Biochemical responses and DNA damage in red sea bream from coastal Fujian Province, China

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ABSTRACT

Relationships between measures of oxidative stress and adaptive antioxidant defenses were studied in red sea bream (*Pagrus major*, RSB) caged at eight locations along the Fujian coast, China. Oxidative damage in tissues was investigated by measuring concentrations of protein carbonyl (CP) and reactive oxygen species (ROS), lipid peroxidation (LPO), acetyl cholinesterase activity (AChE), concentration of thiol groups and activities of enzymes associated with antioxidant defense. Magnitudes of antioxidant defense parameters were significantly, positively correlated with oxidative stress. Significantly greater ROS, LPO and CP content was observed in most tissues of RSB caged at three locations relative to those at the other locations. RSB caged at one location, Meizhou, exhibited significantly more DNA damage than did RSB caged at other locations. AChE activities in liver, kidney and muscle were statistically different among locations. Multivariate analyses were used to categorize the environmental quality of different locations. The battery of measures of oxidative stress proved to be a useful integrator of oxidative stresses that could be compared among locations.

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1. Introduction

In coastal China there is a large human population juxtapositioned with intensive industrial development, transportation and food production. This has lead to contamination of fish with the potential to cause adverse effects on the fish or the people and wildlife that consume fish (Li et al., 2007).

There are several approaches to monitoring the environment for chemical stressors and their effects. The first involves measuring concentrations of residues in abiotic and biotic matrices and then predicting the potential effects based on the results of controlled laboratory studies or relationships inferred from other field monitoring. While this approach has been useful for specific contaminants, it is limited in that it does not measure all of the possible contaminants and does not consider the interactions among residues under ecologically relevant conditions. The alternative method is to monitor biological functions in sentinel organisms.

Monitoring for changes in the biochemistry of organisms, especially fish has been useful in assessing functional changes in populations exposed to toxicants (van der Oost et al., 2003). Measurements of biochemical responses, including adaptive responses can detect effects of stressors before they are manifested in changes in populations or ecosystems. Furthermore, these biomarkers can serve as an integrative measure of the effects of mixtures of chemical stressors in concert with other abiotic factors. Fish are useful in biomonitoring because they can accumulate persistent contaminants, and exhibit adaptive responses to these exposures (Fang et al., 2009). Physiological impairment in fish caused by xenobiotics can be used as an integrative, functional measure of exposure to chemical stressors (Bervoets et al., 2009). Thus, fish can serve as sentinels (Fernandes et al., 2008; Linde-Arias et al., 2008; Cazenave et al., 2009).

Antioxidants play a crucial role in maintaining homeostasis and, when these defenses are impaired, oxidative stress products, specifically reactive oxygen species (ROS), can cause DNA damage, and peroxidation of cell constituents and enzymatic inactivation. In response to exposure to oxidative stressors, to prevent damage caused by ROS, fish would be predicted to have greater levels of antioxidants enzymes, such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), as well as non-enzymatic

free radical scavengers like reduced glutathione (GSH) and biotransformation enzymes glutathione-S-transferase (GST). Therefore, changes in levels of these antioxidants can serve as indicators of exposure to xenobiotics (Doyotte et al., 1997). In addition to these markers, measurement of acetyl choline esterase (AChE) is widely used as a biomarker of exposure to several classes of neurotoxic compounds (Matozzo et al., 2005). Because many types of pollutants to which fish might be exposed are often unknown, use of a battery of multiple biomarkers is useful in identifying potential exposure to hazardous contaminants (Moore and Simpson, 1992).

Due to increased domestic fish consumption and foreign exports marine fish farming in China has rapidly increased over the last decade especially in Fujian province, which has the largest number of marine fish cages in China (Chen et al., 2006; Chang and Chen, 2008). Moreover, this same area has undergone dramatic industrial development, which has resulted in contamination of fish mariculture of the region. Contaminants can be accumulated by fish and exert a range of adverse biochemical and physiological effects. In a previous study concentrations of the metals Ag, As, Cd, Co, Cu, Fe, Mn, Se and Zn (Onsanit et al.,2010) and organic residues (un published data) were measured in two marine cultivated species (*Lateolabrax japonicus* and *Pagrus major*) collected from caged fish at eight locations along the coast of Fujian province, China. That data suggested that there was a range of contamination in fish along the coast of Fujian. Therefore, a follow-up study of the oxidative stress and the antioxidant status in tissues of red sea bream (RSB) (*P. major*; order Perciformes, family Sparidae) was conducted.

The RSB is widely distributed in South East Asia and is one of the most important commercial species, especially in China. Because it can be exposed to xenobiotics for a relatively long life span (15–20 years) accumulation by and effects on the RSB could be greater than other fish of similar trophic position that inhabit coastal areas. However, no information on the current status of ROS damage or adaptive responses was available for this widespread and important commercial species. Because it is frequently cultured it can serve as an integrative sentinel of stress.

Since one biomarker may not reflect the overall status of individuals it has been suggested that a range of biomarkers be used to measure the adaptive responses to environmental conditions (Cajaraville et al., 2000; Regoli et al., 2004; UNEP/MAP, 2005). Information on background values under field conditions is scarce, which makes it difficult to interpret environmental data (Zorita et al., 2005). This study was conducted to determine the current status of a battery of biomarkers in caged RSB from eight locations, representing different types and degrees of urbanization and industrial development, along the coast of Fujian Province in southeastern China. The study examined oxidative stress and levels of antioxidants in tissues of RSB. The biomarkers included xenobiotic metabolizing phase II enzyme GST and its associated enzymes; GSH, and antioxidant enzymes involved in detoxifying ROS including SOD, CAT, GPx, and markers of oxidative tissue damage such as lipid peroxidation (LPO) and strand breaks in DNA. Indicators of oxidative stress, including protein carbonyl (CP), ROS in tissues, and thiol content (L-SH and H-SH) were analyzed.

The study was conducted to provide baseline information on a range of biomarkers that have been previously demonstrated to respond to chemical stressors in a wide variety of fishes. The information collected in this initial study will be used to determine the status and trends in responses to chemical contamination. The studies of biomarkers are part of an integrated study of environmental changes along the coast of Fujian Province. This portion of the study was designed to answer the question: "Are there differences in the levels of the selected biomarkers among locations along a gradient of urbanization and industrial and transportation activities". The study was not designed to be related to specific contaminants measured in the ongoing monitoring program. Based on this initial study, differences among locations will be further studied to determine the likely causes of the responses at those locations. The approach proposed will include a combination of chemical screening and also bioassay-directed fractionation and identification to determine the cause or causes of the responses and then the source or sources of the most likely contaminants.

2. Materials and methods

2.1. Site selection and fish collection

During May 2008 RSB were collected from eight locations along the coast of Fujian Province (Fig. 1). From south to north, the sampling stations were at Dongshan (23°44.539′N, 117°31.081′E) [DS], Fotan (24°10.60′N, 117°55.0′E) [ZP], Pozao (24°21.353′N, 118°04.342′E) [XM], Tong'an (24°34.735′N, 118°11.021′E) [TA], Meizhou (25°11.526′N, 118°58.782′E) [MZ], Fuqing (25°41.169′N, 119°35.167′E) [FQ], Luoyuan (26°21.615′N,119°43.163′E) [LY] and Sandu-ao (26°37.906′N, 119°47.016′E) [SD]. A total of 10 fish of marketable size were collected from cages at each location. The weights ranged from 0.28 to 0.37 kg, while the lengths varied from 15.4 to 20.6 cm. To minimize differences resulting from size, fish of similar size were used for the biochemical analysis. Sampling was not stratified by gender. Fish were anesthetized by use of tricane methanesulfonate and approximately 5 g of each tissue of interest (brain, muscle, gill, kidney and liver) was removed immediately in the field. Tissues were first rinsed with double-distilled water and were placed in liquid nitrogen and transported to the laboratory, where they were stored at -80 °C until further analyses. All studies were conducted in accordance with national and institutional guidelines for the protection of human subjects and animals welfare approved by the university animal use committee, China.

2.2. Sample preparation

Samples were homogenized in 100 mM sodium phosphate buffer, pH 7.0. An aliquot of this homogenate was then mixed with 0.5 ml of 10% trichloroacetic acid (TCA) and centrifuged for 30 min at 12,000g. CP levels were measured in the resulting pellets, and LPO was spectrophotometrically assayed in the supernatants by use of an Ultrospec 2100 pro UV/visible spectrophotometer and plate reader (TECAN A-5082, Genios, Austria). For other biochemical analyses, samples were homogenized as mentioned above but without addition of TCA. The supernatant was stored at -80 °C until assayed. Protein content was determined by the method of Bradford (1976) with bovine serum albumin used as a standard. Positive controls were used for all the bioassay to ensure and avoid any error in the methodology for all the assays wherever necessary.

2.3. Biomarkers

2.3.1. AChE determination

AChE activity was determined in microtiter plates by the method of Ellman et al. (1961), which uses acetylthiocholine iodide as the substrate. Quantification was based on formation of yellow color due to the reaction of thiocholine with dithiobisnitrobenzoate ions. The reaction was initiated by adding 0.1 ml aliquot of
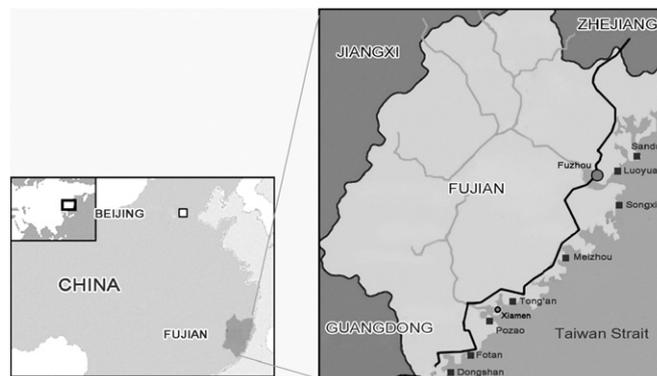

Fig. 1. Locations along the coastal region in Fujian Province of China from which RSB were collected.

enzyme solution to 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.5, 50 μl of DTNB (10 mM) and 50 μl of acetylthiocholine iodide (12.5 mM). The reaction was followed spectrophotometrically at 412 nm at room temperature for 3 min. The change in absorbance per minute was calculated, and activity expressed as the rate of enzyme activity in micromoles of substrate hydrolyzed/min/mg protein.

2.3.2. Evaluation of DNA strand breaks

DNA strand breaks were quantified by use of the alkaline unwinding assay, slightly modified from the method described by Ching et al. (2001). Briefly, tissues were incubated with 1 ml of TNE buffer (50 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% SDS, pH 8.0) at 37 °C for 24 h and after incubation 150 μl of saturated NaCl was added and centrifuged at 12,000g for 20 min at 4 °C and supernatant was added with equal volumes of buffered phenol/chloroform/isoamyl alcohol (PCI) (24:25:1,v/v/v, pH 8.0) and centrifuged further at 12,000g for 5 min. The aqueous layer was transferred to a new tube and the PCI extraction was repeated. The aqueous layer was then digested with 5 μl of Ribonuclease A (10 mg ml^{-1}) for 30 min at 37 °C and extracted successively by equal volumes of PCI. DNA was precipitated from the resulting aqueous layer by adding 2 volumes of cold absolute ethanol and 1/10 volume of 3 M sodium acetate buffer (pH 5.2). The mixture was centrifuged at 12,000g for 15 min and the supernatant decanted. The pellet was rinsed with 500 μl of 70% ethanol, air dried and dissolved in 400 μl of TE buffer (10 mM Tris, 1 mM EDTA). DNA was separated into two equal portions for quantification of double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). The fluorescence of dsDNA and ssDNA was measured with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Data concerning DNA unwinding technique were expressed as an *F*-value determined by dsDNA divided by dsDNA plus ssDNA in the sample.

2.3.3. Oxidative stress markers

ROS was measured with slight modifications to the method of 2-7-dichlorodihydrofluorescein diacetate (DCFH-DA) transformation described by Driver et al. (2000). Homogenates (20 μL), 100 μL physiological saline and 5 μL of DCFH-DA were added to each well and the plates were incubated at 37 °C for 30 min. Background fluorescence (conversion of DCFH to dichlorodihydrofluorescine (DCF) in the absence of sample) was corrected by the inclusion of parallel blanks. Conversion of DCFH to the fluorescent product DCF was measured using a TECAN spectrophotometer with excitation at 485 nm and emission at 530 nm. The rate of conversion is directly proportional to the concentration of ROS.

Protein carbonyl (CP) content was measured by reaction with 2,4-dinitrophenylhydrazine (DNPH) as described in Lushchak et al. (2005). CP was quantified spectrophotometrically at 370 nm using a molar extinction coefficient of 10^3 M^{-1} cm^{-1}. Concentrations of CP were expressed as nanomoles of CP per milligram protein in the guanidine chloride solution. LPO was measured according to the method of Devasagayam and Tarachand (1987). The color developed was measured at 532 nm and the malondialdehyde (MDA) content of the sample was expressed as nmol of MDA formed/mg protein. Free thiols were measured by the Ellman procedure with DTNB (Ellman, 1959) as described earlier (Lushchak and Bagnyukova, 2006). Absorption was read at 412 nm and a molar extinction coefficient of 14×10^3 M^{-1} cm^{-1} was used for calculation of thiol concentration. The L-SH and H-SH thiol concentrations were expressed as micromoles of SH-groups per gram wet mass of tissue.

2.3.4. Antioxidants and associated enzyme markers

CAT activity was determined according to the method of Sinha (1972) and expressed as μmol of H$_2$O$_2$ consumed/min/mg protein. SOD activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at an alkaline pH by the method of Marklund and Marklund (1974). One unit of SOD activity is defined as the amount of enzyme (per protein milligram) that inhibits the quercetin oxidation reaction by 50% of maximal inhibition. GPx was assayed by measuring the amount of GSH consumed in the reaction mixture according to the method of Rotruck et al. (1973). GSH was estimated by the method of Moron et al. (1979) by reading the optical density of the yellow substance formed when 5,5′-dithio-2-nitrobenzoic acid (DTNB) is reduced by glutathione at 412 nm. GST activity of the fraction obtained with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically at 37 °C by following conjugation of the acceptor substrate with glutathione as described by Habig et al. (1974) and Jakoby (1985). Results were expressed as the formed conjugate/min/mg protein.

2.4. Statistical analyses

Statistical comparisons were performed using two-way analysis of variance (ANOVA) and SPSS Version 10.0 software. Briefly, triplicate 3 fish were taken for each location and the results are reported as mean ± S.D. of three individuals per group per station. Data were first tested for normality and homogeneity using Bartlett's test. Since all data were normally distributed, two-way analysis of variance (ANOVA) was used to determine if there were differences among locations. A *p*-value less than 0.05 was considered to be significant. The Tukey's multiple comparison *post hoc* test was performed to identify the statistical

difference between individual treatment groups. Spearman correlation analysis was used to test for associations among parameters or tissues. Principal component analyses (PCA) were performed using the R project language (http://www.r-project.org/) as previously described (Zhang et al., 2008a). PCA was performed on a RSB data set consisting of 12 biomarkers measured in RSB collected at each of the eight locations along the coastal region of Fujian Province.

3. Results

3.1. AChE activity

Acetyl cholinesterase activity (AChE) varied among tissues and locations. AChE activity was present predominantly in brain. There was no statistically significant difference in AChE in brain among locations. AChE activity of liver was greatest in RSB from stations MZ, DS and FQ while RSB from station LY, SD and TA exhibited the least AChE activity. In gill of RSB AChE activity was greater at MZ and least at LY compared to other station in kidney of RSB (Fig. 2). In muscle AChE activity was greatest at LY and ZP and least at TA.

3.2. DNA strand breakage

There was little difference in DNA strand breakage in liver among locations. The results on the integrity of DNA extracted from the liver of RSB were expressed as the fraction of double-stranded DNA (*F*-value). DNA strand breaks in RSB from MZ and LY exhibited the greatest and least *F*-values, respectively. *F*-values for RSB from these two locations were significantly different from those in fish from other locations (Fig. 3).

3.3. Oxidative stress markers

Generation of ROS was greater in kidney than in other tissues of RSB (Table 1). ROS generation was greatest in kidney of RSB from DS, and least at LY. Mean ROS generation in gill was greater at ZP and DS than other locations, but the difference was not statistically significant. There was significantly less ROS in brain and muscle than in other tissues. ROS production in brain and muscle from MZ was significantly greater than that in these tissues from RSB from LY and XM. The greatest ROS in liver was observed at DS and least at ZP.

Concentrations of CP varied among tissues of RSB and among locations (Table 2). CP was present predominantly in gill. The concentration of CP in gill was greatest at DS, while RSB from station XM exhibited the least concentration of CP in gill. The greatest CP content was observed in brain of RSB at ZP while the least was observed in fish from XM. CP concentration in muscle was greater in fish from DS and less at LY, SD and MZ. The greatest concentration of CP in liver was observed in RSB from SD, followed by TA and LY while the least concentration of CP was observed at XM (Table 2).

LPO varied among tissues and locations. The greatest amount of LPO was observed in liver. The greatest LPO level in liver of RSB was observed at station SD, while the least was observed in livers of RSB from TA. The LPO level in muscle of RSB was significantly greater at LY than MZ, DS or ZP (Table 2). LPO of brain, gill and kidney was not significantly different among locations.

Concentrations of L-SH in tissues of RSB varied among locations. The greatest concentration of L-SH was observed in liver of fish from ZP, followed by DS and TA. RSB from MZ and XM exhibited the least L-SH (Fig. 4). In gill of RSB L-SH content was greater at ZP and FQ among the stations. L-SH content in brain was significantly greater at XM than at SD, TA, ZP and FQ. The L-SH content remained significantly less at FQ and SD compared
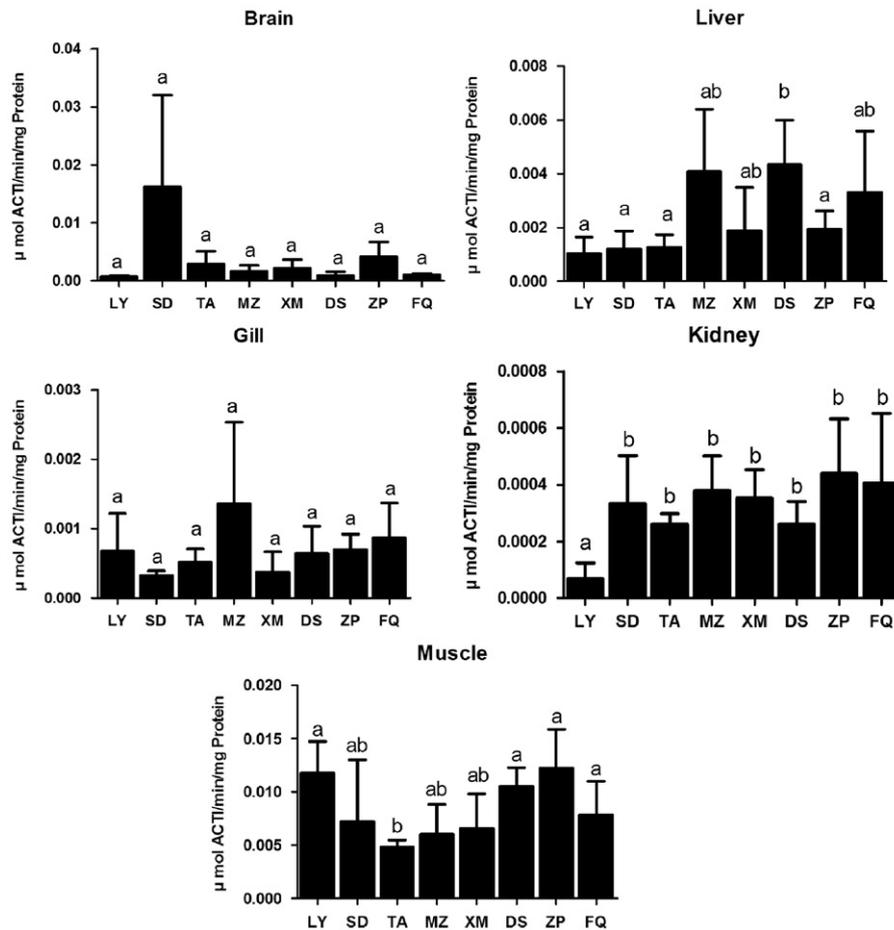
Fig. 2. AChE activity in different tissues of RSB collected from locations along the coast of Fujian Province, China. Each bar represents mean ± standard deviation of three samples. Locations denoted by the same letters (a, b, c) indicate no significant difference between locations (two-way analysis of variance followed by Tukey's post hoc test), whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.
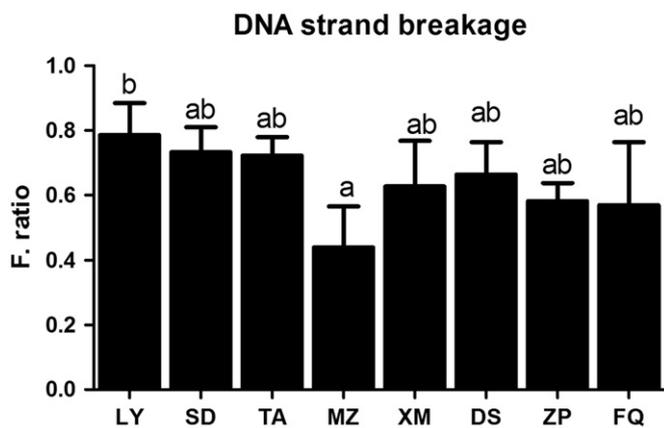
Fig. 3. DNA strand breaks in liver of RSB collected from the coastal region in Fujian Province of China. Each bar represents the mean ± standard deviation of three samples. Locations denoted by the same letters (a, b, c) indicate no significant difference between those locations (two-way analysis of variance followed by Tukey's post hoc test), whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.

to DS, in kidney of RSB. L-SH content of muscle was greatest in RSB from LY, while fish from MZ exhibited the least.

H-SH content varied among tissues and locations. H-SH content was present predominantly in liver of RSB. H-SH content of liver was greatest in RSB from ZP, while RSB from FQ contained the least. After liver, kidney contained the next greatest H-SH content, but the difference was not statistically significant among locations. H-SH content in brain of RSB from SD was significantly greater than from LY, while the gill of RSB from LY was greater than those from all other stations. The greatest H-SH content of RSB muscle was seen at LY and the least in RSB from MZ (Fig. 5).

3.4. Antioxidant markers

SOD activity in different tissues varied among tissues and locations (Table 3). The SOD activity in gill was greatest in RSB from ZP while RSB from XM exhibited the least. The mean SOD activity for brain of RSB collected at station TA was greater than that in RSB from other locations. SOD activity in liver was significantly greater in RSB from FQ than LY, ZP, TA or DS. SOD activity in kidney was greater in RSB from ZP and less in RSB from LY. SOD activity in muscle was significantly greater in RSB from FQ than from TA, XM or DS (Table 3).

CAT activity in gill of RSB was significantly different among locations with the greatest activity observed in RSB from TA, followed by SD and MZ. CAT activity in brain was significantly greater at TA and MZ than other stations (Table 3). CAT activity in liver was greatest in RSB from station LY while RSB from station ZP exhibited the least. CAT activity in kidney of RSB from DS, ZP and FQ was significantly less than in those from other locations. CAT activity of muscle was greater in RSB from LY, SD and TA than from XM, DS, ZP and FQ.

The activity of glutathione-mediated antioxidant enzymes such as GSH, GPx and GST in different tissues of RSB varied

Table 1
ROS level in different tissues of *P. major* collected from coastal region in Fujian province of China.

	LY	SD	TA	MZ	XM	DS	ZP	FQ
ROS								
Brain	0.413 ± 0.28[a]	2.11 ± 0.43[b]	3.82 ± 3.61[abc]	5.93 ± 2.82[b]	0.18 ± 0.014[a]	0.90 ± 0.089[c]	7.77 ± 5.15[b]	0.596 ± 0.34[ac]
Liver	23.59 ± 11.72[a]	32.29 ± 30.10[ab]	52.31 ± 17.04[a]	47.83 ± 35.98[ab]	22.63 ± 17.21[ab]	77.28 ± 63.98[ab]	10.70 ± 0.36[b]	36.06 ± 17.80[a]
Gill	34.80 ± 28.08[a]	28.68 ± 8.69[a]	35.04 ± 19.11[a]	40.16 ± 16.17[a]	34.24 ± 31.08[a]	43.04 ± 32.89[a]	43.25 ± 21.81[a]	37.31 ± 21.83[a]
Kidney	31.13 ± 22.93[a]	77.36 ± 24.13[abc]	79.67 ± 47.49[abc]	69.12 ± 42.86[abc]	76.22 ± 6.51[bc]	133.5 ± 70.97[c]	98.64 ± 44.94[ac]	60.87 ± 7.46[bc]
Muscle	0.943 ± 0.29[a]	2.86 ± 0.44[b]	0.739 ± 0.454[a]	5.92 ± 4.17[b]	0.789 ± 0.209[a]	0.674 ± 0.268[a]	1.27 ± 0.238[ab]	1.5 ± 0.80[b]

ROS: reactive oxygen species expressed as DCF/mg protein. Each bar represents mean ± standard deviation of three samples, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between the sampling stations, whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.
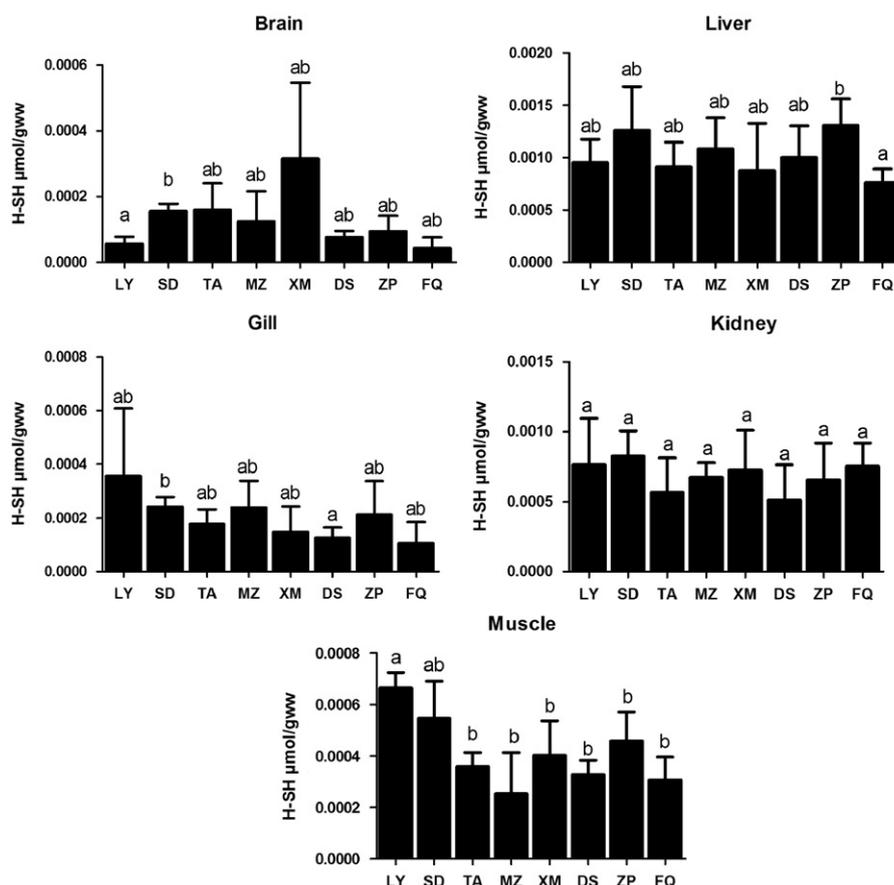
Table 2
Carbonyl protein and lipid peroxidation level in different tissues of *P. major* collected from coastal region in Fujian province of China.

	LY	SD	TA	MZ	XM	DS	ZP	FQ
CP								
Brain	6.39 ± 4.91[abc]	7.51 ± 1.33[ab]	9.05 ± 4.31[abc]	9.47 ± 3.62[ac]	3.56 ± 2.34[b]	6.14 ± 5.72[abc]	11.70 ± 1.94[c]	5.96 ± 5.11[abc]
Liver	15.20 ± 6.33[ab]	8.84 ± 2.69[a]	20.90 ± 4.77[b]	14.06 ± 0.45[b]	3.30 ± 0.06[c]	13.66 ± 3.80[ab]	12.71 ± 5.38[ab]	21.72 ± 5.59[b]
Gill	19.63 ± 4.37[a]	26.90 ± 11.78[ac]	33.15 ± 15.19[a]	16.15 ± 9.39[ab]	11.68 ± 1.84[b]	35.00 ± 6.06[c]	32.24 ± 8.03[ac]	20.94 ± 6.88[ab]
Kidney	18.74 ± 6.49[ac]	14.80 ± 3.78[ac]	11.37 ± 5.71[ab]	5.43 ± 2.92[b]	11.99 ± 3.57[ab]	22.70 ± 5.44[c]	22.49 ± 3.04[c]	9.86 ± 4.39[ab]
Muscle	4.68 ± 0.52[a]	7.99 ± 3.42[ab]	8.67 ± 5.67[abc]	5.29 ± 3.36[a]	15.23 ± 3.19[c]	23.15 ± 9.51[c]	15.82 ± 10.37[abc]	17.52 ± 7.34[bc]
LPO								
Brain	0.033 ± 0.031[a]	0.203 ± 0.192[a]	0.070 ± 0.052[a]	0.040 ± 0.02[a]	0.018 ± 0.012[a]	0.017 ± 0.008[a]	0.047 ± 0.032[a]	0.050 ± 0.019[a]
Liver	0.970 ± 0.277[a]	1.17 ± 0.692[ab]	0.363 ± 0.188[b]	0.836 ± 0.453[ab]	0.723 ± 0.637[ab]	0.771 ± 0.672[ab]	0.468 ± 0.317[ab]	0.410 ± 0.335[ab]
Gill	0.152 ± 0.106[a]	0.227 ± 0.202[a]	0.092 ± 0.033[a]	0.168 ± 0.116[a]	0.142 ± 0.135[a]	0.312 ± 0.247[a]	0.094 ± 0.021[a]	0.197 ± 0.106[a]
Kidney	0.213 ± 0.153[a]	0.287 ± 0.132[a]	0.102 ± 0.064[a]	0.128 ± 0.105[a]	0.251 ± 0.178[a]	0.281 ± 0.183[a]	0.184 ± 0.075[a]	0.231 ± 0.157[a]
Muscle	0.093 ± 0.019[a]	0.058 ± 0.021[ab]	0.082 ± 0.058[ab]	0.020 ± 0.019[b]	0.076 ± 0.068[ab]	0.031 ± 0.009[b]	0.050 ± 0.014[b]	0.080 ± 0.029[ab]

CP: carbonyl protein; LPO: lipid peroxidation. Each bar represents mean ± standard deviation of three samples, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between the sampling stations, whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.
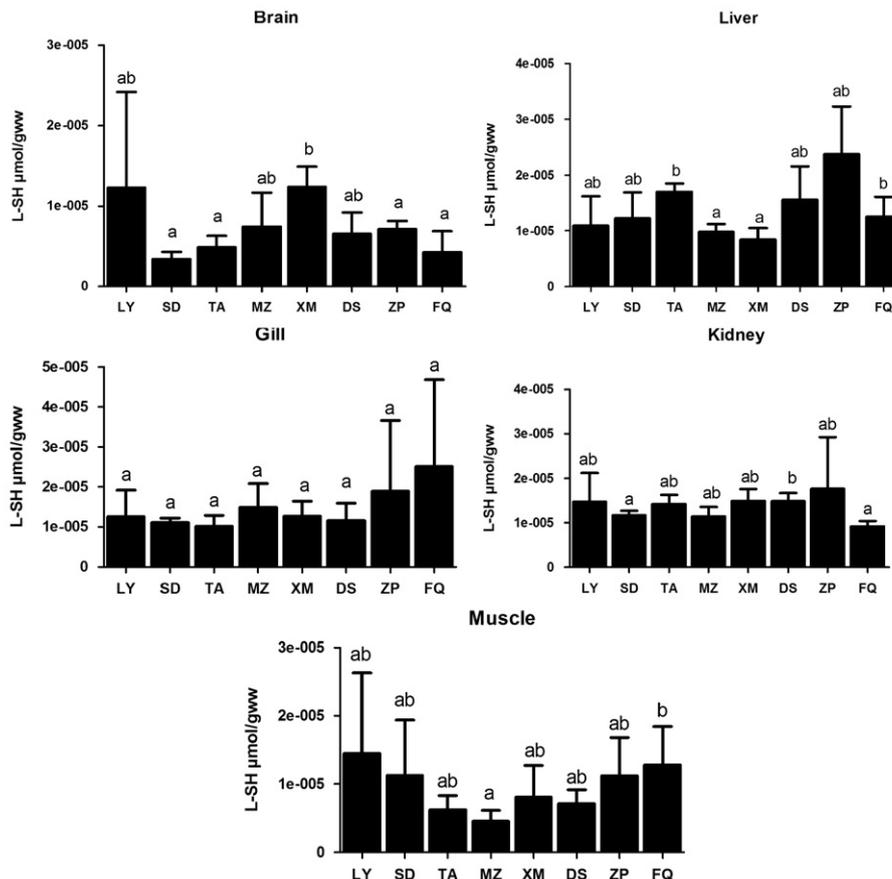
Fig. 4. L-SH concentrations in tissues of RSB collected from locations along the coast of Fujian Province, China. Each bar represents the mean ± standard deviation of three samples. Locations denoted by the same letters (a, b, c) indicate no significant difference between those locations (two-way analysis of variance followed by Tukey's post hoc test), whereas different letters indicate statistically significant differences ($p < 0.05$) between locations.

Fig. 5. H-SH concentrations in tissues of RSB collected from the coastal region in Fujian Province of China. Each bar represents the mean ± standard deviation of three samples. Locations denoted by the same letters (a, b, c) indicate no significant difference among locations (two-way analysis of variance followed by Tukey's post hoc test), whereas different letters indicate statistically significant differences ($p < 0.05$) between locations.

Table 3
Superoxide dismutase and catalase activity in different tissues of *P. major* collected from coastal region in Fujian province of China.

	LY	SD	TA	MZ	XM	DS	ZP	FQ
SOD								
Brain	0.057 ± 0.014^a	0.158 ± 0.097^{ab}	0.212 ± 0.158^{ab}	0.181 ± 0.06^b	0.058 ± 0.034^a	0.067 ± 0.025^a	0.074 ± 0.014^a	0.149 ± 0.054^b
Liver	0.114 ± 0.016^a	0.150 ± 0.050^{ab}	0.080 ± 0.041^a	0.166 ± 0.037^b	0.102 ± 0.035^{ab}	0.079 ± 0.029^a	0.069 ± 0.011^a	0.229 ± 0.070^b
Gill	0.130 ± 0.043^a	0.225 ± 0.025^b	0.190 ± 0.044^{ab}	0.145 ± 0.041^a	0.104 ± 0.021^a	0.163 ± 0.062^{ab}	0.314 ± 0.07^b	0.274 ± 0.086^b
Kidney	0.071 ± 0.016^a	0.078 ± 0.046^{ab}	0.107 ± 0.007^b	0.125 ± 0.016^b	0.113 ± 0.022^{ab}	0.136 ± 0.019^b	0.168 ± 0.035^b	0.130 ± 0.020^b
Muscle	0.16 ± 0.002^a	0.131 ± 0.046^{ab}	0.112 ± 0.008^b	0.201 ± 0.12^{ab}	0.088 ± 0.006^b	0.111 ± 0.015^b	0.185 ± 0.042^a	0.241 ± 0.048^a
CAT								
Brain	0.206 ± 0.048^a	0.315 ± 0.205^{ab}	0.510 ± 0.215^b	0.519 ± 0.199^b	0.156 ± 0.006^a	0.052 ± 0.015^c	0.055 ± 0.015^c	0.099 ± 0.034^c
Liver	0.362 ± 0.034^a	0.310 ± 0.131^{ab}	0.267 ± 0.105^{ab}	0.294 ± 0.062^{ab}	0.194 ± 0.085^{bc}	0.112 ± 0.034^c	0.067 ± 0.031^c	0.167 ± 0.083^{bc}
Gill	0.389 ± 0.142^{ab}	0.688 ± 0.151^b	0.742 ± 0.268^b	0.483 ± 0.190^b	0.162 ± 0.016^a	0.177 ± 0.105^a	0.117 ± 0.113^a	0.207 ± 0.095^a
Kidney	0.217 ± 0.052^a	0.216 ± 0.022^a	0.241 ± 0.057^a	0.370 ± 0.198^a	0.176 ± 0.011^a	0.117 ± 0.004^b	0.107 ± 0.028^b	0.090 ± 0.011^b
Muscle	0.453 ± 0.027^a	0.331 ± 0.107^{ab}	0.246 ± 0.039^b	0.774 ± 0.691^{abc}	0.118 ± 0.021^c	0.083 ± 0.015^c	0.090 ± 0.053^c	0.111 ± 0.064^c

SOD: superoxide dismutase expressed as Unit/mg protein; CAT: catalase expressed as μmol of H_2O_2 consumed/min/mg protein. Each bar represents mean ± standard deviation of three samples, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between the sampling stations, whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.

among tissues and locations. GSH content was greatest in brain of RSB from FQ while RSB from XM exhibited the least. There was significantly less GSH in liver of RSB from SD and TA compared with DS, while GSH content in gill of RSB from DS, ZP and FQ was significantly greater than in RSB from LY, SD, TA and MZ. GSH content of kidney was greater in RSB from DS and was significantly different from that at LY, SD and TA. GSH content of muscle was significantly less in RSB from LY and SD with other stations (Table 4). No significant variation in GPx activity was seen in almost the tissues except kidney at the different locations. The GPx activity in kidney was greatest in RSB from DS while RSB

from SD exhibited the least. GST activity was less in brain of RSB at LY than all other stations (Table 4). No significant variation in GPx activity was seen in liver and gill of RSB at different locations. For kidney, GPx activity was significantly greater at DS and FQ than at LY. GST activity of muscle was significantly greater in RSB from LY than from XM, DS and ZP (Table 4).

3.5. Correlation and PCA analysis

To investigate the relationships between indicators of oxidative stress and adaptive responses, correlations were calculated

Table 4

Glutathione-mediated antioxidant enzyme level in different tissues of *P. major* collected from coastal region in Fujian province of China.

	LY	SD	TA	MZ	XM	DS	ZP	FQ
GSH								
Brain	18.85 ± 0.88[a]	18.63 ± 14.79[a]	22.68 ± 15.46[ab]	29.85 ± 5.81[b]	16.48 ± 15.40[ab]	23.13 ± 2.44[ab]	20.52 ± 9.01[ab]	49.04 ± 22.17[b]
Liver	5.91 ± 1.46[ab]	3.06 ± 1.30[a]	3.01 ± 1.23[a]	6.47 ± 3.17[ab]	6.47 ± 3.59[ab]	5.37 ± 0.011[b]	5.81 ± 1.67[ab]	8.81 ± 3.57[ab]
Gill	7.75 ± 2.00[a]	6.17 ± 3.53[a]	5.32 ± 3.68[a]	7.22 ± 1.67[a]	10.01 ± 4.41[ab]	12.32 ± 2.11[b]	15.45 ± 5.22[b]	12.30 ± 2.02[b]
Kidney	5.69 ± 1.75[a]	4.07 ± 2.86[a]	5.80 ± 1.52[a]	8.85 ± 1.95[ab]	8.73 ± 1.09[ab]	10.20 ± 1.16[b]	8.23 ± 3.03[ab]	6.99 ± 3.54[ab]
Muscle	1.47 ± 0.90[a]	1.25 ± 0.74[a]	4.72 ± 0.14[b]	7.51 ± 5.30[ab]	4.45 ± 0.64[b]	7.60 ± 2.60[b]	8.33 ± 2.15[b]	9.41 ± 3.40[b]
GPx								
Brain	21.98 ± 17.50[a]	11.28 ± 10.27[a]	20.23 ± 13.23[a]	24.18 ± 16.39[a]	15.91 ± 13.60[a]	7.46 ± 4.00[a]	10.35 ± 6.86[a]	19.11 ± 18.46[a]
Liver	38.88 ± 18.77[a]	26.63 ± 12.98[a]	23.32 ± 18.56[a]	15.35 ± 12.97[a]	29.94 ± 14.24[a]	25.11 ± 18.25[a]	14.57 ± 10.70[a]	34.95 ± 28.66[a]
Gill	33.77 ± 30.87[a]	65.90 ± 45.57[a]	42.96 ± 34.25[a]	44.89 ± 29.37[a]	10.46 ± 7.82[a]	35.19 ± 14.28[a]	20.12 ± 19.44[a]	25.38 ± 5.13[a]
Kidney	26.53 ± 17.68[ab]	9.42 ± 9.16[a]	17.48 ± 12.01[ab]	28.60 ± 9.46[ab]	24.31 ± 16.59[ab]	51.22 ± 26.04[b]	23.27 ± 19.64[ab]	31.02 ± 17.01[ab]
Muscle	37.51 ± 31.41[a]	6.36 ± 5.34[a]	6.57 ± 3.61[a]	19.38 ± 14.51[a]	9.47 ± 6.88[a]	7.46 ± 5.47[a]	11.37 ± 9.26[a]	28.41 ± 17.50[a]
GST								
Brain	0.159 ± 0.10[a]	0.336 ± 0.11[ab]	0.664 ± 0.54[ab]	0.616 ± 0.38[ab]	0.299 ± 0.25[ab]	0.218 ± 0.05[ab]	0.299 ± 0.06[b]	0.38 ± 0.29[ab]
Liver	0.928 ± 0.75[a]	2.01 ± 0.62[a]	1.67 ± 1.41[a]	1.97 ± 1.51[a]	1.53 ± 1.26[a]	1.72 ± 0.75[a]	0.915 ± 0.57[a]	2.21 ± 1.84[a]
Gill	2.92 ± 2.45[a]	2.24 ± 1.14[a]	0.62 ± 0.59[a]	1.41 ± 1.06[a]	1.14 ± 1.03[a]	1.97 ± 1.82[a]	1.47 ± 0.89[a]	1.98 ± 0.54[a]
Kidney	0.301 ± 0.22[a]	1.00 ± 0.56[ab]	0.75 ± 0.59[ab]	1.01 ± 0.53[ab]	0.825 ± 0.43[ab]	0.73 ± 0.17[b]	0.922 ± 0.41[ab]	1.06 ± 0.26[b]
Muscle	1.65 ± 0.35[a]	0.971 ± 0.66[ab]	0.88 ± 0.82[ab]	1.08 ± 0.72[ab]	0.735 ± 0.36[b]	0.54 ± 0.17[b]	0.766 ± 0.44[b]	1.30 ± 0.53[ab]

GSH: reduced glutathione expressed in µg/mg protein; GPx: glutathione peroxidase expressed as micromoles of GSH oxidized/(min mg protein); GST: glutathione-*S*-transferase expressed as Unit/mg protein. Each bar represents mean ± standard deviation of three samples, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between the sampling stations, whereas different letters indicate statistically significant differences ($p < 0.05$) between different stations.

Table 5

Significant correlations identified in *P. major* collected from coastal region in Fujian province of China.[a]

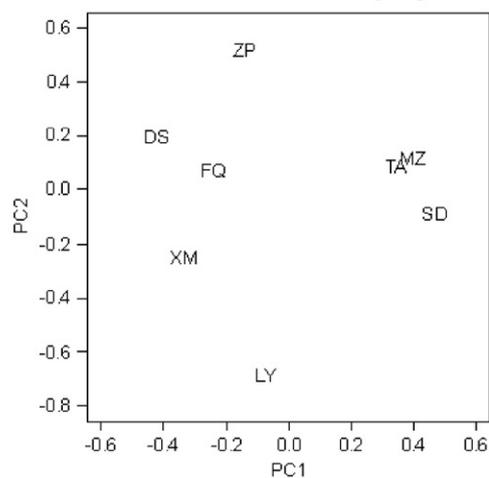| Biomarker I	Biomarker II	R^2
Protein carbonyl (muscle)	CAT (muscle)	0.639
ROS (gill)	AchE (gill)	0.70
GPx (kidney)	GSH (kidney)	0.597
GSH (muscle)	SOD (kidney)	0.633
GST (kidney)	AchE (kidney)	0.585
CAT (brain)	CAT (kidney)	0.699
CAT (muscle)	CAT (liver)	0.654

[a] The acceptable *p*-value is $< 2.33e-05$ after Bonferroni Correction.

Table 6

Rotated component matrix developed by principal component analysis of the biomarker data[a] on sampling sites.

1st principal component		**2nd principal component**	
Biomarkers	**Rotated component**	**Biomarkers**	**Rotated component**
Brain. "Lipid peroxidation"	0.275	Brain. "ROS"	0.503
Brain. "ROS"	0.372	Muscle. "GSH"	0.311
Muscle. "ROS"	0.223	Muscle. "CAT"	−0.201
Brain. "CAT"	0.349	Liver. "CAT"	−0.230
Muscle."CAT"	0.289	Kidney. "AchE"	0.255
Gill. "CAT"	0.279		
Brain. "AchE"	0.278		

[a] Only values > 0.20 are listed here.

Scatter Plot of Differnt Sampling Sites

Fig. 6. Scatter plot of locations ordinate along the first two principal components of the biomarker values at coastal Fujian Province, China locations.

protein carbonyl content was correlated with CAT activity in muscle and the ROS activity of gill was correlated with gill AChE activity.

In the PCA analysis, the first two principal components accounted for 52.82% of overall variance of the data. The percentage of variance explained by different principal components is as follows: 31.00%, 21.82%, 15.73%, 10.72%, 7.84%, 6.93% and 5.95%. The rotated component matrix developed by PCA of the biomarker data on sampling sites is given in Table 6. Based upon the scatter plot of the first two principal components of the biomarker data, the pattern in relative magnitudes of parameters was similar among locations. Specifically, sites MZ, TA and SD form a group with the most similarity (Fig. 6).

4. Discussion

Environmental pollutants can cause oxidative damage in biological systems and their measurement has become a useful

for each endpoint-tissue combination. A total of 61 endpoint-tissue combinations at 8 locations were investigated. Thus, there were a total of 2850 pairs of *x–y* correlation analyses. To avoid errors in inference introduced by multiple testing, the acceptable significance level was adjusted by applying a Bonferroni Correction. The majority of the significantly correlated biomarkers were from the same tissue type (Table 5). For example, the muscle

measure of exposure of aquatic organisms to pollutants (Gray, 1992). Both organic and inorganic pollutants can produce superoxide anion radicals by redox cycling. Some transition metals can catalyze the reaction of superoxide anion radicals with hydrogen peroxide to produce hydroxyl radicals by Fenton reactions (Winston and Di Giulio, 1991). Thus, oxidative stress can occur in tissues of fish exposed to both organic and inorganic xenobiotics that can result in oxidative radicals. To counter the superoxide anion radicals generated, vertebrates produce adaptive antioxidant defenses. The discrepancy between production of oxyradicals and the antioxidant adaptive response determines the level of adverse effects on organisms. Here we have presented the results of an evaluation of oxidative stress in RSB caused by xenobiotic existing in culture regions along the coast of Fujian Province in southeastern China. The results of this study suggest that RSB survived well in different regions with different types and magnitudes of environmental contamination that can be attributed to a balance between the adaptive antioxidant defense and oxidative stress that maintains homeostasis.

Previous studies revealed that station TA was polluted with organochlorine pesticides (DDT 43.9 ng/L; HCH 6.8 ng/L in surface water and 17.5 ng/g of DDT in sediment) and PAH (32.8 ng/L in surface water; 56.8 ng/g in sediment (Wang et al., 2005)). Station LA was reported to exhibit relatively great concentrations of polycyclic aromatic hydrocarbons (PAH) in sediment (784 mg/kg). Concentrations of PAH in the off shore marine environment of Fujian coast throughout the year varied from 5.8 to 37.3 μg/L, with a mean of 14.5 μg/L; and the average concentration in sediment was 1.33×10^2 mg/kg for less polluted sediment (Wang et al., 2003). Station MZ has been reported to have concentrations of 13–15 ng PAH/L in surface water and station SD with 10–12 ng PAH/L offshore water (Wang et al., 2003).

Xenobiotics can induce adaptive changes such as phase I enzymes that can oxidize xenobiotics to make them more polar and thus possible to be conjugated and excreted, but this process involves redox reactions that can generate free radicals, especially ROS which modulate physiological functions in fish (Bainy et al., 1996). ROS production can be increased by the presence of a range of organic and inorganic contaminants. ROS can result in damage to cellular constituents, including oxidation of lipids, protein and DNA (Almeida et al., 2007). In the present study, there was little indication of differential generation of ROS in tissues of RSB among locations. The observation that ROS damage was greater at the more contaminated locations of MZ, TA, DS and SD was similar to those of other studies reporting that production of ROS was greater in fish collected from polluted regions (Moore, 1992; Livingstone, 2001).

The significant variations in AChE activity among liver, kidney and muscle of RSB from LY, SD or TA indicate the possible effect upon exposure to organophosphate or carbamate insecticides (Cajaraville et al., 2000). AChE inhibition has been used widely to monitor for the toxic action of organophosphate and carbamate pesticides. No information is currently available for concentrations of organophosphate or carbamate pesticides at these locations. However, current and historical uses of a variety of pesticides used in these regions likely included large quantities of both types of insecticides (Calamari and Reyes, 1997; Klumpp et al., 2002).

Protein carbonylation results in protein oxidation. The protein attacked by ROS will lead to the formation of carbonyl (Bainy et al., 1996) which is non-reversible and causes a conformational change. The change will reduce catalytic activity in enzymes and ultimately results in breakdown of proteins (Zhang et al., 2008b). Protein carbonyl levels of 3–50 nmol carbonyl/mg protein measured in the current study were greater than those observed in mammals under normal, non-disease conditions which are in the range of 1–4 nmol (Fagan et al., 1999; Das and Chainy, 2004). But these values were within the range of 80 nmol/ g ww observed in fish under stressful conditions such as exposure to carbamazepine (Li et al., 2010). Severely oxidized proteins can be stabilized due to aggregation, cross-linking and deceased solubility (Grune et al., 2003). Therefore, modulation of both an increase and a decrease in protein carbonyl levels in fish from different locations could be indicative of exposure of fish to xenobiotics. Of the two possible responses, an increase would likely be the more severe. CP in gill and liver of RSB was greater at some locations. However, such levels likely do not produce any adverse effects that affect survival of RSB.

Several studies have demonstrated enhancements of LPO in various tissues of fish exposed in vivo to different chemicals (Doyotte et al., 1997; Almeida et al., 2007). The greater level of LPO in different tissues of RSB collected from SD or MZ is consistent with greater contamination at these two locations. LPO products such as malonaldehyde have been shown to be more reactive and an important mediator of DNA damage (Wang and Liehr, 1995). The significantly greater amounts of DNA damage in the liver of RSB collected from station MZ is consistent with intermediates formed during biotransformation of xenobiotics that can cause DNA damage (van der Oost et al., 2003). Such a decrease in DNA integrity can be associated with degenerative processes initiated by LPO (Wang and Liehr, 1995).

Free thiols can protect cells against ROS by regulating redox-sensitive processes (Dröge, 2002). Thiol groups, function in the detoxification of H_2O_2, other peroxides and free radicals, which are considered to be the predominant defense against ROS (DeLeve and Kaplowitz, 1991; Deneke, 2000). Thiols are also involved in detoxifying xenobiotics, which are eventually excreted in the form of mercapturic acids (Pastore et al., 2003). In the present study, levels of thiol groups in different tissues of RSB showed a similar pattern. The lesser concentration of L-SH observed in RSB collected from station MZ and lesser concentration of H-SH at station TA are consistent with the presence of greater concentrations of xenobiotics at these locations that could exhaust the L-SH and H-SH levels in tissues of RSB. Modulation of L-SH depends on synthesis of glutathione, which is the main lower-molecular weight antioxidant (Halliwell and Gutteridge, 1999). Oxidation of thiol compounds usually leads to the loss of biological activity. Modulation of glutathione often occurs during oxidative stress (Halliwell and Gutteridge, 1999; Ali et al., 2004) and reduced glutathione can be consumed by direct interactions with xenobiotics or due to increased consumption of glutathione as a ROS scavenger (Lushchak et al., 2007). While there were no consistent trends in all of the various measures of oxidative stress, responses of RSB in the present study it is possible to speculate that the more industrialized regions of SD, MZ and TA may release more pollutants that cause adverse effects than the others.

Glutathione which is synthesized in the liver and exported to other tissues (Deneke and Fanburg, 1989) functions in detoxification of free radicals and organic peroxides created during lipid peroxidation. GSH reacts with organic free radicals to regenerate damaged molecules. Depletion of GSH observed in liver, muscle and gill of RSB collected from SD and TA is consistent with greater utilization of GSH to mitigate the effects of pro-oxidants present in these more contaminated regions. The fact that there was no significant difference in GSH except in the gill and muscle among locations might be due to the capability of cells to manage oxidative stress, by using GSH as a co-factor for antioxidant enzymes. Fish living in more polluted environments exhibit a tendency to modulation of GST activity due to detoxification of contaminants by biotransformation enzymes, which enable the fish to survive from the heavy exposure of xenobiotics in the contaminated environment (Chatterjee and Bhattacharya, 1984).

ROS production in cells can result in greater concentrations of peroxides, which can be metabolized by the action of GPx (Awasthi et al., 2004). Similar to CAT activity, GPx also catalyzes reduction of hydrogen peroxide to water and also reduction of lipid or hydroperoxides to alcohols (Winston and Di Giulio, 1991). One function of the biotransformation enzyme GST is to protect against oxidative damage and peroxidation products of DNA and lipids (George, 1994). The susceptibility of fish to some xenobiotics can be modulated by GST (Varanasi et al., 1987). Greater GPx and GST activities in several tissues of RSB collected from SD, TA, MZ and FQ was recorded in the present study. The greater GST activity was probably due to metabolic adaptation to exposure to contaminants. Alternatively, in order to mitigate toxicity of xenobiotics present in the environment, RSB exhibited greater GSH contents and increased GPx and GST activity in their tissues. These interdependent pathways suggest that GSH utilization may be exhausted or inhibited in fish from more polluted environments.

Antioxidant defenses include enzymes, such as SOD, CAT, GPx and associated enzymes such as GSH. ROS and other oxygen reactive species generated can be removed by the antioxidant defense system under normal physiological conditions (Livingstone, 2001). SOD is the first antioxidant enzyme to scavenge superoxide radicals (O^{-2}). CAT is responsible for detoxification of hydrogen peroxide (H_2O_2) that is formed as a result of the reaction catalyzed by SOD. Greater SOD and CAT activities observed in the present study were likely associated with generation of ROS during metabolism of xenobiotics. Induction of SOD in brain, muscle and liver of RSB from SD, TA MZ and FQ was consistent with superoxide radical production due to exposure to xenobiotics at these locations. Similarly, an increase in SOD activity in the whole body tissue of mussels collected from polluted Hong Kong waters has been reported by Lau and Wong (2003). Contrary to the previous report that CAT activity is often not affected by exposure to xenobiotics (van der Oost et al., 2003). In this study greater CAT activities were observed in several tissues of RSB from SD, TA MZ, which is consistent with greater oxidative stress in RSB from these areas.

Multivariate analysis helped elucidate relationships among tissues, biomarkers and sampling stations. The eight stations along the Fujian Coast exhibit diverse patterns of pollution, ranging from less to more polluted regions. PCA performed with the antioxidant and oxidative stress parameters proved to be an effective method to distinguish among locations. The parameters associated with the first principal component (1st PC) (Table 2) included "ROS" and "CAT", in brain, muscle and gill. These parameters provided the greatest discrimination power among locations while LPO, GSH and AChE were less discriminatory. Thus, using the first principal component, the eight sample sites can be ranked as following: SD > MZ > TA > LY > ZP > FQ > XM > DS. Since fish will have greater activity of ROS and CAT, when exposed to PAHs and PCBs, the results suggested that RSB from SD, MZ and TA were more possibly affected by exposure to these types of organic xenobiotics. A previous study found that the Tong'an location (TA) was more polluted. Thus, the area may not be suitable for aquaculture in coming years (Wang et al., 2005).

5. Conclusion

The present study provides an assessment of current conditions of antioxidative responses of caged RSB from different locations along Fujian coast. Oxidative stress and antioxidant responses in organs of fish were dependent on organ-specific physiology and there were no clear trends among locations when parameters were considered individually. All of the organs of RSB exhibited a similar resistance to oxidative damage. However, we observed a rise in ROS, CP, LPO content and modulation of antioxidant enzymes in RSB collected at station SD, MZ and TA, possibly due to poor water quality at these stations compared to other stations. This may have stressed the population of RSB, resulting in modulation of oxidative and antioxidant parameters. Brain and gill were the most sensitive organs to oxidative damage, particularly for ROS. However, brain seems to be more specific for ROS and the biomarker such as AChE, L-SH and H-SH were more cost-effective biochemical response, which may not be sensitive enough for pollution monitoring program and may not be suitably recommended for future use. Also, this study showed effectiveness of PCA potential in evaluating the exposure of the organisms to xenobiotics in different stations. The present preliminary study will provide essential thoughtful information on the environment based on the biomarkers studied using RSB. In future studies, targeted measurements of specific known contaminants will be related to the observed differences in some of the biomarkers, specifically those relating to DNA damage and ROS. In addition, to determine the causative agents, a bioassay-directed fractionation and identification approach using extracts of fish tissues and *in vitro* assays, coupled with instrumental analyses will be applied at those locations where the effects of ROS and DNA damage were greatest.

Acknowledgment

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