

1 **Multiple biomarkers responses in *Prochilodus lineatus* allowed assessing changes in**
2 **the water quality of Salado River basin (Santa Fe, Argentina).**

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1 **ABSTRACT**

2 This field study assessed water quality of Salado River basin by using a set of
3 biomarkers in the fish *Prochilodus lineatus*. Multiple biomarkers were measured,
4 including morphological indexes (condition factor, liver somatic index), hematological
5 (red and white blood cells) and biochemical (glucose, total protein and cholinesterase
6 activity) parameters. Besides, detoxication and oxidative stress markers (antioxidant
7 enzymes, lipid peroxidation) were measured in liver, gills and kidney. Despite water
8 quality assessment did not show marked differences among sites, biomarkers responses
9 indicate that fish are living under stressful environmental conditions. According to
10 multivariate analysis glucose, glutathione S-transferase activity, lipid peroxidation
11 levels and the count of white blood cells are key biomarkers to contribute to
12 discrimination of sites. So, we suggest use those biomarkers in future monitoring of
13 freshwater aquatic systems.

14

15 **Keywords:** biomonitoring, fish, hematology, oxidative stress, pollution

16

17 **Capsule:** A battery of biomarkers was successfully applied to assess the health of the
18 fish *Prochilodus lineatus* from Salado River basin.

19

1 **1. Introduction**

2 The development of anthropogenic activities is the main factor leading to the increasing
3 levels of contaminants in aquatic environments. In order to assess water quality, a
4 combination of physical, chemical and microbiological indicators have been
5 traditionally used (Chapman, 1992). However, in recent years there has been a growing
6 awareness to the need to detect and assess the adverse effects of contaminants in
7 inhabiting fauna in order to lead to an integrated water quality assessment with respect
8 to its suitability for aquatic life. The biological responses (biomarkers) of aquatic
9 organisms may reflect the integrated effects of all impacts on the water body, and can be
10 used to compare relative changes in water quality from site to site, or over a period time
11 (Friedrich et al., 1992).

12 Currently, the use of biomarkers for monitoring environmental quality has gained
13 considerable interest in the assessment of river condition in many places around the
14 world (Pandey et al., 2003; Tejada-Vera et al., 2007; Linde-Arias et al., 2008a,b; Ruas
15 et al., 2008; Falfushynska and Stolyar, 2009). Biomarkers complement and enhance the
16 reliability of the chemical analysis data, offering more integral and biologically relevant
17 information on the potential impact of toxic pollutants on the health of organisms (van
18 der Oost et al., 2003; Parvez and Raisuddin, 2005; Parvez et al., 2006). Therefore, the
19 use of biomarkers can offer an integrated evaluation of the effects of pollutants in
20 wildlife and give a clear picture of the “health status” of a system under investigation
21 (Hansen, 2003; Ferreira et al., 2005). Moreover, the use of biomarkers has the
22 advantage of providing early warning signals of possible damage in aquatic ecosystems
23 (van der Oost et al., 2003).

1 In natural environments, contaminants usually are present as very complex mixtures and
2 there is no single biomarker that can give a complete diagnosis of environmental
3 degradation. To overcome this difficulty, the use of a set of complementary biomarkers
4 may be useful to evaluate the various responses to mixtures of pollutants in organisms
5 under stress (Lavado et al., 2006; Tejeda-Vera et al., 2007; Frenzilli et al., 2008). This
6 multiparametric approach is being now broadly utilized for the assessment of the health
7 of river biotic community and has been incorporated into several pollution monitoring
8 programs in Europe and the USA (Cajaraville et al., 2000; Ferreira et al., 2005; Mayon
9 et al., 2006; Parvez et al., 2006; Sanchez et al., 2008).

10 The present study focus on the Salado River basin (a tributary of the middle Parana
11 River). This river runs through regions with different land uses, receiving industrial,
12 agricultural and domestic wastes. Previous reports indicated the existence of potentially
13 toxic chemicals (heavy metals) in water and sediments (Gagneten et al., 2007; Marchese
14 et al., 2008). Besides, the catchment area is surrounded by intensive crop farming,
15 which uses chemical products (fertilizers and/or pesticides) with ecotoxic activity. This
16 deterioration of water and sediment quality may lead to a decrease in natural resources.
17 For instance, benthos and zooplankton structure communities are affected by
18 anthropogenic disturbances occurring in the Salado River basin (Gagneten and Ceresoli,
19 2004; Zilli and Gagneten, 2005; Marchese et al., 2008; Gagneten and Paggi, 2009).
20 Despite fish kill episodes have frequently occurred on this water body, there are no
21 studies on the health status of fish living under these field stressful conditions. Within
22 this context, the development of tools for monitoring water quality and to assess the
23 biological effects of pollution on fish inhabiting this river basin is an imperative need.

1 Fish are widely used to evaluate the health of aquatic systems and their biochemical and
2 physiological changes serve as biomarkers of environmental pollution (Beliaeff and
3 Burgeot, 2002; Dautremepuits et al., 2004; Lavado et al., 2006). *Prochilodus lineatus* is
4 a widely distributed Neotropical fish and one of the most important species of the
5 Parano-Platense ichthyofauna, comprising 60% of the total ichthyomass of the middle
6 Parana River (Bonetto et al., 1970). This native species is relatively abundant along the
7 study area, which allows the comparison between sites. Adult fish are bottom feeder, so
8 they may be in contact with xenobiotics in both water and sediment, representing a well
9 suited species for environmental monitoring (Camargo and Martinez, 2006; Langiano
10 and Martinez, 2008; Simonato et al., 2008). Besides its ecological value, this species is
11 also economically important for the region (subsistence and commercial fishing).

12 Previous studies have demonstrated that *P. lineatus* is an appropriate species for
13 studying the effect of different pollutants using a wide range of biomarkers both in
14 laboratory and field studies (Leite da Veiga et al., 2002; Parma et al., 2007; Vanzella et
15 al., 2007; Winkaler et al., 2007; Langiano and Martinez, 2008; Simonato et al., 2008).

16 Besides, normal values for some hematological parameters and cholinesterase activity
17 have been previously reported (Parma de Croux, 1994; Cazenave et al., 2000). Thus,
18 deviations from normal ranges as consequence of environmental stress can now be
19 assessed.

20 The biomarkers used during this study should give information on the impact of
21 anthropogenic activities on fish health. Within this context, we included general health
22 status indicators, such as morphological indexes and hematological parameters, as well
23 as metabolic parameters (i.e., glucose, protein). Additionally, we assessed biomarkers
24 that could suggest the probable existence of xenobiotics, which could exert oxidative

1 stress (measured by activation of antioxidant enzymes or lipid peroxidation levels).
2 Finally, cholinesterase activity was selected as a specific marker, which inhibition is
3 linked to exposures of organophosphate and carbamate pesticides (Chovanec et al.,
4 2003).

5 The main goal of the present study was evaluate the use of a battery of biomarkers in *P.*
6 *lineatus* to assess stressful environmental conditions on the Salado River basin as well
7 as pointing out which biomarkers and tissues to use during future monitoring programs
8 of this and similar aquatic systems.

9

10 **2. Materials and Methods**

11 *2.1 Sampling sites and fish collection*

12 Four sites were selected in the lower Salado River basin, according to different land
13 uses and the location of potential sources of pollutants (urban, industrial, and
14 agricultural). Site 1 (31°31'S, 60°45'W) is an urban area located in the city of Santo
15 Tomé, near to the mouth of this river (Fig. 1). Site 2 (31°22'S, 60°54'W) is located
16 downstream of an industrial area, next to the city of Esperanza. Site 3 (30°44'S,
17 60°37'W), is located near to the city of San Justo, which is mainly an agricultural area.
18 As reference site we selected El Bonete lagoon (Site 4 or Reference) (29°23', 60°33'W),
19 located in the upper portion of the basin, which is part of the Golondrinas-Calchaquí
20 system, a tributary of the Salado River. This last station is a relatively pristine area,
21 without industrial or urban influences, being extensive livestock the main activity of the
22 region.

23 Two surveys to the study sites were conducted during non-reproductive season and
24 during a dry hydrologic season (May and August 2007). A total of 59 adult specimens

1 of *P. lineatus* were collected with the help of local fishermen at the Salado River basin
2 using gill nets. Fish were transported alive to the laboratory into 100 L tanks containing
3 aerated river water. In order to minimize stress from capture and transportation, fish
4 were held in laboratory until the next morning (within 24 h after catch). Then, fish were
5 sampled for biomarkers measurements.

6

7 *2.2 Water quality assessment*

8 Simultaneously with fish collection we evaluate the water quality at each study site by
9 recording in situ: water temperature, dissolved oxygen (DO), conductivity, and pH.
10 Additionally, 2 L of water samples were taken for each station for chemical analyses;
11 water samples were transported to the laboratory at 4°C in clean plastic bottles and
12 analyzed according to standard procedures (APHA et al., 1998). The following
13 parameters were measured: biochemical oxygen demand (BOD), chemical oxygen
14 demand (COD), nitrates, nitrites, ammonia, total phosphorus, calcium, magnesium,
15 chlorides, sulfates, hardness, turbidity, dissolved solids, and total coliforms by the More
16 Probable Number (MPN) method (Pesce and Wunderlin, 2000).

17 In order to evaluate changes in the water quality, due to combined effects of many
18 parameters, we calculated a water quality index (WQI) proposed by Pesca and
19 Wunderlin (2000). Briefly, the construction of WQI requires first a normalization step,
20 where each parameter is transformed into 0-100% scale, with 100% representing the
21 highest quality. The next step is to apply weighting factors that reflect the importance of
22 each parameter as indicator of the water quality. The constructed WQI gives an
23 adimensional number that can be associated with a quality percentage, easy to
24 understand for everyone, and based on scientific criteria for water quality.

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2.3 Biomarkers

2.3.1 Morphological parameters

Prior to blood sampling and dissection, fish were anaesthetized in benzocaine as described by Parma de Croux (1990). Fish were measured (total length, cm) and weighed (total and liver weights, g), calculating the condition factor (CF) and liver somatic index (LSI) (Goede and Barton, 1990). Gills, liver and kidney were immediately frozen in liquid nitrogen and stored at -80°C until biochemical determinations.

2.3.2 Hematological and biochemical parameters

Blood was rapidly extracted from the caudal vessel by dissection of the caudal peduncle (Reichenbach-Klinke, 1980), using heparinized syringes. Red blood cells (RBC) counts were performed with a Neubauer chamber, using physiological solution for dilution. Haematocrit (Ht) values were determined by the micromethod using capillary tubes and centrifuged at 1409 g for 10 min. Haemoglobin concentration (Hb) was measured by the cyanomethaemoglobin method at wavelength of 546nm on a spectrophotometer (HACH, DR 5000) (Houston, 1990). Mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated from primary indices (Cazenave et al., 2005).

A drop of freshly collected blood was smeared on clean slides to estimate the total white blood cells (WBC) counts and for determination of leukocyte frequency according to Tavares-Dias and de Moraes (2007). The air-dried blood smears were fixed in absolute methanol for 10 min and stained by May-Grünwald-Giemsa (Houston, 1990). Total

1 WBC was performed in relation to the number of erythrocytes counted in randomly
2 selected fields and recalculated per unit volume: $\text{WBC}/\mu\text{L} = \text{number of WBC in blood}$
3 $\text{smear} \times (\text{RBC}/\mu\text{L}/4000 \text{ RBC counted in smear})$. Differential leukocytes counts were
4 performed by identifying 100 white blood cells en each blood smear.
5 Additionally, plasma was separated from whole blood by centrifugation at 1409 g for 10
6 min. Glucose and total protein concentrations were determined colorimetrically using
7 commercial kits (Wiener Lab®). The remaining plasma was stored at -20°C until
8 cholinesterase assay.

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10 *2.3.3 Cholinesterase activity*

11 Plasmatic cholinesterase (ChE) activity was determined colorimetrically (Ellman et al.,
12 1961) using Cholinesterase® test provided by Wiener S.A. Lab Group (Argentina),
13 which uses the butyrylthiocholine as substrate for the cholinesterase determination in
14 human plasma. This test kit has been previously validated by Cazenave et al. (2000) for
15 its use in fish plasma.

16

17 *2.3.4 Oxidative stress markers*

18 Antioxidant enzymes activities were determined in the cytosolic fractions of gills, liver
19 and kidney. Enzyme extracts from each tissue were prepared according to Wiegand et
20 al. (2000), with fewer modifications proposed by Cazenave et al. (2006a). Briefly,
21 tissues were homogenized using 0.1 potassium phosphate buffer, pH 6.5 containing
22 20% glycerol, 1 mM EDTA, and 1.4 mM dithioerythritol.. After removal of cell debris
23 (10 min at 13 000 g), the membrane fraction was separated by centrifugation at 105 000
24 g for 60 min (ultracentrifuge Sorvall® Utraspeed). The remaining supernatant (cytosolic

1 fraction) was used for enzymatic assays, which carried out using a spectrophotometer
2 (MultiSpec 1501, Shimadzu) equipped with a multiple cell holder and temperature
3 control. The activity of Glutathione S-transferase (GST) was determined according to
4 Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.
5 Glutathione reductase (GR) activity was assayed in according to Tanaka et al. (1994).
6 The activity of peroxidase (POD) was measured using guaiacol and hydrogen peroxide
7 (Bergmeyer, 1983). The enzymatic activity was calculated in terms of the protein
8 content of the sample (Bradford, 1976), and is reported in nanokatals per milligram of
9 protein ($\text{nkat}\cdot\text{mg prot}^{-1}$), where 1 kat is the conversion of 1 mol of substrate per second.
10 Each enzymatic assay was carried out in triplicate.

11 Additionally, lipid peroxidation (LPO) in gills, liver and kidney was determined by
12 measuring the formation of thiobarbituric reactive substances (TBARS), in according to
13 Fatima et al. (2000). LPO was expressed as nanomoles of TBARS formed per hour, per
14 milligram of proteins ($\text{nmol TBARS}\cdot\text{mg prot}^{-1}$). Protein content of each sample was
15 determined in according to Bradford (1976).

16

17 *2.4 Statistical analysis*

18 All data are reported as mean \pm standard error. Data obtained for different biomarkers
19 were first tested for normality and homoscedasticity using Shapiro-Wilks and Levene
20 tests, respectively. Variables that had not a normal distribution and/or homogeneity of
21 variance were transformed using Ln and tested again, prior to parametric analysis. A
22 MANOVA test was carried out to check for statistical differences in global biomarker
23 responses among seasons (May and August), sites, and interactions considering both
24 seasonal and spatial responses. We did not find significant seasonal variations. Thus, we

1 decided to pool data from different seasons during further statistical analysis.
2 Comparison of each biomarker among sites was performed using ANOVA ($p < 0.05$). A
3 Tukey posteriori test was used, where necessary, to distinguish among groups. When
4 variables could not be normalized, the Kruskal-Wallis test was used. Then, we selected
5 mainly those variables that showed significant differences among sites to carry out
6 stepwise Linear Discriminant Analysis (LDA) with Jackknife cross validation method to
7 point out which parameters allow differentiating among studied stations.

8

9 **3. Results**

10 *3.1 Water quality assessment*

11 Results obtained with physical and chemical parameters measured in water at all
12 sampling sites are shown in Table 1. After normalization, parameters such as
13 conductivity, sulfates, turbidity and dissolved solids showed values lower than 50,
14 which mean less than 50% of the value optimal for aquatic life preservation, at all
15 sampling sites. Besides, water samples at sites 1, 2 and 3 showed also higher ammonia
16 concentrations, bellow 50% acceptability, whereas ammonia concentrations were within
17 suitable values at reference site (Station 4). The remaining parameters were within
18 normal ranges for natural surface waters, with normalized values higher than 50. On the
19 other hand, the WQI values, constructed with the eighteen measured parameters, were
20 similar throughout studied sampling areas.

21

22 *3.2 Biomarkers*

23 *3.2.1 Morphological parameters*

1 Main morphometric characteristics of collected fish are given in Table 2. The length,
2 weight and condition factor were rather homogeneous, indicating similar nutritional
3 conditions of fish at all the sampling stations. On the contrary, LSI of specimens at sites
4 1, 2 and 3 were significantly lower than specimens from reference site.

5

6 *3.2.2 Hematological and biochemical parameters*

7 The mean values of hematological parameters of *P. lineatus* collected from four sites on
8 the Salado River basin are showed in Table 2. RBC, Ht, MCV, MCHC values did not
9 differ among sampling sites. In contrast, fish at sites 1, 2 and 3 showed lower Hb
10 content than reference site. Similarly, fish MCH was significantly lower at site 1 than
11 those from site 4.

12 The count of WBC ranged from 5,100 to 17,430 in specimens from reference site. Fish
13 at sites 1 and 3 showed higher mean values of WBC (Table 2). In the case of site 1, the
14 mean value of WBC was 3-fold to that recorded for reference fish. The WBC recorded
15 in *P. lineatus* were of four types, namely: lymphocytes, monocytes, neutrophils and
16 eosinophils, while no basophils were identified. Leukocyte frequency was altered in fish
17 at sites 1 and 3, with a lower percentage of lymphocytes and a marked increasing of
18 neutrophils, eosinophils and monocytes (Table 2).

19 On the other hand, plasma concentrations of glucose and total protein were significantly
20 increased in fish at site 1 in comparison with other monitoring areas (Table 2).

21

22 *3.2.3 Cholinesterase activity*

23 The activity of plasmatic cholinesterase enzyme did not show significant differences
24 among sampling sites (Table 2).

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2 3.2.4 Oxidative stress markers

3 Figure 2 shows the mean values for the activity of antioxidant enzymes and lipid
4 peroxidation levels. Hepatic GST activity of fish at site 1 was increased 2.3-fold
5 compared to fish at reference site (Fig. 2 A). GST activity, measured in kidney of fish at
6 site 1, was also increased with respect to reference fish, while GST activity in gills did
7 not show significant differences among studied sites. The activity of hepatic GR was
8 significantly increased in *P. lineatus* at site 1, compared with fish at reference site (Fig.
9 2 B). The activity of GR was also significantly increased in fish kidney at site 3,
10 whereas it did not show significant changes in gills throughout the studied basin. POD
11 activity (Fig. 2 C) was only increased in liver of fish at sites 1, 2 and 3 in comparison
12 with reference specimens.

13 Finally, fish at site 1 showed higher LPO levels in liver, gills and kidney (Fig. 2 D).
14 High LPO levels were also observed in gills of fish at sites 2 and 3 and in liver at site 3.

15

16 3.2.5 Chemometrics

17 In order to examine the discriminating power of the set of studied biomarkers, we
18 carried out stepwise Linear Discriminant Analysis (LDA). To do that, we constructed a
19 data matrix including 20 analyzed biomarkers as independent variables and 4
20 monitoring stations as grouping variables. Figure 3 shows a simple graphical
21 representation of the values of canonical discriminant functions for the sampled
22 individuals. The first two canonical discriminant functions (axis 1 and 2) were enough
23 to account for almost all of the differences (97.68%). Significant differences between
24 studied areas were described primarily by the first canonical axis, which explained

1 68.32% of the total variance, being glucose, LPO-liver, GST-liver, WBC, GST-kidney,
2 total protein, LPO-kidney the parameters presenting the highest influence. On the other
3 hand, the variables that most contributed to the second discriminant function (having the
4 highest scores) were LPO-gill, LPO-liver and LSI. In other words, canonical functions,
5 constructed on the base of studied biomarkers, show complete separation among
6 different studied areas (100% of right assignation) and it is clear that samples from site
7 1 were separated from other sites based on high values of Glucose, LPO-liver, GST-
8 liver and WBC, mainly.

9

10 **4. Discussion**

11 Water quality can be determined using different physical, chemical and biological
12 parameters. Despite the usefulness of physical and chemical parameters to evaluate
13 pollution trends and sources (Pesce and Wunderlin, 2000; Wunderlin et al., 2001), they
14 do not accurately predict changes in aquatic living systems, giving only partial
15 information of the overall water quality (Hued and Bistoni, 2005). Therefore, the use of
16 a set of biomarkers for assessment of environmental quality has been recommended by
17 many researchers (Cajaraville et al., 2000; Lionetto et al., 2003; Mayon et al., 2006;
18 Fernandes et al., 2007; Sanchez et al., 2007, 2008; Tejada-Vera et al., 2007; Linde-Arias
19 et al., 2008b; among other).

20 Our current results show that most of physical and chemical parameters measured are
21 within normal ranges reported for freshwater systems throughout the studied basin
22 (Chapman, 1992). However, parameters such as conductivity, sulfates, turbidity and
23 dissolved solids showed high values at all sampling sites, which is characteristic of the
24 Salado River basin. Similar results have been previously reported by Gagneten et al.

1 (2007) and Marchese et al. (2008), who suggested that Salado River is characterized by
2 hard waters, with high conductivity and suspended material. Those authors have also
3 reported heavy metals (Cr, Cu, Pb, Cd) in water and sediments of Esperanza and San
4 Justo areas (corresponding to sites 2 and 3 of the present study) of the Salado River
5 basin, but no information is available about other kind of compounds that probably
6 could be in this basin. For example, current use pesticides (pyrethroids, organochlorates,
7 etc) are applied in the catchment area of the Salado River and could enter to the aquatic
8 environment through direct application, urban and industrial discharges and surface
9 runoff from non point sources, including agricultural soil, aerosol, particulate deposition
10 and rainfall, etc.

11 The use of a water quality index (WQI) (Pesce and Wunderlin, 2000) did not allow in
12 this case pointing out significant differences between sampling sites. However, several
13 biomarkers measured in *P. lineatus* showed clear differences among sites (Table 2 and
14 Fig. 2).

15

16 *4.1 Morphological parameters*

17 Among the set of measured biomarkers, the condition of the liver (LSI) and of the
18 whole body (CF), can provide information on potential pollution impacts (van der Oost
19 et al., 2003). Although these parameters are not very sensitive and may be affected by
20 non-pollutant factors (e.g., season, disease, nutritional level), they may serve as an
21 initial screening biomarker to indicate effects induced upon exposure to environmental
22 toxics, or to provide information on energy storage (Goede and Barton, 1990; van der
23 Oost et al., 2003). Particularly, the liver somatic index (LSI) can reflect both metabolic

1 energy demand and short-term nutritional status and can be considered a general health
2 indicator, sensitive to environmental contaminants (Almeida et al., 2005).

3 In the present study, fish at sites 1, 2 and 3 showed a reduction in LSI in comparison
4 with fish at reference site (Table 2), which could be interpreted as reflecting of a
5 depletion of energy reserves stored as liver glycogen. In the case of fish captured at site
6 1, the significant increase in plasma glucose may reinforce this hypothesis. Besides,
7 changes in LSI could be indicating biochemical, physiological and histological
8 alterations.

9 Significantly decreased LSI values were observed in laboratory studies with fish
10 exposed to OCPs, PCBs and PAHs and in field studies at polluted sites (van der Oost et
11 al., 2003). For instance, Coimbra et al (2007) observed that endosulfan (a widely used
12 pesticide in our region) induced a significant lost in liver mass, together with liver
13 lesions, in tilapia after dietary exposure during 21 days. In order to explain LSI
14 alteration observed in the present study, further studies are necessary on
15 histopathological features of hepatic tissues as well as on the assessment of energy
16 storage (glycogen content) of fish living at sublethal pollution levels in Salado River
17 basin, where multiple toxic compounds could be occurring.

18

19 *4.2 Hematological and biochemical parameters*

20 Changes in the composition of blood can be used as biomarkers of dysfunctional
21 physiological conditions (Leatherland et al., 1998). In this study, the main changes in
22 red blood cells included a decreasing in Hb and MCH. However, mean values recorded
23 in *P. lineatus* for these variables are within the normal range reported by Parma de
24 Croux (1994) for this species.

1 On the other hand, white blood cells play a major role in the defense system of fish.
2 Leucocytes are centrally involved in phagocytic and immune responses to parasitic,
3 bacterial, viral and similar challenges (Houston, 1990; Leatherland et al., 1998).
4 Information on leukocyte types and numbers is a valuable tool in assessing general
5 condition (Houston, 1990). In our study, fish at sites 1 and 3 were characterized by a
6 significant induction of leukocyte counts as well as an altered leukocytes frequency,
7 compared with those of the reference site. The increase in the number of leucocytes
8 (leucocytosis) is a normal reaction against infections of foreign substances, which can
9 alter the normal physiological processes in fish (Nussey et al., 1995). Furthermore, the
10 increase in leukocytes can be correlated with an increase in antibody production that
11 helps in survival and recovery of the fish exposed to various stressors, such as pesticides
12 (i.e. cypermethrin, monocrotophos) (Agrahari et al., 2007; Adhikari et al., 2004;
13 Mgbenka et al., 2005) and metals (i.e. Cu, Pb) (van Vuren et al., 1994; Nussey et al.,
14 1995; Oliveira Ribeiro et al., 2006). Thus, leucocytosis observed in fish at site 3 could
15 be caused by heavy metals exposure (as reported by Gagneten et al., 2007) and,
16 probably, by pesticide contamination because of the intensive agriculture dominant in
17 the catchment. In the case of site 1, possibly a complex mixture of contaminants could
18 be occurring because of a confluence of both urban and agricultural pollution.

19 With respect to leukocyte frequency, fish at site 1 had a higher neutrophils percentage,
20 in contrast with lower lymphocytes, eosinophyls and monocytes percentages. Similar
21 tendency was also observed in fish at site 3. Each type of leukocyte has a specific
22 function. Granulocytes and monocytes function as phagocytes to salvage debris from
23 injured tissue, while lymphocytes are the most important cells in immune response
24 (produce antibodies) (Oliveira Ribeiro et al., 2006). So, neutrophilia observed in fish

1 could indicate an increased phagocytic action. On the other hand, blood lymphocyte
2 counts in fish are usually highest in the white blood cells, but the numbers tend to vary
3 widely with the condition of fish, especially when diseased. For instance, it is known
4 that stress causes lymphocyte decrease (lymphopenia) (Takashima and Hibiya, 1995), as it
5 was observed in fish at sites 1 and 3. In terms of lymphocyte and neutrophil responses,
6 our results agree with a previous study on fish exposed to Cu (Dethloff and Bailey,
7 1998), heavy metal previously detected in the studied basin (Gagneten et al., 2007).
8 Finally, measurement of blood serum biochemical parameters is commonly used as
9 diagnostic tool in fish toxicology and biomonitoring, mainly because they are quite easy
10 to measure, low cost and sensitive to environmental changes. In the current work, we
11 observed increased glucose and total protein levels in fish at site 1. Particularly,
12 hyperglycaemia was among the most evident changes compared with those of the
13 reference site. This classic stress response could be attributed to the mobilization of
14 glucose (glycogenolysis) linked to changes in carbohydrate metabolism of fish and
15 ultimately increasing energy availability and utilization to improve the response to toxic
16 stress (Cazenave et al, 2006b), or increased secretion of cortisol due to stressful
17 conditions. Many studies have demonstrated that the stressful environment brings about
18 an increase in the energy requirement of fish, causing an increase in serum glucose
19 level. Our results are in line with other studies which observed hyperglycaemia in fish
20 exposed to pesticides (i.e., cypermethrin, glyphosate), heavy metals (i.e., lead,
21 chromium), as well as complex field situations (i.e., paper mill effluents) (Al-Alkel and
22 Shamsi, 1996; Martinez et al., 2004; Sepúlveda et al., 2004; Borges et al., 2007;
23 Langiano and Martinez, 2008).

1 In the case of plasma proteins, they are central to several vital blood activities, including
2 homeostasis and blood coagulation, vitamin and hormone transport, and specific
3 immunity to pathogens (Leatherland et al., 1998). Total protein is additionally used as
4 an indicator of liver impairment. Increased concentrations can be caused by structural
5 liver alterations (e.g., cirrhosis) reducing aminotransferase activity with a concurrent
6 reduction in the deamination capacity (Bernet et al., 1999). Probably, fish from site 1
7 could be exposed to toxics that affect the liver tissue, producing ultimately an increase
8 in plasmatic protein levels.

9

10 *4.3 Oxidative stress*

11 The metabolism of toxic compounds frequently results in the formation of reactive
12 oxygen species (ROS), which significantly contribute to their toxicity (Chovanec et al.,
13 2003). Fish are susceptible to the effects of ROS and have developed effective
14 antioxidant defenses, including antioxidant substances (vitamin E, uric acid, glutathione
15 and carotenoids) and enzymes (CAT, GR, POD) (Winston and Di Giulio, 1991).
16 Oxidative stress occurs when the rate of ROS generation exceeds the antioxidant
17 defense system (Martinez-Alvarez et al., 2005). Its deleterious effects include oxidation
18 of proteins, DNA, and steroid components, as well as peroxidation of unsaturated lipids
19 in cell membranes (van der Oost et al., 2003).

20 This study assessed the induction of oxidative stress in different tissues of fish living in
21 the Salado River basin by the measurement of both the activity of antioxidant enzymes
22 and lipid peroxidation (measured through TBARS).

23 GST conjugates xenobiotics or their metabolites with glutathione and so makes them
24 less toxic and more easily excretable (van der Oost et al., 1996). As observed in various

1 studies, changes in GST activity reflect detoxification process occurring in different
2 organs of fish exposed to toxic compounds (Cazenave et al., 2006a; Monferran et al.,
3 2008; Pesce et al., 2008; Ballesteros et al., 2009). We observed a noticeable increase of
4 GST activity in liver of fish at site 1. Similarly, the activity of GST in fish kidney at site
5 1 was also enhanced in comparison with reference fish. This induction in GST activity
6 could indicate a defense of fish against oxidative stress damage produced by adverse
7 environmental conditions.

8 We also observed increased levels of POD and GR, mainly in liver of fish at site 1. Fish
9 at sites 2 and 3 showed also enhanced hepatic POD activity with respect to the reference
10 site. The induction of antioxidant enzymatic activity in liver of fish could be indicating
11 a response towards increased ROS generation. However, induced ROS could be not
12 totally scavenged by the antioxidant enzymes due elevated levels of LPO (TBARS)
13 observed in different organs (liver, gills and kidney) of fish at sites 1, 2 and 3. LPO
14 alters the normal structural and functional properties of the cell, ultimately leading to
15 cytotoxicity by dismantling the membrane structure in association with various adaptive
16 reactions and changes in physiological status (Bhattacharya et al., 2007). Increased
17 levels of LPO have been observed in fish under experimental conditions, upon exposure
18 to different xenobiotics as well as stressful field conditions (López-López et al., 2006;
19 Santos et al., 2006; Ballesteros et al., 2009).

20 So far, our current results suggest the probable existence of xenobiotics in the Salado
21 River basin, which could exert oxidative stress in different organs of *P. lineatus*. There
22 are evidences that some heavy metals (i.e., Cr, Cu), like those detected in the studied
23 basin, produced increased LPO levels in liver of freshwater fish species (Tagliari et al.,
24 2004; Vutukuru et al., 2006; Talas et al., 2008). Besides, pesticides of current use in our

1 region, such as some pyrethroids (i.e. cypermethrin) and organochlorides (i.e.
2 endosulfan), have shown to induce oxidative stress in fish (Üner et al., 2001; Ballesteros
3 et al., 2009).

4 On the other hand, different tissues did not respond in the same way (Cazenave et al.,
5 2006a). Oxidative stress markers were mainly induced in liver (i.e. fish from site 1),
6 whereas small changes were observed in both gills and kidney of fish sampled at the
7 same site. These results as well as LPO damage could indicate highest sensitivity of the
8 liver in comparison with other organs. So far, these findings point out the convenience
9 of focusing on fish liver during future monitoring.

10

11 *4.4 Integrated analysis*

12 Many authors have highlighted the importance of the use of a wide battery of
13 biomarkers when assessing the biological effects in impacted environments, since a
14 single biomarker may not reflect the health status of a sentinel species. Beliaeff and
15 Burgeot (2002) suggest that the selection of an appropriate battery of biomarkers can
16 avoid false-negative responses obtained with a single biomarker (showing no impact
17 when an actual anthropogenic impact exist). They also pointed out that careful selection
18 of an appropriate combination of biomarkers can provide information about global
19 adverse environmental effects. Thus, the concurrent use of several biomarkers is
20 important to minimize misinterpretation in cases of complex situations of pollution
21 (Minier et al., 2000; Flammarion et al., 2002; Linde-Arias et al., 2008b).

22 Salado River basin is impacted by different anthropogenic disturbances, which are not
23 fully studied at present. Thus, this complex situation required to apply a set of
24 biomarkers in a native species, in addition to assessing physical and chemical

1 parameters, to evaluate changes in the water quality. Our current results clearly
2 evidence that the single use of physical and chemical analysis was not enough to detect
3 changes in the water quality throughout the studied basin. However, the assessment of
4 several hematological, biochemical and physiological parameters, involved in various
5 metabolic processes (energy, oxidative stress, biotransformation, etc), in the native fish
6 *P. lineatus* enabled to clearly differentiate between the reference area and other stations
7 presumably affected by pollution. Multivariate statistical analysis indicated that fish at
8 site 1 exhibited a distinctly response than those from other sites and that several
9 biomarkers significantly contribute to discriminate among four studied areas. All in all,
10 our present results could suggest that *P. lineatus* inhabiting at site 1 may have
11 undergone sublethal stress, possibly due to exposure to different chemical contaminants.
12 It is also evident that the health status of fish inhabiting stations 2 and 3 is rather
13 different (worse) than the corresponding to the reference area (mainly by oxidative
14 damage evidences). However, our current evaluation of changes in physical or chemical
15 variables did not evidence a clear cause for such differences. Therefore, further studies
16 would be needed in order to identify the stressful agents which are affecting the health
17 of these fish within the studied basin.

18

19 **5. Conclusions**

- 20 • The present study showed that, despite water quality assessment of Salado River
21 did not show a severe anthropogenic impact, the use of a set of non-specific
22 biomarkers in *P. lineatus* represented a sensitive and effective tool for reflecting
23 adverse environmental conditions for fish health.

- 1 • Based on the biomarker responses, which are related to some functions such as
2 metabolism (glucose, total protein, LSI), defense (WBC, antioxidant enzymes)
3 and detoxification (GST), as well as evident oxidative damage (LPO-TBARS), it
4 was possible to establish that fish at site 1 were living under stress.
- 5 • According to multivariate statistical analysis, parameters such as glucose, lipid
6 peroxidation, glutathion S-transferase and white blood cells, among other, were
7 key biomarkers to discriminate fish of different sites. So, we suggest using these
8 biomarkers in future biomonitorings.
- 9 • Overall, the present study provides additional evidences for the use of a set of
10 biomarkers in assessing the health of aquatic systems, and corroborates the
11 suitability of *P. lineatus* as sentinel species, pointing out the need of further
12 studies to corroborate the presence of xenobiotics or other environmental toxics
13 affecting this species within the studied basin.

14

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21

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1 **Captions.**

2 **Table 1.** Physical and chemical parameters measured as well as water quality index
3 (WQI) calculated at each sampling site on the Salado River (site 1: Santo Tomé, site 2:
4 Esperanza, site 3: San Justo) and Reference site (El Bonete lagoon, site 4).

5 **Table 2.** Mean values (\pm standard error) of morphometric data, hematological
6 parameters and plasma determinations in *Prochilodus lineatus* at each sampling site on
7 the Salado River basin. (*) The significance levels observed are $p < 0.05$ in comparison
8 with reference site.

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10 **Figure 1.** Studied area and location of sampling sites on the Salado River basin (site 1:
11 Santo Tomé, site 2: Esperanza, site 3: San Justo) and Reference site (El Bonete lagoon).

12 **Figure 2.** Oxidative stress markers in liver, gills and kidney of *Prochilodus lineatus* at
13 each sampling site on the Salado River basin. (A) Glutathione S-transferase (GST)
14 activity. (B) Glutathione reductase activity, GR. (C) Peroxidase activity, POD. (D)
15 Lipid peroxidation (LPO). The values are expressed as means \pm S.E. (*) The
16 significance levels observed are $p < 0.05$ in comparison to Reference site.

17 **Figure 3.** Discriminant analysis: Plotting of the values of canonical discriminant
18 functions showing separation of the sampling sites based on biomarker responses.

19

1 **Table 1**

Parameter	Site 1	Site 2	Site 3	Reference (Site 4)
Ammonia (mg·L ⁻¹)	1.03	0.50	0.40	0.10
Biological Oxygen Demand (mg·L ⁻¹)	5.7	4.5	5.4	4.1
Calcium (mg·L ⁻¹)	69.0	126.5	172.0	154.0
Chemical Oxygen Demand (mg·L ⁻¹)	43.3	40.3	62.3	68.0
Chloride (mg·L ⁻¹)	104.9	150.8	151.7	75.0
Conductivity (μS·cm ⁻¹)	2987	4633	7013	8500
Dissolved oxygen (mg·L ⁻¹)	9.3	8.4	9.4	10.8
Hardness (mg·L ⁻¹)	345	580	900	950
Magnesium (mg·L ⁻¹)	41.8	63.9	118.0	121.0
Nitrates (mg·L ⁻¹)	0.63	0.45	0.60	0.19
Nitrites (mg·L ⁻¹)	0.10	0.02	0.01	0.02
pH	8.4	8.1	7.7	7.3
Sulfates (mg·L ⁻¹)	483.8	1093	651.7	500.0
Temperature (°C)	15.5	13.4	12.1	14.4
Total coliforms (MPN 100 ml ⁻¹)	3100	400	50	1300
Total dissolved solids (mg·L ⁻¹)	1912	3155	7010	6813
Total phosphorus (mg·L ⁻¹)	3.8	4.4	1.8	2.3
Turbidity (NTU)	96	103	113	110
WQI	57	60	56	63

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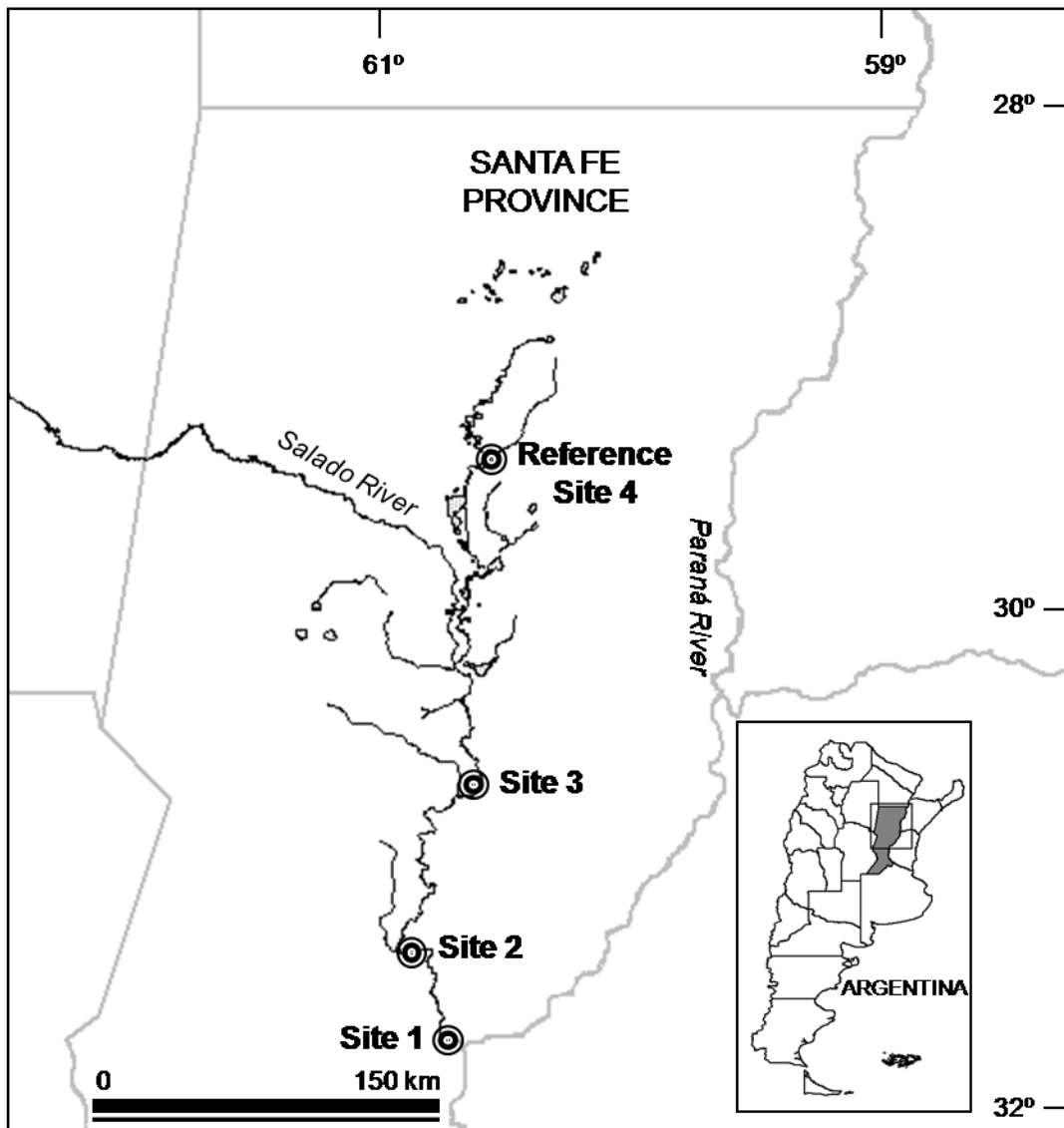
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1 **Table 2.**

	Site 1 (n=18)	Site 2 (n=18)	Site 3 (n=14)	Reference (Site 4) (n=9)
<i>Morphometric data</i>				
Weight (g)	1118.8 ± 38.7	1206.3 ± 39.4	1253.1 ± 41.2	1206.7 ± 58.1
Length (cm)	41.50 ± 0.48	42.75 ± 0.61	43.36 ± 0.79	42.61 ± 0.89
CF	1.56 ± 0.03	1.55 ± 0.04	1.54 ± 0.04	1.56 ± 0.05
LSI	1.41 ± 0.09*	1.45 ± 0.07*	1.51 ± 0.09*	2.08 ± 0.13
<i>Blood cells</i>				
RBC (10 ⁶ /μL)	2.31 ± 0.12	2.45 ± 0.13	2.25 ± 0.16	2.74 ± 0.09
Ht (%)	42.03 ± 3.05	41.89 ± 1.90	40.51 ± 2.63	44.74 ± 2.17
Hb (g/dL)	8.36 ± 0.46*	10.87 ± 0.50*	9.62 ± 0.76*	13.53 ± 0.67
MCV (μm ³)	185.82 ± 12.57	174.59 ± 7.59	183.05 ± 7.85	164.62 ± 9.92
MCH (pg)	37.39 ± 2.08*	45.78 ± 2.46	42.66 ± 1.50	49.50 ± 2.46
MCHC (%)	22.84 ± 1.40	21.18 ± 0.85	24.06 ± 2.02	18.37 ± 0.60
WBC (μL)	36052 ± 5955*	7953 ± 1858	17265 ± 3175*	12019 ± 1831
Lymphocytes (%)	34.94 ± 2.51*	58.85 ± 2.71	36.73 ± 4.50*	52.80 ± 5.77
Neutrophils (%)	47.88 ± 2.64*	11.54 ± 1.75*	32.73 ± 4.50*	18.40 ± 2.04
Eosinophils (%)	2.50 ± 0.45*	3.69 ± 0.72	2.18 ± 0.52*	6.40 ± 0.93
Monocytes (%)	14.63 ± 2.31*	25.77 ± 3.03	28.45 ± 3.25	22.60 ± 4.03
<i>Plasma determinations</i>				
Glucose (g/L)	1.46 ± 0.18*	0.46 ± 0.04	0.50 ± 0.05	0.40 ± 0.07
Total Protein (g/dL)	3.51 ± 0.18*	1.85 ± 0.20	2.36 ± 0.21	2.56 ± 0.24
ChE (μkat/L)	51.68 ± 5.15	43.58 ± 4.54	60.34 ± 13.84	76.58 ± 10.06

2

1 Figure 1.

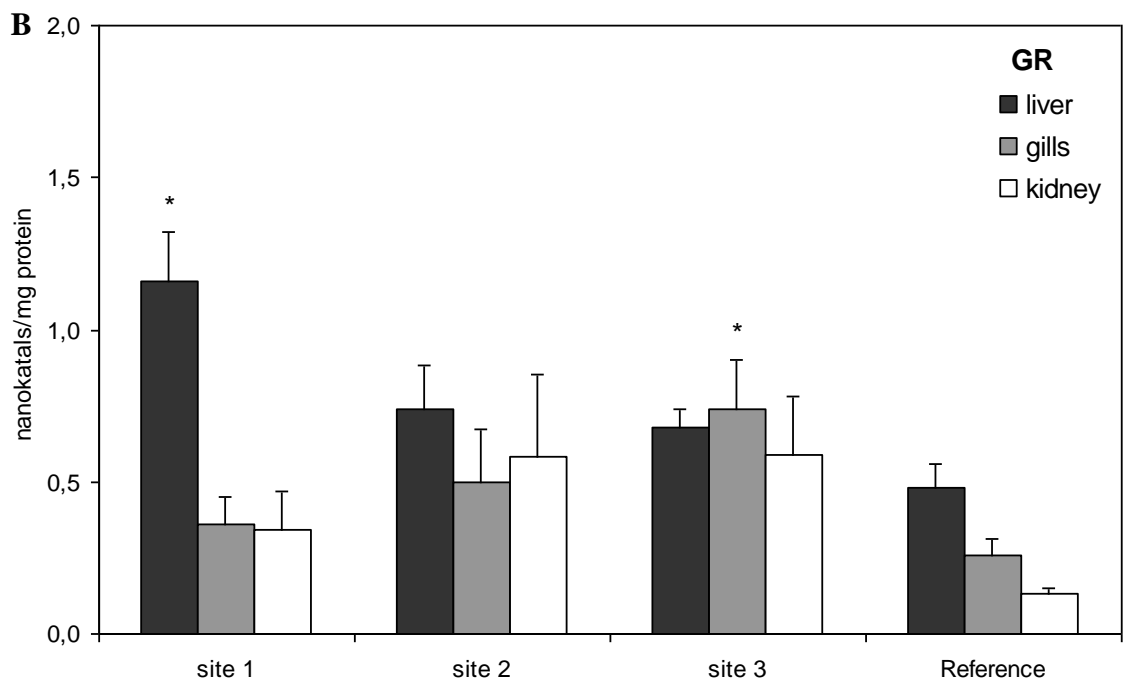
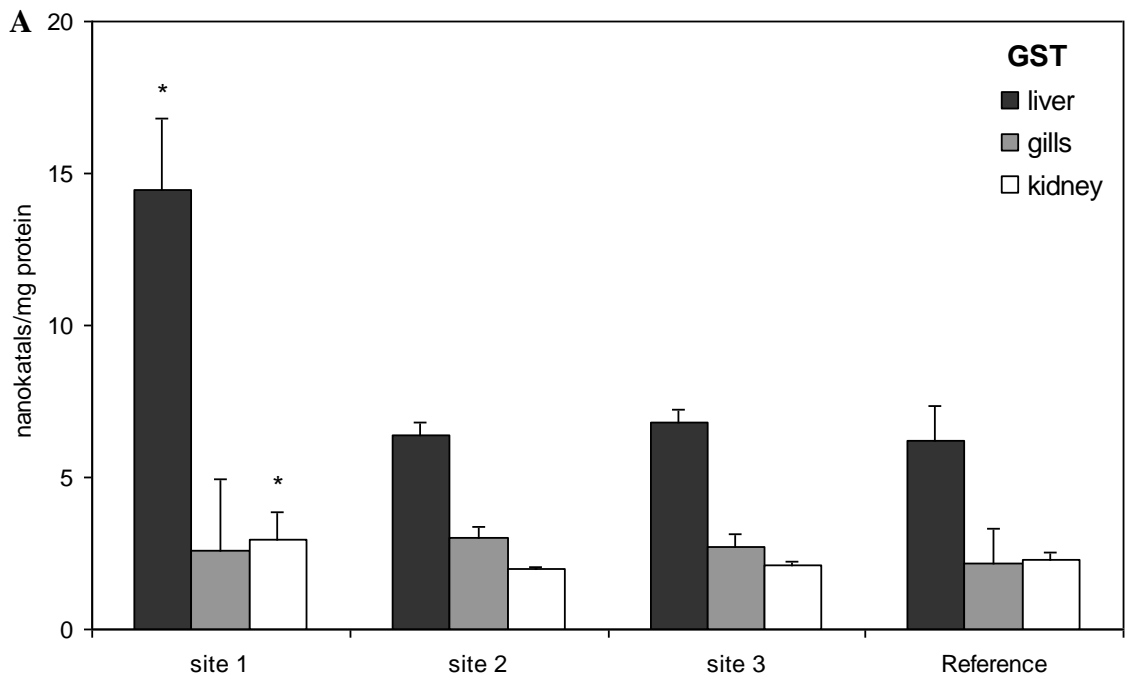


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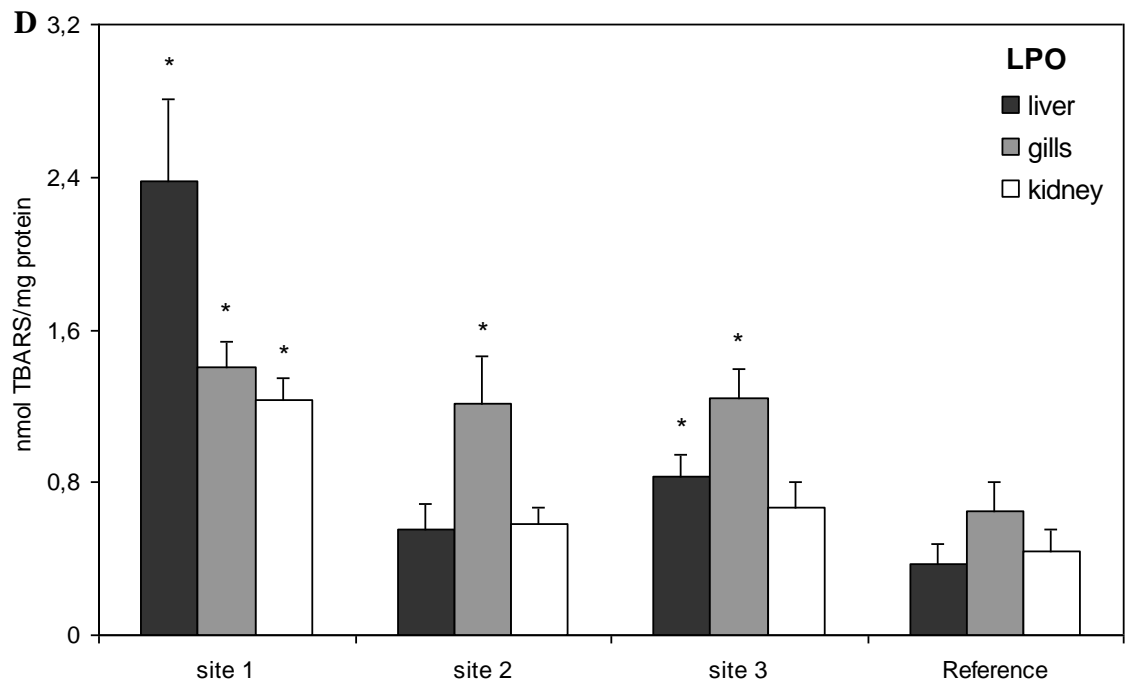
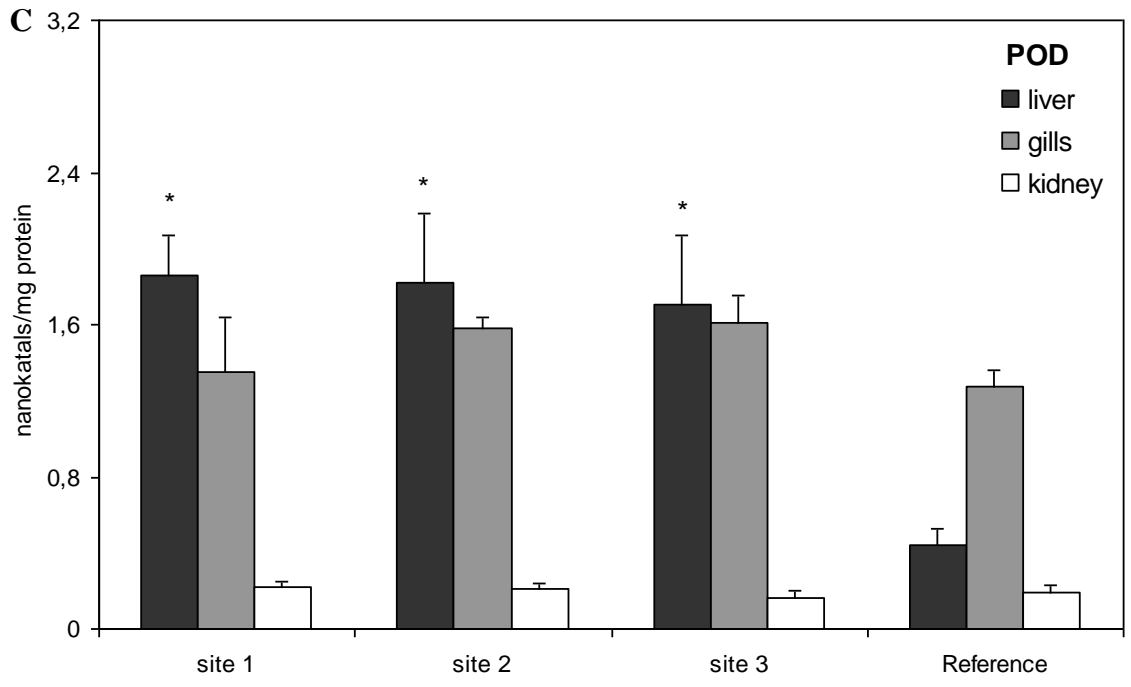
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1 **Figure 2.**

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1 **Figure 3.**

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