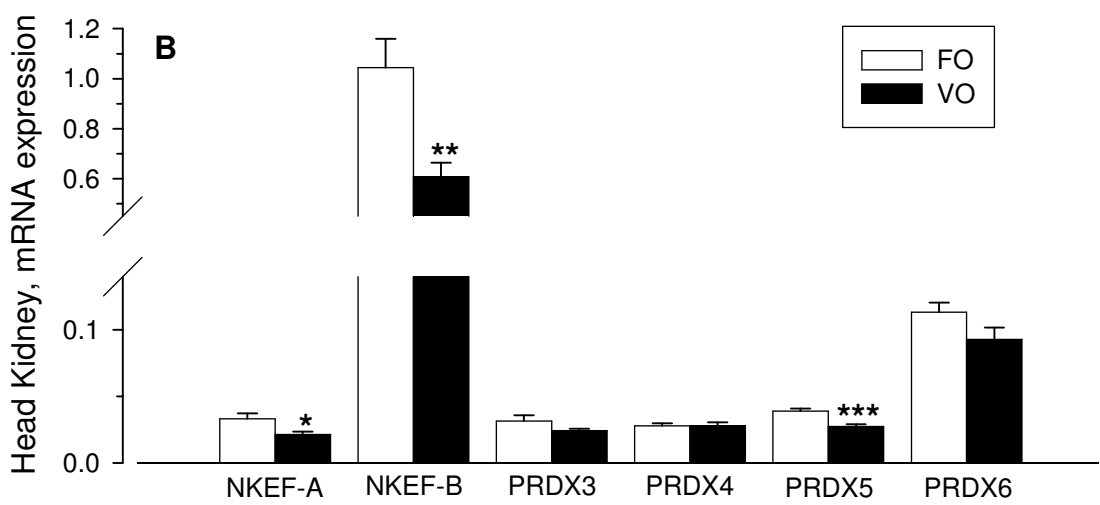
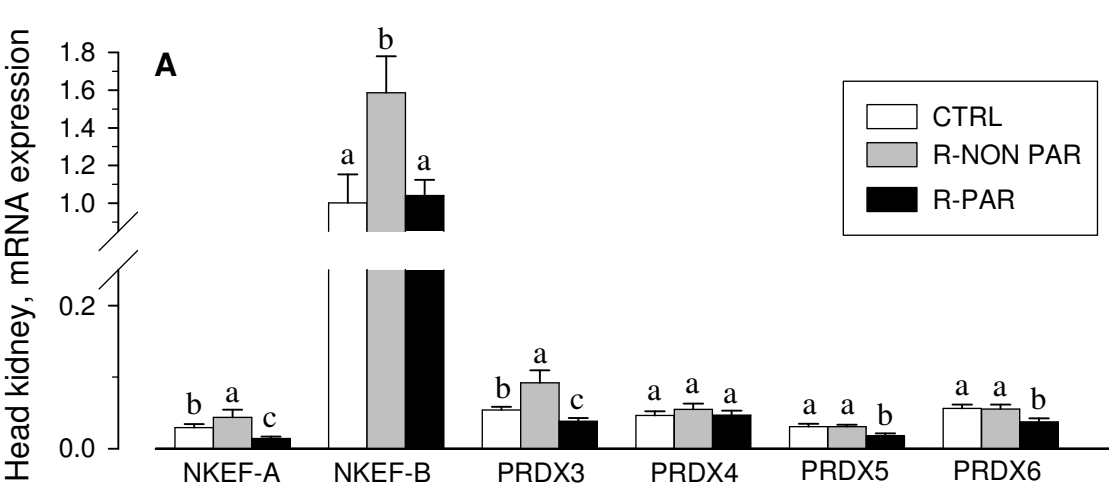


FIGURE 4

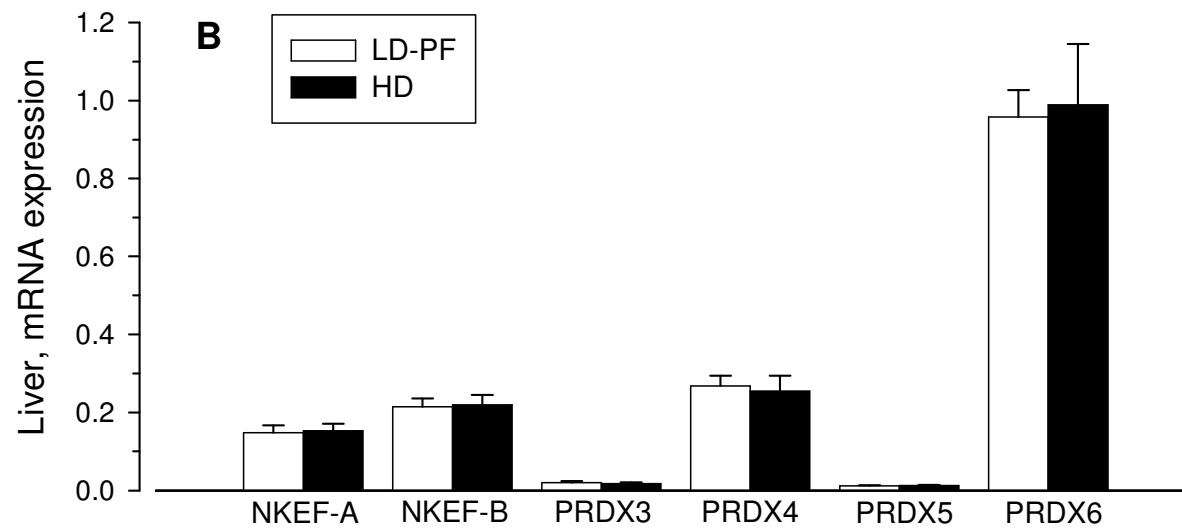
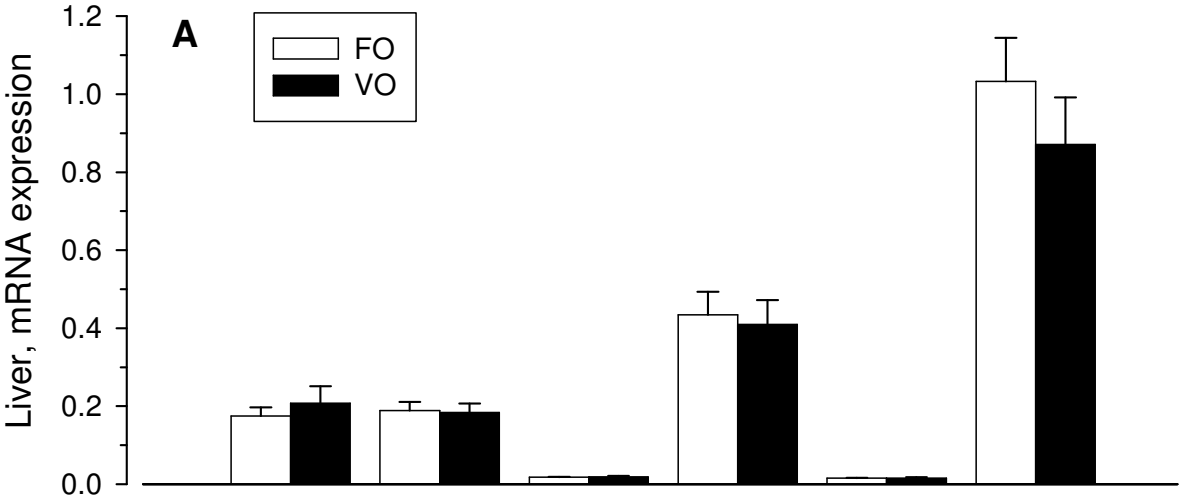


FIGURE 5