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Enzyme activities of brown trout liver and kidney peroxisomes after subacute paraquat exposure

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Abstract: Paraquat, a potent herbicide, can be highly toxic to fish, causing morphological and biochemical alterations in several organs, including induction of oxidative stress that may result in cellular damage. Since peroxisomes are vital regulators of reactive oxygen species, it was hypothesized that a subacute exposure of brown trout – a sensitive bioindicator – to a waterborne environmentally relevant concentration (0.3 mg L^{-1}) of paraquat may impact some liver and kidney peroxisomal enzyme activities. Spectrophotometric measurements of urate oxidase, catalase, and D-amino acid oxidase activities were assayed in liver and renal homogenates from immature trout, control and exposed, at 0, 7, and 15 days. In the liver, none of those enzymes showed significant differences between the control and exposed fish at 7 and 15 days. In the kidney, urate oxidase activity was not detectable, and no significant statistical difference was found for the other enzymatic activities between groups. However, an increase in catalase and a simultaneous decrease in D-amino acid oxidase activities were detected during the experiment. At the concentration tested, paraquat did not affect the targeted hepatic and renal peroxisomal enzymes. Additionally, the study revealed changes over time, warning that captivity or experimental stress influenced the enzyme activities.

Keywords: enzyme activities; kidney peroxisomes; liver peroxisomes; pesticide; teleost

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Introduction

Peroxisomes are deeply implicated in detoxification mechanisms such as xenobiotic and reactive oxygen species (ROS) metabolism. Specific peroxisomal enzymes involved in those pathways are responsible for deactivating and neutralizing toxic compounds because they can transform ROS, such as superoxide and hydroxyl radicals, into non or less toxic products [1,2]. Environmental pollution has continuously increased because of industrial and agricultural developments and the vast array of daily urban activities. The aquatic environment is especially affected since it is the endpoint of many contaminants, such as pesticides and herbicides. These toxicants impose health hazards for many animals, including humans [3-5].

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a non-selective herbicide with a broad spectrum of activity widely used for crop desiccation and weed control [2]. This toxic reaches natural waters via runoff from agricultural fields and accumulates in different aquatic organisms. It causes several deleterious effects [6-10] since it is easily reduced into radical ions, generating superoxide anions, which seem to react with unsaturated membrane lipids [11]. Paraquat has been prohibited in several countries by the Environmental Protection Agency (2022) [12] due to its negative impact. However, it is permitted

under strict regulations and continues to be widely used worldwide [13-15]. Environmental concentrations in surface waters have ranged from less than 1 to over 100 µg L^{-1} [16,17] and may reach over 4 mg L^{-1} [18].

Particularly in fish, it is known that paraquat may induce histological alterations in several organs, including the gill, liver, and kidney [9,19]. Biochemical alterations are also documented in those organs and in different species [4,20]. Catalase, D-amino acid oxidase, and urate oxidase are typical peroxisomal enzymes. As an antioxidant enzyme, catalase was investigated in several fish toxicological studies, generally showing higher activities in the toxic-exposed groups [3,21]. Literature about the effects of toxicants on the activities of peroxisomal oxidases is very scarce. Still, an increase in the activities of acyl-CoA and D-amino acid oxidases was reported in mussels exposed to diverse pollutants [22], as well as an increase in urate oxidase activity in rainbow trout exposed to pesticides [23].

In a preliminary approach and to increase the knowledge on the effect of chemicals that may induce oxidative stress on fish peroxisomes, brown trout (*Salmo trutta fario*) were subacutely exposed to the herbicide paraquat. In the peroxisome's richest organs (liver and kidney), some peroxisomal enzyme activities, namely catalase and two oxidases (D-amino acid and urate oxidases), were measured to detect eventual alterations caused by subacute paraquat exposure. This aligns with regulatory efforts to mitigate chemical pollution and protect biodiversity under international and national guidelines. Indeed, paraquat is banned in many countries but is still in free use in others, so it is pertinent to continue exploring the toxicity *vs.* tolerance of fish to the compound. The integration of these frameworks reinforces the study's relevance to both ecological preservation and responsible research practices. This manuscript was previously published as a preprint on Research Square, DOI: https://doi.org/10.21203/rs.3.rs-4953663/v1.

Materials and Methods

Animals and experimental design

The study followed Directive 2010/63/EU, and the expertise and ethical accreditation for animal use was approved by Direção-Geral de Veterinária (DGAV) (ref. 0421/000/000, 2018-02-27). Fish were provided by Circunscrição Florestal do Norte (CFN), Direção Regional de Agricultura de Entre Douro e Minho (DRAEDM). Groups of immature (1-year-old) brown trout were maintained in four tanks (two per each experimental condition) at an aquaculture station (Estação Aquícola do Rio Ave, Portugal) for 15 days. The body parameters of those fish included weight and length. Fish were acclimatized for one week in tanks with recirculating dechlorinated water and fed commercial trout food. The experiment was started by adding paraquat to the water of exposed fish tanks (0.3 mg L^{-1} nominal concentration). Fish in control tanks were unexposed. During the 15 days of exposure, the water circulated in closed systems, with cleaning maintenance. Total water substitution and renewing paraquat in the exposed group was carried out every 2-3 days. Every other day, water quality physical-chemical parameters were measured with commercial kits (chlorine, ammonia, nitrite, nitrates, hardness) or portable meters (pH, dissolved oxygen), confirming that they were optimal along the assay. To establish baseline conditions and quantify the pre-existing variability, ensuring that the animals' physiological and biochemical parameters were thoroughly documented before exposure, ten fish were collected on the first day of the experience as control at day 0. After the beginning of the paraquat exposure, ten fish from each assayed condition, control and exposed tanks (five specimens per tank), were collected on days 7 and 15.

Preparation of tissue homogenates

Fish were killed by over-anesthesia, immersed in a 1 ml L^{-1} aqueous solution of 2-phenoxyethanol, and then weighed and measured in length. Before tissue collection for homogenization, the liver and kidney were perfused with a heparinized (5 IU/ml) isosmotic buffer for salmonids at 4ºC and with a physiological flow rate of about 5 ml min^{-1} Kg^{-1} of body weight [24]. To improve the perfusion, the most posterior part of the fish was cut, allowing a direct cannulation of either the vein or the artery with simultaneous escape of both perfusate and blood. After removal, the liver, trunk kidney, and gonads were immediately weighed. Subsequently, the organs were homogenized according to Rocha *et al.* (2003) [24]. Organs were minced in chilled homogenization buffer with a pH of 7.4 (250 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.1% PMSF and 0.5% Triton X-100) and then homogenized in the same buffer using a Potter-Elvejhem homogenizer at 1,000 rpm and held at 4ºC. The homogenized volumes were adjusted to 10 ml g^{-1} of liver and 5 ml g^{-1} of kidney and filtered through an about 95-µm mesh net. Subsequently, centrifugation was carried out at 10,000*g* for 10 min. The supernatants were collected and stored at -80ºC until enzymatic measurements were done.

Biochemical measurements

All assays were performed in a spectrophotometer connected to a circulating water system for temperature regulation in the cuvette compartment. Total protein content was determined using bovine serum albumin (BSA) as standard, and results are expressed in BSA equivalents. Enzymatic assays were performed in duplicate for each sample, with two different dilutions made with homogenization medium, to calculate a mean value and ensure repeatability. Appropriate sample dilutions were used to obtain the time linearity of enzymatic activities and proportionally to the amount of protein in the sample. The activities of

peroxisomal enzymes were assayed at 25ºC, except for catalase, whose activity was determined at 20ºC. The evaluation of catalase enzymatic activities was based on the methodology applied to brown trout by Aebi (1984) [25]. The measurement of peroxisomal oxidase activities in liver and kidney total homogenates was based on H_2O_2 production [24]. The enzymatic reaction was started by adding the correct substrate at 20 mM of D-alanine and 1 mM of uric acid. The production of H_2O_2 by peroxisomal oxidases during 10 min was calculated considering a calibration curve [24] with the mathematical expression $[H_2O_2] = 185.07 \times$ Absorbance (500 nm).

Statistical analysis

The Statistica 6.0 for Windows software was used. After confirming the normality and the homogeneity of variances for each parameter, a one-way ANOVA was used to observe the kinetics along the 15 days in both control and paraquat-exposed groups. In some cases, data transformation ensured the normality and homogeneity of variances. Standard post-hoc testing was done after a significant ANOVA. The significance level was set at 5%. Data are presented as means (CV), where CV is the coefficient of variation (CV = standard deviation ÷ mean).

Results

During the assay, i.e., from day 0 to 15, control (days 0, 7, and 15) and paraquat (days 7 and 15) exposed groups had similar mean values in their body and organ parameters, namely fish weight, standard fish length, gonadal-somatic index (GSI), hepato-somatic index (HSI) and reno-somatic index (RSI) (Table 1).

Table 1. Trout morphometric data, given as the means of all sampling days (CV).

	Weight	Length	GSI	HSI	RSI
Control (*n*=30)	51.54 (0.27)	15.8 (0.09)	0.001 (0.52)	0.011 (0.16)	0.009 (0.10)
PQ (*n*=20)	51.60 (0.21)	15.9 (0.07)	0.002 (0.87)	0.011 (0.19)	0.009 (0.06)

Weight and length values are expressed as g and cm, respectively, whereas the GSI, HSI, and RSI are expressed as % of total body weight. GSI – gonadal-somatic index; HSI – hepato-somatic index; *n* – number of animals; PQ – paraquat-exposed group; RSI – reno-somatic index. One-way ANOVA did not disclose significant differences.

Liver peroxisomal enzyme activities

Data on some peroxisomal enzymatic activities (catalase, D-amino acid oxidase, and urate oxidase) are presented in Table 2. None of these liver enzymes showed significantly different activities between control and paraquat-exposed groups in each sampling period. Additionally, after 15 days, the activities of these hepatic enzymes did not show significant differences in control or paraquat groups.

Table 2. Liver peroxisomal enzyme activities, given as means (CV).

Liver	Day 0	Day 7		Day 15	
	Control (*n*=10)	Control (*n*=10)	PQ (*n*=10)	Control (*n*=10)	PQ (*n*=10)
Catalase	1.080 (0.09)	1.090 (0.10)	1.048 (0.13)	1.108 (0.22)	1.124 (0.15)
D-Amino acid oxidase	3.119 (0.23)	3.559 (0.21)	2.803 (0.21)	3.712 (0.23)	3.373 (0.21)
Urate oxidase	2.386 (0.33)	2.115 (0.64)	1.948 (0.40)	1.764 (0.78)	1.924 (0.33)

Values are presented as enzyme activities, expressed as nmol min^{-1} mg^{-1} of protein for oxidases and s^{-1} mg^{-1} of protein for catalase. *n* – number of animals; PQ – paraquat-exposed group. One-way ANOVA did not disclose significant differences.

Kidney peroxisomal enzyme activities

Regarding the enzymatic activities of brown trout kidney, no significant differences were detected between the control and the paraquat groups (Table 3). However, when data were analyzed from a kinetics perspective in both control and paraquat-exposed groups, results revealed a significant increase in catalase activity and a significant decrease in D-amino acid oxidase activity during the 15 days of the assay. No urate oxidase activity was found in kidney samples.

Table 3. Peroxisomal enzymatic activities from kidney control and PQ-exposed groups.

Kidney	Day 0	Day 7		Day 15	
	Control (*n*=10)	Control (*n*=10)	PQ (*n*=10)	Control (*n*=10)	PQ (*n*=10)
Catalase	0.069 (0.18)[a]	0.063 (0.33)[a]	0.083 (0.20)	0.095 (0.25)[b]	0.089 (0.17)[b]
D-Amino acid oxidase	1.891 (0.19)[a]	1.329 (0.38)[b]	1.830 (0.21)[a]	1.333 (0.27)[b]	1.355 (0.33)[b]
Urate oxidase	n.d.	n.d.	n.d.	n.d.	n.d.

Values are enzyme activities, expressed as nmol min^{-1} mg^{-1} of protein for oxidases and s^{-1} mg^{-1} of protein for catalase. n.d. – not detectable; *n* – number of animals; PQ – paraquat-exposed group. Means with lowercase superscript letters represent significant one-way ANOVA post-hoc differences among sampling periods within the same group and for each enzyme.

Discussion

There is vast literature reporting that waterborne exposure of fish to many toxicants, including the widely used herbicide paraquat, triggers changes in xenobiotic metabolism enzymes and induces oxidative stress. Moreover, it is known that peroxisomes are organelles involved in detoxification and in balancing ROS. In aquatic animals, toxicants may alter the enzyme activities in those organelles, serving as biomarkers of pesticide exposure [19,26,27]. Accordingly, we questioned if one subacute exposure of brown trout juveniles to paraquat at an environmentally relevant concentration could impact liver and kidney peroxisomal enzymes.

Here, the concentration tested was 2.3% of the median lethal concentration (LC_{50}) for brown trout (13 mg L^{-1}) [28]; studies referenced by Tomlin proved that paraquat concentrations as low as 0.05-0.1 ppm (i.e., mg L^{-1}) can have detectable ecological impacts on certain aquatic species [28]. However, in this study, and at least for the measured enzymes, the results indicate that 0.3 mg L^{-1} paraquat does not induce enzymatic alterations in the brown trout's liver and kidney peroxisomes after 7 and 15 days of exposure. The concentration of paraquat was chosen based on published data, for which no documented cellular necrosis or cellular death signals were found, and because that pesticide concentration corresponds to a more realistic situation, as seen in significantly polluted surface waters [29] and according to the Guidance on the use of herbicides on nature conservation sites (2003) [30]. Our data contrasts with other studies, revealing increased catalase activity in both organs under herbicide exposure. However, those investigations were performed with much higher paraquat concentrations, which do not translate into frequent or realistic environmental conditions. Therefore, we can assume that the trout's normal hepatic peroxisome function was sufficient or eventually unnecessary to maintain the ROS content in homeostasis for this paraquat amount. The same assumption can be applied to the kidney, even though it is more active in excreting physiological compounds and metabolites derived from itself and other organs, such as the liver. However, the absence of paraquat effects agrees with morphological data mentioned by Rojik *et al.* (1983) [31] in renal tubular cells (where the peroxisomes can be found), contrary to the observed in the climbing perch (*Anabas testudineus*) treated with 12.0-15.0 mg L^{-1} of paraquat, which presented structural alterations in renal corpuscles and tubules [17].

The present study revealed that the peroxisomal enzymatic activities in the kidney varied with time, not only in the paraquat-treated animals but also in the control ones. This fact calls attention to the use of juvenile brown trout kidney samples in toxicological studies integrating enzymatic measurements since the time of sample collection could indicate differences in those activities, not due to the toxicant effects but otherwise to temporal variations reflecting the animals' normal physiology and continuous adaptations. On the contrary, the liver revealed higher stability concerning its peroxisomal enzymatic content, which was not influenced by time. As to factor(s) causing the changes in the kidney, and given the absence of chemical or other sources of stress (e.g., water quality was always optimal), the most likely explanation falls on functional effects of the well-known phenomenon of the continued growth of the fish kidney by addition of new nephrons, occurring in juveniles and adults [32]. In brown trout, it was specifically shown that the kidney is very plastic structurally, with marked histological and ultrastructural changes during the lifecycle and seasons [33-35].

The absence of urate oxidase activity in kidney peroxisomes coincided with prior results in this type of salmonid [36]. In the liver, the urate oxidase activities agree with its former detections in juvenile and adult brown trout [22,36] and other species [37,38].

In conclusion, the present subacute toxicity test showed that, under an environmentally relevant concentration of the herbicide paraquat, there were no changes in the activities of some brown trout peroxisomal enzymes. Nevertheless, the study unveils and reinforces that common biomarker approaches (such as catalase activity) may not detect exposure to low concentrations of toxicants for a relatively short period. We further showed that, for subacute (and likely subchronic) testing and with the selected

peroxisome marker enzymes, kidney studies must account for the potential changes occurring over time, both in control and exposed animals. This aspect deserves further study, including testing other concentrations and targets to refine the assessment of paraquat's toxicological potential to induce peroxisome changes.

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Author Contributions

ALC and ER contributed to the study's conception and design. ADR, ALC, and ER performed material preparation, data collection, and analysis. ALC and ER were responsible for funding acquisition. ADR wrote the first draft of the manuscript, and all authors commented on its previous versions. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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