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Estrés oxidativo, metabolismo lipídico y neurotransmisión en el caracol dulceacuícola *(Pomacea patula)* expuesto a la fracción hidrosoluble de petróleo crudo

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ABSTRACT

Background. Crude oil is a super mixture of chemical compounds and is commonly found in aquatic environments. The *tegogolo* (*Pomacea patula* Baker, 1922) is a Mexican freshwater snail endemic to Lake Catemaco in Veracruz; currently, however, its distribution has expanded to many freshwater ecosystems that suffer the impact of crude oil spills and oil byproducts like fuels. **Goals.** To assess a series of biomarkers involved in oxidative stress, neurotransmission, and fatty acid metabolism in tegogolos exposed to the water-accommodated fraction (WAF) of Maya crude oil (MCO). **Methods**. Tegogolo specimens were exposed to WAF of MCO obtained from loads of 0.1, 1, 10 and 100 mg/L. We evaluated ROS (O_2^{-*} and H_2O_2), oxidative stress (TBARS and RC=O), enzymes involved in antioxidant defense (SOD, CAT, and GPx), some enzymes involved in neurotransmission (AChE, GDA, and CbE activities), and biomarkers of fatty acids metabolism (fatty acids levels and AOX activity). **Results.** Clear biomarkers responses were observed only in some tissues. ROS were clearly higher than controls in the foot, head, and kidney; however, others biomarkers of oxidative stress remain statistically unchanged. SOD response was irregular with respect to controls and treatments. In contrast, CAT (foot) and GPx (foot and intestine) were the more active enzymes and their activities were higher than in controls. The responses of some enzymes involved in neurotransmission suggest that compensation mechanisms exist between AChE and GDA in the foot and head. Fatty acids metabolism increased with exposure to WAF; however, these types of biomarkers seem unsuitable for monitoring the toxic effects produced by WAF at low environmental concentrations. **Conclusions.** We can conclude that under the exposure conditions discussed herein, the tegogolos showed acclimation to WAF of Maya crude oil by complex mechanisms.

Keys words: Crude oil, fatty acid metabolism, neurotransmission, oxidative stress, snails.

RESUMEN

Antecedentes. El petróleo crudo es una supermezcla de compuestos químicos y su presencia en ambientes acuáticos es común. El tegogolo (*Pomacea patula* Baker, 1922) es un caracol dulceacuícola endémico del lago de Catemaco, Veracruz, pero su distribución se ha expandido a muchos ecosistemas de agua dulce que sufren el impacto de los derrames de crudo y sus derivados. **Objetivos.** Evaluar un conjunto de biomarcadores involucrados en el estrés oxidativo, neurotransmisión y metabolismo de ácidos grasos en especímenes expuestos a la fracción hidrosoluble (FH) de petróleo crudo maya (PCM). **Métodos.** Los caracoles se expusieron a la FH del PCM a partir de las cargas de 0.1, 1, 10 y 100 mg/L. Se midieron biomarcadores como ROS (O_2^{-*} y H_2O_2), daño oxidativo (TBARS y RC=O) y enzimas involucradas en defensa antioxidante (SOD, CAT y GPx), neurotransmisión (AChE, GDA y CbE) y biomarcadores del metabolismo de ácidos grasos (niveles de ácidos grasos y actividad AOX). **Resultados.** En algunos tejidos del tegogolo se observó una clara respuesta de los biomarcadores. Las concentraciones de ROS fueron superiores a los controles en el pie, la cabeza y el riñón; sin embargo, otros biomarcadores del estrés oxidativo no presentaron cambios significativos. La SOD fue irregular con respecto a los controles y entre tratamientos. Por el contrario, la CAT (pie) y la GPx (pie e intestino) fueron más activas. Las respuestas de las enzimas involucradas en la neurotransmisión sugieren un mecanismo compensatorio entre AChE y GDA en el pie y la cabeza. El metabolismo de los ácidos grasos aumentó con los tratamientos, aunque estos biomarcadores no parecen ser adecuados para monitorear los efectos de la FH del PCM a bajas concentraciones ambientales. **Conclusiones.** Es posible que en las condiciones de exposición los tegogolos mostraran aclimatación a la FH del PCM por mecanismos complejos.

Palabras clave: Caracoles, estrés oxidativo, metabolismo de ácidos grasos, neurotransmisión, petróleo crudo.

INTRODUCTION

Petroleum is a super complex mixture of chemical compounds with an estimated minimum of 50,000 different substances (Marshall & Rodgers, 2004). The main groups of compounds are aliphatic hydrocarbons, polyaromatic hydrocarbons (PAHs), heterocyclic of nitrogen and sulphur, endocrine compounds, and saturated fatty acids (Benassi *et al*., 2013). In addition, alkenes and heavy metals have been identified (Cote, 1976). Petroleum and its products are common pollutants in the aquatic environment (Crunkilton & Duchrow, 1990). As an example of the impacts of crude oil spills during one such spill in 2010, approximately 200 million gallons of South Louisiana crude oil were released into the northern Gulf of Mexico over the course of 87 days (Crone & Tolstoy 2010). Communities of aquatic macro-invertebrates have been suitable for environmental risk assessment in streams and rivers following spills of crude oil (Poulton *et al*., 1998). Recently, numerous studies have reported that snails are appropriate for toxicity testing because they are benthic and have reduced mobility (Ma *et al*., 2014a). In addition, it is possible to perform tests under laboratory conditions to evaluate the potential damage provoked by environmental pollutants (Whitehead, 2013). One of the most common toxic effects in aquatic organisms is related to oxygen metabolism. Under aerobic conditions, cells can produce reactive oxygen species (ROS); in addition, the antioxidant system in aquatic animals includes specific enzymes to reduce ROS levels (Hermes-Lima, 2004; Lushchak, 2011). Nevertheless, if antioxidant systems do not reduce ROS, their concentrations could be deleterious and induce oxidative stress. Oxidative stress is characterized by oxidation of biomolecules such as lipids, proteins, and nucleic acids (Lushchak, 2011). These oxidative damages caused by crude oil or its components could compromise energy provision as well as high energy demanding organs, such as the nervous system (Beal, 1995). Fatty acids (FA) are essential as energy sources and their concentrations could be suitable as biomarkers (Kowalczyk-Pecka *et al*., 2017). The acyl-CoA oxidase (AOX) is an enzyme that belongs to peroxisomal β-oxidation, which is the metabolic pathway to obtain energy from fatty acids (Cajaraville *et al*., 2003). Further, some enzymes, such as acetylcholinesterase (AChE) and glutamate decarboxylase (GDA), are involved in neurotransmission, i.e., a key function in the nervous system (Basu, 2015). In addition, carboxylesterases (CbE) are enzymes involved in the detoxification of compounds that can inhibit the activity of AChE (Fukuto, 1990).

The tegogolo *Pomacea patula* (Baker, 1922) is an edible freshwater mollusk, endemic to Catemaco Lake in Veracruz, Mexico (Carreón-Palau *et al*., 2003). However, due to the economic importance of this species, its culture has been developed in Central and Southern Mexico. The southern region of Mexico is an important supplier of oil resources (CNH, 2017). In addition, pollution associated with the petroleum industry has been documented in this area (PROFEPA, 2017). The objective of this study was to select and assess a series of biomarkers involved in oxidative stress (contents of O_2^{-*} and H_2O_2, lipid peroxidation, and protein oxidation), antioxidant defense activity (SOD, CAT, and GPx), fatty acid metabolism indicators (fatty acid levels and acyl-CoA oxidase), and neurotransmission enzymes in the *Pomacea patula* mollusk exposed under controlled conditions to the WAF of Maya crude oil. The study compared some of the widely documented aspects related to toxic effects caused by petroleum, such as the oxidative stress response and other less-studied aspects, such as fatty-acid metabolism and neurotransmission indicators.

MATERIALS AND METHODS

Animals and experimental design. Cultured specimens of the Mexican freshwater snail tegogolo (*Pomacea patula*) were obtained from an aquaculture center located in Zacatepec (Morelos, México). Snails were maintained in glass aquariums with a capacity of 145 L using semi-hard synthetic water (0.22 g $MgSO_4$, 0.18 g $NaHCO_3$, 0.08 g KCl and 0.13 g $CaSO_4.2H_2O$ per L) with constant aeration at 24±1 °C under natural light-dark cycle at Mexico City latitude for three months until the experiments started. The snails were feed twice a week with lettuce obtained from a local supermarket. Healthy tegogolos of similar size (51.5 ± 2.96 mm) and weight (26.85 ± 3.87 g) were selected for the test. Groups of seven tegogolos were exposed for 96 h to the four concentrations of WAF obtained from different loads (0.1, 1, 10 and 100 mg/L) of Maya crude oil. The WAF fraction was obtained by the Singer *et al.* (2000) method. Maya crude oil was supplied by the Instituto Mexicano del Petróleo (Mexico). Specimens of *P. patula* were treated in glass aquariums protected from the light in a total volume of 10 L. The control group was exposed to semi-hard synthetic water.

Chemical analysis. A sample of 1 L of exposure medium was collected after the snails were euthanized. The quantification of PAHs was undertaken with a Biotek SynergyMX spectrofluorometer, using certified analytical standards obtained from Chem Service, Inc. (West Chester, PA), as previously documented (Dzul-Caamal *et al*., 2016).

Biomarker evaluation. Test specimens were measured with a Vernier caliper and weighed in analytical balance with a sensitivity of 0.1 g. Organisms were euthanized by fast-freezing them at -20 °C/30 min, as previously reported (Nica *et al*., 2015). Tissues of tegogolo (foot, head, intestine, mantle, digestive gland, and kidney) were obtained according to the anatomy of gastropods (Barnes, 1980). All tissues were weight and homogenized in a Glas-Col™ homogenizer in an ice bath as follows: foot and head in 10 mL of PBS 1X; the rest of the tissues in 3 mL of PBS 1X. 1.0 mL of the homogenates was centrifuged at 4,980 X g (9,000 rpm) and 4° C for 15 min in a Hermle Labnet Z216MK centrifuge to obtain the cytosolic fraction. The uncentrifuged and cytosolic fractions were stored at -70°C until the biomarker assay was performed (less than 2 weeks).

Table 1 summarizes the methods used to assess biomarkers.

Statistical analysis. Biomarker results were compared to controls and treatments by ANOVA, followed by post-hoc Dunnet´s Comparison Test. Statistical significance was set at $p \leq 0.05$ for all tests. Analyses were done in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). To determine the impact of the WAF of MCO in tissues of tegogolos, we calculated the IBRv2 according to Sanchez *et al.* (2013) and employed the general IBRv2 (gIBRv2) proposed by Dzul-Caamal *et al.* (2016) to integrate the IBRv2 values by tissue and treatment.

RESULTS

PAHs concentrations in the exposure medium. The concentration of the PAHs increased in relation to the load of Maya crude oil at the end of the experiment (Table 2). The proportion of individual compounds between loads did not show large variations, except for phenanthrene, benzo[a]pyrene (BaP), and fluoranthene. BaP was the PAH with the hig-

Table 1. Methods for biomarker evaluation in *Pomacea patula* (Baker, 1922) exposed to the water-accommodated fraction (WAF) of Maya crude oil

Biomarker	EC number	Tissue under study	Studied fraction	Reference
O_2^{-*} levels	NA	F, H, I, M, DG, K	Cytosolic	Dzul-Caamal *et al.* (2016)
H_2O_2 levels	NA	F, H, I, M, DG, K	Cytosolic	Dzul-Caamal *et al.* (2016)
TBARS	NA	F, H, I, M, DG, K	Uncentrifuged	Buege & Aust (1978)
RC=O	NA	F, H, I, M, DG, K	Uncentrifuged	Levine *et al.* (1994)
SOD	1.15.1.1	F, H, I, M, DG, K	Cytosolic	Misra & Fridovich (1972)
CAT	1.11.1.6	F, H, I, M, DG, K	Cytosolic	Radi *et al.* (1991)
GPx	1.11.1.9	F, H, I, M, DG, K	Cytosolic	Lei *et al.* (1995)
FA levels	NA	F, H, I, M, DG, K	Uncentrifuged	Cheng *et al.* (2011)
AOX	1.3.3.6	F, H, I, M, DG, K	Cytosolic	Holth *et al.* (2011)
AChE	3.1.1.7	F, H	Cytosolic	Ellman *et al.* (1961)
GDA	4.1.1.15	F, H	Cytosolic	Yu *et al.* (2011)
CbE	3.1.1.1	F, H	Cytosolic	Hotta *et al.* (2002); Kumar *et al.* (2010)

O_2^{-*}: superoxide anion; H_2O_2: hydrogen peroxide; TBARS: lipid peroxidation evaluated as thiobarbituric acid reactive substances; RC=O: protein oxidation evaluated as carbonyls (RC=O) content; SOD: superoxide dismutase activity (CuZn-SOD plus Mn-SOD); CAT: catalase activity; GPx: selenium-dependent glutathione peroxidase activity; FA: fatty acids; AOX: acyl-CoA oxidase activity; AChE: acetyl cholinesterase activity; GDA; glutamate decarboxylase activity; CbE: carboxylesterase activity. EC: Enzyme Commission number; NA: not applicable. F: foot; H: head; I: intestine; M: mantle; DG: Digestive gland; K: kidney.

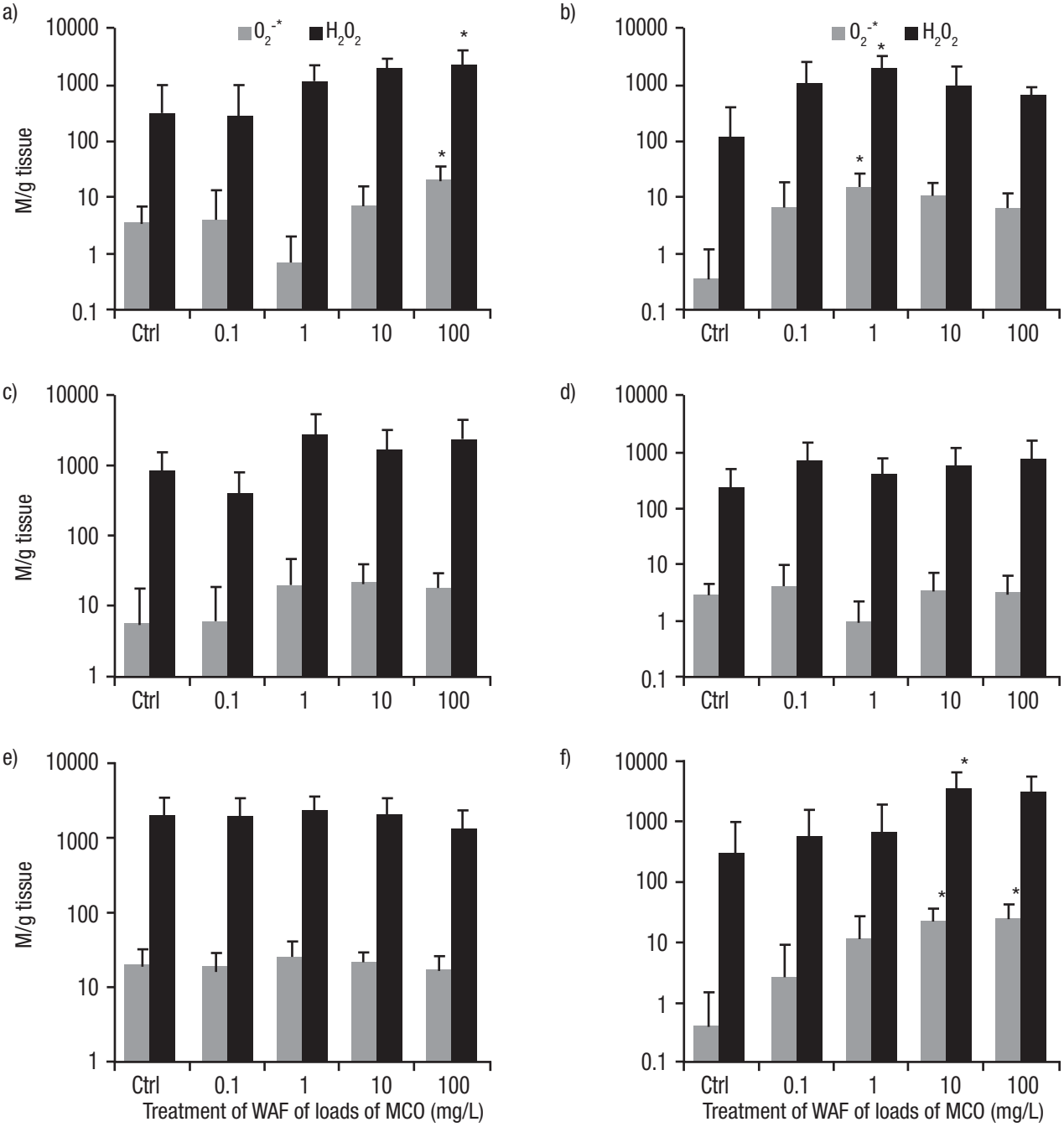
hest proportion in all loads. There was a lower percentage of total low molecular weight (LMW) PAHs than the high molecular weight (HMW) PAHs, due to their volatility and the characteristics of heavy crude oil.

Biomarker responses. Most of the treatments show that the content of ROS was higher in the tissues of *P. patula* exposed to the WAF of Maya crude oil, as compared to controls. The tendency of O_2^{-*} and H_2O_2 was similar in the tissues under study. Wider differences were observed in the higher concentration of WAF particularly for the foot and kidney (p <0.05, Fig. 1a,f). A higher content of ROS was found in the head at 1 mg/L load (p <0.05, Fig 1b). In the intestine, the higher concentration of H_2O_2 was recorded at 1 mg/L; meanwhile, the high levels of O_2^{-*} was detected at 10 mg/L (Fig. 1c). In the tegogolo mantle, the content of ROS was irregular in treatments and was higher at the lower load of Maya crude oil (0.1 mg/L, Fig. 1d). Notably, in the digestive gland, the levels of ROS were similar to controls at 0.1 and 1 mg/L. However, from 1 to 100 mg/L, a slight reduction was observed (Fig. 1e).

Table 2. Concentration of polyaromatic hydrocarbons (PAHs) in µg/L medium of exposure after 96 h of exposure. Mean ± standard deviation.

PAH	Treatment (WAF of load of MCO in mg/L)			
	0.1	1	10	100
Naphthalene	3.53±0.22	2.91±0.27	3.79±0.13	2.78±0.39
Acenaphthene	36.11±12.56	36.45±9.83	41.07±1.59	60.31±20.17
Anthracene	0.06±0.82	0.61±0.20	0.75±0.30	0.78±0.25
Phenanthrene	58.30±23.02	74.30±21.65	45.82±3.67	148.23±23.85
LMW-PAHs (2-3 rings)	97.78±16.68	114.28±30.63	91.06±10.96	212.12±52.32
Pyrene	16.57±6.06	22.06±2.35	24.97±1.28	33.74±7.48
Fluoranthene	24.76±14.90	21.17±4.74	24.64±4.64	60.32±10.31
Benzo[a]anthracene	1.05±0.42	1.24±0.16	1.54±0.16	2.41±0.48
Chrysene	3.86±1.06	3.35±0.54	3.66±0.83	4.88±0.60
Benzo[b]fluoranthene	1.34±1.65	2.20±1.02	2.52±1.49	4.27±0.93
Benzo[a]pyrene	204.75±585.30	263.70±331.30	444.70±528.98	694.44±158.82
Indeno[1,2,3-*c,d*]pyrene	1.38±0.25	1.21±0.16	1.25±0.15	1.97±0.41
HMW-PAHs (≥3 rings)	250.69±32.03	311.19±145.6	498.73±238.47	488.91±247.84
Total PAHs	348.47±44.06	452.47±330.44	589.76±536.17	701.03±637.03
%LMW	28.06	25.26	15.44	30.26
%HMW	71.94	68.78	84.56	69.74
LMW:HMW	3	3	5	2

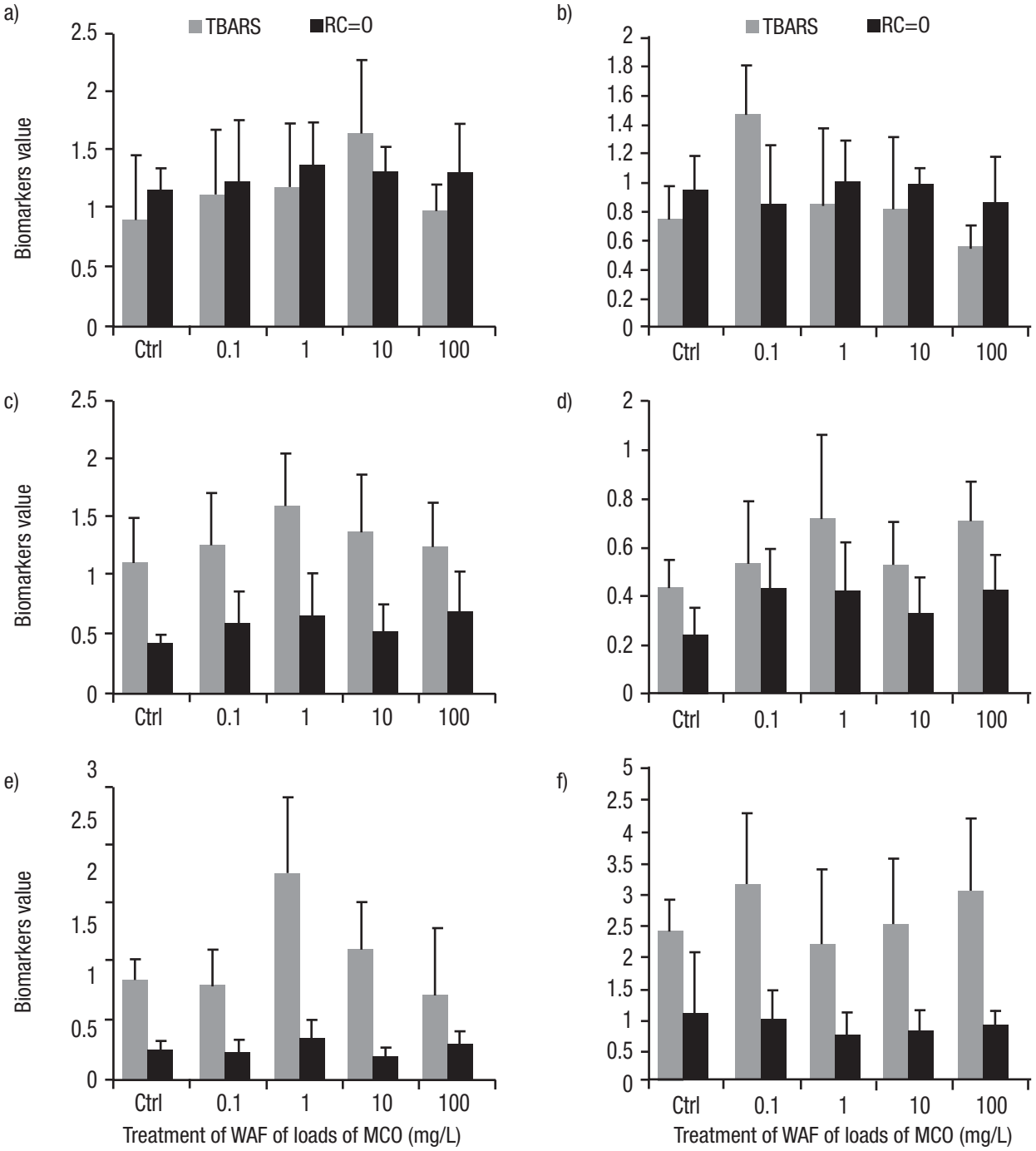
LMW: Low molecular weight; HMW: High molecular weight.



Figures 1a-f. Content of ROS (O_2^{-*} y H_2O_2) in tissues of *Pomacea patula* exposed under controlled conditions to the WAF of MCO for 96 hours. Bars represent standard error of the mean. a) Foot, b) Head, c) Intestine, d) Mantle, e) Digestive gland, f) Kidney.

Oxidative stress. The response of lipid peroxidation and protein oxidation in WAF treated gastropods was inconsistent compared to controls. In addition, the responses of these biomarkers were erratic among tissues. We observed no significant differences in the foot (Fig. 2a). In the head, differences among TBARS and RC=O were found. The lipid peroxidation was higher at 0.1 mg/L; however, this oxidative damage diminished at greater WAF concentrations. Likewise, protein oxidation was similar to controls (Fig. 2b). Of importance, in the intestine, mantle, and digestive gland of *P. patula* treated at 1 mg/L, we detected higher oxidative damage (Figs. 2c-e). In the kidney, the greater level of lipid peroxidation was observed at 0.1 mg/L of crude oil load. In contrast, the protein oxidation in this tissue was lower than controls in all treatments (Fig. 2f).

Activities of enzymes involved in antioxidant defense. Several patterns of responses were detected in the activities of enzymes involved in antioxidant defense (SOD, CAT, and GPx) in *P. patula* exposed to the WAF of Maya crude oil. In the tegogolo foot and head, the SOD activity did not show a trend compared to controls and treatments. In the foot, the activity of CAT increased with WAF concentration. In contrast, in the head, this response was inversely linked with the load of crude oil from 1 to 100 mg/L. The activity of GPx was clearly induced by exposure to the WAF of Maya crude oil compared to controls. Significant differences were found in this snail's foot treated at 10 and 100 mg/L (p <0.05); in the head, significant differences were documented at 10 mg/L (p <0.05; Figs. 3a-b). In the intestine, the activities of CAT and GPx were



Figures 2a-f. Oxidative damage measured as lipid peroxidation (TBARS) and protein oxidation (RC=O) in tissues of *Pomacea patula* exposed under controlled conditions to the WAF of MCO for 96 hours. TBARS were presented as mmol TBARS/g tissue and RC=O as mmol RC=O/mg protein/g tissue. Bars represent standard error of the mean. a) Foot, b) Head, c) Intestine, d) Mantle, e) Digestive gland, f) Kidney.

higher than control specimens with the exception of CAT at 100 mg/L; however, in this tissue, the catalysis of SOD was irregular compared to controls and treatments (Fig. 3c). CAT activity in the mantle was induced at 0.1 and 1 mg/L. Nevertheless, at high concentrations (10 and 100 mg/L), CAT activity was reduced. SOD and GPx showed an irregular tendency in their metabolisms (Fig. 3d). In the digestive gland, higher activities of CAT and GPx were observed at 100 mg/L; meanwhile, SOD was similar and lower than controls (Fig. 3e). In the kidney, the catalysis of these enzymes reached a peak at 1 mg/L of a load of Maya crude oil. However, these activities were reduced at 10 and 100 mg/L (Fig. 3f).

Fatty acid metabolism. The concentration of FA in the foot and intestine of *P. patula* exposed to the WAF of Maya crude oil were higher than in controls (Figs. 4a,c). However, in the head, levels of FA were similar among exposed and unexposed snails (Fig. 4b). In the digestive gland and kidney of *P. patula* treated with the WAF, the concentration of FA was lower than controls, except those observed in the treatment with the WAF obtained from 1 mg/L of Maya crude oil (Figs. 4e-f). In the mantle, the content of these biomolecules was irregular compared to treatment (Fig. 4d).

The activity of AOX was higher in the foot and in the kidney of *P. patula* than in the control in all treatments, with exception of the catalysis of this enzyme detected in the heads of snails exposed to the WAF of 0.1 mg/L of MCO (Figs. 5a-f). In contrast, in the mantle and in the digestive gland, an irregular activity of AOX was observed compared to controls (Figs. 5d-e). The maximum activities of AOX were detected at the WAF at the higher load of MCO (100 mg/L) solely in the snails' foot and digestive gland.

Activity of enzymes involved in neurotransmission. In the head and foot of *P. patula* exposed to the WAF of MCO, AChE was higher than controls in all cases and was related with WAF concentration. In addition, significant differences compared to controls were noted at WAF loads of MCO at 1, 10, and 100 mg/L ($p \leq 0.05$) (Figs. 6a-b).

The catalysis of GDA in the head was lower than controls with a concentration-dependent response with statistical differences at WAF of 10 and 100 mg/L of MCO (Fig. 6d). Similarly, the activity of this enzyme in the foot was lower than control; however, at the WAF of 100 mg/L of MCO, an increase in this enzyme was found, even greater than controls (Fig. 6c).

In general, the activity of CbE in the head and foot of *P. patula* treated with the WAF of Maya crude oil was irregular compared to the treatments. Nevertheless, at the WAF of the lower load of MCO, an increase of this enzyme was detected in both tissues (Figs. 6e-f).

Integrated biomarker response. Higher values of gIBRv2 were found in the head and foot of *P. patula* exposed to the WAF of MCO followed by the digestive gland, intestine, kidney, and mantle. In terms of treatments, the higher value of gIBRv2 was found in specimens exposed to the WAF at 1 mg/L and by treatments of the WAF at 0.1, 100, and 10 mg/L, respectively (Table 3).

DISCUSSION

Several natural sources of pro-oxidants forces have been documented in aquatic organisms such as the electron-transport chain, oxygenases, auto-oxidation, and dependent systems of NADPH oxidases (Lushchak, 2011). Although the pro-oxidant/antioxidant balance in snails exposed to diverse pollutants has been studied, a lack of information about the content of ROS prevails. In this study, following exposure to the WAF of Maya crude oil, the levels of ROS in the tissues under study were higher than in their respective controls in the head of *P. patula* treated with the WAF from 1.0 mg/L, followed by the kidney (10 and 100 mg/L), and the foot (100 mg/L). Since crude oil contains more than 50,000 chemicals (Marshall & Rodgers, 2004), it is not possible to attribute the generation of ROS solely by decoupling the electron flux during the catalysis of specific isoforms of the CYP450 superfamily (Arzuaga & Elskus, 2010). The WAF has high bioavailability to organisms and its chemical characteristics are related to the type of crude oil. There is a large variation in the chemical composition of different oils. The WAF of heavy crude oil, such as the Maya type, contains large amounts of water-soluble heavy molecules and microscopic oil droplets that are associated with HMW PAHs (Couillard *et al.*, 2005). Chemical transformations occur in the soluble molecules allowing the decrease of the concentrations of the LMW compounds within a period of 24 h (Nebo et al., 1998), because of these transformations; in this study, the amounts of HMW PAHs overcame the

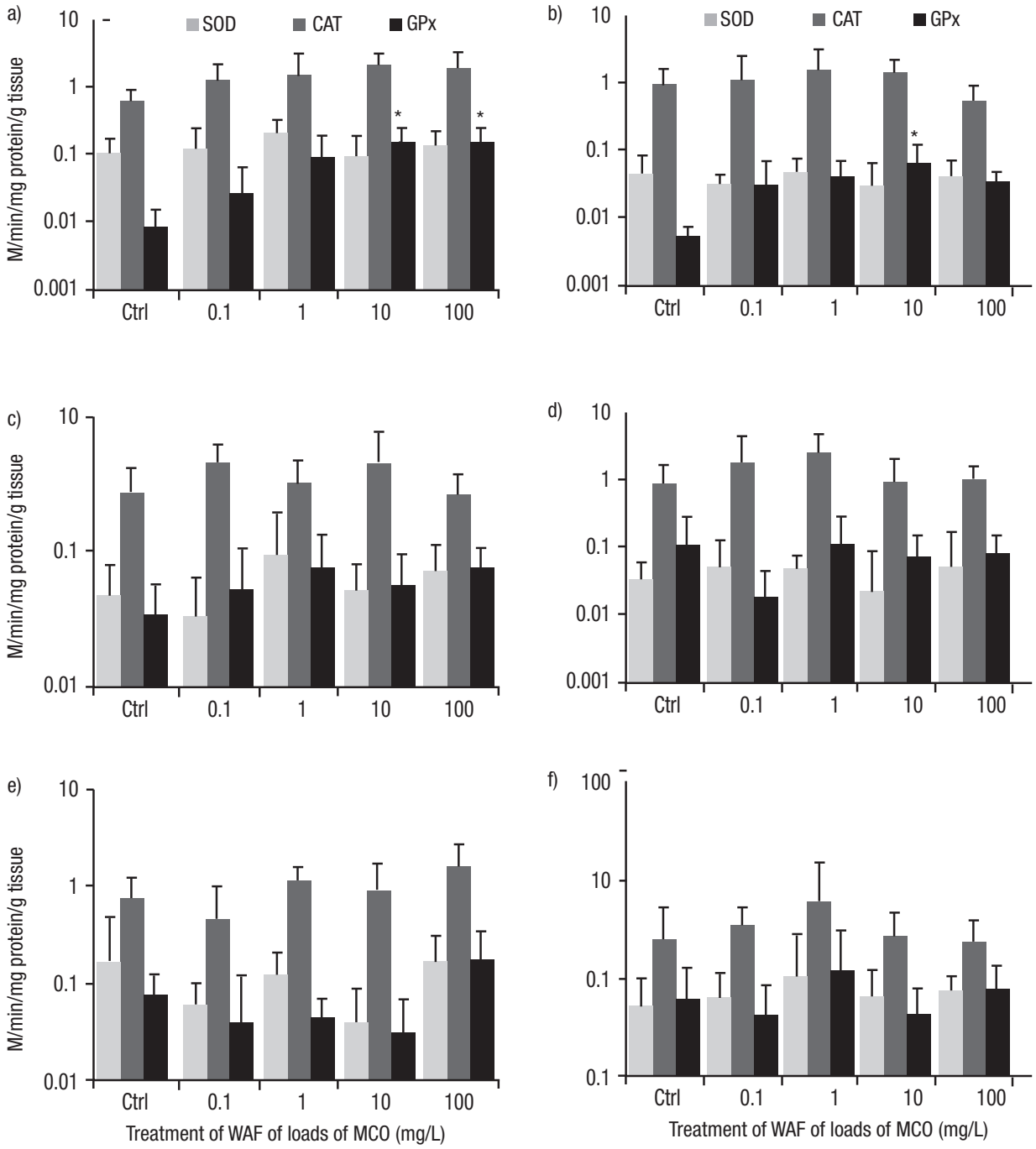
Table 3. A values of the Integrated Biomarker Response index, version 2 (IBRv2) and general Integrated Biomarker Response index, version 2 (gIBRv2) for biomarkers in tissues of *Pomacea patula* exposed to water-accommodated fraction (WAF) of different loads of Maya crude oil for 96 h.

Loads of Maya crude	Biomarker	Foot	Head	Intestine	Mante	Digestive gland	Kidney
0.1 mg/L	O_2^{-*}	-0.920	2.612	-1.126	-0.193	-0.619	2.685
	H_2O_2	-3.423	-0.584	-4.803	-1.164	-2.567	-1.889
	TBARS	-0.212	0.203	-0.156	0.336	0.771	-0.440
	RC=O	-0.606	-0.614	0.702	1.139	1.252	-0.607
	SOD	0.551	0.499	0.147	1.998	-1.379	0.118
	CAT	0.877	-0.336	1.313	1.303	-0.334	-0.383
	GPx	3.474	3.351	1.979	-0.880	0.159	-0.808
	FA	0.549	0.062	1.452	1.315	1.307	-0.651
	AOX	-1.110	-1.550	0.737	-0.733	-1.205	-1.500
	AChE	1.530	-1.921				
	CbE	0.509	0.346				
	GDA	-4.092	-3.090				
	IBRv2	17.851	15.169	12.415	9.060	9.592	9.081

Table 3 (continuation).

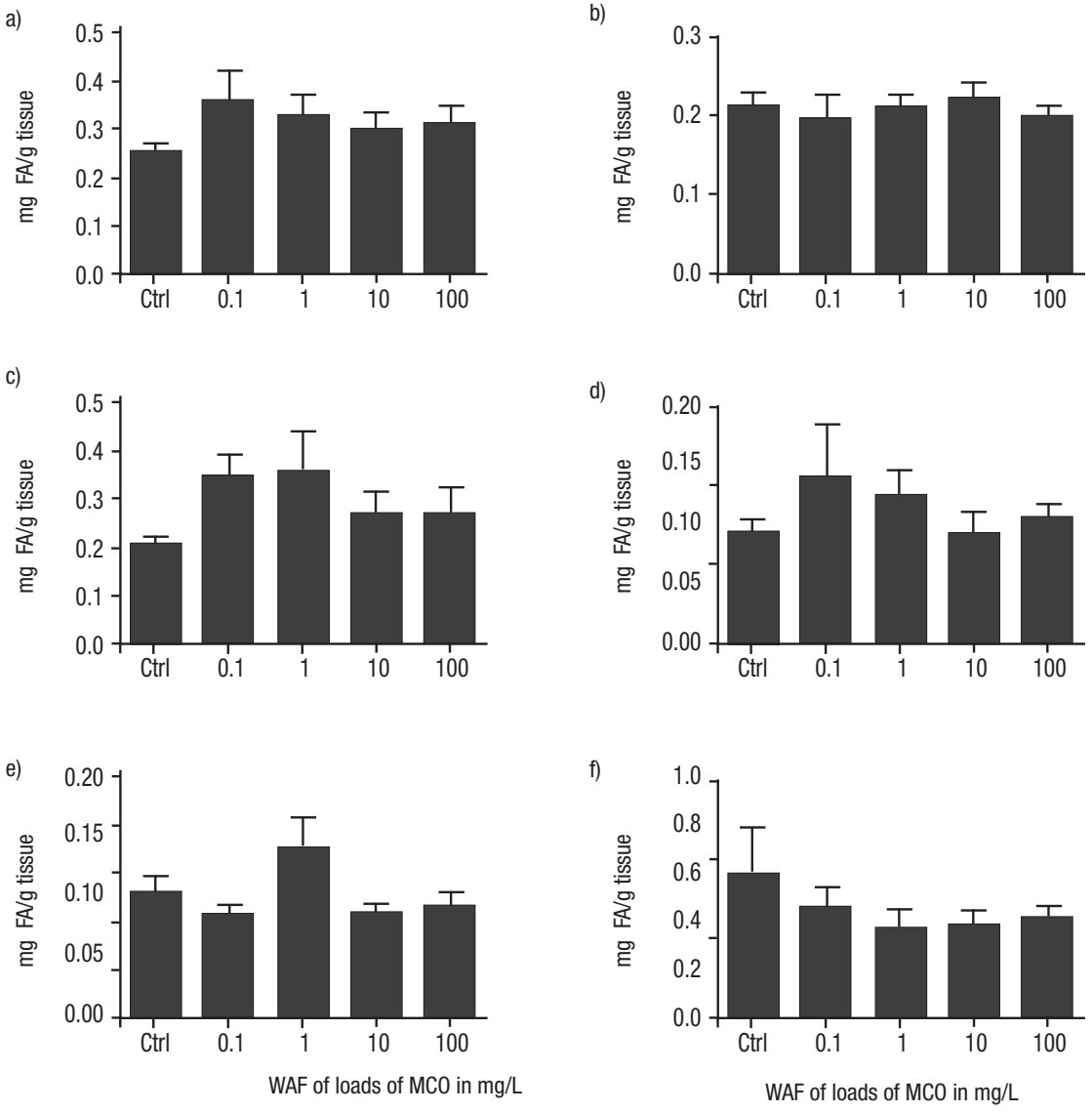
Loads of Maya crude	Biomarker	Foot	Head	Intestine	Mante	Digestive gland	Kidney
1 mg/L	O_2^{-*}	-2.509	2.696	0.632	-2.909	-1.022	2.700
	H_2O_2	1.666	0.540	1.583	1.940	3.417	2.254
	TBARS	-0.122	-0.333	-0.418	0.818	1.457	-1.062
	RC=O	-0.322	-0.489	-1.109	1.184	0.910	-0.943
	SOD	1.199	0.856	1.623	1.954	-0.521	0.351
	CAT	0.659	-0.167	-0.208	1.756	0.860	-0.200
	GPx	3.987	3.189	2.002	1.096	-0.726	0.221
	FA	0.423	0.104	0.745	1.079	1.376	-0.810
	AOX	-0.813	-1.288	-1.439	0.084	-1.534	-1.478
	AChE	0.221	1.723				
	CbE	-0.347	-0.085				
	GDA	-3.584	-3.241				
	IBRv2	15.850	14.712	9.760	12.820	11.822	10.020
10 mg/L	O_2^{-*}	-0.530	2.574	1.363	-0.296	-0.695	2.482
	H_2O_2	1.433	0.958	2.315	0.216	2.888	1.276
	TBARS	-0.069	-0.339	-0.812	0.638	0.982	-0.840
	RC=O	-0.635	-0.467	-0.423	1.175	0.673	-0.697
	SOD	0.168	0.523	0.225	0.652	-1.372	-0.045
	CAT	0.769	-0.184	0.571	0.591	0.980	-0.842
	GPx	4.285	3.662	1.388	0.383	0.001	-0.512
	FA	0.077	0.183	0.090	0.625	1.144	-0.535
	AOX	-1.192	-0.792	0.158	-0.431	-1.440	-1.208
	AChE	0.138	1.813				
	CbE	-0.554	0.069				
	GDA	-3.898	-3.480				
	IBRv2	13.748	15.044	7.346	5.008	10.175	8.439
100 mg/L	O_2^{-*}	0.343	2.490	0.780	-0.959	-1.898	2.613
	H_2O_2	1.416	0.968	1.571	0.202	4.655	1.402
	TBARS	-0.650	-0.314	-0.990	0.827	-0.628	-0.748
	RC=O	-0.721	-0.259	0.326	1.249	0.629	-0.663
	SOD	0.424	1.077	1.139	1.945	0.268	0.030
	CAT	0.514	-0.597	-0.699	0.346	1.583	-0.963
	GPx	4.076	3.506	2.213	0.261	2.640	-0.128
	FA	0.003	0.409	0.151	0.609	0.387	-0.525
	AOX	-0.898	-1.595	-1.104	-0.953	-0.941	-1.690
	AChE	0.106	1.348				
	CbE	-1.044	0.522				
	GDA	3.397	-3.945				
	IBRv2	13.593	17.031	8.971	7.351	13.630	8.761
	gIBRv2	61.042	61.956	38.492	34.239	45.219	36.301

temporary decline. The low variation of most individual PAHs between the different loads showed a reduced WAF weathering during the experiment. Besides, crude oil contains transition metals such as Fe, Mn, and Cr, among others, which are involved in ROS induction by interference of a metal-related process also brought about by generation of free radicals (Lushchak, 2011). Thus, we may speculate that HMW PAHs in addition to transitions metals and other compounds were responsible for ROS generation. With regard to the organ-specific response, it has been documented that some organs of the central nervous system are located in the head of the snails (Battonyai *et al.*, 2012; Battonyai *et al.*, 2014). In addition, the foot is responsible for locomotion (Miyamae *et al.* 2010; Longley, 2014). Thus, the cells which make up this system require large amounts of energy in order to function (Rigon *et al.*, 2010; Panov *et al.*, 2014). However, during the generation of energy, ROS



Figures 3a-f. Activity of enzymes involved in antioxidant defense (SOD, CAT, and GPx) in tissues of *Pomacea patula* exposed under controlled conditions to WAF of MCO for 96 hours. Bars represent standard error of mean. a) Foot, b) Head, c) Intestine, d) Mantle, e) Digestive gland, f) Kidney.

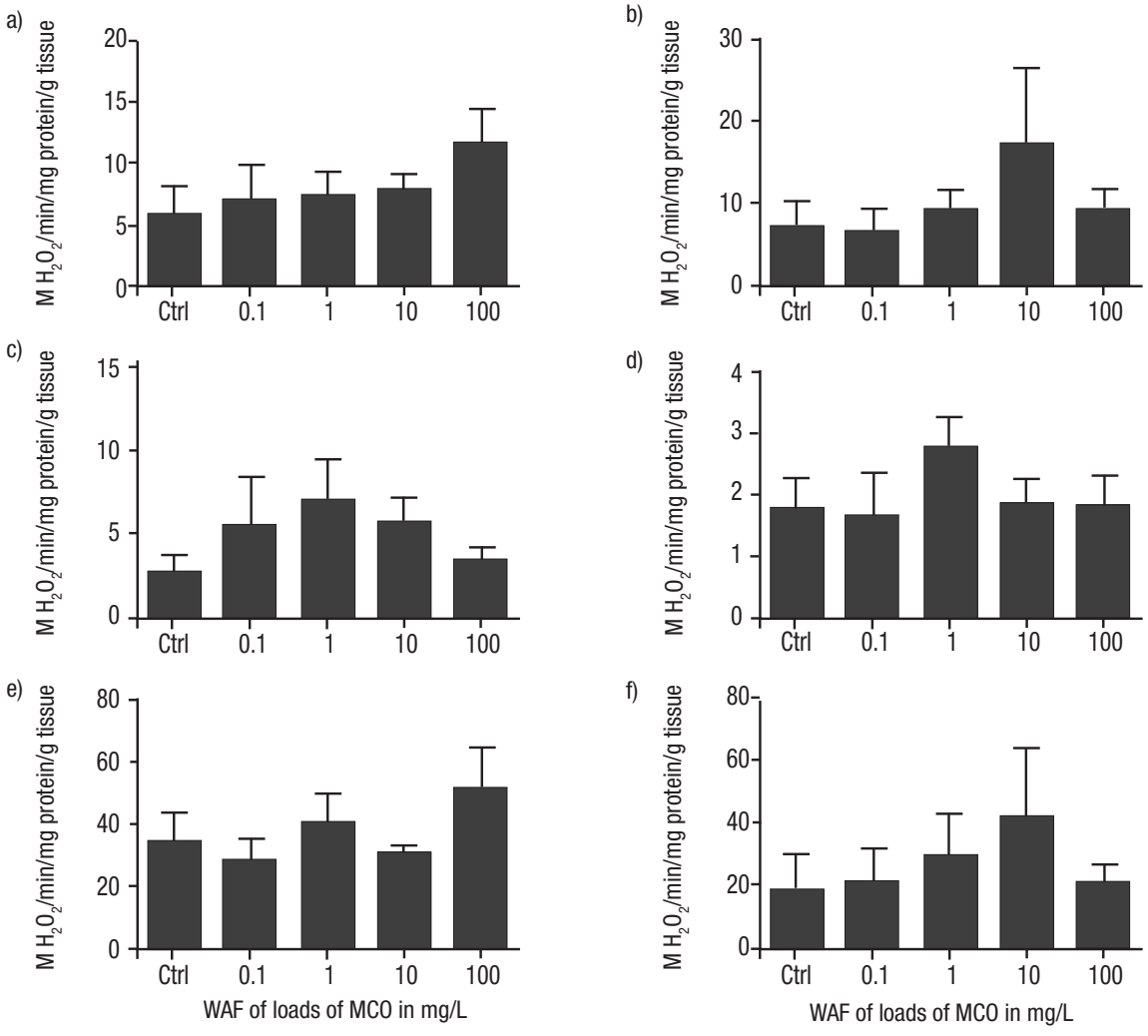
could be induced in the mitochondria which is the principal organelle related to energy production (Sharp & Haller, 2014). The results in this study could be associated to these events; however, more studies are needed to clarify this point. Despite the lack of information about ROS levels in the snails' kidneys, in fish a positive and negative selection of hematopoietic progenitor cells occurs (Davidson & Zon, 2004; Stachura *et al.*, 2009) that involve the generation of ROS required for the extrinsic pathway of apoptosis. Besides, ROS induction is a defense mechanism of some immunocompetent mature cells, which are plentiful in the kidney (Janeway & Medzhitov, 2002).Oxidative stress response is the most reported biological response in snails exposed to several chemical compounds. In this study, the lipid peroxidation assessed as TBARS and protein oxidation evaluated as carbonyl proteins were greater in the tissues of *P. patula* exposed to the WAF of Maya crude oil compared to controls. Nevertheless, the differences observed were not statistically significant. Similar responses were found in some gastropods exposed to compounds different from crude oil (Cochón *et al.*, 2007; Zheng *et al.*, 2013). However, in other mollusk species, contrasting findings have been documented (Ansaldo *et al.*, 2005; Kaloyianni *et al.*, 2009; Itziou *et al.*, 2011a; Itziou *et al.*, 2011b; Ali *et al.*, 2012; Ma *et al.*, 2014a; Wang *et al.*, 2014). Results of this study indicate the presence of efficient processes to reduce the induction of ROS in the foot, head, and kidney of *P. patula*, probably by unspecific antioxidant systems, as well as an efficient process mediated through the ATP-dependent ubiquitination, via endogenous proteases such as cathepsin c, calpain, and trypsin for degradation of RC=O (Hermes-Lima, 2004). This process is aimed at auto-regulating the oxidative damage induced by the WAF of Maya crude oil.



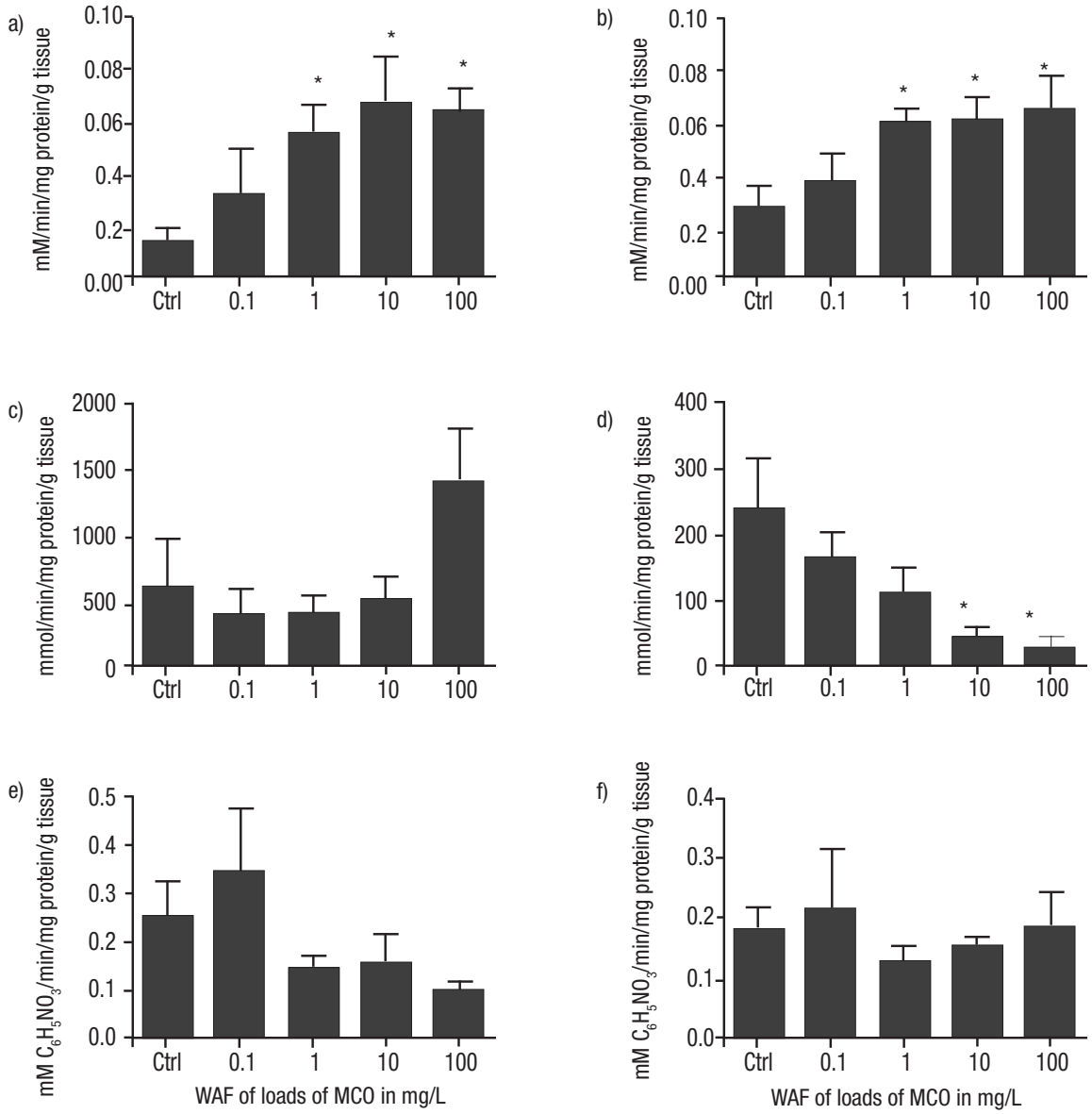
Figures 4a-f. Concentration of FA in tissues of *Pomacea patula* exposed under controlled conditions to WAF of MCO for 96 hours. Bars represent standard error of the mean. a) Foot, b) Head, c) Intestine, d) Mantle, e) Digestive gland, f) Kidney.

No significant changes in the catalysis of enzymes involved in antioxidant defense (SOD, CAT, and GPx) compared to controls were observed in tissues of tegogolos exposed to the WAF of MCO, with the exception of GPx in the foot and head of *P. patula* exposed to high concentration of WAF (10 and 100 mg/L). In addition to oxidative stress, the activity and presence of antioxidant systems are also widely studied in snails treated with pollutants (Ismert *et al.*, 2002; Li *et al.*, 2008; El-Gendy *et al.*, 2009; Radwan *et al.*, 2010; Ali *et al.*, 2012; Bouétard *et al.*, 2013; Zheng *et al.*, 2013; Ma *et al.*, 2014a; 2014b; Wang *et al.*, 2014). Inactivity of SOD, particularly at high concentrations of the WAF of Maya crude oil, could be due to the oxidative stress induced by the accumulation of ROS (Liesivuori & Savolainen, 1991). Additionally, the damage to this enzyme could be due to reactive and oxidant metabolites produced by biotransformation of many compounds, as is the case of PAHs (Gao *et al.*, 2005; Vondrácek *et al.*, 2009). In contrast, significant increases in GPx activity in the foot and head of *P. patula* exposed to high concentrations of WAF are likely due to the presence of H_2O_2 in the cells, since this ROS is the main substrate for these enzymes (Hermes-Lima, 2004). The induction of ROS could be different among tissues due to contact with the environment, but also to their energy demands obtained through fatty-acid metabolism, among others sources. Consequently, the activity of enzymes involved in antioxidant defense could be linked to these pro-oxidant forces. It has not been possible to substantiate that oxidative stress participates in depleting the activity of these antioxidant defenses.

In this study, we found different patterns of response due to the concentration of FA in *P. patula* exposed to the WAF of MCO; however, in no case did we find significant results. In contrast, in other mollusk species, significant results were found (El-Wakil & Radwan, 1991; Radwan *et al.*, 1993; Radwan *et al.*, 2008; Lyssimachou *et al.*, 2009). Research



Figures 5a-f. Metabolism of AOX in tissues of *Pomacea patula* exposed under controlled conditions to the WAF of MCO for 96 hours. Bars represent standard error of mean. a) Foot, b) Head, c) Intestine, d) Mantle, e) Digestive gland, f) Kidney.



Figures 6a-f. Metabolism of enzymes involved in neurotransmission of *Pomacea patula* exposed under controlled conditions to the WAF of MCO for 96 hours. Bars represent standard error of mean. a) Activity of AChE in the foot, b) Activity of AChE in the head, c) Activity of GDA in the foot, d) Activity of GDA in the head, e) Activity of CbE in the foot, f) Activity of CbE in the head.

suggests that the changes of FA content in snails exposed to stressing agents could be explained by their synthesis to repair and prevent damage to organelle and cells, whereas its decrease could be due to utilization of energy requirements (Padmaja & Rao, 1994). Similarly to FA, the activity of AOX showed a different pattern of response among treatments and tissues, even though significant differences were not found. However, in other snail species exposed to inducers of the peroxisome proliferator activated receptor alpha (PPARα), the response was irregular under laboratory conditions (Lyssimachou *et al*., 2009) or amplified in specimens from polluted sites (Cajaraville *et al*., 2003; Regoli *et al*., 2006). The lack of response to FA levels and AOX activity in *P. patula* treated with the WAF of Maya crude oil may have an adaptive significance as documented in other snail species (Arakelova *et al*., 2004) as a protective mechanism for reducing the toxic effects of WAF, as suggested by Padmaja & Rao (1994) in other species. The current results and previous reports denote the need of more studies aimed at increasing knowledge about fatty-acid metabolism in snails exposed to pollutants.

CbE activity, which is present in a range of organism including Bacteria, Eukaryota, and Archaea, is responsible for the hydrolysis of carboxylic esters, carboxylic thioesters, and esters of about 1684 substrates (BRENDA, 2017) in the head and foot of *P. patula* exposed to the WAF of Maya crude oil, was irregular with regard to treatments and tissues.

However, these findings were not significant, probably due to the variable bioavailability of carboxylic compounds in the WAF of Maya crude oil, as well as to the role of CYP450 isoenzymes involved in metabolism of PAHs as documented in snails (Wilbrink *et al*., 1991; Ismert *et al*., 2002). However, it is more likely that the specific aging or stimulation of the AChE will occur after the exposure to soluble compounds present in Maya crude oil. Increases in the catalysis of AChE in head and foot of *P. patula* treated with the WAF of MCO were found. Similar findings were documented in the Senegal sole *Solea senegalensis* (Kaup, 1858) exposed to the WAF of "Prestige" crude oil under laboratory conditions (Solé *et al*., 2008). These results suggest that compounds present in the WAF of Maya crude oil stimulate the activity of this enzyme. Likewise, it is probable that the degradation of acetylcholine overcomes the basal levels provoking deficiencies in this neurotransmitter. Since the acetylcholine participates in the activation of neuromuscular function, it is likely that this function in *P. patula* is inactive. Little information is available regarding the activity of AChE in snails exposed to petroleum hydrocarbons. However, inhibition has been documented in the catalysis of this enzyme in some snail species exposed mainly to pesticides (Singh & Agarwal, 1983; Coeurdassier *et al*., 2001; Radwan & Mohamed, 2013; Khalil *et al*., 2015; Zheng & Zhou, 2017). The different responses documented in previous reports and in this study could be attributed to the presence of bioavailable compounds in WAF that are able to stimulate this enzyme, despite the lack of information about the complete characterization of the WAF obtained from Maya crude oil. Nevertheless, it is probable that the degradation of acetylcholine caused by WAF exposure could modify the response of *P. patula*, probably provoking the apparent lack of sensitivity of this snail species linked to reduced motility.

The catalysis of GDA in the head and foot of *P. patula* was reduced compared to the control, with exception of the activity detected in the foot at the higher WAF concentration. There are few reports regarding the activity of this enzyme in snails exposed to hydrocarbons. However, in the ganglia of a feral freshwater mussel *Elliptio complanata* (Lightfoot, 1786) exposed to dilutions of primary-treated effluent, decreases were documented in GABA catalysis, suggesting glutamatergic stimulation (Gagné *et al*., 2007), which exerts excitatory effects. This neurophysiological process probably occurs as a compensatory mechanism for depression of locomotion activity related with low levels of acetylcholine. Nevertheless, more studies are required to explore the neurotoxicity of petroleum hydrocarbons in freshwater snails as well as specific studies about the composition of the WAF obtained from Maya crude oil.

Comparing the tissues and concentrations of WAF, it is probable that two factors are involved in increased values of glBRv2 in the head and foot of *P. patula* detected in this study: *i)* both are the main tissues in contact with the medium that contains petroleum hydrocarbons, and *ii)* the numerical effect of the three additional biomarkers involved in neurotransmission which was only measured in these tissues, mainly through their high nervous innervation in the foot and by the presence of some organs of the central nervous system in the head. However, the first hypothesis is the more likely, considering their regular contact with the polluted medium. Yet, the digestive gland showed higher values of glBRv2 compared to intestine, mantle, and kidney. This could de due to its high capacity to uptake and concentrate contaminants, which suggests the usefulness of this organ for monitoring biochemical responses (Abdel-Halim *et al*., 2013).

Given the results of this study, we can conclude that the tegogolo foot was the most sensitive organ in terms of the biological response to exposure to the WAF of Maya crude oil. However, more studies are required in order to clarify the biotransformation, bioaccumulation, and detoxification involved in oxidative stress in gastropods exposed under controlled conditions to diverse pollutants. The alterations of some enzymes involved in neurotransmission (AChE and CbE) seem to be suitable biomarkers for monitoring the toxic effects of hydrosoluble compounds present in the Maya crude oil found in this type of organism that also possesses mechanical defenses (shell and operculum) against environmental pressures. Research also confirms that crude oil is one of the most complex contaminants in the aquatic environment and the knowledge of its effects in aquatic organism should be increased.

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