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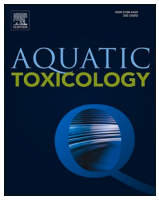

A trait-based approach to determine the risks of Zn to the overall health status of native fish species *Barbus meridionalis*

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ABSTRACT

Fish adapt to changing environments by maintaining homeostasis or making energy trade-offs that impact fitness. We investigated the effect of Zn on the fitness and physiology of *Barbus meridionalis*, a native cyprinid fish species, under two exposure scenarios. The Osor stream's mine-effluent reach represented long-term (chronic) exposure, while the upstream reach served as a control/acute exposure. Acute exposure involved exposing *B. meridionalis* to 1mg/L Zn for 96 h in the laboratory. We examined physiological traits (Standard metabolic rate SMR, Maximum metabolic rate MMR, Absolute Aerobic scope AAS, Critical swimming capacity U_{crit}) and antioxidant system, AS (Superoxide dismutase, SOD; Catalase, CAT; Glutathione peroxidase, GPX; Glutathione-*S*-transferase, GST; Glutathione, GSH; Thiobarbaturic acid reactive substances, TBARS) biomarkers. The results indicated that Zn had no significant effect on osmoregulatory cost (SMR) in either exposure scenario but impaired energetically costly exercise (low MMR). AAS reduction in both exposure groups suggested compromised energy allocation for life-history traits, evidenced by decreased locomotor performance (U_{crit}) after acute exposure. Tissue-specific and time-dependent responses were observed for AS biomarkers. The fish exhibited ineffective control of oxidative damage, as evidenced by high TBARS levels in the liver and gills, despite increased CAT and GSH in the liver under acute conditions. Our findings demonstrate differential responses at the subcellular level between the two exposure scenarios, while trait-based endpoints followed a similar pattern. This highlights the utility of a trait-based approach as a supplementary endpoint in biomonitoring studies, which provides insights into impacts on individual fitness and population demography.

1. Introduction

Agricultural activities, urbanization, and mining activities are sources of trace metal enrichment in aquatic ecosystems (Wood et al., 2012). Once introduced, these metals persist and disperse based on their bioavailability and mobility. Zinc (Zn), in particular, is an essential element, with approximately 10% of proteins in the proteome dependent on it, enabling Zn to enter fish bodies unhindered (Hogstrand, 2011). However, ecological concerns regarding Zn have been supported by empirical evidence, leading to the classification of water bodies into different categories (I to V) based on Zn concentrations ranging from 70 μg/L to >1163 μg/L (OECD., 2007).

Aquatic pollution has a significant impact on fish health as it is a top-level predator and experience an integrated effect of surrounding environment and organisms (Rubio-Gracia et al., 2022). Despite the physiological ability of fish to quickly adapt to changing environments, exposure to metals can cause cellular damage through the generation of reactive oxygen species (ROS) and interference with antioxidant responses (Loro et al., 2012). Numerous studies have investigated the impact of metals on subcellular biomarkers, offering valuable insights into the underlying mechanisms. However, the ecological implications of subcellular disturbances remain uncertain (Vieira et al., 2012). ROS can serve as a biomarker to understand the detoxification and repair costs experienced by organisms exposed to pollutants. While this molecular effect can mechanistically explain the higher maintenance costs, it does not fully elucidate the effects on life-history traits, such as

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growth, locomotion, and reproduction, resulting from increased maintenance costs experienced by fish inhabiting polluted environments (Kooijman, 2018).

Examining metabolic rates provides valuable insights into the fitness and behavior of fish. The rate of oxygen consumption is a commonly used measure of metabolic rate (Chabot et al., 2016). Three key types of metabolic rates are considered: standard metabolic rate (SMR), maximum metabolic rate (MMR), and absolute aerobic scope (AAS). SMR represents the energy needed for maintenance during post-absorptive and inactive states, while MMR represents the upper limit of metabolic activity (Norin and Clark, 2016). The difference between these two rates defines the aerobic scope, which determines the organism's capacity for simultaneous activities (Clark et al., 2013). In a stressful environment and based on the organism's physiological needs, the organism makes energy trade-offs between traits by partitioning the aerobic scope (Fu et al., 2022). For example, Abdel-Tawwab et al. (2018) observed behavioral changes in fish exposed to waterborne Zn pollution, resulting in reduced feed utilization and impaired growth performance. Similarly, Mager and Grosell (2011) studied the effects of lead (Pb) on fathead minnows (*Pimephales promelas*) during acute and chronic exposures. Pb significantly decreased the aerobic scope and locomotor performance (U_{crit}) of the fish. To compensate for the energy limitation, fathead minnows (*Pimephales promelas*) reduced their budget for locomotion. Such compromises can impact population fitness and potentially influence species' geographic distributions or home ranges. Fish, for instance, tend to prefer low-flow areas when their locomotor performance is compromised (Binning et al., 2015). Therefore, a trait-based approach reveals diverse responses within the same species under different conditions and provides insights into the functional performance of the population, as traits are linked to ecological activities (Kolok, 2001).

In our study we used Mediterranean barbel (*Barbus meridionalis*); a native cyprinid species which is currently at an increased risk of extinction (Crivelli, 2006). Its population is most abundant in fast flowing, well oxygenated and shallow waters at high altitudes having rich growth of aquatic vegetation and halophytes. The species adapts a benthic eating strategy with primary diet consists of larvae of chironomids, ephemeropterans, terrestrial prey, and amphipods. Females of *B. meridionalis* are generally observed to have higher growth rates and can live up to 10 years, while males have a maximum lifespan of 7 years. The structure of the population is primarily dominated by age with an average total length often below 10 cm (Zamora, 2017). As an endemic species, *B. meridionalis* is particularly sensitive to habitat degradation and pollution, which are the main drivers of its population decline (Merciai et al., 2016).

Our study focused on the streams of the Osor River, where water quality has been significantly degraded by mining runoff, with zinc (Zn) being the primary pollutant that exceeded established water quality criteria (Bonet et al., 2014; Bori et al., 2016). Previous in vitro studies on the *B. meridionalis* from the Osor River demonstrated an imbalanced antioxidant system (Atli et al., 2020). Additionally, Rubio-Gracia et al. (2022) reported Zn accumulation in periphyton and macroinvertebrates, suggesting a potential bottom-up impact on the population structure of the *B. meridionalis*. Considering these findings, the primary objective of our study was to evaluate the effects of pollution on the physiology and overall fitness of *B. meridionalis*. For this purpose, we examined the activity of first-line antioxidant enzymes and biomarkers of lipid peroxidation to assess oxidative damage and understand the sub-cellular responses. Additionally, we used metabolic traits (SMR, MMR, and AAS) and critical swimming speed (U_{crit}) to gain insights into bioenergetics and locomotor performance of fish. We focused on a case of Zn pollution, resulting from long-term exposure due to mining activities affecting the *B.meridionalis* population. To validate our results, we also conducted an acute exposure under a laboratory-controlled environment. We anticipated that both laboratory and field exposures would yield similar effects on the selected traits, enabling us to establish a connection between two exposure conditions. These outcomes were expected to align with early responses of antioxidant enzymes, as well as the damage to cell membranes caused by reactive oxygen species (ROS)-mediated lipid peroxidation.

2. Materials and methods

2.1. Study area

Our study area was a second-order stream of the Osor River in the northeastern Iberian Peninsula (40.4830°N, 4.0876°W). This river is 23.5km long with a drainage area of 88 km^2 and enters the Ter River (Fig. 1; Rubio-Gracia et al., 2022). The area, located 35km SE of Girona, was heavily exploited by mining operations for sphalerite (Zn, Fe, S) and galena (PbS) until 1980. However, after the termination of mining activities, significant amounts of ore waste were generated, resulting in the contamination of downstream water bodies with Cd, Pb, Zn, and other metals (Bori et al., 2016). Therefore, the ecological quality of the river has been greatly affected by hydrological alteration and metal pollution from mining activities.

Monitoring studies were conducted, and the seasonal concentrations of metals were analyzed in aquatic compartments. Zn levels were found to be as high as 450μg/L and therefore categorized as the primary pollutant of the river (Bonet et al., 2014; Rubio-Gracia et al., 2022). The concentration was, however, dependent on the fluctuating river discharge and changing seasons, with the highest values coinciding with summer droughts (Bonet et al., 2013).

2.2. Fish sampling, holding and exposure condition

Sampling was performed in July 2019. We selected two reaches along the stream for sample collection: a reference location used as a field control (3.7 km upstream from the mine effluent) and a polluted location to assess the field-chronic toxicity (below the mine effluent and water diversion channel) (Fig. 1). These two reaches are hereafter referred to as upstream (UP, reference location) and mine (M, polluted location). There were three groups: a control and laboratory-simulated acute-exposure group, both of which were sampled from upstream, and a field chronic-exposure group that was sampled from the polluted location (M). Fish sampling was conducted in both locations using electrofishing equipment (LR-24 Smith-Root Ltd. 120 V DC-0.6 A). In total, 46 individuals were collected for all respirometry trials, with a standard body length (BL) of 9.28 ± 0.78 cm and wet mass (WM) of 12.1 ± 3 g. Additionally, 21 individuals were collected for oxidative stress analysis (BL = 13.89 ± 1.54 cm and WM = 30.5 ± 9.4 g), corresponding to both chronic exposure and control groups (dissected on-site) and for laboratory exposure, 15 individuals (acute and control group) were collected with BL = 13.49 ± 0.67 cm and WM = 21 ± 3.7 g. The Zn bioaccumulation in tissues was studied in the 21 individuals which were sampled from field for oxidative stress.

Sampling campaign extended for two days, covering both the M and UP reach; water was also collected from the various points to analyze the Zn concentrations. Similarly, during acute exposure, water was periodically sampled for the same purpose. Table 1 details the Zn concentrations of the water. The fish sampled for oxidative stress from the M reach and their control counterparts were immediately dissected, whereas dissections of fish in the laboratory acute-exposure group were performed after 96 h of exposure. Prior to starting, fish were acclimatized to the laboratory conditions for two weeks. Stream water from each location was collected using a pump, stored in drums (30 L) and transported to our facilities. This procedure was repeated every week to simulate similar environmental conditions in our treatments. Water replacements were performed (60%) every 3 to 4 days with the corresponding stream water to maintain the water quality. Continuous aeration was conducted (O_2 concentration range: 7.53-8.85 mg/L) and was maintained at 18±1 °C with a natural photoperiod cycle. The concentrations of NH_4^+, NO_3^-

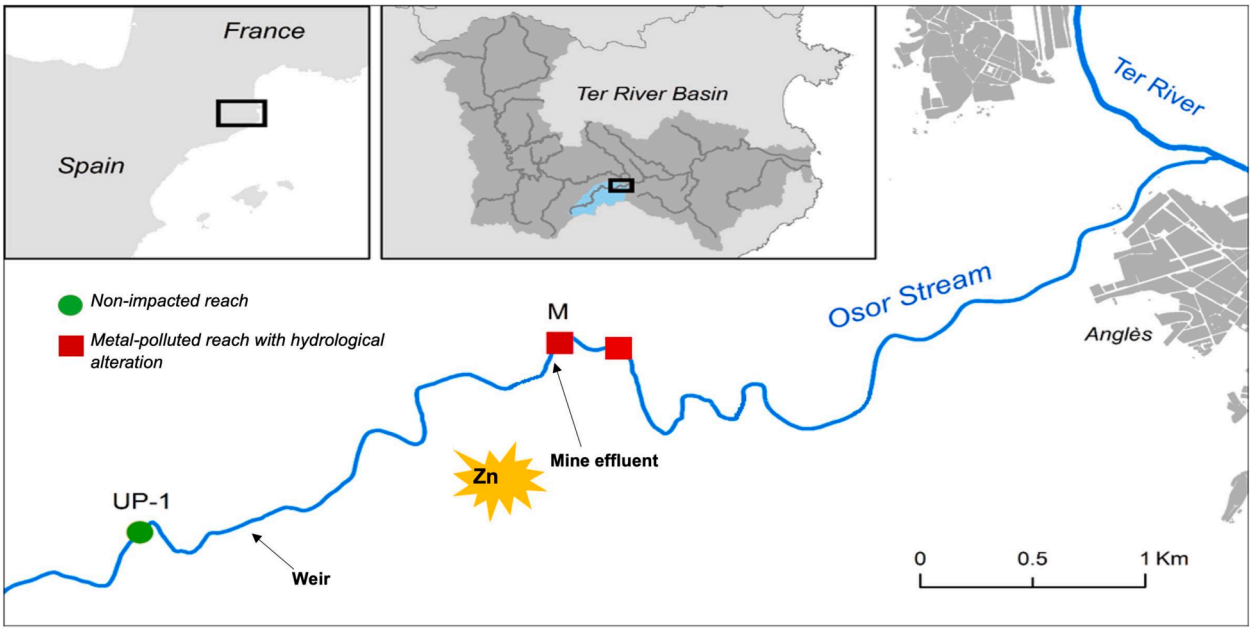

Fig. 1. Map illustrates the locations of the mine effluent/metal-contaminated (M) reaches, highlighted in red, and the non-impacted reach (UP-1) highlighted in green. Fish sampled from the M reach were analyzed for chronic effects, while fish sampled from the upstream reach (UP) served as controls and used for laboratory experiments (Rubio-Gracia et al., 2022).

Table 1
The concentration of Zn observed in the water samples (μg/L) collected from mine (M) and upstream (UP) reach and during 96h for acute exposure.

Location			Zn concentrations (μg/L)	
			Day 1	*Day 2*
Chronic exposure	**M reach**	*L1*	564.56	345.12
		L2	754.3	262
	UP reach	*L1*	5.1	7.85
		L2	4.21	12.26
Acute exposure	*Exposure duration*		*Aquaria 1*	*Aquaria 2*
	Start (0 hour after exposure)		615	624
	48h (before water change)		252.74	306.25
	48h (after water change)		742	613.73
	96h		360	502

and NO_2^- were maintained at <0.5 mg/L, <10 mg/L, and <0.25 mg/L, respectively, and pH 8.0-8.7. From the second day of acclimation, fish in all treatment groups were fed to satiation once a day with frozen bloodworms (*Chironomus* spp.). However, before the respirometry trials, they were starved for 24 h to avoid the postprandial effects. Very few mortalities ($N = 2$) occurred during the acclimation period, and the health condition of all individuals was good. At the end of the trials, all fish were kept in quarantine for at least one week and then returned to the capture sites.

2.2.1. Control group

The fish were kept in aquaria filled with upstream water during the whole period. Once acclimated, the fish were individually placed into 40 L glass aquaria. For the respirometry trials, 15 fish (BL = 13.3 ± 1.12 cm; WM = 28.1 ± 6.86 g) were used as controls for both chronic and acute groups. However, in the case of oxidative stress analysis, 12 individuals from the UP reach (BL = 13.27 ± 1.11 cm; WM = 28.1 ± 6.85 g) were utilized as controls of chronically exposed fish, and 8 individuals (BL = 13.18 ± 0.35 cm; WM = 19.65 ± 1.61 g) were used as controls for acutely exposed group.

2.2.2. Laboratory-acute group

The fish were kept in aquaria filled with upstream water during the acclimation period. After acclimation, the fish were distributed in 40 L glass aquaria with Zn (1 mg/L for 96). A Zn concentration of 1 mg/L was achieved by diluting the 80 mL of the 500 mg/L stock solution (Zn Titrisol®, $ZnCl_2$ in 0.06% HCl) with distilled water however the highest concentration observed was 742 μg/L (Table 1). The pH was kept within optimal range of 7.0-7.5, additionally CO_2 pumps along with pH regulators were attached to the aquaria to maintain the water pH throughout the exposure duration. Water was changed (100%) after 48 hours, and another 80 mL of 500 mg Zn/L was added to the refilled water until acute exposure termination (96 h). Respirometry trials were performed on 14 fish (BL = 13.5 ± 0.68 cm; WM = 21.0 ± 3.71 g) and 7 fish (13.84 ± 0.8 cm; WM = 22.65 ± 4.83 g) were used for oxidative stress analysis.

2.2.3. Field-chronic group

Fish sampled from the mine (M) were kept in aquaria filled with mine water (14 days) in the laboratory to eliminate any effect of handling stress. A total of 15 fish (BL= 14.7 ± 1.70 cm; WM = 33.3 ± 9.89 g) were used for respirometry trials and 9 fish (BL= 14.7 ± 1.71 cm; WM = 33.7 ± 11.65 g) were used for oxidative stress analysis.

2.3. Zn analysis

The water samples were filtered using Whatman nylon filters (pore size of 0.2 μm) and acidified using 1% HNO_3 (65% suprapure, Merck). The concentration of dissolved Zn was analyzed using inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies). As for fish, tissues were lyophilized, weighted with an analytical balance (Menttler-Toledo AX205), and digested in nitric acid (4 mL HNO_3, 65% suprapure, Merck) and hydrogen peroxide (1 mL H_2O_2, 30% suprapure, Merck) in a high-performance microwave digestion unit (Milestone, Ethos Sel). Digested samples were analysed by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies) and by inductively coupled plasma mass (ICPOES 5100 Agilent Technologies). Analytical accuracy was determined using certified reference material of the Joint Research Centre (European Commission), i.e., standard trace elements in fish muscle tissue (ERM-BB422).

2.4. Respirometry

Before the swimming trials, individuals were measured (BL) to the nearest 1mm and weighed (wet mass, WM) to the nearest 0.1mg. We set up the same intermittent-flow respirometry system as in our previous work with *B. meridionalis* (Rubio-Gracia et al., 2020). Swimming speeds and oxygen consumption rates were measured using a Blazka-style swimming tunnel respirometer (Loligo® Systems, Viborg, Denmark). It consisted of a rectangular measuring recirculation system equipped with a swimming chamber of 5L (30cm length × 7.5cm height × 7.5cm width). The external water bath (25L) connected to the swim tunnel respirometer was equipped with an automated pump (Eheim) that flushed constantly aerated water at a rate of 10 L/min. Thus, complete mixing of the respirometer water between flush cycles was ensured. Additionally, an external water bath was connected to a supply plastic tank containing 300L of air-saturated freshwater and equipped with an automated liquid cooler (85W, 972.46 BTU/h, J. Selecta®) to maintain the temperature at 18°C. A honeycomb plastic screen was placed at the entrance of the swimming section to make the flow inside the swim tunnel respirometer rectilinear. For calibration purposes, water flow velocities were measured in the center of the flume and above the substratum using a handheld digital flow meter (Höntzch, 4–20mA/0–10V input).

An optical fiber instrument (Witrox 1; Loligo® Systems, Tjele, Denmark) was used to determine dissolved oxygen concentration in the water (mg O_2/L) and a temperature probe (Pt1000 temperature sensor; Witrox 1; Loligo® Systems, Tjele, Denmark) was used for the automated compensation of oxygen data to changes in temperature and barometric pressure in real time. A two-point calibration with the oxygen sensor was used to record the highest water concentration value as 100% air saturation and the lowest water concentration value as 0% using a solution of sodium sulfate (Na_2SO_3, 0.159M). Oxygen consumption rates ($\dot{M}O_2$) were measured using computerized, intermittent flow respirometry, AutoResp™ software (Loligo® Systems, Tjele, Denmark). Intermittent-flow respirometry consists of a series of linear declines in oxygen content during the measurement period interrupted by exponential increases during the flush period.

2.5. Standard metabolic rate (SMR) measurement

The fish were placed individually into the swim tunnel respirometer and left overnight to allow for $\dot{M}O_2$ measurements of a resting, non-digestive state with a loop of 120s flush, 180s wait and 600s measurement period. The current speed was set to 1cm/*s* to provide a higher level of water mixing during respirometry measurements. The overnight experimental trial to estimate SMR lasted approximately 18h. The SMR was calculated as the mean value of the lowest 10% MO_2values from the entire 18h period of data collection. Only the measurement periods that yielded linear regressions with $r^2 > 0.70$ were included in the analysis. Oxygen consumption by fish was calculated by fitting a linear regression of the oxygen concentration decline over time at each measuring period. Slopes of linear regressions were then used to calculate oxygen consumption rates ($\dot{M}O_2$, in mgO_2/h), following the equation:

$$\dot{M}O_2 = -(\Delta Of - \Delta Ob) \times V,$$

where ΔOf and ΔOb are the rates of oxygen consumption in mg O_2 /*L*/min due to fish and microbial respiration, respectively, and V is the volume of the swim tunnel respirometer (after subtracting the fish volume). Oxygen concentration measurements without any fish in the swim tunnel respirometer were conducted before and after the experiment to quantify background microbial respiration. Values of background respiration were then averaged and subtracted from $\dot{M}O_2$ measurements with fish.

2.6. Locomotor performance (U_{crit}), maximum metabolic rate (MMR) and absolute aerobic scope (AAS)

The next morning, once the SMR protocol finished, the duration of the loop was changed to a 120s flushing period, followed by a wait period of 120s and then a measurement period of 1200s. The current speed inside the respirometer was gradually increased every 24min with 1 BL/*s*, starting from 0.5 BL/*s* until the fish were swept against the rear end of the respirometer. When this occurred, the fish were considered fatigued, and the locomotor performance in terms of critical swimming speed (U_{crit}) was calculated following Beamish (1978):

$$U_{crit} = U_f + U_i T_f T_i^{-1}$$

where U_f is the highest velocity maintained for the entire swimming period, U_i is the speed increment (BL/*s*), T_f is the time elapsed at fatigue speed and T_i is the set interval time (min). The MMR was defined as the highest oxygen consumption rate during the swimming trial, which was usually close to the highest velocity (Rubio-Gracia et al., 2020). The AAS was calculated as the difference between the MMR and SMR (Clark et al., 2013).

Background microbial respiration was measured in the empty respirometer after each swimming trial at a current speed of 1cm/*s* (to ensure appropriate oxygen mixing) using a wait period of 120s and a measurement period of 1200s. Background microbial respiration was measured before and after the trial and then averaged and subtracted from metabolic rate measurements.

2.7. Antioxidant enzyme system (AES)

The dissection was performed by transection of the spinal cord following the best ethical and practice guidelines of the Ethics Committee of the University of Girona. Total lengths and weights were measured prior to dissection. Fish tissues (liver, gill, and kidney) were dissected using clean equipment, pooled in hermetic plastic bags on ice, and stored at −80°C in the laboratory until analysis. The optimal conditions of incubation media were used according to our antioxidant enzyme characterization study (Atli et al., 2020) for antioxidant enzyme analyses. Assays were run at least in duplicate for each sample.

LPO was measured by using a thiobarbituric reactive substances (TBARS) assay, which quantifies the LPO products reacting with thiobarbituric acid. TBARS analysis was performed based on the incubation of supernatants and thiobarbituric acid (TBA) in aerobic conditions at a temperature of 100°C. The formation of a pink colored complex at 532nm determined its concentration (Wills, 1996), which was calculated from an external standard curve of 1,1′,3,3′-tetramethoxypropane. The values were expressed in nanomoles/mg protein.

CAT activity was measured in 1mL of the substrate solution containing 25mM H_2O_2 in 100mM potassium phosphate buffer (pH 7.5) and 20 μL of supernatant. The H_2O_2 concentration (25mM) was quantified with the specific absorption coefficient (0.0392 cm^2/μmol H_2O_2) at 240nm of this molecule. The absorbance decrease was monitored at 240nm for 1min and expressed as μmol H_2O_2 decomposed/mg prot/min (Lartillot et al., 1988).

SOD activity was measured using the indirect method involving the inhibition of cytochrome c reduction at 550nm for 1min (McCord and Fridovich, 1969). The reaction buffer in a final volume of 1mL contained 50mM potassium phosphate buffer (pH 7.0), 0.1mM EDTA, 10μM cytochrome c, 0.05mM hypoxanthine, and 10 μL supernatant. The reaction was started by adding 1.88 mU/mL xanthine oxidase (XOD). An XOD blank was used to adjust the XOD concentration. A unit of SOD activity was expressed as unit/mg prot and was defined as the amount of enzyme that caused 50% inhibition of cytochrome c reduction.

GPX activity was measured in a 1mL reaction buffer consisting of 100mM potassium phosphate buffer (pH 7.0), 2mM reduced glutathione (rGSH), 0.12mM NADPH, 2U glutathione reductase (GR), 10 μL

supernatant, and 3mM *cumene* hydroperoxide (Livingstone et al., 1992). The activity was expressed in μmol/mg prot/min to estimate the decrease in NADPH for 1min at 340nm (specific absorption coefficient is 6.22 */M/*cm NADPH at 340nm).

GR activity was also expressed in μmol/mg prot/min following the NADPH decrease at 340nm for 1min (Carlberg and Mannervik, 1975). One milliliter of reaction medium contained 100mM potassium phosphate buffer (pH 7.5), 0.1mM NADPH, and 30 μL of supernatant. The reaction was initiated by the addition of 1mM oxidized glutathione (GSSG).

GST activity was evaluated as the increase in absorbance for 1min at 340nm that resulted from rGSH and CDNB (1-chloro-2,4-dinitrobenzene) conjugation (Habig et al., 1974). The specific absorption coefficient of the conjugate *S*-(2,4-dinitrophenyl)-glutathione was 9.6/mM/cm at 340nm. One milliliter of reaction buffer contained 100mM potassium phosphate buffer (pH 7.5), 1mM GSH, 1mM CDNB, and 10 μL of supernatant. GST activity was expressed in μmol/mg prot/min.

GSH levels were analyzed by measuring the absorbance increase at 412nm for 1min and expressed as micromoles of GSH equivalents per milligram of protein (Griffith, 1980). GSH was used as a standard. The reaction buffer in a final volume of 1mL contained 100mM sodium phosphate buffer (pH 7.5), 4.2mM NADPH, 0.8mM DTNB, 75U/mL GR, and 25 μL supernatant.

Total protein levels of the supernatants were measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

2.8. Statistical analyses

All statistical analyses were performed using the SPSS package (SPSS 15.0). All variables were $\log_{10}$-transformed to linearize the data, and residual plots of dependent variables were used to confirm the homoscedasticity and normality of residuals. Linear regression analysis was used to evaluate the degree of association between dependent variables (U_{crit}, SMR, MMR and AAS) and fish BL and WM as covariates. The covariates that were significantly related to dependent variables were then used in subsequent analyses of covariance (ANCOVA). In the models, we treated Zn treatment as a fixed effect, U_{crit}, MMR and AAS as dependent variables, and BL or WM as covariates. Similarly, to test for differences in body condition (WM-BL relationship) between the field-control group (UP) and the field-chronic group (M), we applied ANCOVA with Zn treatment as a fixed effect, BL as a covariate and WM as a dependent variable. The ANCOVA assumptions were tested by analyzing the interactions between the covariates and the categorical factor. If the assumption of homogeneity of regression slopes was satisfied, the interactions were removed from the model to improve the statistical power of the ANCOVA analysis.

The SMR did not correlate with either covariate (WM, BL); therefore, an analysis of variance (ANOVA) was performed to compare the differences between Zn treatments. Tukey's multiple comparison tests (Bonferroni correction) were applied to assess the differences in covariate-adjusted means of the dependent variables among Zn treatments. One-way ANOVA was also used to compare the levels of oxidative stress biomarkers among treatments. When significant differences were found, the Bonferroni test was used as a post hoc test, and Tukey's multiple comparison tests (Bonferroni corrected) were applied to analyze differences between treatments. Spearman rho correlation was also applied to compare the antioxidant parameters' correlations ($P<0.05$).

Orthogonal contrasts were used to determine the effect of Zn on the studied endpoints/variables. The effect was assessed by comparing the control group (no Zn) to the mean value of Zn-exposed groups (chronic and acute), whereas the time- and concentration-dependent effect was determined by comparing the two Zn-exposed groups (field-chronic and lab-acute). Cohen's *d* was then calculated for each physiological variable and used to compare the magnitudes of the effects between groups, with confidence intervals computed following Nakagawa and Cuthill (2007).

3. Results

3.1. Metabolic and locomotor performance traits

Neither type of Zn exposure showed prominent changes in BL and WM, reflecting the same size range at the capture site and after both types of exposure (Fig. 2). However, Zn exposure influenced the metabolic and locomotor performance-related endpoints (Fig. 3). There were no significant regressions between U_{crit} and BL ($F_{2.43} = 1.617$, $P = 0.212$) and between U_{crit} and weight ($F_{2,43} = 0.691$, $P = 0.507$). However, differences among groups were observed (control, chronic, acute). After the means were adjusted for the covariate (BL) ($F_{2,43}$=4.94, P=0.012), Zn showed to have lowered the $U_{crit,}$ and we observed a significant difference between the lab-acute group and controls (P=0.013), but the effect was not found in the field-chronic group.

The MMR showed no interaction with WM ($F_{2,43}$= 1.086, P=0.348) and BL ($F_{2,43}$= 1.719, P=0.193). After adjusting the means (WM), the groups showed significant differences ($F_{2,43}$= 12.935, $P<0.001$). Zn reduced the MMRs, and the effect of field-chronic exposure was validated by the lab-acute group, indicating an effect undoubtedly caused by Zn. Significantly lower values were obtained in both Zn-exposed groups than in the control group ($P_{acute\text{-}con} < 0.001$, $P_{chronic\text{-}con} = 0.022$).

The AAS also showed no relationship with WM ($F_{2,43}$= 0.925, P=0.405) and BL ($F_{2,43}$= 1.386, P=0.262). After adjusting for means, the acute and chronic means were significantly lower ($P<0.001$) than that of the control, again suggesting a Zn-dependent effect.

The SMR was significantly related to WM ($F_{2,43}$=4.633, P=0.016) and SL ($F_{2,43}$=4.093, P=0.025). The comparison of all groups revealed no significant differences ($P>0.05$) (Fig. 2).

3.2. Oxidative stress biomarkers

Fish from the field-chronic group exhibited elevated levels ($P<0.05$) of TBARS in gills (0.24±0.09nmol/mg prot) and liver (0.15±0.05nmol/mg prot) compared to their respective controls (TBARS gills: 0.17 ±0.04nmol/mg prot; TBARS liver: 0.08±0.03nmol/mg prot). LPO observed in the field-chronic group indicated oxidative stress-mediated damage. Liver TBARS levels was negatively correlated with SOD activity (r^2 = -0.86, $P<0.05$) though positively correlated with GPX activity (r^2 = 0.86, $P<0.05$) under chronic conditions. The effect, however, was not observed with the lab-acute group (0.10±0.01nmol/mg prot) (Fig. 4A).

Zn increased the activity of CAT ($P<0.05$) in the kidneys of the field-chronic (56.3±39.0μmol H_2O_2/mg prot/min) and in the liver of lab-acute (1160.9±760.3μmol H_2O_2/mg prot/min) groups compared to their control counterparts. There is a positive correlation between CAT and SOD activities in the kidney of the lab-acute (r^2 = 0.93, $P<0.05$). The intergroup differences between exposed fish showed lower CAT in gills after lab-acute exposure (22.1±5.20μmol H_2O_2/mg prot/min) compared to field-chronic exposure (33.0±11.5μmol H_2O_2/mg prot/min) ($P<0.05$) (Fig. 4B).

Increased SOD activity in the kidney was observed in the field-chronic group (48.7±15.9U/mg prot) compared to its control (31.0 ±7.50U/mg prot). The same effect was not observed in the lab-acute group, as the difference from its respective control was insignificant ($P<0.05$). The comparison of field-chronic and lab-acute conditions, however, suggested increased SOD activity ($P<0.05$) in the kidneys of fish in the field-chronic group only. The other tissues remained unaffected in both exposed groups (Fig. 4C).

Fish from the field-chronic group showed elevated GPX activity in the kidneys (0.09±0.02μmol/mg p/min), while fish from the lab-acute group showed higher activity in the gills (0.12±0.02μmol/mg p/min) in comparison to their controls. The intergroup differences between both exposures showed that field exposure repressed the activity of GPX

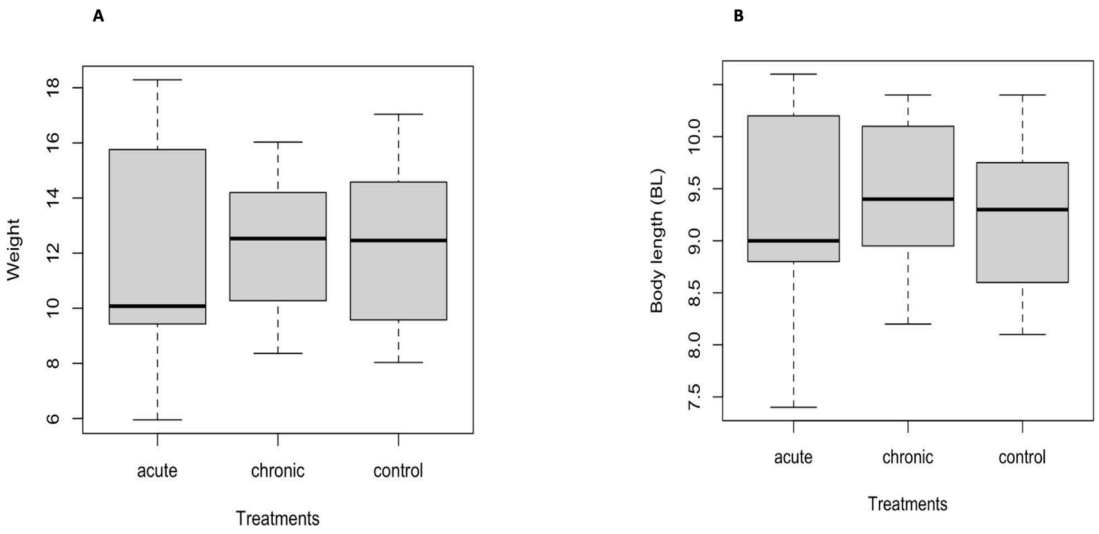

Fig. 2. Graph shows the wet mass (WM) and body length (BL) of *B. meridionalis*. The individuals exhibited similar sizes in all the groups (P>0.05).

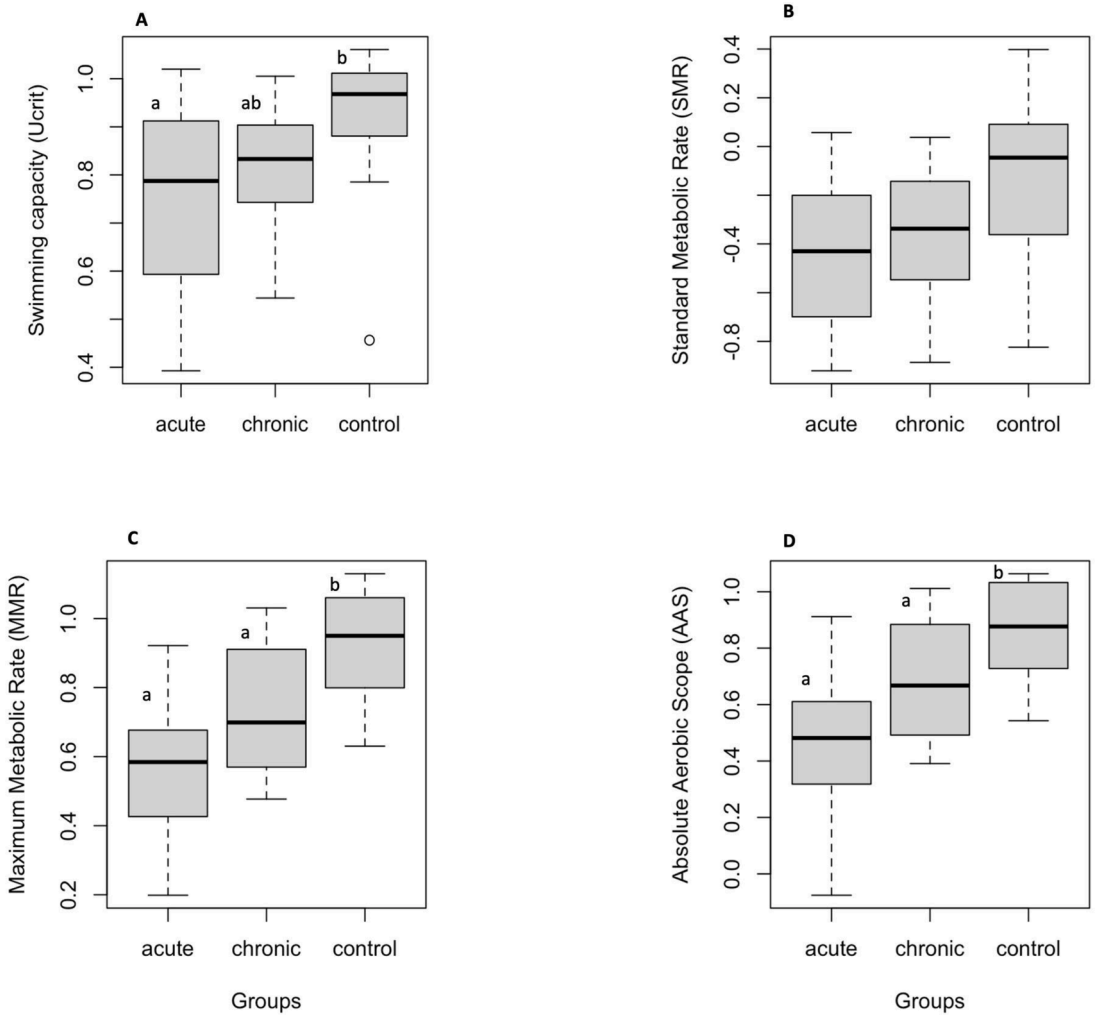

Fig. 3. Results of critical swimming speed (U_{crit}), standard metabolic rate (SMR), maximum metabolic rate (MMR), and absolute aerobic scope (AAS) observed in different groups (N_{acute}= 14, $N_{chronic}$= 15, and $N_{control}$= 15). The alphabets correspond to statistical difference (P<0.05)

(P<0.05) in the liver and gills of fish (GPX_{liver} 0.22±0.04μmol/mg prot/min; GPX_{gills} = 0.08 ± 0.04 μmol/mg prot/min) and increased the activity in the kidney (0.09±0.02μmol/mg p/min) in comparison to lab-acute exposure (GPX_{liver}: 0.79±0.50μmol/mg prot/min; GPX_{gills}: 0.12 ±0.01μmol/mg p/min; GPX_{kidney}: 0.07±0.01μmol/mg p/min) (P<0.05) (Fig. 4D).

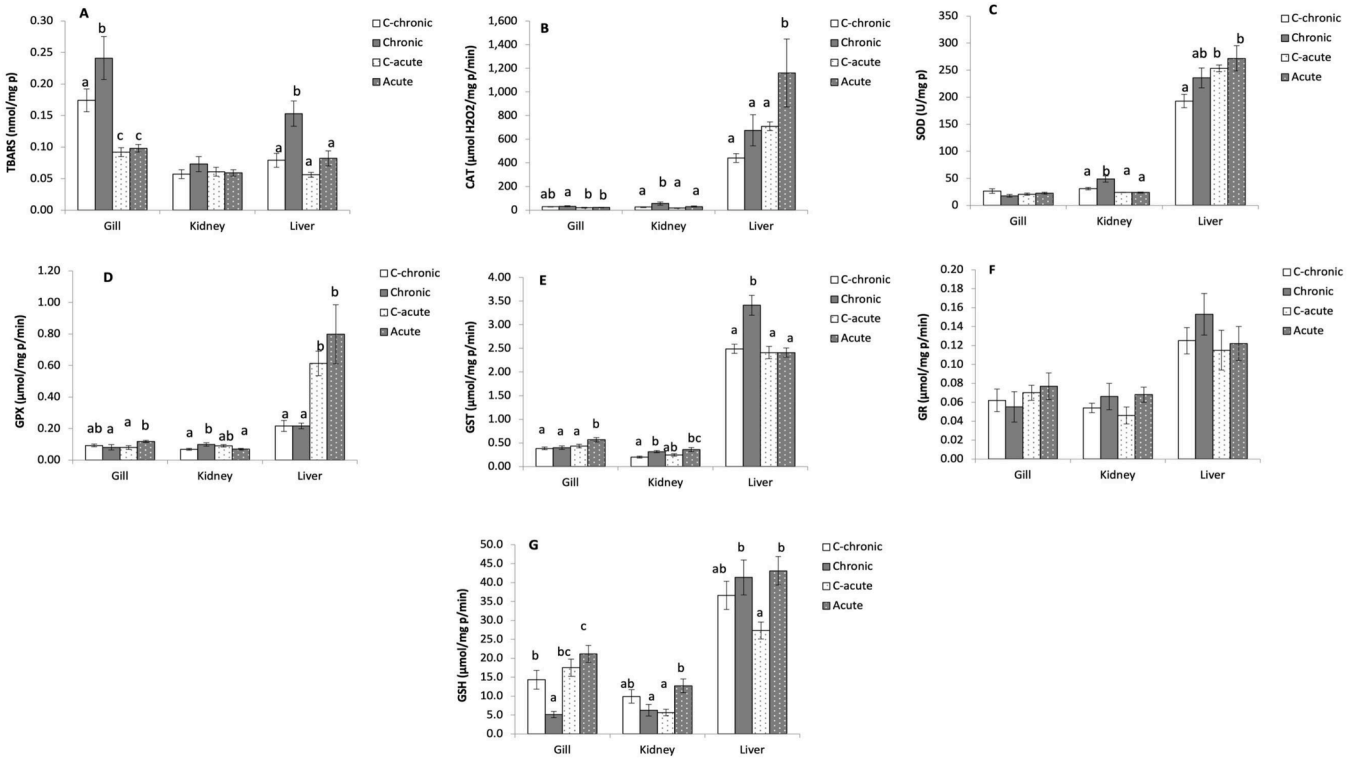

Fig. 4. Antioxidant system parameters (CAT, SOD, GPX, GST, GR, and GSH levels) and lipid peroxidation (TBARS) levels in the tissues of B. meridionalis (N_{acute}= 7; $N_{acute\text{-}conttol}$= 8; $N_{chronic}$= 9, and $N_{field\text{-}control}$= 12) Different letters indicate the statistical differences (P<0.05)

The field-chronic group exhibited a higher activity of GST in the liver (3.41±0.62µmol/mg prot/min) and kidney (0.31±0.06µmol/mg prot/min) compared to its respective controls (GST_{liver}: 0.25±0.31µmol/mg prot/min; GST_{kidney}: 0.2±0.6µmol/mg prot/min). The same effect of increased GST activity in the kidney was observed in the lab-acute group (0.36±0.11µmol/mg prot/min) compared to its control counterparts (0.24±0.07µmol/mg prot/min). The intergroup differences between exposure groups showed that field exposure elevated the activity of GST in the liver but reduced it in the gills (0.39± 0.1µmol/mg prot/min) compared with lab-acute exposure (GST_{liver}: 2.41±0.23µmol/mg prot/min; GST_{gills}: 0.56± 0.12µmol/mg prot/min) (P<0.05) (Fig. 4E).

Zn in both exposure groups, did not affect GR activity in any of the studied tissues (P>0.05) (Fig. 4F).

The GSH levels showed a marked decline (P<0.05) in the gills of the field-chronic group (5.1±2.4µmol/mg prot/min) compared to its control group (14.3±7.90µmol/mg prot/min); however, an opposite effect was seen in the lab-acute group (P<0.05) by observing a noticeable increase in GSH activity in the liver (43.1±10.0µmol/mg prot/min) and kidney (12.7±4.40µmol/mg prot/min) compared to its control (GSH_{liver}: 27.3 ±5.90µmol/mg prot/min; GSH_{kidney}: 5.60±2.40µmol/mg prot/min). Kidney GSH levels showed a positive correlation with GR activity (r^2 = 0.96, P<0.05). The intergroup differences between exposures showed that the field-chronic group had lower GSH activity in the gills and kidneys (GSH_{kidney}: 6.22±1.51µmol/mg prot/min), but Zn in the lab-acute group increased GSH in both tissues (GSH_{gills}: 21.1±5.9µmol/mg prot/min) (P<0.05) (Fig. 4G).

3.3. Tissue concentration

Higher levels of Zn were observed in the tissues of fish sampled from the M reach. The concentration of Zn in the sampled fish's gills was six times higher, four times higher in the liver, and twice as high in the kidneys compared to their control counterparts from the UP reach, as shown in Table 2.

Table 2
The concentration ranges of Zn found in the body tissues (µg/kg) of fish sampled from the upstream (UP) and mine (M) reach

Tissues	Zn accumulation(µg/kg)	
	UP stream reach (controls)	*Mine reach (Chronic)*
Liver	23.26 - 61.83	135.31-273.56
Kidney	44.18 - 95.80	117.66 - 229.77
Gill	49.33 - 81.41	267.33 – 638.25

3.4. Cohen's d

The Zn concentration effect sizes (Cohen's *d*) of 16 of the variables analyzed were large, 4 variable had medium effect sizes and 5 had small effect sizes Supp. Fig. 1 Most of the oxidative stress biomarkers increased in the presence of Zn (Supp. Fig. 1 Cohen's d negative values) in comparison to their control groups (Fig. 4), primarily in the liver and kidneys (i.e., TBARS, CAT, SOD, GPX and GST). The stimulatory effect sizes of Zn (Supp. Fig. 1 Cohen's d negative values) were large (≥ 0.8) in 11 of the variables analyzed, medium (≥ 0.5) in 2 and small (≥ 0.2) in 4. The presence of Zn in the water had an inhibitory effect (Supp. Fig. 1, Cohen's d positive values) on fish locomotor performance, as suggested by the lower MMR, AAS and U_{crit}. Finally, the presence of Zn also had a negative effect on the body condition of *B. meridionalis* irrespective of insignificant changes (Supp. Fig. 1, Supp. Fig. 2).

The effect size of exposures of different duration to different Zn concentrations (acute vs. chronic) in the physiological variables was large in many variables Supp: Fig. 2. It was slightly greater, as a whole, than the magnitude of the effect of the presence of Zn (mean d_{Znconc} = 0.995±0.139 SE vs. mean d_{Zn} = 0.764±0.089).

4. Discussion

4.1. Metabolic and locomotor performance traits

Functional integrity, in terms of swimming performance and metabolic rates, is a proxy of the general health of fish, it is well known that metal contamination alters the physiological traits (SMR, MMR, AAS) of fish and reduces their locomotor performance (U_{crit}) (Couture and Kumar, 2003).

In our study, reduced MMR and AAS were observed in the field-chronic group, and a noticeable effect on these variables was validated by lab-acute conditions, suggesting that Zn conspicuously caused an energy deficit in the fish. The SMR showed no significant differences in any of the groups. We predicted a high maintenance and osmoregulatory cost due to increased expenditure and energy demand associated with homeostasis impairment after Zn exposure, but our results showed that the SMRs of *B. meridionalis* remained unaltered. Nevertheless, our findings were consistent with those of (Bessemer et al., 2015) who found that Zn oxide nanoparticles (nZnO) had no effect on the resting metabolic rates of white suckers (*Catostomus commersonii*). These results can be explained in two ways: either the amplitude of the physiological perturbations was insufficient to cause a detectable increase in the SMR, or the physiological alterations halted the processes involved in energy production. (Dieni et al., 2013)supported the latter conclusion by observing a decrease in the activity of enzymes involved in energy production after nZnO exposure. The manifestation of this effect was observed in both exposure groups, as the MMR was found to be low, validating Zn-mediated toxicity, suggesting that the fish were unable to meet an increased energy demand during strenuous exercise (reduced MMR).

The MMR serves as a crucial endpoint in calculating AAS, and its reduction can have ecological implications. However, understanding its ecological role requires determining how frequently fish can achieve their MMR and reach their full metabolic scope which relies on knowledge about the species' lifestyle and habitat. Unfortunately, the lack of literature on *B. meridionalis* makes it challenging to determine the frequency of achieving energetic extremes (MMR) for this species. For instance, (Binning et al., 2014), observed that fish species adapt to higher flow regimes by enhancing their aerobic capacity through an increased aerobic ceiling. Since *B. meridionalis* is listed as a near-threatened species in the International Union for Conservation of Nature (IUCN), the primary reason for which is the sensitivity of *B. meridionalis* to habitat alteration and pollution (Crivelli, 2006). Our study site (stream of the Osor River) is a habitat of *B. meridionalis,* but the stream has been subjected to water diversion, pollution, habitat loss and changes in the flow regime(Bonet et al., 2013, 2014). However, during drought conditions, the species is frequently seen carrying forced migration to find refuge pools when the streams begin to dry up. (Merciai et al., 2016)d discovered that *B. meridionalis* undergoes ineffective migration consequently high mortality, indicating that the fish is incapable of expending energy for strenuous exercise. This behavioral constraint of *B. meridionalis* in the field strengthens the ecological implications of the reduced MMR that we observed in fish from the metal-polluted reach. A reduction in ceiling aerobic scope leaves individuals with a smaller safety margin for unexpected tasks and therefore depletes the energy reserves for essential tasks (survival).

Reduced AAS due to Zn implies a restricted capacity of the fish to perform aerobic functions concurrently, such as growth, locomotion, reproduction and the rapid recovery from anaerobic activity after exhaustive exercises (Clark et al., 2013). The same metabolic responses (low MMR and AAS) were reported by Callaghan et al. (2016) in white suckers after acute exposure to nZnO.

The reduction in metabolic rates (MMR and AAS) is also indicative of hypoxic conditions and respiratory distress (Callaghan et al., 2016), the effect of which could certainly reduce the aerobic scope (Bessemer et al., 2015) and therefore cannot be disregarded. Empirical evidence suggests that Zn alters gill morphology and compromises the tissue's respiratory ability. Zn toxicity at high concentrations causes edema which causes the gill cell mass to thicken, significantly reducing the oxygen consumption of fish (Hussan et al., 2016). Furthermore, the production of a thick layer of mucus due to Zn has also been reported to clog the gills, damage the cells, and alter gas exchange (Brito Carvalho et al., 2020). This condition is found to be more conspicuous in short exposures because it is a quick and easy adaptation to polluted environments (Hemalatha and Banerjee, 1997). As a result of these changes, respiratory efficiency suffers, making the fish's ability to extract oxygen from water a limiting factor. Such an effect consequently reduces the fish's ability to recover from metabolic expenditure and jeopardizes oxygen allocation to muscles (McKenzie et al., 2021).

The locomotor performance (U_{crit}) after acute exposure in the lab (lab-acute group) was affected, which could be a consequence of a limited energy budget. The results were consistent with the findings of Beaumont et al. (1995) and Cunningham and McGeer (2016), who reported a 31% and 38% reduction in swimming capacity in brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*), respectively, due to cadmium (Cd) toxicity. It is evident that Zn had induced the same effect, but fish living in the metal-polluted reach (field-chronic group) were seemingly unaffected. This analysis, however, did not take into account the likelihood that metabolic resources were diverted from other physiological processes or significant roles in the wild fish to cover the additional expenditure. For instance, reduced growth performance, body condition indicators, consumption rates and feed utilization have been reported in fish species after metal exposure (Abdel-Tawwab et al., 2018).

4.2. Repercussions for the antioxidant system

The way of the response of *B. meridionalis* to oxidative stress under both exposure conditions of Zn was varied in the manner of parameter level and tissue type. In general, acute, and chronic effects cause an enhancement in the AS status of this species. Increased oxidative stress biomarkers (TBARS, CAT, SOD, GPX, and GST) in the liver, kidney and gills were observed in the field-chronic group, and increased GPX, GST, and GSH, mainly in the gills, were observed in the lab-acute group. Antioxidant potential and oxidative stress response vary according to fish species, habitat, and feeding behavior. Inhibition of antioxidant defense mechanisms (e.g., CAT), and increased oxidative damage (e.g., LPO) in the liver and other tissues of fish are reported after Zn exposure (500 μg/L) (Loro et al., 2012).

Our study on lab-acute exposure indicates that Zn induced a tissue-specific antioxidant response, working cooperatively to neutralize ROS after a short-term exposure to high Zn concentrations, this response is similar to the effects seen after exposure to other metals (Vieira et al., 2012). We observed an enhanced response of antioxidant enzymes in all tissues (gills, liver, kidney), and the high CAT levels in the liver supported the findings of Atli et al. (2006), suggesting that Zn has a stimulatory effect on the liver. Furthermore, it is well-established that GPX and CAT play a role in regulating the oxidative stress induced by metal exposure (Vieira et al., 2012). Moreover, increased GSH levels in the liver and kidney suggest its effectiveness in coping with the acute toxicity of Zn. Furthermore, lack of significant changes in other antioxidant enzymes, except for the stimulation of liver CAT activity, implies that the increased GSH levels provide sufficient defense in these tissues (Atli et al., 2020). As a result, we can conclude that the fish exhibited increased detoxification activity in all tissues as an adaptive strategy to cope with the acute exposure.

In contrast, chronic exposure to lower Zn concentrations in the field resulted in increased kidney CAT, SOD, GPX, GST and liver GST activities but decreased GSH levels in the gills. This was an expected physiological response because it shows organ-specific Zn toxicity due to anatomic location and elucidates organ distribution, biotransformation, and detoxification capacity (Atli et al., 2020). In addition, we noticed a

significant increase in the responsivity of AES parameters during the chronic exposure, which could be indicative of the possible overwhelming of tolerant mechanisms. Moreover, we observed more correlations between antioxidant enzyme levels and TBARS during this period, suggesting that the threshold levels of Zn might have been exceeded, triggering the defense system. Additionally, the kidney exhibited a higher impact on AES parameters during the chronic duration, with increased enzyme activities. Given that the kidney receives the greatest amount of branchial blood, our findings suggest that it responded strongly to an increased plasma Zn concentration, (Table 2) that the concentration of Zn was seen two times higher than its control counterparts therefore to eliminate the threat of ROS-mediated damage a pronounced activity of the antioxidant enzymes in the kidney was observed. High GPX activity is associated with a protective role against LPO which presented a positive correlation with GPX activity in the liver. Our findings also revealed high levels of GPX in the kidney, which indicates that the response of this tissue protected it from ROS damage. Another possible explanation of the current situation could be free Zn^{2+}acting a significant role in the pathways of cell signaling in relation to oxidative stress. ROS may lead to a Zn^{2+} release from metal binding-protein metallothionein which in turn can start chain reactions through intracellular zinc sensor (Mtf-1) affecting the target genes by either inducing or downregulating them (Loro et al., 2012).

The non-enzymatic antioxidant parameter GSH plays a crucial role in maintaining the thiol-redox status by acting as a ROS scavenger and serving as a substrate for GPX and GST enzymes. Our data revealed a decline of GSH in the gill under chronic exposure. This decrease indicates its consumption due to increased oxidative stress, as evidenced by the elevated TBARS levels in the gill, which is directly exposed to the aquatic environment.

The liver and gills showed a reduced responsiveness to ROS (reactive oxygen species). One potential reason for this is that both tissues had higher concentrations of Zn, which might had caused resource exhaustion. Although the difference was slight, the continuous exposure of gills to waterborne Zn suggests the possibility of ongoing resource depletion. In the case of the liver, poor response could be attributed to high basal levels of antioxidant enzymes, a physiological adaptation that benefits the tissue when oxidative stress is induced (Atli et al., 2006). However, it is also a vital organ for detoxification, which increases the encounter of tissue with toxicants, consequently overwhelming the protective machinery and causing damage. When the antioxidant system is unable to eliminate or neutralize excess ROS and H_2O_2, there is an increased risk of oxidative damage due to LPO and protein carbonylation, which can reduce enzyme activities or even degrade enzymes (Zhang et al., 2008). The findings revealed that Zn negatively affected the AES status in native fish *B. meridionalis*, despite its essential role as an element in organisms. Even redox-inactive metals like Zn can disrupt AES by causing structural and functional changes to enzymatic and non-enzymatic antioxidants through their interactions with thiol groups (Hogstrand et al., 2011; Loro et al., 2012).

4.3. Conclusion

Zn is widely recognized as an essential element which helps in maintaining metabolic processes by acting as a co-factor in various enzymes, but it has detrimental effects on biological pathways, impacting biochemical, physiological, and molecular mechanisms across a wide range, if it exceeds a threshold limit. Our study concludes that both acute and chronic exposure to Zn modified the energy dynamics and AES status of native fish. In the lab-acute group, we observed a compensatory reduction in locomotor performance after short-term exposure. However, under chronic exposure, there was no observable reduction in locomotor performance, but the possibility of trade-offs cannot be ignored as we found lower AAS which can influence other life-history traits and affect the population fitness consequently. Zn also interfered with the antioxidant metabolic pathway of *B. meridionalis*, resulting in significant alterations in most antioxidant enzymes and GSH levels. Chronic exposure to Zn led to high LPO levels.

The novelty of our findings lies in highlighting the independent interrelationship of energy metabolism and AS stimulation associated with oxidative stress, despite the observed physiological inadequacies. Studying biochemical and physiological parameters with a trait-based approach in this endemic fish habitat exposed to both chronic (field) and acute (lab) conditions provides a holistic and realistic view of its health status. This approach offers fundamental information, from a subcellular level to organismal levels, in an ecological perspective. Based on our results, we suggest the use of various sensitive biomarkers belonging to different mechanisms as endpoints of effects, following a trait-based approach, to get pragmatic result on the functional integrity of species and to establish a more concise protection goal.

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CRediT authorship contribution statement

Amina Khalid: Writing – original draft, Visualization, Data curation. **Cristina Galobart:** Investigation, Methodology. **Francesc Rubio-Gracia:** Investigation, Methodology. **Guluzar Atli:** Conceptualization, Methodology, Writing – review & editing. **Helena Guasch:** Conceptualization, Investigation, Methodology, Visualization, Writing – review & editing. **Anna Vila-Gispert:** Conceptualization, Methodology, Validation, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106661.

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