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RESEARCH PAPER

Biomarker Responses to Polycyclic Aromatic Hydrocarbons in the Native Fish *Ramnogaster arcuata,* South America

Ana Carolina Ronda[1,2] · Ana Laura Oliva[1] · Andrés Hugo Arias[1,3] · Melina Mirta Orazi[1] · Jorge Eduardo Marcovecchio[1,4,5,6]

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Abstract

Quantification of polycyclic aromatic hydrocarbons (PAHs) in Bahía Blanca Estuary (BBE, Argentina) fish samples (*Ramnogaster arcuata*) was performed to evaluate the environmental impact through anthropogenic activity. In addition, several metabolic enzyme activities (Aspartate aminotransferase—AST, Alanine aminotransferase—ALT, Lactate dehydrogenase—LDH, Creatine kinase—CK and, Alkaline phosphatase—ALP), protein content and lipid peroxidation as oxidative stress biomarker were analyzed in muscle and liver and related to tisular PAHs levels. Results showed low to moderate PAHs levels in *R. arcuata* muscle (9.34–41.25 ng/g, wet weight) with a marked predominance of two/three ringed compounds (phenanthrene > naphthalene > acenaphthene > acenaphthylene > fluoranthene). Fluoranthene, pyrene, benzo-[b]fluoranthene and benzo-[a]pyrene concentrations correlated positively with hepatic AST and ALT and negatively with muscular proteins and hepatic lipid peroxidation. 2-metil-naphthalene and acenaphthene levels correlated negatively with LDH in muscle and positively with lipid peroxidation in liver tissue. Correlation of PAHs with metabolic enzymes, proteins and lipid peroxidation indicated a differential metabolization and suggesting that hepatic AST/ALT could be used as PAHs biotransformation biomarkers and muscular LDH as a stress oxidation biomarker in *R. arcuata*. In addition, CK activity was suggested as a good index of muscular health. The obtained results highlighted the significance of using a set of integrated biomarkers to assess PAHs toxicity in fish inhabiting their natural ambient and confirm that *R. arcuata* could be used as a good bioindicator for marine areas.

Article Highlights

- ***Ramogaster arcuata* is proposed as a bioindicator.**
- **AST and ALT serve as biomarkers of PAHs-induced liver biotransformation.**
- **Muscular LDH in *R. arcuata* was in relation to LPO induced by PAHs.**
- **CK in muscle could be used to verify the muscular health status of fishes.**

Keywords Polycyclic aromatic hydrocarbons · Fish · Biomarkers

✉ Ana Carolina Ronda
acronda@criba.edu.ar

[1] Instituto Argentino de Oceanografía (IADO, CONICET/UNS), Camino La Carrindanga km 7.5, 8000 Bahía Blanca, Argentina

[2] Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina

[3] Departamento de Química, Area III, Química Analítica, Universidad Nacional del Sur, Av Alem 1253, 8000 Bahía Blanca, Argentina

[4] Universidad de la Fraternidad de Agrupaciones Santo Tomás de Aquino, Gascón 3145, 7600 Mar del Plata, Argentina

[5] Universidad Tecnológica Nacional, FRBB, 11 de Abril 445, 8000 Bahía Blanca, Argentina

[6] Academia Nacional de Ciencias Exactas, Físicas y Naturales (ANCEFN), Av. Alvear 1711, 4to piso, 1014 Buenos Aires, Argentina

Introduction

Aquatic environments are subjected to different types of pollutants which enter into the water bodies through several routes such as industrial, domestic and agricultural wastewaters, atmospheric depositions and urban runoff. Once in the aquatic media, most of these pollutants are able to severely affect the health of organisms. Since fish are inevitably affected by coastal and marine pollution, research on its biological response is being proposed as a complementary diagnostic tool to evaluate the ecosystem quality and health status (Duarte et al. 2017). Bioassays' studies in different fish species have demonstrated several responses to a single pollutant, increasing the interest in determining these biological alterations to provide an idea of what is happening environmentally (Richardson et al. 2008; Tkachenko et al. 2013; Samanta et al. 2014). A usual research target is to define specific responses triggered by specific xenobiotics; however, the inherent disadvantage of in vitro bioassay conditions is precisely the particularly controlled environment (e.g. temperature, salinity, turbidity, dissolved oxygen, presence of other compounds), which can often lead to a limited interpretation of what is actually happening in natural ecosystems. Pollutants usually occur as complex mixtures in real-world scenarios, which may pose synergic/antagonistic effects over fish species and the physicochemical environment affects several processes of the xenobiotic fate, including its bioavailability, absorption, metabolization and depuration. Therefore, it is a challenge to analyze several fish responses to environmental/anthropic impacts in their natural habitat, in order to use them as early diagnostic tools to evaluate the health status of an environment.

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants with two or more fused benzene rings and are typically characterized by low water solubility and high lipid solubility. PAHs appear in most urbanized coastal areas of the world, accumulating in sediments and biota that are unable to effectively eliminate them. PAHs can bioconcentrate and bioaccumulate in fishes through different mechanisms: uptake by contaminated prey food ingestion, uptake through respiration via gills or direct contact with the skin (Meador et al. 1995; Streit 1998). Fishes have the ability to metabolize these pollutants; indeed, the primary detoxifying pathway of PAHs metabolization is through cytochrome P450 oxidase (CPY1) localized in the hepatic mitochondria. Biotransformation leads to less toxic and harmful chemical compounds which can be easily eliminated (Meador et al. 1995). However, some PAHs metabolites can be more toxic than their precursor (Varanasi et al. 1989; Johnson-Restrepo et al. 2008). During this process, reactive oxygen species (ROS) are produced as by-products which should be rapidly removed through various antioxidant systems. However, under high pollutants levels or prolonged exposure time, ROS concentrations exceed the antioxidant capacity of the fish leading to oxidative stress. The latter occurs when ROS molecules readily react with proteins, DNA and lipids, ultimately causing deterioration and cell death (Abarikwu et al. 2017; Duarte et al. 2017; Kumar et al. 2017).

For both biotransformation and antioxidant defences, organisms use different metabolic processes regulated by enzymes. For instance, aminotransferases—alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—participate in the interconversion among carbohydrate and protein metabolism during stress-imposed conditions to meet the high energy demand of the fish (Ballantyne 2001; Gabriel and George 2005). Secondly, lactate dehydrogenase (LDH) forms the center for a delicately balanced equilibrium between catabolism and anabolism of carbohydrates throughout environmental stress conditions to supply energy to fish metabolism (Banaee 2012; Osman et al. 2010; Gabriel et al. 2012). Thirdly, alkaline phosphatase (ALP) is a polyfunctional enzyme that plays a significant role in phosphate hydrolysis and its determination has been used to assess the effect of contaminants in fishes (Banaee and Ahmadi 2011). Finally, creatine kinase (CK) catalyzes the reversible regeneration of ATP and has been considered as an indirect marker of muscle damage due to environmental stress (Kori-Siakpere et al. 2011; Li et al. 2011).

Although research on fish enzyme levels as potential biomarkers of pollution has been performed in the past (Osioma et al. 2013; Li et al. 2011; Kumari et al. 2011), their assessment in liver and muscle tissue and its relationship with PAHs tissue levels has never been assayed for *Ramnogaster arcuata*. This fish species is a small pelagic zooplankton consumer that lives in coastal zones along the south-western Atlantic and it has been proposed as a bioindicator of coastal regions due to its resident habits and short life cycle (Cazorla and Sidorkewicj 2009). *Ramnogaster arcuata* has a wide spatiotemporal distribution in Bahía Blanca estuary, the area of study where completes its life cycle (López Cazorla 2004).

The Bahia Blanca Estuary (EBB) (38°45′–39°25′S, 61°15′–62°30′W), area of study, is the second largest estuary of Argentina and exhibits a continuous and well documented environmental impact due to anthropic activity. The presence of heavy metals and organic compounds such as polycyclic aromatic hydrocarbons (PAHs) have been recorded in sediments and water column, ranging from low to very high concentrations in particular hotspots, according to the international guidelines and regulations (Botté et al. 2007; Arias et al. 2009, 2010a, b, 2013; Oliva et al. 2015; La Colla et al. 2015). Although precedent research has been used to assess the general environmental quality of the area, there is no previous information addressing associated ecotoxicological

effects using biomarkers. Moreover, in comparison with the northern hemisphere, information concerning biomarkers responses to persistent organic compounds burden in South American coasts is lacking. Highly fragmented data have been reported for biomarkers in other selected Argentinean marine environments (Duarte et al. 2011; Scarcia et al. 2012; Polizzi et al. 2014) and there are no precedents assessing the distribution of biomarkers in native marine fish *Ramnogaster arcuata*.

Then, the first aim of this research is to assess for the first time the mapping of fish liver and muscle biomarkers in order to test their employment as an early diagnostic tool to evaluate PAHs effects pollution in coastal environments. In second place, we pretend to test *R. arcuata* as biochemical sentinel/bioindicator for South American coastal environments.

Materials and Methods

Sampling

Two areas for fish capture were chosen within the area of study in order to ensure adequate geographical representation of the estuary: Galván Port (GP) and Embudo Channel (EC) (Fig. 1); a total of four sampling journeys (S1 and S3 at GP; S2 and S4 at EC) were realized. *R. arcuata* capture was made in spring of 2015 using two shrimp-type nets, and fishing manoeuvres were diurnal in downward tide. Physicochemical parameters (salinity, pH and temperature) were measured in situ with a multisensor Horiba U-10. Collected samples were immediately placed in cold (0 °C) and then transported to the laboratory. As fish metabolic enzymes can vary according to size and growth rate (Pelletier et al. 1993), the same stage of *R. arcuata* was carefully selected from the samples. To compare between the four sampling journeys, we specifically

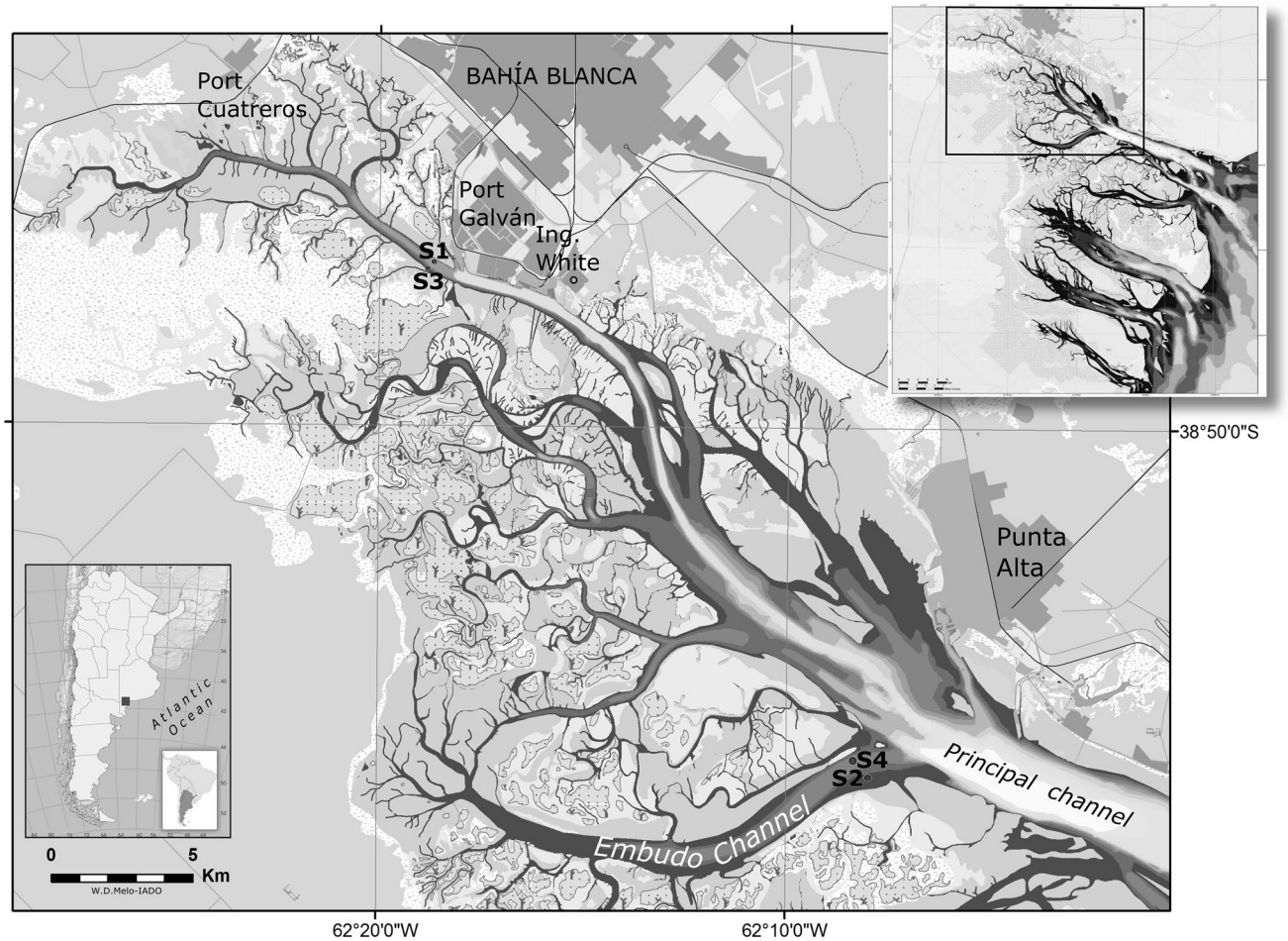


Fig. 1 Map of the Bahía Blanca Estuary, indicating the samplings S1 and S3 in Galvan Port; S2 and S4 in Embudo Channel

selected individuals from class III, according to the length species (ages between 1 and 2 years—advanced maturation) following the López Cazorla and Sidorkewicj criteria (2009). A total of 254 individuals from 80 to 100 mm length were captured and arbitrarily divided into pools to perform the corresponding determinations, as explained below. Samples were not divided by gender since there are no significant differences between the length–weight relationships of males and females in the same stage and positive allometric growth was observed for juveniles, males, females and sexes combined (Cazorla and Sidorkewicj 2009)

For biochemical parameters, samples taken from each sampling journey were divided into three pools of 8–10 individuals approximately (total $n = 94$). For PAHs analysis, one pool of about 38–40 individuals (total $n = 160$) for each sampling was processed. Liver and muscle tissues were carefully dissected by stainless steel knife and stored at − 20 °C for posterior analysis.

Biochemical Analyses

Sample Preparation

Frozen tissue samples were weighed and homogenized (1:10 w/v) in an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA at 0 °C. Homogenate was firstly centrifuged at 1500 *g* and 4 °C for 10 min, and an aliquot of supernatant was subsampled for lipid peroxidation analyses. For enzymatic determination, the homogenate was further centrifuged at 10,000*g* and 4 °C for 30 min to obtain the postmitochondrial fraction. Protein levels were determined spectrophotometrically at 750 nm by Lowry method (Lowry et al. 1951) using bovine serum albumin as standard.

Lipid Peroxidation

Oxidative damage to lipids was determined recording the production of malondialdehyde (MDA) following Ohkawa et al. (1979), slightly modified. Briefly, an aliquot of the homogenate was incubated for 30 min at 100 °C with trichloroacetic acid, thiobarbituric acid and butylated hydroxytoluene (BHT) solution at a final concentration of 0.3, 4, and 0.01%, respectively. BHT was added to prevent the induction of unspecific thiobarbituric acid reactive substances by heat (Pikul and Leszczynski 1986). Subsequently, samples were incubated on ice for 5 min and centrifuged at 10,000*g* for 15 min. The absorbance of the supernatant was read at 535 nm on a Shimadzu UV/visible spectrophotometer. The concentration of colored complex was calculated using the molar extinction coefficient of the MDA-TBA complex under working conditions (156 $mmol^{-1}$ cm^{-1} L). Results were referred to proteins determined in the sample (µmol/g prot).

Metabolic Enzymes Activities

Glutamic pyruvic transaminase (ALT), glutamic oxaloacetic transaminase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and creatine kinase (CK) activities were measured by means of commercial kits from Wiener Lab Group (Rosario, Argentina), following the manufacturer's instructions. Briefly, an aliquot of each sample was incubated with the specific substrates for each enzyme at 25 °C. ALT, AST and LDH activities were measured by NADH consumption that was spectrophotometrically recorded at 340 nm using a Shimadzu UV/visible spectrophotometer. ALP activity was measured through p-nitrophenol production at alkaline pH and spectrophotometrically recorded at 405 nm. CK activity was measured by coupling the enzymatic reaction with the activity of hexokinase and then glucose-6-phosphate dehydrogenase. NADPH decrement was spectrophotometrically recorded at 340 nm. In all cases, Δabsorbance/min was determined. ALT, AST, LDH and CK activities were calculated using the NADH/NADPH molar extinction coefficient (6317 mol^{-1} cm^{-1} L) and ALP activity was calculated using the p-nitrophenolate molar extinction coefficient (18,000 mol^{-1} cm^{-1} L). Results were expressed as International Units per mg of protein (U/mg) in each sample (U = one micromol of subtract produced or consumed per minute).

PAHs Quantification

Analytical Procedure

Muscle subsamples were lyophilized for 48 h, smashed in a mortar and stored in desiccators prior to analyses. Extraction according to the method of UNEP/IAEA/FAO/IOC (1993) was performed using 100 µl of the mixture of four perdeuterated PAHs (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}) as subrogate standards. Muscle tissue from each sampling (5 g dry weight) was digested under reflux with methanol for 8 h, and then potassium hydroxide (0.7 M) and tri-distilled water were added and left to reflux for 2 more hours. The non-saponifiable fraction was extracted with n-hexane; the organic phase was dried with anhydrous sodium sulphate and concentrated close to 5 mL in a rotary evaporator with a low-temperature thermostatic bath. Furthermore, the concentrate was reduced to 1.5 mL under a gentle high purity nitrogen flow. The extract was seeded in an alumina-silica gel (2:1) column to carry

out the sample clean-up. PAHs were eluted with 70 mL of hexane-dichloromethane (9:1) and the volume of elutes was then reduced to 5 mL by rotary evaporator and further to 1.5 mL under nitrogen flow. Finally, just before the GC/MS injection, 100 µl of deuterated internal standard (benzo-[a]-anthracene-d_{12}) was added to the extract vials for recovery asses.

PAHs were quantified using a gas chromatograph (Agilent 7890 B, Santa Clara, USA) coupled with a mass spectrometer (Agilent 5977A, Santa Clara, USA), equipped with a fused silica column (HP-5MS; 30 m; 0.25 mm i.d.; 0.25 µm film thickness). Helium was used as a carrier gas. The mass spectrometer was operated in selected ion monitoring mode (SIM) and electron impact mode (70 eV). The samples were injected in the splitless mode at 250 °C and the temperature program used was as follows: initial temperature 70 °C for 2 min; heated to 150 °C at 30 °C min^{-1} then to 310 °C at 4 °C min^{-1}, and held for 10 min. Each individual PAH compound was confirmed by the retention time and the abundance of quantification/confirmation ions with respect to authentic PAHs standards. Quantification of individual compounds was based on the ratios analyte peak areas/surrogate standards areas (naphthalene-d_{12}, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, internal standard method) using the corresponding calibration curves.

Quality Control and Assurance

To ensure quality control, procedural blanks were regularly performed during the extraction process. Blanks were prepared following the same procedure but without adding the fish tissue. Quality controls for the PAHs analyses were carried out by monitoring the recovery of the internal standard (Benzo-[a]-anthracene-d_{12}) spiked just before GC injection; recoveries ranged from 76 to 107%.

Sample concentrations were expressed as nanograms per gram wet weight (ng/g w.w.). Results were normalized to a water content of 70% (Soclo et al. 2008). The laboratory detection limits of the method (DLs) for individual PAH ranged from 0.15 to 0.39 ng/g w.w.. DL was set at five times the detected amount of the procedural blank. The deuterated internal standard solution, benzanthracene-d_{12} and PAHs external certificated standard solution of 17 PAHs were used and purchased from Supelco (Bellefonte, Pennsylvania, USA). This mixture contained: naphthalene [NA], 2-Methyl-naphthalene [2-M-NA] acenaphthylene [ACY], acenaphthene [ACE], fluorene [FL], phenanthrene [PHE], anthracene [AN], fluoranthene [FLU], pyrene [PY], benzo[a] anthracene [BaA], chrysene [CHR], benzo[b]fluoranthene [BbF], benzo[k]fluoranthene [BkF], benzo[a]pyrene [BaP], indeno[1,2,3-cd]Pyrene [IP], dibenzo[a,h]anthracene [DBA], and benzo[ghi]perylene [BPE]. All solvents used for sample processing and analyses (hexane, methanol and dichloromethane) were of analytical and chromatographic grade from Merck (Darmstadt, Germany). Merck silica gel 60 (70–230 mesh ASTM) and aluminum oxide activated at 450 °C were heated at 120 °C for 12 h prior to use. Glassware was washed with non-ionic detergent, rinsed with ultrapure water and acetone/hexane and dried at 120 °C prior to use.

Statistical Analysis

For biochemical parameters, data were obtained in triplicate and results were expressed as mean ± SEM. Comparison between groups was performed by analysis of variance (Duncan's multiple range test), after testing normality though Kolmogorov–Smirnov test and homogeneity of variance by Levene's test. If necessary, data were previously transformed to meet the required assumptions of homogeneity and normality for the parametric tests. One-way ANOVA test was performed to assess differences between the sampling sites and enzymes. Analyses of correlation between biochemical parameters and PAHs were performed using Pearson´s coefficient test when normality of data was demonstrated or using Spearman´s test when normality could not be demonstrated. The acceptable level of statistical significance used throughout the study was $p < 0.05$. PAHs concentrations that were below the laboratory detection limit (DLs) were substituted by one half of the DL for statistical analyses (Jones and Clarke 2005).

Table 1 Concentration of PAHs in *R. arcuata* from Bahía Blanca Estuary (ng/g wet weight)

	PAHs (ng/g w. w.)			
	S1	S2	S3	S4
NA (2)	0.73	2.34	1.98	1.36
2-M-NA (2)	0.76	1.90	1.40	0.71
ACY (2)	<LOD	0.74	<LOD	<LOD
ACE (2)	1.89	10.39	0.61	0.59
FL (2)	<LOD	0.99	1.57	<LOD
PHE (3)	5.44	11.66	11.55	6.75
AN (3)	0.27	0.62	0.59	0.60
FLU (3)	<LOD	<LOD	3.20	2.00
PY (4)	<LOD	<LOD	4.06	0.66
BaA (4)	<LOD	<LOD	<LOD	<LOD
CHR (4)	<LOD	12.23	<LOD	<LOD
BbF (4)	0.25	0.38	0.87	0.70
BkF (4)	<LOD	<LOD	<LOD	<LOD
BaP (5)	<LOD	<LOD	0.29	0.15
IP (5)	<LOD	<LOD	<LOD	<LOD
DBA (5)	<LOD	<LOD	<LOD	<LOD
BPE (5)	<LOD	<LOD	<LOD	<LOD
ΣPAHs	9.34	41.25	26.12	13.52

Numbers in parentheses indicate ring number of the compound

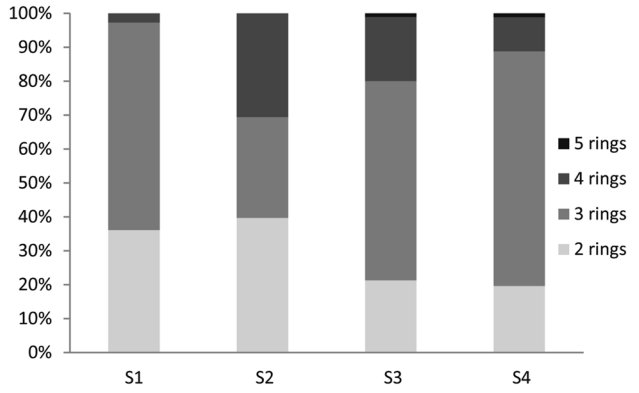


Fig. 2 Average percentual composition of PAHs congeners in fish muscle tissue in each sampling

Results and Discussion

PAHs in *Ramnogaster arcuata*: Levels, Distribution and Toxicity

Within polycyclic aromatic hydrocarbons, 16 are agreed in having the highest priority as targets for pollution monitoring (USEPA 2000). As shown in Table 1, 16 priority PAHs + 2-Methyl-naphthalene (2-M-NA) were studied. Results showed that all samples presented detectable PAHs levels. Total PAHs concentration ranged (sum of 17 PAHs analyzed) from 9.34 to 41.25 ng/g w.w. Considering all samples, NA, 2-M-NA, ACE, PHE, AN and BbF were present in concentrations ranging from 0.25 to 11.66 ng/g w.w. PHE showed the most important contribution to the total PAHs (average 8.85 ± 1.61 SEM ng/g w.w.) (Table 1). This compound is one of the most water-soluble PAH (Meador et al. 1995), consecuently it has a considerable bioavailability for aquatic organisms.

In terms of frequency of detection, low-molecular weight hydrocarbons (2 and 3 rings) were mainly detected compared to those of high molecular weight (more than 3 rings). As shown in Fig. 2, PAHs with 2 and 3 rings were present in all the samples: the most abundant compounds were those of 3 rings with a percentage that was from 30 to 70% and then those of 2 rings with a percentage from 20 to 40%. It is worth mentioning that the only compound of 5 rings detected was BaP, which is considered one of the most carcinogenic PAHs (IARC 2010). Accordingly, Brown (2002) have shown that while high-molecular weight PAHs (HMW-PAHs) are associated with sediment, low-molecular weight PAHs (LMW-PAHs) tend to partition into the dissolved phase. The higher muscle concentrations of LMW-PAHs in *R. arcuata* could be due to the continuous water/gills contact favouring the partition between the dissolved LMW-PAHs and fish tissues. PAHs in the marine environment may come from petrogenic sources or pyrogenic sources. Compounds with 2–3 rings

Table 2 Mean body weight and mean total body length of fish collected and physicochemical variables of sea water

	pH	Temperature (°C)	Salinity (PSU)	*R. arcuata* Length (cm)	*R. arcuata* Weight (g)
S1	7.78	15.1	33.9	8.61 ± 0.24	5.68 ± 0.27
S2	7.99	20.8	33.6	8.66 ± 0.18	5.41 ± 0.41
S3	7.49	21.4	34.5	9.58 ± 0.09	7.86 ± 0.05
S4	7.79	21	34.9	9.23 ± 0.08	6.24 ± 0.22

are considered to be from petrogenic sources, while those of pyrogenic sources have 4–6 rings. Results suggest that PAHs found in the muscle of *R. arcuata* could be mainly from petrogenic sources. However, it must be taken into consideration the PAHs dynamics not only in the environmental but also in the organism metabolization. Low-molecular weight PAHs have higher solubility in water, and greater bioavailability, being assimilated more easily by ingestion, absorption or passive diffusion compared to high-molecular weight PAHs. Also, higher molecular weight PAHs are more rapidly depured than low-molecular weight PAHs in fishes (Meador et al. 1995). Therefore, PAHs origin determination only based on PAHs muscle concentration analyses may not be a true reflection of which source they come from.

Moreover, a group of specific PAHs apparently had higher tissue levels when water temperature was warmer. In particular, FLU, PY, BbF and BaP showed higher muscle concentrations when the water temperature increased (Table 2). This observation could be related to an increase in the absorption of these compounds with temperature. Actually, Jimenez et al. (1987) have been reported an increase in BaP uptake with temperature in *Lepomis macrochirus* (Jimenez et al. 1987). Moreover, Kennedy et al. (1989) found a high correlation between the QIO value (the factor reflecting a change in a process for a 10 °C change) for ventilatory rate and BaP uptake in gulf toadfish (*Opsanus beta*). The authors argument that temperature increases the ventilation rate which results in higher PAH uptake and an accelerated metabolism, suggesting that temperature may be one of the factors that can have an influence on uptake rate for certain PAHs (Kennedy et al. 1989). Although there were no sufficient data to achieve a statistical analysis of correlations, results allowed setting this new hypothesis which will require additional research.

With regard to the potential toxicity impacts of the PAHs body burden in *R. arcuata*, the National Oceanic and Atmospheric Administration (NOAA) has proposed different thresholds of total PAHs in order to set the level of contamination: not polluted (< 10 ng/g w.w.), minimally polluted (10–99 ng/g w.w.), moderately polluted (100–1000 ng/g w.w.) and highly polluted (> 1000 ng/g w.w.) (Varanasi et al. 1993; Soares-Gomes et al. 2010).

Then, following the NOAA criteria, and taking into account total PAHs levels (9.34–41.25 ng/g w.w) found in the samples, we can classify 75% of the samples as minimally polluted whereas the remaining 25% were tagged as not polluted (Table 1). It is noteworthy that PAHs can be rapidly metabolized by some fish species and in consequence half-lives of PAHs are generally shorter than in invertebrate species. In fact, it has been reported half-lives of 6–9 days for FL, PHE, AN, and FLU and of 1–4 days for ACY in rainbow trout (*Salmo gairdneri*) (Niimi and Palazzo 1986; Niimi and Dookhran 1989). Then, considering this, PAHs levels in *R. arcuata* could indicate a recent exposure to these pollutants.

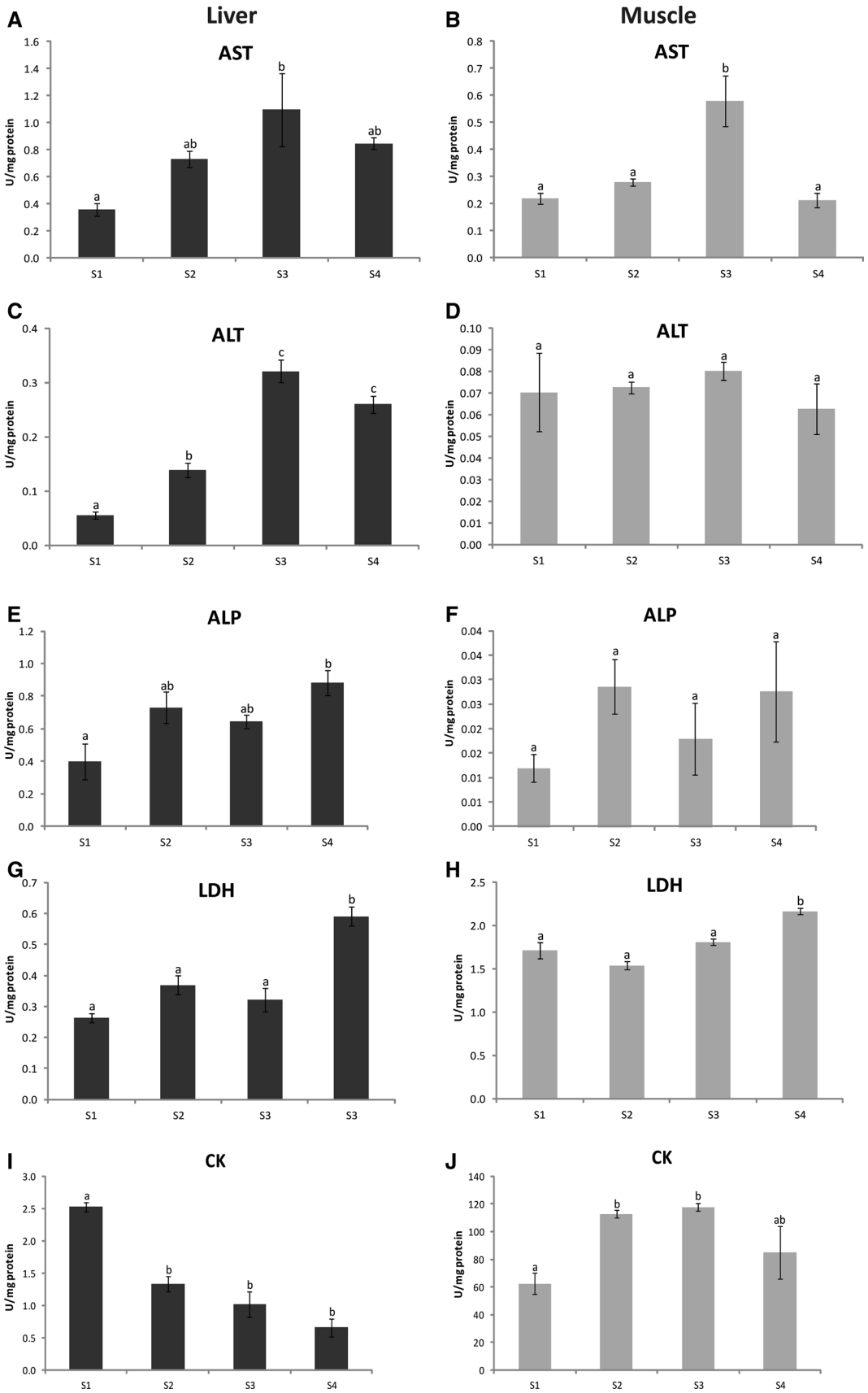
Biomarker Responses vs. PAHs Levels

Ast and Alt

In the present work, AST and ALT enzyme activities, as well as the protein content, were analyzed in muscle and liver of *R. arcuata*. As shown in Fig. 3a, b, AST average activity in liver (0.76 ± 0.10 U/mg, $n = 12$) was higher than in muscle (0.32 ± 0.05 U/mg, $n = 12$) and both isoforms were statistically significant correlated ($p = 0.77$, $p < 0.01$), showing the major activity at S3 sample in both tissues. In addition, ALT average activity (Fig. 3c, d) was also greater in liver (0.19 ± 0.03 U/mg, $n = 12$) than in muscle (0.07 ± 0.005 U/mg, $n = 12$) and no correlation between isoforms was found. Although significant differences between samples were noted for liver, no differences were observed for muscle tissue. Literature results for vertebrate organisms show that AST is found mainly in liver, muscle and heart while ALT is mostly located in the liver (Wróblewski 1958). Correlation analysis between AST and ALT activity in liver showed a positive and significant relationship between these hepatic transaminases ($p = 0.82$; $p < 0.01$). Simultaneous increase of ALT and AST activities in liver fish has been demontrated before as stimulatory effects on gluconeogenic mechanisms (Banaee 2012, 2013). Actually, transamination regulated by aminotransferases is one of the main routes that allow the interconversion between carbohydrates and proteins metabolism promoting gluconeogenesis (Knox and Greengard 1965). Since it has been reported that this process also occurs in fishes during various stress conditions that demand high amounts of energy (Van Waarde and Henegouwen 1982; Tiwari and Singh 2004) and, then, it is possible that ALT and AST increased activities observed in some samples in *R. arcuata* were a consequence of cortisol stimulation in the course of the mentioned process (Banaee 2013) indicating amino acids interconversion and hepatic gluconeogenesis activation.

Our results also showed that the liver had greater protein content (40.1 ± 1.3 mg/g) than muscle (20.8 ± 1.1 mg/g) (Fig. 4). Moreover, a significant decrease in protein content was markedly observed for some muscle samples (S3 and S4). A rationale for this could be found in the probable muscle fish metabolic decomposition under stress conditions cited by Banaee et al. (2014) and others, which is a common metabolic response to pollutants/stressors in order to maintain equilibrium in the organism (Siva Prasada Rao 1980). In gluconeogenesis process, degradation of muscle proteins plays a fundamental role in the generation of gluconeogenic amino acids that are transformed into pyruvate in liver tissue by the action of hepatic transaminases, which is the promoter of energy. In fact, fish under stress mobilizes triglycerides and protein to meet the increased energy demand to cover an increased physical activity, biotransformation and excretion of xenobiotics (Alkahem et al. 1998).

Secondly, results demonstrated a positive and significant correlation between FLU, PY, BbF and BaP with AST and ALT enzymes in hepatic tissue, and a negatively and significant correlation of these compounds with muscular proteins (Table 3). At the light of our settled hypothesis by which the presence of contaminants triggers the gluconeogenic processes, results suggest that *R. arcuata* manage its energy gap by the degradation of muscle proteins, promoting ALT and AST activity in the liver which in turn triggers gluconeogenesis. On the other hand, FL was not correlated with protein content and then its relationship vs. muscle AST and liver ALT were not taken into account. Moreover, the negative correlation of ACE with liver ALT and positive with muscle proteins could be indicating that the necessary energy for the biotransformation/elimination of this compound would be provided by other mechanisms different from gluconeogenesis. Although there are diverse literature reporting an increase in the activity of AST and ALT due to liver damage with the concomitant release of the hepatic enzymes into the bloodstream as a result of fish exposition to PAHs (Sarhadizadeh et al. 2014; Fern et al. 2016; Shirmohammadi et al. 2017), there is a scarcity information about the activity of these transaminases within the fish liver tissue and its relationship with PAHs. The unique precedent reported an acute toxicity bioassay of *Labeo Rohita* with AN, which led to ALT and AST increased activities in liver (Vasanth et al. 2012). Besides, there are studies of these enzymes in fish liver tissue in relation to other contaminants such as pesticides. In e.g., a decrease in the activity of ALT and AST has been reported for *Clarias gariepinus, Oreochromis mossambicus* and *Cyprinus carpio* (Gabriel et al. 2012; Rao 2006; Muralidharan 2014). In general, decrease in ALT/AST activity has been associated with cellular injury. Then, the positive correlations between liver enzymes activity and some PAHs activity in *R. arcuata* in this study point to the triggering of a biotransformation mechanism rather than


A
Liver
AST
U/mg protein
S1
S2
S3
S4
B
Muscle
AST
C
ALT
D
ALT
E
ALP
F
ALP
G
LDH
H
LDH
I
CK
J
CK

◂**Fig. 3** Aspartate aminotransferase (AST) (**a**, **b**), Alanine aminotransferase (ALT) (**c**, **d**), Lactate dehydrogenase activity (LDH) (**e**, **f**), Alkaline phosphatase (ALP) (**g**, **h**) and Creatine kinase (CK) (**i**, **j**) activities (U/mg protein), in muscle and liver of *R. arcuata*. Values are mean ± SEM. Different letters indicate significant differences between means (One-way ANOVA, $p < 0.05$). Black and gray bars present enzymatic activity in liver and muscle, respectively

hepatotoxicity. As a conclusion, we postulate that AST and ALT activity can be used as biomarkers of PAHs-induced liver biotransformation in *R. arcuata*.

Ldh

Results showed that LDH activity in muscle (1.81 ± 0.07 U/mg) was always higher than in liver tissue (0.39 ± 0.04 U/mg) (Fig. 3g, h). Statistical analyses demonstrated significant differences among samplings and a statistical and positive correlation between muscular and hepatic LDH ($p = 0.67$; $p < 0.05$). Since it has been reported that LDH activation reflects the metabolic capacity of fish tissues after long-term exposure to anoxic conditions caused by contaminated water (Osman et al. 2010; Osioma et al. 2013) and then the higher activity of muscular LDH in S3 and S4 could indicate an increase in anaerobic metabolism to obtain extra energy for PAHs metabolization. Despite this, none of the PAHs congeners showed a positive and significant correlation with the enzyme (Table 3). On the opposite, results showed a negative and significant correlation of LDH muscular activity vs. two compounds. Precisely, in muscular tissue LDH correlated statistically and negatively with 2-M-NA and ACE. A first explanation may be attributed to the accumulation of 2-M-NA, ACE and/or their toxic metabolites in muscle tissue which could affect the synthesis of the enzyme protein. In agreement with this, there are several reports demonstrating LDH activity inhibition in fish muscle due to exposition to pollutants (Asztalos et al. 1990; Li et al. 2004; Gabriel et al. 2012). On the other hand, it has been reported that metabolization of certain contaminants in fish can perform through an aerobic pathway. For instance, Kumari et al. (2011) reported that the aerobic cycle is predominant over the anaerobic metabolism under chromium toxicity in *Labeo rohita*. Indeed, they demonstrated a decrease in LDH muscular activity with the concomitant exponential increase in hepatic SDH activity, which suggests that a high ATP production is needed to meet the stress condition due to chromium toxicity. Therefore, these results allowed setting two new hypotheses for *R. arcuata*: the LDH decrement associated with PAHs exposure could be due to either the aerobic metabolization of those pollutants or the direct inhibition/reduction of the enzyme activity by PAHs.

ALP and CK

Results showed that ALP activity in liver (0.66 ± 0.06 U/mg) was always greater than in muscle (0.02 ± 0.003 U/mg) (Fig. 3e, f). This trend is in agreement with the reported ALP activity in other fish species (Obomanu et al. 2009). There was a statically and positive correlation between ALP isoenzymes ($p = 0.62$, $p < 0.05$) and significant differences were detected between ALP activities in liver samples (Fig. 3e). With regard to PAHs levels, results showed a positive correlation between ALP and AN in liver and muscle (Table 3), suggesting a cause–effect relation. Indeed, literature shows several alterations in ALP activity for fish exposed to varying concentration of toxicants: in general comprising an elevation on its activity in different tissues (Rao 2006; Banaee and Ahmadi 2011; Samanta et al. 2014).

On the other side, CK results showed a much higher activity of the enzyme in muscle for all samples (in

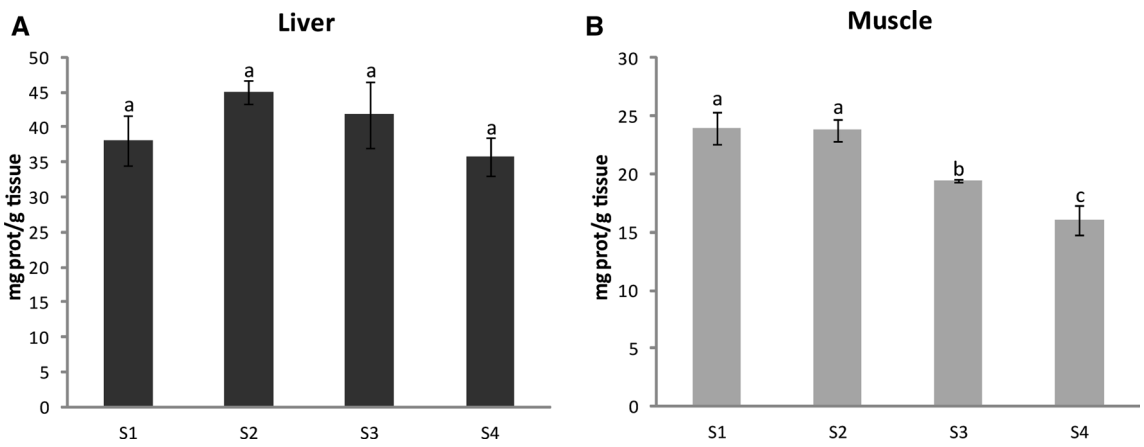


Fig. 4 Protein content (mg protein/g tissue w.w.) in liver (**a**) and muscle (**b**) of *R. arcuata*. Values are mean ± SEM. Different letters indicate significant differences between means (One-way ANOVA, $p < 0.05$)

Table 3 Rho Spearman between PAHs and Aspartate aminotransferase, (AST), Alanine aminotransferase (ALT), Lactate dehydrogenase (LDH), Alkaline phosphatase (ALP), Creatine kinase (CK), protein content (Prot) and lipid peroxidation (LPO) in liver and muscle tissues from *R. arcuata*

	AST liver	AST muscle	ALT liver	ALT muscle	LDH liver	LDH muscle	ALP liver	ALP muscle	CK liver	CK muscle	Prot liver	Prot muscle	LPO liver	LPO muscle
NA	0.37	0.57	0.38	−0.04	0.30	−0.45	0.40	0.44	−0.28	0.47	0.63	0.06	0.49	−0.44
2-M-NA	−0.09	0.57	−0.02	0.18	−0.26	−0.80**	−0.14	0.15	0.26	0.31	0.73	0.56	0.73**	−0.28
ACE	−0.56	0.11	−0.60*	0.15	−0.45	−0.89**	−0.41	0.02	0.65*	0.13	0.56	0.86**	0.84**	0.21
FL	0.40	0.85**	0.59*	0.21	−0.11	−0.34	0.05	0.07	−0.17	0.55	0.56	0.03	0.22	−0.74
PHE	0.37	0.57	0.38	−0.04	0.3	−0.45	0.40	0.44	−0.28	0.47	0.63	0.06	0.49	−0.44
AN	0.35	0.11	0.21	−0.29	0.67*	−0.19	0.67*	0.61*	−0.43	0.22	0.35	−0.13	0.29	0.04
FLU	0.74**	0.50	0.91**	0.03	0.26	0.55	0.34	−0.02	−0.65*	0.40	−0.13	−0.72*	−0.59	−0.76**
PY	0.74**	0.50	0.91**	0.03	0.26	0.55	0.34	−0.02	−0.65*	0.40	−0.13	−0.72*	−0.59	−0.76**
BbF	0.82**	0.58	0.96**	−0.02	0.41	0.41	0.50	0.16	−0.73*	0.50	0.04	−0.69*	−0.5	−0.79**
BaP	0.74**	0.50	0.91**	0.03	0.26	0.55	0.34	−0.02	−0.65*	0.40	−0.13	−0.72**	−0.59	−0.76**

Level of statistical significance: $^{*}p < 0.05$; $^{**}p < 00.1$

average 97.39 ± 7.79 U/mg) than in liver tissue (in average 1.4 ± 0.2 U/mg) (Fig. 3i, j). In fact, it has been reported for various organisms including fishes that CK is mainly a muscular enzyme (Lee et al. 1999). Liver CK activity correlated positively with ACE and negatively with FLU, PY, BbF and BaP (Table 3). Although a decrement of CK activity has been associated with the reduction of fish muscle in other species (Banaee et al. 2014), our data did not show any negative correlation between PAHs and muscle CK. This result might lead to interpreting that the negative correlation of FLU, PY, BbF and BaP vs. muscle protein (Table 3) could not be associated with muscle deterioration and would be related to gluconeogenesis. Similarly, the negative correlation between PAHs and liver CK could be related to the same hepatic metabolism by which FLU, PY, BbF and BaP were positively correlated with hepatic AST and ALT activities.

Lpo

Oxidative stress in organisms, including fish, causes oxidation of polyunsaturated fatty acids known as lipid peroxidation (LPO). It has been reported that LPO determination in fish is a useful biomarker of exposure to organic pollution (Ji et al. 2012; Otitoloju and Olagoke 2011). Results showed that LPO in liver (13.1 ± 2.8 µmol MDA/g prot) was always greater than in muscle tissue (6.4 ± 1.4 µmol MDA/g prot) (Fig. 5). This was consistent as the liver is the principal organ of metabolism and has a critical role in many body processes including the detoxification of chemical compounds. This finding is in agreement with those found in other fish species. Indeed, while LPO concentrations of 1–8 µmol/g of protein were found in the liver, concentrations of 0.3–1 µmol/g of protein were found in the muscle of *Oreochromis niloticus*, (dos Santos Carvalho et al. 2012). Similarly, LPO ranging from 0.31 to 1.31 µmol/g of protein for liver and of 0.17–0.43 µmol/g of protein for muscle was found for *Dicentarchus labrax* (Ferreira et al. 2010). Results also showed that while in liver tissue the minimum peroxidation was found at S3 sampling and in muscle tissue the minimum was found at S4. Since increased levels in LPO in aquatic organisms were observed related to an increase of water temperature (Pellerin-Massicotte 1997), we can discard temperature driven LPO in *R. arcuata*, as the minimum LPO was recorded at maximum water temperatures (Table 2). Then, correlation analyses between MDA levels and PAHs were performed. Results showed a significant and positive correlation of 2-M-NA and ACE with LPO levels in liver (Table 3), pointing these PAHs as oxidative stressors in such tissue. Consistently, low muscle LDH activity, which was inversely correlated with these hydrocarbons, was probably due to an inhibitory effect of these compounds. On the other hand, there was a negative correlation between FLU, PY, BbF and BaP with muscular LPO concentrations. This

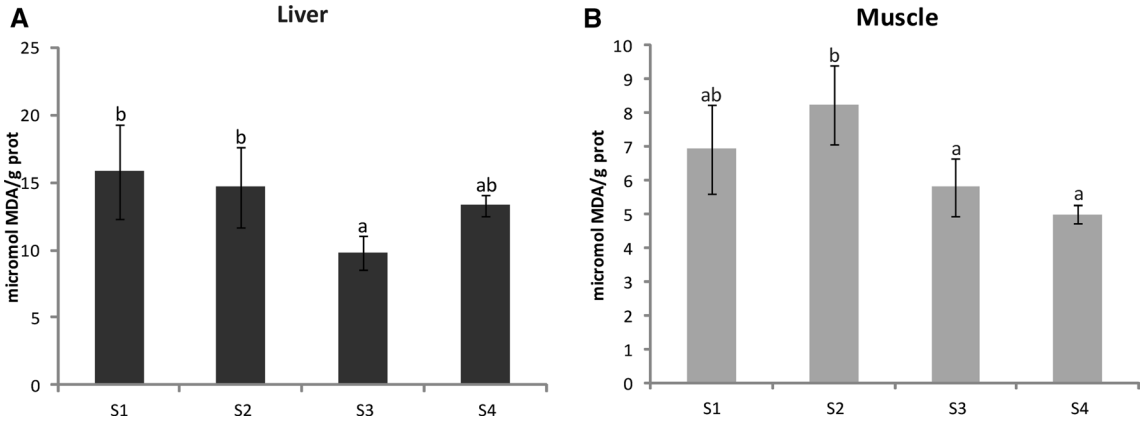


Fig. 5 Lipid peroxidation expressed as malondialdehyde concentration (MDA) (µmol/g prot) in liver (**a**) and muscle (**b**) of *R. arcuata*. Values are mean ± SEM. Different letters indicate significant differences between means (One-way ANOVA, $p < 0.05$)

could indicate that, apart from the liver, PAHs were not triggering oxidative stress in muscle. Mechanisms underlying lipid peroxidation are complex and, furthermore, variable among chemical compounds. PAH-induced lipid oxidative damage has been attributed to the formation of ROS during the detoxification process followed by antioxidant defences failure (Livingstone 2001; Shimada 2006). We can conclude that 2-M-NA and ACE metabolization by *R. arcuata* generated lipid peroxidation as a result of antioxidant system protection saturated, while FLU, PY, BbF and BaP biotransformation did not produced oxidative stress. We base our conclusion due to the fact that LMW-PAHs have higher uptake and lower depuration rates compared to HMW-PAHs (Meador et al. 1995).

Conclusions

In this work, for the first time, fish enzymatic biomarkers were related to muscle PAHs concentration, providing the first record in this topic for the area (EBB, South America).

According to the differential biotransformation of PAHs by fishes, we postulate to use different biomarkers to diagnostic PAHs effects in *R. arcuata*. An increase in hepatic AST and ALT in conjunction to a decrease in muscular protein can be used as biomarkers of a successful liver PAHs biotransformation while muscle LDH inhibition in conjunction with lipid peroxidation levels could be employed as an early indicator of PAHs-induced oxidative stress. Moreover, the activity of the CK in muscle would serve to verify the muscular health status of the fish. Despite our results do not rule out the possibility that other contaminants might be associated with those variations, we conclude that *R. arcuata* serves as an efficient bioindicator organism for South American coastal environments.

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